# Mycaminose metabolism and control of tylosin synthesis in *Streptomyces fradiae*

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

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Sept 2000





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## Acknowledgements

I would like to thank my supervisor, Professor Eric Cundliffe, for the opportunity to work in his laboratory and for regular and illuminating discussion during my time in Leicester. Thanks are also due to the BBSRC for funding my research.

The collective help and support of "Lab 103/104" members has been invaluable to me during this project. In particular, I would like to thank Andrew Butler for providing an excellent starting point from which my work could continue and Neil Bate for his guidance and numerous 'cowboy' methods. Friendship, technical expertise, useful advice and lively debate were also provided by Joanna Bacon, Steven Fish, Ian Smith and George Stratigopolous. Much fun and games were also had outside of the lab !!

Thanks are also due to the many other members of the department, too numerous to mention, who helped me out in a host of different ways and to Joanne Carter for the loan of a computer on which to 'write up'.

During the early part of my PhD, Jo Parish provided much vital love and support for which I am extremely grateful.

For helping to keep reality at bay I would particularly like to thank my housemates, Marky and Joe with whom many excellent barbarian adventures were had and their replacement, (picklin') Steph, an oasis of insanity in a crazy world.

I would also like to thank the Wednesday night scrotes, notably John, Pete, Mark, Craig, Janis, Cameron, Fiona, Martin and anyone else who joined us for curry, beers, midweek cheer and more beers, all available, on draught, on a weekly basis. Thanks also to the many other friends I made in Leicester, in particular Loz and Shirls and especially Billy for being essential parts of my social lifeline and to 'the Fanclub' for Fridays.

Craig and Jo deserve special (extra) mentions for (apart from being ace fun, obviously) giving me a place to go when I needed tea and sympathy. I hope there's always a space in their parking lot.

Finally, thanks to all my family and especially Mum for her love, help and support.

"Look at the size of that thing"
Luke Skywalker

"Now for the science bit..... concentrate"
Jennifer Aniston

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## Abstract

Production of the macrolide antibiotic, tylosin, occurs by a combination of polyketide synthesis and deoxyhexose sugar metabolism in *S. fradiae*. Polyketide synthesis was stimulated by glycosylated tylosin-precursors, and by tylosin itself, in an *S. fradiae* strain disrupted in the glycosyltransferase responsible for the addition of mycaminose (Fish and Cundliffe, 1997). The addition of mycaminose is the essential first step in the conversion of the polyketide ring, tylactone, to tylosin. In the absence of glycosylated material, the strain disrupted in mycaminose addition produced no significant amounts of any tylosin precursor. The possibility that this effect might have been the result of downstream effects on a gene, *ccr*, thought to be involved in polyketide synthesis was refuted by analysis of a strain disrupted in *ccr*. The behaviour of strains disrupted in orf1*, putatively encoding an isomerase and in *tylMI*, encoding a methyltransferase involved in mycaminose synthesis were also analysed. orf1* was revealed to contain an authentic mycaminose-specific gene, designated *tylMIII*. Both these disrupted strains and the *tylB* mutant, altered in an aminotransferase gene also required for mycaminose synthesis but located over 45 kb from its functional counterparts, behaved similarly to the mycaminosyltransferase disrupted strain with respect to stimulation of polyketide synthesis. The stimulation of polyketide synthesis was shown to be a catalytic effect which could also be provoked by other glycosylated macrolides, rosaramicin and spiramycin but not by erythromycin, carbomycin or chalcomycin. The possible involvement of recently discovered regulatory genes associated with the tylosin biosynthetic cluster was investigated and interesting preliminary data were obtained.

The three *tylM*, mycaminose-specific genes are located immediately downstream of the polyketide synthase genes *tylGI* - *tylGV* in the tylosin biosynthetic gene cluster. Construction of a strain disrupted in *tylGV* and complementation analysis of the *tylM* disrupted strains proved the *tylM* genes to be co-transcribed, independantly of *tylG*.

# Abbreviations

| | |
|---|---|
| A | adenine |
| ACP | acyl carrier protein |
| AS-1 | solid growth medium for *Streptomyces* spp. (Baltz, 1980) |
| AT | acetyltransferase |
| ATP | Adenosine triphosphate |
| C | cytosine |
| °C | degree Celsius |
| cAMP | adenosine 3':5' cyclic monophosphate |
| Ccr | crotonyl CoA reductase |
| CIAP | calf intestinal alkaline phoshatase |
| CoA | coenzyme A |
| dATP | 2'-deoxyadenosine 5'-triphosphate |
| dCTP | 2'-deoxycytidine 5'-triphosphate |
| dGTP | 2'-deoxyguanosine 5'-triphosphate |
| dNTP | deoxynucleoside triphosphate |
| dTTP | 2'-deoxythymidine 5'-triphosphate |
| DH | dehydratase |
| DMSO | Dimethyl sulphoxide |
| DMT | demycinosyltylosin |
| ds | double-stranded |
| *E* | *Escherichia* |
| EDTA | Diaminoethanetetra-acetic acid disodium salt |
| ER | enoyl reductase |
| *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 |
| HPLC | High Performance Liquid Chromatography |
| KS | β–keto acyl synthase |
| KSQ | β–keto acyl synthase-like domain (section 1.2.7) |
| kb | kilobase(s) or 1000 bp |
| LB | Luria-Bertani |

| | |
|---|---|
| *m* | mass |
| *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) |
| MS | mass spectrometry |
| NTG | *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine |
| mRNA | messenger RNA |
| NaCl | sodium chloride |
| $Na_2EDTA$ | the disodium salt of ethylenediamine tetraacetic acid |
| NaOAc | sodium acetate |
| NaOH | Sodium hydroxide |
| nm | nanometre |
| NMR | nuclear magnetic resonance |
| OD | optical density |
| -OH | hydroxyl group |
| OMT | 5-*O*-mycaminosyl-tylonolide |
| ORF | open reading frame |
| -P | phosphate group |
| PBP | pencillin-binding protein |
| PCR | polymerase chain reaction |
| PKS | polyketide synthase |
| pmol | picomole |
| R | resistance |
| RNase | ribonuclease |
| rpm | revolutions per minute |
| rRNA | ribosomal RNA |
| rrt | relative retention time |
| S | svedberg unit |
| *S* | *Streptomyces* |
| SAM | *S*-adenosyl-L-methionine |

| | |
|---|---|
| SDS | Sodium dodecyl sulphate |
| sec | seconds |
| ss | single-stranded |
| SSC | standard saline citrate |
| SSQ | Sterile Millipore SuperQ water |
| T | thymine |
| Taq | *Thermus aquaticus* |
| TAE | Tris-acetate- EDTA |
| TBE | Tris-borate-EDTA |
| TDP | Thymidine 5'-diphosphate |
| TE | Tris-EDTA |
| TES | [N-tris(hydroxymethyl)methyl-2-aminoethane-sulphonic acid] |
| *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*. |
| UV | ultraviolet |
| v/v | ratio of volume to volume |
| V | volt |
| w/v | ratio of weight to volume |
| *z* | charge |
| µl | microlitre |

# CHAPTER 1

# INTRODUCTION

## 1.1 *Streptomyces* and antibiotics

### 1.1.1 The genus *Streptomyces*

The genus *Streptomyces* is a member of the family *Streptomycetaceae* in the bacterial order *Actinomycetales*. The name *Streptomyces* translates from Greek as 'bent-fungus' and similarly, *Actinomyces* translates as 'ray-fungus'. As their names suggest, these organisms were originally considered to be fungi but, more recently, a host of biochemical and physiological evidence has been accumulated to the contrary. Important distinguishing features of actinomycetes and therefore also *Streptomyces* include the lack of a membrane boundary separating the nucleus from the cytoplasm, ribosomes of the 70S type (as opposed to 80S in fungi) and peptidoglycan as a cell wall constituent (typical of bacteria). As with all other major prokaryotic and eukaryotic groups, the precise phylogeny of *Streptomyces* has been determined by ribosomal ribonucleic acid sequencing. Their taxonomic status is therefore now clearly established in the kingdom *Prokaryotae*.

*Streptomyces* are aerobic, Gram positive, soil-colonising bacteria and most are non-fastidious chemoorganotrophs. Despite their unequivocal classification as bacteria, *Streptomyces* along with the other actinomycetes can be considered to be 'boundary' organisms in a number of ways. The morphological development of *Streptomyces* on agar is more characteristic of fungi than bacteria. *Streptomyces* grow from spores into complex vegetative mycelia, formed from branching hyphae with infrequent septae. Growth of hyphae mainly occurs at the tips while branching is more characteristic of older hyphae. Nutrients are obtained by the secretion of hydrolytic enzymes which solubilise organic material. Hyphal development involves differentiation into two types of long branching cells, first non-fragmenting substrate mycelia then aerial mycelia which subsequently undergo fragmentation and/or sporulation. The aerial hyphae form long chains of uninucleate spores which are subsequently disseminated. A number of aspects of actinomycete primary metabolism are also more reminiscent of fungi than bacteria. For example in the cells of *Saccharopolyspora erythraea* (originally known as *Streptomyces erythraeus*), higher fatty acids are similar to those found in typical bacteria, palmitic acid being the major component. In contrast the fatty acid condensing enzyme (3-ketoacyl-[acyl carrier protein] synthetase) of this organism is a multienzyme complex that is typical of yeast and filamentous fungi.

*Streptomyces* DNA has a GC content of 70-75%. This characteristically high, narrow range suggests close similarity among the micro-organisms of this genus.

### 1.1.2 Antibiotics and *Streptomyces*

The most striking feature of the actinomycetes, and in particular the genus *Streptomyces*, is their ability to elaborate a wide variety of antibiotic compounds. Antibiotics are secondary metabolites which are harmful to other organisms. Being the products of secondary metabolism their production is non-essential and they are often excreted. There are a large number of chemically diverse classes of antibiotics produced by mant different species. Antibiotics typically have a complex chemical structure and their biosynthesis from modified intermediates of primary metabolism is typically catalysed by specific enzymes which are expressed under certain physiological conditions. Nutrient limitation and the passage from rapid growth into stationary phase are generally the key factors associated with the onset of secondary metabolism.

A number of hypotheses have been put forward to explain why secondary metabolites in general and antibiotics in particular, are produced. One interesting hypothesis (Davies, 1990) suggests that some antibiotics, such as those that bind to ribosomes, are ancient effector molecules, the precursors of proteins in the evolution of cellular life. These molecules were gradually replaced by more efficiently acting protein complexes. However the presence of active sites, in modern macromolecular structures, conserved from that earlier era means that in some cases, now non-essential secondary metabolites can antagonise the selective and specific actions that they used to catalyse. This gives them a new, destructive role as antibiotics. Thus, antibiotic inhibitors of protein synthesis could be the functional precursors of modern ribosomal proteins. Many of these antibiotic inhibitors show extremely specific and strong binding to ribosomal target sites (Cundliffe, 1987).

It has also been proposed that antibiotic secondary metabolites are used by the producing organism to convey a selective advantage in competitive environments (reviewed in Stone and Williams, 1992). In antibiotic producing *Streptomyces*, this argument is consistent with the observation that antibiotic production generally reaches its maximum as the organism encounters nutrient depletion and enters stationary phase. At this time the organism is also channelling energy into the production of aerial mycelium and spores. This is when an organism is at its most vulnerable. The production of antibiotic may therefore give it some selective advantage at this time (Katz and Demain, 1977). There is however little direct evidence to support this theory. No *Streptomyces* have ever been shown to produce antibiotics in greater than trace amounts in their natural environment. There is only circumstantial evidence that antibiotic production ever confers a selective advantage, this comes from antibiotics believed to play a role in plant/microbe interactions (Thomashow and Weller, 1982).

In most antibiotic-producing *Streptomyces* the biosynthesis of antibiotics occurs at its maximum as the level of primary metabolism decreases and the associated period of vigourous growth comes to an end. In liquid fermentation cultures this production can be maintained for a considerable time, in some cases weeks. This biosynthesis is temporally associated with the differentiation to form aerial mycelium and initiation of spore formation on agar. It has been suggested (Hopwood, 1988) that these two 'specialised' properties of *Streptomyces* may be genetically linked as mutation of certain genes appears to affect both aerial mycelia formation and secondary metabolite biosynthesis. Such a pleiotropic switch would be compatible with the idea that antibiotic production confers a selective advantage on an organism at a time when the demands of differentiation make it vulnerable. It is interesting to note that some spores themselves contain antibiotic. It is not inconceivable that the purpose of this is to confer some small advantage on it when it lands on its new environment. It has also been proposed (Davies and Chater, 1990) that the production of antibiotics may actually exert control over spore formation.

While many possibilities have been suggested, the precise reasons why certain organisms produce secondary metabolites and in particular antibiotics is not clear. It is apparent however that the wide variety of compounds produced, of chemically diverse structural types, implies an equally wide variety of different, distinct, functions. None or all of the above possibilities could be represented by the production of secondary metabolites in individual cases. The purpose of the production of a metabolite may have altered on an evolutionary time scale. For instance, what was once a pre-biotic effector may have had its role altered to become an antibiotic giving selective advantage over competitors. It is not impossible that particular metabolites may have several different functions in a single example of production or have different functions when produced in different organisms.

Regardless of the advantage that the biosynthesis of an antibiotic secondary metabolite confers on the producing organism, and whether the antibiotic properties are the reason for production or just an incidental side effect, these compounds have proved extremely useful to humankind. *Streptomyces* are responsible for the production of about 50% of the approximately ten thousand antibiotic compounds which are known. Only a few hundred of the total known antibiotic compounds have proved to have practical application and, of these, approximately 90% are produced by *Streptomyces*.

These natural products have found a wide variety of different uses. In medicine they are used as antibacterial compounds and antitumour agents. They have also been used

to treat animal, fish and plant diseases and as promoters of animal growth. In addition, antibiotics have proved to be invaluable tools in biochemical research.

The medical use of antibiotics, along with improvements in food quality and public health have been the major factors in the increase in human life expectancy in the developed world over the last fifty years.

### 1.1.3 Biosynthesis and resistance in the producing organism

In all known cases of antibiotic production, the genes associated with production have been found in a single biosynthetic cluster. In addition there is typically at least one gene associated with the cluster capable of conferring resistance to the antibiotic produced. In some cases this resistance gene also plays an essential role in biosynthesis. The presence of resistance genes alongside biosynthetic clusters clearly makes sense from the point of view of producing organisms. In order to avoid suicide they must be resistant to their own antibiotic. This is a useful characteristic that has been exploited many times. Finding genes from the producing organism that are capable of conferring resistance is often the first step towards locating the biosynthetic cluster.

Resistance to an antibiotic may be achieved in a number of ways (Cundliffe, 1989):

- Modification or replacement of the target site
- Inactivation of the antibiotic
- Intracellular sequestration and/or efflux
- Barriers to entry of the antibiotic

The nature of the resistance mechanism can give valuable clues regarding the target site and mode of action of the antibiotic.

The resistance mechanism employed by the producing organism is likely to depend on whether the antibiotic is synthesised intracellularly in an active or inactive form. In the latter case activation would occur either during export or extracellularly. Thus modification or replacement of the target site would be unnecessary, unless the antibiotic could re-enter the organism, in which case it would become preferable. A futile cycle of antibiotic synthesis and excretion followed by re-entry and either inactivation or sequestration and/or efflux would then be avoided. Clearly this would be energetically inefficient.

### 1.1.4 Regulation of antibiotic production in Streptomyces

The G-C rich nature of their DNA (Frontali *et al.*, 1965) compared to other organisms has regulatory implications for *Streptomyces*. Transcriptional control sequences are typically A-T rich in other organisms and although the initiation of transcription in *Streptomyces* appears similar to that in other prokaryotes (Buttner and Brown, 1985), *Streptomyces* utilise an unusually large variety of sigma factors. Differences in promoter sequences and the use of multiple sigma factors by *Streptomyces* might explain why *Streptomyces* genes are rarely expressed in *E. coli.*, despite the fact that *E. coli* genes are generally expressed in *Streptomyces* sp.

One result of the high G-C content of *Streptomyces* DNA is that GUG is a more common translational start codon than AUG, unlike the situation in most other organisms in which AUG predominates. The high G-C content also manifests itself in a very strong codon bias at the third position. This bias is a useful feature in the analysis of *Streptomyces* DNA as it facilitates the identification of possible open reading frames (Bibb *et al.*, 1984). A common feature of resistance and regulatory genes in *Streptomyces* is the use of TTA codons encoding leucine. This codon is rarely used elsewhere in *Streptomyces* and its presence is almost certainly indicative of a regulatory impact (Leskiw *et al.*, 1991). In *S. coelicolor* , a series of mutants with a variety of phenotypic characteristics were generated (reviewed in Champness and Chater, 1994). One of these, the pleiotropic mutant *bldA*, was incapable of actinorhodin production. The pathway specific activator for actinorhodin production, *actII* orf4 contains the rare TTA leucine codon (as does *actII* orf2, thought to be involved in export of the antibiotic). The *bldA* gene was found to encode a $tRNA^{LEU}$ which translates the rare codon UUA and its expression was found to be up-regulated in response to nutrient deprivation (Leskiw *et al.*, 1993). Thus when the TTA codon in *actII* orf4 was substituted with TTG, the negative influence of the mutation on actinorhodin production was released (Fernandez - Moreno *et al.*, 1991).

The transition from primary to secondary metabolism is in general growth-phase dependent and temporally linked with morphological development and differentiation in actinomycetes (Demain and Fang, 1995). Antibiotic production is thus usually associated with an ill-defined idiophase following vegetative growth in liquid culture and with the development of aerial hyphae and sporulation on solid media. Nutritional status and a variety of environmental conditions , often also influencing growth rate, are the factors initially responsible for triggering antibiotic production (Champness *et al.*, 1994; Chater and Bibb, 1995; Doull and Vining, 1994).

Small diffusable compounds belonging to the γ-butyrolactone family of chemicals have been isolated from many *Streptomyces*. They have become known as '*Streptomyces* hormones' because of their influence on morphological differentiation and antibiotic production (Barabas *et al.*, 1994; Horinouchi and Beppu, 1992; 1993). Typically, strain-specific γ-butyrolactones bind to and release repressor proteins bound to the promoter regions of genes involved in the regulation of morphological differentiation or secondary metabolism. Transcription of these genes is thus derepressed. It has been shown that *S. coelicolor* produces at least seven γ-butyrolactone autoregulators (Kawabuchi *et al.*, 1997). Of these, one resembles the famous A-factor from *S. griseus* (Horinouchi and Beppu, 1993), four resemble the virginiae butanolides (VBs) and two are of the IM-2 type.

Actinomycete gene clusters for the biosynthesis of other types of antibiotic are generally found to contain pathway specific regulators, sometimes themselves part of a cascade that also influences other aspects of secondary metabolism or development (reviewed in Chater and Bibb, 1995). Most pathway specific regulatory genes seem to be responsible for positive control of production. A number of examples have been characterised including *actII* orf4 (Gramajo *et al.*, 1993) and *redD* (Takano *et al.*, 1992), controlling actinorhodin and undecylprodigiosin production respectively in *S. coelicolor* and *dnrR* 1 controlling daunorubicin production in *S. peucetius* (Madduri and Hutchinson, 1995; Stutzman-Engwall *et al.*, 1992) These genes all encode proteins that belong to the SARP family (as described by Weitzorrek and Bibb, 1997).

Examples of negative regulation in the control of antibiotic production are less common. The gene from *S. peucetius*, *dnrO* at the *dnrR* 2 locus seems to exert a negative effect on daunorubicin production (Otten *et al.* 1995) and the methylenomycin producer, *S coelicolor* contains a gene encoding a repressor (Fisher *et al.* 1987) of the TetR family (Chater and Bibb, 1995).

As has already been pointed out, production of antibiotics (and other secondary metabolites) production usually coincide temporally with morphological development. There is evidence from an increasingly large number of sources that these processes might be integrated into similar regulatory cascades (Hopwood *et al.*, 1994). Genes with pleiotropic regulatory effects have been isolated from a number of organisms. Thus in *S. coelicolor*, the two pathway-specific regulatory genes *redD* and *actII* orf4 mentioned earlier, controlling undecylprodigiosin and actinorhodin production respectively, are themselves activated by AfsR (reviewed in Bibb, 1996; Beppu, 1995). This is the target protein of a serine/threonine kinase AfsK. This system seems to be part of a signal transduction cascade regulating secondary metabolism in *S. coelicolor* (Beppu 1995;

Isuzuka, 1992). Homologues of *afsR* and *afsK* found in *S. griseus* exert an influence over morphological development (Umeyama *et al.*, 1999). γ-butryolactone receptor proteins can also exert multiple effects. The IM-2 receptor, Far A from *Streptomyces* sp., FarA controls production of a blue pigment and also the antibiotics showdomycin and minimycin (Kitani *et al.*, 1999).

Thus the activities of pathway-specific regulatory genes are controlled by other genes with more widespread influence in the regulatory hierarchy, which in turn might be controlled in response to environmental conditions (Doull and Vining, 1994).

### 1.1.5 Macrolide antibiotics

Tylosin (Figure 1.1) is one of a group of antibiotics known as the macrolides McGuire *et al.*, 1961; Seno *et al.*, 1977). Chemically these are comprised of a macrocyclic lactone ring, usually with attached amino or neutral sugar moieties. Interest in the macrolides derives mainly from their antimicrobial properties against Gram +ve bacteria (including anaerobes), Gram -ve cocci and mycoplasmas. The macrolide antibiotics were first identified in 1950 with the discovery of pikromycin. Since that time many more have been identified, produced mainly by *Streptomyces* (Omura, 1984). The mode of action of the macrolides is to inhibit protein synthesis by interacting with the 50S subunit of the bacterial ribosome, possibly by binding directly to the 23S rRNA. The lactone ring that forms the 'core' of a macrolide's structure is a product of polyketide synthesis. Polyketide synthases found in actinomycetes produce a structurally diverse range of secondary meatabolites possessing a broad spectrum of biological activities (Hopwood and Sherman, 1990; Cane *et al.*, 1998). These range from antitumour aromatic compounds such as mithramycin to antibacterial macrolide antibiotics.

The manipulation of polyketide synthesis offers great potential for the artificial creation of novel macrolides (section 1.2.7). Macrolide antibiotics can be classified according to the size of the lactone ring. For instance methymycin has a 12 membered ring, erythromycin a 14 membered ring and spiramycin and tylosin 16 membered rings. The attachment of sugars is often critical to their biological activity (Liu and Thorson, 1994). The manipulation of this aspect of macrolide synthesis also provides great scope for the development of novel compounds.

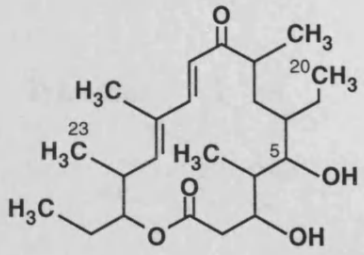
## 1.2 Biosynthesis of tylosin in *S. fradiae*

### 1.2.1 The macrolide antibiotic, tylosin

Tylosin, although best known and most extensively studied as a product of *S. fradiae* is also produced by *S. rimosus* (Pape and Brillinger, 1973) and *S. hygroscopicus* (Jensen *et al.*, 1963). Tylosin is an industrially important macrolide antibiotic, produced on a large scale by *S. fradiae* and is used as an animal growth promotant and in veterinary medicine. Tylosin consists of a branched lactone (tylonolide with three attached sugar groups, mycaminose, mycinose and mycarose (Morin *et al.*, 1970).

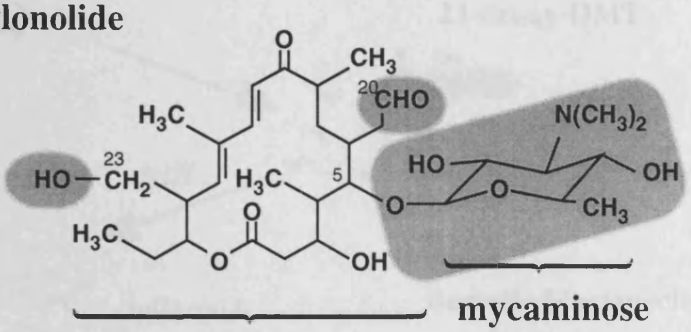
### 1.2.2 Biosynthesis of tylosin

Production of the macrolide antibiotic tylosin, by *Streptomyces fradiae*, proceeds via a combination of polyketide and 6-deoxyhexose metabolism. The first tylosin intermediate excreted by *S. fradiae* is a 16 membered polyketide lactone, tylactone, known also as protylonolide (Figure 1.1). This has methyl groups at C20 and C23 and has no attached sugars. It is biologically inert. Three deoxyhexose sugar moieties are subsequently added to produce tylosin (Figure 1.2). These are mycaminose (an amino sugar), mycarose and mycinose (neutral sugars). Synthesis of the cyclised polyketide, tylactone, is always followed in the biosynthesis of tylosin by the addition of mycaminose at C5-OH. This elaboration of the polyketide lactone confers the anti-ribosomal activity on the molecule which is the basis of the industrial value of tylosin. The addition of mycaminose, which is the obligatory first step in the conversion of tylactone to tylosin, is then followed by oxidation at C20 and hydroxylation at C23 to produce *O* -mycaminosyl tylonolide (OMT) (Figure 1.1). Further glycosylation then occurs in a preferred but not obligatory order, usually beginning with the addition of 6-deoxyallose at the hydroxylated C23 position. Mycarose is then substituted onto C4-OH of mycaminose and the conversion to tylosin is completed by stepwise bis *O* -methylation of 6-deoxyallose at C2-OH and C3-OH to form mycinose (Baltz *et al.*, 1983). In fermentation tylosin is then slowly reduced to the less active and industrially undesirable antibiotic, relomycin (20-dihydrotylosin) (Huang *et al.*, 1993). Tylosin non-producing mutants of *S. fradiae* , generated using NTG (Baltz and Seno, 1981a), facilitated this initial characterisation of the tylosin biosynthetic pathway in co-synthesis and bioconversion analyses (Baltz *et al.*, 1983; Omura *et al.*, 1982a, b). The mutants were found to exhibit nine distinct phenotypes.



**Tylactone (protylonolide)**

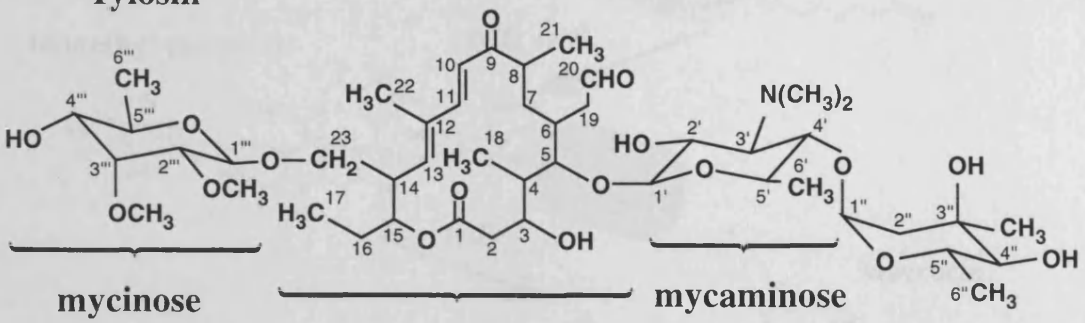
**5-*O*-mycaminosyl tylonolide (OMT)**



**mycaminose**

**tylonolide**

**Tylosin**



**mycinose** **tylonolide** **mycaminose** **mycarose**

**Figure 1.1 The structures of tylactone, OMT and tylosin.** The 16-membered polyketide ring, tylactone (also known as protylonolide) is the first intermediate of tylosin excreted by *S. fradiae*. 5-*O*-mycaminosyl tylonolide is modified from tylactone by substituition of the sugar mycaminose onto C5 (the obligatory first step in the biosynthesis of tylosin) and ring oxidation on C20 and C23 as indicated (shaded areas). Further elaboration with the sugar moieties mycinose and mycarose then completes the biosynthesis of tylosin.

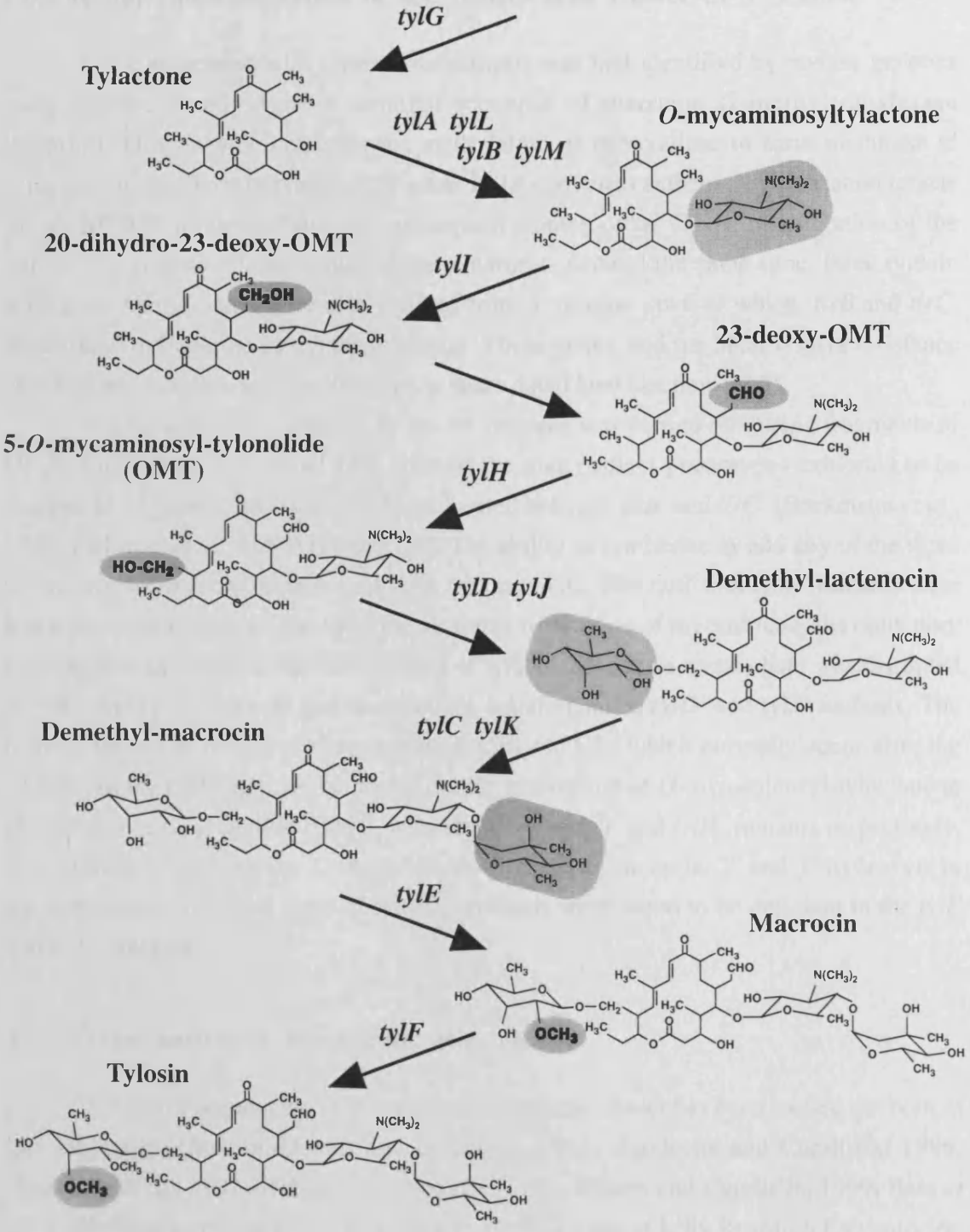



**Figure 1.2 The prefered biosynthetic route from tylactone to tylosin.** The 13 *tyl* loci associated with steps in the biosynthetic route are indicated above the arrows. The modifications made to each tylosin precursor at each stage have been highlighted (shaded areas).

### 1.2.3 Initial characterisation of the tylosin gene cluster in *S. fradiae*

DNA associated with tylosin biosynthesis was first identified by reverse genetics using a probe based on the *N*-terminal sequence of macrocin *O*-methyltransferase (MOMT). This enzyme catalyses the methylation of deoxyallose to form mycinose to complete the synthesis of tylosin. *S. fradiae* DNA contained authentic hybridisation targets for the MOMT probe and with the subsequent cloning of *tyl* DNA, investigation of the tylosin biosynthetic cluster could begin in earnest. Around the same time, three tylosin resistance determinants were also isolated from *S. fradiae* , two of which, *tlrB* and *tlrC*, were found to flank the biosynthetic cluster. These genes, and the other tylosin resistance determinants isolated, will be discussed in more detail later (section 1.2.5).

Complementation analysis of the *tyl* mutants was carried out, using fragments of DNA cloned from *S. fradiae*. This allowed the nine distinct phenotypes exhibited to be mapped to 13 genetic loci (*tylA-tylM*), all located between *tlrB* and *tlrC* (Beckmann *et al.*, 1989; Fishman *et al.*, 1987) (Figure 1.2). The ability to synthesise or add any of the three tylosin sugars was deficient in *tylA* and *tylL* mutants. The *tylB* and *tylM* mutants were found to be deficient specifically in the synthesis or addition of mycaminose, the obligatory first sugar to be added in the biosynthesis of tylosin. Mycarose metabolism was disrupted in *tylC* and *tylK* mutants and deoxyallose metabolism in *tylD* and *tylJ* mutants. The hydroxylations of the polyketide lactone at C20 and C23 which normally occur after the addition of the first sugar, mycaminose, in the conversion of *O*-mycaminosyl-tylactone to *O*-mycaminosyl-tylonolide (OMT) were deficient in *tylI* and *tylH* mutants respectively. The abilities to perform the *O*-methylations of deoxyallose on its 2" and 3" hydroxyls in the penultimate and final steps of tylosin synthesis were found to be deficient in the *tylE* and *tylF* mutants.

### 1.2.4 Organisation of the tylosin gene cluster

Extensive sequencing of the tylosin biosynthetic cluster has been carried out both in this laboratory (Merson-Davies and Cundliffe, 1994; Gandecha and Cundliffe, 1996; Gandecha *et al.*, 1997; Wilson and Cundliffe, 1998; Wilson and Cundliffe, 1999; Bate *et al.*, 1999; Bate and Cundliffe, 1999; Bate *et al.*, 2000) and at Lilly Research Laboratories, Indianapolis (not all data published), including the ends of the cluster (Rosteck *et al.*, 1991) and the *tylG* polyketide synthase region (Genbank accession number U78289). Most of the open reading frames found have been at least assigned putative functions on the basis of database comparisons and many have been more extensively characterised. Further complementation analysis of the *tyl* mutant strains (Baltz and Seno, 1981), and an

increasingly extensive program of targeted gene disruptions and studies on purified gene products have all added to this process. The tylosin biosynthetic cluster as defined thus far is approximately 85 kb in length and contains 43 open reading frames including the two resistance determinants *tlrB* and *tlrC* which currently define its outer limits (Figure 1.3). The cluster is split unequally by the *tylG* polyketide synthase complex, *tylG*, which spans approximately 41 kb. As in all other cases where antibiotic biosynthesis has been studied in *Streptomyces*, all the genes specific to production of tylosin are located in a single biosynthetic cluster. The cluster apparently contains almost all the structural genes necessary specifically for the biosynthesis of tylosin as well as a number of resistance, ancillary and regulatory genes along with other open reading frames whose functions remain unclear. The various elements of the *tyl* cluster and their impact on tylosin biosynthesis will be discussed below.

Many of the functionally related genes in the *tyl* cluster are grouped together in blocks, often similarly oriented and tightly packed. This may be to facilitate co-regulation of these genes, possibly through co-transcription.

### 1.2.5 Resistance determinants in *S. fradiae*

Three tylosin resistance determinants were originally isolated from *S. fradiae* , two of which, *tlrB* and *tlrC*, were found to flank the biosynthetic cluster ≅ 85 kb apart (Birmingham *et al.*, 1986; Beckmann *et al.*, 1989). The location of *tlrA* remains unknown but it is not associated with the other *tyl* genes. A fourth resistance gene, *tlrD* was later located in the middle of the cluster (Zalacain and Cundliffe, 1991) (Figure 1.3). Such an association of resistance determinants with the biosynthetic gene cluster is typical of known antibiotic clusters. The products of *tlrA* and *tlrD* are both methylases that act upon the same target nucleotide in 23S rRNA. Their mode of actions do however differ. Whereas *tlrD* is constituitively expressed and renders ribosomes only partially resistant to tylosin (and other macrolides) by momomethylation (Zalacain and Cundliffe, 1991), *tlrA* is induced in response to glycosylated metabolites of tylosin and confers higher levels of resistance by virtue of a second methylation (Zalacain and Cundliffe, 1989; Keleman *et al.*, 1994). The product of *tylB* recently became the first of a new subclass of rRNA modifying methyltransferases conferring resistance to macrolide antibiotics to be described (Liu *et al.*, 2000). The other resistance determinant, *tlrC* displays similarity to ATP-binding transport proteins and may therefore cause resistance by means of tylosin efflux from the cell. (Rosteck *et al.*, 1991).

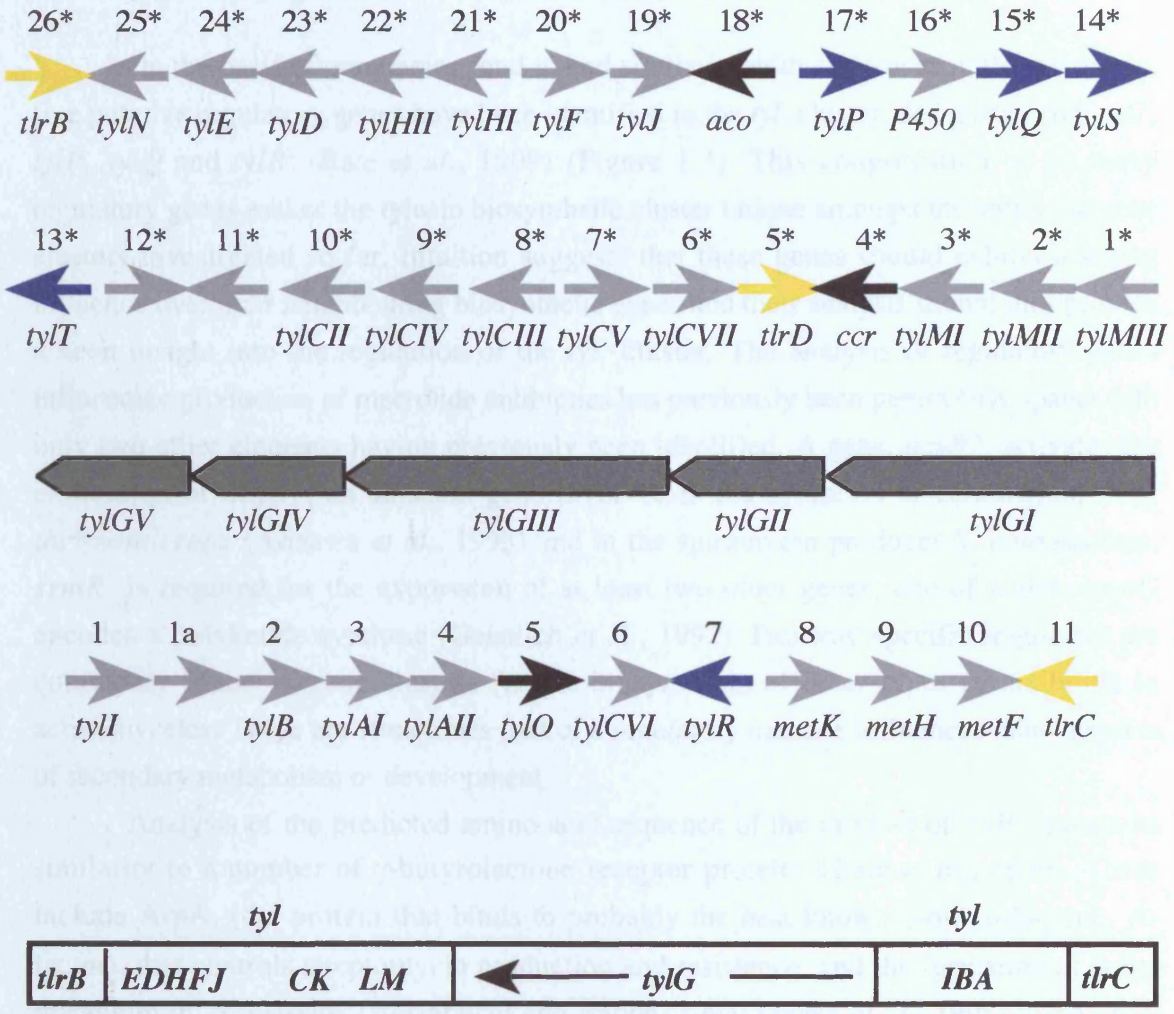



**Figure 1.3 The tylosin gene cluster of *S. fradiae*.** The relative positions of each of the 13 *tyl* loci identified within the ≅ 85kb tylosin biosynthetic cluster are shown on the bar (not to scale). This represents a contiguous portion of the *S. fradiae* genome containing the structural genes required for tylosin biosynthesis. The *tyl* gene cluster, including the flanking resistance determinants *tlrB* and *tlrC* contains 43 open reading frames arranged as shown. The *tylG* locus, situated in the middle of the cluster covers ≅ 41 kb and contains five mega-genes encoding polyketide synthase. Other genes thought to be associated with polyketide synthesis, *ccr*, *aco* and *tylO* are indicated (shaded black). The open reading frames located upsteam of *tylG* are denoted numerically as are those downstream which are additionally distinguished with an '*'.
The regulatory genes *tylP*, *tylQ*, *tylR*, *tylS* and *tylT* are indicated (shaded blue). The resistance determinants *tlrB*, *tlrC* and *tlrD* are also indicated (shaded yellow). *tlrA* is located elsewhere in the genome.

### 1.2.6 Regulatory genes in the tylosin biosynthetic cluster

On the basis of convincing, end to end similarity with sequences in the databases, five putative regulatory genes have been identified in the *tyl* cluster, designated *tylS, tylT, tylP, tylQ* and *tylR* (Bate *et al.*, 1999) (Figure 1.3). This congregation of so many regulatory genes makes the tylosin biosynthetic cluster unique amongst the antibiotic gene clusters investigated so far. Intuition suggests that these genes should exhibit a strong influence over their neighbouring biosynthetic genes and their analysis should thus provide a keen insight into the regulation of the *tyl* cluster. The analysis of regulatory genes influencing production of macrolide antibiotics has previously been particularly sparse with only two other elements having previously been identified. A gene, *acyB2*, activates the expression of *acyB1*, an adjacent gene involved in the synthesis of carbomycin in *S. thermotolerans* (Arisawa *et al.*, 1993) and in the spiramycin producer *S. ambofaciens*, *srmR* is required for the expression of at least two other genes, one of which, *srmG* encodes a polyketide synthase (Geistlich *et al.*, 1992). Pathway specific regulators are commonly found in gene clusters for the biosynthesis of other types of antibiotic in actinomycetes. These are sometimes part of a regulatory cascade influences many aspects of secondary metabolism or development.

Analysis of the predicted amino acid sequence of the product of *tylP* reveals its similarity to a number of γ-butyrolactone receptor proteins (Bate *et al.*, 1999). These include ArpA, (the protein that binds to probably the best known γ-butyrolactone, A-factor), that controls streptomycin production and resistance, and the formation of aerial mycelium in *S. griseus* (Horinouchi and Beppu, 1994; Onaka *et al.*, 1995), FarA from *Streptomyces* sp. that binds to the γ-butyrolactone, IM-2 (Waki *et al.*, 1997) and BarA from the virginiamycin producer, *S. virginiae* (Okamoto *et al.*, 1995). In *S. virginiae*, BarA dissociates from the promoter of the downstream gene *BarB* in response to specific γ-butyrolactones known in this case as the virginiae butanolides (VB's). The dissociation of BarA induces transcription of *barB* (Kinoshita *et al.*, 1997; Nakano *et al.*, 1998). Downstream of *tylP* in *S. fradiae* (separated by orf16*) lies *tylQ* (Figure 1.3) the proposed protein product of which bears considerable similarity to BarB (Bate *et al.*, 1999). Consistent with its putative designation as a DNA binding protein, TylP and the other γ-butyrolactone binding proteins mentioned, show particular sequence similarity in the amino terminal region where highly conserved DNA binding helix-turn helix motifs are located (Bate *et al.*, 1999).

The *tylQ* predicted protein sequence resembles proteins such as BarB (Kinoshita *et al.*, 1997) as mentioned above and also JadR2 from the jadomycin B producer *S. venezulae*. (Yang *et al.*, 1995). Expression of *BarB*, caused by the dissociation of BarA

from its promoter and antibiotic synthesis in *S. virginiae* both occur in response to the presence of VBs. It is thus possible to speculate in the absence of detailed functional analysis of BarB that it might be a transcriptional activator of virginiamycin biosynthetic genes. In contrast, the biosynthetic genes for jadomycin production in *S. venezulae* appear to be negatively regulated by JadR2 (Yang *et al.*, 1995). From these sequence comparisons it is logical to predict that *tylQ* might encode a transcriptional regulator of tylosin biosynthetic genes and might be the target gene for the product of *tylP*, a possible γ-butyrolactone-responsive regulator.

The deduced amino acid sequences of *tylS* and *tylT* display considerable similarity to each other and to slightly lesser extents, to members of the *Streptomyces* antibiotic regulatory proteins (SARPs) family (Weitzorrek and Bibb, 1997). This family includes DnrI from the daunorubicin producer *S. peucetius* (Stutzman-Engwall *et al.*, 1992) and ActII-orf4, the actinorhodin pathway specific activator of *S. coelicolor* (Fernandez-Moreno *et al.*, 1991) to which the proposed product of *tylS* bears particular similarity. Also from *S. coelicolor*, the proposed product of *tylT* displays significant similarity to RedD (Narva and Feitelson, 1990), the pathway specific activator undecylprodigiosin and to the amino terminal of the regulatory protein AfsR that promotes both undecylprodigiosin and actinorhodin production in *S. coelicolor* (Horinouchi and Beppu, 1990). These similarities strongly suggest that *tylS* and *tylT* might encode pathway specific activators of tylosin biosynthesis. Presumably because of the high GC content in actinomycetes the leucine codon TTA is rarely used and is usually found only in resistance and secondary metabolism regulatory genes. In common with other SARP-encoding genes (reviewed in Bibb, 1996; Beppu, 1995), both *tylS* and *tylT* each contain a TTA codon. This characteristic may reveal a mechanism by which *tylS* and *tylT* are regulated.

The proposed amino acid sequence for *tylR* shows similarity to one of the few other regulatory genes previously identified in a macrolide producer. This gene, *acyB2*, is thought to encode a positive regulator of carbomycin in *S. thermotolerans* (Arisawa *et al.*, 1993). The likely functional similarity of TylR to AcyB2 was reinforced when a strain disrupted in *tylR* was found to be incapable of either polyketide synthesis or the addition of tylosin sugars to precursors (Bate *et al.*, 1999). Reintroduction of the *tylR* gene restored these abilities leading to the unequivocal conclusion that it is indeed a positive regulator of tylosin biosynthesis.

The majority of genes found in the *tyl* cluster are extremely tightly packed, to the extent that many open reading frames are terminally overlapping. Despite this fact, each of the regulatory genes identified in the cluster has a comparatively large non-coding region

located directly upstream. These upstream regions are probably indicative of the necessity for independent expression of these genes for successful regulation of the tylosin gene cluster.

### 1.2.7 Polyketide biosynthesis

Polyketide synthesis closely resembles fatty acid synthesis and like the fatty acid synthases (FASs), polyketide synthases (PKSs) fall into two categories. Type II polyketide synthases, responsible for aromatic polyketides such as those in the antibiotic actinorhodin (Fernandez-Moreno *et al.*, 1992) produced by *S. coelicolor* and the anti-tumour compound mithramycin (Lombo *et al.*, 1997), consist of several, usually monofunctional enzyme subunits. These most closely resemble typical type II FASs such as those of *E. coli*. Complex polyketide antibiotics, such as tylosin, erythromycin and the other macrolides are synthesised by large multifunctional type I PKSs that resemble eukaryote type I FASs. Each module of these large multifunctional proteins contains three to six discrete catalytic domains each involved in a single condensation/processing cycle (Donadio *et al.*, 1991). The nascent polyketide is thus sequentially manipulated through successive rounds of polyketide chain extension as it is passed from one domain to the next in production line fashion (Staunton and Wilkinson, 1998). The structure of the polyketide product is linearly specified by the module arrangement (Donadio *et al.*, 1991).

Each module in a type I polyketide synthase is responsible for one round of chain extension. The essential domains contained in each module are an acyltransferase (AT), a β-keto-acyl synthase (KS) and an acyl carrier protein (ACP). In each round of chain extension a condensation reaction occurs between a carboxylated coenzyme A activated extender unit and a decarboxylated, enzyme bound, growing polyketide chain. A flexible phosphopantetheine prosthetic unit attached via a serine side chain to the ACP is responsible for transfer of the substrates from one active site to another. This giant prosthetic group acts like a 'macro CoA'. Modules often contain other catalytic domains such as ketoreductases (KR) (to reduce keto to hydroxyl groups), dehydratases (DH) (to remove water and form double bonds) and enoyl reductases (ER) (to saturate double bonds).

The AT domain is responsible for the selection and transfer to the ACP of carboxylated, CoA activated extender units via the oxygen atom in its serine side chain. The extender unit is thus transferred from the sulfhydryl terminus of CoA to the sulfhydryl terminus of the phosphopantetheine-ACP prosthetic group and CoA is released. The nascent polyketide is attached to the thiol group of the catalytic domain responsible for condensation, the KS. Elongation of the carbon chain attached to the KS occurs by

condensation of it and the extender unit attached to the ACP. $CO_2$ is released, an acyl-*S*-phosphopantetheine is formed on the ACP and the active site sulfhydryl of the KS 'condensing enzyme' is restored in preparation for another round of chain extension. The growing carbon chain is then delivered to the active sites of the other catalytic domains in the module by the phosphopantetheine prosthetic arm to which it is attached. This allows great variation in the structures produced by PKSs. The nascent polyketide is then moved to the next module, where it is transferred from the phosphopantetheine-ACP to the active site cysteine sulphur of the next KS domain. The next round of chain extension can then begin, with the AT domain of the next module selecting the next extender unit for addition to the ACP. Having proceeded through the appropriate number of chain extension cycles, as specified by the modular structure of the PKS, the final module contains a terminal thioesterase domain. This is responsible for the termination of synthesis and cyclisation of the polyketide product.

The best studied example of a type I PKS is the 6-deoxyerythronolide B synthase (DEBS) from the erythromycin producer *Saccharopolyspora erythraea*. This consists of three large multifunctional proteins (Caffrey *et al.*, 1992), each containing two modules responsible for as many extension cycles of the polyketide chain (Leadley *et al.*, 1993). The DEBS PKS has a discrete loading module containing an AT and an ACP domain (Staunton and Wilkinson, 1998). The starter units which are the substrates of this type of loading module are non-carboxylated acyl CoA species (Wiessman *et al.*, 1995; Peiper *et al.*, 1995). More typically of modular PKSs, the tylosin PKS differs from DEBS in that it begins with a KS-like domain prior to the AT and ACP of the loading module. Until recently the function of such domains was unknown as they do not contain the essential, conserved, active site cysteine (Kim *et al.*, 1995; Meurer and Hutchinson, 1995). In place of this they contain a glutamine residue (Kao *et al.*, 1996). It was recently demonstrated that these KS-like domains, denoted KSQ nevertheless probably play an essential role in the PKSs in which they are found (Bisang *et al.*, 1999). The glutamine residue was shown to render the domain an effective decarboxylase and KSQ probably functions as a chain initiation factor. Thus it specifically decarboxylates ACP-bound extender units to generate suitable starter units in situ on the PKS. The KSQ domain and its accompanying AT and ACP domains therefore constitute an essential loading module.

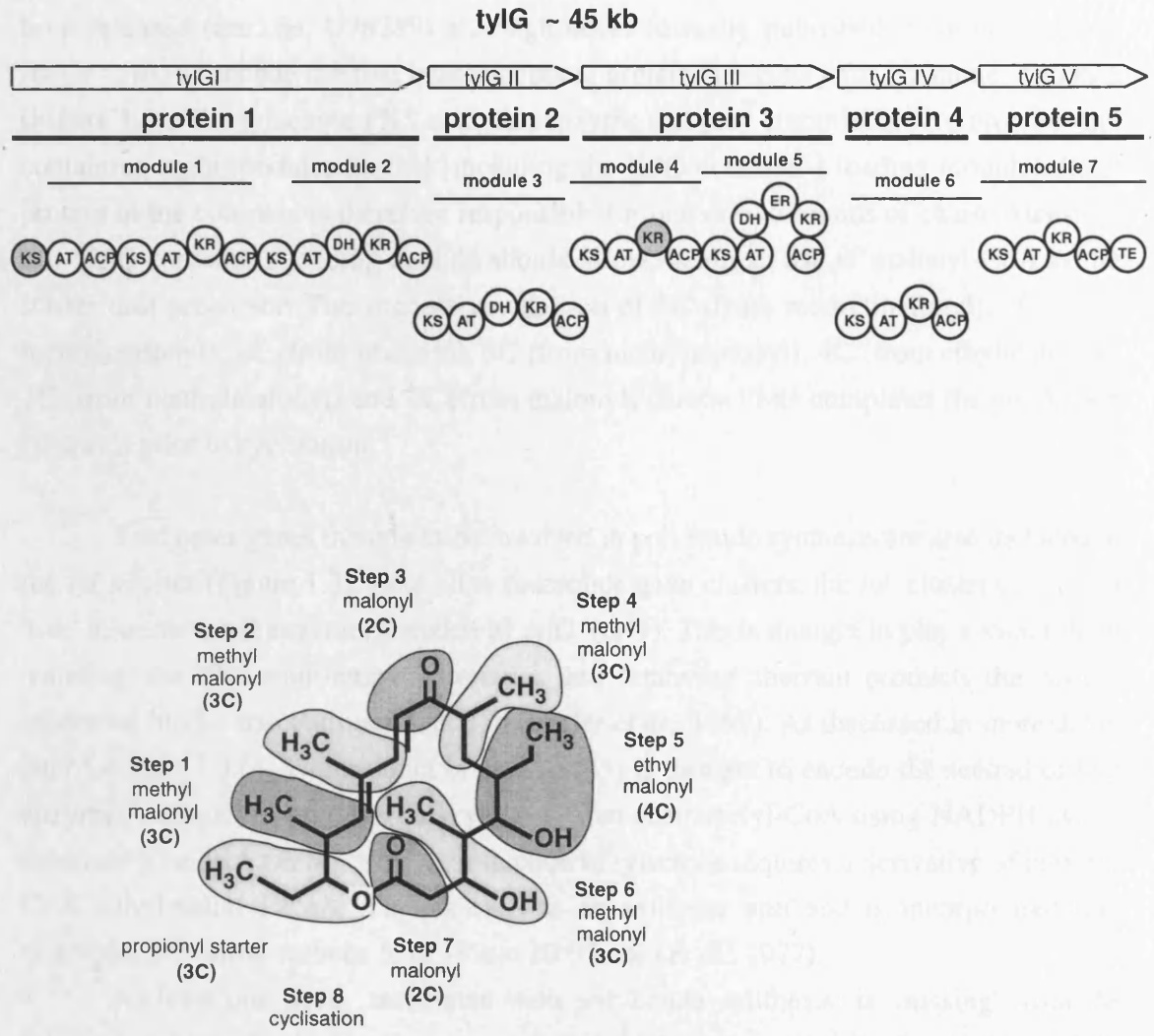



**Figure 1.4 The structure and biosynthesis of tylactone.** Polyketide synthase modules are represented by strings of circles. Each circle represents an enzymatic domain. KSQ, a 'KS-like' domain with decarboxylase function acting as a chain initiation factor; ACP, acyl carrier protein; AT, acyl-transferase catalysing the transfer of an acyl group from acyl CoA to form acyl-ACP; KS, ketosynthase catalysing the decarboxylative condensation of two adjacent ketoacyl-ACPs resulting in ketoacyl chain elongation on the downstream ACP; KR, ketoreductase hydroxylating the ketoacyl-ACP; DH, dehydratase (double bond formation); ER, enoylreductase (double bond saturation); TE, thioesterase releasing and cyclising the acyl chain. Tylactone is formed by the successive addition of 3C (from methylmalonyl), 3C (from methylmalonyl), 2C (from malonyl), 3C (from methylmalonyl), 4C (from ethylmalonyl), 3C (from methylmalonyl) and 2C (from malonyl) carbon units prior to cyclisation.

The tylactone polyketide synthase genes have been fully sequenced and the results have been released (acc. no. U78289) although never formally published. Five mega-genes (*tylGI - tylGV*) encode the five multifunctional proteins necessary for tylactone synthesis (Figure 1.4). The tylactone PKS is a multienzyme complex comprising five proteins and containing eight modules in total (including the KSQ-containing loading module). Each protein in the complex is therefore responsible for one or two rounds of chain extension. The KSQ containing loading module should allow the direct use of malonyl-CoA as the starter unit precursor. The successive addition of 3C (from methylmalonyl), 3C (from methylmalonyl), 2C (from malonyl), 3C (from methylmalonyl), 4C (from ethylmalonyl), 3C (from methylmalonyl) and 2C (from malonyl) carbon units completes the polyketide synthesis prior to cyclisation.

Two other genes thought to be involved in polyketide synthesis are also included in the *tyl* cluster (Figure 1.3). Like other macrolide gene clusters, the *tyl* cluster contains a 'free' thioesterase II enzyme, encoded by *tylO* (orf5). This is thought to play a vital role in 'valeting' the PKS multienzyme complex and removing aberrant products that would otherwise hinder its continued function (Butler *et al.*, 1999). As discussed in more detail later (section 1.3.6), The product of *ccr* (orf4*) is thought to encode the second of two enzymes required to produce butyryl-CoA from acetoacetyl-CoA using NADPH as co-substrate (Gandecha *et al.*, 1997). Production of tylactone requires a derivative of butyryl-CoA, ethylmalonyl CoA. This is used as an extender unit and is incorporated into tylactone, providing carbons 5, 6, 19 and 20 (Omura *et al.*, 1977).

At least one gene, associated with polyketide synthesis, is 'missing' from the tylosin biosynthetic cluster. Post translational modification, namely the addition of 4'-phosphopantethenyl cysteine to the ACP domains to form the phosphopantetheine prosthetic unit, is clearly necessary for a PKS multienzyme complex to function. Whether a separate enzyme is required to perform each addition (eight in total, in the tylactone PKS) or whether a single enzyme is competent for them all is unknown. Such enzymes have not yet been found to be associated with biosynthetic clusters and the *tyl* cluster is thus no exception in this respect.

The modular organisation of polyketide synthases is currently being exploited in the search for new and novel variations on polyketide drugs (Marsden *et al.* 1998; Zierman and Betlach, 2000). In particular the potential of combinatorial libraries for creating diverse analogs of polyketide products is increasingly being demonstrated (Lal *et al.*, 2000).

### 1.2.8 Deoxyhexose sugar metabolism

The antibiotic potential of tylosin precursors is only realised once the polyketide ring has been glycosylated and differently glycosylated precursors have different potencies (Fish *et al.*, 1996). All the genes required for tylosin-specific deoxyhexose sugar metabolism have been at least putatively identified and are located within the tylosin cluster (Figure 1.5). In addition the *tylA* genes, *tylAI* and *tylAII*, thought to synthesise the common dTDP activated precursor all of the tylosin sugars are derived, are also found in the cluster (Merson-Davies and Cundliffe, 1994). The *tylA* genes *tylAI* (orf3) and *tylAII* (orf4) encode dTDP-glucose synthase and dTDP-glucose dehydratase respectively. These enzymes are responsible for the first two steps, which are common to the synthesis of all three tylosin sugars. Thus glucose-1-phosphate is converted to the common intermediate dTDP-4-keto, 6-deoxyglucose, via dTDP-glucose (Figure 1.6). These two genes almost certainly have orthologues elsewhere in the genome involved in primary metabolism. Their inclusion in the *tyl* cluster makes it unusual. Although functionally identical genes are required for the synthesis of other macrolide antibiotics such as erythromycin, no examples of equivalent genes have yet been found associated with other biosynthetic clusters in the producing organisms. Thus the tylosin cluster is unique in this respect among the antibiotic gene clusters so far analysed.

Most of the genes specific to each sugar are located in blocks, although the genes for mycaminose and mycarose biosynthesis are split. In each case one gene is located, separate to the others, at the other end of the tyl cluster on the other side of the*tylG* polyketide synthase genes. Interestingly, these genes far from being isolated away from other sugar genes are situated not only in the same block of DNA as each other, but also along with *tylAI* and *tylAII*. They form part of a set of seven, similarly oriented open reading frames located upstream and divergent from the polyketide synthase genes, *tylG*. The mycaminose specific gene *tylB* lies downstream of *tylI* (a cytochrome P450 involved in polyketide ring hydroxylation (section 1.2.9), and orf1a (unassigned) and immediately upstream of the common tylosin-sugar genes *tylAI* and *tylAII*. Downstream of *tylAII* lies *tylO* (a thioesterase important in polyketide metabolism, (section 1.2.7) followed by the mycarose specific gene *tylCVI*. The regulatory gene *tylR* (section 1.2.6) lies downstream of *tylCVI* but is oriented in the opposite direction. The reason for the mycaminose and mycarose genes being split in this manner is unknown, however their particular situation is suggestive of a possible regulatory implication.

