**Journal of Industrial Microbiology & Biotechnology (1999) 23**, 118–122

http://www.stockton-press.co.uk/jim

# The mycinose-biosynthetic genes of *Streptomyces fradiae*, producer of tylosin

N Bate and E Cundliffe

*Department of Biochemistry, University of Leicester, Leicester LE1 7RH, UK*

**The *tylE-J* region of the tylosin-biosynthetic gene cluster of *Streptomyces fradiae* contains six open reading frames. The products of *tylJ* and *tylD* are nucleoside diphospho (NDP)-deoxyhexose 3-epimerase and NDP-deoxyhexose 4-ketoreductase, respectively, involved in the synthesis of NDP-6-deoxyallose from NDP-4-keto, 6-deoxyglucose. After incorporation of deoxyallose at C23-OH of the polyketide lactone, tylosin biosynthesis is completed by the products of *tylE* and *tylF*, which convert the deoxyallosyl moiety to mycinose via bis-*O*-methylation at 2‴-OH and 3‴-OH, respectively. Hydroxylation of the polyketide lactone at C23 is catalysed by the cytochrome P450 enzyme, TylHI. The product of *tylHII* is a ferredoxin of unknown specificity that could conceivably act together with TylHI.**

**Keywords:** *Streptomyces fradiae*; tylosin production; mycinose; antibiotic-biosynthetic genes

## Introduction

The tylosin-biosynthetic gene cluster, as presently defined, occupies a contiguous segment (about 85 kb) of the *Streptomyces fradiae* genome, and is flanked by the resistance genes *tlrB* and *tlrC* [3,7]. Co-synthesis and bioconversion studies, with mutants of *S. fradiae* blocked in tylosin production, revealed the likely biosynthetic route to tylosin [2,4] and, together with complementation analysis using cloned DNA fragments [7,8], allowed the mapping of 13 genetic loci (*tylA–M*) within the *S. fradiae* genome (Figure 1). Thus, the TylG polyketide synthase produces tylactone (Figure 2), to which three 6-deoxyhexose sugars (d-mycaminose, 6-deoxy-d-allose and l-mycarose) are subsequently added. In the final two steps of tylosin biosynthesis, the deoxyallose moiety is converted to d-mycinose via bis-*O*-methylation. Synthesis or addition of all three sugars is blocked in *tylA* and *tylL* mutants, whereas the defects in other strains are more selective. Thus, *tylB* and *tylM* mutants are blocked in the synthesis or addition of mycaminose, *tylC* and *tylK* mutants are defective in mycarose metabolism, whereas *tylD* and *tylJ* strains cannot produce deoxyallose. More specifically, *tylI* and *tylH* strains do not hydroxylate the polyketide lactone at C20 and C23, respectively, whereas *tylE* and *tylF* mutants respectively lack the penultimate and terminal *O*-methylation activities [2].

The *tyl* gene cluster of *S. fradiae* was first addressed via reverse genetics based on the sequence of macrocin *O*-methyltransferase (MOMT), the enzyme that catalyses the terminal step in tylosin biosynthesis. Deoxyoligonucleotide probes designed from knowledge of the *N*-terminal sequence of MOMT found hybridisation targets in an *Streptomyces fradiae* genomic library [8] and a 2.3-kb fragment of the target DNA expressed MOMT activity when cloned in *Streptomyces lividans*. Moreover, that same DNA fragment also restored tylosin production when introduced into a *tylF* mutant of *S. fradiae* that normally accumulated macrocin (3‴-*O*-demethyl-tylosin). The purified MOMT protein introduced a single methyl group into macrocin using *S*-adenosyl-methionine (SAM) as co-substrate, but did not act on demethylmacrocin [6]. The latter compound (2‴, 3‴-*O*-demethyltylosin) accumulated in *tylE* mutants, extracts of which still contained MOMT activity, suggesting that two separate *O*-methyltransferases (the products of *tylE* and *tylF*) must catalyse the final two steps in tylosin production [24]. Consistent with this proposal, a SAM-dependent 2‴ *O*-methyltransferase was subsequently purified from *S. fradiae* and shown to convert demethylmacrocin to macrocin [16].

