Regulation of tylosin production in

*Streptomyces fradiae*

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

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Abstract

The tylosin biosynthetic (tyl) gene cluster of S. fradiae contains at least five regulatory genes (tylP,Q,R,S,T). During promoter-probe analysis, TylP inhibited expression from tylPp in S. lividans, raising the possibility of autoregulation. TylP also negatively regulated the expression of tylQ in S. lividans, although tylQ was still switched off in a S. fradiae strain disrupted in tylP. The latter strain produced more tylosin and sporulated a day earlier, whereas over-expression of tylP resulted in reduced levels of antibiotic. Expression analysis by RT-PCR performed on the latter strain revealed barely detectable transcription from multiple genes, including tylS. This was probably a direct effect since TylP repressed tylSp in S. lividans. Meanwhile, expression analysis before and after the onset of tylosin production implied that TylQ is a key, negative regulator of tylosin production. The latter hypothesis was substantiated by expression analysis of a tylosin non-producing strain wherein tylQ was over-expressed. Moreover, expression analysis of a tylR-disrupted strain confirmed that TylQ controls tylosin production by repressing tylR. TylS, on the other hand, is essential for tylR activation and maximal expression of tylGIII-MIII, as shown by expression analysis of a strain disrupted in tylS. In the same and other genetically modified strains, expression analysis revealed that tylG and/or tylM genes are not necessarily co-transcribed or even co-regulated. This finding was supported by promoter probing in S. lividans and S. fradiae.

During an extensive portion of the empirical strain improvement program carried out at Lilly Laboratories, few changes of significance seem to have occurred within the tyl cluster. No mutations were found in the tyl promoters analyzed. Of regulatory genes, only tylQ had undergone a single point mutation that resulted in inactivation of its product. Enhancement of tylosin yields in empirically improved strains appears to have mainly occurred by mutations present outside the tyl cluster.
### Abbreviations

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<td>αα</td>
<td>amino acid</td>
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<td>A</td>
<td>adenine</td>
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<tr>
<td>ACP</td>
<td>acyl carrier protein</td>
</tr>
<tr>
<td>act</td>
<td>genes involved in actinorhosin production in <em>S.coelicolor</em></td>
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<tr>
<td>AS-1</td>
<td>solid growth medium for <em>Streptomyces</em> spp. (Baltz, 1980)</td>
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<tr>
<td>AT</td>
<td>acetyltransferase</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>BARE</td>
<td>BarA Recognition Element</td>
</tr>
<tr>
<td>bld</td>
<td>bald</td>
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<td>C</td>
<td>cytosine</td>
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<td>degree Celsius</td>
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<tr>
<td>cAMP</td>
<td>adenosine 3':5' cyclic monophosphate</td>
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<td>Ccr</td>
<td>crotonyl CoA reductase</td>
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<td>CIAP</td>
<td>calf intestinal alkaline phosphatase</td>
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<tr>
<td>CoA</td>
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<td>deoxynucleoside triphosphate</td>
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<td><em>Escherichia</em></td>
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<tr>
<td>EDTA</td>
<td>Diaminoethanetetra-acetic acid disodium salt</td>
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<td>enoyl reductase</td>
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<td>erm</td>
<td>family of genes encoding resistance to MLS antibiotics</td>
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<tr>
<td>ery</td>
<td><em>erythromycin</em></td>
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<td>EtBr</td>
<td>ethidium bromide</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>FARE</td>
<td>FarA Recognition Element</td>
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<td>fatty acid synthase</td>
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<td>g</td>
<td>gram</td>
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<td>G</td>
<td>guanine</td>
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<td>GRF</td>
<td>George’s RNA extraction Fermentation medium</td>
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<td>GTP</td>
<td>Guanosine triphosphate</td>
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<tr>
<td>h</td>
<td>hour</td>
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<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<tr>
<td>IM</td>
<td>inducing material</td>
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<tr>
<td>KS</td>
<td>β-keto acyl synthase</td>
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<td>KSQ</td>
<td>β-keto acyl synthase-like domain (section ?)</td>
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<td>α helix-turn-α helix</td>
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<td>KR</td>
<td>keto reductase</td>
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<tr>
<td>kb</td>
<td>kilobase(s) or 1000 bp</td>
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<td>LB</td>
<td>Luria-Bertani</td>
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<tr>
<td>μl</td>
<td>microlitre</td>
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<td>m</td>
<td>mass</td>
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<td><em>Micrococcus</em></td>
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<tr>
<td>M</td>
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<td>Megabases</td>
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<tr>
<td>MCS</td>
<td>multiple cloning site</td>
</tr>
<tr>
<td>MIC</td>
<td>minimal inhibitory concentration</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
</tr>
<tr>
<td>MLS</td>
<td>macrolide-lincosamide-streptogramin B</td>
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<tr>
<td>mM</td>
<td>millimolar</td>
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<td>MM-1</td>
<td>Tylosin production medium (Gray <em>et. al.</em>, 1980)</td>
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<tr>
<td>MNNG</td>
<td>N-methyl-N'-nitro-N-nitrosoguanidine</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>Na₂EDTA</td>
<td>the disodium salt of ethylenediamine tetraacetic acid</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>NaOAc</td>
<td>sodium acetate</td>
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<td>NaOH</td>
<td>Sodium hydroxide</td>
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<td>nm</td>
<td>nanometre</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>-OH</td>
<td>hydroxyl group</td>
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<tr>
<td>OMT</td>
<td>5-O-mycaminosyl-tylonolide</td>
</tr>
<tr>
<td>orf</td>
<td>open reading frame</td>
</tr>
<tr>
<td>-P</td>
<td>phosphate group</td>
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<tr>
<td>PBP</td>
<td>pencillin-binding protein</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>polyketide synthase</td>
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<td>pmol</td>
<td>picomole</td>
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<td>R</td>
<td>resistance</td>
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<tr>
<td>Red</td>
<td>undecylprodigiosin</td>
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<tr>
<td>red genes</td>
<td>involved in undecylprodigiosin production</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
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<td>rpm</td>
<td>revolutions per minute</td>
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<td>rRNA</td>
<td>ribosomal RNA</td>
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<td>reverse transcriptase-PCR</td>
</tr>
<tr>
<td>rrt</td>
<td>relative retention time</td>
</tr>
<tr>
<td>S</td>
<td>svedberg unit</td>
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<td>S</td>
<td><em>Streptomyces</em></td>
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<tr>
<td>SAM</td>
<td>S-adenosyl-L-methionine</td>
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<td>Sac</td>
<td><em>Saccharopolyspora</em></td>
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<tr>
<td>SCB</td>
<td><em>S. coelicolor</em> butanolide</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>sec</td>
<td>seconds</td>
</tr>
<tr>
<td>Sm</td>
<td>streptomycin</td>
</tr>
<tr>
<td>ss</td>
<td>single-stranded</td>
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<tr>
<td>SSQ</td>
<td>Sterile Millipore SuperQ water</td>
</tr>
<tr>
<td>T</td>
<td>thymine</td>
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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>Taq</td>
<td><em>Thermus aquaticus</em></td>
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<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate-EDTA</td>
</tr>
<tr>
<td>TDP</td>
<td>Thymidine 5'-diphosphate</td>
</tr>
<tr>
<td>TEA</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TE</td>
<td>Thioesterase</td>
</tr>
<tr>
<td>TES</td>
<td>[N-tris(hydroxymethyl)methyl-2-aminoethane-sulphonic acid]</td>
</tr>
<tr>
<td>tlr</td>
<td>Tylosin resistance gene from <em>S. fradiae</em></td>
</tr>
<tr>
<td>Tm</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>TSB</td>
<td>Tryptic soy broth</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>tyl</td>
<td>Tylosin biosynthetic gene(s) from <em>S. fradiae</em></td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VB</td>
<td>Virginiae butanolide</td>
</tr>
<tr>
<td>VS</td>
<td>Virginiamycin S</td>
</tr>
<tr>
<td>VM</td>
<td>Virginiamycin M1</td>
</tr>
<tr>
<td>v/v</td>
<td>Ratio of volume to volume</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>whi</td>
<td>White</td>
</tr>
<tr>
<td>w/v</td>
<td>Ratio of weight to volume</td>
</tr>
<tr>
<td>Xgal</td>
<td>5-bromo-4-chloro-3-indoly-β-D galactoside</td>
</tr>
<tr>
<td>z</td>
<td>Charge</td>
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Chapter 1

Introduction
1.1 *Streptomyces* and antibiotics

1.1.1 The genus *Streptomyces*

A member of the bacterial order, Actinomycetales, *Streptomyces* are aerobic, *Gram* positive bacteria that are naturally found in soil. Their phylogeny, determined by ribosomal RNA sequence, places them in the prokaryotic group. They display typical prokaryotic features such as the lack of a nuclear membrane and the presence of peptidoglycan in their cell wall. Their morphological development though is more characteristic of fungi in that they derive from spores as mycelial units and develop into a complex, multinucleal, vegetative network (Figure 1.1; reviewed in Chater, 1998). Growth mainly occurs by cell wall extension of the hyphal tips. The resulting substrate gives birth to a white layer of aerial hyphae that eventually subdivide and fragment to uninucleate spores. Under favourable conditions, mature spores germinate and the life-cycle is repeated.

1.1.2 Antibiotics as natural products

Actinomycete species produce a number of compounds that are not essential for cell growth and viability, thus named ‘secondary’ metabolites. Antibiotics are biologically active secondary metabolites that are known to kill or inhibit the growth of living organisms. In liquid culture, *Streptomyces* produce antibiotics on entry to stationary phase although synthesis sometimes overlaps with the growth phase. On solid media, secondary metabolism tends to coincide with the morphological step of aerial hyphae formation and sporulation (Figure 1.1). This apparent delay in antibiotic production can be manipulated by varying the environmental conditions on liquid and solid media. For example, the onset of secondary metabolism can be induced by limitation of certain carbon or phosphate sources (section 1.4). In that context, one can define the delay in secondary metabolism as the period during which the cell primarily utilizes material for growth and, when they are depleted, the growth rate is reduced and the cell enters secondary metabolism.

In some cases, the presence of antibiotics early in growth can kill the producing organism implying that early utilization of resistance mechanisms could be essential for
Figure 1.1 The life-cycle of *Streptomyces*. A cross section of the streptomycete colony as seen throughout development in *Streptomyces coelicolor*. From left to right, pictures depict *S. fradiae* growing on solid AS1 media at 24 and 48 hours. The life cycle of a streptomycete starts with spore germination that leads to hyphal growth via elongation and branching. This occurs along the surface and inside the layer of agar. It eventually transforms into a complex layer of substrate mycelium. As aerial hyphae grow upwards, many cells from the substrate mycelium die. The birth of aerial hyphae usually coincides with the onset of secondary metabolism. The end of aerial hyphal elongation is followed by the formation of sporulation septa, consisting of two membrane layers separated by a double layer of cell-wall material. The rounding up of spores then follows at the same time as their wall thickens. Finally, mature spores are released, ready for germination.
survival (Cundliffe, 1989). In that respect, a delay in the onset of secondary metabolism ensures that producing organisms avoid suicide by rendering themselves resistant. Conversely, some antibiotic producing organisms are resistant on a constitutive basis.

Actually, it is not clear why organisms produce antibiotics. Although one can hypothesize that evolutionary pressures have contributed toward developing antibiotics as effective weapons against competitors, there is no evidence to support this. In fact, streptomycetes have not yet been shown to produce antibiotics in their natural habitat.

1.1.3 Macrolide antibiotics

Antibiotics possess unusual chemical structures that derive from modified intermediates of primary metabolism. Their synthesis is often catalyzed by enzymatic pathways. Such biosynthetic pathways are those involved in the synthesis of peptides (e.g. bacitracin), β-lactam rings (e.g. penicillin), aromatic compounds (e.g. chloramphenicol) and polyketides (e.g. actinorhodin).

Macrolides are structurally complex antibiotics. They contain large, variable, lactone rings (the core of the macrolide structure) connected to amino or neutral sugar moieties (Figure 1.2). Since the discovery of the first macrolide (pikromycin) in 1950, many more have been identified (Omura, 1984). They can be classified according to the size of the lactone ring. Erythromycin A, the most extensively studied macrolide, has a 14-membered ring (Figure 1.2A) as does oleandomycin. Tylosin and spiramycin contain 16-membered rings (Figure 1.2B, 1.2C). Macrolide antibiotics also differ in the number and type of sugars and their positions of attachment to the ring. The manipulation of both polyketide rings and their attached sugars has offered great potential for the artificial creation of novel antibiotics (reviewed in Hopwood, 1997, Liu and Thorson, 1994).

Macrolide compounds display antimicrobial activity against Gram positive bacteria and Gram negative cocci and mycoplasmas. They achieve this by inhibiting protein synthesis via interaction with the 50S subunit of the ribosome.
Figure 1.2 The structures of erythromycin, spiramycin and tylosin. A. Erythromycin A from *Sac. erythraea* consists of a 14-membered macrolactone ring and two deoxysugars, L-cladinose (a) and D-deosamine (b). B. Structure of spiramycin produced by *Streptomyces ambofaciens*. It has a 16-membered ring substituted by three sugars, mycaminose (a), mycarose (b) and forosamine (c). C. Tylosin consists of a 16-membered ring known as tylactone. 5-O-mycaminosyl tylonolide (OMT) is formed via the addition of the sugar mycaminose at C5 and oxidation at C20 and C23 of the polyketide ring. Addition and modification of mycinose and mycarose complete the synthesis of tylosin.
1.2 Tylosin biosynthesis and the tyl gene cluster in *S. fradiae*

1.2.1 Tylosin

Tylosin is a macrolide antibiotic consisting of a 16-membered polyketide ring substituted with three deoxyhexose sugars: mycaminose, mycinose and mycarose (Morin et al., 1970) (Figure 1.2C). It is industrially produced from *Streptomyces fradiae* and used in veterinary applications as well as a growth promotant. Tylosin is also made by *S. hydroscopicus* (Jensen et al., 1963) and *S. rimorus* (Pape and Brillinger, 1973).

1.2.2 Biosynthesis of tylosin

Early co-fermentation and bioconversion studies on *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)-induced mutants (Baltz et al., 1982) and cell-free extracts blocked in tylactone synthesis (Ômura et al., 1982a,b) helped in the characterization of the tylosin biosynthetic pathway (Figure 1.3). Synthesis starts with the assembly of the lactone ring which, by itself, is biologically inactive (section 1.2.7). The next obligatory step is the addition of mycaminose at C5-OH which is followed by oxidation and hydroxylation of the ring at C20 and C23 respectively, to produce 9-mycaminosyl tylactonolide (OMT). The addition of the first sugar confers on this intermediate antiribosomal activity. Tylosin synthesis proceeds with the favoured but not obligatory step of 6-deoxyallose addition at the C23 position. This step is followed by substitution of the third sugar, mycarose, at C4-OH of mycaminose. The final two steps of tylosin biosynthesis are bis-*O*-methylation at C2-OH and C3-OH of deoxyallose to form mycinose. Under certain fermentation conditions, tylosin can be further reduced to 20-dihydrorylosin (relomycin), a less active antibiotic (Jensen et al., 1963).

1.2.3 Biosynthetic gene clusters

A characteristic that separates streptomycetes and other microbes from eukaryotes is the closely packed clustering of genes, usually with related functions and sometimes co-regulated. In the case of antibiotic biosynthetic genes, this became apparent soon after methods for gene cloning were developed (Bibb et al., 1980, Thompson et al., 1980, Suarez et al., 1980). This breakthrough in *Streptomyces* molecular biology
The *tyl* loci

<table>
<thead>
<tr>
<th><em>tlrB</em></th>
<th>EDHFJ</th>
<th>CK</th>
<th>LM</th>
<th>← <em>tylG</em></th>
<th>← <em>tylF</em></th>
<th>← <em>tylE</em></th>
<th>← <em>tylD</em></th>
<th>← <em>tylH</em></th>
<th>← <em>tylI</em></th>
<th>← <em>tylA</em>, <em>tylL</em>, <em>tylB</em>, <em>tylM</em></th>
<th>← O-mycaminosyltylactone</th>
</tr>
</thead>
</table>

Tylactone

20-dihydro-23-deoxy-OMT

5-O-mycaminosyl-tylonoide (OMT)

Demethyl-macrocin

Demethyl-lactenocin

Macrocin

Tylosin

**Figure 1.3 The preferred biosynthetic route from tylactone to tylosin.** Mutations that block tylosin biosynthetic steps were all mapped in *S. fradiae* to a ~85 kb region flanked by the resistant determinants *tlrB* and *tlrC*. The tylosin precursor modifications associated with the 13 *tyl* loci have been highlighted (shaded areas). In addition, tylactone synthesis undergoes positive (+ve) feedback regulation by glycosylated precursors. Tylosin blocks the last step of its synthesis catalyzed by macrocin-O-methyltransferase (-ve).
facilitated the isolation of whole antibiotic biosynthetic gene clusters, mainly by complementation of blocked mutants via shot-gun cloning. One of the earliest demonstrations of this was the isolation of the actinorhodin biosynthetic genes on a 25 kb fragment of chromosomal DNA from \textit{S. coelicolor} (Malpartida & Hopwood, 1984). When that piece of DNA was introduced into a heterologous host, it produced actinorhodin, which suggested the tight packaging of biosynthetic, resistance and possibly regulatory genes.


1.2.4 Identification and initial characterization of the \textit{tyl} gene cluster from \textit{S. fradiae}

The first piece of DNA that was cloned from the tylosin biosynthetic (\textit{tyl}) gene cluster carried the gene encoding macrocin-O-methyltransferase, involved in the final step of tylosin biosynthesis (section 1.2.10). This was achieved via reverse genetics using the N-terminal sequence of the enzyme in order to design a hybridization probe (Fishman \textit{et al.,} 1987). Thus, the positively identified and other co-linear cosmids were isolated. Subsequent complementation of \textit{tyl} mutants followed. This led to the mapping of 13 loci (\textit{tylA-tylM}; Figure 1.4), flanked by the resistance determinants \textit{tlrB} and \textit{tlrC} (Beckmann \textit{et al.,} 1989, Fishman \textit{et al.,} 1987). \textit{tylA} and \textit{tylL} mutants were deficient in synthesizing all three sugars. The \textit{tylB} and \textit{tylM} loci were responsible for the synthesis and/or addition of mycaminose while \textit{tylD} and \textit{tylJ} loci controlled mycarose metabolism. The \textit{tyll} and \textit{tylH} loci were involved in hydroxylation of the lactone ring at position C20 and C23 while \textit{tylE} and \textit{tylF} mutants were respectively defective in the penultimate and ultimate steps of tylosin synthesis. Between the \textit{tylM} locus and the resistance determinant \textit{tlrC} there was a structurally unstable DNA segment (~30 kb) consisting of multiple direct repeats (Beckmann \textit{et al.,} 1989). Comparative studies related to the erythromycin
Figure 1.4 Organization of the tylosin biosynthetic gene cluster of *S. fradiae*. The diagram represents a contiguous piece of *S. fradiae* DNA depicting open reading frames (orfs) and their position relative to each of the 13 tyl loci, as identified by computer analysis, within the ~85 kb tylosin biosynthetic cluster (not drawn to scale). Between the resistance determinants *tlrB* and *tlrC* (shaded black) lie 41 orfs including all structural genes needed for tylosin biosynthesis. The other resistance determinant, *tlrD*, is also indicated (shaded black). *tlrA* is not associated with the biosynthetic cluster. In the middle of the cluster, the *tylG* locus covers ~41 kb and encodes a type I PKS responsible for tylactone synthesis. Each orf located upstream of *tylG* was assigned a successive number as were those downstream which were additionally distinguished with an asterisk. Those orfs with a tylosin-associated function were given a tyl name.
modular polyketide synthase genes from Sac. erythraea and sequencing of the region by P.R. Rosteck and co-workers revealed that the region encoded the polyketide tylactone synthase genes (tylG) required for production of tylactone.

1.2.5 Organization of tyl genes

The sequencing of the biosynthetic gene cluster was conducted on both sides of the Atlantic (Bate et al., 1999, Bate & Cundliffe, 1999, Bate et al., 2000, Butler et al., 2001b, Gandecha & Cundliffe, 1996, Gandecha et al., 1997, Merson-Davies & Cundliffe, 1994 Rosteck et al., 1991, Wilson and Cundliffe, 1998, Wilson and Cundliffe, 1999). The polyketide synthase genes were sequenced at Eli Lilly Laboratories but the data were not formally published (Genbank accession number U78289). Until now, most of the open reading frames (orfs) discovered were assigned functions according to similarity of their deduced protein sequence with other proteins of known function in the database. The function of some of the orfs has also been assessed by complementation analysis of tyl mutant strains (Baltz & Seno, 1981) and gene disruption studies (Bate et al., 1999, Bate et al., 2000, Bate et al., 2002, Butler et al., 2001a-2001b, Butler & Cundliffe, 2001, Stratigopoulos & Cundliffe 2002a, Stratigopoulos et al., 2002). In the current model, the tylosin biosynthetic gene cluster is ~85 kb in length and contains 41 orfs sandwiched between tlrB and tlrC (Figure 1.4). The cluster includes most of the structural genes required for tylosin production, another resistance determinant (tlrD, see below), other ancillary and regulatory genes plus orfs of unassigned function.

1.2.6 Tylosin resistance genes.

There are two more tylosin resistance determinants found in S. fradiae, in addition to tlrB and tlrC. One of them is tlrD, which is located within the cluster (Zalacain and Cundliffe, 1991) (Figure 1.4); the other, tlrA is not linked to the tyl cluster and its location has not been mapped (Birmingham et al., 1986). Although tlrA and tlrD encode methylases that target the same site in 23S rRNA, tlrA confers higher levels of tylosin resistance than tlrD by adding a second methyl group at the same residue (Zalacain and Cundliffe 1989, Kelemen et al., 1994). Moreover, tlrA is regulated through induction in response to glycosylated macrolides (Kelemen et al., 1994) whereas tlrD
was expressed constitutively when cloned in *S. lividans* (Zalacain & Cundliffe, 1991) and might be similarly expressed in *S. fradiae*. The other cluster-associated resistance gene, *tlrB*, encodes a methyltransferase that monomethylates an alternative base in 23S rRNA (Liu *et al.*, 2000). Finally, the deduced product of *tlrC* resembles an ATP-binding transport protein of unknown function (Rosteck *et al.*, 1991).

### 1.2.7 Polyketide biosynthesis

The cloning and sequencing of various polyketide synthase (PKS) genes in *Streptomyces* revealed two different types: type II PKSs resemble fatty acid synthase genes (FASs) found in prokaryotes (Figure 1.5A). They catalyze the synthesis of aromatic polyketide rings such as actinorhodin from *S. coelicolor* (Hallam *et al.*, 1988, Fernandez-Moreno *et al.*, 1992). Although in type II PKS systems each gene encodes a monofunctional enzyme, type I PKSs are similar to FASs found in fungi and vertebrates but are more complex (Figure 1.5B). They encode multifunctional proteins that contain separate domains, each with individual catalytic activity. Subgroups of these domains form modules, each responsible for one round of chain extension (Donadio *et al.*, 1991).

In each extension round, a condensation reaction occurs between a carboxylated extender unit and a decarboxylated growing polyketide chain (Figure 1.6). The acyltransferase (AT) domain selects and transfers the extender unit to the acyl carrier protein (ACP) domain via attachment to the oxygen atom at its serine side chain. ACP has a flexible, phosphopantetheine prosthetic unit that mediates transfer of the nascent polyketide from one active site to another. Thus, the extender unit exchanges the sulfhydryl terminus of CoA for the sulfhydryl terminus of the phosphopantetheine-ACP prosthetic group. The polyketide chain is attached to the thiol group of the catalytic domain, β-keto-acyl synthase (KS). Elongation occurs via KS-mediated condensation with the ACP-attached extender unit during which CO₂ is released. Then, an acyl-S-phosphopantetheinyl unit is formed on ACP and the KS active site is restored and ready for another round of chain elongation. If other catalytic domains such as ketoreductases (KR), dehydratases (DH) or enoyl reductases (ER) are part of the module, then the phosphopantetheine arm of ACP delivers the polyketide to each respective active site and the chain is modified accordingly. At the end of each round, the polyketide is translocated to the KS of the next module that includes a separate AT domain responsible
Figure 1.5 Examples of type I and type II polyketide synthase (PKS) genes. Open reading frames are shown as block arrows. Domains with individual catalytic activity are represented as circles. Each string of circles constitutes a module (for abbreviations see Figure 1.6). A. Type II PKS genes from actinorhodin resembling in organization those encoding the *E. coli* fatty acid synthase (FAS). In both examples, each gene encodes a monofunctional protein. B. Type I PKSs are encoded by multiple genes, each resembling that of a vertebrate FAS gene. Each open reading frame codes for a multifunctional protein with various catalytic domains. A group of domains constitutes a module that independently catalyze one round of polyketide chain extension (section 1.2.7).
for selecting and charging an extender unit of its choice to the ACP. The polyketide is passed from one domain to the other in production-line fashion (Staunton and Wilkinson, 1998) until a thioesterase (TE) domain fused to the terminal PKS module terminates synthesis and cyclizes the product.

The 6-deoxyerythronolide B synthase (DEBS) from the erythromycin producer *Sac. erythraea* is the best studied type I PKS (Caffrey *et al*., 1992, Leadlay *et al*., 1993) (Figure 1.5B). It consists of three multifunctional proteins and six modules that account for all six extension cycles of the polyketide chain. The tylactone polyketide synthase genes encode five multifunctional proteins (TylGI-GV) that contain eight modules in total (Figure 1.6). Although the mode of synthesis of tylactone is presumed to proceed in a similar fashion to that of DEBS, there is a distinct difference in the sequence of the domains present in their respective starter modules. The DEBS starter module consists of an AT and an ACP domain (Staunton and Wilkinson, 1998). For this type of module, the starting substrate is a non-carboxylated acyl CoA species (Wiessman *et al*., 1995, Peiper *et al*., 1995). On the other hand, the starter module for the tylactone PKS has a KS-like domain followed by an AT and an ACP domain. For a while, it was thought that this KS-like domain, along with those found in other modular PKSs, were inactive because they lacked the essential cysteine residue in their active site (Meurer & Hutchinson, 1995a, 1995b). In place of that residue, they contained a conserved glutamine residue. However, a recent study (Bisang *et al*., 1999) demonstrated that the glutamine-carrying KS-like domains are essential for chain initiation through specific decarboxylation of enzyme-bound extender units. In addition, site-specific mutagenesis of the glutamine residue highlighted its importance in the process.

Except for the five multifunctional TylG proteins, there are four other enzymes encoded in the *tyl* cluster that are related to the synthesis and modification of the polyketide ring. *tylO*, whose product was deduced to be a thioesterase (TE), is not associated with the polyketide synthase genes (Figure 1.7). When *tylO* was disrupted in *S. fradiae*, the strain produced very low levels of macrolide (Butler & Cundliffe, 1999). The phenotype was not rescued by the introduction of the TE domain from the TylGV multifunctional protein. It was suggested that this 'free' TE (or known as TE-II) is
Figure 1.6 Tylactone and its proposed synthesis catalyzed by a type I PKS. The tylosin PKS region is ~45 kb in length. It consists of five mega open reading frames (orfs) with small non-coding DNA gaps between them. Domains from each module are represented as circles. KSQ is a 'KS-like' chain initiation factor with decarboxylase activity. ACP is the acyl carrier protein while AT is the acyl-transferase catalyzing the transfer of an acyl group from acyl CoA to form acyl-ACP. KS is the ketosynthase that catalyzes the condensation of two adjacent ketoacyl-ACPs resulting in ketoacyl chain elongation on the downstream ACP. KR (ketoreductase), DH (dehydratase) and ER (enoylreductase) modify the chain by mediating the addition of a hydroxyl group as well as the formation or saturation of a double bond respectively. Each module catalyzes in a line fashion the successive addition of 3C (from methyl malonyl), 3C (from methylmalonyl), 2C (from malonyl), 3C (from methylmalonyl), 4C (from ethyl malonyl), 3C (from methylmalonyl) and 2C (from malonyl) carbon units. At the end, the extended product is cyclized by the TE (thioesterase) domain. Drawn by E. Cundliffe.
needed for physiological levels of tylactone synthesis because, presumably, it removed aberrant PKS products that would otherwise block its synthesis.

The supply of ethylmalonyl-CoA, derived from butyryl-CoA, provides the cell with building blocks for polyketide synthesis. An enzyme that catalyzes the formation of butyryl-CoA from crotonyl-CoA is encoded by the *tyl* gene, *ccr*, as deduced from database comparisons (Gandecha *et al.*, 1997) (Figure 1.7). Some *Streptomyces* species also use butyryl-CoA for straight chain fatty acid synthesis during primary metabolism. *ccr* though is not an essential gene for growth since its absence is not a lethal event (Beckmann *et al.*, 1989). Targeted disruption of *ccr* later confirmed this (Butler *et al.*, 2001b).

Database comparisons identified the *tylHI* product as a putative cytochrome P450 (Figure 1.7; Bate & Cundliffe, 1999). At Lilly Research Laboratories (Indianapolis), mutations mapped at the *tylH* locus (Fishman *et al.*, 1987) led to failure in the addition of 6-deoxyallose (section 1.2.10) (Baltz *et al.*, 1981). The product of *tylHI* was assigned the function of oxidizing the tylactone ring at position C23 before 6-deoxyallose was added. In addition, a small, adjacent orf downstream of *tylHI*, named *tylHII*, encoded a putative ferredoxin (Bate & Cundliffe 1999). Previous examples of ferredoxins accompanying P450 enzymes have been found in other *Streptomyces* species (Trower *et al.*, 1998). Whether the product of TylHII functions together with *tylHI* still remains to be established. The *tyll* gene encodes a putative P450 hydroxylase. The *S. fradiae* mutants mapped at the *tyll* locus (Baltz *et al.*, 1982) failed to convert 5-O-mycaminosyl tylactone to 5-O-mycaminosyl tyronolide by hydroxylation of the methyl group at the C20 position and subsequent dehydrogenation of the hydroxymethyl to a formyl group (Figure 1.3). Thus, Tyll was assigned this role. Although TylG post-translational modification requires at least one more gene encoding phosphopantetheneinyl transferase, such a gene is not associated with the *tyl* gene cluster.

1.2.8 A common pathway to deoxyhexose sugar synthesis

Tylactone is semi-sequentially substituted with three deoxyhexose sugars (Figure 1.3). It has been demonstrated that *S. fradiae* has the capacity to produce excess sugars, enough to substitute at least four times the amount of tylactone naturally
Figure 1.7 Genes associated with polyketide and sugar metabolism in the tylosin biosynthetic gene cluster. The *tylA* genes that are involved in the synthesis of the common sugar precursors are shaded purple. The *tylM* genes responsible for the synthesis and addition of mycaminose are shaded green. The other mycaminose-related gene, *tylB*, is found directly upstream of the *tylA* genes, on the other side of the *tylG* region. The *tylC* genes associated with mycarose synthesis and addition are clustered together with the exception of *tylCVI* that is found at the right-hand side of the cluster (shaded blue). The genes involved in the synthesis and addition of 6-deoxyallose (*tylN, tylD* and *tylI*) and its conversion to mycinose (*tylE* and *tylF*) are all clustered together (shaded orange). Genes implicated in polyketide metabolism and ring modification are also highlighted (shaded black). Genes of unknown function are indicated as white.
Figure 1.8 The biosynthetic route to the three tylosin sugars. D-mycaminose, L-mycarose and 6-deoxy-D-allose derive from the same precursor, β-D-glucose-1-phosphate. The modifications catalyzed by respective gene products are shaded gray. Drawn by E. Cundliffe and modified by S.A. Flint.
produced (Butler & Cundliffe, 2001). The biosynthesis of all three deoxyhexose sugars proceeds via a common precursor, whose synthesis is catalyzed by the products of two genes, *tylAI* and *tylAII* (Merson-Davies & Cundliffe, 1994) (Figure 1.7). These genes encode dTDP-glucose synthase and dTDP-glucose dehydratase respectively. TylAI converts glucose-1-phosphate to dTDP-glucose and TylAII in turn converts dTDP-glucose to dTDP-4-keto,6-glucose, the common intermediate for the three sugars (Figure 1.8).

1.2.9 Mycaminose metabolism

In previous studies (Fishman *et al.*, 1987), the *tylM* locus was mapped at the left-hand side of the *tylG* mega genes (in the orientation of Figure 1.4). When DNA from that region was sequenced, four orfs were identified (Gandecha *et al.*, 1997). *tlrD* (see section 1.2.6), the resistance determinant, was divergent from the *tylG* genes while the other three (*tylMI-III*) were also co-directional with *tylGI-GV* (Figure 1.7). *In silico* analysis revealed a significant similarity between the deduced product of *tylMIII* and that of *eryCII* found in *Sac. erythraea* (Salah-Bey *et al.*, 1998). EryCII is thought to catalyze the isomerization of dTDP-4-keto,6-deoxy glucose to dTDP-3-keto,6-deoxyglucose, so a similar role was assigned to the TylMIII orthologue (Figure 1.8). Targeted disruption of *tylMIII* led to a tylosin ‘null’ phenotype since no mycaminose was synthesized prior to the obligatory step of mycaminose addition (Flint, 2000).

