# Impact of thioesterase activity on tylosin biosynthesis in *Streptomyces fradiae*

Andrew R Butler, Neil Bate and Eric Cundliffe

**Background:** The polyketide lactone tylactone is produced in *Streptomyces fradiae* by the TylG complex of five multifunctional proteins. As with other type I polyketide synthases, the enzyme catalysing the final elongation step (TylGV) possesses an integral thioesterase domain that is believed to be responsible for chain termination and ring closure to form tylactone, which is then glycosylated to yield tylosin. In common with other macrolide producers, *S. fradiae* also possesses an additional thioesterase gene (*orf5*) located within the cluster of antibiotic biosynthetic genes. The function of the Orf5 protein is addressed here.

**Results:** Disruption of *orf5* reduced antibiotic accumulation in *S. fradiae* by at least 85%. Under such circumstances, the strain accumulated desmycosin (demycarosyl-tylosin) due to a downstream polar effect on the expression of *orf6*, which encodes a mycarose biosynthetic enzyme. High levels of desmycosin production were restored in the disrupted strain by complementation with intact *orf5*, or with the corresponding thioesterase gene, *nbmB*, from *S. narbonensis*, but not with DNA encoding the integral thioesterase domain of TylGV.

**Conclusions:** Polyketide metabolism in *S. fradiae* is strongly dependent on the thioesterase activity encoded by *orf5* (*tylO*). It is proposed that the TylG complex might operate with a significant error frequency and be prone to blockage with aberrant polyketides. A putative editing activity associated with TylO might be essential to unblock the polyketide synthase complex and thereby promote antibiotic accumulation.

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**Key words:** antibiotic production, macrolides, *Streptomyces fradiae*, thioesterase, tylosin biosynthesis

Received: **18 January 1999**
Revisions requested: **1 February 1999**
Revisions received: **8 February 1999**
Accepted: **9 February 1999**

Published: **8 April 1999**

**Chemistry & Biology** May 1999, **6**:287–292
http://biomednet.com/elecref/1074552100600287

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## Introduction

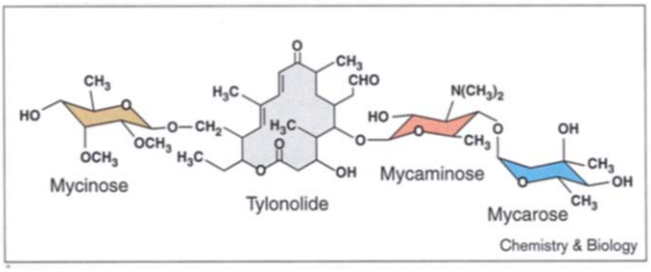
Macrolide antibiotics consist of polyketide lactones substituted with one or more 6-deoxyhexose(s). During tylosin production in *Streptomyces fradiae* [1] the primary product of polyketide synthase, tylactone, is subjected to ring hydroxylation and also acquires three sugar molecules (Figure 1). The polyketide synthase (PKS) complexes used in macrolide production are oligomers of giant multifunctional proteins, with discrete catalytic domains responsible for each step *en route* to the end product — the so-called 'assembly line' model. Thus, the erythromycin-producing PKS complex (otherwise known as EryA or 6-deoxyerythronolide B synthase; DEBS) of *Saccharopolyspora erythraea* consists of three multifunctional proteins (encoded by *eryAI-III*), each of which contains two complete sets (modules) of chain extension domains [2–5]. Polyketide intermediates are attached to the PKS as thioesters, and the enzyme catalysing addition of the final extender unit typically possesses a carboxy-terminal putative thioesterase (TE) domain that ostensibly terminates chain extension and cyclises the product. This role was confirmed when the *eryA* genes of *S. erythraea* were engineered so that the TE domain normally associated with module 6 of the DEBS3 protein was transplanted onto other DEBS modules. Such strains produced truncated, cyclised products, the sizes of which were determined by the location of the transplanted TE domain within the respective PKS enzymes [6,7]. These studies opened up the prospect of 'designer' macrolides, made possible through judicious relocation of PKS TE domains. But they also raised certain questions, because additional thioesterase genes are commonly found adjacent to the PKS-encoding genes in macrolide producers, for example, *orf5* in *S. fradiae* [8] and, co-incidentally, *orf5* in *S. erythraea* [9]. Because the putative product of *eryA orf5* was clearly unable to duplicate the function(s) of the PKS TE domain in the above experiments, is there any role at all for 'free' thioesterase activity in the production of erythromycin or other macrolides, such as tylosin? In earlier work [8], the product of the *tyl orf5* was deduced from DNA sequence analysis to be a thioesterase (Mr ~ 32,150) with 44% sequence identity to the product of *ery orf5*. Here we show that the free thioesterase activity of *S. fradiae* Orf5 does indeed play an important role in tylosin biosynthesis.

