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Stimulation of polyketide metabolism in *Streptomyces fradiae* by tylosin and its glycosylated precursors

Steven A. Fish† and Eric Cundliffe

Author for correspondence: Eric Cundliffe. Tel: +44 116 252 3451. Fax: +44 116 252 3369. e-mail: ec13@le.ac.uk

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

Three glycosyltransferases are involved in tylosin biosynthesis in *Streptomyces fradiae*. The first sugar to be added to the polyketide aglycone (tylactone) is mycaminose and the gene encoding mycaminosyltransferase is *orf2 (*tylM2*). However, targeted disruption of *orf2** did not lead to the accumulation of tylactone under conditions that normally favour tylosin production; instead, the synthesis of tylactone was virtually abolished. This may, in part, have resulted from a polar effect on the expression of genes downstream of *orf2**, particularly *orf4** (*ccr*) which encodes crotonyl-CoA reductase, an enzyme that supplies 4-carbon extender units for polyketide metabolism. However, that cannot be the entire explanation, since tylosin production was restored at about 10% of the wild-type level when *orf2** was re-introduced into the disrupted strain. When glycosylated precursors of tylosin were fed to the disrupted strain, they were converted to tylosin, confirming that two of the three glycosyltransferase activities associated with tylosin biosynthesis were still intact. Interestingly, however, tylactone also accumulated under such conditions and, to a much lesser extent, when tylosin was added to similar fermentations. It is concluded that glycosylated macrolides exert a pronounced positive effect on polyketide metabolism in *S. fradiae*.**

Keywords: tylosin production, glycosyltransferase, polyketide metabolism, *Streptomyces fradiae*

INTRODUCTION

The macrolide antibiotic, tylosin (Fig. 1), is produced by *Streptomyces fradiae* via a combination of polyketide and 6-deoxyhexose metabolism. Glycosylation of tylactone, the cyclized polyketide product, always begins with the addition of mycaminose followed, in a preferred but not obligatory order, by deoxyallose and then mycarose to generate demethyl-macrocin. Stepwise bis *O*-methylation then converts the deoxyallose moiety to mycinose as macrocin is produced and converted to tylosin (Baltz *et al*., 1983). Characterization of the tylosin biosynthetic pathway (Fig. 2) was facilitated by the availability of non-producing mutants of *S. fradiae*, generated using NTG (Baltz & Seno, 1981). Collectively, these exhibited nine distinct phenotypes in cross-feeding experiments and allowed 13 genetic loci (*tylA–M*) to be mapped (Fig. 3) following complementation studies using cloned fragments of *tyl* DNA (Beckmann *et al*.,

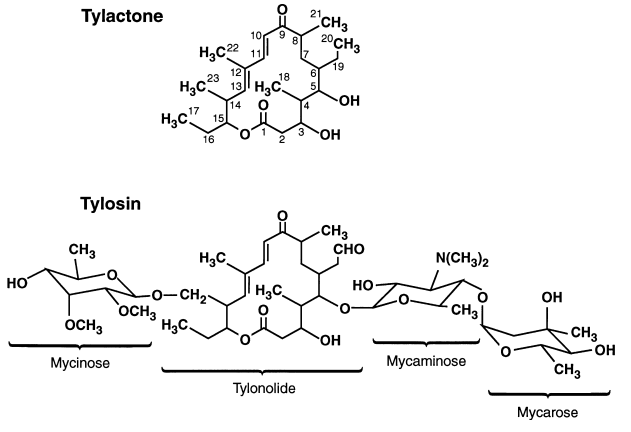

Fig. 1. Structures of tylactone and tylosin.

Abbreviations: OMT, *O*-mycaminosyl-tylonolide; DMT, demycinosyl-tylosin.

†**Present address:** Department of Microbiology and Immunology, University of Leicester, Leicester LE1 7RH, UK.