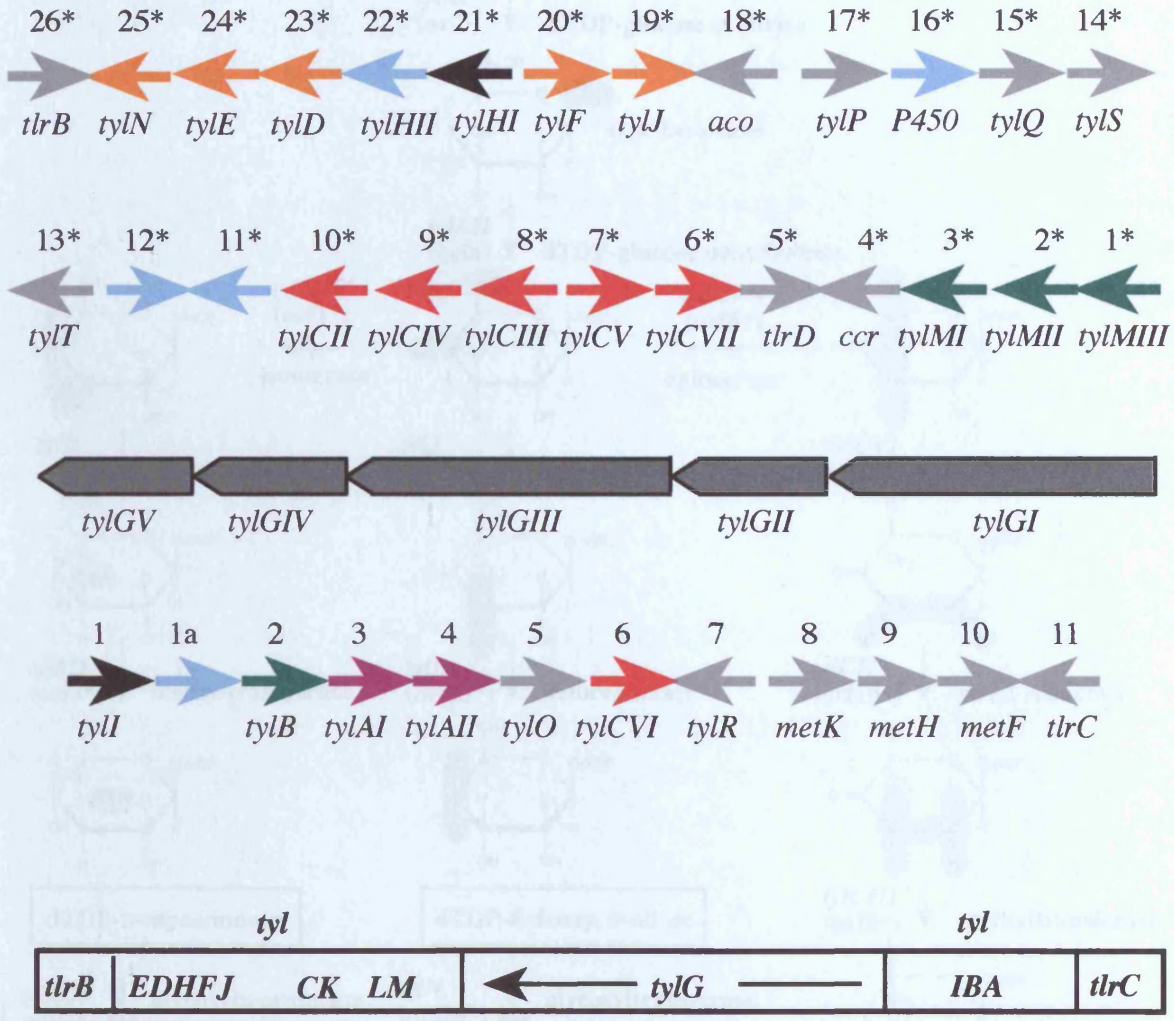



**Figure 1.5 Location of the sugar genes in the tylosin biosynthetic cluster.** The bar representing the *tyl* gene cluster and the open reading frames contained within it are shown as previously ( Figure 1.3). In this case the genes associated with synthesis of the tylosin-sugars are indicated. These include: the *tylA* genes associated with synthesis of the common sugar precursors (shaded purple); the *tylM* and *tylB* genes associated with mycaminose synthesis and addition (shaded green); the *tylC* genes associated with mycarose synthesis and addition (shaded red) and genes associated with the synthesis and addition of 6-deoxyallose (*tylD*, *tylH*, *tylJ* and *tylN*) and its subsequent conversion to mycinose (*tylE* and *tylF*) (shaded red). Interestingly, unlike the mycinose genes, those for mycaminose and mycarose synthesis and addition are separated with one gene being located separate from the rest, on the other side of *tylG* in each case. Genes responsible for ring hydroxylations are shaded black. Genes of unknown function are shaded blue.

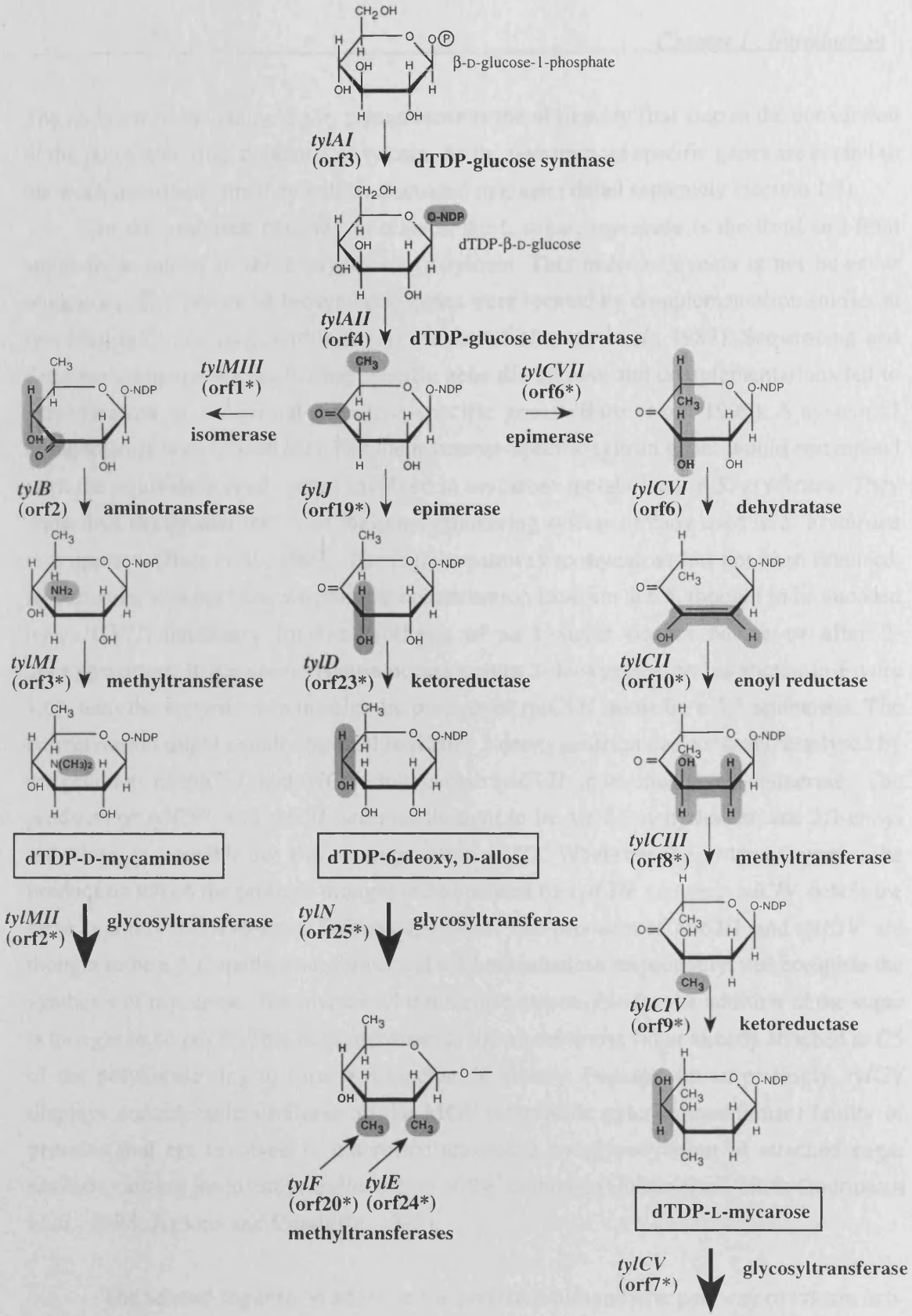



**Figure 1.6 The biosynthetic route to the three tylosin sugars.** The three tylosin sugars, D-mycaminose, L-mycarose and 6-deoxy-D-allose (converted to mycinose following addition to the polyketide) are derived from the same precursor β-D-glucose-1-phosphate. The modification catalysed by each enzymatic function has been indicated in each case (shaded).

The addition of the aminosugar, mycaminose is the obligatory first step in the conversion of the polyketide ring, tylactone, to tylosin. As the mycaminose specific genes are central to the work described here they will be discussed in greater detail separately (section 1.3).

In the preferred pathway to tylosin, the L-sugar, mycarose is the third and final sugar to be added in the biosynthesis of tylosin. This order of events is not however obligatory. The mycarose biosynthetic genes were located by complementation studies at two loci, *tylC* and *tylK* within the *tyl* cluster (Fishmann *et al.*, 1987). Sequencing and database comparisons facilitating specific gene disruptions and complementations led to identification of individual mycarose-specific genes (Bate *et al.*, 1999). A system of nomenclature was applied such that the mycarose-specific tylosin genes would correspond with the equivalent *eryB* genes involved in mycarose metabolism in *S. erythraea*. They were thus designated *tylC* and the same numbering system already used in *S. erythraea* was applied. (Bate *et al.*, 1999). The precise pathway to mycarose has not been finalised. In particular it is not clear whether the epimerisation function at C5, thought to be encoded by *tylCVII*, necessary for the synthesis of an L-sugar occurs before or after 2-deoxygenation. If the epimerisation occurs before 2-deoxygenation (as shown in Figure 1.6), then the enzyme responsible, the product of *tylCVII* must be a 3,5-epimerase. The epimerisation might equally be catalysed after 2-deoxygenation has occurred, catalysed by the products of *tylCVI* and *tylCII*. In this case *tylCVII* must encode a 5-epimerase. The products of *tylCVI* and *tylCII* are thus thought to be the 2,3-dehydratase and 2,3-enoyl reductase responsible for the deoxygenation at C2. Whatever the order of events, the product on which the proteins thought to be encoded by *tylCIII* and then *tylCIV* acts is the same, namely dTDP-4-keto, 6-deoxy-L-altrose. The products of *tylCIII* and *tylCIV* are thought to be a 3-*C*-methyltransferase and a 4-ketoreductase respectively, that complete the synthesis of mycarose. The mycarosyl transferase responsible for the addition of the sugar is thought to be *tylCV*. This adds mycarose to the mycaminose sugar already attached to C5 of the polyketide ring to form a disaccharide moiety. Perhaps not surprisingly, *tylCV* displays considerable similarity to the MGT (macrolide gylcosyltransferase) family of proteins that are involved in macrolide resistance by glycosylation of attached sugar residues causing inactivation and/or efflux of the antibiotic (Quirós *et al.*, 1998; Gourmelen *et al.*, 1998; Jenkins and Cundliffe, 1991).

The second sugar to be added in the preferred biosynthetic pathway to tylosin is 6-deoxyallose although the final steps in this process involve its conversion to mycinose. The mycinose specific genes were located within the cluster and identified by complementation studies carried out in mutant strains (Beckman *et al.*, 1989; Fishmann *et al.*, 1987) followed by sequencing and database comparisons (Bate and Cundliffe, 1999). The

product of *tylJ* (orf19*) is a 3-epimerase acting on the common tylosin-sugar precursor dTDP-4-keto, 6-deoxyglucose and the product of *tylD* (orf23*) encodes the NADPH-dependant 4-ketoreductase activity necessary for synthesis of 6-deoxyallose (Figure 1.6). The glycosyltransferase responsible for addition of this sugar to the C23-OH position of tylactone is the product of *tylN*. Subsequent modification of the sugar to convert it to mycinose are encoded by *tylE* (orf24*) and *tylF* (orf20*). These genes encode the 2''' (*tylE*) and 3''' (*tylF*) *O*-methyltransferase activities that are the penultimate and final steps respectively in the production of tylosin.

### 1.2.9 Other genes in the *tyl* cluster

Four of the five regulatory genes identified are located in the same region of the tylosin cluster, although it is not suggested that this is of any particular regulatory significance *per se*. It seems unlikely, for instance, that *tylP*, *tylQ* and *tylS* should be co-transcribed, especially bearing in mind the upstream non-coding regions with which they are associated. It is interesting however that also located in this particular block of DNA are a number of genes whose function is either completely unknown or whose role in the biosynthesis of tylosin is at best speculative. Of the eight open reading frames between orf11* and orf18* inclusive, which includes the four putative tylosin-regulatory genes *tylP*, *tylQ*, *tylS* and *tylT*, no tylosin structural genes are present.

On the basis of sequence similarities, orf18*, located upstream of, but divergent from *tylP*, has been proposed to encode an acyl CoA oxidase, perhaps involved in the provision of short chain acyl CoA substrates for the synthesis of γ-butyrolactones or polyketide metabolism (Bate *et al.*, 1999). Positioned upstream of *tylQ* and *tylS*, downstream of *tylP* and similarly oriented to these neighbouring regulatory genes lies orf16*, which sequence similarities suggest must encode a cytochrome P450 (Bate *et al.* 1999). Such a gene has no apparent role in tylosin biosynthesis since the ring hydroxylations necessary during the conversion of tylactone to tylosin are catalysed by *tylI* and *tylHI* at C20 and C23 respectively (Merson-Davies and Cundliffe, 1994; Bate and Cundliffe, 1999; Baltz and Seno, 1981). However the possible function of orf16* is of considerable interest as it does provide a candidate gene for the production of uncharacterised material that is associated with tylactone accumulation in fermentations of some *S. fradiae* strains. It will therefore be discussed in more detail later, in Chapter 6.

Upstream of *tylT*, but divergent from it, lies orf12*. Together with orf1a (Figure 1.5) and orf9 (B. S. DeHoff and P. R. Rosteck Jr., personal communication) this open reading frame remains functionally unassigned not only with respect to tylosin biosynthesis but also regarding the specific type of protein, if any, encoded. Downstream of, and

convergent with, orf12* lies another open reading frame designated orf11*. This is similarly oriented to the three mycarose genes located immediately upstream, *tylCII*, *tylCIV* and *tylCIII*. The deduced product of orf11*, a GTP binding protein, can only speculatively be suggested to be another possible regulatory element, perhaps involved in regulated proteolysis (Bate *et al.*, 1999).

Upstream of *tylG* and oriented in the opposite direction is *tylI* (Merson-Davies and Cundliffe, 1994). The gap separating these divergent coding sequences is relatively large (447 bp) presumably containing promoters of considerable impotance in tylosin biosynthesis. Downstream of tylI and and similarly oriented lies orf1a whose role is as yet unassigned and the sugar genes *tylAI*, *tylAII*, *tylB* and *tylCVI* (section 1.2.8). The latter two genes are separated by *tylO* (section 1.2.7). The product of this *tylI* is responsible for hydroxylation of the methyl group on C20 (Merson-Davies and Cundliffe, 1994). This is the first step in the process of converting the polyketide ring from tylactone to tylonolide following the addition of mycaminose. The enzyme responsible for the second modification at this position, dehydrogenation of the hydroxymethyl to a formyl group, remains unidentified. This function was also blocked in the *tylI* mutant strain, GS77 (Baltz *et al.*, 1982) but it seems unlikely that the product of *tylI* should be responsible for both activities, although they might be closely coupled. These modifications C20 follow the addition of mycaminose to the polyketide and thus convert 5-*O*-mycaminosyl tylactone to 5-*O*-mycaminosyl tylonolide (Baltz *et al.*, 1982). The gene whose product performs the second modification at C20 does not appear to be located within the *tyl* cluster. Interestingly the aldehyde reductase which performs the reverse of this modification in the slow conversion of tylosin to relomycin is also not located in the cluster. Mutant strains which did not retain any other tylosin-related enzyme function could still perform this function, and at increased levels (Baltz and Seno, 1981).

## 1.3 Mycaminose metabolism in *S. fradiae*

### 1.3.1 The location of mycaminose specific genes

The DNA sequence of the *tylLM* region (Gandecha *et al.*, 1997) revealed four open reading frames situated downstream and similarly oriented to the five mega gene polyketide synthase region (*tylGI-tylGV*) (Figure 1.5). The resistance determinant, *tlrD* (orf5*) was found to border the *tylM* region at the other end, reading in the opposite direction. Some of these open reading frames are potentially terminally overlapping. In this situation, the TGA 'stop' codon of one gene overlaps with the GTG 'start' codon of the next (GTG is

commonly utilised as a 'start' codon in *Streptomyces* sp.). This generates the DNA sequence motif GTGA. This is a frequently encountered occurrence in *Streptomyces* and has been suggested to be evidence of translational coupling of the respective genes (Arrowsmith et al., 1992; Sherman et al., 1989). The sequence of *tylG* (encoding polyketide synthase) terminally overlaps with orf1*, which in turn terminally overlaps with *tylMII* (orf2*). Downstream, *tylMI* (orf3*) and *ccr* (orf4*) are similarly terminally overlapping. The deduced products of genes in the *tylLM* region and their proposed functions (Gandecha *et al.*, 1997) are described below.

The remaining mycaminose gene, *tylB*, is located away from its functional partners ≅ 45 kb away on the other side of *tylG*. It is similarly oriented to and downstream of tylI and orf1a and upstream of the common tylosin-sugar genes *tylAI* and *tylAII*, *tylO* (polyketide editing) and the mycarose gene *tylCVI*.

### 1.3.2 The *tylL* phenotype

The *tylA* and *tylL* phenotypes, correspond to the inability to synthesise any of the tylosin sugars and these two loci were mapped, one on either side of the *tylG* polyketide synthase region. As discussed above, the two *tylA* genes were located and their probable functions verified by sequence comparisons. The *tylL* locus, which was mapped alongside the *tylM* locus, proved more elusive. Sequencing in this region, described in detail below, revealed no *tylL* genes (Gandecha *et al.*, 1997). Sequencing the *tylL* mutant strain, GS33, revealed a mutation in the mycaminosyltransferase gene,*tylMII* (Clark, 1997). A G-C to A-T transition (consistent with the effect of the mutagen used) was discovered within the sequence. The effect of this would be the creation of an in-frame stop codon TGA which would truncate the proposed mycaminosyl transferase protein after 142 amino acids (normally 452 amino acids total). Sequencing of *tylMIII* revealed no changes but *tylMI* was not sequenced. Further analysis of the *tylL* mutant, GS33, led to no positive conclusions regarding its apparently misleading phenotype (Clark, 1997). Fermentation analysis of GS33 with DNA from the *tylM* region re-introduced was carried out, the results of which were consistent with those previously produced in this laboratory (Clark, 1997). Namely, the mutation could be complemented by all three *tylM* genes but only partially by introduction of *tylMII* alone. Thus no further light was shed on the paradoxical phenotype of the mutant. As the mutation found could not alone be responsible for the *tylL* phenotype, it was therefore concluded to be due either to physiological constraints or, more likely, multiple mutations. The *tylL* mutant GS33 was therefore discarded as a "red herring". Since the '*tylLM* ' region of the *S. fradiae* geneome clearly contains no *tylL*

gene it was deemed inappropriate to maintain the misnomer and it has thus been more correctly referred to here as the *tylM* region.

### 1.3.3 orf1* - an isomerase required for mycaminose synthesis ?

A putative function can only be tentatively assigned to the deduced protein product of orf1*, as it does not display convincing resemblance to any enzymatically characterised protein in the databases. It does display some similarity to the deduced products of genes *eryCII* (38% identity) and *dnrQ* (35% identity) found in the erythromycin and daunomycin macrolide biosynthetic clusters of *Saccharopolyspora erythraea* (Salah-Bey *et al.*, 1998) and *Streptomyces peucetius* (Otten *et al.*, 1995) respectively. At the time the sequence of orf1* was published, no definite function could be assigned to it (Gandecha *et al.*, 1997). Subsequently it was proposed (Salah-Bey *et al.*, 1998) that EryCII catalyses the isomerisation of dTDP-4-keto-6-deoxyglucose to dTDP-3-keto-6-deoxyglucose in the biosynthetic pathway to desosamine and that DnrQ and Orf1* might fulfill analogous roles in their respective pathways. A similar 3-keto-hexose intermediate is also thought to be required in the synthesis of the nucleotide activated aminosugar necessary for both tylosin (Figure 1.6) and daunomycin production. The mechanism by which this isomerisation might be catalysed by this possible new class of enzymes remains unclear. All three of these putative protein sequences also display similarity to cytochrome P450 hydroxylases. However, none of them contain a haem binding motif and, in particular, lack the active site cysteine residue that is an essential element of an authentic P450.

### 1.3.4 *tylMII* (orf2*), the mycaminosyltransferase

On the basis of similarity to other deduced protein sequences in the databases it was concluded that the computer translated *tylMII* (orf2*) product represented a glycosyltransferase (Gandecha *et al.*, 1997). Among others, it displayed convincing end to end similarity to the deduced sequence of Mgt from *S. lividans*, the first authenticated glycosyltransferase from actinomycetes (Jenkins and Cundliffe, 1991). Then deduced product of *tylMII* also displayed 50% similarity to the deduced protein product of *ery* orf8 from the erythromycin biosynthetic cluster of *Saccharopolyspora erythraea* (Salah-Bey *et al.*, 1998). It is postulated that this protein product adds 4-deoxymycaminose to a 16 membered lactone ring during the biosynthesis of erythromycin. This is consistent with the product of *tylMII* being the mycaminosyltransferase required to add mycaminose to tylactone (Figure 1.6). Likewise, the DnrS protein from the daunomycin biosynthetic cluster of *Streptomyces peucetius* displays similarity to these sequences and has also been

reported as a putative glycosyltransferase (Otten *et al.*, 1995). These putative protein sequences all contain a consensus sequence motif thought to be diagnostic of a sub-group of glycosyltransferases (Cundliffe *et al.*, 1997). Disruption and complementaion studies of *tylMII* showed its designated role, encoding the mycaminosyltransferase in tylosin biosynthesis, to be secure (Fish and Cundliffe, 1997).

### 1.3.5 *tylMI* (orf3*), the methyltransferase in mycaminose synthesis

The deduced protein product of *tylMI* (orf3*) also shows similarity to the putative products of orfs found in other macrolide biosynthetic gene clusters (Gandecha *et al.*, 1997), namely SrmX of the spiramycin biosynthetic pathway of *S. ambofaciens* (47% identity) and OrfX from the nogalomycin gene cluster of *S. nogalater*. In addition TylMIII bears even greater similarity to the deduced product of *rdmD* from *S. purpurascens* which is involved in the production of anthrocyclines. In common with *tylMI* (orf3*), these sequences posses all three of the characteristic consensus sequence motifs of methyltransferases that utilise *S*-adenosyl methionine (SAM) as a co-substrate (Kagan and Clarke, 1994). The ability of TylMI to bind SAM was confirmed when it was produced as a maltose binding protein fusion in *E. coli* and was shown to bind radiolabelled SAM in gel filtration chromatography (Gandecha *et al.*, 1997).

*tylMI* (orf3*) was found to complement *in trans* the *tylM* mutation in GS62 (Gandecha *et al.*, 1997) when introduced on an integrative vector, thus proving that *tylMI* is a mycaminose specific gene. It was therefore concluded that *tylMI* (orf3*) encodes a methyltransferase that introduces one or both of the methyl groups on to the amino group of dTDP-3-amino, 6-deoxyglucose during the biosynthesis of mycaminose (Figure 1.6).

### 1.3.6 *ccr* (orf4*), a crotonyl-CoA reductase

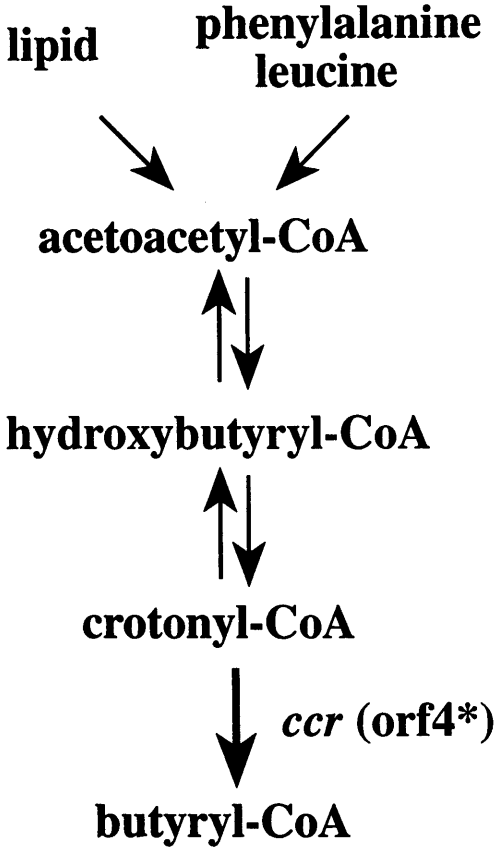
When *ccr* (orf4*) is translated *in silico* (Gandecha *et al.*, 1997) it displays 78% identity with the similarly translated crotonyl-CoA reductase (Ccr) of *S. collinus* (Wallace *et al.*, 1995). This deduced protein product is thought to produce butyryl-CoA using NADPH as co-substrate. Production of tylactone requires a derivative of butyryl-CoA, ethylmalonyl CoA. This is used as an extender unit and is incorporated into tylactone, providing carbons 5, 6, 19 and 20 (Omura *et al.*, 1977).

The butyryl-CoA for tylactone production is largely derived from acetoacetyl-CoA which is in turn derived from lipid degradation or leucine and phenylalanine catabolism. The deduced protein product of *ccr* (orf4*) was thus proposed to be the crotonyl-CoA reductase responsible for the production of butyryl-CoA from hydroxybutyryl-CoA, in turn

derived from acetoacetyl-CoA (Figure 1.7). In certain *Streptomycetes* butyryl-CoA is also used in primary metabolism for the biosynthesis of straight chain fatty acids. Since the entire tylosin biosynthetic cluster can be deleted in *S. fradiae* with no loss of viability (strain GS93, (Beckmann *et al.*, 1989), it is therefore likely that a second crotonyl-CoA reductase is present in its genome and *ccr* is therefore viewed as an ancillary gene.

### 1.3.7 *tylB*, an aminotransferase in mycaminose synthesis

On the basis of database comparisons, in particular with an enzyme, the product of *eryCI* of the erythromycin biosynthetic cluster (Dhillon *et al.*, 1989), the product of this gene was thought to be the aminotransferase responsible for the conversion of dTDP 3-keto, 6 deoxyglucose to dTDP 3-amino, 6-deoxyglucose in the biosynthesis of mycaminose (Merson-Davies and Cundliffe, 1994) (Figure 1.8). A *tylB* mutant strain, GS50 (Baltz and Seno, 1981), was also sequenced at this time and found to contain an in-frame stop codon (TGA) which would lead to a truncated version of the deduced product (262 amino acids compared to 388 in the wild type).



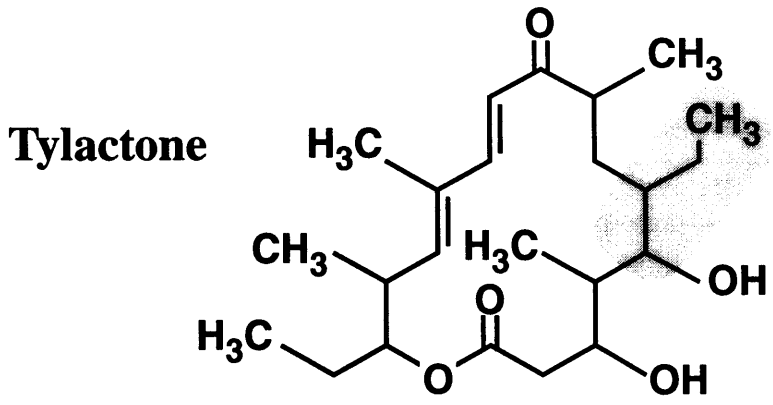




**Figure 1.7 The proposed role of the enzyme encoded by *ccr* .** The synthesis of the polyketide, tylactone, requires a supply of four carbon ethylmalonyl-CoA extender units derived from butyryl-CoA. The gene *ccr* (orf4*) is proposed to encode the enzymatic activity responsible for the supply of butyryl-CoA as shown. The 4-carbon unit, derived from butyryl-CoA, incorporated into the polyketide ring is shaded. The supply of butyryl-CoA is largely derived from lipid degradation or phenylalanine and leucine catabolism.

### 1.3.8 The influence of mycaminose metabolism on polyketide synthesis

One gene from the *tylM* region, *tylMII* (ort2*), the glycosyltransferase responsible for the addition of mycaminose to the polyketide tylactone, had previously been disrupted in this laboratory (Fish and Cundliffe, 1997). This disrupted strain was designated SF01 at the time. However, in the interests of clarity, I propose to refer to this, and all the other disrupted strains generated, using a more intuitive system of nomenclature that has the name of the disrupted gene in question intrinsic to it. The name of each disrupted strain will therefore be composed of the letter code of the gene disrupted and the suffix -KO (for 'knockout' strain). The *tylMII* disrupted strain, formerly known as SF01, will be thus referred to as *tylMII*-KO.

Contrary to expectation, when strain *tylMII*-KO was fermented and its products analysed, tylactone did not accumulate, except perhaps in, barely detectable, quantities (Fish and Cundliffe, 1997). Equally, no other intermediates of tylosin biosynthesis could be identified in the fermentation extracts. This result was surprising as *tylMII*-KO, deficient in the ability to add mycaminose, the essential first step in the further conversion of tylactone, might logically have been expected to accumulate this unglycosylated polyketide. Glycosylated intermediates of tylosin were fed to *tylMII*-KO during fermentation, in order to test the ability of the strain to perform the glycosylations downstream of the addition of mycaminose in the biosynthesis of tylosin. When this was done, full conversion of the fed intermediate to tylosin occurred, indicating that all the other glycoslytransferase and other activities necessary for tylosin production remained intact. In addition however, a wholly unexpected phenomenon was observed. Tylactone accumulated in these fermentations in large quantity. In contrast, when genes involved in the biosynthesis of the other tylosin sugars, mycinose (Wilson and Cundliffe, 1998) and mycarose (Bate *et al.*, 2000) were disrupted the expected products (demycinosyl-tylosin and demycarosyl-tylosin respectively) were accumulated.

It is highly unlikely that the tylactone accumulated in the *tylMII*-KO strain could have originated from degradation of the fed intermediates which would require the reversal of lactone ring hydroxylations. Instead the fed glycosylated compounds (including tylosin itself) appear to have stimulated polyketide metabolism , leading to *de novo* synthesis of tylactone. Since the strain is deficient in the ability to add mycaminose, neither this, or any other glycosylation could occur and thus the tylactone, unable to be converted any further, accumulated.

A complicating factor in this suggestion was the possibility that the disruption in *tylMII* may have exerted a downstream polar effect, in particular on the expression of *ccr* (orf4*). The gene was proposed to encode a crotonyl CoA reductase responsible, perhaps

not exclusively, for the conversion of acetoacyl CoA to butyryl CoA, the four carbon extender unit providing C5, 6, 19 and 20 in the synthesis of tylactone (Gandecha *et al.*, 1997). Any such downstream consequences on ccr might clearly influence polyketide synthesis although it is difficult to rationalise the observed phenomenon simply on this basis.

## 1.4 Investigation of the genes found in the tylosin biosynthetic cluster

### 1.4.1 Generation of targeted gene disruptions by gene replacement

The creation and analysis of mutant strains has been instrumental in the study of the tylosin biosynthetic cluster so far. As knowledge of the *tyl* cluster has become more detailed and in particular, the sequence and arrangement of the genes involved determined, their usefulness has been superseded to some extent. These strains almost certainly contain multiple mutations, the nature and position of which are unknown and are unlikely to be localised entirely in the region of interest. Many of these mutations might have little or no effect. However, a particular mutant strain might contain several different significant changes which conspire to produce a given phenotype. Also, mutations might lead to 'leaky' expression of genes rather than an absolute effect. These are complicating factors which are clearly undesirable and are bound to lead to some strains being isolated (such as the *tylL* mutant, GS33) whose phenotype is difficult to explain.

A more directed approach to the analysis of gene function is to use targeted gene disruptions. This approach avoids the possible complications of working with mutant strains. An effective way of creating such disruptions is by insertion of a resistance cassette such as hygromycin or streptomycin/spectinomycin, flanked by transcriptional/translational terminators into the gene of interest by homologous recombination (Blondelet-Rouault *et al.*, 1997). The transcriptional terminators used are derived from the fd coliphage and have been shown to function effectively in *Streptomyces* (Ward *et al.*, 1986)

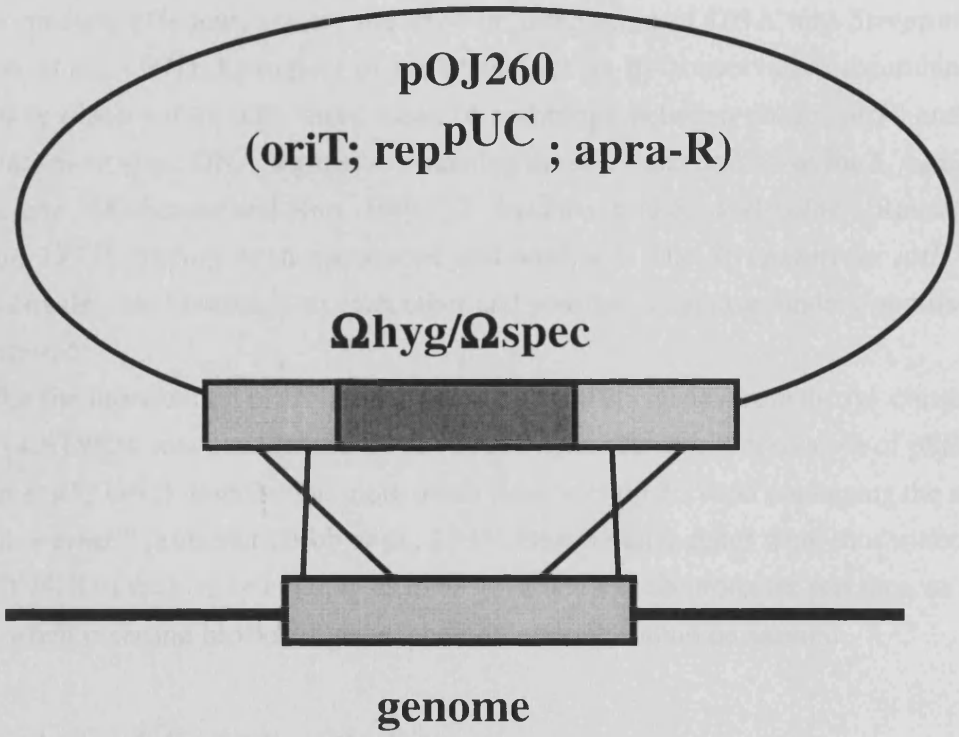
Gene disruption is achieved by inserting the resistance cassette approximately centrally into a fragment of DNA so as to interrupt a the coding region of interest at an appropriate place. This construct is then be introduced into the wild type organism on a suitable vector containing a second antibiotic resistance marker. In this case the suicide vector pOJ260 was used (section 2.2). This can be introduced into *S. fradiae* by conjugation and contains an apramycin resistance gene. An homologous recombination event occurring either side of the resistance cassette should then introduce this cassette into the genome at the desired position (Figure 1.8). A double recombinant can then be

identified by being resistant only to hygromycin, not the antibiotic for which the vector contains a resistance marker. A single recombinant would be resistant to both antibiotics since it would also contain the vector sequence and therefore its associated resistance characteristics.

It is important to consider the effects on downstream genes that a gene disruption of this nature may have. The transcription stops sites in the resistance cassette should ensure that any genes downstream of the disruption that are dependent on the same promoter will not be expressed. Although this approach might initially lead to complications when interpreting the phenoype of a disrupted strain, it is ultimately a useful device for obtaining extra information. Unlike when dealing with mutant strains, the luxury of knowing the precise nature of the disruption and its possible effects should allow the phenotype observed to be accurately interpreted. If necessary, downstream genes can be introduced to a strain in order to obtain an accurate picture of the effects of a disruption, in isolation of downstream consequences. In addition the re-introduction of downstream genes, necessitated by gene disruptions, can lead to examples of co-regulation being identified.

### 1.4.2 Introduction of DNA into *S. fradiae* strains

A useful way of analysing the function of genes is often to introduce the gene into strains under conditions in which it will be strongly and constituitively expressed. In this case the effect on tylosin biosynthesis of over-expressing a gene from the cluster might prove useful in determining its functional significance in the biosynthesis. Also, by introducing extra copies of genes, it might be possible to enhance antibiotic production yields. The re-introduction of genes is likely to be particularly informative in the wake of targeted gene disruptions. The restoration of the original phenotype of disrupted strains provides a useful control. Also the relative orientation of many of the *tyl* genes means that a disruption in one particular gene might have downstream polar effects on others. Thus reintroduction of more than just the disrupted gene itself might be required in order to restore the original phenotype. Determining which, if any, downstream genes are required in such a case is a useful tool for revealing the transcriptional organisation within the candidate operons. Under other circumstances it might be informative to introduce other DNA into *Streptomyces* strains, such as genes from other organisms or possible promoter sequences in promoter-probe vectors.




**single crossover** **hyg-R, apra-R**

**double crossover** **hyg-R, apra-S**

**Figure 1.8 Targeted gene disruptions by insertion of a resistance cassette.** The hygromycin resistance cassette Ωhyg or the spectinomycin/streptomycin resistance cassette Ωspec and their flanking transcriptional terminators can be inserted into genes by encouraging a double crossover to occur. Two separate homologous recombination events between the chromosome and the plasmid borne homologous sequence occuring either side of the disruption cassette inserted therein, will cause the chromosomal copy of the gene to be dirupted. Strains in which a successful disruption has been achieved can then be distinguished from those in which only a single crossover has occured, on the basis of their antibiotic resistance phenotype, as shown (where R is resistant and S is sensitive). Disrupted strains will maintain resistance to hygromycin but lose resistance to apramycin.

Cloning vectors based on the actinophage ØC31 facilitate the introduction of DNA into *Streptomyces* and have been used extensively in this project. Such vectors have been found to mediate efficient, stable, site-specific integration of DNA into *Streptomyces* (Kuhstoss *et al.*, 1997). Lysogeny of the phage occurs by conservative recombination which takes place within only three bases of homology between phage (*attP*) and host (*attB*) attachment sites, DNA segments containing the *aatP* and *attB* sites for *S. fradiae*, *S. ambofaciens* (Kuhstoss and Rao, 1991), *S. lividans* and *S. coelicolor* (Rausch and Lehmann, 1991), having been sequenced and analysed. The *Streptomyces attB* sites display considerable homology to each other and possible 'integrase binding' motifs have been identified.

For the introduction of DNA containing biosynthetic genes from the *tyl* cluster, the plasmid pLST9828 was used (Butler *et al.*, 1999). This vector is a derivative of pSET152 (Bierman *et al.*, 1992) described in more detail later (section 2.2) and containing the strong constitutive *ermE** promoter (Bibb *et al.*, 1994). Biosynthetic genes were thus introduced into pLST9828 in such an orientation as to be governed by this promoter and thus, as far as possible when inserting blocks of genes, their expression should be assured.

### 1.4.3 Analysis of *S. fradiae* strains

The main tools employed for the analysis of engineered *S. fradiae* strains (section 2.9) was via analysis of fermentation extracts by HPLC (High Performance Liquid Chromatography) and mass spectrometry. Strains were sometimes supplemented with tylosin metabolites during fermentation in order to demonstrate their bioconversion and/or to stimulate tylactone accumulation. The bioactivity of certain fermentation extracts was also determined in plate assays. Analysis of DNA fragments containing possible promoter elements was carried out using promoter probe vectors.

## 1.5 Thesis aims

The initial aim of this work was to investigate the genes *S.fradiae tylM* genes by obtaining disruptions by gene replacement. Analysis of these and mycaminose-specific mutant strains (*tylM* and *tylB*) was intended to provide an insight into mycaminose metabolism and its possible importance in the regulation of polyketide synthesis in *S. fradiae* (Chapter 3). In addition it was hoped that the patterns of expression in the *tylM* region might be determined (Chapter 4). During the course of this project, regulatory genes were identified in the *tyl* cluster. The intriguing possibility that some or all of these regulatory elements might be involved in the stimulation of polyketide synthesis was

therefore investigated (Chapter 5). Various compounds, some previously identified and some not, were found to accumulate in various *S. fradiae* strains. The nature and origin of these compounds was therefore investigated (Chapter 6).

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Sources and Preparation of materials

### 2.1.1 Standard reagents, bacterial culture media and molecular biology enzymes

Unless otherwise stated, chemicals were purchased from either Sigma Chemical Company Ltd. or from Fisher Scientific UK. Bacterial culture media was purchased from Difco Laboratories or Oxoid Ltd. unless otherwise stated. Agar was obtained from Oxoid Ltd. (Bacteriological agar) for *E. coli* culture and Fisher Scientific UK Ltd. (agar-agar) for *Streptomyces* culture.

Enzymes were purchased from GIBCO BRL, Promega, New England Biolabs UK. Ltd or Boehringer Mannheim. Oligonucleotides for sequencing and PCR applications were purchased from GIBCO BRL.

### 2.1.2 Preparation of solutions and media

Solutions were prepared using glass distilled deionised water, further purified by passage through a SuperQ water purification system (Millipore). The pH of solutions was measured using an Ultrolab 2100 pH meter. Where appropriate, sterilisation was achieved by either filtration through a 0.22 micron filter or by autoclaving for 15 minutes at 121 °C and 25 $lb/in^2$ pressure.

For composition of solutions refer to section 2.10, for composition and preparation of bacterial media refer to section 2.11

### 2.1.3 Growth and manipulation of bacteria

Strains, growth conditions, storage conditions and manipulation of bacteria are described in section 2.5 (*E. coli*) and section 2.6 and 2.7 (*Streptomyces*).

## 2.2 General purpose plasmids

Two plasmids were used for general purpose manipulation of DNA in *E.coli*, pUC18 and pIJ2925 (shown and described in Figures 2.1 and 2.2 respectively). For the conjugal transfer of of DNA from *E. coli* to *S. fradiae*, the plasmids pOJ260 and pLST9828 (shown and described in Figures 2.3 and 2.4 respectively) were used. For promoter-probe analysis the *redD* -containing conjugative vector pIJ2585 was used (shown and described in Figure 2.5). DNA from the *tylG* and *tylM* region was obtained from the

cosmid pMOMT4 (Beckman et al., 1989) and the pUC18 based plasmid pLST97A (Clark, 1997).

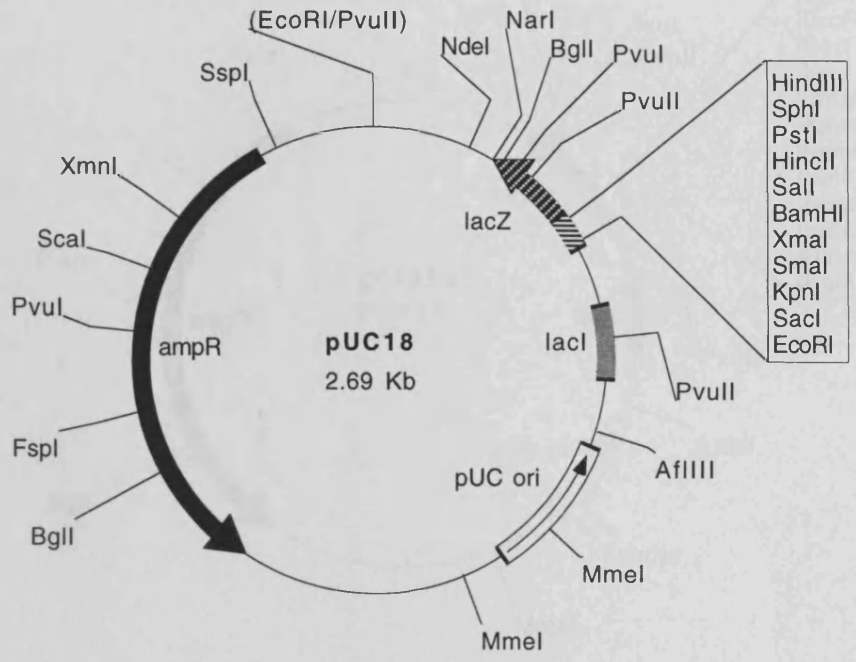



**Figure 2.1. Map of pUC18.** Plasmid pUC18 is a 2.69 kb, high copy number, *E. coli* cloning vector with ampicillin resistance (ampR). Universal primer sequences are present either side of the polylinker which facilitate PCR cycle sequencing (Yanisch-Perron *et al.*, 1985).

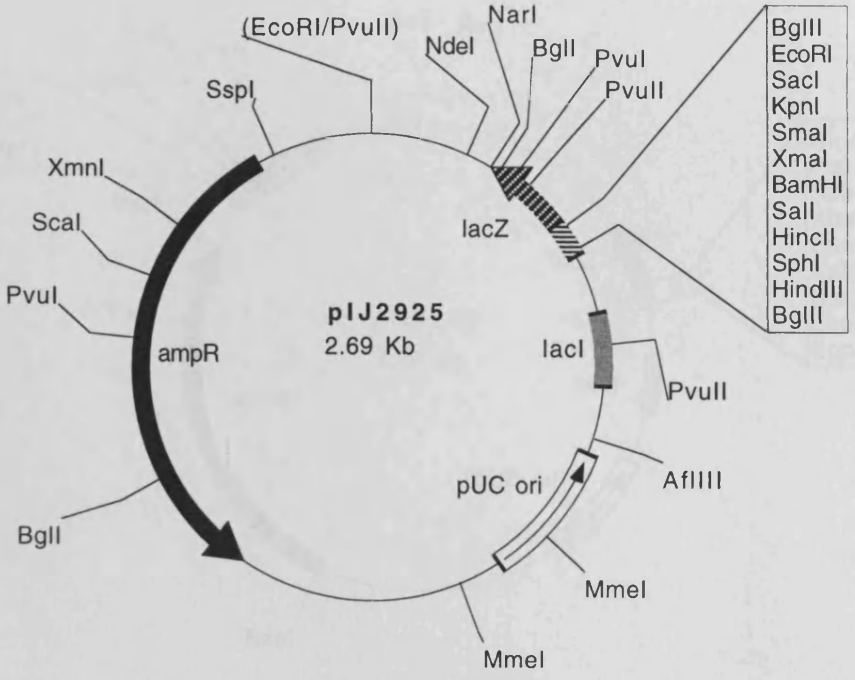



**Figure 2.2. Map of pIJ2925.** Plasmid pIJ2925 is a derivative of pUC18 with *Bgl* II sites introduced so as to flank the polylinker (Janssen and Bibb, 1993).

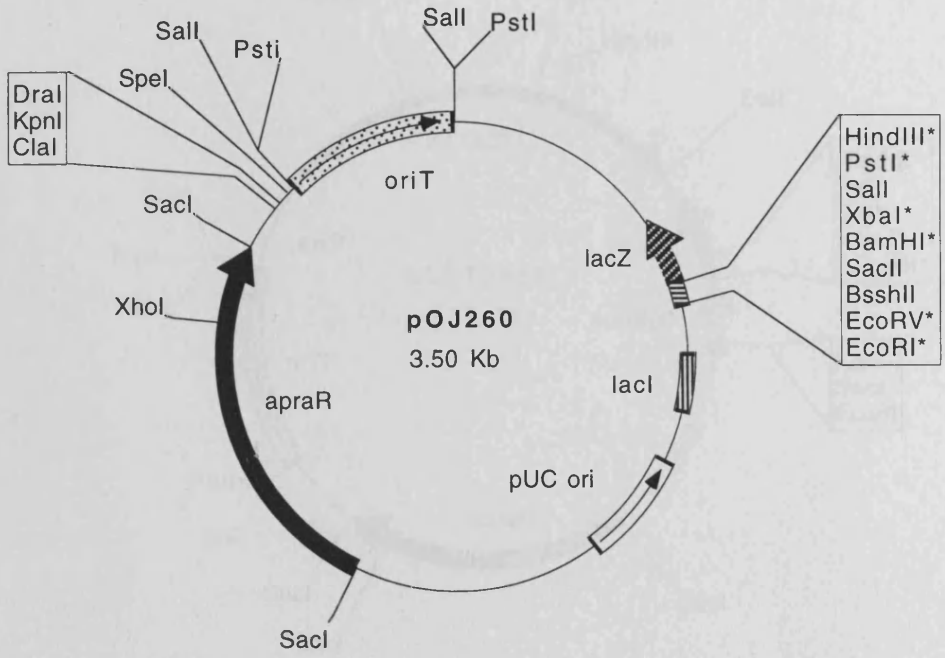



**Figure 2.3. Map of pOJ260.** Plasmid pOJ260 is a 3.5kb vector designed for the conjugal transfer of DNA from *E. coli* into *Streptomyces* spp. for subsequent homologous recombination. It therefore has no *Streptomyces* origin of replication. It has an apramicin resistance gene (apraR) for selection in *E. coli* and *Streptomyces* (Bierman *et al.*, 1992). Unique sites in the polylinker are marked '*'.

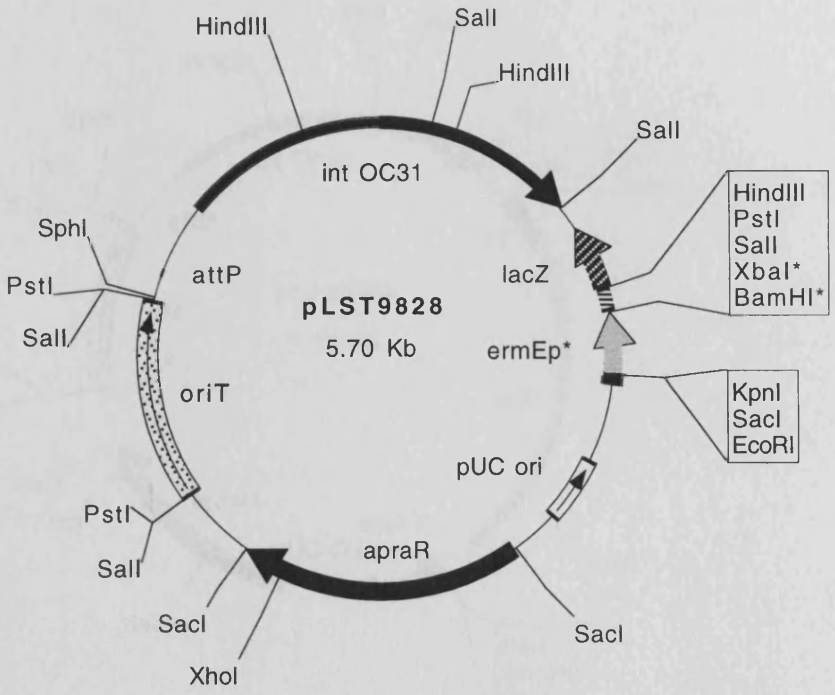



**Figure 2.4. Map of pLST9828.** Plasmid pLST9828 is a derivative of pSET152 (Bierman *et al.*, 1992), a 5.7 kb *E. coli* cloning vector designed for the conjugal transfer of DNA into *Streptomyces* spp. and subsequent integration into the øC31 *attB* site, carrying the strong constituitive *ermE** promoter inserted into the polylinker (Butler *et al.*, 1999).

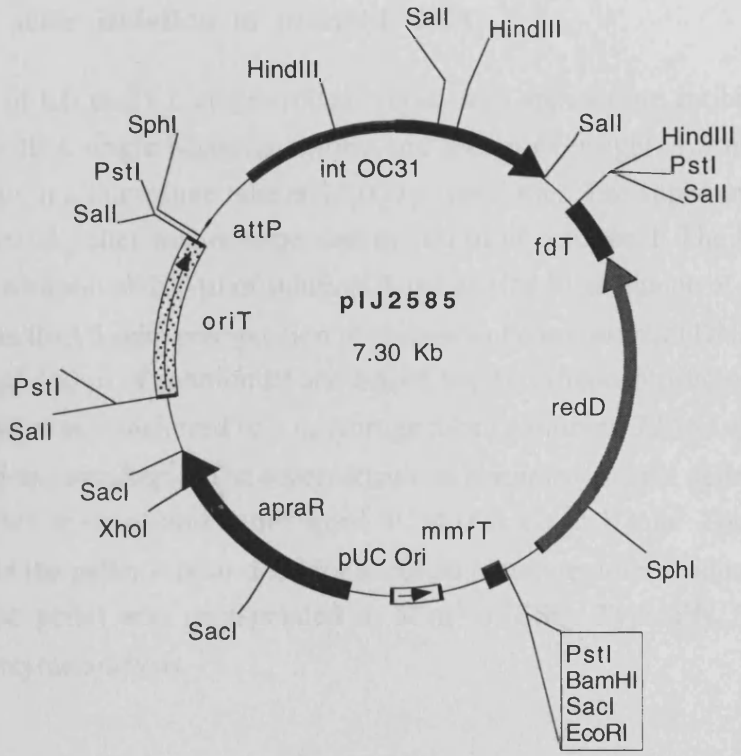



**Figure 2.5. Map of pIJ2585.** Plasmid pIJ2585 is a derivative of pSET152 (Bierman *et al.*, 1992), designed for the conjugal transfer of DNA into *Streptomyces* spp. and subsequent integration into the øC31 *attB* site. Additionally it contains a *redD* cassette for promoter-probe analysis (M. J. Bibb, personal communication).

## 2.3 Isolation of plasmid DNA

### 2.3.1 Mini scale isolation of plasmid DNA

3 ml of LB or 2YT in a sterile universal with appropriate antibiotic selection was inoculated with a single bacterial colony and grown overnight. 1.5 ml was pelleted by centrifugation in a microfuge tube at 14,000 *g* for 5 min. The supernatant was discarded and the bacterial pellet was resuspended in 100 µl of solution I. The bacteria were then lysed by the addition of 200 µl of solution II and mixing by inversion of the tube 3-4 times. After no more than 5 min, precipitation of protein and chromosomal DNA was achieved by the addition of 150 µl of solution III and a brief vortex. After centrifuging as before, 360 µl of supernatant was transferred to a microfuge tube containing 720 µl of absolute ethanol, vortexed and re-centrifuged. The supernatant was discarded and the pellet washed with 100 µl of 70% v/v ethanol and centrifuged at 14,000 *g* for 1 min. The supernatant was discarded and the pellet was air dried for about 30 min to remove residual traces of ethanol. Once dry the pellet was resuspended in 50 µl of SSQ. Typically 5 µl was used per restriction enzyme analysis.

### 2.3.2 Midi scale isolation of plasmid DNA

In order to obtain larger quantities (approximately 50 µg) of good quality plasmid DNA from high copy number plasmid vectors (such as pUC18), the following method was employed. 50 ml of LB or 2YT in a sterile 250 ml conical flask containing appropriate antibiotic selection was inoculated with 50 µl of bacterial culture from a positive clone identified by mini-scale isolation of plasmid DNA. The bacteria were harvested by centrifugation for 10 min. at 10,000 *g* in 50 ml polypropylene tubes. The supernatant was discarded and the pellet was resuspended in 2 ml of solution I. The addition of 3 ml of solution II followed by gentle inversion 3-4 times facilitated lysis of the bacteria. 2.5 ml of solution III was then added and briefly vortexed, thus allowing the precipitation of chromosomal DNA and protein. The precipitate was removed by a combination of centrifugation at 10,000 *g* for 5 min followed by decanting of the resulting viscous supernatant through myra cloth (Calbiochem) into a clean 50 ml polypropylene tube. To isolate the remaining nucleic acid, an equal volume of isopropanol was added and mixed by inversion, followed by centrifugation at 10,000 g for 10 min at 4 °C. The supernatant was removed and the pellet resuspended in 300 µl SSQ and transferred to a 1.5 ml microfuge tube.

The removal of RNA from the preparation was achieved in two stages. First by the addition of 300 µl of 8M LiCl, vortexing and centrifugation at 14,000 g for 5 min. The supernatant, from which large RNA molecules had thus been removed, was then transferred to a clean microfuge tube and the remaining nucleic acids were again isolated by the addition of an equal volume of isopropanol and centrifugation, this time at 14,000 g for 5 min. Then 10 µl of RNAse A (20 mg/ml) was added to the resuspended pellet (500 µl SSQ), mixed and incubated at 37 °C for 1 hr.

500 µl of phenol/chloroform was added, vortexed and centrifuged at 14,000 g for 5 min. The top, aqueous layer was transferred to into a clean microfuge tube to which 100 µl of 1 M ammonium acetate and 2 volumes of absolute ethanol were added, vortexed, centrifuged as before and the supernatant removed. The pellet was washed with 200 µl of 70% v/v absolute ethanol, centrifuged and the supernatant removed as before. The resulting pellet was dried under vacuum to remove residual ethanol and resuspended in an appropriate volume of SSQ.

### 2.3.3 High quality plasmid preparation

High quality plasmid DNA for sequencing was prepared by alkaline lysis/QiagenR anion-exchange chromatography as recommended by the supplier (Qiagen Ltd.).

## 2.4 Manipulation and analysis of DNA

### 2.4.1 Digestion of plasmid DNA

Plasmid DNA was digested with restriction endonucleases in order to generate compatible ends for cloning and to verify newly created plasmids. The reaction conditions were set up according to manufacturers instructions. Typically, digests were set up in a 30 µl reaction volume consisting of :

| | |
|---|---|
| DNA | x µl |
| restriction endonuclease (10 units/µl)* | 1 µl |
| 10x manufacturers buffer | 3 µl |
| RNAse (2 mg/ml)** | 1 µl |
| SSQ | x µl to a total volume of 30 µl |

Digests were normally carried out at 37 °C for 3-4 hours unless advised otherwise in the manufacturers instructions.

* When two or more enzymes were used simultaneously the total reaction volume was increased proportionally to prevent the glycerol concentration from exceeding 5%, which is problematical when using certain restriction endonucleases

** RNAse was used only in digests of mini-scale prepared plasmid in which there is no RNA removal incorporated into the isolation protocol.

### 2.4.2 Removal of 5' terminal phosphate groups from cleaved plasmid DNA

In order to reduce the efficiency with which plasmid DNA cleaved by a single restriction endonuclease would re-ligate itself without any insert DNA, the 5' terminal phosphate groups were removed by treatment with calf intestinal alkaline phosphatase (CIAP). Digests were set up in twice the normal reaction volume and incubated. 1 µl of CIAP (1 unit/µl) was then added for the last hour of incubation.

### 2.4.3 End filling 5' overhangs

DNA digested so as to leave 5' overhangs could be end-filled to obtain blunt ended fragments by setting up the following reaction mix:

| | |
|---|---|
| 0.5 mM dNTPs | 1 µl |
| klenow fragment (5 units/µl) | 1 µl |
| digested DNA | 0.5 µg |
| SSQ | x µl to a 20 µl total volume |

The reaction was incubated at 30 °C for 15 min and then 75 °C to heat incativate the enzyme.

### 2.4.4 Ligation of DNA fragments

Recombinant plasmids were created by annealing cut fragments together using T4 DNA ligase. Typically 25 ng of cut vector was used. The quantity of insert for each ligation was calculated using a vector : insert ratio of 1:3 when performing cohesive end ligations and 1:1 when ligating blunt ends according to the following equations:

Compatible cohesive ends;

vector size/insert size x 1/3 x ng of vector = ng of insert

Blunt ends;

vector size/insert size x 1/1 x ng of vector = ng of insert

Standard ligations were set up as follows and incubated at room temperature next to Neil's magic ligation budgie for 2 hours or overnight:

| | |
|---|---|
| vector | x µl |
| insert | x µl |
| 5x T4 DNA ligation buffer | 2 µl |
| T4 DNA ligase (5 units/µl) | 1 µl |
| SSQ | x µl to a total volume of 10 µl |

Vector only controls were set up as above except without the addition of insert DNA.

### 2.4.5 Agarose gel electrophoresis

DNA was size fractionated on neutral agarose gels between 1 and 2% as appropriate to the size of the fragments being resolved. Gels containing 125 ng/ml ethidium bromide were made and run in 1x TAE buffer as described in standard protocols (Sambrook *et al.*, 1989). DNA samples were loaded in a 1x gel loading buffer. Following electrophoresis gels were visualised by transillumination with ultra violet light and photographed. The size (kb) and amount (ng) of DNA fragments was determined by comparison with a reference DNA molecular weight standard kb ladder.

### 2.4.6 Purification of DNA fragments from agarose gel slices

DNA fragments were purified from agarose gel slices using a 'Jetsorb DNA Extraction from Agarose Gels' kit as recommended by the vendor (Genomed).

### 2.4.7 Spectrophotometric quantification of DNA

DNA was quantified either by estimation based on comparison of various dilutions of DNA samples with molecular weight markers of known concentration following separation by gel electrophoreisis (section 2.4.5) or by measuring its absorbence in an Unicam SP1800 Ultraviolet spectrophotometer. At 260 nm an optical density of one is equivalent to 50 µg/ml of DNA. Pure DNA has an $A_{260}/A_{280}$ absorbence ratio of greater or equal to 2.0. The concentration of resuspended oligonucleotides used for PCR amplification of DNA fragments and for automated cycle sequencing were calculated according to the following formula:

$O.D._{260}$ x 20 x dilution = mg/ml

## 2.5 Culture, storage and transformation of *E. coli*

### 2.5.1 *E. coli* strains

*E. coli* strain DH5α (Hanahan, 1983) was used for all molecular cloning applications.

For the conjugal transfer of DNA from *E. coli* to *Streptomyces* spp. *E. coli* strain S17.1 (Simon *et al.*, 1983) was used.

### 2.5.2 Growth of *E. coli*

*E. coli* was grown on solid LB or 2YT media at 37 °C overnight or until colonies were clearly visible. Bacteria could then be stored at 4 °C for up to a month.

In liquid culture *E. coli* was grown in LB broth or 2YT at 37 °C on an orbital shaker at 200 rpm for whatever time as prescribed in each individual protocol.

### 2.5.3 Preparation of competent *E. coli*

Preparation and transformation of *E. coli* was carried out by the following method (based on that of Hanahan, 1983).

