The tylosin biosynthetic cluster from Streptomyces fradiae: genetic organization of the left region

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The genetic organization of the left edge (tylEDHFJ region) of the tylosin biosynthetic gene cluster from Streptomyces fradiae has been determined. Sequence analysis of a 12.9 kb region has revealed the presence of 11 ORFs, 10 of them belonging to the biosynthetic cluster. The putative functions of the proteins encoded by these genes are as follows: peptidase (ORF1, ddcA), tylosin resistance determinant (ORF2, tlrB), glycosyltransferase (ORF3, tylN), methyltransferase (ORF4, tylE), ketoreductase (ORF5, tylD), ferredoxin (ORF6, tylH2), cytochrome P450 (ORF7, tylH1), methyltransferase (ORF8, tylF), epimerase (ORF9, tylJ), acyl-CoA oxidase (ORF10, tylP) and receptor of regulatory factors (ORF11, tylQ). The functional identification of the genes in the proposed tylosin biosynthetic pathway has been deduced by database searches and previous genetic complementation studies performed with tylosin idiotrophic mutants blocked at various stages in tylosin biosynthesis. The tlrB gene has been shown to be useful as a tylosin resistance marker in Streptomyces lividans, Streptomyces parvulus and Streptomyces coelicolor and the effect of tylF on macrocin depletion has been confirmed. A pathway for the biosynthesis of 6-deoxy-D-allose, the unmethylated mycinose precursor, involving the genes tylD, tylJ and tylN is proposed.

Keywords: glycosyltransferase, ketoreductase, cytochrome P450, methyltransferase, mycinose

INTRODUCTION

Tylosin is a macrolide antibiotic used in veterinary medicine to treat infections caused by Gram-positive bacteria and as an animal growth promoter in the swine industry. It is produced by several Streptomyces species including S. fradiae (Seno et al., 1977), S. rimosus (Pape & Brillinger, 1973) and S. hygroscopicus (Jensen et al., 1964), but S. fradiae is the micro-organism of choice for its industrial production. As with other macrolides, the antibiotic activity of tylosin is due to the inhibition of protein biosynthesis by a mechanism that involves the binding of tylosin to the ribosome, preventing the formation of the mRNA–aminoacyl–tRNA–ribosome complex (Kageyama et al., 1971).

The presence of gene clusters is a common phenomenon in antibiotic-producing micro-organisms. The genes involved in the biosynthetic pathway of antibiotics such as erythromycin, clavulanic acid, cephemycin, actinorhodin, tylosin, nogalamycin, puromycin and daunorubicin are clustered in the chromosome of different Streptomyces species (Diez et al., 1997). Tylosin biosynthesis has been extensively studied by both physiological (Fishman et al., 1987; Baltz & Seno, 1981, 1988) and genetic (Merson-Davies & Cundliffe, 1994; Gandecha et al., 1997; Cox et al., 1997) approaches. From the results of co-synthesis studies with idiotrophic mutants blocked at different stages in tylosin biosynthesis, 13 different loci (tylA to tylM) have been mapped. As a result, the most probable pathway for tylactone conversion to tylosin has been deduced (Baltz et al., 1983) (Fig. 1): the biosynthetic pathway proceeds from two acetate, one butyrate and five propionate units to the tylactone moiety to which sugar residues are attached (Baltz & Seno, 1981). The tylosin gene cluster extends over about 85 kb in the genome of S. fradiae; it
Fig. 1. Proposed biosynthetic pathway for mycinose synthesis and attachment to the tylosin molecule. A probable sequence of reactions leading to the mycinose moiety is shown. The involvement of the genes tyIA1, tyIA2, tyIJ, tyID, tyIN, tyIE and tyIF in the different steps is indicated.
is flanked by the tylosin-resistance determinants tlrB and tlrC, and can be divided into four different regions. The tylI-BA region (right end) is located between the polyketide synthase genes (tylG) and the tlrC resistance gene (Fig. 2). The nucleotide sequence of around 7 kb of this region contains five ORFs involved in early steps of the tylosin pathway: tylE encodes a cytochrome P450 responsible for macrolide ring hydroxylation at C-20; tylB is involved in the biosynthesis or addition of mycaminose; tylA1 and tylA2 encode two enzymes (TDP-glucose synthase and sTDP-glucose dehydratase) involved in the biosynthesis of TDP-4-keto-6-deoxyglucose, the biosynthetic precursor of tylosin sugars; and finally, ORF5 encodes a thiosterase (Merton-Davies & Cundliffe, 1994). Recently, the sequence of the tylLM region, located downstream of the tylG genes (Fig. 2), has been shown to include five ORFs (Gandecha et al., 1997): tlrD, encoding a tylosin-resistance determinant; ccr, encoding a crotolyl-CoA reductase that converts acetacetyl-CoA to butyryl-CoA for use as a C5 extender unit during tylactone production; tylM1 and tylM2, encoding respectively an enzyme involved in N-methylation during mycaminose biosynthesis and a glycosyltransferase which adds mycaminose to the 5-hydroxyl group of tylactone; and another ORF with an unknown function. The two remaining regions of the cluster, tylCK and tylEDHFJ, are located at the left edge (Fig. 2) and, according to complementation of mutants blocked in tylosin biosynthesis, genes involved in the last steps of the pathway should be located in this area (Baltz & Seno, 1988). Prior to our study, the nucleotide sequence of these regions remained unknown and only the tylF and tylN genes had been described. The macrinic O-methyltransferase-encoding gene (tylF), which catalyses the final step in the tylosin pathway, had been partially sequenced (Fishman et al., 1987) and more recently, the tylN gene, encoding a glycosyltransferase involved in mycinose attachment to O-mycaminosyltylactone, has been characterized (Wilson & Cundliffe, 1998). The tylC and tylK genes seemed to be involved in the biosynthesis or attachment of mycarose, whereas tylD mutants accumulated demycinosyl tylosin (lacking mycinose) because they were blocked in the biosynthesis or addition of 6-deoxy-D-allose, a precursor of mycinose. tylF and flanking DNA was also postulated to be involved in the biosynthesis of 6-deoxy-D-allose. tylE mutants accumulated demethylmacrinin and were unable to achieve the methylation of the 2-hydroxy position of the attached mycinose residue. tylH mutations resulted in accumulation of 23-deoxymycinosyl tylosin and these mutants were unable to oxidize the C-23 methyl position of lactone (Baltz & Seno, 1981).