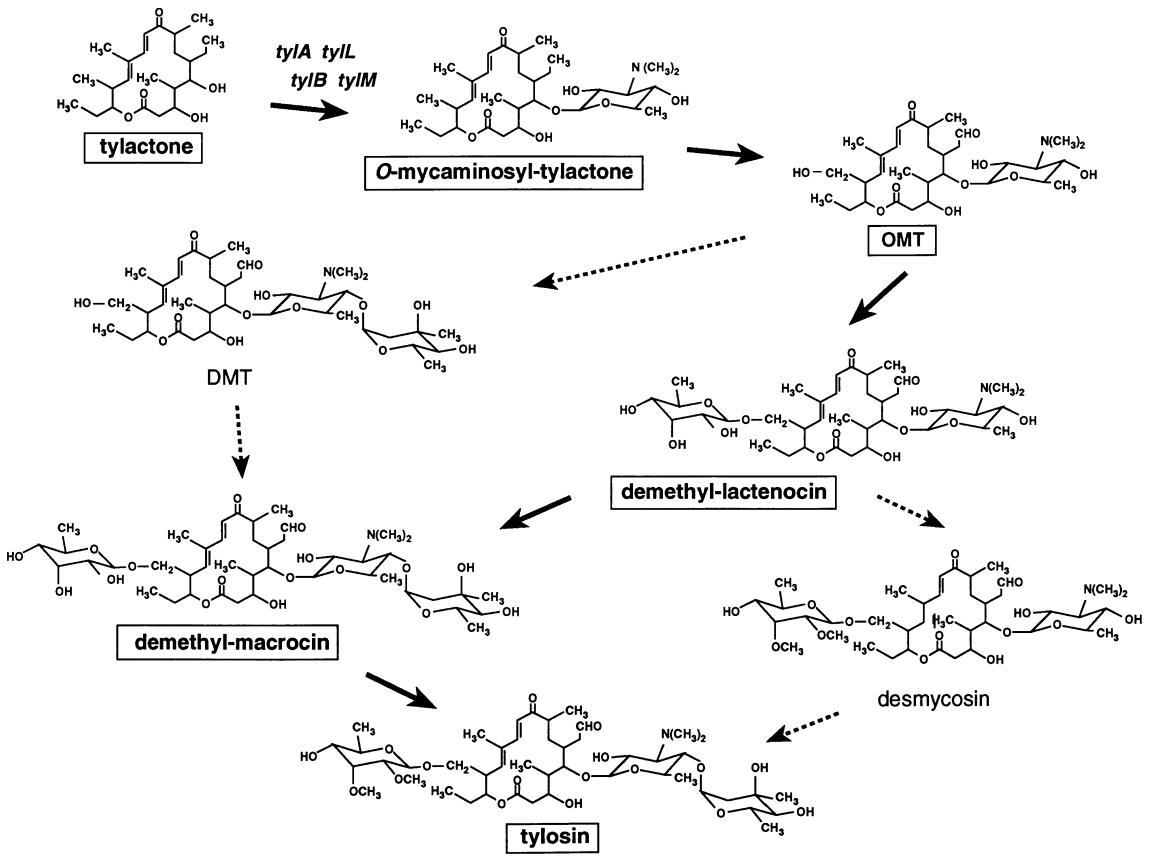

Fig. 2. The biosynthetic route from tylactone to tylosin. The preferred pathway from OMT to tylosin (solid arrows) goes via demethyl-lactenocin and demethyl-macrocin (Baltz *et al.*, 1983). However, this route is not obligatory since DMT and desmycosin accumulate in blocked mutants of *S. fradiae* and can be bioconverted to tylosin (dashed arrows). The step at which various *tyl* mutations block the pathway is indicated.