10 ml of 2YT in a sterile universal was inoculated with a single colony of DH5α or S17-1 and grown overnight. 500 µl of the overnight bacterial culture was used to inoculate 50 ml of pre-warmed LB or 2YT in a 250 ml conical flask. This was then grown until the $O.D._{600}$ of the bacterial culture had reached 0.2. (approximately 2 hours) when sterile 1M $MgCl_2$ was added to a final concentration of 20 mM. The bacterial culture was then grown further until it reached an $O.D._{600}$ of 0.45-0.55 (approximately 40 min). The culture was then poured into 50 ml sterile tubes and incubated on ice for 2 hours. Bacteria were pelleted at 3000 *g* for 5 min at 4 °C, the supernatant discarded, the pellet resuspended in 50 ml of freshly prepared, pre-chilled, filter sterilised $Ca^{2+}$ $Mn^{2+}$ solution and the cells incubated on ice for a further 40 min. The bacteria were pelleted as before, the supernatant discarded and the cells resuspended in 5 ml $Ca^{2+}$ $Mn^{2+}$ solution containing 15 % v/v glycerol. The cells were quickly aliquoted into pre-chilled 1.5 ml microfuge tubes, flash frozen in dry ice/IMS and stored at -70 °C where they remained viable for several months.

### 2.5.4 Transformation of competent *E. coli*

50 µl aliquots of competent cells prepared as described above were used for each transformation. In the case of previously constructed plasmids, 1 ng of DNA was sufficient to ensure a good yield of successfully transformed cells. When transforming ligation mixtures, 2 µl of a 10 µl ligation was used. A total volume of 25 µl of DNA solution was added to each 50 µl aliquot of cells, previously thawed on ice. Cells and DNA were mixed by flicking the tube and incubated on ice for 30 min. The transformation mixture was then heat shocked by incubation at 37 °C for 5 min. The cells were then transferred to a sterile universal containing 0.9 ml of LB or 2YT and grown for 1 hour prior to being aliquoted onto agar plates containing appropriate antibiotic selection. These were then dried and incubated overnight. When selecting with certain antibiotics such as ampicillin, pre-growth

of cells prior to exposure to the antibiotic was unnecesary, and the cells were aliquoted directly onto plates immediately after heat shocking.

The transformation efficiency of the competent cells could be calculated simply by transforming with a known (small, i.e. 10 pg of pUC18) amount of plasmid, in the following way:

Number of colonies obtained x dilution x 1/amount of DNA used (μg)

If the transformation efficiency of the competent cells was $1x10^{-6}$ - $1x10^{-7}$, and if no bacterial colonies were obtained in a no DNA control then the competent cells were deemed suitable for use.

### 2.5.5 Antibiotics used for selection in *E. coli*

| Antibiotic | Concentration (μg/ml) |
|---|---|
| Ampicillin | 100 |
| Apramicin | 50 |
| Hygromycin | 75 |
| Streptomycin (in DH5α) | 100* |
| Streptomycin (in S17-1) | 30* |

* This antibiotic was used for selection of plasmids containing the streptomycin/spectinomycin resistance gene in DH5α. *E. coli* strain S17-1 possesses non-plasmid mediated resistance and a lower concentration of antibiotic was used.

### 2.5.6 Long term storage of bacterial cultures

Bacterial cultures were maintained for long periods of time by transferring 0.5 ml of a dense bacterial culture to a cryogenic storage tube. An equal volume of sterile 50 % v/v glycerol was then added to the culture, the contents were vortexed, flash frozen (IMS/dry ice) and stored at -70 °C.

## 2.6 Culture, storage and manipulation of *Streptomyces*

### 2.6.1 Streptomyces strains

*S. fradiae* strain C373.1 (T59235) 'wild type' provided by E.T. Seno, Eli Lilly and Co.)

*S. fradiae* GS14, GS33, GS50, GS62 and GS77 (mutant strains defective in tylosin biosynthesis, generated using NTG (Baltz *et al.*, 1981) provided by E. T. Seno, Eli Lilly and Co.).

*S. lividans* 66 strain TK21 (Ward *et al.*, 1986).

### 2.6.2 Routine growth of *Streptomyces*

*S. fradiae* was grown on solid AS-1 plates for 3 days at 37 °C or until sporulation had occurred. Sporulated plates could then be kept at 4 °C for several months. In liquid culture, inoculated from freshly harvested spores (section 2.6.3) or 1 ml mycelial stocks, *S. fradiae* was propagated in 50 ml TSB at 30 °C in sprung 250 ml conical flasks for 2-4 days on a orbital shaker at 250 rpm, depending on the strain and the antibiotic selection applied, until the culture became thick.

*S. lividans* was grown on solid NEF plates at 30 °C for 3 days or until sporulation had occurred. As for *S. fradiae*, plates could then be stored temporarily at 4 °C. In liquid culture inoculated from freshly harvested spores or 1 ml mycelial stocks, *S. lividans* was grown in 50 ml YEME at 30 °C in baffled 250 ml conical flasks on at orbital shaker at 250 rpm.

### 2.6.3 Harvesting *Streptomyces* spores from plates

Spores were harvested from the surface of thickly grown plates by the addition of 5 ml of SSQ and scraping with a sterile loop. The spore-containing water was then removed to a sterile universal along with further 5 ml 'washings' of the plate until the majority of spores had been removed. Following a thorough vortex, spore suspensions were centrifuged at 3,000 *g* for 15 min, in order to pellet the spores prior to removal of the supernatant.

### 2.6.4 Antibiotics used for *Streptomyces* selection

| Antibiotic | Concentration (μg/ml) |
|---|---|
| Apramicin sulphate | 50 |
| Hygromycin B | 75 |
| Nalidixic acid | 60 |
| Spectinomycin* | 1 |
| Streptomycin* | 100 |

* These antibiotics were only ever used in combination for the selection of *Streptomyces fradiae* strains.

### 2.6.5 Long term storage of bacterial cultures

*Streptomyces* bacterial cultures were maintained for long periods of time in 5 % v/v DMSO by transferring aliquots of dense cultures cryogenic storage tubes and adding an appropiate volume of DMSO in each case. These were then flash frozen (IMS/dry ice) and stored at -70 °C.

## 2.7 Genetic manipulation of *Streptomyces*

### 2.7.1 Conjugation of DNA from *E. coli* S17-1 into *S. fradiae*

Conjugation of DNA from the donor strain *E. coli* S17-1 into *S. fradiae* (modified from Bierman *et al.*, 1992; Mazodier *et al.*, 1989), is the most efficient way of achieving such a transfer. Plasmid pLST9828 (based on pSET152, Bierman *et al.*, 1992), containing the apramycin resistance determinant was used to introduce DNA with high efficiency into the *S. fradiae* ØC31 *attB* chromosomal integration site. Plasmids based on the vector pOJ260, unable to replicate in actinomycetes, were used to introduce resistance cassettes into chromosomal genes by homologous recombination events (as described in section 1.4.1). Since this latter process occurs with far less efficiency than the former, slightly differing protocols were applied for the conjugation of DNA and transconjugant selection.

In both cases, *E. coli* S17-1 single colonies containing the appropriate plasmid were used to inoculate 10 ml 2YT or LB which was grown overnight under streptomycin (S17-1 selection) and apramycin (plasmid maintenance) selection in order to coincide with the growth and sporulation of *S. fradiae*. 1 ml of overnight culture was then used to

inoculate either 10 ml (for a pLST9828 based conjugation) of media which was grown for around 8 hours or 50 ml (for a pOJ260) of media grown for about 12 hours under the same dual selection described above in both cases.

Spores were harvested from the surface of AS-1 plates and following removal of the supernatant, were resuspended in 10 ml pre-germination media in sterile universals and grown at 30 °C for no longer than four hours. The growth of S17-1 donor cultures and the pre-germinating *S. fradiae* recipient cultures were timed so as to coincide.

All further resuspensions were carried out using LB or 2YT. Both sets of cultures were centrifuged at 3,000 *g* for 10 min, the supernatant was discarded and the pellet resuspended in 1 ml in 1.5 ml microfuge tubes. Bacteria were then twice pelleted by centrifugation at 14,000 *g* for 2 min, the supernatant discarded and the culture resuspended. The first time in 1 ml and the second time in a volume appropriate to performing the mating.

For pLST9828 based conjugations, up to 10 conjugations into different *S. fradiae* strains could be successfully carried out from each 10 ml culture. The *E. coli* was therefore resuspended and 100 µl aliquots were placed in 1.5 ml microfuge tubes. Similarly, many pLST9828 based constructs could be conjugated into the pre-germinated *S. fradiae* derived from a single plate of spores. Thus the spore pellet was resuspended and 100 µl aliquots were added to the appropriate, resuspended S17-1 donor cells and mixed by inversion, before being plated on AS-1.

When attempting pOJ260 based conjugations, the S17-1 donor cells were resuspended in 1 ml aliquoted into ten, 1.5 ml microfuge tubes and mixed with the pre-germinated spores obtained from 3-4 sporulating plates, pooled, resuspended in 1 ml and thus similarly aliquoted. Each mixture of donor and recipient cells was plated onto separate AS-1 plates.

For both types of conjugation, AS-1 plates were incubated at 37 °C for 16-20 hours.

### 2.7.3 Selection of successful transconjugants

After 16-20 hours of incubation, AS-1 plates were overlaid to select for successful transconjugants. Conjugations involving integration of DNA into the chromosomal ØC31 *attB* site, mediated through pLST9828 based plasmids were overlaid with 5 ml of AS-1 media made with 0.7 % w/v agarose and containing nalidixic acid to inhibit donor cell growth and apramycin to select for *S. fradiae* strains having successfully accepted the integrative vector. Plates were grown for a further 3 days or until colonies had grown through the overlay and sporulated. Single colonies were picked and re-plated onto antibiotic-containing AS-1.

Conjugations using pOJ260 based plasmids designed for targeted disruption via gene transplacement were similarly overlaid with 0.7 % w/v agarose AS-1 containing nalidixic acid and antibiotic appropiate to the resistance marker being used in the gene disruption. Thus transconjugants in which the disrupting cassette had been integrated by homologous recombination into the genome would be selected for. Single colonies were picked and re-plated onto AS-1 containing antibiotic appropiate to the resistance marker being used in the disruption. In order to select specifically for transconjugants in which the desired double homologous recombination event had occurred, colonies were replica plated onto apramycin. *S. fradiae* colonies sensitive to apramycin yet resistant to the antibiotic for which the disrruption cassette contained the corresponding determinant were selected as being promising candidates for authentic gene disrupted strains.

## 2.8 Confirming the authenticity of disrupted strains by Southern analysis

### 2.8.1 Isolation of total DNA from *S. fradiae*

*S. fradiae* was grown without selection for 2-3 days in sprung TSB flasks at 30 °C. The culture was harvested in a 50 ml polypropylene tube (10 min, 3000 *g*) and resuspended in 5 ml SET. Lysozyme was added to a final concentration of 2 mg/ml and incubated for 10 min or until lysis was evident. 1/10 volume of 10% w/v SDS and proteinase K to a final concentration of 0.5 mg/ml were added and mixed by inversion. The preparation was then incubated at 55 °C for 1 hour with occasional inversion. An equal volume of phenol/chloroform was added and the tube was inverted reasonably vigorously until an emulsion was formed. This was centrifuged at 10,000 *g* for 10 min and the aqueous phase was removed using a 1.5 ml plastic pastuer pipette. DNA was precipitated by addition of 1/10 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of absolute ethanol. This was spooled out of solution using a glass rod and washed with 70% v/v ethanol and then absolute ethanol. The DNA was allowed to dry (15 min at room temperature) and then re-dissolved in 0.5 ml water in a microfuge tube. The DNA was RNAse A treated (final concentration 30 mg/ml) for 2 hours at 37 °C before being treated with phenol/chloroform and ethanol extracted as before, using a glass rod to spool out the DNA. The DNA was then washed with 70% ethanol and then absolute ethanol, allowed to dry as before and dissolved in 0.5 ml water. Total DNA preparations were stored at 4 °C. Pipetting was carried out using 'cut off' tips to avoid shearing genomic DNA.

### 2.8.2 Digestion and Southern blotting of total DNA

Total DNA was digested with appropiate restriction endonucleases to generate fragments which could be probed in order to confirm the authenticity of disrupted strains, when compared to the similarly digested wild type. The reaction conditions were set up according to manufacturers instructions. Typically, digests were set up in a 100 µl reaction volume consisting of :

| | |
|---|---|
| DNA | x µl |
| restriction endonuclease (10 units/µl)* | 5 µl |
| 10x manufacturers buffer | 10 µl |
| SSQ | x µl to 100 µl total volume |

Digests were normally carried out at 37 °C for 3-4 hours unless advised otherwise in the manufacturers instructions. Fragments were separated overnight by gel electrophoreisis on a 1x TAE agarose gel (section 2.4.5) with appropiate controls and DNA size markers. Approximately 0.5 µg of total DNA was loaded for each hybridisation analysis. Prior to blotting, gels were photographed alongside a ruler to facilitate interpretation. Southern blotting (based on Southern, 1975) onto Hybond-N membrane was carried out according to manufacturers instructions (Amersham pharmacia biotech).

### 2.8.3 Probe labelling and hybridisation analysis

Probe labelling and Southern blot hybridisation was acheived using the DIG High Prime DNA Labelling and Detection Starter Kit II (Boehringer Mannheim) using the 'standard hybridisation buffer' according to the manufacturers instructions. Approximately 1 µg of DNA, purified from an agarose gel slice (section 2.4.6) was DIG labelled in a 20 hour reaction in each case.

## 2.9 Fermentation analysis of *S. fradiae*

### 2.9.1 Fermentation of *S. fradiae*

All fermentations were carried out in the absence of antibiotic selection, in the following manner. A 1 ml mycelial fragment stock was added to 10 ml TSB in a sterile universal and incubated for 1 day at 30 °C on an orbital shaker at 250 rpm. The mycelia was then allowed to settle at the bottom of the tube for 30 min. Any contamination of this culture was usually obvious at this stage. 1 ml of material was then removed from the base

of the universal and used to inoculate 30 ml pre-fermentation medium which was incubated for 3 days at 28 °C in a 100 ml conical flask on an orbital shaker at 250 rpm. A healthy culture should be thick (like pea soup) and have an orangey-brown complexion. 5 ml of pre-fermentation culture was then used to inoculate each 50 ml MM-1 fermentation media which was incubated for 7 days at 28 °C in a 250 ml conical flask on an orbital shaker at 250 rpm.

Whenever feeding of fermentations with biosynthetic intermediates was necessary, this was carried out after 2 days into the 7 day fermentation in MM-1. Routinely 10 mg of intermediate was added in 100 µl of DMSO although occasionally, when induction of polyketide synthesis was under investigation rather than bioconversion of intermediates only 1 mg of intermediate was used in order to preserve limited stocks. Control flasks were fed with 100 µl DMSO.

Tylosin intermediates used in feeding experiments and as standards in HPLC analysis (section 2.9.3) were supplied by Eli Lilly research laboratories, Indianapolis.

### 2.9.2 Extraction of the beer

An equal volume of chloroform (50 ml) was added to the fermentation and shaken for 10 min at 200 rpm. This was then poured into two 50 ml falcon tubes which were spun at 3000 *g* for 10 min. Following centrifugation the top aqueous layer is separated from the lower chloroform layer by a 'plug' of solid material (mycelia etc.) The lower organic phase was removed and stored at -20 °C at least overnight. Chloroform extracts were allowed to equilibrate to room temperature before being concentrated by rotary flash evaporation at 30° C until no further reduction in volume could be achieved. The material remaining was then re-suspended in HPLC grade chloroform to a final volume of 1 ml where possible. In some cases the volume of material following rotary evaporation exceeded 1 ml in which case the extract was made up to some other suitable volume (never exceeding 2 ml) taken into account during its analysis. The fermentation extract was stored in cryotubes at -20 °C until required for analysis.

### 2.9.3 HPLC analysis of the fermentation products

Fermentation products were analysed by reverse phase HPLC analysis (as described by Huber *et al.*, 1990). Chloroform extracts in elution buffer were applied to a 3.9 x 300 mm C18 µBondapak column protected by a C18 µBondapak guard column (Waters Associates). Products were eluted in 0.3% (w/v) ammonium formate (pH 4.0) buffer with a 15 min linear concentration gradient of 50-80 % methanol v/v at a flow rate of

1.75ml/min.The UV absorbance of the eluate was measured at 282 nm unless stated otherwise. Tylosin metabolites were identified on the basis of retention time in comparison to standard compounds. The elution of compounds was referred to on the basis of retention time relative to that of tylosin (rrt), where: rrt = $\frac{\text{retention time of compound}}{\text{retention time of tylosin}}$

### 2.9.4 Analysis of the fermentation products by mass spectrometry

Fermentation extracts were analysed by electrospray ionisation mass spectrometry using a Micromass platform II (Fisons instruments) in the positive ion mode by PNACL Services, Leicester University. The solvent used was 50 % acetonitrile v/v in 0.3 % v/v formic acid.

### 2.9.5 Bioassay of fermentation extracts

The biological activity of fermentation extracts was assessed by comparison of zones of inhibition on *Micrococcus luteus* plates. *M. luteus* was grown overnight in 10 ml LB at 37 °C on an orbital shaker at 250 rpm. 200 µl of this culture was added to 5 ml 1% agar LB medium kept at 50 °C and mixed by inversion. This was then poured onto LB plates and allowed to set.

2 µl of fermentation extract was spotted onto small discs of Whatman 3M paper and allowed to soak in and dry. Negative control discs were spotted with 2 µl choroform. Discs were then carefully placed on the *M. luteus* plates which were kept at 4 °C for 2 hours to allow diffusion of the antibiotic before incubation at 37 °C overnight.

## 2.10 Composition of solutions

Agarose gel loading buffer (10x)
0.5 % w/v orange G, 50% v/v glycerol

$Ca^{2+}$ $Mn^{2+}$ solution
100 mM $CaCl_2$, 70 mM $MnCl_2$, 40 mM Na acetate (pH 5.5 with 1M HCl)

SET
75 mM NaCl, (25 mM EDTA pH 8.0), 20 mM Tris-HCl pH 7.5

Solution I
50 mM Glucose, 25 mM Tris/HCl (pH 8.0), 10 mM EDTA (pH 8.0)

Solution II
1% w/v SDS, 0.2M NaOH

Solution III
5M Potassium acetate (pH 4.8)

Tris-acetate-EDTA (TAE)
40 mM Tris base, 40 mM glacial acetic acid, 1 mM EDTA (pH 8.0)

## 2.11 Composition and preparation of bacterial media

2YT media
1.6 % w/v tryptone, 1.6 % w/v yeast extract, 0.5 % w/v NaCl (pH 7.0 NaOH)
Solidified where required by the addition of 2 % w/v bacteriological agar.

AS-1 media
0.1 % w/v yeast extract, 0.02 % w/v L-alanine, 0.02 % w/v L-arginine, 0.05 % w/v L-asparagine, 0.5 % w/v soluble starch, 0.25 % w/v NaCl, 1 % w/v $Na_2SO_4$, 2 % w/v agar-agar
pH to 8.0 with KOH.
Solidified where required by the addition of 2 % w/v agar-agar unless used for overlaying in which case 0.8 w/v % was used.

Luria-Bertoni (LB) media
1 % w/v tryptone, 0.5 % w/v yeast extract, 1 % w/v NaCl (pH 7.0 NaOH)
Solidified where required by the addition of 2 % w/v bacteriological agar.

MM-1 (tylosin production medium)
1 % v/v methyl oleate, 0.5 % w/v betaine HCl, 0.23 % w/v di potassium hydrogen orthophosphate trihydrate, 0.2 % w/v sodium chloride, 0.3 % w/v calcium chloride dihydrate, 0.5 % w/v magnesium sulphate, 0.0001 % w/v cobalt chloride, 0.001 % w/v zinc sulphate heptahydrate, 0.3 % w/v ferric ammonium citrate, 1.75 % w/v monosodium glutamate, 0.5 % w/v glucose, 0.26 % Potassium hydroxide.
The methyl oleate was added individually to 250 ml conical flasks. The rest of the media was prepared as a batch pH 7.0 which was checked and altered if necessary by the addition of a small amount of 1M KOH

NEF
0.5 % w/v glucose, 0.1 % w/v yeast extract, 0.05 % w/v beef extract, 0.1 % w/v casamino acids.
pH to 8.0 with KOH
Solidified by the addition of 2 % w/v agar-agar.

Pre-fermentation medium

1.0 % w/v corn steep liquor or 0.5 % corn steep solids, 0.5 % w/v yeast extract, 0.5 % w/v soya bean meal (Hopkin and Williams), 0.3 % w/v calcium carbonate, 0.5 % w/v methyl oleate

pH to 7.8 with NaOH

The non soluble components were added individually to 100 ml conical flasks. All other components were made as a batch and brought to pH 7.8 with 1M NaOH. The pH of the media does not alter when added to the insoluble components.

Pre-germination media

0.5 % w/v yeast extract, 0.5 w/v % casamino acids, 0.025 M TES, 10 mM $CaCl_2$

The yeast extract and casamino acids were made up to double strength, the TES was made as a 0.05 M solution, pH 8.0 with NaOH and the $CaCl_2$ was made as a 5 M stock. Each component was made and autoclaved separately. Prior to use the double strength media and TES were sterily mixed in equal quantities and made up to 10 mM $CaCl_2$.

R2YE

10.3 % w/v sucrose, 0.025 % w/v $K_2SO_4$, 1.012 % w/v $MgCl_2.6H2O$, 1% w/v glucose, 0.01 % w/v casamino acids solidified with 2.2 % agar-agar and autoclaved (all final concentations after addition of supplements). At time of use the media was remelted and supplemented with 1 ml $KH_2PO_4$ (0.5 % w/v), 8 ml $CaCl_2.H_20$ (3.68 % w/v), 1.5 ml L-proline (20 % w/v), 10 ml TES (5.73 % w/v pH7.2), 0.2 ml trace element solution, 0.5 ml NaOH (1 M), 5 ml yeast extract (10 % w/v).

Make up to 1L SQ

Tryptic soy broth

3 % w/v Tryptic soy broth.

YEME

0.5 % w/v peptone, 0.3 % w/v yeast extract, 0.3 % w/v malt extract, 1 % w/v glucose, 34 % w/v sucrose, 0.1 % w/v $MgCl_2$, 0.5 % w/v glycine.

# CHAPTER 3

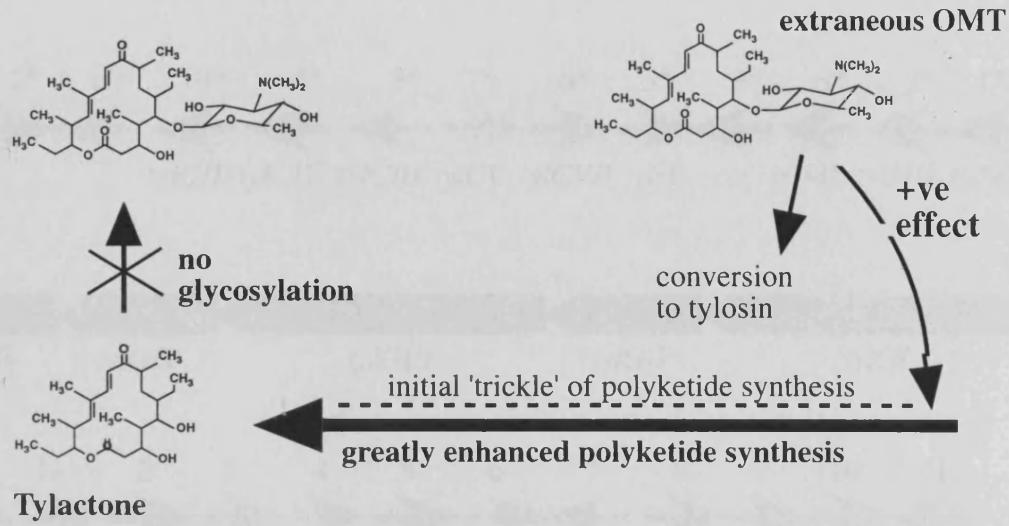
# CONTROL OF POLYKETIDE SYNTHESIS VIA MYCAMINOSE METABOLISM IN *S. FRADIAE*

## 3.1 Introduction

Analysis of the fermentation products of *tylMII*-KO (disrupted in the mycaminosyltransferase, *tylMII* and formerly known as SF01) by reverse phase HPLC had previously revealed that the strain accumulated no tylosin intermediates in any significant detectable quantities. A possible trace of tylactone only, was reported when analysis was carried out at high sensitivity (Fish and Cundliffe, 1997). This inability of *tylMII*-KO, deficient in the obligatory first step in the glycosylation of the polyketide, to produce the polyketide itself, was unexpected. It certainly did not accumulate this precursor in anything remotely approaching the expected amounts. However, when fermentations of *tylMII*-KO were fed with the glycosylated tylosin-intermediates OMT (5-*O*-mycaminosyltylonolide), DMT (demycinosyltylosin), desmycosin (demycarosyltylosin) and tylosin itself, tylactone did accumulate. It was therefore speculated that in wild type strains the products of tylactone glycosylation exert strong, positive feedback on polyketide synthesis. This leads to the phenomenon observed in *tylMII*-KO in which the obligatory first glycosylation function (mycaminose addition) has been abolished and thus no glycosylated compounds can be made. Presumably in this case low levels of polyketide synthase (*tylG*) expression, leading to a very low initial background level of tylactone can be stimulated many-fold by feeding with the glycosylated polyketide. This causes the accumulation of large quantities of tylactone which cannot be converted any further in this mycaminosyltransferase-deficient strain (Figure 3.1).

This speculation was complicated by the possibility that the disruption in *tylMII*-KO might have had downstream polar effects on *ccr* (Figure 3.1), a gene thought to be involved in the supply of precursors for polyketide synthesis (section 1.3.6). Any downstream consequences on *ccr* might influence polyketide synthesis, although it is difficult to rationalise the observed 'tylactone-stimulation' phenomenon (whereby the mycaminosyltransferase-deficient strain has a non-producing phenotype unless tylactone accumulation is stimulated by the addition of glycosylated intermediates of tylosin biosynthesis) with a downstream effect on *ccr*. This issue can now be fully resolved, primarily by disruption of the *ccr* gene. Additional evidence was supplied by analysis of an *S. fradiae* strain, GS50 (Baltz and Seno, 1981) which contains a mutation in *tylB*, an aminotransferase gene involved in mycaminose synthesis (section 1.3.7) (Merson-Davies and Cundliffe, 1994). This gene is located approximately 45 kb distant from the *tylM* region, on the other side of the polyketide synthase domain *tylG* (Figure 3.2). The mutation in *tylB* in strain GS50 is a point mutation leading to a in-frame stop codon, truncating the protein (262 residues as opposed to 388 in the wild type) (section 1.3.7) (Merson-Davies and Cundliffe, 1994).

Is the phenotype of *tylMII*-KO (SF01) due to a positve feedback loop regulating polyketide synthase expression being broken ?




Or a downstream effect on a gene essential for polyketide synthesis ?

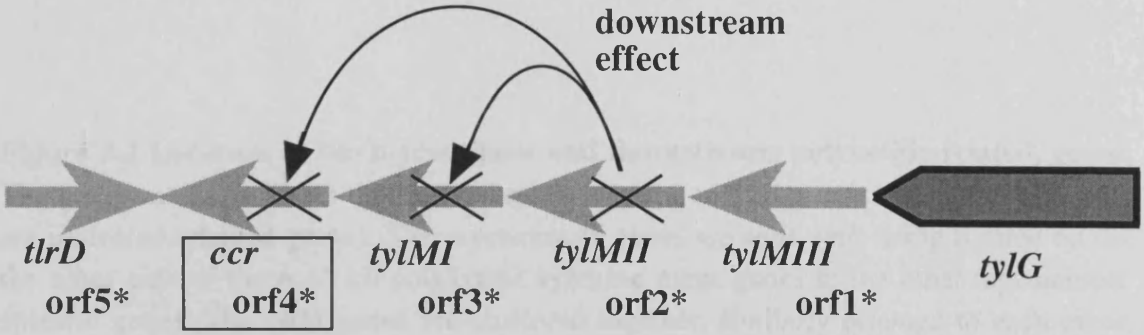



**Figure 3.1 Explaining the phenotype of *tylMII*-KO (SF01).** Strain *tylMII*-KO is disrupted in the glycosyltransferase that adds the first sugar, mycaminose, to tylactone. This is the essential first step in the conversion of tylactone to tylosin. The unexpected phenotype of the *tylMII*-KO is most likely due to a positive feedback loop being broken. Glycosylated polyketide causes a great enhancement of polyketide synthesis from an initial 'trickle'. The ability of *tylMII*-KO to perform the essential first glycosylation has been abolished. Thus tylactone only accumulates when stimulated by exogenous glycosylated tylosin-precursor. Alternatively it is possible that the disruption in *tylMII* might adversely affect the expression of downstream genes, in particular the gene *ccr*, proposed to be involved in the supply of polyketide precursors.

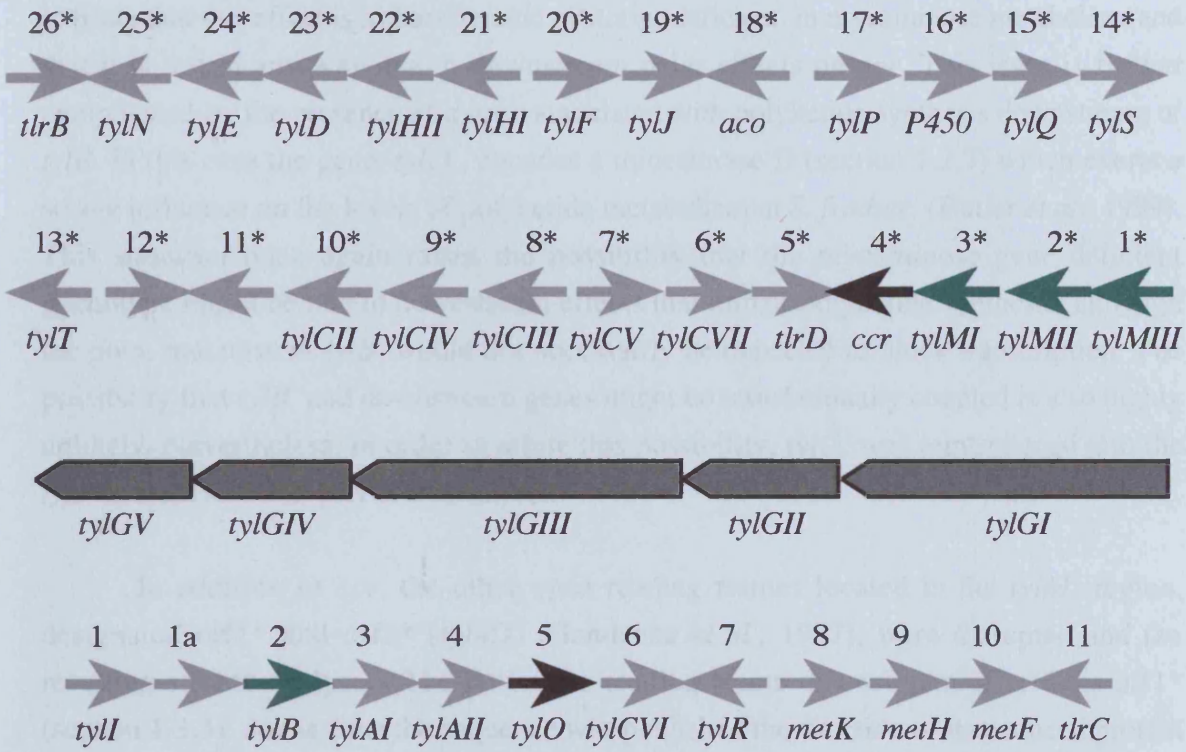



**Figure 3.2 Location of the mycaminose and downstream, polyketide-related, genes.** The genes associated with the biosynthesis and addition of the amino sugar mycaminose are indicated (shaded green). The mycaminose genes are split, *tylB* being located on the the other side of the ≅ 45 kb polyketide synthase mega-genes to the other mycaminose specific genes. The *tylM* genes are clustered together, similarly oriented to each other, and to the polyketide synthase genes (*tylG*) immediately upstream. In each case, downstream of the mycaminose specific genes, there lies a gene associated with polyketide synthesis (shaded black). Thus *ccr* (thought to be involved in the supply of precursors for polyketide synthesis) lies immediately downstream of the *tylM* genes and *tylO* (an editing thioesterase important in efficient polyketide synthesis) lies downstream of *tylB*.

A repetition of the 'tylactone stimulation' phenomenon in the *tylB* mutant should indicate that this effect is a characteristic of strains deficient in mycaminose metabolism and that it is not in any way due to downstream polar effects on *ccr*. This issue is further complicated by the presence of a gene associated with polyketide synthesis downstream of *tylB*. In this case the gene, *tylO* , encodes a thioesterase II (section 1.2.7) which exerts a strong influence on the levels of polyketide metabolism in *S. fradiae* (Butler *et al.*, 1999). This situation once again raises the possibility that the mycaminose gene deficient phenotype might be due to downstream effects that impair polyketide synthesis, although the point mutation in *tylB* would not necessarily be expected to block transcription. The possibility that *tylB* and downstream genes might be translationally coupled is also highly unlikely. Nevertheless, in order to refute this possibility, *tylO* was reintroduced into the *tylB* mutant strain as part of its analysis.

In addition to *ccr*, the other open reading frames located in the *tylM* region, designated orf1* and orf3* (*tylMI*) (Gandecha *et al.*, 1997), were disrupted and the resulting strains analysed. The first open reading frame downstream of *tylG*, is orf1* (section 1.3.3). At the time the sequence was published the function of its deduced protein product remained unclear, although its sequence similarity at the deduced protein level to the proposed product of *dnrQ* (35% identity) from the daunomycin biosynthetic cluster of *S. peucetius* (Otten *et al.*, 1995) and in particular to EryCII (*ery* orf9) (38% identity) from the erythromycin biosynthetic cluster of *Saccharopolyspora erythraea* was noted. It was proposed that the product of this gene catalysed the isomerisation of dTDP-4-keto-6-deoxyglucose to dTDP-3-keto-6-deoxyglucose in the synthesis of desosamine and suggested that Orf1* and DnrQ might perform analogous roles in the biosynthesis of mycaminose (Figure 3.3) and desosamine respectively (Salah-Bey *et al.*, 1998). In order to determine whether orf1* is in fact a mycaminose specific gene, a strain disrupted in orf1* was generated (A. R. Butler personal communication) and analysed.

A strain disrupted in the *tylMI* (orf3*) gene, located immediately downstream of the mycaminosyl transferase, was also generated. This gene, *tylMI* , had originally been characterised as a mycaminose specific gene on the basis of its ability to complement the *tylM* mutant strain GS62 (Gandecha *et al.*, 1997). Sequence comparisons and the ability of an Orf3*-maltose binding protein fusion to bind *S*-adenosyl methionine provided the evidence that this gene encoded the *N*-methyltransferase responsible for one or both of the methylations on the amino group of dTDP-3-amino, 6-deoxyglucose during mycaminose biosynthesis (Gandecha *et al.*, 1997) (Figure 3.3). Analysis of the *tylMI* disrupted strain, *tylMI*-KO provided further evidence of the mycaminose specific nature of the gene.

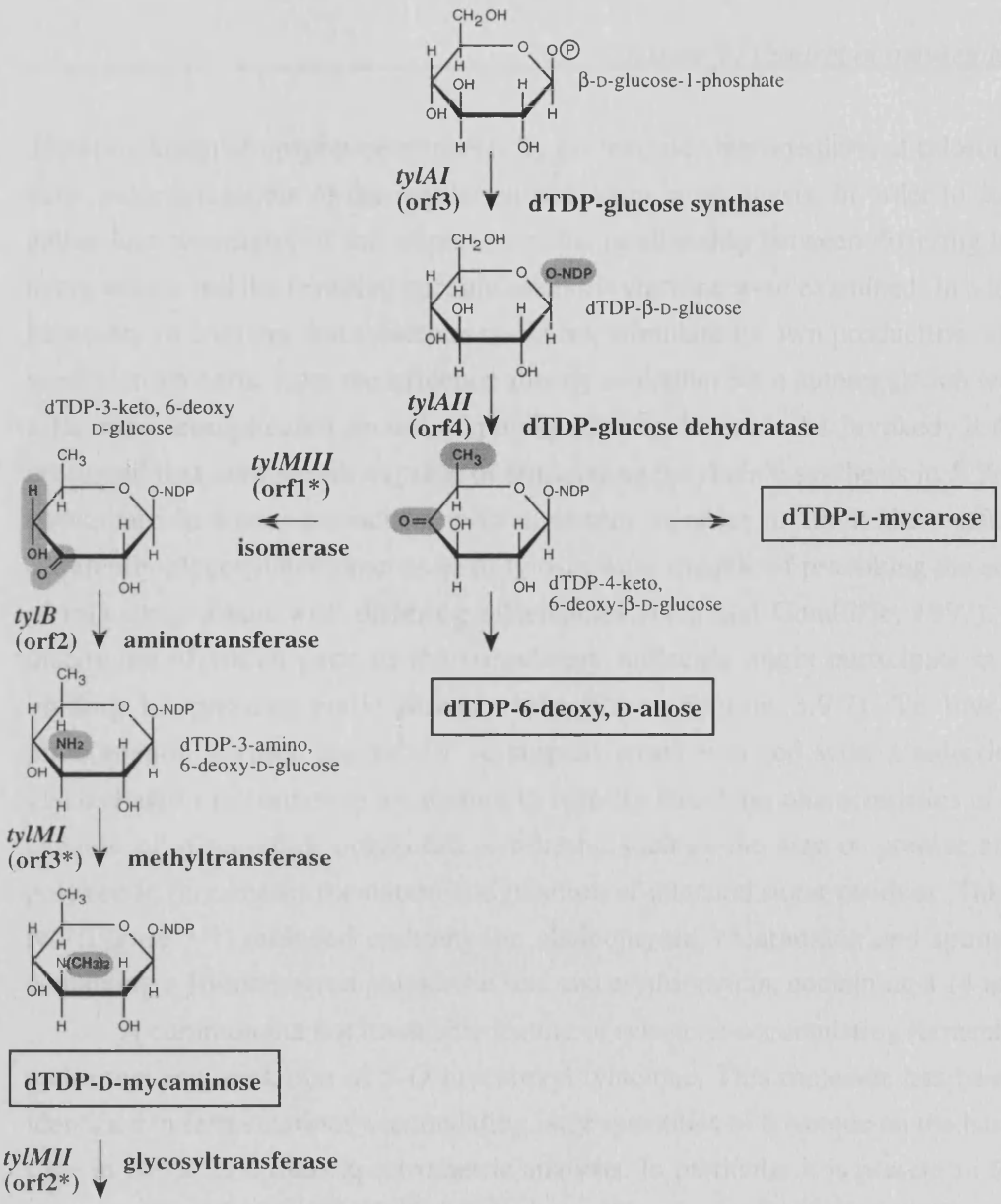



**Figure 3.3 The biosynthetic route to mycaminose.** The biosynthesis of mycaminose from β-D-glucose-1-phosphate, involves the action of dTDP-glucose synthase and dTDP-glucose dehydratase functions encoded by *tylAI* and *tylAII* respectively. These steps are common to the synthesis of all three tylosin sugars. The last common intermediate, 4-keto, 6-deoxy-D-glucose is then converted to mycaminose by the action of a 3, 4 - isomerase (*tylMIII*), an aminotransferase (*tylB*), and a *N*-methyltransferase (*tylMI*). The modification catalysed by each enzymatic function has been indicated in each case (shaded). The glycosyltransferase responsible for the addition of mycaminose to tylactone is encoded by *tylMII*.

The stimulation of polyketide synthesis by glycosylated intermediates of tylosin is clearly a very important aspect of the regulation of tylosin biosynthesis. In order to determine the nature and sensitivity of this stimulation, the relationship between differing levels of fed intermediate and the resulting accumulation of tylactone were examined. In addition it was necessary to confirm that tylactone could not stimulate its own production, although this seemed improbable from the evidence already available. Such autoregulation would require a far more complicated model of polyketide regulation to be invoked. It can only be presumed that compounds capable of stimulating polyketide synthesis in *S. fradiae* must participate in some interaction with a protein in order to illicit their effect. Various differently glycosylated precursors of tylosin were capable of provoking the accumulation of tylactone, albeit with differing efficiencies (Fish and Cundliffe, 1997). Thus some discussion of which parts of the stimulatory molecule might participate in the protein binding in question could already take place (Section 3.9.7). To investigate this phenomenon further the *tylMII* disrupted strain was fed with a selection of other glycosylated macrolides in an attempt to identify structural characteristics of compounds capable of stimulating polyketide synthesis, such as the size or precise nature of the polyketide ring and/or the nature and position of attached sugar residues. The macrolides fed (Figure 3.4) included carbomycin, chalcomycin, rosaramicin and spiramycin, each containing a 16-membered polyketide ring and erythromycin, containing a 14-atom ring.

A common but not invariable feature of tylactone-accumulating fermentations is the additional accumulation of 5-*O*-mycarosyl tylactone. This molecule has been positively identified in fermentations accumulating large quantities of tylactone on the basis of elution time in HPLC and mass spectrometric analysis. In particular it is present in fermentation extracts of *tylMI*-KO fed OMT (3.7.4), the *tylM* mutant (GS62) fed OMT (section 5.3.2) and in similarly supplemented fermentations of the *tylI/tylD* mutant GS77 in which *tylMI* has been additionally disrupted (GS77:*tylMI*-KO) (Chapter 6). The presence of 5-*O*-mycarosyltylactone in such fermentations is odd for a number of reasons. It has no apparent role in the biosynthesis of tylosin *per se*. The addition of mycarose obviously prevents subsequent conversion to tylosin. If 5-*O*-mycarosyltylactone is not simply a spuriously produced molecule (perhaps incorrectly glycosylated due to the vast excess of tylactone accumulated) then it seems possible that it may play a role as a regulatory molecule. Perhaps the most obvious implication of the discovery of this compound in tylosin fermentations is the possibility that mycarosyltylactone might, like other glycosylated tylosin-metabolites, stimulate tylactone accumulation. When strains deficient in mycaminose metabolism were fed with OMT, or other glycosylated tylosin-precursors more advanced along the biosynthetic route, accumulated tylactone is highly unlikely to be derived from the extraneous compound. Feeding with 5-*O*-mycarosyltylactone poses a

slightly different problem. Although the polyketide ring has been glycosylated, it has not undergone any of the ring hydroxylations at C20 and C23 which convert the ring from tylactone to tylonolide. The re-formation of tylactone from 5-*O*-mycarosyltylactone would therefore be relatively easy and it was necessary to demonstrate that any accumulated tylactone was not derived from the fed compound.

Before these issues are addressed, analysis of standard compounds by HPLC and mass spectrometry will be presented. Following this, data gathered from the analysis by reverse phase HPLC of wild type tylosin-accumulating fermentations will be shown.

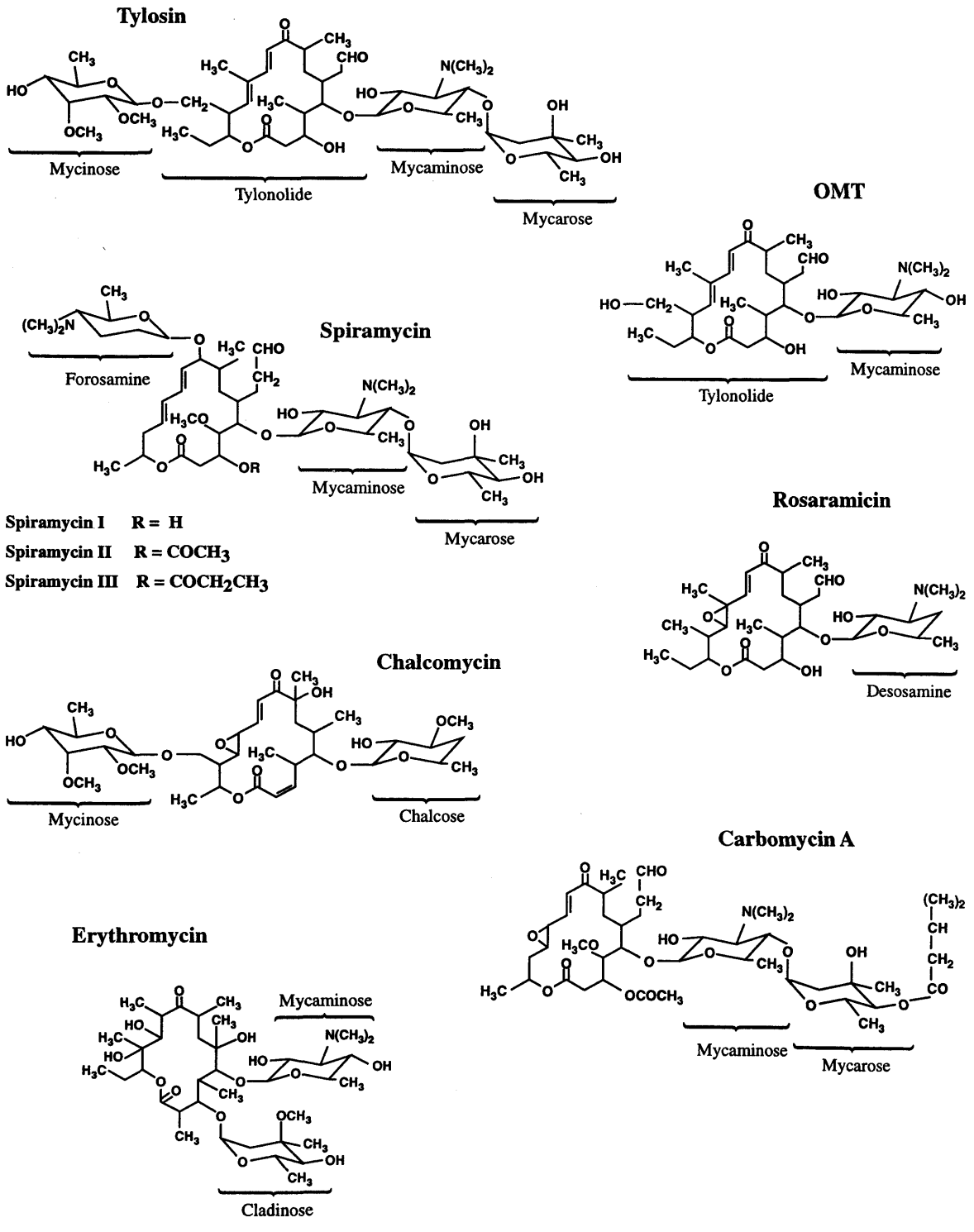



**Figure 3.4 Structures of the macrolides used to feed *tylMII*-KO.** All the macrolides used to feed *tylMII*-KO have a 16 membered polyketide ring (except erythromycin, which is 14-membered) with attached sugar moieties. The spiramycin used to feed was probably a mixture of the three forms shown in unknown proportions (see section 3.8.5).

## 3.2 Analysis of standard compounds

### 3.2.1 Analysis by HPLC

A range of purified tylosin-metabolites (kindly supplied by Eli Lilly and Co.) was available for use as standard compounds and for *S. fradiae* strain feeding experiments. The HPLC system applied here (section 2.9.3) achieved excellent separation between the compounds commonly identified in this report (Figure 3.5). The nature of DMT (demycinosyltylosin) and desmycosin will be described in detail later (Chapter 6). The absorbance of each compound at 282 nm was excellent. The retention time of the various compounds relative to tylosin were OMT (rrt 0.5), DMT (rrt 0.6), desmycosin (rrt 0.8), tylactone (rrt 1.4), 5-*O*-mycarosyltylactone (1.7).

### 3.2.2 Analysis by mass spectrometry

Standard compounds OMT, desmycosin, tylosin, tylactone and 5-*O*-mycarosyltylactone were also analysed by mass spectrometry (Figure 3.6a, b and c). The predicted *m/z* value for tylosin was 916 ($[915 + H]^+$). When analysed, the *m/z* value assigned to tylosin was 916.5 (Figure 3.6a). This slight deviation from the precise *m/z* value expected (916) illustrates the slight inaccuracy inherent in the system used. This could lead to peaks being assigned *m/z* values one unit higher or lower than the 'real' value. Thus, especially for compounds for which standards are not available, some flexibility of interpretation is required. The mass spectral data for OMT (Figure 3.6a) and desmycosin (Figure 3.6b) each displayed peaks at the expected *m/z* values (598 and 772 respectively). Analysis of tylactone and 5-*O*-mycarosyltylactone appeared more complicated (Figure 3.6c). In each case the major peak had the expected *m/z* value (tylactone, 395 and 5-O-mycarosyltylactone, 539). Other peaks were also of significant size, some m/z value 257, 618/619 appearing in both traces. Peaks with *m/z* values of 377, 474, 790 (twice 395) and 811 appear to be characteristic of tylactone mass spectral data and will be referred to elsewhere. Some trace of tylactone (*m/z* 395) was also evident in the 5-*O*-mycarosyl tylactone standard.

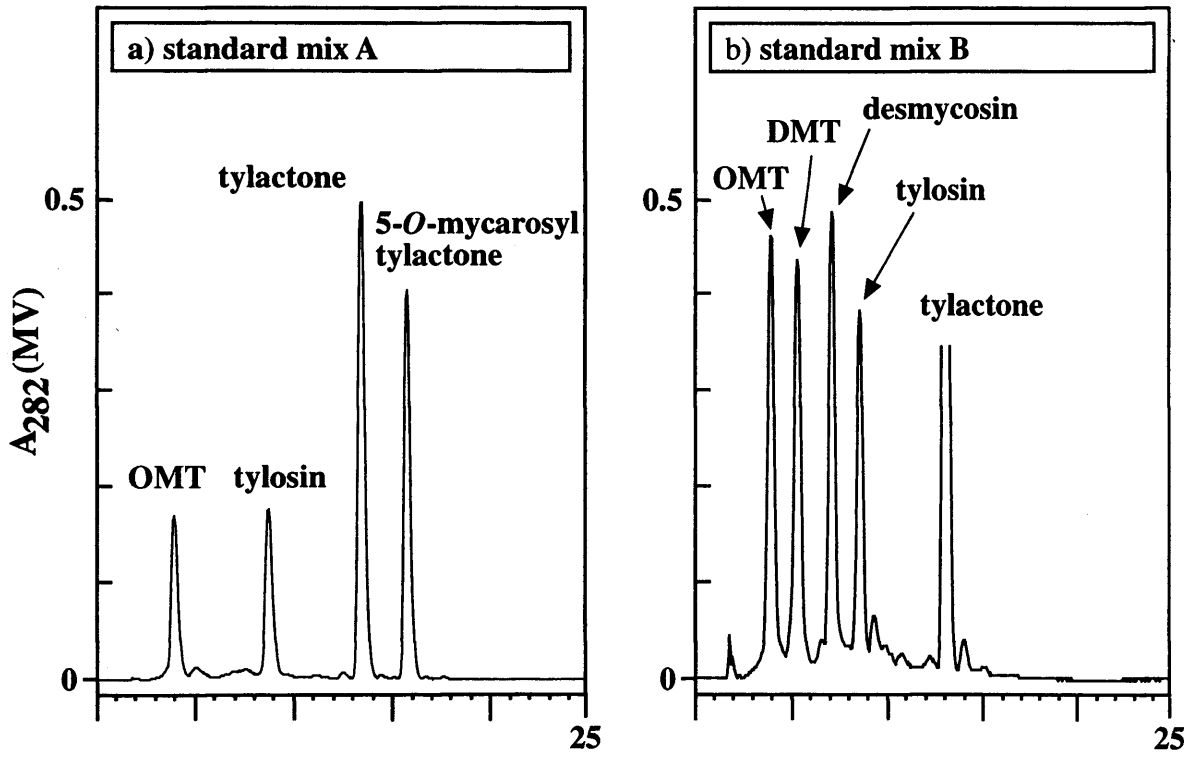



**Figure 3.5 HPLC analysis of standard compounds.** Standard compounds OMT, tylosin, tylactone and 5-*O*-mycarosyltylactone (standard mix A) and OMT, DMT, desmycosin, tylosin and tylactone (standard mix B) as visualised by HPLC at 282 nm. The separation between the compounds used is good facilitating the identification of tylosin metabolites in fermentation extracts.

As indicated, the scale used here is 0 - 0.5 V.

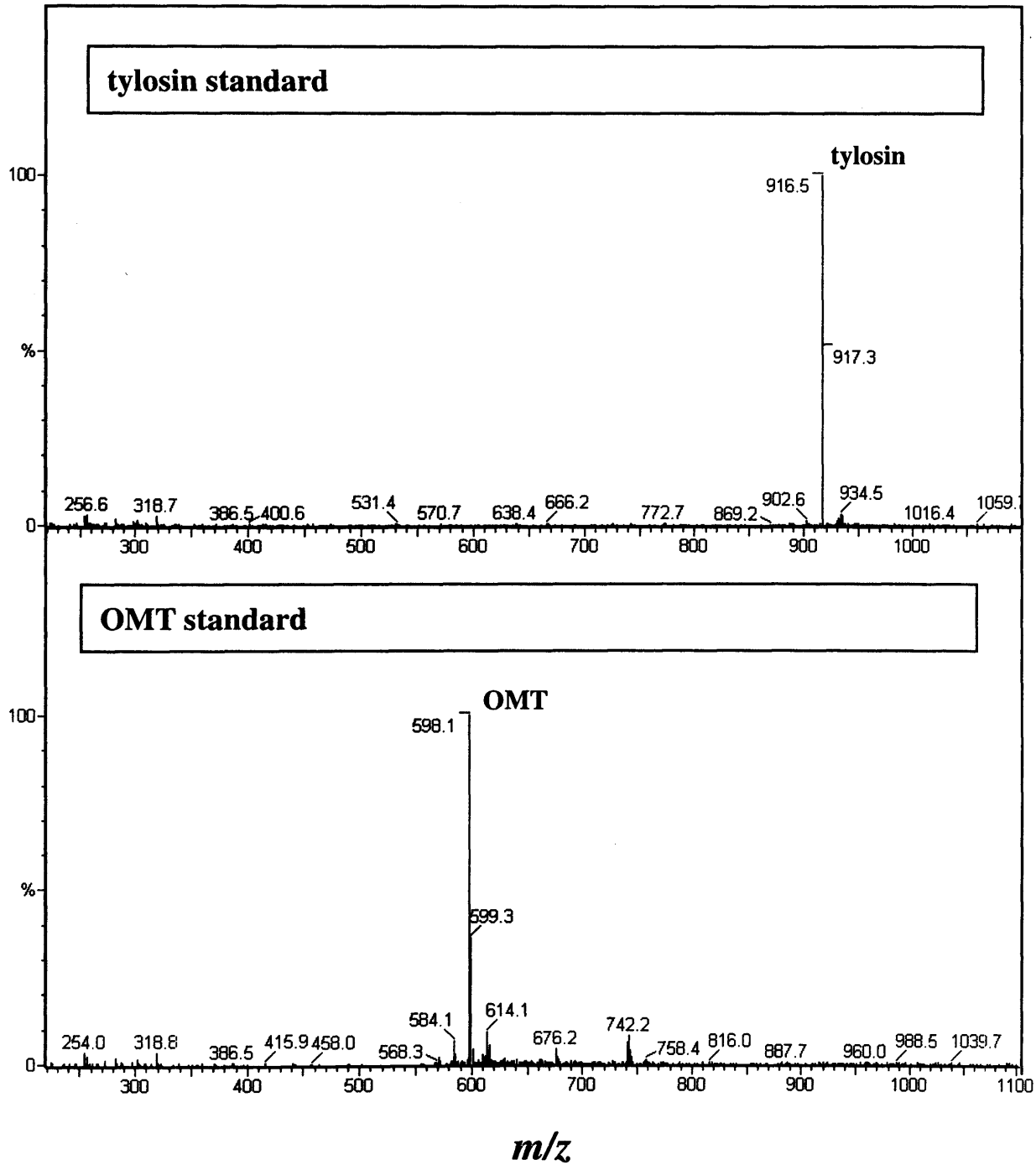



**Figure 3.6 (a) Analysis of tylosin and OMT standards by mass spectrometry.** Analysis of tylosin (predicted *m/z* 916) and OMT (predicted *m/z* 598) standard compounds in each case yielded a single major species with the expected *m/z* value.

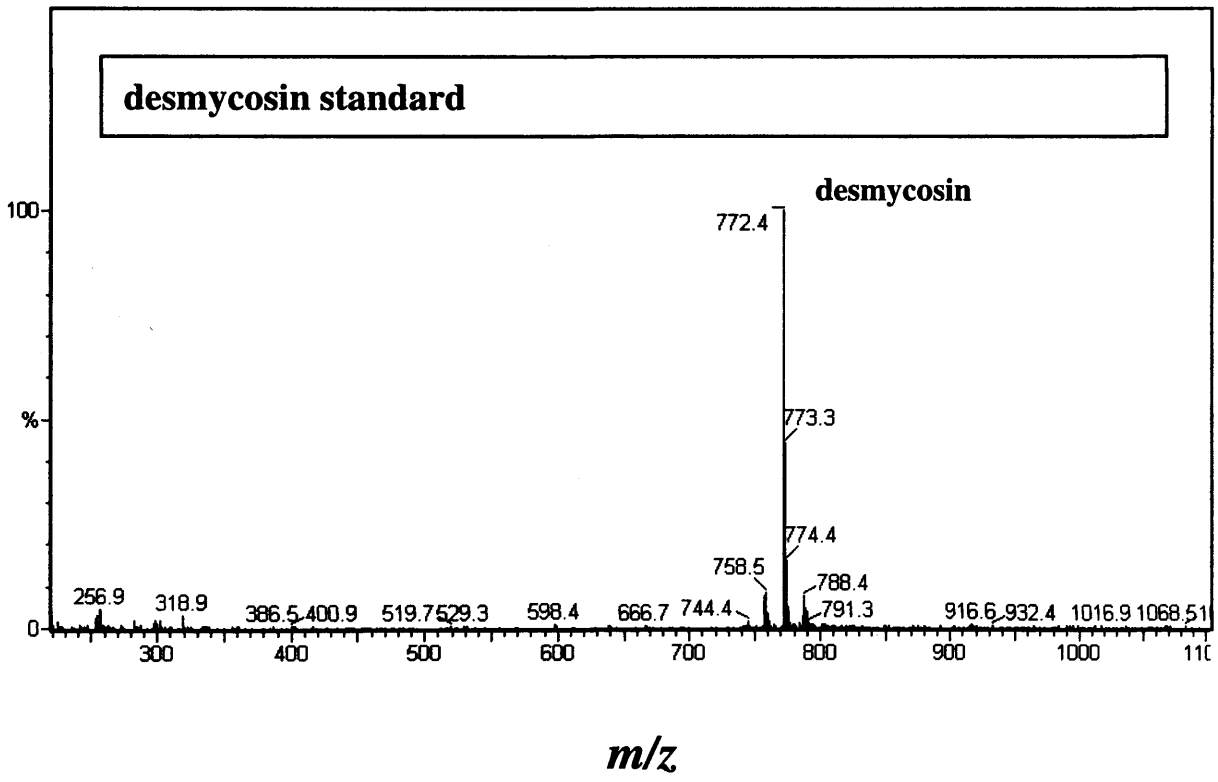



**Figure 3.6 (b) Analysis of the desmycosin standard by mass spectrometry.** As predicted, the desmycosin standard contained a single major species with an *m/z* value of 772.

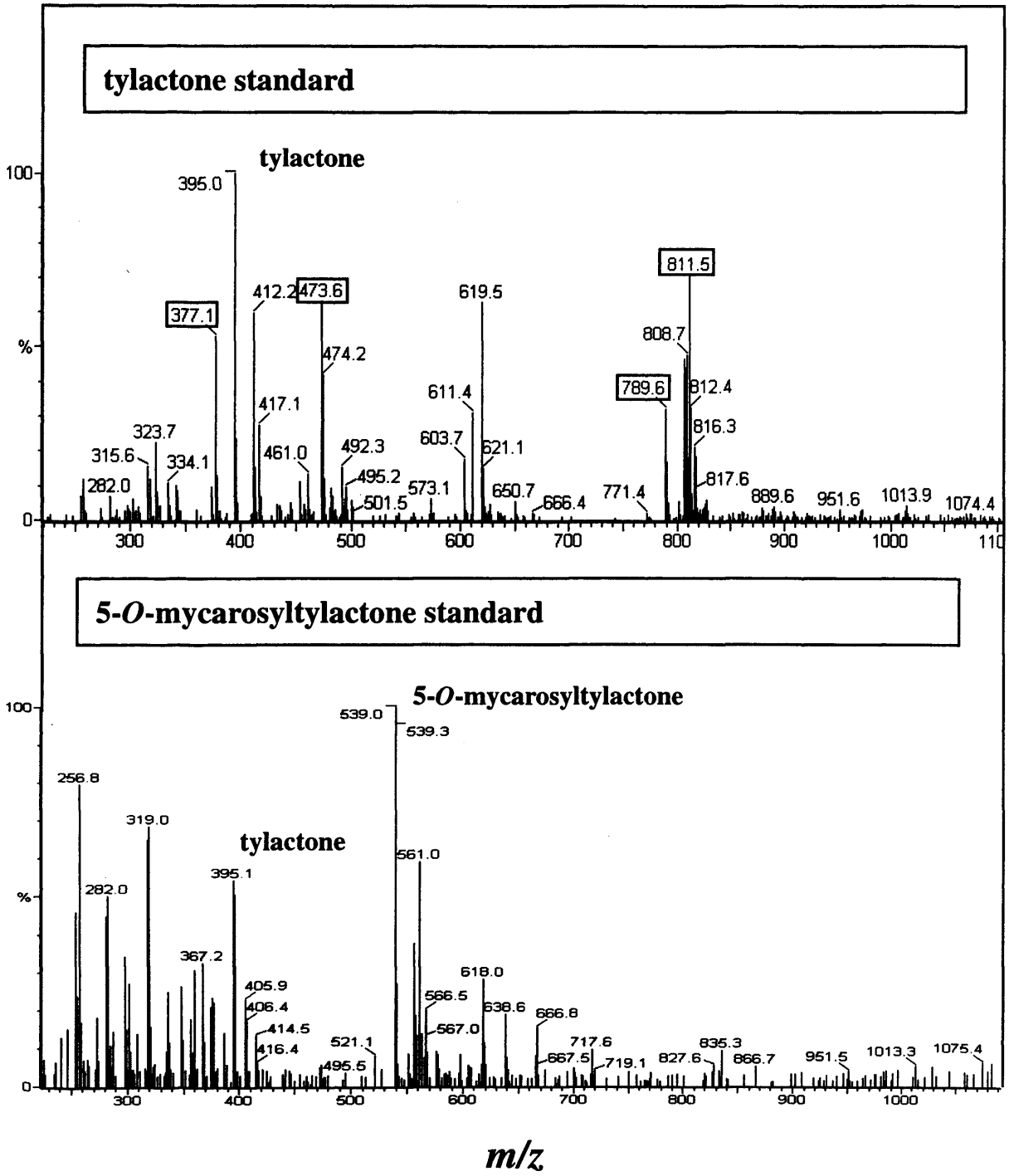



**Figure 3.6 (c) Analysis of tylactone and 5-*O*-mycarosyltylactone standards.** Both the tylactone (predicted m/z 395) and 5-*O*-mycarosyltylactone (predicted *m/z* 539) standards revealed complicated mass spectra. Tylactone (*m/z* 395) was accompanied by peaks with *m/z* values of 377, 474, 790 and 811 (boxed). These peaks are present whenever tylactone accumulates in fermentation extracts. The 5-*O*-mycarosyltylactone standard (*m/z* 539) also appears to contain some tylactone.