Sequencing of *tyl* DNA began at Lilly Research Laboratories, Indianapolis, in the late 1980s although the data have not all been released. Both ends of the *tyl* cluster were sequenced ([23]; P Szoke and PR Rosteck Jr, personal communication; BS DeHoff and PR Rosteck Jr, personal communication) and also the *tylG* region (GenBank accession number U78289). More recently, the rest of the *tyl* cluster has been sequenced in this laboratory ([5,11,12,17,30,31]; N Bate *et al*, submitted) and the functions of specific gene products have been identified by a combination of database comparisons, targeted gene disruptions, studies with purified gene products, and complementation of *tyl* mutants. In the present work, we have analysed the genes involved in deoxyallose/mycinose biosynthesis.

## Materials and methods

The *S. fradiae tyl* DNA sequenced here was obtained from plasmid pHJL315 [8] as a 5.7-kb *Bam*HI fragment (Figure 1). This was subcloned in plJ2925 [14] and sequenced directly as double-stranded DNA, by primer walking, using *Taq* FS polymerase and dye terminator chemistry (Perkin Elmer, Warrington, UK) on an ABI 377 automated sequencer. Both strands of the DNA were sequenced independently in overlapping fashion. At the left-hand end in

Correspondence: E Cundliffe, Department of Biochemistry, University of Leicester, Leicester LE1 7RH, UK
Received 17 March 1999; accepted 20 June 1999

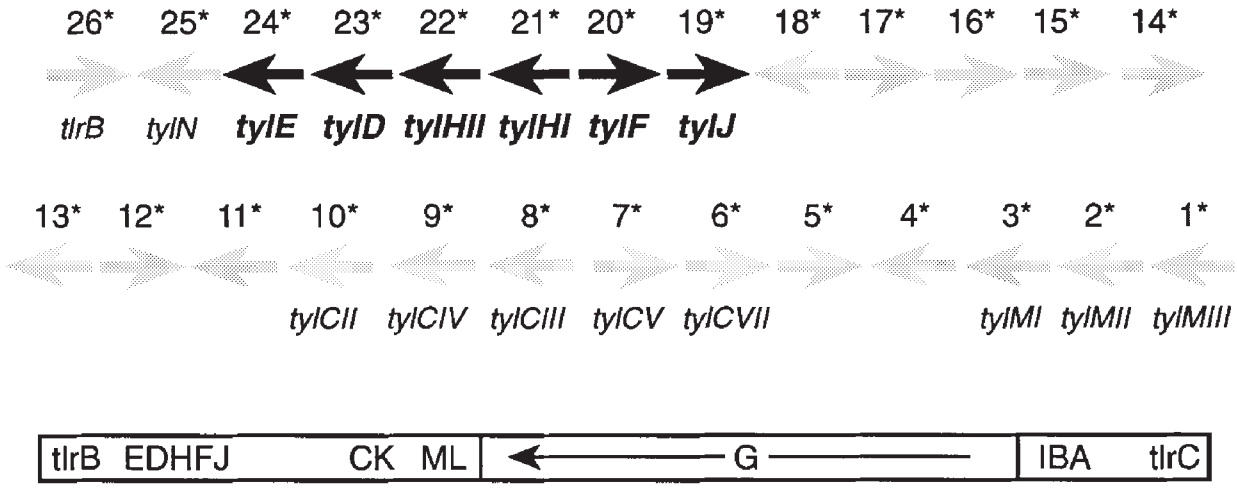


**Figure 1** The *tyl* gene cluster of *Streptomyces fradiae*. Not drawn to scale. The bar showing the thirteen *tyl* loci (*A*–*M*) represents a contiguous portion of the genome (~85 kb including the flanking resistance genes *tlrB* and *tlrC*). The *tylG* locus covers ~41 kb and contains five polyketide synthase mega genes reading right to left. Genes of the *tyl* cluster (orfs 1*–26*) located downstream of *tylG* are shown as arrows, with those analysed here shown in black. The use of '*' distinguishes these genes from others (not shown) located upstream of *tylG* in the *tylIBA* region. Data are taken from references [5,11,12,30,31]; N Bate *et al*, manuscript submitted; present work.

