The *tylLM* region of the *Streptomyces fradiae* genome.

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by

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Abstract.

The *tylLM* region of the *Streptomyces fradiae* genome.

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Streptomyces fradiae produces the macrolide antibiotic tylosin which is composed of a polyketide lactone, tylactone, and three deoxyhexose sugars. The addition of the first sugar, mycaminose, confers antimicrobial activity onto the inert polyketide. The *tylIBA* and *tylLM* loci from the tylosin gene cluster are involved in the production and addition of mycaminose. *tylL* and *tylA* mutants are defective in the biosynthesis or addition of all three sugars, in contrast to the TylM and TylB lesions which are mycaminose specific. Little was known about the *tylLM* region, but the *tylL* and *tylM* mutants had both been complemented with an 8 kb fragment of *tyl* DNA (Fishman *et. al.*, 1987).

Sequence has been generated for the *tylLM* region, identifying four ORFs (*1*-4**), which could have roles in deoxyhexose metabolism (Gandecha *et. al.*, 1997). The *tylM* mutant was complemented by integrating a wild-type copy of *orf3** into the mutant genome; this gene is thought to be the methyltransferase which acts in the mycaminose pathway. The wild-type *orf2** integrated into the *tylL* mutant chromosome restored tylosin biosynthesis, and an in-frame stop codon was located part way through *orf2** amplified from the *tylL* genome. *orf2** was a surprising candidate for the *tylL* gene because it is the mycaminosyl-glycosyltransferase. The *tylL* mutant could not convincingly convert OMT, DMT or desmycosin to tylosin. We therefore believe that the TylL phenotype is the result of multiple mutations or a physiological effect. The function of *orf1** is still unknown, although matches with other genes involved in the biosynthesis of amino sugars suggest that it is involved in mycaminose production.

Analysis of the *orf1** transcript suggested that, during antibiotic biosynthesis, *orf1** may be co-transcribed with *tylG*, with two additional transcripts initiating independently of *tylG*. Promoter-probe vectors have been of limited use in identifying promoters.

Abbreviations.

A	adenine
ACP	acyl carrier protein
AS-1	solid growth medium for *Streptomyces* spp.(Baltz, 1980)
AT	acetyltransferase
ATP	Adenosine triphosphate
AUD	amplifiable unit of DNA
C	cytosine
°C	degree Celsius
cAMP	adenosine 3':5' cyclic monophosphate
CCC	covalently closed circular
Ccr	crotonyl CoA reductase
Ci	Curie
CIAP	calf intestinal alkaline phosphatase
CoA	coenzyme A
CTAB	cetyl trimethyl ammonium bromide
(d)dNTP	(di) deoxynucleoside triphosphate
7-deaza dATP	2'-deoxy-7-deazaadenosine 5'-triphosphate
7-deaza dGTP	2'-deoxy-7-deazaguanosine 5'-triphosphate
DEPC	diethylpyrocarbonate
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dITP	2'-deoxyinosine 5'-triphosphate
dNTP	deoxynucleoside triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
DMSO	Dimethyl sulphoxide
DMT	demycinosyl-tylosin
ds	double-stranded
E	*Escherichia*
erm	family of genes encoding resistance to MLS antibiotics
EtBr	ethidium bromide
FAS	fatty acid synthase
g	gram
G	guanine
GTP	Guanosine triphosphate
h	hour

Hepes	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
IPTG	isopropylthio-ß-D-thiogalactoside
kb	kilobase(s) or 1000 bp
LB	Luria-Bertani
LMP	low melting point
M	*Micrococcus*
M	molar
Mb	Megabases
MCS	multiple cloning site
MIC	minimal inhibitory concentration
min	minute
ml	millilitre
MLS	macrolide-lincosamide-streptogramin B
mM	millimolar
MM-1	Tylosin production medium (Gray *et. al.*, 1980)
MNNG	*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
MOPS	3-(*N*-morpholino) propanesulfonic acid
mRNA	messenger RNA
NaCl	sodium chloride
Na_2EDTA	the disodium salt of ethylenediamine tetraacetic acid
NaOAc	sodium acetate
NaOH	Sodium hydroxide
nm	nanometre
OD	optical density
-OH	hydroxyl group
OMT	5-*O*-mycaminosyl-tylonolide
ORF	open reading frame
-P	phosphate group
PAB	*p*-aminobenzoic acid
PBP	pencillin-binding protein
PCR	polymerase chain reaction
PEG	Polyethylene glycol
Pipes	1,4-piperazinebis (ethane-sulphonic acid)
PKS	polyketide synthase
pmol	picomole
ppGpp	guanosine 5'-diphosphate 3'-diphosphate
R	resistance
rRNA	ribosomal RNA

RNase	ribonuclease
rpm	revolutions per minute
S	svedberg unit
S	*Streptomyces*
Sac	*Saccharopolyspora*
SAM	*S*-adenosyl-L-methionine
SDS	Sodium dodecyl sulphate
sec	seconds
ss	single-stranded
SSC	standard saline citrate
SSPE	standard saline phosphate-EDTA
SQ	Millipore SuperQ water
T	thymine
Taq	*Thermus aquaticus*
TAE	Tris-acetate- EDTA
TBE	Tris-borate-EDTA
TDP	Thymidine 5'-diphosphate
TE	Tris-EDTA
TEMED	*N, N, N', N'*-tetramethylenediamine
TES	[N-tris(hydroxymethyl)methyl-2-aminoethane-sulphonic acid]
TLC	Thin-layer chromatography
tlr	tylosin resistance gene from *S. fradiae*
T_m	melting temperature
TSB	tryptic soy broth
tRNA	transfer RNA
tyl	tylosin biosynthetic gene(s) from *S. fradiae*.
UDP	uridine diphosphate
UV	ultraviolet
v/v	ratio of volume to volume.
V	volt
w/v	ratio of weight to volume.
Xgal	5-bromo-4-chloro-3-indolyl-ß-D galactoside
µl	microlitre
µV	microvolt
UWGCG	University of Wisconsin Genetics Computer Group

Chapter 1 Introduction

Antibiotics.

Antibiotics are secondary metabolites which are produced during the growth of one organism and interfere with the growth of another. Although actinomycetes and fungi are the predominant producers of antibiotics, it has been recognised that antibacterial substances are produced by a wider range of cells. For example, in the mammalian immune system, neutrophils produce defensins which are antibacterial peptides (Selsted *et. al.*, 1995).

Antibiotics exhibit a diverse and complicated chemistry which incoporates a wide range of biochemical compounds. Aminoglycosides, such as kanamycin, are composed of amino sugars which are held together by glycosidic linkages, in contrast to thiostrepton which is a large modified peptide antibiotic. The macrolide antibiotic erythromycin is based on a polyketide ring, which is synthesised in a similar way to fatty acids, with sugars attached.

The use of antibiotics in medicine.

The application of antibiotics in medicine did not occur until the twentieth century. Before this time various chemicals were used to treat infectious diseases. An early form of chemotherapy was to use cinchona bark to treat malaria, a practice introduced from South America to Europe in the 17th century (Franklin *et. al.*, 1989). Quinine, the active component in the bark, was not isolated until 1820. Such treatments were therefore based on the use of natural products to treat symptoms, without any understanding of the underlying mechanism behind the cure. Chemotherapy did not develop as a science until the work of Paul Ehrlich at the beginning of the twentieth century. Ehrlich developed the idea of selective toxicity, recognising that an antimicrobial compound must bind to the infectious agent and have a toxic effect, but leave the host unharmed. He also recognised that the ability of a drug to disciminate between microorganism and host was linked to the level of dosage (Franklin *et. al.*, 1989).

The first natural antibiotic, penicillin G, was discovered by Fleming in 1929 with his famous observation that *Penicillium notatum* produced a diffusible substance which inhibited staphylococcal growth on an agar plate. This antibiotic was not successfully extracted until the 1940's, after which great efforts were made to synthesise it on a large scale (Franklin *et. al.*, 1989). This initial success encouraged Waksman to search for new antibiotics by screening soil samples for antibiotic producers (Franklin *et. al.*, 1989). New antibiotic activities have since been discovered by modifying existing antibiotics either chemically or genetically. Many of the Beta-lactams used today are semisynthetic variants of original natural molecules (Donowitz *et. al.*, 1988).

The search to find new antibiotics has become increasingly important as their use has been compromised by the appearance of resistant strains (Cohen, 1992). Resistance in a pathogenic bacterium may be the result of a chromosomal mutation, or acquisition of aresistance gene from the environment; transfer of resistance determinants between bacteria can occur by transduction, transformation, conjugation and transposition. Drug resistance may be combated in a number of different ways. The World Health Organisation now encourages the the treament of tuberculosis with a cocktail of drugs (streptomycin, isoniazid, rifampicin, and pyrazinamide), a strategy called DOTS (directly observed treatment, short-course), in order to fight drug-resistant strains of *Mycobacterium tuberculosis* (Spinney, 1996). Many argue, however, that the use of antibiotics needs to be restricted in order to limit the selection of resistant strains.

Selective toxicity; antibiotic targets.

Antibiotics have specific targets which can either be structural or metabolic components of the microbial cell. The action of antibiotics is selective; they can be used to attack the infectious agent, not the mammalian host.

Beta-lactams interfere with the biosynthesis of the bacterial cell wall, which is composed of peptidoglycan (Donowitz *et. al.,* 1988). Peptidoglycan is a polysaccharide unique to bacteria, explaining the drugs ability to discriminate between host and microorganism. Two alternating sugars, *N*-acetylglucosamine and *N*-acetylmuramic acid are polymerised to form an unbranched chain of peptidoglycan. The muramic acid residues may be attached to a short chain of amino acids (a pentapeptide) which may be linked to other pentapeptides by a pentapeptide bridge. Beta-lactams bind to, and inhibit, specific penicillin-binding proteins (PBP's) which perform different functions in cell wall synthesis (Jawetz *et. al.*, 1991). Penicillin is thought to prevent the cross-linking of peptidoglycan chains by specifically binding to, and inhibiting, a transpeptidase (a PBP) (Rogers Yocum *et. al.*, 1980). The transpepidation reaction involves the loss of a D-alanine from the pentapeptide, so that crosslinked chains of amino acids are in fact tetrapeptides (Franklin *et. al.*, 1989).

The specifity of antibiotic action may also be the result of structural variation between targets in the host and bacterium. Many antibiotics act by inhibiting protein synthesis, either by preventing the transcription of genetic material, or the translation of mRNA into proteins. The 70S ribosome is the protein synthesis factory in a bacterial cell and is a popular target for antibiotics, in contrast to the 80S ribosome in mammals. Within the 70S ribosome, the 30S subunit is the target of many aminoglycosides (Franklin *et. al.*, 1989), where as macrolides (Menninger *et. al.*, 1982) interact with the peptidyl transferase center of the 50S subunit. These inhibitors of protein synthesis may interact with either ribosomal proteins and or rRNA (Cundliffe, 1987). Bacterial

DNA-dependent RNA polymerase transcribes the DNA blue print into an RNA message, which migrates to the ribosome during protein synthesis. This enzyme is also a focus of certain antibiotics. Rifamycin is thought to interact with the ß subunit in the core enzyme of RNA polymerase, and is also reported to inhibit reverse transcriptase from retroviruses (Lal *et. al.*, 1993).

Some antibiotics interfere with DNA. The bleomycins make vicious single-stranded and double-stranded breaks in DNA, which are sequence dependent (Stubbe *et. al.*, 1996). Topoisomerases alleviate topological constraints during DNA replication and transcription. DNA gyrase (prokaryote topoisomerase II) is blocked by coumarin and quinolone antibiotics. Novobiocin (a coumarin) targets the ATPase (B subunit), blocking its interaction with ATP (Sugino *et. al.*, 1978), and nalidixic acid (a quinolone) targets the subunit responsible for strand breakage and reunion (Sugino *et. al.*, 1977; Gellert *et. al.*, 1977).

Antibiotics may also mimic intermediates in microbial metabolism. Sulphonamides (synthetic antibacterials) are structural analogues of a component of folic acid, *p*-aminobenzoic acid (PABA), and therefore compete with PABA. Sulphonamides are incorporated into the structure of folic acid, so that this coenzyme, which is important in synthesis of nucleic acids, is no longer active. Animals remain unaffected as they do not synthesise their own folic acid; they obtain it from the environment (Jawetz *et. al.*, 1991).

Resistance to antibiotics.

Resistance mechanisms against antibiotics, exhibited by their producers, are diverse (Cundliffe, 1989). Modifying enzymes may convert an antibiotic from an active to an inactive form; for example aminoglycosides may be adenylated, phosphorylated or acetylated (Cundliffe, 1989). A bacterium may be impermeable to a drug, or able to pump the antibiotic out of the cell by an efflux mechanism (Levy, 1992). Drug targets may be modified, for example methylation of rRNA, or an alteration to a ribosomal protein (Cundliffe, 1987), and the modified target may be inducibly expressed, being switched on during antibiotic biosynthesis. The novobiocin producer, *Streptomyces sphaeroides,* produces two forms of DNA gyrase, one which is resistant to novobiocin and one which is sensitive (Thiara *et. al.*, 1989). The expression of the resistant B subunit only occurs when novobiocin is being synthesised, transcription from the $gyrB^R$ promoter occurring in response to changes in DNA topology which result from novobiocin impeding the sensitive form of gyrase (Thiara *et. al.*, 1989).

Streptomyces.

Streptomyces are Gram positive, filamentous bacteria, which are classified within the actinomycetes, which contains the principal genera *Streptomyces*, *Actinomyces*, *Nocardia* and

Micromonospora. *Streptomyces* are soil organisms which do not cause disease, with the exception of the plant pathogen *Streptomyces scabies*. They are of interest because they are able to produce a wide range of secondary metabolites and also undergo complex morphological development. Extracellular enzymes are often synthesised by *Streptomyces*, including hydrolytic enzymes, proteases, amylases, and cellulases.

Streptomyces are strange bacteria. Many aspects of their morphology and metabolism are analogous to fungi, which historically caused confusion over their classification (Hopwood, 1988). Although they are of bacterial dimensions, filaments ranging between 0.5 and 1.5 μm in diameter, *Streptomyces* have a complicated life cycle. A vegetative mycelium produces aerial hyphae which subsequently develop spores, in contrast to bacteria such as *Escherichia coli* whose cell cycle is equivalent to their life cycle (Chater, 1989). However, a closer inspection of *Streptomyces* cell structure indicates that these organisms are true bacteria; their cell wall is composed of peptidoglycan, not chitin; and they possess a single chromosome, and plasmids. Yet, the bacterial chromosome is typically circular, but *Streptomyces* are an exception. A linear chromosome has been identified in *Streptomyces lividans* 66 and *Streptomyces coelicolor* A3(2) with ends resembling telomeres found at the termini of eukaryote chromosomes (Lin *et. al.*, 1993).

These organisms produce a wide range of secondary metabolites, whose synthesis is normally restricted to the later stages of growth. Of particular significance are the antibiotics which are clinically important. In batch culture, primary metabolism occurs during the period of active growth and cell division (the trophophase) allowing the production of essential macromolecules such as DNA, RNA and proteins. The stationary phase (the idiophase) involves the production of chemically diverse molecules, known as secondary metabolites, although the timing of the switch between primary and secondary metabolism is not absolute (Martin and Demain, 1980). Secondary metabolites do not appear essential for the producers survival, and their functions in nature are much debated. It has been proposed that secondary metabolites do not have a "single, universal function" but instead provide an opportunity to produce something useful to an individual organism (Vining, 1990; 1992). The enzymes involved in secondary metabolism are not so substrate specific as catalytic proteins involved in primary metabolism, and can therefore be more flexible, allowing acceptance of alternative substrates and therefore the evolution of new biochemical pathways. Genes for secondary metabolism may have evolved from genes for primary metabolism; for example polyketide synthase genes (PKSs) and fatty acid synthase genes (FASs) are likely to have a common evolutionary origin (Hopwood *et. al.*, 1992).

As well as being interesting academically, an understanding of antibiotic biosynthesis has practical applications. New antibiotic activities may be created in response to the growing

problem of widespread resistance amongst clinical isolates (Hopwood, 1989). Chemical modification of existing antibiotics, for example the development of the semi-synthetic penicillins and cephalosporins, has occurred, but is limited in the variety of activities it can produce. Antibiotic yields in the soil are thought to be low, but antibiotic production may be increased by either isolating better producers or improving fermentation conditions. Upregulating levels of antibiotic gene expression by overexpressing a positive regulator gene in a producing strain could allow an increase in antibiotic yield (Chater, 1990). Also, it is possible to create novel "hybrid" antibiotics by introducing genes for a chemically related antibiotic from one *Streptomycete* into another (Hopwood et al., 1985), or genetically engineer biosynthetic enzymes (Katz *et. al.*, 1993).

Why do *Streptomyces* produce antibiotics?

Historically, it was presumed that antibiotics were produced in the struggle to compete with other soil organisms. There has been much debate, however, as to whether antibiotic production can be demonstrated in the soil, and if so, whether it has an ecological role (Gottlieb, 1976). Considering the amount of DNA committed to antibiotic biosynthesis and the clustering of biosynthetic and resistance genes, their functions must have provided a selectable advantage and hence a role in defence would not be surprising (Maplestone *et. al.*, 1992). However, the production of antibiotics appears to be maximal in the stationary phase when organism would not be competing for space, athough morphological differentiation occurs. Genes for sporulation and antibiotic biosynthesis are often switched on together during *Streptomyces* development. It has been proposed that antibiotics may have a regulatory role in sporulation. The *whiE* locus isolated from *S. coelicolor* A3(2) shares sequence similarities with polyketide genes, and plays a role in determining spore colour (Davis *et. al.*, 1990). Antibiotic specificity and the popularity of rRNA and ribosomal proteins as drug targets, reflects the proposition (Davies, 1990) that antibiotics may have been part of the cell machinery in more primitive times. Antibiotic binding sites in rRNA are conserved between eukaryotes and prokaryotes, and lie in conserved domains of rRNA suggesting that antibiotics were involved in the evolution of the translational mechanism. During biochemical evolution, antibiotics may have been replaced by more effective proteins.

The *Streptomyces* genome.

The genome of *S. lividans* 66 has been estimated to be at least twice the size (8 Mb) of the *E. coli* genome (Lin *et. al.*, 1993). Not all of this additional coding capacity has been explained, although the complexity of *Streptomyces* morphological and physiological development requires many genes. The chromosomes of *S. lividans* 66 and *S. coelicolor* A3(2) are thought to be physically linear, although they also exist as circular molecules, with the origin of replication (*oriC*) in the centre (Lin *et. al.*, 1993; Musialowski *et. al.*, 1994). The ends of the chromosome

are thought to have a similar structure to the termini of eukaryote chromosomes; there are telomeric sequences which contain inverted repeats and have covalently associated proteins (Lin *et. al.*, 1993). Large linear plasmids have been identified in *Streptomyces* species (Kinashi *et. al.*, 1987). pSLA2, a giant linear plasmid isolated from *Streptomyces rochei,* also has an internal origin suggesting a similar mechanism of replication to the *Streptomycete* chromosome (Chang *et. al.*, 1994). Processes involved with genetic instability in *Streptomyces* are not fully understood, but transposable elements (Chater *et. al.*, 1985a; Lydiate *et. al.*, 1986) and DNA rearrangements involving deletions and amplifications have been recognised (Rauland *et. al.*, 1995).

Streptomyces DNA is G+C rich (73%) (Frontali *et. al.*, 1965), in contrast to that of other prokaryotes, and eukaryotes. The transcriptional control signals in *Streptomyces* may therefore be very different to other organisms, whose consensus promoters are typically A+T rich. This may explain the constraints on expressing *Streptomyces* genes in heterologous hosts. *E. coli* has a lower G+C ratio, (49%), and therefore when constructing vectors from both *Streptomycete* and *E. coli* DNA, it is often difficult to find compatible, unique restriction sites, which allow the insertion of DNA. Also the high G+C ratio in *Streptomyces* makes DNA sequencing difficult due to compressions. Despite these difficulties, many antibiotic biosynthesis and resistance genes have been isolated from *Streptomyces*, and more is being understood about how these genes are regulated.

Differentiation.

Both morphological and physiological differentiation require different genes to be switched on at different times. Exactly which signals in the environment are recognised, and how such messages are communicated to the level of the gene, is not fully understood. However, different aspects of this intricate network have been elucidated in certain species of *Streptomyces*. An understanding of what controls antibiotic biosynthesis might suggest ways in which antibiotic yields could be increased, which has obvious commercial significance (Chater, 1990). However, there are also certain features of *Streptomyces* gene expression which suggest that there may be interesting, and perhaps novel, mechanisms of regulation.

Organisation and expression of genes involved in antibiotic production in *Streptomyces*.

Antibiotics are genetically complex; many genes are required to encode all the necessary steps in their synthesis. Twenty or thirty genes may be required for the production of tylosin in *Streptomyces fradiae*. The genes that encode antibiotics are normally clustered together on the chromosome, with resistance genes, providing an opportunity for their coregulation. This can be rationalised since an antibiotic producer must be resistant to the compound it synthesises

(Cundliffe, 1989) and grouping of these genes favours their combined selection (Seno *et. al.*, 1989).

In bacteria, genes are often arranged into operons which encode polycistronic messenger RNA, which allows synchronous expression of genes in response to a particular signal. However, the complexity of *Streptomyces* development means that genes in different parts of the chromosome are switched on at the same time. Sporulation and antibiotic production are coordinated events, yet evidence from *Streptomyces coelicolor* A3(2) suggests that genes involved in morphological development are scattered around the chromosome, in contrast to genes encoding antibiotics (Hopwood, 1986). Greater flexibility of gene expression is therefore required, and this may be facilitated by sigma factors and/or small diffusible molecules. Within antibiotic gene clusters there is transcriptional complexity, with both divergent and convergent transcription units. In *S. fradiae,* the *tylIBA* and *tylG* loci appear to be transcribed in opposite directions (Figure 2, page 28). The DNA inbetween these two regions may contain several promoters, which, because of their close proximity, may be coregulated.

Morphological differentiation and the transition from primary to secondary metabolism are linked by a regulatory network as well as having separate controls. Antibiotics are normally produced during nutrient starvation, which is associated with sporulation on plates, or when liquid cultures reach the stationary phase. Most of the information about such control mechanisms comes from studies of *S. coelicolor* A3(2). This species produces two pigmented antibiotics, actinorhodin and undecylprodigiosin, in addition to methylenomycin and the so-called calcium-dependent antibiotic, (CDA). Identification of sequences thought to encode proteins that regulate antibiotic biosynthesis in *S. coelicolor* A3(2), has occurred through the screening and analysis of mutants, (Champness, 1988, Adamidis *et. al.*, 1990, and Adamidis *et. al.*, 1992).

Mechanisms to coordinate expression.

Global control may allow coordinated production of several antibiotics, or even coregulation of secondary metabolism and morphological development. Loci associated with different levels of global control have been identified in *Streptomyces coelicolor* A3(2). In contrast to *bld* mutants which can't sporulate or produce antibiotics (Champness, 1988), *absA* and *absB* mutants can sporulate, but are unable to synthesise antibiotics (Adamidis, *et. al.*, 1990; 1992). Other genes may be selective as to the number of antibiotics they regulate. Genes encoding putative two-component regulatory systems have been identified in *S. coelicolor* A3(2); *afsk* and *afsR* (Horinouchi *et. al.*, 1992; Matsumoto *et. al.*, 1994); and *afsQ2* and *afsQ1* (Ishizuka *et. al.*, 1992). In *S. lividans* , pairs of proteins encoded by these genes, a sensor kinase and a response regulator,

stimulate the production of the pigmented antibiotics, actinorhodin and undecylprodigiosin, and A factor.

"Hormone-like substances" have been isolated from actinomycetes, and have been implicated in the control of differentiation (Barabas *et. al*, 1994; Horinouchi *et. al.*, 1993). Sporulation and the production of streptomycin in *Streptomyces griseus* is thought to be controlled by A factor, which triggers a cascade of regulatory genes (Horinouchi *et. al.*, 1992; 1993). Reduced GTP and raised ppGpp levels have also been correlated with the onset of the stationary phase in this species (Ochi, 1987). This suggests that the stringent response, which occurs during nutrient starvation, may also regulate metabolic and morphological differentiation. Exactly how the physiological effector, ppGpp, functions in the stringent response is not known. It may bind RNA polymerase changing the conformation of the enzyme, and therefore affect the recognition of different promoters.

Streptomyces genes exhibit a codon bias (Bibb *et. al.*, 1984), a further repercussion of a high G+C ratio. This has been exploited by *Streptomyces coelicolor* A3(2), which uses A+T rich codons as a regulatory device. Mutants designated "bald" have been recognised which are incapable of sporulation and antibiotic biosynthesis (Champness, 1988). The *bldA* locus is thought to encode a $tRNA^{leu}$ which translates a rare codon, UUA. The expression of this gene is upregulated during nutrient deprivation, late in the bacterial growth cycle (Leskiw, 1993). The TTA codon has been identified in two open reading frames in the actinorhodin cluster of *Streptomyces coelicolor* A3(2) (Férnandez-Moreno *et. al.*, 1991), suggesting that their expression is regulated at the level of translation. These genes, *act*II-ORF4 and *act*II-ORF2, have putative roles in antibiotic biosynthesis, possibly being involved in transcriptional activation and antibiotic export, respectively (Férnandez-Moreno *et. al.*, 1991).

Many sigma factors have been recognised in *Streptomyces coelicolor* A3(2) (Westpheling *et. al.* 1985; Lonetto *et. al.*, 1994), such as WhiG which is thought to regulate sporulation (Chater *et. al.*, 1989a). Their association with a common core RNA polymerase may allow recognition of different classes of promoter. Transcription and translation signals in *Streptomyces* are not well defined, but a convenient distinction can be made between promoters which look like consensus sequences from *E. coli* (Jaurin *et. al.*, 1985) and those which do not (Bibb *et. al.*, 1982). This diversity of promoter sequence, perhaps a consequence of the high G+C ratio, may explain the variety of sigma factors, and also explain the ease with which genes from *E. coli* are expressed in *Streptomyces*, rather than the other way round (Bibb *et. al.*, 1982). Multiple tandem promoters have been identified upstream of many *Streptomyces* genes, and may be recognised by different forms of RNA polymerase. For example, four promoters have been identified upstream of the agarase gene (*dagA*) of *S. coelicolor* A3(2) (Buttner *et. al.*, 1987), and two promoters upstream

of the thiostrepton resistance gene in *Streptomyces azureus* (Janssen *et. al.*, 1990), which would facilitate differential gene expression.

These multiple levels of regulation reflect the complexity of the life cycle and metabolism of *Streptomyces*, and the subsequent interplay between different genes.

Pathway specific regulatory genes.

Local control of antibiotic biosynthesis involves pathway specific regulatory genes, which may be located within the antibiotic biosynthetic cluster. Most of those identified are positive activators, such as *strR*, a positive regulatory gene in the streptomycin biosynthetic cluster of *S. griseus* (Distler *et. al.*, 1987). StrR has been shown to interact with DNA sequences upstream of the promoter of the biosynthetic gene *strB*, activating its transcription (Retzlaff *et. al.*, 1995). A region involved in negative regulation has been identified in the methylenomycin cluster of *Streptomyces coelicolor* A3(2) (Chater *et. al.*, 1985b).

Physiological factors influencing antibiotic biosynthesis in *Streptomyces*.

Carbon sources, especially glucose, can have a repressive effect on antibiotic biosynthesis. In enteric bacteria the suppressive effects of glucose are mediated by carbon catabolite repression. The mechanisms operating in *Streptomyces* are not so well understood; levels of cAMP (adenosine 3':5' cyclic monophosphate) do not appear to correlate with different carbon sources (Paulsen, 1996). It has been observed, however, that high intracellular levels of cAMP improve yields of tylosin in fermentations cultures of *S. fradiae* (Tata *et. al.*, 1994). Sources of nitrogen and phosphate can also be repressive. Ammonium ions (NH_4^{+}) inhibit tylosin biosynthesis by preventing valine and threonine catabolism, which is a source of carboxylic acids which are used in the construction of macrolides (Tang *et. al.*, 1994; Nguyen *et. al.*, 1995). Inorganic phosphate represses the biosynthesis of candicidin in *S. griseus*, through its interaction with *p*-Aminobenzoic acid synthase (Gil *et. al.*, 1985).

Polyketides.

Polyketides are secondary metabolites produced in a similar way to fatty acids by a wide range of creatures, including the actinomycetes which incorporate polyketides into the structure of certain antibiotics (O'Hagan, 1992). Polyketides are chains of carboxylic acids joined together by condensation reactions. The building units are often acetyl, propionyl, butyryl, malonyl, and methylmalonyl thioesters, each monomer contributing two carbon atoms to the backbone of the chain, alternating carbon atoms bearing keto groups which may be modified or removed.

Polyketide synthesis is like fatty acid biosynthesis (Hopwood *et. al.*, 1992).

The starter unit, for example an acetate residue, is moved from the thiol group of coenzyme A (CoA) to the thiol group of the acyl carrier protein (ACP) by an acetyltransferase (AT). The acetyl group is then transferred to the thiol of the condensing enzyme, and the ACP is then free to receive malonyl extender units. A condensation reaction occurs between the extender and starter unit on the ACP to produce a four carbon chain. Keto groups are often removed after the condensation reactions have taken place; this requires the reduction of the keto group to hydroxyl group (by a ketoreductase), the removal of water to produce a double bond (by a dehydrase), and saturation of the double bond (by an enoylreductase). The chain of carbon units then returns to the thiol of the condensing enzyme so that the ACP can receive more extender units. Once the polyketide chain is complete, it is released from the PKS (polyketide synthase), either by being transferred to CoA or by the action of a thioesterase. In contrast to fatty acid biosynthesis, the production of polyketides involves greater variation in structure and must therefore be more highly programmed (Hopwood *et. al.*, 1990). Choices have to be made as to the number, order and identity of extender units, aswell as a greater diversity of starter unit (Cane, 1994). The chemistry which occurs during chain extension also differs; fewer keto groups are reduced during polyketide biosynthesis, so that the chain of carbon atoms is a more reactive structure. This structure can therefore undergo a diversity of condensations to produce macrocyclic or aromatic polyketides (Vining, 1992). Different moieties may be added such as methyl groups and deoxyhexose sugars (Hopwood *et. al.*, 1990), and genes encoding these functions are normally clustered with PKS genes. The erythromycin A producer, *Saccharopolyspora erythraea,* produces a 14-membered lactone which is cyclised to form a ring, 6-deoxyerythronolide B (6-DEB), which is then modified by hydroxylation, methylation and the addition of two sugars, desosamine and mycarose.

Polyketide synthases involved in antibiotic biosynthesis in *Streptomyces*.

Different types of PKSs have been associated with the biosynthesis of polyketide antibiotics in *Streptomyces*. Simple polyketide antibiotics are produced by type II PKSs, which consist of monofunctional proteins. The aromatic polyketide antibiotic, actinorhodin, is synthesised by individual enzymes (Férnandez-Moreno *et. al.*, 1992) in *S. coelicolor* A3(2) which are similar to *E. coli* (Type II) FASs (fatty acid synthases). In contrast, complex polyketide antibiotics are produced by several large multifunctional proteins, which like vertebrate (Type I) FASs, have different domains for each catalytic function. In *Sac. erythraea*, three giant proteins make up the 6-deoxyerythronolide B synthase; DEBS 1, 2, and 3 (Caffrey *et. al.,* 1992) which are the deduced products of *eryA*I, *eryA*II, and *eryA*III (Cortes *et. al.*, 1990; Bevitt *et. al.*, 1992; Donadio *et. al.*, 1991). Each of these proteins displays many potential active sites and is responsible for two extension cycles of the polyketide chain (Leadlay, 1993). Similarly, ORFB from *Streptomyces antibioticus,* which produces oleandomycin (Swan *et al.*, 1994), is also predicted to encode a

multifunctional polyketide synthase. The starter unit for simple polyketides is normally derived from acetate, which is often the obligatory extender unit; actinorhodin is composed of eight acetates. The choice of extender units in the production of complex polyketides can vary and are in a precise order, requiring 'programming" (Hopwood *et. al.,* 1990; Katz *et. al.*, 1993; and Cane, 1994) of the PKS; 6-deoxyerythronolide B is drived from one propionyl-CoA starter unit and six methylmalonyl residues.

Increased knowledge about polyketide biosynthesis has allowed new forms of the erythromycin lactone to be produced via genetic interference with the PKS genes (Katz *et. al.*, 1993). Different domains have been inactivated, altering the final product; for example the ketoreductase in module 5 of DEBS has been deleted, which prevents the reduction of the keto group on carbon 5 (Donadio *et. al.*, 1991). Modifications can occur to the polyketide once it has been synthesised; a *Sac. erythraea* strain disrupted in *eryF* (encodes the cytochrome P450 for C-6 hydroxylation of 6-deoxyerythronolide B) synthesises 6-deoxyerythromycin A as opposed to erythromycin A (Weber *et. al.,* 1991). Recombinant PKSs may also be constructed via gene replacement. The ACP encoded by the actinorhodin PKS from *S. coelicolor* has been replaced by a *Sac. erythraea* FAS ACP, forming an active PKS (Khosla *et. al.*, 1992).

Macrolide antibiotics.

Macrolides antibiotics are based on a macrocyclic lactone, and the first macrolide antibiotic, pikromycin, was isolated by Brockmann and Henkel in 1950; there are now more than ninety macrolides recognised (Omura, 1984), which are mainly produced by *Streptomyces*. These antibiotics are particularly potent to Gram positive bacteria, Gram negative cocci such as *Haemophilus influenzae*, and mycoplasmas. Macrolides are classified according to the number of carbon atoms in the lactone ring; tylosin is 16-membered (Figure 1, page 25), erythromycin A 14-, and Methymycin 12-. Macrolides often have other functional groups attached, such as deoxyhexose sugars.

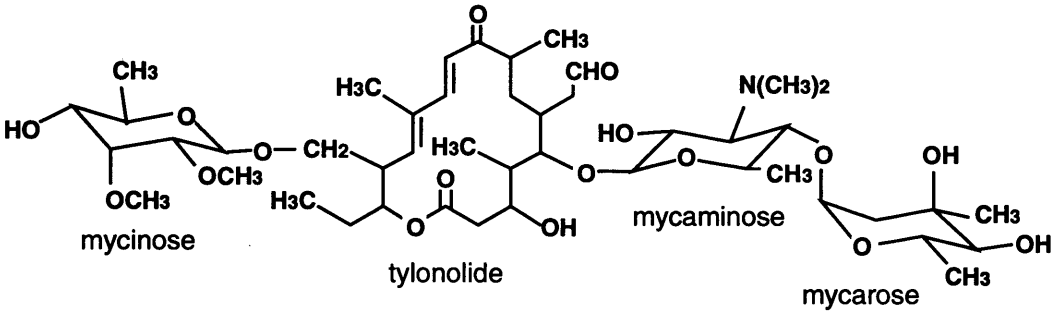
Macrolides interact with the 50S ribosome, probably binding to 23S rRNA. Erythromycin, carbomycin, and spiramycin are thought to provoke dissociation of peptidyl-tRNA from the ribosome (Menninger *et. al.,* 1982). Resistance to these antibiotics often involves methylation of the target site in 23S rRNA (Cundliffe, 1990); *tlrA* is a ribosomal methylase from *S. fradiae*, which acts at A^{2058} in 23S rRNA (Zalacain *et. al.*, 1989).

Tylosin.

Tylosin is a veterinary antibiotic used to treat Gram positive and mycoplasma infections, aswell as being used as a growth promotant. *S. fradiae* is the most well known producer of this macrolide antibiotic, although it also produced by *Streptomyces rimosus* and *Streptomyces*

hygroscopicus. Tylosin consists of a polyketide lactone, tylactone, and three deoxyhexose sugars, mycaminose, mycinose, and mycarose which are added sequentially to the lactone ring (Figure 1, page 25).

Figure 1 The structure of tylosin



Tylactone is a 16-membered polyketide; its synthesis is inhibited by cerulenin which also interferes with fatty acid biosynthesis (Omura *et. al.*, 1978). Omura et. al. (1977) demonstrated that tylactone is constructed from two acetates, five propionates, and one butyrate unit by labelling precursors with carbon-13. Studies of the *S. fradiae tylA* mutant (GS14) demonstrated that tylactone precursors are derived from amino acids (Doltzlaf *et. al.* 1984). Branched chain fatty acids have also been identified as possible precursors of tylactone by examining the fermentation products of *S. fradiae* mutants (Huber *et. al.*, 1990). Mycarose and mycinose are both neutral sugars attached to the C-4 hydroxyl position of mycaminose and the C-23 hydoxyl group of tylactone, respectively. Mycaminose is an amino sugar which is attached to tylactone at the C-5 hydroxyl position.

Elucidating *S. fradiae* genetics and tylosin biosynthesis.

***S. fradiae* genetics.**

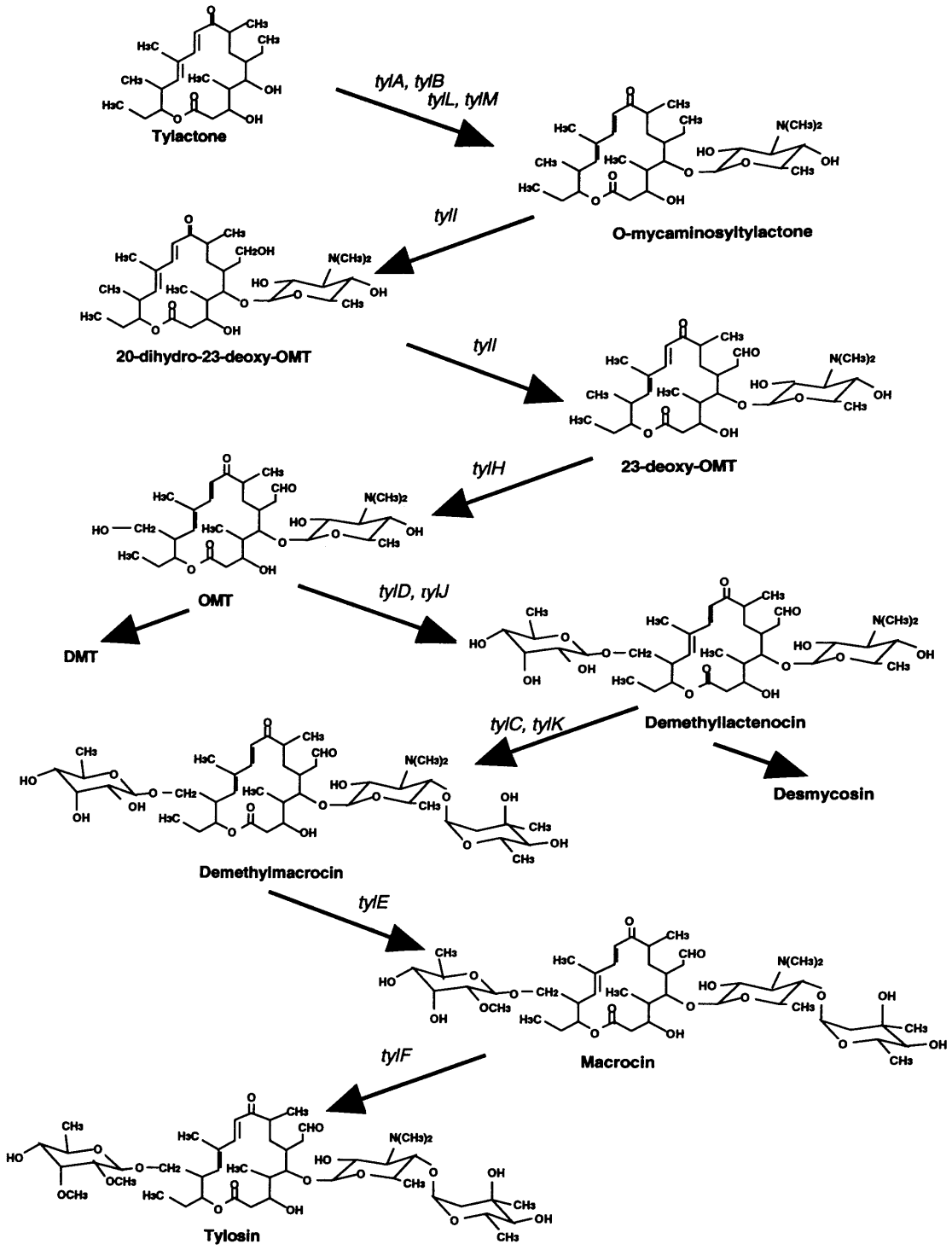
Manipulation of *Streptomyces* DNA has allowed strain improvement and a greater understanding of their biology. However, genetic manipulation of *Streptomyces* is not as straightforward as that of laboratory strains of *E. coli*. Not only have they received less attention, but they have awkward cell walls, and therefore protoplasts have to be generated for transformation (Okanishi, 1974). Hopwood et. al. (1977) reported the development of a protocol for protoplast fusion in *Streptomyces*. The genetics of *S. fradiae* was first investigated in the 1970's (Baltz, 1988) and protoplast fusion was used to introduce DNA into strains of *S. fradiae* (Baltz, 1978 and 1980). These experiments (Baltz, 1980) allowed an attempt at formulating a genetic map of the *S. fradiae* chromosome, although the tylosin biosynthetic genes appeared genetically unstable, suggesting that they could be on a plasmid. Experiments were designed to confirm or refute the possiblity that the tylosin genes were located on an extrachromosomal element. Two *S. fradiae*

hosts defective in tylosin biosynthesis, JS82 and JS87, were isolated by protoplast regeneration, which cured the strains of plasmid (Baltz *et. al.*, 1985). These strains were used as recipients in conjugation experiments, in order to demonstrate the transfer of tylosin genes from a wild-type strain on a mobilisable plasmid. However, the loss of the ability to synthesise antibiotic was eventually associated with deletion and amplification of sequences in close proximity to the tylosin genes. JS82 contains an amplification (500 times) of a 10.5 kb sequence, for which JS87 is deleted (Fishman *et. al.,* 1983; Baltz *et. al.*, 1985), and the wild-type contains a single copy which is flanked by two additional copies of a 2.2 kb direct repeat (Fishman *et. al.*, 1985). A derivative of JS82, JS85, was shown to be a good recipient of tylosin genes by conjugation, and results suggested that the tylosin biosynthetic and resistance genes, and the amplifiable unit of DNA (AUD) were linked on a self-transmissable element (Stonesifer *et. al.*, 1986). JS85 was eventually mutated using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), and restriction deficient derivatives were subsequently selected for increased ability to be transformed with plasmid DNA (Matsushima *et. al.*, 1987).

Potent restriction systems have made the introduction of DNA into other strains of *S. fradiae* by transformation difficult (Cox *et. al,* 1984). A highly transformable strain of *S. fradiae,* M1, was created using the mutagen MNNG from *S. fradiae* c373 (Baltz, 1978), and has been used to optimise transformation procedures for *S. fradiae* protoplasts (Matsushima *et. al.*, 1985). However, the limitations of transformation have been overcome by the use of conjugal transfer to introduce plasmid into *Streptomyces* (Bierman *et. al.*, 1992).