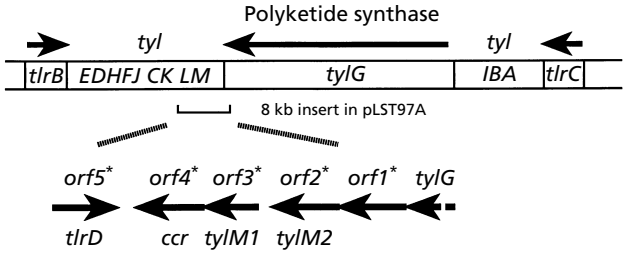

Fig. 3. The tylosin biosynthetic gene cluster of *Streptomyces fradiae*.

1989; Fishman *et al.*, 1987). Sequence analysis of DNA spanning the *tylLM* loci (Gandecha *et al.*, 1997) revealed four ORFs (*orf1**–*4**) located between the terminal thioesterase gene of the *tylG* polyketide synthase cluster and the resistance determinant, *tlrD* (Fig. 3). The function of the Orf1* protein is not known, but the deduced products of the other three genes displayed convincing similarities to authentic enzymes in end-to-end sequence comparisons. Thus, the *orf2** product is a glycosyltransferase, the Orf3* protein is a methyltransferase and *orf4** encodes crotonyl-CoA reductase. The latter appears to be an ancillary gene, ostensibly recruited into the *tyl* cluster to provide butyryl-CoA for polyketide metabolism. Sequence analysis of *orf3** in *S. fradiae* GS62 (a *tylM* mutant defective in mycaminose metabolism), revealed an amino acid substitution in the presumed cofactor binding site of the enzyme, and reintroduction of *orf3** into this organism resulted in restoration of tylosin biosynthesis (Gandecha *et al.*, 1997). However, *orf2** and its product were less easy to characterize. A *tylL* mutant, *S. fradiae* GS33 (unable to synthesize or add any of the three tylosin sugars), harbours a single mutational change (a GC to AT transition) in *orf2** that generates an in-frame stop codon early in the coding sequence (Clark, 1997). However, attempts to restore tylosin production in this strain by complementation with fragments of *tylLM* DNA were inconclusive, suggesting that the TylL phenotype probably results from multiple mutations. We therefore generated a strain of *S. fradiae* specifically disrupted in *orf2** in a further attempt to characterize the action of Orf2*. Unexpectedly, polyketide metabolism was also affected in this strain.

METHODS

Bacterial strains and plasmids. *S. fradiae* T59235 (also known as C373.1; referred to here as wild-type) and derivatives thereof were routinely grown at 30 °C in Tryptic Soy Broth (TSB; Difco) or at 37 °C on AS-1 agar plates (Baltz, 1980) and were maintained at −70 °C as mycelial fragments following addition of 5% (v/v) DMSO (final concn) to TSB-grown cultures. *tyl* DNA (11 kb) from *S. fradiae* was initially excised from cosmid pMOMT4 (Beckmann *et al.*, 1989) and introduced into pUC18 to generate pLST97 (Gandecha *et al.*, 1997) from which an 8 kb *Kpn*I–*Bam*HI fragment spanning the *tylLM* loci was excised and subcloned, again in pUC18, to generate pLST97A. This contained *tyl orfs 1*–4** plus *tlrD* and the terminal portion of *tylG* (see Fig. 3). pUC-derived plasmids were manipulated in *Escherichia coli* using standard protocols (Sambrook *et al.*, 1989).