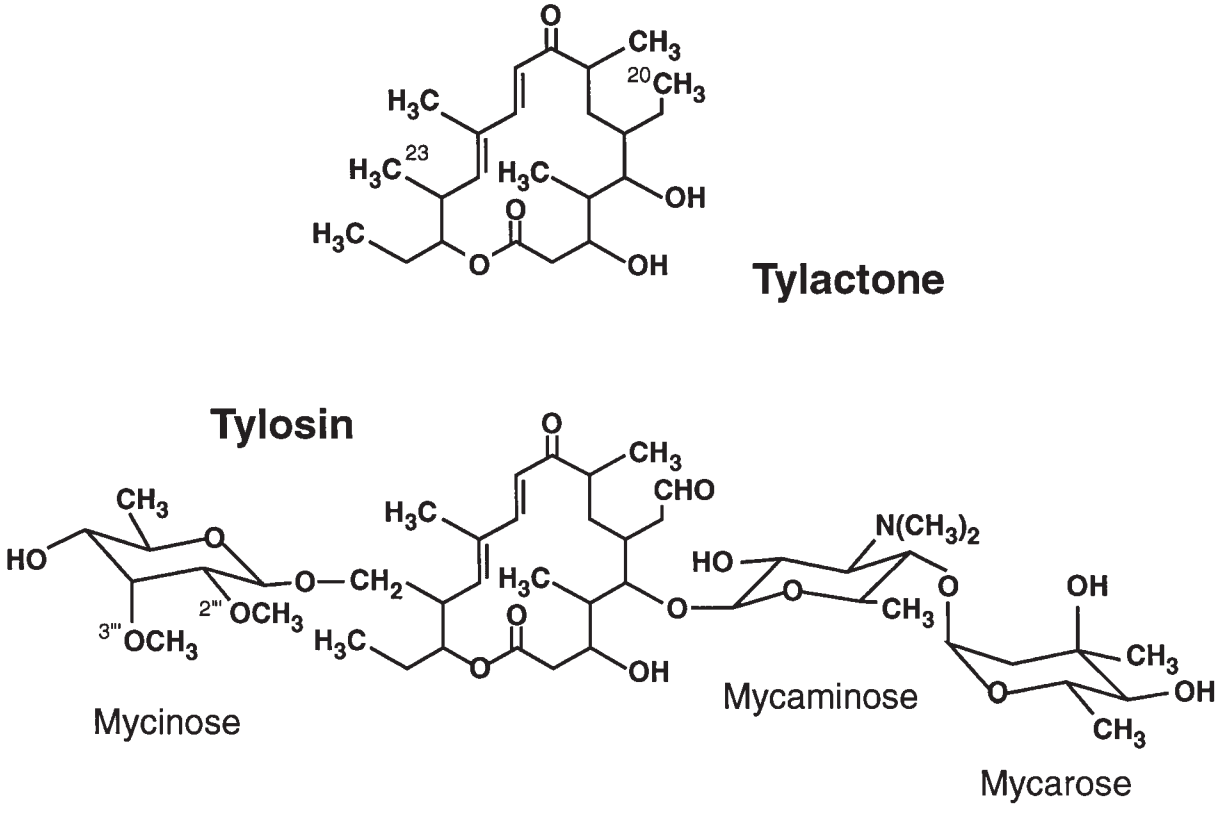


**Figure 2** Structure and synthesis of tylosin. The polyketide lactone, tylactone, is substituted with mycaminose, and then oxidised at C20 and C23, prior to addition of deoxyallose and mycarose. Finally, the deoxyallose moiety is converted to mycinose via methylation of the 2‴- and 3‴-OH groups.

the orientation of Figure 1, the sequence of the *Bam*HI fragment adjoined that for *tyIN* (accession number AJ005397) and the two sequences were then linked by primer extension. DNA sequences together with the corresponding chromatograms were imported into SEQ ED v 1.0.3 and aligned using AUTO ASSEMBLER (Applied Biosystems, Warrington, UK). Open reading frames were identified using BLASTX and six-frame translation with DNA STRIDER. Deduced gene products were analysed using BLASTP.

