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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).

Abbreviations: MLS, macrolide-lincosamide-streptogramin B; PBP, penicillin-binding protein.

The GenBank accession number for the 12905 bp sequence reported in this paper is AF055922.

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, cephamycin, actinorhodin, tylosin, nogalamycin, puromycin and daunorubicin are clustered in the chromosome of different *Streptomyces* species (Díez *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 steps 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

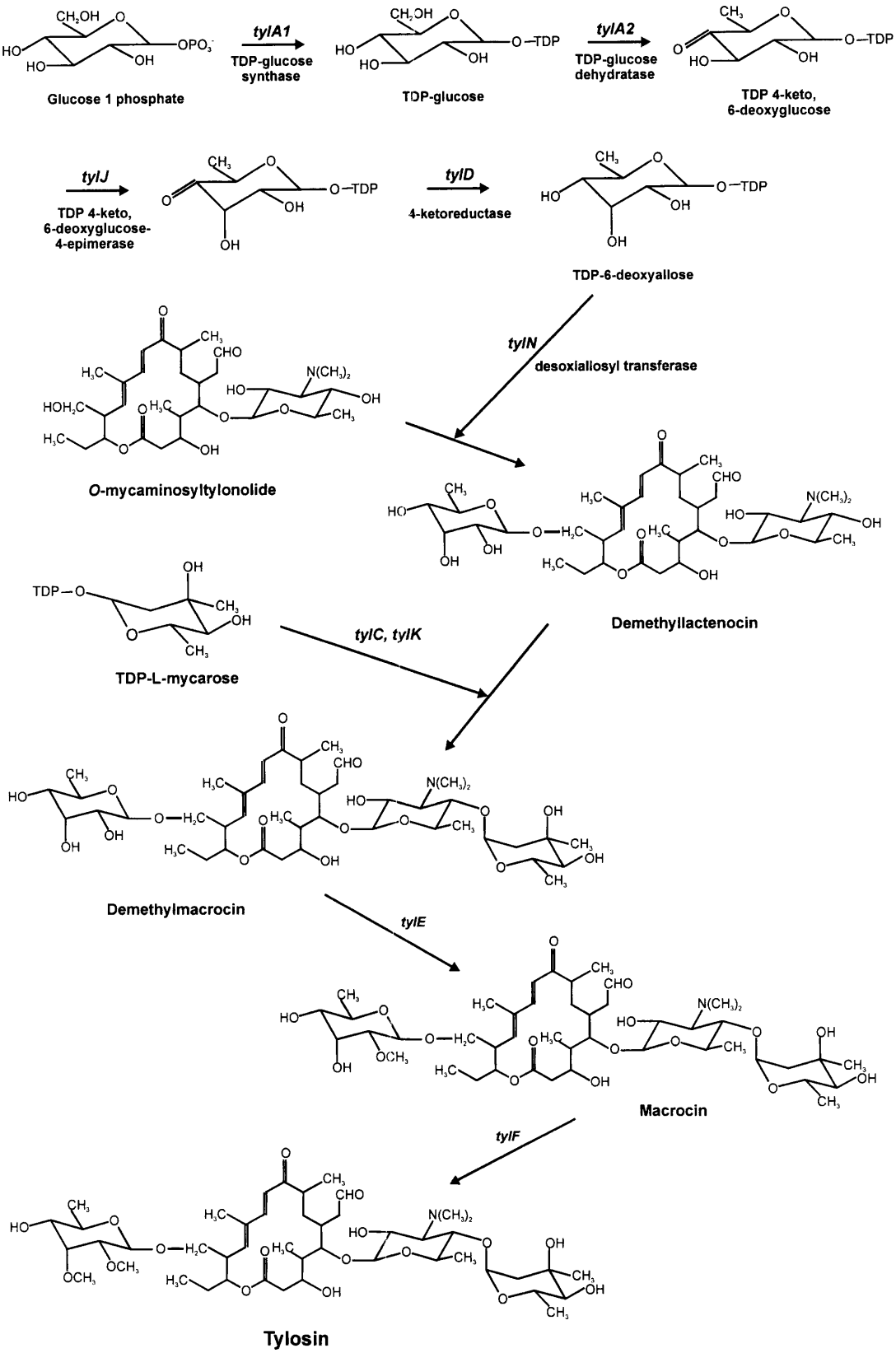

Fig. 1. Proposed biosynthetic pathway for mycinose synthesis and attachment to the tylosin molecule. A probable sequence of reactions leading to the mycinose moiety is shown. The involvement of the genes *tylA1*, *tylA2*, *tylJ*, *tylD*, *tylN*, *tylE* and *tylF* in the different steps is indicated.

is flanked by the tylosin-resistance determinants *tlrB* and *tlrC*, and can be divided into four different regions. The *tylIBA* 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: *tylI* 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 δTDP-glucose dehydratase) involved in the biosynthesis of TDP-4-keto-6-deoxyglucose, the biosynthetic precursor of tylosin sugars; and finally, ORF5 encodes a thioesterase (Merson-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 crotonyl-CoA reductase that converts acetoacetyl-CoA to butyryl-CoA for use as a C_4 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 macrocin *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*-mycaminosyltylonolide, 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. *tylJ* and flanking DNA was also postulated to be involved in the biosynthesis of 6-deoxy-D-allose. *tylE* mutants accumulated demethylmacrocin and were unable to achieve the methylation of the 2-hydroxy position of the attached mycinose residue. *tylH* mutations resulted in accumulation of 23-deoxydemycinosyl 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 (macrolide-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* idiotrophic 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 macrocin. Recombinant strains expressing higher levels of macrocin *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 I KS(+), pBluescript II SK(+) and pBC KS(+) phagemids (Stratagene) were selected for routine subcloning and ssDNA preparation with the helper phage M13K07. pULVK99 (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 García-Domínguez *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.