Tylosin biosynthetic and self-resistance genes are closely linked in the genome of Streptomyces (Beckmann et al., 1989). S. fradiae has been reported to possess four tylosin resistance genes designated as tlrA, tlrB, tlrC and tlrD. tlrA (Zalacain & Cundliffe, 1991; Cundliffe et al., 1993) and tlrD (Gandecha et al., 1997) encode methyltransferases responsible for methylation of a specific residue of adenine in the 23S rRNA. The deduced amino acid sequence of tlrD shows homology to erythromycin-resistance methylases (Gandecha et al., 1997). tlrC, located at the right end of the cluster (Fig. 2), is an ATP-binding protein probably constituting a subunit of a multicomponent export system for the energy-dependent efflux of tylosin from the producing organism (Rosteck et al., 1991). The presence of the tlrB resistance gene at the left end of the biosynthetic cluster (Fig. 2) has also been reported (Birmingham et al., 1986; Birmingham & Seno, 1988); it has been suggested that it may confer the MLS (macroleide-lincosamide-streptogramin B) resistance phenotype (Fujisawa & Weisblum, 1981).

This paper describes the nucleotide sequence of the left edge of the tylosin gene cluster (Fig. 2), and discusses the putative functionality of the genes found in relation to the previously described S. fradiae idiothetic mutants. This information can be used to guide strain improvement programmes, combining random mutagenesis and molecular cloning to optimize the yield of tylosin. An industrial-scale application based on the increase of the tylF gene dosage has been performed with highly productive strains of S. fradiae that accumulate relatively high levels of macrinic. Recombinant strains expressing higher levels of macrinic O-methyltransferase showed an improvement in tylosin yield (Solenberg et al., 1996; Baltz et al., 1997).

**METHODS**

**Microbial strains, plasmids and microbiological procedures.** S. fradiae ATCC 19609 was used as source of DNA. Escherichia coli DH5α (Hanahan, 1983) and E. coli WK6 (Kramer et al., 1984) were used for subcloning and ssDNA purification respectively. pBluescript KI(+) and pBluescript II SK(+) as well as phagemids (Stratagene) were selected for routine subcloning and ssDNA preparation with the helper phage M13K07. pUL.VK99 (Chary et al., 1997) was utilized as E. coli–Streptomyces shuttle vector. Protoplast transformation of S. lividans, S. parvulus and S. coelicolor was performed according to Hopwood et al. (1985), whereas S. clavuligerus was transformed as described by Garcia-Dominguez et al. (1987). Tylosin production was tested by flask fermentation according to previously described methods (Baltz & Seno, 1981). Nucleic acid purification and manipulation were performed by standard procedures (Sambrook et al., 1990; Hopwood et al., 1985) with minor modifications. Plasmids were propagated in S. lividans ATCC 1326 prior to being introduced into S. fradiae ATCC 19609, S. parvulus DSM 40048, S. coelicolor DSM 40233 and S. clavuligerus ATCC 27064. Streptomyces transformants were selected on R5 plates (Hopwood et al., 1983) supplemented with thiostrepton (50 μg ml⁻¹), kanamycin (50 μg ml⁻¹) or tylosin (from 100 to 500 μg ml⁻¹ depending on the strain).

**DNA sequencing and Southern analysis.** Sequencing clones were constructed with the Erase-a-Base kit (Promega), converted into ssDNA by standard procedures (Sambrook et al., 1989) and sequenced by the dideoxynucleotide method using Sequenase 2.0 (Amersham). Deazanucleotides and/or high annealing temperature (42°C) were employed to eliminate compression problems. Southern blotting was carried out by

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standard procedures (Hopwood et al., 1985; Sambrook et al., 1989).

**Construction of a S. fradiae genomic library.** Total DNA from *S. fradiae* ATCC 19609 was isolated as previously described (Hopwood et al., 1985). Fragments of 17–22 kb were purified from Sau3AI partially digested DNA and ligated to GEM12 phage vector (Promega) by standard procedures (Sambrook et al., 1989). Ligation products were packaged in vitro with the Gigapack Gold kit (Stratagene), used to infect *E. coli* LE392 and plated to obtain about 5 x 10^6 p.f.u. Recombinant phases were amplified in liquid medium in order to purify their DNA (Sambrook et al., 1989).

**Tylosin- and erythromycin-resistance assays in S. lividans.** Tylosin (Tailan) was purchased from Elanco and erythromycin from Sigma. Expression of the tlrB gene conferring the tylosin-resistance phenotype was achieved using the plasmid pALF250, consisting of pULVK99 with a 1.4 kb KpnI-Sac11 fragment carrying the tlrB gene. This plasmid was introduced into *S. lividans* ATCC 1326, *S. parvulus* DSM 40048, *S. coelicolor* DSM 40233 and *S. clavuligerus* ATCC 27064. Antibiotic resistance tests of these Streptomyces species were performed in R5 medium supplemented with either 0–3500 μg tylosin ml⁻¹ or 0–200 μg erythromycin ml⁻¹.