The deduced amino acid sequence from *tylMII* was similar to that of *ery orf8* from *Sac. erythraea* (Salah-Bey *et al.*, 1998) and *dnrS* from the daunorubicin producer, *S. peucetius* (Otten *et al.*, 1995). These proteins are thought to encode enzymes similar to the characterized glycosyltransferase, MGT, from *S. lividans* (Cundliffe, 1992). Disruption analysis added to the notion that *tylMII* encoded the mycaminosyltransferase in tylosin biosynthesis (Figure 1.8; Fish & Cundliffe, 1997).

The third orf, *tylMI*, is thought to encode a methyltransferase that adds one or, most likely, both of the methyl groups onto the amino group of dTDP-3-amino-6-deoxyglucose during mycaminose biosynthesis (Figure 1.8). TylMI is also similar to SrmX from *S. ambofaciens* and OrfX from *S. nogalater* although only TylMI was shown to bind S-adenosylmethionine (SAM) (Gandecha *et al.*, 1997). All three possessed consensus sequence motifs of methyltransferases that utilize SAM as a co-substrate.
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(Kagan & Clarke, 1994). *tylMI* was disrupted and the resultant strain had a similar phenotype to the *tylMIII* and *tylMII* disrupted strains (Butler *et al.*, 2001a).

Apart from the three closely clustered *tylM* genes, *tylB* is also involved in mycaminose synthesis (Figure 1.7). Database comparisons suggested that *tylB* encoded the aminotransferase responsible for the conversion of dTDP 3-keto,6-deoxyglucose to dTDP 3-amino,6-deoxyglucose during mycaminose biosynthesis (Merson-Davies & Cundliffe, 1994) (Figure 1.8). The *tylB* disrupted strain displays a tylosin null phenotype, similar to that of the three *tylM* genes (Butler *et al.*, 2001a).

1.2.10 Mycinose metabolism

In the preferred biosynthetic pathway to tylosin, 6-deoxyallose is the second sugar to be added. In early studies, mutations that affected the synthesis and/or addition and modification of 6-deoxyallose were mapped at the *tylF* and *tylJ* loci (Beckmann *et al.*, 1989, Fishman *et al.*, 1987). Sequence analysis of the left-hand region from the tylosin biosynthetic cluster conducted in this laboratory revealed five genes (*tylN,E,D,F,J*) involved in mycinose synthesis and addition (Figure 1.7; Bate & Cundliffe, 1999). Database comparisons helped identify *tylJ* as the 3-epimerase acting on dTDP-4-keto,6-deoxyglucose and *tylD* as the NADPH-dependant 4-ketoreductase that catalyzed the final step of 6-deoxyallose synthesis (Figure 1.8). Characterization and targeted disruption identified *tylN* as the gene encoding the glycosyltransferase responsible for the addition of 6-deoxyallose to the C23-OH position of tylactone (Wilson & Cundliffe, 1998). The N-terminal sequence of the *tylE* deduced product was identical to that of the demethylmacorcin 2''-O-methyltransferase that had previously been purified. Thus, the product of *tylE* was presumed to catalyze the penultimate step of tylosin biosynthesis which is the addition of a methyl group to C20 of 6-deoxyallose (Figures 1.3, 1.8). The N-terminal sequence of the *tylF* deduced product was similar to that of the purified macrocin-O-methyltrasferase (Fishman *et al.*, 1987) that displayed absolute specificity to macrocin and SAM as co-substrates (Bauer *et al.*, 1988). Therefore, TylF most likely catalyzed the final step during tylosin production which is the addition of a methyl group at C23-OH of 6-deoxyallose to form mycinose (Figures 1.3, 1.8).
1.2.11 Mycarose biosynthesis

Mutations that were later mapped at the tylC and tylK loci (Fishman et al., 1987) were found to abolish mycarose biosynthesis (Baltz et al., 1981). Since then, a combination of database comparisons, targeted gene disruption and complementation experiments helped identify the orfs (tylCII-CVII) that encode the mycarose biosynthetic enzymes (Figure 1.7; Bate et al., 2000). Specifically, the tylCVI and tylCII products are believed to encode the 2-3-dehydratase and 2,3-enoyl reductase that are involved in the deoxygenation step of mycarose biosynthesis (Figure 1.8). At this point, the pathway has not yet been finalised since it is not known whether deoxygenation occurs before or after epimerisation that is thought to be catalyzed by the putative product of tylCVII. If epimerisation occurs before deoxygenation, then TylCVII must be a 3,5-epimerase. In the reverse occurrence of events, tylCVII encodes a 5-epimerase. In either case, synthesis involves the sequential action of a methyltransferase and a ketoreductase, thought to be encoded by tylCII and tylCIV respectively. Finally, the product of tylCV encodes the glycosyltransferase that adds mycarose to mycinose.

1.2.12 Other orfs in the tyl biosynthetic cluster

At the left-hand end of the tylosin cluster and divergent to orf7* lies orf8* (Figure 1.7), whose deduced product encodes an acyl CoA oxidase (Bate et al., 1999), perhaps providing short chain acyl CoA substrates for polyketide (section 1.2.7) or γ—
butyrolactone synthesis (section 1.5).

The orf16 and orf11* deduced products resemble a cytochrome P450 and a hypothetical ATP/GTP-binding protein, respectively (Figure 1.3; Bate et al., 1999). Their roles remain elusive. Database comparisons showed no convincing similarities for the deduced products of orf1a and orf9 (Merson-Davies & Cundliffe, 1994, Butler et al., 2001b). Recently, two submitted sequences (PgaK, Acc. No. AY034378; Aur10, Acc. No AY033654.1) from other Streptomyces species matched the deduced product of orf12* (36% identity). The role of Orf12* remains elusive.

At the tlrC end of the tyl gene cluster, the orf8 (metk) gene product displayed convincing end-to-end similarity to a large number of authentic and putative S-
adenosylmethionine synthases (Butler et al., 2001b). These enzymes catalyze the synthesis of S-adenosylmethionine, a major donor of methyl groups. Methyl groups are known to be added in all tylosin-associated sugars and they are also utilized in ribosome modification by the resistant determinants (section 1.2). In addition, the downstream gene, orf10 (metF) codes for a putative N⁵,N¹⁰-methylenetetrahydrolate reductase, potentially involved in the synthesis of N⁴-methyltetrahydrofolate which acts as a methyl donor during the conversion of homocysteine to methionine. Methionine is the substrate for the S-adenosylmethionine synthase (MetK), which suggests a synergistic role between MetF and MetK.

The putative regulatory genes present in the tyl gene cluster are discussed later (section 1.6.7).

1.3 Characteristic features of S. fradiae genetics

1.3.1 General genetic features

The development of Streptomyces genetics started more than forty years ago. From the understanding of molecular biology and physiology to the advances in cloning and studying molecular genetics, Streptomyces research now enjoys the in silico advancements that marked the turn of the century. The recent completion of the genome sequence from Streptomyces coelicolor A3(2), the best-studied streptomycete to date, allows us to have a more global view of Streptomyces genetics (Bentley et al., 2001). Similarly, at least one more Streptomyces genome has been sequenced (Omura et al., 2001) and hopefully the sequence will soon be released in the public domain.

The S. coelicolor genome is linear and 8,667,507 bp long. It displays high density of encoding DNA (7846 orfs), but not higher than other microbial genomes. On the other hand, a striking characteristic is the GC richness of the genome (72.1%), especially the almost exclusive occurrence of G or C at the third position of streptomycete gene codons (Bibb et al., 1984). Although putative functions have not yet been assigned to all orfs, the S. coelicolor genome contains 65 genes encoding sigma factors (section 1.3.2), an unusually large number considering that the E. coli genome contains only 7. Also in the S. coelicolor genome, 4% of the total number of orfs are
dedicated to secondary metabolism and are localized mostly at the two ends of the linear chromosome.

Once the *S. coelicolor* genomic sequence is fully annotated and the results of the project officially published, more genetic trends will doubtless emerge.

1.3.2 Sigma factors

Sigma (σ) factors are bifunctional in that they interact with the core RNA polymerase as well as recognize specific promoter sequences (-35, -10 regions), thus increasing RNA polymerase specificity. In brief terms, the role of a σ factor is to bind to the core polymerase, scan along and identify (in a one-dimensional manner) specific promoter DNA sequences. A large numbers of σ factors (with variable sizes) have been discovered both in *Gram* negative and *Gram* positive bacteria involved in diverse cellular responses such as heat shock (σ32) and nitrogen starvation (σ54) in *E. coli*. In *B. subtilis*, σ factors are expressed in response to developmental signals, such as σ37 and σ32 that switch on genes expressed at the onset of sporulation.

The *Streptomyces* RNA polymerase core enzyme structure (ββ′α2) is similar to that of other prokaryotic core enzymes (Jones, 1979, Buttner & Brown, 1985). In contrast, *Streptomyces* species are characterized by phenomenal RNA polymerase holoenzyme heterogeneity that is dictated by the ever growing number of versatile sigma factors isolated and studied. For example, *Streptomyces coelicolor* contains four genes (*hrdA,B,C,D*) that code for σ factors similar to the essential, housekeeping σ70 factor family, whereas *E. coli*, *B. subtilis* and *Myxococcus xanthus* contain only one (reviewed in Buttner, 1989). Other σ factors from *S. coelicolor* are responsible for the expression of genes for metabolic pathways such as agarose utilization (σ49, σ28, σ35) (Buttner *et al.*, 1988). The gene encoding σ28 was later cloned (Lonetto *et al.*, 1994) and served as the founder member of the ECF (extracytoplasmic function) subfamily. Members of this subfamily are now found in several microbial species. They generally facilitate a variety of functions such as iron uptake, nickel-cobalt efflux and synthesis of outer membrane proteins. σ28 is a homologue of the extensively studied σE from *E. coli*. It is known to
stimulate the expression of genes as a response to imbalanced synthesis of outer membrane proteins (reviewed in Missiakas & Raina, 1998).

At least two sigma factors, $\sigma^{\text{whiG}}$ (reviewed in Chater, 1998) and $\sigma^F$ (Potuckova et al., 1995), are involved in morphological differentiation of S. coelicolor. Mutant S. coelicolor strains that lack $\sigma^{\text{whiG}}$ produce 'white' colonies and are deficient in sporulation. This $\sigma$ factor is involved in initiation of spore formation and presumably exerts its control on spore maturation via the expression of $\sigma^F$. In other whi mutants containing wild type whiG genes, transcription of $\sigma^F$ was abolished, so $\sigma^{\text{whiG}}$ is not the only regulatory determinant of $\sigma^F$.

As yet, little is known about $\sigma$ factors that control the expression of genes involved in secondary metabolism. They will be discussed later (section 1.6.6).

### 1.3.3 Streptomyces promoters

The pioneering study of Bibb & Cohen (1982) showed that various heterologous promoters from E. coli and elsewhere functioned in Streptomyces lividans. However, in the same study, a variety of promoters from S. lividans were not transcriptionally active in E. coli, suggesting promoter heterogeneity among streptomycetes. Since then, a large number of promoters have been identified mainly via promoter-probing and transcript mapping, and some have been further characterized via directed-mutagenesis and RNA-polymerase assays. On the DNA sequence level, most of the promoters analyzed are quite diverse (Strohl, 1992). Most of them display poorly-conserved or no distinct $-10$ and $-35$ regions known to be recognized by the principal factor $\sigma^7$ in E. coli (Dehasth et al., 1998). Mutation analysis at the $-10$ and $-35$ region of aphDp1 (a promoter of the aminoglycoside phosphotransferase gene from S. fradiae) and ermEp1 (a promoter of the erythromycin resistant gene from Sac. erythraea) showed that only the $-10$ region was essential for promoter activity (Janssen et al., 1989, Bibb et al., 1994).

Another feature which is present in ermE and aph is that of multiple transcriptional start sites at their promoter region, some of them shown to overlap with promoters of directly divergent genes. It can be conceived that the occurrence of overlapping promoters may serve as a regulatory mechanism. For example, the
promoters of \textit{scbR} and \textit{scbA} from \textit{S. coelicolor} have recently been mapped and found to overlap (section 1.5.5; Figure 1.13). It was proposed that this occurrence served a regulatory role in the expression of these genes. The \textit{scbR} product, by targeting a single site downstream of the \textit{scbR} transcriptional start but upstream of the \textit{scbA} promoter, acted both as a transcriptional repressor of \textit{scbR} and an activator of the divergent \textit{scbA}.

Promoters have also been found to lie within coding region of adjacent genes such as \textit{afsBp} of \textit{S. coelicolor} (Horinouchi et al., 1990). In addition, it has been shown that transcription of streptomycete genes such as \textit{aph} from \textit{S. fradiae} and \textit{afsA} from \textit{S. griseus} (Horinouchi et al., 1989) share a common site for the initiation of transcription and translation. It was later demonstrated by Bibb and co-workers transcription of \textit{ermE} gene begins one nucleotide before the translational start and that translation occurs in the absence of a ribosome-binding Shine-Dalgarno sequence (Bibb et al., 1994).

Finally, the relatively frequent occurrence of tandem or inverted repeats in \textit{Streptomyces} promoters and their significance in regulation will become apparent in later sections of this thesis.

### 1.4 Physiological aspects of the regulation of secondary metabolism

#### 1.4.1 Carbon sources and secondary metabolism

Early studies have shown that the production of a number of antibiotics is inhibited by the presence of high levels of glucose (reviewed in Martin & Demain, 1980). Glucose is rapidly metabolized by microbes as a carbon source, preferably during vegetative growth. Certain organisms such as \textit{Sac. erythraea} or \textit{S. griseus} do not produce erythromycin and streptomycin, respectively, until glucose is depleted and other less readily available carbon sources are utilized instead (reviewed in Demain & Fang, 1995). In \textit{S. fradiae}, carbon sources such as vegetable oil, soybean meal or even fish meal are readily metabolized by the organism for the synthesis of secondary metabolites. When glucose is added to defined batch media during the tylosin synthetic phase, antibiotic production is reduced dramatically but resumes upon glucose depletion (Gray & Bhuwapathanapum, 1980). In other \textit{Streptomyces} species, glucose repression occurs at the level of transcription. In \textit{Streptomyces antibioticus}, repression of actinomycin D
production in the presence of glucose involves transcriptional repression of
phenoxazinone synthase in a growth-phase-dependent manner (Jones, 1985).

Pioneering studies on glucose catabolite repression in *E. coli* revealed that the
predominantly extracellular factor adenosine 3',5' cyclic monophosphate (cAMP)
reversed glucose repression by binding to the catabolite gene activator protein, thus
increasing (on the transcriptional level) the expression of enzymes such as β-
galactosidase, arabinose isomerase and tryptophanase that catalyze the utilization of
alternative carbon sources. Despite the fact that cAMP and its binding protein are present
in streptomycetes (Gersch et al., 1978), for many years cAMP was eliminated from
playing a role in glucose catabolite repression since a number of studies showed that
glucose levels did not affect cAMP concentration (for example Chatterjee & Vining,
1982; note though that they measured only intracellular levels). However, when Charles
Thompson and colleagues (Susstrunk et al., 1998) disrupted the adenylate cyclase gene
(*cya*) responsible for the synthesis of cAMP from ATP in *S. coelicolor*, the resultant
mutant was defective in spore germination and, under nitrogen deprivation, was
incapable of producing actinorhodin. In wild type *S. coelicolor* growing on solid media,
cAMP intracellular levels increased with growth while extracellular levels peaked prior to
aerial mycelium formation and then was dramatically reduced. In the presence of
exogenously added cAMP, actinorhodin was overproduced, and so was undecylprodigiosin but at much higher cAMP concentrations. These data suggested that
CAMP affects secondary metabolism after all, although via repression of glucose
catabolism or some other unknown mechanism(s) remaining to be explored.

It has recently been reported that cAMP levels change under certain conditions in
*S. griseus* (Horinouchi et al., 2001). In the streptomycin producer, antibiotic production
is initiated when A-factor binds to ArpA and relieves the repression of the pathway-
specific activator, StrR (section 1.5.2). In an *arpA*-disrupted strain, it was found that
cAMP (only at high concentrations) blocked aerial mycelium formation on solid media and
streptomycin production on solid and liquid media.

The role of cAMP has also been briefly studied in *S. fradiae* (Tata & Menawat,
1994). Although it was claimed that exogenously added cAMP increased tylosin yields
and partially relieved the glucose effect, the results were far from convincing from the
statistical point of view.
1.4.2 The nitrogen source and regulation of secondary metabolism

Certain types of nitrogen source, such as ammonium salts and certain amino acids that are readily utilized for growth, interfere with secondary metabolism. For example, the presence of ammonium salts represses actinorhodin production in *S. coelicolor* while actinomycin D production in *S. antibioticus* is affected by the presence of L-alanine or L-valine in the medium (reviewed in Demain & Fang, 1995).

In *S. fradiae*, high levels of NH$_4^+$ resulted in depression of tylactone synthesis (Ômura *et al.*, 1983-1984, Lee & Lee, 1991-1993). In such extracts, the levels of valine dehydrogenase, threonine dehydratase and aspartate aminotransferase activities were markedly reduced. This effect was attributed to inhibition of the catabolism of amino acids that normally yielded precursors for polyketide (i.e. tylactone) synthesis.

1.4.3 Regulation by the phosphate source

The presence of excess phosphate was shown to have a negative effect on the synthesis of certain antibiotics. In *S. griseus*, inorganic phosphate inhibits the phosphatase activity of the enzyme that is needed for the cleavage of dihydrostreptomycin-6-phosphate (Mansouri *et al.*, 1989). In *S. fradiae*, addition of excess phosphate early in batch fermentations led to the reduction of tylosin biosynthesis (Ômura *et al.*, 1984). This was attributed in part to reduction of the specific activity of enzymes involved in the interconversion of propionyl-CoA and methylmalonyl-CoA, two polyketide precursors. Also, the activities of the sugar biosynthetic enzymes dTDP-glucose-4,6-dehydratase, dTDP-mycarose synthase and macrocin-O-methylase were reduced in tylosin fermentation extracts in the presence of excess phosphate (Madry & Pape, 1980).

Phosphate can also regulate antibiotic production on the level of transcription. In *S. coelicolor*, the presence of 11mM phosphate in nitrogen-limited media led to transcriptional repression of the *actIII* structural gene and subsequent loss of actinorhodin production (Hobbs *et al.*, 1992). In *S. lividans*, the presence of phosphate (above 0.1 mM) resulted in reduction of *p*-aminobenzoic acid synthase (an enzyme needed for candidin production in *S. griseus*), presumably via interference of upstream promoter regions (Rebollo *et al.*, 1987).
Presumably, phosphate control of antibiotic production could also be exerted via phosphorylation and/or dephosphorylation of regulatory proteins. For example, studies on the AfsK/AfsR system in \textit{S. coelicolor} pointed to the importance of phosphorylation in the regulation of antibiotic biosynthesis. When the putative serine/threonine kinase AfsK was expressed in \textit{E. coli}, it was autophosphorylated and also phosphorylated the product of afsR, a global regulator of antibiotic production (Matsumoto \textit{et al.}, 1994). Disruption of afsK resulted in reduction (but not abolition) of actinorhodin production, an occurrence rationalized by the capability of the disrupted strain to phosphorylate AfsR. Using a similar mechanism, the AbsAl/AbsA2 two-component system down-regulates the production of at least three antibiotics in \textit{S. coelicolor} (Anderson \textit{et al.}, 2001). In this context, the presence of a phenomenal number of two-component regulatory systems in the \textit{S. coelicolor} genome reinforces the role of phosphate in the regulation of secondary metabolism.

1.4.4 Feedback regulation

Many antibiotics are known to block their own synthesis by repressing or inhibiting biosynthetic enzymes (reviewed in Demain & Fang, 1995). Antibiotics such as chloramphenicol and kanamycin repress the expression of acrylamine synthetase and acetyltransferase respectively. Also, tylosin inhibits the last step of its own synthesis which is catalyzed by macrocin-\textit{O}-methyltransferase (see Figure 1.3) (Seno & Baltz, 1981).

In addition to negative feedback regulation, the tylosin biosynthetic pathway is also regulated by glycosylated intermediates in a feedback-loop fashion. Evidence supporting this model came when genes involved in the biosynthesis or addition of the first sugar, mycaminose, were disrupted (section 1.2.9) (Fish & Cundliffe, 1997, Butler \textit{et al.}, 2001a). The resultant strains failed to produce detectable amounts of ty lactone, an effect that was not evidently due to polar effects on downstream genes (Butler \textit{et al.}, 2001a). Instead, copious amounts of ty lactone accumulated when glycosylated precursors from the tylosin synthetic pathway were added exogenously. Since disruption of mycarose genes did not have the same effect on ty lactone biosynthesis (Bate \textit{et al.}, 2000) it was concluded that addition of the first sugar onto ty lactone acts as an inducer of polyketide metabolism in \textit{S. fradiae}. 31
1.4.5 Influence of dimethylsulphoxide on antibiotic production

Dimethylsulphoxide (DMSO) is a chemically synthesized antioxidant that is widely used in pharmacological applications. It is also produced as a natural product mainly by anaerobic bacteria. A recent study demonstrated the stimulatory role of DMSO on antibiotic production in several actinomycetes and bacilli (Chen et al., 2000). At a concentration of 3%, exogenously added DMSO had a stimulatory effect on the synthesis of various antibiotics such as chloramphenicol and tetracenomycin C produced by *S. venezuelae* and *S. glaucescens* respectively. In the same study, pleiotropic effects of DMSO associated with morphological differentiation and growth rate were also reported, such as the occurrence of earlier and heavier sporulation in *S. lividans*. In an independent study conducted in this laboratory, the addition of 3% DMSO to *S. fradiae* batch cultures stimulated tylosin production markedly (Butler & Cundliffe, 2001). Moreover, adding 1% DMSO into an *S. fradiae* mutant strain that produced tylactone caused an increase in the accumulating levels of the aglycone. This suggested a direct role of DMSO on polyketide metabolism. On the other hand, no morphological or growth rate effects caused by the addition of DMSO were observed on liquid or solid media.

1.5 Regulation of antibiotic biosynthesis by γ-butyrolactones

1.5.1 Autoregulators and antibiotic biosynthesis

More than thirty years ago, Khokhlov and co-workers discovered a low molecular weight, diffusible molecule that restored streptomycin production and sporulation in *S. griseus* mutants (reviewed in Khokhlov, 1980). They speculated that this molecule was part of an autoregulatory cascade, therefore naming it A-factor. Later, in the early 1980s, the structure of A-factor was correctly determined as 2-(6′-methylheptanoyl)-3R-hydroxymethyl-4-butanolide (reviewed in Yamada, 1999; Figure 1.9A).

Other molecules with structures similar to that of A-factor were found to induce secondary metabolism in other streptomycetes such as *S. virginiae*, *S. lavendulae* and *S. coelicolor* (Figure 1.9) (reviewed in Yamada, 1999). Next, we describe the role of A-factor and A-factor homologues in *S. griseus* and three other antibiotic producing organisms.
Figure 1.9 The structures of various γ-butyrolactones. γ-butyrolactones have a similar core but they mainly vary in the length of the tail. A. A-factor from *S. griseus*. B. Virginiae butanolides (VB-A to D) from *S. virginiae*. They contain a hydroxyl instead of a keto group that is present in A-factor (shaded). VB-A is synthesized from one glycerol C₃, two acetate C₂, and one isovalerate C₅ units (Sakuda et al., 1990). C. IM-2 γ-butyrolactone from *S. lavendulae*. D. The structure of the *S. coelicolor* γ-butyrolactone, SCB1. The absolute conformation has not yet been determined.
1.5.2 A-factor from *S. griseus*

In 1982, Beppu and co-workers (Hara & Beppu, 1982a) found that A-factor induced streptomycin (Sm) production and the formation of spores in mutants deficient in secondary metabolism and sporulation. A-factor accumulated in a growth-phase-dependent manner and levels were reduced once streptomycin production was initiated. They also reported that Sm resistance was restored in the presence of A-factor via induced expression of the Sm-6-phosphotransferase gene (Hara & Beppu, 1982b). It was later discovered that A-factor enhanced transcription of the streptomycin resistance determinant *aphD* via read-through from the *strR* promoter (Vujaklija *et al.*, 1991; Figure 1.10). The *strR* gene encodes a DNA-binding protein that targets the partially palindromic operator site upstream of the structural *strBl* gene (Retzlaff & Distler, 1995) and positively regulates its transcription (Distler *et al.*, 1987, 1992). Similar palindromic sequences are present in various locations within the Sm biosynthetic gene cluster. It was shown via footprinting analysis that *strR* binds to one of these sites located within the *strR* gene (suggesting auto-repression) and to another within the promoter of the Sm structural gene, *stsC* (Retzlaff & Distler, 1995).

Four unidentified proteins interacted with the *strR* promoter, one of which was A-factor responsive (Vujaklija *et al.*, 1993). By using the *strR* upstream activation sequence as bait, the DNA-binding protein was isolated and partially sequenced. The encoding gene (*adpA* for A-factor-dependent-DNA protein) was eventually cloned by reverse genetics (Ohnishi *et al.*, 1999). When *adpA* was disrupted by in-frame deletion, so that transcription of downstream genes was not affected, the mutant failed to produce Sm and was defective in aerial mycelial formation and sporulation. When *adpA* was overexpressed, streptomycin production increased 10-fold and expression of *aphD* was also enhanced. It was also confirmed that *adpA* transcription was A-factor-dependent since no *adpA* transcript was present in an A-factor-deficient mutant.

So, how does A-factor exert its positive control on *adpA* transcription? In earlier experiments, tritium-labelled A-factor was used to ‘fish-out’ a cytoplasm-associated protein that had the capacity to bind A-factor (Miyake *et al.*, 1989). This Δ-factor receptor protein (ArpA) was assigned a negative, pleiotropic role in Sm production and mycelial formation since a mutant that could carry out both functions in the absence of
A-factor had a defective receptor protein (Miyake et al., 1990). ArpA was purified and the encoding gene cloned (Onaka et al., 1995). The deduced protein was 276 amino acids long and contained a DNA-binding domain with a classic helix-turn-helix (H-T-H) motif similar to those originally found in the structures of Cro, CAP and λ-repressor, and later in other DNA binding proteins among prokaryotes (reviewed in Brennan & Matthews, 1989). During the purification process, it was also suggested that ArpA functioned as a homodimer while the formation of multidimer aggregated at high concentrations affected the binding affinity of the protein. A binding/immunoprecipitation/PCR amplification procedure coupled with further binding/gel retardation/PCR amplification rounds led to the identification of a small stretch of palindromic DNA sequence that ArpA recognized only in the absence of A-factor (Onaka & Horinouchi, 1997). It was proposed that one subunit of ArpA recognized one half of the palindromic sequence via its H-T-H domain while the other interacted with the other half of the sequence in a two-fold symmetrical fashion. It was later found that the promoter sequence of adpA contained a similar palindromic sequence that ArpA could physically bind to as shown by gel-shift assays (Ohnisi et al., 1999).

A piece of DNA found to complement an A-factor-deficient mutant contained a gene designated afsA (Horinouchi et al., 1989). When this DNA was expressed in E. coli, the purified extracts displayed A-factor activity (Ando et al., 1997) suggesting that afsA encoded an enzyme involved in A-factor biosynthesis.

Another DNA fragment from S. griseus was found to complement a mutant that was deficient in aerial mycelium formation (Ueda et al., 1993). A gene carried by that piece of DNA, amfR, was responsible for the phenotype and encoded a putative response regulator. Transcription of amfR was induced in response to A-factor although AdpA did not control its expression in S. griseus (Ueda et al., 1998). It was concluded that A-factor controls morphological differentiation in an AdpA-independent manner via the action of AmfR (Figure 1.10).
Figure 1.10 Regulation of streptomycin production in *S. griseus*. The presence of A-factor at critical levels relieves *adpA* repression by binding to ArpA and releasing it from the *adpA* promoter. AdpA initiates aerial mycelium formation in an AmfR-independent manner. It also promotes transcription of the pathway-specific regulator *strR* and the co-transcribed resistance determinant, *aphD*. StrR activates the expression of structural genes needed for the synthesis of streptomycin.
1.5.3 Virginiae butanolides from *S. virginiae*

Low molecular weight material that induced virginiamycin production in *S. virginiae* (Yanagimoto & Terui, 1971) was later found to contain a mixture of five related 2,3-disubstituted butyrolactone derivatives named virginiae butanolides (VB) (Kondo *et al*., 1989). The absolute conformation of VBs A,B&C was later corrected (Figure 1.9B) (Mori & Chiba, 1990). VBs differ from A-factor on the structural level since they carry a 6-α-hydroxyl group. They also differ on the functional level. Firstly, they don’t control morphological differentiation. Secondly, they also exert a positive as well as a negative effect on secondary metabolism. Naturally, VBs are produced after 12 h of cultivation and induce antibiotic production by 14 h. When exogenous VB was added 2 hours before it was naturally produced, it caused early initiation of virginiamycin synthesis. But when exogenous VB was added at the start of the culture, it reduced antibiotic production dramatically.

Following the A-factor example, radioactively-labelled VB-C was used to isolate its cognate protein (Kim *et al*., 1989). The protein, named BarA (butyrolactone autoregulator receptor A), was later sequenced, thereby facilitating isolation of the *barA* gene via reverse genetics (Okamoto *et al*., 1995). The *barA* gene was situated between the divergently transcribed *barX* that encoded an AfsA orthologue and the downstream, co-directionally transcribed *barB* that encoded an ArpA orthologue carrying a highly conserved H-T-H motif (Figure 1.11) (Kinoshita *et al*., 1997). The *barA* transcript was detectable at early hours and barely detectable at later stages in fermentation, only to show up again when endogenous VB was produced. The presence of basal expression of *barA* was later rationalized as the consequence of transcription initiation from a site independent of negative regulation. *barAp* and *barBp* were transcriptionally fused to *xylE* and introduced in *S. lividans*. The introduction of BarA led to dramatic reduction of *barBp* activity and less dramatic reduction of *barAp* activity. When *barA* was disrupted, it caused early production of virginiamycin, a complex of two structurally different compounds with synergistic bactericidal activity, virginiamycin S (VS) and virginiamycin M1 (VM). Accordingly, it was concluded that BarA acted as a repressor of virginiamycin biosynthesis (Nakano *et al*., 1998). Moreover, it had previously been shown that BarA could bind to two sites within the *barB* promoter *in vitro* and that it
dissociated from the DNA once VB was added (Kinoshita et al., 1997). In a later study, the DNA target sequence for BarA was later identified (BARE for BarA recognition element) and shown to be similar to the palindromic sequence recognized by ArpA (section 1.5.2). Thus a BARE-like sequence was deduced (Kinoshita et al., 1999; Figure 3.15).

Early evidence that barB was part of a polycistron came from Northern blotting analysis (Kinoshita et al., 1997). It was later demonstrated that barB was co-transcribed with varS, a resistance determinant (Figure 1.11) (Lee et al., 1999). varS also had its own promoter that was induced by the presence of VS but not VM₁.

The function of BarX was revealed when the respective gene was disrupted. The resulting strain did not produce antibiotics or VBs and was sensitive to VM₁ (Kawachi et al., 2000a). In the same study, a piece of DNA on the left-hand side of barX (Figure 1.11) was sequenced. The region contained two structural genes: orf4, an orthologue of the actinorhodin structural gene, actHI from S. coelicolor, and orf5 that was deduced to encode a protein similar to dTDP-glucose-4,6-dehydratase from S. fradiae (tylAI). The sequenced DNA also carried barZ that coded for a BarB orthologue, orf2 that encoded a putative protein similar to members of the family of Streptomyces antibiotic regulatory proteins (SARPs, section 1.6.3), and varM, a putative resistant determinant that encoded and ABC transporter (similar to TlrC). Transcriptional analysis of the region using RT-PCR revealed that orf4, barX and barA were constitutively expressed. The orf2, varM, orf5, barB and varS transcripts were detectable only when VBs were present in the culture and barZ expression coincided with VS and VM₁ production. In the barX disruptant, transcription of barZ, orf2, varM and orf5 was abolished, suggesting that BarX controlled their expression. On the other hand, barB and varS transcription became constitutive, suggesting that they were negatively controlled by BarX. Since BarA recognizes the barB promoter, it was proposed that BarX and BarA worked synergistically in the control of barB expression.