Construction of disrupted *orf2*.* PCR-amplified *orf2** (1·4 kb), with engineered terminal *Bam*HI and *Pst*I sites, was generated using the following primers: 5′ GGATCCGTGCGTCGTGCACTGGATGAC 3′ and 5′ CTGCAGCTACCTTTCCGGCGCGGATCG 3′. Reaction mixtures (50 µl) were specifically designed for G+C-rich DNA templates and contained 50 ng template (pLST97A), 250 ng each primer, 1 mM dNTPs and 1 U *Taq* DNA polymerase in buffer containing 60 mM Tris/HCl, pH 8·5 (adjusted at 20 °C), 3·5 mM $MgCl_2$, 15 mM $(NH_4)_2SO_4$ and 10% DMSO. After an initial hot start at 80 °C for 5 min, the mixture was subjected to 35 cycles of amplification, each consisting of 1 min at 94 °C, 2 min at 60 °C and 3 min at 72 °C, before the thermal cycle was terminated with 7 min at 72 °C. The PCR product was captured in pGEM-T (Promega) and introduced into *E. coli* NM522 (Gough & Murray, 1983). *orf2** was excised from the plasmid using the terminal *Bam*HI and *Pst*I sites, ligated into the respective sites in pRSET A (Invitrogen) and disrupted at the unique (and slightly off-centre) *Aat*II site by insertion of the 2·3 kb hygromycin-resistance cassette, Ωhyg (Blondelet-Roualt *et al.*, 1997). This involved blunt-end ligation using the Boehringer Mannheim Rapid DNA Ligation Kit following treatment of linearized plasmid with mung bean nuclease (Promega). Disrupted *orf2** (3·7 kb) was then removed by digestion with *Nhe*I and *Pst*I and ligated into pOJ260, which cannot replicate in actinomycetes (Bierman *et al.*, 1992). The resultant plasmid (pLST9734) was initially propagated in *E. coli* NM522, before being introduced into *E. coli* S17-1, the donor strain used for conjugal transfer to *Streptomyces* spp. (modified from Bierman *et al.*, 1992; Mazodier *et al.*, 1989).

Targeted disruption of *orf2 in *S. fradiae*.** Following growth of *S. fradiae* C373.1 on AS-1 medium at 37 °C for 3 d, spores from one plate were resuspended in 10 ml pre-germination medium (Hopwood *et al.*, 1985) and incubated at 37 °C for 3–4 h before centrifugation and resuspension in 500 µl LB broth. An overnight culture (5 ml) of *E. coli* S17-1 (containing pLST9734) grown in LB medium containing spectinomycin (30 µg ml^{-1}; Sigma) and apramycin (25 µg ml^{-1}; Lilly Research Laboratories) was harvested and resuspended in 500 µl LB medium. Then 200 µl of the germinating spore suspension was mixed with 100 µl of the *E. coli* donor cells, plated onto AS-1 agar and incubated at 37 °C. After 18 h, the plate was overlaid with 4 ml soft R2 agar (Hopwood *et al.*, 1985) containing hygromycin B (100 µg ml^{-1}; Calbiochem) to select for transconjugants and nalidixic acid (60 µg ml^{-1}; Sigma), to inhibit donor cell growth, and incubation was continued at 37 °C for 7 d or until colonies had grown through the overlay. Colonies were picked and dually inoculated onto AS-1 medium containing hygromycin B (100 µg ml^{-1}) or apramycin (25 µg ml^{-1}) to screen for recombinants containing double cross-overs in which chromosomal *orf2** had been replaced with the disrupted gene. Such strains were expected to be hygromycin-resistant and apramycin-sensitive. Southern blot hybridization analysis was used to confirm the gene replacement and one such strain, SF01, was chosen for further analysis.

Complementation of strain SF01 with PCR-amplified *orf2*.* PCR-amplified *orf2** was ligated into the *Bam*HI site of pLST9829, a low-copy-number replicating plasmid (A. R. Butler & E. Cundliffe, unpublished), downstream of the strong, constitutive promoter *ermE*p* (Bibb *et al.*, 1994). The resultant plasmid, pLST9735, was then introduced into *S. fradiae* SF01 by conjugal transfer from *E. coli* S17-1, thereby generating strain SF02.