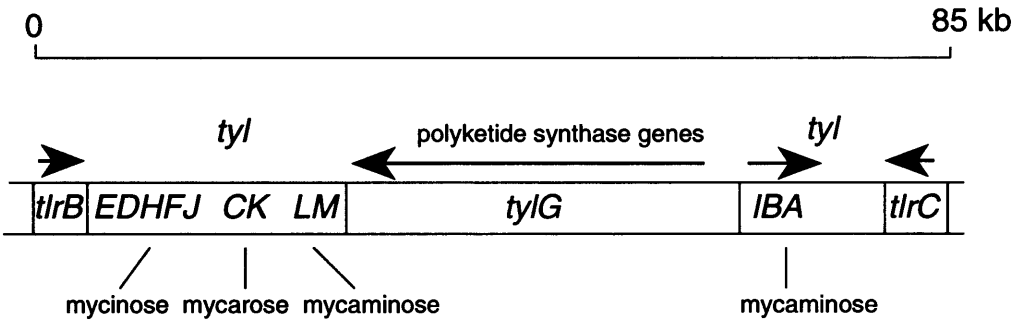
S. fradiae mutants were created via (MNNG) mutagenesis (Baltz *et. al.*, 1981), allowing the elucidation of the catalytic steps involved in tylosin biosynthesis (Figure 3, page 27). Pairs of blocked mutants have been cofermented and screened for tylosin production (Baltz *et. al.*, 1981), and mutants complemented with fragments of DNA from the wild-type (Fishman *et. al.*, 1987). In total, nine groups of mutants were generated (Baltz *et. al.,* 1981), 80% being *tylG* mutants which could not synthesise tylactone. Mutants blocked in the production or addition of the tylosin sugars were split into four groups; the *tylA* mutant which could not synthesise or add any of the three sugars; the *tylB* mutant which could not synthesise or add mycaminose, although it did produce 5-*O*-mycarosyl-tylactone as a shunt product; the *tylC* mutant which could not synthesise or add mycarose; and the *tylD* mutant which was blocked in 6-deoxy-D-allose synthesis or addition. *tylH* and *tylI* mutants were blocked in specific oxidations of tylactone, and *tylE* and *tylF* strains were blocked in specific *O*-methylations of demethylmacrocin and macrocin, respectively. Four additional mutant loci (*tylJ*, *tylK, tylL*, and *tylM*) were identified by Fishman et. al. (1987) by complementing mutants with *tyl* DNA.

Figure 3 Pathway to tylosin, based on Baltz et. al. (1983)



The tylosin genes were initially located using a DNA probe generated from the amino-terminal sequence of the purified Macrocin-*O*-methyltransferase, which catalyses the last step in tylosin biosynthesis (Seno *et. al.*, 1977). This probe was used to screen bacteriophage and cosmid libraries containing *S. fradiae* DNA (Fishman *et. al.*, 1987). *tyl* DNA from these cosmids was introduced into shuttle plasmids which were transformed into *S. fradiae* M-1 (Matsushima, 1985), a non restricting or modifying host, before being introduced into mutants blocked in tylosin production. These experiments resulted in the identification of tylosin genes inbetween the resistance gene *tlrB* and an amplifiable unit of DNA (AUD) (within *tylG*), representing the left hand side of the cluster (Figure 2, page 28). These genes are largely responsible for the later

Figure 2 The tylosin biosynthetic gene cluster from *S. fradiae*



steps of tylosin biosynthesis (Figure 3, page 27). The results suggested that genes with related functions were clustered. *tylE*, *tylD*, *tylH*, *tylF*, and *tylJ* loci are all involved in the biosynthesis, attachment or methylation of 6-deoxy-D-allose, and are situated together on a 6 kb *Bam*H I fragment; the *tylC* and *tylK* mutations which are specific to mycarose production are also closely linked (Cox *et. al.*, 1986). However, the genes for mycaminose production are separated. The *tylLM* genes are located between *tylG* and *tlrB* (Fishman *et. al.*, 1987; Cox *et. al.*, 1986). In contrast the *tylI*, *tylB*, and *tylA* loci were identified with the rest of the cluster, by probing cosmids containing *tyl* DNA, adjacent to the preliminary cluster, with *tlrC* and AUD probes (Beckman *et. al.*, 1989). The *tylI*, *tylB*, and *tylA* mutations were eventually complemented with cosmid DNA (Beckman *et. al.*, 1989) which extends out towards *tlrC* (Figure 2, page 28). The tylosin genes are flanked by two resistance genes, *tlrB* and *tlrC*, the whole region spanning 85 kb. These individual loci may each represent a number of open reading frames.

The pathway to tylosin, Figure 3, page 27.

Cofermentation of mutants blocked in tylosin biosynthesis (Baltz *et. al.*, 1981), and feeding experiments with the mutant GS22 (*tylG*) (Baltz *et. al.*, 1983), has allowed the elucidation of a preferred order of reactions in tylosin biosynthesis (Figure 3, page 27). The *tylA, tylL, tylB and tylM* loci are all involved in the biosynthesis and addition of mycaminose, which is the first sugar

to be added to tylactone at C-5 hydroxyl group (see page 31). Tylactone is then oxidised at C-20 (*tylI*), followed by hydroxylation at C-23 (*tylH*). Before the addition of mycarose, 6-deoxy-D-allose (precursor of mycinose) is added to 5-*O*-mycaminosyl-tylactone (*tylD*, *tylJ*); the order of addition of the two neutral sugars was determined by comparing the efficiency of conversion of demycinosyl-tylosin (DMT), which lacks mycinose, and demethyllactenocin which lacks mycarose, to tylosin by GS22. Conversion of demethyllactenocin to tylosin was more efficient, DMT being classified as a shunt metabolite (Baltz, 1983). The *tylC* and *tylK* loci are required for the synthesis and transfer of mycarose to mycaminose. Demethylmacrocin is converted to macrocin by the demethylmacrocin *O*-methyltransferase which is encoded by *tylE*. The final step in tylosin biosynthesis involves the conversion of macrocin to tylosin by the macrocin *O*-methyltransferase (*tylF*). During tylosin fermentations other compounds also accumulate; macrocin which precedes tylosin, and relomycin which is produced via the reduction of tylosin (Seno *et. al.*, 1977).

Regulation of tylosin biosynthesis.

The biochemical and genetic details of how tylosin production in *S. fradiae* is regulated has not been elucidated. However, the biosynthetic genes are clustered with resistance genes, and divergent and convergent transcription units have been identified suggesting opportunities for co-regulation. For example, *tylG* and the *tylIBA* regions appear to be transcribed in opposite directions (Figure 2, page 28). Questions need to be answered concerning the organisation of genes into operons, and the regulation of gene expression at the level of transcription and translation. The *tylG* mutants GS40 and GS41 could not coferment with other tylosin mutants, or sporulate, suggesting that they might contain mutations in regulatory genes (Baltz *et. al.*, 1981).

Tylosin resistance genes in *S. fradiae*; *tlrA, tlrB, tlrC and tlrD.*

Four determinants have been associated with resistance to tylosin in *S. fradiae*. Three of these resistance genes have been associated with the tylosin gene cluster; *tlrD* has been located within the *tylLM* region (Gandecha *et. al.*, 1996) and *tlrB* and *tlrC* flank the biosynthetic cluster (Baltz, 1988; Beckman *et. al.*, 1989). When *tlrA* was introduced on a 28 kb DNA fragment into mutants blocked in tylosin biosynthesis antibiotic production was not restored, suggesting that this gene is not so closely associated with tylosin genes (Birmingham *et. al.*, 1986).

Both *tlrD and tlrA* exhibit the MLS-resistance phenotype (resistance to macrolides, lincosamides and streptogramin B-type antibiotics). These drugs have a similar site of action on the 50S subunit of the bacterial ribosome, probably through interactions with 23S rRNA (Cundliffe, 1990). Both genes encode ribosomal methylases, but have different resistance profiles in strains of *S. lividans,* and modes of expression. *tlrA* confered high levels of resistance to tylosin (Birmingham *et. al.*, 1986) in constrast to *tlrD* which confered high levels of resistance to

lincomycin, but only moderate levels of resistance to macrolides and streptogramin B antibiotics (Zalacain *et. al.*, 1991). Both *tlrA* and *tlrD* encode ribosomal methylases which act on the N^6 amino group of A^{2058} within 23S rRNA (the *E. coli* numbering system is used for easy reference). *tlrD* is responsible for N^6 monomethylation (Zalacain *et. al.*, 1991) and TlrA is a N^6, N^6-dimethylase, reflecting its higher levels of resistance to tylosin and all MLS antibiotics compared to *tlrD* (Zalacain *et. al.*, 1989). The differences in MLS-resistance profile between *tlrA* and *tlrD* reflect their recent classification as examples of MLS-II and MLS-I genes, respectively (Pernodet *et. al.*, 1996). The expression of *tlrD* is constitutive (Zalacain *et. al.*, 1991) in contrast to *tlrA* whose expression is inducible via a transcriptional attenuation mechanism (Keleman *et. al.*, 1994). A full length *tlrA* transcript, and therefore gene expression, is induced according to the methylation state of the ribosome, via a conformational change in *tlrA* mRNA. Methylation, via the action of TlrD, has been shown to alter the response of ribosomes to inducers of *tlrA*, causing tylosin and the latter intermediates to induce *tlrA* expression (Keleman *et. al.*, 1994).

tlrC and *tlrB* flank the tylosin gene cluster (Figure 2, page 28), and their resistance phenotypes are more specific to tylosin than the aforementioned determinants (Baltz, 1988; Beckman *et. al*, 1989). TlrC is thought to be a membrane associated, ATP-binding protein, probably acting as an efflux mechanism (Rosteck *et. al.*, 1991). This protein was thought to act in concert with the product of *tlrB* (Cundliffe *et. al.*, 1993), however, TlrB is now known to be a separate resistance determinant with its own particular function (Dr Vanessa Wilson, personal communication).

Deoxyhexose sugar biosynthesis.

Deoxysugars have diversity of biological roles in plants, fungi and bacteria (Liu *et. al.*, 1994). The incorporation of deoxyhexose sugars into the structure of many antibiotics, for example in the structure of tylosin (Fish *et. al.*, 1996), contributes to their potency, and therefore information about how they are synthesised could lead to other avenues for the creation of new antibacterials (Liu *et. al.*, 1994). However, whilst polyketide biosynthesis has been researched extensively, there is a scarcity of information about the production of deoxyhexose sugars, which are often incorporated into macrolide structures. Manipulation of the glycosyltransferases which transfer sugars onto polyketides could allow different sugars to incorporate into pre-existing antibiotics (Piepersberg, 1994).

Deoxyhexose sugars incorporated into the structure of many antibiotics, are often modified by the addition of functional groups. Amino sugars, where one or more hydroxyl group(s) are replaced with amino group(s) (Liu *et. al.*, 1994), are common in antibiotics; aminoglycosides contain amino sugars; the amino sugars mycaminose and desosamine (Figure 14, page 66, Chapter 3) are incorporated into the structures of the macrolide antibiotics tylosin and

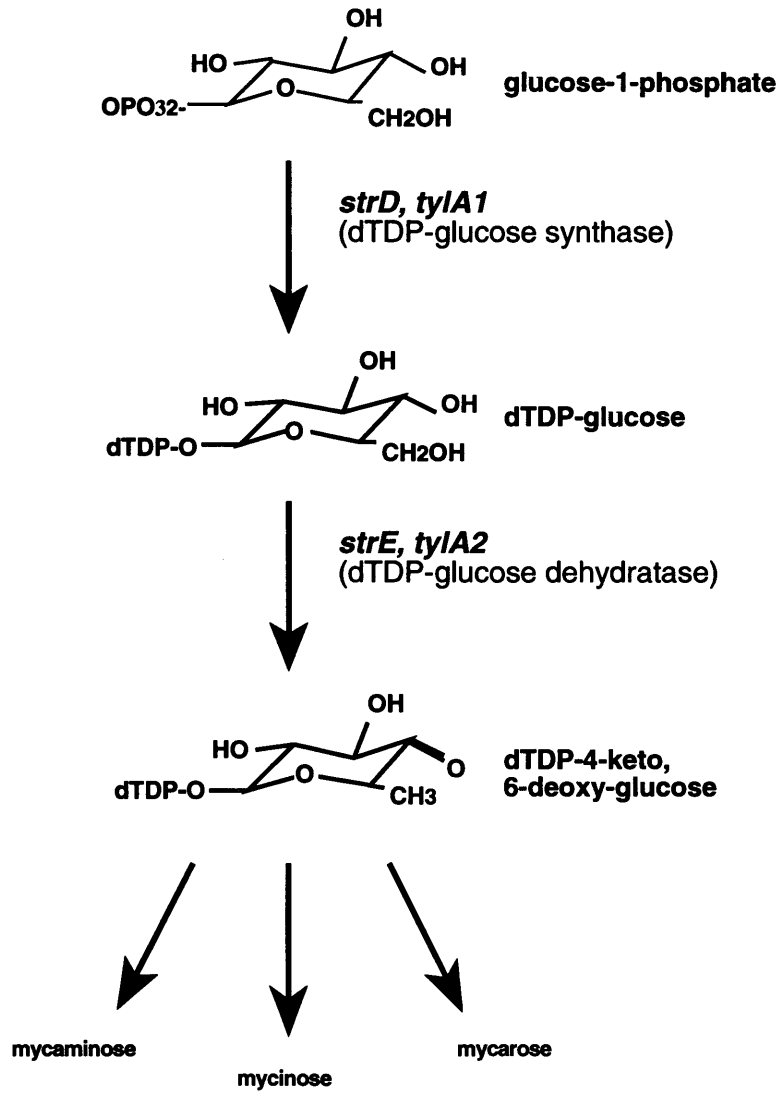
erythromycin, respectively; and the amino sugar daunosamine (Figure 14, page 66, Chapter 3) is present in the anthracycline antibiotic daunorubicin.

Mycaminose is the first sugar to be added to tylactone during the synthesis of tylosin; the *tylB* mutant, which is defective in the biosynthesis or addition of mycaminose, can't add the other sugars to tylactone, although it coferments with *tylC* (defective in the biosynthesis or addition of mycarose) and *tylD* (defective in the biosynthesis or addition of 6-deoxy-D-allose) mutants (Baltz *et. al.*, 1981). This sugar is of particular interest because if confers antimicrobial activity on tylactone (Kirst *et. al.*, 1982), which is inactive. Understanding mycaminose biosynthesis requires investigation of at least the *tylLM* and *tylIBA* loci, which flank the polyketide synthase genes (Figure 2, page 28).

The *tylA* and *tylL* phenotypes, an inability to synthesise or add the three tylosin sugars to tylactone, suggests that there is a common intermediate from which mycaminose, mycinose and mycarose are produced (Baltz *et. al.*, 1981).

Deoxyhexose sugars are thought to be derived from dTDP-glucose via the common intermediate dTDP-4-keto, 6-deoxyglucose (Grisebach, 1978; Liu *et. al.*, 1994). The generation of dTDP-4-keto, 6-deoxyglucose from glucose-1-phosphate requires two steps; a dTDP-glucose synthase; and a dTDP-glucose dehydratase (Figure 4, page 32). In order to find these genes in *S. fradiae*, *strD* (dTDP-glucose synthase) and *strE* (dTDP-glucose dehydratase), from the streptomycin producer *S. griseus* (Pissowotzki *et. al.*, 1991), were used to probe *tyl* DNA in cosmids which had complemented the *tylA* mutation (Merson-Davies *et. al.*, 1994). These genes are involved in the synthesis of dTDP-dihydrostreptose, in the production of streptomycin. Probing experiments revealed a target within the *tylA* locus, and 7 kb of *S. fradiae* DNA was sequenced revealing five ORFs (Merson-Davies *et. al.*, 1994). Two of these genes were homologues of *strD* and *strE* (Figure 4, page 32); *tylA1* (ORF 3, a dTDP-glucose synthase); and *tylA2* (ORF 4, a dTDP-glucose dehydratase). The other three ORFs were identified as *tylI* (ORF1), *tylB* (ORF2), and ORF5. *tylI* encodes a cytochrome P-450 which would oxidise tylactone at C-20 (Merson-Davies *et. al.*, 1994), and *tylB* is a putative aminotransferase (Thorson *et. al.*, 1993), and as suggested by the *tylB* mutant phenotype, is specific to mycaminose biosynthesis. *tylB* shows a high level of sequence similarity with *eryCI* isolated from *Sac. erythraea* (Dhillon *et. al.*, 1989), and *dnrJ* isolated from *S. peuceticus* (Stutzman-Engwall *et. al.*, 1992) which both incoprorate amino sugars into their respective antibiotics, erythromycin and daunorubicin. ORF5 may encode a thioesterase which releases and cyclises the linear polyketide, tylactone, and or removes abberant products produced during polyketide synthesis (P. F. Leadlay, personal communication).

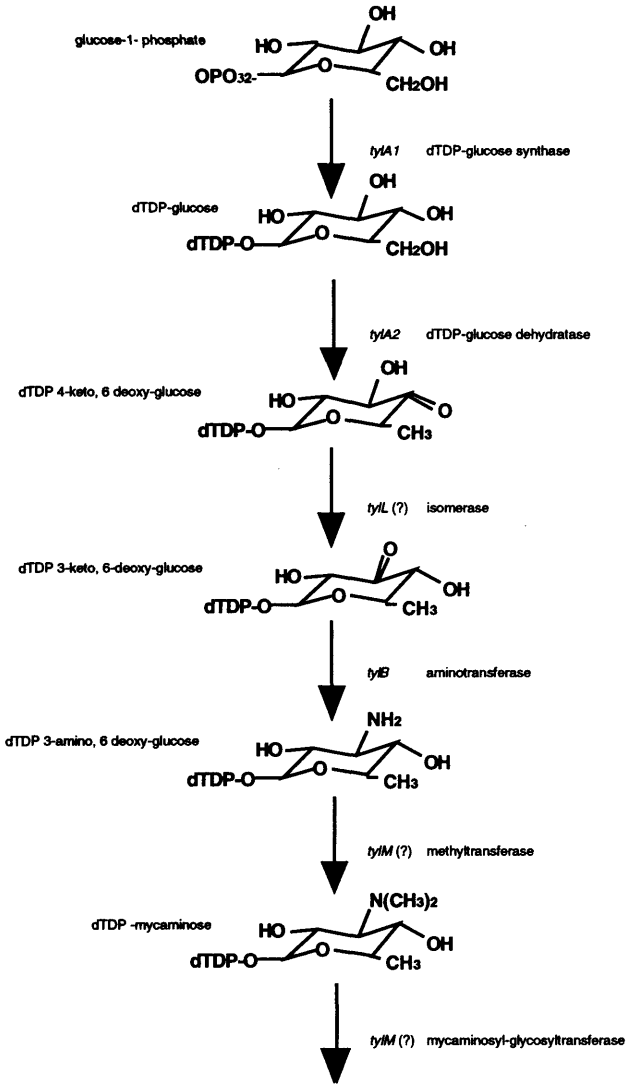
Figure 4 The conversion of glucose-1-phosphate to dTDP-4-keto, 6-deoxyglucose.



Still, as regards mycaminose biosynthesis, there remains to be identified the putative isomerase that converts TDP-4-keto, 6-deoxyglucose to TDP-3-keto, deoxyglucose; the methyltransferase which methylates TDP-3-amino, 6-deoxyglucose; and the enzyme that adds mycaminose to tylactone (Figure 5, page 33). The *tylM* and *tylL* mutants are both unable to add mycaminose to tylactone, and these loci, on the other side of the polyketide synthase genes to the *tylIBA* region, may contain the relevant genes. The *tylLM* region is not so well understood as the *tylIBA* region. The TylM phenotype is specific to mycaminose biosynthesis, in contrast to *tylL* mutants that cannot synthesise any of the three sugars. Could *tylL* be the isomerase which allows the conversion of dTDP 4-keto, 6-deoxyglucose to dTDP 3-keto, 6-deoxyglucose, an alternative candidate for the last common intermediate in the production of tylosin sugars (Figure 5, page 33)? This sequence of events is unlikely, however, as mycarose is presumed to be derived from dTDP 4-keto, 6-deoxyglucose (Grisebach, 1978). Also, dTDP-glucose dehydratases which produce dTDP 4-keto, 6-deoxyglucose, have been purified from *Sac. erythraea* (Vara *et. al.*,

1988) and *Streptomyces peucetius* (Thompson *et. al.*, 1992). The intermediate dTDP 3-keto, 6-deoxyglucose is not thought to form part of the hypothetical pathway to daunosamine, the only sugar incorporated into daunorubicin (Otten *et. al.*, 1995), or a common intermediate in the biosynthesis of desosamine and mycarose in the biosynthesis of erythromycin A (Vara *et. al.*, 1989). How can the *tylL* phenotype, therefore, be explained if all of the early steps in tylosin sugar biosynthesis have been accounted for? *tylL* may be a regulatory mutation which affects the expression of genes which are involved in early stages of sugar biosynthesis. For example, it could be a promoter mutation which has polar effects on downstream genes which are co-transcribed and or co-translated, or alternatively, a mutation in a regulatory gene which might, for example, affect expression of the *tylIBA* region.

Figure 5 The hypothetical pathway to mycaminose.



The *tylLM* region has been delimited to a *Kpn* I-*Bam*H I fragment which has been shown to complement *tylL* and *tylM* mutants (Fishman *et. al.*, 1987). A sequencing project has therefore been undertaken to identify open reading frames in the *tylLM* region (Gandecha *et. al.*, 1997). This thesis includes details of the sequencing and analysis of one of these open reading frames (*orf1**), and a summary of the rest of the *tylLM* region (Chapter 3, page 55). Complementation of the *tylL* and *tylM* mutants with single ORFs has also revealed information about the functions of genes within the *tylLM* region (Chapter 4, page 71), and further elucidation of the *tylL* mutant has occurred by sequencing *tylL* DNA, and analysing and comparing the fermentation products of different *tylL* strains (Chapter 5, page 92).

Also, we have been interested in patterns of gene expression within the *tylLM* region, both temporal and spatial. Experiments were therefore carried out in an attempt to locate key promoters within this region (Chapter 6, page 112). Antibiotic biosynthesis is switched on late in batch cultures, and therefore there is a high probability that these *tyl* loci will contain inducible promoters. From sequence data generated from the *tylLM* region, four open reading frames were identified which could be sugar genes; *orf1**, *orf2**, *orf3** and *orf4** (Figure 7, page 55, Chapter 3) which appear to be transcribed in the same direction (Gandecha *et. al.*, 1997). Are these genes co-transcribed from a promoter either upstream of *orf1** or *tylG*, or are they independently transcribed?

The aims of this thesis are therefore to
(1) Contribute to the completion of double-stranded sequencing and sequence analysis of the *tylLM* region.
(2) Complement *tylL* and *tylM* mutants with individual genes form the *tylLM* region, and therefore reveal information about *tyl* gene functions.
(3) Attempt to locate key promoters and transcripts within the *tylLM* region.

Chapter 2 Materials and Methods.

(A) Bacterial strains and growth conditions.

Antibiotics.

Apramycin, tylosin, and the tylosin intermediates 5-*O*-mycaminosyl-tylonolide (OMT), demycinosyl-tylosin (DMT), and desmycosin were supplied by Eli Lilly research laboratories, Indianapolis, Ind.. Ampicillin, kanamycin, neomycin, and nalidixic acid were obtained from Sigma. Thiostrepton was supplied by the Squibb Institute, Princeton, N.J..

Bacterial growth conditions.

Strains of *Escherichia coli* and *Micrococcus luteus* T194 were grown in Luria-Bertani (LB) medium [1% NaCl; 0.5% yeast extract; 1% tryptone (Sambrook *et. al.*, 1989)] overnight, and were shaken, (300 rpm), at 37°C. Bacteria containing constructs based on pUC18 (Norrander *et. al.*, 1983) and pGEM-T (Promega) were selected for using ampicillin (100 µg/ µl). An apramycin resistant strain of *M. luteus* and strains containing plasmids based on pSET152 and pKC1218 (Bierman *et. al.*, 1992), were grown in the presence of apramycin (25 µg/ ml). S17-1, the *E. coli* donor strain used in conjugation experiments, was supplied by Dr P. Mazodier (Pasteur Institute, Paris).

Streptomyces fradiae was grown in tryptic soy broth (3% TSB; Difco) at 30°C, and was shaken to allow good aeration, (160 rpm). Baffled flasks or flasks with stainless steel springs were used in order to promote dispersed growth and fragmentation of mycelium. Tylosin production medium MM-1 [20 ml of 70% methyloleate (Aldrich); 2.5 g betaine-hydrochloride; 1.15 g dipotassium hydrogen phosphate; 1g NaCl; 1.5 g $CaCl_2$; 2.5 g $MgSO_4$; 0.5 mg CoCl; 5 mg $ZnSO_4$; 1.5 g Ferric amonium citrate; 8.75 g monosodium glutamate; 2.5 g glucose; per litre, adjusted to pH 7 with 1 M KOH] was used to promote tylosin production when culturing *Streptomyces fradiae* strains (Gray *et. al.*, 1980). Culture conditions for batch fermentations of *S. fradiae* were based on Baltz et. al. (1981). A frozen stock of *S. fradiae* (100 µl) was inoculated into 30 ml of modified complex vegetative medium [1% w/ v corn steep liquor (Sigma); 0.5% yeast extract; 0.5% soya bean meal; 0.3% calcium carbonate; 0.45% methyloleate; adjusted to pH 7.8 with 1 M NaOH] in a 100 ml flask lacking baffles or springs. After 2-3 days growth at 28°C, this culture was used as a 10% inoculum in 50 ml of tylosin production medium MM-1, in 250 ml flasks, and incubated for 7 days. These cultures were shaken at 300 rpm. Strains carrying plasmids based on pSET152 and pKC1218 (Bierman *et. al.*, 1992) were grown in apramycin (25 µg/ ml). Solidified medium, AS-1 [0.1% yeast extract; 0.02% L-alanine; 0.02% L-arginine; 0.05% L-asparagine; 0.5% soluble starch; 0.25% sodium chloride; 1% Na_2SO_4; 2% agar; pH 7.5 adjusted with KOH (Baltz, 1980)] was used as a solid

medium for strains of *S. fradiae,* which were incubated at 37°C, allowing plenty of sporulation. However, the addition of 10 mM $MgCl_2$ to AS-1 after autoclaving, was omitted as it appeared to make no difference to the growth *S. fradiae* or the efficiency of conjugation experiments. Tylosin production medium MM-1 was solidified with 2% agar for growing exconjugants for plug assays (Chapter 4, page 74).

Strains of *Streptomyces lividans* OS456 were grown in liquid culture in YEME/ sucrose [0.5% peptone; 0.3% yeast extract; 0.3% malt extract; 1% glucose; 34% sucrose; 0.1% $MgCl_2$; 0.5% glycine (Hopwood *et. al.,* 1985) and on the solid medium NEF [0.5% glucose; 0.1% yeast extract; 0.05% beef extract (Lab Lemco, Oxoid); 0.1% casamino acids (Difco); 2% agar; pH 7.0, adjusted with KOH (Dr Steven Fish, personal communication)].

Strains of bacteria are described in Figure 6, page 36, and plasmids containing *S. fradiae tylLM* DNA derived from the cosmid pMOMT4 (Beckman *et. al.*, 1989), are represented in Chapter 4, Figure 20, page 77.

Figure 6 Strains of bacteria.

Organism	Genetics		Reference
Escherichia coli			
NM522	*supE thi Δ(lac - proAB) hsd5 F' [proAB$^+$ lacI^q lacZΔM15]*		Gough et. al. (1983).
S17-1	*pro res$^-$ mod$^+$* RP4		Simon et. al. (1983).
M. luteus T149			Department of Microbiology, University of Leicester.
M. luteus T149	Apramycin resistant strain		Dr. A. R. Butler.
NM522	DNA fragments from the *tylLM* region in pUC18.		
	*Bam*H I-*Bam*H I	pLST97	Dr. Louise Merson-Davies.
	Kpn I-*Bam*H I	pLST97A	
	Kpn I-*Sst* I	pLST97B	
	Kpn I-*Sal* I	pLST973	Dr. Louise Merson-Davies.
	Kpn I-*Sph* I	pLST97C	

Nco I-*Xho* I	pLST973A (orientations 1 and 2)
Xho I-*Xho* I	pLST973B
*Eco*47 III-*Xho* I	pLST973C

Nested deletions derived from pLST973

DNA fragments from the *tylLM* region in pSET152.

Kpn I-*Bam*H I	pLST97A1
Kpn I-*Sst* I	pLST97B1
Kpn I-*Sal* I	pLST9731
Nco I-*Xho* I	pLST973A1

DNA fragments from the *tylLM* region in pKC1218.

Kpn I-*Bam*H I	pLST97A2
Kpn I-*Sst* I	pLST97B2
Kpn I-*Sph* I	pLST97C2
Kpn I-*Sal* I	pLST9732
Xho I-*Xho* I	pLST973B2
Eco47 IIII- *Xho* I	pLST973C2

PCR products amplified from the *tylLM* region of c373.1, in pLST9828.

*orf2**
*orf3**

PCR products amplified from the *tylLM* region in pLST9829.

*orf2**

DNA fragments from the *tylLM* region in pGEM-T.

*orf1** from the *tylL* mutant
*orf1** from the *tylM* mutant
*orf2** from the *tylL* mutant
*orf2** from the *tylM* mutant
*orf3** from c373.1

Streptomyces

c373.1	wild-type *S. fradiae*.

c373.1	pSET152	
tylL mutant (GS33)		Dr E. T. Seno
tylL mutant	pSET152	
	pKC1218	
	pLST9828	
	pLST9829	
	pLST97A1	
	pLST97C2	
	pLST9731	
	pLST9732	
	pLST973A1	
	pLST973B2	
	pLST973C2	
	pLST9828+*orf2**from c373.1	
	pLST9829+*orf2**from c373.1	
tylM mutant (GS62)		Dr E. T. Seno
tylM mutant	pSET152	
	pKC1218	
	pLST9828	
	pLST9829	
	pLST97B1	
	pLST97B2	
	pLST97C2	
	pLST9828 *orf3** from c373.1	
tylD + *tylA/L* (GS78)		Dr E. T. Seno
tylD + *tylA/L* (GS79)		Dr E. T. Seno
S. lividans 0S456		Pernodet et. al. (1996)
S. lividans TK24	pIJ486	Ward et. al. (1986), supplied by Mervin Bibb, John Innes Institute, Norwich, UK.

Streptomyces fradiae strains c373.1 (T59235), GS33, GS62, GS78, and GS79 were provided by Dr E. T. Seno (Eli Lilly and Co.). These mutants are defective in tylosin biosynthesis and were

generated (Baltz *et. al.*, 1981) using the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). The *tylM* (GS62) and *tylL* (GS33) mutants were distinguished from the *tylB* and *tylA* mutants, respectively, by complementation studies (Fishman *et. al.*, 1987).

S. lividans 0S456 is a MLS-sensitive (macrolides, lincosamides and streptogramin B) derivative of *Streptomyces lividans* TK21. It was generated by disrupting the resistance genes *lrm-mgt* with the hygromycin resistance cassette (Pernodet *et. al.*, 1996)

Storage of bacteria.
Streptomyces spores were stored at -20°C in 20% (v/v) glycerol, and mycelial fragments were stored at -70°C in 5% DMSO. Strains of *E. coli* were stored at -20°C in 50% (v/v) glycerol.

Autoclaved Millipore SuperQ water (SQ) was used exclusively for preparing solutions.

(B) Manipulation of DNA.

1. Gel electrophoresis of DNA fragments.

Fragments of DNA were separated on polyacrylamide or agarose gels. DNA was visualised by staining gels with ethidium bromide (EtBr) which fluoresces in the UV, or by labelling the DNA with radioactivity and positioning fragments by exposure to X-ray film (Fuji RX). Agarose gels were composed routinely of 0.7% agarose (Seakem HGT) melted in 1x Tris-acetate electrophoresis buffer [1x TAE (0.04 M Tris-acetate; 0.001 M Na_2EDTA)], to which 0.01% EtBr was added. However, the percentage of agarose in the gel was varied according to the sizes of DNA fragments to be resolved. For example, a 0.7% agarose gel allowed resolution of fragments up to about 6 kb, whereas a 0.5% agarose gel allowed better separation of fragments of 6 to 20 kb. Each gel was poured into a taped gel plate, with a comb to form wells, and left to set at room temperature (20°C) for 1 h. Gels were run at 100 volts for 1-3 h, or at 15 volts overnight, in 1x TAE.

Size markers were used during gel electrophoresis to allow estimation of the size of DNA fragments. A 1 kb ladder (Gibco BRL) was used to size DNA fragments up to 12 kb. This ladder could also be end labelled with ^{32}P-radioactivity and visualised by autoradiography (Chapter 6, page 112). When attempting to visualise DNA fragments larger than 10 kb, lambda DNA (Gibco BRL) was cut with *Eco*R I and *Hind* III and could size pieces of DNA of up to 21 kb.

Preparation of DNA ladders.
The 1 kb ladder (20 µg) was mixed with 180 µl SQ and 200 µl blue gel dye [200 µl 50x TAE; 5 ml glycerol; 4 ml SQ; 0.01% Xylene Cyanol FF (Sigma); and 0.01% Bromophenol blue

(Sigma)], and 10 µl was loaded on a gel. The lambda ladder (33.75 µg) was digested with *Eco*R I and *Hin*d III, in a total volume of 250 µl, at 37°C for 3 h, before the digest was heated to 70°C for 15 min and 100 µl of loading dye was added. A loading of 10 µl of the lambda ladder could also be visualised on a gel stained with EtBr.

2. Estimation of DNA, oligonucleotides or RNA in solution.

The concentration of DNA in a solution was measured by UV absorbance at 260 nm. One OD_{260} unit represents 50 µg of ds DNA, 44.1 µg of RNA, or 33 µg of oligonucleotides. The purity of the DNA solution was estimated from the A_{260}/ A_{280} ratio. The optimum A_{260}/ A_{280} values for DNA and RNA are 1.8 and 2.0, respectively. Oligonucleotides, RNA and plasmid DNA were stored at -20°C, and chromosomal DNA at 4°C.

3. DNA restriction.

Restriction endonucleases (Gibco BRL) were used according to the manufacturers' guidelines.

4. DNA ligation (based on Sambrook *et. al.*, 1989).

DNA ends with 5' or 3' overhangs were ligated together in a molar ratio of insert to vector of 3:1. The concentration of vector and insert was estimated by gel electrophoresis against standards of known concentration. The vector and insert DNA was dissolved in 15 µl of SQ and incubated at 65°C for 2 minutes to separate DNA fragments. The ligation mixture required the addition of 4 µl of 5x T4 DNA ligase buffer and 1 µl of T4 DNA ligase (1 unit/ µl Gibco - BRL), and was incubated overnight at 16°C. Blunt ended ligations were carried as described above, except that three times as much ligase and high concentrations of both insert and vector DNA were used.

5. Dephosphorylation of 5'-ends (based on Sambrook *et. al.* 1989).

To prevent self ligation of a linearised plasmid, calf intestinal alkaline phosphatase (CIAP) was used to remove 5' phosphates. DNA (8 µl), 1 µl of 10x CIAP buffer, and 1 µl of CIAP (1 unit/ µl, Boehringer) were incubated at 37°C for 1 h.

6. Converting a 3'-recess to a blunt end terminus.

The Klenow fragment of *E. coli* DNA polymerase I was used to fill in the 3' recessed termini of DNA fragments using procedure B (Linearization of circular DNA: end-protection by thionucleotides) from the double-stranded nested deletion kit (Pharmacia). DNA (5-10 µg) was digested in a volume of 10 µl with an enzyme that provided a 3' recessed end. The Klenow fragment was diluted in 1x Klenow buffer to 0.05 units/ µl. The end filling reaction was set up using 7 µl of DNA, 1 µl 10x Klenow buffer, 1 µl dNTPαS Mix and 1 µl of diluted Klenow, and was centrifuged briefly before being incubated at 37°C for 15 min. The mixture was heated at

65°C for 20 min, and then precipitated at -70°C with 20 µl of NaCl/ glycogen and 75 µl of ethanol for 10 min. The DNA was then pelleted by centrifugation (13, 000 rpm, 10 min) and the supernatant was removed. The pellet was washed with 70% ethanol, dried and dissolved in 10 µl of SQ.

Isolation of DNA fragments from agarose gels.

7. Jetsorb kit protocol (Genomed).

For each 100 mg of agarose, 300 µl of solution A1 and 10 µl of JETSORB suspension was added into a 1.5 ml eppendorf vial. This mixture was then mixed and incubated at 50°C for 15 min to melt the gel, during which the eppendorf vial was inverted approximately every three minutes. After centrifugation (13, 000 rpm, 30 sec) the supernatant was completely removed and the pellet was resuspended in 300 µl of solution A1. The mixture was again centrifuged (13, 000 rpm, 30 sec) and the pellet was washed twice with 300 µl of solution A2. Once all the supernatant had been removed, the pellet was air dried for 10 min before being resuspended in 20 µl of SQ. The suspension was then incubated at 50°C for 5 min and centrifuged (13, 000 rpm, 30 sec), before the supernatant (DNA) was transferred to a fresh tube.

8. Isolation of DNA fragments from low melting point agarose (based on Langridge *et. al.*, 1980).

DNA fragments were separated on a 1% low melting point agarose gel (LMP agarose, Gibco BRL), and the required DNA fragment cut from the gel. Gel fragments were then incubated in a 1.5 ml eppendorf vial at 70°C for 30 min, and the following manipulations were performed at 37°C to prevent the gel from solidifying. An equal volume of water/CTAB (cetyl trimethyl ammonium bromide, Sigma) and butanol/CTAB were added, and the mixture mixed for 30 sec. After centrifugation (13, 000 rpm, 1 min, at 37°C), the upper butanolic phase was transferred to another tube, and the lower aqueous phase was extracted with half the volume of butanol/CTAB. Both butanolic phases were then pooled and manipulations could then proceed at room temperature (20°C). DNA was extracted by vortexing with 1/4 volume 0.3 M sodium acetate, pH 7.0, and the different phases were separated by centrifugation (13, 000 rpm, 1 min). The lower aqueous phase was then extracted with an equal volume of chloroform, after which DNA in the upper phase was precipitated with 3 volumes of 100% ethanol at -70°C for 30 min. The DNA was pelleted (13, 000 rpm, 10 min), washed in 70% ethanol, and dried in a vacuum dryer. The DNA was then dissolved in 10 µl SQ.

Preparation of Butanol/ CTAB and water/ CTAB.

CTAB (1 g) was dissolved in 100 ml of butantol, to which 100 ml of SQ was added and mixed well. The phases were allowed to separate at room temperature (20°C) for 16-24 h. The upper butanolic phase was removed, and both solutions were kept, tightly capped, at 37 °C.

9. Preparation of total DNA from *Streptomyces*.

A 50 ml culture of *Streptomyces* was harvested in universal bottles by centrifugation (3500 x g; 10 min; at 4°C in a Heraeus megacentrifuge) and washed twice in 10% sucrose. The cells were resuspended in 10 ml of lysozyme solution [2 mg/ ml lysozyme (Sigma); in 0.3 M sucrose; 25 mM Tris-HCl, pH 8; 25 mM Na_2EDTA, pH 8] and incubated at 37°C for 15 min. Once lysis was evident, 2.4 ml of 0.5 M Na_2EDTA was immediately mixed with the lysate by inversion, and incubated at 37°C for 15 min. To disrupt cell membranes 1.4 ml of 10% SDS was added, and incubated at 37°C for 2 h. The lysate was mixed with an equal volume of Tris-HCl (pH 8)-equilibrated phenol/chloroform using a small magnetic flea (12 x 3 mm) in a glass beaker on a magnetic stirrer. Once homogeneous and white, the DNA solution was centrifuged in 30 ml glass COREX tubes, at 10 K for 15 min at 4°C (Sorvall RC-5B centrifuge and SS34 rotor). If the aqueous phase appeared cloudy this process was repeated. Once the aqueous phase cleared it was transferred to a sterile universal bottle and inverted with an equal volume of chloroform. Care was taken to avoid shearing, and the sample was centrifuged (3500 x g, 10 min, at 4°C). The DNA was precipitated by adding 2.5 volumes of ice cold 100% ethanol and 1/10 volume of 3 M sodium acetate, pH 6 to the aqueous phase. The DNA was isolated by spooling with a glass rod and was then washed with 70% ethanol followed by 100% ethanol. The DNA was left to dry in air before it was dissolved in 5 ml SQ at 4°C overnight. The following day the DNA was incubated at 37°C for 1 h with ribonuclease A (Sigma) to give a final concentration of 30 μg/ ml. The DNA was again extracted with Tris-HCl (pH 8)-equilibrated phenol/chloroform and precipitated, spooled and washed as described above. The DNA was finally resuspended in a volume of 1 ml SQ and quantified by spectrophotometry.

10. Southern blotting, based on Southern et. al. (1975).

Preparing the gel.

Chromosomal DNA was digested at 37°C overnight. Digests (4 μg of DNA) were loaded with an appropriate size ladder, onto a 100 ml agarose gel, and run overnight at a low voltage to ensure good separation of DNA fragments. The gel was photographed with a ruler on an UV transilluminator to provide a record of the positions of different tracks and different size DNA bands. The DNA was partially hydrolysed by washing the gel in 250 ml of 0.25 M HCl for 7 min, in a sealable sandwich box on a gently shaking water bath. The gel was then washed briefly in SQ, before the DNA was denatured in 250 ml of 0.5 M sodium hydroxide and 1.5 M sodium chloride for 30 min. Following a further wash in SQ, the gel was neutralised in 3 M sodium chloride and 0.5 M Tris HCl, pH 7.4 for 30 min.

Assembly of the blotting apparatus.

Two glass plates, (larger than the gel to be blotted) were washed, dryed and wrapped in Saran wrap (Dow). Three sheets of Whatman 3 MM chromatography paper, cut the same size as the

plates, were dipped in 20x SSC (17.53% NaCl; 8.82% sodium citrate; adjusted to pH 7.0 with NaOH). Each sheet was smoothed out with a sterile glass pipette, in order to remove any air bubbles, onto one of the glass plates. The gel was then place wells side down into the centre of the Whatman paper and surrounded by folded strips of Saran wrap to prevent buffer from short circuiting the gel. A piece of Hybond-N membrane (Amersham International, UK), was cut to fit the gel exactly, before being dipped in 3x SSC. Care was taken when placing the membrane onto the gel as it could not be replaced once it was in contact with the gel. Three more sheets of Whatman 3MM paper (cut the same size as before) were dipped in 3x SSC and assembled as previously described, but on top of the nitrocellulose membrane. Paper towels were then stacked 5-10 cm high on top of the sheets of Whatman, followed by a glass plate which was weighed down. The Southern blot was then left overnight to ensure transfer of DNA from the gel to the membrane, after which the equipment was dismantled and the nitrocellulose washed in 3x SSC and left to dry at room temperature (20°C). The membrane was then wrapped in a single layer of Saran wrap and exposed to UV on the transilluminator for 30 sec, DNA side down, to allow cross-linking of DNA to the membrane.

Hybridisation

Hybridisation was carried out under conditions of high stringency as described by Hopwood et. al. 1985, i.e. greater than 70% sequence identity. Hybridisation buffer was freshly prepared [0.25 g of dried milk powder (Cadbury's "Marvel"); 3 g of PEG 8000; 3.75 ml 20x SSPE (17.4% NaCl; 2.76% $NaH_2PO_4.H_2O$; 0.74% Na_2EDTA; pH adjusted to 7.4 with NaOH); 5 ml 10% SDS; made up to 50 ml with SQ]. The hybridisation buffer was prewarmed to 65°C, and the membrane was prehybridised in 20 ml hybridisation buffer (65°C for 1 h) in a water bath that allowed gentle shaking. Purified probe was boiled for 10 min and then immediately chilled on ice, before it was added to the hybridisation chamber and left to hybridise at 65°C overnight. The following day the membrane was washed at high stringency; a brief wash with 3x SSC; twice with 200 ml 2x SSC, 0.1%SDS for 10 min at 65 °C; and twice with 0.5x SSC, 0.1%SDS for 10 min at 65°C. The membrane was then wrapped in Saran wrap before exposing it to X-ray film, between intensifying screens for 48 h.