The role of orf2 (later named vmsR) was further investigated (Kawachi et al., 2000b). When the gene was deleted, it resulted in loss of VS and VM₁ but not VB synthesis. None of the mapped genes known to be involved in virginiamycin regulation and biosynthesis were transcriptionally controlled by the product of vmsR.
Figure 1.11 Function of virginiiae butanolides in *S. virginiiae*. Virginiae butanolide VB-C binds to the BarA repressor and relieves the repression of the regulator barB needed for virginiamycin production. Also, BarX temporally represses the transcription of barB.varS, a virginiamycin S resistance determinant is co-transcribed with barB but it also has its own inducible promoter. BarX acts positively on the transcription of the pathway-specific activator vmsR and the resistant determinant for virginiamycin M1, varM. BarX also activates barZ that encodes a SARP of unknown function.
1.5.4 IM-2 from *S. lavendulae*

*Streptomyces lavendulae* produces D-cycloserine as a secondary metabolite and also excretes blue pigments under certain nutrient-depletion conditions. An A-factor-like substance was found to induce blue pigment production (and suppress D-clucoserine synthesis) and was therefore referred to as inducing material (IM) (Yanagimoto & Enatu, 1983). Its structure was later determined as 2(1'-hydroxymethyl)-3-hydroxymethyl butyrolactone and the molecule named IM-2 (Figure 1.9C). The absolute configuration was later determined (Mizuno *et al.*, 1994). During growth, IM-2 appeared at the mid-exponential phase and induced the production of blue pigmented material, including the nucleoside antibiotics showdomycin and minimycin (Hashimoto *et al.*, 1992). An IM-2 receptor protein, isolated the same way as with A-factor and VB-C (Ruengjitchatchawalya *et al.*, 1995), bound IM-2 specifically and did not recognize A-factor or VB, while under non-denaturing conditions, the protein size doubled suggesting the formation of a dimer.

*barX* from *S. virginiae* was successfully used as a probe to isolate a piece of DNA from *S. lavendulae* that, when expressed in *E. coli*, gave rise to a protein able to bind IM-2 (Waki *et al.*, 1997). The DNA fragment was sequenced and found to contain a gene deduced to encode an orthologue of BarX, therefore named FarX (Figure 1.12). 184 bp downstream and co-directional with *farX* lies *farA*, whose deduced product was similar to BarA and ArpA. During fermentation, exogenous addition of IM-2 resulted in elevation of *farA* transcription, suggesting autoregulation. Later, it was demonstrated *in vitro* that FarA was a DNA-binding protein (Kitani *et al.*, 1999) that recognized a partially palindromic sequence (FARE, for FarA recognition element) similar to those recognized by ArpA and BarA. This sequence was positioned between the mapped transcriptional and translational start points of *farA*. It was therefore suggested that FarA represses its own synthesis (Figure 1.12).
Figure 1.12 Blue pigment inducing material (IM) in *S. lavendulae*. IM-2 induces the production of blue pigments. The orthologue of BarA in *S. levendulae* (FarA) represses its own expression. IM-2 presumably binds to FarA and relieves repression of the gene *farX* that encodes an AfsA orthologue, possibly implicated in IM-2 synthesis.
1.5.5 γ–butyrolactones from *S. coelicolor*

*S. coelicolor* was originally found to produce six γ–butyrolactones whose structures were partially determined (Efremenkova *et al.*, 1985). Twenty five years later, at least four γ–butyrolactones were purified from stationary phase extracts of *S. coelicolor* and one of them, SCB1, was structurally characterized (Figure 1.9D; Takano *et al.*, 2000). The determined structure did not correspond to any of the six γ–butyrolactones previously described (Efremenkova *et al.*, 1985). SCB1 (at a narrow concentration range) induced antibiotic production when spotted onto *S. coelicolor* growing on solid media but at a much higher concentration than that needed for induction of streptomycin synthesis by A-factor in *S. griseus*. No effect of SCB1 on morphological differentiation was detected.

Horinouchi and co-workers first set out to identify A-factor receptor orthologues in *S. coelicolor* (Onaka *et al.*, 1998), using a 150 bp fragment from *arpA* to hybridize with digested chromosomal DNA from *S. coelicolor*. A mini library was created and re-screened in order to identify two clones, each containing ArpA orthologues. One of them, named CprA, displayed 35% identity to ArpA while the other, CprB, was 39% identical to ArpA. Interestingly, CprA was ~91% identical to CprB. When the chromosomal copy of *cprA* was insertionally disrupted, the resultant strain produced low levels of actinorhodin (section 1.6.4) and caused a one day delay in sporulation. Conversely, disruption of *cprB* resulted in overproduction of actinorhodin and early initiation of sporulation. As expected, over-expression of either gene had the opposite phenotypic effect respectively. The production of undecylprodigiosin, another antibiotic produced by *S. coelicolor* (section 1.6.5), was not affected by disruption of either gene.

In a recent attempt to isolate a BarX orthologue from *S. coelicolor*, an 189 bp DNA fragment was amplified using degenerate primers based on the *barX* sequence (Takano *et al.*, 2001) and later used to probe a cosmid library. As a result, a positive clone was identified that carried a gene encoding a BarX orthologue, *scbA* (Figure 1.13). A divergently transcribed gene that shared the upstream region of *scbA* was also identified and named *scbR*. The putative product of *scbR* was 56% identical to FarA and 47% to BarA. Transcript mapping pin pointed the *scbR* and *scbA* transcript starts. The two transcripts were found to overlap (Figure 1.13).
In the same study, the ScbR protein was purified and found to bind to two sites at the scbR-scbA intergenic region, having greater affinity for one than the other. The binding affinity of ScbR was markedly reduced in the presence of SCB-1. An in-frame deletion introduced in scbA led to over-production of actinorhodin and undecyprodigiosin and reduction of the scbA transcript while the phenotype was reversed by the addition of exogenous SCB-1. This also resulted in the elevation of scbR transcript. In a scbR mutant, actinorhodin production was delayed. While the scbR transcript was abundant in the scbR mutant strain, there was no scbA transcript detected. These data suggested that ScbR was a negative regulator of its own expression and, together with ScbA, was a positive activator of scbA. The presence of SCB-1 seemed to neutralize the activity of ScbR, perhaps by binding to it, thus releasing it from the DNA (Figure 1.13).

Clearly, the genetic mechanism controlled by SCB-1 seemed to be more complicated than the ones previously described and raised more questions than it answered.
Figure 1.13 γ-butyrolactones and antibiotic production in *S. coelicolor*. ScbR, like its orthologue in *S. lavendulae* (FarA), represses its own expression by binding to BS1 (preferred Binding Site to BS2). The repression is relieved by the presence of the γ-butyrolactone, SCB1. ScbR also acts as an activator of *scbA* which is needed for SCB1 synthesis. Curiously though, ScbA negatively controls the production of both undecylprodigiosin and actinorhodin, unlike SCB1 and ScbR that act as positive regulators. There are at least two more ScbR orthologues in *S. coelicolor* with opposite roles. CprA positively regulates actinorhodin production while CprB does the reverse (section 1.5.5). There is one more ScbR orthologue as revealed by the *S. coelicolor* genome project. Its function has not yet been studied.
1.6 Pleiotropic and pathway-specific regulators of secondary metabolism

1.6.1 Control of secondary metabolism and morphogenesis

Pioneering work from D.A. Hopwood and K.F. Chater carried out in *S. coelicolor* revealed the existence of genetic links between antibiotic production and morphological differentiation. Mutations affecting both cellular functions were later mapped and the genes were identified and further studied (Chater, 1998). One of these genes, *bldA*, acted at the interface of morphological development and secondary metabolism but was unique in exerting its control at the translational, rather than the transcriptional, level. It is further discussed below.

1.6.2 *bldA*

Bald (*bld*) is the name assigned to mutants that are defective in aerial mycelia formation. In *S. coelicolor*, 12 classes of viable *bld* mutants have been isolated so far (reviewed in Chater, 1998). These mutants are seldom unconditional since they produce secondary metabolites under certain conditions. When grown on low phosphate medium, *bldE* and *bldF* mutants produce undecylprodigiosin in abundance while actinorhodin production is not affected under any conditions. *bldB* mutants produce actinorhodin after a longer incubation period while in *bldH* mutants, undecylprodigiosin synthesis is restored in response to a change in carbon source (Champness, 1988). The phenotype of the *bldA* mutants is carbon source-dependent too. If grown on glucose, no antibiotic production or aerial mycelial formation occurs. When the main carbon source is replaced by mannitol or maltose, *bldA* mutants produce aerial mycelia but still fail to make antibiotics (Merrick, 1976).

A DNA fragment that restored the BldA phenotype was cloned (Piret & Chater, 1985). It carried a gene, named *bldA*, that coded for a leucyl tRNA<sub>TTA</sub>. The *bldA* product appears to be the principal means by which UUA codons are translated in *S. coelicolor* (Leskiw *et al.*, 1991a,b). So far, no TTA codons have been found within coding regions of genes involved in vegetative growth. They were rarely found in resistance determinants such as the *hyg* gene from *S. hygroscopicus* conferring resistance to
hygromycin (Zalacain et al., 1986) and the actinorhodin export gene actII-ORF2 (section 1.6.4). TTA codons have also been found in genes related to morphological differentiation such as the amfR gene that complemented a bld mutant in S. griseus (section 1.5.2).

Intriguingly, TTA codons are also found in genes that regulate secondary metabolism such as the strR activator of streptomycin production in S. griseus (section 1.5.2). The actinorhodin pathway-specific activator actII-ORF4 from S. coelicolor also contains one (section 1.5.2). In a bldA mutant, the actinorhodin structural genes controlled by actII-ORF4 were silenced (Bruton et al., 1991). The replacement of the TTA codon by TTG restored actinorhodin production in the bldA mutant (Fernandez-Moreno et al., 1991). This suggested that the tRNA_{TTA} is essential for actII-ORF4 expression. But did the relative abundance of tRNA_{TTA} dictate the efficient expression of actII-ORF4 under physiological conditions? It was found that translation of actII-ORF4 in the wild-type strain was not limited by the presence of the TTA codon, since an engineered gene with an altered version of the codon (TTG) did not give rise to higher protein levels (Gramajo et al., 1993). In the same study, it was reported that transcription of bldA was constitutive, thus excluding temporal regulation of bldA on the transcriptional level. On the other hand, an independent study showed that the bldA promoter was temporally controlled and that processing at the 5' end of the primary transcript to produce mature leucyl tRNA increased in intensity as the growth rate dropped (Leskiw et al., 1993). Controversy still surrounds this point.

bldA mutants were conditionally deficient in undecylprodigiosin (Red) synthesis in S. coelicolor. They produced Red but only under low-phosphate conditions (Guthrie & Chater, 1990). Under those circumstances, the transcription of Red-associated genes (red) was restored. All this suggested that the bldA product was essential for the expression of red genes but not under low-phosphate conditions. The fact that Red production was at all affected in the absence of the tRNA_{TTA} was enigmatic since none of the known genes associated with the production of the antibiotic contained a TTA codon including the pathway specific activator redD (section 1.6.5). Later, Chater and co-workers isolated a bld mutant that was capable of producing undecylprodigiosin at normal levels of phosphate (Guthrie et al., 1998). They used it to create a DNA library in
order to isolate a gene that could suppress the Red non-producing phenotype of the \textit{bldA} mutant. That way, they isolated \textit{redZ}.

The deduced N-terminal sequence of RedZ was similar to other response regulators, normally found as part of two-component regulatory systems. The deduced C-terminal region contained a H-T-H motif. Most interestingly, \textit{redZ} contained a TTA codon. White & Bibb (1997) showed that transcription of the Red pathway-specific activator \textit{redD} depended on \textit{bldA} and \textit{redZ}, thus identifying the genetic link between the \textit{bldA} gene and Red production in \textit{S. coelicolor}.

1.6.3 Regulators of one or more antibiotic biosynthetic pathways

In various \textit{Streptomyces} species, the synthesis of secondary metabolites is regulated in a cascade-like manner via the action of pathway-specific activators. StrR from \textit{S. griseus} is a DNA-binding protein that targets promoters of the streptomycin biosynthetic genes and induces their expression (section 1.5.2). Similarly, DnrI is a positive activator of daunorubicin biosynthesis in \textit{S. peucetius} (Stutzmann-Engwall et al., 1992), SrmR controls spiramycin biosynthesis in \textit{S. ambofaciens} (Geistlich et al., 1992) and the previously mentioned ActII-ORF4 and RedD induce actinorhodin and Red production in \textit{S. coelicolor}, respectively.

The DNA-binding domain of SrmR contains a H-T-H, a DNA-binding motif well conserved among \textit{Gram} negative (Perez-Rueda & Collado-Vides, 2000) and \textit{Gram} positive bacteria including streptomycetes. On the other hand, ActII-ORF4, RedD, DnrI and other pathway-specific regulators such as CcaR from \textit{S. clavuligerus} and MtmR from \textit{S. argillaceus} have similar deduced secondary structures which are more complex (Wietzorrek & Bibb, 1997). The N-terminally-located region of these proteins was found to be structurally similar to the C-terminal end of OmpR, which is the DNA-recognition domain of the protein (Martinez-Hackert & Stock, 1997). OmpR is a transcription factor that regulates the synthesis of genes that encode the major outer membrane porin proteins in \textit{E. coli} (Csonka & Hanson, 1991). All \textit{Streptomyces} proteins that share a similar DNA-binding domain are members of the SARP (\textit{Streptomyces} Antibiotic Regulatory Protein) family (Wietzorrek & Bibb, 1997). In addition, DNA comparisons among known promoter sequences recognized by SARPs revealed a certain degree of similarity (Wietzorrek & Bibb, 1997). Specifically, they contained 2 or 3 similar heptameric
repeats separated from adjacent copies by 11 bp (1 complete helix turn) or 22 bp gaps, thus facing at the same side of the DNA helix. It was proposed that SARPs recognize and bind to those regions as dimers.

Not all SARPs are pathway-specific regulators. AfsR was identified as a pleiotropic regulator of actinorhodin and Red biosynthesis from its ability to induce both actinorhodin and Red production in *S. coelicolor* (Floriano & Bibb, 1996). The N-terminal region of AfsR is orthologous to other SARPs (Wietzorrek & Bibb, 1997). However, this protein is unusual in that it contained two H-T-H motifs at the C-terminus and two ATP-binding consensus sequences at its approximate centre. These regions were found by disruption analysis to be essential for the function of the protein (Horinouchi *et al.*, 1990). AfsR seems to control antibiotic production independently of ActII-ORF4 and RedD, since an *afsR* mutant still expressed both of these pathway-specific activators (Floriano & Bibb, 1996). In addition, AfsR is thought to be controlled by Afsk, a eukaryotic type Ser/Thr kinase that is capable of phosphorylating AfsR *in vitro* (Matsumoto *et al.*, 1994).

### 1.6.4 actII-ORF4

The occurrence of tight clustering among genes responsible for the biosynthesis and resistance of antibiotics in *Streptomyces* was first demonstrated when a 25 kb fragment from *S. coelicolor* was introduced in *S. parvulus* and induced heterologous expression of the blue-pigmented antibiotic actinorhodin (Malpartida & Hopwood, 1984). Subsequent subcloning led to the mapping of 7 classes of mutants (*actI-actVII*) that were previously isolated for their actinorhodin null phenotype (reviewed in Hopwood *et al.*, 1985) (Figure 1.14). The *actI-III, actIV-VI* and *actVII* loci contained the actinorhodin structural genes (Hallam *et al.*, 1988, Fernandez-Moreno *et al.*, 1992, Fernandez-Moreno *et al.*, 1994). *actII* was the regulatory region consisting of 4 *orfs* (Fernandez-Moreno *et al.*, 1991). *actII-ORF2* and *actII-ORF3* were associated with actinorhodin export, *actII-ORF1* encoded a DNA-binding protein that repressed the co-transcribed *actII-ORF2* and *actII-ORF3* as well as the expression of its own gene (Caballero *et al.*, 1991). *actII-ORF4* was the pathway-specific activator. Disruption of the latter gene resulted in loss of actinorhodin production while over-expression of the
gene led to early onset, and over-production, of the antibiotic (Fernandez-Moreno et al., 1991).

actII-ORF4 is normally expressed in a growth-phase-dependent manner (Gramajo et al., 1993). Transcript levels were low throughout the early and mid-exponential phase and were markedly increased at the end of the exponential phase, closely followed by an increase in transcription of the synthetic genes actIII and actVI-ORF1. A recent study utilizing DNA microarray analysis showed that transcription of all act structural genes is affected in an actII-ORF4 mutant suggesting a broader range of activity for the pathway-specific regulator (Huang et al., 2001). In addition, a number of putative orfs that were not associated with the biosynthetic cluster were temporally coordinated with act gene expression. Nevertheless, actIII-ORF4 has so far been shown to bind to multiple sites at the actVI-ORFA-actVI-ORF1 and actIII-actI intergenic regions (Arias et al., 1999).
Figure 1.14 The actinorhodin biosynthetic gene cluster of *S. coelicolor*. The actII locus includes the pathway-specific activator ActII-ORF4 that controls, directly or indirectly, the expression of all the actinorhodin biosynthetic genes located at the actI and actIII-actVII regions. So far, it has been demonstrated that ActII-ORF4 targets the actII-ORFA-actII-ORF1 and actIII-actI intergenic regions. The actII locus also includes the actinorhodin transport genes ORF2 and ORF3 that are repressed by the product of ORF1. ORF1 also represses its own synthesis. ActII-ORF4 and ActII-ORF2 synthesis depends on the presence of the rare tRNA\_\text{TTA} that is encoded by *bldA*. The actII-ORF4 promoter is recognized by at least two RNA polymerase holoenzymes, Eo\_\text{HrdB} and the principal Eo\_\text{HrdA}. 

 loci

| loci | actVI | actV | actII | actIII | actI | actVII | actIV |

ORFB ORFA ORF1 ORF2 ORF3

• P P X

Pi [p]

ORF1 ORF2 ORF3 ORF4

tRNA\_\text{Leu}_\text{TTA}

ActII-ORF1

ActII-ORF4

bldA
1.6.5 redD

Clones identified for their ability to complement mutations associated with the production of the red-pigmented undecylprodigiosin (Feitelson et al., 1985) were successfully used for the cloning of the entire Red biosynthetic cluster (Malpartida et al., 1990). Further complementation analysis led to identification of a subclone that carried two divergent orfs (Narva & Feitelson, 1990), only one of which (redD) could restore Red production. Although in vitro transcript analysis indicated that the transcripts of the two orfs overlapped, it was later shown that their in vivo transcript start points did not overlap when mapped by S1 nuclease protection experiments (Takano et al., 1992).

RedD positively regulates Red production in a pathway-specific manner, since overexpression of redD led to early initiation and overproduction of the Red antibiotic (Narva & Feitelson, 1990, Takano et al., 1992). The expression of redD was shown to occur in a growth-phase-dependent manner. Transcript started accumulating at the exponential phase and increased markedly during the stationary phase. This increase coincided with elevation of transcription of one of the Red early structural genes, redX. In addition, overexpression of redD resulted in early accumulation of redX transcript. Although these early data suggested that RedD might positively regulate redX, cDNA microarray analysis on a redD mutant suggested that RedD does not regulate redX nor the redW and redY structural genes (Huang, 2001). Instead, redD upregulates the remainder of the Red structural genes. Additionally, expression of the red genes was coordinated with orfs (mostly putative two-component regulators) that mapped at positions distant from the red biosynthetic cluster. In the same study, Northern blot analysis in a redZ mutant suggested that RedZ could alternatively be regulating the transcription of redX, W, Y. RedZ, whose expression relies on the presence of the rare tRNA_{TTA} (section 1.6.5), is a positive regulator of redD and a repressor of its own synthesis (White & Bibb, 1997; Figure 1.15).
Figure 1.15 The pathway leading to undecylprodigiosin (Red) biosynthesis in *S. coelicolor*. The autoregulated response activator RedZ is needed for the transcription of the structural genes redX, W & Y. It also regulates the expression of the other Red biosynthetic genes by upregulating the pathway-specific activator redD. RedZ translation is subject to availability of tRNA\textsubscript{TTA} encoded by bldA. The redD promoter is recognized by at least two sigma (\(\sigma\)) factors, \(\sigma^{\text{HrdD}}\) and \(\sigma^{\text{HrdB}}\). Under low-phosphate conditions, Red production is bldA independent.
1.6.6 Sigma factors associated with antibiotic regulatory genes

A limited amount of information is available on $\sigma$ factors involved in the expression of genes specific to antibiotic production, since previous studies have mainly focused on $\sigma$ factors that recognize genes needed for primary metabolic pathways or morphogenesis (section 1.3.2). In 1996, Fugii et al. reported that $\sigma^{\text{HrdD}}$ can drive transcription of the redD and actII-ORF4 regulators in vitro. $\sigma^{\text{HrdD}}$ is not essential for viability of the cell but it is very similar to the essential $\sigma^{70}$-like factor, $\sigma^{\text{HrdB}}$ (Buttner, 1989). In addition, $\sigma^{\text{HrdD}}$ was not essential for expression of the two pathway-specific activators in vivo, suggesting the presence of at least one more $\sigma$ factor capable of recognizing their promoters. In a later study, complementation analysis of a mutant deficient in actinorhodin and undecylprodigiosin production in S. coelicolor (afsB; Hara et al., 1983) revealed a mutation at the encoding gene of the sigma factor, $\sigma^{\text{HrdB}}$ (Aigle et al., 2000). It was also shown that transcription of actII-ORF4 and redD was markedly reduced in the afsB mutant, while addition of the wild-type copy of hrdB restored their transcription to wild-type levels. These data suggested that transcription of the pathway specific activators for actinorhodin and undecylprodigiosin was dependent on $\sigma^{\text{HrdB}}$ in vivo.
Figure 1.16 Regulatory genes in the tyl gene cluster of *S. fradiae*. Diagram not drawn to scale. Four of the five regulatory genes are situated at the left-hand side of the cluster (shown in blue). The fifth one, tylR, is found on the other side, sharing a ~1 kb upstream, non-coding region with orf8 (annotated as a striped box). Each of tylP, Q and tylS have a ~400 bp upstream gap with their respective upstream genes while tylT shares a ~350 bp gap with orf12*. Except for upstream gaps of regulatory genes and the tylHI-tylF, tylCIII-tylCV and tylGI-tyll intergenic regions, genes are closely packed and, in some cases, they even overlap (e.g. tylGV-tylMIII).
1.6.7 Regulatory genes in the tylosin biosynthetic gene cluster

There are five putative regulatory genes present in the tyl gene cluster (Figure 1.16). Four are clustered together on the left-hand side of the polyketide synthase genes (in the orientation of Figure 1.16) and all four possess unusually long non-coding upstream sequences, which could serve as targets for regulatory elements. The deduced products of tylS and tylT are very similar to each other and showed significant similarity to other members of the SARP family (section 1.6.3). They each contain a TTA codon, which suggests that their expression could depend on a BldA homologue (section 1.6.2). Disruption of tylS at the putative DNA-binding domain shared by other SARPs (Wietzorrek & Bibb, 1997) resulted in loss of tylosin production (Bate et al., 2002). The disruptant in question partially converted exogenously added demethylmacrocin and macrocin to tylosin (Figure 1.3), suggesting that TylS was not essential for the expression of the two respective enzymes. The tylT disruptant still accumulated tylosin, suggesting that the two SARPs have differential roles in tylosin biosynthesis.

Upstream of tylS lie the other two putative regulatory genes, tylP and tylQ. The product of tylP resembles other γ-butyrolactone receptor proteins such as BarA and FarA (sections 1.5.3, 1.5.4; Figure 1.17). The deduced product of tylQ is similar to BarB (section 1.5.3) and JadR2 from the jadomycin B producer S. venezulae (Yang et al., 1995). JadR2 is a repressor of jadomycin production that was recently found to regulate negatively the expression of the positive activator JadR1, a putative response regulator (Yang et al., 2001).

tylR is located a considerable distance away from the 'regulatory region' and shares a ~1 kb intergenic region with the divergently transcribed metK gene, suggesting independent regulation. Its deduced product is similar to that of acyB2, a pathway-specific regulator that is known to activate the expression of acyB1, an adjacent gene involved in the synthesis of the macrolide antibiotic carbomycin in S. thermotolerans (Arisawa et al., 1993). The product of tylR is essential for tylosin production since tylR disruptants are unable to synthesize the antibiotic or even bioconvert any exogenously added precursors. Evidently, tylR is essential for tylactone biosynthesis and synthesis or addition of all three sugars (Bate et al., 1999).
Figure 1.17 Comparison of the N-terminal sequences of TylP and TylQ with their respective orthologues. A. The TylP sequence represents the N-terminal portion, as encoded by the shortest possible orf, compared with the experimentally determined N-terminal sequences of BarA, FarA and ArpA (section 1.3). B. The TylQ sequence derived from the longest possible orf is compared with the N-terminal portions of BarB and JadR2 (sections 1.3.2 & 1.6.6?). The translational starts of TylP, FarA, TylQ, BarB and JadR2 have not been experimentally determined. The helix-turn-helix motifs are indicated. The second helix that is known to recognize and bind into the DNA major groove is well conserved. The conserved primary sequence of the recognition helices in A and B shows distinct diversity.
1.7 Experimental approach

1.7.1 Generation of *Streptomyces* recombinant strains

In previous work conducted in this laboratory, putative regulatory genes were disrupted in *S. fradiae* using the hygromycin B resistance cassette Ωhyg (Blondelet-Rouault *et al.*, 1997). Ωhyg is flanked by transcriptional terminators from the *fd* coliphage, previously shown to function effectively in *Streptomyces* species (Ward *et al.*, 1986). The resistance cassette was routinely introduced into orfs via double homologous recombination between the chromosome and suicide vectors (see section 2.2.5) carrying part of the respective orfs cloned on either side of the Ωhyg (Figure 1.18).

In this work, *S. fradiae* recombinant strains were constructed, where regulatory genes were over-expressed. Firstly, each gene was transcriptionally fused with the strong constitutive promoter ermEp⁺ (Bibb *et al.*, 1994) and cloned into the suicide vector pOJ260 (section 2.2.5). The resultant construct was then integrated into the respective chromosomal locus via a single, homologous recombination event (Figure 1.19). Alternatively, DNA was integrated into the *S. lividans* genome using facilitated integration at the pSAM2 or (|)C31 _attB_ sites (section 2.3.6).

1.7.2 Confirmation of disrupted strains

DC-PCR (Disruption-Confirmation PCR) was utilized for the convenient and definitive confirmation of disrupted strains. The technique involves hemi-nested, step-down PCR using primers that amplify DNA fragments of definitive sizes around and within the disrupted region, provided Ωhyg was successfully introduced at the right locus (Figure 1.18). It is described in more detail later (section 3.6.2).

1.7.3 Promoter-probe analysis in *Streptomyces*

Heterologous genes are often poorly expressed in streptomycetes due to the highly biased codon usage displayed by these organisms. In consequence, only a limited number (e.g. _xylE_) have been used as reporter genes in *Streptomyces* species. Originally isolated from *Pseudomonas putida*, _xylE_ encodes a catechol dioxygenase that converts
Figure 1.18 Disruption of genes via insertional deactivation. Two independent homologous recombination events occurring either side of the hygromycin resistance cassette (Ωhyg) mediate its 'clean' insertion at the chromosomal copy of the gene. The resulting knock-out strain is hygromycin resistant (hyg-R; conferred by the Ωhyg) and apramycin sensitive (apra-S) because the plasmid backbone of pOJ260 is not integrated into the genome. The disrupted strains were confirmed by DC-PCR (Disruption-Confirmation PCR; section 3.6.2) using primers specific to Ωhyg DNA (small arrows shaded gray) and primers to chromosomal DNA flanking the region (small arrows shaded blue).
Figure 1.19 **Targeted integration of over-expression cassettes.** The strong constitutive promoter \( ermEp^* \) is cloned upstream of an open reading frame \((orf)\) in the suicide vector pOJ260. A single cross-over event occurring between the cloned and the chromosomal copy of the \( orf \) will result in the integration of the over-expression cassette at the respective chromosomal locus. The deduced strain is apramycin resistant (apra-R), a marker carried by the plasmid backbone which is, too, integrated into the genome.
colourless catechol into an easily detectable yellow product (Ingram et al., 1989). The *E. coli* chloramphenicol acetyltransferase (CAT) gene, used extensively as a reporter gene in *Gram* positive and negative bacteria, has also been used successfully in *Streptomyces* (Bibb & Cohen, 1982). A more recently discovered reporter gene, *gfp*, encodes a green fluorescent protein that can be visualized when irradiated with ultraviolet light of a certain wavelength. A recombinant version of this gene encoding a modified version of the green fluorescent protein (EGFP) has been used in the analysis of spatial and temporal regulation in *Streptomyces coelicolor* (Sun et al., 1999).

In this work, a promoter-probe vector was constructed utilizing the *aphII* reporter gene from the *E. coli* transposon Tn5 that encodes an aminoglycoside 3'-phosphotransferase (Beck et al., 1982). AphII confers resistance to kanamycin by inactivating the drug. Promoters were routinely cloned upstream of the reporter gene and introduced into *S. fradiae* and/or *S. lividans* via facilitated integration into their respective chromosomal attB sites. The resistance of each resultant strain to kanamycin was measured using antibiotic gradient plates (section 2.3.8).

1.7.4 Gene expression analysis

A number of techniques have been developed over the years that facilitate the detection of messenger RNA (mRNA). In the present work, the aim was to analyze the expression of ~40 genes (i.e. the entire *tyl* cluster) in various strains. Techniques such as Northern blotting or RNA dot blot analysis are more suitable for detecting a small number of transcripts from a larger number of RNA samples. In addition, the use of total RNA limits the clarity of the blots. Other quantitative methods include S1 nuclease or RNase protection analysis. These techniques are time-consuming and unsuitable when dealing with a large number of transcripts. On the other hand, the recent development of cDNA microarrays allows the detection of mRNA from a large number of genes in a large number of total RNA samples. In this technique, DNA fragments are PCR amplified from respective genes and spotted onto glass slides. Then, cDNA samples derived from two comparative strains are labelled with different fluorescent dyes, combined and hybridized onto the slide. The variation of coloration in each spot gives a comparative, quantitative measure of transcript present. Although this technique offers a quick way of analyzing the expression of a large number of genes, it is mostly aimed at
facilitating total transcriptome studies and that is fully reflected in the cost of setting up such a system.

A more suitable method for analyzing expression of the *tyl* genes would be Real-Time PCR. In this approach, mRNA-specific PCR amplification is quantitatively assayed as product accumulates. This can be achieved by the use of a device that measures the increasing fluorescence of the reaction, caused by a gene-specific, probe-bound molecule that emits only when it is displaced as a result of primer extension. Again, this technique is suitable for quick, quantitative expression analysis but it is costly. Other quantitative RT-PCR methods have been developed such as the use of DNA or RNA competitors but their use is excessively time-consuming.

In the present study, One-Step RT-PCR (section 2.5.4) was used for the fast and convenient screening of expression patterns from the tylosin biosynthetic gene cluster. The system allows reverse transcription and amplification to take place sequentially in the same tube. The technique is semi-quantitative because different primers bind with different efficiencies and the exponential phase of amplification was not vigorously determined for each set of primers, but differences in transcript levels can still be reliably measured when detection of amplified products depends on different numbers of cycles.

1.7.5 Phenotypic analysis of *S. fradiae* strains

Levels of tylosin or its biosynthetic precursors were measured by means of high performance liquid chromatography (HPLC; section 2.8.1) and, where needed, by mass spectrometry (section 2.8.2). Occasionally, extracts from tylosin fermentations where assayed for biological activity using ‘plugs’ on confluent *Micrococcus luteus* plates (section 2.8.3).

1.8 Aims of this thesis

The aim of this work was to give a first insight into the temporal expression of *tyl* genes, prior to and after the onset of tylosin production. The function and expression patterns of the putative regulatory genes *tylP,Q,S* and *tylR* were specifically addressed. In addition, a comparative study between wild-type *S. fradiae* and an empirically improved
production strain was aimed at revealing key mutations that might have caused improvements in tylosin yields.
Chapter 2

Materials & methods
2.1 General material, solutions and media

2.1.1 Sources

The Sigma Chemical Company Ltd. and Fischer Scientific UK were the providers of all chemicals, unless stated otherwise. Culture media were purchased from Oxoid Ltd. and Difco Laboratories. Bacteriological agar (used in media for growing *E. coli*) was purchased from Oxoid Ltd. while agar-agar (used in media for growing *Streptomyces*) was provided by Fischer Scientific UK. Enzymes used in this work were purchased from GIBCO BRL, unless stated otherwise.
2.1.2 Preparation of culture media and stock solutions

All solutions and media were made up with Super Q (Millipore)-quality water. Adjustment of the pH was monitored using an Ultrolab 2100 pH meter. Solutions were either filter sterilized through a 0.22 micron filter or autoclaved for 15 min at 121 °C under pressure (15 lb/in²).

2.1.3 Stock solution recipes

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

Ca²⁺  Mn²⁺ solution
100 mM CaCl₂, 70 mM MnCl₂, 40 mM Na acetate (pH 5.5 with 1M HCl)

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)
2.1.4 Media recipes

2xYT media
1.6 % w/v tryptone, 1.6 % w/v yeast extract, 0.5 % w/v NaCl
Solidified when 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 Na₂SO₄, 2 % w/v agar-agar.
pH adjusted to 8.0 with 1M KOH.
Solidified when required by the addition of 2 % w/v agar-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.
The methyl oleate was added individually to 250 ml conical flasks. The rest of the media were prepared as a batch and the pH was adjusted to 7.0 with 0.26 % w/v KOH.