## Results

When genes of the *tyl* cluster were first identified using deoxyoligonucleotide probes based on the sequence of the

purified MOMT enzyme, a 5.7-kb *Bam*HI fragment was found to restore tylosin production in *tylD*, *tylE*, *tylF*, *tylH* and *tylJ* mutants, and further analysis using nested DNA fragments allowed the gene order to be established as EDHFJ reading left to right in the orientation of Figure 1 [7,8]. In the present work, 5833 bp of *S. fradiae tyl* DNA, including that *Bam*HI fragment, has been sequenced (GenBank accession number AF147703) and, interestingly, the sequence contains six complete open reading frames, designated orfs 19*–24* (Figure 1) according to the systematic nomenclature previously adopted [12]. (The use of '*' denotes orfs located downstream of *tylG*.) One end of the present sequence overlaps (by 127 bp) the sequence containing the deoxyallosyltransferase gene *tylN* (accession number AJ005397; [30]). At the right-hand end in the orientation of Figure 1, the present sequence overlaps (by 159 bp) the downstream end of a convergent gene (orf18*; accession number AF145049). Each of the orfs characterised here displays the biased codon usage characteristic of *Streptomyces* genes, with 87–94% usage of G or C in the third codon position. The analysis of their functions is summarised in Table 1.

### *tylJ (orf19*)*

This orf must be *tylJ* since it is the only gene downstream of *tylF* within the *Bam*HI fragment alluded to above. The deduced product strongly resembles various putative 3,5- (or 5-) epimerases involved in deoxyhexose metabolism, including StrM from streptomycin-producing *Streptomyces griseus* [21] and EryBVII from *Saccharopolyspora erythraea* [10,28]. It is also similar to TylCVII, proposed to be the nucleoside diphospho (NDP)-deoxyhexose 5- (or 3,5-) epimerase involved in mycarose biosynthesis during tylosin production (N Bate *et al*, submitted). Originally, *tylJ* mutants were described as being unable to synthesise or add 6-deoxyallose during tylosin production, but the latter possibility can now be eliminated since the deoxyallose-addition enzyme is encoded by *tylN* [30]. Therefore *tylJ* must encode a deoxyallose-biosynthetic enzyme, proposed to be NDP-deoxyhexose 3-epimerase (Figure 3).

### *tylF (orf20*)*

The product of this orf could not be deduced accurately from the DNA sequence. Starting 17 triplets after the most-upstream candidate start codon is an encoded sequence of 256 amino acids with the same N-terminus (accession number J03008) as purified macrocin-*O*-methyltransferase, MOMT, except that the latter undergoes post-translational processing at the N-terminus [8]. The authentic product of orf20* displays a very close end-to-end match (70% amino acid sequence identity) to MycF, encoding mycinamicin III *O*-methyltransferase from *Micromonospora griseorubida* [13]. Such activity corresponds to macrocin 3‴-*O*-methyltransferase (tylosin and mycinamicin both contain mycinose) and was expressed in *E. coli* from DNA containing *mycF*. MOMT-encoding DNA restored tylosin production when introduced into *tylF* mutants of *S. fradiae* that normally accumulate macrocin [8], and purified MOMT displayed absolute specificity for macrocin and SAM as co-substrates [6]. It is concluded that orf20* is *tylF* and that MOMT, the 3‴-*O*-methyltransferase that catalyses the final step in tylosin production, is the product of this gene.

**Table 1** The mycinose-biosynthetic gene cassette of *S. fradiae*

| Gene | Product (kDa) | Function |
|---|---|---|
| *tylJ* | 22.9 | NDP-deoxyhexose 3-epimerase |
| *tylF* | 28.7 | macrocin 3‴-*O*-methyltransferase |
| *tylHI* | 47.4[a] | P450 hydroxylase; ring oxidation at C23 |
| *tylHII* | 8.3 | tylodoxin, a ferredoxin of unknown specificity |
| *tylD* | 36.1 | NDP-deoxyhexose 4-ketoreductase |
| *tylE* | 43.2 | demethylmacrocin 2‴-*O*-methyltransferase |
| *tylN* | 46.6[b] | deoxyallosyltransferase |

[a]Maximal size; translational start codon not known unequivocally.
[b]Reference [30]; see AJ005397, revised.