**Computer analysis of sequences.** Sequence analyses were performed with Dnastar and Winstar packages, including the following programs: Mapseq for restriction analysis, Geneplot for ORF location, Geneman for database searching and Megalign for alignment. Alignments to determine protein similarities were performed using the CLUSTAL method. Comparisons of the deduced polypeptides were furthermore accomplished using the FASTA and T-FASTA programs against the SWISS-PROT and NBRF-PIR databases.

**RESULTS AND DISCUSSION**

**Molecular cloning of the tylosin gene cluster**

In order to isolate the tylosin gene cluster, a *S. fradiae* library was constructed and screened with the oligonucleotide 5’ GCTCGATGTAGAGATCG 3’ designed according to the nucleotide sequence of the 5’ region of the previously described *tylF* gene (Fishman et al., 1987). After three purification cycles, eight recombinant phages were characterized by restriction mapping and Southern analysis. All the phages shared common restriction fragments corresponding to the same genomic region. An 11·5 kb SacI fragment was purified and subcloned in pBluescript I KS(+) producing the plasmid pALF1A (Fig. 2). Likewise, a PvuII fragment including a portion of the phage vector DNA was purified. The genomic fragment (around 16 kb) was subcloned in pBluescript II SK(+) generating the plasmid pALF2A (Fig. 2). Using the above-mentioned oligonucleotide as a probe, the *tylF* gene was located in 5·7 kb BamHI and 4·1 kb BamHI–BglII fragments.

To construct sequencing clones, several DNA fragments of the left edge of the tylosin gene cluster were subcloned in pBluescript I KS(+) and pBC KS(+) : (I) a 5·5 kb BglII fragment generating pALF17 and pALF18; (II) a 5·7 kb BamHI fragment including the *tylF* gene and generating pALF32 and pALF33; (III) a 1·0 kb BamHI fragment generating pALF13 and pALF15; and (IV) a 2·1 kb BamHI fragment generating pALF14 and pALF20. Afterwards, pALF17 and pALF18 were digested with BamHI and SacI and the resulting fragments were subcloned in pBluescript I KS(+) to yield pALF71, pALF72, pALF73, pALF74, pALF76 and pALF77. Similarly, pALF32 was digested with BamHI and BglIII and the resulting fragments (4·1 kb and 165 bp) were subcloned in pBluescript I KS(+) generating pALF2, pALF10 and pALF21 (Fig. 2). The above-mentioned plasmids were treated with the Erase-a-Base kit, generating sequential deletions of about 300–500 bp.

**Nucleotide sequence determination of the left edge of the tylosin gene cluster**

A total of 12905 bp of DNA, spanning the *tylEDHFJ* region of the tylosin biosynthetic cluster, was sequenced. Computer analysis of the sequence using the Geneplot program revealed the presence of 11 complete ORFs, named from ORF1 (left) to ORF11 (right) (Fig. 2). All the ORFs detected showed the typical biased codon usage of *Streptomyces* genes (Bibb et al., 1984) and a mean G+C content of 71±8 mol%. Whereas ORF2, ORF8, ORF9 and ORF11 were oriented from left to right, ORF1, ORF3, ORF4, ORF5, ORF6, ORF7 and ORF10 were transcribed in the opposite direction. The close proximity of ORFs 3–7 and 8–9 suggested two potential co-transcription units: ORFs 3–7 were spaced 45, 17, 0 and 35 bp and ORFs 8–9 were 36 bp apart (Fig. 3). The presence of three bidirectional promoter regions between ORFs 1 and 2 (356 bp), ORFs 7 and 8 (263 bp) and ORFs 10 and 11 (346 bp) is proposed.

**Deduced products of the sequenced ORFs**

In order to ascertain the putative functions of the previously determined ORFs, the deduced amino acid sequences were compared to the protein databases SWISS-PROT and NBRF-PIR. In the light of previous work on the genetics of tylosin biosynthesis and database search results, a function is proposed for each ORF. The results are summarized in Table 1.

**ORF1 (ddCA).** The predicted product (398 amino acid residues, 42.6 kDa, pl 9·7) showed the highest similarity (31·4%) to an extracellular D-endopeptidase from *Bacillus cereus* with ß-lactamase activity against ampicillin and penicillin G (Asano et al., 1996). Lower similarities were found to a serine D0-peptidase from *Streptomyces R61 (27·4%) (Duez et al., 1987)* and a class 4 penicillin-binding protein (PPB) (19·4%) (Coque et al., 1993). The sequence FRIGSLTK agrees with the consensus motif FXXSXXK found in class A ß-lactamases (Table 2). This gene, named ddca, may play a role in the transpeptidation reaction of the peptide intermediate in peptidoglycan biosynthesis.

**ORF2 (tlrB).** The deduced amino acid sequence (280 residues, 30.4 kDa, pl 8.1) showed high similarity (50·4%) to the mycaminicin-resistance determinant...
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**Fig. 2.** Genetic map of the tylosin biosynthetic gene cluster. The region described in this work, including the genes *ddpA*, *tlrB*, *tylN*, *tylE*, *tylD*, *tylH2*, *tylH1*, *tylF*, *tylI*, *tylP* and *tylQ*, is shown in greater detail. The restriction map of the 12.9 kb sequenced is marked as a thick line. Plasmids used in this work are indicated as pALFs. The ORFs revealed by Geneplot analysis and the direction of transcription are shown by arrows. The regions *tyLM* and *tylBA*, and the genes *tylG* and *tlrC* (Merson-Davies & Cundliffe, 1994; Gandecha et al., 1997; Rosteck et al., 1991), are also indicated.