## 3.3 HPLC analysis of the products of *S. fradiae* strains

All the targeted gene-disruptions discussed here were made in the tylosin-producing strain, C373.1 which will be referred to as 'wild type'. The mutant strains studied were however created from a descendant of this strain, C373.10. This strain was derived from C373.1 but altered by multiple rounds of random mutagenesis followed by selection for improved tylosin production characteristics. It is important to bear in mind that the total tylosin biosynthetic potential in the mutant strains and their derivatives is therefore far greater than that of the disrupted strains and their derivatives. HPLC data will be presented using different scales on the y-axis, representing UV absorbance at 282 nm as shown in Figure 3.7. The scale used in each case will be clearly shown and, in most cases, will be the same for each for each particular figure representing the data for each experiment.

### 3.3.1 HPLC analysis of un-inoculated media

Un-inoculated media was extracted and analysed by HPLC as normal (section 2.9). The results revealed that when viewed at the same scale usually applied to tylosin fermentations no material absorbing at 282 nm was evident (Figure 3.7). When viewed on a greatly expanded scale however, peaks of UV absorbing material are present that could be mistaken for tylosin intermediates. This illustrates the danger of examining HPLC data at this level, and while very small quantites of tylosin-related material might well be present and might be of some significance in *S. fradiae* fermentations, HPLC analysis alone is clearly not the best way of identifying such compounds.

### 3.3.2 Analysis of the wild type and other tylosin producing strains

Reverse phase HPLC analysis of the wild type strain C373.1 following fermentation and extraction (section 2.9), produces a characteristic chromatogram (Figure 3.7). Always apparent are a group of initial peaks that elute between 1.5 and 3.5 min, closely following the solvent front. These peaks are extremely small and since they appear in all fermentation extracts, they have not been analysed further. The major peak in a wild type fermentation is tylosin. This typically elutes at approximately 8 min depending on slight fluctuations in the precise conditions of the HPLC system (temperature variations, minor differences in buffer composition). The elution time of any given component is, as would be expected, very consistent within each batch of consecutively run samples.

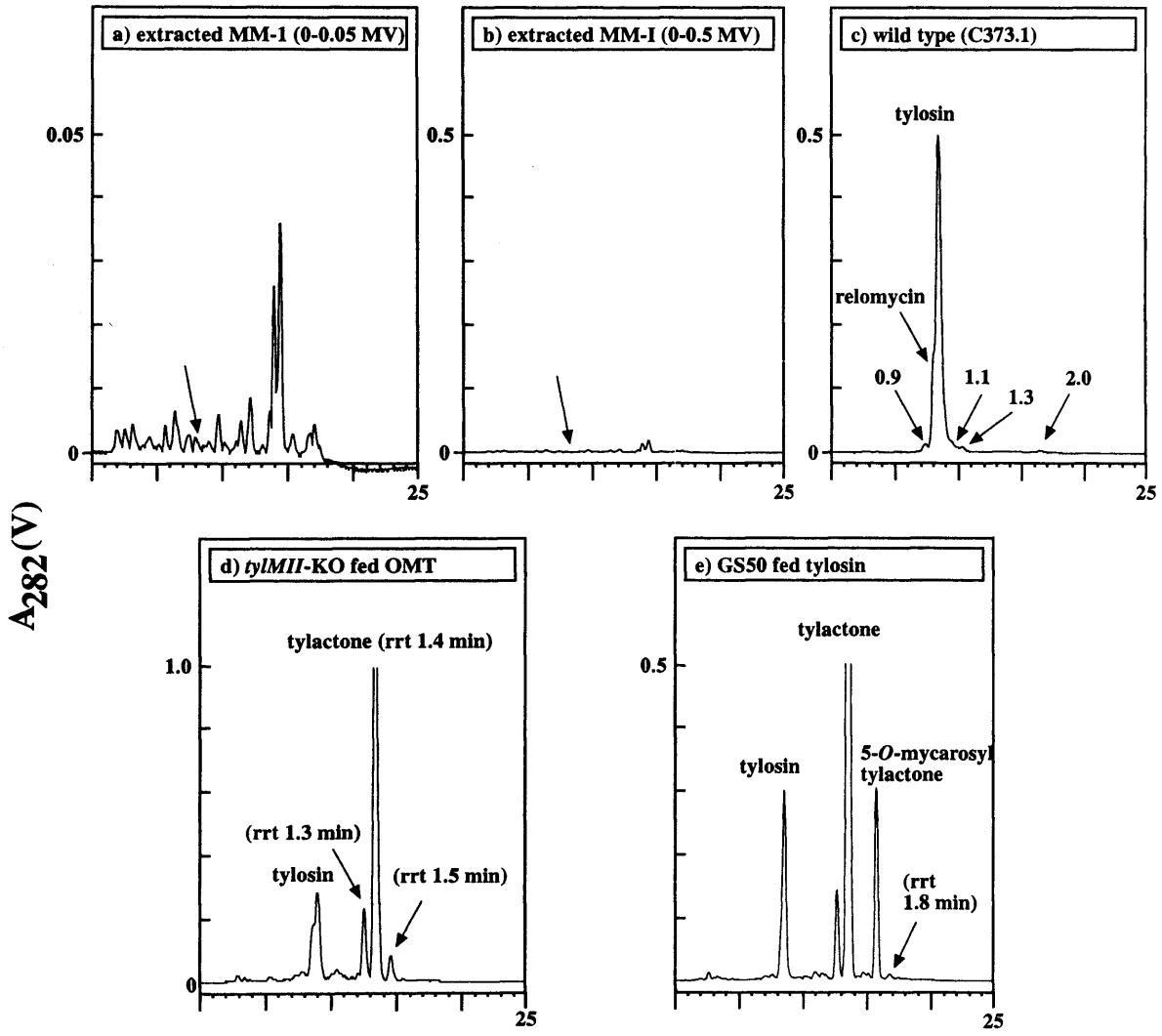



**Figure 3.7 HPLC analysis of *S. fradiae* strains.** Extracted fermentation media (MM-1) is shown on two scales, 0 - 0.05 V, (10% of that used to display normal wild type fermetentation products) and 0 - 0.5 V (that usually applied to wild type fermentation products) and the similarly extracted fermentation products of (c) the wild type tylosin-producer and two fermentations in which tylactone accumulates (d) *tylMII*-KO fed OMT and (e) the *tylB* mutant GS50 fed tylosin, as visualised by HPLC at 282 nm.

The scales used on individual traces has been indicated.

Material visualised by HPLC analysis was identified on the basis of relative retention time with respect to tylosin (rrt) (section 2.9.3) The tylosin peak is always preceded by a far smaller peak (rrt 0.9). This compound of unknown origin can be up to 2 % by area of the tylosin peak.

Although the HPLC conditions used are excellent for separating the majority of commonly seen tylosin precursors, they have limitations when separating tylosin from relomycin. Relomycin is a reduced form of tylosin (20-dihydrotylosin) to which tylosin is slowly converted during fermentation (Seno *et al.*, 1977). It has less potent antimicrobial properties than tylosin and is an unwanted metabolite in production strains. Relomycin elutes slightly prior to tylosin but separation in this system is so poor that it is usually only seen as a leading edge on the tylosin peak. Nevertheless, it clearly constitutes a significant proportion of the absorbant material that elutes at this point. However, since the only biosynthetic route to relomycin is through tylosin and their relative absorbancies at the wavelength used (282 nm) are similar, the peak is usually integrated and considered as if it were tylosin alone.

The tylosin peak invariably trails slightly and usually two peaks can be identified in its wake. The first of these, (rrt 1.1) is almost always distinguishable and is usually a little less than 10% of the area of the tylosin peak. A second peak which is not always obvious has a rrt of 1.3 and is present in much smaller quantities, usually amounting to approximately 1% of the area of the tylosin peak. These peaks eluting within, and thus partially forming, the trailing edge of the tylosin peak are difficult to integrate as single components and thus their relative sizes may be slightly exaggerated.

As well as being present in wild type fermentations, these flanking peaks are always associated with fermentations that produce tylosin in identifiable quantities and it seems most likely that they are closely related metabolites of tylosin (Chapter 6). In addition to these peaks, a far smaller peak can be identified eluting with a relative retention time of 2.0 min. This peak is usually less than 1% by area of the size of the tylosin peak. This can be seen in almost all the fermentations analysed and is therefore not considered to be an important factor in the interpretation of the data presented. Its nature is unknown but no intermediate of tylosin is known to elute this late. No convincing trace of tylactone or any of the glycosylated precursors of tylosin has yet been found in a wild type fermentation under the conditions applied here, indicating that the conversion of tylactone to tylosin must be a very tightly regulated process.

### 3.3.3 Analysis of strains producing tylactone

Tylactone is frequently identified in the fermentation extracts of genetically altered *S. fradiae* strains. It should be noted that tylactone-containing fermentation extracts contain certain other materials that also absorb at 282 nm. Two typical examples of this are shown here. Like tylosin, tylactone is also invariably accompanied by two flanking peaks that do not co-elute with any known intermediates of tylosin biosynthesis (Figure 3.7). Tylactone has a relative retention time of 1.4 with respect to tylosin. A peak that elutes immediately prior to tylactone (rrt 1.3) is always obvious in chromatograms of tylactone-accumulating strains and has an area of 10-25% that of tylactone. The second peak, that elutes immediately after tylactone (rrt 1.5) is of more variable size. In some tylactone accumulating fermentations it is only just visible, whereas in others it can be as large as the peak that immediately precedes tylactone. These two peaks were the subject of some investigation and will be discussed in more detail in Chapter 6.

Another peak often but not always associated with tylactone accumulation is the tylosin-related metabolite 5-*O*-mycarosyltylactone (rrt 1.7). This metabolite is not an intermediate of tylosin production and its significance, if any, is unknown. The properties and origin of this interesting molecule will be investigated later (section 3.8). A further peak of material of unknown origin is asscociated with mycarosyltylactone accumulation and elutes immediately after it (rrt 1.8). Again this will be discussed later (Chapter 6).

## 3.4 Construction of vectors for the targeted disruption of genes in the *tylM* region

Vectors suitable for the targeted disruption of orf1*, *ccr* (orf4*) (A. R. Butler, personal communication) and *tylMI* (orf3*) were constructed as follows. Fragments of cloned *tyl* DNA each containing a roughly central, unique restriction site, suitably positioned to facilitate interruption of a particular open reading frame, were ligated into pIJ2925 (Janssen and Bibb, 1993). After insertion of the hygromycin resistance cassette Ωhyg (Blondelet-Rouault *et al.*, 1997), together with its flanking transcriptional / translational terminators into this site, the disruption construct was excised using the *Bgl*II sites which flank the pIJ2925 polylinker and ligated into the *Bam*HI site of pOJ260 (Bierman *et al.*, 1992) ready for conjugal transfer into *S. fradiae*. The cloning of DNA into pIJ2925 to create the disruption construct ready for subcloning into pOJ260 will be described in each case. Construction of the vector previously used to create *tylMII*-KO (SF01) used a different approach (Fish and Cundliffe, 1997).

### 3.4.1 Construction of a vector for the disruption of orf1*

A 2.11 kb *Age*I fragment was subcloned from pLST973 into pIJ2925 (A. R. Butler, personal communication) (Figure 3.8). This fragment contained a slightly off-centre *Mlu*I site suitable for the insertion of the 2.3 kb hygromycin resistance cassette, Ωhyg (Blondelet-Rouault *et al.*, 1997). This would disrupt in the early part of orf1*, 286 bp downstream of the proposed translational start and 986 bp upstream of the translational stop (interruping the deduced product 95 amino acids into its proposed 423 amino acid total length).

### 3.4.2 Disruption in *tylMII*

A strain disrupted in *tylMII* (orf2*) was already available for analysis (Fish and Cundliffe, 1997). This was named SF01 but will be referred to here as *tylMII*-KO for clarity. The gene was disrupted 530 bp downstream of the proposed translational start and 829 bp upstream of the translational stop (interruping the deduced product 176 amino acids into its proposed 452 amino acid total length).

### 3.4.3 Construction of a vector for the disruption of *tylMI* (orf3*)

A 1.5 kb *Hin*cII fragment was excised from pLST97A and ligated into the similarly digested vector pIJ2925 (Figure 3.8). Digestion with *Msc*I provided a suitable, blunt ended disruption site 250 bp downstream of the proposed translational start and 514 bp upstream of the translational stop into which the *Bam*HI digested, end-filled hygromycin resistance cassette was inserted (interruping the deduced product 84 amino acids into its proposed 254 amino acid total length).

### 3.4.4 Construction of a vector for the disruption of *ccr* (orf4*)

A 1.9 kb *Sty*I fragment was isolated from pLST97A and ligated into pIJ2925 (Figure 3.8) (A. R. Butler, personal communication).. An almost central *Bsa*AI site provided blunt ends into which the *Bam*HI digested, end filled hygromycin resistance cassette was inserted, interrupting *ccr* 167 bp downstream of the proposed translational start and 1104 bp upstream of the translational stop (interruping the deduced product 56 amino acids into its proposed 423 amino acid total length).

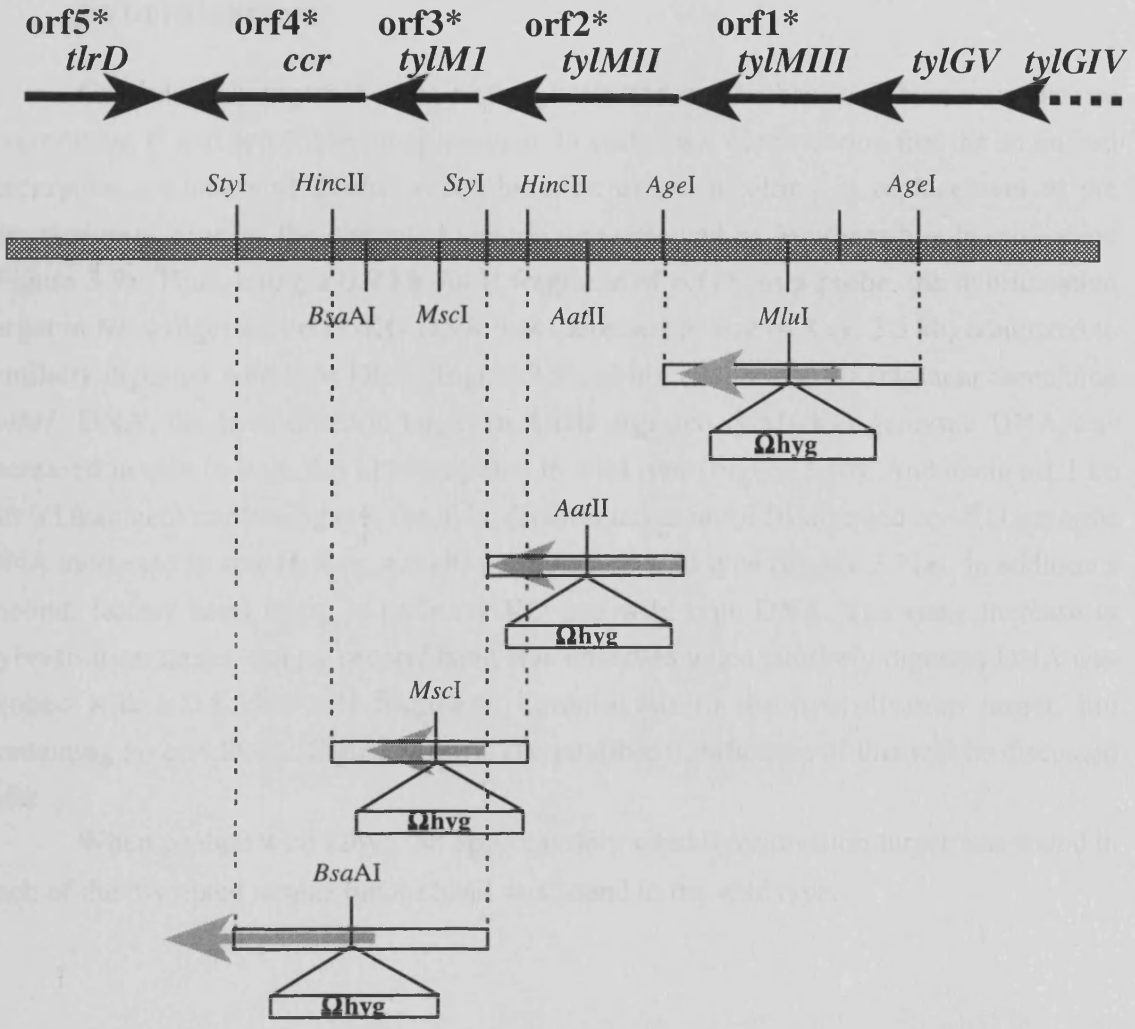



**Figure 3.8 Disruptions in the *tylM* genes and *ccr*.** The hygromycin cassette was inserted into cloned fragments of DNA in order to disrupt specific genes. Fragments of DNA of suitable size containing roughly central restriction sites appropiately positioned for gene disruptions were cloned. *tylMIII* (orf1*) and *ccr* (orf4*) (Dr A. R. Butler, personal communication) and *tylMI* were obtained from cloned DNA as restriction fragments and inserted into pOJ260 via pIJ2925. The *tylMII* disruption construct was obtained as a PCR amplified fragment and cloned via a different route (Fish and Cundliffe, 1997).

## 3.5 Confirming the authenticity of disrupted strains by Southern hybridisation

Candidate disruptants were initially selected on the basis of their resistance to hygromycin B and sensitivity to apramycin. In each case, confirmation that the strain had undergone a successful double recombination event, resulting in replacement of the chromosomal gene by the disrupted version was obtained by Southern blot hybridisation (Figure 3.9). Thus, using a 0.9 kb *Sac*II fragment of orf1* as a probe, the hybridisation target in *Nco*I digested orf1*-KO DNA was increased in size (4.8 vs. 2.5 kb) compared to similarly digested wild type DNA (Figure 3.9). Using a 0.8 kb *Aat* II fragment containing *tylMI* DNA, the hybridisation target in *Afl*III digested *tylMI*-KO genomic DNA was increased in size (6.9 vs. 4.6 kb) compared to wild type (Figure 3.10). And using a 1.1 kb *Bst* YI fragment containing *ccr*, the hybridisation target in *Afl* III digested *ccr*-KO genomic DNA increased in size (6.9 vs. 4.6 kb) compared to wild type (Figure 3.11a). In addition a second, fainter band lit up in both *ccr*-KO and wild type DNA. The same increase in hybridisation target, but no second band was observed when similarly digested DNA was probed with a 0.8 kb *Aat*II fragment, homologous to the hybridisation target, but containing no *ccr* DNA (Figure 3.11b). The possible significance of this will be discussed later.

When probed with Ωhyg an appropriately sized hybridisation target was found in each of the disrupted strains but no band was found in the wild type.

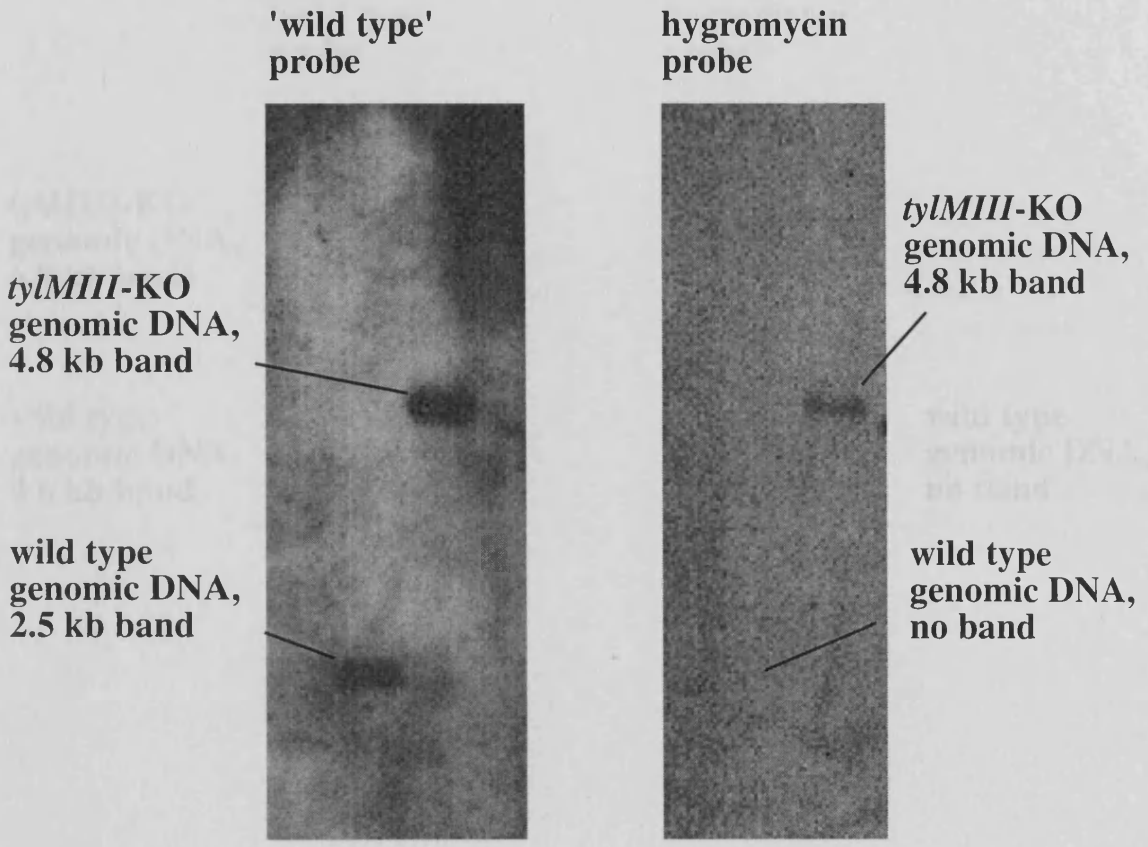



**Figure 3.9 Confirmation of the orf1* (*tylMIII*) disrupted strain.** Southern hybridisation analysis confirmed that the putative orf1* (*tylMIII*) disrupted strain was authentic. Wild type and disrupted strain geneomic DNA were digested with *Nco*I. Probes were prepared from the hygromycin cassette and from a 0.9 kb *Sty* I fragment of cloned DNA containing part of orf1*. The hygromycin probe lit up a band of the predicted size (4.8 kb) in the disrupted strain but no band in the wild type. The 'wild type' probe lit up bands indicating that the predicted increase in fragment size (from 2.5 to 4.8 kb) due to insertion of the hygromycin cassete had occurred in the disrupted strain, relative to wild type.

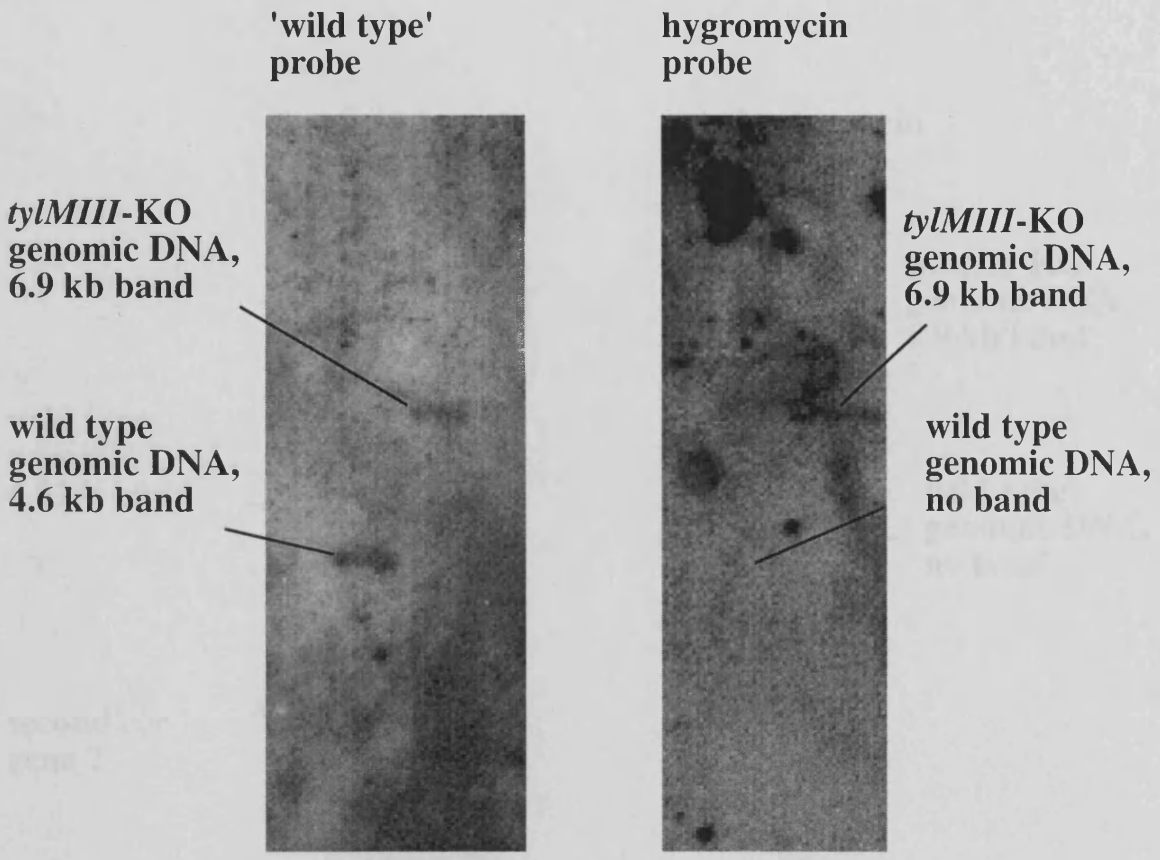



**Figure 3.10 Confirmation of the *tylMI* disrupted strain.** Southern hybridisation analysis confirmed that the putative *tylMI* disrupted strain was authentic. Wild type and disrupted strain geneomic DNA were digested with *afl* III. Probes were prepared from the hygromycin cassette and from a 0.8 kb fragment of cloned DNA containing part of *tylMI*. The hygromycin probe lit up a band of the predicted size (6.9 kb) in the disrupted strain but no band in the wild type. The 'wild type' probe lit up bands indicating that the predicted increase in fragment size (from 4.6 to 6.9 kb) due to insertion of the hygromycin cassete had occureed in the disrupted strain, relative to wild type.

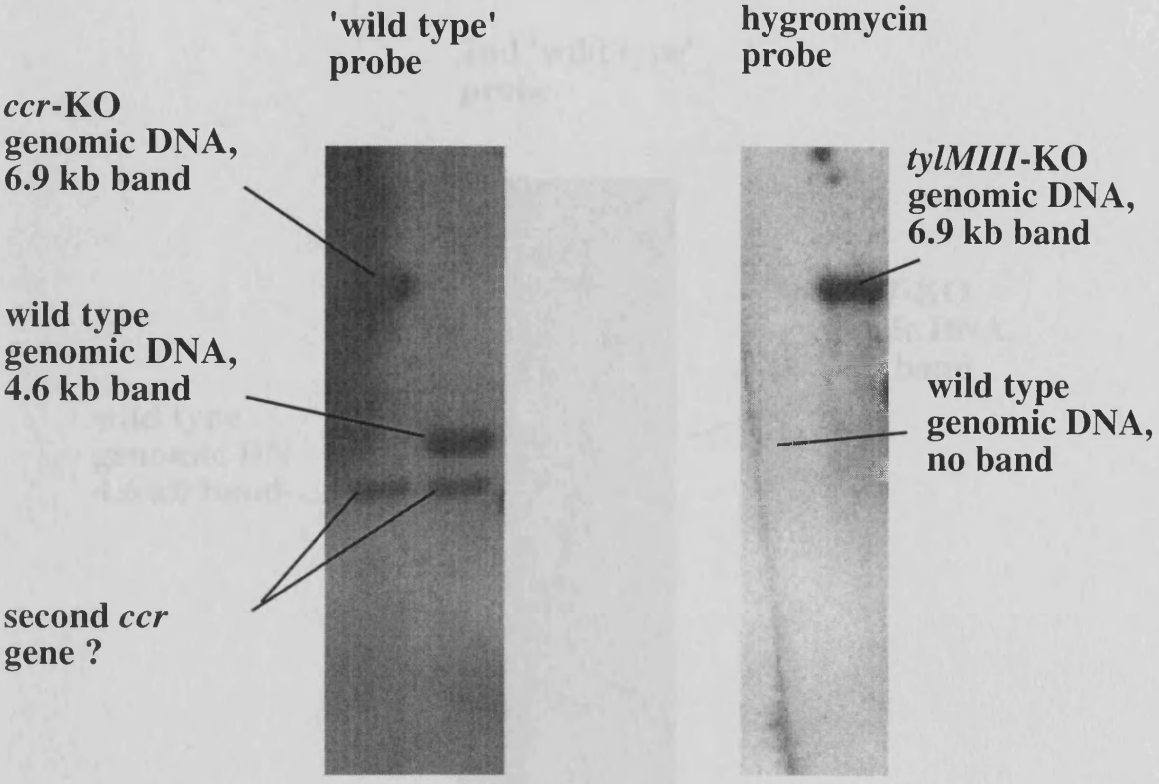



**Figure 3.11a Confirmation of the *ccr* disrupted strain.** Southern blot hybridisation analysis confirmed that the putative *ccr* disrupted strain was authentic. Wild type and disrupted strain genomic DNA were digested with *afl* III . Probes were prepared from the hygromycin cassette and from a 1.1 kb *Bst*YI fragment of cloned DNA containing part of ccr. The hygromycin probe lit up a band of the predicted size (6.9 kb) in the disrupted strain but no band in the wild type. The 'wild type' probe lit up bands indicating that the predicted increase in fragment size (from 4.6 to 6.9 kb) due to insertion of the hygromycin cassete had occurred in the disrupted strain, relative to wild type. The second, fainter band possibly represents a second *ccr* gene present in the *S. fradiae* genome. An alternative Southern analysis is shown in figure 3.11b.

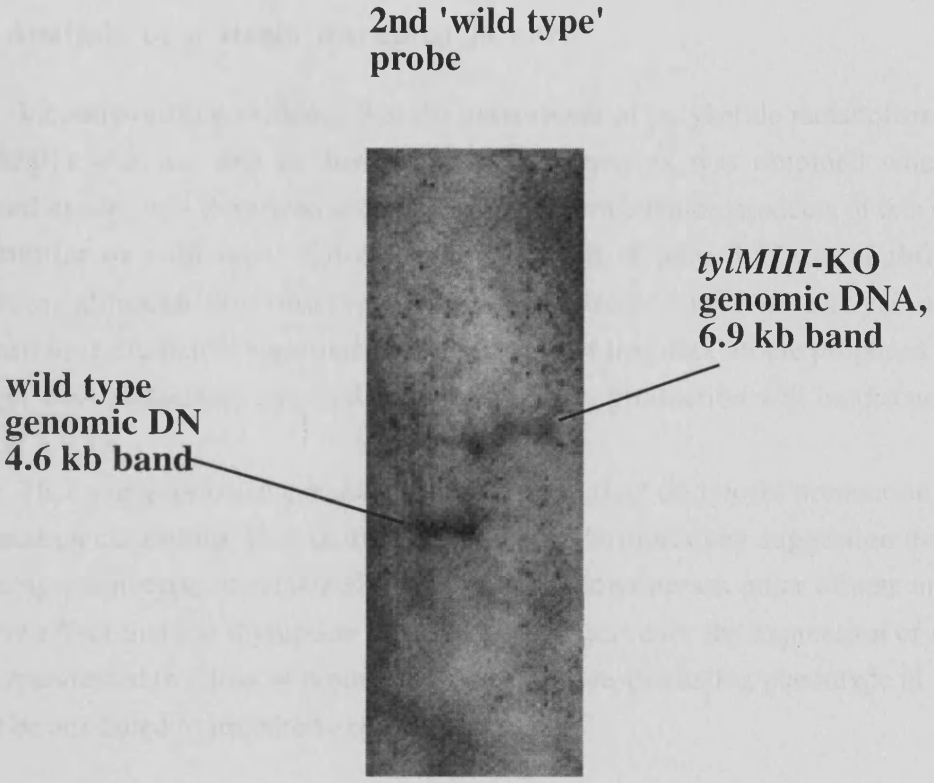



**Figure 3.11b Confirmation of the *ccr* disrupted strain.** Southern blot hybridisation analysis had previously confirmed that the putative *ccr* disrupted strain was probably authentic. However, a second band lighting up gave cause for concern. A second hybridisation was therefore carried out using a different probe, homologous to the hybridisation target but not containing any *ccr* DNA. The disruption in *ccr* was confirmed when the predicted shift in band size (4.6 to 6.9 kb) from the disrupted to the wild type strain occurred but no second band was evident.

## 3.6 The phenotype of *tylMII*-KO cannot be due to downstream effects on *ccr*

### 3.6.1 Analysis of a strain disrupted in *ccr*

Incontrovertible evidence that the impairment of polyketide metabolism in *tylMII*-KO (SF01) was not due to downstream consequences was obtained when a strain disrupted in *ccr* was generated and analysed. The fermentation products of this strain were very similar to wild type. Tylosin was produced (Figure 3.12) in slightly reduced quantities, although this observation was not followed up with analysis capable of demonstrating statistical significance. The impact of this data on the proposed role of the crotonyl CoA reductase, encoded by *ccr*, in tylosin production will be discussed below (section 3.9.1).

That a disruption in *ccr* has no qualitative effect on tylosin production, under the fermentation conditions used in these analyses, undermines any suggestion that the non-producing phenotype of *tylMII*-KO is a result of downstream polar effects on *ccr*. Any negative effect that the disruption in *tylMII* does exert over the expression of *ccr* would not be manifested in a loss of production. Thus the non-producing phenotype of *tylMII*-KO cannot be attributed to impaired expression of *ccr*.

### 3.6.2 The role of *ccr* in tylosin biosynthesis

In a crude attempt was made to determine whether *ccr* might play a more important role in tylactone synthesis under conditions in which polyketide precursors were not so readily available. Thus the wild type strain and *ccr*-KO were fermented in media not containing methyl oleate (pre-fermentation was carried out as normal i.e. with methyl oleate present). No significant difference between the two strains could be identified under these conditions as in both cases only very small quantities of tylosin were accumulated (Figure 3.12).

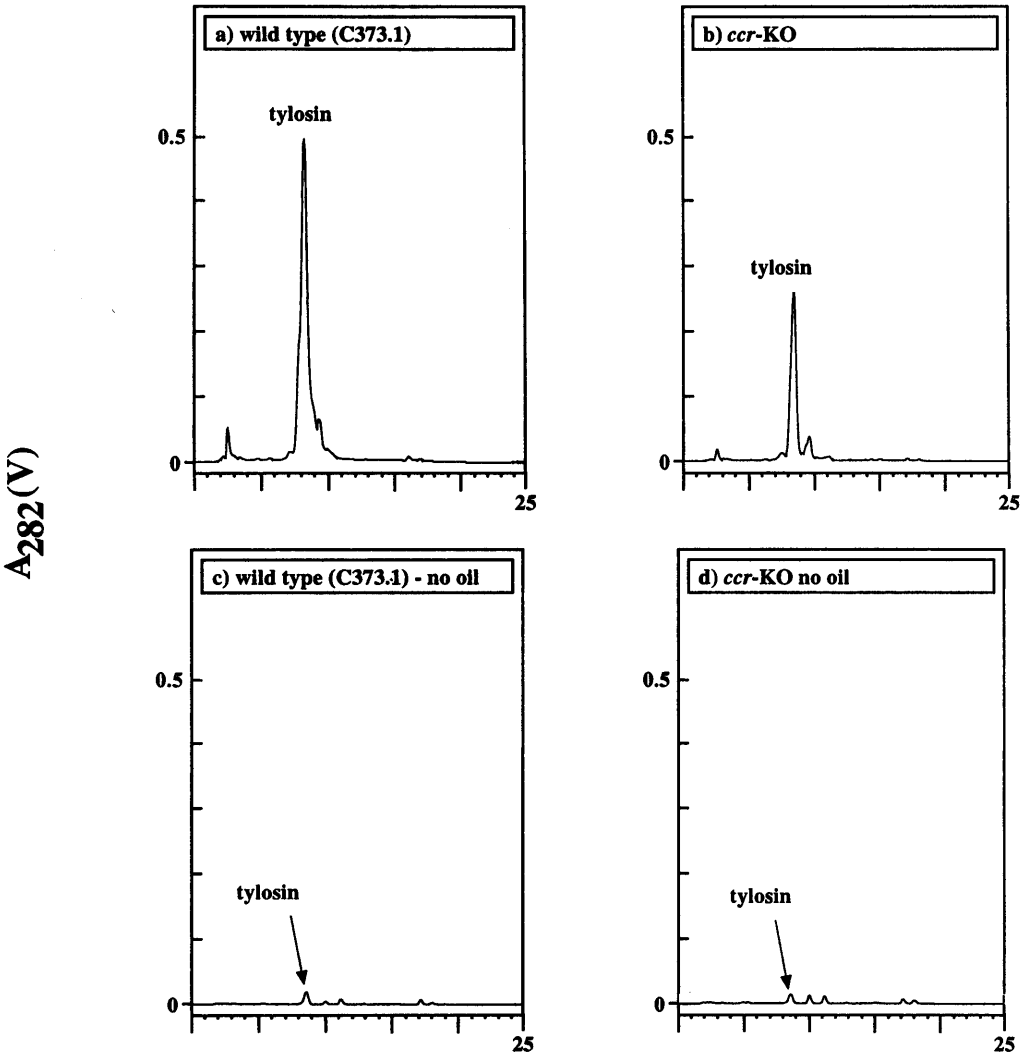



**Figure 3.12 HPLC analysis of *S. fradiae* wild type and *ccr*-KO.** The fermentation products of wild type (a and c) and *ccr*-KO (b and d) strains, fermented under normal conditions and without methyl oleate oil in the media respectively, as visualised by HPLC at 282 nm.

As indicated, the scale used here is 0 - 0.5 V.

## 3.7 Mycaminose genes and stimulation of polyketide metabolism

### 3.7.1 Analysis of the *tylB* mutant, GS50

Further evidence (if any were needed) that downstream consequences on *ccr* played no part in the failure of *tylMII*-KO to accumulate tylactone was obtained when a *tylB* mutant strain (GS50) was analysed. The *tylB* gene encodes the aminotransferase responsible for conversion of dTDP 3-keto, 6 deoxyglucose to dTDP 3-amino, 6-deoxyglucose in the biosynthesis of mycaminose and is located approximately 45 kb away from *tylMII* and oriented in the opposite direction (Merson-Davies and Cundliffe, 1994). It therefore seems highly unlikely that a mutation affecting the expression of *tylB* should exert any effect over *ccr*. When the OMT-fed and control fermentation products of this strain were analysed they displayed qualitatively exactly the same phenotypic pattern as *tylMII*-KO (Figure 3.13). Thus, no detectable production of tylosin intermediates occurred unless the accumulation of tylactone was stimulated by feeding with OMT. Under these conditions, as expected, the OMT fed was converted to tylosin. Being derived from a strain selected for its greater tylosin producing potential, GS50 accumulated tylactone in even larger quantities than *tylMII*-KO when fed OMT. One complicating factor remaining to cloud the issue was the presence of a gene, downstream of *tylB*, associated with polyketide synthesis. This gene, *tylO*, encodes a thioesterase II shown to be important in polyketide synthesis and a construct competent to introduce *tylO* into the chromosomal ØC31 *attB* site (governed by the strong, constitutive promoter, *ermE*p*) was available in the laboratory (Butler *et al.*, 1999). The integration of *tylO* into GS50 was thus facillitated and the resulting strain was fermented in the presence and absence of OMT. The reintroduction of *tylO* did not alter the non-producing phenotype of GS50 and the substantial accumulation of tylactone when fed with OMT also remained unaffected (Figure 3.13). The possibility that the non-producing phenotype of GS50 might be due to a downstream effect on *tylO* was therefore discounted.

The phenotype of *tylMII*-KO, when fermented in the presence and absence of glycosylated tylosin precursors is thus mirrored in the *tylB* mutant strain, GS50. Given the disparate location of these two genes it is clear that the distinctive phenotype displayed is a feature characteristic of strains disabled in the ability to synthesise or add mycaminose. The accumulation of tylactone, triggered by the presence of glycosylated tylosin precursors, is presumably caused by a significant enhancement of polyketide synthesis. The presence of *ccr* downstream of the mycaminose specific gene *tylMII* and *tylO* downstream of *tylB* have no significant impact on this phenomenon.

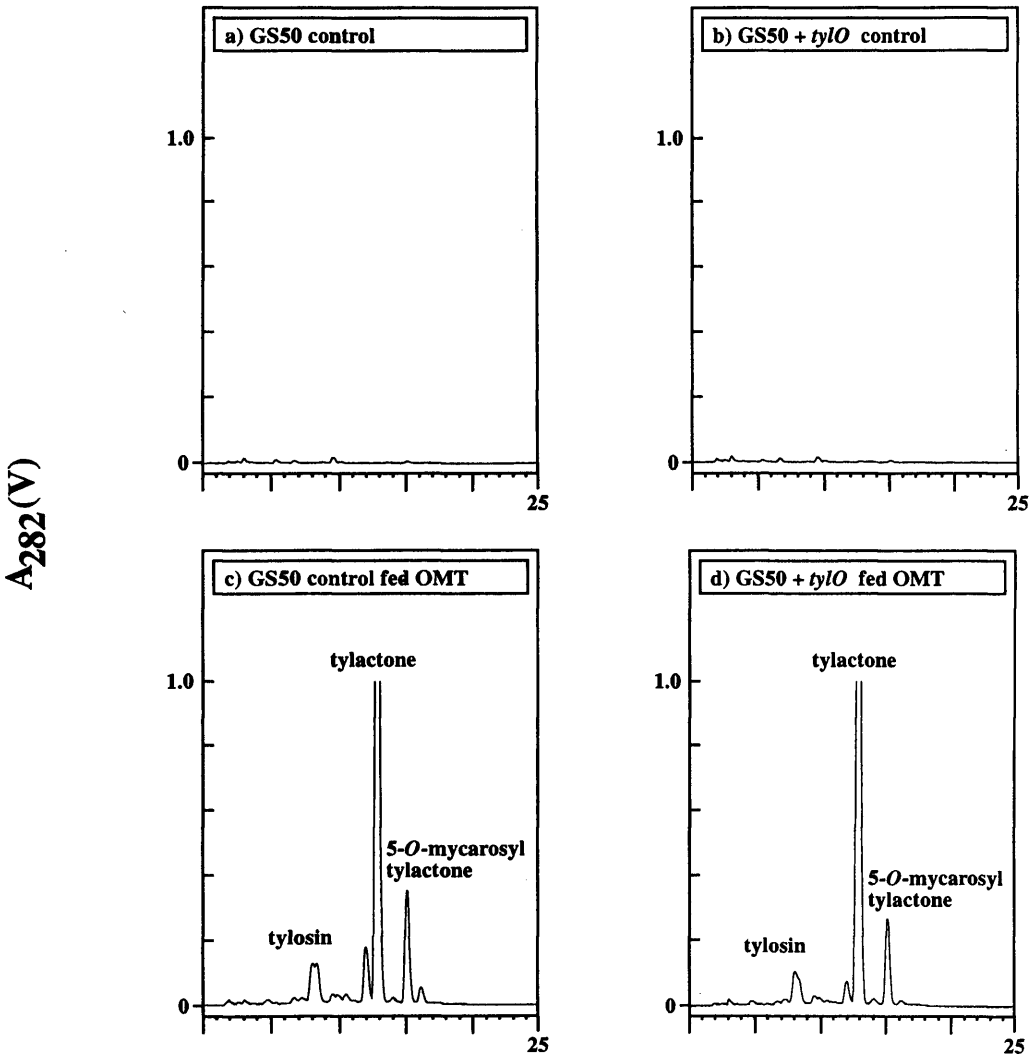



**Figure 3.13 HPLC analysis of the *tylB* mutant strain, GS50.** The fermentation products of (a) GS50 control (with pLST 9828 alone introduced and fed DMSO), (b) GS50 with *tylO* integrated, control (fed DMSO alone), (c) GS50 control (with pLST9828 alone integrated) fed OMT and (d) GS50 with *tylO* integrated fed OMT, as visualised by HPLC at 282 nm.

As indicated, the scale used here is 0 - 1.0 V.

### 3.7.2 Analysis of the orf1* disrupted strain (orf1*-KO)

Confirmation of the mycaminose specific nature orf1* was obtained by examination of the fermentation products of the orf1* disrupted strain. Its behaviour matched that of *tylMII*-KO (SF01) and the *tylB* mutant, GS50. In un-supplemented fermentation orf1*-KO produced no metabolites of tylosin biosynthesis in significant detectable quantities (Figure 3.14). Additionally added glycosylated precursors of tylosin (in this case OMT) were fully converted, indicating that all other tylosin sugar functions had been maintained in the orf1* disrupted strain. A greater than usual proportion of relomycin can identified eluting slightly prior to tylosin. This is characteristic of similar, fed strains (Figure 3.14). The relative conversion of exogenous material from tylosin to relomycin is presumably exaggerated by having been being fed at an early stage and the limited amount of material present. In addition to these compounds, tylactone accumulated. As discussed later (section 4.3.3), re-introduction of *tylM* DNA into this strain proved that this phenotype is a genuine effect of the disruption in orf1* and is not due to any downstream polar effects on the other mycaminose genes. The phenotype of orf1*-KO is thus consistent with that of the other mycaminose-deficient strains. This evidence therefore confirmed for the first time not only that orf1* is in fact a *tyl* gene but in particular that it is mycaminose specific. It is thus entitled to be designated *tylMIII* and the disrupted strain will be called *tylMIII*-KO.

### 3.7.3 Analysis of *tylMII*-KO (SF01)

Analysis of *tylMII*-KO showed that it behaved as described previously (Fish and Cundliffe, 1997) when fermented in the presence and absence of OMT (Figure 3.14). No tylosin metabolites could be identifed by HPLC analysis unless the fermentation was supplemented with OMT. The OMT was converted to tylosin (and relomycin) and tylactone accumulated. The fed and unfed fermentation extracts were also analysed by mass spectrometry (Figure 3.15 and 3.16). The major species present in the unfed *tylMII*-KO fermentation extract had an *m/z* value of 311. A similar peak is evident in all the *S. fradiae* fermentation extracts examined so far, although not always as the major species. The nature of this material is unknown. No trace of any other tylosin metabolites was distinguishable from background, even when the relavant part of the trace was expanded by five times. As expected, mass spectra characteristic of tylactone was obtained from the OMT-fed fermentation extract (Figure 3.16). In addition to the predicted *m/z* value of 395, species of m/z 377, 474, 790 and 811 can be identified, just as in the standard tylactone preparation (section 3.2.2).

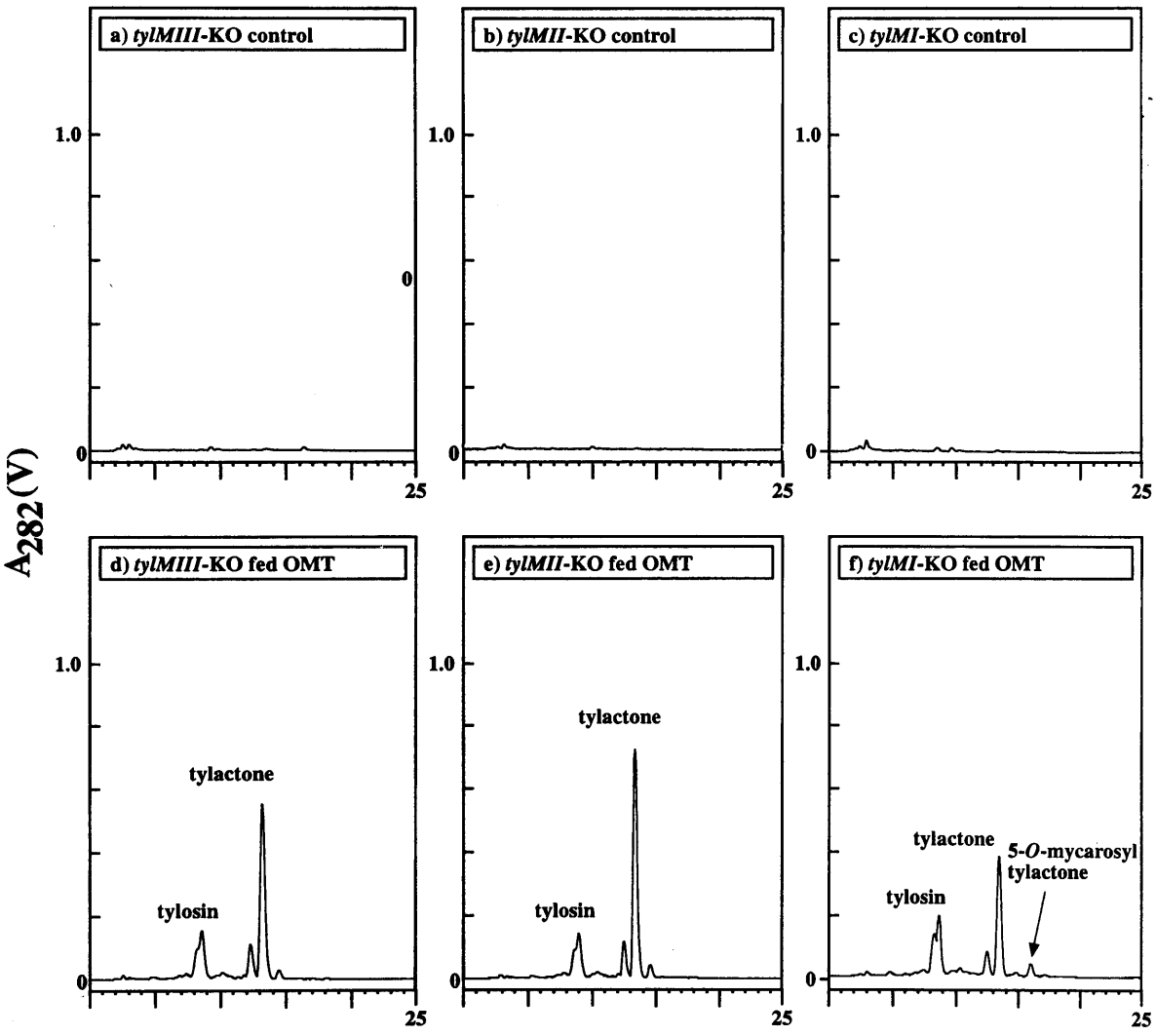



**Figure 3.14 HPLC analysis of *tylM* disrupted strains fed OMT.** The fermentation products of (a) *tylMIII*-KO control, (b) *tylMII*-KO control, (c) *tylMI*-KO control, (d) *tylMIII*-KO fed OMT, (e) *tylMII*-KO fed OMT and (f) *tylMI*-KO fed OMT, as visualised by HPLC at 282 nm. Control fermentations were fed DMSO.

As indicated, the scale used here is 0 - 1.0 V.

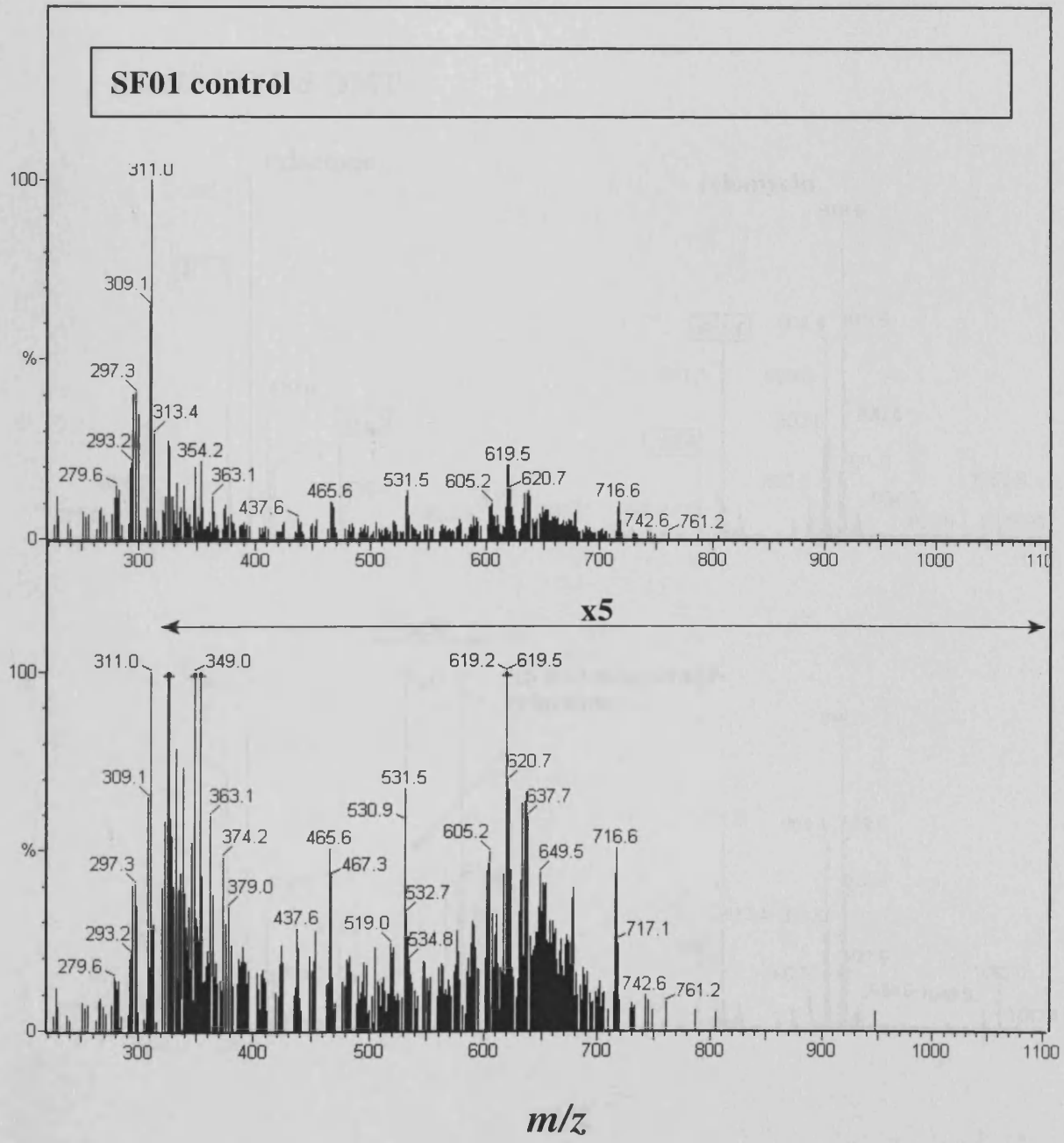



**Figure 3.15 Analysis of *tylMII*-KO by mass spectrometry.** No species with *m/z* values equivalent to those predicted for tylosin related metabolites could be identified in the fermentation extract even when part of the trace was expanded five times (lower panel). The major species has an *m/z* value of 311. This is a consistent feature of all the *S. fradiae* fermentation extracts analysed so far. Its nature is unknown. Although less prominent, the species with *m/z* values of 531 and 619 (and the surrounding material) are also common features of other fermentation extracts.

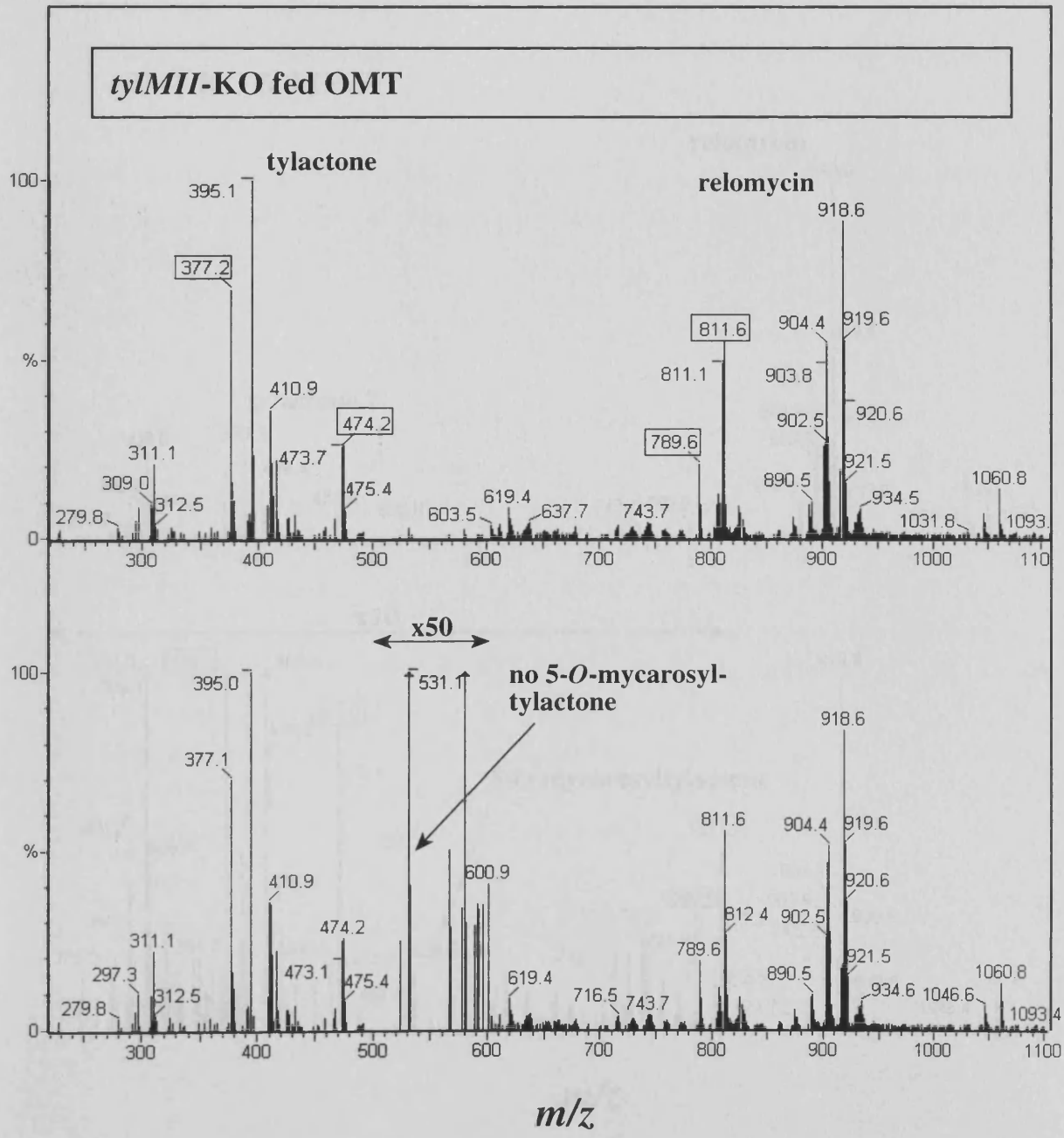



**Figure 3.16 Analysis of *tylMII*-KO by mass spectrometry.** As expected from the HPLC data, mass spectra characteristic of tylactone was obtained when *tylMII*-KO was analysed. Tylactone (*m/z* 395) constiutes the major species present accompanied as always by peaks with *m/z* values of 377, 474, 790 and 811 (boxed). Material with an *m/z* value of 311 appears to be ubiquitous in fermentation extracts. The major product of OMT conversion appears to be relomycin (predicted *m/z* 918), in place of tylosin (*m/z* 916). Despite being magnified by 50 times in the relevant region (lower panel), no peak with an *m/z* value consistent with 5-*O*-mycarosyltylactone (539) is present.