### *tylHI (orf21*)*

The deduced product of this orf is a protein of maximum size 436 amino acid residues. However, there are two other candidate start codons located seven and 16 triplets downstream, and the coding sequence could plausibly begin at any of the three. The gene product is evidently a cytochrome P450. It matches many such sequences in the database, including the well characterized cytochrome P-450$_{SU1}$, synonym P-450CVA1, from *Streptomyces griseolus* [20], and displays highly conserved sequence motifs [22] characteristic of such enzymes, including the haem-binding pocket that contains the invariant cysteine involved in haem attachment (**FG**Y**G**PHQ**C**L**G**QN**LA**MF**E**LE**V**; consensus sequence given in bold). During the original analysis at Lilly, the *tylH* locus was mapped by complementation of null mutants using cloned DNA fragments [8] and *tylH* mutants failed to oxidise the polyketide ring at C23 [2], a process that requires a cytochrome P450 hydroxylase. However, the DNA sequence reveals the presence of two genes between *tylD* and *tylF*. Accordingly, orf21* has been designated *tylHI*.

### *tylHII (orf22*)*

Beginning 36 bp downstream of *tylHI* lies a small orf, the deduced product of which (81 amino acid residues) gives a large number of sequence matches to ferredoxins, including ferredoxin$_{soy}$ (product of *soyB* [29]), and Fd-1 plus Fd-2 (products of *suaB* and *subB*, respectively) from *S. griseolus* [19]. These well characterized ferredoxins contain unusual [3Fe–4S] centres and the orf22* product, TylHII, is deduced to resemble them in this respect. In *S. griseolus*, the three ferredoxin genes are each adjacent to 'partner' genes that encode P450 enzymes [20,29]. Likewise, it is possible that TylHII (tylodoxin) functions together with TylHI, although this remains to be established.

### *tylD (orf23*)*

The deduced product of this orf, a protein of 336 amino acid residues, possesses a candidate NADP-binding motif (GAGAAV), with a downstream cluster of arginines that could interact with the 2′-phosphate of NADP (NS Scrutton, personal communication). The closest sequence match was given by *E. coli* fucose synthetase, the product of