11. Labelling DNA probes.

Primed synthesis of DNA probes, based on Feinberg et. al. (1984).

DNA probes (ranging between several hundred bp and 2 kb) were released from plasmids by restriction endonuclease digestion, and were gel isolated (Chapter 2, page 41, Jetsorb). DNA (25 ng) was dissolved in a total volume of 10 µl and boiled in a water bath for 10 min. The denatured DNA was then immediately chilled on ice to prevent renaturation. The labelling reaction [4 µl of 5x *OLB (oligonucleotide labelling buffer), 0.6 µl of 10 mg/ ml BSA (bovine serum albumin, Sigma); 1.5 µl of ^{32}P α-dCTP (10 mCi/ ml, Dupont); and 0.6 µl of the Klenow

Fragment of DNA Polymerase I (8.6 units/ µl Gibco - BRL)] was set up on ice, and incubated for 1 h at 37°C, and then overnight at room temperature (20°C).

*OLB was composed of three solutions (A, B, and C) in the ratio of 100:250:150. OLB, solutions A and C were stored at -20°C, and solutions O and B were stored at 4°C.
Solution A: 1 ml of solution O (1.25 M Tris-HCl; 0.125 M $MgCl_2$; adjusted to pH 8.0), 18 µl 2-mercaptoethanol, 5 µl dATP, 5 µl dGTP, 5 µl dTTP (each dNTP was previously dissovled in 3 mM Tris-HCl; 0.2 mM Na_2EDTA; adjusted to pH 7.0 at a concentration of 0.1 M).
Solution B: 2 M Hepes, titrated to pH 6.6 with 4 M NaOH
Solution C: Hexadeoxyribonucleotides suspended in TE at 90 OD units / ml.

The following day any unincorporated radioactivity was removed using a mini column of G50-Sephadex (Pharmacia Biotech) as described by Sambrook et. al. (1989). The end of the barrel of a sterile 1 ml syringe was plugged with sterile siliconised glass wool. Sterile Sephadex was dropped into the syringe using a sterile pasteur pipette, until the column had completely packed down. The syringe was placed in an 11.5 ml Röhre tube and spun in an MSE bench top centrifuge for 1 min. The column was washed with SQ; 100 µl of SQ was added to the top of the column, which was then recentrifuged. The volume of the probe was made up to 100 µl before it was spun through the column into an 1.5 ml eppendorf vial (with the lid removed) placed at the bottom of the column.

Preparation of a single-stranded, end labelled DNA probe for S1 mapping-see Chapter 6, page 117.

12. Large scale plasmid preparation from *Streptomyces*.
A spore suspension (100 µl) was grown in 50 ml YEME/ sucrose at 30°C for 34 h. The culture was transferred to universal bottles and diluted with a 1/3 volume SQ before they were pelleted by centrifugation, (3500 x g, 10 min, at 4°C). The harvested mycelium was washed twice with 10% sucrose and centrifuged as before, discarding the supernatant. Cell walls were removed by resuspending the cell pellet in 5 ml lysozyme solution (10% sucrose; 25 mM Tris-HCl, pH 8.0; 25 mM Na_2EDTA, pH 8.0; and 2 mg/ ml lysozyme) and incubating at 30°C for 30 min. Chromosomal DNA was then removed by incubation with 0.5 volume alkaline SDS (0.3 M NaOH; 2% SDS) at 70°C for 15 - 20 min. As soon as the alkaline SDS was added the sample was mixed to ensure complete removal of chromosomal DNA. The DNA solution was then left to cool at room temperature (20°C) for 5 min, before being mixed with 1/3 volume of acid phenol/chloroform. The sample was centrifuged (3500 x g, 10 min, at 4°C), and the upper aqueous phase was then extracted twice with an equal volume of Tris-HCl (pH 8)-equilibrated phenol/chloroform. DNA from the aqueous phase was then precipitated with 0.1 volume 3 M

sodium acetate and 0.54 volume isopropanol on ice for 10 min. After centrifugation (3500 x g, 10 min, at 4°C) the pelleted DNA was washed with 70% ethanol and dried, before being dissolved in 100 µl of SQ.

13. Small scale plasmid preparation from *Streptomyces*.

A spore suspension (20 µl) was grown in 10 ml YEME/ sucrose in a sterile universal bottle at 30°C for 34 h. The cells were pelleted by centrifugation, (3500 x g, 10 min, at 4°C) and washed with 10% sucrose. The harvested mycelium was resuspended in 400 µl lysozyme solution (refer to large scale protocol) and incubated at 30°C for 30 min. Chromosomal DNA was then removed by incubation with 0.5 volume alkaline SDS (refer to large scale protocol), which was mixed immediately to ensure complete removal of chromosomal DNA, at 70°C for 15-20 min. The DNA solution was then left to cool on the bench, before being mixed with 1/3 volume of acid phenol/chloroform. The sample was microcentrifuged (13, 000 rpm, 10 min, at 4°C), and the upper aqueous phase was then extracted twice with an equal volume of Tris-HCl (pH 8)-equilibrated phenol/chloroform. DNA from the aqueous phase was precipitated with 0.1 volume 3 M sodium acetate and 0.54 volume isopropanol on ice for 10 min. After centrifugation (13, 000 rpm, 10 min) the pelleted DNA was washed with 70% ethanol and dried, before being dissolved in 50 µl of SQ.

14. Large scale plasmid preparation from *Escherichia coli* by alkaline lysis (based on Birnboim *et. al.*, 1979).

A 50 ml overnight culture of *E. coli* was harvested by centrifugation (3500 x g, 5 min, at 4°C). The pelleted cells washed in 10 ml of SQ, before being resuspended by vortexing in 1 ml of TEG (25 mM Tris-HCl, pH 8.0; 10 mM Na_2EDTA, pH 8.0; 50 mM glucose). After 5 min at room temperature (20°C), 2 ml of 0.2 M NaOH, 1% SDS (made up freshly) was added and the tube inverted before incubation on ice for 10 min. After lysis, the alkaline pH was neutralised by the addition of 1.5 ml of KAc solution (60 ml 5 M potassium acetate; 11.5 ml glacial acetic acid; 28.5 ml SQ; pH 4.8) and the preparation was mixed for 2 min, after which the tube was returned to ice for 15 min. The alkaline pH denatures linear DNA so that on addition of KAc solution the chromosome forms insoluble debris. Plasmid DNA has a different conformation to chromosomal DNA. It exists as a covalently closed circle and therefore remains in the supernatant. The supernatant was separated from the insoluble mass by centrifugation (3500 x g, 10 min, at 4°C) and incubated with RNase (30 µg/ ml; heated to 100°C before use) at 37°C for 30 min. The DNA solution was then mixed with an equal volume of Tris HCl (pH 8)-equilibrated phenol/chloroform and centrifuged (3500 x g, 10 min, at 4°C). The top layer was removed and precipitated with 0.6 volumes of isopropanol and 1/10 volume 3 M NaAc, pH 4.8 on ice for 10 min. The DNA was pelleted (3500 x g, 15 min, at 4°C) and washed with 70% ethanol to remove salts. The plasmid was then dried for 10 min under vacuum and dissolved in 200 µl of SQ.

15. Small scale plasmid preparation from *Escherichia coli* by alkaline lysis (based on Birnboim *et. al.*, 1979).

Cells from 5 ml overnight culture of *E. coli* were harvested by centrifugation (3500 g, 10 min, at 4°C). The pellet was washed in 1 ml of SQ and pelleted in an 1.5 ml eppendorf vial (13, 000 rpm, 1 min). The bacteria were resuspended by mixing in 100 µl of ice cold TEG (refer to large scale protocol) and were incubated for 5 min at room temperature (20°C). Cell lysis involved the addition of 200 µl of 0.2 M NaOH, 1% SDS, and the tube inverted before incubation on ice for 10 min. The DNA lysate was mixed with 150 µl KAc solution and then incubated on ice for 15 min. After centrifugation (13, 000 rpm, 5 min) the supernatant was transferred to an equal volume of Tris HCl (pH 8)-equilibrated phenol/chloroform, mixed, and then centrifuged (13, 000 rpm, 5 min). The top layer was removed and precipitated with 2 volumes of ethanol at -70°C for at least 10 minutes. The DNA was pelleted (13, 000 rpm, 1 min) and washed with 70% ethanol to remove salts. Plasmid was dried for 10 min in a vacuum dryer and dissolved in 50 µl of SQ. RNA was removed by adding RNase to restriction endonuclease digests.

(C) Sequencing *tyl* DNA.

Streptomyces DNA is over 70% G+C rich, and therefore secondary structure can be problem when sequencing DNA. Nested deletions (Henikoff, 1984) were used to generate overlapping sequence, as opposed to using progressive primers (Chapter 3, page 57). Also, both strands of DNA were sequenced with nucleotide analogues which base pair weakly with normal nucleotides (Mizusawa *et. al.*, 1986).

1. Generation of a set of nested deletion mutants (Double - Stranded nested deletion kit, Pharmacia Biotech).

Nested deletions were generated using plasmid purified by QIAGEN midi preps (protocol page 47). Exonuclease III (Exo III) will digest a single strand of a double-stranded DNA molecule which has either a blunt end or a 5' overhang.

Plasmid DNA (5-10 µg) was first digested with an enzyme which cuts adjacent to the DNA insert providing a site that is susceptible to Exo III digestion. The vector was then cut with a second enzyme that created a 3' overhang in order to protect the rest of the plasmid from digestion. Both of these enzymes were chosen to cut at unique sites within the multiple cloning site (MCS) of the plasmid.

The conditions for exonuclease III digestion, ie the incubation temperature, and the number and spacing of time points, were calculated by considering the appropriate spacing of deletions and the size of largest deletion required. As Exo III functions optimally at 75 mM NaCl, it was necessary to prepare 2x Exo III buffer of the appropriate NaCl concentration that it took into

account the salt concentration in the second digest. The digested DNA was dissolved in SQ at a concentration of 0.1 µg/ µl, and 20 µl was mixed with 20 µl of Exo III buffer. This mixture was incubated at the selected temperature for 2-3 min, before 2 µl was transferred into a tube containing 3 µl of S1 nuclease/ buffer mix (33 µl of SI buffer, 66 µl of SQ and 1 µl of SI nuclease). The remaining DNA was then incubated with 1 µl of exonuclease III. As each selected time point was reached, 2 µl was transferred to a fresh tube of SI nuclease/ buffer, mixed well and left on ice. Once all the samples had been taken they were incubated at room temperature (20°C) for 30 min to allow the S1 nuclease to digest any remaining single-stranded DNA (ssDNA). S1 stop solution (1 µl) was then added to each sample and they were incubated at 65°C for 10 min.

The deleted plasmids (3µl) were religated at room temperature (20°C) for 2 h with 17 µl of ligation mix (2 µl T4 DNA ligase; 80 µl 25% PEG; 40 µl 10x ligation buffer and 218 µl SQ). *E. coli* NM522 was then transformed with these ligations, and plasmid prepared from transformants. Deletion products were analysed by restriction digests to check the size of the plasmid insert.

Preparation of plasmid DNA for sequencing.

2. PEG precipitation of plasmid DNA.

PEG precipitation of plasmid DNA allows the removal of impurities that can intefere with sequencing reactions. Large scale plasmid preparations were therefore purified for non automated and automated sequencing by precipitation of 80 µl DNA with 100 µl 13% PEG 8000 (Sigma) and 20 µl 4 M NaCl, on ice for 30 min. After the DNA was spun down (13, 000 rpm, 10 min) and washed with 75% ethanol, the DNA pellet was dried under a vacuum and dissolved in 40 µl of SQ.

3. QIAGEN plasmid Midi protocol

QIAGEN plasmid Midi protocol was used to prepare plasmid for automated sequencing and generating nested deletions. QIAGEN columns allow the preparation of plasmid DNA which is as pure as CsCl preparations, but the procedure is much quicker. A QIAGEN Midi column is expected to yield between 75-100 µg of DNA, however, the column must not be overloaded with cells as this reduces plasmid yields dramatically. For high copy plasmids such as pUC18 and pGEM-T a 25 ml overnight culture was harvested. The cell pellet was resuspended in 4 ml of buffer P1 (removes RNA and other low MW impurities), before 4 ml of lysis buffer was added and inverted After incubation on ice for 5 min, 4 ml of chilled neutralisation buffer was mixed with the lysate and incubated for a further 15 minutes on ice. To remove chromosomal DNA the mixture was centrifuged (13 K, 30 min, at 4°C) and the supernatant immediately transferred to another tube. A QIAGEN-tip 100 was equilibrated with 4 ml equilibration buffer. The supernatant was then applied to the column, after which the tip was washed twice with 10 ml of

wash buffer. The DNA was then eluted with 5 ml of elution buffer and precipitated with 0.7 volumes of isopropanol. DNA was pelleted by centrifugation (11 K for 30 min at 4°C), before it was washed with 70% ethanol and air dried for 5 min. The DNA was dissolved and quantified by spectrophotometry.

Dideoxy-chain termination sequencing (Sanger *et. al.*, 1977).

4. Non automated sequencing using the ^{T7}Sequencing Kit (Pharmacia).

Plasmid DNA was prepared by PEG precipitation, page 47. Double-stranded DNA template was denatured by briefly mixing 1.5-2 µg of DNA (in a total volume of 36 µl) with 4 µl of 2 M NaOH and incubating at room temperature (20°C) for 5 min. The DNA was reprecipitated with 4 µl 3 M sodium acetate, pH 4.8 and 90 µl of 100% ethanol at -70°C for 15 min, and centrifuged (13, 000 rpm, 10 min). After the pellet was washed with 75% ethanol, the DNA was dried and dissolved in 10 µl of sterile water.

Annealing the primer to the DNA template.

Universal primer [5'-GTAAAACGACGGCCAGT-3', (Heidecker *et. al.*, 1980)] was annealed to a region flanking the insert in the multiple cloning site of pUC18. The denatured DNA (1.5-2 µg DNA in 10 µl SQ) was incubated with 2 µl of primer (5-10 pmol) and 2 µl annealing buffer at 37°C for 20 min, followed by 10 min at room temperature (20°C). Other primers were used in conjunction with the sequencing kit ie reverse primer (5'-AACAGCTATGACCATG-3') and oligonucleotides complementary to *tyl* DNA.

Sequencing reactions.

Due to the high rate of polymerisation, the primer extension reactions were carried out in two stages. Initially, labelling allowed extension from the primer in limiting concentrations of all four nucleotides with the efficient incorporation of the one radioactively labelled nucleoside triphosphate, [α -^{35}S] dATP, so that bands could be visualised. The annealing reaction was therefore incubated with 3 µl of labelling mix, 1 µl of [α -^{35}S] dATP, and 2 µl of diluted T7 DNA polymerase (3 units) at room temperature (20°C) for 5 min.

The termination stage involved four separate reactions in which high concentrations of all four deoxynucleoside triphosphates and a specific dideoxynucleoside triphosphate were present. Dideoxynucleoside triphosphates lack the 3' OH group of conventional deoxynucleoside triphosphates and can therefore be added to the nascent DNA strand, but block further synthesis. The A, C, G, T short mixes were pipetted (2.5 µl) into separate eppendorf vials and were prewarmed at 37°C during labelling. From the labelling reactions 4.5 µl volumes were transferred to each of the A, C, G, T mixes. These samples were mixed thoroughly and incubated at 37°C for 5 min, before 5 µl of stop solution was added.

Gel electrophoresis of sequencing reactions.

The products of sequencing were then separated by electrophoresis on a 6% polyacrylamide gel under denaturing conditions (7 M urea). Gel preparation involved dissolving 16.8 g of urea into 30 ml SQ, and adding 4 ml 10 x Tris-borate electrophoresis buffer [1x TBE (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M Na_2EDTA)] and 6 ml acrylamide solution (40% w/ v Photogel^{TM} National diagnostics) in a side arm flask. Once cooled to room temperature (20°C), the gel was degased using a vacuum line. To polymerise the gel, 640 µl of freshly prepared 1.6% ammonium persulphate (Fissons) and 40 µl TEMED (*N', N', N', N'*, - tetramethylethylenediamine, Sigma) was added and immediately mixed gently. The gel was poured between two gel plates (200 mm x 360 mm x 0.35 mm), one of which had been silicone treated (Repelcote water repellant, BDH). The plates were separated by narrow spacers (20 mm x 360 x 1 mm) and secured with tape and large bull dog clips. A comb was inserted with the flat side in the gel (teeth up), to make a large well in the top of the gel.

The gel was assembled in a vertical tank with the comb inserted so that the teeth just touched the surface of the well in the gel. The top tank was filled with 0.5x TBE and the bottom tank with 1x TBE. To reduce the chances of DNA secondary structures occurring, the gel was pre-warmed at 39-40 watts for 30 min. The samples were boiled for 2 min and placed on ice before 2 µl was loaded into each well, in the order A, C, G, T. Just before loading the samples the wells were washed with 0.5x TBE buffer using a needle and syringe. The gel was run until the xylene blue dye front reached 5 cm from the bottom of the gel. At that point a second loading of sequencing reactions could occur into other wells, and the gel was run for a further 40 min. The gel apparatus was dismantled, and the plate with the gel attached was placed in a bath of 10% glacial acetic acid and 10% methanol for 20 min. The gel was then transferred to a piece of Whatman 3MM paper, covered in Saran wrap and dried under vacuum at 80°C for 1 h. The sequence ladders were visualised by autoradiography by exposing the gel for 48 h to X-ray film at room temperature (20°C)

Nucleotide analogues.

7-deaza dGTP, and 7-deaza dATP, were used to resequence each nested deletion in an attempt to resolve possible compressions (Deaza G/A ^{T7}Sequencing Mixes, Pharmacia).

Primers.

Oligonocleotides of 17/ 18 bases in length, were used to bridge any gaps between nested deletions. These primers were designed so that they were located approximately 30 bp away from the region of interest, and contained an equal ratio of G+C to A+T bases (T_m 55-60°C).

Inverted repeats which might give rise to secondary structure were avoided, as were A's or T's as the first or last base of the primer which can weaken primer template interactions.

5. Automated sequencing.

Plasmid DNA from QIAGEN columns was sequenced using the ABI (Applied Biosystems) automated procedure, with fluorescent dye-labelled dideoxynucleoside triphosphate terminators and Ampli*Taq* DNA polymerase. Different fluorescent groups are attached to the four dideoxynucleoside triphosphates which emit at different wavelengths when excited by a laser (Prober *et al.*, 1987). The G analogue inosine (dITP) is used in the sequencing mix to reduce the likehood of compressions. Several sequencing protocols were used during different sequencing projects. Firstly, a modified form of the ABI automated sequencing protocol, which was specifically designed to cope with GC rich templates, was used. The amounts of premix, primers and DNA were doubled (19 µl of premix; 2 µg of template; and 6.4 pmol of primer), and the reaction mix was made up to a total volume of 40 µl, and overlayed with 50 µl mineral oil. An initial hot start of 98°C for 1 min was followed by 30 cycles of 30 sec denaturation at 98°C, 15 sec annealing at 50°C, and 4 min polymerisation at 60°C in a thermal cycler (Perkin-Elmer).

A protocol was subsequently made available to us from Paul R. Rosteck, Jr. (Eli Lilly and Co.) which uses dimethyl sulphoxide (DMSO), which helps sequencing of GC rich templates. The reaction mix (7.5% DMSO; 700 ng DNA; 9.5 µl of premix; 3.2 pmol primer; in a total volume of 20 µl) was subjected to 25 cycles of 1 min denaturation at 95°C, 15 sec annealing at 50°C, and 4 min polymerisation at 60°C, with no hot start.

The most recent advance was the use of the ABI PRISM™ dye terminator cycle sequencing ready kit, which uses Ampli*Taq* DNA polymerase, FS. Less template is required, and the reaction mix consisted of 8.0 µl terminator ready reaction mix, 250-500 ng ds DNA, 3.2 pmole primer, in a total volume of 20 µl. The thermal cycler programme recommended by Paul R. Rosteck, Jr. (Eli Lilly and Co.) was used. Also, there was less variation in peak height in chromatograms, and purification of extension products was easier because there is less dye terminators to remove.

Purification of extension products.

The reduction in the amount of dye terminators in the terminator ready reaction mix has forfeited the need for phenol extracting the sequence reaction before precipitating of DNA, during the purification procedure. The sequence reactions were mixed with 2 µl of 3 M sodium acetate, pH 4.6, 50 µl ethanol and precipitated on ice for 10 min. The DNA was pelleted (13, 000 rpm, 10 min), washed with 70% ethanol and dried under vacuum.

Electrophoresis of sequencing reactions (Protein and Nucleic Acid Chemistry Laboratory, Leicester University).
Samples were analysed on an automated DNA sequencer (Applied Biosystems Inc.) using a 48 cm, 4.5% polyacrylamide gel. As many as 650 bp of good sequence data could be read off one track.

(D) Introduction of DNA into bacteria.

1. Preparation of competent *Escherichia coli*, and identification of transformants and recombinants.

Transformations were performed using a modified protocol based upon the standard calcium chloride/rubidium chloride procedure (Sambrook *et. al.*, 1989). A single colony of *E. coli* NM522 was inoculated into 10 ml of LB broth in a sterile universal bottle and shaken overnight at 37°C. This culture of NM522 (0.5 ml) was used to inoculate 50 ml of LB broth, which was then grown at 37°C for 2 h until the cells reached an OD_{600} of 0.4-0.5, (not a turbid culture). The culture was split between two sterile universal bottles, and the cells were harvested by centrifugation (3500 x g, 10 min, 4°C). The cells were pooled and resuspended in 4 ml of fresh buffer A (8 ml SQ; 1 ml 100 mM MOPS, pH 7; 1 ml 100 mM rubidium Chloride), and divided between four 1.5 ml eppendorf vial tubes. The cells were pelleted in a microcentrifuge (13, 000 rpm, 30 sec) before each pellet was resuspended in 1 ml of buffer B (7 ml SQ; 1 ml 1 M MOPS pH 6.5; 1 ml 100 mM rubidium chloride; 1 ml 500 mM $CaCl_2$) and placed on ice for 30 -60 min. The bacteria were again harvested by centrifugation (13, 000 rpm, 30 sec) and resuspended in 500 µl buffer B. DNA was added to 100 µl of competent cells and incubated on ice for a further 30-60 min. The cells were then heated by placing them in a 50°C water bath for 2 min before they were transferred to ice for a further 2 min. LB broth (1 ml) was added to each tube and incubated at 37°C for 1 h to allow expression of plasmid markers. The transformation (100 µl) was plated out onto LB agar with the appropriate antibiotic for the selection of plasmid.

Identification of transformants.

Successful transformation of a bacterial cell with plasmid DNA can be detected by selecting transformants on an antibiotic to which the plasmid confers resistance, but the original strain is sensitive. For example, pUC18 encodes resistance to ampicillin (100 µg/ ml) to which *E. coli* NM522 is sensitive.

Identification of recombinants

It is necessary to be able to differentiate between cells carrying recombinant plasmid and those carrying religated vector. Insertional inactivation of a plasmid marker gene allows such discrimination. For example, the *lacZ* gene in pUC18 encodes the alpha subunit of the ß-galactosidase enzyme which can function with the omega subunit produced by NM522 to form a

functional enzyme. ß-galactosidase production is inducible by IPTG and converts colourless X-gal into a blue compound. Recombinant plasmids were identified loss of a complementation (white colonies), on LB agar supplemented with 40 µg Xgal/ ml (5-bromo-4-chloro-3-indolyl-beta-D-galactosidase, Novabiochem), 30 µg IPTG/ ml (isopropylthiogalactoside, Sigma), and ampicillin (100 µg/ ml).

2. Preparing *Streptomyces* protoplasts (Hopwood *et. al.*, 1985).

YEME/ sucrose (50 ml) was inoculated with *Streptomyces livdans* OS456 (100 µl of a spore stock) and shaken at 300 rpm at 30°C for 38-41 h . The culture was diluted with SQ to help pellet the cells, and split into sterile universal bottles. The cells were centrifuged (3500 x g, 10 min, at 4°C) and washed twice with 10% sucrose. The harvested mycelium was then resuspended in 4 ml lysozyme solution [1 mg/ ml lysozyme in L buffer (10 ml 10.3% sucrose; 250 µl 22.92% TES, pH 7.2; 100 µl 2.5% K_2SO_4; 20 µl *trace elements; 100 µl 0.5% KH_2PO_4; 10 µl 50% $MgCl_2$; 25 µl 1M $CaCl_2$)] and incubated at 30°C for 30-45 minutes, inverting gently every 15 min. A sample was taken with a sterile pipette to check protoplast formation had occurred under the microscope, and the mix was left for a further 15 min at 30°C. Sterile P buffer (10.3 g sucrose; 1 ml 2.5% K_2SO_4; 0.4 ml 50% $MgCl_2$; 0.2 ml trace element solution; in 90-95 ml SQ. Before use 1 ml 0.5% KH_2PO_4; 2.5 ml 1 M $CaCl_2$; 2.5 ml 22.92 % TES, pH 7.2, was added asceptically) was then added (5 ml) and gently pipetted with a sterile transfer pipette, before the protoplasts were filtered through a sterile p5000 tip filled with cotton wool. The filtrate was then transferred to sterile plastic tubes which were spun at 3000 rpm for 5 min at 4°C. The supernatant was discarded and the pellet of protoplasts was washed with P buffer. Protoplasts were finally resuspended in 3-400 µl P buffer, and were stored in 50 µl volumes (containing 5 x 10^7 protoplasts) at -70°C. The concentration of protoplasts was measured by spectrophotometry, one OD_{600} unit representing 1.5 x 10^9 protoplasts/ ml.

*Trace element solution (40 mg $ZnCl_2$; 200 mg $FeCl_3.6H_2O$; 10 mg $CuCl_2.2H_2O$; 10 mg $MnCl_2.4H_20$; 10 mg $Na_2B_4O_7.10H_20$; 10 mg $(NH_4)_6Mo_7O_{24}.4H_2O$; per litre).

3. Rapid small scale procedure for the transformation of *Streptomyces* protoplasts with plasmid DNA (Hopwood *et. al.*, 1985).

DNA was added to 50 µl of protoplasts and mixed by tapping the tube, before 200 µl of **transformation buffer was then mixed with the protoplast solution. The transformation was plated out on dried ***R2YE plates and incubated at 30°C for 14-20 h. Plates were overlayed with 1 ml SQ containing the appropriate antibiotic and incubated at 30°C for 3 days.. The amount of antibiotic added to the overlay also took into account the volume of agar in the plate.

**Transformation buffer: 7.5 ml of SQ was mixed with 2.5 ml 10.3% sucrose, 20 µl of trace elements and 100 µl 2.5% K_2SO_4, before 700 µl of the mixture was discarded. To the remaining 9.5 ml, 0.2 ml 5 M $CaCl_2$ and 0.5 ml 1 M Tris-maleic acid, pH 8, were added. From this mixture 3 ml was transferred to 1 g of autoclaved PEG 1000, which was kept molten in a 37°C water bath. This buffer was stored in the -20 °C freezer in 250 µl volumes which were not refrozen.

***R2YE plates: 124 g sucrose; 12 g glucose; 0.3 g K_2SO_4; 12.14 g $MgCl_2.6H_20$; 0.12 g Difco casaminoacids; SQ added to 1 litre. 270 ml was added to 6.6 g of agar and autoclaved. Prior to use add to each bottle, asceptically; 0.6 ml trace elements; 3 ml 0.5% KH_2PO_4; 4.5 ml 20% L-proline; 1.5 ml 1 M NaOH; 5.7 ml 1 M $CaCl_2$; 6.54 ml 22.92% TES, pH 7.2; 7.5 ml 20% yeast extract. Plates were dried for 3 h in the laminar flow hood.

4. Replica plating, based on Lederberg et. al. 1952.

Replica plating was used to transfer *Streptomyces* colonies from one solidifed medium to another. Velvet squares (20 cm x 20 cm) were autoclaved and placed, nap side up, on top of a cylindrical wood support (9 cm diameter), and held in place by a wooden ring. The agar plate supporting colony growth was placed face down onto the fabric and pressure was applied. The deposited spores would then provide inoculum for other plates which were pressed onto the suface of the velvet. Each plate was marked and orientated to line up with corresponding marks on the wooden block, allowing the position of colonies on different plates to be determined. The procedure was carried out close to a bunsen flame.

(E) Amplifying *Streptomyces fradiae* genes by PCR.

The conditions for amplifying DNA fragments from *S. fradiae* were developed using Invitrogen's PCR optimiser™ kit (Dr Atul Gandecha, personal communication). PCR reactions (50 µl) contained 2 µg of chromosomal DNA or 50 ng of plasmid, 250 ng of each primer (27- or 26-mers), 1 mM dNTPs 1 unit of *Taq* DNA polymerase (Gibco BRL) in 60 mM Tris-HCl pH 8.5 (adjusted at 20°C), 0.7 mM $MgCl_2$, 15 mM $(NH_4)_2SO_4$ and 10% $^{v}/_{v}$ DMSO (Varadaraj *et. al.*, 1994), and were overlayed with mineral oil in a 0.6 ml eppendorf vial. Primers were designed so as to achieve as near 50:50 AT:GC as possible. However, the T_m for each primer did vary, and therefore so did the annealing temperature. After an initial hot start at 80°C for 5 min followed by 94°C for 2 min, the reaction mix was subjected to 35 cycles of amplification, each consisting of 1 min at 94°C, 2 min at 60°C and 3 min at 72°C, before the thermal cycle was terminated with 7 min at 72°C. The PCR product was gel isolated, (Jetsorb, page 41), before being ligated into pGEM-T (Promega). The identity of the DNA fragment was confirmed by restriction digest and or automated sequencing.

(F) Determination of the Minimum Inhibitory Drug Concentrations (MIC values).

The resistance profile of a *Streptomyces* strain was analysed by calculating the MIC. This was determined by plating fresh spores onto NEF agar plates containing a range of antibiotic concentrations. Plates were incubated at 30°C for 3 days.

(G) Gradient plates [based on Bryson et. al. (1952) with modifications from Ward et. al., (1986)].

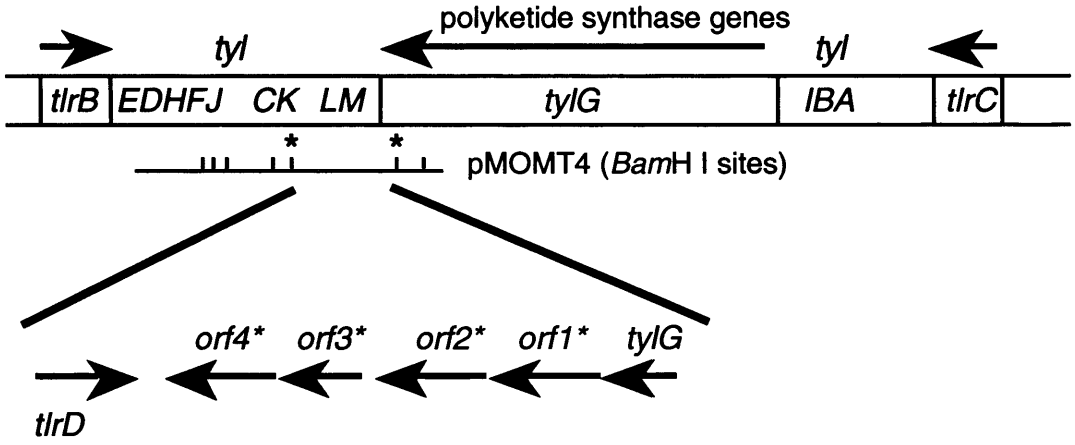
Plates were set up with a gradient of antibiotic from 0-100 μg/ ml. NEF agar (25 ml) containing antibiotic (100 μg/ ml) was poured in a square petri dish (100 mm x 100 mm) which was then tilted so that it set at an angle for 1 h at room temperature (20°C) Agar (25 ml) was then poured on top and the antibiotic was allowed to diffuse up into the agar for 2 h at 30°C. Spores (5×10^5 in 5 μl) were then spread across the gradient plate from a low to a high concentration using a sterile tooth pick.

Chapter 3 Sequencing *tylLM* DNA.

Introduction.

The sequence of the *tylLM* region from the genome of *Streptomyces fradiae*, (accession number X81885, Gandecha *et. al.*, 1997), was generated by sequencing about 6 kb of DNA located between the end of the polyketide synthase genes (*tylG*) and the termination of the resistance gene *tlrD* (Figure 7, page 55). Four open reading frames were identified downstream of *tylG*, *orfs1-4** (Figure 7, page 55). Double-stranded DNA sequence was generated for *orf1** in a non automated fashion by the dideoxynucleoside triphosphate chain termination method (Sanger *et. al.*, 1977) as described in the manual sequencing protocol in Chapter 2, page 48.

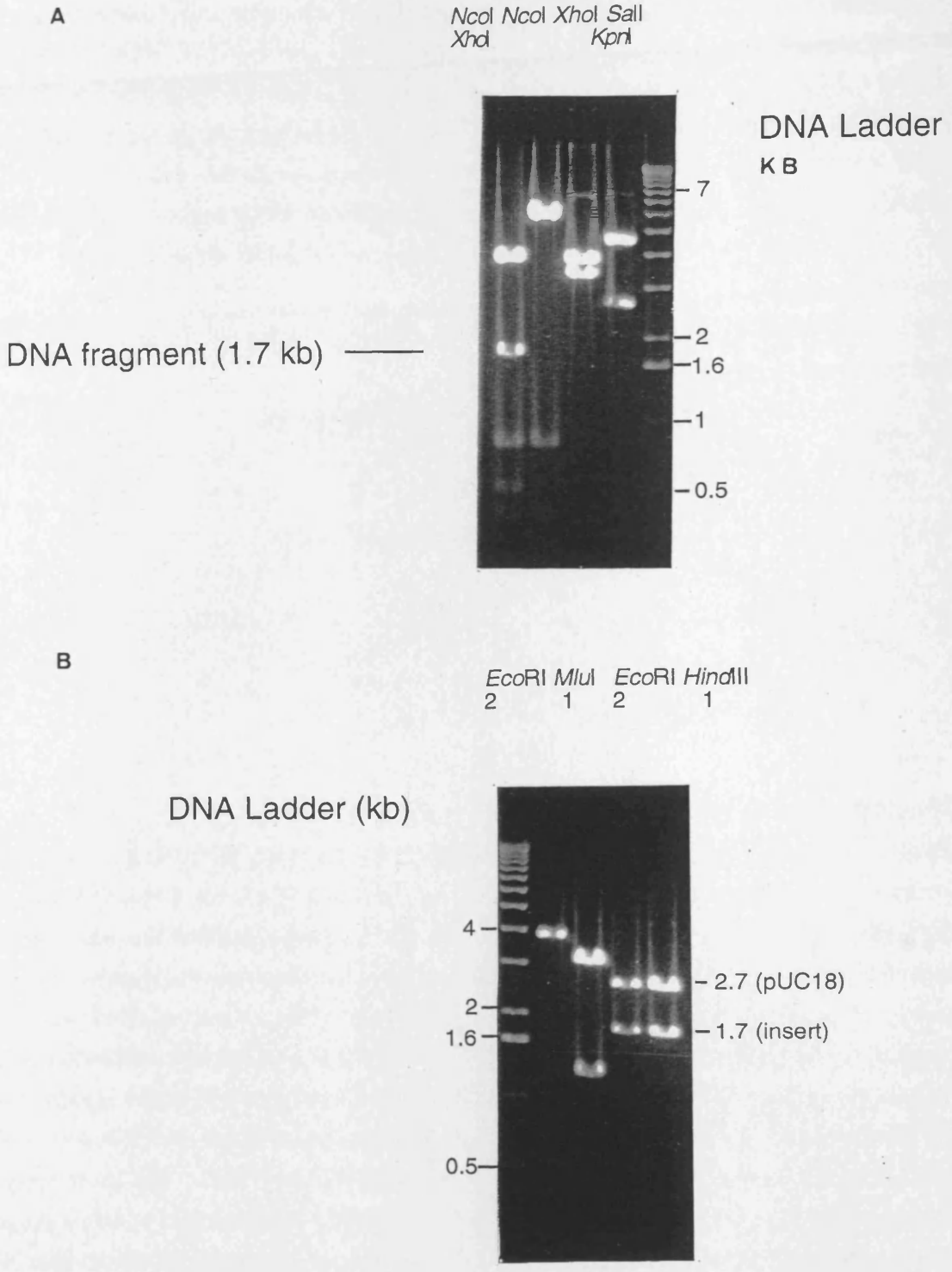
Figure 7 Map of the tylosin biosynthetic gene cluster showing the location of the *tylLM* region and respective genes.



The difficulties of sequencing *Streptomyces* DNA.

Streptomyces DNA is over 70% G+C which makes sequencing difficult due to compressions, which result from secondary structure in DNA. Hairpin loops in the DNA template can impede the progression of the sequencing enzyme, and during electrophoresis secondary structure can make DNA bands shorter than they really are causing them to migrate together. In order to avoid secondary structure it is therefore necessary to sequence both strands of DNA, and to resolve compressions using nucleotide analogues which base pair weakly with normal nucleotides (Mizusawa *et. al.*, 1986). These analogues can only be used to resequence regions of DNA, as the resulting gels are not as clear as those using conventional bases. Additionally, DNA was sequenced using nested deletions as opposed to progressive primers. This involves digestion of the cloned insert in order to generate a series of plasmids with an insert which is progressively deleted in from one end (Henikoff, 1984). Sequencing of each plasmid occurs from the same primer at one end of the multiple cloning site (MCS) from which the DNA has been deleted. This ensures that much of the DNA is sequenced more than once, and avoids the need to synthesise primers which are complementary to the hypothetical sequence.

Figure 8 Digestion of pLST973 with *Nco* I and *Xho* I generated a 1.7 kb DNA fragment (A), which was ligated into pUC18 in both orientations, 1 and 2 (B).



Subcloning the required DNA template.

S. fradiae DNA derived from c373.10 in the cosmid pMOMT4 (Beckman *et. al.*, 1989) was digested with *Bam*H I, and the largest DNA fragment (11 kb) was ligated into pUC18 (Norrander, *et. al.*, 1983) to generate the plasmid pLST97 (Dr Louise A Merson-Davies). The *Bam*H I DNA fragment in pLST97 contains the *tylLM* region (Fishman *et. al.*, 1987) and was digested with a number of restriction enzymes allowing the creation of a series of subclones which would aid DNA sequencing. One of these plasmids, pLST973 (Dr Louise A Merson-Davies), is composed of a 4.5 kb *Sal* I-*Kpn* I DNA fragment from the *tylLM* region, (Figure 20, page 77, Chapter 4). This DNA contains the end of *tylG* and downstream DNA. An *Nco* I site was located 212 bp inside the end of *tylG* (as confirmed by P. R. Rosteck, Jr., personal communication). In order to look for an ORF downstream of *tylG,* a *Nco* I-*Xho* I DNA fragment (1.7 kb) was identified in pLST973 (Figure 8 (A), page 56). Neither *Nco* I or *Xho* I are compatible with unique sites in the MCS of pUC18. Therefore, the Klenow fragment of *E. coli* polymerase I was used to fill in the 3' recessed termini of the 1.7 kb fragment after gel isolation (Chapter 2, page 40), so that the DNA could be ligated into the *Sma* I site of pUC18 (Chapter 2, pages 40) to form the plasmid pLST973A. The vector was treated with calf intestinal alkaline phosphatase (Chapter 2, page 40), which removes 5' phosphates thereby limiting self ligation of the vector DNA.

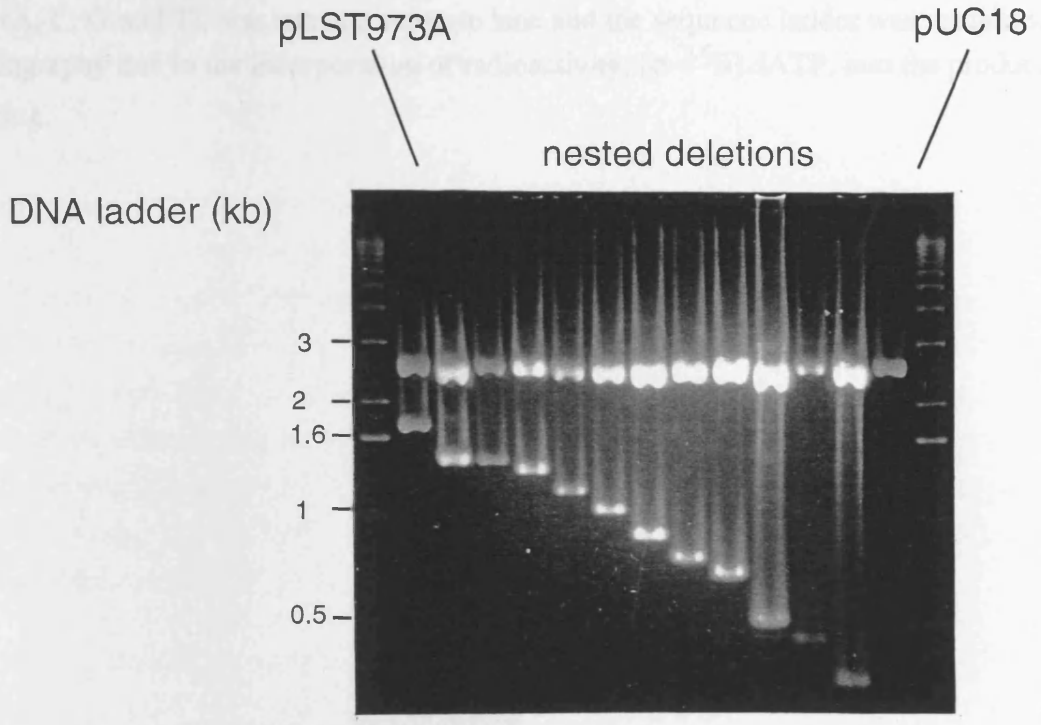
E. coli NM522 was then transformed with this ligation using a modified protocol based upon the standard calcium chloride/ rubidium chloride procedure (Chapter 2, page 51), and colonies containing recombinant plasmid were distinguished by blue-white selection (Chapter 2, page 51). Plasmid DNA was isolated using the alkaline lysis method (Chapter 2, page 51) and was then digested with *Eco*R I and *Hin*d III, which cut at opposite ends of the MCS of pUC18, in order to check the size of the DNA insert (Figure 8 (B), page 56). Recombinant plasmids which contained a fragment of 1.7 kb were then digested with *Eco*R I and *Mlu* I to check the orientation of the insert (Figure 8 (B), page 56). *Mlu* I cuts asymmetrically within the insert, approximately 500 bp from the *Nco* I site. Plasmid was identified with the insert in both orientations (1 and 2) allowing the generation of double-stranded sequence.

Creation of a nested set of deletion mutants.

DNA polymerases have a tendency to dissociate from the DNA template, and will only reliably sequence several hundred bases. Therefore, in order to sequence a large piece of DNA progressive primers or nested deletions can be used (Henikoff, 1984). A series of nested deletions was generated for pLST973A as described in Chapter 2, page 46. Exonuclease III digests a single strand of a double-stranded DNA molecule, which is either blunt ended or has a 5' overhang. For example, pLST973A (orientation 1) was digested with *Bam*H I and *Pst* I, these sites lying between the *Nco* I end of the insert and the universal primer binding site. This

provided a 5' overhang (*Bam*H I) which is susceptible to digestion by exonuclease III, and a 3' overhang (*Pst* I) which protects the vector DNA from digestion. At various time intervals samples were treated with SI nuclease which digested the remaining single stranded DNA before a stop solution was added. The deleted plasmids were then religated according to the kit protocol, introduced into *E. coli* NM522 by transformation, and analysed to check the size of the insert. A series of subclones was generated with inserts of progressively diminishing size compared to the original plasmid (Figure 9, page 58).