## Results

### The *tyl* gene cluster of *S. fradiae*

The tylosin biosynthetic gene cluster occupies about 85 kilobases (kb) of the *S. fradiae* genome [1] and includes over 40 genes, including and surrounding the

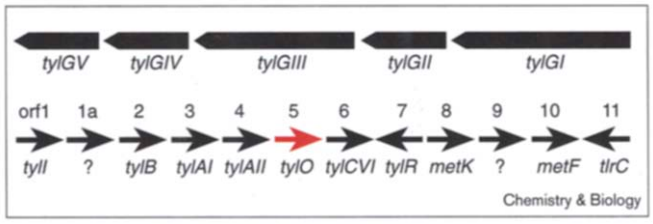
**Figure 1**




Structure of tylosin.

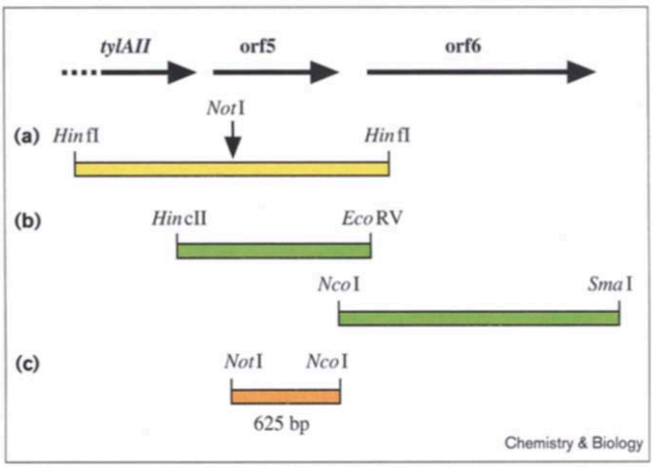
*tylG* (PKS-encoding) sub-cluster. Upstream of *tylG*, and divergent from it, are 11 such genes that are flanked by the resistance determinant *tlrC* and include the object of the present exercise, *orf5* (Figure 2). In the following experiments, the possible involvement of *orf5* in tylosin production was addressed through targeted gene disruption using the hygromycin-resistance cassette, Ωhyg [10]. The Ωhyg cassette is flanked by transcription terminators, however, and can cause downstream polar effects when inserted into operons. As we demonstrate below, that clearly happened in this case. Even though the earliest possible start codon for *orf6* is separated by a gap of 126 bp from the termination codon of *orf5*, the two genes are apparently co-transcribed. Elsewhere (A.R.B. and E.C., unpublished observations) we have shown that *orf6* is a mycarose-biosynthetic gene (designated *tylCVI*),

**Figure 2**




Part of the *tyl* cluster of *S. fradiae,* not drawn to scale. The five *tylG* genes occupy ~41 kb; *orfs 1–11* occupy about 14.8 kb. The divergent *tylGI* and *tylI* genes are separated by about 750 bp. The putative functions of the deduced gene products are: Orf1 (TylI), cytochrome P450 hydroxylase involved in oxidation of tylactone at C20; Orf1a, function unknown; Orf2 (TylB), 3-aminotransferase involved in mycaminose biosynthesis; Orf3 (TylAI), NDP-glucose synthase involved in the biosynthesis of all three tylosin sugars; Orf4 (TylAII), NDP-glucose dehydratase produces NDP-4-keto, 6-deoxyglucose, involved in the biosynthesis of all three tylosin sugars; Orf5 (TylO), thioesterase (subject of the present work); Orf6 (TylCVI), enzyme involved in mycarose biosynthesis; Orf7 (TylR), tylosin regulatory gene; Orf8 (MetK) *S*-adenosyl-methionine synthase; Orf9, a protein of unknown function; Orf10 (MetF) 5,10-methylenetetrahydrofolate reductase; Orf11 (TlrC), ATP-binding protein involved in resistance to tylosin. References: *orfs 1–5* [8]; *orfs 6* and *7* (A.R.B. and E.C., unpublished observations); *orfs 8–10* (B.S. DeHoff and P.R. Rosteck Jr., personal communication); *orf11* [27].