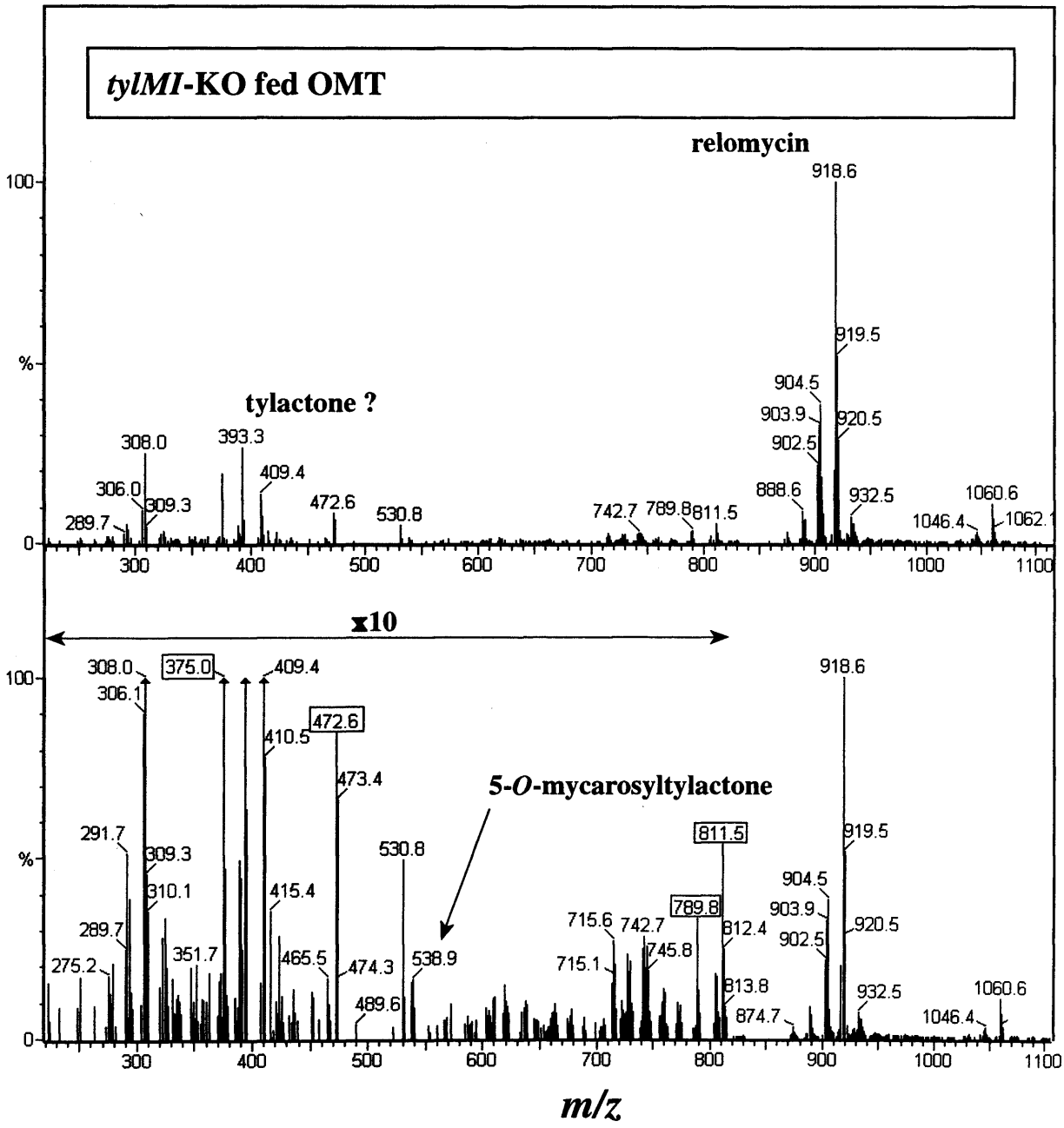



**Figure 3.17 Analysis of *tylMI*-KO by mass spectrometry.** The major species detectable in this extract had an *m/z* value of 918, consistent with the OMT fed to the fermentation having been converted mainly to relomycin.

Mass spectra characteristic of tylactone was also obtained (although some minor problem with calibration was encountered at the lower end of the spectrum when this sample was analysed with the commonly encountered species with m/z values of 311, 395 and 474 being assigned slightly lower *m/z* values than expected). The lower trace shows a region expanded by 10 times. The peaks that invariably accompany tylactone accumulation have been marked (boxed) in this region. A species with the predicted *m/z* value of 5-*O*-mycarosyltylactone (539) becomes obvious at this magnification.

Next to tylactone, (*m/z* 395) material with an *m/z* value of 918 is the second most prominent peak. This probably represents relomycin (20-dihydrotylosin, predicted *m/z* 918). As shown by HPLC analysis, relomycin forms a large proportion of the material to which OMT is converted. 5-*O*-mycarosyltylactone (predicted *m/z* 539) is a compound that commonly co-accumulates with tylactone, yet no trace of it can be detected in this fermentation extract even when the relevant area of the trace is greatly expanded (Figure 3.16).

### 3.7.4 Analysis of *tylMI*-KO

The *tylMI* (orf3*) disrupted strain, *tylMI*-KO, behaved similarly to the other mycaminose deficient strains (Figure 3.14). In unfed fermentation it produced no identifiable tylosin metabolites when analysed by HPLC. When fed with OMT, analysis of the fermentation products showed that, as expected, the OMT had been fully converted to tylosin/relomycin and that in addition, tylactone accumulation had been stimulated. Also, significant quantities of material co-eluting with 5-*O*-mycarosyltylactone were evident. In this respect the phenotype of *tylMI*-KO is inconsistent with the other *tylM* disrupted strains but resembles that of the *tylB* mutant, GS50. Mass spectral data obtained from the *tylMI*-KO, OMT-fed fermentation extract was similar to that obtained from *tylMII*-KO (Figure 3.17). It differs significantly only in that material with an *m/z* value consistent with 5-*O*-mycarosyltylactone (539) is visible when the relevant area of the trace is expanded.

## 3.8 Stimulation of polyketide synthesis

### 3.8.1 Stimulation of polyketide synthesis by very low levels of OMT

It became interesting to determine the nature and sensitivity of the polyketide stimulation that occurs when strains disrupted in mycaminose biosynthesis are fed with glycosylated polyketide. Strain *tylMII*-KO was therefore fed with varying amounts of OMT in a dose-response experiment (Figure 3.18). Duplicate fermentations, were fed with quantities of OMT from 100 μg - 10.0 mg and the levels of tylactone accumulated were assessed. The amount of tylactone accumulated in each duplicate is shown using the same absorbance scale applied to HPLC traces. This experiment revealed two important characteristics of the tylactone stimulation phenomenon. Firstly, the level of tylactone accumulated was not proportional to the amount of OMT fed during fermentation. Rather, an all or nothing response was observed.

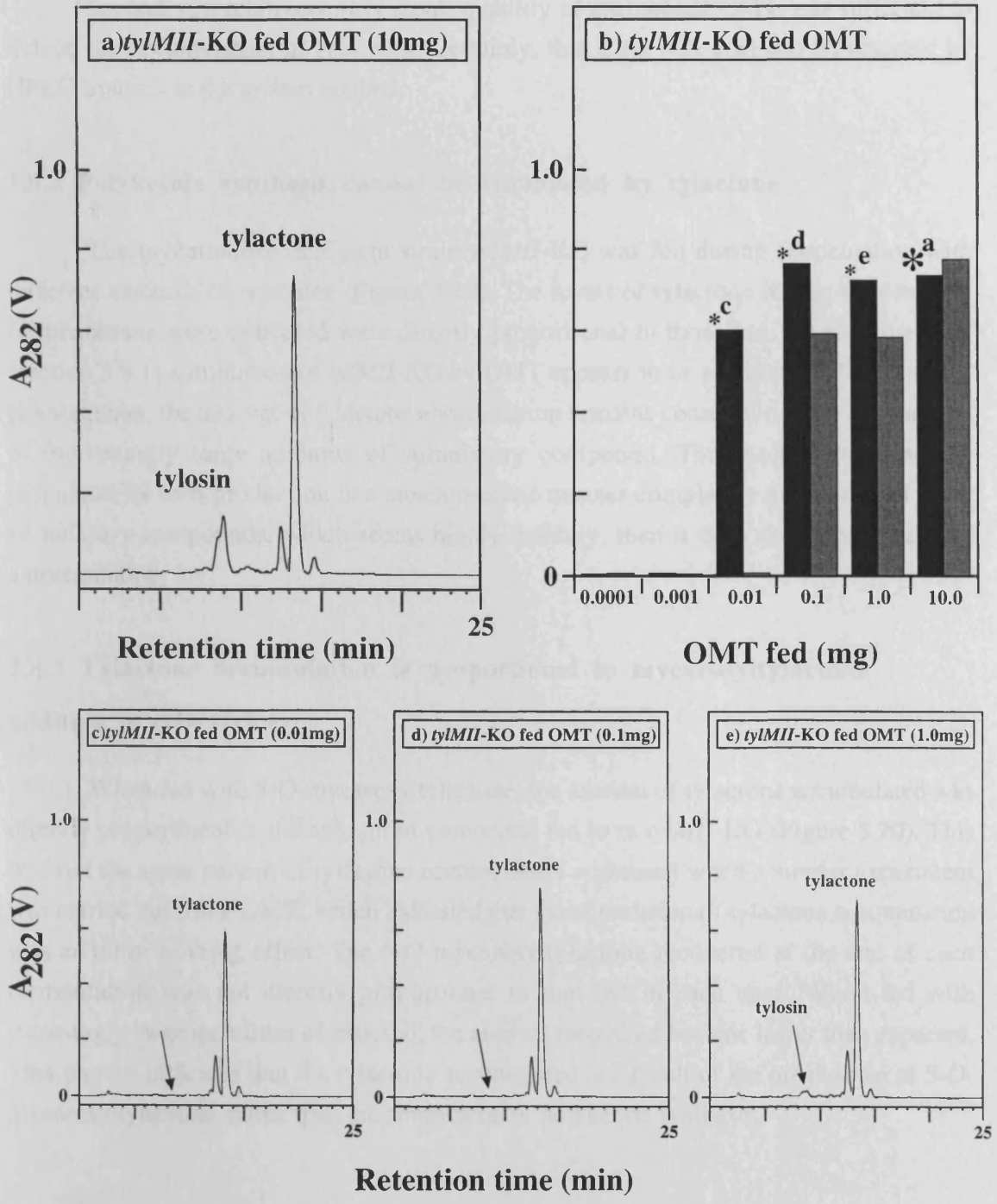



**Figure 3.18 Accumulation of tylactone in response to varying amounts of OMT fed during fermentation.** The levels of tylactone accumulation are shown (b) when duplicate *tylMII*-KO fermentations were fed varying quantities OMT. The graph shows the peak heights obtained at 282 nm, and the scale used corresponds to that used for the HPLC traces displayed alongside (0-1.0 V). HPLC data is shown for the peaks marked with an '*' Thus the fermentation products of *tylMII*-KO fed (a) 10 mg OMT (marked with a large, bold '*' on panel (b), (c) 0.01 mg OMT, (d) 0.1 mg OMT, (e) 1.0 mg OMT, as visualised by HPLC at 282 nm.

Secondly, a relatively very small quantity of extraneous OMT was sufficient to induce the accumulation of tylactone. Certainly, this is far less than can be detected by HPLC analysis in the system applied.

### 3.8.2 Polyketide synthesis cannot be stimulated by tylactone

The mycaminose defficient strain *tylMII*-KO was fed during fermentation with different amounts of tylactone (Figure 3.19). The levels of tylactone recovered when the fermentations were extracted were directly proportional to those fed. As shown earlier (section 3.8.1) stimulation of *tylMII*-KO by OMT appears to be a catalytic 'all or nothing' phenomenon, the amount of tylactone accumulation remains constant, despite the addition of increasingly large amounts of stimulatory compound. Thus unless the tylactone stimulates its own production in a stoichiometric manner completely unlike that of other stimulatory compounds, which seems highly unlikely, then it does not display positive autoregulation.

### 3.8.3 Tylactone accumulation is proportional to mycarosyltylactone addition in *tylMII*-KO

When fed with 5-*O*-mycarosyltylactone, the amount of tylactone accumulated was directly proportional to the amount of compound fed to in *tylMII*-KO (Figure 3.20). This was not the same pattern of tylactone accumulation witnessed when a similar experiment was carried out using OMT, which indicated that the stimulation of tylactone accumulation was an all or nothing effect. The 5-*O*-mycarosyltylactone recovered at the end of each fermentation was not directly proportional to that fed in each case. When fed with incresingly large quantities of material, the amount recovered became lower than expected. This pattern indicates that the tylactone accumulated is a result of the breakdown of 5-*O*-mycarosyltylactone, rather than the stimulation of polyketide synthesis.

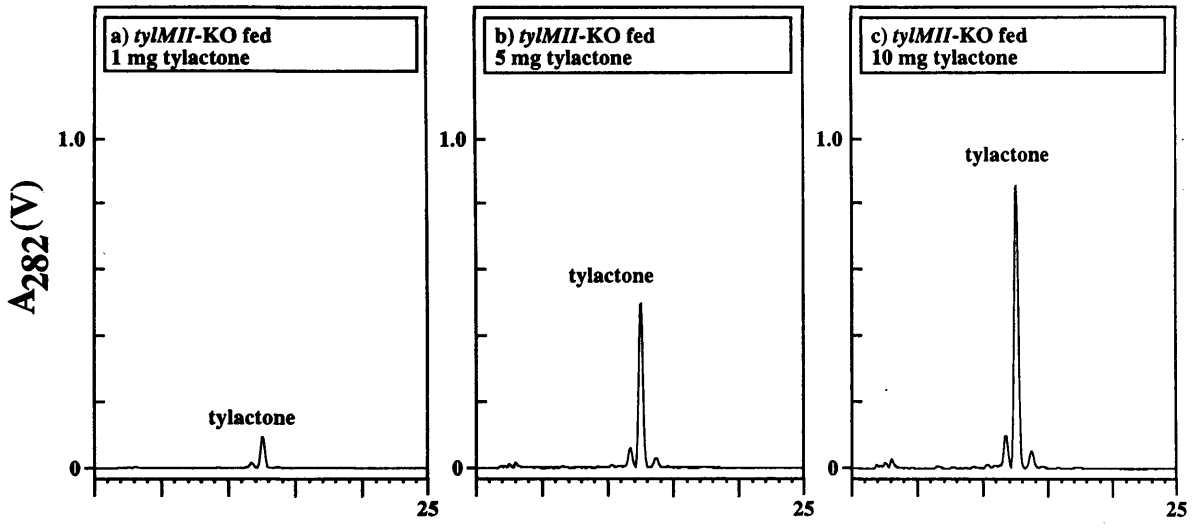



**Figure 3.19 HPLC analysis of *tylMII*-KO fed varying quantities of tylactone.** The fermentation products of *tylMII*-KO fed (a) 1 mg, (b) 5 mg and, (c) 10 mg of tylactone, as visualised by HPLC at 282 nm. The amount of tylactone fed was proportional to the amount recovered in each case.

As indicated, the scale used here is 0 - 1.0 V.

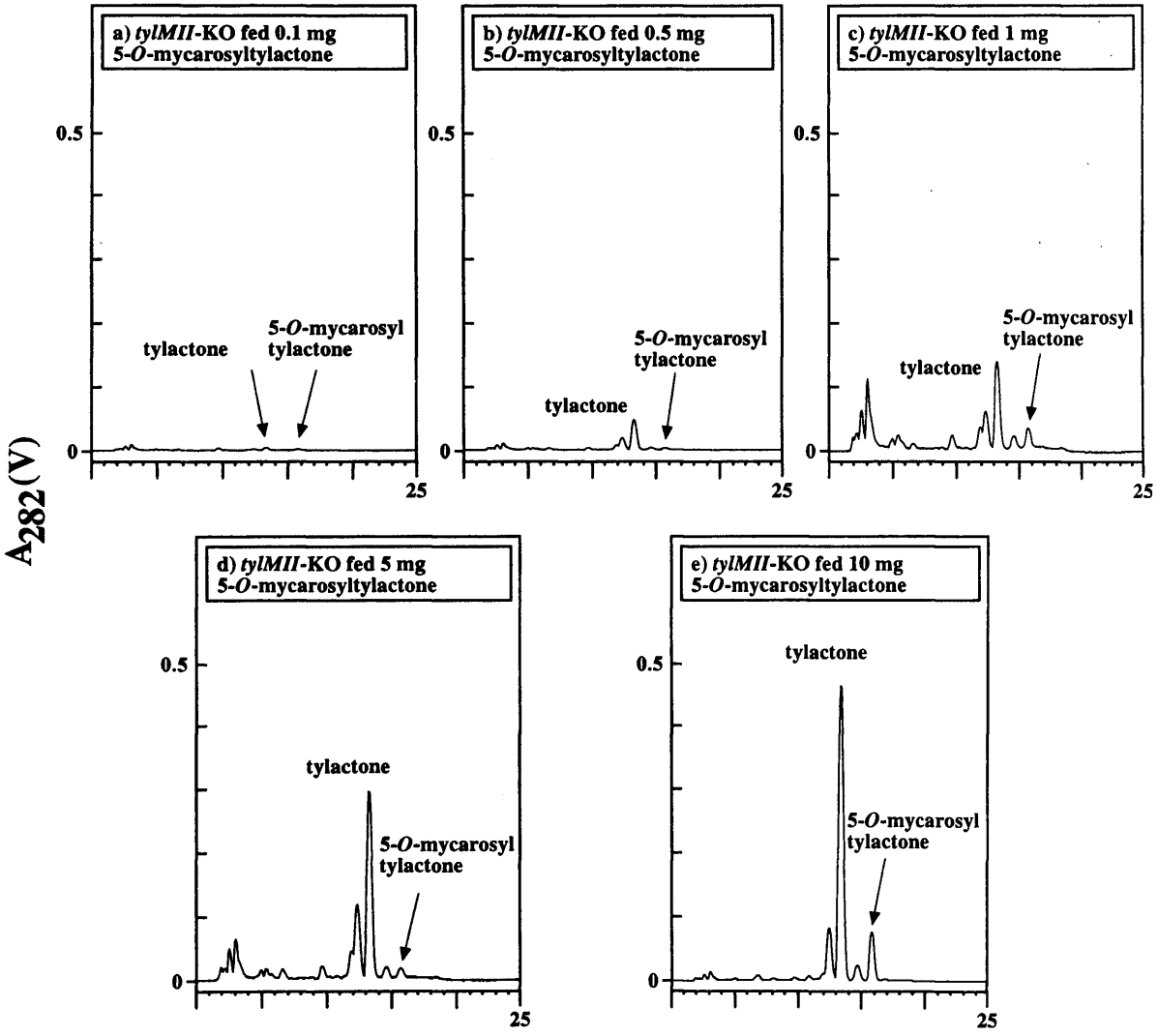



**Figure 3.20 HPLC analysis of *tylMII*-KO fed varying quantities of 5-*O*-mycarosyltylactone.** The fermentation products of *tylMII*-KO fed (a) 0.1 mg, (b) 0.5 mg, (c) 1.0 mg, (d) 5.0 mg and (e) 10.0 mg of 5-*O*-mycarosyltylactone, as visualised by HPLC at 282 nm. The amount of tylactone accumulated in each case is roughly proportional to the amount of 5-*O*-mycarosyltylactone fed.

As indicated, the scale used here is 0 - 0.5 V.

### 3.8.4 Mycarosyltylactone is not broken down in tylactone deficient strains

5-*O*-mycarosyltylactone was fed to three different *S. fradiae* strains during fermentation, all unable to synthesise tylactone. These were *tylR*-KO, *tylGV*-KO and the mutant strain GS5. Strain *tylR*-KO is a wild type strain disrupted in *tylR* (Bate *et al.*, 1999). This strain is unable to perform any tylosin biosynthetic functions including synthesis of tylactone. Strain *tylGV*-KO, disrupted in the KS domain of the final module (*tylGV*) of the tylactone biosynthetic gene complex, was constructed as part of the analysis of transcriptional organisation in the *tylM* region (described in Chapter 4). Strain GS5 (Baltz and Seno, 1981) is thought to be a clean mutant in the promoter upstream of *tylG*.

In each case, as expected, control fermentations accumulated no material absorbing at 282 nm (Figure 3.21). Fermentations were supplemented with 5-*O*-mycarosyltylactone which was recovered at the end of fermentation. Some small traces of tylactone appear to be present in the resulting fermentation extracts, particularly GS5. This material presumably must derive from breakdown of the exogenous compound although a trace of tylactone was shown to be present in the standard preparation of 5-*O*-mycarosyltylactone. Nevertheless the amount of tylactone detected was very small, far less than would be required to fully explain the accumulation of tylactone in other strains (*tylMII*-KO) fed with 5-*O*-mycarosyltylactone.

### 3.8.5 Stimulation of polyketide synthesis by other glycosylated macrolides

Interestingly, it was found that tylactone accumulation could be provoked by both rosaramicin and spiramycin. The other glycosylated macrolides fed, carbomycin, chalcomycin and erythromycin failed to produce such a response (Figure 3.22). Fermentations of *tylMII*-KO fed rosaramicin accumulated tylactone at levels comparable to those of OMT fed fermentations. The level of tylactone accumulation was lower in fermentations supplemented with spiramycin, comparable to that witnessed in *tylMII*-KO fermentations fed tylosin (Figure 3.22). No significant detectable trace of any tylosin intermediate could be found in *tylMII*-KO fermentations to which carbomycin, chalcomycin or erythromycin had been added (Figure 3.23).

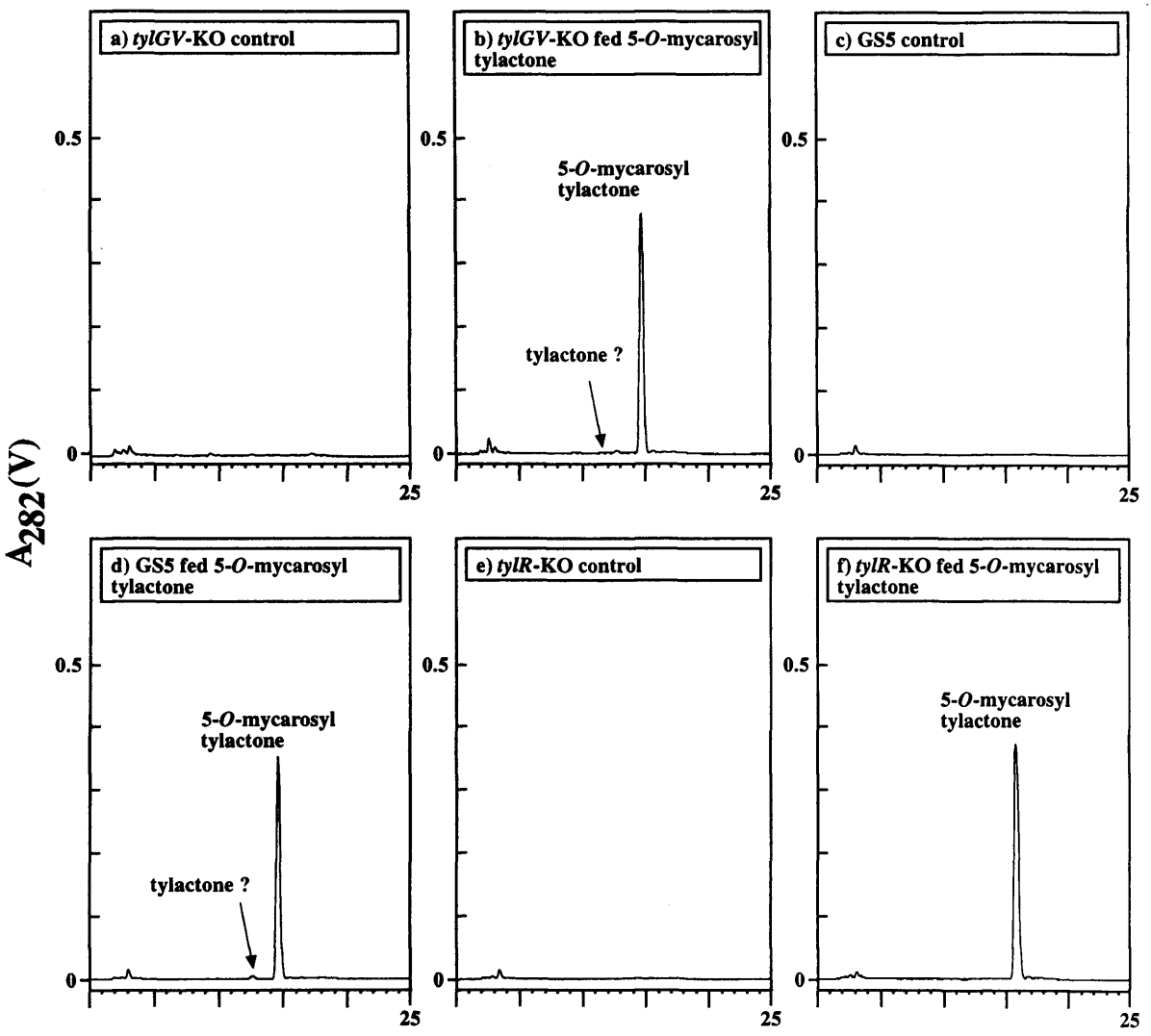



**Figure 3.21 HPLC analysis of polyketide synthesis deficient strains, fed mycarosyl-tylactone.** The fermentation products of *tylGV*-KO (a) control (fed DMSO) and (b) fed 5-*O*-mycarosyltylactone, GS5 (b) control (fed DMSO) and (c) fed 5-*O*-mycarosyltylactone, and *tylR*-KO (d) control (fed DMSO) and (b) fed 5-*O*-mycarosyltylactone, as visualised by HPLC at 282 nm.

As indicated, the scale used here is 0 - 0.5 V.

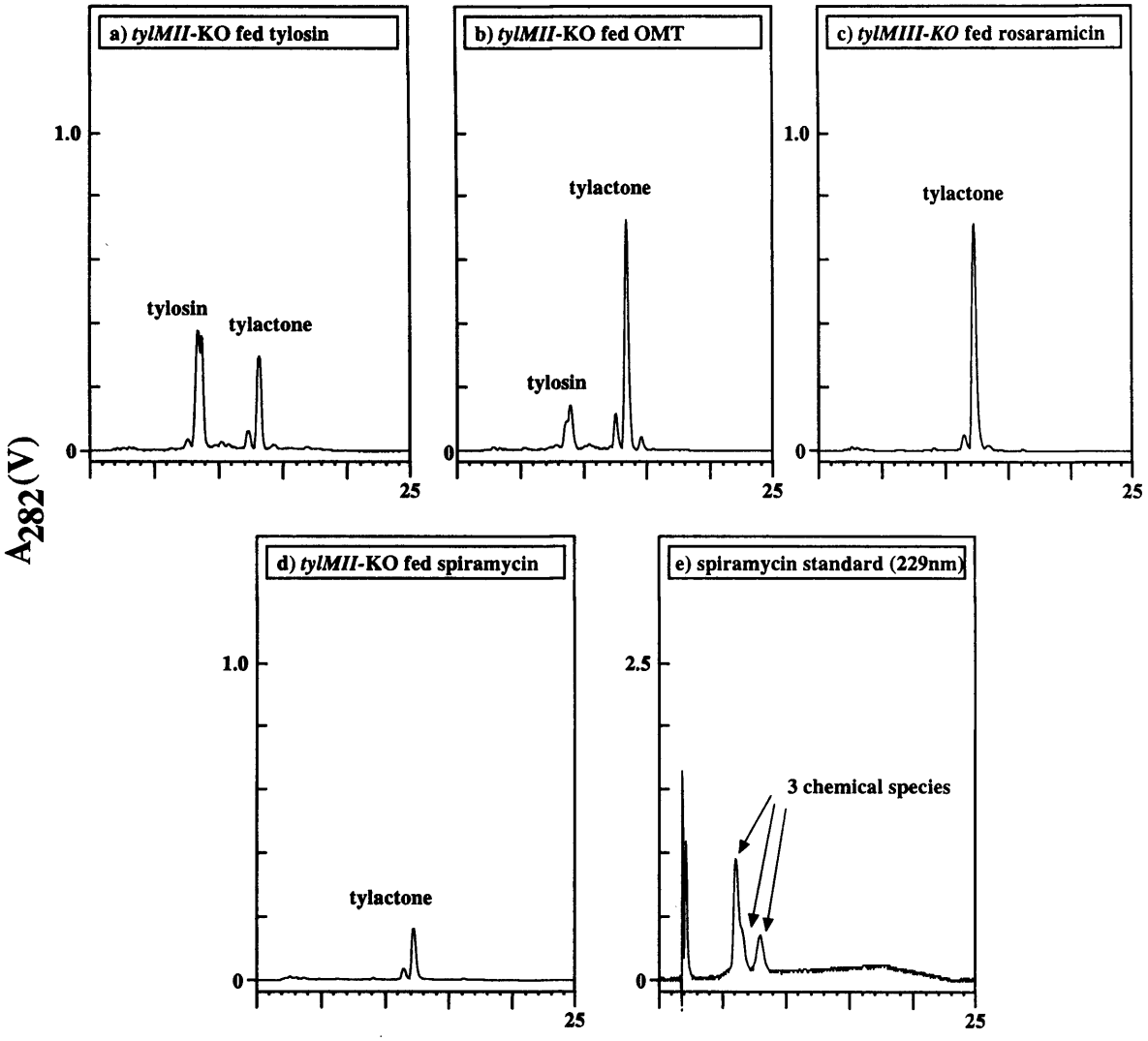



**Figure 3.22 HPLC analysis of *tylMII*-KO fed macrolide antibiotics that stimulate tylactone accumulation.** The fermentation products of *tylMII*-K fed 10 mg:
(a) tylosin, (b) OMT, (c) rosaramicin and (d) spiramycin, as visualised by HPLC at 282 nm. Both rosaramicin and spiramycin stimulated tylactone accumulation.
The spiramycin that was used to feed is shown visualised at 229 nm (e).

As indicated the scale used is 0 - 1.0 V (except for the spiramycin std, 0 - 2.5 V).

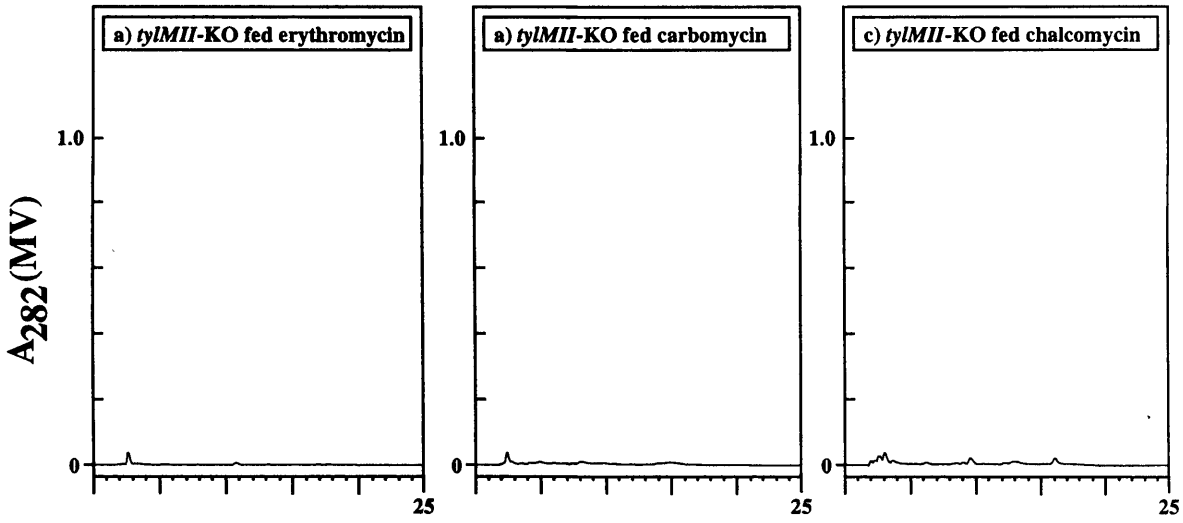



**Figure 3.23 HPLC analysis of *tylM*-KO fed macrolides that do not stimulate tylactone accumulation.** The fermentation products of *tylMII*-KO fed 10 mg of (a) erythromycin (b) carbomycin and (c) chalcomycin, as visualised by HPLC at 282 nm. None of these compounds stimulated tylactone accumulation.

As indicated, the scale used here is 0 - 1.0 MV.

## 3.9 Discussion

### 3.9.1 The role of *ccr* in tylosin biosynthesis

The disruption in *ccr* had no discernable effect on the phenotype of the wild type. This result obviates the possibility that the phenotype of strains disrupted in the upstream *tylM* genes could be due to an effect on *ccr*. Its expression appears to be largely irrelevant in the biosynthesis of tylosin under the fermentation conditions used. This does not necessarily imply that it is not a functionally important gene under other circumstances. The fermentation conditions used are designed to supply the fermenting strain as far as possible with all the necessary components to facilitate the production of tylosin. The role of *ccr* in the supply of four carbon units for the extension of the polyketide may be reduced if the requirement for such precursors was easily supplied by the media itself, for instance by the methyl oleate in the fermentation media. In a crude attempt to address this consideration the wild type strain and *ccr*-KO were fermented in media not containing methyl oleate, probably the primary source from which polyketide precursors are derived. In both cases only a very small quantitiy of tylosin was accumulated under these conditions (section 3.6.1). Although this result is unhelpful in determining the importance of *ccr*, it does illustrate the reliance upon methyl oleate in these fermentations and that the media used might not be the best one to identify the importance of such a gene, whose real value might only be revealed under less 'perfect' conditions.

Superimposed on these considerations, an orthologue of *ccr* may be required in primary metabolism for the biosynthesis of straight chain fatty acids as it is in other *Streptomyces* spp. As the entire tylosin biosynthetic cluster can be deleted from *S. fradiae* (strain GS93) with no loss of viability (Beckmann *et al.*, 1989) it is likely that a second crotonyl CoA reductase is present in its genome. When probing the genome of wild type and the disrupted strain with DNA taken from *ccr*, in addition to the bands relating to *ccr*, a second, fainter band was noticed (section 3.5). It was assumed that this represented the second crotonyl CoA reductase. It is thus possible, that this second gene is capable of functionally complementing the *ccr* disrupted strain under the fermentation conditions used. It is therefore best to regard *ccr* as an ancillary gene in the tylosin biosynthetic cluster.

### 3.9.2 orf1* contains a mycaminose specific gene

Sequence similarities with other putative proteins had previously raised the possibility that the product of orf 1*, the first open reading frame found downstream of

*tylG* might catalyse the isomerisation of dTDP-4-keto-6-deoxyglucose to dTDP-3-keto-6-deoxyglucose in the biosynthesis of mycaminose (section 1.3.3) (Salah-Bey *et al.*, 1998). The phenotype of a strain disrupted in this open reading frame was consistent with a strain in which a gene involved in mycaminose synthesis or addition had been disrupted. Data presented in Chapter 4 involving re-introduction of *tylM* DNA into this strain proved that this phenotype is a genuine effect of the disruption in orf1* and is not due to any downstream polar effects on other mycaminose genes. The position of orf1* as, not only an essential tylosin gene, but one whose product is specifically involved in mycaminose metabolism was therefore confirmed. It was therefore re-named *tylMI* and its product proposed to catalyse the conversion of dTDP-4-keto-6-deoxyglucose to dTDP-3-keto-6-deoxyglucose in the biosynthesis of mycaminose.

### 3.9.3 *tylMI* is a mycaminose specific gene

Complementation analysis in strain GS62 had previously revealed a role for the product of *tylMI* in mycaminose biosynthesis (Gandecha *et al.*, 1997). On the basis of sequence comparisons and the ability of a TylM fusion protein to bind *S*-adenosyl methionine, TylMI was evidently the *N*-methyltransferase necessary fro the synthesis of mycaminose. The phenotype of *tylMI*-KO, being similar to the other 'mycaminose deficient' strains was consistent with this evidence. It demonstrates that strains disrupted in mycaminose specific genes all display the same characteristic phenotype.

### 3.9.4 The stimulation of polyketide synthesis

Feeding *tylMII*-KO with varying quantities of OMT revealed two important characteristics of the tylactone stimulation phenomenon. Firstly, the level of tylactone accumulated was not proportional to the amount of OMT fed during fermentation (section 3.8.1), a catalytic rather than stoichiometric response was observed. Presumably, tylactone is initially maintained at only a very low level, such that it is undetectable by HPLC at the scale of fermentation carried out here. The level of polyketide synthesis becomes greatly enhanced when the level of glycosylated precursor rises above a particular threshold. This threshold spans a relatively narrow range of glycosylated precursor concentration. The increased accumulation of tylactone, presumably caused by an increase in polyketide synthase expression, then appears to be free from any further attenuation based on the level of glycosylated, stimulating compound present. Secondly, a relatively very small quantity of extraneous OMT was sufficient to induce the accumulation of tylactone. Certainly, this is far less than can be detected by HPLC analysis in the system applied. This additional

information also reinforces the point that the accumulation of tylactone cannot have been derived from breakdown of the fed tylosin precursors.

The similar behaviour of all the mycaminose disrupted strains and the absence of any downstream effects on polyketide related genes means that the previously proposed model for stimulation of polyketide synthesis in *S. fradiae* holds true (Figure 3.24). Thus polyketide synthesis can occur at only an imperceptable level in the absence of the ability to glycosylate the polyketide. Polyketide synthesis is thus regulated by a intricate, positive feedback mechanism when conversion of tylactone to tylosin can proceed. The profound response of polyketide synthesis to glycosylated polyketide is catalytic rather than stoichometric. It is perhaps important to bear in mind that different intermediates of the tylosin biosynthetic pathway were found to stimulate tylactone accumulation to varying levels (Fish and Cundliffe 1997). In general the further advanced along the route to tylosin, the lower the level of tylactone accumulation resulting, with OMT thus being the most and tylosin the least efficient.

### 3.9.5 5-*O*-mycarosyltylactone does not stimulate polyketide synthesis

The possibility that 5-*O*-mycarosyltylactone, might be capable of stimulating the accumulation of tylactone can be discounted at least partly on the basis of indirect evidence. Tylactone does not accumulate in any of the unfed mycaminose-defficient strains and yet whatever mechanism is involved in the production of 5-*O*-mycarosyltylactone should be capable of functioning. It is however possible that disruptions in the pathway to mycaminose might exert a negative influence on the synthesis or addition of mycarose to produce 5-*O*-mycarosyltylactone and no stimulation would occur for this reason. The data obtained from feeding 5-*O*-mycarosyltylactone to tylactone-deficient strains and OMT to *tylMII*-KO seem to be contradictory. Although breakdown of 5-*O*-mycarosyltylactone to tylactone is shown to occur (section 3.8.4), it does not occur in quantities large enough to explain the levels of tylactone in *tylMII*-KO when similarly fed (section 3.8.3). If the tylactone accumulation in the latter case was due to stimulation of polyketide synthesis, then the mechanism would have to be different than that caused by OMT. Another possibility is that the breakdown of 5-*O*-mycarosyltylactone occurs more efficiently in *tylMII*-KO than the tylactone-deficient strains. Whichever is the case it seems that 5-*O*-mycarosyltylactone either cannot be produced independantly of other stimulatory factors (such as OMT) or, more likely, it is produced only when tylactone accumulates in large quantities. Either way the accumulation of 5-*O*-mycarosyltylactone is an effect not the cause of the accumulated tylactone.

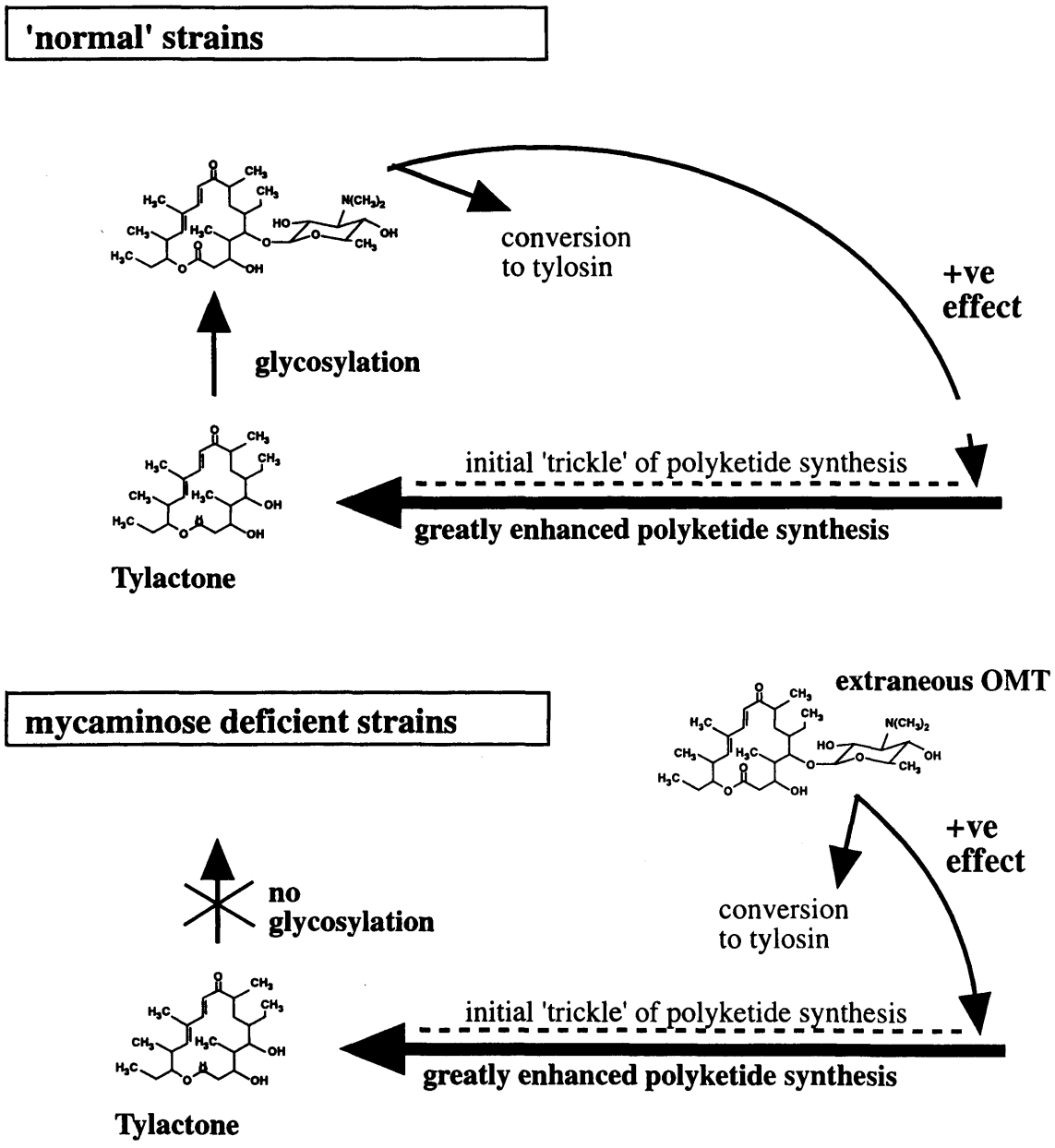



**Figure 3.24 The positive feedback of glycosylated tylactone on polyketide synthesis.** The behaviour of strains deficient in mycaminose metabolism suggests that polyketide synthesis occurs at only very low levels if glycosylation of the polyketide can not occur. The addition of glycosylated tylosin precursors to fermentations of such strains causes polyketide synthesis to be dramatically up-regulated and tylactone accumulates. In 'normal' strains, glycosylation of the low levels of polyketide can occur the products of which presumably stimulate further polyketide synthesis at greatly enhanced levels, the positive feedback loop would thus not be broken and the tylactone produced, rather than accumulate, would be converted to tylosin, leading to the 'normal' tylosin-producing phenotype. The mechanism by which the proposed stimulation occurs is unknown.

### 3.9.6 Is synthesis of 5-*O*-mycarosyltylactone a function of TylMII ?

It is not clear which glycosyltransferase might be responsible for the addition of mycarose to tylactone. The two most obvious candidates are the mycaminosyltransferase or the mycarosyltransferase. In the first instance it is necessary to envisage the unlikely situation in which the wrong sugar, the neutral, L - sugar as opposed to amino D- sugar is recognised by the mycaminosyltransferase, TylMII, and added to the normal substrate, tylactone. Relaxed specificity for sugar substrate has been demonstrated for other glycosyltransferases such as those encoded by *gtfE* (from the vancomycin producer, *Amycolatopsis orientalis*) (Solenberg *et al.*, 1997) and *oleG2* (from the oleanomycin producer, *Streptomyces antibioticus* (Olano *et al.*, 1998; Doumith *et al.*, 1999) when expressed in heterologous strains. More importantly in this context, relaxed specificity of glycosyltransferases has also been demonstrated when deoxyhexose sugar genes have been inactivated, such as in the pathways to erythromycin (Gaisser *et al.*, 1998) and methymycin/neomethymycin (Zhao *et al.*, 1998) from *Saccharopolyspora erythraea* and *S. venezulae* respectively. Of particular interest is a report (Wohlert *et al.*, 1998) of a glycosyltransferase from *Streptomyces olivaceous* whose flexibility in the choice of sugar substrate added to an aglycone ranges from D- to L- sugars and even includes disaccharides.

Alternatively, if the mycarosyltransferase, TylCV, is responsible for producing mycarosyl tylactone then it must add the correct sugar, mycarose, to the wrong substrate (a polyketide ring as opposed to a sugar). Certainly, relaxed specificity for macrolactone substrate has previously been demonstrated (Doumith *et al.*, 1999; Spagnoli *et al.*, 1983). Either situation would require a remarkably versatile enzyme and it is thus a likely possibility that this conversion is the product of a different enzyme, perhaps even one specific for the task, as yet unidentified.

It is interesting to note that whereas 5-*O*-mycarosyltylactone can be identified in fermentation extracts of *tylMI*-KO fed OMT, no trace can be found in fermentation extracts of *tylMII*-KO similarly fed, by either HPLC or mass spectrometry (section 3.7.3). This evidence while being far from unequivocal, does provide some evidence to support the unlikely possibility that *tylMII* encodes the enzyme responsible for the synthesis of 5-*O*-mycarosyltylactone and is thus capable of utilising both L- and D- sugars as substrate. If this was the case then it could explain the contradictory feeding data. In the tylactone-deficient strains, any 5-*O*-mycarosyltylactone degraded to tylactone could be converted straight back and thus would not accumulate. This could not occur in *tylMII*-KO. Repeating the same experiment in *tylMI*-KO might therefore produce a different result. In

*Saccharopolyspora erythraea* a system was designed for testing the activity of polyketide glycosyltransferases against the activated sugars L-mycarose and D-desosamine (whose structure is closely related to that of D-mycaminose) (Gaisser *et al.*, 2000). This was done by generating a strain disrupted in its normal polyketide synthase and glycosyltransferase functions in which heterologous glycosyltransferases could be expressed. When *tylMII* was expressed in this strain, which was fed tylactone, 5-*O*-desosaminyltylactone was produced (Gaisser *et al.*, 2000). This indicated that, not unexpectedly, *tylMII* could utilise desosamine in place of mycaminose. No trace of 5-*O*-mycarosyltylactone or 5-*O*-cladinosyltylactone was reported (in the synthesis of erythromycin mycarose is modified following addition to the polyketide ring by *O*-methylation of the mycarosyl group (Haydock *et al.*, 1991). This indicates that if *tylMII* can utilise L-mycarose, then it does so extremely inefficiently in the presence of alternative substrates.

### 3.9.7 Stimulation of polyketide synthesis by glcosylated tylosin precursors

The stimulation of polyketide synthesis by glycosylated intermediates of the tylosin biosynthetic pathway raised the intriguing possibility that other glycosylated macrolides might also be capable of stimulating a similar effect. In order to address this question, *tylMII*-KO was fed with a number of different macrolide antibiotics, with varying similarities to tylosin (Figure 3.4). It was hoped that differences in the ability of these compounds to stimulate might give interesting clues regarding the chemical characteristics important in the protein binding that must occur as the first step in mediating polyketide stimulation. The characteristics of the glycosylated intermediates of the tylosin is the obvious starting point for such a discussion

It has been shown that tylactone does not stimulate its own biosynthesis. The compound that stimulates tylactone synthesis with the greatest efficiency, OMT, differs from tylactone in three ways (Figure 3.25). Firstly, and most obviously it is glycosylated on C5 with the amino sugar mycaminose. In addition it is oxidised at C20 and C23 to convert the polyketide ring into tylonolide and produce OMT.The oxidation at C23 allows the addition of 6-deoxyallose at this position and since compounds thus glycosylated still stimulate polyketide synthesis, albeit with a lower efficiency this oxidation cannot be responsible for bestowing any stimulatory ability on the molecule. Indeed the fact that the addition of a sugar moiety does not abolish, merely reduces, the stimulatory ability of the molecule indicates that this portion of the polyketide ring is unlikely to be integral to the proposed protein interaction that occurs. The oxidation at C20 might however be more important in this respect. This alteration and addition of the amino sugar, mycaminose which lies very close to it, thus seem to be the key to a molecule capable of polyketide

stimulation. The protein binding which motivates this process must therefore be presupposed to involve this region of the molecule. Interestingly, the compound 5-*O*-mycarosyltylactone, which does not appear to stimulate polyketide synthesis is substituted with the neutral sugar mycarose instead on the amino sugar mycaminose and remains un-oxidised at the C20 position. It would be interesting to determine whether 5-*O*-mycaminosyltylactone (unmodified at C20) was capable of stimulation. Unfortunately, no pure samples of this compound are currently available for this analysis and it is thus not possible to determine whether the amino sugar alone bestows stimulatory characteristics on the macrolide. The subsequent substituition of a sugar, mycarose, onto mycaminose does not abolish stimulatory activity. Thus, the amino sugar (or at least a sugar group) while probably essential, is unlikely to be integral to the protein interaction proposed. Post-mycaminose glycosylation of the molecule only reduces the efficiency of the stimulatory effect, with tylosin itself being least efficient (Fish and Cundliffe, 1997).

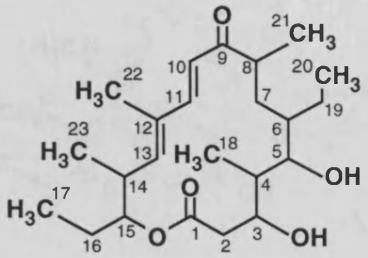
### 3.9.8 Glycosylated macrolides that stimulate polyketide synthesis

Rosaramicin differs only slightly to OMT and was therefore a likely candidate to mimic its effect on polyketide synthesis. When compared to OMT (using the same ring numbering system as applied to tylosin) it differs in containing an epoxy group between C12 and C13 in its polyketide ring and being substituted with the amino sugar desosamine in place of mycaminose (Figure 3.26). Similarly it is not surprising that spiramycin can mimic tylosin and stimulate polyketide metabolism. The 16-membered polyketide ring is similar to tylonolide and the sugar groups mycaminose and mycarose are attached as they are in tylosin. The carbon at position 3 (again using the same ring numbering system as applied to tylosin) can be attached to various groups, OH as it is in the tylosin metabolites or a two or three carbon chain attached by an ester bond ($OCOCH_3$ or $OCOCH_2CH_3$). The spiramycin polyketide also contains a single carbon aldehyde group on the un-oxidised carbon at position 20 compared to tylonolide. This difference, although fairly minor perhaps suggests that oxidation of the tylactone ring at this position, might not be an important characteristic of a stimulatory molecule. This would indicate (with reference to the stimulatory ability of tylosin intermediates) that addition of the amino sugar is the single crucial difference separating a molecule capable of stimulation (OMT) and one that is not (tylactone). Thus, in the case of rosaramicin, the aminosugar desosamine can mimic mycaminose in provoking the stimulatory response. Again, unlike tylosin, spiramycin has a sugar moiety, forosamine substituted on to the carbon at position 9. This is a far more major difference and might be expected to disrupt any protein interaction in which this part of the molecule played an important part. Nevertheless spiramycin, like tylosin, is still

capable of stimulating polyketide synthesis. This indicates that this part of the molecule is probably not important in the protein interaction that mediates the polyketide stimulation. There remain two other differences between spiramycin and tylosin. At C12, the spiramycin polyketide is unmethylated compared to tylonolide and C17 is absent. Despite these many differences, spiramycin clearly resembles tylosin in whichever aspects are most important for causing the enhancement in tylactone production. Indeed, it is even possible to speculate that the similarity of rosaramicin to OMT and spiramycin to tylosin might account for the differing efficiencies with which these molecules stimulate tylactone production, since OMT and rosaramicin stimulate with greater efficiency than tylosin and spiramycin.

### 3.9.9 Glycosylated macrolides that do not stimulate polyketide synthesis

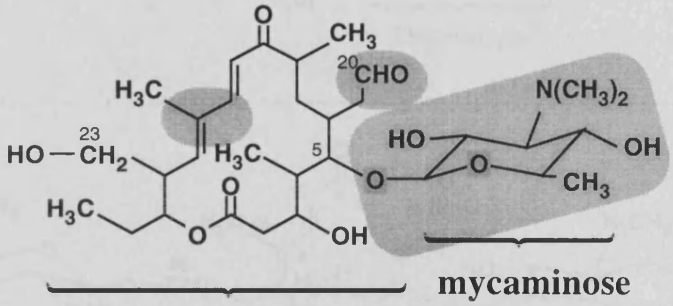
The other glycosylated macrolides fed, carbomycin, chalcomycin and erythromycin, did not stimulate tylactone accumulation. Of these, the inability of carbomycin to stimulate polyketide synthesis is the most informative. Carbomycin is similar to tylosin and spiramycin in that the sugar groups mycaminose and mycarose are substituted onto its 16-membered polyketide ring in the same relative positions. The mycarose sugar is however modified on C4" by addition of a branched five carbon chain attached by an ester bond ($OCOCH_2CH_2(CH_3)_2$) (Figure 3.27). The addition of mycarose (and mycinose) to precursors of tylosin reduces the efficiency of polyketide stimulation (Fish and Cundliffe, 1997). It is thus possible that a group additional to mycarose would reduce this efficiency to nothing. The protein binding, which by some undefined mechanism then provokes polyketide synthesis, has been speculated to involve the amino sugar. When mycarose is subsequently added the efficiency of stimulation is only reduced. Thus the presence of an additional carbon chain, on the far side of the mycarose sugar seems unlikely to interfere with the protein interaction to any much greater extent than the addition of mycarose itself. It is therefore unlikely that the presence of the five carbon chain attached to mycarose at C4" would completely destroy the stimulatory capacity of the molecule. Carbomycin resembles spiramycin at C20 of its polyketide ring (using the same ring numbering system as applied to tylosin). This difference from the tylosin metabolites is thus unlikely to be responsible for its lack of stimulatory activity. On the other side of the polyketide ring, carbomycin resembles rosaramicin in that C12 and C13 are epoxidised but spiramycin in that C12 is unmethylated and C17 of the ring is absent. These three differences in the ring from tylonolide are thus also unlikely to be the cause of carbomycins' failure as a stimulatory molecule.



**Tylactone (protylonolide)**

**vs.**

**OMT**



**mycaminose**

**tylonolide**

**Figure 3.25 Comparison of the structures of tylactone and OMT.** The un-elaborated polyketide ring, tylactone (also known as protylonolide) is incapable of stimulating its own synthesis in *S. fradiae*. The tylosin intermediate OMT (5-*O*-mycaminosyltylonolide), which is capable of stimulating tylactone synthesis, differs from tylactone in a limited number of ways. Substituition of the amino sugar, mycaminose, onto C5 is the most obvious difference but the ring is also oxidised at C20 and C23. These modifications are indicated here (shaded boxes).

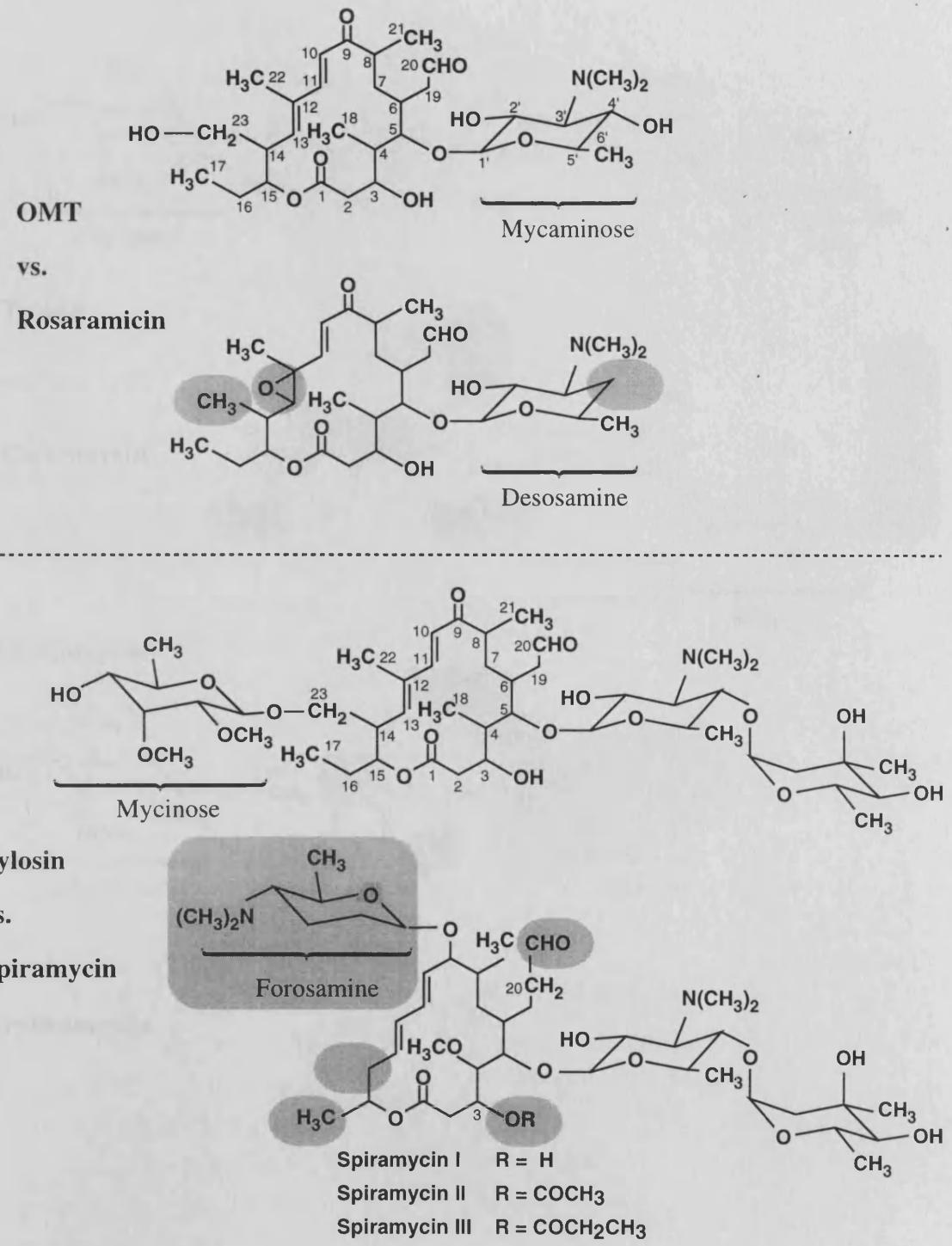



**Figure 3.26 Structures of the macrolides that stimulate polyketide synthesis in *tylMII*-KO.** The macrolide antibiotics rosaramicin and spiramycin, not produced in *S. fradiae* but nevertheless shown to stimulate tylactone accumulation in *tylMII*-KO are structurally comparable with OMT and tylosin respectively. The differences between the molecules have been indicated (shaded boxes) in each case.

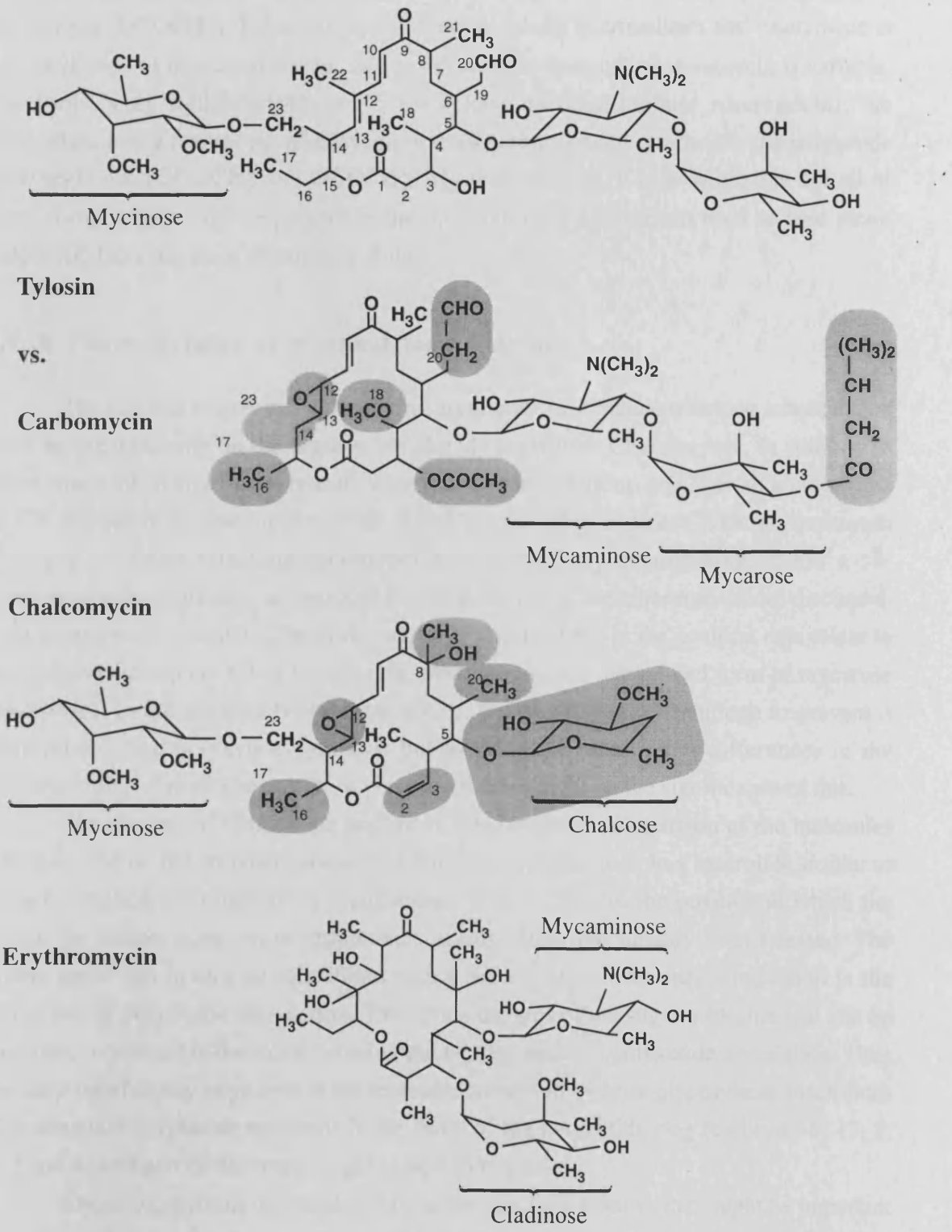



**Figure 3.27 Structures of the macrolides that do not stimulate polyketide synthesis in *tylMII*-KO.** The macrolides carbomycin, chalcomycin and erythromycin, which do not stimulate polyketide synthesis, in *S. fradiae* are compared with tylosin (which does). The difference between tylosin and the other macrolides are indicated (shaded boxes).