Figure 9 A series of nested deletions was generated for pLST973A.



Sequencing *tyl* DNA from pLST973A.

DNA was sequenced manually using the dideoxy-chain termination method (Sanger *et. al.*, 1977), as described in Chapter 2, page 48. T7 DNA polymerase was used as the sequencing enzyme as it is more processive and has a higher rate of polymerisation than the Klenow fragment, allowing a greater length of sequence to be generated (Sambrook *et. al.*, 1989). It is also low in exonuclease activity and tolerant of nucleotide analogues (Pharmacia, T^7 sequencing kit). Due to the high rate of polymerisation, the primer extension reactions were carried out in two stages. Initially, labelling allowed extension from the primer in limiting concentrations of all four deoxynucleoside triphosphates (dNTPs) with the efficient incorporation of the one radiolabelled deoxynucleoside, [α-^{35}S] dATP, so that bands could be visualised by autoradiography. The termination stage involved four separate reactions in which high

concentrations of all four dNTPs and a specific dideoxynucleoside triphosphate (ddNTP) were present. ddNTP's lack the 3' OH group of conventional dNTPs and can therefore be added to the nascent DNA strand, but block further synthesis. These four sequencing reactions were then separated by electrophoresis.

Electrophoresis of sequencing products was carried out on 6% polyacrylamide gel under denaturing conditions (7 M urea). The sequence ladders (Figure 10, page 59) were visualised by autoradiography by exposing the gel for 48 h to X-ray film (Fuji RX).

Figure 10 A sequencing ladder corresponding to residues 75-153 of the nucleotide sequence of the coding strand of pLST973A (Figure 11, page 60). Each reaction, containing a different ddNTP (A, C, G and T), was run in a separate lane and the sequence ladder was visualised by autoradiography due to the incorporation of radioactivity, [α-^{35}S] dATP, into the products of sequencing.

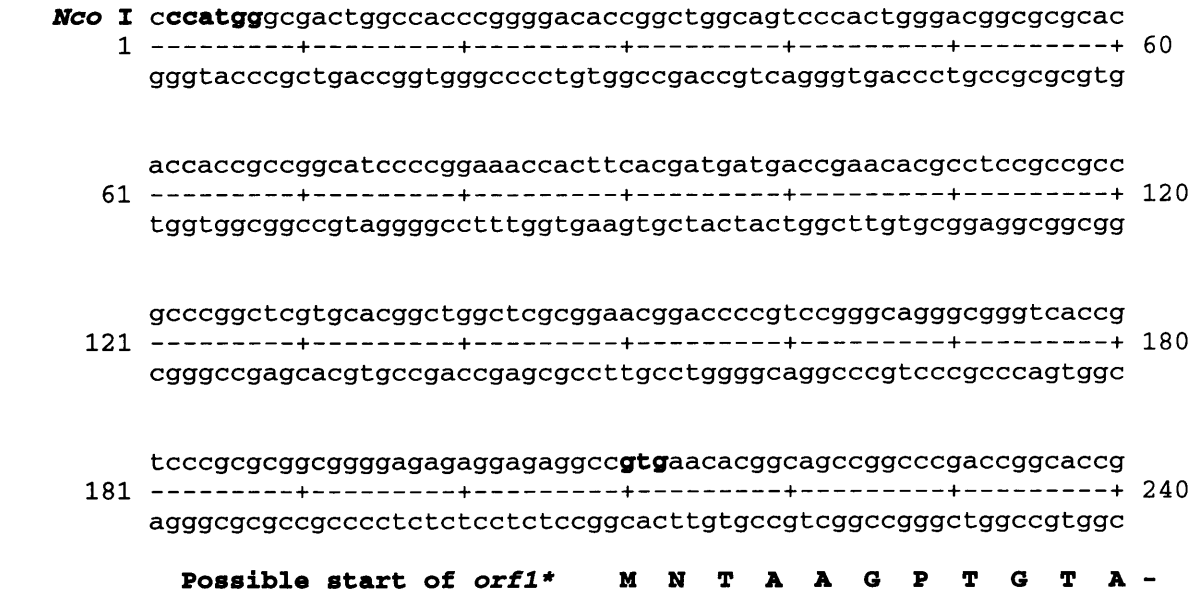
In order to reduce the likelihood of secondary structure in DNA during sequencing, nucleoside analogues, 7-deaza dGTP, and 7-deaza dATP (Mizusawa *et. al.*, 1986), were used to resequence each nested deletion in an attempt to resolve possible compressions (Deaza G/A ^{T7}Sequencing Mixes, Pharmacia). Each nested deletion overlapped with adjacent deletions for at least 50 base pairs. Primers were used to fill in any remaining gaps in the sequence, and were designed to be complementary to sequences which had been checked with deaza analogues. These oligonocleotides were at least 17 bases in length and contained an equal ratio of G+C to A+T bases. Primers containing inverted repeats, which might give rise to secondary structure, were avoided.

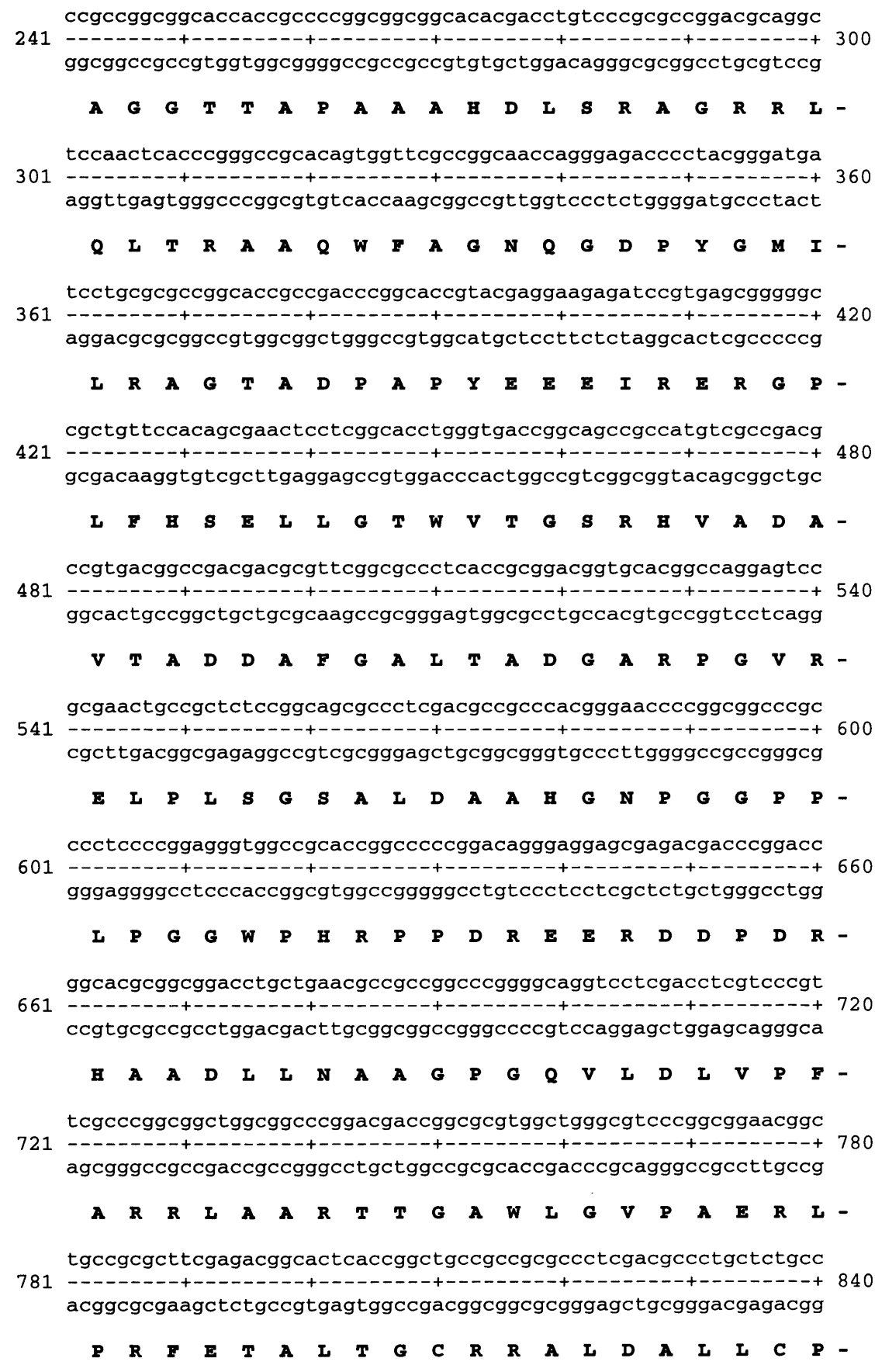
Results.

(a) Sequence analysis; identification of a putative open reading frame, *orf1.**

Double-stranded DNA sequence was generated from *tyl* DNA in pLST973A (Figure 11, page 60). Open reading frames are defined as stretches of DNA sequence devoid of stop codons. It is difficult to identify putative coding sequences in *Streptomyces* DNA because of the lack of out of frame, A+T rich, stop codons which means that potential open reading frames may be identified in a number of the six possible frames (Bibb *et. al.*, 1984). However, due to the degeneracy of the genetic code and the G+C richness of *Streptomyces* DNA, it is possible to identify putative open reading frames by codon preference computer analysis (Gribskov *et. al.*, 1984). Within a codon there are different amounts of flexibility in each nucleotide position. The most "wobble" occurs in the third position, and consequently in *Streptomyces* the frequency of G+C nucleotides in the third position is over 90%. This information can also indicate the direction of transcription due to the asymmetry of G+C frequencies across the codon (Bibb *et. al.*, 1985).

Figure 11 Sequence generated from *tyl* DNA in pLST973A, from the *Nco* I site to the *Xho* I site.




ccgccggcggcaccaccgccccggcggcggcacacgacctgtcccgcgccggacgcaggc
241 ---------+---------+---------+---------+---------+---------+ 300
ggcggccgccgtggtggcggggccgccgccgtgtgctggacagggcgcggcctgcgtccg
A G G T T A P A A A H D L S R A G R R L -
tccaactcacccgggccgcacagtggttcgccggcaaccagggagacccctacgggatga
301 ---------+---------+---------+---------+---------+---------+ 360
aggttgagtgggcccggcgtgtcaccaagcggccgttggtccctctggggatgccctact
Q L T R A A Q W F A G N Q G D P Y G M I -
tcctgcgcgccggcaccgccgacccggcaccgtacgaggaagagatccgtgagcgggggc
361 ---------+---------+---------+---------+---------+---------+ 420
aggacgcgcggccgtggcggctgggccgtggcatgctccttctctaggcactcgcccccg
L R A G T A D P A P Y E E E I R E R G P -
cgctgttccacagcgaactcctcggcacctgggtgaccggcagccgccatgtcgccgacg
421 ---------+---------+---------+---------+---------+---------+ 480
gcgacaaggtgtcgcttgaggagccgtggacccactggccgtcggcggtacagcggctgc
L F H S E L L G T W V T G S R H V A D A -
ccgtgacggccgacgacgcgttcggcgccctcaccgcggacggtgcacggccaggagtcc
481 ---------+---------+---------+---------+---------+---------+ 540
ggcactgccggctgctgcgcaagccgcgggagtggcgcctgccacgtgccggtcctcagg
V T A D D A F G A L T A D G A R P G V R -
gcgaactgccgctctccggcagcgccctcgacgccgcccacgggaaccccggcggcccgc
541 ---------+---------+---------+---------+---------+---------+ 600
cgcttgacggcgagaggccgtcgcgggagctgcggcgggtgcccttggggccgccgggcg
E L P L S G S A L D A A H G N P G G P P -
ccctccccggagggtggccgcaccggcccccggacagggaggagcgagacgacccggacc
601 ---------+---------+---------+---------+---------+---------+ 660
gggaggggcctcccaccggcgtggccgggggcctgtccctcctcgctctgctgggcctgg
L P G G W P H R P P D R E E R D D P D R -
ggcacgcggcggacctgctgaacgccgccggcccggggcaggtcctcgacctcgtcccgt
661 ---------+---------+---------+---------+---------+---------+ 720
ccgtgcgccgcctggacgacttgcggcggccgggccccgtccaggagctggagcagggca
H A A D L L N A A G P G Q V L D L V P F -
tcgcccggcggctggcggcccggacgaccggcgcgtggctgggcgtcccggcggaacggc
721 ---------+---------+---------+---------+---------+---------+ 780
agcgggccgccgaccgccgggcctgctggccgcgcaccgacccgcagggccgccttgccg
A R R L A A R T T G A W L G V P A E R L -
tgccgcgcttcgagacggcactcaccggctgccgccgcgccctcgacgccctgctctgcc
781 ---------+---------+---------+---------+---------+---------+ 840
acggcgcgaagctctgccgtgagtggccgacggcggcgcgggagctgcgggacgagacgg
P R F E T A L T G C R R A L D A L L C P -

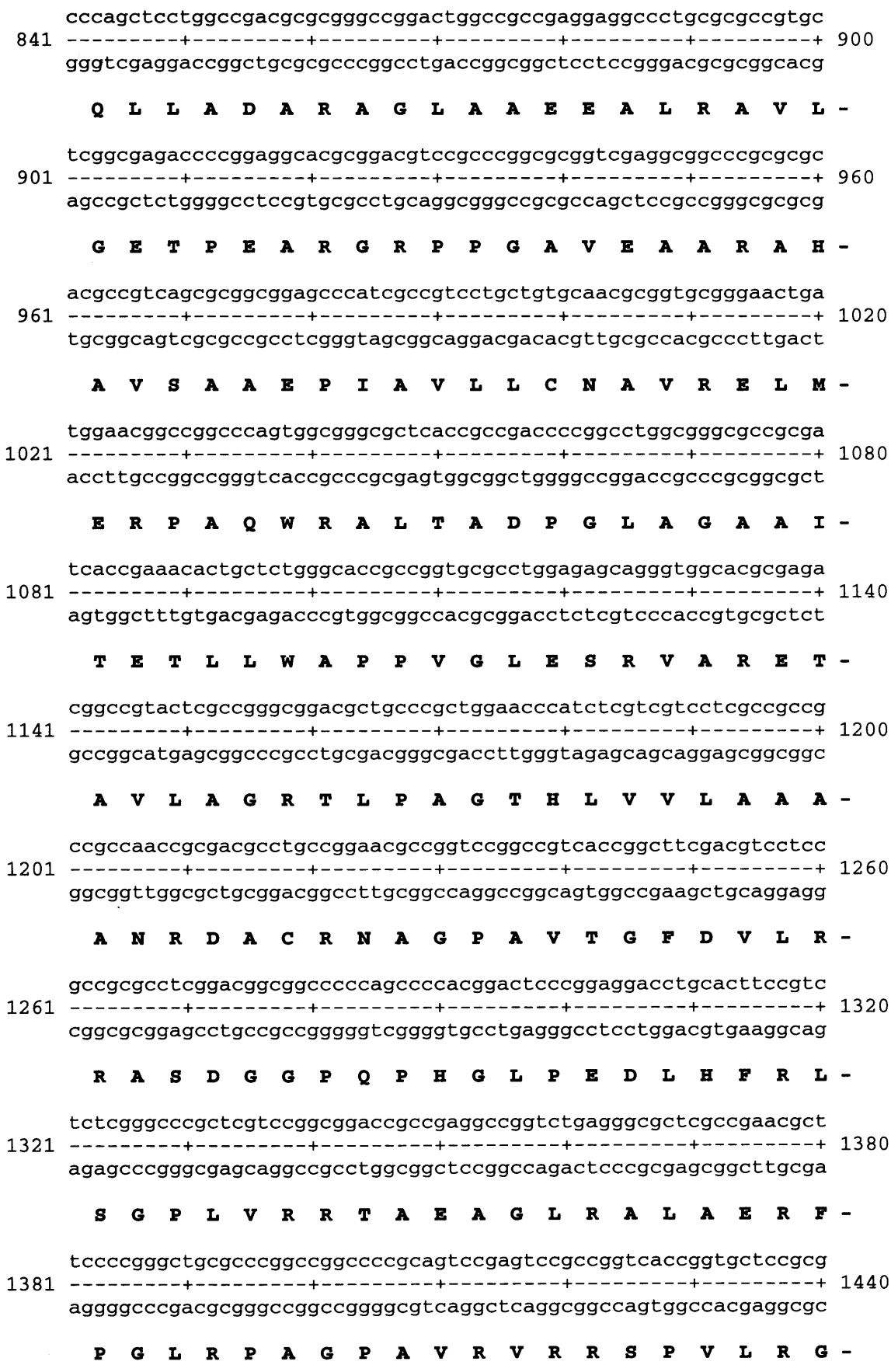
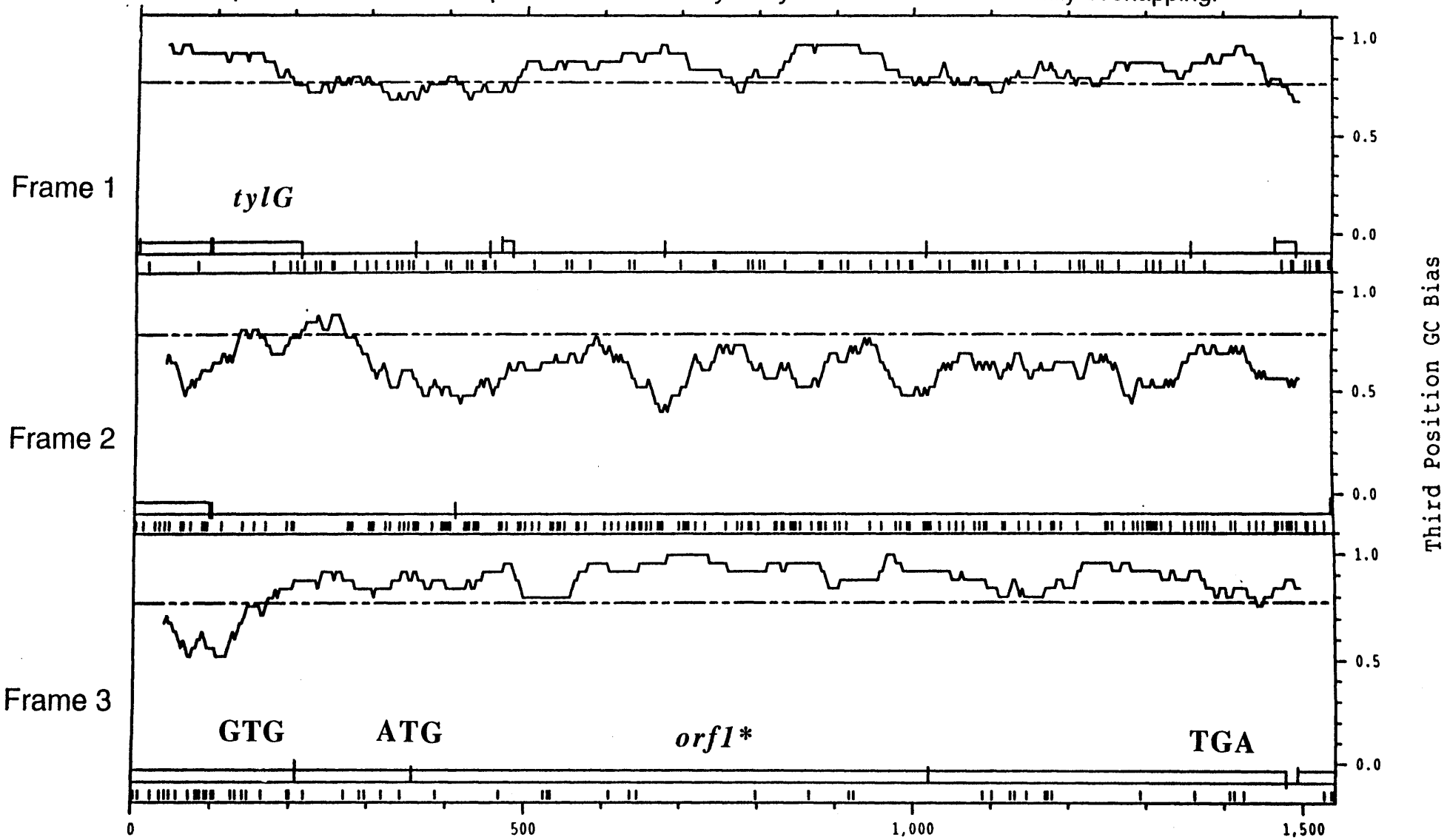

cccagctcctggccgacgcgcgggccggactggccgccgaggaggccctgcgcgccgtgc
841 ---------+---------+---------+---------+---------+---------+ 900
gggtcgaggaccggctgcgcgcccggcctgaccggcggctcctccgggacgcgcggcacg
Q L L A D A R A G L A A E E A L R A V L -
tcggcgagaccccggaggcacgcggacgtccgcccggcgcggtcgaggcggcccgcgcgc
901 ---------+---------+---------+---------+---------+---------+ 960
agccgctctggggcctccgtgcgcctgcaggcgggccgcgccagctccgccgggcgcgcg
G E T P E A R G R P P G A V E A A R A H -
acgccgtcagcgcggcggagcccatcgccgtcctgctgtgcaacgcggtgcgggaactga
961 ---------+---------+---------+---------+---------+---------+ 1020
tgcggcagtcgcgccgcctcgggtagcggcaggacgacacgttgcgccacgcccttgact
A V S A A E P I A V L L C N A V R E L M -
tggaacggccggcccagtggcgggcgctcaccgccgaccccggcctggcgggcgccgcga
1021 ---------+---------+---------+---------+---------+---------+ 1080
accttgccggccgggtcaccgcccgcgagtggcggctggggccggaccgcccgcggcgct
E R P A Q W R A L T A D P G L A G A A I -
tcaccgaaacactgctctgggcaccgccggtgcgcctggagagcagggtggcacgcgaga
1081 ---------+---------+---------+---------+---------+---------+ 1140
agtggctttgtgacgagacccgtggcggccacgcggacctctcgtcccaccgtgcgctct
T E T L L W A P P V G L E S R V A R E T -
cggccgtactcgccgggcggacgctgcccgctggaacccatctcgtcgtcctcgccgccg
1141 ---------+---------+---------+---------+---------+---------+ 1200
gccggcatgagcggcccgcctgcgacgggcgaccttgggtagagcagcaggagcggcggc
A V L A G R T L P A G T H L V V L A A A -
ccgccaaccgcgacgcctgccggaacgccggtccggccgtcaccggcttcgacgtcctcc
1201 ---------+---------+---------+---------+---------+---------+ 1260
ggcggttggcgctgcggacggccttgcggccaggccggcagtggccgaagctgcaggagg
A N R D A C R N A G P A V T G F D V L R -
gccgcgcctcggacggcggcccccagccccacggactcccggaggacctgcacttccgtc
1261 ---------+---------+---------+---------+---------+---------+ 1320
cggcgcggagcctgccgccgggggtcggggtgcctgagggcctcctggacgtgaaggcag
R A S D G G P Q P H G L P E D L H F R L -
tctcgggcccgctcgtccggcggaccgccgaggccggtctgagggcgctcgccgaacgct
1321 ---------+---------+---------+---------+---------+---------+ 1380
agagcccgggcgagcaggccgcctggcggctccggccagactcccgcgagcggcttgcga
S G P L V R R T A E A G L R A L A E R F -
tccccgggctgcgcccggccggccccgcagtccgagtccgccggtcaccggtgctccgcg
1381 ---------+---------+---------+---------+---------+---------+ 1440
aggggcccgacgcgggccggccggggcgtcaggctcaggcggccagtggccacgaggcgc
P G L R P A G P A V R V R R S P V L R G -

Figure 12 CODONPREFERENCE analysis for *tyl* DNA sequence generated from pLST973A (the coding strand). The positions of two possible translational start codons (GTG/ ATG) and a stop codon (TGA) for *orf1** are indicated in frame 3. The incomplete ORF in frame 1 represents the end of *tylG*. *tylG* and *orf1** are terminally overlapping.



```
     gtctcggccggctgcccgtcgccccgtatgtccccgagtgagaagggcactggatgaccg
1441 ---------+---------+---------+---------+---------+---------+ 1500
     cagagccggccgacgggcagcggggcatacaggggctcactcttcccgtgacctactggc

      Possible start of orf2*              M  R  R  A  L  D  D  R  -
       L  G  R  L  P  V  A  P  Y  V  P  E  *  stop of orf1*

     ccgccgaggaccgcacggaccggaagggaaaccgccgatgcgggtactgctgacctgtat
1501 ---------+---------+---------+---------+---------+---------+ 1560
     ggcggctcctggcgtgcctggccttccctttggcggctacgcccatgacgactggacata

      R  R  G  P  H  G  P  E  G  K  P  P  M  R  V  L  L  T  C  I  -

     cgcgcacaacacccactactacaacctggtgccggtcgcctgggccctgagagcggccgg
1561 ---------+---------+---------+---------+---------+---------+ 1620
     gcgcgtgttgtgggtgatgatgttggaccacggccagcggacccgggactctcgccggcc

      A  H  N  T  H  Y  Y  N  L  V  P  V  A  W  A  L  R  A  A  G  -

     acacgaggtgcgggtggccgcgcagcccgccctcaccgacacgatcaccgcctccggact
1621 ---------+---------+---------+---------+---------+---------+ 1680
     tgtgctccacgcccaccggcgcgtcgggcgggagtggctgtgctagtggcggaggcctga

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

     gaccgccgtgccggtcggcggcaacgagtccgtgctcgag Xho I site
1681 ---------+---------+---------+---------+ 1720
     ctggcggcacggccagccgccgttgctcaggcacgagctc

      T  A  V  P  V  G  G  N  E  S  V  L  E
```

CODONPREFERENCE (University of Wisconsin Genetics Computer Group program) was used to analyse third position codon bias in different reading frames of the sequence generated from pLST973A (Figure 12, page 63). Putative coding regions were correlated with regions of strong G+C bias in the third position, and appropriate positioning of start and stop codons. This programme only recognises ATG start codons, however, and in *Streptomyces* GTG is used more often than ATG as a start signal (Hopwood *et. al.,* 1985). The upstream sequence, beyond the first ATG, did contain an in frame GTG codon which overlapped the TGA stop codon of *tylG* . This GTG codon was at position 210, at the start of the region of strong G+C bias in the third position, and was therefore assumed to be the translational start point. The overlap between the stop codon of *tylG* and the start codon of *orf1** suggests that *tylG* and *orf1** may be transcriptionally and, perhaps, translationally coupled. The next in frame TGA, at position 1481, was assumed to be the *orf1** stop codon. The sequence was deduced to encode a 423 amino acid protein using TRANSLATE (UWGCG program).

(b) What could *orf1 encode?**

Protein sequence data bases were examined with ORF1* using a BLAST (Basic Local Alignment Search Tool) search (Altschul *et. al.*, 1990). No significant matches to the *orf1** protein sequence were identified. Most of the higher scoring matches were cytochrome

P450's, such as EryK from the erythromycin cluster of *Saccharopolyspora erythraea*, (Stassi *et al.*, 1993), and TylI from *Streptomyces fradiae* (Merson-Davies *et. al.*, 1994). However, BESTFIT and MOTIF analysis (UWGCG program) revealed that both the cysteine haeme-iron ligand signature and the oxygen binding pocket were not present in the *orf1** protein. The absence of these motifs was reflected by the moderate levels of identity between these matches and the *orf1** protein (TylI 21%; and EryK 25%). However, comparison of the *orf1** protein with the amino acid sequence of *eryCII* (ORF9) from the erythromycin cluster of *Sac. erythraea* (P. F. Leadlay, unpublished data), identified a closer match, of 38% identity. Also, a match was found with the protein sequence of *dnrQ* (35% identity, Figure 13, page 65), a gene isolated from the daunorubicin (DNR) producer *Streptomyces peucetius* (Otten *et al.*, 1995). Functions of these genes are not known, but they are present in *Streptomyces* species that incorporate amino sugars into the antibiotics they produce (Figure 14, page 66).

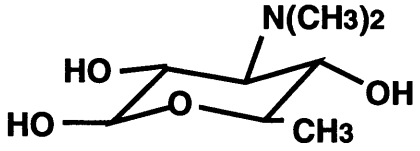
Figure 13 Bestfit of *dnrQ* and *orf1** amino acid sequences.

```
 12 AGGTTAPAAAHDLSRAGRRLQLTRAAQWFAGNQGDPYGMILRAGTADPAP 61
 2  PTPTSAPPAAPTDSELGRHLLTVRGFHFVFGALGDPYARRLR.GEADHLS 50

 62 YEEEIRERGPLFHSELLGTWVTGSRHVADAVTADDAFGALTADGARPGVR 111
 51 LGELVRDRGPL.HGSALGTWVTADGGISARLLDDPLLGPRHPASEGPQEH 99

112 EL.............PLSGSALDAAHGNPGGPPLPGGWPHRPPDREERDD 148
100 VLENVWETWRTCHVTPLGEDLLTPAAADSDRLAALLGPVLGPRTCTAWQV 149

149 PDRHAADLLNAAGPGQVLDLVPFARRLAARTTGAWLGVPAERLPRFETAL 198
150 DAGRAVHRVLDGLPPHFDVVSDLARPAIAGSLAAVLGLPDEARAELPDLL 199

199 TGCRRALDALLCPQLLADARA.....................GLAAEEALR 228
200 AACGPVLDSALCPPRLPVARAMTQALRRVRELMAAAVANHLTAPADGAVS 249

229 AVLGETPEARGRPPGAVEAARAHAVSAAEPIAVLLCNAVRELMERPAQWR 278
250 ALLAVDPGGGRDPGDTVTAAVLSTVVGAETAITTVANAVMALLKHDEQWS 299

279 ALTADPGLAGAAITETLLWAPPVRLESRVARETAVLAGRTLPAGTHLVVL 328
300 LLRADPGRAADAVEETLRWAPPVTLRSLITQGEVQIGGETLEADQHVVVL 349

329 AAAANRDACRNAGPAVTGFDVLRRASDGGPQPHGLPEDLHFRLSGPLVRR 378
350 VDAAQRDPALYEDPDRFRLD...RPRSPGFTHMALAGRDHLGLVAPLVRV 396

379 TAEAGLRALAERFPGLRPAGPAVRVRRSPVLRGLGRLPVAP 419 ORF1*
397 QCTAVLRALAERLPGLRAEGEPLRRGRSPVVRAPLSLRLAQ 437 DNRQ
```

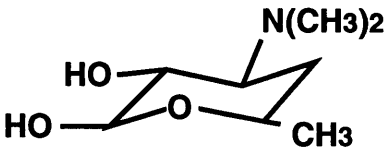
Sac. erythraea adds TDP-desosamine to 3-α–mycarosyl-eryronolide B; *S. peucetius* transfers daunosamine to the aglycone part of DNR (ε-rhodomycinone); and mycaminose is the first sugar to be added to tylactone during the biosynthesis of tylosin in *S. fradiae*.

Figure 14 Structures of three amino sugars found in certain polyketide antibiotics; mycaminose from tylosin; desosamine from erythromycin; and daunosamine from daunorubicin.

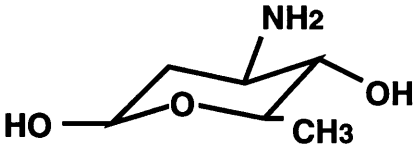
mycaminose



desosamine



daunosamine



orf1, eryCII,* and *dnrQ* are located in similar positions in their respective gene clusters. Both *orf1** and *eryCII* are located immediately downstream of polyketide synthase genes in *S. fradiae* and *Sac. erythraea*, respectively. All three genes are thought to be located upstream of the glycosyltransferase that is specific to each amino sugar. In *S. peucetius dnrQ* is located upstream of *dnrS* which is thought to encode the enzyme that adds TDP-daunosamine to ε-rhodomycinone (Otten *et. al.*, 1995). *orf2** (Figure 7, page 55) which encodes the mycaminosyl-glycosyltransferase in *S. fradiae* (page 67) is located downstream of *orf1**, and *eryCIII* (ORF 8) may encode the glycosyltransferase that interacts with TDP-desosamine in the biosynthesis of erythromycin (Katz, personal communication). None of these three amino acid sequences revealed matches in the MOTIF file (UWGCG program), but the function of *orf1** could be elucidated through complementation of *tylL* and *tylM* mutants, and more directly through gene disruption experiments. However, analysis of a strain of *Streptomyces peucetius* disrupted in

dnrQ has not yet determined the exact role of DNRQ in daunosamine biosynthesis (Otten *et. al.*, 1995), as the insertion of a kanamycin resistance gene into *dnrQ* has a polar effect on *dnrS*.

(c) Other genes in the *tylLM* region (Gandecha *et. al.*, 1997).

Downstream of *orf1**, three other tylosin genes (Figure 7, page 55) have been identified in the *tylLM* region by sequence analysis; *orfs 2*-4** (Gandecha *et. al.*, 1997). *tylG* and *orf1**, *orfs 1** and *2**, and *orfs 3** and *4** are all terminally overlapping, suggesting that these genes may be transcriptionally and possibly translationally coupled. The gap between *orfs 2** and *3** is only 8 bp. Genes involved in deoxyhexose sugar metabolism in antibiotic producers have often been investigated by comparing their encoded amino acid sequences with protein seqences, or characterised gene products, derived from other biosynthetic clusters (Lui *et. al.*, 1994). This kind of analysis has also been applied to genes identified in the *tylLM* region of *S. fradiae*, although additional data has been obtained through complementation studies (Chapter 4, page 71), and protein purification (Gandecha *et. al.*, 1997).

The Orf2* protein (452 amino acids) is the glycosyltransferase which adds mycaminose to tylactone. Evidence that *orf2** encodes a glycosyltransferase includes convincing matches with a number of characterised or putative *Streptomyces* glycosyltransferase protein sequences. ORF2* exhibits end to end similarity with MGT (macrolide glycosyltransferase, accession number M74717; Jenkins *et. al.* 1991), an enzyme produced by *Streptomyces lividans* TK21 which inactivates macrolide antibiotics using UDP-glucose (Cundliffe, 1992). The most convincing match (50% identity) is between the *orf2** protein and the *eryCIII* product from *Sac. erythraea* (P. F. Leadlay, personal communication) reflecting the strong possibility that both proteins interact with amino sugars; the *eryCIII* (ORF8) product with desosamine in the biosynthesis of erythromycin; and ORF2* with mycaminose in the production of tylosin. Fermentation studies carried out with a strain of *S. fradiae* disrupted in *orf2** (Dr Steven Fish, personal communication) have shown that this leision is specific to the addition of mycaminose to tylactone.

The *orf3** product (254 aminoacids) is thought to be the methyltransferase which methylates mycaminose (dTDP-3-amino, 6-deoxyglucose). Convincing matches have been made with other methyltransferase protein sequences for example, ORF3* shares 47% sequence identity with SrmX (Geistlich *et. al.*, 1992) from the spiramycin producer *S. ambofaciens;* and three consensus sequence motifs have been identified in both methyltransferases, which use S-adenosyl-

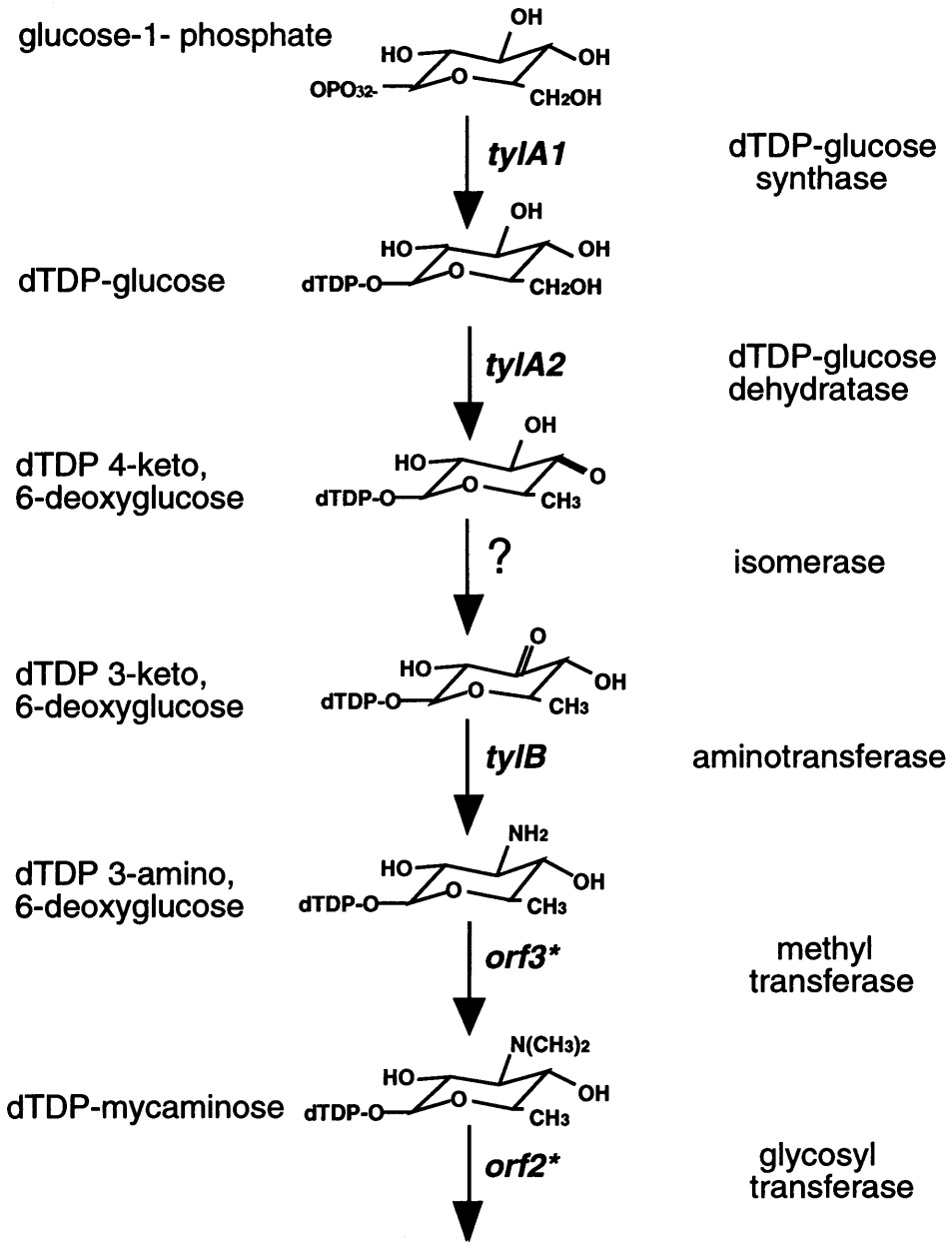
methionine (SAM) as co-substrate (Kagan *et. al.*, 1994). *orf3** was shown to be a mycaminose specific gene, by integrating a copy of *orf3** downstream of a constitutive promoter in the *tylM* mutant and restoring tylosin biosynthesis in *trans* (Figure 22, page 80, Chapter 4; Figure 41a, page 107, Chapter 5). The Orf3* protein was also purified, and its ability to bind [methyl-^{3}H]SAM was demonstrated by gel filtration chromatography (Gandecha *et. al.*, 1997).

*orf4** is thought to be a crotonyl-CoA reductase gene (*ccr*)which encodes a product of 423 amino acids. The protein sequence shows 78% identity to the crotonyl-CoA reductase isolated from *S. collinus* (Wallace *et. al.*, 1995). Crotonyl-CoA reductases are involved in the biosynthesis of butyryl-CoA which is a substrate for polyketide biosynthesis. ORF4* is probably involved with the conversion of acetoacetyl-CoA to butyryl-CoA, which is then converted to ethylmalonyl-CoA (Omura *et. al.*, 1977) and used as an extender unit in polyketide metabolism.

(d) Genes from the *tylLM* region involved in mycaminose biosynthesis.

Biosynthesis of the tylosin sugars occurs via the conversion of glucose-1-phosphate to dTDP-d-glucose and then to dTDP-4-keto, 6- deoxyglucose, a likely common intermediate in the synthesis of all three sugars.

Figure 15 The pathway to mycaminose.



The *tyllBA* region encodes these early steps (Figure 15, page 69); *tylA1* encodes a dTDP-glucose

synthase; *tylA2* encodes a dTDP-glucose dehydratase; and *tylB* is a putative aminotransferase which is specific to mycaminose biosynthesis (Merson-Davies *et. al.*, 1994).

Proteins encoded by *orfs2** and *3** from the *tylLM* region are also involved in the biosynthesis of the sugar mycaminose; *orf2** encodes the enzyme that adds mycaminose to tylactone, and ORF3* methylates dTDP-3-amino, 6-deoxyglucose.

Chapter 4 Complementation of *tylL* and *tylM* mutants.

Introduction.

Complementation of mutant strains can reveal information about the functions of different genes, and may also disclose patterns of gene expression; for example, do a series of genes have their own promoters or are they co-transcribed? The mutagen MNNG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) has been used to generate mutants of *S. fradiae* blocked in tylosin biosynthesis (Baltz *et. al.*, 1981). These strains were characterised by analysing the products of both cofermentation experiments with other mutant strains, and compounds generated when mutants were fed tylosin intermediates (Baltz *et. al.*, 1981). Fishman et. al. (1987) were able to distinguish further, mutants unable to biosynthesise or add mycaminose to tylactone (the *tylM* and *tylB* mutants) and mutants that cannot produce or add any of the three tylosin sugars (the *tylL* and *tylA* mutants) by complementation studies. Wild-type *S. fradiae* DNA carried by the cosmid pHJL309 complemented the *tylM* and *tylL* strains, but not the *tylB* or *tylA* mutants. From this cosmid the *tylLM* region was delimited to a *Kpn* I-*Bam*H I DNA fragment (Fishman *et. al.*, 1987) of approximately 8 kb (Figure 20, page 77).

Our aim was to complement the *tylL* and *tylM* mutants with single open reading frames from the *tylLM* region. *orfs 1*-4** had been identified by sequence analysis of the *tylLM* region (Gandecha *et. al.*, 1997) from *S. fradiae* c373.10 (Figure 7, page 55, Chapter 3), which was derived from the cosmid pMOMT4 (Beckman *et. al.*, 1989).

Complementing mutants; introducing DNA into strains of *Streptomyces fradiae*.

Genetic manipulation of *Streptomyces* is not as straightforward as with laboratory strains of *E. coli,* however. For example, they have awkward cell walls and therefore protoplasts must be generated to allow transformation. Different strains harbour different restriction modification systems and will therefore recognise and digest foreign DNA (Matsushima *et. al.*, 1987), with the exception of *Streptomyces lividans* 66. Fishman et. al. (1987) introduced plasmids into strains of *S. fradiae* by transformation. A highly transformable strain of *S. fradiae* (M-1) had to be used to passage plasmid before further transformation of mutant hosts could occur. M-1 was created using the mutagen MNNG from *S. fradiae* c373 (Baltz, 1978), and has been used to optimise transformation procedures for *S. fradiae* protoplasts (Matsushima *et. al.*, 1985). However, the limitations of transformation have been overcome by the use of conjugal transfer. Shuttle vectors have been constructed which are stably maintained in diverse hosts, for example allowing transfer of DNA from *E. coli* to *Streptomyces* (Mazodier *et. al.*, 1989). During conjugal transfer the DNA enters the recipient single-stranded, preventing cleavage by endogenous restriction endonucleases. Double-stranded DNA is then synthesised in the recipient and subsequently modified, thereby preventing digestion. Initial manipulations of cloned DNA may

therefore be carried out, more conveniently, in *E. coli* prior to conjugation into mutants of *S. fradiae*. Restoration of the ability to synthesise tylosin in mutants was measured in the following experiments using a simple plate assay which relies on the sensitivity of *Micrococcus luteus* T194 to tylosin.