**Fig. 3.** Geneplot analysis revealing the ORFs in the 12905 bp sequenced region. The putative transcriptional units are indicated by arrows. Co-transcription of ORFs 3–7 and ORFs 8–9 is proposed. The numbering above the ORFs indicates spacing in bp between each pair.

encoded by the *myrA* gene from *Micromonospora griseorubida* (Fig. 4). The *myrA* gene confers both mycinamicin and tylosin resistance upon *S. lividans* (Inouye et al., 1994a). The deduced products of *myrA* and *tlrB* do not show significant similarity to other antibiotic-resistance proteins in the databases. The
Table 1. Putative products and deduced functions of the genes analysed in this work

<table>
<thead>
<tr>
<th>ORF</th>
<th>Gene</th>
<th>Product size (kDa)</th>
<th>Putative function</th>
<th>Gene with high similarity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORF1</td>
<td>ddcA</td>
<td>42.6</td>
<td>Carboxypeptidase</td>
<td>adp</td>
<td>Asano et al. (1996)</td>
</tr>
<tr>
<td>ORF2</td>
<td>tlrB</td>
<td>30.4</td>
<td>Tylosin resistance</td>
<td>myrA</td>
<td>Inouye et al. (1994a)</td>
</tr>
<tr>
<td>ORF3</td>
<td>tylN</td>
<td>46.6</td>
<td>Glycosyltransferase</td>
<td>sgt</td>
<td>Warnecke et al. (1997)</td>
</tr>
<tr>
<td>ORF4</td>
<td>tylE</td>
<td>43.2</td>
<td>Methyltransferase</td>
<td>ORFY</td>
<td>Ylihonko et al. (1996)</td>
</tr>
<tr>
<td>ORF5</td>
<td>tylD</td>
<td>36.0</td>
<td>4-Ketoreductase</td>
<td>eryBL</td>
<td>Gaiser et al. (1997)</td>
</tr>
<tr>
<td>ORF6</td>
<td>tylH2</td>
<td>8.2</td>
<td>Ferredoxin</td>
<td>soyB</td>
<td>Trower et al. (1992)</td>
</tr>
<tr>
<td>ORF7</td>
<td>tylH1</td>
<td>45.5</td>
<td>Cytochrome P450</td>
<td>sauC</td>
<td>Omer et al. (1990)</td>
</tr>
<tr>
<td>ORF8</td>
<td>tylP</td>
<td>28.6</td>
<td>Methyltransferase</td>
<td>mycF</td>
<td>Inouye et al. (1994b)</td>
</tr>
<tr>
<td>ORF9</td>
<td>tylJ</td>
<td>22.8</td>
<td>Epimerase</td>
<td>strM</td>
<td>Pissowitzki et al. (1991)</td>
</tr>
<tr>
<td>ORF10</td>
<td>tylQ</td>
<td>71.5</td>
<td>Acyl-CoA oxidase</td>
<td>aco</td>
<td>GenBank AF013216</td>
</tr>
<tr>
<td>ORF11</td>
<td>tylQ</td>
<td>24.7</td>
<td>Receptor of butyrolactones</td>
<td>farA</td>
<td>Waki et al. (1997)</td>
</tr>
</tbody>
</table>

Table 2. Conserved motifs in PBPs

Motif I corresponds to the β-lactamase active centre; motifs II and III are secondary elements present in PBPs.

<table>
<thead>
<tr>
<th>Micro-organism</th>
<th>Gene</th>
<th>Motif I</th>
<th>Motif II</th>
<th>Motif III</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptomyces fradiae</em></td>
<td>ddcA</td>
<td>91FRIGSLTK</td>
<td>188YSNT</td>
<td>351FRIGS</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>adp</td>
<td>100FRIVSTK</td>
<td>201YSNT</td>
<td>341GHSG</td>
</tr>
<tr>
<td><em>Streptomyces lividans</em></td>
<td>dd-Peptidase</td>
<td>89FRIVSVTK</td>
<td>191YSNT</td>
<td>328GHTG</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em></td>
<td>mtcY</td>
<td>88FIRGAVAI</td>
<td>188YSNT</td>
<td>328GBML</td>
</tr>
<tr>
<td><em>Nocardia lactamdurans</em></td>
<td>pbp4</td>
<td>56FQGSVAK</td>
<td>152YCTST</td>
<td>201GHDG</td>
</tr>
</tbody>
</table>

Fig. 4. Dot-plot analysis of six tylosin biosynthetic polypeptides versus their similar proteins using the program Dotplot with a window size of 30 and a percentage match of 50. The following proteins were analysed: TlrB versus MyrA from *M. griseorubida*, TylE versus ORFY product from *S. nogalater*, TylF versus MycF from *M. griseorubida*, TylH2 versus SoyB from *S. griseus*, TylJ versus StrM from *S. glaucescens* and TylQ versus FarA from *Streptomyces* spp.

The clustering of biosynthetic and resistance genes for the same antibiotic has been reported in several microorganisms (Epp et al., 1987; Distler et al., 1985;
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Richardson et al., 1987). Four tylosin-resistance genes named *tlrA* (Zalacain & Cundliffe, 1991), *tlrB* (Birmingham et al., 1986; Birmingham & Seno, 1988), *tlrC* (Rosteck et al., 1991) and *tlrD* (Gandecha et al., 1997) have been isolated and phenotypically characterized from tylosin-producing strains of *S. fradiae*. The presence of two of these resistance genes (*tlrB* and *tlrC*) in the vicinity of the tylosin cluster suggests a functional interaction among them.