GRF (George’s RNA extraction Fermentation medium)
Same as MM-1 with the exception that ferric ammonium citrate was replaced by 4 ml of Hopwood’s trace elements (Hopwood et al., 1985)

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 adjusted to 8.0 with 1M KOH
Solidified by the addition of 2 % w/v agar-agar.
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Pre-fermentation medium
0.5 % w/v corn steep solids, 0.5 % w/v yeast extract, 0.5 % w/v soya bean meal (Hopkin and Williams Biochemicals), 0.3 % w/v calcium carbonate, 0.5 % w/v methyl oleate

The pH of the medium was adjusted to 7.8 with 1M NaOH in the absence of corn steep solids and methyl oleate. The latter components were added individually to each flask.

Pre-germination medium
0.5 % w/v yeast extract, 0.5 w/v % casamino acids, 0.025 M TES, 10 mM CaCl₂

The yeast extract and casamino acids were made up to double strength. The TES was made as a 50 mM solution (pH to 8.0 with NaOH) and the CaCl₂ was made up as a 5 M stock. Prior to use, the double strength media and TES were mixed in equal quantities and made up to 10 mM CaCl₂.

R2YE
10.3 % w/v sucrose, 0.025 % w/v K₂SO₄, 1.012 % w/v MgCl₂.6H₂O, 1 % w/v glucose, 0.01 % w/v casamino acids.

Solidified with 2.2 % agar-agar. Prior to use, the molten medium was supplemented with 1 ml KH₂PO₄ (0.5 % w/v), 8 ml CaCl₂·H₂O (3.68 % w/v), 1.5 ml L-proline (20 % w/v), 10 ml TES (5.73 % w/v pH7.2), 0.2 ml Hopwood’s trace elements, 0.5 ml NaOH (1 M) and 5 ml yeast extract (10 % w/v).

TSB (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 MgCl₂, 0.5 % w/v glycine.
Before use, all media were autoclaved or filter sterilized, as described elsewhere (section 2.1.2)

2.2 Strains, growth conditions and manipulation of E. coli

2.2.1 E. coli strains and growth conditions

Plasmid DNA was routinely propagated in E. coli strain DH5α. Foreign DNA was introduced into Streptomyces species via transconjugation with the F+ E. coli strain S17.1 (Simon et al., 1983).

All E. coli strains were grown in 2xYT liquid media with orbital shaking (200 rpm) at 37 °C for as long as specified by each protocol. E. coli strains were alternatively grown on 2xYT solid media at 37 °C overnight.

2.2.2 Preparation of competent E. coli

A modified version of the method described elsewhere (Hanahan, 1983) was routinely utilized for the preparation of competent E. coli strains ready for subsequent transformation with plasmid DNA.

A 10 ml 2xYT pre-culture was inoculated with a single colony of E. coli and grown overnight. The following day, 500 μl of the pre-culture was used to inoculate 50 ml of 2xYT in a 250 ml Pyrex flask. Bacterial cells were then grown to an OD₆₀₀ of 0.2 and supplemented with 1 M MgCl₂ to a final concentration of 20 mM. The culture was grown further until it reached an OD₆₀₀ of 0.45-0.55. The broth was then poured into a prechilled, 50 ml sterile tube and incubated on ice for ~2 h. Cells were collected at the bottom of the tube by centrifugation at 3000 g for 5 min (at 4 °C) and its pellet resuspended in prechilled Ca²⁺ Mn²⁺ solution (section 2.1.3). After 40 min, they were again harvested as before, resuspended in 5 ml of Ca²⁺ Mn²⁺ solution containing 15 % v/v glycerol and aliquoted into 1.5 ml microcentrifuge tubes. These were quick frozen in liquid N₂ and stored at −60 °C for several months. The transformation efficiency of each given strain was calculated as follows:

No. colonies on plate x dilution factor x 1 μg / amount of DNA used (μg)
In this study, competent cells used in subsequent transformation experiments (section 2.2.3) displayed transformation efficiency within the range of $1 \times 10^6 - 1 \times 10^7$.

### 2.2.3 Transformation of *E. coli* strains

1 ng of plasmid DNA or 2 µl of a 10 µl ligation mixture (section 2.4.4) were individually added to a prechilled, 1.5 ml microcentrifuge tube and made up to a total volume of 25 µl with SSQ. Then, 50 µl of competent cells (section 2.2.2) were added to the DNA mixture and incubated on ice for 30 min. Cells were then heat-shocked at 37 °C for 5 min and incubated in 0.925 ml of 2xYT in a 10 ml sterile universal for 1 h with shaking (250 rpm). 200 µl of the mini culture was then spread on 2xYT agar containing the appropriate antibiotic selection (section 2.2.4) and grown overnight.

### 2.2.4 Antibiotic selection in *E. coli*

- Ampicillin 100 µg/ml
- Apramycin 50 µg/ml
- Spectinomycin 30 µg/ml
- Streptomycin 100 µg/ml

### 2.2.5 General plasmids.

The pUC18-based vector pIJ2925 was used for general purpose cloning of *Streptomyces* DNA in *E. coli* (Figure 2.1). The plasmids pSET152 (Figure 2.2) and pIJ487 (Figure 2.3) were utilized for the construction of the promoter-probe vector pLST920 (section 3.3.1). A derivative of pSET152, pLST9828 (Figure 2.4), was routinely used for the cloning of *tyl* genes downstream of the constitutive promoter *ermEp*. *Streptomyces* DNA was also cloned into the suicide vector pOJ260 (Figure 2.5) for its conjugal transfer from *E. coli* to *S. fradiae* and subsequent integration of DNA into the *S. fradiae* genome via homologous recombination (section 1.7.1). pPM925 (Figure 2.6) was also utilized for the integration of foreign DNA at the pSAM2 *attB* site of the *S.*
"lividans" chromosome. pMOMT4 and its subclones (Beckman et al., 1989) were also used as templates in PCR amplification.
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Figure 2.1 Map of pIJ2925. A derivative of pUC18 (Yanisch-Perron et al., 1985), this high-copy-number vector carries a multiclonal site flanked with BgIII sites, which are ideal for multi-purpose cloning (Janssen & Bibb, 1993). The plasmid was also used to generate sequence of cloned DNA by utilization of the following primers flanking the multiclonal site: 5'-CAGGAAACAGTATGACC-3' and 5'-TGTTAAAAACGACGCGCCAGT-3'. pIJ2925 confers ampicillin resistance (ap gene) and replicates in E. coli (oriR).
Figure 2.2 Map of pSET152. A conjugative (oriT), integrative (int phiC31; Kuhstoss et al., 1991a,b) vector with an origin of replication (oriR) for *E. coli* (Bierman et al., 1992). It confers resistance to apramycin (*apra* gene). Unique sites are indicated with an asterisk.
Figure 2.3 Map of pIJ487. A high-copy-number, promoter-probe vector with an origin of replication for Streptomyces (Ward et al., 1986). It contains the aphII cassette consisting of the fd transcriptional terminator, a multiclonal site and the aphII gene that confers resistance to kanamycin. pIJ487 carries the tsr gene that confers resistance to thiostrepton. Unique sites are indicated with an asterisk.
Figure 2.4. Map of pLST9828. Based on pSET152 (Figure 2.2), this vector carries the strong, constitutive promoter \textit{ermEp*} (Bibb \textit{et al.}, 1994), previously cloned at the polylinker (Butler \textit{et al.}, 1999).
Figure 2.5 Map of pOJ260. A conjugative (oriT), suicide (no *Streptomyces* oriR) vector (Bierman et al., 1992) used for the integration of DNA into the *S. fradiae* genome via homologous recombination. It carries the *apra* gene that confers resistance to apramycin.
Figure 2.6 Map of pPM925. An integrative (int pSAM2), conjugative (oriT) vector used for the insertion of foreign DNA into *S. lividans* (Smokvina *et al.*, 1990). It also contains the *fd* transcriptional terminator (Ward *et al.*, 1986) cloned directly upstream of the multiclonal site to prevent transcriptional read-through from the 'back-bone'. pPM925 confers resistance to spectinomycin/streptomycin (*spec/strep* gene) and thiostrepton (*tsr* gene).
2.2.6 Mini-scale plasmid preparation from *E. coli*

A crude, alkaline lysis-based method was utilized for extracting plasmid DNA from *E. coli* strains. Routinely, a single colony was used to inoculate 5 ml of 2xYT in a 30 ml sterile universal for overnight growth in the presence of appropriate antibiotic selection. The following day (and no more than 16 h later), 1.5 ml of the culture was pipetted into an 1.5 ml microcentrifuge tube and spun down at 14,000 g for 5 min. The supernatant was discarded and the pellet resuspended in 100 µl of Solution I (section 2.1.3). 200 µl of Solution II was then added and the contents mixed by inversion. Solution III (150 µl) was subsequently added and the contents immediately mixed by gentle inversion. The tube was centrifuged at 14,000 g for 5 min and the supernatant transferred to a 1.5 ml microcentrifuge tube containing 700 µl of 100 % ethanol. The contents of the tube were vortexed and re-centrifuged as above. The pellet was then washed with 150 µl of 70 % v/v ethanol, dried at 37 °C for 30 min and resuspended in 50 µl of SSQ.

If the purified DNA was destined for restriction analysis (section 2.4.1), 10 µl was routinely used per reaction. In the case where low yields of high quality plasmid DNA were needed for sequencing analysis or transformation in *E. coli* (or *S. lividans*), plasmid preparation was conducted using a Qiagen Spin Miniprep kit, according to the manufacturer’s instructions.

2.2.7 Midi-scale isolation of plasmid DNA from *E. coli*

Occasionally, high yields of plasmid DNA were produced for multiple cloning experiments or for the purpose of long-term storage. This was achieved by inoculating a larger culture [50 ml of 2xYT in a 250 ml sterile conical flask (Pyrex) in the presence of appropriate antibiotic selection] with 50 µl from a 5 ml 2xYT, overnight pre-culture of plasmid-carrying *E. coli*. Cells were harvested the following day in a 50 ml polypropylene tube by centrifugation at 10,000 g for 5 min. The pellet was resuspended in 2 ml of Solution I (section 2.1.3) followed by the addition of Solution II (3 ml). The contents were mixed by inversion and 2.5 ml of Solution III were immediately added. The contents were again mixed and centrifuged at 10,000 g for 10 min. At that point, the supernatant was filtered through Miracloth (Calbiochem) into a clean, 30 ml
polypropylene tube. Total nucleic acid precipitation and collection was achieved by adding an equal volume of isopropanol, vortex and centrifugation at 10,000 g for 10 min at 4 °C. The pellet was then resuspended in 300 μl of SSQ and mixed with an equal volume of 8 M LiCl₂. The resulting large-RNA-species precipitate was removed by centrifugation at 10,000 g for 5 min and the supernatant introduced into a 1.5 ml microcentrifuge tube. The remainder of the nucleic acid was re-precipitated via the addition of an equal volume of isopropanol, collected by centrifugation at 14,000 g for 10 min and re-dissolved in 500 μl of SSQ. 10 μl of RNase A (20 mg/ml) was then added and the mixture incubated at 37 °C. After 1 h of incubation, residual protein matter was removed by the addition of 500 μl phenol/chloroform/isoamyl alcohol (25:24:1, pH 8.0) and centrifugation at 14,000 g for 5 min. The top layer was then transferred into a clean, 1.5 ml microcentrifuge tube to which 100 μl of 5 M ammonium acetate and 2 volumes of 100 % ethanol had previously been added. The contents were vortexed and centrifuged as before. The supernatant was discarded, the pellet was washed with 250 μl 70 % v/v ethanol, dried at 37 °C for 30 min and resuspended in 50 μl of SSQ. The yield and quality of the plasmid DNA was crudely determined by agarose gel electrophoresis (section 2.4.5).

2.3 Strains, growth conditions and manipulations of Streptomyces

2.3.1 Streptomyces strains

The *S. fradiae* strain T59235 (also known as c373.1; referred to here as wild type or ancestral strain) was provided by E.T. Seno (Eli Lilly and Co). The *S. fradiae* tylR-disrupted (Bate *et al.*, 1999) and tylS-disrupted (Bate *et al.*, 2002) strains were provided by A.R. Butler and N. Bate respectively. Equally, the tylP-disrupted strain (Stratigopoulos *et al.*, 2002) was provided by A.R. Gandecha. The tylQ-disrupted strain was generated by A.R. Butler and A.R. Gandecha (Stratigopoulos & Cundliffe, 2002a). The *S. lividans* strain TK21 was also utilized in this study (Ward *et al.*, 1986).
2.3.2 Growth conditions for *Streptomyces*

*S. fradiae* spores were routinely propagated on AS-1 solid media (section 2.1.4) at 37 °C for three days. The generation of *S. fradiae* mycelial biomass for the purpose of stock storage (section 2.3.5) was achieved by the inoculation of a 30 ml TSB liquid culture with ~10^8 spores (prepared as described in section 2.3.3) and grown in a sprung, 100ml conical flask at 37 °C for 40 h with orbital shaking (250 rpm). For the purpose of total RNA extraction, 150 ml of TSB was inoculated with ~10^8 pre-germinated spores (prepared as described in section 2.3.3) and grown in a 150 ml conical flasks at 28 °C with orbital shaking (250 rpm).

*S. lividans* spores were propagated on NEF solid media (section 2.1.4) at 30 °C for three days. *S. lividans* protoplasts were re-generated on R2YE solid media (section 2.1.4) under the same conditions. Mycelial biomass was generated in sprung,150 ml conical flasks containing 50 ml of YEME liquid media (section 2.1.4). The cultures were then inoculated with *S. lividans* spores and grown at 30 °C with orbital shaking at 250 rpm.

2.3.3 Preparation of *Streptomyces* spores

*S. lividans* spores were displaced from the agar surface using a sterile loop and resuspended in 5 ml of SSQ. They were then filtered through non-absorbent cotton wool so that mycelial moieties were removed from the spore-containing suspension. The spores were then collected at the bottom of a 30 ml plastic universal tube by centrifugation at 3,500 g for 10 min, washed once with SSQ, centrifuged as above and resuspended in 4 ml of SSQ. The concentration was determined by UV absorbance at 600 nm using a UNICAM SP1800 ULTRAVIOLET spectrophotometer. A spore suspension with an absorbance (A_600) value of 1 corresponded to ~10^8 spore units.

*S. fradiae* spore suspensions used for the inoculation of cultures destined for generation of stocks (section 2.3.5) were prepared as in *S. lividans*. Alternatively, *S. fradiae* spores destined for the inoculation of cultures used in total RNA isolation were originally propagated from the contents of a mycelial stock (section 2.3.5) grown on AS-1 solid media at 37 °C for 3 days. They were then harvested, filtered and washed (with 0.001 % Triton X) and used for re-inoculation of AS-1 solid media and propagation of
new spores after incubation at 37 °C for 3 days. The spores were then re-harvested, filtered and washed as before. The concentration was determined by UV absorption at 600 nm and each aliquot containing ~5x10^7 spores was transferred into a 30 ml sterile, plastic universal to which 10 ml of pre-germination media (section 2.1.4) had previously been added. Thus, spores were allowed to pre-germinate at 37 °C for 1 h prior inoculation of the TSB culture.

### 2.3.4 Antibiotic selection in *Streptomyces*

- **Apramycin**: 50 μg/ml
- **Nalidixic acid**: 60 μg/ml
- **Spectinomycin**: 100 μg/ml
- **Streptomycin**: 10 μg/ml

### 2.3.5 Storage of *Streptomyces* strains

*S. fradiae* and *S. lividans* spore suspensions (section 2.3.3) were sustained at -20 °C in Cryotubes (SARSTEDT) as 500 μl aliquots in the presence of 20 % v/v glycerol. Mycelial cultures were stored in Cryotubes as 1 ml aliquots at -60 °C in 5 % v/v DMSO. Storage was indefinite although viability was confirmed prior inoculation.

### 2.3.6 Conjugal transfer of plasmid DNA into *S. fradiae*

A previously reported method (Bierman *et al.*, 1992; Mazodier *et al.*, 1989) was modified for successful transfer of plasmid DNA from *E. coli* S17-1 (section 2.2.1) into *S. fradiae*. For this purpose, DNA introduced in *S. fradiae* was previously cloned into either pLST920 or pOJ260 (section 2.2.5).

A 10 ml 2xYT culture was inoculated with a single colony of *E. coli*, S17-1 carrying the appropriate plasmid and grown overnight in the presence of spectinomycin (selection for the S17-1 strain; 100 μg/ml) and apramycin (50 μg/ml) for maintaining either pLST920 or pOJ260-based constructs. The following day, 1 ml from that culture was used to inoculate 10 ml (for pLST920-based constructs) or 100 ml (for pOJ260-based constructs) of 2xYT under dual selection. These were grown in a 30 ml plastic
universal tube or a 250 ml conical flask respectively for ~8 h or until mid-exponential phase had been reached. During donor incubation, spores of the recipient \textit{S. fradiae} strain were harvested and prepared for pre-germination (section 2.3.3). The spore suspension was then introduced into a 30 ml sterile, plastic universal containing 10 ml of pre-germination media (section 2.1.4) and grown at 28 °C for ~4 h or until the donor strain had reached the appropriate growth phase. Both donor and recipient strains were then centrifuged at 3,000 g for 10 min. The \textit{S. fradiae} strain was then resuspended in 500 µl of 2xYT while the \textit{E. coli} strain was washed twice with 5 ml of 2xYT for the complete removal of residual antibiotic, and resuspended in 500 µl of 2xYT. For pLST920-based conjugations, all the recipient and donor were pipetted into a Cryotube, mixed and spread onto an AS-1 agar plate containing no antibiotic selection. In pOJ260-based conjugations, 100 µl portions of donor were individually mixed with 100 µl portions of recipient in Cryotubes and each mixture was spread onto an AS-1 agar plate in the absence of antibiotic selection. The plates were incubated at 37 °C for 16-20 h and each was overlaid with 5 ml of AS-1 agar (containing 0.7 % w/v multipurpose agarose BOEHRINGER) at ~40 °C. The overlay contained nalidixic acid (inhibitor of donor cell growth; 60 µg/ml) as well as apramycin (plasmid selection; 50 µg/ml). Plates were incubated for 3 more days or until individual colonies were visible. Single colonies were then picked and spores propagated on apramycin-containing (50 µg/ml) AS-1 agar plates.

\subsection*{2.3.7 Transformation of \textit{S. lividans} with plasmid DNA}

Introduction of plasmid DNA into \textit{S. lividans} was achieved via PEG-based transformation as described elsewhere (Hopwood \textit{et al.}, 1985). Prior to this, DNA fragments intended for insertion into the \textit{S. lividans} chromosome were cloned into either pLST920 (section 3.3.1) or pPM925 (section 2.2.5). Both plasmids are capable of integrating into the \textit{S. lividans} genome via the φC31 or pSAM2 \textit{attB} sites respectively. Typically, ~500 ng of plasmid DNA was used for each \textit{S. lividans} transformation.

\subsection*{2.3.8 Preparation of gradient plates}

Preparation of antibiotic gradient plates was based on a method reported elsewhere (Bryson \textit{et al.}, 1952). For this purpose, 30 ml of pre-melted NEF (\textit{S. lividans})
or AS-1 (S. fradiae) agar was poured in a square petri dish (9 x 9 cm) lying with one edge elevated at 0.9 cm, thus generating an angle of 5° (Figure 2.7). The agar layer was left to dry for ~10 min before another layer of NEF or AS-1 agar of equal volume containing an appropriate amount of kanamycin was poured over while the plate rested horizontally. The agar plates were incubated at 30 °C (NEF) or 37 °C (AS-1) for 1 h. During that time and throughout further incubation, diffusion of the antibiotic occurred in proportion to the ratio of the agar-layer thickness, thus creating an antibiotic gradient. ~10⁸ spores of each S. fradiae or S. lividans strain carrying a pLST920-derived construct were spread along the gradient from lower to higher concentration. Plates were incubated at 30 °C (NEF) or 37 °C (AS-1) for another 3 days. Pictures were taken using a Kodak DC260 digital camera.
Figure 2.7 Promoter-probe assay. Preparation of gradient plates. See text for details.
2.3.9 Extraction of genomic DNA from *S. fradiae*

A modified version of a method described elsewhere (Hopwood *et al.*, 1985) was developed for quick and convenient extraction of genomic DNA from *S. fradiae*. Firstly, a 1 ml mycelial stock (section 2.3.5) was thawed out and centrifuged at 14,000 g for 3 min. The supernatant was discarded and the mycelia were then resuspended in 0.5 ml of lysozyme solution (containing 0.35 ml of 10 mg/ml lysozyme, 1.8 ml of 0.5 M w/v sucrose, 70 µl of 1 M Tris-Cl pH 8.0, 150 µl of 0.5 M EDTA Na₂ pH 8.0 and 0.63 ml of SSQ). The mixture was incubated at 37 °C for ~40 min with occasional shaking. After the lysis event had been confirmed by inspection under the microscope, 120 µl of 0.5 M EDTA Na₂ was added to a final concentration of 0.1 M. Cryotube contents were mixed by inversion and incubated at 37 °C. After 5 min, 70 µl of 10 % w/v SDS was added together with 70 µl of 1 mg/ml Proteinase K. The contents were mixed by inversion and incubated at 50 °C for 1 h. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, pH 8.0) was then added and contents were gently mixed by inversion until a confluent, milky liquid was formed. Centrifuged at 14,000 g for 10 min then followed and the resulting aqueous phase was transferred into a 1.5 ml microcentrifuge tube. Total nucleic acid was precipitated via the addition of 0.7 volumes of isopropanol and was subsequently collected at the bottom of the tube by centrifugation at 14,000 g for 5 min. The pellet was washed with 250 µl of 70 % v/v ethanol and the sample dried at 37 °C for ~1 h. Nucleic acid was then dissolved in 200 µl of SSQ at 4 °C overnight. At that point, the sample was RNase A-treated (final concentration 30 mg/ml) at 37 °C for 1 h and subsequently treated with phenol/chloroform, gently mixed and centrifuged as before. Again, the aqueous layer was transferred to a 1.5 ml microcentrifuge tube and total DNA was precipitated, washed with 70 % v/v ethanol and resuspended as before. The yield and integrity of the genomic DNA was crudely determined by restriction analysis (section 2.4.1) and agarose gel electrophoresis (section 2.4.5).

2.3.10 Extraction of total RNA from *S. fradiae*

*S. fradiae* TSB cultures were inoculated with spores as described elsewhere (section 2.3.3) and grown at 28 °C with orbital shaking (250 rpm). Mycelia were then separated from the fermentation broth by filtration through Miracloth (CALBIOCHEM).
and immediately frozen by immersion into liquid $N_2$. They were then broken by shearing in a Hughes Press (Type A1, APEX CONSTRUCTIONS) and the frozen lysate was left to thaw out in the presence of Buffer RLT (QIAGEN) that contained 1% v/v β-mercaptoethanol (SIGMA). Total RNA was then isolated using the RNeasy Midi kit (QIAGEN), according to manufacturer's instructions. The preparation was treated with DNase I using the DNA-free kit (AMBION), according to manufacturer's recommendations.

The integrity of total RNA was crudely determined via gel electrophoresis (section 2.4.6). The quality of messenger RNA (mRNA) was determined via RT-PCR (section 2.5.4). An indication to the successful separation of RNA from impurities was given by the measure of UV absorption at 260 nm and 280 nm. A $A_{260}/A_{280}$ ratio of $\sim 1.8$ or above was deemed adequate. The UV absorption reading at 260 nm was also used for the quantification of total RNA using the following formula:

$$A_{260} \times \text{dilution factor} \times \mu\text{g per OD}$$

Knowing that an absorbance reading of 1 at 260 nm equals $\sim 40 \mu\text{g/ml}$ ($\mu\text{g per OD}$), one could measure the amount of total RNA present in each sample. In order to improve accuracy, each RNA sample was sequentially diluted and the absorbance of each diluted sample was determined.

All solutions used for the extraction of total RNA were made up in DEPC (SIGMA)-treated water, prepared as specified by the manufacturer. All hardware used had previously been sterilized by their corresponding manufacturer via UV treatment. Alternatively, equipment were immersed in SSQ containing 0.1% v/v DEPC, incubated at 37°C for 12 h, autoclaved (section 2.1.2) and baked at 170°C for 8 h prior usage.

2.4 Nucleic acid manipulations

2.4.1 Digestion of DNA

Genomic and plasmid DNA as well as DNA fragments amplified by PCR were routinely digested with restriction endonucleases. Restriction analysis of genomic DNA
was used as an indicator of nucleic acid integrity. Plasmid DNA or PCR-amplified DNA fragments were digested for subsequent cloning. In addition, restriction analysis of plasmid DNA was used for authentication of plasmid inserts.

For each digest, the following reaction mixture was prepared:

- DNA: x μg
- Enzyme: 10 units
- 10xReaction Buffer: 3 μl
- RNase A*: 2 ng
- SSQ: x μl

Total volume: 30 μl

*RNase A was included in the reaction mixture only when plasmid DNA was extracted using the crude, mini-scale preparation method (section 2.2.6).

Digestion took place in a 1.5 ml microcentrifuge tube at the appropriate temperature for as long as specified by the manufacturer and depending on the restriction enzyme(s) added. When partial digests were attempted, a serial dilution of the enzyme(s) was prepared and added to multiple reaction mixtures containing the same plasmid DNA. They were then incubated at the appropriate temperature and digestion was successively stopped every 10 min by placing on ice or adding 1 x loading buffer (section 2.1.3).

2.4.2 Removal of 5' phosphate termini

The 'staggered' or 'blunt' ends created by digestion of plasmid DNA with a single restriction endonuclease were routinely treated with calf intestinal alkaline phosphatase (CIAP). This aimed at removing 5' terminal phosphate groups in order to avoid re-ligation of the two ends during subsequent cloning steps. In each case, 6 μg of plasmid DNA were digested using twice the amount of enzyme referred to elsewhere (section 2.4.1) in a total volume of 60 μl. Digestion was confirmed via gel electrophoresis (section 2.4.5) and the remainder of the mixture (50 μl) was incubated with 1 unit of CIAP together with 5 μl of 10 x phosphatase buffer at 37 °C for 30 min.
Protein matter was then removed by the addition of an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, pH 8.0) and centrifugation at 14,000 g for 5 min. The aqueous layer was then transferred into a new 1.5 ml microcentrifuge tube. Digested, dephosphorylated DNA was precipitated by mixing with an equal volume of 100 % ethanol and 10 μl of 3 M sodium acetate (pH 5.2). It was collected at the bottom of the tube by centrifugation at 14,000 g for 20 min, washed with 70 % v/v ethanol, air-dried for 30 min at 37 °C and rehydrated in 30 μl of SSQ. DNA was then crudely quantified via agarose gel electrophoresis (section 2.4.5).

2.4.3 End-filling of ‘staggered’ ends

Occasionally, ‘staggered’ ends created by digestion were converted to ‘blunt’ ends by using the Klenow fragment (BOEHRINGER) in the following reaction:

DNA 0.1-0.4 μg
Klenow fragment 1-5 units
0.5 mM of each dNTP 3 μl
SSQ x μl

20 μl total volume

The components were individually transferred and mixed in a 0.5 ml microcentrifuge tube. They were then incubated at 30 °C for 15 min followed by 10 min at 75 °C for the inactivation of the enzyme. The contents of the tube were transferred into a 1.5 ml microcentrifuge tube and the volume was made up to 200 μl with SSQ followed by the addition of an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, pH 8.0). The contents were mixed by vortex and centrifuged at 14,000 g for 5 min. DNA precipitation then followed by the addition of an equal volume of 100 % ethanol and 10 μl of 3 M sodium acetate (pH 5.2). DNA was collected at the bottom of the tube by centrifugation at 14,000 for 20 min, washed with 70 % v/v ethanol, air-dried at 37 °C for 25 min and re-dissolved in 30 μl of SSQ. The yield of the purified DNA was crudely determined by gel electrophoresis (section 2.4.5).
2.4.4 Ligation of DNA

DNA fragments with 'blunt' or compatible 'staggered' ends were ligated using T4 DNA ligase (BOERHINGER). Typically, 25 ng of the larger DNA fragment (vector) was used at a 1/3 ratio in respect to the smaller fragment (insert). The reaction mixture was made up as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector</td>
<td>25 ng</td>
</tr>
<tr>
<td>Insert</td>
<td>x ng</td>
</tr>
<tr>
<td>5xT4 DNA ligase buffer</td>
<td>2 µl</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>5 units</td>
</tr>
<tr>
<td>SSQ</td>
<td>x µl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td>10 µl</td>
</tr>
</tbody>
</table>

The amount of insert was calculated using the following formula:

\[
\text{Vector length (bp)} / \text{Insert length (bp)} \times \frac{1}{3} \times 25 \text{ ng}
\]

For each ligation, a negative control in which the insert had been omitted was set up for each cloning step. Ligation mixtures were incubated at room temperature for 1 h and 2 µl of that was used for subsequent transformation in E. coli (section 2.2.3).

2.4.5 DNA gel electrophoresis

Multipurpose agarose powder (BOEHRINGER) was routinely added at a final concentration of 0.7-2 % (w/v) in 1x TAE buffer (section 2.1.3). The powder was then dissolved and the confluent mixture was poured into a gel case and left to set at room temperature (combs of various sizes were used). DNA samples were loaded in the gel in the presence of 1x loading buffer (section 2.1.3) and fragments were fractionated according to size by gel electrophoresis as described elsewhere (Sambrook et al., 1989). Ethidium bromide had previously been added to the molten agarose at a final concentration of 500 ng/ml to facilitate the visualization of DNA by UV excitation at 320 nm. Gels were photographed using a UVP gel documentation system.
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This method was routinely used for the crude estimation of DNA fragment length. This was achieved by comparing the migration point of the DNA fragment in question with that of an appropriate size marker (1 kb Ladder [GIBCO BRL]). This method was also utilized for the crude quantification of DNA by comparing the intensity of a band (of unknown quantity) with the intensity displayed by a band of known quantity present in the ladder.

2.4.6 RNA gel electrophoresis

The integrity of total RNA was quickly assessed by gel electrophoresis through a standard, non-denaturing 1% agarose gel as described elsewhere (Farrell, 1998). In this method, 9 μl containing ~1 μg of total RNA was pipetted into a 0.5 ml thin-walled, microcentrifuge tube. The RNA was denatured by incubation at 60 °C for 5 min and quickly cooled by placing on ice in order to avoid re-formation of secondary structure. This was then followed by the addition of 1 μl of 10x loading buffer (section 2.1.3) containing 1 mM EDTA. The sample was then electrophoresed at a low voltage to avoid high temperature build-up.

Prior to agarose gel electrophoresis, all hardware were soaked in 0.1 M NaOH overnight and rinsed with DEPC-treated SSQ. All solutions used in this process were made up in DEPC-treated SSQ.

2.4.7 Extraction of DNA from agarose gel slices

DNA fragments separated by gel electrophoresis and destined for cloning were routinely purified from agarose gel slices using the ‘JETSORB DNA EXTRACTION’ kit (GENOMED), according to the manufacturer’s recommendations.

2.5 PCR

2.5.1 Quantification of oligonucleotides

Oligonucleotides used for PCR were provided as dried pellets and were reconstituted according to the manufacturer’s instructions (GIBCO BRL). A UNICAM
SP1800 ULTRAVIOLET spectrophotometer was utilized for the measurement of oligonucleotide concentration via absorption at 260 nm using the following formula:

$$A_{260} \times \text{dilution factor} \times \mu g \text{ per OD}$$

The ‘μg per OD’ value was unique for each oligonucleotide and was determined by the manufacturer.

2.5.2 Amplification of DNA by PCR

PCR was performed using plasmid, cosmid, genomic DNA (Genomic PCR) or even spore suspension (Colony PCR) as template. Whenever needed, primers were designed to introduce specific restriction sites at both ends of the amplified DNA to accommodate subsequent cloning. Each primer was manually designed to have a melting point (Tm) of ~60 °C.

A typical PCR reaction consisted of the following components:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA template</td>
<td>100 μg (or a minute amount of spores)</td>
</tr>
<tr>
<td>5’ primer</td>
<td>250 ng</td>
</tr>
<tr>
<td>3’ primer</td>
<td>250 ng</td>
</tr>
<tr>
<td>25 mM of each dNTP</td>
<td>0.8 μl</td>
</tr>
<tr>
<td>Pfu DNA pol. (STRATAGENE)</td>
<td>2.5 units</td>
</tr>
<tr>
<td>10xCloned Pfu DNA pol. buffer</td>
<td>10 μl</td>
</tr>
<tr>
<td>DMSO</td>
<td>5 μl</td>
</tr>
<tr>
<td>SSQ</td>
<td>x μl</td>
</tr>
</tbody>
</table>

100 μl final volume

Dimethylsulfoxide (DMSO) was added at a final concentration of 5 % v/v as it has been reported to aid PCR amplification of GC-rich templates (Gandecha et al., 1997).