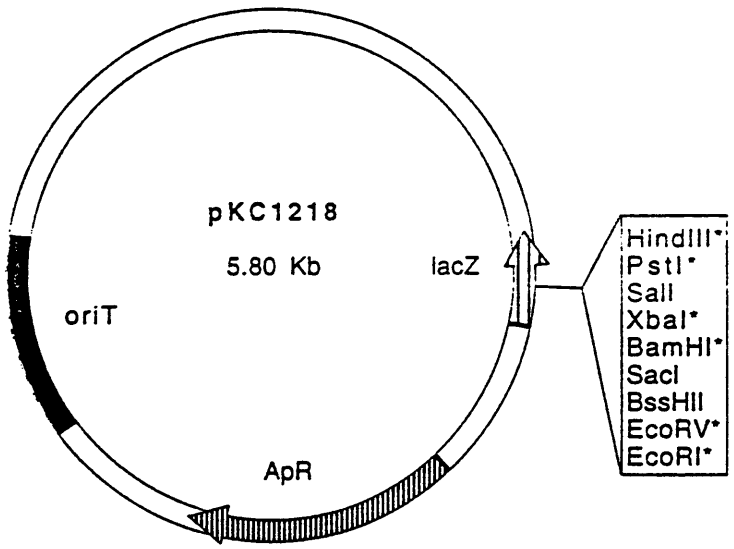
Conjugation.

Conjugal transfer was attempted between *E. coli* and strains of *Streptomyces fradiae*. The donor strain, *E. coli* S17-1, has a derivative of plasmid RP4 integrated into the chromosome which encodes the transfer functions necessary for conjugation (Simon *et. al.*, 1983).

(i) Conjugal plasmids.

The vectors pSET152 and pKC1218 have been used sucessfully in our laboratory for conjugation from *E. coli* to *S. fradiae* strains (Bierman *et. al.*, 1992). pSET152 is an integrative vector which uses the ϕC31 attachment site to insert into the host chromosome (Kuhstoss *et. al.*, 1991a). In contrast, pKC1218 (Figure 16, page 72) is a replicating plasmid which is maintained at a low copy number by the *Streptomyces* SCP2* origin of replication (Larson *et. al.*, 1986).

Figure 16 pKC1218 is a free replicating conjugal vector that can be used to transfer DNA from an *E. coli* donor strain, S17-1, to *Streptomyces*. * - unique sites in MCS.



Both of these plasmids contain pUC replication functions (Norrander *et. al.*, 1983) that are recognised in *E. coli*, and an origin of transfer (*oriT*) derived from RK2 (Guiney *et. al.*, 1983). The apramycin resistance gene (ApR) is used as a selectable marker in both genera, and recombinant plasmids can be identified in *E. coli* by blue-white selection. Other than different origins of replication, pSET152 is similar in structure to pKC1218 (Figure 16, page 72), except that the *Hind* III site in the MSC is not unique. pLST9828 and pLST9829 were generated by introducing the ermEp* promoter, which was derived from *ermE*p1 by deletion of TGG from the putative -35 region (Bibb *et. al.*, 1985, 1994), into both pSET152 and pKC1218 (Butler *et. al.*, manuscript in preparation). This promoter would be expected to allow constitutive expression of downstream DNA fragments in *Streptomyces* strains.

Methods.

Conjugation was carried out using a combination of protocols outlined by Bierman et. al. (1992) and Mazodier et. al. (1989). Mutant and wild-type strains of *S. fradiae* sporulated successfully on AS-1 media at 37°C. Spores were harvested and then pregerminated according to the protocol of Hopwood et. al. (1985).

Pre-germination of *Streptomyces* spores (Hopwood *et. al.*, 1985).

S. fradiae strains were incubated on AS-1 for 2 to 3 days until they had sporulated sufficiently. Spores were harvested by flooding the surface of a plate with 5 ml of SQ and scraping the spores off with a sterile loop, before transferring them to a sterile universal bottle. The spores were pelleted by centrifugation (3500 x g, 10 min, at 4°C), and were then resuspended in 5 ml 0.05 M TES buffer, pH adjusted to 8 with NaOH, and heated at 50°C for 10 min before they were cooled under the cold tap. An equal volume of double strength Pre-germination medium (1% yeast extract; 1% Difco casaminoacids; and 0.01 M $CaCl_2$ which had been autoclaved separately) was mixed with the spores and incubated at 37°C for 2-3 h. After centrifugation (3500 x g, 10 min, at 4°C) pregerminated spores were washed in 2 ml of TSB, and were finally resuspended by mixing, at a concentration of 10^8 cells per ml. The concentration of spores was intially estimated by using either a haemocytometer or by plating serial dilutions of spores onto AS-1 and incubating overnight at 37°C.

Conjugation.

E. coli S17-1 donor strains were grown at 37°C overnight in 10 ml LB broth in the presence of apramycin (25 μg/ml). Flocculation of bacteria was seen in overnight cultures of S17-1, and under the microscope pairing, presumably associated with conjugal transfer, was observed. Donors were pelleted and washed in LB before they were resuspended in 2 ml of LB broth. These *E. coli* cells were plated onto AS-1 agar at 10^8 (100 μl) and recipients (100 μl) at 10^7 colony forming units. After incubation of conjugation plates at 37°C overnight, plates were

overlayed with 4 ml *soft R2 agar containing 1.8 mg nalidixic acid to select against donors and 0.75 mg apramycin to select for exconjugants containing the plasmids pSET152/ pKC1218. These plates were incubated at 37°C for seven days. Putative exconjugants were lifted with a sterile toothpick onto AS-1 plates containing apramycin (25 μg/ml) and nalidixic acid (60 μg/ml), and were incubated at 37°C for three days.

*soft R2 agar: 103 g sucrose; 10 g glucose; 0.25 g K_2SO_4; 10.12 g $MgCl_2.6H_20$; 0.1 g Difco casaminoacids; 800 ml SQ. 0.7% agar was added to 80 ml volumes which were autoclaved. Before use, 0.2 ml trace elements; 1 ml 0.5% KH_2PO_4; 1.5 ml 20% L-proline; 8 ml 3.68% $CaCl_2.2H_2O$; and 10 ml 5.73% TES, pH adjusted to 7.2 with NaOH; were added asceptically to each bottle (Baltz, 1980).

Assay for the addition of mycaminose to tylactone.

Mycaminose is the first sugar to be added to tylactone and produces the first biologically active intermediate. Antibiotic activity can be measured using a simple plate assay which relies on the sensitivity of *Micrococcus luteus* T194 to tylosin and its active intermediates. *M. luteus* T194 was grown overnight at 37°C in LB broth, and 10 μl from this culture was then added to 4 ml of sloppy agar (1% tryptone; 0.5% yeast extract and 0.75% agar) which was poured onto an LB agar plate. When growing *S. fradiae* in liquid tylosin production medium MM-1 (Chapter 2, page 35), antibiotic production was assayed by moistening paper disks (6 mm in diameter, Whatman) with 20 μl of supernatant from the fermentation culture (Bauer *et. al.*, 1966). Supernatant was obtained by placing a sample of the fermentation culture into a 1.5 ml eppendorf vial and spinning the sample in a microcentrifuge (13, 000 rpm, 10 min), and sampling the top layer. Disks were carefully placed onto the surface of the sloppy agar, and the plates were incubated overnight at 37°C. When screening exconjugants, plugs of solidified tylosin production medium MM-1 (2% agar) were inoculated with exconjugants and incubated for seven days after which the plugs were placed on plates seeded with *M. luteus* T194 (Butler *et. al.*, manuscript in preparation). In cases where the free replicating plasmid pKC1218 was used, antibiotic selection had to be maintained. Therefore apramycin (25 μg/ ml) was added to MM-1, and an apramycin resistant strain of *M. luteus* T194 (generated by Dr Andrew Butler) was required.

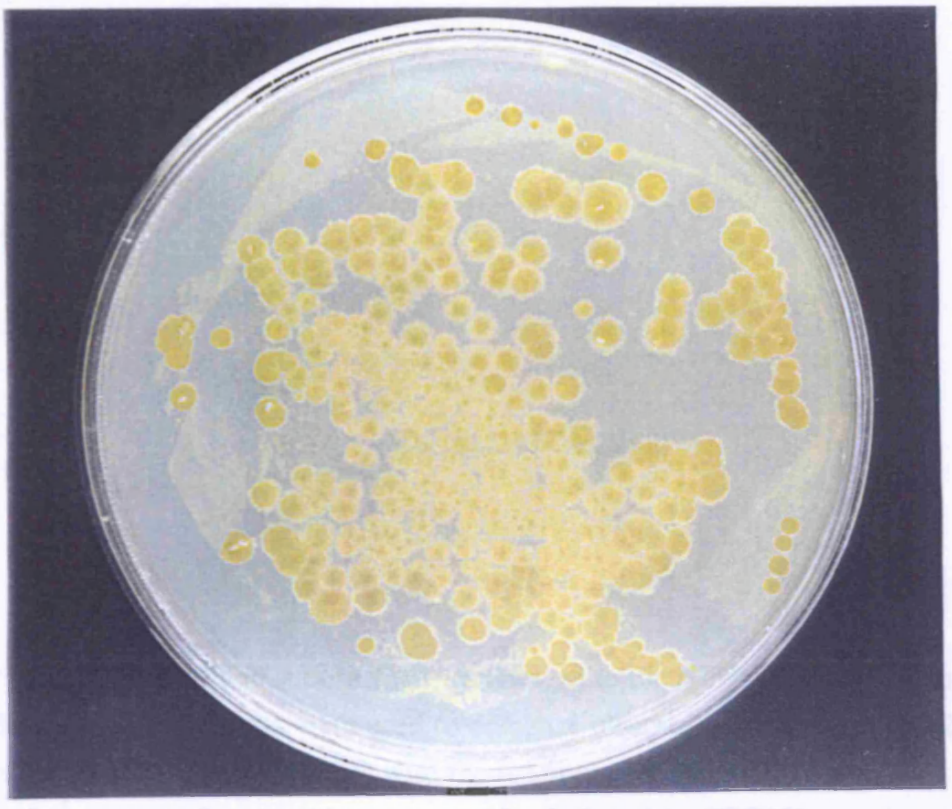
Results.

Demonstrating conjugation.

Initial control experiments to demonstrate conjugation involved introducing pKC1218 and pSET152 into c373.1, the *tylL* mutant and the *tylM* mutant (Figure 17, page 75). A number of controls were set up; plates with either only S17-1 or *Streptomyces* (which did not produce growth on the surface of the overlay); plates with no overlay (which allowed growth of both

organisms); and plates where the overlay contained nalidixic acid but no apramycin (only *Streptomyces* grew to the surface). Many more exconjugants were obtained with pSET152 than with pKC1218, perhaps because the integrated plasmid is more stable than the replicative form.

Figure 17 Exconjugants surfacing above the overlay. pKC1218 has been successfully conjugated into the *tylM* mutant from S17-1.



In order to demonstrate that exconjugants contained plasmid, pSET152 was detected by Southern hybridisation (Figure 18, page 76), and pKC1218 by reisolating the plasmid (Figure 19, page 76). Genomic DNA for Southern blotting was isolated from c373.1, with and without pSET152, and was totally digested with *Sst* I. These digests were transferred to a nitrocellulose membrane and hybridised (Chapter 2, pages 42) with the apramycin resistance gene, a 900 bp *Sst* I fragment derived from pSET152. This probe found itself in the strain carrying pSET152, but detected no target in the c373.1 DNA (Figure 18, page 76). Strains carrying pKC1218 were grown in 10 ml of YEME/ sucrose in the presence of apramycin (25 µg/ ml) for 3 days at 30°C, before plasmid was prepared as a small scale preparation (Chapter 2, page 45). It was difficult to visualise pKC1218, isolated from *Streptomyces,* by gel electrophoresis, probably because its low copy number in *Streptomyces* meant that plasmid yields were low. Therefore *E. coli* NM522 was transformed with pKC1218, isolated from the *tylM* mutant, to allow further restriction analysis. A satisfactory preparation of pKC1218 was obtained from *E. coli* NM522, and was digested with *Eco*R I and *Sst* I, and compared to digests of the original plasmid by gel electrophoresis (Figure 19, page 76).

Figure 18 pSET152 has been integrated into the genome of *Streptomyces fradiae* c373.1. *Sst* I total digests of genomic DNA which had been isolated from c373.1, with and without pSET152, were probed with the apramycin resistance gene (a 900 bp *Sst* I fragment, derived from pSET152).

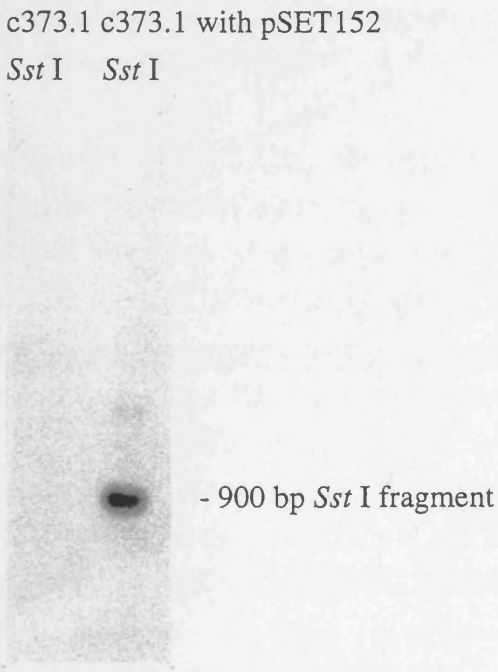


Figure 19 pKC1218 has been successfully transferred to the *tylM* mutant by conjugation. *Eco*R I (E) and *Sst* I (S) digests were used to compare plasmid isolated from GS62 and retransformed into *E. coli* NM522 (B) with the original pKC1218 (A).

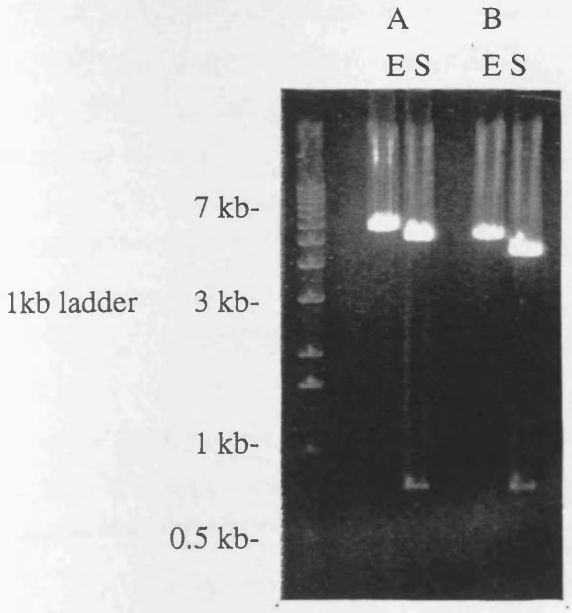
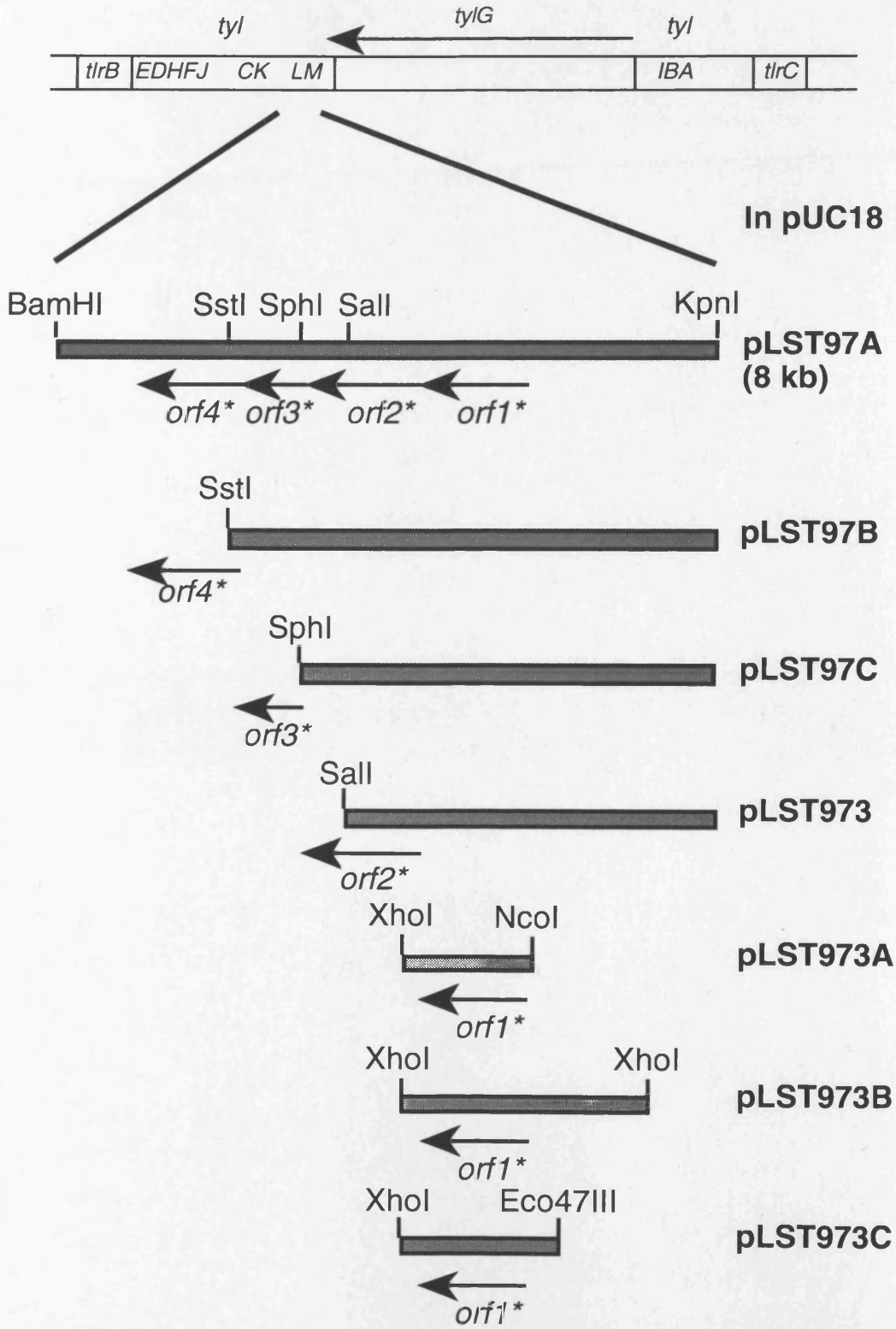


Figure 20 DNA fragments from the *tylLM* region used when complementing *tylL* and *tylM* mutants.



Introducing *tylLM* DNA into conjugal plasmids (Figure 6, Chapter 2, page 36).
A series of plasmids, based on pSET152 and or pKC1218, was constructed containing pieces of *S. fradiae* DNA from the wild-type *tylLM* region (Figure 20, page 77). These DNA inserts have been cut back, progressively, from the *Bam*H I site downstream of *tlrD,* towards the *Kpn* I site within the polyketide synthase genes. This would allow preservation of a promoter upstream of *orf1** which may allow cotranscription of *orf1*, orf2*, orf3*,* and *orf4**.

Pieces of DNA were derived from the pUC18-based plasmid pLST97 which carries an 11 kb *Bam*H I fragment containing the *tylLM* region (Figure 7, Chapter 3, page 55), which was derived from the cosmid pMOMT4 (Beckman *et. al.,* 1989). The respective pieces of DNA were introduced into pSET152 and or pKC1218 as shown in Figure 6, page 36, Chapter 2. ORFs were inserted so that the direction of transcription would be the same as that of the *lacZ* gene in both plasmids. Creation of such plasmids occurred in *E. coli* NM522 which allowed blue-white selection, before being used to transform the S17-1 donor strain. Generation of these constructs sometimes required filling in 3' recessed ends of DNA fragments (Chapter 2, page 40) and or an extra cloning step in pUC18 in order to generate the appropriate sites for insertion into the conjugal vectors. Plasmid DNA was prepared both large scale and small scale from *E. coli* by alkaline lysis (Chapter 2 page 45-46), and DNA was digested, isolated from gels and ligated according to standard protocols (Chapter 2, page 40).

An 8 kb *Kpn* I-*Bam* HI DNA fragment containing the *tylLM* region (Figure 20, page 77) was cleaved from pLST97, and ligated into pUC18 which had been digested with *Kpn* I and *Bam*H I. The resulting construct (pLST97A) was cleaved with *Eco*R I and *Bam*H I to release the same DNA fragment, except that it had different flanking restriction sites, allowing it to be inserted into the *Eco*R I and *Bam*H I sites in the MCS's of pKC1218 (to form pLST97A2) and pSET152 (to form pLST97A1). The *Kpn* I-*Sst* I DNA fragment (Figure 20, page 77) was isolated by cutting pLST97A with *Sst* I, which cuts both within the insert and upstream of the *Kpn* I site in the MCS of pUC18, before being inserted into the *Sst* I site of pUC18 (pLST97B). pLST97B (in the orientation whereby the *Kpn* I site was nearest to the *Eco*R I end of the pUC18 MCS), could then be cleaved with *Eco*R I and *Bam*H I, and the DNA insert ligated into pKC1218 (pLST97B2) and pSET152 (pLST97B1). The plasmid pLST97C was generated by digesting pLST97A with *Sph* I, which cuts both within the DNA insert and downsteam of the *Bam*H I site in the MCS of pUC18, and religating the remaining plasmid together. The *Kpn* I-*Sph* I fragment (Figure 20, page 77) was then released by cutting pLST97C with *Eco*R I and *Hin*d III, before it was inserted into the unique *Eco*R I and *Hin*d III sites in pKC1218 (pLST97C2). The *Kpn* I-*Sal* I fragment (Figure 20, page 77) was released from pLST973 by digesting with *Eco*R I and *Hin*d III, and was inserted into the *Eco*R I and *Hin*d III sites of pKC1218 to form pLST9732. The 3' recessed ends of this DNA fragment were also converted to blunt end termini (Chapter 2, page 40), and ligated

into pSET152, digested with *Eco*R V which had been dephosphorylated to limit religation of the vector DNA (pLST9731). pLST973A (orientation 2, Figure 8 (B), page 56, Chapter 3) was cut with *Eco*R I and *Bam*H I (Figure 20, page 77) and inserted into pSET152 (pLST973A1). An *Xho* I-*Xho* I fragment (Figure 20, page 77) released from pLST973 was blunt ended and ligated into the *Sma* I site of pUC18, which had been terminally dephosphorylated, to create the plasmid pLST973B. This plasmid was then digested with *Eco*R I and *Hin*d III, and the DNA fragment inserted into pKC1218 (pLST973B2). pLST973B was also digested with *Eco47* III and *Hin*d III, and the fragment of *tylLM* DNA released was ligated into pKC1218 cut with *Eco*R V and *Hin*d III (pLST973C2).

Conjugal plasmids containing DNA inserts from the *tylLM* region were then introduced into the *E. coli* donor strain by transformation before being transferred from S17-1 by conjugation into c373.1, the *tylL* mutant, and the *tylM* mutant. Plugs of tylosin production medium were inoculated with exconjugants and incubated for seven days at 30°C, after which tylosin production was assayed microbiologically (page 74).

Complementation of mutants.

The 8 kb *Kpn* I-*Bam*H I DNA fragment complemented both the *tylL* and *tylM* mutants, confirming the findings of Fishman et. al. (1987).

The *tylM* mutant.

The *tylM* mutant was complemented in *trans* by a fragment containing *orf3** and upstream DNA (pLST97B2), but complementation in *trans* was lost when *orf3** was deleted in pLST97C2 (Figure 21, page 80). This result was eventually strengthened by restoring tylosin biosynthesis with *orf3** downstream of a constitutive promoter, suggesting that it is involved in the biosynthesis or addition of mycaminose (Figure 22, page 80). *orf3** was amplified by PCR (Chapter 2, page 53) from the genome of c373.1 using two primers; P-TCTAGAAGATGCTTGCCGAACCGA -OH complementary to residues 2736-2753 of the *tylLM* DNA sequence, with an *Xba* I site located at the 5' end; and P-GTCCCACTTCACCGGGTTTCTCCCTT -OH complementary to residues 3617-3592 of the *tylLM* sequence. The PCR product was captured in pGEM-T (Promega) and the orientation was chosen that allowed *orf3** to be released by cleavage with *Xba* I (*Xba* I cuts at the 5' end of the first primer, but not in pGEM-T) and *Spe* I (*Spe* I which cuts the MCS of pGEM-T). The *Xba* I-*Spe* I DNA fragment was ligated into the *Xba* I site of pLST9828. The correct orientation of *orf3** downstream of ermEp* was confirmed by digesting the construct with *Xho* I which cuts the insert asymetrically (position 1715 of the *tylLM* sequence), and also cuts pLST9828 once (1.8 kb away from the *Eco*R I site).

Figure 21 c373.1 produced tylosin (1) in contrast to the *tylL* (2) and *tylM* mutants (6) when grown on solidified MM-1 medium. The *tylM* mutant was complemented in *trans* by a DNA fragment containing *orf3** and upstream DNA (5), but complementation was lost in this mutant when *orf3** was removed (4). The *tylL* mutant was complemented in *trans* by a fragment including the entire *orf2** and upstream DNA (3).

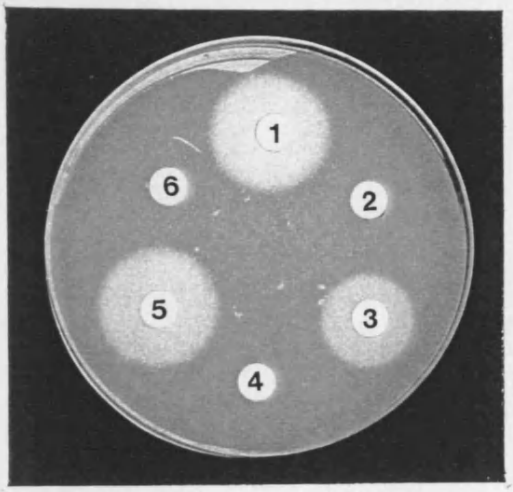
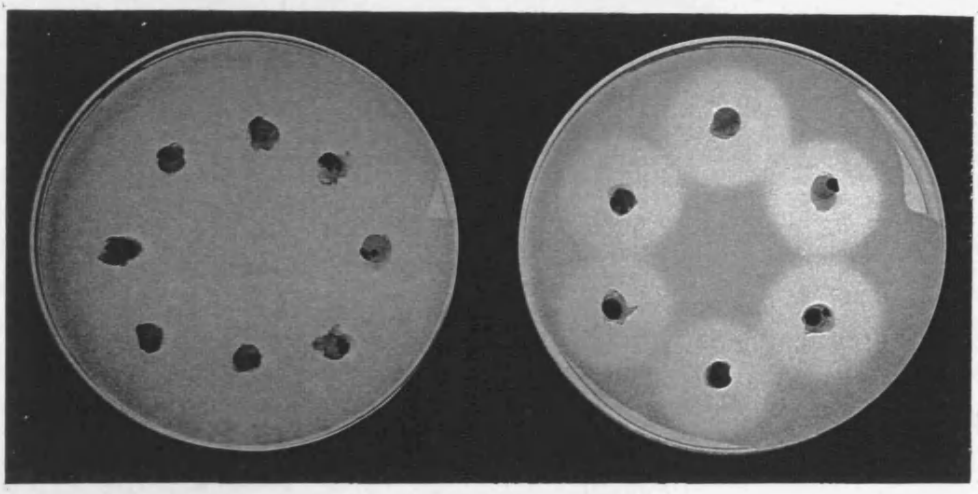


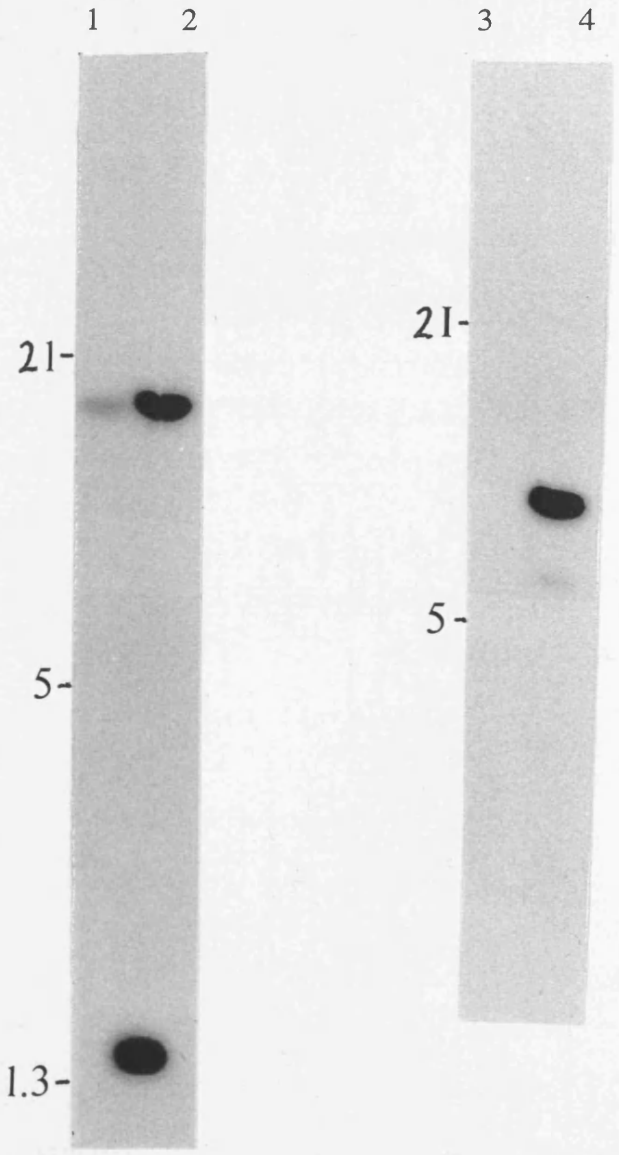
Figure 22 The *tylM* mutant is complemented in *trans* by *orf3** expressed from a constitutive promoter in the integrative plasmid pLST9828. *tylM* strains carrying the integrative plasmid pLST9828 do not produce zones.



pLST9828 pLST9828+*orf3**

tylM mutant

Figure 23 The presence of two copies of *orf3**, and the apramycin resistance gene in the complemented *tylM* mutant was confirmed by Southern hybridisation. Only a single copy of *orf3** was found in the *tylM* mutant. Total DNA digests probed with *orf3**; Lane 1, *tylM Bam*H I/ *Hin*d III; Lane 2, *tylM* with pLST9828+*orf3* Bam*H I/ *Hin*d III. Total DNA digests probed with the apramycin resistance gene; Lane 3, *tylM Bam*H I/ *Hin*d III; Lane 4, *tylM* with pLST9828+*orf3* Bam*H I/ *Hin*d III. Bacteriophage lambda digested with *Eco*R I and *Hin*d III was used as a size marker.



The presence of two copies of *orf3** in the *tylM* mutant complemented by pLST9828+*orf3** was confirmed by Southern hybridisation (Figure 23, page 81). Genomic DNA isolated from this strain (Chapter 2, page 42) and the *tylM* mutant, was digested with both *Bam*H I and *Hin*d III, which would release the *tylLM* region on a 11 kb *Bam*H I fragment, and *orf3** from pLST9828+*orf3** as a *Bam*H I-*Hin*d III fragment of about 1.3 kb. These digests were separated on a 0.5% agarose gel with a lambda ladder (digested with *Eco*R I and *Hin*d III), as a size marker. The DNA was transferred to a nitrocellulose membrane (Chapter 2, page 42) and probed with the *orf3** PCR product (released from pGEM-T by an *Xba* I/ *Spe* I digest), and the apramycin resistance gene (a 900 bp *Sst* I fragment from pSET152). Both DNA probes were labelled by the random primer method (Chapter 2, page 43) and hybridisation was carried out at 65°C overnight (Chapter 2, page 43). Two targets of 11 kb and 1.3 kb were found when the complemented strain was probed with *orf3**, corresponding to *orf3** within the *tylLM* region, and the integrated *orf3** PCR product (*Hin*d III cuts pSET152 a few hundred bp away from the *Xba* I site), respectively. In contrast, one target of 11 kb (*orf3** from the *tylLM* region) was found in the *tylM* mutant (Figure 23, page 81). The apramycin resistance gene hybridised to pLST9828, integrated in the genome of the complemented strain, but did not hybridise to *tylM* mutant DNA (Figure 23, page 81).

The *tylL* mutant.

Complementation of the *tylL* mutant has not been straightforward. A plasmid carrying the *Kpn* I-*Sph* I fragment (pLST97C2), which contains part of *orf3**, both *orf2** and *orf1**, and part of *tylG*, complemented this mutant *in trans* (Figure 21, page 80). However, when part (400 bp) of *orf2** was deleted (the *Kpn* I-*Sal* I fragment in pLST9732), the complementation frequency dropped from 100% to 20% (Figure 24, page 83). This implied that *orf1** might be responsible for complementing the mutation as it was the only intact ORF on this piece of *tyl* DNA, other than DNA upstream which contains polyketide synthase genes which are unlikely to be involved in sugar biosynthesis.

pLST973B2, pLST973C2 and pLST973A1, which contain *orf1** and varying amounts of upstream sequence (Figure 20, page 77) did not complement the mutation. In case any regulatory sequences relating to *orf1** expression had been removed in these plasmids, progressive deletions were made from the *Sal* I site, whilst preserving the *Kpn* I site. pLST973 was digested with *Hin*c II (an exonuclease sensitive site) and *Sph* I (to protect the rest of the vector from digestion), and a series of deletions were made (Chapter 2, page 46). Six of these deletions were sequenced in pUC18 using universal primer, so that the end of the deletion could be positioned within the *tylLM* sequence. These plasmids were then cut with *Eco*R I and *Hin*d III, and the deleted DNA inserted into into the *Eco*R I and *Hin*d III sites of pKC1218. These DNA inserts ranged from 800 bp to approximately 4.1 kb, the latter deletion retaining the first

600 bp of *orf2**. None of these plasmids appeared to restore tylosin production, with the exception of the largest DNA fragment which complemented at a frequency of 2%. Truncation of *orf2** in pLST9732 removes a motif which is common to a number of glycosyltransferases (Professor Cundliffe, personal communication), and without this highly conserved sequence the protein may not be expected to be functional. ORF2* encodes the enzyme that puts mycaminose onto tylactone and would therefore be expected to be a *tylM* gene (Chapter 3, page 67). Why is part of *orf2** therefore required to complement the *tylL* mutant? Has the DNA insert recombined with the mutant tylosin genes allowing restoration a defective part of the *orf2** gene, or a promoter inside *orf2** which controls the transcription of the downstream genes, *orf3** and *orf4**? This phenomena of 'incomplete complementation' has been observed by Malpartida et. al. (1986) in mutants blocked in actinorhodin biosynthesis, and was attributed to homologous recombination between wild-type and mutant DNA .

Homologous recombination in the *tylL* mutant.

The *tylL* mutant was complemented *in cis* by a DNA fragment lacking the complete *orf2** in a free replicating plasmid (pLST9732). *In cis* complementation occurred in 0-20% of exconjugants and producing strains exhibited a range of zone sizes (Figure 24, page 83); out of 45 exconjugants ten produced zones which ranged in size between 8 to 16 mm. The variation in zone size could be the result of differences in the timing of a homologous recombination event that occurred between the wild-type and mutant copies of the *tylLM* genes during the growth of exconjugants on solidified MM-1. It was important to establish whether complementation was operating *in trans,* or *in cis* via a recombination event.

Figure 24 The *tylL* mutant is complemented *in cis* by DNA fragments lacking the complete *orf2** in both the free replicating plasmid pKC1218 (plates A and B) and the integrative plasmid pSET152 (plate C).

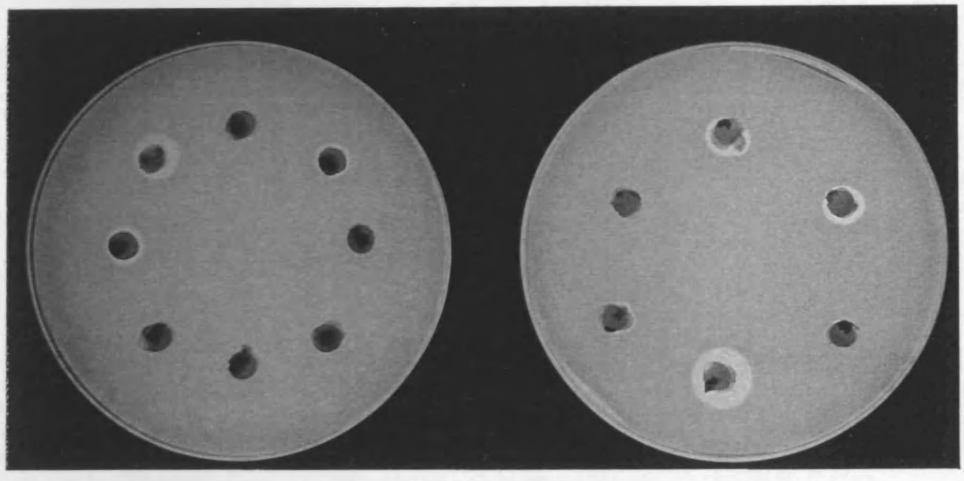
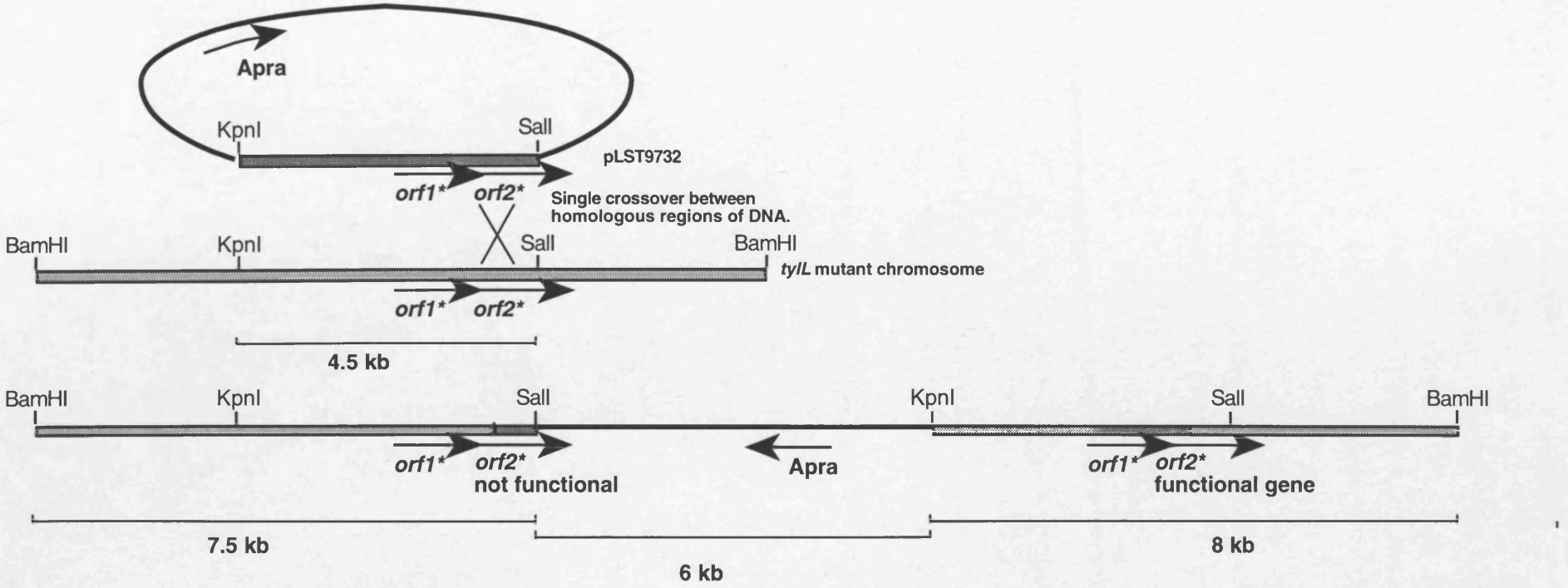


Plate A Plate B

Figure 25 Rescue of the defective *orf2 in the *tylL* mutant by recombination.**



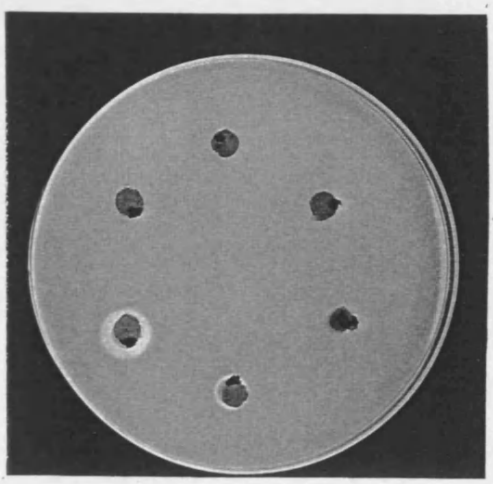
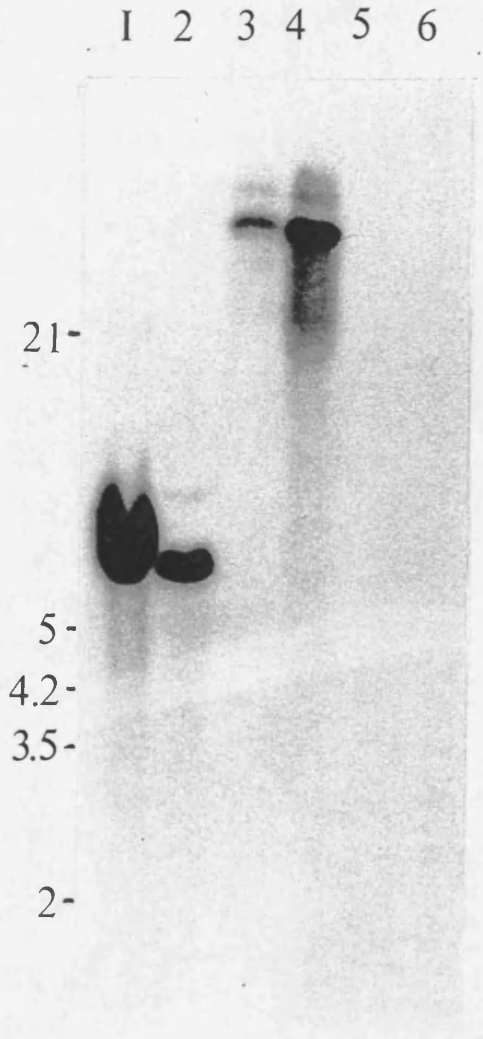


Plate C

In order to confirm that the wild-type phenotype is rescued by recombination, Southern hybridisation (Chapter 2, page 42) was carried out to demonstrate a single cross-over between *tylLM* DNA in pLST9732 and the genomic copy of the *tylLM* region in *tylL* strains producing tylosin (Figure 26, pages 86-87). Genomic DNA was prepared from producing *tylL* strains carrying pLST9732, which had been isolated after growth on plugs of solidified MM-1 medium containing apramycin (25 μg/ml). *Bam*H I digests of DNA from these *tylL* strains, c373.1, and the *tylL* mutant were compared by probing DNA with the apramycin resistance gene and the *orf3** PCR product. Both probes targeted a fragment of about 22 kb in the *tylL* strains carrying pLST9732 (Figure 26, pages 86-87). The *orf3** probe would be expected to light up an 11 kb *Bam*H I fragment (the *tylLM* region), as demonstrated by probing wild-type, *tylL* (Figure 26, page 87), and *tylM* DNA (Figure 23, page 81). The plasmid, pLST9732 does not contain *orf3** and would therefore remain undetected. The addition of pLST9732 DNA into the *tylL* genome through a single cross-over event, however, would double the size of the *tylLM* region to approximately 22 kb (Figure 25, page 84) as *Bam*H I does not cut pLST9732. Also, the apramycin resistance gene hybridises to a fragment of 22 kb in these strains, indicating that the plasmid has recombined with the genomic copy of the *tylLM* region (Figure 26, page 86).