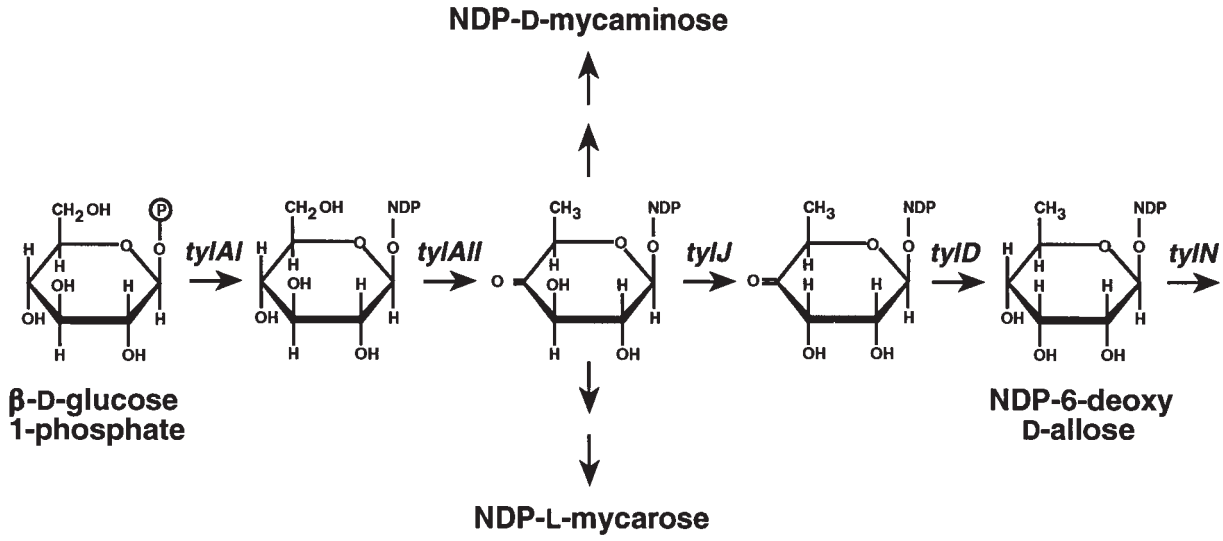


**Figure 3** Deoxyhexose metabolism during tylosin production. The three deoxyhexose sugars are transferred from their respective NDP-adducts into the emerging tylosin molecule by specific glycosyltransferases. Following such transfer by TylN, the deoxyallose moiety is converted to mycinose, via bis-*O*-methylation catalysed by TylE followed by TylF, in the last two steps of tylosin production.

*wcaG*, now re-named *fcl* [1,27]. This bifunctional protein, involved in GDP-l-fucose biosynthesis, possesses 3,5-epimerase and, significantly, NADPH-dependent 4-ketoreductase activities. Since *tylD* mutants fail to synthesise 6- deoxyallose, and since DNA fragments from this region of the *S. fradiae* genome restored tylosin production in such strains [8], it is proposed that orf23* is *tylD* and encodes NADPH-dependent 4-ketoreductase activity required for deoxyallose biosynthesis (Figure 3).

### *tylE (orf24*)*

Beginning 18 bp downstream of *tylD* is an encoded sequence of 395 amino acids with the same N-terminus as purified demethylmacrocin 2‴-*O*-methyltransferase (P Szoke and PR Rosteck Jr, poster H-10, American Society for Microbiology Annual Meeting, 1989). This enzyme catalyses the penultimate step in tylosin biosynthesis, and extracts of *S. fradiae tylE* mutants that normally accumulate demethylmacrocin (2‴, 3‴-*O*-demethyltylosin) lack such activity [24]. Tylosin production was restored in *tylE* mutants by a 5.7-kb *Bam*HI fragment contained within the present sequence and further analysis using a nested series of DNA fragments placed *tylE* at the left end of that *Bam*HI fragment in the orientation of Figure 1 [7,8]. Therefore, orf24* must be *tylE*, although it is interesting that the gene extends 50 bp leftwards beyond the *Bam*HI site. When demethylmacrocin-*O*-methyltransferase was purified [16], it demonstrated an absolute requirement for SAM as co-substrate although TylE does not display the sequence motifs characteristic of SAM-binding proteins [15]. The TylE sequence may therefore be useful in allowing 'cryptic' *O*-methyltransferases in the database to be recognised.

## Discussion

The mycinose biosynthetic genes of *S. fradiae* present a rare example of a natural uninterrupted sugar-biosynthetic cassette. Since genes equivalent to *tylAI* and *tylAII*, encoding initial steps common to the biosynthesis of many deoxyhexoses, are widely distributed among actinomycetes, it may be relatively simple to engineer the expression of mycinose-biosynthetic capability in surrogate hosts. Moreover, this block of genes has other novel features.

The majority of bacterial cytochromes P450 receive electrons from ferredoxins, which in turn are supplied by FAD-containing reductases that utilise NADPH as electron donor. The archetype is the P450cam system from *Pseudomonas putida* in which genes encoding the three components are co-transcribed from a single operon (for review, see [18]). In contrast, in antibiotic-biosynthetic gene clusters, it is commonplace to encounter isolated P450-encoding genes, the products of which are thought to be serviced by housekeeping ferredoxins and reductases encoded elsewhere in the respective genomes. In that context, the *tylHI-tylHII* pairing is unprecedented and it will be pertinent to discover whether the TylHII ferredoxin (tylodoxin) exclusively serves one or other of the cytochromes P450 of the tylosin pathway. Interestingly, there appears to be no NADPH:ferredoxin oxidoreductase gene anywhere within the entire *tyl* cluster.

Genetic studies based on the sequence of the TylF protein provided the means by which the tylosin-biosynthetic gene cluster was first found within the *S. fradiae* genome and the *tylF* gene has recently featured in a successful strategy for rational strain improvement. Following the observation [25] that MOMT activity was sub-optimal for efficient conversion of macrocin to tylosin in certain strains of *S. fradiae*, an extra copy of *tylF* was introduced into the genome of an industrial producing-organism. The resultant strain produced elevated levels of tylosin relative to macrocin [26].