A two carbon chain is attached by an ester bond to C3 in the polyketide ring of carbomycin ($OCOCH_3$). The equivalent position in tylosin intermediates and rosaramicin is hydroxylated. As discussed earlier, this position in the structure of spiramycin is variable, one form being hydroxylated (as in the tylosin metabolites and rosaramicin), the alternatives being two (as in carbomycin) or three carbon chains attached to the polyketide by ester bonds ($OCOCH_3$ and $OCOCH_2CH_3$ respectively). It is possible that not all of these forms which may be present in the preparation of spiramycin used to feed strain *tylMII*-KO have the same stimulatory ability.

### 3.9.10 Characteristics of a stimulatory molecule

The fact that neither chalcomycin nor erythromycin stimulate tylactone accumulation have no great bearing on the arguements already presented. Chalcomycin, in addition to other minor differences from tylosin possesses a hydroxyl group on C3 or an amino sugar on C5. Instead it is substituted with the deoxyhexose sugar, chalcose. That erythromycin does not stimulate tylactone accumulation is particularly unsurprising. It has a 14-membered polyketide ring, as opposed to 16-membered in the other macrolides discussed. Like rosaramicin it contains the amino sugar desosamine but in the position equivalent to the hydroxyl group on C3 of tylosin it is substituted with a methylated form of mycarose (cladinose). In the arguments presented above, this alone would be enough to prevent it from stimulating polyketide synthesis but as there are many other differences in the polyketide ring it would be unwise to place too much weight on the significance of this.

The process of elimination applied in this structural comparison of the molecules which do and do not stimulate polyketide synthesis is suggestive. In a macrolide similar to tylosin, position C3 might be of significance. This is close to the position at which the amino the amino sugar whose importance in this matter has already been stressed. The amino sugar can in turn be substituted with a second sugar with only a reduction in the efficiency of polyketide stimulation. This limits the area of the sugar molecule that can be supposed to interact in the initial protein binding that mediates polyketide stimulation. Thus the only significantly large area of the molecule conserved in those glycosylated macrolides that stimulate polyketide synthesis is the 'base' of the polyketide ring (carbons 16, 17, 1, 2, 3 and 4) and part of the amino sugar closest to this area.

These experiments do therefore hint at the structural features that might be important in a glycosylated macrolide in order to elicit the large enhancement of tylactone synthesis observed in mycaminose deficient strains. In addition they also demonstrate unequivocally that the accumulation of tylactone cannot have been derived from breakdown of the fed compounds and must have been the result of *de novo* polyketide synthesis.

# CHAPTER 4

# TRANSCRIPTIONAL ORGANISATION OF THE *tylM* REGION

## 4.1 Introduction

As described in Chapter 3, strains disrupted in all the mycaminose genes have now been generated and shown to behave similarly with respect to production of the polyketide, tylactone, in the presence and absence of its glycosylated forms. Three of these mycaminose genes, *tylMIII*, *tylMII* and *tylMI* , and the polyketide ancillary gene *ccr* are located immediately downstream and similarly oriented to the polyketide synthase genes (*tylGI-tylGV*). The resistance determinant, *tlrD* (orf5*) borders the *tylM* region at the other end, reading in the opposite direction. As is typical of this and other antibiotic gene clusters found in actinomycetes the genes are tightly packed. Indeed the open reading frames of *tylGV* (the final module in the polyketide synthase cluster), *tylMIII* and *tylMII*; and *tylMI* and *ccr* are terminally overlapping (Figure 4.1). In this extreme example of tight clustering, the TGA 'stop' codon of one open reading frame overlaps with the GTG or, less commonly in *Streptomyces,* ATG 'start' codon of the next. This generates the DNA sequence motif GTGA (or ATGA). The terminal overlap of open reading frames is a commonly encountered occurrence in *Streptomyces* and although protein coding sequences do not necessarily begin at the earliest possible candidate start, it has been suggested that such overlap implies translational coupling of the genes (Arrowsmith *et al.*, 1992; Sherman *et al.*, 1989).

It has recently been proposed that only four major polycistronic transcripts, two of which are nested, cover the erythromycin gene cluster of *Saccharopolyspora erythraea* (Reeves *et al.*, 1999). These major polycistronic transcripts, along with four monocistronic ones, account for the entire cluster. Apart from the nested transcript, this is the minimum number of trnascripts and promoters that would be theoretically required, given the relative arrangement of the genes. This model requires that a single promoter drives transcription of the entire *eryA* polyketide synthase complex and four downstream genes, approximately 35 kb in length. If a similar model applies to the tylosin cluster then *tylGV* (and indeed the upstream *tylG* domains) should be co-transcribed with the *tylM* genes and *ccr*. In order to test this model, a strain disrupted in *tylGV* was generated. Alternatively, if the *tylM* genes are transcribed independently of *tylGV*, then a promoter must be located within *tylGV* itself. The disruption site was therefore positioned relatively early in the sequence of *tylGV* such that any promoter embedded within the upstream gene would most likely remain intact.

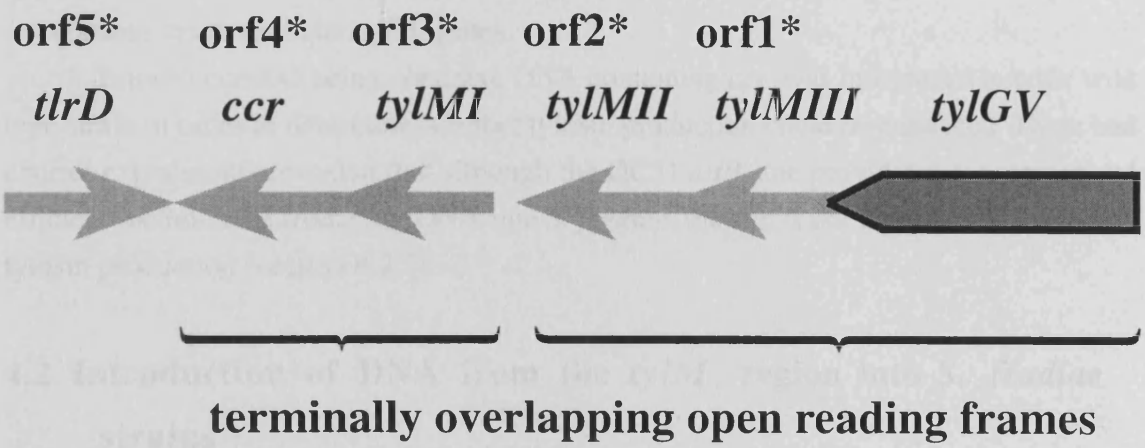



**Figure 4.1 Terminally overlapping open reading frames in the *tylM* region.** The *tylGV* open reading frame terminally overlaps with that corresponding to *tylMIII* (orf1*) which in turn overlaps with that of *tylMII* (orf2*). Similarly the *tylMI* open reading frame (orf3*) overlaps with that of *ccr* (orf4*). The resistence determinant *tlrD* is oriented in the opposite direction. It has been suggested that terminally overlapping coding regions implies translational coupling.

The *tylM* genes encode methyltransferase (*tylMI*), mycaminosyltransferase *tylMII*) and isomerase activities (*tylMIII*) involved in the synthesis and subsequent addition of mycaminose to tylactone. DNA fragments from the *tylM* region, containing complete open reading frames, were introduced into the *tylM* disrupted strains in order to complement the disruptions. This was particularly important in the case of the *tylMIII* (orf1*) disrupted strain, *tylMIII*-KO, in order to demonstrate unambiguously that *tylMIII* is a mycaminose specific gene (as suggested in section 3.7.2) and that the disruption had not merely affected the expression of a downstream mycaminose gene. It was hoped, that this approach would also provide information regarding the transcriptional organisation of the *tylM* genes. A strain disrupted in *ccr* (*ccr*-KO) produced tylosin (section 3.6.1) and thus these experiments were not capable of revealing any information regarding its transcriptional relationship to the upstream *tylM* genes.

Prior to *ccr*-KO being obtained, DNA containing *ccr* was introduced into the wild type strain in order to determine whether tylosin production could be enhanced. These and control experiments revealed that although the ØC31 *attB* site provides a convenient and efficient method of introducing DNA into *S. fradiae*, the site is not neutral with respect to tylosin production (section 4.2.3).

## 4.2 Introduction of DNA from the *tylM* region into *S. fradiae* strains

### 4.2.1 Integration of genes into the ØC31 *attB* site

It was originally intended that constructs containing particular genes, governed by the *ermE** promoter, might be used to overexpress genes in the wild type to see if antibiotic production yields could be enhanced. This was attempted speculatively with *ccr*, prior to *ccr*-KO having been generated. The dissappointing results obtained indicated that the integration site might not be neutral with respect to tylosin biosynthesis (section 4.3). Nevertheless the ability to stably introduce genes by integration into the ØC31 *attB* site still provides a useful tool in the investigation of *S. fradiae* genes. By reintroducing appropriate genes, it is possible to determine whether the phenotypic effects of gene disruptions are due to loss of the disrupted gene alone or are influenced by possible downstream ettects on other genes. Extending this theme, the patterns of transcription within a particular region can be identified by determining which downstream genes, if any, need to be introduced into a disrupted strain in order to restore its original phenotype. In order to facilitate analysis of the *tylM* region, a library of plasmids was constructed in pLST9828 containing open reading frames from the *tylM* region (A. R. Butler, personal communication), these

are described in the next section. Use of these plasmids allowed fragments of DNA from the *tylM* region to be integrated site specifically into the ØC31 *attB* site in the genome of *S. fradiae*. Plasmids were constructed so as to allow expression to be driven by the strong constitutive promoter *ermE*p*. In each case fragments were excised from *S. fradiae* using appropriate restriction endonucleases (Figure 4.2) as described in the next section.

### 4.2.2 Vectors for the integration of DNA from the *tylM* region

Vectors based on pLST9828 were constructed to facilitate the introduction of seven different fragments from the *tylM* region to various strains (Figure 4.2) (A. R. Butler, personal communication). The fragments generated were:

(i) a 1.5 kb *Bss* SI fragment containing *tylMIII* (orf1*) and flanking DNA (83 bp upstream and 146 bp downstream);

(ii) a 1.7 kb *Bst* EII - *Msc* I fragment containing *tylMII* (orf2*) and flanking DNA (55 bp upstream and 254 bp downstream);

(iii) a 1.4 kb *Sal* I - *Sst* I fragment containing *tylMI* (orf3*) and flanking DNA (389 bp upstream and 228 bp downstream);

(iv) a 1.4 kb *Bss* HII fragment containing *ccr* and flanking DNA (101 bp upstream and 25 bp downstream);

(v) a 3.0 kb *Msc* I - *Sph* I fragment containing *tylMIII* and *tylMII* (orfs 1* and 2*) and flanking DNA (195 bp upstream and 194 bp downstream);

(vi) a 4.3 kb *Psh* AI - *Sst* I fragment containing *tylMIII, tylMII* and *tylMI* (orfs 1*, 2* and 3*) and flanking DNA (538 bp upstream and 228 bp downstream); and

(vii) a 5.9 kb *Msc* I - *Sph* I fragment containing *tylMIII, tylMII, tylMI* and *ccr* and flanking DNA (195 bp upstream and 1012 bp downstream) (a strain incorporating this fragment is described in chapter 5).

### 4.2.3 The ØC31 *attB* site is not neutral with respect to tylosin biosynthesis

The *ccr* gene was introduced into the wild type strain in a speculative experiment to determine whether tylosin yields could be improved by its overexpression (carried out before *ccr*-KO was obtained). Both the strain with *ccr* introduced and the control strain (wild type with the 'empty vector' pLST9828 alone introduced) displayed a reduced yield of tylosin relative to wild type (Figure 4.3). This provided the first evidence that introduction of pLST9828 into the ØC31 *attB* site is not neutral with respect to tylosin biosynthesis under the conditions used, resulting in a reduction in fermentation yield.

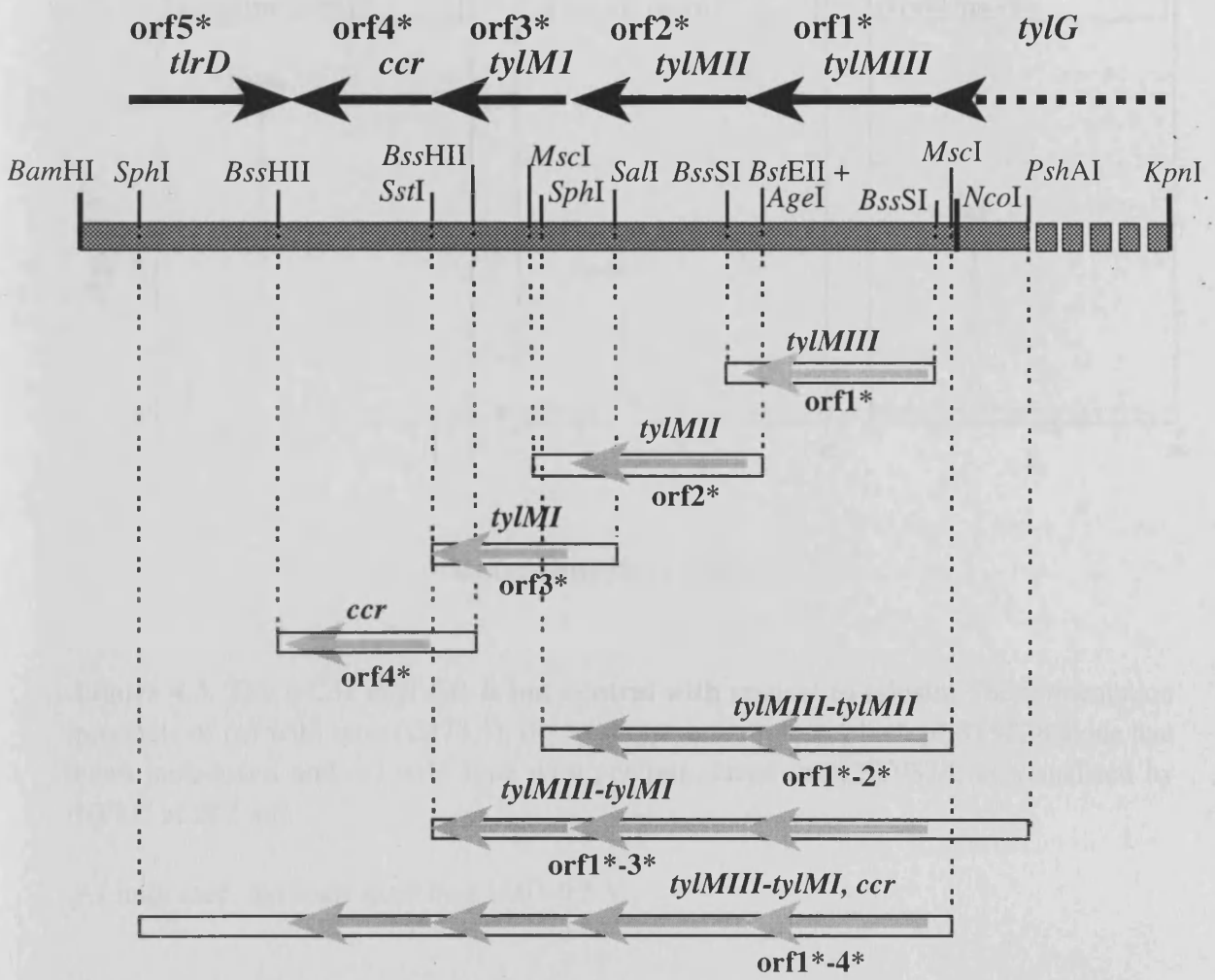



**Figure 4.2 Fragments from the *tylM* region integrated into *S. fradiae* strains.** Fragments of DNA from the *tylM* region, designed to contain complete open reading frames, were cloned into pLST9828 to facilitate their introduction into *S. fradiae* strains (A. R. Butler, personal communication). Fragments were oriented to ensure that the genes within would be governed by the *ermE** promoter.

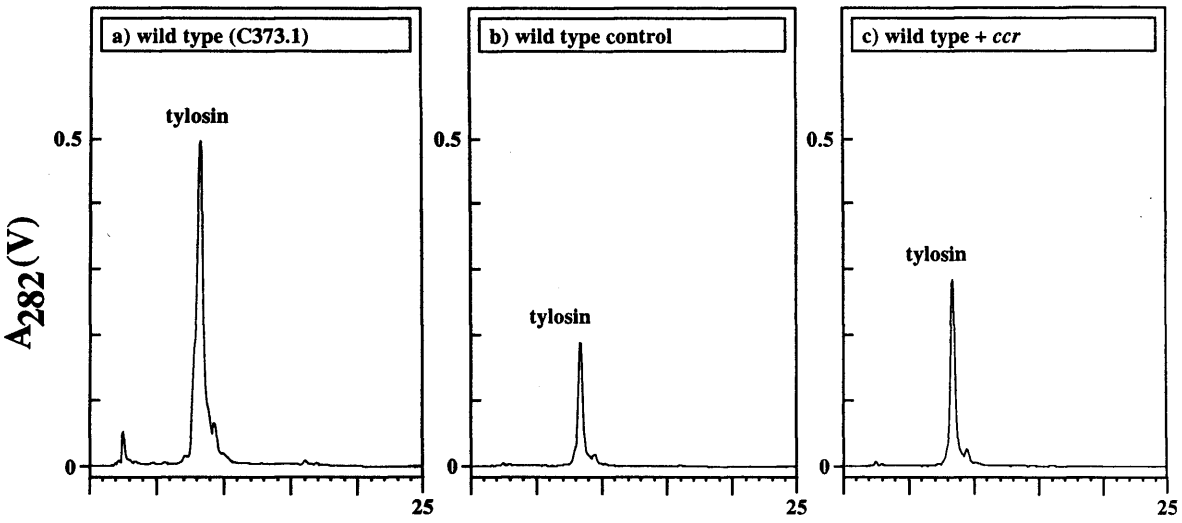



**Figure 4.3. The ϕC31 *attB* site is not neutral with respect to tylosin.** The fermentation products of (a) wild type (C373.1), (b) wild type control into which pLST9828 alone had been introduced and (c) wild type with *ccr* introduced in pLST9828, as visualised by HPLC at 282 nm.

As indicated, the scale used here is 0 - 0.5 V.

Although this observation was never formally tested in experiments likely to produce statistically significant data, it was repeated in many other strains in a variety of other experiments during this project. Similar observations have also been made elsewhere in the laboratory (N. Bate, personal communication). Thus although the ØC31 *attB* site provides a fast and efficient means of introducing DNA into *S. fradiae* to obtain qualitative data, quantitative observations arising from such experiments are unlikely to be reliable unless extremely pronounced changes are evident.

## 4.3 Transcriptional organisation in the *tylM* region

### 4.3.1 Complementation of *tylM*-KO strains

Each of the *tylM* disrupted strains, was incapable of accumulating significant quantities of any tylosin metabolite unless tylactone accumulation was stimulated with exogenous glycosylated macrolide (Chapter 3). Tylosin production was restored in each of the three *tylM* disrupted strains when DNA containing the whole of *tylMIII, tylMII* and *tylMI* was introduced into the ØC31 *attB* site governed by the *ermE* * promoter (Figure 4.4). It was not necessary to include *ccr* in the fragment of complementing DNA. This was expected, since strains specifically disrupted in *ccr* continue to produce tylosin (section 3.6.1). Thus if disruptions in the *tylM* genes do exert downstream effects on *ccr* then they would not be manifested under the experimental conditions applied here.

### 4.3.2 The *tylM* genes are co-transcribed

Tylosin production was restored in *tylMI*-KO (orf3* disrupted strain) into which DNA containing the whole of *tylMI* was reintroduced (Figure 4.5). Re-introduction of DNA containing the whole of *tylMII* into *tylMII*-KO did not however result in an equivalent restoration of tylosin production. Unexpectedly, analysis of the fermentation products of this strain revealed the accumulation of tylactone in a manner reminiscent of a *tylM* disrupted strain fed with a glycosylated precursor of tylosin. No glycosylated compounds could be identified by HPLC which could have accounted for this unexpected polyketide accumulation. Nevertheless, the inability of the strain to produce tylosin suggests that *tylMII* must be transcriptionally linked to *tylMI*. This was confirmed a fragment of DNA containing both *tylMII* and *tylMI* proved capable of complementing the *tylMII* disrupted strain and restoring tylosin biosynthesis (Figure 4.5).

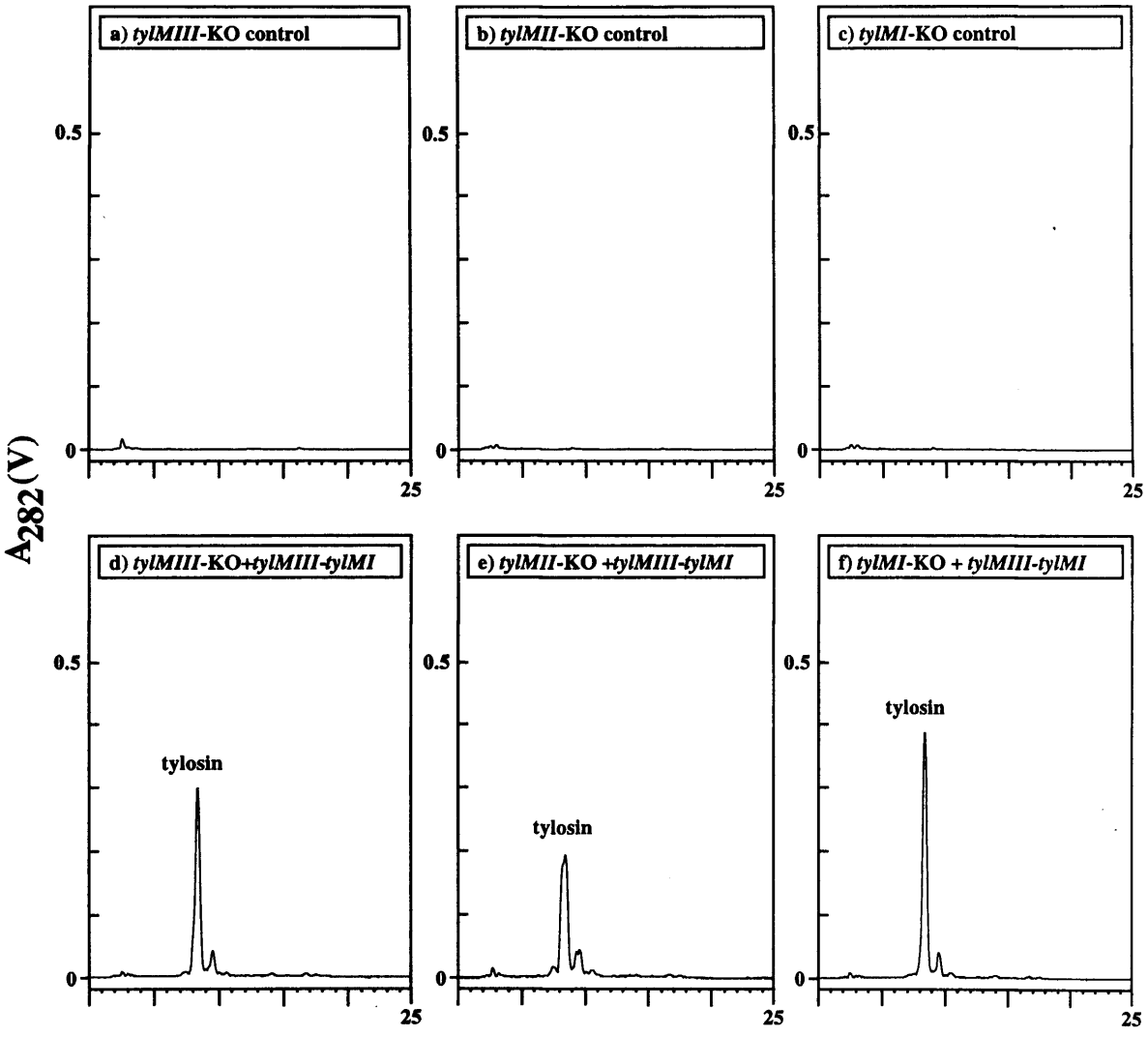



**Figure 4.4. HPLC analysis of *tylM* disrupted strains complemented with all three tylM genes.** The fermentation products of (a) *tylMIII*-KO, (b) *tylMII*-KO, (c) *tylMI*-KO control strains (with pLST 9828 alone introduced) and (d) *tylMIII*-KO, (e) *tylMII*-KO and (f) *tylMI*-KO each with *tylMIII*, *tylMII* and *tylMI* introduced in pLST9828, as visualised by HPLC at 282 nm.

As indicated, the scale used here is 0 - 0.5 V.

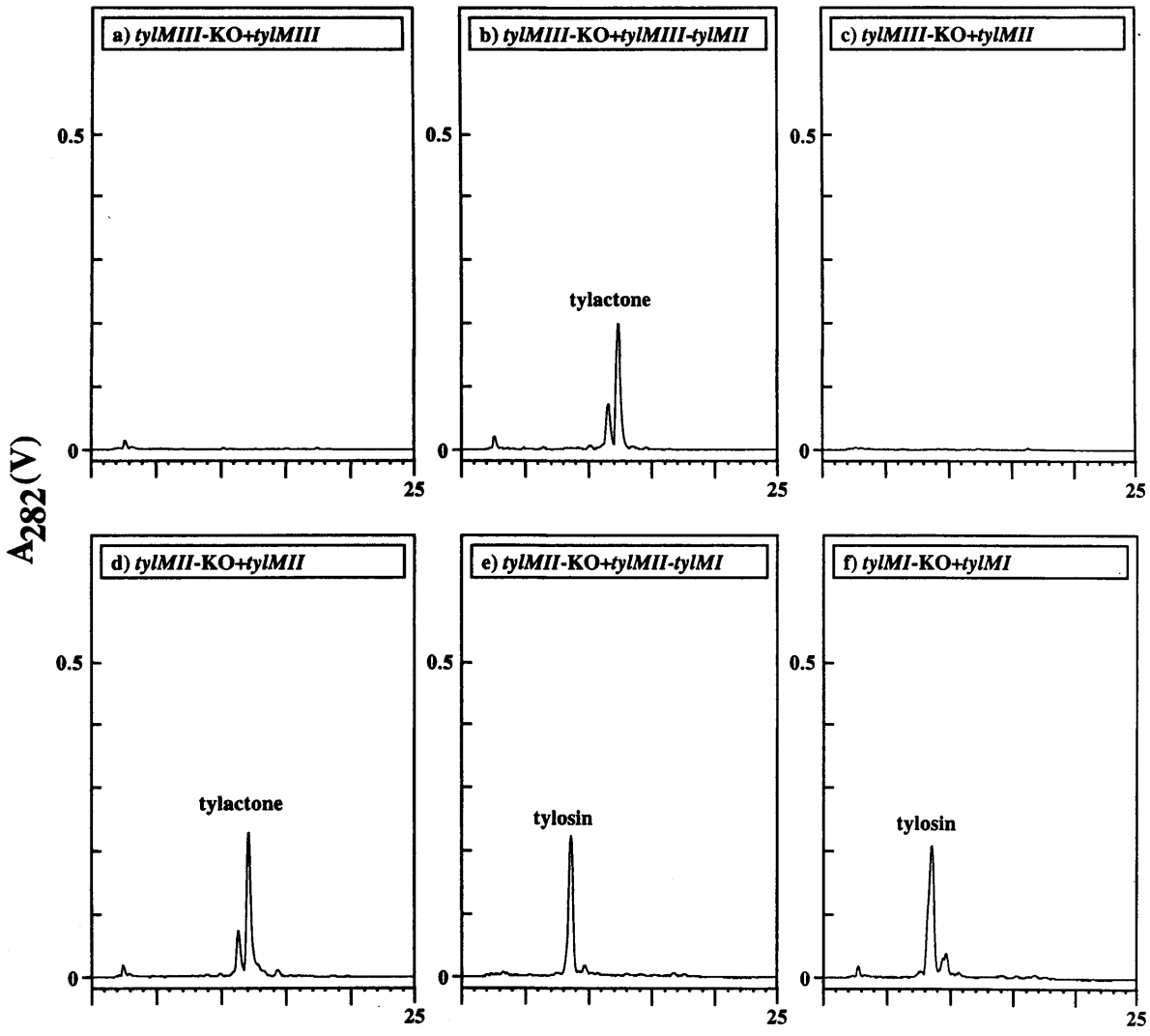



**Figure 4.5. HPLC analysis of *tylM* disrupted strains with *tylM* DNA introduced.** The fermentation products of *tylMIII*-KO with (a) *tylMIII* re-introduced, (b) *tylMII* introduced and (c) *tylMII* introduced, and *tylMII*-KO with (d) *tylMII* re-introduced, (e) *tylMII* and *tylMI* introduced, and (f) *tylMI*-KO with *tylMI* re-introduced, as visualised by HPLC at 282 nm.

As indicated, the scale used here is 0 - 0.5 V.

Re-introduction of DNA containing the whole of *tylMIII* into *tylMIII*-KO had no discernable effect on its non-producing phenotype in fermentation, but introduction of both *tylMIII* and *tylMII* caused the accumulation of tylactone. Again, no significant trace of tylosin or any other glycosylated product could be detected by HPLC, just the accumulation of tylactone. That *tylMIII*-KO and *tylMII*-KO remained incapable of glycosylating the accumulated tylactone in detectable amounts, despite the re-introduction of the disrupted gene indicated that mycaminose metabolism had not been restored. Thus, the disruptions in *tylMIII* and *tylMII* must exert polar effects on downstream mycaminose genes and therefore be co-transcribed. That all the downstream *tylM* genes need to be introduced for tylosin synthesis to be restored indicates that all three *tylM* genes must be expressed from the same transcript.

### 4.3.3 *tylMIII* (orf1*) is a mycaminose specific gene

From the above results, it was not clear that *tylMIII* had any importance in mycaminose metabolism in *S. fradiae*. It was important to introduce *tylMII* alone into *tylMIII*-KO in order to demonstrate that the unexpected alteration in phenotype when *tylMIII* and *tylMII* together were introduced (accumulation of tylactone) would not be replicated. This would imply that the mycaminose specific phenotype (non-production unless exogenously fed with glycosylated tylosin intermediates) of *tylMIII*-KO was due to downstream polar effects on *tylMII* and not a disruption in a genuine mycaminose gene. This did not prove to be the case. When the fermentation products of *tylMIII*-KO with *tylMII* re-introduced were analysed no tylosin metabolites were evident (Figure 4.5). Since both *tylMIII* and *tylMII* need to be introduced for any effect on tylosin metabolism to be manifested in *tylMIII*-KO, *tylMIII* must be a genuine mycaminose gene and transcriptionally linked to *tylMII*. That all three *tylM* genes need to be introduced in order to achieve restoration of tylosin synthesis (Figure 4.4) indicates that it must also be transcriptionally linked to *tylMI*.

### 4.3.4 The unexpected accumulation of tylactone in some *tylM* strains

Tylactone unexpectedly accumulated in *tylMIII*-KO with *tylMIII* and *tylMII* introduced and *tylMII*-KO with *tylMII* re-introduced. Both of these strains should contain functional copies of *tylMIII* and *tylMII* but, by deduction, must be adversely affected in *tylMI* in order to cause a blockage in the glycosylation of tylactone. These two strains might be expected to behave like the *tylMI* (orf3*) disrupted strain itself, *tylMI*-KO. This strain should similarly contain functional copies of *tylMIII* and *tylMII* but be disrupted in

*tylMI* . However, like the other *tylM* disrupted strains, *tylMI*-KO does not accumulate any identifiable tylosin intermediates unless the fermentation is supplemented with glycosylated tylosin intermediates thereby stimulating tylactone accumulation.

Feeding *tylMII*-KO with varying amounts of OMT during fermentation revealed two important characteristics of the tylactone stimulation phenomenon (section 3.8.1). Firstly, the level of tylactone accumulated was not proportional to the amount of OMT fed during fermentation, rather, an 'all or nothing' response was observed. Secondly, a relatively very small quantity of extraneous OMT was sufficient to induce the accumulation of tylactone. Certainly, this is far less than can be convincingly detected by HPLC analysis in the system applied.

If low level expression of *tylMI* could occur, despite disruptions in upstream genes, it would be possible to reconcile the unexpected accumulation of tylactone in the strains described with the model of co-transcription of the *tylM* genes. A 'promoter' capable of driving low levels of *tylMI* expression might thus cause a small amount of mycaminose to be produced. This could then be added to the low levels of tylactone proposed to exist in mycaminose deficient strains. Thus sufficient glycosylated polyketide might be produced to stimulate tylactone accumulation at the much higher level. The small amount of glycosylated compound produced would of course be converted to tylosin, but must be present in such small quantity as to be undetectable by HPLC. Thus although the effects of the glycosylated compound are clearly manifested, the compound itself is not. The postulated 'promoter' would have to be located downstream of the site of the gene disruption in *tylMII* and (obviously) upstream of the translational start of *tylMI*. Promoter probe analysis of this fragment of DNA was therefore carried out (section 4.4.1).

## 4.4 Promoter probe analysis of the region upstream of *tylMI*

### 4.4.1 Construction of promoter-probe vectors

The possibility that there may be promoter activity associated with the DNA upstream of the beginning of *tylMI* was investigated by promoter-probe analysis. A 0.85 kb *Fok*I fragment containing the region in between the *Aat*II site at which the hygromycin cassette was inserted in order to generate the *tylMII* disruption construct (Fish and Cundliffe, 1997) and the proposed translational start of *tylMI* (Gandecha *et al.*, 1997) was isolated from pLST97A, end-filled and ligated into the *Hin*cII site of pIJ2925. This fragment contains 10 bp upstream of the point at which the hygromycin cassette was inserted into *tylMII* and the first base pair of the ATG start codon of *tylMI*. The fragment was excised from pIJ2925 using *Bgl*II and ligated into the *Bam*HI site upstream of the

*redD* reporter gene of the promoter probe vector pIJ2585 (M. J. Bibb, personal communication). The plasmid was obtained with the insert in both orientations with respect to *redD*. These vectors, along with pIJ2585 itself and pIJ2585 containing *ermE*p* oriented so as to force expression of *redD* (G. Stratigopoulos, personal communication) (Figure 4.6) were then introduced into *S. lividans*.

### 4.4.2 The region immediately upstream of *tylMI* has promoter activity

Colonies containing the 0.85 kb region immediately upstream of *tylMI* in each orientation were isolated at random in triplicate and grown on R2YE alongside pIJ2585 with no insert and containing *ermE*p* (Figure 2.7). As expected, very strong red colouration was associated with the vector containing *ermE* p* after two days. The vector with no insert displyed very little red colouration after two days and some colouration after three. In both orientations the fragement immediately upstream of tylMI displayed limited red colouration after two days in all three isolates. However, after both two and three days the colouration caused by the *tylMI* upstream fragment in the 'correct' orientation was marginally more intense.

## 4.5 Transcription of the *tylM* genes is independant of *tylG*

### 4.5.1 Organisation of the *tylM* region

As discussed earlier (section 1.3.1) the five mega gene polyketide synthase cluster (*tylG*) lies immediately upstream of, and similarly oriented to, the *tylM* genes already shown to be co-transribed. If the pattern of transcription within the tylosin gene cluster fits that proposed for the erythromycin cluster (Reeves *et al.*, 1999), the entire *tylG* mega gene complex and the four genes of the *tylM* region should all be expressed from the same enormous transcript (45 kb). Alternatively transcription in the *tylM* region might be independent of *tylG*. In this case a promoter for these downstream genes must be located within *tylGV*. A vector was designed, competent to generate a disruption early in *tylGV*, so as to minimise the chances of inadvertently disrupting or separating any such promoter from the downstream genes.

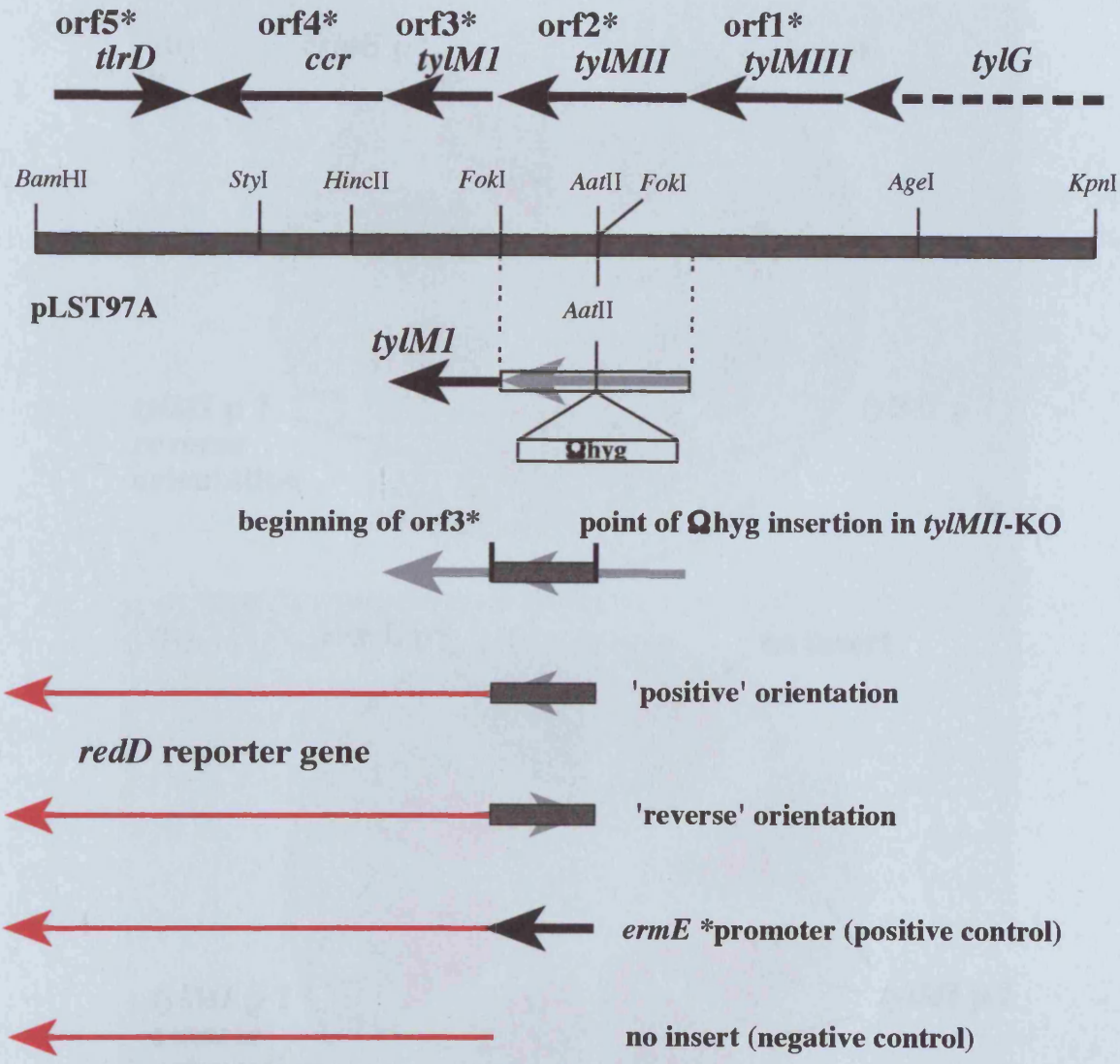



**Figure 4.6 Investigation of the promoter activity upstream of *tylMI*.** A 0.85 kb *Fok*I fragment of DNA almost exactly spanning the region in between the translational start of *tylMI* and the point of hygromycin cassette insertion in *tylMII*-KO was obtained. This was inserted in both orientations into the *redD* promoter-probe vector pIJ2585 (M. J. Bibb, personal communication) These constructs, along with the 'empty' vector (no insert) and pIJ2585 containing ermEp* oriented so as to drive *redD* expression (G. Stratigopolous, personal communication) were then integrated into *S. lividans*.

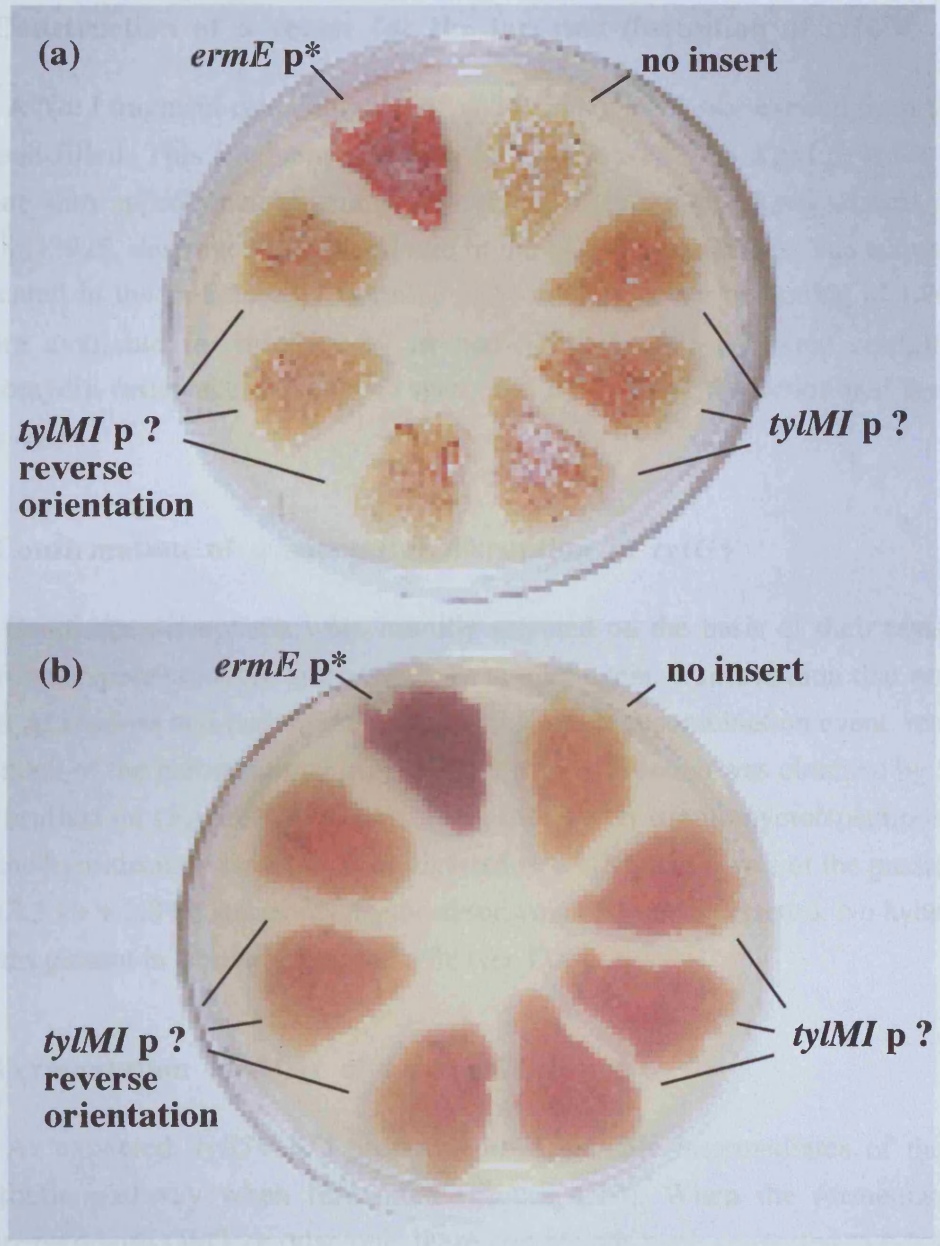



**Figure 4.7 The DNA directly upstream of *tylMI* has promoter activity.** The *S. lividans* strains, transfomed with the integrative promoter probe vectors (described in section 4.4.1), were plated onto R2YE media. Panel (a) shows the plate from above after 2 days. Panel (b) shows the plate from below after three days, by this time the colonies had sporulated, obscuring the red pigmentation produced. The picture has been flipped about its vertical axis for ease of comparison. The DNA contained in the promoter probe vector has been indicated in each case.

### 4.5.2 Construction of a vector for the targeted disruption of *tylGV*

A *Not* I fragment containing *tylGV* and flanking DNA was excised from pMOMT-4 and end-filled. This fragment was then further cleaved with *Kpn*I to leave a 2.1 kb fragment with approximately central *Hin*cII site. Ligation into *Kpn*I -*Hin*cII prepared vector, pIJ2925, destroyed the *Hin*cII site in the pIJ2925 polylinker. The central *Hin*cII site, located in the β–keto acyl synthase (KS) domain at the beginning of *tylGV*, was therefore available for insertion of an end-filled *Bam*HI fragment containing the spectinomycin resistance cassette (Ω spec) and its flanking transcriptional terminators (Figure 4.8).

### 4.5.3 Confirmation of a successful disruption in *tylGV*

Candidate disruptants were initially selected on the basis of their resistance to streptomycin/spectinomycin and sensitivity to apramycin. Confirmation that one isolate selected At random had undergone a successful double recombination event, resulting in replacement of the chromosomal gene by the disrupted version was obtained by Southern blot hybridisation (Figure 4.9). Thus, using the 2.0 kb streptomycin/spectinomycin as probe, the hybridisation target in *Kpn*I digested *tylGV*-KO DNA was of the predicted size, 5.3 kb (3.3 kb + 2.0 kb streptomycin/spectinomycin resistance cassette). No hybridisation target was present in similarly digested wild type DNA.

### 4.5.4 Fermentation analysis of *tylGV*-KO

As expected, *tylGV*-KO produced no detectable intermediates of the tylosin biosynthetic pathway when fermented (Figure 4.10). When the fermentation was supplemented with OMT or tylactone, these compounds were converted to tylosin. Thus although polyketide metabolism had been successfully disrupted sugar metabolism remained unaffected. Clearly, exogenously added tylactone could only be converted to tylosin if the first sugar, mycaminose could be successfully synthesised and added to the polyketide. The implication of this is that in this instance the *tylM* genes must be transcribed independently of *tylG*, from a promoter, presumably located within it. The relative strength of this promoter was subsequently assessed indirectly by the addition of increasingly large quantities of tylactone to *tylGV*-KO in fermentation. It was found that *tylGV*-KO could convert exogenous tylactone to tylosin, considerably in excess of normal wild type tylosin production levels (Figure 4.10).

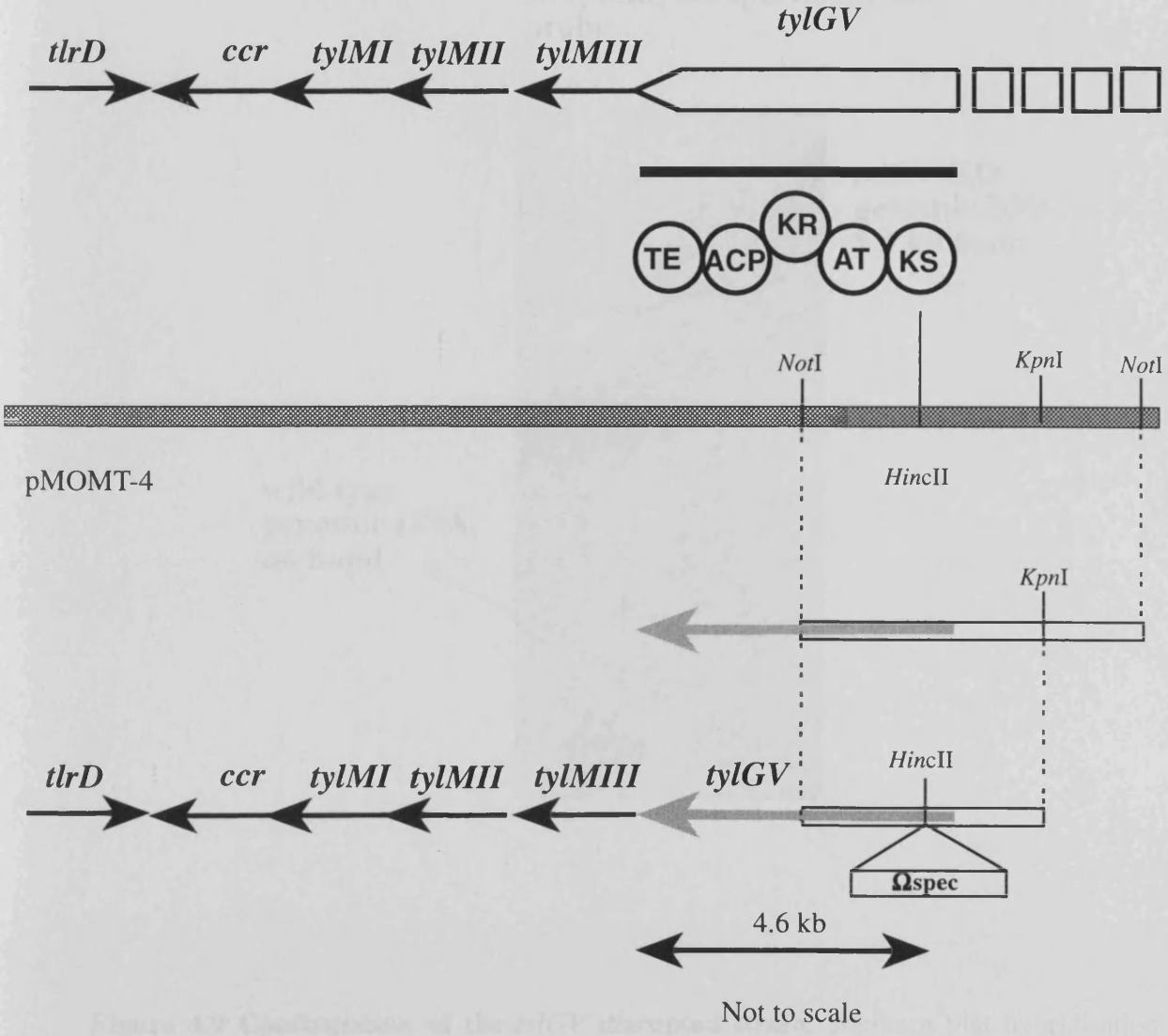



**Figure 4.8 Generating a disruption in *tylGV*.** The Ω streptomycin / spectinomycin cassette was inserted approximately centrally into a 2.1 kb cloned fragment of *tylG* DNA in pLST2925. This was done in order to disrupt early in the region encoding *tylGV*, the final module in the tylactone synthase. The resistance cassette was inserted into the region encoding the first domain of *tylGV*, the keto-acyl-synthase (KS), 4.6 kb away from the proposed translational start of *tylMIII*. The construct was then transfered into the unique *Bam*HI site of pOJ260 as a *Bgl*II fragment, ready for conjugal transfer into *S. fradiae*.

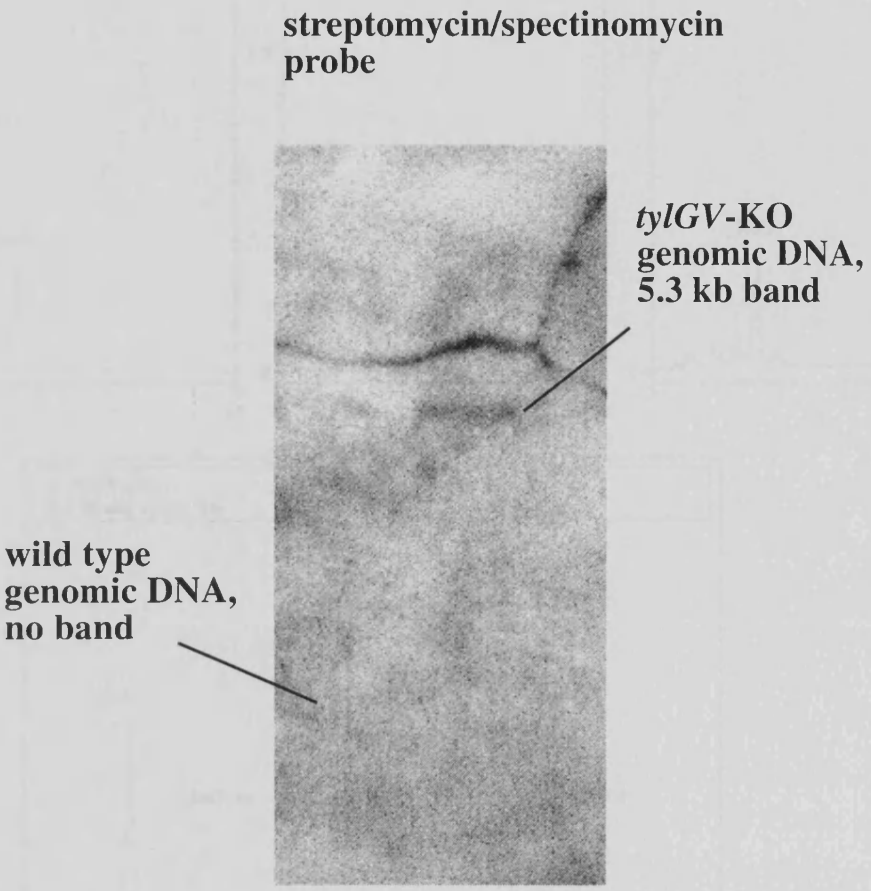



**Figure 4.9 Confirmation of the *tylGV* disrupted strain.** Southern blot hybridisation analysis confirmed that a putative *tylGV* disrupted strain was authentic. Wild type and disrupted strain genomic DNA were digested with *Kpn*I. Probe was prepared from the streptomycin/spectinomycin resistence cassette. The probe lit up a band of the predicted size (5.3 kb) in the disrupted strain but no band in the wild type.

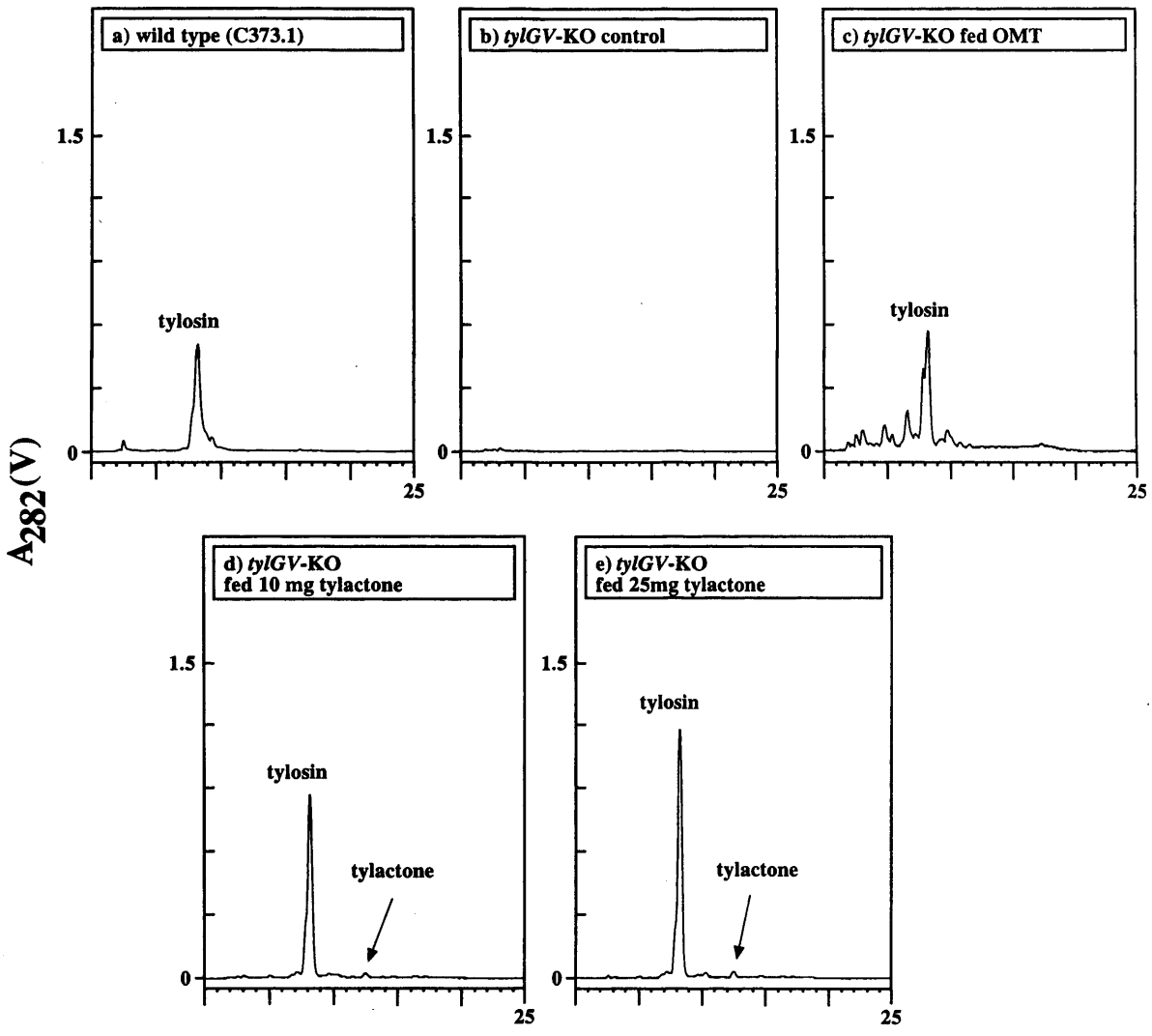



**Figure 4.10 HPLC analysis of *tylGV*-KO.** The fermentation products of (a) wild type (C373.1), (b) *tylGV*-KO control (fed DMSO), (c) *tylGV*-KO fed OMT, (d) *tylGV*-KO fed 10 mg tylactone and (e)*tylGV*-KO fed 25 mg tylactone as visualised by HPLC at 282 nm.

As indicated, the scale used here is 0 - 1.5 V.

Tylactone-feeding experiments carried out on the wild type strain had demonstrated that tylosin accumulation could be increased to at least twice normal levels (A. R. Butler personal communication). A similar increase in tylosin accumulation was acheived by feeding *tylGV*-KO, demonstrating unequivocally that the co-transcribed *tylM* genes must be expressed independantly of *tylG* at physiological levels.

## 4.6 Discussion

### 4.6.1 How could *tylMII* have previously complemented *tylMII*-KO

The inability of *tylMII* alone, when reintroduced into *tylMII*-KO, to restore tylosin production (section 4.3.4) was initially puzzling. Tylosin production in this strain, *tylMII*-KO (SF01), had previously been restored by reintroduction of *tylMII* , albeit at a reduced level (10% of wild type) (Fish and Cundliffe, 1997). Rationalisation of this apparent contradiction requires a comparison of the way in which the reintroduction of *tylMII* was achieved in either case. In both cases the DNA fragment in question was governed by the strong, constitutive *ermE** promoter. However in the experiments performed previously, the fragment used was a PCR generated fragment and was re-introduced to the disrupted strain on the low copy number, free replicating plasmid pLST9829 (a derivative of the pKC1218 (Bierman *et al.*, 1992) containing the *ermE** promoter (A. R. Butler, personal communication). This plasmid required antibiotic selection during fermentation in order to ensure its continued maintenance in the bacterial population. Conversely, in the experiments described here, all the DNA fragments (including *tylMII* ) were obtained as cloned fragments and were introduced into *S. fradiae* strains on the integrative vector pLST9828 (Butler *et al.*, 1999). This vector, based on pSET152 (Bierman *et al.*, 1992), facilitates highly efficient, stable integration into the chromosomal ØC31 *attB* site. Thus, once obtained, strains did not require antibiotic selection during fermentation. Although it is possible that a PCR-generated error might have occurred in the *tylMII* fragment used in the previous experiments, this seems unlikely to have caused the diverse nature of the results seen in this case. Its seems far more probable that in the previously performed experiments, at some point during the 10 day fermentation an homologous recombination event may have occurred between the disrupted chromosomal copy of *tylMII* and the uninterrupted plasmid borne version, leading to the disruption being repaired. A single crossover in the *tylMII* gene could lead, not only to an intact copy of *tylMII* being reformed, but the re-formed copy being driven by *ermE* p*. Such recombination events allow the generation of disrupted strains such as *tylMII*-KO in the first place and the rescue of a defective gene by recombination with an authentic, plasmid borne copy has been witnessed previously in this

laboratry (Clark, 1997). A copy of *tylMII* re-formed in this way would thus be certain of being expressed and any downstream polar effects that the disruption in *tylMII* might have exerted on *tylMI* would also be negated. The apramycin resistance marker carried on the plasmid would also be maintained (now inserted into the chromosome) allowing the newly formed strain to survive under selection. That only a fraction of the normal levels of tylosin were produced can be explained if the 'repair' of *tylMII* occurred late in the fermentation. The use of apramycin during fermentation, necessary for maintenance of the plasmid, might also have retarded the growth, or reduced the antibiotic producing potential of the strain in fermentation. This hypothesis is difficult to test easily because of the destructive nature of the extraction process. Thus no biological material exists from the final fermentation phase which could be investigated. It would of course be possible to try to re-create this result by multiple fermentation of the strain under the same conditions but this was not attempted.