Tylosin-production fermentation. Conical flasks (100 ml capacity) containing 25 ml pre-fermentation medium were inoculated with 100 µl *S. fradiae* stock mycelial fragments and incubated at 28 °C with rotary shaking at 300 r.p.m. for 3 d (wild-type and strain SF01) or 5 d (strain SF02, grown in the presence of apramycin, see below). Pre-fermentation medium (pH adjusted to 7·8 with NaOH) contained 1% (w/v) corn steep liquor, 0·5% (w/v) yeast extract, 0·5% (w/v) soya bean meal, 0·3% (w/v) calcium carbonate and 0·5% (v/v) methyl oleate. Then 5 ml portions of each culture were used to inoculate 45 ml batches of MM-1 tylosin production medium (Gray & Bhuwapathanapun, 1980) and incubation was continued under similar conditions for a further 7 d. [To maintain pLST9735 in strain SF02, apramycin (25 µg ml^{-1}) was added to batches of pre-fermentation medium and tylosin production medium used for this strain.] Fermentation products were extracted by shaking the entire culture with an equal volume of chloroform, the extracts were dried by rotary flash evaporation at 30 °C and the residues re-dissolved in 500 µl HPLC grade chloroform.

Analysis of fermentation products. This was done by reverse phase HPLC (as described by Huber *et al.*, 1990) using a 3·9 × 300 mm C18 µBondapak column protected by a C18 µBondapak guard column (Waters Associates). Chloroform extracts were applied to the column in 0·3% (w/v) ammonium formate (pH 4·0) containing 50% (v/v) methanol and products were eluted using a similar buffer with a linear concentration gradient (50–80%) of methanol at a flow rate of 1·75 ml min^{-1}. The absorbance of the eluate was monitored at 282 nm.

Bioconversion of tylosin precursors by *S. fradiae* SF01. *O*-Mycaminosyl-tylonolide (OMT), demycinosyl-tylosin (DMT), demycarosyl-tylosin (desmycosin) and tylosin (10 mg each) were added separately to fermentation cultures (50 ml) in MM-1 medium after 2 d at 28 °C and incubation was continued for a further 3 d prior to extraction with chloroform and HPLC analysis of the products, as above.

RESULTS AND DISCUSSION

The glycosyltransferase sequence motif

When *orf2** was first sequenced, its deduced product was suggested to be a glycosyltransferase (Gandecha *et al.*, 1997). It displayed end-to-end similarity to the deduced sequences of the Mgt protein from *S. lividans* (Jenkins & Cundliffe, 1991) and RhlB from *Pseudomonas aeruginosa* (Ochsner *et al.*, 1994), both of which are authentic glycosyltransferases. More recently, another related protein, GtfB from glycopeptide-pro-

TylM2	LLPTCSAVVHHGGAGTCFTATLNGLPQIVVA
EryCIII/Orf8	LLPTCAATVHHGGPGSWHTAAIHGVPQVILP
DnrS	VLPSCAAVVHHGGAGTWATAALHGVPQLALA
DauH	LLPSCSGIIHHGGSGTFMTALAHATPQLIVP
RhlB	LLPSCAGLVHPGGIGAMSLALAAGVPQVLLP
GtfB	LFGRVAAVIHHGGAGTTHVAARAGAPQILLP
Consensus	LLP*CAAhhHHGGAGT**hAhh*GhPQhhhP

Fig. 4. The glycosyltransferase consensus sequence motif. Sequences: TylM2 from *S. fradiae* (Gandecha *et al.*, 1997; GenBank accession no. X81885); EryCIII/Orf8 from *Saccharopolyspora erythraea* (Haydock, 1992); DnrS from *S. peucetius* (Otten *et al.*, 1995; L47164); DauH from *Streptomyces* sp. strain C5 (Dickens *et al.*, 1996; U43704); RhlB from *P. aeruginosa* (Ochsner *et al.*, 1994; L28170); GtfB from *Amycolatopsis orientalis* (Solenberg *et al.*, 1997; U84349). 'h' denotes a hydrophobic residue.