**ORF3 (ty/N).** The deduced polypeptide (422 residues, 46.6 kDa, pl 8.0) displayed similarity to glycosyltransferases from different sources, including a UDP-glucose:sterol-glycosyltransferase from *Arabidopsis thaliana* (24.2%) (Warnecke et al., 1997) and a glycosyltransferase from the vancomycin producer *Amycolatopsis orientalis* (23.3%) (Solenberg et al., 1997). As occurs with the product of the *tylM* gene, located in the *tylLM* region of the tylosin cluster from *S. fradiae* (Gandecha et al., 1997), slight similarity was detected to other glycosyltransferases from antibiotic-producing *Streptomyces* spp. A recent report by Wilson & Cundliffe (1998) describes the characterization and targeted disruption of the *tylN* gene from *S. fradiae* encoding a glycosyltransferase (GenBank AJ005397). In the tylosin biosynthetic pathway proposed by Baltz et al. (1983), the synthesis of the macrolide proceeds by sequential glycosyltransfer reactions, each catalysed by a specific transferase. Previous complementation experiments (Fishman et al., 1987; Baltz & Seno, 1981) revealed that the products of the genes grouped in the region studied in the present work are involved in reactions leading to biosynthesis and/or addition of the mycinose moiety to the aglycone. According to Wilson & Cundliffe (1998), the glycosyltransferase encoded by *tylN* is responsible for the transfer of the 6-deoxy-d-allose, the unmethylated precursor of mycinose, to the tylactone ring. The ORF3 corresponds to the reported *tylN* gene, but significant differences have been found at the sequence level. (1) The ATG translation start codon is different because ORF3 includes 62 additional amino acids in the N-terminal region. Both the length and N-terminal sequence of the ORF3 deduced protein agree better with other transferases, and codon preference analysis with the Genetools program shows a clear bias in this region. (2) A frame shift between amino acids 341 and 359 (corresponding to residues 278–296 in the sequence AJ005397) is detected. In this case, the ORF3 product agrees better in this region with other transferases, and Genetools analysis shows the frame shift in the sequence AJ005397. (3) There are 12 additional single-residue disagreements at the amino acid sequence level.

**ORF4 (ty/E).** The deduced amino acid sequence (395 residues, 43.2 kDa, pl 5.5) showed 51.8% similarity to the unknown product encoded by ORF4 of the nogalamycin biosynthetic gene cluster from *Streptomyces nogalater* (Ylihonko et al., 1996) (Fig. 4) and a remote similarity to the *mdmc* gene from the midecamycin producer *Streptomyces mycarofaciens*, encoding a 4-O-methyltransferase (Hara & Hutchinson, 1992). According to previous complementation studies using idio-trophic mutants (Fishman et al., 1987; Baltz & Seno, 1981), and characterization of the activity of demethyl-macrocin-O-methyltransferase (Kreuzman et al., 1988), the *tylE* locus was proposed to be located in this group of genes and to be involved in the methylation of the 2-OH position of the attached 6-deoxy-d-allose residue. The ORF4 location would fit the phenotype of *tylE* mutants; however similarity to O-methyltransferases, including the *tylF* product (macrocin O-methyltransferase) is minimal. Furthermore, the consensus binding region common to O-methyltransferases is lacking in ORF4 and *mdmc* (Ingrosso et al., 1989). Nevertheless, Ingrosso et al. (1989) analysed a series of *S*. adenosylmethionine-dependent methyltransferases observing that, despite sequence divergence, a glycine-rich motif (VLE/DXGXXG) involved in *S*. adenosylmethionine binding was conserved. As in the products of ORFY from *S*. nogalater, *tylM1* and *tylF* from *S. fradiae*, and *mdmc* from *S. mycarofaciens*, the sequence **196**VEIIGGY**201** was found in the product of *tylE*.

**ORF5 (ty/D).** The deduced protein (336 residues, 36.0 kDa, pl 8.8) showed significant sequence identity to glucose 4,6-dehydrogases from various organisms as does the deduced product of eryBIV from *Saccharopolyspora erythraea* (Gaisser et al., 1997). Since an *eryBIV* deletion mutant synthesizes erythromycins containing a 4-keto derivative of mycarose, EryBIV is likely to be the 4-ketoreductase required for mycarose biosynthesis. In spite of the divergence present in the primary structure of sugar oxidoreductases, all members have two rigorously conserved motifs involved in cofactor binding: GXXGXXG and YXXXKXXXD/E. Motif I is located within the first 20 residues of their N-termini, resembling the Rossmann fold characteristic of nucleotide-binding sites (Wierenga & Hol, 1983). Motif II is about 100 residues downstream of motif I, and tyrosine and lysine residues are involved in cofactor binding (Bauer et al., 1992). Similar motifs are also present in NDP sugar 4,6-dehydrogases (Table 3). In the ORF5 product motif I was located at the N-terminal edge (163YLSKIFCE171). Likewise, in EryBIV there are 120 residues between the two motifs (Table 3). This suggests that, as eryBIV encodes a 4-ketoreductase involved in mycarose biosynthesis, ORF5 could be involved in a similar ketoreductase step for mycinose biosynthesis. Another gene encoding a TDP-glucose dehydratase, designated *tylA2*, located in the *tylBA* region of the tylosin cluster of *S. fradiae*, has also been described (Merson-Davies & Cundliffe, 1994). According to these authors, *tylA2* is responsible for the formation of TDP-4-keto-6-deoxy-d-glucose, a common intermediate in the biosynthetic pathway of the three tylosin sugars: mycinose, mycarose and mycinominose. This assignment was made according to the phenotype of *tylA* mutants, which are defective in the biosynthesis of all three deoxysugars. In contrast, *tylD* mutants are blocked only in mycinose biosynthesis. In the present work, the ORF5 encoding a putative 4-ketoreductase activity which could direct the synthesis of 6-deoxy-d-allose, the
Two rigorously conserved motifs involved in cofactor binding are shown.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Gene</th>
<th>Motif I</th>
<th>Motif II</th>
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<tr>
<td><em>Streptomyces fradiae</em></td>
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<td><em>viridochromogenes</em></td>
<td>strE</td>
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</table>

**Table 3. Conserved motifs in glucose dehydratases**

Two rigorously conserved motifs involved in cofactor binding are shown.