*, 1985) 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

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 *Sau*3AI partially digested DNA and ligated to λGEM12 phage vector (Promega) by standard procedures (Sambrook *et al.*, 1989). Ligation products were packaged *in vitro* with the Gigapack II Gold kit (Stratagene), used to infect *E. coli* LE392 and plated to obtain about 5×10^4 p.f.u. Recombinant phages 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 *Kpn*I–*Sac*II 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 *Sac*I fragment was purified and subcloned in pBluescript I KS(+) producing the plasmid pALF1A (Fig. 2). Likewise, a *Pvu*II 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 *Bam*HI and 4·1 kb *Bam*HI–*Bgl*II 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 *Bgl*II fragment generating pALF17 and pALF18; (II) a 5·7 kb *Bam*HI fragment including the *tylF* gene and generating pALF32 and pALF33; (III) a 1·0 kb *Bam*HI fragment generating pALF13 and pALF15; and (IV) a 2·1 kb *Bam*HI fragment generating pALF14 and pALF20. Afterwards, pALF17 and pALF18 were digested with *Bam*HI and *Sac*I and the resulting fragments were subcloned in pBluescript I KS(+) to yield pALF71, pALF72, pALF73, pALF74, pALF76 and pALF77. Similarly, pALF32 was digested with *Bam*HI and *Bgl*II 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, pI 9·7) showed the highest similarity (31·4%) to an extracellular D-endopeptidase from *Bacillus cereus* with β-lactamase activity against ampicillin and penicillin G (Asano *et al.*, 1996). Lower similarities were found to a serine DD-peptidase from *Streptomyces* R61 (27·4%) (Duez *et al.*, 1987) and a class 4 penicillin-binding protein (PBP) (19·4%) (Coque *et al.*, 1993). The sequence ^{89}FRIGSLTK^{98} agrees with the consensus motif FXXXSXXK found in class A β-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, pI 8·1) showed high similarity (50·4%) to the mycinamicin-resistance determinant