Figure 26 To demonstrate complementation *in cis*, genomic DNA from *tylL* strains, and c373.1 were probed with the apramycin resistance gene and *orf3**. Genomic DNA was isolated from *tylL* exconjugants containing either pLST9731 or pLST9732 (both containing the incomplete *orf2** and upstream sequence), from producing and non-producing strains. The DNA was digested with enzymes so that a single cross over would be detected by probing with the apramycin resistance gene (Lanes 1-6) and the *orf3** PCR product (Lanes 7-12). Lanes 1 and 7, *tylL* producing strain carrying pLST9731 *Bam*H I/ *Eco*R I; Lanes 2 and 8, *tylL* non-producing strain carrying pLST9731 *Bam*H I/ *Eco*R I; Lanes 3 and 9, *tylL* producing strain carrying pLST9732 *Bam*H I; Lanes 4 and 10, *tylL* producing strain carrying pLST9732 digested with *Bam*H I; Lanes 5 and 11, c373.1 *Bam*H I; Lanes 6 and 12, *tylL* mutant *Bam*H I. Bacteriophage lambda digested with *Eco*R I and *Hin*d III was used as a size marker.

Digests probed with the apramycin resistance gene



Digests probed with the *orf3** PCR product.

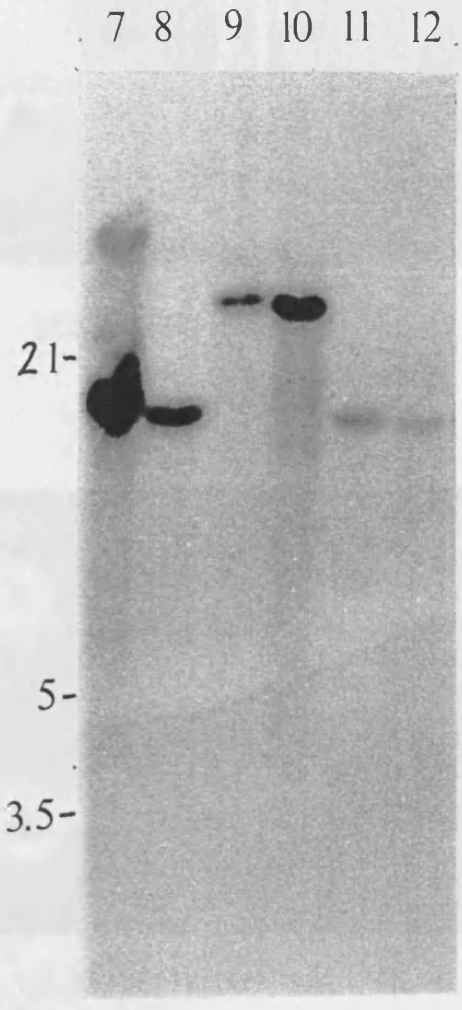
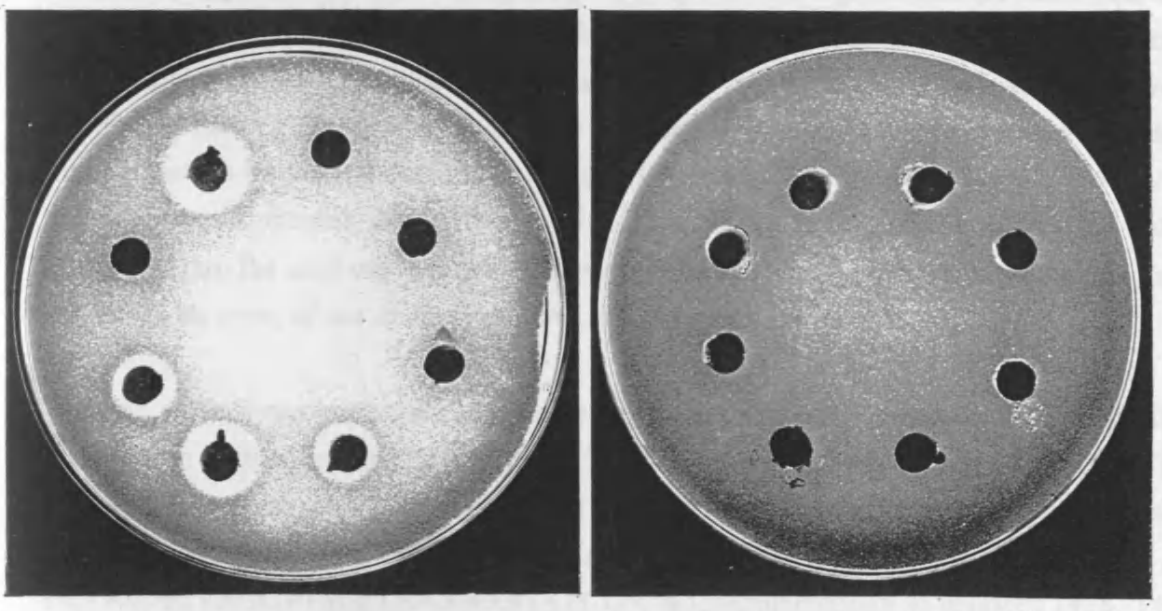
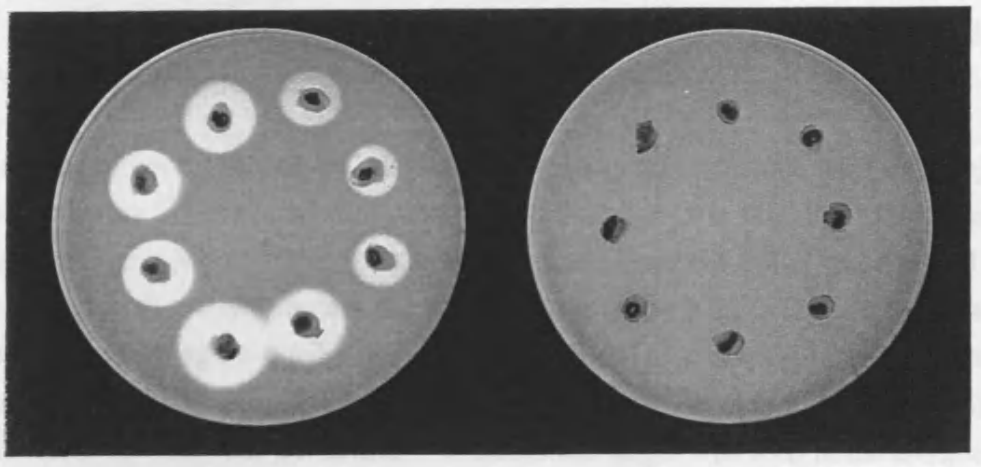


Figure 27 The *tylL* mutant was complemented *in trans* by *orf2** expressed from a constitutive promoter in both the integrative plasmid pLST9828 and the free replicating plasmid pLST9829. *tylL* strains carrying pLST9828 or pLST9829 do not produce zones. Both plasmids containing *orf2** appeared to exhibit instability; resistance to apramycin was often lost during 7 day incubation on solidified MM-1.



pLST9828+*orf2** pLST9828

tylL mutant



pLST9829+*orf2** pLST9829

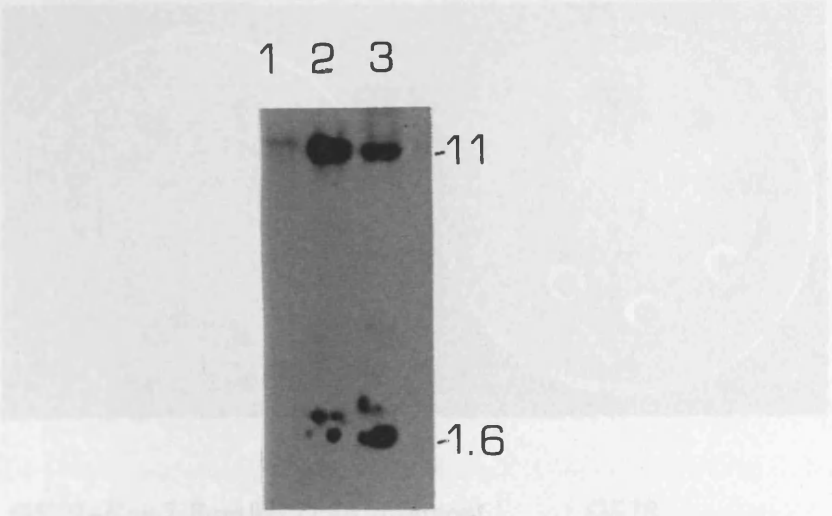
tylL mutant

Unexpectedly, *in cis* complementation also occurred when this DNA fragment was inserted into the *attB* site of the *tylL* mutant chromosome via the plasmid pLST9731 (Figure 24, page 85). DNA isolated from producing and non-producing *tylL* strains containing pLST9731 was digested with *Bam*H I and *Eco*R I (which would release the *tyl* DNA insert), before being probed with the apramycin resistance gene and the *orf3** PCR product. However, a single cross-over could not be demonstrated in the producer strain by Southern hybridisation (Figure 26, pages 86-87); *orf3** targeted an 11 kb DNA fragment (the *tylLM* region); an 8 kb fragment would be targeted as the result of a single cross-over event. The apramycin resistance gene detected pSET152, and no difference was observed between the producer and non-producer strains. This result implies that restoration of tylosin biosynthesis may have resulted from a double cross-over. However, unpublised data (E. T. Seno) refered to by Bierman et al. (1992), and experimental evidence of transformation frequencies obtained with ϕC31-based vectors (Kuhstoss *et. al.*, 1991b), suggests that site specific recombination mediated by the ϕC31 site is very efficient. Has the vector integrated into the *attB* site and then wild-type DNA has recombined with the mutant genes via a double-cross over, or has a single cross-over event been reversed?

The *tylL* mutant was eventually complemented *in trans* by *orf2** downstream of a constitutive promoter in both an integrative vector (pLST9828), and a free replicating vector (pLST9829) (Figure 27, page 88). *orf2** was amplified from the genome of C373.1 by PCR using the following primers; P-GAATTCGTGAGAAGGGCACTGGATGAC-OH and P-TCTAGACTACCTTTCCGGCGCGGATCG-OH (complementary to residues 1478 -1498 and 2815-2836 of the *tylLM* sequence, respectively) and was introduced into the vectors pRSETA (Invitrogen) and pGEM-T by Dr Steven Fish. pRSETA with *orf2** was cut with *Bam*H I and *Hin*d III releasing *orf2** on a 1.5 kb DNA fragment which could then be inserted into pLST9829 digested with *Bam*H I and *Hin*d III. This construct appeared to be unstable in the *tylL* mutant, and so the *orf2** PCR product was inserted as a *Bam*H I-*Spe* I fragment (derived from *orf2** inserted into pGEM-T) into the *Bam*H I/ *Xba* I sites of pLST9828.

The presence of two copies of *orf2** in the *tylL* mutant complemented with the integrative vector was confirmed by Southern hybridisation (Figure 28, page 90). The *orf2** PCR product released from pGEM-T as a *Bam*H I-*Spe* I fragment was used to probe *Bam*H I and *Hin*d III digests of DNA isolated from the *tylL* mutant and two strains carrying pLST9828+*orf2**. The digests were separated on a 0.7% agarose gel before being transferred to a nitrocellulose membrane (Chapter 2, page 42). The 11 kb *Bam*H I fragment containing the *tylLM* region produced a signal in the mutant and complemented *tylL* strains. An additional target was seen in the complemented strains, corresponding to a 1.6 kb *Bam*H I-*Hin*d III fragment, released from pLST9828+*orf2**.

Figure 28 The presence of two copies of *orf2** in the complemented *tylL* mutant was confirmed by Southern hybridisation. Genomic DNA isolated from *tylL* mutant (Lane 1), and two *tylL* mutant strains complemented with pLST9828+*orf2** (Lanes 2 and 3) were digested with *Bam*H I, and *Hin*d III, and were probed with the *orf2** PCR product. 1 kb ladder was used as a size marker.

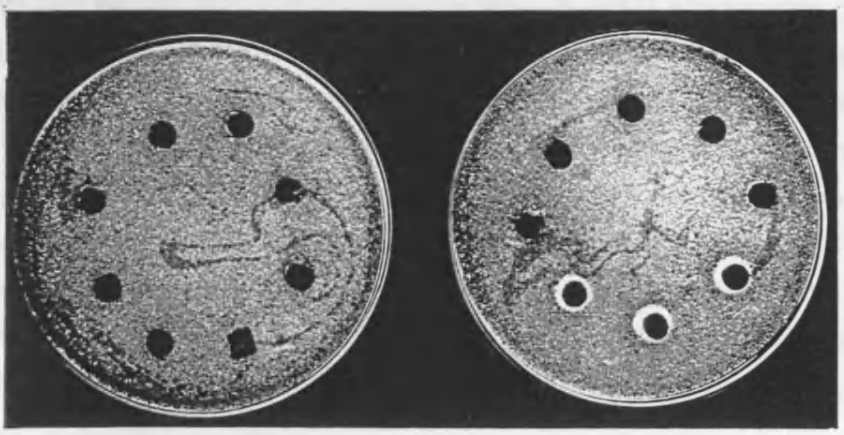


The most obvious conclusion is that *orf2** is the *tylL* gene. Yet, *orf2** is thought to encode the mycaminosyl-glycosyltransferase and therefore must be a mycaminose specific gene (Chapter 3, page 67). Whenever the *tylL* strain was complemented the zone sizes were smaller than those produced by the complemented *tylM* strain, suggesting that it may be producing either lower levels of tylosin or a different, but antibiotically active intermediate. For example, zones produced by the *tylL* mutant carrying pLST9828+*orf2** averaged at 14 mm diameter, in contrast to the *tylM* mutant complemented with *orf3** in pLST9828 where the average zone size was larger (26 mm).

Are there any more *tylL* mutants?

Experiments were carried out to complement two other potential *tylL* mutants, GS78 and GS79, which are known to be *tylD + A/L* mutants (Dr E. T. Seno). *tylD* mutants are defective in the addition or biosynthesis of TDP-6-deoxy-D-allose. Yet if these mutants were *tylL* strains they would still produce an active biosynthetic intermediate if complemented by *tylLM* DNA, because they would be able to add mycaminose to tylactone.

Figure 29 GS79 was not complemented by *tylLM* DNA (*Kpn* I - *Bam*H I DNA fragment) and the GS78 mutant phenotype was leaky.



GS79+*Kpn* I-*Bam* HI DNA fragment GS78

However, GS79 was not complemented by the *Kpn* I-*Bam*H I DNA fragment (pLST97A1), and the GS78 mutant phenotype was leaky and was therefore unreliable to work with (Figure 29, page 91).

Chapter 5 Explaining the *tylL* phenotype.

Introduction.

Eventually, the *tylL* mutant was complemented in *trans* by *orf2** downstream of a constitutive promoter (Chapter 4, page 89), indicating that *orf2** is the *tylL* gene. *orf2** from the *tylL* mutant was sequenced in search of the *tylL* mutation, and the data were compared to sequence generated from the 'wild-type' strain c373.10 (Gandecha *et. al.*, 1997). Sequencing template was provided by amplifying *orf2** by PCR from chromosomal DNA isolated from the *tylL* mutant. PCR products were captured in the vector pGEM-T (Promega), and were sequenced by automated DNA sequencing, using primers and nested deletions. An in-frame stop codon (TGA) was identified in three *orf2** PCR clones. This mutation truncates the *orf2** protein to 142 amino acids in the *tylL* mutant, compared to the wild-type protein of 452 amino acids.

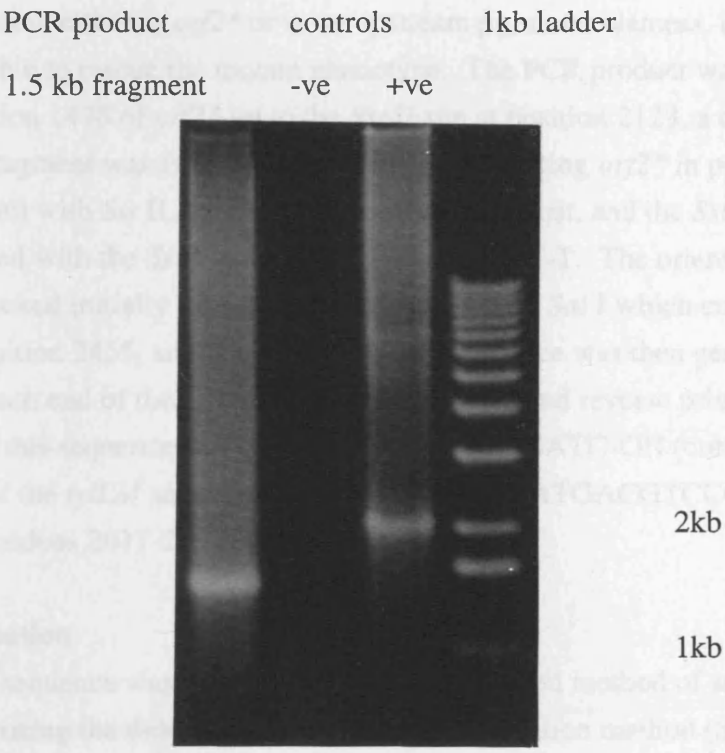
However, *orf2** was a surprising candidate for the *tylL* gene as it is thought to be the mycaminosyl-glycosyltransferase. The amino acid sequence produces significant matches with other glycosyltransferases (Chapter 3, page 67) revealing a common motif (Professor Cundliffe, personal communication) of unknown function within the C-terminus. The enzyme is therefore unlikely to be involved in the biosynthesis or addition of all three sugars unless it acts as part of a larger protein complex with the other glycosyltransferases. However, the action of the deoxyallosyl-glycosyltransferase appears to be specific to the addition of 6-deoxy-D-allose (Dr Vanessa Wilson, personal communication). *tylN* encodes the deoxyallosyl-glycosyltransferase. A 'wild-type' *S. fradiae* strain disrupted in this gene cannot produce tylosin, but is still able to ferment DMT (demycinosyl-tylosin) and can therefore add both mycaminose and mycarose to tylactone (Dr Vanessa Wilson, personal communication). If *orf2** is a mycaminose specific gene, is the *tylL* mutant complemented with *orf2** producing tylosin, and if not, what active intermediate does it produce? In plug assays it was observed that complemented *tylL* exconjugants always produced smaller size zones than those produced by complemented *tylM* strains (Chapter 4, page 90). Was this because the complemented *tylL* strains were producing less tylosin or a different but biologically active intermediate? This question was addressed by fermenting mutant and complemented strains and analysing their fermentation products by high-performance liquid chromatography (HPLC) and thin layer chromatography (TLC).

The aim of these experiments were to distinguish two possibilities; either the *tylL* mutant is a multiple mutant, or the *orf2** protein is somehow responsible for the addition of all three sugars to tylactone.

Obtaining the sequence of *orf1 and *orf2** from the *tylL* mutant.**

In addition to *orf2**, *orf1** was also amplified from the genome of the *tylL* mutant and sequenced, in case it contained mutations contributing to the *tylL* phenotype. Both *orf1** and *orf2** were amplified from *tylL* mutant DNA by PCR (Chapter 2, page 53) using 27mer deoxyoligonucleotides as primers. Primer sequences were chosen to minimise secondary structure and require a similar annealing temperature; *orf1**was amplified using the two primers P-AACCACTTCACGATGATGACCGAACAC-OH and P-GCACCAGGTTGTAGTAGTGGGTGTTGT-OH (complementary to residues 82-108 and 1566-1592 of the *tylLM* sequence, respectively); and *orf2** was amplified using two primers P-GAATTCGTGAGAAGGGCACTGGATGAC-OH and P-TCTAGACTACCTTTCCGGCGCGGATCG-OH (complementary to residues 1478 -11498 and 2815-2836 of the *tylLM* sequence, respectively).

Figure 30 *orf1** was amplified by PCR from *tylL* mutant genomic DNA as a 1.5 kb DNA fragment. No product was produced in the absence of template, and a 2 kb fragment was amplified from the control template 2000 using control primers (PCR optimizer™ kit, Invitrogen)



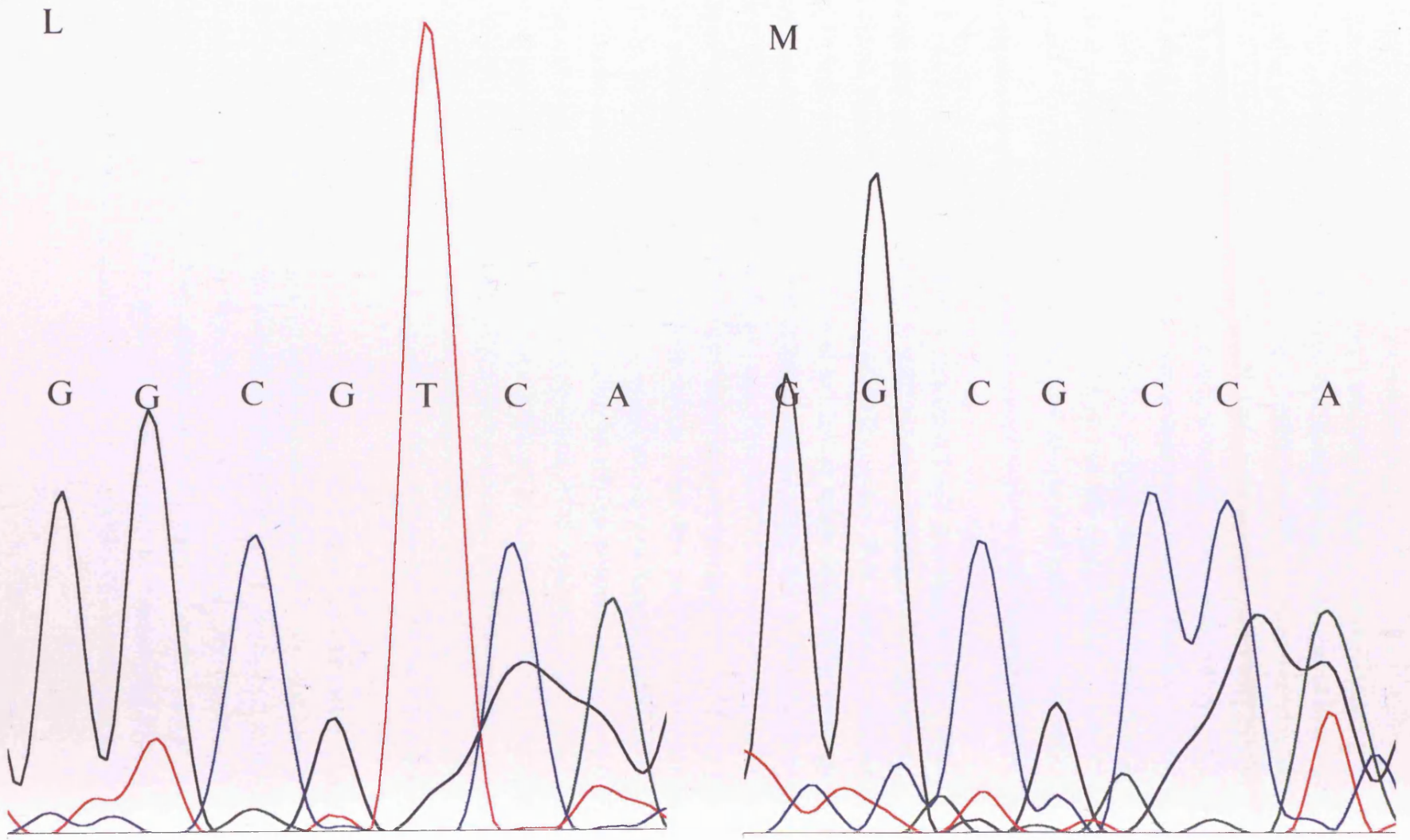
The *orf1** PCR product (Figure 30, page 93) was gel isolated (Chapter 2, page 41, jetsorb protocol) to remove residual chromosomal DNA and ligated into the PCR cloning vector pGEM-T (Promega). The ligation mixture consisted of 7 µl PCR product, 1 µl 10x ligase buffer, 1 µl pGEM-T, and 1 µl T4 DNA ligase and was incubated at 4°C overnight. *E. coli* NM522 was transformed (Chapter 2, page 51) with the ligation mix. Recombinant constructs were identified by blue-white selection, and the insert was inserted in both orientations. The restriction endonuclease sites in the MCS of pGEM-T are particularly suitable for generating nested deletions. However, many of these sites are common in *Streptomyces* DNA and would therefore also disrupt the DNA insert. However, this allowed the creation of certain deletions by simply cutting and religating the recombinant vector, so that sites in the DNA insert would be ligated to sites in the MCS (*Apa* I, *Mlu* I, *Sfi* I). A family of deletions was also produced as described previously (Chapter 2, page 46) and sequence was therefore generated in an overlapping fashion. No difference was found between the *tylL orf1** sequence, and the *orf1** from c373.10 (Figure 11, page 60, Chapter 3).

The *orf2** PCR product was also ligated into pGEM-T, but only part of the DNA was sequenced. One of the smallest fragments of *tylLM* DNA that complemented the *tylL* mutant in *cis* terminated at residue 2068 within *orf2** (Chapter 4, page 82). The *tylL* mutation must therefore be upstream of this point, either in *orf2** or in an upstream promoter element, in order for this piece of DNA to be able to rescue the mutant phenotype. The PCR product was therefore sequenced from position 1478 of *orf2** up to the *Sst* II site at position 2123, a distance of about 600 bp. This DNA fragment was isolated for sequencing by cutting *orf2** in pGEM-T (in the appropriate orientation) with *Sst* II, so that the C-terminus was lost, and the *Sst* II site at position 2123 could be religated with the *Sst* II site in the MCS in pGEM-T. The orientation of the *orf2** PCR product was checked initially by cutting the construct with *Sal* I which cuts the insert asymmetrically at position 2455, and once in the MCS. Sequence was then generated by sequencing in from each end of the DNA insert with universal and reverse primers. Two primers were used to confirm this sequence; P-GAGTTCGTCACCGAGATC-OH (complementary to residues 1718-1735 of the *tylLM* sequence); and P-GTTGAGGATGACGTCCG-OH (complementary to residues 2017-2001 of the *tylLM* sequence).

Finding the *tylL* mutation.

The 'wild-type' *orf1** sequence was generated by a non automated method of sequencing (Chapter 2, page 48), using the dideoxynucleotide chain termination method (Sanger *et. al.*, 1977). In contrast, the *tylL* mutant *orf1** and *orf2** sequences were determined by automated sequencing (Chapter 2, page 50) which provided the following advantages (Prober *et. al.*, 1987). *Taq* DNA polymerase was used as the sequencing enzyme at higher temperatures (60 - 80°C) than T7 polymerase (37 - 45°C). This was particularly advantageous when considering

Figure 31 Chromatograms showing DNA sequence generated from *orf2** amplified by PCR from the *tylL* and *tylM* mutants. C to T transition in at position 1906 (anti-coding strand) in *tylL* DNA results in an in-frame stop codon.



Streptomyces DNA which is prone to secondary structure because of the high G+C ratio. Use of nucleotide analogues to resequence DNA was avoided as G analogues were included in the premix, although the use of nested deletions and double-stranded sequencing was still regarded as essential. Radioactivity was not used, each dideoxynucleoside triphosphate being labelled with one of four fluorescent labels which differ in their emission frequency. The sequence was read by an optical detector which monitors the wavelength of fluorescence whilst the labelled fragments were excited by a laser, allowing the products of sequencing to be run in a single lane as opposed to four lanes. Automated sequencing data were displayed on a computer as a chromatogram (Figure 31, page 95) which could analysed by a variety of sequencing programs such as sequence editor (Applied Biosystems), in contrast to manual sequencing where autoradiogram are read by eye. More than 600 bases may be read off one gel in contrast to a maximum of 300 on an autoradiogram. Plasmid DNA was prepared using QIAGEN Midi kits (Chapter 2, page 47) which provided DNA of sufficient quality that it could be used directly for generating nested deletions and sequence.

The G-C to an A-T transition at position 1906 is typical of the mutagen MNNG (Coulondre *et. al.*, 1977), and produces an in-frame stop codon, TGA (Figures 31 and 32, pages 95-96). This mutation would truncate the protein to 142 amino acids (Figure 32, page 96), and remove the conserved sequence motif identified at the C-terminus of the protein. As the sequence was generated from a PCR product, three independent PCR clones from the *tylL* mutant were sequenced to ensure that any difference observed was not the result of a PCR error (Tindall *et. al.*, 1988). The *tylM* mutant was used as a 'wild-type' control because both the *tylL* and *tylM* mutants were derived from c373.10.

Figure 32 MNNG mutagenesis resulted in a characteristic G-C to A-T transition at nucleotide 1906 of the *tylLM* sequence which would result in a truncated version of the Orf2* protein.

```
     gtctcggccggctgcccgtcgccccgtatgtccccgagtgagaagggcactggatgaccg
1441 ---------+---------+---------+---------+---------+---------+ 1500
     cagagccggccgacgggcagcggggcatacaggggctcactcttcccgtgacctactggc

      Possible start of orf2*              M  R  R  A  L  D  D  R  -
       L  G  R  L  P  V  A  P  Y  V  P  E  *  stop of orf1*

     ccgccgaggaccgcacggaccggaagggaaaccgccgatgcgggtactgctgacctgtat
1501 ---------+---------+---------+---------+---------+---------+ 1560
     ggcggctcctggcgtgcctggccttccctttggcggctacgcccatgacgactggacata

      R  R  G  P  H  G  P  E  G  K  P  P  M  R  V  L  L  T  C  I  -

     cgcgcacaacacccactactacaacctggtgccggtcgcctgggccctgagagcggccgg
1561 ---------+---------+---------+---------+---------+---------+ 1620
     gcgcgtgttgtgggtgatgatgttggaccacggccagcggacccgggactctcgccggcc

      A  H  N  T  H  Y  Y  N  L  V  P  V  A  W  A  L  R  A  A  G  -
```

```
     acacgaggtgcgggtggccgcgcagcccgccctcaccgacacgatcaccgCctccggact
1621 ---------+---------+---------+---------+---------+---------+ 1680
     tgtgctccacgcccaccggcgcgtcgggcgggagtggctgtgctagtggcGgaggcctga

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

     gaccgccgtgccggtcggcggcaacgagtccgtgctcgagttcgtcaccgagatcggcgg
1681 ---------+---------+---------+---------+---------+---------+ 1740
     ctggcggcacggccagccgccgttgctcaggcacgagctcaagcagtggctctagccgcc

      T  A  V  P  V  G  G  N  E  S  V  L  E  F  V  T  E  I  G  G  -

     cgacccgggcccctaccagcgaggcatggacttcgccgagacgtgcggcgaaccgctgtc
1741 ---------+---------+---------+---------+---------+---------+ 1800
     gctgggcccggggatggtcgctccgtacctgaagcggctctgcacgccgcttggcgacag

      D  P  G  P  Y  Q  R  G  M  D  F  A  E  T  C  G  E  P  L  S  -

     ctacgagcacgcgctcggccagcagaccgccatgtcggcgctgtgcttcgccccgttcaa
1801 ---------+---------+---------+---------+---------+---------+ 1860
     gatgctcgtgcgcgagccggtcgtctggcggtacagccgcgacacgaagcggggcaagtt

      Y  E  H  A  L  G  Q  Q  T  A  M  S  A  L  C  F  A  P  F  N  -

     ctgcgactccaccatcgacgacatggtcgccctcgcccgctcctgAcgccccgacctcgt
1861 ---------+---------+---------+---------+---------+---------+ 1920
     gacgctgaggtggtagctgctgtaccagcgggagcgggcgaggacTgcggggctggagca

      C  D  S  T  I  D  D  M  V  A  L  A  R  S  *
```

Analysis of fermentation products from the wild-type (c373.1), mutants, and complemented mutant strains.

Tylosin compounds were extracted from fermentation cultures into a solvent and then dried down by rotary evaporation. The concentrated samples were analysed by high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC).

Culture conditions for fermentation were based on Baltz et. al., (1981) (Chapter 2, page 35). A frozen stock of *S. fradiae* (100 µl) was inoculated into 30 ml of modified complex vegetative medium in a 100 ml flask which had no baffles or springs. After 2-3 days growth, 5 ml of this culture was used to inoculate tylosin production medium MM-1, to give a final volume of 50 ml in 250 ml flasks. These cultures were shaken at 300 rpm at 28°C for 7 days. Strains carrying derivatives of pSET152 and pKC1218 were grown with apramycin (25 µg/ ml).

Methods.

Bioconversion studies.

Feeding experiments were carried out as described by Baltz et. al. (1981). Strains were cultured for 2 days in MM-1 before a compound was added and the culture then incubated for a further 3 days prior to extraction. Each compound was added at a lower concentration than the 2 mM

described by Baltz et. al. (1981) in order to conserve supplies. The amount of OMT (5-*O*-mycaminosyl-tylonolide) added was in fact more appropriate (0.15 mM) as it has been observed that intermediates lacking mycarose and 6-deoxy-D-allose are unstable in fermentation cultures at high concentration (Baltz *et. al.*, 1983). This was reflected by the HPLC trace for the *tylL* mutant fed OMT; OMT was successfully extracted at the end of the fermentation (Figure 41a, page 107). DMT and desmycosin were both added at a concentration of 0.12 mM.

Extraction procedure (Dr Steven Fish, personal communication).
An equal volume (50 ml) of chloroform was added to fermentation cultures (mycelium and supernatent) which were then briefly shaken before being filtered through Whatman No.1 disks (55 mm in diameter), and centrifuged (3500 x g, 10 min, 4°C) to remove any further debris. The filtrate was then dried down to a volume of 1 ml by rotary evaporation. Previously, only the supernatent had been extracted, which was sufficient to detect high levels of tylosin production, by c373.1 and the *tylM* mutant complemented with *orf3** (Figures 35 and 36, page 102), but not in the elucidation of fermentation products produced by the *tylL* strains.

Conditions for HPLC (Huber *et. al.*, 1990).
HPLC was carried out on a reverse phase column (compounds with a higher polarity came off earliest), and the compounds (10 µl) were eluted using a linear gradient from 50% to 80% methanol in 0.3% ammonium formate (pH 4.0) at a flow rate of 2 ml/ min at an absorbance of 282 nm. I thank Dr Vanessa Wilson for running the HPLC equipment. It was observed that apramycin, which was added to certain cultures, does not absorb at 282 nm (Dr Steven Fish, personal communication).

Retention times for tylosin and its precursors (Dr Vanessa Wilson).
Retention times for tylosin compounds did show slight variations between runs on different days, and therefore standards were therefore always analysed during each run.

Compound	Retention time (min).
Tylactone	12.14
5-*O*-mycarosyl-tylactone	14.68
5-*O*-mycaminosyl-tylactone	10.93
20-dihydro, 23-deoxy-OMT	7.99
23-deoxy-OMT	7.99
20-deoxo-demycinosyl-tylosin	7.73
OMT	2.79
DMT	3.79

O-demethylmacrocin	4.72
lactenocin	4.21
macrocin	5.51
relomycin	7.07
desmycosin	5.51
tylosin	6.92

Thin-layer chromatography (TLC).
Tylosin compounds were separated using the solvent system of ethyl acetate-diethylamine (95:5) (Baltz *et. al.*, 1981). Tylosin compounds (1 µg of drug in 5 µl chloroform) and fermentation culture extracts were spotted onto a TLC plastic sheet coated with silica gel 60 F_{254} (MERK), and stood in 1 cm of solvent in a glass beaker which was covered with a glass plate. Once the solvent front reached 2 cm below the top edge of the TLC sheet, the sheet was removed and left to dry in air. Compounds were positioned by ultraviolet absorbtion. In this solvent system the *Rf* values for tylactone, relomycin, and tylosin were 0.93, 0.38, and 0.67, respectively (Baltz *et. al.*, 1981).

Results.

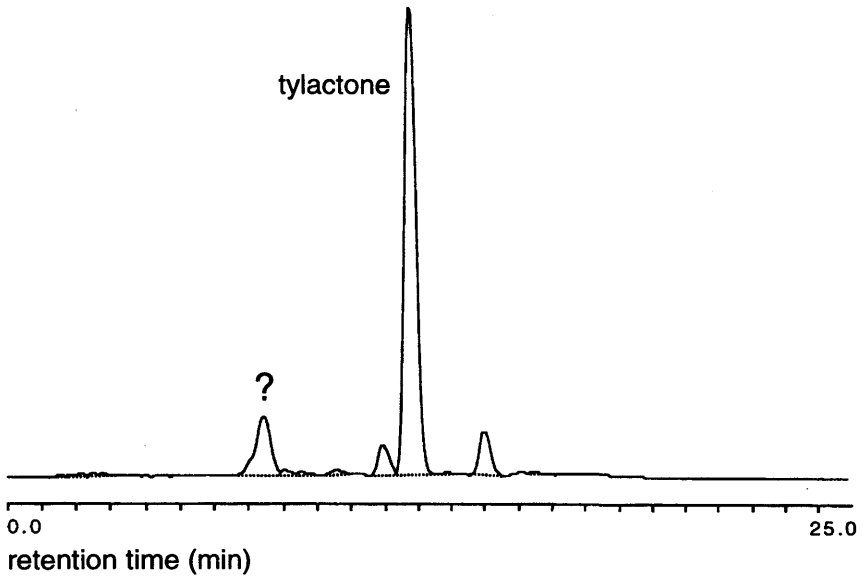
Fermentation products synthesised by wild-type and mutant strains of *S. fradiae*.

Products of fermentation were analysed by HPLC, and in some cases by using TLC. Tylosin compounds were monitored at 282 nm, and peak areas (µV/ sec) are shown in Figure 43, page 111.

The *tylL* and *tylM* mutants did not produce biologically active material (Figure 33, page 101), but when the *tylL* fermentation products were analysed by HPLC, a small peak of presumably inactive material was observed where tylosin elutes, (Figure 34, page 100).

Figure 34 HPLC traces of (a) the fermentation products synthesised by the *tylL* mutant showing a small peak at the point at which tylosin comes off, and (b) 50 μg of tylosin.

(a)



(b)

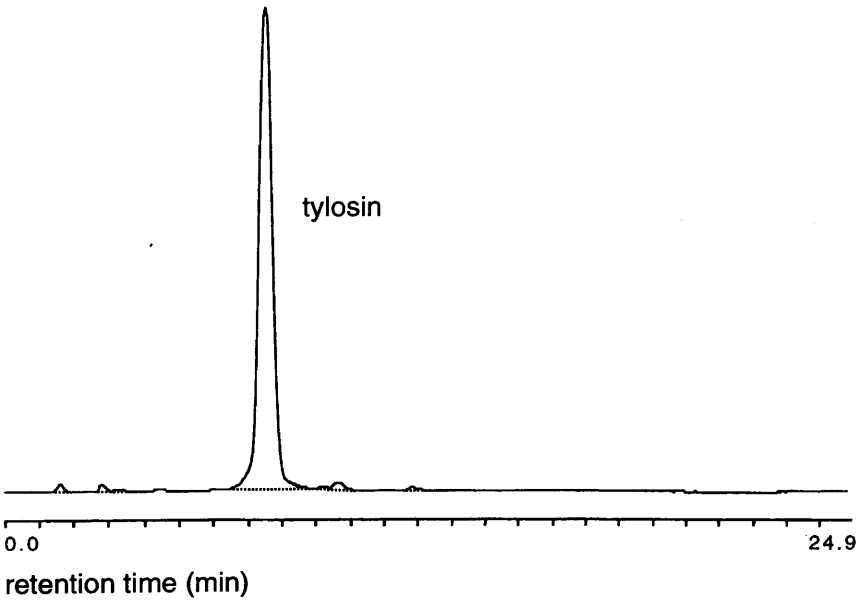
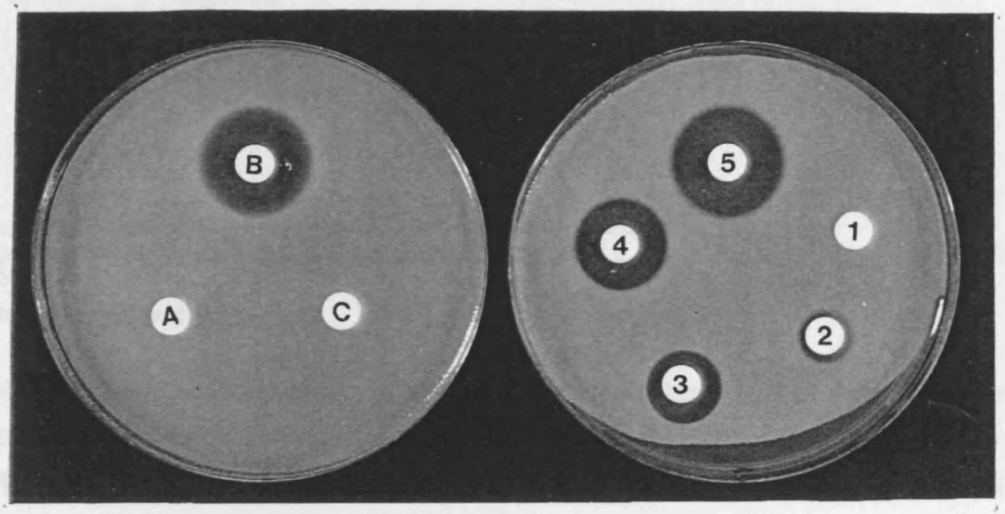
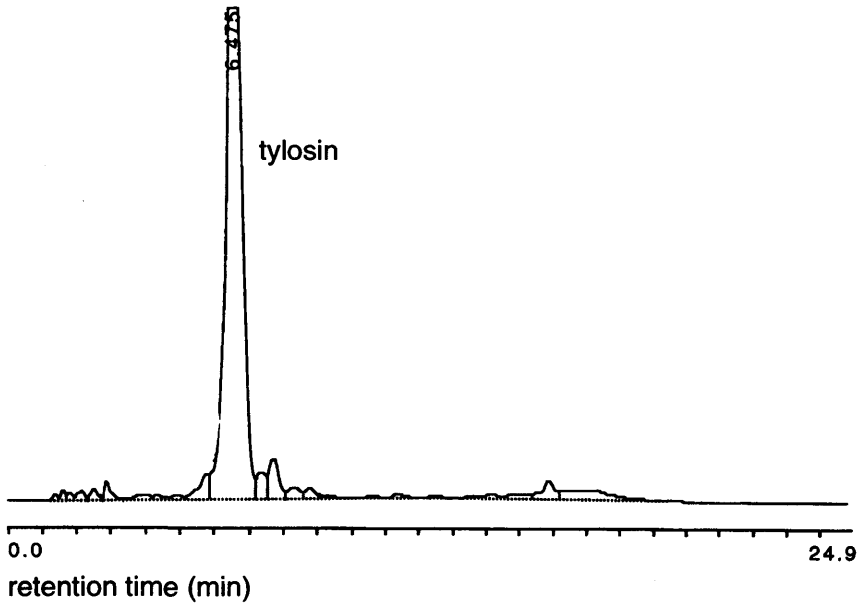


Figure 33 The *tylL* (A) and *tylM* (C) mutants did not produce biologically active compounds in contrast to the wild-type strain, c373.1 (B). These three strains were fermented in MM-1 medium for 14 days, after which the cultures were spun down and 20 µl of supernatant was placed on a antibiotic disk (diameter 6 mm, Whatman) and assayed micobiologically using the sensitive strain *Micrococcus luteus* T149. A control plate was set up to demonstrate how an increase in the amount of tylosin on antibiotic disks increased the size of zone of inhibition, (1, 0 ng; 2, 50 ng; 3, 100 ng; 4, 200 ng; 5, 400 ng).