**Fig. 5.** Alignment of amino acid sequences of ferredoxins encoded by the following genes: *tylH2* from *S. fradiae*, *soyB* from *S. griseus*, *subB* from *S. griseolus* and *suaB* from *S. griseolus*. The alignment was performed with the program Megalign using the Clustal method and the following parameters: ktuple 1, gap penalty 3 and window 5. Three critical Cys residues, conserved in all ferredoxins and involved in iron chelating, are marked by asterisks; they are located at positions 10, 16 and 54 of the TylH2 polypeptide.

mycinose precursor, has been found in the map position of tyID locus (related to mycinose biosynthesis). We propose ORF5 to be the tyID gene involved in mycinose biosynthesis, as previously characterized by complementation studies of tyID mutants (Fishman et al., 1987) (Fig. 1).

**ORF6 (tylH2).** This gene has the highest G + C percentage (77.7 mol%) among the ORFs analysed in this work and encodes a predicted 81-residue polypeptide (molecular mass 8.2 kDa, pl 4.4). Database searches showed high similarity to [3Fe-4S]-type ferredoxins from several *Streptomyces* spp. and also to the N-terminal end of both a [4Fe-4S]-type ferredoxin from *Rhodococcus fascians* (Crespi et al., 1994) and a homologous thioesterase from *S. griseus* (Criadó et al., 1993). The closest similarity (43.1%) was found to the *soyB* gene from *S. griseus* (Trower et al., 1992) (Fig. 4), encoding a ferredoxin-like protein, suggesting this function for the ORF6 product. Additional support comes from the high degree of conservation of the three critical cysteine residues necessary for chelating iron at positions 10, 16 and 54 (Fig. 5). Ferredoxins are small acidic electron-transfer proteins that contain Fe-S clusters attached to the polypeptide via cysteine residues. Little is known about the in vivo assembly of these clusters and the role that the sequence motif plays in that process (Buschi & Guerlesquin, 1988). As in several other organisms, this gene, which we designate as *tylH2*, is located downstream of and adjacent to ORF7 (encoding a cytochrome; see below). Hence, these two genes form an oxidoreduction system, probably implicated in the C-23 ring oxidation during tylosin biosynthesis.

**ORF7 (tylH1).** The deduced protein (420 residues, 45.5 kDa, pl 5.0), showed close similarities to cytochrome P450 proteins encoded by the genes *suaC* (40.9%) from *Streptomyces griseolus* (Omer et al., 1990) and *sca-2* (39.5%) from *Streptomyces carbophilus* (Watanabe et al., 1995). In most actinomycetes, a protein with monoxygenase activity is encoded in the vicinity of an iron-sulfur redox protein (O’Keefe & Harder, 1991). The alignment of the ORF7 product with other reported cytochromes, including the CYP450 encoded by the *tyl* gene of the tylosin cluster responsible for C-23 ring oxidation of O-mycaminsosytylactone (Merson-Davis & Cundliffe, 1994), is shown in Fig. 6. The residues forming the oxygen-binding site (256AHEETK66) in helix I, including the invariant G260 and T263 residues common to all cytochrome P450 proteins, were also present. Furthermore, a very strong conservation of the residues that constitute the haem-binding domain (260FGYFHPQCLGQ272) is present. This domain is thought to be involved in the folding of the haem-binding pocket (Poulos et al., 1987). Conservation at the C260, which provides the thiolate ligand to the haem...
group, was also observed (Fig. 6). Since tylH mutants were unable to oxidize the C-23 methyl position of the lactone (Balz & Seno, 1981; Bauer et al., 1988), the cytochrome P450 and ferredoxin could be responsible for the oxidation at the C-23 methyl position of the lactone. Consequently, we propose the designation of ORF7 as tylH1. The tylH1 and tylH2 gene products strongly resemble the enzymic complex found in bio-

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**Fig. 6.** Alignment of amino acid sequences of cytochromes P450 encoded by the following genes: tylH1 from *S. fradiae*, soyC from *S. griseus*, suA from *S. griseolus*, sca2 from *S. carboxphilus*, eryF from *S. erythraea*, and tyl from *S. fradiae*. The alignment was performed as in Fig. 5. The structural features identified (secondary structures, oxygen-binding site and haem pocket) and key amino acids involved in either stabilizing the polypeptide conformation or providing hydrogen bonding to the haem and substrate interactions are from the reported crystal structure for the cytochrome P450cam from *Pseudomonas putida* (Poulos et al., 1987). Residues conserved in both the oxygen-binding site (Gly-260 and Thr-263) and the haem pocket (Phe-362, Tyr-363, Cys-366, and Gly-371) which are invariant in most P450 species are highlighted in bold. Residues marked O (Gln-189, Arg-197, Glu-262 and Ser-266) represent amino acids providing ligands for haem and substrate interactions.
degradative and biosynthetic pathways of actinomyctes (Sarialsani & Omer, 1992). However, we have not found the third component of this system, a ferredoxin reductase, in the region of the tylosin biosynthetic cluster analysed. The frequent absence of these reductases within P450-ferredoxin operons agrees with the reductase, in the region of the tylosin biosynthetic pathway and with the product of the ORF8 (tylF) gene in the 5. fradiae genome by transposon exchange, resulting in a 30% increase in tylosin yield. It has been reported and assumed that the methylation of the two hydroxyl groups at the 2''-OH and 3''-OH positions of 6-deoxyxallose is catalysed by separate enzymes with narrow substrate specificity (Seno & Balz, 1982). As we describe below, the expression of additional copies of the tylF gene in S. fradiae results in a dramatic macrocin depletion.