ducing *Amycolatopsis orientalis*, displayed glucosyltransferase activity with a glycopeptide-aglycone as acceptor substrate (Solenberg *et al*., 1997). Other entries in the protein identification databases were also found in routine searches using the Orf2* sequence, including DnrS and DauH from daunorubicin (daunomycin)-producing *S. peucetius* (Otten *et al*., 1995) and *Streptomyces* sp. strain C5 (Dickens *et al*., 1996), respectively, but the closest match (50% identity) was found with the product of *eryCIII* (*orf8*) from the erythromycin producer, *Saccharopolyspora erythraea* (Haydock, 1992). These proteins all contain a consensus sequence motif (Fig. 4) that is also present in eukaryotic enzymes and appears to be diagnostic of a sub-group of glycosyltransferases (Cundliffe *et al*., 1997), although it is not present in all such enzymes. This sequence motif has been used to design (by reverse genetics) deoxyoligonucleotides that find specific hybridization targets in the genomes of antibiotic-producing *Streptomyces* spp. (A. R. Gandecha & E. Cundliffe, unpublished data). The location of *orf2** within the tylosin biosynthetic (*tyl*) gene cluster of *S. fradiae* suggested that it probably encoded one of the three glycosyltransferase activities involved in tylosin production. This hypothesis was addressed by a combination of gene disruption and bioconversion studies.

Targeted disruption of *orf2** via gene replacement

Initially, *orf2** was amplified by PCR from wild-type *tyl* DNA and disrupted by insertion of the hygromycin B-resistance cassette, Ωhyg (Blondelet-Roualt *et al*., 1997). The disrupted *orf2** was then ligated into pOJ260, a suicide vector unable to replicate in actinomycetes (Bierman *et al*., 1992), and introduced into *S. fradiae* via conjugal transfer from *E. coli* S17-1. Hygromycin B-resistant transconjugants that lacked the apramycin-resistance gene of pOJ260 were selected phenotypically and Southern blot hybridization analysis (data not shown) confirmed that one such strain (designated SF01) had undergone double recombination, resulting in replacement of chromosomal *orf2** by the disrupted gene. Thus, using *orf2** as probe, the hybridization target in *Afl*III digests of the SF01 genome was increased in size compared with that in the wild-type (6·9 vs 4·6 kb) and was also found by an Ωhyg probe. However, strain SF01 had lost the remainder of pOJ260, including the apramycin-resistance gene. In negative controls, genomic DNA from *S. fradiae* wild-type did not light up with the Ωhyg probe or with the apramycin resistance gene.

Fermentation and bioconversion analysis

The fermentation products of *S. fradiae* wild-type and strain SF01 were compared by reverse phase HPLC, following 7 d incubation at 28 °C in tylosin production medium. As expected, the wild-type produced tylosin (Fig. 5a) but, surprisingly, strain SF01 produced very little material that absorbed at 282 nm (Fig. 5b) although traces of tylactone could be detected when the sensitivity of analysis was increased 100-fold (data not shown). In contrast, no glycosylated precursors of tylosin were evident. However, when glycosylated macrolides were fed to strain SF01 during similar fermentations, OMT was converted to tylosin (Fig. 5d), as were DMT and desmycosin, revealing that strain SF01 still possesses the deoxyallosyl- and mycarosyl-transferase activities normally associated with tylosin biosynthesis. When PCR-amplified *orf2**, governed by the strong, constitutive *ermE*p* promoter (Bibb *et al*., 1994), was re-introduced into strain SF01 using pLST9735 to generate strain SF02, tylosin production was restored but only at about 10% of the wild-type level (Fig. 5f).