A large tylactone peak was visible in extracts from the *tylL* mutant (Figure 34, page 100) in contrast to virtually no tylactone in extracts from c373.1 (Figure 35, page 102). When the 'wild-type' strain c373.1 was fermented and analysed by HPLC a large peak occurred were relomycin and tylosin elute (Figure 35, page 102). When a sample of this extract was analysed by TLC, tylosin was apparent (Figure 39, page 104). c373.1 is an ancestor of c373.10; c373.10 is a very efficient tylosin producer and was not available for use in this study.

Figure 35 A large relomycin/ tylosin peak is present in the HPLC trace of the fermentation products produced by c373.1.



The *tylM* mutant was complemented in *trans* by an integrated copy of *orf3** downstream of a constitutive promoter (Figure 22, page 80, Chapter 4). An extract from this strain was analysed by HPLC and showed an obvious peak at the expected elution position of tylosin (Figure 36, page 102). TLC data confirmed this conclusion (Figure 39, page 104).

Figure 36 HPLC trace of the fermentation products produced by the *tylM* mutant complemented by an integrated version of *orf3**. A large peak is observed where tylosin elutes.

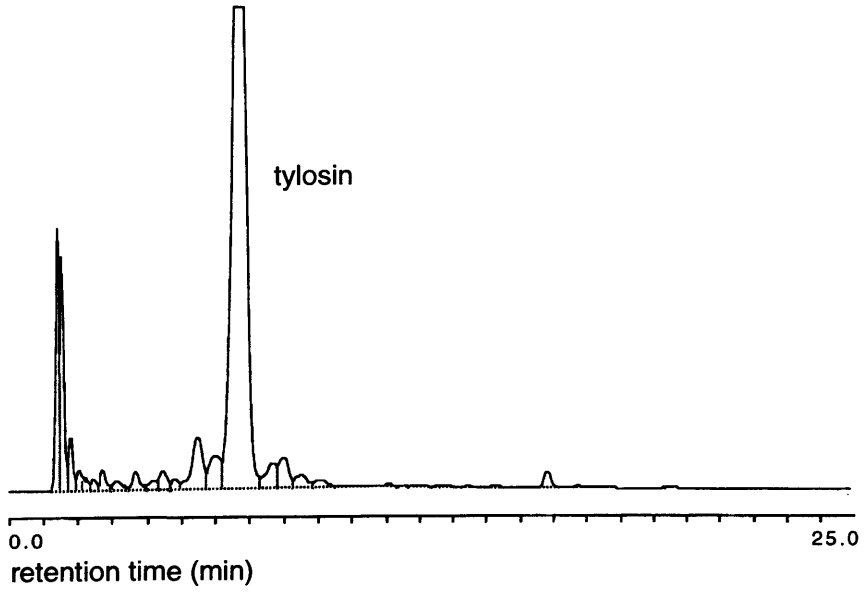


Figure 37 A number of *tylL* strains were fermented and 5 µl of the concentrated extracts were tested for biological activity on *M. luteus*. When the *tylL* mutant was fermented on its own (4) and fed tylactone (2) it did not produce a zone of inhibition. The complemented *tylL* strain carrying an integrated version of *orf2** produced zones when fermented on its own (1) and when it was fed tylactone (3) and OMT (5). The *tylL* strain which was complemented by the *Kpn* I - *Bam* HI fragment also produced zones when it was fed tylactone (6) or OMT (7).

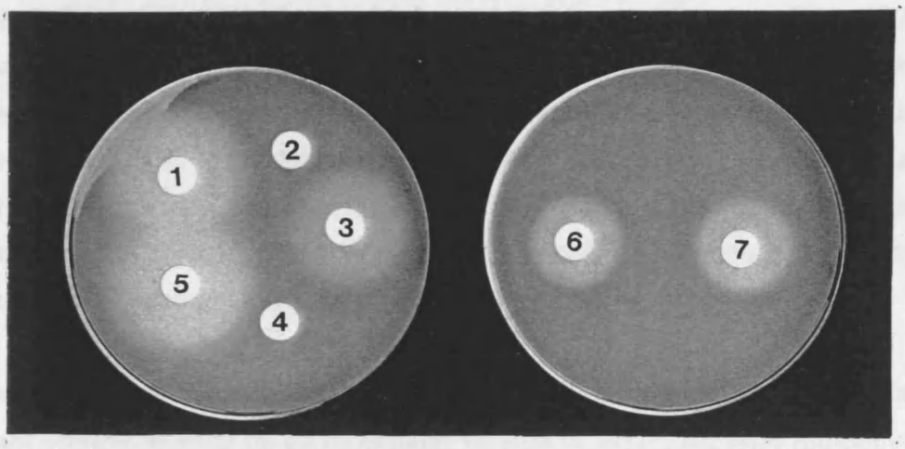
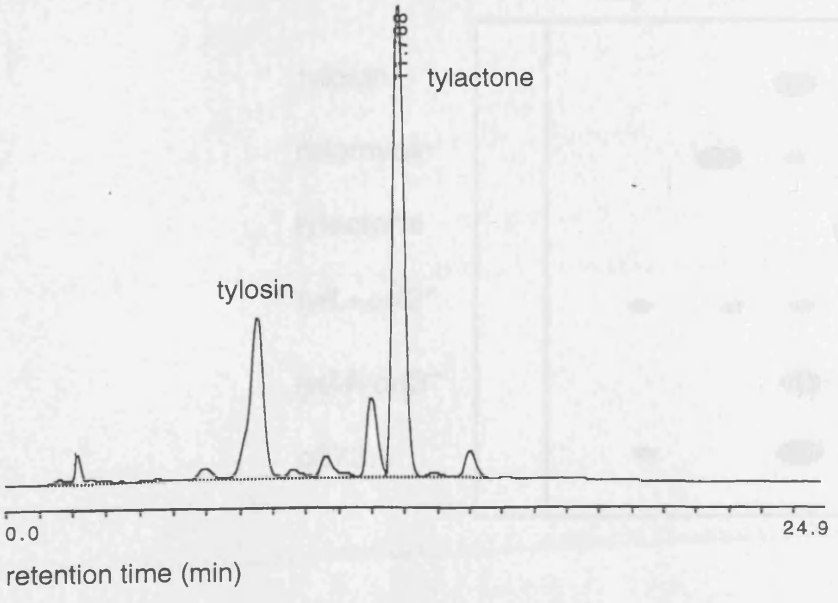
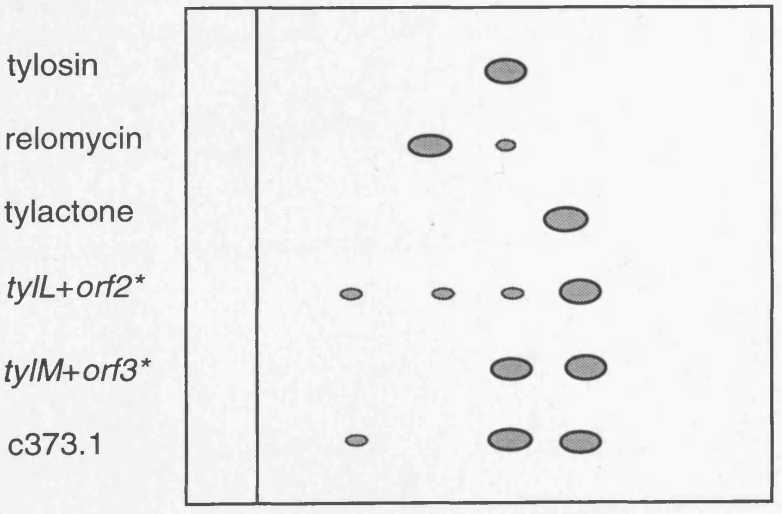


Figure 38 HPLC trace of the fermentation products produced by the *tylL* mutant complemented by an integrated version of *orf2**. A substantial tylosin peak and a large amount of tylactone are present.



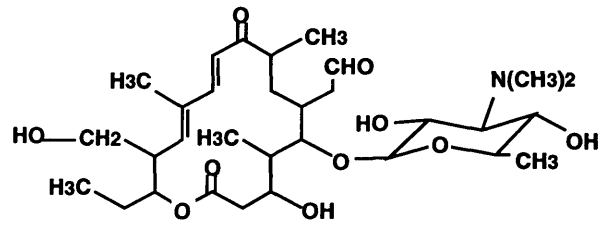
The *tylL* mutant was eventually complemented in *trans* by both integrated and free replicating vectors carrying *orf2** downstream of a constitutive promoter (Chapter 4, page 89). However, in biological assays the zones produced by the complemented *tylL* strains were always significantly smaller than those produced by the complemented *tylM* mutant (Chapter 4, page 90). Could the complemented *tylL* mutant be producing a biologically active intermediate as opposed to tylosin? The *tylL* strain carrying pLST9828+*orf2** was used in fermentation studies as the integrated plasmid appeared to be generally more stable than the replicative form (pLST9829+*orf2**). Extracts from this strain showed biological activity on *M. luteus* (Figure 37, page 103), and the HPLC trace (Figure 38, page 103) showed a peak at the expected elution position of tylosin and a large tylactone peak. Why does the complemented *tylL* mutant accumulate so much tylactone in contrast to c373.1? Perhaps the tylactone is not completely converted because the integrated copy of *orf2**, a PCR product, is only partially active. This sample was also analysed by TLC, and three spots corresponding to relomycin, tylosin and tylactone, were apparent (Figure 39, page 104). Tylosin, relomycin and tylactone (1 μg) were spotted onto the TLC plate as controls.

Figure 39 Diagramatic representation of TLC of the fermentation products produced by the *tylL* mutant complemented by an integrated version of *orf2**, the *tylM* mutant complemented by an integrated version of *orf3**, and c373.1. Purified compounds (tylosin, relomycin and tylactone) were run as controls (1 μg). The relomycin standard was contaminated with tylosin.

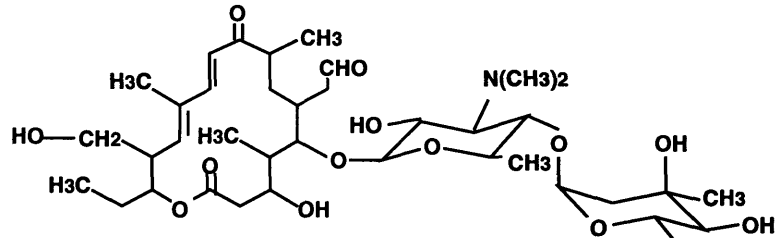


An *orf2** disruption has been made in c373.1 by Dr. Steven Fish using the hygromycin resistance cassette. This strain can not bioconvert tylactone, but can convert the fed intemediates

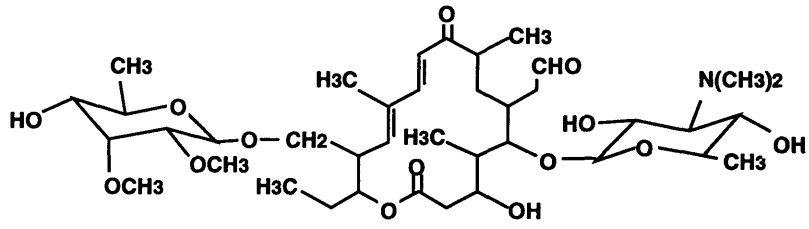
Figure 40 Structures of tylosin intermediates, OMT, DMT, and desmycosin fed to the *tylL* mutant.



5-*O*-mycaminosyl-tylonolide (OMT)



Demycinosyl-tylosin (DMT)

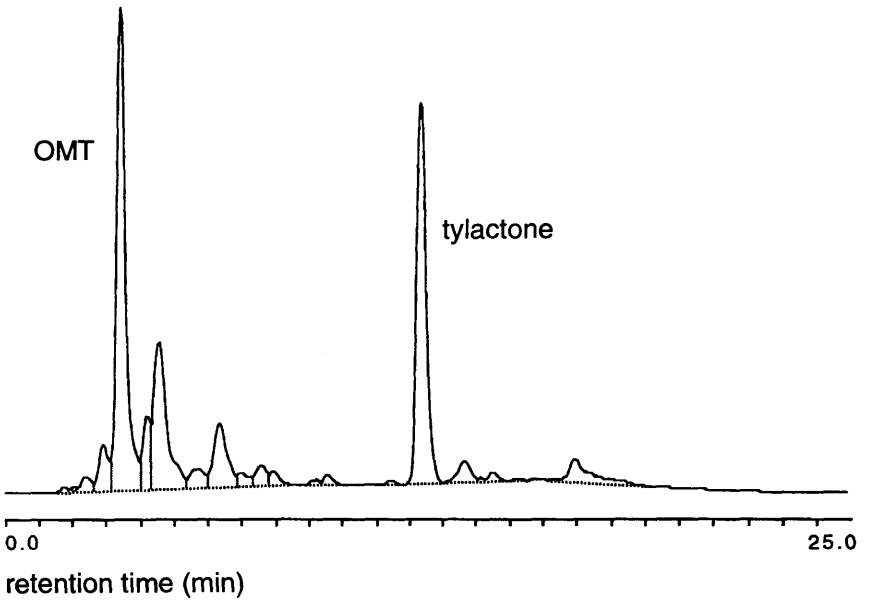


Desmycosin

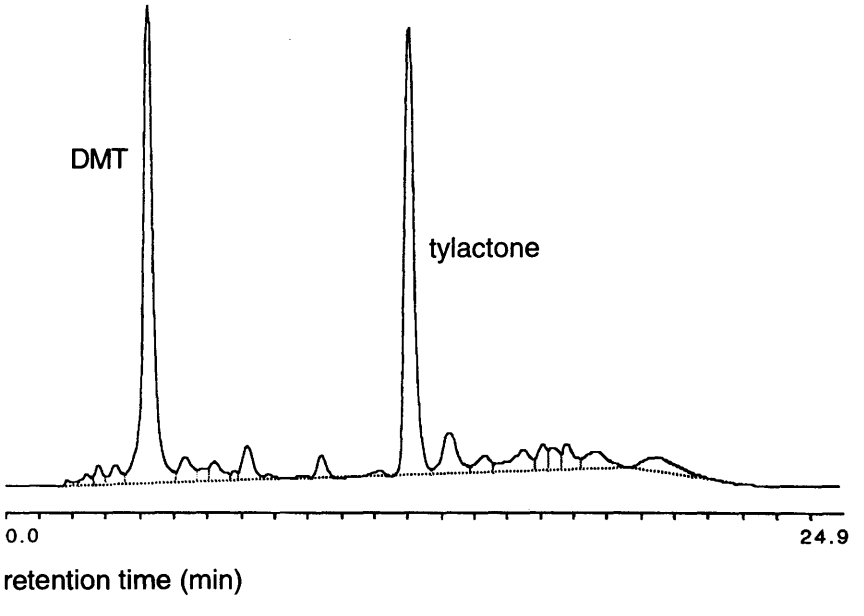
OMT, DMT (lacks mycinose), desmycosin (lacks mycarose) to tylosin, (Dr. Steven Fish, personal communication). These results indicate that the *orf2** disrupted strain is blocked only in the addition of mycaminose. Why, therefore, does the *tylL* strain exhibit a different phenotype when it is also defective in the expression of *orf2**? The TylL phenotype was checked by feeding the intemediates tylactone, OMT, desmycosin and DMT to the *tylL* mutant (Figure 40, page 105). None of these intermediates were indisputably converted to tylosin, (Figure 41, pages 107-109). When the mutant was fed OMT, obvious OMT and tylactone peaks were present (Figure 41a, page 107), although there are some small peaks which could correspond to small amounts of DMT and desmycosin suggesting that the *tylL* mutant maybe able to convert OMT, if only inefficiently, to DMT and desmycosin. The extract from the *tylL* mutant fed DMT exhibits distinct DMT and tylactone peaks, only (Figure 41b, page 107). When fed desmycosin the *tylL* mutant appears to produce a peak where tylosin would be expected to elute, (Figure 41c, page 108), but the stock of desmycosin was contaminated with a small amount of tylosin (Figure 41e, page 109). The ratio of desmycosin:tylosin peak areas (μV/sec, Figure 43, page 111) in traces of the desmycosin standard (Figure 41e, page 109) and the *tylL* mutant fed desmycosin (Figure 41c, page 108), were approximately the same (3:1). However, these strains might not be expected to convert these compounds with maximum efficiency, as it was noted by Baltz et. al. (1983) that DMT fed to the *tylG* mutant, GS22, was not converted efficiently to tylosin, in contrast to demethyllactenocin which lacks mycarose. Also desmycosin was not bioconverted efficiently to tylosin, suggesting that DMT and desmycosin are not true intermediates, but are 'shunt metabolites' in the tylosin pathway (Baltz *et. al.*, 1983; Figure 3, page 27, Chapter 1). Yet, feeding OMT, DMT and desmycosin to the *orf2** disrupted strain provided unequivocal data that this strain could convert these compounds to tylosin (Dr Steven Fish, personal communication).

Figure 41 HPLC traces of the fermentation products produced by the *tylL* mutant fed different tylosin intemediates (a) OMT, (b) DMT, and (c) desmycosin, and the standards (d) 10 μg each of OMT, DMT, desmycosin, tylosin and tylactone and (e) 100 μg of desmycosin.

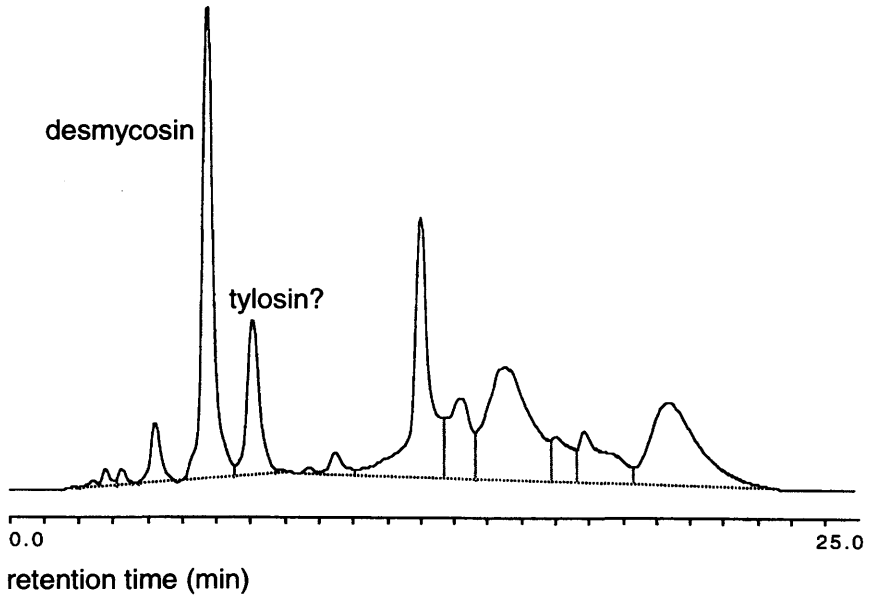
(a)



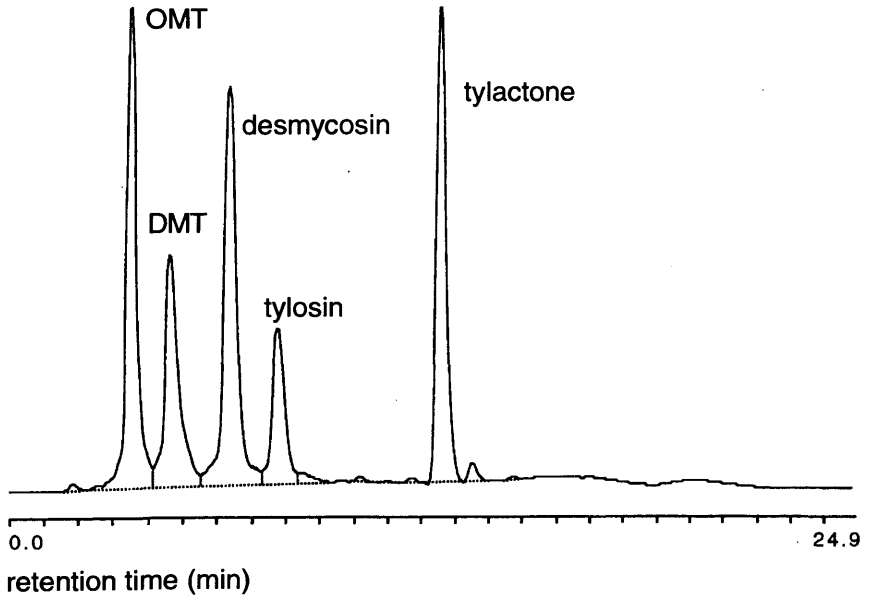
(b)



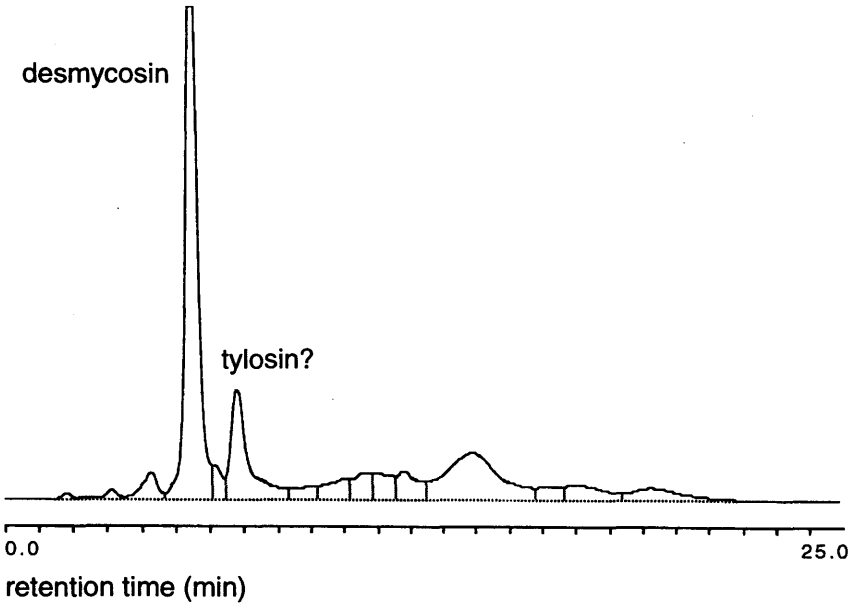
(c)


desmycosin
tylosin?
0.0
25.0
retention time (min)

(d)

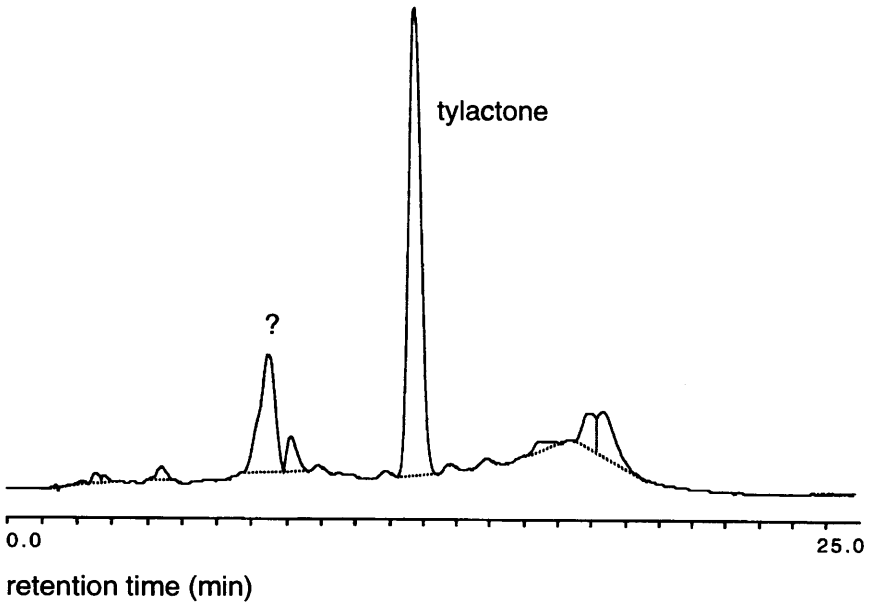

OMT
desmycosin
tylactone
DMT
tylosin
0.0
24.9
retention time (min)

(e)



Fishman et. al. (1987) reported that the *Kpn* I-*Bam*H I DNA fragment from the *tylLM* region restored tylosin production in the *tylL* mutant. Complementation of the *tylL* strain with this DNA fragment was repeated, but growing this strain appeared to be problematic. Extracts were obtained from this strain fed OMT (Figure 42, page 110) and tylactone, but only a small peak was observed where tylosin elutes, although these extracts were active in biological assays (Figure 37, page 103).

Figure 42 HPLC trace of the fermentation products produced by the *tylL* mutant complemented with the *Kpn* I-*Bam*H I DNA fragment from the *tylLM* region fed OMT. Only a small peak is present with the same retention time as tylosin.



Dr. Steven Fish sucessfully restored tylosin production in the *orf2** disruption with pLST97B1 (*orfs1*-3**), but not pLST97C2 (*orfs1* and 2**). The disruption of *orf2** must therefore have a polar effect on *orf3** which must either share the same transcript as *orf2** or be transcribed from a promoter within *orf2** upstream of the disruption. The *tylL* mutant was complemented by pLST97C2 (Figure 21, page 80, Chapter 4), however, which demonstrates a difference between the *tylL* mutant and the disrupted strain. Either the *tylL* mutant is a multiple mutant, or the inability to add the three sugars is due to physiological factors. However, the observation that *orf2** restores tylosin production in the *tylL* mutant (Figures 38 and 39, pages 103 and 104, repectively) suggests that the TylL phenotype is not a result of more than one mutation. Unlike the disrupted strain, the *tylL* mutant has a functional *orf3** and would therefore accumulate TDP-mycaminose as opposed to the unmethylated sugar. Could this explain the phenotypic differences observed between these two strains? For example, could the mycaminose produced by the *tylL* mutant sequester the cofactor TDP, which needs to be recycled, and therefore prevent the other two glycosyltransferases from adding the other sugars?

Figure 43 Table to show areas (μV/ sec) of peaks eluted during HPLC analysis of the fermentation products extracted from different *S. fradiae* cultures.

	Area (μV/ sec)					
Figure	Tylosin	Tylactone	OMT	DMT	Desmycosin	?
34a		8602097				1399220
34b	12432340					
35	39482810					
36	35042428					
38	6373752	14281960				
41a		5740799	6721060	3316204	1427457	
41b		6083971		6800201		
41c	2648864				6793156	
41d	2962202	6726220	7070470	4906391	7808337	
41e	9064950				28777934	
42	685852	2162966				
tylM mutant		800027				

Chapter 6 Promoters and transcripts.

Introduction.

Once potential ORFs had been located in the *tylLM* region (Chapter 3, page 55), questions were asked about location of transcripts, and the temporal and spatial regulation of their expression. Two approaches were used in an attempt to answer some of these questions.

(1) RNA was prepared from *S. fradiae* grown both in TSB and tylosin production medium, MM-1. These two media, TSB and MM-1, provided contrasting culture conditions under which transcript could be analysed. Samples of supernatant (20 µl from a 50 ml culture) taken from *S. fradiae* cultures grown in TSB did not exhibit biological activity when placed on antibiotic disks (6 mm diameter, Whatman) and tested against *M. luteus,* in contrast to cultures grown in MM-1 (Chapter 5, Figure 33, page 101). The integrity of total RNA was assessed by gel electrophoresis, and dot blot hybridisation. Dot blots were probed with a fragment of the resistance gene *tlrA* , which is complementary to a truncated transcript which is constitutively expressed in cultures of *S. fradiae* (Kelemen *et. al.*, 1984). *tylG*, *orf1**, *orf2**, *orf3**, and *orf4** are all orientated in the same direction and are terminally overlapping, except for a gap of 8 bp between *orfs2** and *3**, suggesting that these genes may be transcriptionally, and possibly translationally, coupled (Figure 7, page 55, Chapter 3). The start (5' end) of the *orf1** transcript was analysed by S1 mapping.

(2) Identifying promoter elements in *tylLM* DNA using promoter-probe vectors.
Attempts were also made to identify promoters by shot-gun cloning fragments of *tylLM* DNA into a promoter-probe vector, pIJ486. Promoter strength was assessed by quantifying resistance to kanamycin, to which the product of the reporter gene conferred resistance.

(1) RNA work

Methods.

Equipment was doubled baked to inactivate ribonucleases, and sterile water and RNA solutions were DEPC-treated (Diethyl pyrocarbonate, Sigma), with the exception of Tris buffers. Samples and equipment were kept cool on ice.

1. Isolation of RNA from *S. fradiae* grown in TSB (protocol based on Hopwood *et. al.*, 1985).

An overnight culture of *S. fradiae* grown in TSB was spun down in sterile universal bottles (3500 x g, 5 min, at 4°C) and the supernatant discarded. The pelleted cells were resuspended in 3 ml P buffer containing 5 mg/ ml lysozyme, and were incubated at 37°C for 10 min. The lysate was then mixed with 3 ml of double strength modified "Kirby mix" (2% SDS; 100 mM Na_2EDTA;

pH 8; 12% phenol; 100 mM Tris-HCl, pH 8.3) in a universal bottle containing glass balls (2 mm diameter), to disrupt the mycelial cell walls by vigorous shaking. The lysate was then mixed with 5 ml Tris-HCl (pH 8)-equilibrated phenol/chloroform, and centrifuged (3500 x g, 5 min, at 4°C). The upper phase was removed and extracted twice with an equal volume of Tris-HCl (pH 8)-equilibrated phenol/chloroform. Nucleic acids in the aqueous phase were precipitated with an equal volume of isopropanol and 1/10 volume 3 M sodium acetate in a 30 ml COREX tube at room temperature (20°C) for 10 min. After centrifugation (8 K rpm, 10 min, at 4°C), the pellet was rinsed with 100% ethanol, dried and dissolved in 1 ml of DEPC-treated SQ. RNA was purified by a high salt precipitation with three volumes of 4 M sodium acetate at -20°C overnight. The RNA was pelleted (8 K rpm, 10 min, at 4°C), before being washed in 70% ethanol, dried and dissolved in 500 µl of DEPC-treated SQ.

Extracting RNA from *S. fradiae* cultures grown in TSB was relatively straightforward. However, preparing RNA from cultures grown in MM-1 was more difficult. The previous protocol was therefore developed to allow extraction of RNA from *S. fradiae* grown in tylosin production medium MM-I. This would provide RNA for mapping transcripts synthesised during antibiotic production.

2. Extraction of RNA from cultures of *S. fradiae* grown in tylosin production medium, MM-1 (S. L Clark and Dr Andrew Butler).

Extraction of RNA from cultures of *S. fradiae* grown in tylosin production medium MM-1 was problematic. Low yields of RNA appeared to be the result of inefficient lysis, as the aqueous phase during phenol extractions was remarkably clear. These difficulties could be due to the oil (methyloleate) and precipitates in MM-1 which might interfere with the lysis process. Also, the cultures were harvested after 3 to 5 days growth, when tylosin production is high, the mycelium at this stage being more difficult to lyse due to age. Spooning in excessive amounts of lysozyme did not alleviate this problem, and incubation with this enzyme at 37°C could not be prolonged because of the risk of RNA degradation. The original protocol (1) was therefore adapted to cope with these difficulties.

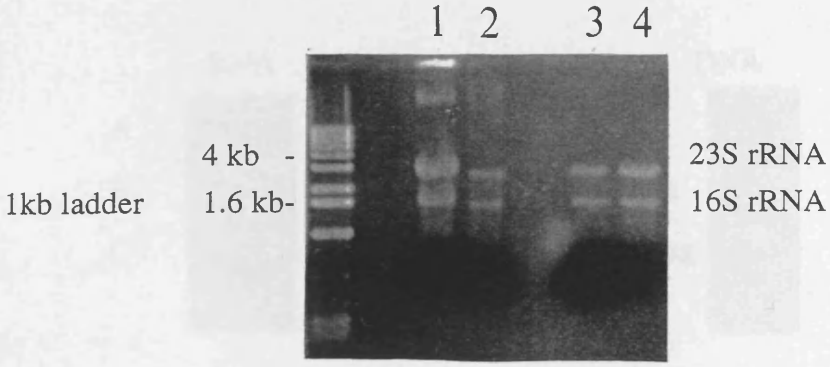
A wash step with a high salt buffer was incorporated into the protocol, and an alternative procedure was used to break the cells open. Biomass was harvested on sterile muslin which allowed any precipitate in the medium to filter through, but retained the mycelium. The muslin was supported in a sterile Buchner funnel, positioned in a Buchner flask, and the supernatant was drawn through under vacuum. The cells were immediately washed with a high salt buffer [200 ml of 1 M Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH adjusted to 7.5 with KOH; 200 ml 1 M $MgCl_2$; 5 ml 4 M NH_4Cl; 7.8 µl ß-mercaptoethanol (Sigma); and 14.6 ml DEPQ-treated SQ] to remove any surface ribonucleases. The mycelium was scraped off the

muslin using a sterile spatula into a mortar before liquid nitrogen was added and the material was ground up using a pestle. This material was then added directly to the Kirby mix.

3. Gel electrophoresis of RNA.

The gel tank, gel tray and comb were bleached with a dilute solution of hydrogen peroxide (Sigma) to denature any ribonucleases, and rinsed with DEPC-treated SQ before use. RNA samples were run routinely on a 1% agarose gel in 1 M MOPS buffer (20 mM MOPS; 5 mM NaOAc; 0.1 mM Na_2EDTA; pH adjusted to 7.0 with NaOH). Samples of RNA (5 µl) were denatured by the addition of 7.5 ml formamide and 2.5 ml formaldehyde (38% w/v) at 55°C for 10 min, before gel dye (40% v/v formamide; 50% glycerol; and 10% 10x MOPS) was added, and the samples were placed on ice. Samples were run at 100 volts for 1 h, before the gel was stained in a solution of EtBr (0.2 µg/ ml) for 20-30 min. RNA made from TSB cultures was quantified using a spectrophotometer (Chapter 2, page 40), but samples from tylosin production medium often gave false readings because the samples were coloured. These samples of RNA were therefore quantified by comparing dilutions run on a gel with known amounts of RNA isolated from cultures grown in TSB. Bands of ribosomal RNA could clearly be identified and were used to approximate the total amounts of RNA present.

Figure 44 RNA isolated from *S. fradiae* can be visualised by gel electrophoresis. Lane 1, RNA prepared from *S. fradiae* grown overnight in TSB; Lanes 2-4, RNA prepared from *S. fradiae* grown in MM-I for 5 days. Both the 23S rRNA and 16S rRNA bands are visible.



4. RNA dot/ slot blots.

RNA (3 µl) was incubated with 200 µl of ice cold denaturing solution (10 mM NaOH, 1 mM Na_2EDTA) at 60°C for 10 min. DNA controls (0.1 µg) were treated identically, except that they were denatured at 100°C. RNA and DNA samples were then chilled on ice whilst the vacuum blotter (Bio-dot Microflitration apparatus, Bio-Rad) was assembled with Zeta-probe GT membrane (Bio-Rad) and filter paper, both pre-wetted in DPEC-treated SQ. Samples were loaded and a vacuum applied so that the samples were sucked onto the membrane. The wells

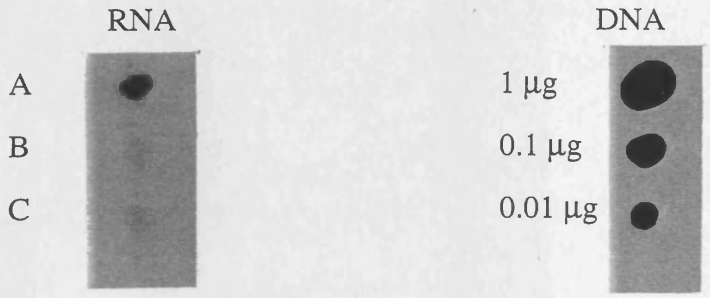
were washed with denaturing solution and and the vacuum reapplied. The membrane was washed in 2x SSC, 0.1% SDS and baked for 30 min at 80°C and stored at room temperature (20°C).

Hybridisation.

The membrane was prehybridised in 25 ml of hybridisation buffer (1 mM Na_2EDTA; 0.25 M Na_2HPO_4, pH 7.2; 7% SDS) for 5 min at 65°C. The probe, which was labelled by primed synthesis (Chapter 2, page 43), was denatured in a boiling water bath for 10 min and then chilled on ice. The probe was then added to the hybridisation buffer and hybridised overnight at 65°C. The membrane was then washed briefly in 2x SSC, wrapped in Saran wrap, and exposed to X-ray film overnight at -70°C.

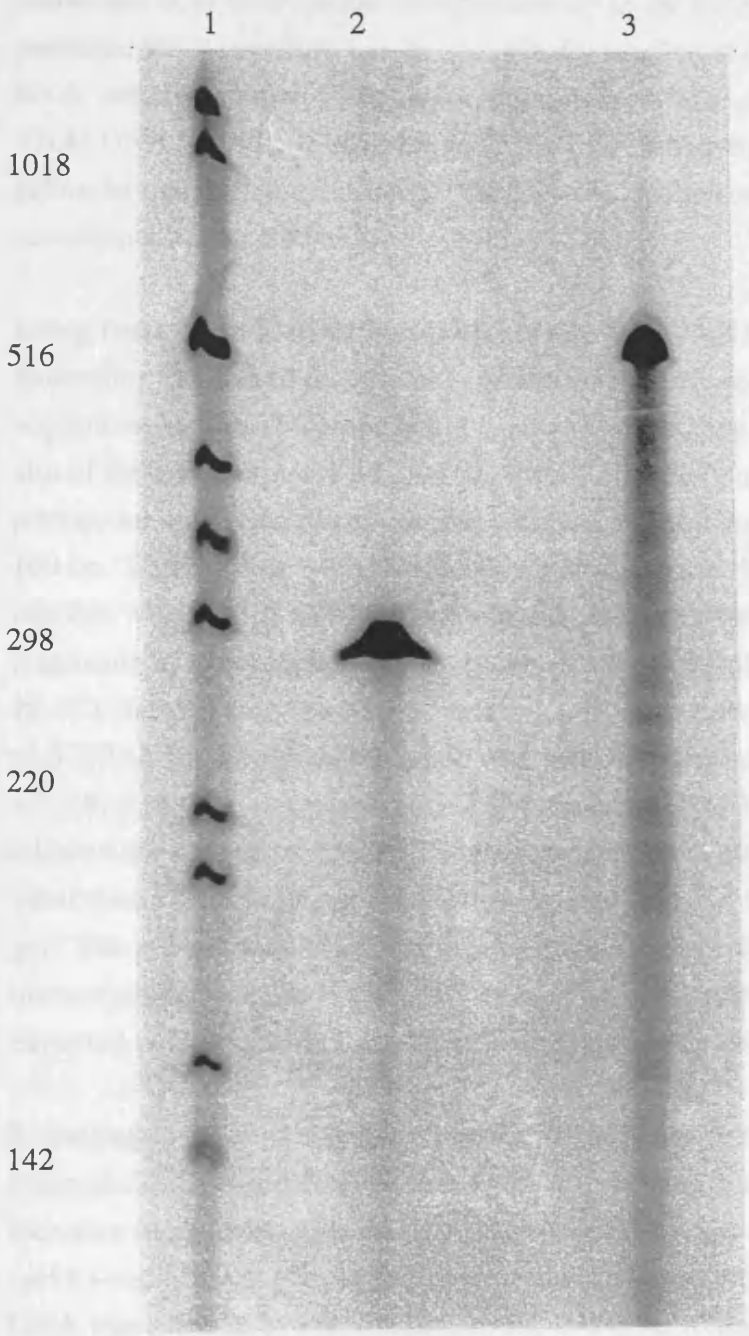
The integrity of RNA prepared from *S. fradiae,* grown in TSB and MM-I, was checked by probing with a gene that is constitutively expressed. The resistance gene, *tlrA,* was chosen because its transcript, although truncated, is constitutively expressed in uninduced and induced conditions (Kelemen *et. al.*, 1994).

Figure 45 In order to check the integrity of RNA preparations, RNA dot blots were probed with the resistance gene *tlrA* (a *Kpn* I-*Sst* I DNA fragment derived from pSVB2). RNA prepared from *S. fradiae* grown A, overnight in TSB; B, grown for 5 days in MM-I; C, grown for 3 days in MM-I. DNA controls (pSVB2); 1 µg; 0.1 µg; 0.01 µg.



The probe was a 1 kb *Kpn* I-*Sst* I DNA fragment, derived from pSVB2 which is a pIJ702 based plasmid which contains *tyl* DNA, including the resistance gene *tlrA* (Birmingham *et. al.*, 1986). The *Kpn* I-*Sst* I probe would hybridise to the truncated *tlrA* transcript (Kelemen *et. al.*, 1994). The probe produced a signal with all three samples of RNA, although more transcript appeared to be present in the RNA prepared from *S. fradiae* grown overnight in TSB, than in the RNA isolated from cultures grown in MM-1 for 3 and 5 days (Figure 45, page 114). Positive controls consisted of serial dilutions of pSVB2 (Figure 45, page 115).

Figure 46 Demonstration of the use of a radioactively labelled 1 kb ladder to estimate the size of [γ-^{32}P]ATP labelled DNA fragments. Lane 1, 1 kb ladder; Lane 2, ermEp* (200 bp); Lane 3, *Bam*H I-*Mlu* I DNA fragment from pLST973A (500 bp).



5. Mapping transcripts by S1 mapping (protocol based on Hopwood et. al.. 1985, with modifications by Kelemen et. al., 1991).

S1 mapping (Berk *et. al.*, 1977).

S1 mapping is a technique which is used to determine both the 5' and 3' termini of a RNA transcript. A ss DNA probe (complementary to the RNA transcript), which overlaps the predicted RNA terminus, can be specifically labelled at one end. The probe is then hybridised to RNA, and treated with S1 nuclease which is specific to ss RNA and ss DNA. Any resulting RNA/ DNA hybrid can be sized against a DNA ladder by electrophoresis, on a polyacrylamide gel under denaturing conditions. The DNA is labelled on the anti-coding strand, only, which is complementary to mRNA.

Using the 1 kb ladder (Gibco-BRL) to size DNA/ RNA hybrids (S.L.C and J. Bacon).

Estimating the size of the products generated in SI mapping normally requires the use of a sequencing ladder, the probe being used as a sequencing template (Keleman *et. al.*, 1991). The aim of these experiments was not high resolution mapping to the nearest nucleotide, but to provide an indication of the number and position of transcripts in the *tylLM* region, to the nearest 100 bp. Experiments were therefore carried out to test the accuracy of using a 1 kb ladder, end labelled with [γ-^{32}P]ATP (protocol page 117), to size transcripts (Figure 46, page 116). DNA fragments of known size, ermEp* (Bibb *et. al.*, 1985; 1994) derived from pLST9828 as 200 bp *Eco*R I-*Bam*H I fragment (approximate size); and a *Bam* HI-*Mlu* I DNA fragment from pLST973A (500 bp), were labelled with radioactivity, boiled for three minutes, and separated on a 3.5%, 7 M urea, polyacrylamide gel with the labelled 1 kb ladder. A 3.5% acrylamide gel allowed the separation of DNA fragments of between 100-1000 base pairs in size, and was run at 39-40 watts for 60 min, until the bromophenol blue dye front was 5 cm from the bottom of the gel. The gel was then fixed and dried (Chapter 2, page 49) and exposed to X-ray film between intensifying screens, at -70°C for 2 days. The labelled DNA fragments were positioned as expected with respect to the 1 kb ladder (Figure 46, page 116).