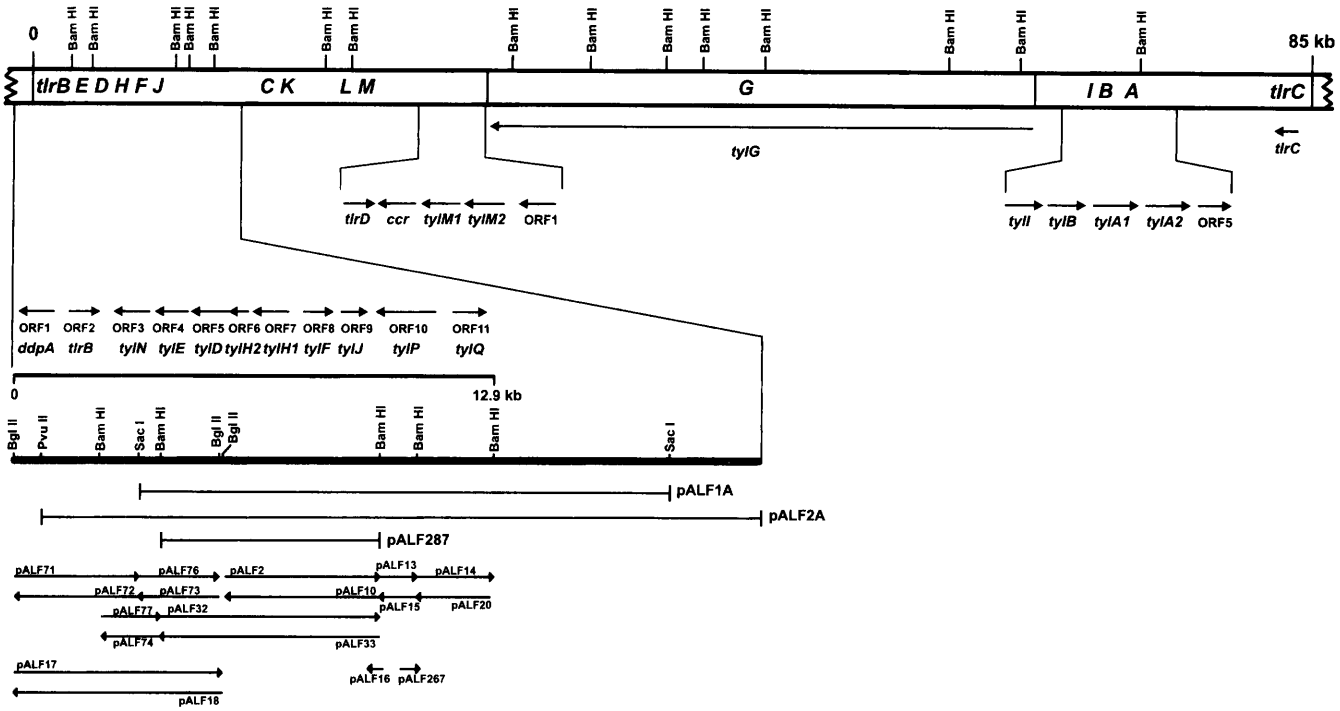

Fig. 2. Genetic map of the tylosin biosynthetic gene cluster. The region described in this work, including the genes *ddpA*, *tlrB*, *tylN*, *tylE*, *tylD*, *tylH2*, *tylH1*, *tylF*, *tylJ*, *tylP* and *tylQ*, is shown in greater detail. The restriction map of the 12·9 kb sequenced is marked as a thick 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 *tylLM* and *tylIBA*, and the genes *tylG* and *tlrC* (Merson-Davies & Cundliffe, 1994; Gandecha *et al.*, 1997; Rosteck *et al.*, 1991), are also indicated.