The predicted polypeptide (205 residues, 22.8 kDa, pl 6.7) showed similarity to many proteins with TDP-4-keto-6-deoxyxyllosu 3,5-epimerase activity. The closest similarity (50%) was found to the homologous proteins encoded by the strM genes from Streptomyces glaucescens (Fig. 4) and Streptomyces griseus (Pissoworzki et al., 1991). An epimerase is required in the biosynthetic pathway to convert TDP-glucose to mycinose and mycarose. We propose to allocate ORF9 to the tylF gene, which would code for an epimerase activity involved in mycinose biosynthesis. This result is consistent with the description of the tylF locus postulated to be involved in the biosynthesis or attachment of 6-deoxy-d-allose, accumulating demycinosyltylosin (Fishman et al., 1987; Baltz & Seno, 1988).

The glycosyltransferase activity encoded by tylN and involved in mycinose biosynthesis showed no significant similarity to the protein with same function encoded by tylM2 (Gandecha et al., 1997) and involved in the biosynthesis of mycinose. However, tylJ encoded a protein with 43.0% similarity to the product of erylBVI, which is thought to encode a 3,5-epimerase involved in the biosynthesis of the erythromycin sugar 1-mycarose (Summers et al., 1997).

The deduced product (658 residues, 71.5 kDa, pl 7.1) showed similarity to several acyl-CoA oxidases (26.6% to Myxococcus xanthus acyl-CoA oxidase), some of them located in the peroxisomes of eukaryotic organisms. These enzymes catalyse the initial step in fatty acid β-oxidation, introducing a double bond into the saturated carbon chain bound to coenzyme A. Synthesis of tylactone appears to be carried out by a mechanism similar to the long-chain fatty acid biosynthesis: condensation of simple carboxylated CoA derivatives of acetate, propionate and butyrate (Robinson, 1991). A crotonyl-CoA reductase encoded by the ccr gene, that converts acetoacetyl-CoA to butyryl-CoA, has been reported to be involved in the supply of precursors for tylactone biosynthesis (Gandecha et al., 1997). TylP could catalyse the synthesis of crotonyl-CoA from butyryl-CoA and Ccr could reduce it back. Complementary work is required to determine whether the tylP putative product performs some of these functions.

The protein deduced (224 residues, 24.7 kDa, pl 7.9) was named TylQ and showed significant similarity to several receptors of regulatory factors such as F-atom from Streptomyces sp. (Waki et al., 1997) (Fig. 4), BarA from Streptomyces virginiae (Okamoto et al., 1995) and A-factor receptor from Streptomyces griseus (Onaka et al., 1995). A-factor is a bacterial pheromone widely employed to trigger morphological development or antibiotic biosynthesis in Streptomyces, which is thought to elicit these effects through interactions with A-factor-binding proteins (Hara & Beppu, 1982; Yamada et al., 1987; Horinouchi & Beppu, 1990). Butyrolactone receptors are a sub-group of the tetR family of regulatory proteins which have a common α-helix–turn–α-helix (HTH) DNA-binding motif for switching off the expression of key genes. This HTH motif was found at the N-terminal edge of TylQ (GYEATTIAEIKRSVGTVKAGYHFTSKELQ) (44). The potential involvement of TylQ in regulation of tylosin production is proposed. To our knowledge, this is the first report of a regulatory gene within a type I polyketide biosynthetic gene cluster.

**Functional characterization of tlrB and tylF**

In order to express the tlrB gene in several Streptomyces strains, a 1.4 kb KpnI-SacII fragment including this tylosin-resistance gene was cloned in the shuttle vector pULV99 (Chary et al., 1997) generating the plasmid pALF250, which carries thioestrepton-, kanamycin- and
The mycinose biosynthetic pathway

Many antibiotics contain partially deoxygenated sugar components essential for biological activity. Since mycinose, mycarose and mycaminose are key components of the tylosin molecule, identification of the genes involved in their biosynthesis and knowledge of the enzymatic pathway leading to them are fundamental for the construction of recombinant strains overproducing this antibiotic. All three of these sugars seem to be synthesized from glucose, which is converted into TDP-glucose by TDP-glucose synthase (Grisebach, 1978), encoded by tylA1 (Merson-Davies & Cundliffe, 1994). Subsequently, TDP-glucose is converted into TDP-4-keto-6-deoxy-d-glucone, a common intermediate in the biosynthetic pathway of most deoxysugars (Liu & Thorson, 1994). This irreversible intramolecular oxidation-reduction is catalysed by an NAD'-dependent TDP-glucose dehydratase. According to Merson-Davies & Cundliffe (1994), the tylA2 gene is responsible for this enzymatic conversion in the three tylosin sugars.

The genes involved in mycinamino biosynthesis have recently been described (Gandeche et al., 1997) and only an isomerase-encoding gene remains unidentified in the route. Likewise, another pathway was proposed for the formation of mycarose, a sugar component of both tylosin and erythromycin antibiotics (Liu & Thorson, 1994; Summers et al., 1997; Gaisser et al., 1997; Salah-Bey et al., 1998). Five eryB genes (eryBVII, eryBVI, eryBIII, eryBIII and eryBVII) have been proposed to accomplish the biosynthetic pathway from TDP-4-keto-6-deoxyhexose to TDP-mycarose in S. erythraea (Gaiser et al., 1998; Salah-Bey et al., 1998). A similar pathway with homologous genes should exist in S. fradiae.