### *Note added*

After this manuscript had been submitted for publication, another group [9] published similar, but not identical,

sequences (accession number AF055922) for the genes analysed here.

## Acknowledgements

This work was supported by project grant 91/T08195 from BBSRC (UK), and by Eli Lilly and Co, Indianapolis (USA). We thank Paul Rosteck and colleagues at Lilly for generously sharing unpublished data, and Nigel Scrutton (of this department) for insightful analysis of protein sequences.

## References

1 Andrianopoulos K, L Wang and PR Reeves. 1998. Identification of the fucose synthetase gene in the colanic acid gene cluster of *Escherichia coli* K-12. J Bacteriol 180: 998–1001.
2 Baltz RH and ET Seno. 1981. Properties of *Streptomyces fradiae* mutants blocked in biosynthesis of the macrolide antibiotic tylosin. Antimicrob Agents Chemother 20: 214–225.
3 Baltz RH and ET Seno. 1988. Genetics of *Streptomyces fradiae* and tylosin biosynthesis. Annu Rev Microbiol 42: 547–574.
4 Baltz RH, ET Seno, J Stonesifer and GM Wild. 1983. Biosynthesis of the macrolide antibiotic tylosin. A preferred pathway from tylactone to tylosin. J Antibiot 36: 131–141.
5 Bate N, AR Butler, AR Gandecha and E Cundliffe. 1999. Multiple regulatory genes in the tylosin-biosynthetic cluster of *Streptomyces fradiae*. Chem Biol 6: 617–624.
6 Bauer NJ, AJ Kreuzman, JE Dotzlaf and W-K Yeh. 1988. Purification, characterization, and kinetic mechanism of *S*-adenosyl-l-methionine: macrocin *O*-methyltransferase from *Streptomyces fradiae*. J Biol Chem 263: 15619–15625.
7 Beckmann RJ, K Cox and ET Seno. 1989. A cluster of tylosin biosynthetic genes is interrupted by a structurally unstable segment containing four repeated sequences. In: Genetics and Molecular Biology of Industrial Microorganisms (Hershberger CL, Queener SW and Hegeman G, eds), pp 176–186, American Society for Microbiology, Washington, DC.
8 Fishman SE, K Cox, JL Larson, PA Reynolds, ET Seno, W-K Yeh, R Van Frank and CL Hershberger. 1987. Cloning genes for the biosynthesis of a macrolide antibiotic. Proc Natl Acad Sci USA 84: 8248–8252.
9 Fouces R, E Mellado, B Diez and JL Barredo. 1999. The tylosin biosynthetic cluster from *Streptomyces fradiae*: genetic organization of the left region. Microbiology 145: 855–868.
10 Gaisser S, GA Böhm, J Cortés and PF Leadlay. 1997. Analysis of seven genes from the *eryAI–eryK* region of the erythromycin biosynthetic gene cluster in *Saccharopolyspora erythraea*. Mol Gen Genet 256: 239–251.
11 Gandecha AR and E Cundliffe. 1996. Molecular analysis of *tlrD*, an MLS resistance determinant from the tylosin producer, *Streptomyces fradiae*. Gene 180: 173–176.
12 Gandecha AR, SL Large and E Cundliffe. 1997. Analysis of four tylosin biosynthetic genes from the *tylLM* region of the *Streptomyces fradiae* genome. Gene 184: 197–203.
13 Inouye M, H Suzuki, Y Takada, N Muto, S Horinouchi and T Beppu. 1994. A gene encoding mycinamicin III *O*-methyltransferase from *Micromonospora griseorubida*. Gene 141: 121–124.
14 Janssen GR and MJ Bibb. 1993. Derivatives of pUC18 that have *Bg*/II sites flanking a modified multiple cloning site and that retain the ability to identify recombinant clones by visual screening of *Escherichia coli* colonies. Gene 124: 133–134.
15 Kagan RM and S Clarke. 1994. Widespread occurrence of three sequence motifs in diverse *S*-adenosylmethionine-dependent methyltransferases suggests a common structure for these enzymes. Arch Biochem Biophys 310: 417–427.