### 4.6.2 Regulation of the *tylM* region

From the evidence presented in this chapter it is clear that the three *tylM* genes are co-transcribed. No comment can be made on the transcription of *ccr* because, although its location relative to the *tylM* genes is suggestive of a transcriptional link, its expression or not appears to be largely irrelevant in the biosynthesis of tylosin under the fermentation conditions used. It was also postulated that *tylMI* could be expressed, to an extremely limited degree, independantly of the upstream genes (*tylMII* and *tylMIII*) due to promoter activity somewhere in the 0.85 kb upstream of its proposed translational start. The discussion of promoter activity upstream of *tylMI* is not intended to imply that it is thought to have any physiological significance in the biosynthesis of tylosin. The amount of mycaminose produced as a result of the proposed TylMI activity is thought to be very small. No glycosylated precursors of tylosin were detectable by HPLC. It is most likely that the activity witnessed here has no real significance in *S. fradiae* strains under normal conditions. This fortuitous promoter activity upstream of *tylMI* thus has no impact on the proposal that *tylMIII* , *tylMII* and *tylMI* are transcriptionally linked and therefore co-regulated.

### 4.6.3 Location of the *tylM* promoter

The phenotype of the *tylGV*-KO strain demonstrates that a promoter for the *tylM* genes must be embedded within *tylGV*. Moreover, it is not merely fortuitous promoter activity such as that found upstream of *tylMI*, capable of expressing the gene at low level.

This promoter is capable of driving expression such that mycaminose synthesis and addition can occur at the same high level as that in the wild type strain. This level of activity is in itself considerably in excess of that witnessed under normal fermentation conditions. There seems to be considerable excess capacity in the ability of *S. fradiae* to glycosylate tylactone. The unavoidable conclusion must be that this promoter is most likely the normal physiological promoter for the *tylM* genes. The disruption in *tylGV* was positioned so as to limit the possibility of separating the *tylM* genes from such a promoter 4.6 kb upstream of the proposed translational start *tylMI*. The *tylM* promoter must therefore be located somewhere in this region.

### 4.6.4 Terminally overlapping orfs are not necessarily co-transcribed

The fact that transcription of the *tylM* genes is initiated from a promoter embedded in *tylGV* at wild type levels and above, makes it unnecesary to invoke the involvement of any other promoter. There is therefore no need to suppose that the downstream *tylM* genes might be co-transcribed along with *tylGV*, despite being linked by the GTGA sequence that has been thought of as diagnostic of translational coupling. Actinomycetes have extremely GC rich DNA and correspondingly biased coding usage. The translational stop codon TGA is used almost exclusively. Terminal overlap sequences, GTGA or less commonly ATGA, therefore appear to link open reading frames far more frequently than they do in other organisms which use TGA far less often. Examples of open reading frames which terminally overlap in this way are therefore considerably more likely to be of significance in other organisms.

# CHAPTER 5

# REGULATORY GENES AND POLYKETIDE SYNTHESIS IN *S. FRADIAE*

## 5.1 Introduction

In Chapter 3, the profound stimulatory effect exerted by glycosylated forms of tylactone, on the synthesis of tylactone itself, was investigated. This positive feedback mechanism clearly has a significant impact, initially on the regulation of expression of *tylG*, the polyketide synthase gene complex, and thus ultimately on the regulation of tylosin synthesis as a whole. In addition, the presence of glycosylated polyketide might have other regulatory consequences for tylosin production which currently remain unknown. In the model presented earlier (section 3.9.4), it was proposed that *S. fradiae* strains maintain expression of polyketide synthase at only very low level, unless glycosylation can occur. The presence of glycosylated polyketide then stimulates polyketide metabolism and the trickle of *tylG* expression becomes a flood. The nature of the regulation exerted here is currently unknown, in particular whether the glycosylated polyketide causes repression of the polyketide synthase genes to be relieved, or by positively activating their expression. It is also not known whether glycosylated tylosin intermediates act directly as inducers or transcriptional co-activators, or whether their presence set in motion a more complex series of interactions leading to tylactone accumulation.

It is evident that at least one regulatory protein must interact with glycosylated polyketides in order to mediate their stimulatory effect. As discussed in the introduction (section 1.2.6) a number of regulatory genes have been found associated with the tylosin biosynthetic cluster. Although the precise functions of these regulatory genes have yet to be elucidated it is intuitive that they should exert at least some influence over their neighbouring tylosin-biosynthetic genes. One way of determining the effect and importance of these regulatory genes with respect to tylosin biosynthesis is to engineer their disruption or over-expression and analyse the fermentation products of the resulting strains. Such studies are currently underway in the laboratory in wild type strains. Strains already disrupted in mycaminose metabolism provide an excellent opportunity to test whether a regulatory gene exerts an influence specifically over polyketide synthesis or forms part of the mechanism by which polyketide synthesis can be stimulated in response to glycosylated tylosin precursors. The phenotype of a strain disrupted in mycaminose metabolism is non-production unless tylactone accumulation is stimulated by an exogenous glycosylated polyketide. If this phenotype could be altered by the additional disruption of a regulatory gene, the implication would be that the product of that gene must be involved either directly or indirectly in the positive feedback regulation of *tylG*. The manner in which the phenotype of the strain was altered might also be indicative of the precise function of the regulatory gene. Thus, a mycaminose deficient strain disrupted in a regulatory gene involved in repression of *tylG* might accumulate tylactone even in the absence of a

stimulatory molecule. Alternatively, if tylactone accumulation could not be stimulated by exogenous OMT, the implication would be that the regulatory gene was essential to the stimulation process. These experiments were performed in the *tylM* mutant strain, GS62.

The *tylM* mutation in strain GS62 was located within *tylMI* by complementation analysis and the specific change from wild type was determined by sequence analysis (Gandecha *et al.*, 1997). This revealed an amino acid substitution in the proposed SAM binding pocket of the methyltransferase. The resulting replacement of an alanine residue, with the bulkier valine might well affect the ability of the enzyme to bind its co-factor. Analysis of this strain, demonstrating its suitability for this role, is presented in this chapter (section 5.3). As expected, GS62 was found to behave entirely consistently with a strain disrupted in a mycaminose specific gene and therefore provided an excellent background in which this analysis could be performed. Disruption of regulatory genes in strain GS62 and analysis of the fermentation products was performed concurrently with experiments to disrupt the same genes in the wild type. Vectors compatible with achieving this aim in the wild type had therefore already been constructed in the laboratory using the hygromycin resistance cassette (Ω hyg). Since the *tylM* mutant, GS62, does not already contain the hygromycin resistance cassette, the same vectors could also be used in these experiments. Authenticated *tylQ, tylS* and *tylT* disruptants and putative *tylP* and *tylR* disruptants in GS62 were generated and preliminary analysis was carried out.

Another approach is to introduce candidate regulatory genes into mycaminose deficient strains under the control of a strong constitutive promoter. Most genes involved in regulation might be expected to have their own expression regulated in some way by the products of other genes. A regulatory gene introduced into a mycaminose deficient strain so as to escape its own normal regulation, might thus reveal its influence by some phenotypic change. Specifically, if a gene involved in the regulation of polyketide biosynthesis has its own expression controlled by the presence or absence of glycosylated polyketide then introduction of the gene, released from its normal regulation might be manifested in a modification of the typical mycaminose-deficient phenotype usually exhibited by the strain. Once again, these experiments were facilitated by the fact that suitable vectors were already available in the laboratory having been constructed for use in the wild type. The mycaminose deficient strain used in this analysis was *tylMI*-KOl, disrupted in the methyltransferase gene *tylMI* (section 3.4 and 3.5).

## 5.2 Overexpression of regulatory genes in *tylMI*-KO

### 5.2.1 Vectors used to introduce regulatory genes

In each case the integrative vector pLST9828 was used to introduce the putative regulatory genes into *S. fradiae* strains. The vectors in question were constructed by other members of the laboratory and had the following characteristics.

The *tylP* vector contained a 0.99 bk fragment including 302 bp upstream and 8 bp downstream of the proposed coding region (A. R. Gandecha, personal communication).

The *tylQ* vector contained a 0.87 kb fragment including 109 bp upstream and 116 bp downstream of the proposed coding region (A. R. Gandecha, personal communication).

The *tylR* vector contained a 1.69 kb fragment including 188 bp upstream and 210 bp downstream of the proposed coding region (Bate *et al.*, 1999).

The *tylS* vector contained a 1.23 kb fragment including 218 bp upstream and 214 bp downstream of the proposed coding region (N. Bate, personal communication).

The *tylT* vector contained a 1.21 kb fragment generated by PCR so as to contain the largest possible coding region (N. Bate, personal communication).

### 5.2.2 Analysis of *tylMI*-KO with over-expressed regulatory genes

The regulatory genes found associated with the tylosin biosynthetic cluster (section 1.2.6), *tylP*, *tylQ*, *tylR* and *tylS* were introduced into the *tylMI* disrupted strain *tylMI*-KO governed in each case by the *ermE** promoter. When fermented, all of these strains behaved similarly to the *tylMI*-KO control (into which the 'empty vector' pLST9828 alone, had been introduced) (Figure 5.1). Thus no detectable levels of any tylosin intermediate were accumulated. The over-expression of any of these regulatory genes failed to elicit any interesting response from this mycaminose deficient, non-producing strain. When these strains were fermented with exogenous OMT, the results again mirrored those of control strains (Figure 5.2). In each case tylactone accumulation was stimulated and the OMT was converted to tylosin. Thus, the over-expression of these regulatory genes could neither suppress nor provoke the stimulation of tylactone accumulation that normally occurs in response to glycosylated intermediates of tylosin in mycaminose deficient strains.

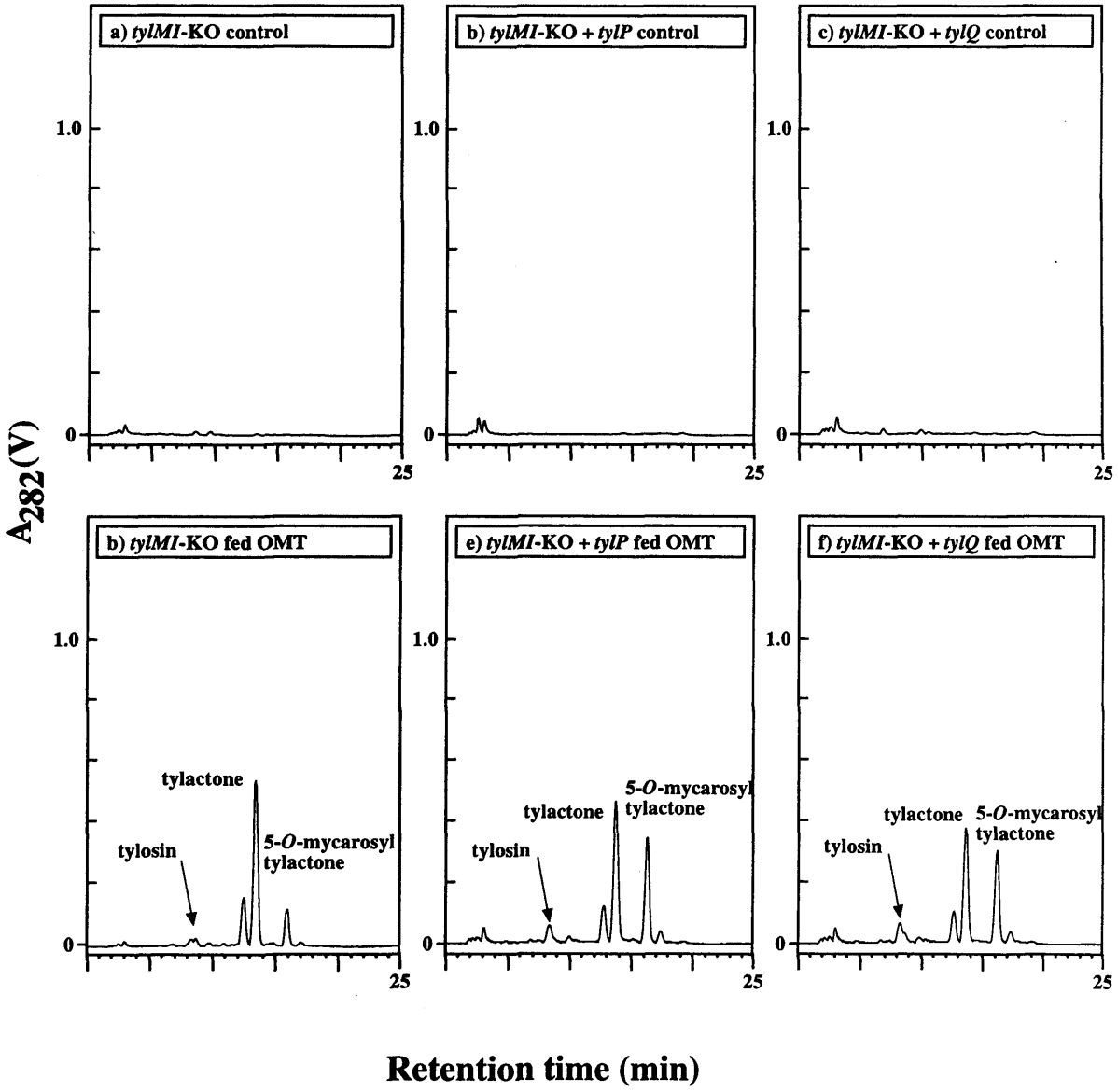



**Figure 5.1 HPLC analysis of *tylMI*-KO with regulatory genes *tylP* and *tylQ* introduced.** The fermentation products of *tylMI*-KO (a) control (with pLST9828 introduced and fed DMSO), (b) with *tylP* introduced control (fed DMSO), (c) with *tylQ* introduced control (fed DMSO), (d) control (with pLST9828 alone introduced) fed OMT, (e) with *tylP* introduced fed OMT and (f) with *tylQ* introduced fed OMT, as visualised by HPLC at 282 nm.
To preserve stocks, strains were fed 1 mg of OMT only.

As indicated, the scale used here is 0 - 1.0 V.

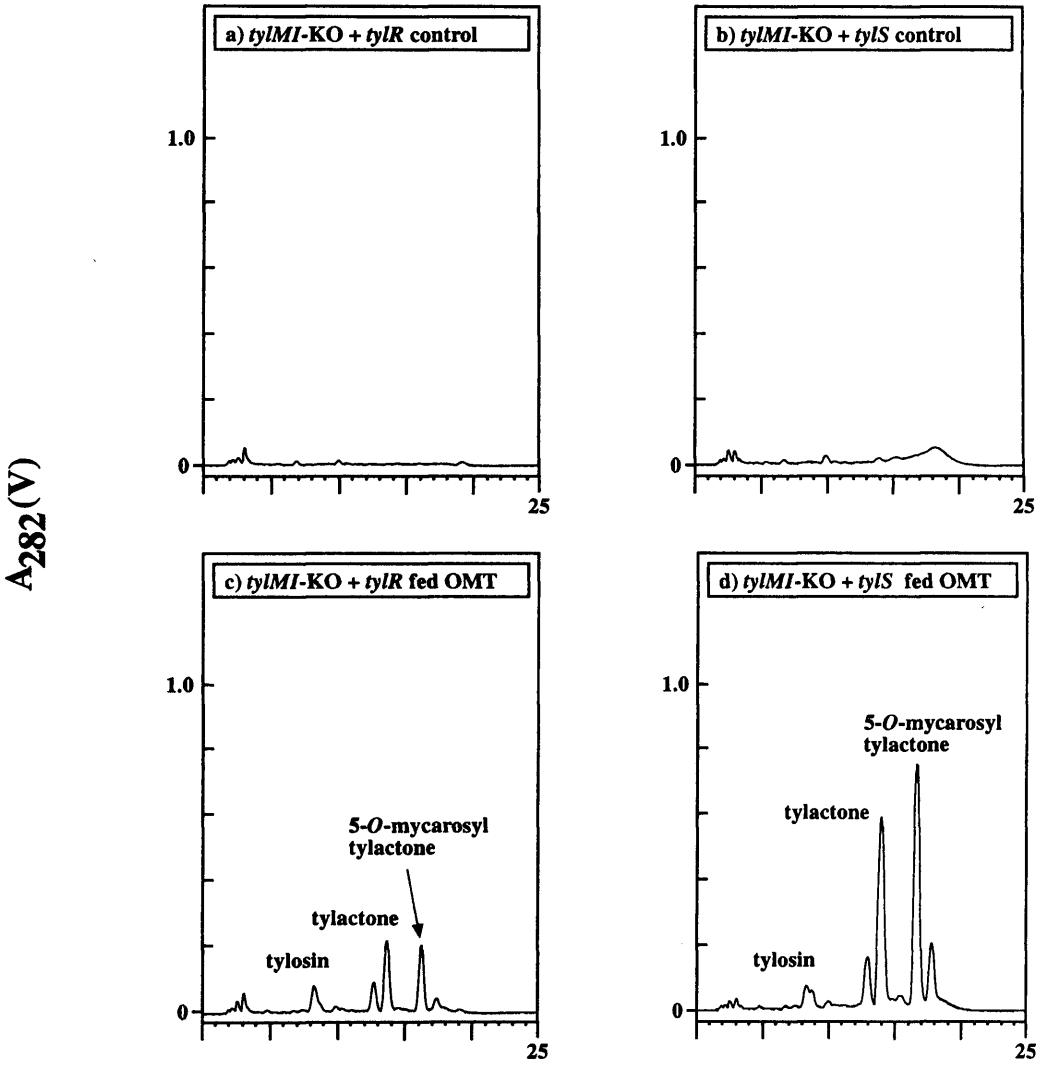



**Figure 5.2 HPLC analysis of *tylMI*-KO with regulatory genes *tylR* and *tylS* introduced.** The fermentation products of *tylMI*-KO (a) with *tylR* introduced control (fed DMSO), (b) with *tylS* introduced control (fed DMSO), (c) with *tylR* introduced fed OMT and (d) with *tylS* introduced fed OMT, as visualised by HPLC at 282 nm. To preserve stocks, strains were fed 1 mg of OMT only.

As indicated, the scale used here is 0 - 1.0 V.

## 5.3 Analysis of the *tylM* mutant, GS62

### 5.3.1 Complementation analysis of GS62

In fermentation, strain GS62 produced no identifiable metabolites of tylosin biosynthesis (Figure 5.3). When *tylMI* was reintroduced into GS62 the resulting high level of tylosin accumulation occurred as expected. This analysis differs only from that previously carried out in this laboratory in the exact nature of the DNA fragment used to complement the mutation. In the previous analysis a PCR amplified DNA fragment containing *tylMI* was used (Clark, 1997). In the experiments carried out here the fragment used was derived from cloned DNA (section 4.2.2). In all other respects the analysis carried out and the results obtained were the same and serve only as further confirmation.

### 5.3.2 Feeding the *tylM* mutant with precursors of tylosin

In experiments further to those previously carried out in this laboratory fermentations of the *tylM* mutant, GS62, were supplemented with OMT. As expected the strain converted the exogenously added material to tylosin and in addition, accumulated large quantities of tylactone (Figure 5.4). This data illustrates that the *tylM* mutant GS62 behaves entirely consistently with the *tylM* disrupted strains (section 3.7) under these conditions. This makes it suitable for further experiments designed to investigate the tylactone stimulation phenomenon.

## 5.4 Disruption of regulatory genes in the *tylM* mutant, GS62

### 5.4.1 Vectors for the disruption of regulatory genes

The vectors used to generate regulatory gene disruptions were generated by other members of the laboratory and had the following characteristics.

The vector for disrupting *tylP* was designed to interrupt the coding region 129 bp downstream of the predicted translational start (43 amino acids into the 226 amino acid total length) (A. R. Gandecha, personal communication).

The vector for disrupting *tylQ* was designed to interrupt the coding region 120 bp downstream of the predicted translational start (40 amino acids into the 213 amino acid total length) (A. R. Gandecha, personal communication).

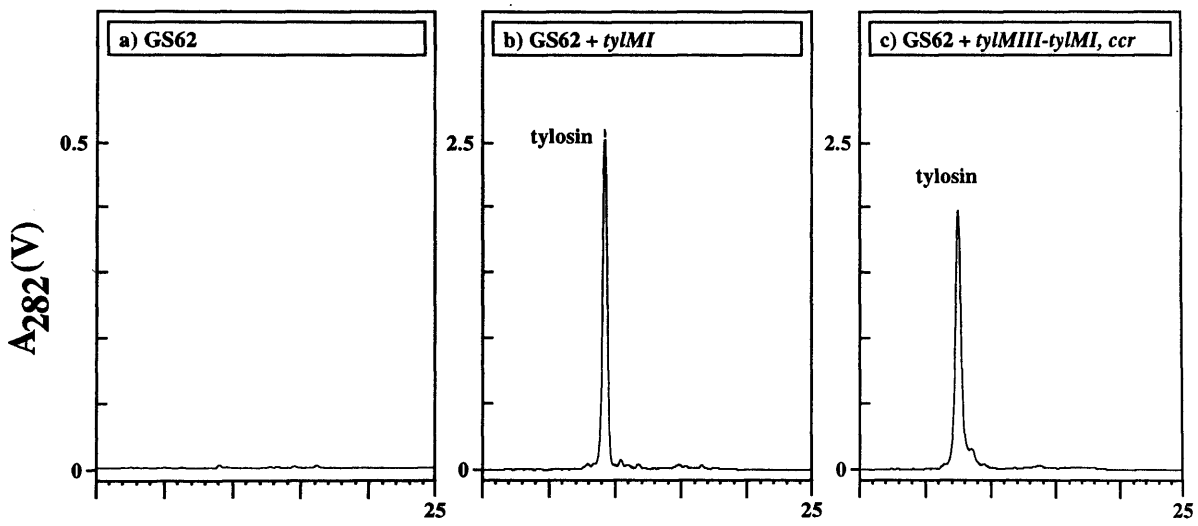



**Figure 5.3. HPLC analysis of the *tylM* mutant strain, GS62.** The fermentation products of (a) GS62 control (with pLST9828 alone introduced), (b) GS62 with *tylMI* introduced and (c) GS62 with *tylMIII, tylMII tylMI* and *ccr* introduced, as visualised by HPLC at 282 nm.

As indicated, the scale used here is 0 - 2.5 V (except a, shown at 0 - 0.5 V).

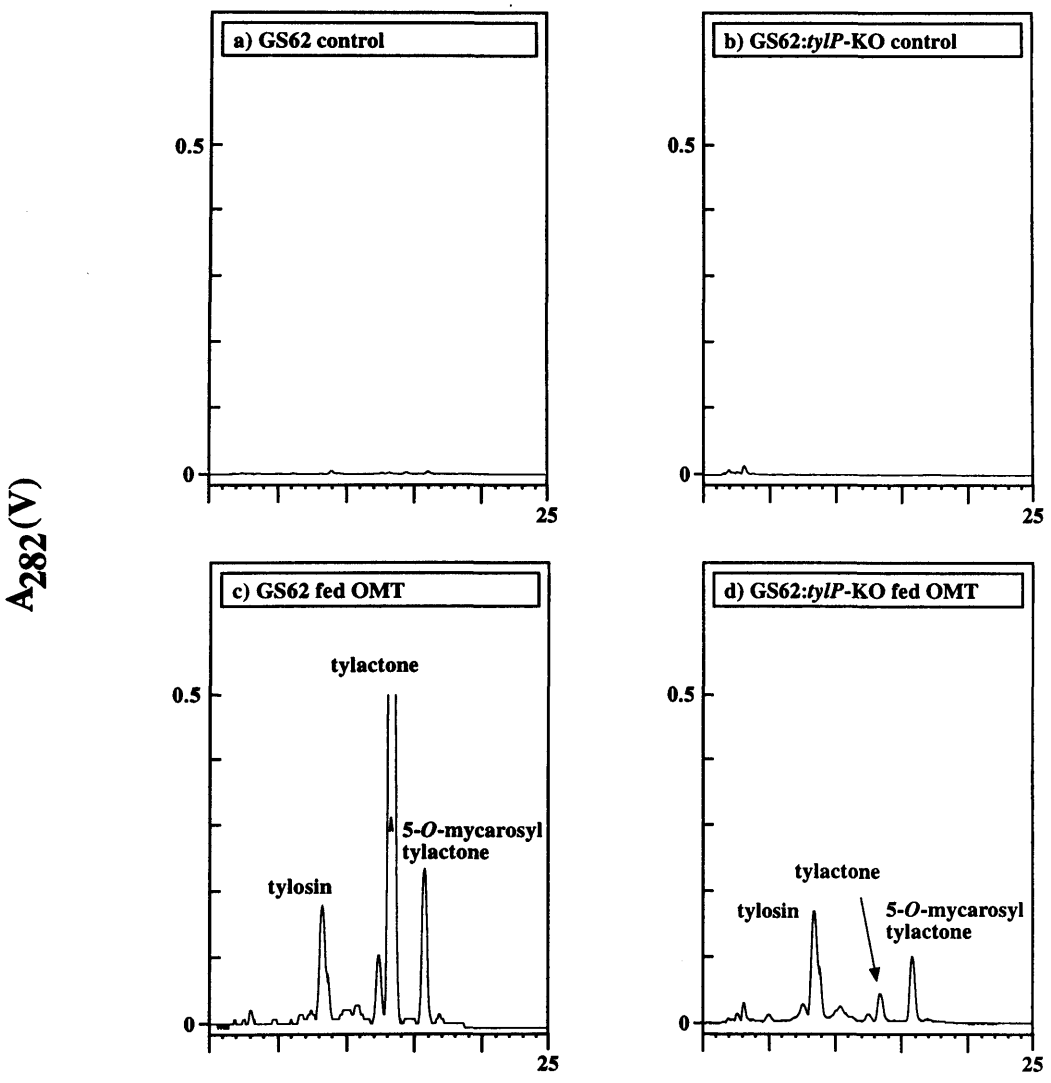



**Figure 5.4 HPLC analysis of GS62:*tylP*-KO.** The fermentation products of (a) the *tylM* mutant strain, GS62 control (fed DMSO) with, (b) with *tylP* disrupted (*tylP*-KO) control (fed DMSO), (c) fed OMT, (d) *tylP*-KO fed OMT, as visualised by HPLC at 282 nm.

As indicated, the scale used here is 0 - 0.5 V.

The vector for disrupting *tylR* was designed to interrupt the coding region 378 bp downstream of the furthest possible upstream translational start (126 amino acids into the 430 amino acid total possible length) (Bate *et al.*, 1999).

The vector for disrupting *tylS* was designed to interrupt the coding region 122 bp downstream of the predicted translational start (40 amino acids into the 277 amino acid total length) (N. Bate, personal communication).

The vector for disrupting *tylT* was designed to interrupt the coding region 524 bp downstream of the furthest possible upstream translational start (174 amino acids into the 404 amino acid total possible length) (N. Bate, personal communication).

### 5.4.2 Confirmation of the regulatory-gene disruptions in GS62

Candidate disruptants were initially selected on the basis of their resistance to hygromycin B and sensitivity to apramycin. In each case, confirmation that the strain had undergone a successful double recombination event, resulting in replacement of the chromosomal gene by the disrupted version was obtained by Southern blot hybridisation (Figure 5.5). In each case the 2.3 kb hygromycin resistance cassette was used as probe. The hybridisation target in *Bst*XI digested GS62:*tylQ*-KO DNA was of the predicted size, 4.3 kb (2.0 kb+ 2.3 kb hygromycin cassette). The hybridisation target in *Afl*III digested GS62:*tylS*-KO DNA was of the predicted size, 2.8 kb (0.5 kb+ 2.3 kb hygromycin cassette). The hybridisation target in *Nco*I digested GS62:*tylT*-KO DNA was of the predicted size, 4.3 kb (2.0 kb+ 2.3 kb hygromycin cassette). No band was evident in GS62 DNA (*kpn*I digested).

### 5.4.3 Analysis of the *tylM* mutant, GS62, disrupted in *tylP*

The gene, *tylP* is thought to encode a γ-butyrolactone receptor, possibly regulating the transcription of other genes (section 1.2.6). A likely target for TylP is the downstream gene, *tylQ* with precedent suggesting that it might function as a repressor. The wild type strain when disrupted in *tylP* showed very little change in its tylosin-producing phenotype in preliminary experiments (A. R. Gandecha, personal communication). GS62:*tylP*-KO behaved like its undisrupted predecessor, GS62, producing no detectable tylosin intermediates (Figure 5.3). The disruption in *tylP* did not therefore have any discernible effect under these conditions. When GS62:*tylP*-KO was fed with OMT, this exogenous material was converted to tylosin indicating that there were no detectable adverse effects on sugar metabolism.

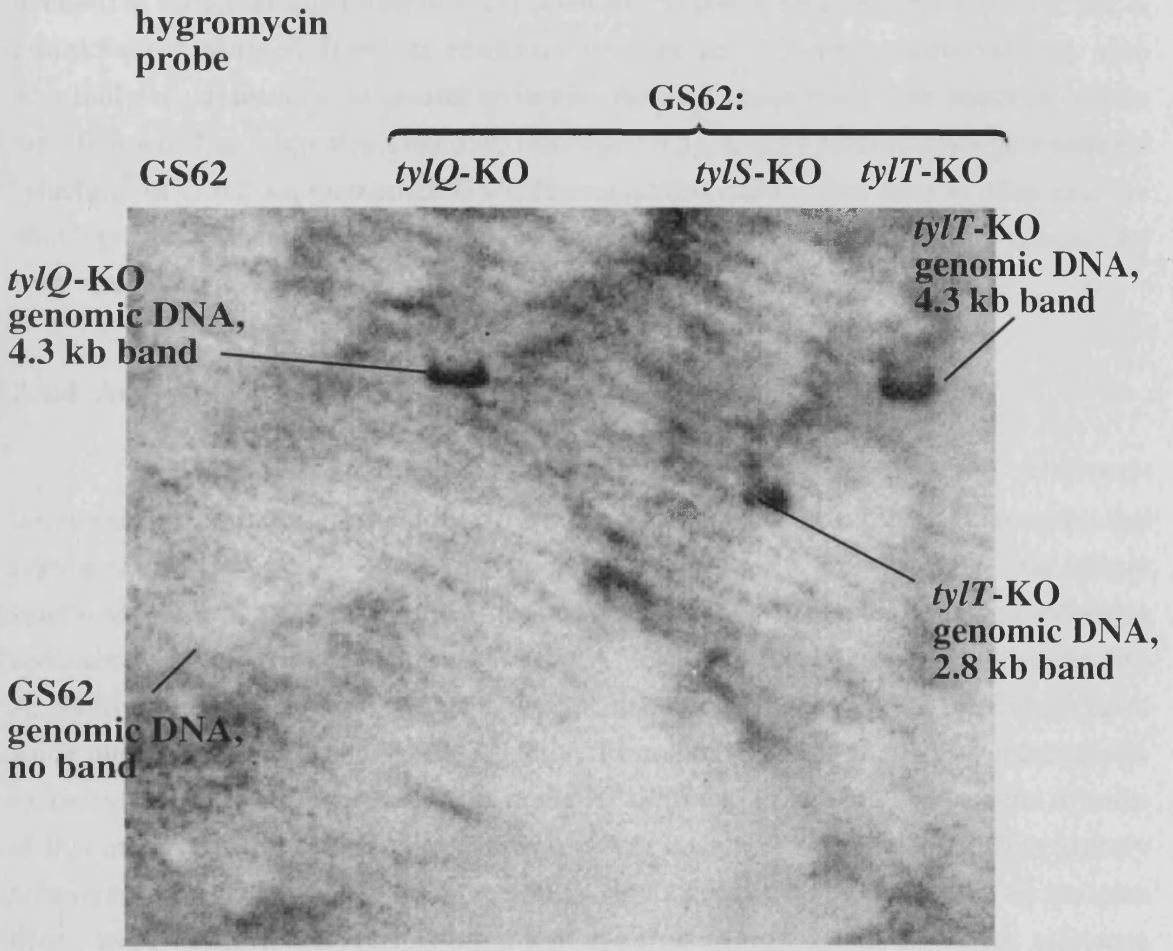



**Figure 5.5 Confirmation of GS62 strains disrupted in *tylQ, tylS* and *tylT*.** Southern blot hybridisation analysis confirmed that strains GS62:*tylQ*-KO, GS62:*tylS*-KO andGS62:*tylT*-KO were authentic. Wild type and disrupted strain genomic DNA were digested with *Kpn*I (wild type), *Bst*YI (*tylQ*-KO), *Afl*III (*tylS*-KO) and *Nco*I (*tylT*-KO). Probe was prepared from the hygromycin cassette lit up a bands of the predicted sizes in the disrupted strains but no band in the wild type.

The tylactone accumulation in GS62:*tylP*-KO fed with OMT, while significant, occured in far lower quantities than expected when compared to the undisrupted strain. A compound presumed from its retention time to be 5-*O*-mycarosyltylactone also accumulated, unusually, in greater quantities than tylactone itself. This material, whose significance if any remains unknown (section 3.9.5), always accompanies accumulated tylactone in OMT-supplemented GS62 fermentations and is often seen in other cases in which tylactone accumulates in excessive amounts. It usually constitutes only a fraction (by peak size) of the tylactone accumulated, rather than the major component.

### 5.4.4 Analysis of the *tylM* mutant, GS62, disrupted in *tylQ*

The gene *tylQ* is thought to be the transcriptional target for the putative γ-butyrolactone receptor encoded by *tylP*. It is possible that it too encodes a transcriptional regulator (section 1.2.6). Preliminary analysis in the wild type revealed that tylosin synthesis was not affected by a disruption in *tylQ* in this strain and tylosin production continued at approximately normal levels (A. R. Gandecha, personal communication). Analysis of the fermentation products of GS62 disrupted in *tylQ* (GS62:*tylQ*-KO) revealed some interesting data. In un-supplemented fermentations various unexpected material was extracted, represented by small peaks in the HPLC trace (Figure 5.6). The nature of some of this material, represented by peaks with rrt's of 0.8 and 1.2 remain difficult to identify although they may have been seen previously. Small peaks with the same relative retention times were identified in fermentations of *tylM* disrupted strains in which tylactone accumulation had been stimulated. Of more interest here, simply because its identity can be positively determined, is a relatively small peak presumbed from elution time to be 5-*O*-mycarosyltylactone. Mass spectral data confirmed this observation, revealing a species with the predicted *m/z* value as 5-*O*-mycarosyltylactone (539) along with the presence of tylactone (*m/z* - 395) (Figure 5.7). This compound must be present in very small quantities, only just detectable by HPLC (Figure 5.6). The very low concentration of these tylosin metabolites means that the trace is dominated by other peaks representing unknown species. Some material with *m/z* values between 899 and 976 can be seen. Within this cluster some material that could be mistaken for tylosin can be identified (916). It is highly unlikely that this strain could produce tylosin. The parent strain, GS62, has been demonstrated as far as possible to be mycaminose-deficient. No trace of tylosin can be found by mass spectrometry when fermentation extracts of it were analysed (H. Kirst, personal communication). It can only be conluded that the material seen in this trace is not tylosin. When *tylQ* was reintroduced into the disrupted strain, all these peaks disappeared from the fermentation products (Figure 5.6 and 5.7).

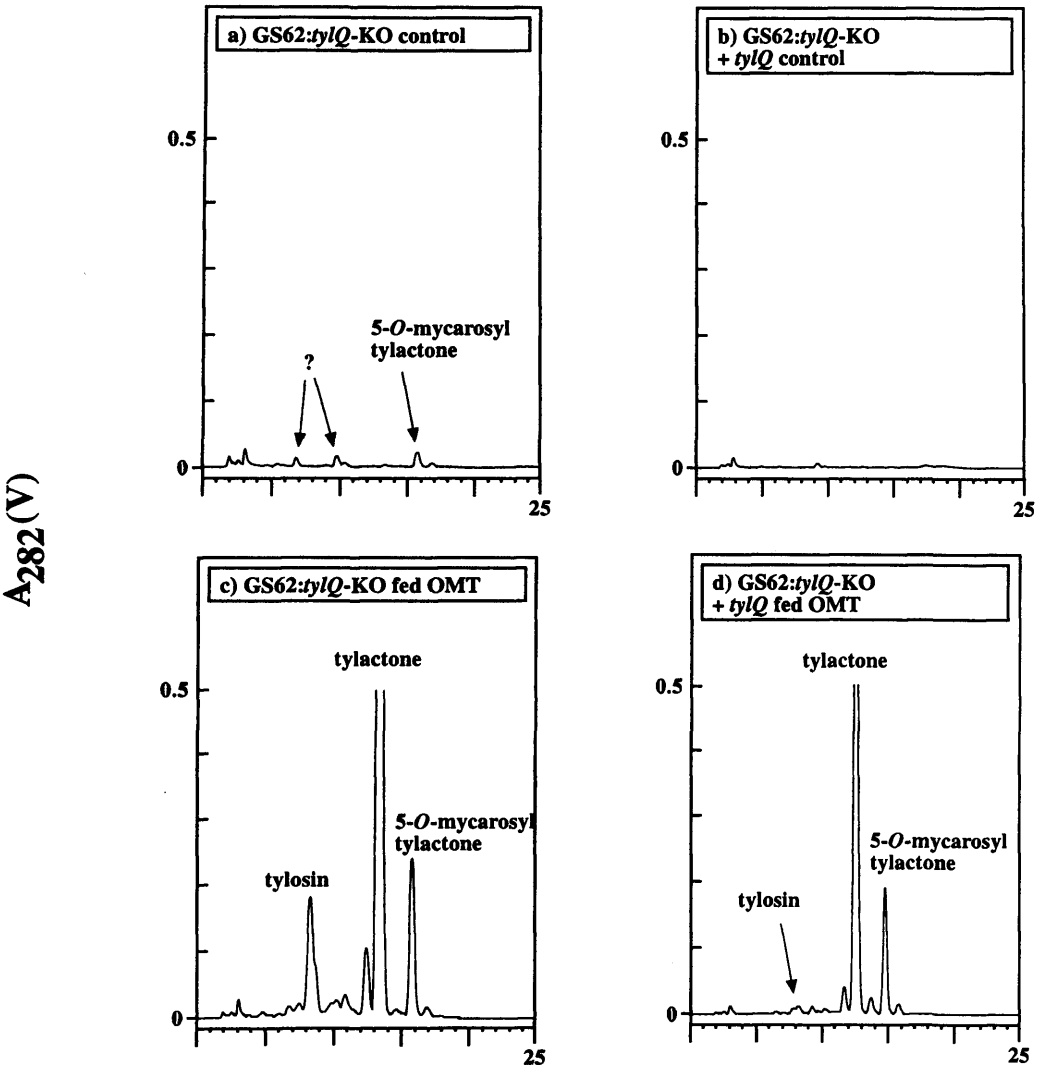



**Figure 5.6 HPLC analysis of GS62:*tylQ*-KO.** The fermentation products of the *tylM* mutant strain, GS62 in which *tylQ* has been disrupted (a) control (fed DMSO) (b) with *tylQ* reintroduced control (fed DMSO), (c) fed OMT, (d) with *tylQ* reintroduced fed OMT, as visualised by HPLC at 282 nm.

As indicated, the scale used here is 0 - 0.5 V.

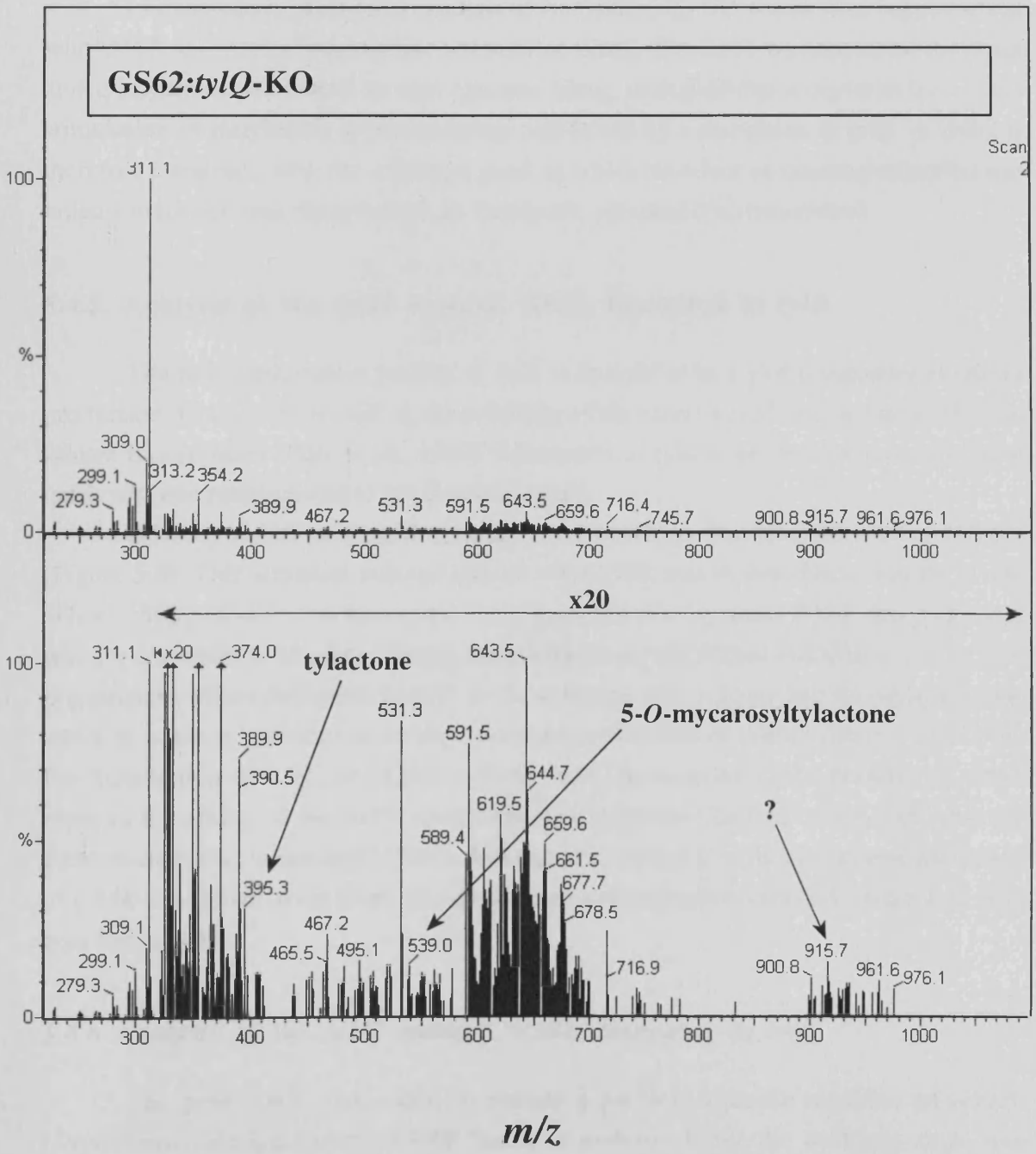



**Figure 5.7 Analysis of GS62:*tylQ*-KO by mass spectrometry.** When GS62:*tylQ*-KO was analysed, apart fromt the ubiquitous *m/z* 311 material found in fermentation extracts, no tylosin related metabolites could initially be identified. When magnified 20 times, both 5-*O*-mycarosyltylactone (*m/z* 598) and tylactone (*m/z* 395) were shown to be present. A species with an *m/z* value equivalent to tylosin could also be seen although the presence of this compound seems unlikely.

In either case, when fermentations of the GS62:*tylQ*-KO strains were supplemented with OMT, the strains behaved like unmodified GS62. The OMT was converted to tylosin and tylactone accumulated in vast quantity along with 5-*O*-mycarosyltylactone. This stimulation of polyketide synthesis being unaffected by a disruption in *tylQ* in GS62 is therefore consistent with the wild type result in which no effect on tylosin production was noted when *tylQ* was disrupted (A. R. Gandecha, personal communication).

### 5.4.5. Analysis of the *tylM* mutant, GS62, disrupted in *tylR*

The proposed protein product of *tylR* is thought to be a global regulator of tylosin production. Disruption of *tylR* in the wild type strain exerts a profound negative effect on tylosin biosynthesis (Bate et al., 1999). All aspects of tylosin production were abolished until *tylR* was reintroduced to the disrupted strain.

GS62:*tylR*-KO produced nothing in fermentation that absorbs well at 282 nm (Figure 5.8). This situation was not altered when *tylR* was re-introduced into the strain. When GS62:*tylR*-KO was fermented in the presence of extraneous OMT, this compound was not converted to any other tylosin metabolite to any significant identifiable extent. This is consistent with a disruption in *tylR* in the wild type strain destroying the capacity of the strain to produce tylactone or to bioconvert any precursors of tylosin (Bate *et al.*, 1999). Re-introduction of *tylR* into GS62:*tylR*-KO and fermentation in the presence of OMT restored the ability of the strain to convert the extraneous OMT to tylosin and tylactone accumulation was stimulated. This is thus entirely consistent with the reported phenotype of a *tylR* disrupted strain when transposed into a mycaminose deficient, rather than wild type background.

### 5.4.6 Analysis of the *tylM* mutant, GS62, disrupted in *tylS*

The gene, *tylS* is thought to encode a pathway specific regulator of tylosin biosynthesis belonging to the SARP family of proteins. When the wild type strain was disrupted in tylS it proved unable to produce or convert any intermediates of tylosin (N. Bate, personal communication). When disrupted in *tylS*, GS62 produced no detectable products of tylosin biosynthesis or any other material in fermentation (Figure 5.9). Reintroduction of *tylS* to GS62:*tylS*-KO had no detectable impact on this phenotype. However, an apparent anomaly was uncovered when these two strains were supplemented with OMT during fermentation. GS62:*tylS*-KO seemed incapable of bioconverting the fed compound in any significant detectable amount and no accumulation of any other tylosin intermediate was stimulated. This is consistent with the wild type strain when disrupted in

*tylS*. As expected, GS62:*tylS*-KO into which *tylS* had been reintroduced was competent to convert OMT into tylosin. Unexpectedly though, tylactone did not accumulate (Figure 5.9). This result might prove to be of considerable interest in determining the precise function of the product of *tylS*. Clearly the strain described above, containing a reintroduced copy of *tylS*, when fed with OMT should have manifested greatly enhanced tylactone accumulation.

### 5.4.7 Analysis of the *tylM* mutant, GS62, disrupted in *tylT*

Like *tylS*, the putative regulatory gene, *tylT* is thought to encode a pathway specific regulator of tylosin biosynthesis belonging to the SARP family of proteins.

The data obtained from analysis of GS62 disrupted in *tylT* closely resembled that of GS62 disrupted in *tylQ*. In unfed fermentation GS62:*tylT*-KO produced presumably the same three species of material absorbing at 282 nm as GS62-*tylQ*-KO and in similar quantities (Figure 5.10). Analysis by mass spectrometry also produced a trace similar to that of GS62:*tylQ*-KO, confirming the presence of 5-*O*-mycarosyltylactone and tylactone (Figure 5.11). Again, material that could be mistaken for tylosin was also evident in the trace although, as argued previously, this is highly unlikely. Also similar to GS62:*tylQ*-KO, none of these compounds could be identified in fermentation extracts of GS62:*tylT*-KO when the disrupted gene (*tylT* ) was reintroduced. When GS62 with and without the introduction of the disrupted gene were fermented in the presence of OMT, tylactone accumulation in large quantity occured along with 5-*O*-mycarosyltylactone. This accumulation of tylactone was consistent with the fact that wild type *tylT*-KO fermentations were found to produce tylosin at approximately wild type levels (N. Bate, personal communication).

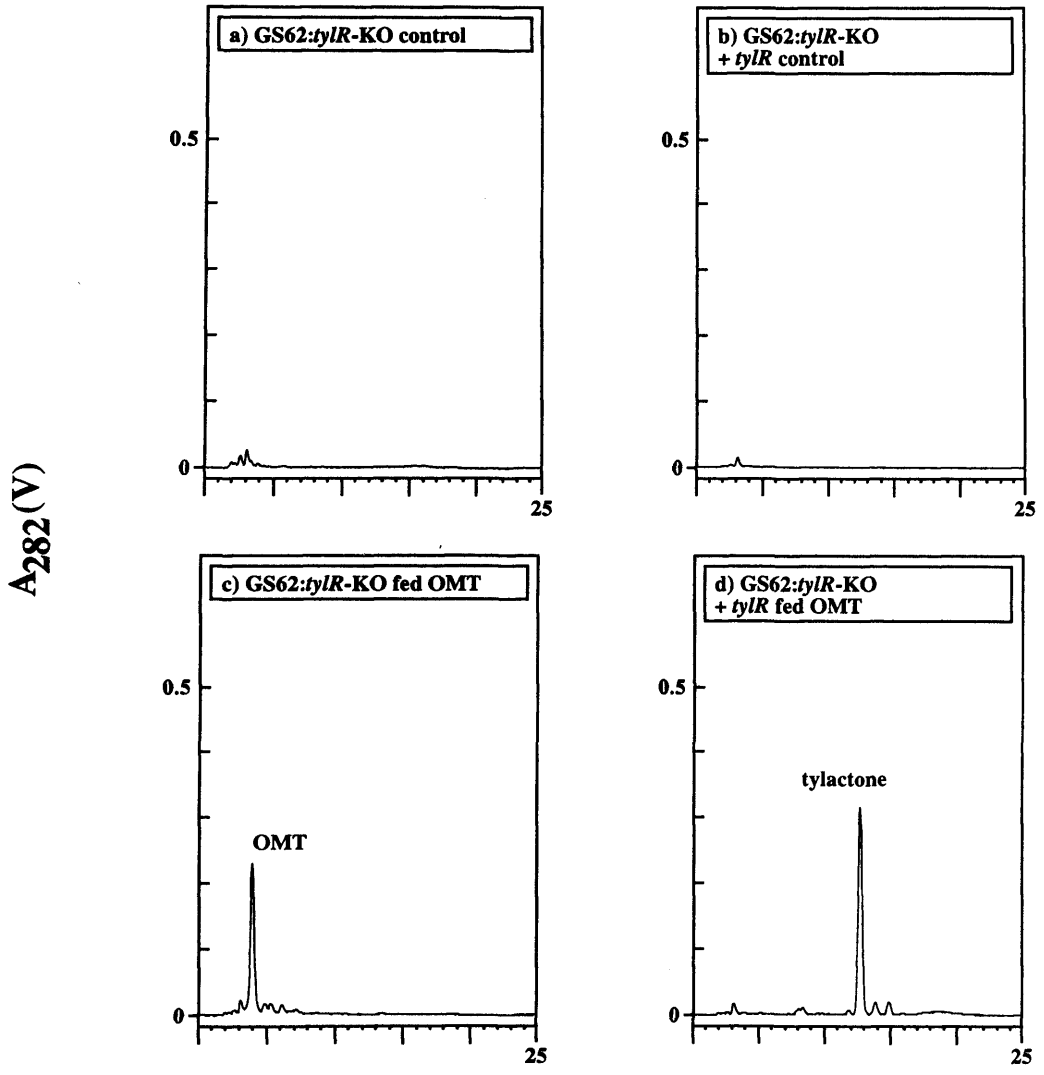



**Figure 5.8 HPLC analysis of GS62:*tylR*-KO.** The fermentation products of the *tylM* mutant strain, GS62 in which *tylR* has been disrupted,(a) control (fed DMSO), (b) with *tylR* reintroduced control (fed DMSO), (c) fed OMT (10 mg), (d) with *tylR* reintroduced fed OMT (1 mg), as visualised by HPLC at 282 nm.

As indicated, the scale used here is 0 - 0.5 V.

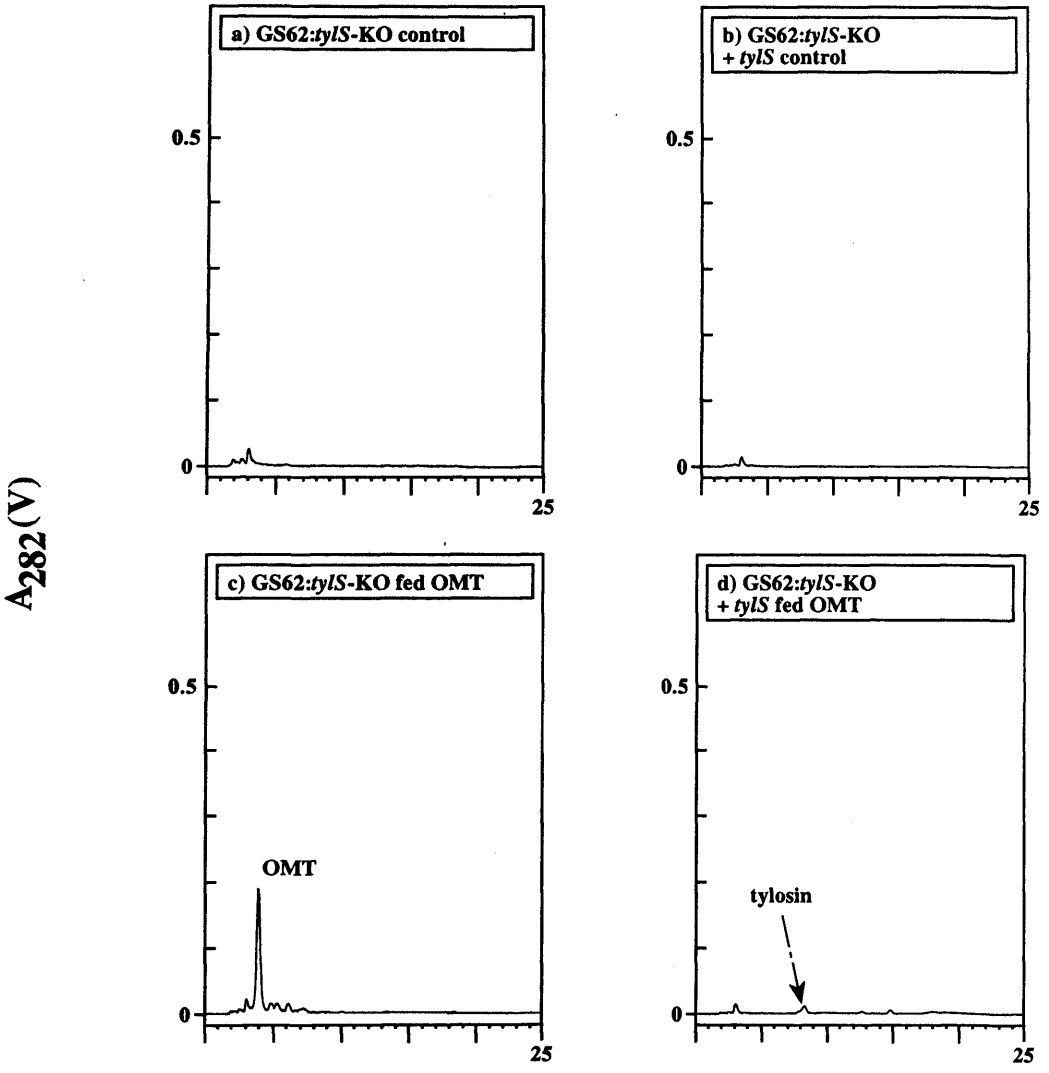



**Figure 5.9 HPLC analysis of GS62:*tylS*-KO.** The fermentation products of the *tylM* mutant strain, GS62 in which *tylS* has been disrupted, (a) control (fed DMSO), (b) with *tylS* reintroduced control (fed DMSO), (c) fed OMT (10 mg), (d) with *tylS* reintroduced fed OMT (1 mg), as visualised by HPLC at 282 nm.

As indicated, the scale used here is 0 - 0.5 V.

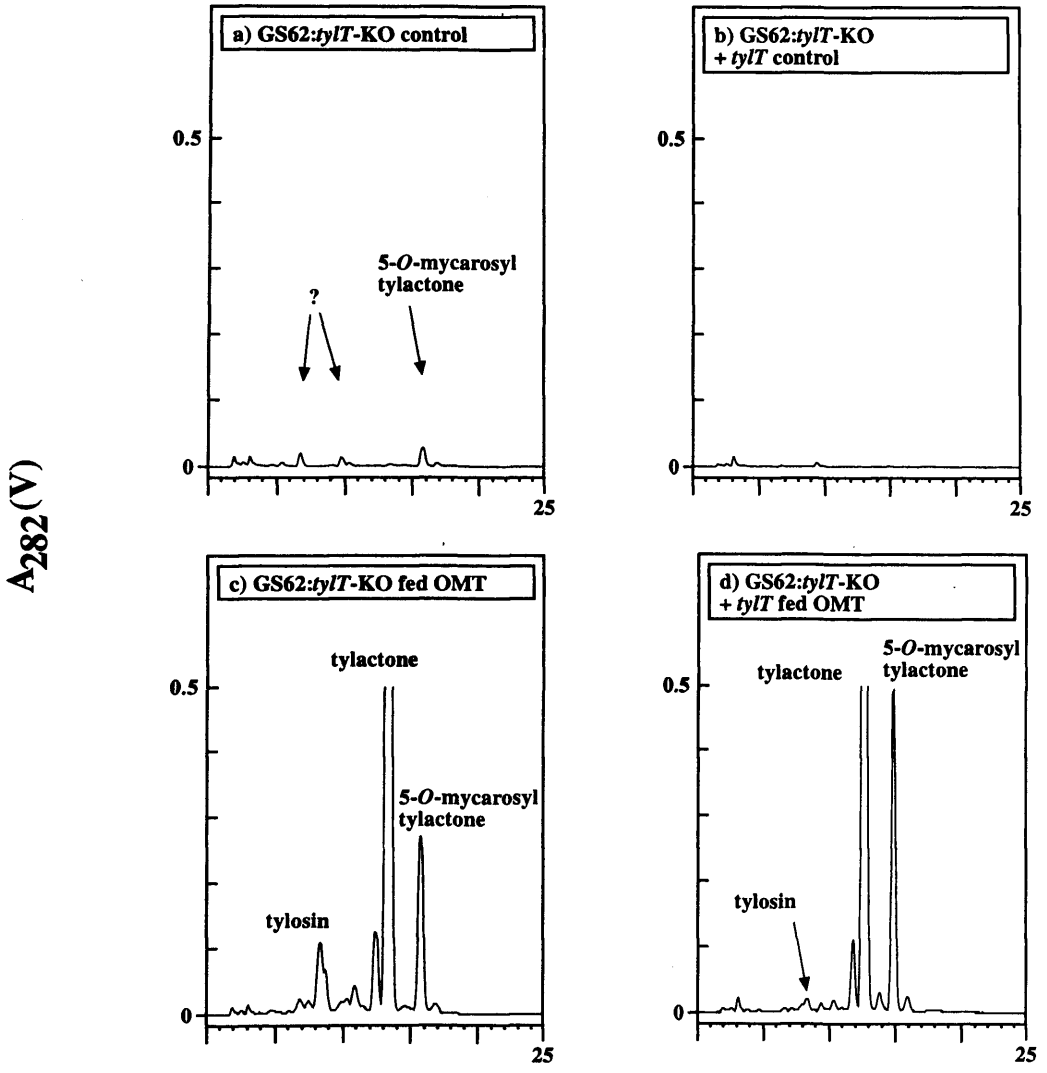



**Figure 5.10 HPLC analysis of GS62:*tylT*-KO.** The fermentation products of the *tylM* mutant strain, GS62 in which *tylT* has been disrupted,(a) control (fed DMSO), (b) with *tylT* reintroduced control (fed DMSO), (c) fed OMT (10 mg), (d) with *tylT* reintroduced fed OMT fed (1 mg), as visualised by HPLC at 282 nm.

As indicated, the scale used here is 0 - 0.5 V.

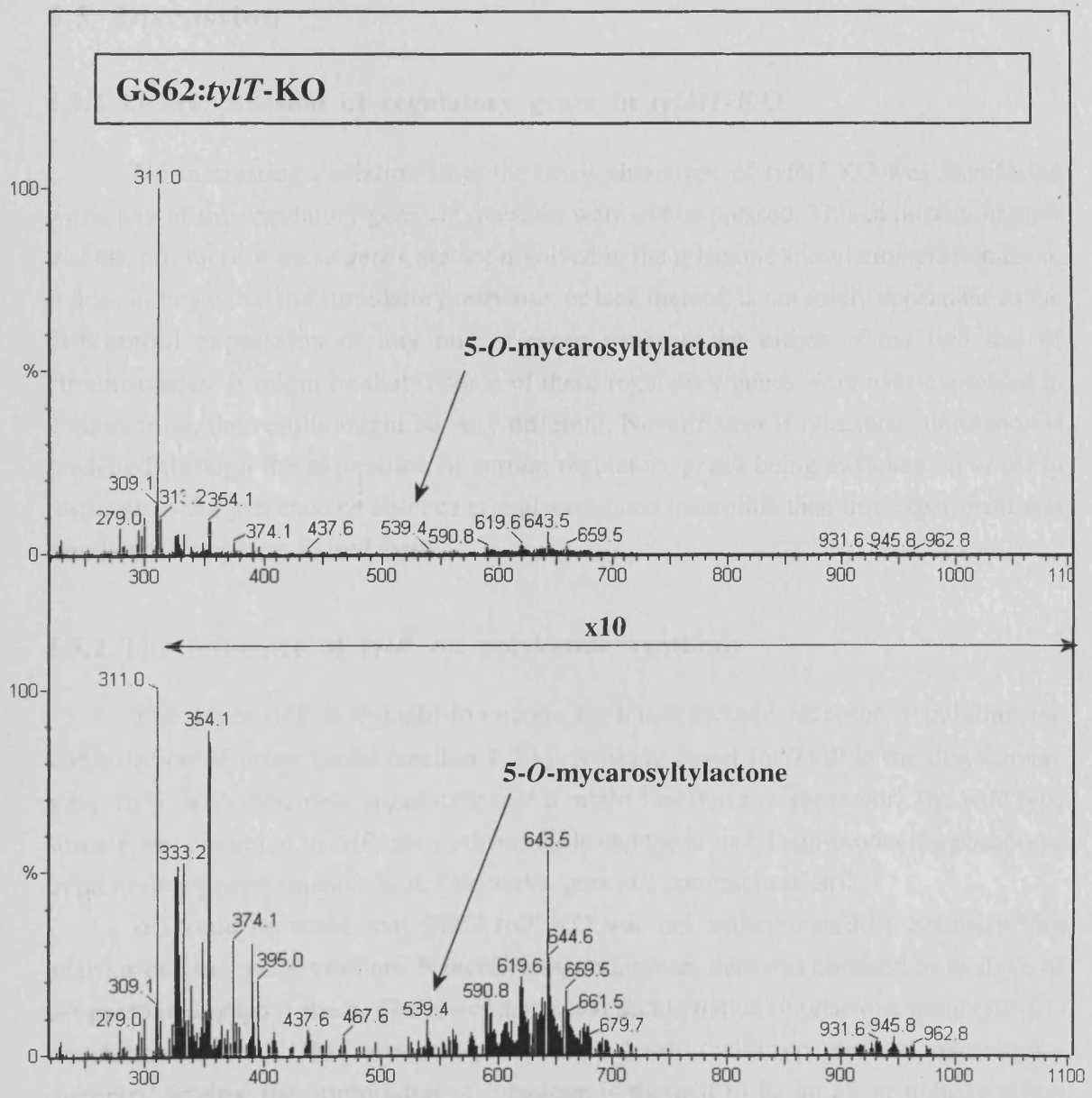



**Figure 5.11 Analysis of GS62:*tylT*-KO by mass spectrometry.** A species with an *m/z* value equivalent to that predicted for 5-*O*-mycarosyltylactone seems to confirm the presence of this compound in the fermentation extract. Tylactone (*m/z* 395) can also be identified when the part of the trace is expanded 10 times (lower panel). Some species are are also present with *m/z* values in the region of tylosin (predicted *m/z* 916) although these might be misleading.