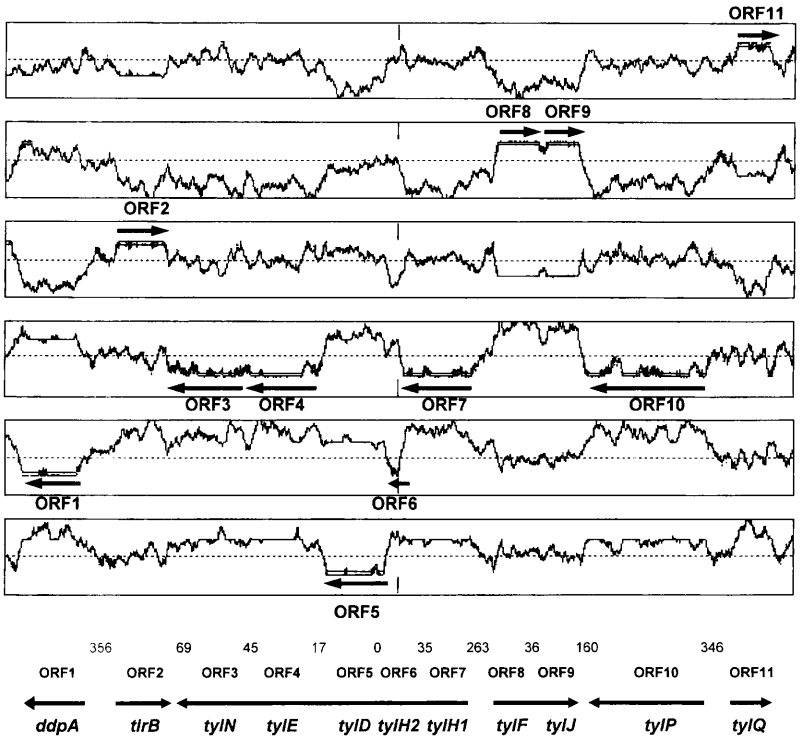

Fig. 3. Geneplot analysis revealing the ORFs in the 12905 bp sequenced region. The putative transcriptional units are indicated by arrows. Co-transcription of ORFs 3–7 and ORFs 8–9 is proposed. The numbering above the ORFs indicates spacing in bp between each pair.

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

ORF	Gene	Product size (kDa)	Putative function	Gene with high similarity	Reference
ORF1	*ddcA*	42·6	Carboxypeptidase	*adp*	Asano *et al.* (1996)
ORF2	*tlrB*	30·4	Tylosin resistance determinant	*myrA*	Inouye *et al.* (1994a)
ORF3	*tylN*	46·6	Glycosyltransferase	*sgt*	Warnecke *et al.* (1997)
ORF4	*tylE*	43·2	Methyltransferase	ORFY	Ylihonko *et al.* (1996)
ORF5	*tylD*	36·0	4-Ketoreductase	*eryBIV*	Gaisser *et al.* (1997)
ORF6	*tylH2*	8·2	Ferredoxin	*soyB*	Trower *et al.* (1992)
ORF7	*tylH1*	45·5	Cytochrome P450	*suaC*	Omer *et al.* (1990)
ORF8	*tylF*	28·6	Methyltransferase	*mycF*	Inouye *et al.* (1994b)
ORF9	*tylJ*	22·8	Epimerase	*strM*	Pissowotzki *et al.* (1991)
ORF10	*tylP*	71·5	Acyl-CoA oxidase	*aco*	GenBank AF013216
ORF11	*tylQ*	24·7	Receptor of butyrolactones	*farA*	Waki *et al.* (1997)

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.