Labelling the 5' end a single stranded DNA probe for S1 mapping.

Plasmid DNA (10 µg) was digested with a restriction enzyme that gave a 5' overhang and could therefore be dephoshorylated. The digest was extracted with with an equal volume of Tris-HCl (pH 8)-equilibrated phenol/chloroform and the aqueous phase was precipitated with ethanol. The DNA was pelleted by centrifugation (13, 000 rpm, 10 min), washed with 70% ethanol, and dried under vacuum.

The 5' end was dephosphorylated with calf intestinal alkaline phosphatase (CIAP) using a method based on a Promega protocol. The reaction (50 µl) was set up using 5 µl 10x CIAP buffer (Boehringer), 10 µg of digested DNA in 1 µl of 10 mM Tris-HCl, pH 8.0, and 0.1 units of

CIAP (Boehringer), and incubated at 37°C for 30 min. The reaction was extracted with phenol, precipitated, washed and dried as described above. The DNA was then digested with a second enzyme to release the probe, and the products were run on a 1% low melting point agarose gel and isolated from the agarose (Chapter 2, page 41). The 1 kb DNA ladder (10 µg) was extracted with Tris-HCl (pH 8)-equilibrated phenol/chloroform, and DNA in the aqueous phase precipitated, washed and dried. The 5' phosphates were removed by CIAP as previously described.

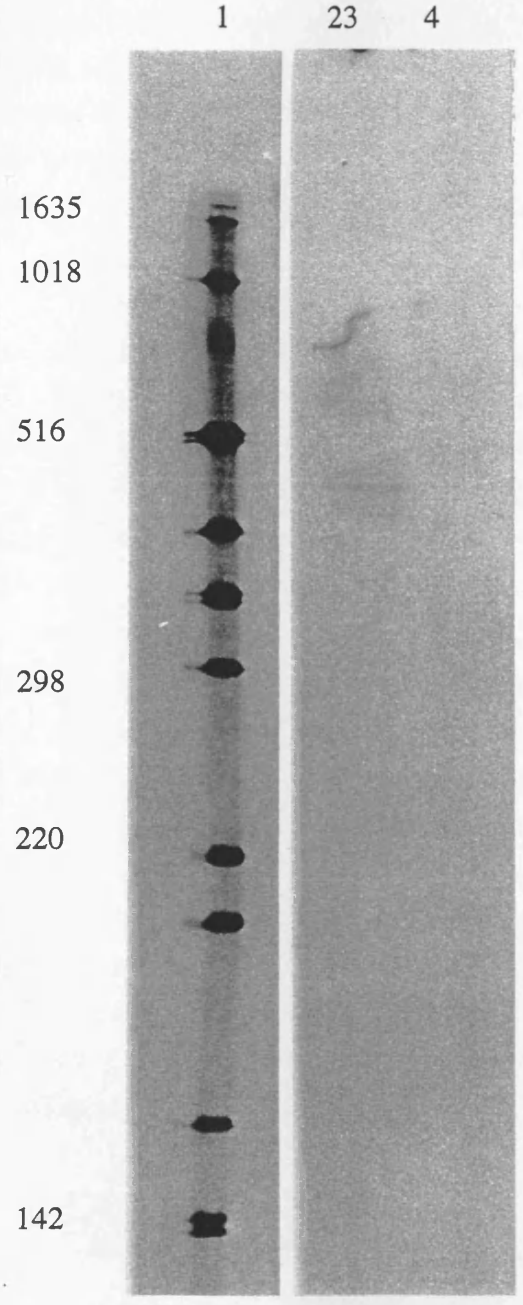
DNA was resuspended in 34 µl of DEPC-treated SQ and labelled with 10 µl 5x T4 polynucleotide kinase exchange buffer (Gibco BRL), 2 µl T4 polynucleotide kinase (10 units/ µl, Gibco BRL), 10 µl [γ-^{32}P]ATP (3,000 Ci/ mmole, 10 mCi/ ml, Dupont). The labelling reaction was incubated at 37°C for 2 h, and the probe was precipitated with 240 µl 5 M ammonium acetate, and 700 µl of 100% ethanol. The 1 kb ladder was treated identically, but always labelled more efficiently than the probe.

Hybridisation of RNA and DNA probe, and S1 treatment.

RNA (100-200 µg) was precipitated with the DNA probe (1 µg in 10 µl of DEPC-treated SQ) using 500 µl of isopropanol and 1/10 volume sodium acetate, pH 6 at -70°C for 10 min. The DNA and RNA were then pelleted by centrifugation, (13, 000 rpm, 10 min) before being washed with 70% ethanol. The pellet was air dried and dissolved in 100 µl of hybridisation buffer [80% v/v deionised formamide; 40 mM PIPES, pH 6.4; 400 mM NaCl; 1 mM Na_2EDTA (Favaloro *et. al.*, 1980)]. This involved repeated pipetting of the mixture. The hybridisation mixture was then denatured at 90°C for 5 min before being immediately transferred to a water bath at 75°C for 30 min, before the water bath was left to cool to 63°C over 3 h. The hybrid was then chilled on ice, before 50 µl was incubated with 300 µl of S1 buffer [50 mM sodium acetate, pH 4.6; 280 mM NaCl; 5 mM $ZnSO_4$; 800 units of S1 nuclease (Gibco BRL); and 20 µg/ ml denatured salmon sperm (Favaloro, 1980)] at 23°C for 45 min. S1 nuclease preferentially digests single stranded RNA and DNA. The hybrid was extracted with an equal volume of Tris-HCl (pH 8)-equilibrated phenol/chloroform to stop nuclease digestion. Calf thymus DNA (Sigma) (1-2 µg) was added to the aqueous phase which was then precipitated with 3 volumes of ethanol at -70°C for 10 min. The sample was pelleted (13, 000 rpm, 10 min) and washed in 70% ethanol before being air dried. The pellet was dissolved in 6 µl of DEPC-treated SQ and 4 µl of formamide loading buffer (80% formamide, 10 mM Na_2EDTA pH 8.0, 1 mg/ml xylene cyanol FF and 1 mg/ml bromophenol blue).

Samples were separated by electrophoresis as described for the 1 kb ladder (page 117).

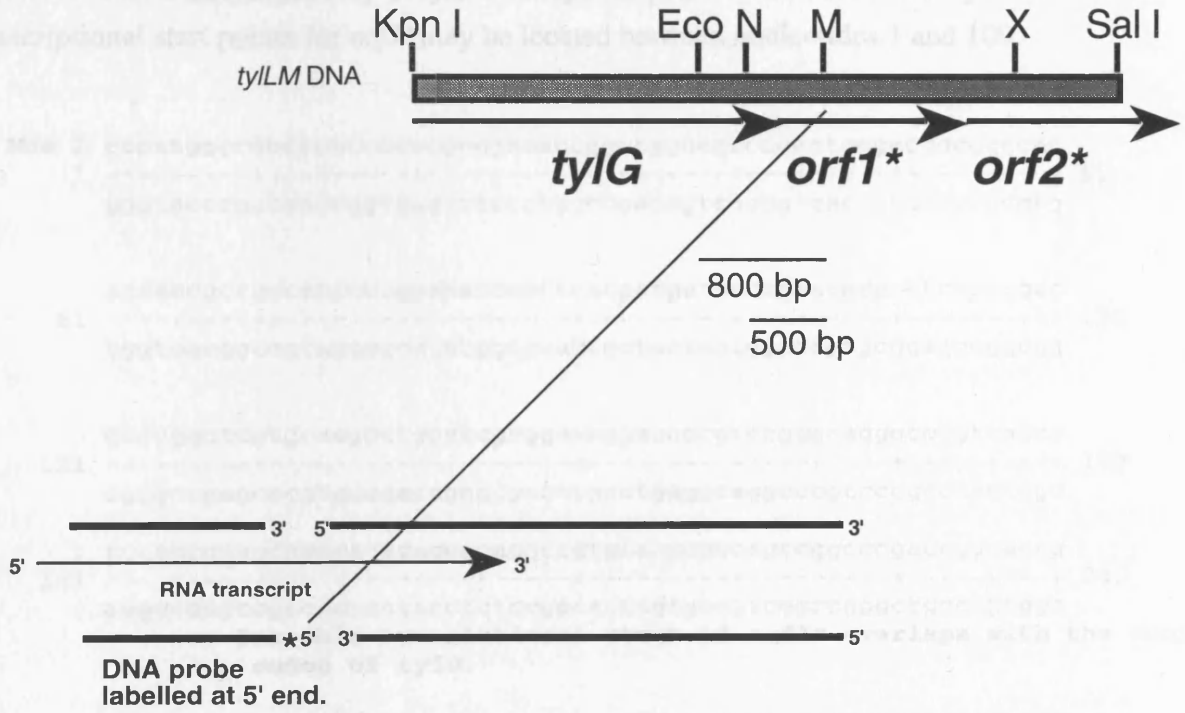
Figure 48 Low-resolution S1 nuclease protection analysis of the 5' end of the *orf1** transcript. RNA was prepared from *S. fradiae* which was harvested after 5 days of growth in MM-1, and was used in an S1 experiment with a probe bridging the translational start of *orf1**. The probe was an 800 bp *Mlu* I-*Bam*H I fragment, labelled at the 5' *Mlu* I end. Lane 1, 1 kb DNA ladder; lanes 2 and 3, probe 2 digested with S1 nuclease in the presence of RNA from *S. fradiae* cultivated for 5 days in MM-1; lane 4 intact probe. The autoradiogram is divided into two parts and the intevening area was used for another experiment.



Mapping the 5' end of the *orf1 transcript.**

tylG and *orf1** are terminally overlapping (Chapter 3, page 64), suggesting that these genes may be co-transcribed. Alternatively, a promoter close to the start of *orf1** might allow transcription independently of *tylG,* and may represent the start of a polycistronic transcript within the *tylLM* region. Attempts were made to map the 5' end of the *orf1** transcript by S1 mapping. Two probes (1 and 2), both spanning the putative translational start of *orf1** (Figure 47, page 120), were used, but a result was only achieved with probe 2 (Figure 48, page 119).

Figure 47 The location of two probes used to map the 5' end of the *orf1** transcript within the *tylLM* region. Restriction sites; Eco, *Eco*47 III; X, *Xho* I; M, *Mlu* I; N, *Nco* I.



Probe 1 was a *Bam*H I (*Nco* I in *tylLM* DNA; *Bam*H I cut in MCS of pUC18, and was used to release the fragment as the *Nco* I site was blunt ended) -*Mlu* I fragment (500 bp), derived from pLST973A and probe 2 a *Bam* HI (*Eco* 47III in *tylLM* DNA) -*Mlu* I fragment (800 bp) derived from pLST973C (Figure 47, page 120). Both probes were uniquely labelled at the 5' *Mlu* I end with [γ-^{32}P] and are complementary to mRNA transcribed from DNA 5' to the *Mlu* I site. Total RNA prepared from two cultures of *S. fradiae*, grown for 5 days in MM-1 medium, was hybridised to probe 2. The production of active compounds by these cultures was confirmed microbiologically (Chapter 4, page 74).

Results

How many transcriptional start points?

Two bands between 400 and 500 bp, and possibly a band the same size as the intact probe were present in SI experiments using RNA prepared from *S. fradiae*, grown for 5 days in MM-1 (Figure 48, page 119). The band the same size as the intact probe (800 bp) could represent a continuous transcript between *tylG* and *orf1**, the other bands corresponding to two transcripts initiating between 100 and 200 bp upstream of the presumed translational start point of *orf1**. Are the bands which indicate less than full length protection degraded or processed transcripts, or 'true' additional transcriptional start points?

Figure 49 The translational stop of *tylG* overlaps the putative start codon of *orf1**. Two transcriptional start points for *orf1**may be located between nucleotides 1 and 100.

```
Nco I cccatggcgactggccacccggggacaccggctggcagtcccactgggacggcgcgcac
    1 ---------+---------+---------+---------+---------+---------+ 60
      gggtacccgctgaccggtgggcccctgtggccgaccgtcagggtgaccctgccgcgcgtg

      accaccgccggcatccccggaaaccacttcacgatgatgaccgaacacgcctccgccgcc
   61 ---------+---------+---------+---------+---------+---------+ 120
      tggtggcggccgtaggggcctttggtgaagtgctactactggcttgtgcggaggcggcgg

      gcccggctcgtgcacggctggctcgcggaacggaccccgtccgggcagggcgggtcaccg
  121 ---------+---------+---------+---------+---------+---------+ 180
      cgggccgagcacgtgccgaccgagcgccttgcctggggcaggcccgtcccgcccagtggc

      tcccgcgcggcggggagagaggagaggccgtgaacacggcagccggcccgaccggcaccg
  181 ---------+---------+---------+---------+---------+---------+ 240
      agggcgcgccgcccctctctcctctccggcacttgtgccgtcggccgggctggccgtggc
              Possible translational start of orf1* overlaps with the stop
               codon of tylG.

      ccgccggcggcaccaccgccccggcggcggcacacgacctgtcccgcgccggacgcaggc
  241 ---------+---------+---------+---------+---------+---------+ 300
      ggcggccgccgtggtggcggggccgccgccgtgtgctggacagggcgcggcctgcgtccg

      tccaactcacccgggccgcacagtggttcgccggcaaccagggagacccctacgggatga
  301 ---------+---------+---------+---------+---------+---------+ 360
      aggttgagtgggcccggcgtgtcaccaagcggccgttggtccctctggggatgccctact

      tcctgcgcgccggcaccgccgacccggcaccgtacgaggaagagatccgtgagcgggggc
  361 ---------+---------+---------+---------+---------+---------+ 420
      aggacgcgcggccgtggcggctgggccgtggcatgctccttctctaggcactcgccccg

      cgctgttccacagcgaactcctcggcacctgggtgaccggcagccgccatgtcgccgacg
  421 ---------+---------+---------+---------+---------+---------+ 480
      gcgacaaggtgtcgcttgaggagccgtggacccactggccgtcggcggtacagcggctgc
```

```
    ccgtgacggccgacgacgcgt Mlu I
481 ---------+---------+-
    ggcactgccggctgctgcgca
```

Possible further work.

S1 mapping is a useful technique for analysing transcripts, but artefacts can occur due to secondary structure in RNA, and therefore other methods could be used to confirm preliminary results. Primer extension involves annealing a primer (labelled with [γ-^{32}P]ATP at the 5' end) to mRNA, and extending from it to the 5' end of the mRNA, using reverse transcriptase (Sambrook *et. al.*, 1989). The length of the synthesised DNA is assessed by electrophoresis on a polyacrylamide gel, and can be use to locate transcriptional start points. Mutational cloning is another method that could be used to delimit transcriptional units (Chater *et. al.*, 1983). DNA from the region of interest can be introduced into a derivative of the bacteriophage ϕC31, with the attachment site deleted, encouraging integration into the corresponding region into the host genome by homologous recombination. Depending on whether the DNA insert is within a transcription unit, or flanks a transcriptional start or stop signal, the host will exhibit a mutant or wild-type phenotype, respectively.

(2) Identifying promoter elements in *tylLM* DNA using promoter probe vectors.

Promoter-probe vectors.

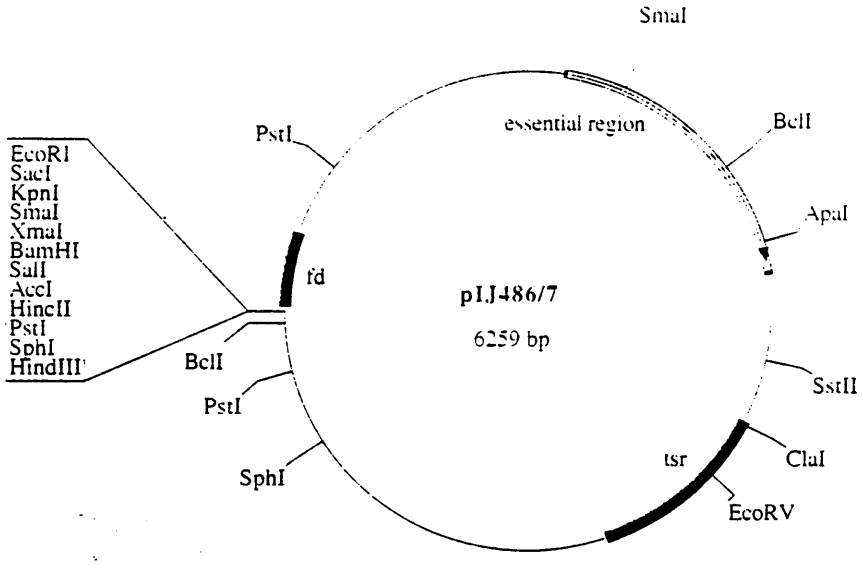
Promoter-probe vectors allow the ability of a DNA fragment to act as a promoter to be assessed by introducing it upstream of a promoterless reporter gene whose gene product can be assayed. A diversity of reporter genes have been used in *Streptomyces*. *xylE,* isolated from *Pseudomonas putida,* has been used as a chromogenic reporter gene (Ingram *et. al.,* 1989). It encodes a catechol dioxygenase that converts colourless catechol into a yellow product. However, levels of yellow product do not necessarily reflect the amount of transcript produced, as confirmed by SI mapping (Dr Gabriella Kelemen, personal communication). In the following studies, an antibiotic resistance gene was used as a reporter gene which allowed easy and rapid estimation of promoter strength by simply calculating the MIC (Chapter 2, page 54) of a particular clone.

pIJ486 has a reporter gene that confers resistance to kanamycin.

pIJ486 (Figure 50, page 123) is a high copy promoter-probe vector (6 kb) that has a reporter gene which confers resistance to neomycin and kanamycin in *S. lividans* and *E. coli* (Ward *et al.*, 1986). The reporter gene (*neo*) is an aminoglycoside 3'-phosphotransferase II gene which was derived from the transposon Tn*5* (Beck *et. al.*, 1982). Upstream of the reporter gene is a transcriptional terminator from the *E. coli* phage *fd* (Gentz *et. al.*, 1981), which prevents transcriptional read-through from promoters within the vector. A translation stop codon downstream of the MCS, but located before the start codon of *neo,* prevents any translational

fusions that could occur between the phosphotransferase and upstream genes. The MCS of pIJ486 is present in the opposite orientation in the sister plasmid pIJ487. The multitude of unique sites in the MCS of pIJ486 means that it is relatively easy to release and introduce DNA inserts, aswell as generate probes for SI mapping (Ward *et. al.*, 1986).

Figure 50 pIJ486 is a promoter-probe vector that has an antibiotic resistance gene as a reporter gene.



Ligation of random fragments of the *tylLM* DNA into the promoter-probe vector pIJ486. pLST97A was digested with *Kpn* I and *Bam*H I to release the 8 kb insert of *tylLM* DNA. *tylLM* DNA was gel isolated (Chapter 2, page 41) before digestion with *Sau*3A I which cuts DNA frequently, as it has a four base pair recognition sequence (GATC). The *Sau*3A I digest was ligated into pIJ486 which had been digested with *Bam*H I (a unique restriction endonuclease site in the MCS, which is compatible with *Sau*3A I) and treated with CIAP (Chapter 2, page 40). This ligation mix was introduced into *S. lividans* OS456 protoplasts by transformation (Chapter 2, page 52). *S. lividans* OS456, derived from *S. lividans* TK21 (Pernodet *et. al.*, 1996), was used as it is relatively easy to transform, and is sensitive to both kanamycin and neomycin (pages 124-125). Transformation plates were incubated overnight at 30°C and were then overlayed with 1 ml SQ containing 0.2 mg of thiostrepton to select for pIJ486. There is no colour selection for recombinant derivatives of pIJ486, and therefore 130 transformants were transferred, using toothpicks, to NEF containing thiostrepton (10 µg/ ml) to select for the presence of pIJ486, and were incubated at 30°C for 3 days (this helped limit the chances of selecting mutants which are resistant to kanamycin/ neomycin). These plates were then used to transfer colonies onto NEF

Figure 51 Gradient plates showing relative promoter strengths of different fragments of DNA inserted into the promoter-probe vector pIJ486 and introduced into *S. lividans* 0S456. Each plate has a control, 486 (0S456 with pIJ486), and contains an approximate gradient of 0-100 μg/ ml antibiotic, either kanamycin (plates A, and C), or neomycin (plates B, and D). Plates containing kanamycin had less background.

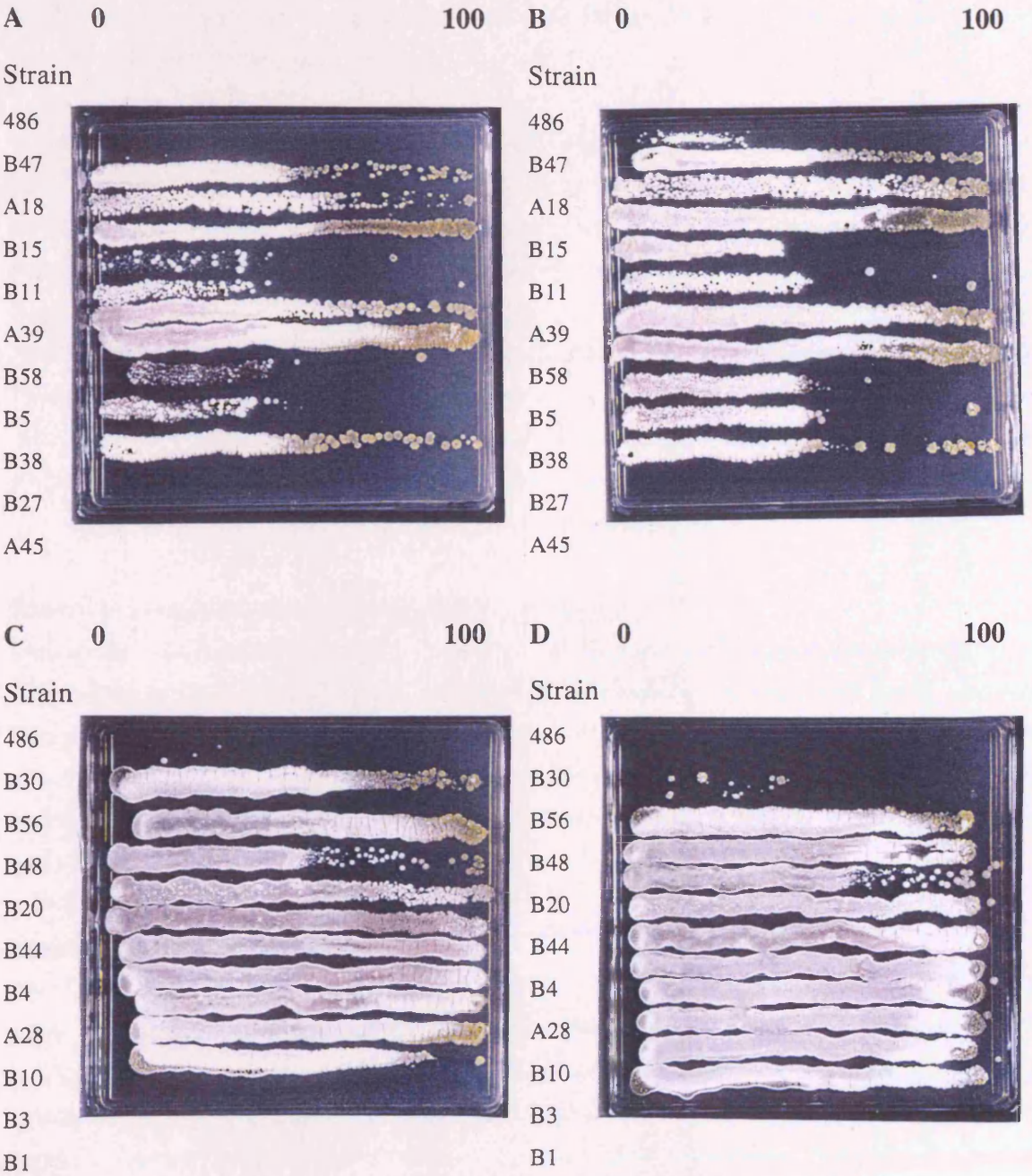


Figure 52 A table summarising the details of ten 0S456 strains containing DNA inserts in pIJ486, which confer high levels of resistance to kanamycin; size of DNA insert (bp) released when pIJ486 was digested with *Bgl* II; MIC on kanamycin (μg/ ml); retransformation data; and restriction patterns in *tylLM* DNA using the *enzymes, *Nco* I, *Sac* II, *Bst*E II, *Sfi* I, and *Msc* I.

Strain.	Size of DNA insert (bp).	MIC on Kanamycin (μg/ ml).	Retransformation of 0S456 still confers resistance.	Digestion of DNA insert*.
A18	300	200-250	Yes	None
B30	200	200-250	Yes	*Nco* I, *Sac* II.
B48	400	200-250	Yes	None
B1	400	90-110	Yes	*Sac* II
B44	500	250-300	Yes	*Msc* I
B5	200	250-300	Yes	*Sac* II
B15	200	200-250	No	
B56	500	200-250	Yes	None
B10	500	200-250	Yes	*Bst*E II, *Sfi* I.
B3	500	200-250	Yes	*Bst*E II, *Sfi* I.

containing thiostrepton (10 μg/ ml) and NEF containing kanamycin (10 μg/ ml) by replica plating (Chapter 2, page 53). Clones that grew on kanamycin (10 μg/ ml) were streaked onto gradient plates (Chapter 2, page 54) containing kanamycin or neomycin (0-100 μg/ ml).

Results.

Analysis of resistant clones.

Out of a total 130 transformants, 20 (15%) grew on low levels of kanamycin (10 μg/ ml). These strains were used to inoculate gradient plates which demonstrated differences in promoter strength between these clones (Figure 51, page 124).

However, higher background growth of 0S456 carrying pIJ486 was observed on neomycin gradients than kanamycin, and therefore MICs were calculated on kanamycin. OS456 with pIJ486 only grew on very low levels of kanamycin (< 5 μg/ ml). Ten clones which gave high levels of resistance to kanamycin, and had DNA inserts of less than 700 bp, were studied in detail (Figure 52, page 126). These plasmids were retransformed into OS456 to confirm that the resistance levels confered were due to the presence of the plasmid, as opposed to a chromosomal mutation. All of the ten plasmids continued to confer the same levels of resistance, except for one (B15) which was subsequently discarded. Plasmid DNA was isolated as small scale preparations (Chapter 2, page 45), which were digested with *Bgl* II to release any DNA insert that might be present. MICs were determined on kanamycin.

Restriction endonuclease digests of DNA inserts.

Due to the multitude of restriction endonuclease sites in pIJ486 which are common in *Streptomyces* DNA, *tyl* DNA was released from pIJ486 by digestion with *Bgl* II, and was ligated into pUC18 digested with *Bam*H I and treated with CIAP (Chapter 2, page 40). Two strains of OS456 (B10 and B3) exhibited the similar levels of resistance to kanamycin (250 - 300 μg/ml) and their DNA inserts were the same size (500 bp) and cut by *Bst*E II and *Sfi* I (Figure 52, page 125; Figure 53, page 127).

Sequencing

The DNA insert in B10 was sequenced in pUC18 using universal and reverse primers (Chapter 2, page 50) and located within the *tylLM* region. The sequence generated identified the DNA as a *Sau*3A I fragment of *tylLM* DNA, which was situated within the translational start of *orf4** (residues 3659-4148, Figure 53, page 126). This DNA fragment could possibly contain a promoter as the designated start codon at position 3606 is putative. There are alternative in-frame translational start signals at positions 3723 and 3735, which lie within the *Sau*3A I fragment. This DNA was released as an *Eco*R I and *Hin*d III fragment, and was inserted into pIJ487 in order to reverse its orientation. This plasmid in OS456, grew on kanamycin (50 - 100

μg/ml) representing a relatively high background. The DNA fragment was orientated in pIJ486 by digesting the plasmid with *Sst* I which cuts within the MCS once, and the *tylLM* DNA insert once asymmetrically (at position 3833). This digest revealed that this piece of DNA is in the opposite orientation than expected for a potential *orf4** promoter. This piece of DNA is unlikely to be a 'true' promoter as codon preference analysis has shown that there are no ORFs on the other DNA strand, and *tlrD* is transcribed in the opposite direction to *orf4**. This data therefore suggests that promoter-probe vectors are limited in their use as identifiers of 'true' promoter fragments.

Figure 53 A *Sau*3A I fragment of *tylLM* DNA, situated within the start of *orf4** (residues 3659-4145), acted as a strong promoter in pIJ486. The DNA insert released from pIJ486 digested with *Bgl* II, isolated from strain B10, was introduced into the *Bam*H I site of pUC18. The DNA was then sequenced using universal and reverse primers. The *orf4** start codon at position 3606 is putative, and alternative in-frame translational start signals at positions 3723 and 3735 are marked.

Translational stop of *orf3 overlaps putative start codon of *orf4***

```
          acccggtgaagtgggaccccgagatcctcaccgctgccggccggccgtcaccggaggaga
    3601  ---------+---------+---------+---------+---------+---------+ 3660
          tgggccacttcaccctggggctctaggagtggcgacggccggccggcagtggcctcctct
                                                                  Sau3A I

                                                BstE II
          tcgccgccctccgactccctgagtcctgccgcgcggtcacctccacaaggcggacaccg
    3661  ---------+---------+---------+---------+---------+---------+ 3720
          agcggcgggaggctgagggactcaggacggcgccagtgggaggtgttccgcctgtggc
```

Other in-frame start codons

```
          gcatgttcgacggcatggccggcccggacaaggacccacgcaagtcgctccacgtggacg
    3721  ---------+---------+---------+---------+---------+---------+ 3780
          cgtacaagctgccgtaccggccgggcctgttcctgggtgcgttcagcgaggtgcacctgc

                                  Sfi I                         Sst I
          aggtcccggtgcccggactcggccccggcgaggccctggtggccgtcatggcgagctcgg
    3781  ---------+---------+---------+---------+---------+---------+ 3840
          tccagggccacgggcctgagccgggggccgctccgggaccaccggcagtaccgctcgagcc

          tgaactacaacaccgtctggtcggccatcttcgagccggttccgaccttcggtttcctgg
    3841  ---------+---------+---------+---------+---------+---------+ 3900
          acttgatgttgtggcagaccagccggtagaagctcggccaaggctggaagccaaaggacc

          agcggtacggccggctctccccgctcaccaagcggcatgacctgccgtaccacatcatcg
    3901  ---------+---------+---------+---------+---------+---------+ 3960
          tcgccatgccggccgagaggggcgagtggttcgccgtactggacggcatggtgtagtagc

          gctccgacctctccggtgtggtgctccgcaccggccccggcgtcaacgcctggaagcccg
    3961  ---------+---------+---------+---------+---------+---------+ 4020
          cgaggctggagaggccacaccacgaggcgtggccggggccgcagttgcggaccttcgggc
```

```
     gtgacgaggtcgtcgcgcactgcctctccgtggagctggagtccgccgacggccacaacg
4021 ---------+---------+---------+---------+---------+---------+ 4080
     cactgctccagcagcgcgtgacggagaggcacctcgacctcaggcggctgccggtgttgc

     acaccatgctcgaccccgagcagcgcatctggggcttcgagacgaacttcggcggcctcg
4081 ---------+---------+---------+---------+---------+---------+ 4140
     tgtggtacgagctggggctcgtcgcgtagaccccgaagctctgcttgaagccgccggagc

     ccgagatc Sau3A I
4141 ---------+
     ggctctag
```

Problems associated with the use of promoter-probe vectors.

These studies have shown that a DNA fragment that acts as a promoter in a promoter-probe vector, may not be a 'true' promoter in vivo. Also, when using promoter-probe vectors the amount of the protein encoded by the reporter gene is used as an indicator of the amount of transcript produced (Ward *et. al.*, 1986). However, regulation of protein expression may also operate at the level of translation, aswell as at the level of transcription. The high copy number of pIJ486 (100-200 copies per chromosome) could cause difficulties when analysing the regulation of transcription from a promoter. It would be useful to be able to insert a reporter gene downstream of the promoter being studied in the wild-type organism, ie as a single copy, and compare levels of reporter gene expression with a single copy in a heterologous host.

Chapter 7 Discussion.

Streptomyces fradiae produces the antibiotic tylosin, which consists of a polyketide lactone with three deoxyhexose sugars, mycaminose, mycarose and mycinose, attached.

Mutants of *S. fradiae* had been generated that were defective in tylosin biosynthesis, in order to help elucidate the pathway from tylactone to tylosin (Baltz, *et. al.*, 1981). We were interested in genes involved in the biosynthesis or addition of mycaminose, the first sugar to be added to tylactone, forming the first biologically active intermediate (Kirst *et. al.*, 1982). Within the *tylIBA* region, two genes catalysing steps common to the biosynthesis of all three sugars were identified, (*tylA1*, and *tylA2*), and a mycaminose specific gene, *tylB,* which is thought to encode the mycaminose aminotransferase (Merson-Davies *et. al.*, 1994). According to a hypothetical pathway to TDP-mycaminose (Figure 5, page 33, Chapter 1), this left an isomerase, methyltransferase, and the enzyme that adds mycaminose to tylactone, to be identified.

Two mutants defective in sugar biosynthesis, *tylL* (GS33) and *tylM* (GS62) were both complemented by a 8 kb *Kpn* I-*Bam*H I DNA fragment, from the tylosin biosynthetic gene cluster (Fishman *et. al.*, 1987). The *tylM* mutant is defective in the biosynthesis or addition of mycaminose, in contrast to the *tylL* mutant which is defective in the production or addition of all three sugars. These mutants do not, therefore, produce active compounds (Figure 21, page 80, Chapter 4; Figure 33, page 101, Chapter 5).

Experiments have therefore been carried out to identify which open reading frame would complement each mutant, in hope of identifying other genes involved in deoxyhexose biosynthesis (Chapter 4, page 71). About 6 kb of the *tylLM* region was sequenced (Gandecha *et. al.*, 1997), and analysed by codon preference (UWGCG program) in order to identify putative tylosin genes. Five ORFs were identified downstream of the polyketide synthase genes (*tylG*); one was the resistance gene *tlrD* (Gandecha *et. al.*, 1996); and the other four were possible candidates for deoxyhexose genes (Figure 7, page 55, Chapter 3). The first gene downstream of *tylG* was sequenced as part of this project. The function of the *orf1** product is not known, although convincing matches with two other genes, *eryCII* (P. F. Leadlay, personal communication) and *dnrQ* (Otten *et. al.*, 1995) suggest that it functions in the biosynthesis of an amino sugar, ie in the case of *orf1**, mycaminose. Could it be the isomerase that converts 4-keto, 6-deoxyglucose to 3-keto, 6-deoxyglucose, suggesting the latter as the last common intermediate shared in the synthesis of the tylosin sugars (Figure 5, page 33, Chapter 1)?

'Wild-type' DNA from the cosmid pMOMT4 (Beckman *et. al.*, 1989) was used in complementation experiments. A series of plasmids was generated which contained *tylLM* DNA

which had been progressively deleted back from the *Bam*H I site, so as to preserve any promoters driving expression of the *tylLM* genes (Figure 20, page 77, Chapter 4). DNA was introduced into the *S. fradiae* mutants by conjugation from *E. coli,* which avoided digestion of foreign DNA by the recipient restriction systems. Exconjugants were then incubated on solidified tylosin production medium MM-1, and the ability of colonies to produce antimicrobial compounds was assayed microbiologically, relying on the sensitivity of *Micrococcus luteus* to tylosin and its intermediates (Chapter 4, page 74).

The *tylM* mutant was complemented in *trans* by a region of *tylLM* DNA which included *orfs1-3**, but not with the same fragment with *orf3** deleted (Figure 21, page 80, Chapter 4). Eventually, this mutant was complemented in *trans* by *orf3** downstream of a constitutive promoter in an integrative vector (Figure 22, page 80, Chapter 4). The presence of two copies of *orf3** in the complemented *tylM* mutant, in contrast to one in the original mutant, was confirmed by Southern hybridisation (Figure 23, page 81, Chapter 4). Restoration of the ability to make tylosin was established by HPLC (Figure 36, page 102, Chapter 5) and TLC (Figure 39, page 104, Chapter 5). As the TylM leision is mycaminose specific, this data, coupled with protein sequence matches between Orf3* and methyltransferase protein sequences, and the ability of the *orf3** protein to bind SAM (Gandecha *et. al.*, 1997), indicated that this gene encodes the methyltransferase which is responsible for 3-*N*-methylation during dTDP-mycaminose biosynthesis.

Complementation of the *tylL* mutant was not so straightforward. This mutant was complemented by a DNA fragment that included the end of *tylG, orf1**and part of *orf2**, in a replicating vector. However, complementation only occurred in 20% of exonjugants, which exhibited varied levels of production (Figure 24, page 83, Chapter 4). These results suggested that complementation was occurring in *cis* due to a homologous recombination event between the *tylLM* genes in the mutant and the wild-type copies in pLST9732. In *cis* complementation also occurred with this DNA fragment in an integrative vector (Figure 24, page 85, Chapter 4), which was unexpected as this plasmid should insert preferentially into the *attB* site of the *Streptomyces* chromosome (Bierman *et. al.*, 1992; Kuhstoss *et. al.*, 1991b). A single-cross over between the *tylLM* genes in the replicative vector (pLST9732) and the *tylL* genome was confirmed by Southern hybridisation (Figure 26, page 86-88, Chapter 4). However, homologous recombination via a single cross-over was not demonstrated in producing strains carrying the integrative vector (pLST9731), suggesting that either a double-cross over event had occurred, or a single-cross over event had been reversed (Figure 26, page 86-88, Chapter 4). Finally, the *tylL* mutant was complemented in *trans* by *orf2** downstream of a constitutive promoter (Figure 27, page 88, Chapter 4). The presence of two copies of *orf2** in the complemented *tylL* mutant was revealed by Southern hybridisation (Figure 28, page 90, Chapter 4) and these results suggested that *orf2** is the *tylL*

gene. However, these findings contradict other data. Convincing protein sequence matches have been made with glycosyltransferases sequences and motifs from other *Streptomyces* species (Chapter 3, page 67). Also, a *S. fradiae* strain disrupted in *orf2** cannot produce OMT (or tylosin), but when fed OMT, it can convert it to tylosin (Dr Steven Fish, personal communication). This indicates that the strain disrupted in *orf2** has the capabilities to add the other two sugars, mycarose and mycinose to OMT. *orf2** is therefore a mycaminose specific gene, and encodes the glycosyltransferase that adds mycaminose to tylactone.

Zones of inhibition of *M. luteus* growth, produced by complemented *tylL* strains, were always significantly smaller than those produced by the complemeted *tylM* mutant (Chapter 4, page 90). We therefore proposed that the complemeted *tylL* strains might produce a biologically active intermediate, for example OMT, as opposed to tylosin. If true, other mutations in the *tylL* strain may be contributing to the TylL phenotype. However, when *orf2** was integrated into the *tylL* mutant it produced tylosin, as confirmed by HPLC (Figure 38, page 103, Chapter 5) and TLC (Figure 39, page 104, Chapter 5). An in-frame TGA stop codon was identified in *orf2**, amplified by PCR from the *tylL* mutant (Figures 31 and 32, pages 95-96, Chapter 5) and no deviations from the wild-type sequence were found in the *tylL orf1**. The possibility that the *tylL* strain is a multiple mutant therefore seems remote. The TylL phenotype has been characterised as an inability to biosynthesise or add any of the three sugars to tylactone. The possibility that this mutant has been incorrectly typed, or has mutated since it was classified, was rejected on the basis of feeding experiments (Chapter 5, page 106). The *tylL* mutant was fed OMT, desmycosin and DMT, but could not, convincingly, convert any of these compounds to tylosin (Figure 41, pages 107-109, Chapter 5).

How can the *tylL* mutant exhibit a different phenotype to the *orf2** disrupted *S. fradiae* strain when they both appear soley defective in *orf2**? Yet, the *orf2** disruption was not complemented by *orfs1*-2** (Dr Steven Fish, personal communication), in contrast to the *tylL* mutant (Figure 21, page 80, Chapter 4). This result implies that the insertion of the hygromycin cassette into *orf2** in the disrupted strain has a polar effect on *orf3**, and therefore *orf3** must be on same transcript as *orf2** or has its promoter within *orf2**. Unlike the *orf2** disrupted strain, the *tylL* mutant has a functional *orf3** and would therefore accumulate TDP-mycaminose as opposed to the unmethylated sugar. Could this explain the phenotypic differences observed between these two strains? For example, could the mycaminose produced by the *tylL* mutant sequester the cofactor TDP, which needs to be recycled, and therefore prevent the other two glycosyltransferases from adding mycinose and mycarose?

The *tylL* locus; does it exist?

The existence of the *tylL* mutant has caused confusion over the possible routes of deoxyhexose metabolism in the biosynthesis of the tylosin sugars. Two genes (*tylA1* and *tylA2*) which catalyse steps common to the biosynthesis of all three sugars have already been identified (Merson-Davies *et. al.*, 1984). *tylA1* encodes a dTDP-glucose synthase, and *tylA2* a dTDP-glucose dehydratase, which between them allows the conversion of glucose-1-phosphate to dTDP-4-keto, 6-deoxyglucose (Figure 5, page 33, Chapter 1). dTDP-4-keto, 6-deoxy-glucose is a common intermediate in the biosynthesis of many sugars incorporated into the structure of antibiotics. The difficulties of reconciling the results of feeding experiments with a strain disrupted in *orf2** (Dr Steven Fish, personal communication), and complementation experiments with the *tylL* mutant calls into question the existence of the *tylL* locus. Two other possible *tylL* mutants were investigated insearch of the *tylL* locus. GS78 and GS79 are both double mutants, (*tylD* + *A/L*), but restoration of the ability to add mycaminose can still be assayed microbiologically (Chapter 4, page 90). The GS78 phenotype was leaky, however, and GS79 was not complemented by the *Kpn* I-*Bam*H I fragment derived from the *tylLM* region. We therefore propose that the *tylL* phenotype may be the result of physiological constraints, perhaps via the sequestration of TDP by accumulating mycaminose.

Gene expression in the *tylLM* region.

Progress has been made in developing a protocol to cope with the difficulties of isolating RNA from *S. fradiae* strains grown in tylosin production medium MM-1. Constitutively expressed mRNA has been demonstrated in RNA preparations from *S. fradiae* cultures grown in TSB and MM-1, by probing RNA dot blots with a fragment of *tlrA* corresponding to a constitutively expressed transcript (Figure 45, page 115, Chapter 6). Many of the genes in the *tylLM* region are terminally overlapping, suggesting the possibility of co-transcription and or translational coupling. S1 mapping experiments have revealed the possibitity of a polycistronic message running through *tylG* into *orf1** and two additonal transcriptional start points upstream of *orf1**, within *tylG*, during antibiotic biosynthesis (Figure 48, page 119, Chapter 6). However, more detailed transcript analysis is required in the *tylLM* region, and the use of additional approaches to mapping transcripts in *S. fradiae* may help limit the possibility of identifying artefacts. Promoter-probe vectors have been limited in their use in identifying promoters in the *tylLM* region. *tylLM* DNA fragments which act as promoters in pIJ486 have been found (Figure 51, page 124, Chapter 6), but there is little evidence from sequence data that they are true promoters (Chapter 6, page 127).

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Appendix.

Publications.
Gandecha, A. R., **Large, S. L.** and Cundliffe. E. (1997) Analysis of four tylosin biosynthetic genes from the *tylLM* region of the *Streptomyces fradiae* genome. *Gene*, 184, 197-203.

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Conferences.
Biology of Streptomycetes Ohrbeck, Germany, April 1996. Poster: Complementation of *Streptomyces fradiae* mutants blocked in tylosin production. **S. L. Large** and E. Cundliffe.