All contents were individually pipetted into a thin-walled, 0.5 ml microcentrifuge tube, mixed by vortex and briefly span down. The reaction mixture was sealed with 70 μl
of mineral oil (SIGMA) and the PCR reaction was conducted in a PERKIN-ELMER 720 thermal cycler. Unless otherwise specified, the reaction proceeded with 5 cycles of:

- 94 °C for 1 min (Denaturing step)
- 55 °C for 1 min (Primer annealing)
- 72 °C for x min (Primer extension step, ~1 min per kb of expected product)

The remaining 25 cycles were conducted as above except that the annealing temperature was raised to 60 °C. The reaction was then stored at 4 °C until further manipulation.

Primarily, the size and quantity of the PCR product was estimated by gel electrophoresis (section 2.4.5). If DNA was amplified only for diagnostic reasons, no further manipulations took place. Alternatively, the reaction mixture was mixed with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, pH 8.0) and centrifuged at 14,000 g for 5 min. The aqueous layer was transferred into a 1.5 ml microcentrifuge tube containing 220 µl of 100 % ethanol and 10 µl of 3M sodium acetate (pH 5.2). The contents were mixed and centrifuged at 14,000 g for 20 min. The pellet was then washed with 100 µl of 70 % v/v ethanol, air-dried for 30 min at 37 °C and resuspended in 30 µl of SSQ. 15 µl of that was used for digestion (section 2.4.1).
2.5.3 Designing primers for RT-PCR

Both forward and reverse primers used in RT-PCR were designed using PRIMER v 1.0 (Ashland, MA). They were generally aimed to amplify gene-specific fragments ~400 bp long. The Tm values ranged from 59 °C to 61 °C while the length of the primers ranged from 20-22 bp (Table 1).

2.5.4 One-Step RT-PCR

Messenger RNA (mRNA) detection was carried out using the SUPERSCRIPT ONE-STEP RT-PCR kit with PLATINUM Taq (GIBCO BRL). This method utilizes SUPERSCRIPT II, the product of a recombinant M-MLV (Moloney murine Leukemia Virus) gene that encodes a reverse transcriptase where alterations have been made leading to reduced levels of RNase H activity. The other DNA polymerase, Platinum Taq, is a thermostable, recombinant DNA polymerase designed to bind to a thermolabile inhibitor carrying monoclonal antibody raised to Taq polymerase. This ensures improved specificity of the enzyme since the inhibitor binds to the enzyme and is released only after heating at 94 °C during the denaturation step (see below).

This system allowed both cDNA synthesis and PCR amplification to occur in the same tube, thus providing us with a convenient method of detecting a large number of transcripts.

Each reaction mixture was assembled in a 0.5 ml microcentrifuge tube previously placed on ice. It contained the following:

- RNA template 500 ng
- 2x Reaction Mix 25 μl
- RNA guard RNase Inhibitor 29.4 units
- Forward primer 200 ng
- Reverse primer 200 ng
- DMSO (see section 2.5.2) 2.5 μl
- RT/Taq mix 1 μl
- SSO x μl

50 μl final volume
RNA guard RNase Inhibitor (Amersham Pharmacia) was the first component to be added in order to minimize RNA degradation throughout the preparation of the reaction mixture and, subsequently, the RT-PCR reaction. Out of convenience and improved accuracy, a ‘master’ mix was created consisting of 2x Reaction Mix, DMSO and SSQ and was aliquoted into each reaction tube. All other components were added individually and the contents were briefly vortexed and centrifuged. 70 µl of mineral oil (SIGMA) were placed on top of each reaction mixture and RT-PCR was performed in a PERKIN ELMER 720 thermal cycle as follows:

50 °C for 30 min (first strand cDNA synthesis)
94 °C for 2 min (inactivation of SUPERSCRIPT II)
followed by 1 cycle of :
94 °C for 1 min (denaturation)
52 °C for 1 min (primer annealing)
72 °C for 1 min (primer extension)
followed by 24-29 cycles of :
94 °C for 1 min (denaturation)
55 °C for 1 min (primer annealing)
72 °C for 1 min (primer extension)

Negative controls were also carried out as before except in the absence of SUPERSCRIPT II. 20 µl of each reaction was then analyzed in a 2 % agarose gel by electrophoresis (section 2.4.5).

2.6 DNA sequencing

DNA sequence was generated using the automated version of the ‘Sanger dideoxy method’ utilizing fluorescent-chain-terminating dideoxy nucleotides (Prober et al., 1987). Each sequencing reaction contained the following components:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Premix*</td>
<td>8 µl</td>
</tr>
<tr>
<td>DNA template (plasmid DNA)</td>
<td>100 ng per kb</td>
</tr>
</tbody>
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Chapter 2: Materials & methods

Sequencing primer (20 mer; Tm 55 °C) 20 ng
DMSO 2 µl
SSQ x µl
20 µl final volume

*The premix contained fluorescent-dye-labeled dideoxynucleotide chain terminators, dNTPs and TaqFS DNA polymerase (Applied Biosystems) provided by PNACL (Leicester University services).

Each component was added into a thin-walled, 0.5 ml microcentrifuge tube, mixed by vortex and overlaid with 40 µl of mineral oil (SIGMA). The sequencing reaction was performed in a PERKIN ELMER 720 thermal cycler as follows:

25 cycles of:
  96 °C for 30 sec
  50 °C for 15 sec
  60 °C for 4 min

Afterwards, the reaction mixture was transferred to a clean 1.5 ml microcentrifuge tube containing 2 µl of 3 M sodium acetate (pH 4.6) and 50 µl of 100 % ethanol. The contents were mixed by vortex and the primer extension products were allowed to precipitate on ice for 10 min. They were then collected at the bottom of the tube via centrifugation at 14,000 g for 25 min. The supernatant was replaced with 250 µl of 70 % v/v ethanol and the contents of the tube were centrifuged at 14,000 g for 5 min. The supernatant was then removed and the pellet was air-dried at 37 °C for 20 min.

DNA sequencing was performed on an ABI 377 automated sequencer by PNACL (Leicester University services). The generated sequence was edited using Seq Ed v 1.0.3 (Applied Biosystems) and aligned in AUTO ASSEMBLER (Applied Biosystems).
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2.7 Fermentation analysis

2.7.1 Growing *S. fradiae* in defined fermentation media

Maximal levels of tylosin production by *S. fradiae* were achieved under the following fermentation condition: Firstly, an *S. fradiae* mycelial stock (section 2.3.5) was used to inoculate 10 ml of TSB in a sterile, 30 ml plastic universal. The mini culture was incubated at 28 °C for 24 h with shaking (250 rpm). The mycelia were then left to settle and 1 ml taken from the bottom of the tube was introduced into a 100 ml conical flask containing 30 ml of pre-fermentation media (section 2.1.4). The culture was then grown under the same conditions for 3 days. The mycelial biomass was then allowed to settle and 5 ml were extracted as previously and used to inoculate a 250 ml conical flask containing 50 ml of MM-1 or GRF fermentation media (section 2.1.4). The culture was then incubated under the same conditions for 7 days. When a tylosin fermentation time-course was performed, *S. fradiae* GRF cultures were also grown for shorter time lengths.

In feeding experiments, tylosin biosynthetic intermediates (supplied by Eli Lilly research Laboratories, Indianapolis) were added to fermentation media after 3 days of growth. Ten micrograms from each intermediate was deemed sufficient for the visualization of bioconversion by HPLC (section 2.8.1).

2.7.2 Extraction of the fermentation broth

Tylosin was extracted from MM-1, GRF or TSB (and its derivatives) fermentation broth by mixing with an equal volume of chloroform at room temperature for 10 min with shaking (200 rpm). The contents were then split into two 50 ml falcon tubes and separated by centrifugation at 3,000 g for 10 min. The chloroform was removed from the bottom of both tubes and placed into a clean, 50 ml falcon tube. The extract was incubated at −20 °C overnight and brought back to room temperature the following day. It was then re-centrifuged as above and half of it (25 ml) was dried down through evaporation using a Rotary Evaporator (ROTAVAPOR R110 [BÜCHI]). The volume of the sample was then made up to 1 ml with HPLC grade chloroform in a Cryotube and stored indefinitely at −20 °C.
2.8 Analysis of purified extracts

2.8.1 High Performance Liquid Chromatography (HPLC) analysis

Reverse phase HPLC (method described in Huber et al., 1990) was used to detect and estimate tylosin yields and its intermediates from *S. fradiae* fermentation extracts. Routinely, 20 μL of a fermentation extract was vigorously mixed with 380 μL of elution buffer (0.3 % w/v ammonium formate, pH 4.0). Half of the confluent mixture was immediately applied to a 3.9 x 300 mm C18 μBondapak column protected by a C18 μBondapak guard column (Waters Associates). A 15 min linear concentration gradient of 50-80 % methanol (v/v) was applied at a flow rate of 1.75 ml/min. The UV absorbance of the eluate was measured at 282 nm. Tylosin and its intermediates were distinguished from each other on the basis of retention time in comparison to standard compounds (Flint, 2000).

2.8.2 Qualitative analysis of *S. fradiae* fermentation extracts by mass spectrometry

Electrospray ionization mass spectrometry (PNACL, Leicester University services) was utilized for the definitive detection of tylosin in TSB broth extracts using a Micromass platform II (Fisons Instruments) in the positive ion mode. Samples were dissolved in 50 % acetonitrile made up in 0.3 % formic acid (v/v).

2.8.3 A bioassay for tylosin detection

Tylosin levels present in TSB broth extracts were assessed by comparing zones of inhibition on *Micrococcus luteus* plates. Firstly, 200 μl of *M. luteus* cells, grown in 10 ml of 2xYT the night before, were added into a 30 ml plastic universal containing 5 ml of 2xYT agar molten at 40 °C. The contents were then mixed by inversion and poured onto the surface of a 2xYT agar plate. An appropriate volume (determined by trial and error) of TSB broth extracts were spotted onto 5 mm-diameter Whatman 3M paper discs and placed on the confluent layer of *M. luteus*. The plate was then incubated at 37 °C overnight.
Chapter 3

The role of a γ-butyrolactone receptor protein in
S. fradiae
Chapter 3: The role of a γ-butyrolactone receptor protein in S. fradiae

3.1 Introduction

The tylosin biosynthetic (tyl) gene cluster contains 43 genes that occupy ~1% of the Streptomyces fradiae genome. Those include all the tylosin structural genes except for a gene encoding a phosphopantetheiny transferase that is needed for post translational addition of ‘arms’ to the polyketide synthase (PKS) enzymes (section 1.2.7).

Most of the tyl genes are grouped according to function (Figure 3.1). Thus, the tylG region contains genes responsible for the extension of the polyketide chain and cyclization generating tylactone (section 1.2.7). The polyketide ring is firstly substituted by mycaminose whose structural genes (tylM; section 1.2.9) are clustered directly downstream of the PKS complex. Curiously, another mycaminose gene, tylB, is situated on the other side of the tylG region and close to another ‘stray’ structural gene, tylCVI. The latter is required for synthesis of the tylosin sugar, mycarose (section 1.2.11). The remainder of the mycarose structural genes (tylCII,CIV,CHI,CV,CVI) are clustered together downstream of the tylM region. Mycinose is the second tylosin sugar that is attached to the tylactone ring (section 1.2.10). The genes responsible for its synthesis (tylN,E,D,F,J) are closely packed near the tlrB end of the cluster. Two other genes included in this sub-cluster, tylHI and tylHII, are required for modification of the lactone ring at C23, the site where mycinose is later attached (section 1.2.7). The tyl gene cluster also includes a number of genes with deduced miscellaneous functions such as orf11* (encoding a GTP-binding protein), aco (encoding an acyl CoA oxidase) and orf16* (encoding a cytochrome P450) (section 1.2.12). Other tyl genes include ccr (encoding a crotonyl CoA reductase) (section 1.2.7), metK (encoding a S-adenosylmethionine synthase) and metF (encoding a N\textsuperscript{5}, N\textsuperscript{10}-methylene tetrahydrofolate) (section 1.2.12). The deduced products of orf12*, orfla and orf9 display unique sequences of unknown function.

Most remarkably, the tyl gene cluster contains five regulatory genes, four of which are clustered together. Database comparisons identified the product of tylP as a putative γ–butyrolactone receptor (section 1.5) and tylQ as a candidate target (section 1.6.7). Both deduced proteins contain H-T-H motifs suggesting that they act as DNA-binding proteins (Figure 1.17). Two other regulatory genes, tylS and tylT, encode proteins of the SARP family (section 1.6.7). As shown by disruption and
Figure 3.1 Genes associated with polyketide, sugar metabolism and regulation in the tylosin biosynthetic gene cluster. The tylA genes that are involved in the synthesis of the common sugar precursors are shaded purple. The mycaminose tylM and tylB genes are green while the mycarose tylC genes are shaded blue. The genes involved in the synthesis and addition of 6-deoxyallose (tylN, tylD and tylJ) and its conversion to mycinose (tylE and tylF) are all clustered together (shaded red). Genes implicated in polyketide metabolism and ring modification are also highlighted (shaded black). Regulatory genes are shaded yellow. The patterned boxes represent upstream gaps of ~400 bp in length. The box upstream of tylMIII lies ~400 bp inside the tylGV coding sequence. These DNA fragments were assayed for promoter activity in this study.
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complementation analysis, only the former is essential for tylosin production in S. fradiae. The fifth regulatory gene, tylR, is ~60 kb distant from the regulatory sub-cluster. TylR has been identified as an activator of tylosin biosynthesis (Bate et al., 1999).

Once the organization of the tyl gene cluster was elucidated, the expression pattern of the tyl genes was investigated before and after the onset of tylosin production in liquid batch media. Results were coupled with promoter-probe analysis of various tyl promoters in strains grown on solid media. Particular attention was paid to the expression patterns of tyl regulatory genes in the wild type and recombinant S. fradiae strains. The following experiments were focussed on tylP and its role in the regulation of tylosin production.

3.2 Expression analysis of the tyl gene cluster by RT-PCR

3.2.1 Setting up an expression analysis system for the study of tyl genes in S. fradiae

In this laboratory, S. fradiae fermentations have routinely been carried in MM-1 batch media containing, among other constituents, a mixture of various non-soluble salts and mineral oil (section 2.1.4). Since various engineered S. fradiae strains had been phenotypically assessed under such conditions, it was desirable to generate a method through which high quality total RNA could be extracted from S. fradiae growing in MM-1.

Given that little is known about the stability of mRNAs in streptomycetes, a protocol was designed whereby mycelia could be washed, frozen and broken within 30 seconds, thus minimizing the possibility of mRNA degradation during RNA extraction (section 2.3.10). However, when total RNA was repeatedly extracted from strains grown in MM-1 medium, the quality was poor and no mRNA could be detected by reverse transcriptase-PCR (RT-PCR; section 2.5.4) using gene-specific primers (Figure 3.2A,D). This could have been due to high salt levels in the medium leading to co-precipitation with the nucleic acid. The prime suspect in this context was ferric ammonium citrate (FAC) and, accordingly, fermentations were repeated in the absence of this salt. However, under those conditions the organism produced only low levels of tylosin as detected by High Performance Liquid Chromatography (HPLC; section 2.8.1) (Figure
Figure 3.2 Tylosin production and RNA extraction from *S. fradiae* grown in MM-1 and GRF media. A. HPLC trace of tylosin produced by *S. fradiae* wild type grown in MM-1 media for 7 days. Total RNA was extracted from a 4-day fermentation (inset). B. Tylosin levels produced by *S. fradiae* wild type in MM-1 media in the absence of ferric ammonium citrate. C. HPLC trace of tylosin produced by *S. fradiae* wild type growing in GRF media for 7 days. Total RNA was extracted from a three day fermentation (inset). D. Detection of *tylS* transcript via RT-PCR using as template total RNA extracted from *S. fradiae* wild type growing in MM-1 and GRF media. The following primers were used: 5' - GCATGGACATTGCTGTACTG -3' and 5' - GTACACAAGCTTGAGCGTCT -3'. The predicted size of the PCR product was ~300 bp. Negative controls were also carried out in the absence of reverse transcriptase.
3.2B). In addition, the absence of FAC had a devastating effect on the growth of the strain. This was obviously undesirable but when FAC in MM-1 medium was replaced with Hopwood's trace elements (to generated GRF medium; section 2.1.4), growth was not affected and the levels of tylosin were similar to those produced in MM-1 (Figure 3.2C). Most importantly, when total RNA was extracted from *S. fradiae* growing in GRF medium, it was obvious that the quality was much improved, as it was now possible to detect gene-specific transcripts by RT-PCR (Figure 3.2C,D).

Unfortunately, however, it became apparent that tylosin was previously produced in pre-fermentation medium used to inoculate GRF medium (data not shown; see section 2.7.1 for protocol). In addition, when a spore inoculum was added directly to GRF medium, *S. fradiae* failed to grow. The former occurrence did not allow the temporal expression analysis of *tyl* genes before and after the onset of tylosin production and the latter method failed to solve the problem. Evidently, another type of liquid medium had to be found wherein *S. fradiae* could produce tylosin in a temporally controlled fashion. When the wild type *S. fradiae* was grown in tryptic soy broth (TSB), it was confirmed by mass spectrometry that tylosin was not produced at early times (Figure 3.3) and, importantly, the quality of total RNA extracted from *S. fradiae* growing in TSB was adequate for the proposed studies (Figure 3.4A). Yet again, however, the organism did not produce high levels of tylosin over a seven-day period (Figure 3.5). Consequently, TSB medium was supplemented with various compounds in an effort to improve tylosin yields (Figure 3.4B) but the resulting modified media did not favour the extraction of RNA (data not shown). It was therefore decided to conduct transcript analysis with total RNA extracted from *S. fradiae* growing in TSB liquid medium because it, firstly, allowed detection of transcripts before and after tylosin production and, secondly, it was a non-complicated medium in which *S. fradiae* were sufficiently grown only for a short period of time (as opposed to MM-1 or GRF).
Figure 3.3 Tylosin analyzed by mass spectrometry. The predicted m/z value is 916 ([915+H]+). Due to the slight inaccuracy inherent in the system, the tylosin m/z value displayed here is ~917.

A. Mass spectroscopy performed on a concentrated extract of *S. fradiae* wild type previously grown in TSB media for 18 h. B. Mass spectroscopy performed using a dilute extract of *S. fradiae* wild type previously grown in TSB for 40 h. Tylosin is depicted as the prominent peak with a m/z value of 917.22.
Figure 3.4 Tylosin produced by *S. fradiae* in TSB-based media. A. A typical tylosin HPLC trace from a seven-day TSB fermentation of *S. fradiae* wild type. Total RNA was extracted from a 3-day culture (inset). B. Levels of tylosin produced after a 7-day fermentation in modified TSB media as determined by HPLC. Abbreviations: OIL, methyl oleate (at the same concentration as in MM-1 media); TE, Hopwood's trace elements (at the same concentration as in GRF media); SPR, springs added to the bottom of the flask.
Figure 3.5 Tylosin time course of *S. fradiae* in MM-1 and TSB media. Multiple batch fermentations of *S. fradiae* wild type were extracted once a day over a period of seven days. Tylosin levels were determined individually by HPLC. Each point on the graph represents the mean value.
3.2.2 Transcript detection by RT-PCR

Various methods were considered for the detection of mRNA from tyl genes (section 1.7.4). In this study, One-Step RT-PCR was utilized whereby cDNA synthesis and PCR amplification were carried out in the same tube (section 2.5.4). The primers used were specific to sequences within tyl genes except for the upstream primer of tylGI, which was located just outside the coding sequence but within the transcribed region. Primers were designed to be of approximately similar length (~20mers) with similar melting points (Tm, section 2.5.3; Table 1). Each set of primers produced cDNA of ~400 bp except those for orf12* that produced a cDNA ~200 bp long, and each product was authenticated by single-stranded sequence generated using one of the two primers.

Not all of the genes from the tyl gene cluster were included in this study. Those omitted were the mini gene tylHII, tlrC and three genes not essential for tylosin production, metK, orf9 and metF (Figure 3.1; Section 3.1). In addition, the polyketide gene tylGII was not included due to technical problems (related to primer design) that were not resolved prior to completion of this study. Negative controls were carried out for each RT-PCR reaction in the absence of reverse transcriptase. This ensured that amplified products were not derived from chromosomal DNA that inevitably contaminates RNA preparations. PCR amplification was typically carried out at 25 cycles, unless no DNA was detected. In that case, the number of cycles was increased (to 28-30) until DNA was detectable in the negative control. Although, in this way it was possible to detect transcript levels that were considerably different, there are generally inherent problems to consider when RT-PCR is used for quantitative analysis. Firstly, primers do not always bind to DNA with equal efficiencies. Moreover, in this study the exponential amplification rate for each set of primers was not rigorously established.

3.2.3 Expression analysis of tyl genes before and after the onset of tylosin production in S. fradiae wild type

Wild type S. fradiae was grown in TSB batch media at 28°C with shaking (section 2.3.2). Total RNA was extracted at an early time point (18 h) when no tylosin was detectable by mass spectrometry and at a later time point (40 h) when tylosin was present (Figure 3.3). Time points in fermentation were defined simply as the time elapsed
between spore inoculation and extraction of RNA since the mycelial density could not be accurately determined at any given time. No attempts were made to define the growth phases or the relative rates of *S. fradiae* growth in TSB so the specific growth phases could not be associated with given times of fermentation. It was clear though that tylosin production was not confined to stationary phase, unlike antibiotic synthesis in other streptomycetes (data not shown).
Figure 3.6  Expression analysis of the ty/l gene cluster by RT-PCR. Total RNA was extracted from S. fradiae wild type (wt) previously grown in TSB for 18 h (no tylosin detected) and 40 h (tylosin produced). RT-PCR was utilized to amplify gene-specific products that were later authenticated by sequence analysis. 25 cycles were routinely used for amplification unless no band was visualized, in which case, the reactions were repeated at 28 cycles. Negative controls were performed for each case in the absence of reverse transcriptase (data not shown).
Prior to this analysis, a limited amount of information was known about the expression pattern of some of the tyl genes. For example, it was previously reported that the resistance determinant tlrD (section 1.2.6) was expressed constitutively when introduced into the heterologous strain, S. lividans. In the present case, RT-PCR showed that tlrD was transcribed in S. fradiae at 18 h prior to tylosin synthesis (Figure 3.6). Although RNA samples were not taken back to time zero, the data left open the possibility that tlrD might also be constitutively expressed in S. fradiae. A piece of evidence against this notion arose from promoter-probe analysis during which the tlrD promoter region was silent in S. fradiae grown on solid media, although, that was not the case when the promoter was tested in S. lividans (Figure 3.7A). Due to the nature of the reporter gene, trickle expression of aphII early in growth is the minimal requirement for survival of the strain in the presence of kanamycin. Since the strain that contained tlrDp cloned directly upstream of aphII did not elevate kanamycin resistance levels, it meant that the promoter in question was silenced early in growth although, in this type of experiment, it was not possible to know whether the promoter was de-repressed at a later stage in growth.

Other transcripts detected at 18 h included that of tlrB and several ‘sugar’ genes such as tylE, tylCV and tylCVII, which might also be constitutively transcribed. It remains to be seen whether their respective products are produced ahead of the other enzymes/partners involved in sugar biosynthesis.

Another interesting occurrence was the early transcription of tylCVI, a gene needed for the synthesis of mycarose (section 1.2.11). Previous studies suggested that tylCVI was co-transcribed with the upstream, co-directional gene tylB needed for mycaminose metabolism (Figure 3.1; see also section 1.2.9). Thus, a tylB-disrupted strain produced tylosin when complemented with the downstream genes tyl[Al-AlI-O-CVl] but not when tylCVI was omitted. Under the latter conditions, exogenously added tylosin precursors were metabolized to desmycosin (demycarosyl tylosin) and not to tylosin (Butler et al., 2001a). In contrast, RT-PCR data suggested that tylCVI was transcribed in a coordinated manner with tylO (section 1.2.7) rather than tylB. Although, this occurrence did not exclude the possibility that tylB and tylCVI might be co-transcribed at another stage during growth, it remains to be confirmed whether tylCVI is expressed at all in the tylB-disrupted strain.
Figure 3.7 Activity of tlrDp and tylMIIIp in S. lividans and S. fradiae. A ~500 bp long, non-coding, promoter region upstream of tlrD was PCR amplified using the following primers: 5'-ACGAGGTACCCATGAACTACCTCTCTTA-3' and 5'-CGGCTCTAGACGGGCCTCACGGGCCTAA-3'. The fragment was digested at the restriction sites introduced by the primers (shown in bold) and then cloned at the respective unique sites of pIJ2925 and subsequently into pLST920 as described elsewhere (Figure 3.10). In addition, a ~500 bp long DNA fragment upstream of tylMIII and withing the coding region of tylGV was PCR amplified using the following primers: 5' - GTGTAATTCCTCCCTCTCCCTGCC -3' and 5' - CACTTCTAGAGCCCAACATGGCCC -3'. As with tlrDp, the putative tylMIII promoter was cloned into pLST920 in a similar fashion (Figure 3.10). A. The tlrD promoter fused with aphII ([tlrDp-aphII]) was introduced into S. lividans and the resultant strain was compared with that carrying ermEp* ([ermEp*-aphII]) by assessing kanamycin levels of resistance on NEF gradient plates (black & white). Background resistance of the wild type was also measured by introducing a promoterless version ([pLST920]). The same constructs were introduced in S. fradiae and kanamycin levels of resistance for each resulting strain were measured on AS1 gradient plates (coloured). B. The S. lividans strains containing [tylMIIIp-aphII], [ermEp*-aphII] and [pLST920] were streaked on NEF, kanamycin gradient plates (black & white) while the respective S. fradiae strains were assessed for kanamycin resistance on AS1 plates (coloured).
Along with tylB, the co-directional genes tyl\(\text{II}\), tyl\(\text{IIa}\), tylAI and tyl\(\text{AII}\) (see Figure 3.6) were also silent at 18 h, as were the tyl\(\text{G}\) genes (note: there are no data relating to tyl\(\text{GII}\)). Although tyl\(\text{GV}\) is terminally linked via the GTGA sequence with tyl\(\text{MIII}\), these two genes were not co-transcribed at 18 h, although they might possibly be co-transcribed when the polyketide genes are normally expressed. Transcript analysis left open the possibility that all three tyl\(\text{M}\) genes are constitutively expressed although promoter-probe analysis showed that tyl\(\text{MIIIp}\) was not active in \(S. \text{fradiae}\) early in growth on solid media, as compared with the activity displayed by the same promoter in \(S. \text{lividans}\) (Figure 3.7B).

The cytochrome P450 gene, orf\(16^*\) was also expressed early, as was orf\(12^*\) and orf\(11^*\). Most importantly though, the regulatory genes tyl\(\text{Q}\) and tyl\(\text{S}\) were also expressed early. TyIS was previously found to act as a positive regulator of tylosin biosynthesis (Bate \textit{et al.}, 2002). Presumably, tyl\(\text{S}\) was transcribed prior to tylosin production in order to ensure the presence of TyIS when tylosin was being made. On the other hand, transcript from the tyl\(\text{R}\) gene, encoding a global activator of tyl genes (section 1.6.7) was non-detectable. Similarly, the non-essential putative regulator of tylosin synthesis tyl\(\text{T}\) (section 1.6.7) was not transcribed before the onset of tylosin production. tyl\(\text{P}\) (section 1.6.7) also followed a similar transcriptional pattern.

The fact that tyl\(\text{P}\) and tyl\(\text{Q}\) transcripts were not detected concurrently was compatible with the hypothesis originally derived from database comparisons that TyIP might negatively control the transcription of tyl\(\text{Q}\). This notion was further strengthened when RNA samples taken from 40 h fermentations contained transcript from tyl\(\text{P}\) but not tyl\(\text{Q}\) (Figure 3.6). At 40 h, all in fact the tyl genes produced transcripts except tyl\(\text{Q}\). This strongly suggested that tyl\(\text{Q}\) encodes a negative regulator of secondary metabolism that must be turned off prior to tylosin production. The role of tyl\(\text{Q}\) was further investigated as described in Chapter 4. Here, we inquire whether the mutually exclusive transcription patterns of tyl\(\text{P}\) versus tyl\(\text{Q}\) reflect a mere coincidence or whether the genes are indeed linked in the regulatory sense.
3.3 Investigation of \( \text{tylP} \) and \( \text{tylQ} \) by promoter-probe analysis

3.3.1 Construction of a promoter-probe vector

An integrative, promoter-probe vector was designed for measuring relative promoter strengths by determining levels of resistance to kanamycin conferred by the aminoglycoside phosphotransferase gene (\( \text{aphII} \)) from Tn5 (Beck \textit{et al.}, 1982). For the construction of this vector, a \(-3.5\) kb fragment was excised from pIJ487 as a \( \text{XhoI} \)-\( \text{EcoRV} \) fragment and ligated in the \( \text{SalI} \)-\( \text{SmaI} \) restriction sites of pIJ2925 (Figure 3.8). The cloned fragment carried the promoterless \( \text{aphII} \) with the pUC18-derived polylinker and the bacteriophage \( \text{fd} \) transcriptional terminator previously shown to work in streptomycetes (Ward \textit{et al.}, 1986). The resultant plasmid was propagated in the \( \text{dam}^- \)XL1 supercompetent \( \text{E. coli} \) strain (STRATAGENE), purified and partially digested with \( \text{BclI} \). The \(-2\) Kb fragment carrying the reporter-gene cassette was ligated into the \( \text{BamHI} \) site of pSET152, creating pLST920 (the unique \( \text{XbaI} \) and \( \text{EcoRI} \) sites carried by pSET152 were previously destroyed through digestion, end filling and religation). pLST920 was capable of efficient and stable integration into the \( \text{S. lividans} \) and \( \text{S. fradiae} \) genomes at the respective \( \phi\text{C31} \) attachment (\( \text{attB} \)) sites and, for ease of cloning, it carried an origin of replication that functioned in \( \text{E. coli} \). It also contained an origin of transfer (\( \text{oriT} \)) for introduction into \( \text{S. fradiae} \) via transconjugation.