Micro-organism	Gene	Motif I	Motif II	Motif III
Streptomyces fradiae	*ddcA*	^{91}**FRIGS**LT**K**^{98}	^{198}**YSNT**^{201}	^{351}F**HGG**^{354}
Bacillus cereus	*adp*	^{100}**FRIGSV**T**K**^{107}	^{201}**YSNT**^{204}	^{341}**GHGG**^{344}
Streptomyces lividans	DD-Peptidase	^{89}**FRVGSV**T**K**^{96}	^{191}**YSNT**^{193}	^{328}**GHTG**^{331}
Mycobacterium tuberculosis	*mtcY*	^{88}**FRNGAV**AI^{95}	^{188}YAHT^{191}	^{339}**GHWL**^{341}
Nocardia lactamdurans	*pbp4*	^{56}**FQSGSV**A**K**^{63}	^{152}YCST^{155}	^{301}**GHDG**^{304}

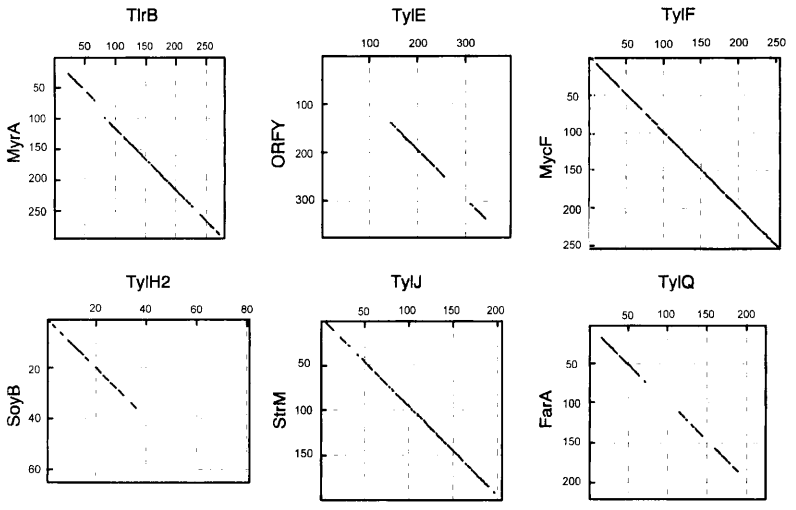

Fig. 4. Dot-plot analysis of six tylosin biosynthetic polypeptides versus their similar proteins using the program Dotplot with a window size of 30 and a percentage match of 50. The following proteins were analysed: TlrB versus MyrA from *M. griseorubida*, TylE versus ORFY product from *S. nogalater*,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

myrA gene does not belong to the MLS resistance phenotype family since it is unable to confer resistance to erythromycin (Inouye *et al.*, 1994a), and, as will be discussed later, the *tlrB* gene behaved in a similar way when it was expressed in *S. lividans* and *S. parvulus*. The plasmid pALF250 carrying the *tlrB* gene was able to confer tylosin resistance upon *S. lividans*, *S. parvulus* and *S. coelicolor*, whereas no resistance could be detected when the same plasmid was introduced into *S. clavuligerus*. The *tlrB* gene has proved to be a useful resistance marker for transformation experiments in several *Streptomyces* spp.

The clustering of biosynthetic and resistance genes for the same antibiotic has been reported in several micro-organisms (Epp *et al.*, 1987; Distler *et al.*, 1985;

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 (*tylN*). The deduced polypeptide (422 residues, 46·6 kDa, pI 8·0) displayed similarity to glycosyltransferases from different sources, including a UDP-glucose sterol-glucosyltransferase 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 *tylM2* 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 Geneplot 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 Geneplot analysis shows the frame shift in the sequence AJ005397. (3) There are 12 additional single-residue disagreements at the amino acid sequence level.

ORF4 (*tylE*). The deduced amino acid sequence (395 residues, 43·2 kDa, pI 5·5) showed 51·8% similarity to the unknown product encoded by ORFY 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 idiotrophic mutants (Fishman *et al.*, 1987; Baltz & Seno, 1981), and characterization of the activity of demethylmacrocin-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/DXGXGXG) 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}VLEIGIGGY^{204} was found in the product of *tylE*.