In rationalizing these results, we considered the proposition that *orf2** encodes a glycosyltransferase to be secure. The level of similarity between its deduced product and authentic glycosyltransferases, such as the GtfB protein, renders other possibilities remote. Since mycaminose is normally the first sugar to be added to the polyketide aglycone during tylosin production and since glycosylated macrolide(s) are not produced by the *orf2**-disrupted strain, SF01, we conclude that *orf2** encodes the mycaminosyl-transferase enzyme. Accordingly, *orf2** has been designated *tylM2* since it is located at, or very close to, the mycaminose-specific *tylM* locus in the genome of *S. fradiae* (Beckmann *et al*., 1989; Fishman *et al*., 1987). Why then does strain SF01 not accumulate significant levels of tylactone? Is tylactone unstable in *S. fradiae* when not glycosylated, or is polyketide metabolism impaired in strain SF01?

Polyketide metabolism and glycosylation

These results call to mind earlier observations with the *tylM* mutant, GS62, in which *orf3** has recently been shown to be altered (Gandecha *et al*., 1997). This gene encodes methyltransferase activity normally involved in mycaminose biosynthesis (and is therefore designated *tylM1*), yet strain GS62 (like SF01) accumu-

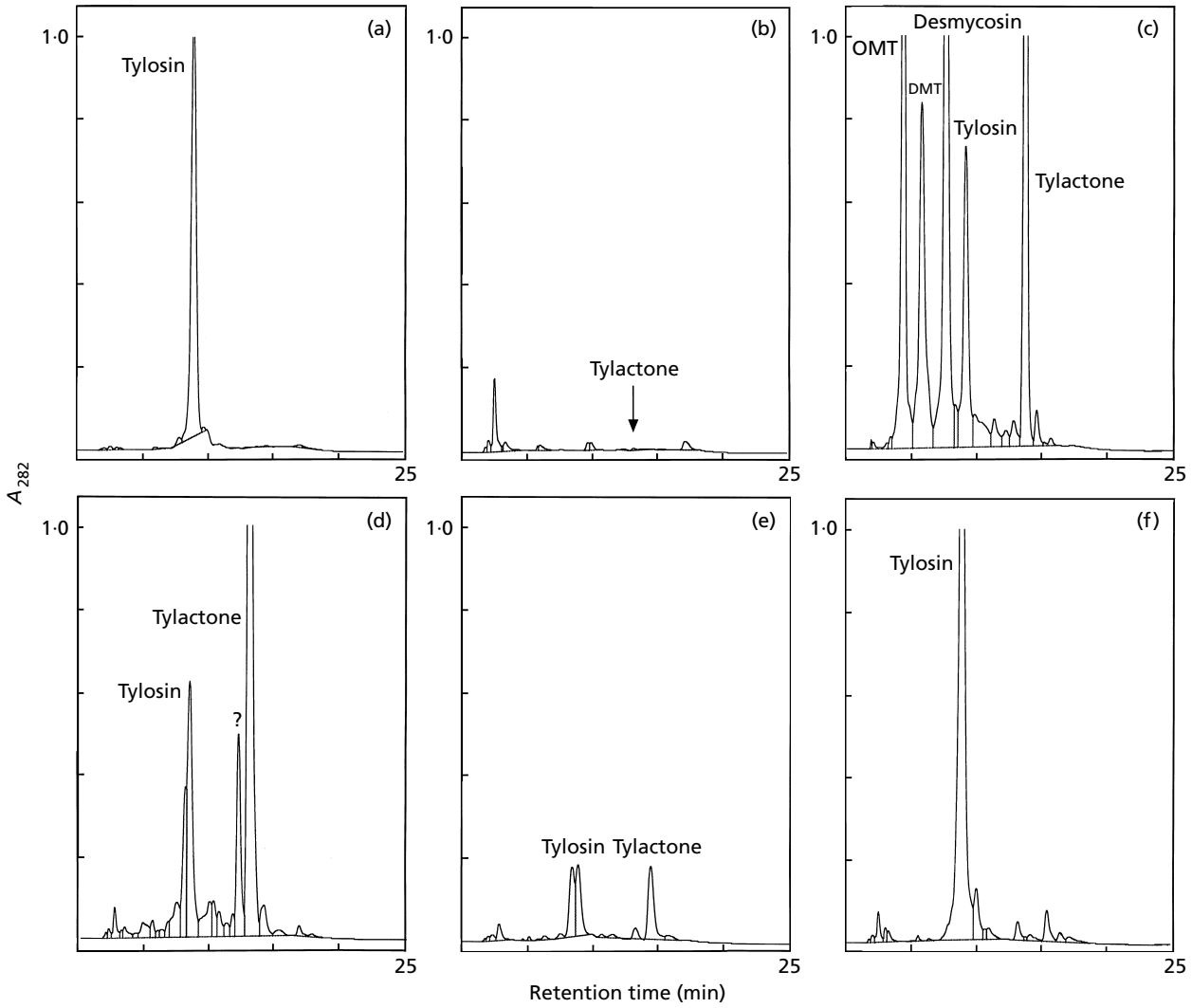

Fig. 5. HPLC analysis of *S. fradiae* fermentation products produced by (a) *S. fradiae* wild-type, (b) strain SF01, (d, e) strain SF01 with exogenously added OMT (d) or tylosin (e) and (f) strain SF02. (c) Standard compounds (50 µg each). Relomycin (20-dihydrotylosin) is co-produced with tylosin in *S. fradiae* and appears as a leading shoulder on the tylosin absorption peak. Relative to the total amounts of fermentation product(s) obtained, the proportionate loading in (a) was 10% of that in (b), (d), (e) and (f).

lated only trace amounts of tylactone, together with fatty acids of intermediate chain length that appeared to be precursors of tylactone rather than degradation products (Huber *et al.*, 1990). Similar products were also seen with other *S. fradiae* mutants, including a *tylB* mutant (blocked in mycaminose biosynthesis) and *tylA* and *tylL* mutants (blocked in the biosynthesis or addition of all three tylosin sugars), but strains blocked beyond OMT in the tylosin biosynthetic pathway (see Fig. 2) accumulated the expected glycosylated intermediates. Those results (Huber *et al.