## 5.5 Discussion

### 5.5.1 Overexpression of regulatory genes in *tylMI*-KO

No interesting deviation from the usual phenotype of *tylMI*-KO was manifested when any of the regulatory genes in question were overexpressed. This in no way implies that the products of these genes are not involved in the tylactone stimulation phenomenon. It does indicate that the stimulatory response, or lack thereof, is not solely dependant on the differential expression of any one of these genes under either of the two sets of circumstances. It might be that if some of these regulatory genes were over-expressed in combination, the results might be very different. Nevertheless if tylactone stimulation is mediated through the expression of certain regulatory genes being switched on or off in response to the presence or absence of a glycosylated macrolide then this experiment was clearly not competent to find them.

### 5.5.2 The influence of *tylP* on polyketide synthesis

The gene, *tylP* is thought to encode a γ-butyrolactone receptor, regulating the transcription of other genes (section 1.2.6). A likely target for TylP is the downstream gene, *tylQ* with precedent suggesting that it might function as a repressor. The wild type strain when disrupted in *tylP* showed very little change in its tylosin-producing phenotype in preliminary experiments (A. R. Gandecha, personal communication).

It should be noted that GS62:*tylP*-KO was not authenticated by Southern blot analysis due to time constraints. Nevertheless, preliminary data was obtained by analysis of the putative disrupted strain. The lower than usual accumulation of tylactone when tylP-KO was fermented with OMT was unexpected as, at least in wild type derived mycaminose disrupted strains, the stimulation of tylactone is thought to be an all or nothing effect (section 3.8.1). The fact that some tylactone accumulated indicates that TylP cannot be essential to the mechanism by which polyketide synthesis is stimulated. The greatly reduced levels of tylactone accumulation witnessed might be due to poor fermentation. Following confirmation of the authenticity of GS62:*tylP*-KO this analysis would require repetition. If this data was confirmed it would indicate some (but not crucial) involvement of the product of *tylP*, either directly or indirectly, in positive regulation of polyketide synthesis. Via receptor proteins, γ-butyrolactones are responsible for the positive regulation of antibiotic synthesis in many instances (see section 1.2.6) although TylP bears more resemblance to repressors.

### 5.5.3 The influence of *tylQ* on polyketide synthesis

The gene *tylQ* is thought to be the transcriptional target for the putative γ-butyrolactone receptor encoded by *tylP*. It is possible that it too encodes a transcriptional regulator (section 1.2.6). Preliminary analysis in the wild type revealed that tylosin synthesis was not affected by a disruption in *tylQ* in this strain and tylosin production continued at approximately normal levels (A. R. Gandecha, personal communication). In the mycaminose deficient background of GS62, the disruption in *tylQ* was at least partly consistent with the wild type result. Unexpectedly, GS62:*tylQ*-KO accumulated 5-*O*-mycarosyltylactone and also a very small amount of tylactone when fermented alone, but the stimulation of polyketide synthesis in response to fed OMT occurred as normal. The latter response indicates that *tylQ* is not essential in the activation of polyketide synthesis or in the mechanism by which glycosylated tylosin precursors stimulate this process. The low levels of polyketide synthesis detected in unfed fermentation requires further analysis. If this preliminary data is verified it would indicate that in the absence of *tylQ* polyketide synthesis must be active at far higher level than the undetectable level proposed to normally occur in un-stimulated, mycaminose-deficient strains. In this case polyketide synthesis would have to be be activated or de-repressed to a limited extent. Given that the product of *tylQ* bears similarity to transcriptional repressors as well as activators, this is a possibility.

### 5.5.4 The influence of *tylR* on polyketide synthesis

The behaviour of *tylR* is entirely consistent with it being, as proposed (Bate *et al.*, 1999), a pathway specific activator necessary for global positive control of tylosin biosynthesis. Nevertheless the strain requires cofirmation of its authenticity which was not obtained due to time constraints.

### 5.5.5 The influence of *tylS* on polyketide synthesis

In the wild type strain when *tylS* was disrupted and then re-introduced, its ability to produce tylosin, having been abolished, was then restored (N. Bate, personal communication) This behaviour is consistent with TylS being an activator of tylosin biosynthesis. Also consistent with this, GS62:*tylS*-KO could neither convert OMT to tylosin or accumulate tylactone in response to the exogenous compound. One aspect of the phenotype of GS62-*tylS*-KO was unexpected. When *tylS* was reintroduced to the strain, tylactone accumulation was not stimulated by OMT although the fed material was converted to tylosin. In the wild type, tylosin production was restored by reintroduction of the

disrupted gene. The failure of GS62:*tylS*-KO to display the equivalent characteristic (restoration of tylactone accumulation in response to OMT) is difficult to explain at this stage. A lower than normal level of OMT was used to feed in all the strains described in this chapter into which regulatory genes were reintroduced into GS62 disrupted strains. This lower level of OMT was used in order to preserve stocks, on the assumption that the level used would still ordinarily produce the same level of tylactone accumulation in mycaminose-disrupted strains (section 3.8.1). As has been shown in this chapter, other GS62 derived strains are quite capable of accumulating tylactone to the usual high degree given the same, reduced level of OMT. Due to time restraints this experiment was not repeated with a higher level of OMT. If confirmed by analysis of further fermentation extracts then this initial observation might lead to an important insight into the function of TylS.

### 5.5.6 The influence of *tylT* on polyketide synthesis

As in the case of GS62:*tylQ*-KO, the most interesting aspect of the phenotype of GS62: *tylT*-KO is the accumulation of 5-*O*-mycarosyl tylactone and, in smaller quantities, tylactone itself. Again, time constraints prevented this initial observation from being confirmed by multiple fermentations. If this data were to be replicated it would indicate that polyketide synthesis is functional in this strain at far higher than the expected 'trickle' of expression proposed to occur in other un-stimulated mycaminose deficient strains. Also, the similarity of the phenotypes of GS62:*tylQ*-KO and GS62:*tylT*-KO would make it tempting to speculate that the two genes might be functionally connected.

### 5.5.7 Regulation of polyketide synthesis

Due to time restraints, this analysis was stopped at a relatively preliminary stage. Some of the observations, particularly those made on the basis of small quantities of material, ideally require considerable further investigation, starting with multiple fermentations to confirm the preliminary findings and confirmation of the authenticity of the *tylP* and *tylR* disrupted strains. However the results obtained are suggestive of particular functional roles for the regulatory proteins associated with the *tyl* cluster and might prove to be of some significance given further analysis.

The preliminary results suggested that none of the regulatory genes investigated (*tylP, tylQ, tylR, tylS* and *tylT*) exerted a crucial influence over the stimulation of polyketide synthesis in mycaminose deficient strains in response to glycosylated tylosin

precursors. Thus, while no great insight into this intricate regulatory process was gained, some interesting preliminary data was nevertheless obtained.

# CHAPTER 6

# INVESTIGATION OF UNCHARACTERISED FERMENTATION PRODUCTS

## 6.1 Introduction

In many of the *S. fradiae* fermentations presented in this dissertation, HPLC analysis revealed the accumulation of tylactone. Invariably, other material absorbing at 282 nm accumulated alongside it. Tylactone has a relative retention time of 1.5 with respect to tylosin. Some material can always be found eluting immediatley after tylactone (rrt 1.6). The size of this peak is variable and in some strains it is less obvious than others. The nature of the material that it represents is unknown. A more consistent feature is a peak of significant size that elutes immediately prior to that representing tylactone (rrt 1.4). This peak is always obvious in chromatograms of tylactone-accumulating strains, representing 10 - 15 % of the size of the tylactone peak it accompanies. Analysis by mass spectrometry of fermentation extracts generated as part of this project led to speculation that this compound might be an oxygenated form of tylactone (H. Kirst, personal communication). Whether this modification arises due to atmospheric oxidation of the tylactone or is an enzyme catalysed function is unknown, as is its possible significance, if any, in the biosynthesis of tylosin. It might be a non-functional shunt metabolite, an aberrantly produced side product or a compound of real physiological significance.

Sequence analysis of the biosynthetic cluster revealed an open reading frame designated orf16*. Comparison with sequence databases showed that the deduced protein product of orf16* displayed end to end similarity with many cytochrome P450 enzymes (Bate *et al.*, 1999). In addition the sequence contains the characteristic conserved sequence motifs necessary for haem attachment and oxygen binding essential for such enzymes (Poulos *et al.*, 1987). In particular orf16* displayed similarity to the product of *mycG* from *Micromonospora griseorubida*. This enzyme possesses the remarkable characteristic of being able to catalyse two specific modifications of a polyketide ring (Inouye *et al.*, 1994). The modifications catalysed by this enzyme are 12-13, epoxidation and 14 hydroxylation of the polyketide ring during the biosynthesis of mycinamicin. Another enzyme similar to both MycG and the proposed product of orf16* is PikC from *S. venezulae*. This enzyme also performs multiple functions, all hydroxylations, but on different substrates in the production of pikromycin, methymycin and neomethymycin by *S. venezulae* (Xue *et al.*, 1998). There is no apparent need for another cytochrome P450 enzyme function in the biosynthesis of tylosin. The oxidation functions necessary for tylosin synthesis have been accounted for by *tylI* (hydroxylation at C20) and *tylHI* (hydroxylation at C23) (see section 1.2.9). It is therefore possible that this gene encodes a protein that for reasons unknown, modifies tylactone to incorporate an extra oxygen atom, for instance by catalysing an epoxydation or hydroxylation. In order to investigate this possibility a construct was made with the intention of disrupting orf16* in a strain that normally produces tylactone (and the

material that immediately precedes it in the HPLC system used) such the *tylA* mutant, GS14. Unfortunately an authenticated strain failed to be generated due to time constraints and the effects of a disruption in orf 16* remain unknown.

Another candidate gene from the biosynthetic cluster for producing the material that flanks tylactone in HPLC analysis is *tylI*. The product of this gene is responsible for hydroxylation of the methyl group on C20. This is the first step in the process of converting the polyketide ring from tylactone to tylonolide following the addition of mycaminose. The enzyme responsible for the second modification at this position, dehydrogenation of the hydroxymethyl to a formyl group, remains unidentified. The *tylI* mutant, GS77, was incapable of performing either modification at C20 which suggests that the two enzymatic functions might be closely coupled, although it is possible but unlikely that the mutant might contain separate mutational blocks. The second oxidation also seems to occur less efficiently than the first (Baltz *et al.*, 1982). Conversion of the polyketide ring, from tylactone, to tylonolide is then completed by hydroxylation on C23 by the product of *tylHI*. These modifications at C20 follow the addition of mycaminose to the polyketide and thus convert 5-*O*-mycaminosyl tylactone to OMT (5-*O*-mycaminosyl tylonolide). They can be bypassed by other tylosin-biosynthetic functions but normally occur first (Baltz *et al.*, 1982). The product of *tylI* might, under conditions in which unusually high levels of tylactone accumulate, be responsible for the oxidation of tylactone, either at C20 or at some other position on the polyketide ring, without the prior addition of mycaminose. The strain containing a mutation in *tylI* , GS77, was thus engineered such that it could be encouraged to accumulate tylactone rather than its usual products, 20-deoxy-20-dihydro-OMT and 20-deoxy-20-dihydro-DMT. The easiest way of achieving this aim was to disrupt a mycaminose specific gene and stimulate tylactone accumulation by the addition of OMT. The strain deficient in mycaminose metabolism was obtained by disrupting *tylMI* using the vector described previously (section 3.4.3). This strain, GS77 has mutations in both *tylI* and *tylD* and therefore should be incapable of producing 6-deoxyallose in addition to the hydroxylation on C20. Since the strain was disrupted in mycaminose activity in any case, the mutation in mycinose metabolism should be of no significance in this context.

In addition, GS14 fermentation extracts containing tylactone and its accompanying compounds were analysed by HPLC at different wavelengths to determine whether or not any of the compounds were likely to be an epoxy form of tylactone. Epoxidation would necesarily destroy the conjugated double bond system that gives tylosin metabolites their absorbance characteristics at 282 nm.

The *tylA* mutant, GS14, when analysed (section 6.2.1) was found to accumulate tylactone, making it suitable for the investigation described above. This matched the

original reports of the phenotype of this strain (Baltz and Seno, 1981), but is inconsistent with the data presented in Chapter 3 with respect to strains deficient in mycaminose metabolism. Sequencing analysis revealed that the mutation in GS14 was a single base pair substituition occuring early in *tylAII* (A. R. Gandecha, personal communication). The resulting protein would contain an isoleucine instead of threonine six amino acids into the sequence, just upstream of the proposed $NAD^+$ binding motif. This mutation clearly exerts a profound effect over sugar metabolism in *S. fradiae*, causing it to accumulate tylactone rather than glycosylated material. A strain deficient in the proposed function of *tylAII*, synthesis of the dTDP-4-keto, 6-deoxyglucose sugar precursor common to all the tylosin sugar pathways, should be unable to glycosylate tylactone and thereby stimulate polyketide synthesis. Thus no tylosin-precursors (including tylactone) should accumulate in fermentation. The *tylA* mutant, GS14 was analysed in an attempt to reproduce and perhaps explain its reported phenotype. This was done by feeding GS14 with differently glycosylated tylosin metabolites during fermentation. These included OMT and compounds desmycosin and DMT (demycinosyltylosin) which do not lie on the preferred biosynthetic route, but can nevertheless be converted, to tylosin (Figure 6.1).

## 6.2 Analysis of the *tylA* mutant, GS14

### 6.2.1 Analysis of GS14 by HPLC

When the fermentation products of GS14 were analysed, tylactone was found to have accumulated in very large amounts (Figure 6.2). In addition to tylactone and the flanking peaks which invariably accompany its accumulation, two further peaks were obvious in the HPLC traces produced. A small peak (rrt 1.2) can be identified, the nature of which is unknown. A small peak with the same relative retention time can sometimes be seen in the fermentation extracts of mycaminose-deficient strains in which tylactone accumulation has been stimulated (section 3.7 and 5.3.2). It has not been referred to previously as the peak is usually less easy to identify, being obscured by other material, and its significance, if any, is unknown. Another relatively small but significant peak of material (rrt 0.8) co-elutes with desmycosin, a tylosin-metabolite glycosylated with mycaminose and mycinose, which does not lie on the preferred biosynthetic route to tylosin in *S. fradiae*. (Figure 6.1). Desmycosin occurs when the 6-deoxy-allose moiety, substituted onto C23 of the polyketide ring is methylated prior to, rather than after the addition of mycarose. This deviant from the preferred biosynthetic route accumulates in some blocked mutants and can be converted to tylosin.

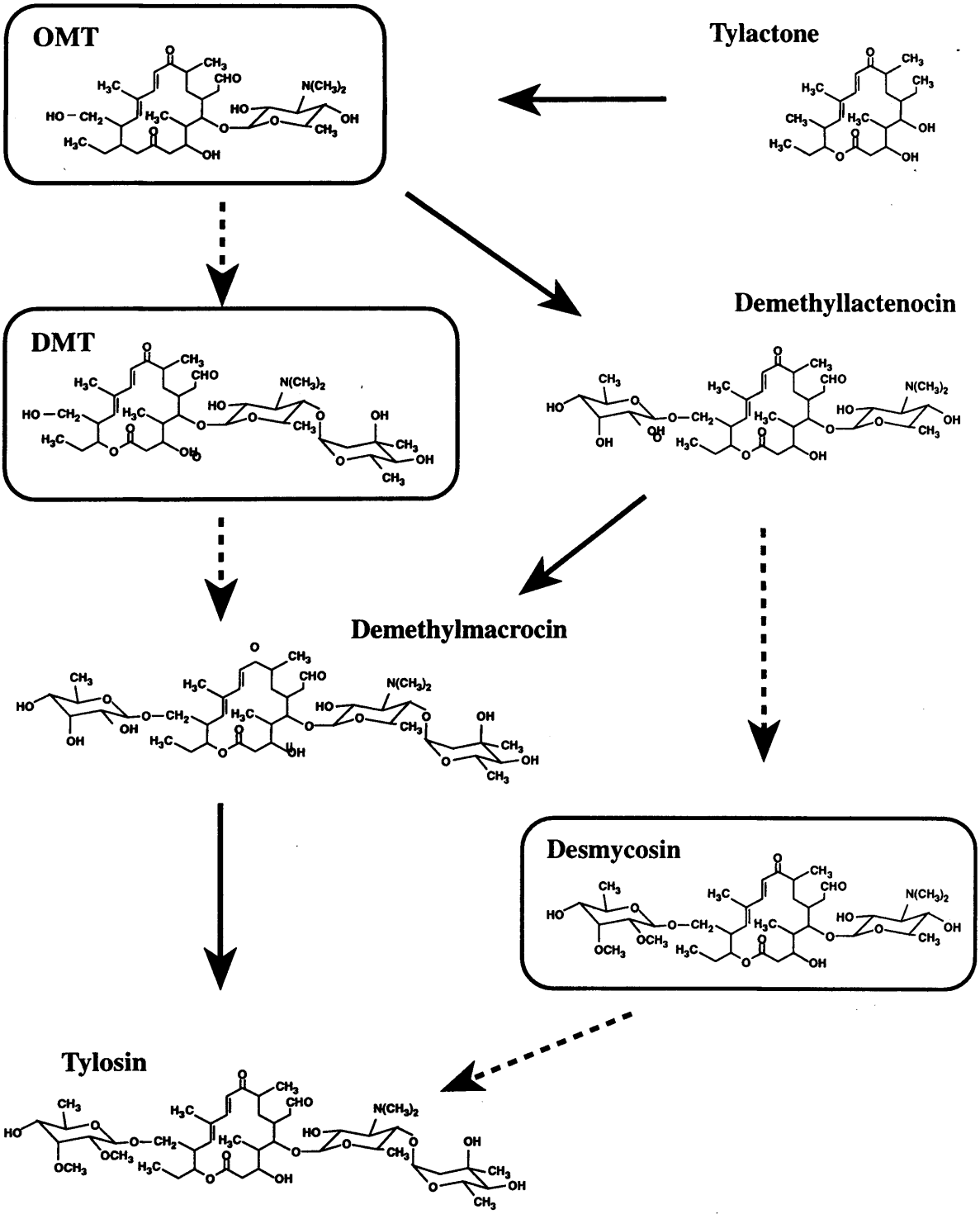



**Figure 6.1 The glycosylated compounds used to feed GS14.** The compounds OMT, DMT and desmycosin used to feed the *tylA* mutant GS14 are shown boxed. The prefered pathway from OMT to tylosin goes via demethyllactenocin and demethylmacrocin (solid arrows). However this route is not obligatory and DMT and desmycosin which can ordinarily both be converted to tylosin (dashed arrows) accumulate in blocked mutants.

The suggestion that desmycosin might accumulate in a strain deficient in one of the genes thought to provide the sugar intermediate common to all the tylosin sugars was surprising. No desmycosin had previously been identified in fermentations of GS14, albeit produced under slightly different conditions (Baltz and Seno, 1981). Fermentations supplemented with the tylosin metabolites OMT, DMT, and desmycosin itself confirmed that despite appearences it was unlikely that the observed material was desmycosin. In OMT-fed fermentation, although no OMT appeared to be present at the end, no increase in the size of peak of material co-eluting with desmycosin was evident (Figure 6.2). Similarly no conversion to tylosin could be seen in fermentations supplemented with DMT or desmycosin, the fed compounds in these cases being evident as an extra peak (rrt 0.6) or a clear increase in the size of the 'desmycosin' peak respectively. That the amount of material co-eluting with desmycosin appears to be independent of the addition of compounds which should be converted to desmycosin suggests that it might not be what it seems.

### 6.2.2 Analysis by mass spectrometry shows GS14 does not make desmycosin

The fermentation extract of GS14 was analysed by mass spectrometry (Figure 6.3). No trace of any species with an *m/z* value equivalent to that expected for desmycosin (772), or any other glycosylated tylosin metabolite, was present. If the material seen in HPLC traces of GS14, was desmycosin, the quantity present should make it easily detectable by this sensitive technique. This provides compelling evidence that GS14 is not capable of producing desmycosin and that the material eluting with a rrt 0.8 represents other material of unknown origin. The presence of tylactone was confirmed by the range of peaks that seem to be characteristic of its mass spectra (*m/z* - 377, 395 (tylactone), 474, 790 and 811) (section 3.2.2). The material seemingly charateristic of fermentaion extracts generally (*m/z* - 311) was also represented.

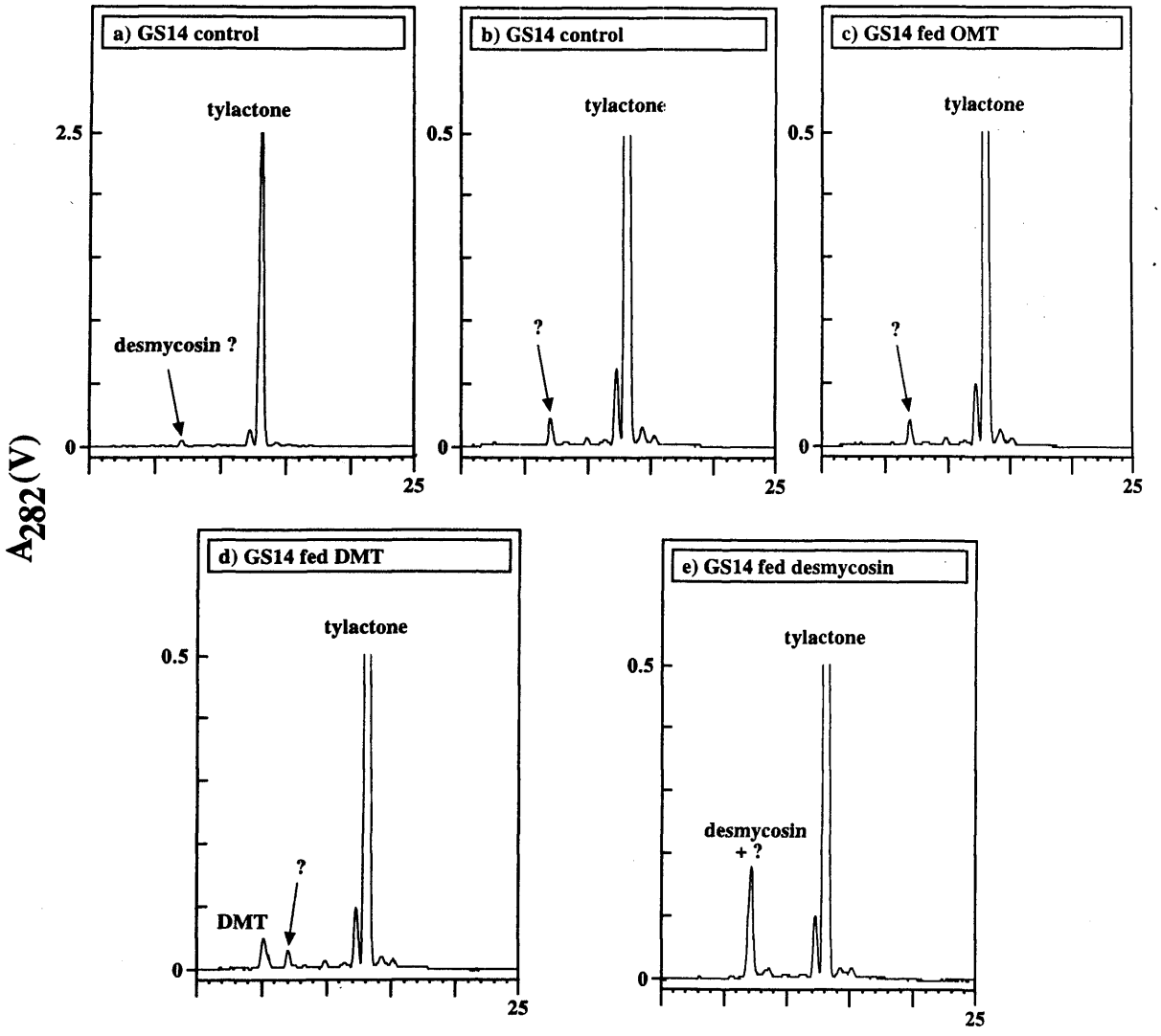



**Figure 6.2 HPLC analysis of the *tylA* mutant strain, GS14.** The fermentation products of GS14 (a) and (b) control (fed DMSO), (c) fed OMT, (d) fed DMT and (e) fed desmycosin, as visualised by HPLC at 282 nm.

As indicated, the scale used here is 0 - 0.5 V (on all panels except (a), 0 - 2.5 V).

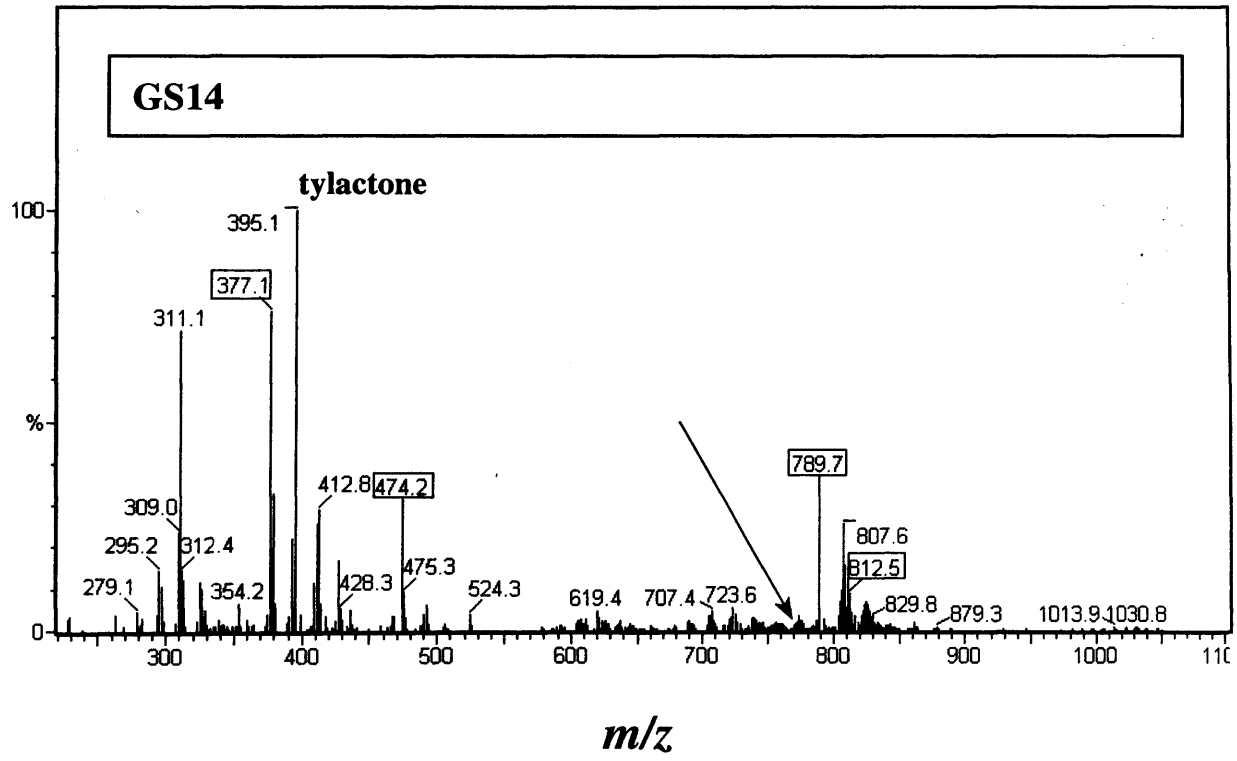



**Figure 6.3 Analysis of GS14 by mass spectrometry.** As expected, mass spectra characteristic of tylactone was obtained when GS14 was analysed. Apart fromt the ubiquitous *m/z* 311 material, tylactone (*m/z* 395) constiutes the major species present accompanied as always by peaks with *m/z* values of 377, 474 and 811 (boxed). No species with an *m/z* value equivalent to to desmycosin (the predicted position of which is marked with an arrow) could be convincingly identified.

### 6.2.3 GS14 does produce bioactive material

Extracts of GS14 fermentations were investigated for bioactive material by determining their ability to inhibit the growth of *Micrococcus luteus* (Figure 6.4). This analysis revealed that un-supplemented GS14 extracts did produce bioactive material. A small zone of inhibition is visible around the disc representing GS14. Another disc soaked in the equivalent amount of desmycosin as the peak with which it co-elutes would represent in a GS14 fermentation, produced a far larger zone. Thus although GS14 did not accumulate desmycosin or any other glycosylated tylosin intermediates detectable by the methods applied, it did produce bioactive material whose nature is unknown.

## 6.3 Analysis of GS14 by HPLC at 238 nm

In a crude attempt to investigate the suggestion that the material that co-accumulates with tylactone might be epoxytylactone, HPLC of GS14 fermentation extract was carried out at 238 nm (Figure 6.5). Because of the use of methanol in the mobile phase, the HPLC system applied here is not well suited to analysis at this wavelength, resulting in an unavoidably noisy baseline. The tylactone present in each sample clearly absorbs far less well under these conditions. Immediately preceding tylactone and including the area in which, at 282 nm its preceding peak (rrt 1.4) would elute, some material is evident, possibly representing a range of different species covering as it does a relatively large and diffuse area. This material is not represented by a sharp isocratic peak more an ill-defined 'smudge' and thus cannot represent the same material that absorbs at 282 nm which displays entirely different separation characteristcs. The tylactone-preceding peak is wholly obscured so any effect upon it is hard to judge. A more defined peak elutes immediately prior to the diffuse peak, rrt 1.2. This could easily represent the same material evident at 282nm in GS14 fermentation extracts and those of other tylactone-accumulating strains. A peak rrt 1.6 is visible eluting after tylactone at both wavelengths in the GS14 fermentation extract. The peaks visualised at these different wavelengths although co-eluting, might not represent the same material.

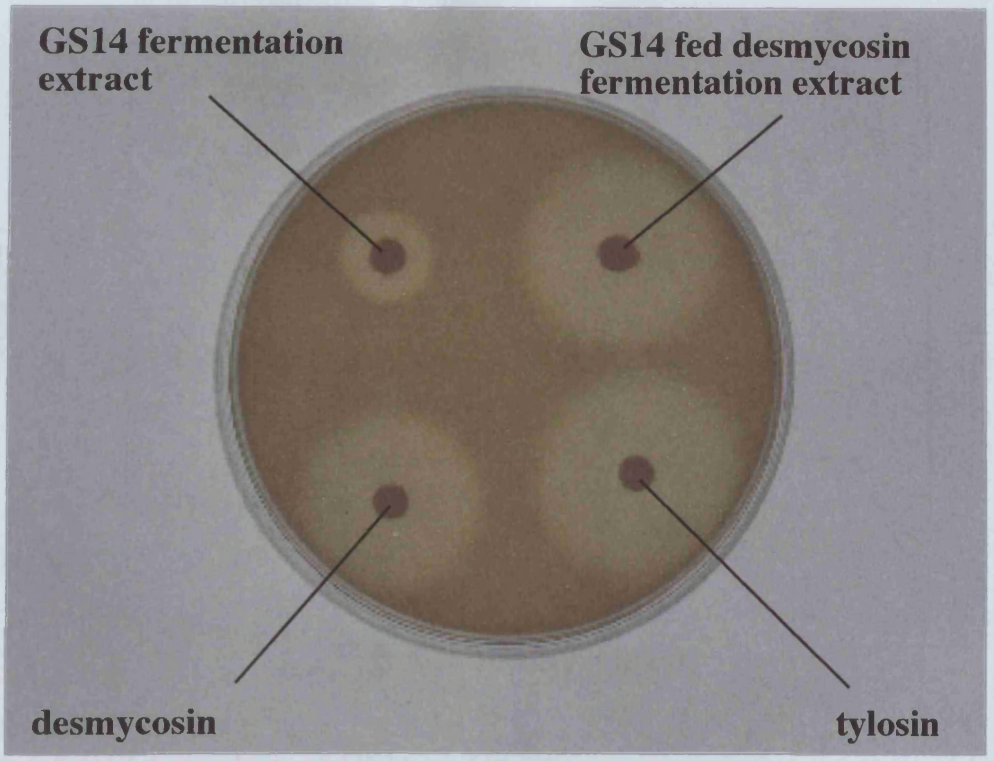



**Figure 6.4 GS14 fermentation extracts contain bioactive material.** The bioactivity of GS14 and GS14 fed desmycosin fermentation extracts were assayed on a *Micrococcus luteus* plate (section 2.9.5). An amount of desmycosin, which would be equivalent to the material that co-elutes with desmycosin and a similar quantity of tylosin were used as controls. The GS14 fermentation extract clearly displays bioactivity but not as great as if it contained desmycosin in the quantity indicated by the peak that co-elutes with desmycosin.

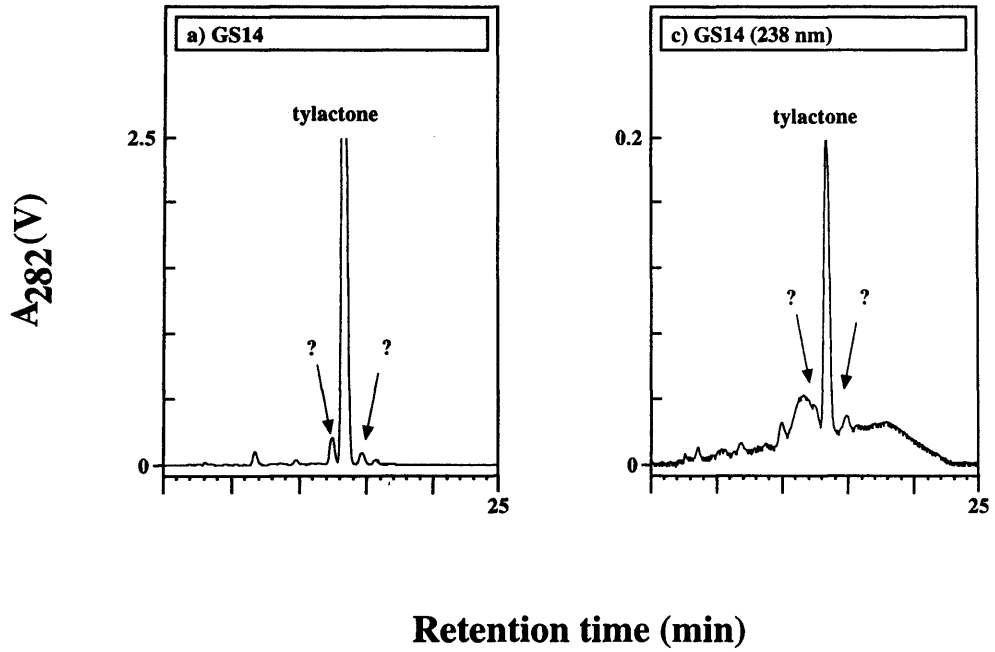



**Figure 6.5 HPLC analysis of GS14 at 238 nm.** The fermentation products of GS14, as visualised by HPLC at (a) 282 nm and (b) 238 nm respectively.

As indicated, the scale used are 0 - 2.5 V (282 nm) and 0 - 0.2 V (238 nm).

## 6.4 Analysis of the *tylI* mutant, GS77, disrupted in *tylMI*

### 6.4.1 Generation and confirmation of a *tylMI* disruption in GS77

The construct used to generate *tylMI*-KO (section 3.4.3) was also used to create GS77:*tylMI*-KO. Candidate disruptants were initially selected on the basis of their resistance to hygromycin B and sensitivity to apramycin. Confirmation that one isolate chosen at random had undergone a successful double recombination event, resulting in replacement of the chromosomal gene by the disrupted version was obtained by Southern blot hybridisation. When the hygromycin resistance cassette was used as probe the hybridisation target in *Afl*III digested GS77:*tylMI*-KO genomic DNA was of the predicted size, 6.9 kb, but no band was found in the wild type (Figure 6.6).

### 6.4.2 Analysis of GS77:*tylMI*-KO by HPLC

The *tylI/D* GS77 was expected to produce 20-deoxy-20-dihydro-*O*-mycaminosyltylonolide and 20-deoxy-20-dihydro-demycinosyltylosin (Baltz and Seno, 1981) when fermented. Two major peaks of material were produced which were presumed to represent these species (Figure 6.7). This was impossible to confirm by HPLC analysis as standard compounds were not available. When disrupted in *tylMI* (GS77:*tylMI*-KO), as expected, the strain did not accumulate any intermediates of tylosin in significant detectable amounts. This was consistent with a strain incapable of performing the first glycosylation in the biosynthetic route. When the strain was supplemented with OMT in fermentation, HPLC analysis indicated that this compound had, as expected, been converted to DMT. This indicated that apart from the engineered inability of the strain to add mycaminose, the strain maintained the same glycosylating abilities (mycarose synthesis and addition, 6-deoxy-allose deficient) as its ancestor, GS77. In addition, as intended, tylactone accumulated in large quantity.

Eluting immediately prior to tylactone a small 'bump' can be identified in the position where a definate peak has always previously been encountered whenever tylactone has accumulated. A GS14 fermentation is shown at the same scale for comparison (Figure 6.7). This evidence seems to indicate that the product of *tylI* might be responsible for producing this tylactone-preceding peak as a definate alteration in the tylactone-accumulating phenotype has occurred. Following tylactone again a small 'bump' can be seen. The significance of this is less easy to establish as this peak is more variable in tylactone-accumulating fermentations.

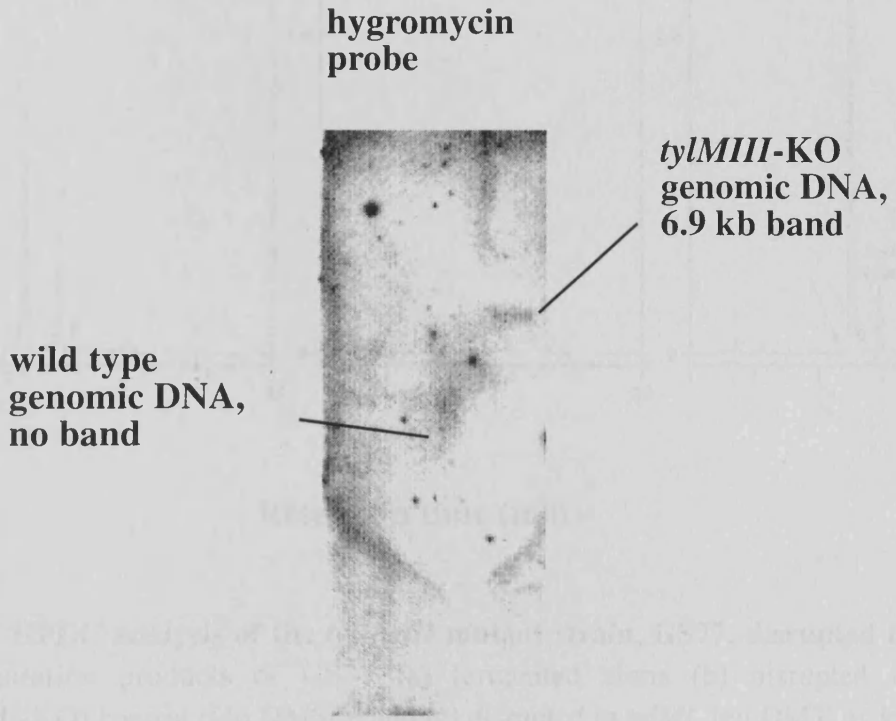



**Figure 6.6 Confirmation of the GS77:*tylMI* disrupted strain.** Southern blot hybridisation analysis confirmed that the putative *tylMI* disruption in strain GS77 was authentic. Wild type and disrupted strain genomic DNA were digested with *Afl*III . A probe was prepared from the hygromycin cassette and lit up a band of the predicted size (6.9 kb) in the disrupted strain but no band in undisrupted parent strain, GS77.

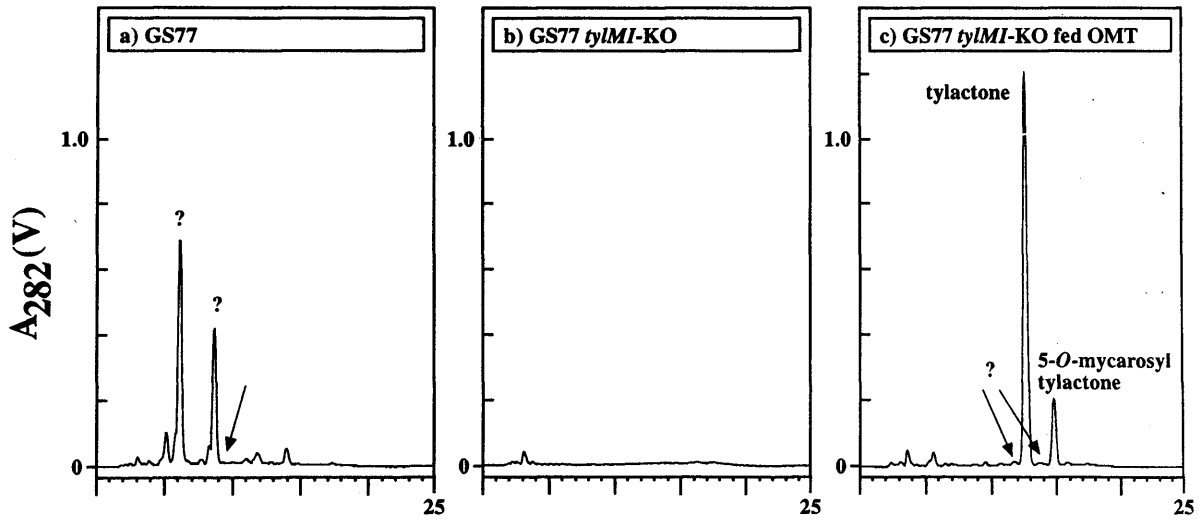



**Figure 6.7 HPLC analysis of the *tylI/tylD* mutant strain, GS77, disrupted in *tylMI*.** The fermentation products of GS77 (a) fermented alone (b) disrupted in *tylMI* (GS77:*tylMI*-KO) control (fed DMSO) and (c) disrupted in *tylMI*, fed OMT, as visualised by HPLC at 282 nm.

As indicated, the scale used here is 0 - 1.0 V.

### 6.4.3 Analysis of GS77:*tylMI*-KO by mass spectrometry

Mass spectral data from analysis of GS77:*tylMI*-KO fed OMT displayed similar characterisitics to other tylactone-accumulating fermentation extracts (Figure 6.8). It contained material with an *m/z* value of 311 as seen in all the fermentation extracts analysed. Consistent with the accumulation of tylactone (section 3.2.2), species with *m/z* values of 377, 395 (tylactone), 474, 790, and 811 are present. Strain GS77 was fed OMT (predicted *m/z* value 598), which should have been converted to DMT (predicted *m/z* value 742). No material of this mass is obvious although it might be present but obscured in a group of peaks centering on *m/z* 744. The GS77:*tylMI*-KO, OMT-fed fermentation extract also contains material with an *m/z* value of 539, consistent with 5-*O*-mycarosyltylactone. No similar material is present in GS14 (Figure 6.3), consistent with a strain unable to synthesise mycarose (or any of the other tylosin sugars). No other convincing differences can be identified between GS77:*tylMI*-KO fed OMT and other tylactone accumulating strains when their fermentation extracts are analysed by this method.

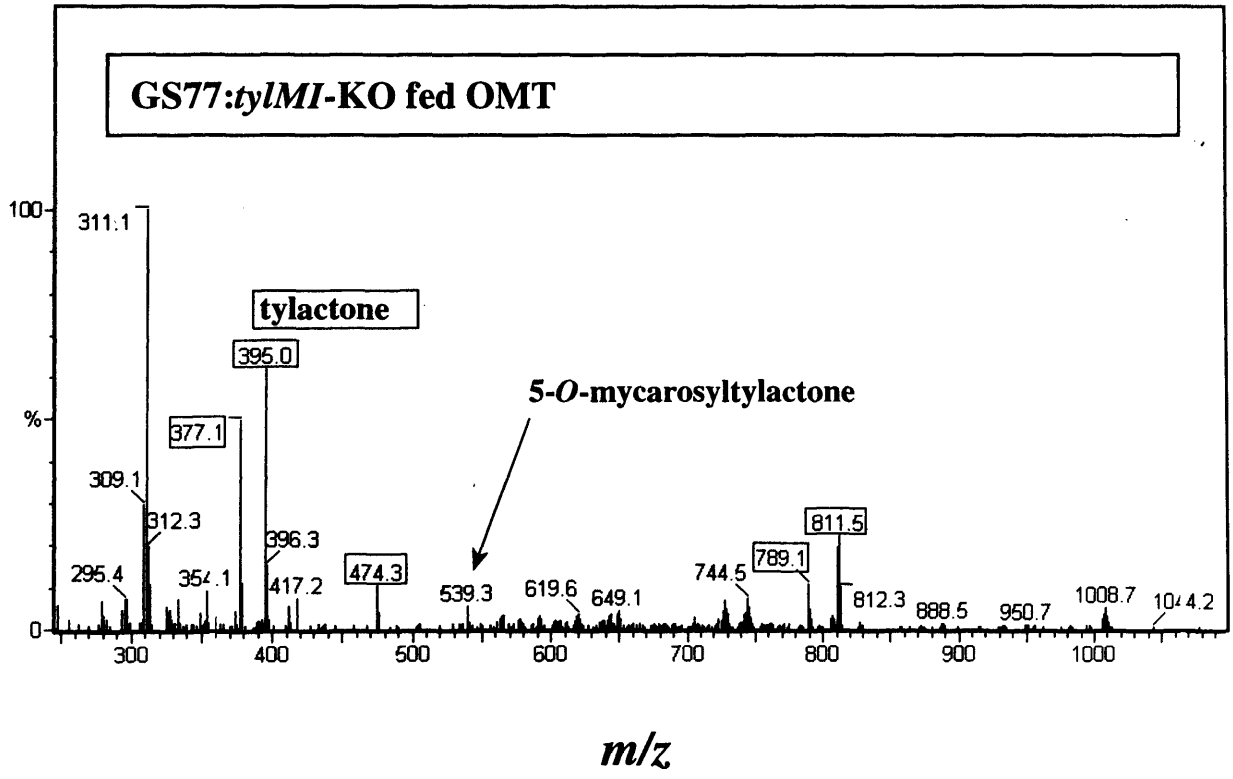



**Figure 6.8 Analysis of GS77:*tylMI*-KO by mass spectrometry.** As expected, mass spectra characteristic of tylactone was obtained when GS77:*tylMI*-KO was analysed. Apart from the ubiquitous *m/z* 311 material, tylactone (*m/z* 395) constiutes the major species present accompanied as always by peaks with *m/z* values of 377, 474, 790 and 811. A peak with an *m/z* value consistent with 5-*O*-mycarosyltylactone is also present.

## 6.5 Discussion

### 6.5.1 The phenotype of GS14

A strain capable of producing desmycosin, when fed with OMT or DMT, should be capable of the synthesis and addition of mycinose (C2'' and C3'' methylated, 6-deoxyallose) to form desmycosin and tylosin respectively. In OMT-fed fermentation, although no OMT appeared to be present at the end, no increase in the size of peak of material co-eluting with desmycosin was evident. The mysterious disappearance of the exogenous OMT has been observed previously in *S. fradiae* fermentations in which it appeared to have been degraded to polar compounds (Baltz *et al.*, 1982). Similarly no conversion to tylosin could be seen in fermentations supplemented with DMT or desmycosin. The amount of material co-eluting with desmycosin appears to be independent of the addition of compounds which should be converted to desmycosin. This and the mass spectral data presented clearly indicate that the peak (rrt 0.8), although co-eluting with, is not desmycosin.

The most likely candidate for a bioactive compound in any *S. fradiae* fermentation has to be a glycosylated intermediate of tylosin. The *tylA* mutant strain is clearly incapable of normal tylosin sugar metabolism but, as shown previously, (3.8.1) a very low concentration of glycosylated polyketide is capable of stimulating tylactone accumulation to a very high level. The accumulation of tylactone and the presence of bioactive material would be consistent with GS14 producing a tiny amount of sugar precursor but no trace of any glycosylated tylosin precursor could be found when the GS14 fermentation extract was analysed by mass spectrometry. In the absence of even trace amounts of such material it is difficult to argue that any glycosylated tylosin metabolites are present. The material that co-elutes with desmycosin makes the matter particularly intrigueing. A strain disrupted in *tylAII* is currently under construction in the laboratory (A. R. Butler, personal communication) which may well shed more light on this matter. What is clear from this data is that if glycosylation can in any way occur in GS14, through very low activity of the *tylAII* mutant protein, an orthologue of *tylAII* elsewhere in the genome or by using other nucleotide activated sugars then it occurs extremely inefficiently such that the products of these activities are undetectable by the methods applied here.

### 6.5.2 GS14 might produce epoxidised tylosin metabolites

One initial suggestion regarding the nature of the compounds that flank tylactone in the HPLC system used was that they could be epoxidised forms of tylactone, the

epoxidation having occurred between C10 and C11 or C12 and C13. One reason why this seems unlikely is that such an epoxidation would disrupt the conjugated double bond system that forms the basis of the excellent absorption at 282 nm that makes tylosin intermediates visible at this wavelength. The macrolide antibiotic rosaramicin is very similar to the tylosin intermediate OMT, the major difference being that it is epoxidised between C12 and C13 (as discussed in section 3.9.7). This compound does not absorb well at 282 nm in the HPLC system used. It can however be visualised well at a lower wavelength (238 nm). The data obtained at 238 nm from the fermentation extracts of GS14 shows that some material can be visualised at 238 nm that is far less obvious at 282 nm, relative to tylactone. The peak eluting with a rrt of 1.2 might be the same material that is visualised at 282 nm as a small peak in tylactone accumulating strains. The larger 'smudge' of material might represent a number of different chemical species. This material would totally obscure the material that at 282 nm can be seen eluting immediately prior to tylosin. It is possible that the compunds which absorb at 238 nm, but not 282 nm are epoxidated metabolites of tylosin. The data is inclonclusive regarding the material that co-accumulates with tylactone and flanks it in the HPLC system used. Further analysis would be required in order to characterise these species. Analysis by HPLC-mass spectometry might prove useful or purification of the peaks and analysis by mass spectrometry or NMR.

### 6.5.3 Is TylI responsible for the tylactone co-accumulating material ?

The data presented in this chapter indicates that the material that immediately precedes tylactone in the HPLC system used and possibly also the material that elutes immediately after, might be the products of TylI. If so, the most likely possibility is that when levels of tylactone accumulation are high, the enzyme is encouraged to perform its normal catalytic role but on the slightly different to normal, un-glycosylated, tylactone substrate. Under normal conditions, the product of *tylI* is proposed to act, following the addition of mycaminose to the polyketide tylactone. TylI performs the hydroxylation on C20 of 5-*O*-mycaminosyl tylactone (Figure 1.2). Under the physiologically unusual conditions contrived in certain strains, in which tylactone to accumulates in extremely high quantities, it is possible that TylI might accept tylactone rather than 5-*O*-mycaminosyl tylactone as its substrate. The products of such activity would be 20-dihydro, 23-deoxy-tylonolide which would molecular weight equivalent to tylactone plus 16. The possibility also exists that this enzyme might act on the polyketide ring in something other than its normal function. The mass spectometry data obtained from analysis of GS77:*tylMI*-KO is inconclusive (section 6.4.3). Some small differences can be identified, compared to mass spectral data obtained from other tylactone accumulating strains such as GS14 (section

6.2.2) but nothing to which any significance can be attached at this stage. Determing the nature and derivation of these compounds would again require further, more sophisticated analysis. Nevertheless the clear reduction in the relative peak sizes of the tylactone-flanking material in a *tylI* deficient strain, as judged by HPLC analysis, is suggestive that *tylI* might be responsible for synthesis of these compounds.

# CHAPTER 7

# DISCUSSION

## 7.1 Mycaminose specific genes

The *tylM* region of the *S.fradiae* genome contains four closely packed and similarly oriented open reading frames, thought to be associated with biosynthesis of the macrolide antibiotic, tylosin (Gandecha *et al.* 1997). These lie immediately downstream and similarly oriented to the five polyketide synthase mega-genes (*tylGI* - *tylGV*). The resistance determinant, *tlrD*, borders the *tylM* region at the other end and is oriented in the opposite direction. One of these genes, designated *tylMII*, encodes the mycaminosyltransferase necessary for tylosin production. This function is the essential first step in the conversion of the biologically inactive polyketide ring, tylactone, to tylosin. A strain disrupted in this gene was generated and its fermentation products were analysed (Fish and Cundliffe, 1997). It displayed an unexpected non-producing phenotype, rather than accumulation of tylactone. In order to determine that other tylosin-specific functions remained unaffected in this strain, it was fed with differently glycosylated precursors of tylosin. In each case, the expected conversion to tylosin took place and, in addition, tylactone was accumulated. A complicating factor in explaining this phenotype was that downstream of *tylMII* in the *tylM* region lies *ccr*, a gene thought to be involved in the supply of carbon precursors for polyketide synthesis. It was therefore possible that the disruption in *tylMII* led to a non-tylactone accumulating phenotype because of a downstream polar effect on this gene. A number of pieces of evidence refuted this suggestion (section 3.6). Most importantly, a strain disrupted in *ccr* itself manifested no significant detectable effect on tylosin production. An intricate positive feedback mechanism was therefore postulated to explain the data obtained. Thus polyketide synthesis occurs at only very low levels unless glycosylation can occur. The presence of glycosylated tylosin precursors exerts a strong up-regulatory effect on polyketide synthesis and tylactone accumulates.

*S. fradiae* strains deficient in three other mycaminose specific genes were analysed. GS50 contains a mutation in *tylB*, the aminotransferase necesary for the synthesis of tylosin. This gene is located on the other side of *tylG*, ≈ 50 kb distant from its functional counterparts. A strains disrupted in *tylMI* was also generated. This gene encodes the methyltransferase that performs the final modification in the synthesis of mycaminose. It lies between and is similarly oriented to *tylMII* and *ccr*. Similar behaviour to *tylMII*-KO with respect to tylactone accumulation confimed the important influence that mycaminose metabolism exerts over polyketide synthesis.

When the sequence of the *tylM* region was published (Gandecha *et al.*, 1997), the function of the product of orf1* remained obscure. This open reading frame lies directly downstream of the polyketide synthase (*tylG*) genes. The behaviour of a strain disrupted in

orf1* in feeding experiments and complementation studies demonstrated orf1* to be a mycaminose specific gene and deserved the designation *tylMIII*.

## 7.2 Transcription in the *tylM* region

Complementation of strains disrupted in *tylM* genes showed that *tylMIII*, *tylMII* and *tylMI* are co-transcribed. No comment could be made about the transcription of *ccr* without RNA analysis being carried out. This was not an unexpected result, the arrangement and functional relation of the *tylM* genes was highly suggestive of co-regulation. Similar operons, containing related genes can be found in other biosynthestic clusters in actinomycetes such as *Saccharopolyspora erythraea* (Reeves *et al.*, 1999). The *tyl* cluster itself contains other potential examples. Some of the mycarose genes seem to be co-transcribed in a similar manner (Bate *et al.*, 1999). Presumably this is purely to facilitate the co-regulation of different aspects of tylosin biosynthesis.

With the exception of *tylMII* and *tylMI*, the biosynthetic genes of the *tylM* region potentially overlap each other and *tylG*. It has been suggested that such terminal overlap implies that the genes involved are not only transcriptionally but translationally coupled. A strain disrupted in *tylGV* showed that the *tylM* genes were expressed independantly of *tylG*. Despite the upstream disruption, the *tylM* genes were expressed such that they were capable of glycosylating tylactone at levels considerably exceeding normal wild type. Clearly a promoter element must be present, embedded within *tylGV*. The fact that this promoter accounts for at least normal levels of expression, suggests that it is the physiological promoter for these genes. In this respect the experiments performed are superior to promoter probe analyses as they are carried out with the promoter in its natural location. There is no need to postulate that the *tylM* genes might be co-transcribed along with *tylGV* under any other circumstances. The GTGA sequence that terminally overlaps these genes is thus more likely to be an artifact of the extremely close packing of genes in the biosynthetic cluster and the biased codon usage of translational stop codons. It would be interesting to determine whether RNA transcript spanning the junction between *tylGV* and orf1* can be found.

## 7.3 Stimulation of polyketide synthesis

The positive feedback loop controlling polyketide synthesis in *S. fradiae* is a fascinating and previously un-encountered mechanism in the regulation of antibiotic biosynthetic pathways. In the model suggested by the evidence presented here it appears to be a catalytic phenomenon. Polyketide synthesis continues at a 'trickle' until the

concentration of stimulatory molecule (glycosylated polyketide) rises above a threshold level, when the level of synthesis becomes greatly increased. The level of synthesis is not then subject to any further attenuation based on increased concentrations of stimulatory molecule. It is interesting that the more advanced along the route to tylosin the glycosylated precursor is, the less stimulatory effect it exerts (Fish and Cundliffe, 1997). This could be a regulatory device to enable polyketide synthesis to remain 'in step' with sugar metabolism in *S. fradiae*. Very limited polyketide synthesis occurs until glycosylation becomes possible. Once OMT is produced, polyketide synthesis is stimulated and tylosin synthesis can continue. However, if polyketide synthesis begins to outstrip the ability of the strain to glycosylate it then OMT levels would fall, relative to levels of tylosin and other 'more advanced' tylosin-precursors. Thus polyketide synthesis would be stimulated to a lesser degree and the accumulation of tylactone would be prevented. Under the fermentation conditions used here, tylosin is the only product detectable by HPLC. No additional accumulation of tylactone of any other product is evident. This is suggestive of the biosynthesic process being relatively tightly shackled to some regulatory device. It would be interesting to confirm that a qualitatively similar but quantitatively different 'all or nothing' response of polyketide synthesis could be replicated to each of the differently glycosylated tylosin-precursors.

It is not known whether the stimulation of polyketide synthesis occurs through positive activation or relief of repression. Promoter analysis experiments using promoter probe vectors are currently being carried out in the laboratory which should be competent to answer this question. Specifically promoter probe vectors containing the region upstream of *tylG*, placed into a foreign background (such as *S. coelicolor* or *S. lividans*) should indicate whether the promoter is active, due to the absence of repressors, or silent due to the absence of positive activators. Other promoter probe vectors, in an *S. fradiae* backgound, could be used to determine the response on this promoter to OMT under different conditions. This would be acheived by using strains disrupted in mycaminose sprecific genes. The control of polyketide synthesis might be mediated in a range of ways. Repression of *tylG* might occur at the downstream end of the coding sequence. Thus, polyketide synthesis might be preventedby the activity of a repressor, despite the promoter upstream of *tylG* being active. In particular the region of *tylGV* in which the promoter for the tylM genes lies would be a candidate for just such a repressor binding site. Strains also disrupted in putative regulatory genes such as those descibed in Chapter 5 might also be subjected to similar promoter probe analysis. The regulation of polyketide synthesis is likely to be extremely complex with many different elements. It is anticipated that proteins capable of binding glycosylated tylosin precursors will be isolated and analysed in time.

Such analysis should help to unravel the complex interactions which are likely to control expression of *tylG* and its stimulation in response to glycosylated tylosin precursors.

## 7.4 5-*O*-mycarosyltylactone and other fermentation products

The significance, if any, of 5-*O*-mycarosyltylactone in tylosin synthesis remains unknown. It is possible that it might have regulatory significance, perhaps acting as a signal to stop the stimulation of tylactone synthesis, in competition with positively acting compounds. Alternatively it might equally be a shunt metabolite of no significance. Similarly the enzyme responsible for the production of 5-*O*-mycarosyltylactone remains unidentified. Investigations directed towards this question, involving the generation of *S. fradiae* strains with multiple gene disruptions in sugar genes were curtailed due to time constraints.

Other species found to accumulate along with tylactone are also of unknown derivation and significance. In addition to 5-*O*-mycarosyltylactone, two distinct species absorbing well at 282 nm, can be identified. Preliminary evidence suggests that the product of *tylI* might be responsible for the production of at least one of these. In addition, material, possibly epoxidised tylosin metabolites, might also be accumulate. Further investigation should reveal the nature and possible significance of these species. NMR and mass spectrometric analysis are likely to be invaluable tools in this respect.

## 7.5 The tylosin biosynthetic cluster

The *tyl* cluster of *S. fradiae* is unique among other similar clusters in its complexity. Of particular interest is the congregation of regulatory elements associated with the biosyntheic genes. This characteristic should facilitate the analysis of tylosin regulation in *S. fradiae* and possibly generate useful paradigms for other systems in which the regulatory genes are not so accessible. The positive feedback mechanism proposed to control polyketide synthesis also makes *S. fradiae* remarkable. The analysis of these and other elements of the tylosin biosynthetic cluster should provide a fascinating future study.

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