ORF5 (*tylD*). The deduced protein (336 residues, 36·0 kDa, pI 8·8) showed significant sequence identity to glucose 4,6-dehydratases 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-dehydratases (Table 3). In the ORF5 product motif I was located at the N-terminal edge (^{16}GALGFIG^{22}) and motif II was located 141 residues downstream (^{163}YVLSKIFCE^{171}). 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 *tylIBA* 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 mycaminose. 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

Table 3. Conserved motifs in glucose dehydratases

Two rigorously conserved motifs involved in cofactor binding are shown.

Organism	Gene	Motif I	Motif II
Streptomyces fradiae	*tylD*	^{16}GAIGFIG^{22}	^{163}YVLSKIFCE^{171}
Streptomyces fradiae	*tylA2*	^{7}GGAGFIG^{13}	^{151}YAATKAASD^{159}
Arabidopsis thaliana	*d18*	^{125}GGAGFVG^{131}	^{261}YDEGKRTAE^{269}
Arabidopsis thaliana	*T30b22.4*	^{127}GGAGFVG^{133}	^{258}YDEGKRTAE^{266}
Methanobacterium thermoautotrophicum	*mth380*	^{10}GGLGFIG^{16}	^{145}YAVSKVTGE^{153}
Synechocystis sp.	rfbB	^{26}GGAGFIG^{32}	^{162}YDEGKRVAE^{170}
Synechocystis sp.	*ORFslr0583*	^{7}GGTGFLG^{13}	^{130}YAMTKRMLY^{138}
Saccharopolyspora erythraea	*eryBIV*	^{16}GASGFVG^{22}	^{142}YAQQKTEAE^{150}
Streptomyces violaceoruber	*graE*	^{7}GAAGFIG^{13}	^{149}YAASKASTT^{157}
Streptomyces viridochromogenes	*aviE*	^{18}GGAGFIG^{24}	^{156}YAASKAGGD^{164}
Streptomyces griseus	*strE*	^{9}GAAGFIG^{15}	^{150}YSASKASGD^{158}

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 *tylD* locus (related to mycinose biosynthesis). We propose ORF5 to be the *tylD* gene involved in mycinose biosynthesis, as previously characterized by complementation studies of *tylD* 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, pI 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* (Criado *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 (Bruschi & Guerlesquin, 1988). As in several other organisms, this gene, which we designated 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, pI 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 monooxygenase 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 *tylI* gene of the tylosin cluster responsible for C-20 ring oxidation of O-mycaminosyltylactone (Merson-Davis & Cundliffe, 1994), is shown in Fig. 6. The residues forming the oxygen-binding site (^{259}AGHETT^{264}) in helix I, including the invariant G^{260} and T^{263} residues common to all cytochrome P450 proteins, were also present. Furthermore, a very strong conservation of the residues that constitute the haem-binding domain ^{362}FGYGPHQCLGQ^{372} is present. This domain is thought to be involved in the folding of the haem-binding pocket (Poulos *et al.*, 1987). Conservation at the C^{369}, which provides the thiolate ligand to the haem