3.3.2 Manipulation of promoter DNA

The putative \( \text{tylP} \), \( \text{tylQ} \) and \( \text{orf12}^* \) promoters were isolated by PCR from cosmid pMOMT4 (Beckmann \textit{et al.}, 1989) (Figure 3.9A,B,C). Each promoter fragment spanned from the translational end or putative start (only in case of divergently transcribed genes) of the upstream gene to the probable translational start of the downstream gene and the fragments were individually cloned at the \( \text{XbaI} \) site of pIJ2925 (Figure 3.10). Single-stranded DNA sequence was generated to confirm that no unexpected mutations were introduced during PCR amplification. The \( \text{tylP} \), \( \text{tylQ} \) and \( \text{orf12}^* \) promoter fragments were separately ligated into the unique \( \text{BamHI} \)-\( \text{EcoRI} \) unique sites of pLST920 situated upstream of the \( \text{aphII} \) reporter gene, thus creating pLST920P, pLST920Q and
pLST92012* respectively. Likewise, the strong constitutive promoter *ermEp* was excised from pLST9828 as a *EcoRI-BamHI* fragment and introduced into pLST920, creating pLST920E* (Figure 3.10). Each promoter-probe construct was then integrated into the *S. lividans* genome at the φC31 *attB* site and the levels of kanamycin resistance for respective strains were measured using antibiotic gradient plates, as described elsewhere (section 2.3.8). As a negative control, in order to eliminate the possibility that multicloning region from pIJ2925 carried along with promoter DNA was by itself active in the promoter sense, the polylinker from pIJ2925 was excised as a *BglII* fragment and introduced into pLST920 in place of the pre-existing polylinker (Figure 3.10). The construct was introduced into *S. fradiae* and *S. lividans* but no subsequent elevation of kanamycin resistance was observed in either case (data not shown).
Figure 3.8 Construction of a promoter-probe vector. The aphII reporter gene together with the bacteriophage fd transcriptional terminator were excised from pIJ487 and cloned into pIJ2925. The DNA fragment was then excised from pIJ2925 as a partial BclI digest and introduced into pSET152 after both XbaI and EcoRI unique sites had been destroyed. The resultant promoter-probe vector, pLST920, replicates in E. coli. It can also integrate into the attB φC31 respective sites of S. lividans and S. fradiae. Introduction into S. lividans and S. fradiae was achieved via transformation and transconjugation respectively. Abbreviations: tsr, thioestrepton resistance gene; oriR, origin of replication in E. coli; int φC31, site responsible for chromosomal integration; apra, apramycin resistance gene; oriT, origin of transfer during conjugation; ap, ampicillin resistance gene.
**Figure 3.9** Nucleotide sequence and chromosomal targets of promoter-specific primers. Primers used for the amplification of A. *tylP*, B. *tylQ*, C. *orf12* and D. *tylS*. The probable start codons of *TylQ* and *TylP* as determined by database comparisons (Figure 1.17) were altered by the introduction of a restriction enzyme target sequence and a translational stop codon respectively. The *Orf12* and *TylS* start codons were also altered. Each primer carries restriction sites for convenient cloning. BARE-like sequences are represented as black boxes (see also Figure 3.14).
Figure 3.10 Cloning strategies of promoter fragments. Each DNA fragment was PCR amplified, digested at the restriction sites introduced by the primers and cloned at the appropriate orientation into pIJ2925. It was then authenticated by sequencing, excised from pIJ2925 and directionally cloned into pLST920. The *ermE* promoter was excised from pLST9828 and introduced at the respective sites of pLST920. Asterisks of the same colour indicate the insertion and excision points of each promoter fragment. As a negative control, polylinker DNA from pIJ2925 was also excised as a *BglII* fragment and introduced into the *BglII* sites of pLST920 in place of the pre-existing polylinker DNA. Abbreviations: *tsr*, thiostrepton resistance gene; *oriR*, origin of replication in *E. coli*; *int* C31, site responsible for chromosomal integration; *apra*, apramycin resistance gene; *oriT*, origin of transfer during conjugation; *ap*, ampicillin resistance gene.
3.3.3 Over-expression of \textit{tylP} in \textit{S. lividans}

The \textit{tylP} gene was PCR amplified from pMOMT4 (Beckman \textit{et al.}, 1989) as a 872 bp long DNA fragment using the following primers: 5'-TTATGGATCCACTGCGTTGACGCGCA -3' and 5'-GCTCTAGACGGCCAGGTCAGTCCCCG GC-3'. Using the restriction sites introduced by the primers (shown in bold), the amplified fragment (lacking the BARE-like sequence normally found upstream of \textit{tylP}) was cloned into pLST9828, thus creating pLST9292 (Figure 3.11). The cloned piece of DNA was then sequenced to confirm that mutations had not been introduced during PCR. Then, \textit{tylP} was excised together with the powerful, constitutive promoter \textit{ermEp*} as an \textit{KpnI}-\textit{XbaI} fragment from pLST9292 and ligated into the respective sites of pPM925, creating pLST925P. The construct was integrated at the pSAM2 \textit{attB} site of the \textit{S. lividans} genome. Transformants that had undergone site-specific integration were resistant to streptomycin and spectinomycin (a marker carried by pLST925P).
Figure 3.11 Creation of two *tylP* over-expression constructs. The *tylP* probable *orf* was PCR amplified and introduced upstream of the strong, constitutive promoter *ermEp* in pLST9828 to generate pLST9292. A piece of DNA containing both *tylP* and *ermEp* was excised from pLST9292 and introduced into pOJ260 for subsequent integration into the *S. fradiae* genome. Alternatively, it was cloned into pPM925 to create pLST925P that was subsequently integrated into the pSAM2 *attB* site of the *S. lividans* genome. Abbreviations: oriR, origin of replication in *E. coli*; int φC31, site responsible for chromosomal integration; *tsr*, thioestreton resistance gene, *apra*, apramycin resistance gene; oriT, origin of transfer during conjugation; *ap*, ampicillin resistance gene; *strep/spec*, gene conferring both streptomycin and spectinomycin resistance; int pSAM2, site responsible for integration into the pSAM2 *attB* site of *S. lividans*. 
3.3.4 Investigation of the \textit{tylP} and \textit{tylQ} promoters in \textit{S. lividans}

Unlike most structural genes of the \textit{tyl} cluster, regulatory genes have large upstream non-coding regions (Figure 3.1). Prokaryotic promoters are generally not found beyond \(\sim 400\) bp upstream of the respective start codon. Thus, \textit{tylP} and \textit{tylQ} have \(\sim 400\) bp upstream sequences that separate them from their respective upstream genes, \textit{aco} and \textit{orf16*} (Figure 3.10). Even though the \textit{tylP} and \textit{tylQ} promoters had not been mapped, it was highly likely that they were located within their respective upstream gaps. Therefore, upstream DNA containing these gaps was PCR-amplified and cloned into pLST920 as described elsewhere (section 3.3.2). When the resulting constructs were integrated into the \(\phi C31\) \textit{attB} site of \textit{S. lividans}, they raised the level of kanamycin resistance relative to the strain containing an integrated, promoterless copy of \textit{aphII} [Figure 3.12(a), (c), (i)]. Having ascertained that the \textit{tylP}, \textit{tylQ} and \textit{orf12*} promoters were functional in \textit{S. lividans}, (and, incidentally, having observed that \textit{tylQp} appeared to be a stronger promoter than \textit{ermEp*} under such circumstances) the possible effect(s) of \textit{tylP} overexpression on such promoter activity was analyzed.

When \textit{tylP} was over-expressed in the presence of the \textit{tylP} promoter in \textit{S. lividans} via integration of pLST925P ([\textit{ermEp*}-\textit{tylP}]) at the \textit{pSAM2} \textit{attB} site (section 3.3.3), kanamycin resistance of the resulting strain was reduced dramatically [Figure 3.12(b)]. TylP had a similar effect when over-expressed in the presence of the \textit{tylQ} promoter, but not \textit{ermEp*} or \textit{orf12*p} [Figure 3.12(d), (f), (h)]. These data suggested that not only did TylP have the ability of specifically repressing the \textit{tylQ} promoter, but it also had the capacity to regulate itself.
Figure 3.12 Activity of tyl$P_p$ and tyl$Q_p$ in S. lividans. Each of the $tylP$, $tylQ$ and orf12* promoters were transcriptionally fused to $aphl$ in pLST920 and integrated into the $\phi$C31 $attB$ site of the S. lividans genome. In a similar fashion, the strong, constitutive promoter $ermEp^*$ was employed as a positive control. The $tylP$ gene, under control of $ermEp^*$, was integrated into the pSAM2 $attB$ site of the S. lividans strains containing either of the three foreign promoters. In order to reveal background resistance to kanamycin, strains containing "empty" pLST920 with and without the additional presence of [ermEp*-tyl$P_p$] were generated. Relative levels of kanamycin resistance were determined on NEF antibiotic gradient plates.
3.4 *tylP* over-expression in *S. fradiae*

### 3.4.1 Construction of the *tylP* over-expression cassette and introduction into *S. fradiae*

The *tylP* gene was excised from pLST9292 (section 3.3.3) together with the powerful, constitutive promoter *ermEp* as an *EcoRI*-*XbaI* fragment and ligated into pOJ260 (Figure 3.11). The resultant plasmid containing the *tylP* over-expression cassette was then integrated into the *tylP* region of the *S. fradiae* genome via a single cross-over (section 1.7.1). The apramycin resistance gene carried by pOJ260 was used to select for the event.

### 3.4.2 Effect of *tylP* over-expression on *tyl* gene expression and tylosin yields

When an extra copy of *tylP* was integrated into the *S. fradiae* genome (section 3.4.1) and over-expressed, the transcription pattern of *tyl* genes was altered. As expected, the *tylP* transcript was now present at 18 h but the *tylQ* transcript was not detectable at any time (Figure 3.13). Moreover, the resistance gene *tlrD*, *tylMIII* and *tylO* were also undetected. Most interestingly, no *tylS* transcript was present at 18 h under these conditions. On the other hand, a number of transcripts from *tyl* genes that were not present at 18 h in the wild type strain were now visible. Of particular interest was the finding that some of the structural genes were de-repressed when *tylP* was expressed constitutively, although transcripts from both pathway-specific activator genes, *tylS* and *tylR*, were not present at 18 h. As will become apparent (section 4.2.2), this effect was probably caused by (or, at least, involved) lack of repression from *tylQ* which, in turn, allowed partial de-repression of *tylR*, although this was not detectable by RT-PCR.

At 40 h, all *tyl* transcripts were detected in the *tylP* over-expression strain (except, of course, *tylQ*), although *tylS*, *tylT* and *orfI2* as well as the *tylG* transcripts were only visualized at a higher cycle number. These data suggested that negative regulation by *tylP* was reduced at later times even though its transcription was driven by the strong, constitutive promoter, *ermEp*. Since *tylP* encodes a putative γ–butyrolactone receptor, it
seems likely that TylP activity is influenced by γ-butyrolactone levels and that these will rise during later stages of fermentation.

Over-expression of *tylP* in the wild type also resulted in the reduction of tylosin production (Figure 3.14A). This was presumably due to the prolonged repression of *tylS*, the positive regulator of tylosin production, as well as negative control of various *tyl* structural genes (Figure 3.13). What remained unclear was which effects attributed to TylP in *S. fradiae* were exerted directly and which resulted indirectly. In this context, it was shown previously (section 3.3.4) that TylP controlled the *tylP* and *tylQ* promoters directly in *S. lividans*—directly in agreement with the presence of BARE-like sequences upstream of *tylP* and *tylQ* in the *S. fradiae* genome (Figure 3.15). Interestingly, there is also a BARE-like sequence present upstream of *tylS* suggesting that TylP could also directly regulate the *tylS* promoter. This finding prompted further investigation.
Figure 3.13 Expression analysis of the *tyl* gene cluster in a *tylP* over-expression strain. Total RNA was extracted from *S. fradiae* wild type (wt) and *S. fradiae* *tylP* over-expression (*tylP*-OE) strains previously grown in TSB for 18 h (no tylosin detected) and 40 h (tylosin produced). RT-PCR was performed to amplify gene-specific products that were later authenticated by sequence analysis. 25 cycles where routinely used for amplification unless no band was visualized, in which case, the reactions were repeated at 28 or 29 cycles. Negative controls were performed for each case in the absence of reverse transcriptase (data not shown).
Figure 3.14 Tylosin time course of *S. fradiae* strains. HPLC analysis of extracts produced from GRF fermentations of: A. wild type and a strain ('OE') constitutively over-expressing an additional copy of *tylP* under control of *ermEp*; B. wild type and a *tylP*-disrupted ('KO') strain. Each point represents the mean tylosin yield of multiple batch fermentations extracted on the same day.
**Figure 3.15** Candidate regulatory promoter regions of *tyl* genes. Alignment of the BARE-like consensus (Kinoshita et al., 1999) with similar, non-coding regions upstream of *tylP*, *tylQ* and *tylS*. Arrows represent inverted repeats.
3.5 Investigation of *tyl*<sub>Sp</sub> by promoter-probe analysis

### 3.5.1 Cloning of the *tylS* promoter

The *tylQ*-*tylS* intergenic region was PCR amplified as a 447 bp long DNA fragment (Figure 3.9D), ligated into pIJ2925 and authenticated by sequencing. It was then excised as a *BglII*-*KpnI* fragment and introduced into the promoter-probe vector pLST920, thus creating pLST920S (Figure 3.10) which was integrated at the φC31 *attB* site of the *S. lividans* genome, as described elsewhere (section 3.3.2).

### 3.5.2 Activity of *tyl*<sub>Sp</sub> in *S. lividans*

When pLST920S was integrated into the *S. lividans* genome (section 3.5.1), the resulting strain displayed higher levels of kanamycin resistance relative to the negative control [Figure 3.16(a), (e)], although these were lower than those seen with equivalent strains harbouring *tylP*, *tylQ* and *orf12*<sup>*</sup> promoters [Figure 3.16 (a); 3.12(a), (c), (e)]. When pLST925P (*ermEr*<sup>-</sup>*-*tylP*) was integrated into the *S. lividans* strain containing pLST920S (*tyl*<sub>Sp</sub>-*aphII*), the levels of kanamycin resistance were reduced implying that TylP recognized *tyl*<sub>Sp</sub> directly in *S. lividans* [Figure 3.16 (a), (b);]. However, TylP more powerfully repressed *tylP*<sub>p</sub> than *tyl*<sub>Sp</sub> (Figures 3.12, 3.16). This can plausibly account for transcript patterns in *S. fradiae* wild type at 18 h when, in the presumed absence (or low abundance) of γ-butyrolactone(s), *tylP* would be preferentially autoregulated, thus allowing expression of *tylS*. This model also suggests that autoregulation of *tylP* would allow expression of *tylQ* at early stages of fermentation. In order to test this hypothesis, *tylP* was disrupted in *S. fradiae* wild type.
Figure 3.16 Activity of tylSp in S. lividans. The tylS promoter was transcriptionally fused to aphII in pLST920 and integrated into the \( \Phi C31 \) attB site of the S. lividans genome. In a similar fashion, the strong, constitutive promoter \( \text{ermEp}^* \) was employed as a positive control. The tylP gene, under control of \( \text{ermEp}^* \), was integrated into the pSAM2 attB site of the S. lividans chromosome also carrying either of the two foreign promoters. In order to reveal background resistance to kanamycin, strains containing "empty" pLST920 with and without the additional presence of \([\text{ermEp}^* \cdot \text{tylP}]\) were generated. Relative levels of kanamycin resistance were determined on NEF antibiotic gradient plates.
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3.6 Expression analysis of a tylP-disrupted strain

3.6.1 Disruption of tylP in S. fradiae

Disruption of tylP (carried out by A.R. Gandecha) was achieved by introducing the hygromycin-resistance cassette (Ωhyg, which includes flanking transcriptional and translational terminators [section 1.7.1]) 132 bp inside the deduced orf, thus disrupting the recognition helix of the H-T-H motif (Figure 3.17). The general method is described elsewhere (section 1.7.1), while the specifics are also given elsewhere (Stratigopoulos et al., 2002b).

Ωhyg was integrated at the target site via a double cross-over using ‘arms’ of approximately equal size (750 and 801 bp) ligated on either end of the disruption cassette. The ‘arms’ were obtained by PCR amplified from tyl DNA derived from pHJL311 (Fishman et al., 1987) using the following primers:

5'-GGAGGAATTCGCCGGCGGGTGCCGGAC -3' plus
5'-CTCCGGATCCCTTGGTCACCCGGACCTCT -3'; and
5'-GGAGGGATCCCGCTGTACTTCCACTTC -3' plus
5'-CGCCAAGCTTGGTACCTCCACCCAGGTGG -3'.

The restriction sites introduced by the primers (shown in bold) allowed fusion at the BamHI sites by ligation into pIJ2925. The 2.3 kb Ωhyg fragment was then inserted into the BamHI site and the overall insert (~3.88 kb in total) was excised from pIJ2925 as a BglII fragment, ligated into pOJ260 at the BamHI site and introduced into S. fradiae via transconjugation (section 2.3.6). Strains that had undergone gene transplacement via double recombination into the genome were resistant to hygromycin and sensitive to apramycin (Figure 1.18).

3.6.2 Confirmation of the tylP disruption

Hemi-nested, step-down PCR was used to confirm the disruption of tylP. This alternative method is more definitive and convenient than Southern analysis. In order to eliminate the barrier of higher order structures (presumably present at either end of the Ωhyg disruption cassette), long primers (30-mer) with high Tm values were used to accommodate PCR at relatively high temperatures. Thus, the conditions for the Step-Down PCR were: primary DNA melting at 96 °C for 5 min followed by 5 cycles of
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°C for 1 min, 65 °C for 1.5 min, 73 °C for 3.5 min], 10 cycles of [96 °C for 1 min, 63 °C for 1.5 min, 73 °C for 3.5 min] and, finally, 10 cycles of [96 °C for 1 min, 61 °C for 1.5 min, 73 °C for 3.5 min].

The set of primers used for the primary PCR with genomic DNA as template were as follows:

1) 5'-AGCAGGCTGCCGAGGAACAGGTTGTAGTGG-3';
2) 5'-CACAATTCCACACAAACATACGAGCCGGAAG-3';
3) 5'-GTACGAGAACATCATGTGTCTGGGCGCGAC-3';
4) 5'-TCACAGCCAAACTATCAGGTCAAGTCTGCT-3'.

Primers (1) and (3) were specific to chromosomal DNA flanking either end of the cloned tyl region used for homologous recombination (Figure 1.18). Primers (2) and (4) were specific to Ωhyg DNA. For Hemi-Nested PCR, internal primer 5'-ACGAGTTCGTTGACGAAACCAGGAGCGGTGCTC-3' together with (2) amplified a definitive 1176 bp upstream sequence and internal primer 5'-CCACCTGCTTCACATACCGGTGGCCGGTCA-3' together with primer (4) amplified a 1117 bp downstream sequence (Figure 3.18). Each PCR product included one end of the Ωhyg cassette plus the 'arm' used for recombination and extended out into untouched DNA. Authentication of the product by sequencing analysis confirmed successful integration of the disruption cassette at the desired locus.
Figure 3.17 Point of disruption at the putative DNA-binding region of TylP. Alignment of the putative helix-turn-helix motifs of \(\gamma\)-butyrolactone receptor proteins orthologous to TylP (section 1.5). The point of disruption at the downstream, well conserved, DNA recognition helix is indicated.
Figure 3.18 Confirmation of tylp disruption by DC-PCR. A combination of hemi-nested and step-down PCR was employed to amplify definitive sizes spanning either end (U, upstream; D, downstream) of the integrated disruption construct. Pairs of primers were complementary to sequences within the Øhyg cassette used to disrupt tylp and within untouched DNA flanking the tylp region of the genome (see also Figure 1.17). Amplified products were authenticated by sequencing. Abbreviations: L, 1 kb ladder (GIBCO BRL).
3.6.3 Transcript detection and phenotypic analysis of the *tylP*-disrupted strain

The conclusion that *tylP* encodes a negative regulator of tylosin synthesis was supported by the finding that the *tylP*-disrupted strain produced more tylosin than the wild-type in various media, including those designed for high yields (Figure 3.14). Moreover, when the strain was grown on solid media, it sporulated at an early time point (Figure 3.19). This suggests that *tylP*, like *arpA* (section 1.6.2), controls both secondary metabolism and morphological differentiation.

Expression analysis of the *tylP*-disrupted strain showed that all the *tyl* transcripts were present at 18 h and 40 h except that of *tylP* and *tylQ* (Figure 3.20). Controversially, the *tylQ* transcript was not detectable at either 18 h or 40 h. This explained why the *tylP*-disrupted strain produced more tylosin than wild type (presumably due to earlier initiation of tylosin synthesis [see also Chapter 4]) but it also implied that *tylP* was not the only regulator of *tylQ*. Plausibly, de-repression of *tylS* [a positive regulator of tylosin production in *S. fradiae* (section 1.6.7)] in the *tylP*-disrupted strain might also contribute to enhanced yields.
Figure 3.19 Early sporulation of a *tylP*-disrupted strain. Spores from *S. fradiae* wild type (wt) and a *tylP*-disrupted (*tylP*-KO) strain were streaked on AS1 media and incubated at 37°C.
Figure 3.20 Expression analysis of the *tyl* gene cluster in a *tylP*-disrupted strain. Total RNA was extracted from *S. fradiae* wild type (wt) and *S. fradiae* *tylP*-disrupted (*tylP*-KO) strains previously grown in TSB for 18 h (no tylosin detected in wt) and 40 h (tylosin produced). RT-PCR was utilized to amplify gene-specific products that were later authenticated by sequence analysis. 25 cycles where routinely used for amplification unless no band was visualized, in which case, the reactions were repeated at 28 cycles. Negative controls were performed for each case in the absence of reverse transcriptase (data not shown).
3.7 Promoter-probe analysis of tylPp, tylQp and tylSp in S. fradiae

When pLST920P ([tylPp-aphII]), pLST920Q ([tylQp-aphII]) and pLST920S ([tylSp-aphII]) were individually integrated at the φC31 attB site of S. fradiae, the strains displayed higher levels of kanamycin resistance relative to their respective negative controls [Figure 3.21(b), (d), (e), (f), (j), (l)]. Interestingly, tylSp activity was 'weaker' than that of tylPp and tylQp, with the latter promoter being the strongest of the three. These data suggested that tylQ was transcribed early during growth on solid media as it was found to be the case in liquid TSB media. Conversely, the occurrence of tylPp activity in S. fradiae wild type under such conditions contradicted the finding that tylP transcript was not detectable in the same strain at 18 h in liquid TSB (Figure 3.6). It is possible that tylPp activity early in growth on solid media reflects the level of TylP expression needed to repress the tylP promoter prior to de-repression by γ-butyrolactone(s). The data regarding tylSp also implied that tylS transcription in S. fradiae occurred early in growth in solid media. This finding agreed with the presence of tylS transcript at 18 h in S. fradiae wild type.

When the tylP and tylS promoters were introduced into the tylP-disrupted strain, there was a discrete de-repression effect [Figure 3.21(c), (k)] that was not proportional to the effect of TylP on the respective promoters in S. lividans (section 3.3.2). The tylQ promoter, on the other hand, was not de-repressed in the tylP-disrupted strain. Instead, it was affected negatively. By now, this was not a surprise because it was consistent with the finding that the tylQ transcript was not present at 18 h when tylP was disrupted. It also reconfirmed the existence of another regulatory factor that repressed the expression of tylQ in S. fradiae.
Figure 3.21 Activity of $tylP_p$, $tylQ_p$, and $tylS_p$ in *S. fradiae*. Each of the $tylP$, $tylQ$ and $tylS$ promoters were transcriptionally fused to $aphl$ in pLST920 and integrated into the $\phi C31$ attB site of the *S. fradiae* wild type genome. In a similar fashion, the strong, constitutive promoter $ermEp^*$ was employed as a positive control. The $tylP_p$, $tylQ_p$ and $tylS_p$ promoter-probe constructs were also integrated into the $\phi C31$ attB site of the $tylP$-disrupted strain ($tylP$-KO). In order to reveal background resistance to kanamycin, *S. fradiae* wild type strains containing "empty" pLST920 were generated. Relative levels of kanamycin resistance were determined on AS1 antibiotic gradient plates.
3.8 Discussion

3.8.1 Close, co-directional genes of related functions are not always co-transcribed

Expression analysis via RT-PCR was proven to be an efficient and reliable method for the detection of numerous tyl transcripts. The results obtained were surprising on account of the differential expression patterns revealed. It became quite clear that, under physiological conditions, tyl genes that were co-directional and close to each other did not necessarily form operons (e.g. tylO, tylCV). Likewise, at 18 h in the strain where tylP was over-expressed the tylMIII transcript was absent while tylMII and tylMI were normally expressed (Figure 3.13). These results called to mind the finding that a tylMIII-disrupted strain complemented with tylMIII could still synthesize TyIMII and TyIMI so that enough OMT was produced to induce tylactone biosynthesis (Flint et al., 2002; see also section 1.2.9). This implied that tylMI and tylMII were transcribed independently of tylMIII under those conditions.

It was also shown in this work that the occurrence of overlapping open reading frames (orfs) does not imply co-transcription, even though it might be an indicator for co-translation. Although no one excludes the possibility that, under certain physiological conditions, this could be the case, there is also a chance that the downstream gene can be transcribed from its own promoter. Thus, tylMIII transcribed independently of tylGV. Early evidence supporting this arose when a tylGV-disrupted strain was generated (Flint et al., 2002). As expected, the strain did not accumulate tylosin but was capable of fully converting exogenously added tylactone to tylosin. Since the insertion of the Ωhyg cassette ensured transcriptional blockage from upstream promoters (i.e. that strain could still express all of the mycaminose genes, including tylMIII), tylMIII must have been transcribed independently of tylGV by a promoter imbedded within tylGV and downstream of the Ωhyg insertion point. The detection of transcript from the tylMIII gene but not tylGV at 18 h in the wild type (Figure 3.6) suggested that the tylMIII promoter within tylGV was also active under physiological conditions. More evidence supporting this was the cloning of a DNA fragment upstream of tylMIII and within tylGV coding sequence that displayed promoter activity when introduced in S. lividans (Figure 3.7). A similar observation has recently been reported in the literature where the
antibiotic structural genes desVIII, VI, VII in S. venezuelae were co-transcribed from a promoter embedded within the upstream gene pikAV whose stop codon overlapped with the start codon of desVIII (Chen et al., 2001). This finding reinforced the notion that this phenomenon is widely common among other Streptomyces species.

3.8.2 TyIP, a negative regulator of tylosin production

The current model for the role of γ-butyrolactones in antibiotic production is that they bind to transcriptional repressors, thus relieving the expression of their target genes (section 1.5). In the tylosin producer S. fradiae, tylP encodes a putative γ-butyrolactone receptor that acts as a negative regulator of tylosin production. Its role is more versatile than the respective orthologues studied elsewhere because it, directly or indirectly, works as a positive (e.g. tylMII) and a negative (eg tylG) regulator of structural genes. It also negatively influences both positive (tylS) and negative (tylP) regulators of tylosin production (Figure 3.22). The autoregulatory aspect of γ-butyrolactone receptors has also been reported in other organisms such as S. coelicolor (scbR; section 1.5.5) and S. virginiae (barA; section 1.5.3). In contrast, this is the first description of a γ-butyrolactone receptor targeting a gene that codes for a protein of the SARP family (section 1.6.3). Like ArpA in S. griseus, tylP also controls morphological differentiation.

Although γ-butyrolactones have not yet been isolated in S. fradiae, a plausible scenario would involve autoregulation of tylP at early stages in growth that would eventually be relieved by the gradual accumulation of γ-butyrolactone(s) in the medium (Figure 3.22). A similar scenario involving barA, the γ-butyrolactone receptor in S. virginiae, was examined in earlier studies by Northern blot analysis (Kinoshita et al., 1997). In that occasion, barA transcript was present early in fermentation but its transcription declined over time and was subsequently relieved by the exogenous addition of VB.

The modulation of tylosin production and morphological differentiation might depend on the relative levels of free TyIP and γ-butyrolactone(s) (Figure 3.22). TyIP would still down-regulate the expression of tylS to some extent and this might serve as a tuning mechanism ensuring the gradual accumulation of tylosin.
Although TyIP has been shown to repress \textit{tylQ}p in a heterologous host, it seems that the presence of another regulator in \textit{S. fradiae} is essential for the silencing of \textit{tylQ} transcription (Figure 3.22). This hypothesis was supported by the fact that \textit{tylQ} expression was not de-repressed in the \textit{tylP}-disrupted strain. In order to explain in detail how \textit{tylQ} is regulated, that other regulator needs to be identified.
Figure 3.22 A proposed role of TyIP in S. fradiae. Prior to tylosin production, enough TyIP is produced to silence \( tylP \) in the absence of \( \gamma \)-butyrolactone(s). This results in normal expression of \( tylQ \) and \( tylS \) as it also prevents sporulation. Gradual accumulation of \( \gamma \)-butyrolactone reaches a critical level leading to de-repression of \( tylP \) and, as a result, free TyIP is present [more than \( \gamma \)-butyrolactone(s)] down-regulating \( tylQ \) and \( tylS \). Protein X then silences \( tylQ \) and tylosin synthesis proceeds (see section 4.7.1). Presumably, TyIP still represses \( tylS \) but only partially since it has a low affinity for \( tylSp \). This may serve as a tuning mechanism that ensures gradual accumulation of tylosin. At a later stage in growth, it is possible that enough \( \gamma \)-butyrolactone is present to remove any residual TyIP from \( tylSp \).
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Chapter 4

Positive and negative control of a global regulator of tylosin production
Chapter 4: Positive and negative control of a global regulator of tylosin production

4.1 Introduction

Three of at least five regulatory genes present in the tylosin biosynthetic (tyl) gene cluster are known to control tylosin biosynthesis (Figure 4.1). One of them, *tylP*, encodes a negative regulator of both secondary metabolism and morphological differentiation (section 3.8.2) while the other two, *tylR* and *tylS*, are essential for tylosin production (Bate et al., 2002, see also below).

Expression analysis of the *tyl* gene cluster revealed a rather intriguing role for *tylQ*, a gene targeted by TyIP in *S. lividans* (section 3.3.4). At 18 h (before the onset of tylosin production), *tylQ* transcript was present in the wild type while, at 40 h (when tylosin was being produced), all *tyl* genes were transcribed except *tylQ* (section 3.2.2). This suggested that the product of *tylQ* exerted negative control on some aspect(s) of tylosin biosynthesis. One way to test this hypothesis was to over-express *tylQ* in *S. fradiae*.

4.2 Phenotypic and expression analysis of a *tylQ* over-expression strain

4.2.1 Construction of a *tylQ* over-expression cassette

*S. fradiae* wild type spores were used in Colony PCR (see section 2.5.2) to generate a 725 bp long fragment containing *tylQ* together with the candidate Shine-Dalgarno motif (GGAG) but lacking the BARE-like sequence normally found upstream of the gene (Figures 4.2, 3.15). The primers used for PCR amplification were as follows: 

5' - GGGGGGATCCTCAGCCAGGAGACAA -3' and 
5' - GGTTAAGCTTATCCCAAAGGACCGC - 3'.

The amplified DNA fragment was inserted into pIJ2925 (see section 2.2.5) via the restriction sites introduced by the primers (shown in bold) and was subsequently authenticated by sequencing (Figure 4.2). The powerful, constitutive promoter *ermEp* (Bibb et al., 1994) was then cloned into pIJ2925 (containing *tylQ*) at the unique EcoRI-BamHI sites (generating pLST9293), excised together with *tylQ* as a *Bgl*II-*Hind*III fragment and ligated into the *Bam*HI-*Hind*III sites of pOJ260 (see section 2.2.5). The resulting construct was introduced into *S. fradiae* wild
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type via transconjugation (section 2.3.6) where it integrated into the genome at the tylQ region via a single cross-over (also refer to Figure 1.9).
Figure 4.1 Regulatory genes and their position relative to genes associated with polyketide and sugar metabolism. The \( tylA \) genes that are involved in the synthesis of the common sugar precursors are shaded purple. The mycaminose \( tylM \) and \( tylB \) genes are green while the mycarose \( tylC \) genes are shaded blue. The genes involved in the synthesis and addition of 6-deoxyallose (\( tylN \), \( tylD \) and \( tylI \)) and its conversion to mycinose (\( tylE \) and \( tylF \)) are all clustered together (shaded red). Genes implicated in polyketide metabolism and ring modification are also highlighted (shaded black). Regulatory genes are shaded yellow. The patterned boxes represent DNA regions of ~500 bp in length, each containing intergenic and coding sequence from the upstream gene (except those upstream of \( tylGI \) and \( tylR \) that are ~1 kb in length and do not include coding sequence). These DNA fragments were assayed for promoter activity in this study.
Figure 4.2 Creation of a tylQ over-expression construct. The tylQ orf was PCR amplified and introduced into pIJ2925. The insert was then authenticated by sequencing. The strong, constitutive promoter ermEp* was cloned upstream of tylQ to generate pLST9293. A piece of DNA containing both tylQ and ermEp* was excised from pLST9293 and introduced into pOJ260 for subsequent integration into the S. fradiae genome. Abbreviations: oriR, origin of replication in E. coli; int ϕC31, site responsible for chromosomal integration; apra, apramycin resistance gene; oriT, origin of transfer during conjugation; ap, ampicillin resistance gene;
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4.2.2 Transcript detection and tylosin production in a \textit{t}y\textit{l}Q over-expression strain

When the \textit{t}y\textit{l}Q over-expression cassette was integrated into the \textit{S. fradiae} genome (see section 4.2.1), the resultant strain failed to produce tylosin when fermented in GRF medium (section 2.7) [Figure 4.3(b)]. To ascertain which aspect of tylosin production was affected, individual GRF fermentation batches of the \textit{t}y\textit{l}Q over-expression strain were supplemented with various biosynthetic intermediates (section 2.7.1) and analyzed by HPLC (section 2.8.1). The strain in question failed to glycosylate tylactone [Figure 4.3(c)], which suggested that the organism was incapable of synthesizing and/or adding mycaminoose (see also section 1.2.9). When OMT (section 1.2.2) was exogenously added, it failed to induce tylactone synthesis [Figure 4.3(d); see also section 1.4.4] and the OMT was not converted to tylosin. This implied that the strain was defective in polyketide metabolism and was also unable to synthesize and/or add mycinose and mycarose (see also sections 1.2.10, 1.2.11). These results strongly suggested that \textit{t}y\textit{l}Q encoded a negative regulator of various \textit{t}y\textit{l} genes. In order to identify which genes were targeted by TylQ, expression analysis was applied to the \textit{t}y\textit{l} gene cluster of the \textit{t}y\textit{l}Q over-expression strain.

Wild type \textit{S. fradiae} together with its \textit{t}y\textit{l}Q over-expression derivative were grown in TSB batch media at 28°C with shaking (section 2.3.2). Total RNA was extracted at an early time point (18 h) when no tylosin was detected and at a late time point (40 h) when tylosin was being produced by the wild type (Figure 3.3). At 18 h, the expression patterns of both strains were similar although the effect caused by constitutive expression of \textit{t}y\textit{l}Q was apparent by 40 h (Figure 4.4). For example, at 40 h no transcript from \textit{t}y\textit{l}AI or \textit{t}y\textit{l}AII (see section 1.2.8) was present in the \textit{t}y\textit{l}Q over-expression strain. This was compatible with bioconversion experiments where none of the deoxyhexose sugars were added to exogenously added tylosin precursors in the \textit{t}y\textit{l}Q over-expression strain (Figure 4.3). In addition, no transcript was detected at 40 h from the mycarose and mycinose biosynthetic genes \textit{t}y\textit{l}CII, \textit{CIV}, \textit{CIII} and \textit{t}y\textit{l}N, \textit{D}, \textit{J} respectively.