16 Kreuzman AJ, JR Turner and W-K Yeh. 1988 Two distinctive *O*-methyltransferases catalysing penultimate and terminal reactions of macrolide (tylosin) biosynthesis. J Biol Chem 263: 15626–15633.
17 Merson-Davies LA and E Cundliffe. 1994. Analysis of five tylosin biosynthetic genes from the *tyllBA* region of the *Streptomyces fradiae* genome. Mol Microbiol 13: 349–355.
18 Munro AW and JG Lindsay. 1996. Bacterial cytochromes P-450. Mol Microbiol 20: 1115–1125.
19 O'Keefe DP, KJ Gibson, MH Emptage, R Lenstra, JA Romesser, PJ Litle and CA Omer. 1991. Ferredoxins from two sulfonylurea herbicide monooxygenase systems in *Streptomyces griseolus*. Biochemistry 30: 447–455.
20 Omer CA, R Lenstra, PJ Litle, C Dean, JM Tepperman, KJ Leto, JA Romesser and DP O'Keefe. 1990. Genes for two herbicide-inducible cytochromes P-450 from *Streptomyces griseolus*. J Bacteriol 172: 3335–3345.
21 Pissowotzki K, K Mansouri and W Piepersberg. 1991. Genetics of streptomycin production in *Streptomyces griseus*: molecular structure and putative function of genes *strELMB2N*. Mol Gen Genet 231: 113–123.
22 Poulos TL, BC Finzel and AJ Howard. 1987. High-resolution crystal structure of cytochrome P450cam. J Mol Biol 195: 687–700.
23 Rosteck Jr PR, PA Reynolds and CL Hershberger. 1991. Homology between proteins controlling *Streptomyces fradiae* tylosin resistance and ATP-binding transport. Gene 102: 27–32.
24 Seno ET and RH Baltz. 1981. Properties of *S*-adenosyl-l-methionine: macrocin *O*-methyltransferase in extracts of *Streptomyces fradiae* strains which produce normal or elevated levels of tylosin and in mutants blocked in specific *O*-methylations. Antimicrob Agents Chemother 20: 370–377.
25 Seno ET and RH Baltz. 1982. *S*-adenosyl-l-methionine: macrocin *O*-methyltransferase activities in a series of *Streptomyces fradiae* mutants that produce different levels of the macrolide antibiotic tylosin. Antimicrob Agents Chemother 21: 758–763.
26 Solenberg PJ, CA Cantwell, AJ Tietz, D McGilvray, SW Queener and RH Baltz. 1996. Transposon mutagenesis in *Streptomyces fradiae*: identification of a neutral site for the stable insertion of DNA by transposon exchange. Gene 168: 67–72.
27 Stevenson G, K Andrianopoulos, M Hobbs and PR Reeves. 1996. Organization of the *Escherichia coli* K-12 gene cluster responsible for production of the extracellular polysaccharide colanic acid. J Bacteriol 178: 4885–4893.
28 Summers RG, S Donadio, MJ Staver, E Wendt-Pienkowski, CR Hutchinson and L Katz. 1997. Sequencing and mutagenesis of genes from the erythromycin biosynthetic gene cluster of *Saccharopolyspora erythraea* that are involved in l-mycarose and d-desosamine production. Microbiology 143: 3251–3262.
29 Trower MK, R Lenstra, C Omer, SE Buchholz and FS Sariaslani. 1992. Cloning, nucleotide sequence determination and expression of the genes encoding cytochrome P-450$_{soy}$ (SoyC) and ferredoxin$_{soy}$ (SoyB) from *Streptomyces griseus*. Mol Microbiol 6: 2125–2134.
30 Wilson VTW and E Cundliffe. 1998. Characterization and targeted disruption of a glycosyltransferase gene in the tylosin producer, *Streptomyces fradiae*. Gene 214: 95–100.
31 Wilson VTW and E Cundliffe. 1999. Molecular analysis of *tlrB*, an antibiotic-resistance gene from tylosin-producing *Streptomyces fradiae*, and discovery of a novel resistance mechanism. J Antibiot 52: 288–296.