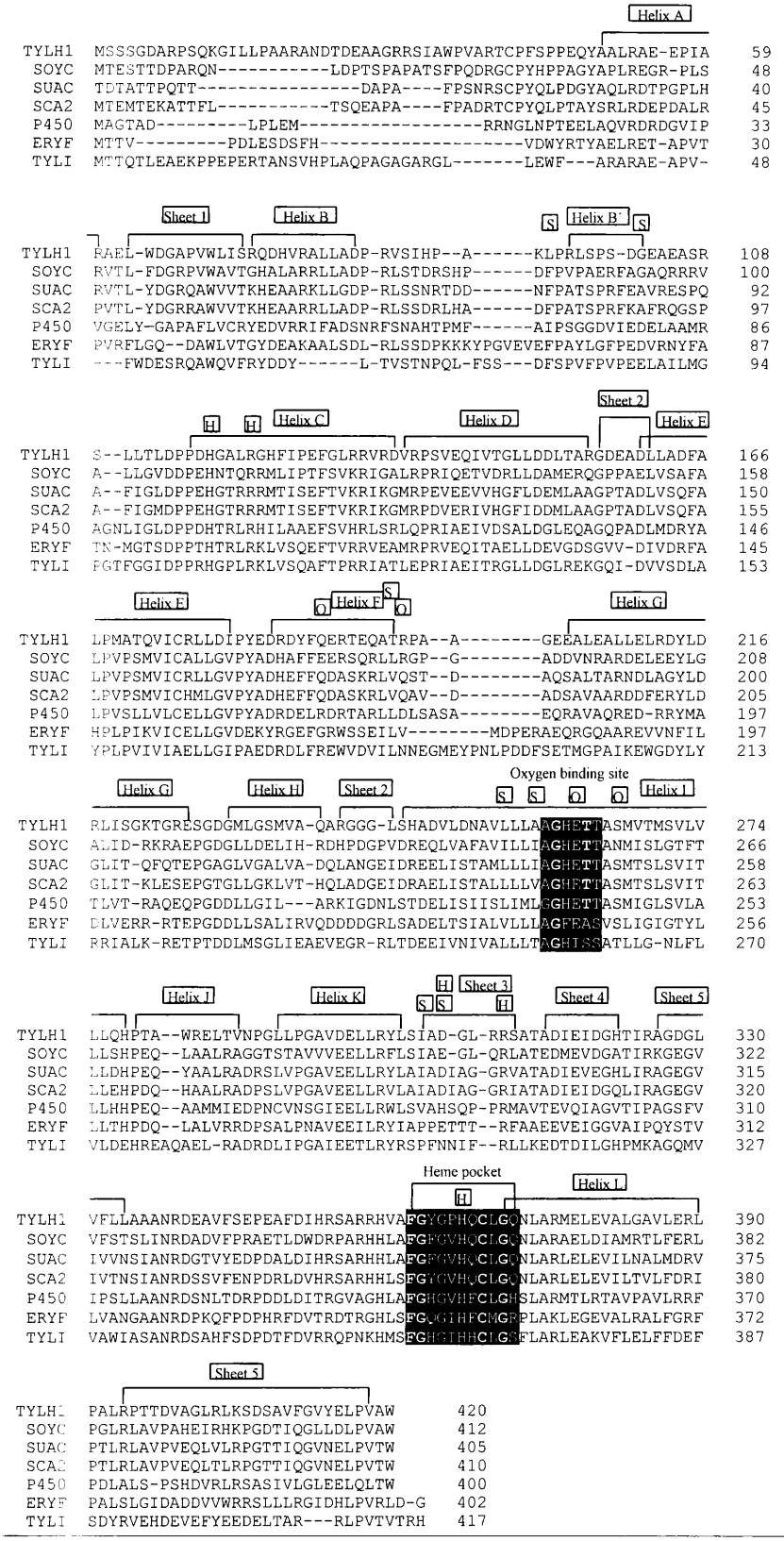

Fig. 6. Alignment of amino acid sequences of cytochromes P450 encoded by the following genes: *tylH1* from *S. fradiae*, *soyC* from *S. griseus*, *suaC* from *S. griseolus*, *sca2* from *S. carbophilus*, P450 from *Rhodococcus fascians*, *eryF* from *S. erythraea*, and *tylI* from *S. fradiae*. The 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 ligands for 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, Gly-363, Cys-369, 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 to stabilize the oxygen-binding site. Residues marked H (His-118, Arg-122, Asp-307, Arg-311 and His-367) represent amino acids providing hydrogen bonding to the haem propionate groups. Residues marked S (Leu-94, Gly-102, Thr-196, Leu-255, Ala-258, Ile-305 and Asp-307) represent amino acids having extensive protein–substrate contacts.

group, was also observed (Fig. 6). Since *tylH* mutants were unable to oxidize the C-23 methyl position of the lactone (Baltz & 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-

degradative and biosynthetic pathways of actinomycetes (Sariaslani & 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 data presented by O'Keefe & Harder (1991).