*, 1990) and the present data suggest that polyketide metabolism in *S. fradiae* is impaired when the primary product, tylactone, cannot be glycosylated. Interestingly, tylactone did accumulate (together with unidentified material eluting slightly earlier during HPLC) in strain SF01 when tylosin or its glycosylated precursors were added to fermentations. This effect (Fig. 5d) was seen most prominently with OMT, and also with DMT or desmycosin (for structures, see Fig. 2), whereas, of the four compounds tested, tylosin was by far the least active in this respect (Fig. 5e). Since tylactone could not have been formed by degradation of the added compounds (unless, as seems unlikely, lactone ring hydroxylation could be reversed), polyketide metabolism appears to have been stimulated in the presence of the glycosylated macrolides.

Superimposed on such considerations is the possibility that disruption of *orf2** in strain SF01 might have exerted a polar effect on the expression of downstream genes, particularly *orf4** (*ccr*) which encodes crotonyl-CoA reductase. This enzyme has been proposed (Gandecha *et al.*, 1997) to participate in the conversion of acetoacetyl-CoA to butyryl-CoA, the extender unit

that provides carbons 5, 6, 19 and 20 of tylactone during polyketide metabolism, although other (perhaps less productive) routes to butyryl-CoA are not excluded. Whether the failure of strain SF01 to accumulate tylactone and/or the low level of tylosin production by strain SF02 are due to impaired expression of *ccr* may become clear when transcripts have been mapped and promoters located within the *orf1*–4** region. It is also possible that the performance of strain SF02 may have been adversely influenced by the state of the PCR-amplified *orf2** construct, which was not sequenced. Studies are in hand to assess these various possibilities together with the likely contributions of genetic regulatory elements to the control of polyketide and/or deoxyhexose metabolism in *S. fradiae*.

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