Another interesting observation at 40 h in the \textit{t}y\textit{l}Q over-expression strain was the absence of transcript from \textit{t}y\textit{l}GI, a gene essential for tylactone synthesis (Baltz & Seno, 1981; see also section 1.2.7). This result was compatible with the previous finding that tylactone synthesis was not induced in the \textit{t}y\textit{l}Q over-expression strain in the presence of
exogenous OMT (Figure 4.3). On the other hand, tylGIII, tylGIV and tylGV transcripts were present at 40 h. This was the first indication of tylG genes being independently transcribed. Further evidence supporting independent transcription of tylG genes (this time under physiological conditions) arose during promoter-probe analysis of tylG promoters in S. fradiae wild type.
Figure 4.3 HPLC analysis of a tylQ over-expression strain. HPLC analysis of extracts recovered from 7-day GRF fermentations of (a) *S. fradiae* wild-type (b) tylQ over-expression strain (tylQ-OE), (c) tylQ over-expression strain fed tylactone and (d) tylQ over-expression strain fed OMT. Intermediates were added after three days of growth in GRF media. When tylactone and OMT were added to batch GRF fermentations of *S. fradiae* wild type, they were converted to tylosin (data not shown).
Figure 4.4 Expression analysis of the *tyl* gene cluster in a *tylQ* over-expression strain. Total RNA was extracted from *S. fradiae* wild type (wt) and *S. fradiae* *tylQ* over-expression (*tylQ-OE*) strains previously grown in TSB for 18 h (no tylosin detected) and 40 h (tylosin produced only in wt). RT-PCR was utilized to amplify gene-specific products that were later authenticated by sequence analysis. 25 cycles were routinely used for amplification unless no band was visualized, in which case, the reactions were repeated at 28 cycles (red arrows). Negative controls were performed for each case in the absence of reverse transcriptase (data not shown).
4.3 Promoter-probe analysis of the tylG region

4.3.1 Manipulation of tylG promoter DNA

Putative tyl[GI-GV] promoters were isolated by PCR from wild type genomic DNA (section 2.5.2) using primers described elsewhere (Table 2). Each of the tyl[GI-GV] DNA fragments included part of the coding region from the upstream convergent gene followed by non-coding intergenic region as far as the translational start of each respective tylG gene (Figure 4.1). On the other hand, the tylGI promoter fragment spanned from the translational start of the upstream gene (tyll) to the translational start of tylGI. All DNA fragments were individually cloned into pIJ2925 by the use of restriction sites introduced by the primers used from PCR amplification (Table 2, shown in bold). Single-stranded sequence was generated from each DNA fragment to confirm that no unexpected mutations had been introduced during PCR amplification. The DNA fragments were then excised from pIJ2925 and ligated separately into pLST920 upstream of the aphII reporter gene (Figure 4.5), thus creating pLST920[GI-GV]. Likewise, the strong, constitutive promoter ermEp* was introduced into pLST920 as described elsewhere (Figure 3.10). Each construct was then integrated into the S. lividans and/or S. fradiae genome at the respective φC31 attB sites. The level of kanamycin resistance for each strain was measured using antibiotic gradient plates as described elsewhere (section 2.3.8). In order to eliminate the possibility that part of the multicloning region (carried along with promoter DNA from pIJ2925) was by itself active in the promoter sense, the polylinker from pIJ2925 was excised as a BglII fragment and introduced at the respective sites of pLST920 in place of the pre-existing polylinker (Figure 3.10). The resultant construct was introduced both S. fradiae and S. lividans but no subsequent elevation of kanamycin resistance was observed in either case (data not shown).
Figure 4.5 Cloning strategies of promoter fragments. Promoter DNA was PCR amplified, digested at the restriction sites introduced by each primer and cloned at the appropriate orientation into pIJ2925. It was then inserted into pLST920, upstream of aphII and integrated into the S. fradiae and/or S. lividans genomes. Asterisks of the same colour indicate insertion and excision points of each promoter fragment. Abbreviations: oriR, origin of replication in E. coli; int φC31, site responsible for chromosomal integration; apra, apramycin resistance gene; oriT, origin of transfer during conjugation; ap, ampicillin resistance gene.
4.3.2 Investigation of tyIG promoters and tyIp in S. lividans

Unlike regulatory genes from the tyl gene cluster (section 3.1), the tyl[GII-GV] polyketide genes have short upstream non-coding regions (Figure 4.1). Thus, the tylGI-GII and tylGIV-GV intergenic regions are 117 and 182 bp long respectively, while the tylGII-GIII and tylGIII-GIV intergenic regions are much shorter (65 and 33 bp respectively). In contrast, tylGI shares a 1,038 bp long upstream region with the divergently transcribed gene, tylI. Because this piece of DNA is sandwiched between two regions containing structural genes involved in all major steps of tylosin biosynthesis (Figure 4.1), it is plausible that it serves as a target for positive and/or negative regulators of tylosin biosynthesis. This hypothesis was also substantiated by the remarkable structure of this piece of intergenic DNA. At the ‘tylGI’ end lies a perfect 8-mer tandem repeat separated by 13 bp (~ one helical turn; Figure 4.6B). Even more remarkable is the occurrence of highly repetitive regions at the ‘tylI end’ (Figure 4.6C). All these observations in addition to the fact that prokaryotic promoters are usually found within ~400 bp of upstream sequence pointed to the high probability that the tylGI and tylI promoters were positioned within that piece of DNA. In fact, transcript mapping by RT-PCR (section 2.5.4) using primer walking helped in the approximate mapping of the tylGI transcriptional start site within its upstream non-coding region, 189-414 bp upstream of the gene (data not shown).

Unlike tylGIp, no attempts were made to map the rest of the tylG promoters. Nevertheless, tylGIIp, tylGIIIp, tylGIVp and tylGVp (if they really existed) would lie within their respective intergenic regions or within part of the coding region from their respective upstream gene. Therefore, ~ 500 bp long putative promoter DNA fragments from tyl[GII-GV] and a ~1 kb long fragment upstream of tylGI were PCR-amplified and cloned into pLST920 as described elsewhere (section 4.3.1). When the resultant constructs were integrated into the φC31 attB site of S. lividans, they raised the level of kanamycin resistance relative to the control strain containing an integrated, promoterless copy of aphII [Figure 4.7A(a),(c),(d),(e),(f)]. The tylGI promoter also raised kanamycin resistance levels but to lower levels than those seen with the other tylG promoters (Figure 4.7A, B). Similar results were also observed when the same piece of DNA (i.e. tylGI-tylI intergenic DNA) was cloned into pLST920 in the opposite orientation (generating
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pLST920I; Figure 4.5) and introduced into *S. lividans* (Figure 4.7B). The fact that both *tylGI* and *tyll* promoters had ‘weaker’ activities in comparison to the *tylGII, III, IV, V* promoters might be explained by the absence of a positive activator for *tylGI* and *tyll* in *S. lividans*. Alternatively, *tylGIp* and *tyllp* are simply weaker promoters. Comparatively, the *tylGII, III, IV, V* promoters displayed ‘strong’ activity in *S. lividans*, an occurrence which, likewise, might be caused by the absence of a negative regulator in *S. lividans* that is otherwise present in *S. fradiae*. In order to identify and compare their behaviour in their natural host, each of the *tylGI, tylGV* and *tyll* promoters was introduced into *S. fradiae* together with *aphII*.
Figure 4.6 Repeats upstream of *tylR*, *tylGI* and *tylII*. A. Heptameric repeats (dashed arrows) found within non-coding sequence upstream of *tylR* resemble those thought to be recognized by SARPs (Wietzorrek & Bibb, 1997). They are separated by 11 bp (one helical turn). Between the putative SARP recognition region and the *tylR* translational start lies a perfect 10-mer repeat (solid arrows) separated by 32 bp (3 helical turns). B. A perfect 8-mer repeat (solid arrows) found within non-coding sequence upstream of *tylGI* are separated by 13 bp. C. Unusually repetitive sequence located within a non-coding region upstream of *tylII*. Six 5-mers (solid arrows) are repeated within a stretch of ~100 bp. In addition, a perfect 16-mer repeat (shown in red) is found close to the translational start of *tylII*. 
Figure 4.7 Activity of *tylG* promoters and *tyllp* in *S. lividans* and *S. fradiae*. A. Each of the putative *tylG* promoter regions were cloned in pLST920 upstream of *aphII* and the resultant constructs were introduced into *S. lividans* as described elsewhere (section). Kanamycin resistance of the deduced strains was assessed on NEF antibiotic gradient plates (black & white). B. The ~1 kb long *tylGI*-tyll intergenic region was cloned in pLST920 at both orientations and introduced into *S. lividans*. The resulting strains ([*tylGlp-aphII*] and [*tyllp-aphII*]) were compared with that carrying ermEp* ([ermEp*-aphII]) and the promoterless version ([pLST920]) by assessing kanamycin levels of resistance on NEF gradient plates (black & white). C. The *S. fradiae* strains containing [*tylGlp-aphII*], [*tyllp-aphII*], [*tylGVp-aphII*], [ermEp*-aphII] and [pLST920] were streaked on AS1 antibiotic gradient plates (coloured).
4.3.3 Performance of \textit{tylGIp}, \textit{tyllIp} and \textit{tylGVp} in \textit{S. fradiae}

When pLST920GI ([\textit{tylGI-aphII}]) and pLST920I ([\textit{tyll-aphII}]) were integrated into the \textit{S. fradiae} genome (section 4.3.1), no elevation of kanamycin resistance was reported relative to the strain containing an integrated, promoterless copy of \textit{aphII} [Figure 4.7C(a), (b), (d), (e)]. Due to the nature of the reporter gene, trickle expression of \textit{aphII} early in growth is the minimal requirement for survival of the strain in the presence of kanamycin. Since the strains that contained \textit{tylGIp} or \textit{tyllIp} cloned directly upstream of \textit{aphII} did not elevate kanamycin resistance levels, the promoters in question were presumably silenced early in growth although, in this type of experiment, it was not possible to know whether they were de-repressed at a later stage in the absence of drug. These findings were also compatible with the absence of \textit{tylGI} and \textit{tyll} transcript in TSB fermentations of \textit{S. fradiae} wild type at 18 h (Figure 4.4).

In contrast, when pLST920GV ([\textit{tylGV-aphII}]) was introduced into the \textit{φC31 attB} site of the \textit{S. fradiae} genome, the resultant strain displayed elevated kanamycin resistance relative to the negative control [Figure 4.7C(g), (h)]. This finding cannot readily be reconciled with the observation that no \textit{tylGV} transcript was present in fermentations of \textit{S. fradiae} wild type after 18 h of growth, although, the transcript might have been present at earlier times (i.e. spores). Equally, it cannot be certified whether \textit{tylGVp} would have been down-regulated at a later stage in growth on solid media in the absence of the drug. In any event, the implication was that \textit{tylGVp} was active at early times on solid media whereas \textit{tylGIp} apparently was not.

4.4 TylQ represses the global activator of \textit{tyl} genes

4.4.1 TylR, a global regulator of tylosin synthesis

The most important finding during expression analysis of the \textit{tylQ} over-expression strain was the absence of \textit{tylR} transcript at 40 h, which suggested that TylQ was repressing such transcription (Figure 4.4). When \textit{tylR} was insertionally disrupted in \textit{S. fradiae} (Bate \textit{et al.}, 1999), the resultant strain failed to produce tylosin, and subsequent bioconversion analysis revealed that the \textit{tylR}-disrupted strain was defective in polyketide metabolism, synthesis and/or addition of all three sugars and terminal bis O-
methylation (see also section 1.2.10). These data suggested that \textit{tylR} controlled most, if not all, aspects of tylosin biosynthesis in a positive manner.

### 4.4.2 Negative regulation of \textit{tylR}

Early evidence that \textit{tylR} transcription was silenced early in growth arose when \textit{tylRp} (cloned upstream of \textit{aphII}) failed to raise kanamycin resistance levels in \textit{S. fradiae} relative to the promoterless control [Figure 4.8A(a), (b)]. In contrast, the same DNA fragment was active as a promoter when introduced into \textit{S. lividans} [Figure 4.8B(a), (b)], albeit at levels as low as previously seen with \textit{tylGIp} and \textit{tyllIp} in the same organism (section 4.3.3). Subsequent gene expression analysis of \textit{S. fradiae} wild type in TSB fermentations before (18 h) and after (40 h) the onset of tylosin production revealed that \textit{tylR} and \textit{tylQ} were not transcribed concurrently (Figure 4.9). It was concluded that \textit{tylQ}, directly or indirectly, represses transcription of \textit{tylR} and that tylosin synthesis is initiated once \textit{tylQp} is silenced and \textit{tylR} is de-repressed. In addition, expression analysis of the \textit{tylQ} over-expression strain showed that the product of \textit{tylQ} acted as a negative regulator of various \textit{tyl} genes. Despite all this, it was not known whether the negative effect of TylQ was exerted via repression of \textit{tylR} or whether TylQ directly controlled the expression of \textit{tyl} structural genes. In order to resolve this, a \textit{tylR}-disrupted strain was subjected to expression analysis involving RT-PCR and transcript patterns were compared with those seen in the \textit{tylQ} over-expression strain.
Figure 4.8 Activity of *tylR* in *S. lividans* and *S. fradiae*. A ~1 kb long, non-coding region upstream of *tylR* was PCR amplified using primers described elsewhere (Table 2). The fragment was digested at the restriction sites introduced by the primers (shown in bold) and then cloned at the respective unique sites of pIJ2925 and subsequently into pLST920 as described elsewhere (Figure 4.5). A. The *tylR* promoter fused to *aphII* ([*tylR*-aphII]) was introduced into *S. fradiae* and the resultant strain was compared with that carrying *ermEp* ([*ermEp*-aphII]) by assessing kanamycin levels of resistance on NEF gradient plates (coloured). B. The *S. lividans* strains containing [*tylR*-aphII], [*ermEp*-aphII] and [pLST920] were streaked on NEF, kanamycin gradient plates (black & white).
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4.4.3 Expression analysis of a tylR-disrupted strain

At 18 h, the expression pattern of a tylR-disrupted strain (section 4.4.2) was almost indistinguishable from that of the tylQ-disrupted strain (Figure 4.9). An exception was the presence of tylT and orf16* transcripts in the tylR-disrupted strain, which were absent in the tylQ over-expression strain. The deduced product of orf16* is a cytochrome P450 but its role in tylosin production (if any) remains elusive.

At 40 h, transcript from orf16* remained undetectable in the tylQ over-expression strain whereas orf16* transcript was present in the tylR-disrupted strain. In addition, transcript from the mycinose biosynthetic gene, tylE, was now detectable at 40 h in the tylR-disrupted strain and so was tylJ. Transcripts from tylE and tylJ were not detectable in the tylQ over-expression strain at 40 h. Nevertheless, both strains were equally capable of transcribing tyl[GI-GV] independently of tylGI.

With the exception of tylE, tylJ and orf16*, these data suggested that TyIQ down-regulates sugar and polyketide genes by silencing the expression of their positive activator, TyIR. The latter controls polyketide metabolism by, directly or indirectly, activating tylGI transcription. TyIR also regulates sugar metabolism by activating the tylIBA region and other sugar genes present in the tyl cluster.
Figure 4.9 Expression analysis of the *tyl* gene cluster in the *tylR*-disrupted and *tylQ* over-expression strains. Total RNA was extracted from *S. fradiae* wild type (wt), *S. fradiae* *tylR*-disrupted (tylR-KO) and *tylQ* over-expression (tylQ-OE) strains previously grown in TSB for 18 h (no tylosin detected) and 40 h (tylosin produced only in wt). RT-PCR was utilized to amplify gene-specific products that were later authenticated by sequence analysis. 25 cycles where routinely used for amplification unless no band was visualized, in which case, the reactions were repeated at 28 cycles (red arrows). Negative controls were performed in each case in the absence of reverse transcriptase (data not shown).
4.5. Confirming the role of \textit{tylQ} by targeted disruption

4.5.1 Disruption of \textit{tylQ} in \textit{S. fradiae}

Disruption of \textit{tylQ} was achieved by introduction of the hygromycin resistance cassette (\textit{\Omega}hyg, which includes flanking transcriptional and translational terminators [section 1.7.1]) 132 bp inside the deduced \textit{orf}, thus disrupting the DNA-recognition helix of the H-T-H motif (Figure 4.10). The general method is described elsewhere (section 1.7.1) while the specifics are also given elsewhere (Stratigopoulos & Cundliffe, 2002a).

The ‘arms’ (authenticated by sequence analysis) were of approximately equal size (750-800 bp) to facilitate insertion of \textit{\Omega}hyg into the \textit{tylQ} region of the \textit{S. fradiae} genome via double recombination. The PCR primers were:

\begin{align*}
5\text{'-CCTGAAGCTTCTGCGCGCCGGCCGACAA-3'} & \text{ plus} \\
5\text{'-GAGCGGATCCCCCGCTGCTGACTCCCGCCC-3'} & \text{; and} \\
5\text{'-GCTCGGATCCATTTCCAATTCGAGAGC-3'} & \text{ plus} \\
5\text{'-GTCTGAATTCCGACCCTGCCGCTCAGGTAT-3'} & \text{'. The template was pLST962, a derivative of pHJL311 (Beckmann \textit{et al.}, 1989). The primers introduced restriction sites (shown in bold) into the arms (\textit{EcoRI} and \textit{BamHI} in the upstream arm, \textit{BamHI} and \textit{HindIII} in the downstream arm) allowing fusion at the \textit{BamHI} sites by ligation into pIJ2925. Then, \textit{\Omega}hyg (a 2.3 kb \textit{BglII} fragment) was introduced into the \textit{BamHI} site. Disrupted \textit{tylQ} plus flanking DNA (~3.84 kb in total) was excised from pIJ2925 as a \textit{BglII} fragment, ligated into the \textit{BamHI} site of pOJ260 and introduced into \textit{S. fradiae}. Transconjugants that had undergone gene transplacement via double recombination into the genome were resistant to hygromycin and sensitive to apramycin (a marker carried by pOJ260).} \\
\end{align*}
<table>
<thead>
<tr>
<th>Protein</th>
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<td>BarB</td>
<td>FDROGFATAS LTAISNS AGVS</td>
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<td>JadR2</td>
<td>FDEHGYALAK LSAISSG AGVS</td>
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Figure 4.10 Point of disruption at the putative DNA-binding region of TylQ. Alignment of the putative helix-turn-helix motifs of proteins orthologous to TylQ (section 1.5). The point of disruption at the downstream, well conserved, DNA recognition helix is indicated.
4.5.2 Confirmation of a successful disruption in \textit{tylQ}

Hemi-nested, step-down PCR (extensively described in section 3.6.2) was used to confirm \textit{tylQ} disruption. The set of primers used for primary PCR with genomic DNA as template were as follows:

\begin{enumerate}
\item 5'-TGTGATCGCCGAGACGATGCGGACCAAGAA-3';
\item 5'-TCACAGCCAAACTATCAGTGCTCTGCT-3';
\item 5'-CCCACAGTTCTCGATCAGCGCTGACGG-3';
\item 5'-CACAATTCCACAACACATACGAGCCGAAG-3'.
\end{enumerate}

Primers (1) and (3) were specific to chromosomal DNA flanking either end of the region used for homologous recombination (Figure 1.8). Primers (2) and (4) were specific to \textit{Ωhyg} DNA. For the hemi-nested PCR, internal primer 5'-GTCTCCTTCGCCGCTCCCGGGCTCGACCCC-3' together with (2) amplified a definitive 1137 bp upstream sequence and internal primer 5'-CGTGCGTGAGTGAGTCGAGTCGCGACGCTCCGAC-3' together with primer (4) amplified a 1199 bp downstream sequence (Figure 4.11). Each PCR product included one end of the \textit{Ωhyg} cassette plus the 'arm' used for recombination together with sequence extending out into untouched DNA, thus confirming successful integration of the disruption cassette at the desired locus.
Figure 4.11 Confirmation of \textit{tylQ} disruption by DC-PCR. A combination of hemi-nested and step-down PCR was employed to amplify definitive sizes spanning either end (U, upstream; D, downstream) of the disruption construct. Pairs of primers were complementary to sequences within \textit{\Omega}hyg used to disrupt \textit{tylQ} and within untouched DNA flanking the \textit{tylQ} region in the genome (see also Figure 1.17). Amplified products were authenticated by sequencing. Abbreviations: L, 1 kb ladder (GIBCO BRL).
4.5.3 Transcript detection and phenotypic analysis of a tylQ-disrupted strain

The conclusion that tylQ encodes a negative regulator of tylosin synthesis was supported by the finding that the tylQ-disrupted strain was capable of initiating tylosin synthesis ahead of the wild type (Figure 4.12). Moreover, when this strain was subjected to expression analysis by RT-PCR, it was clear that tylosin production began prior to 18 h since transcripts from all tyl genes (except tylQ, of course, and also tylP) were present at that time point (Figure 4.13). This was presumably caused by de-repression of tylR whose transcript was also present as early as 18 h.

Although early initiation of tylosin production raised the possibility that tylosin yields might be overall elevated, the tylQ-disrupted strain did not differ significantly from the wild type in this regard when grown in MM-1 (data not shown) or GRF tylosin production media (Figure 4.14). Perhaps this was due to the unaffected expression of TylP which, to some extent, might have restricted tylosin accumulation down to physiological levels by down-regulating tylS (see sections 3.4.2, 4.6 & 5.6.2).
Figure 4.12 Early production of tylosin by the tylQ-disrupted strain. *M. luteus* plug assays using chloroform extracts from TSB fermentations of wild type (wt) and *tylQ*-disrupted (*tylQ*-KO) strains. Extracts were diluted accordingly. Plates were prepared as described elsewhere (section 2.8.3) and incubated at 37 °C overnight. As a negative control, plugs containing an equal volume of chloroform were also incubated because fermentation extracts were dissolved in chloroform (data not shown).
Figure 4.13 Expression analysis of the *tyl* gene cluster in a *tylQ*-disrupted strain. Total RNA was extracted from *S. fradiae* wild type (wt) and *S. fradiae* *tylQ*-disrupted (*tylQ-KO*) strains previously grown in TSB for 18 h (no tylosin detected in the wild type) and 40 h (tylosin produced). RT-PCR was utilized to amplify gene-specific products that were later authenticated by sequence analysis. 25 cycles were routinely used for amplification unless no band was visualized, in which case, the reactions were repeated at 28 cycles (red arrows). Negative controls were performed for each case in the absence of reverse transcriptase (data not shown).
Figure 4.14 HPLC analysis of a tylQ-disrupted strain. HPLC analysis of extracts recovered from 7-day, GRF fermentations of (a) S. fradiae wild-type and (b) tylQ-disrupted strain (tylQ-KO).
4.6 TylS, a positive activator of tyl genes

4.6.1 TylS, a member of the SARP family

The deduced products of tylS and tylT are distinctly similar to each other and are also similar to proteins of the SARP family that have been experimentally identified as positive regulators of secondary metabolism (Bate et al., 1999). When the role(s) of these two genes was addressed by gene disruption, it was discovered that only tylS was essential for tylosin production in S. fradiae (Bate et al., 2002). Bioconversion experiments confirmed that the tylS-disrupted strain did not have the ability to synthesize and/or add any of the three tylosin sugars or to produce tylactone. However, the strain was still capable of converting macrocin to tylosin (the last step of tylosin synthesis; section 1.2.2), albeit at very low levels and, in this way, could be distinguished from a tylR-disrupted strain. In order to address the specific role of TyLS in the regulation of tyl genes, a tylS-disrupted strain was subjected to RT-PCR analysis.

4.6.2 Expression analysis of a tylS-disrupted strain

The expression pattern of tyl genes in the tylS-disrupted strain (see section 2.3.1) was quite similar to that of the tylR-disrupted strain, although there were some differences (Figure 4.15). The tylS-disrupted strain did not produce orf12* transcript at 18 h or 40 h, although orf12* was transcribed at such times both in wild type and tylR-disrupted strains. In addition, no orf11* (section 1.2.12) transcript was present at 18 h or at 40 h when tylS was disrupted, although that was not the case with wild type and tylR-disrupted strains. Most importantly, no tylR transcript was detected in the tylS-disrupted strain, suggesting that TyLS positively regulated the expression of the global activator of tyl genes. This was not a new finding since earlier complementation experiments addressing the hierarchy of those regulatory genes placed tylS above tylR in the tyl regulatory cascade (Bate et al., 2002).
Figure 4.15 Expression analysis of the *tyl* gene cluster in the *tylS* and *tylR*-disrupted strains. Total RNA was extracted from *S. fradiae* wild type (wt), *S. fradiae* *tylS*-disrupted (*tylS*-KO) and *S. fradiae* *tylR*-disrupted (*tylR*-KO) strains previously grown in TSB for 18 h (no tylosin detected) and 40 h (tylosin produced only in wt). RT-PCR was utilized to amplify gene-specific products that were later authenticated by sequence analysis. 25 cycles were routinely used for amplification unless no band was visualized, in which case, the reactions were repeated at 28 cycles (red arrows). Negative controls were performed for each case in the absence of reverse transcriptase (data not shown).
Interestingly, the $tylS$-disrupted strain still produced $tylF$ transcript, unlike the $tylR$-disrupted strain where no $tylF$ transcript was present at 18 h or 40 h. This finding was compatible with previous reports claiming that the $tylS$-disrupted strain was capable of converting macrocin to tylosin, a step catalyzed by the product of $tylF$ (Bate et al., 2002). It also agreed with the finding that the $tylR$-disrupted strain could not perform a similar function (Bate et al., 1999) since $tylF$ transcript was non-detectable in the latter strain.

Most interesting was the finding that no $tylMIII$ transcript was present at 18 h and 40 h in the $tylS$-disrupted strain. This expression pattern was previously seen when $tylP$ was over-expressed in the wild-type (section 3.4.2). On that occasion, excess $tylP$ down-regulated $tylMIII$. Similarly, disruption of $tylS$ apparently caused loss of the $tylMIII$ transcript although more time points would have be taken past 40 h to confirm this. This strongly suggested that TylP regulates the expression of $tylMIII$ by repressing the transcription of $tylS$. Similarly, down-regulation of the $tyl[GIII-GV]$ genes in the $tylP$ over-expression strain (section 3.4.2) might have been due to repression of $tylS$ since the $tylS$-disrupted strain failed to transcribe the polyketide genes in question.

Transcripts from $tylGI$, $tylIBA$ region and other sugar genes were also undetectable in the $tylS$-disrupted strain and were also absent in the $tylR$-disrupted strain. This implied that $tylR$ is the principal activator of their transcription and that TylS is the essential activator of $tylR$. In addition, TylR can activate $tylGIII-GV$ and $tylMIII$ in the absence of TylS since a $tylS$-disrupted strain, complemented with $tylR$, produced tylosin (Bate et al., 2002).

4.7 Discussion

4.7.1 Pivotal role of $tylQ$ in the regulation of tylosin production in S. fradiae

TylQ is a negative regulator of tylosin production in S. fradiae. It down-regulates various $tyl$ genes, mainly by repressing (directly or indirectly) their global activator, TylR (Figure 4.16). This was supported by expression analysis of the $tylQ$ over-expression and $tylR$-disrupted strains (Figure 4.9). Moreover, $tylQ$ transcription had to be silenced prior to the onset of tylosin production, as shown by expression analysis of the wild type at 40
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h (Figure 4.9). Even though promoter-probe analysis coupled with transcript detection identified TylP as a repressor of tylQ (section 3.3.4), the absence of tylQ transcript in the tylP-disrupted strain suggested that TylP is not its sole repressor (Figure 4.16). The other hypothetical regulatory gene has not yet been identified (see section 4.7.5).
Figure 4.16 A proposed role of TylQ in *S. fradiae*. Prior to tylosin production, enough TylP is produced to silence tylP in the absence of γ-butyrolactone(s). This results in normal expression of tylQ and tylS resulting in repression of tylR by TylQ. Gradual accumulation of γ-butyrolactone reaches a critical level leading to de-repression of tylP and, as a result, free TylP is present [more than γ-butyrolactone(s)] down-regulating tylQ and tylS. Protein X then silences tylQ and tylosin synthesis proceeds by activation of tylR by TylS. Presumably, TylP still represses tylS but only partially since it has a low affinity for tylSp. This may serve as a tuning mechanism that ensures gradual accumulation of tylosin. At a later stage in growth, it is possible that enough γ-butyrolactone is present to remove any residual TylP from tylSp.
4.7.2 Differential expression of \textit{tylG} genes

Prior to this work, the only attempt to analyze the expression of type I polyketide synthase (PKS) genes was made in \textit{Sac. erythraea} (Reeves et al., 1999), where the three \textit{eryA} polyketide genes were part of a polycistronic message that also covered downstream sugar genes. The \textit{tylG} polyketide genes of \textit{S. fradiae} are co-directional and are separated by short intergenic gaps (Figure 4.18) suggesting that they may be co-transcribed too. Moreover, \textit{tylGV} overlaps with the sugar gene \textit{tylMIII} by the ‘GTGA’ motif and \textit{tylMIII}, in turn, is co-directional with the functionally related genes \textit{tylMI} and \textit{tylMII}. Data presented so far showed that \textit{tylGV} is not co-transcribed with \textit{tylMIII} prior to the onset of tylosin production in the wild type (Figure 4.13). In addition, expression analysis of the \textit{tylP} over-expression and \textit{tylS}-disrupted strains supported independent transcription of \textit{tylMII} and \textit{tylMI} relative to \textit{tylMIII}. New evidence from this work suggested that four of the \textit{tylG} genes (nothing is yet known about expression of \textit{tylGII}) were not necessarily co-transcribed either. Thus, in the \textit{tylQ} over-expression and \textit{tylR}-disrupted strains, \textit{tyl[GIII-GV]} were transcribed independently of \textit{tylGI} (Figure 4.9).

The possibility of \textit{tylG} and/or \textit{tylM} genes being co-transcribed is not entirely excluded. In fact, when a \textit{tylS}-disrupted strain was complemented with \textit{tylR}, under control of \textit{ermEp*}, the resultant strain produced tylosin (Bate et al., 2002), although a \textit{tylR}-disrupted strain transcribed \textit{tylGIII-MIII} but not \textit{tylGI}. One hypothesis arising from these data is that TylR can recruit a sigma factor that is bound to an RNA polymerase capable of co-transcribing \textit{tylGI-MIII} from the \textit{tylGI} promoter. Nevertheless, it is not certain whether physiological levels of TylR (i.e arisen from \textit{tylR} under control of its native promoter) would still be capable of expressing a sufficient amount of TylG and TylMIII proteins for production of tylosin at the levels seen with \textit{S. fradiae} wild type.

4.7.3 TylR, a global activator of \textit{tyl} genes

Expression analysis of a \textit{tylR}-disrupted strain revealed that TylR is essential for the activation of \textit{tyl} structural genes (Figure 4.17). Most importantly, TylR controls the expression of the \textit{tylIBA} region that is essential for synthesis of all three tylosin sugars. TylR is also needed for the activation of \textit{tylGI} that encodes a multifunctional protein essential for the initiation of polyketide synthesis (section 1.2.7) and can also drive
transcription of all *tylG* and *tylM* genes since a *tylS*-disrupted strain, complemented with *tylR*, produced tylosin (Bate *et al.*, 2002). If *tylR* encodes a DNA-binding protein, it is plausible that it might target (among others) the *tylGI-tylII* intergenic region. In fact, that ~1 kb intergenic DNA fragment displays repetitive sequences at both end (Figure 4.6B, C). It remains to be seen whether TylR recognizes any of these sequences.

Transcription of *tylR* is positively and negatively regulated. Thus, constitutive expression of *tylQ* caused permanent repression of *tylR* (section 4.2.2). Similarly, *tylR* transcription was abolished in the *fyAS*-disrupted strain (section 4.6.2) due to the absence of the transcriptional activator TylS (Figure 4.17).

### 4.7.4 TylS is essential for the activation of *tylR*

The function of TylS is compatible with that of other members of the SARP family (section 1.6.3) in that it acts as a pathway-specific, positive regulator of *tyl* structural genes such as *tylMIII* and *tyl[GIII-GV]* (Figure 4.17). However, unlike other SARPs, *tylS* activates, directly or indirectly, a regulatory gene (*tylR*). This is the first report of such an occurrence in streptomycetes (Bate *et al.*, 2002, Figure 4.15). In addition, TylS (unlike other SARPs such as ActII-ORF4 or RedD and the structural genes they control; section 1.6) is not essential for the activation of *tylMIII* and *tyl[GII-GV]* since a *tylS*-disrupted strain, complemented with *tylR*, produced tylosin (Bate *et al.*, 2002).

In an attempt to identify target DNA sequences for TylS, similar to those generally recognized by SARPs (Wietzorrek & Bibb, 1997), two heptameric repeats were found upstream of *tylR* (Figure 4.6A). Conceivably, these sequences are recognized by TylS, although the only definitive way of establishing that model would be by DNA-binding assays.