ORF8 (*tylF*). The product of this ORF (255 residues, 28·6 kDa, pI 4·6) includes the partial 38 amino acid sequence of the N-terminal edge of the macrocin O-methyltransferase encoded by *tylF* (GenBank accession number J03008) (Fishman *et al.*, 1987). It also showed similarity to the product of the *mycF* gene from *Micromonospora griseorubida* (Fig. 4), encoding the enzyme mycinamicin III O-methyltransferase, involved in the biosynthesis of the macrolide antibiotic mycinamicin (Inouye *et al.*, 1994b). These data confirmed that ORF8 corresponds to the *tylF* gene encoding macrocin O-methyltransferase. Curiously, the similarity to the methyltransferase reported to be encoded by the *tylM1* gene of the tylosin cluster is not significant (Gandecha *et al.*, 1997). The function of the *tylF* product, which catalyses the conversion of macrocin into tylosin, was previously analysed in cell-free extracts from a tylosin-producing strain of *S. fradiae* and its mutants (Seno & Baltz, 1981). This enzymic conversion is known to be the major bottleneck in tylosin biosynthesis due to the large amounts of macrocin accumulated in fermentation broths. The *tylF* gene has been introduced into a tylosin-producing strain of *S. fradiae* on a self-replicating plasmid; conversion of macrocin to tylosin was more efficient, but the overall production of tylosin together with its immediate precursors was reduced (Cox & Seno, 1990). However, Solenberg *et al.* (1996) described the insertion of a second copy of the *tylF* gene into the *S. 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-deoxyallose is catalysed by separate enzymes with narrow substrate specificity (Seno & Baltz, 1982). As we describe below, the expression of additional copies of the *tylF* gene in *S. fradiae* results in a dramatic macrocin depletion.

ORF9 (*tylJ*). The predicted polypeptide (205 residues, 22·8 kDa, pI 6·7) showed similarity to many proteins with TDP-4-keto-6-deoxyglucose 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* (Pissowotzki *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 *tylJ* gene, which would code for an epimerase activity involved in mycinose biosynthesis. This result is consistent with the description of the *tylJ* 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 mycaminose. However, *tylJ* encoded a protein with 43·0% similarity to the product of *eryBVII*, which is thought to encode a 3,5-epimerase involved in the biosynthesis of the erythromycin sugar L-mycarose (Summers *et al.*, 1997).

ORF10 (*tylP*). The deduced product (658 residues, 71·5 kDa, pI 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.

ORF11 (*tylQ*). The protein deduced (224 residues, 24·7 kDa, pI 7·9) was named TylQ and showed significant similarity to several receptors of regulatory factors such as FarA 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 subgroup 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 (^{25}GYEATTIAEILKRSGVTKGALYFH-FTSKELQ^{54}). 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 *Kpn*I-*Sac*II fragment including this tylosin-resistance gene was cloned in the shuttle vector pULVK99 (Chary *et al.*, 1997) generating the plasmid pALF250, which carries thiostrepton-, kanamycin- and

tylosin-resistance markers. 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 *Bam*HI 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 macrocin 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).

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 enzymic 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-glucose, 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 enzymic conversion in the three tylosin sugars.

The genes involved in mycaminose biosynthesis have recently been described (Gandecha *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, 1998; Salah-Bey *et al.*, 1998). Five *eryB* genes (*eryBVII*, *eryBVI*, *eryBII*, *eryBIII* and *eryBIV*) have been proposed to accomplish the biosynthetic pathway from TDP-4-keto-6-deoxyhexose to TDP-mycarose in *S. erythraea* (Gaisser *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-glucose, 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

6-deoxy-D-allose residue, to transform this compound into mycinose, yielding tylosin. The two methyltransferases would be encoded by *tylE* and *tylF* 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 (Díez *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 *tyl* cluster is the synthesis of heterospecific recombinant strains to produce novel hybrid antibiotics (Baltz, 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 macrolide antibiotic structures which might be further modified chemically.

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