According to previous studies on complementation of blocked mutants (Fishman et al., 1987; Baltz & Seno, 1981), a portion of the DNA region described in this work includes genes involved in mycinose biosynthesis. Complementation of idiotrophic mutants blocked in the tylD and tylJ loci showed that they were related to the biosynthesis or attachment of mycinose, whereas tylE and tylF encoded enzymes responsible for methylation of the 2-hydroxy and 3-hydroxy positions of the attached 6-deoxy-d-allose residue (Baltz & Seno, 1988). We propose a putative route for the biosynthesis of mycinose (Fig. 1) where TDP-4-keto-6-deoxy-d-glucone, the product of the dehydratase encoded by tylA2, would be converted to 6-deoxy-d-allose by 3'-epimerization and subsequent reduction of the 4-keto position to an alcohol. Such an epimerase-reductase has been described in the L-fucose biosynthetic route (Chang et al., 1988). The epimerase activity would be encoded by tylJ, whereas the product of tylD, which showed similarity to conserved motifs of reductases, could catalyse the subsequent conversion to 6-deoxy-d-allose. This sugar would be attached to the C-23 OH of the tylactone ring by the glycosyltransferase encoded by tylN. In the final steps of the pathway, two methyl groups are incorporated into the 2'''-OH and 3'''-OH positions of the

Tylosin gene cluster from Streptomyces fradiae

In parallel, the minimal inhibitory concentration of tylosin was determined for S. lividans ATCC 1326 (25 μg ml⁻¹), S. parvulus DSM 40048 (25 μg ml⁻¹), S. coelicolor DSM 40233 (10 μg ml⁻¹) and S. clavuligerus ATCC 27064 (70 μg ml⁻¹). Once the selection conditions were established, pALF250 was used to transform the above-mentioned hosts by standard methods (Hopwood et al., 1985) and thiostrepton-resistant transformants were selected on R5 plates. Tylosin resistance level was subsequently checked in these transformants. S. lividans clones were able to grow at 250 μg ml⁻¹, some of them reaching a resistance level of 3500 μg ml⁻¹. The transformants of S. parvulus were able to grow at 200 μg ml⁻¹ and a few of them at 1000 μg ml⁻¹. Most of the S. coelicolor transformants resisted 10 μg ml⁻¹, some of them growing at 30 μg ml⁻¹. However, the thiostrepton-resistant transformants of S. clavuligerus were unable to grow in a tylosin range from 70 to 100 μg ml⁻¹. In addition, transformants of S. lividans and S. parvulus were directly selected on R5 plates using tylosin concentrations of 250 μg ml⁻¹ and 200 μg ml⁻¹ respectively. Therefore, the tlrB gene can be used as a transformation marker in several Streptomyces species.

The mycinamicin-resistance protein encoded by myrA from M. griseorubida showed the highest similarity to the tlrB gene product. The myrA gene is unable to confer resistance to erythromycin, and therefore cannot be classified as an MLS resistance gene (Inouye et al., 1994a). In order to analyse this feature with the tlrB gene, its ability to confer erythromycin resistance upon S. lividans and S. parvulus was checked. The erythromycin minimal inhibitory concentration was determined for S. lividans (25 μg ml⁻¹) and S. parvulus (200 μg ml⁻¹), and pALF250 transformants of both species were selected by thiostrepton resistance. All of them were unable to grow in the presence of the above-mentioned inhibitory concentrations of erythromycin. According to these data, the tlrB gene does not seem to belong to the MLS group of resistance determinants.

Furthermore, the tylosin biosynthetic genes tylD, tylH2, tylH1, tylF and tylJ were subcloned as a 5.7 kb BamHI fragment (Fig. 2) in the shuttle vector pULVK99 (Chary et al., 1997), generating the plasmid pALF287. Transformants of S. fradiae with additional copies of these genes were selected in R5 plates supplemented with thiostrepton (Hopwood et al., 1985). The effect of these genes on tylosin production was tested by flask fermentation of the transformants in tylosin-producing conditions (Baltz & Seno, 1981). Around 40 transformants were analysed; they showed significant increases of tylosin production (10–50%) and dramatic macrolin depletion (15–30-fold). These results are in agreement with those of Baltz et al. (1997). A remarkable variability in terms of tylosin production level was detected among the transformants tested, probably due to the use of a non-integrative vector instead of the stable insertion by transposon exchange described by Solenberg et al. (1996) or by insertion into a neutral site (Baltz et al., 1997).
6-deoxy-D-allose residue, to transform this compound into mycinose, yielding tylosin. The two methyltransferases would be encoded by tyfE and tyfF respectively.

The complete characterization of the biosynthetic gene cluster will provide a very important tool for the improvement of tylosin production. The development of some antibiotic-producing strains by increasing the copy number of the biosynthetic genes has been reported (Diez et al., 1997). This suggests that transforming tylosin-producing micro-organisms with these biosynthetic genes would improve tylosin productivity. Moreover, an outstanding application of the establishment of the genetic organization of the tyf cluster is the synthesis of heterospecific recombinant strains to produce novel hybrid antibiotics (Balz, 1995). Hybrid antibiotics could be constructed using genes from different clusters (actinorhodin, tetracenomycin, granaticin, etc.). With this approach, one can envision the possibility of producing large numbers of novel macroclide antibiotic structures which might be further modified chemically.

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