As previously shown (section 3.4.2), TylP acted as a repressor of *tylS*. It also affected the expression of *orfI2* and *tyl[GIII-V]*. Although more direct evidence is needed, it appears that TylP influences the expression of those genes via the repression of *tylS*. This was supported by the finding that the *tylS*-disrupted strain also failed to transcribe *orfI2* or *tyl[GIII-V]* (Figure 4.15) and that repression of *tylS* in the *tylP* overexpression strain had a similar effect (Figure 3.13). In addition, TylP was shown to repress *tylSp* in *S. lividans* (section 3.5.2). The fact that *tylGI* expression was also
affected in the \textit{tylP} over-expression strain might be rationalized as the result of \textit{tylS} repression leading to a reduction in \textit{tylR} transcription, which, in turn, is needed for activation of \textit{tylGI}. More direct evidence is needed to substantiate this hypothesis.
Figure 4.17 Proposed role of TylS and TylR in \textit{S. fradiae}. When \textit{tylR} is de-repressed (section 4.7.1), TylS is essential for its activation. At the same time, TylS up-regulates (but is not essential) for the activation of the mycaminose gene \textit{tylMIII} (shaded green) and polyketide genes \textit{tylGIII} and \textit{tyl}[GIV-V] (shaded black). TylR is essential for expression of \textit{tylGI} and the \textit{tylIBA} region. Presumably, TylR is also capable of driving transcription of \textit{tyl}[GII-GV] and \textit{tylM} genes from \textit{tylGIp}. (The \textit{tylA} genes (involved in the synthesis of the common sugar precursors) are shaded purple. The \textit{tylM} genes (synthesis and addition of mycaminose) are shaded green. The other mycaminose-related gene, \textit{tylB}, is found directly upstream of the \textit{tylA} genes. The \textit{tylC} genes associated with mycarose synthesis and addition are clustered together with the exception of \textit{tylCVI} that is found at the right-hand side of the cluster (shaded blue). The genes involved in the synthesis and addition of 6-deoxyallose (\textit{tylN}, \textit{tylD} and \textit{tylJ}) and its conversion to mycinose (\textit{tylE} and \textit{tylF}) are all clustered together (shaded orange). Genes implicated in polyketide metabolism and ring modification are also highlighted (shaded black).)
4.7.5 How is \textit{tylQ} regulated?

So far, in all strains subjected to expression analysis, \textit{tylP} and \textit{tylQ} expression was not concurrent. In addition, promoter-probe analysis demonstrated that TyIP directly repressed \textit{tylQp} in \textit{S. lividans}. These data were consistent with the hypothesis that TyIP represses \textit{tylQ} in \textit{S. fradiae}. Plausibly, this might have been caused by de-repression of \textit{tylP} through the binding of \(\gamma\)-butyrolactone to the autoregulator TyIP. This, in turn, would result in repression of \textit{tylQ} followed by de-repression of \textit{tylR} and activation of the latter by TyIS.

The model became more complicated from the finding that \textit{tylQ} transcript was not present in the \textit{tylP}-disrupted strain. This pointed to the fact that TyIP is not the sole repressor of \textit{tylQ} in \textit{S. fradiae} wild type (Figure 3.20). In fact, promoter-probe analysis in the \textit{tylP}-disrupted strain showed that \textit{tylQ} promoter activity was reduced relative to wild type (Figure 3.21). These results raised the possibility that TyIP might also down-regulate \textit{tylQ} in an indirect fashion (perhaps via the positive regulator TyIS) since TyIP also recognized \textit{tylSp} in \textit{S. lividans}. If that were the case, then a repressor (TyIP) would repress a 'dual' regulatory gene (TyIS) that acts both as an activator of \textit{tylR} and a repressor of \textit{tylQ}. In an effort to find probable binding sites for TyIS at the \textit{tylQ} upstream region, no heptameric DNA repeats similar to those SARPs bind to (Wietzorrek & Bibb, 1997) were identified. Instead, seven heptamers (similar to those thought to be recognized by SARPS) were situated within \textit{tylQ} (Figure 4.16). Two of them were separated by 20 bp (approximately two helical turns apart) thus facing the same side of the DNA. It remains to be seen whether TyIS recognizes this sequence.

Alternatively, TyIR might repress \textit{tylQ} since \textit{tylR} and \textit{tylQ} were not concurrently expressed in any of the strains subjected to expression analysis. This could happen directly or indirectly. On the other hand, TyIR might directly repress the \textit{tylQ} promoter. Alternatively, TyIR might activate expression of an identified repressor gene (perhaps encoding another \(\gamma\)-butyrolactone receptor) whose product might negatively regulate \textit{tylQ}.  

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Figure 4.18 Heptameric repeats within *tylQ*. Heptameric repeats (dashed arrows) found within *tylQ*. Two of them (shown in red) are separated by 20 bp (2 helical turns). The occurrence of the conserved 4-bp palindromic sequence (TCGA) is 1/198 bp across the whole tylosin biosynthetic gene cluster.
Chapter 5

Inactivation of a repressor during empirical strain improvement of *S. fradiae*
Chapter 5: Inactivation of a repressor during empirical strain improvement of S. fradiae

5.1 Introduction

The increasing importance of antibiotics in our multiplex societies was the driving force for their production on an industrial scale. In addition, cost effectiveness ensured that antibiotics were widely available. Since chemical synthesis was, and still is, a time-consuming and expensive process, early efforts concentrated on the production of antibiotics as natural products. Over the years, the advancement of fermentation technologies helped in improving antibiotic yields at a low cost. In parallel, manipulation of antibiotic-producing organisms was a major contributor to the elevation of the antibiotic titre. This was mainly achieved by mutation and selection (otherwise known as empirical or classical improvement). During this process, a strain was randomly mutagenized and the resulting isolates were screened until an improved derivative was discovered. In turn, the improved strain was mutagenized and a new round of screening took place until a newly improved strain was isolated. By using this method, strains were generated producing considerably higher levels of antibiotic relative to their respective progenitors. A striking example of that is production of penicillin by *Penicillium chrysogenum*. When the drug was produced for the first time on an industrial scale, it yielded 1-10 μg/ml. Nowadays, new variants of the same strain generated solely by classical improvement programs produce 50,000 times more penicillin than their ancestors.

However, empirical improvement programs are expensive and time-consuming. For example, it took ~1,000,000 assays and 20 years of effort by people at Lilly Research Laboratories, Indianapolis, to generate economically viable *S. fradiae* strains that produced high levels of tylosin (Zhang *et al.*, 2002). In the same group, alternative methods were developed for the improvement of tylosin yields, such as genetic engineering. In the latter case, one could genetically manipulate an antibiotic-producing organism provided that cloning methods had previously been developed and enough information had been gathered related to the molecular mechanisms involved in the synthesis of the antibiotic. Thus, an extra copy of the *tlyF* gene (see section 1.2.10) was cloned into a specific chromosomal locus of *S. fradiae* that was neutral in respect to tylosin production. This was achieved by transposon exchange or homologous
Chapter 5: Inactivation of a repressor during empirical strain improvement of S. fradiae

recombination and resulted in a 40% or 60% increase of the tylosin titre respectively (Solenberg et al., 1996, Baltz, 1998).

In this laboratory, tylosin yields were elevated when the tylP gene, encoding a repressor of tyl genes, was insertionally disrupted (section 3.7.1). At the same time, another approach was adopted whereby mutations causing elevation in tylosin yields could be discovered. This involved generating DNA sequence from the ancestral strain and comparing it with DNA sequence from an empirically improved derivative. An alternative experimental approach would be comparative genomic analysis although this method wouldn't reveal the significance of specific mutations. Likewise, transcriptome analysis by microarrays would not detect the consequence of mutations that fail to block transcription.

In the present work, a comparative study was carried out on the DNA level that focussed on specific regions of the tyl gene cluster in an empirically improved and the ancestral strain (Figure 5.1). These regions did not include structural genes since it is unlikely that tylosin yields would have been markedly enhanced by mutations that generated super-efficient structural enzymes. Most likely, enhancement of tylosin production was achieved by the generation of mutations affecting promoter regions and/or regulatory genes. A gene with novel sequence and unknown function was also included in this study.
Figure 5.1 Genes and promoter regions used for sequencing. Genes sequenced from the ancestral strain are shown in yellow. Patterned boxes (orf18*-tylP, tylQp, tylS, tylT-orf12*, tylCIII-CV) represent DNA regions of 350-500 bp in length, each containing intergenic DNA. The tylGI-tyll and tylR-orf8 intergenic regions were ~1 kb in length. Each of the tylG promoters (tylGIIp, tylGIIIp, tylGIVp, tylGVp) were ~500 bp in length and they included intergenic preceded by coding sequence from their upstream gene. tylMIIP consisted exclusively of coding sequence from the upstream gene, tylGV.
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5.2 Comparison of an empirically improved S. fradiae derivative with the ancestral progenitor strain

Relatively long, non-coding gaps within the *tyl* gene cluster are exclusively found between divergent genes (*tyl*HI-F, *orf*18*-tyl*P, *tyl*I*-orf*12*, *tyl*CIII-CV, *tyl*GI-*tyl*I, *tyl*R-*orf*8) or upstream regulatory genes (*tyl*Q, *tyl*S) as well as the resistant determinant *tlr*D (Figure 5.1). Therefore, a safe conclusion could be drawn as to where the promoters may lie. Moreover, following the discovery that the *tyl*GII-GV genes may be differentially transcribed (section 4.7.2) and that *tyl*MIII may be expressed from its own promoter (section 3.8.1), DNA regions that lie upstream of these genes were also taken into consideration. In addition, regulatory genes (*tyl*P, Q, S, T, R) and a gene of unknown function (*orf*12*) were included in this study (Figure 5.1).

Since no attempts were made to experimentally identify the start codon of each deduced protein, the longest possible *orf* was selected for each gene. Similarly, at least 340 bp of upstream DNA sequence was chosen so that it spanned from the start of the shortest possible *orf* to the closest start or stop of the upstream gene (*orf*18*-tyl*P, *tyl*I*-orf*12*, *tyl*CIII-CV, *tyl*GI-*tyl*I, *tyl*R-*orf*8, *tyl*Qp, *tlr*Dp). In each of the remaining DNA fragments (*tyl*HI-F, *tyl*Sp, *tyl*GIIp, *tyl*GIIIp, *tyl*GIVp, *tyl*GVp, *tyl*MIIIp), upstream coding sequence was also included.

DNA fragments were amplified from the ancestral strain by genomic PCR (section 2.5.2) using primers described elsewhere (Table 2). Each fragment was cloned into pIJ2925 using restriction sites introduced by the primers (Table 2; shown in bold). For each PCR product, double-stranded DNA sequence was generated from multiple isolates and edited as described elsewhere (section 2.6). It was then compared with DNA sequence from a cosmid library that derived from an advanced production strain (X), again as described elsewhere (section 2.6). The results showed that almost no changes occurred within genes and promoter regions of strain X. The low mutational rate within *tyl* sequence (although the entire cluster was not sequenced) suggested that the cluster remained mostly unchanged. The only difference discovered was within *tyl*Q (a T to A at position 147) which caused a histidine to glutamine change (Figure 5.2). The mutation was located within the well-conserved recognition helix of the H-T-H motif found at the N-terminal portion of TylQ (Figure 5.3). This finding, in combination with the fact that the ‘ancestral TylQ’ (TylQ) acts as a repressor of various *tyl* genes (section 4.7.1),
suggested that the TyIQ mutant version (TyIQ*) may be functionally defective. This prompted further investigation.
Figure 5.2 DNA sequence comparison between *tylQ* and *tylQ*.
Cromatograms as generated by automated sequencing (section 2.6) for part of the *tylQ* gene encoding part of the recognition helix. Template DNA derived from the ancestral strain, improved strain C4 and improved strain X. The point mutation present in strains C4 and X is indicated.
Figure 5.3 Location of the TylQ mutation in respect to the predicted secondary structure. Comparison of the N-terminal region of TylQ from the ancestral strain with other orthologues in the database (see also section 1.6.7). The T147A change caused a H to Q substitution within the well conserved, downstream helix [part of the H-T-H motif (yellow box)]. The position of the mutational change is marked with an asterisk. (Diagram taken from Stratigopoulos & Cundliffe, 2002b)
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5.3 Over-expressing tylQ* in the ancestral strain

5.3.1 Construction of a tylQ* over-expression cassette

The tylQ* over-expression cassette was constructed as described elsewhere (section 4.2.1), with the exception that pMOMT4 (containing DNA from the empirically improved strain X; Beckmann et al., 1989) was used as template for PCR amplification. The resultant construct was then introduced into the tylQ region of the ancestral strain, again as described elsewhere (section 4.2.1). In order to confirm that the point mutation present in tylQ* was not inserted to the chromosomal tylQ copy, DNA sequence was generated of the integrated tylQ* over-expression cassette from the resultant strain.

5.3.2 Phenotypic and expression analysis of a tylQ* over-expression strain

It was previously shown that tylQ is expressed at an early time point in liquid culture and that tylosin production proceeds after tylQ transcription is silenced (section 3.2.2). In addition, over-expression of tylQ in the ancestral strain resulted in loss of tylosin production [Figure 5.4(b)] via repression of tyl genes which persisted even after 40 h of growth (Figure 5.5). In contrast, when tylQ*, under control of ermEp*, was integrated into the genome of the ancestral strain (section 5.3.1), it failed to abolish tylosin production [Figure 5.4(c)] as it also had no profound effect on transcription of any of the tyl genes after 40 h of growth (Figure 5.5). Since repression of most tyl genes by TylQ is known to be achieved via the direct or indirect repression of tylR (encoding the global activator of the cluster; section 4.7.3), it was concluded that TylQ* failed to block tylosin synthesis in the ancestral strain by failing to repress tylR. The latter conclusion led to the prediction that inactivation of tylQ in the ancestral strain would lead to enhanced tylosin yields. Since insertional disruption of tylQ did not result in enhanced tylosin yields (section 4.5.3), an alternative approach was then adopted (described below).
Figure 5.4 Comparison of tylosin levels produced by the *tylQ* and *tylQ*\(^*\) over-expression strains. HPLC analysis of extracts derived from 7-day, GRF fermentations of (a) ancestral strain, (b) an ancestral-derived strain where *tylQ* is over-expressed and (c) an ancestral-derived strain where *tylQ*\(^*\) is over-expressed.
Figure 5.5  Expression analysis of the tyl gene cluster containing tylQ*. Total RNA was extracted from S. fradiae ancestral strain, tylQ over-expression (tylQ-OE) and tylQ* over-expression strains previously grown in TSB for 40 h (tylosin produced only in wt and tylQ*-OE). RT-PCR was utilized to amplify gene-specific products that were later authenticated by sequence analysis. 25 cycles were routinely used for amplification (black arrows) unless no band was visualized, in which case, the reactions were repeated at 28 cycles (gray arrows). Negative controls were performed for each case in the absence of reverse transcriptase (data not shown).
5.4 Replacement of \textit{tylQ} by \textit{tylQ*}

5.4.1 Cloning \textit{tylQ*} in the \textit{tylQ}-disrupted strain

A DNA fragment containing 222 bp of \textit{orf6*}, 400 bp of intergenic region and the whole of \textit{tylQ*} was PCR amplified from pMOMT4 (Beckmann \textit{et al.}, 1989) using the following primers: 5'- CCTGAAGCTTCTGCAGCAGCGGCGCCGACAA -3' and 5'- GGTTCATGAGCAGCGGCGGCGGACAA -3'. The fragment was ligated into pOJ260 using the restriction sites introduced by the primers (shown in bold; Figure 5.6). It was then authenticated by sequencing and introduced into the \textit{tylQ} region of the \textit{tylQ}-disrupted strain via a single cross-over. It was important that the cross-over did not occur between the point of disruption (132 bp from the proposed translational start) and the mutational change (T147A). If the latter had happened, then the mutational change would have been integrated into the disrupted chromosomal copy and, in effect, the \textit{tylQ}-disrupted strain would be complemented with \textit{tylQ} and not \textit{tylQ*} (Figure 5.7). Confirmation that this did not happen was achieved by generating DNA sequence of \textit{tylQ*} from the resultant strain.
**Figure 5.6 Cloning tylQ* in a tylQ-disrupted strain of S. fradiae.** A DNA fragment containing tylQ* with its native promoter was PCR amplified from pMOMT4 (Beckmann et al., 1989) and introduced into pOJ260 using the restriction sites introduced by the primers (section 5.4.1; shown in bold). The construct was then integrated into the tylQ region of the tylQ-disrupted strain (see also Figure 5.7).
Figure 5.7 Integration of *tylQ* into the *tylQ*-disrupted region via homologous recombination. Probable cross-over points between *tylQ* carrying its native promoter and the *tylQ* region of the *tylQ*-disrupted strain. The red box represents the mutation present in *tylQ* as opposed to the ancestral *tylQ* sequence (blue box) A. If the cross-over occurs between the disruption and mutation points, the mutation present in *tylQ* will be integrated into the disrupted *tylQ* copy. If the cross-over occurs upstream (B) or downstream (C) of both the disruption and mutation points, the *tylQ* copy will be integrated un-disrupted. PCR amplification of the integrated *tylQ* copy (primer sites are shown as small, gray arrows) and subsequent sequencing confirmed the successful replacement of *tylQ* with *tylQ*.
5.4.2 Phenotypic analysis of a doubly modified ancestral strain at the \textit{tylQ} region

As discussed previously (section 4.5.3), insertional disruption of \textit{tylQ} in the ancestral strain did not result in elevation of the tylosin titre [Figure 5.8(a), (b)] although it did cause earlier onset of tylosin production (Figure 4.12). Since it was not known whether \textit{tylQ}* still played some role in the regulation of tylosin production, we integrated \textit{tylQ}*, under control of its native promoter, at the \textit{tylQ} region of the \textit{tylQ}-disrupted strain (section 5.4.1), thus, effectively replacing TylQ with TylQ* in the ancestral strain. When the resulting strain was fermented in high tylosin-production media, it failed to raise tylosin production relative to the ancestral strain [Figure 5.8(a), (c)].
Figure 5.8 Assessment of fermentation products from strains disrupted in \( tylQ \).
HPLC analysis of material produced by 7-day, GRF fermentations of (a) ancestral strain, (b) a \( tylQ \)-disrupted strain and (c) a strain derived from the \( tylQ \) disruptant where \( tylQ^* \) is expressed by its native promoter.
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5.5. Over-expression of *tylQ* in an empirically improved strain of *S. fradiae*

5.5.1 Insertion of the *tylQ* over-expression cassette into a high tylosin producing strain

The *tylQ* gene, under control of *ermEp*+, was integrated into the *tylQ*+ region of C4 (Seno & Baltz, 1982; see also section 5.5.2) as described elsewhere (section 4.2.1). In order to confirm that the T147A change present in the chromosomal *tylQ*+ copy was not introduced into the integrated version (*ermEp*+-*tylQ*), the latter was PCR amplified from the resultant strain and authenticated by sequencing.

5.5.2 Phenotypic analysis of an empirically improved strain where the ancestral copy of *tylQ* was over-expressed

Although the improved strain (strain X from which cosmid DNA was derived) was not available, another empirically improved *S. fradiae* strain (C4) lower in the lineage than strain X was obtained. Strain C4 was generated after multiple rounds of random mutagenesis involving UV irradiation, nitrous acid and nitrosoguanidine (Seno & Baltz, 1982). When *tylQ* was PCR amplified from C4 genomic DNA and sequenced, it revealed the *tylQ*+ sequence (Figure 5.2). This finding suggested that the mutation within *tylQ* had been acquired earlier in the lineage.

When *tylQ*, under the control of the strong, constitutive promoter *ermEp*+, was integrated into the *tylQ*+ region of C4 (section 5.5.2), the resulting strain still produced tylosin although at negligible levels relative to C4 (Figure 5.9).
Figure 5.9 Fermentation products from an empirically improved strain and its derivative. HPLC analysis of extracts derived from 7-day, GRF fermentations of (a) C4 and (b) C4 with an extra copy of *tylQ* under control of the strong, constitutive promoter *ermEp*<sup>+</sup>. For the C4-derived extract, only 1/5 of the amount normally used (section 2.8.1) was assessed for the presence of tylosin. The 'C4 plus *tylQ*' extract was loaded as normal.
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5.6 Discussion

5.6.1 Modification of the tyl gene cluster during an empirical strain improvement programme

So far, little is known about genomic alterations in actinomycetes associated with enhanced antibiotic titre. In this respect, the most extensively studied organism is the penicillin producer *Penicillium chrysogenum*. An empirically improved strain of this organism was originally found to contain multiple copies of the entire penicillin biosynthetic cluster (Smith *et al.*, 1989). In the same study was shown that multiplication of a structural gene present in the cluster resulted in a 50 to 60-fold increase in its transcript level which, in turn, led to an increase in the activity of the encoded enzyme. The occurrence of cluster multiplication in other empirically improved strains that produced high levels of penicillin was reported in other studies, as it was also shown that the penicillin biosynthetic gene cluster multiplied in a tandem fashion (Fierro *et al.*, 1995). Later, it was claimed that a correlation existed between copy number of the biosynthetic cluster and penicillin titre (Newbert *et al.*, 1997). In the latter study, the improved strains under investigation had not acquired any mutations within promoter regions. Similarly, the work presented here showed that promoter regions within the tyl gene cluster remained unchanged in the high tylosin producing strain X (although probably not all *tyl* promoters were sequenced). Unlike the empirically improved *Penicillium chrysogenum* strains, there is no evidence to support the presence of gene amplification within high tylosin producing strains in *S. fradiae*. Since various tylosin non-producing mutants and disruptants were generated from strain C4 in Lilly Research Laboratories (Baltz & Seno, 1981) and this laboratory (Flint, 2000) respectively, it is highly unlikely that concurrent mutations/disruption in copies of the same gene took place in each case.

Particularly important was the finding that all *tylG* promoters were unaltered in strain X. Since tylosin production is limited by the rate of polyketide metabolism (Butler & Cundliffe, 2001), increased amounts of tylosin (produced by empirically improved strains such as C4) were probably achieved by increasing the amount of polyketide precursors rather than transcription of *tylG* genes.
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5.6.2 Inactivation of TyIQ in an empirically improved *S. fradiae* strain

As discussed in Chapter 4, TyIQ was found to act as a negative regulator of tylosin production by mainly repressing the global activator of *tyl* genes, TyIR (section 4.7.3). When DNA sequence was generated of regulatory genes present in the *tyl* gene cluster from strain X (*tylP, Q, S, T, R*), only *tylQ* had acquired a mutation that was embedded in the recognition helix of its DNA-binding motif (Figure 5.3). This change resulted in the replacement of a well conserved, acidic histidine with a neutral glutamine residue. Hypothetically speaking, the glutamine residue failed to interact with DNA or even distorted the helical structure. Whatever the reason may be, the mutated version of TyIQ (TyIQ*) failed to repress *tylR* transcription when over-expressed in the ancestral strain (Figure 5.5). Subsequent efforts to enhance tylosin yields by disrupting *tylQ* or replacing it with *tylQ* in the ancestral strain failed to elevate tylosin yields (section 5.4.2). This finding can be explained if the wild type were normally saturated with TyIR, in which case, disruption of its repressor would not have a profound effect. Another possibility is that the limiting step for expression of *tylR* is not the presence of TyIQ but the limited supply of the activator TyIS (section 4.7.4). In this context, one would expect that over-expression of *tylS* or disruption of the gene encoding its repressor, *tylP*, would result in enhanced tylosin yields in the wild type. In the latter case, disruption of *tylP* did result in elevation of the tylosin titre (section 3.7.1), while in the former case, the availability of a neutral site (in respect to tylosin production) on the *S. fradiae* chromosome is essential in order to test the hypothesis.

If indeed the mutation in TyIQ was selected only for elevated tylosin yields during empirical improvement of *S. fradiae* by Lilly Research Laboratories, it is possible that an increase in the tylosin titre was found under specific fermentation conditions or resulted in combination with other mutations that accumulated during previous rounds of mutagenesis. Alternatively, the fact that disruption of *tylQ* in the ancestral strain did not lead to improved tylosin yields could simply be due to the insertional inactivation itself. If we had the appropriate technology, site-directed mutagenesis would have been a preferable means of introducing the TyIQ* sequence in the wild type without integrating heterologous DNA into the chromosome.
5.6.3 Competition between TylQ and TylQ* in strain C4

As shown before, over-expression of *tylQ* in the ancestral strain resulted in loss of tylosin production (section 4.2.2). In contrast, introduction of the same construct into the empirically improved strain C4 did not result in abolition of tylosin synthesis [Figure 5.9(b)]. Since the chromosome of strain C4 carries *tylQ* under its native promoter (Figure 5.2), it can be assumed that the latter gene is expressed early in growth and is silenced at a later time point. Assuming that TylQ binds to DNA as a dimer, one would expect that TylQ-TylQ* heterodimers present early in growth would fail to function as repressors, thus allowing de-repression of *tylR* and subsequent synthesis of tylosin for a brief period of time. Since *tylQ*, under control of *ermEp*+, was expressed constitutively in strain C4, TylQ would eventually silence *tylR* after *tylQ* transcription had been ‘turned off’. This occurrence would prevent tylosin production reaching levels seen with strain C4 (Figure 5.9(a)].
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Chapter 6

Discussion
6.1 Regulatory genes in the *tyl* gene cluster

The tylosin biosynthetic (*tyl*) gene cluster contains an unprecedented number of regulatory genes. At least five have been identified (*tylP, Q, S, T, R;* Figure 1.16). *TylP* encodes a negative regulator of tylosin production that acts as a repressor of *tylQ* and *tylS*. This was supported by phenotypic and expression analysis of a *tylP*-disrupted and *tylP* over-expression strains coupled with promoter probing in *S. lividans* (sections 3.3, 3.4, 3.5, 3.6). *TylQ* is a repressor and *TylS* an activator of *tylR*, the latter encoding the global activator of *tyl* genes. Again, this conclusion was drawn from data produced by phenotypic and expression analysis of strains disrupted in *tylS* or *tylR* and a strain wherein *tylQ* was over-expressed (sections 4.2, 4.4, 4.6). *TylP* and *TylQ* contain helix-turn-helix (H-T-H) motifs at their respective N-termini implying that they exert their control by binding to DNA, thus blocking transcription. In support to this proposal, a recent study (involving a large number of *E. coli* proteins containing H-T-H motifs) showed that repressors tend to have their DNA-binding motif at the N-terminus while activators have it at the C-terminus (Perez-Rueda et al., 2000).

According to others (Wietzorrek & Bibb, 1997), *TylS* displays a complex DNA-binding motif that resembles the structurally resolved, DNA-binding region of OmpR (see also section 1.6.3). On the other hand, *TylR* has a unique sequence with no apparent DNA-binding domains. Therefore, there is no evidence to suggest how *TylR* exerts its control on the expression of *tyl* genes.

This study has pointed to several candidate protein-DNA interactions that can be readily tested by DNA-binding assays. Nevertheless, genetic engineering coupled with expression analysis was shown to be a powerful method for studying the regulation of tylosin production since it revealed aspects of the model that would not have been discovered by DNA-binding assays alone.

6.2 A proposed model for the regulation of tylosin production in *S. fradiae*

From all known regulatory genes present in the *tyl* gene cluster, *TylP* is involved early in the pathway since one would expect to find pleiotropic regulators acting earlier than pathway-specific ones. Thus, *TylP* controls both morphological differentiation and
secondary metabolism. Data produced by expression analysis, promoter probing and database comparisons suggest that, early in fermentation, \textit{tylP} is autoregulated until critical levels of \(\gamma\)-butyrolactone(s) cause de-repression by binding to the TylP protein and releasing it from the BARE-like sequence of the \textit{tylP} promoter. This leads to synthesis of more TylP (relative to \(\gamma\)-butyrolactone) that partially represses \textit{tylQ} and \textit{tylS} (Figure 6.1). Since TylP has a higher affinity for \textit{tylQp} than \textit{tylSp} (section 3.5), TylS levels will be higher than those of TylQ. As a result, TylS successfully competes with TylQ for \textit{tylRp} and the latter is partially activated. Since \textit{tylR} and \textit{tylQ} transcripts were not concurrently transcribed in any of the strains subjected to expression analysis, TylR may be the protein (Protein X) responsible for silencing \textit{tylQ}. In that context, partial activation of \textit{tylR} by TylS leads to complete repression of \textit{tylQ} by TylR. Consequently, TylS freely activates the expression of \textit{tylR} and this, in turn, leads to the full expression of \textit{tyl} structural genes. At the same time, TylS is also needed for maximal expression of \textit{tyl}III-\textit{GV} and \textit{tyl}III. Presumably, TylP still down-regulates \textit{tylS} (thus acting as a tuning mechanism) although gradual accumulation of \(\gamma\)-butyrolactone during growth may lead to complete de-repression of \textit{tylS} from TylP. One would also envisage the possibility that \textit{tylQ} may be de-repressed at later stages in growth, if \(\gamma\)-butyrolactone production comes to a halt and, again, TylP 'shuts down' \textit{tylP}. 
Figure 6.1 Regulation of tylosin production in *S. fradiae*. The regulatory pathway leading to tylosin production based on work presented in this thesis and elsewhere (Bate *et al.*, 1999, Bate *et al.*, 2002). Accumulation of γ-butyrolactone leads to de-repression of *tylP* and subsequent repression of *TylQ* (with the help of protein X; *TylR?*) and partial repression of *TylS*. *TylS* then activates *tylR* and *TylR*, in turn, is essential for the expression of all *tyl* structural genes leading to tylosin production. *TylS* may also contribute to maximal expression of *tylGIII-V* and *tylMIII* genes.
6.3 Enhancement of tylosin yields by genetic engineering

The proposed model predicts that disruption of *tylP* and/or *tylQ* will lead to elevation of tylosin yields in wild type. In the former case, disruption of *tylP* led to enhancement of tylosin production (section 3.7.1). Conversely, disruption of *tylQ* did not have the same effect (section 4.5). Nevertheless, one would expect to improve tylosin yields by over-expressing genes encoding positive regulators acting downstream of TylP and TylQ in the pathway (i.e. TylS and/or TylR; Figure 6.1). In order to test the hypothesis, a vector that integrates into a neutral site (in respect to tylosin production) on the *S. fradiae* chromosome should be constructed. In fact, a region from the *S. fradiae* genome has recently been cloned, which fits this requirement (N. Bate, personal communication), and will presumably be used to insert over-expression cassettes via homologous recombination.

6.4 ‘Filling in the gaps’

RT-PCR is a powerful tool utilized for expression analysis of the *tyl* gene cluster in wild type and genetically engineered strains. Time permitting, one would detect all *tyl* gene transcripts from multiple fermentation time points. Nevertheless, this primary screening revealed regulatory mechanisms that can be further tested. However, a number of issues regarding the regulation of tylosin production still need to be addressed. For example, the role of genes with unique sequences and unassigned functions (*orf12*, *orf1a*, *orf9*) needs to be investigated, primarily, by gene disruption and, secondly, by gene expression analysis. In addition, expression analysis of *tylGII* in wild type and in other genetically engineered strains will help elucidate the expression and regulatory patterns of PKS genes.

The role of *tylT* in the regulation of tylosin production is another issue that needs to be resolved. As shown elsewhere (Bate et al., 2002), targeted disruption of *tylT* led to a mild reduction in tylosin yields but, unlike targeted disruption of *tylS*, tylosin synthesis was not abolished. This finding suggested that a more focused, highly quantitative approach is needed in order to reveal less obvious effects that are, most likely, mediated by TylT.
Another powerful method useful in testing hypotheses that arose from this work is the generation of strains disrupted in more than one gene. For example, it would be interesting to determine tylosin yields in a strain disrupted both in *tylP* and *tylQ* or address the role of multiple genes that are not essential for tylosin production (by themselves) but do exert a collaborative regulatory effect essential for tylosin production.

Perhaps the most intriguing aspect of tylosin production is induction of tylactone synthesis by glycosylated precursors (section 1.4.4). In a model presented elsewhere (Flint, 2000), trickle expression of genes involved in polyketide metabolism as well as synthesis and addition of the first sugar occurs at early stages in growth (perhaps due to partial expression of *tylR*; see section 6.2). This leads to the synthesis of a critical amount of OMT (section 1.2.2) that stimulates polyketide metabolism. It is important to find out whether glycosylated intermediates (such as OMT) stimulate tylactone synthesis by elevating the expression of *tylG* genes. This hypothesis can be tested in a strain that fails to produce glycosylated precursors (e.g. *tylG* or *tylM* mutants/disruptants) by adding OMT and analyzing the expression pattern of the *tyl* gene cluster shortly afterwards. If the stimulatory effect of glycosylated intermediates is indeed exerted on the transcriptional level, then it should also be determined whether this is achieved by activation or relieved repression. To answer this, one should perform DNA-binding assays using regulatory proteins and promoter DNA in the presence and absence of glycosylated precursors.

At this stage, it is useful to determine the transcriptional starts for some of the *tyl* genes in question. This will confirm that *tylG* and/or *tylM* genes are independently transcribed and answer a similar question for other sub-cluster regions such as *tyll, B, A*. It will also help in locating transcript starts in respect to protein binding sites, thus substantiating the role of regulatory proteins.

### 6.5 *S. fradiae* as a model organism for studying the regulation of industrially important antibiotics

Tylosin production in *S. fradiae* is regulated by an intriguing mechanism that other *Streptomyces* species may also employ in order to control synthesis of secondary metabolites. This is the first example of an industrially important antibiotic that has been subjected to extended gene-disruption and expression analysis. The discovery of other
biosynthetic gene clusters resembling the \textit{tyl} gene cluster ensures that the latter is going to serve as a model for the regulation of antibiotic production in studies to follow.
References


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