**Figure 3**




Generation and complementation of targeted gene disruptions in *S. fradiae*. **(a)** Ωhyg [10], the DNA fragment used to disrupt *orf5*, was inserted into the *Not*I site; **(b)** *tyl* DNA fragments used, together with *ermEp**, to complement the *orf5*-disrupted strain; **(c)** the probe used in hybridisation analysis to confirm the disruption of *orf5*. The *Bam*HI sites used to digest chromosomal DNA for hybridisation analysis (Figure 4) lie 511 bp to the right of the left-most *Hin*fI site (i.e. within *tylAII*) and 1925 bp to the right of the *Sma*I site (i.e. beyond *orf7*, which is not shown).

disruption of which causes the accumulation of demycarosyl-tylosin (desmycosin) in amounts equivalent to the levels of tylosin produced by the wild-type strain. Once this was realised, it became possible to rationalise the phenotypes of *orf5*-disrupted strains.

### Mutational analysis of *orf5* via gene transplacement

Targeted disruption of chromosomal *orf5* in *S. fradiae* was confirmed by Southern blot hybridisation analysis. Insertion of Ωhyg (2.3 kb) into plasmid-borne *orf5* (Figure 3a), followed by double recombination, resulted in replacement of chromosomal *orf5* by the disrupted gene. As a result, a 625 bp *Not*I–*Nco*I probe derived from *orf5* (Figure 3c) that normally hybridised to a 4.6 kb fragment in *Bam*HI digests of the wild-type genome (Figure 4a) found a 6.9 kb target in similar digests from the disrupted strain (Figure 4b). When the *orf5*-disrupted strain was fermented in tylosin-production medium, analysis of the products by high performance liquid chromatography (HPLC) revealed only two minor components absorbing at 282 nm (Figure 5a), one of which co-eluted with internal desmycosin standards. The other material has not been identified, although it was clearly distinguishable from tylosin, the major fermentation product of wild-type *S. fradiae* (Figure 5b). To confirm the reproducibility of this unexpected phenotype, 22 separate fermentations were carried out using two independently derived *orf5*-disrupted strains. Consistently, the yields of desmycosin were low but variable, ranging from the equivalent of about 15% of wild-type tylosin yields down to barely

detectable levels. Given such a pronounced influence on tylosin production, *orf5* was designated *'tylO'*.

### Complementation of the orf5-disrupted strain

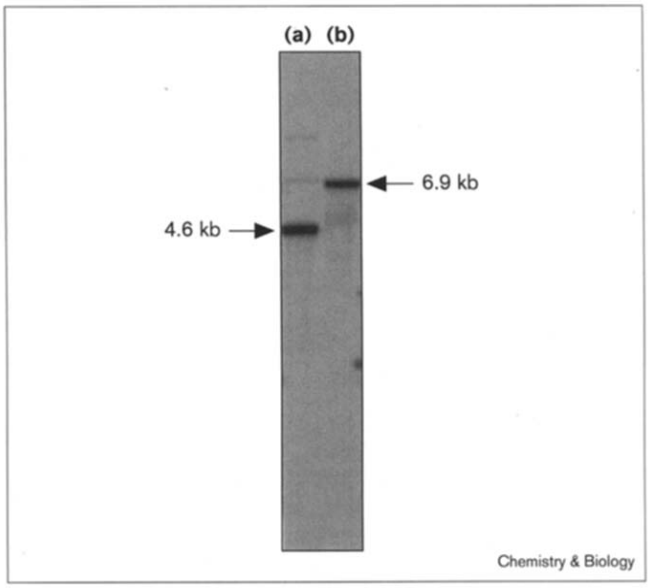
When an intact copy of *orf5* (together with the powerful, constitutive promoter *ermEp** [11]) was integrated into the chromosome of the *orf5*-disrupted strain, the yield of desmycosin was greatly enhanced but tylosin was still not produced (Figure 5c). This was the first indication that *orf6* is normally co-transcribed with *orf5*, so that disruption of *orf5* indirectly blocked mycarose biosynthesis and generated a phenotype that could not be rescued by re-introduction of *orf5* alone. Consistent with this model, equivalent levels of desmycosin were produced by the *orf6*-disrupted strain (data not shown) and by the *orf5*-disrupted strain complemented with *orf5* (Figure 5c). Moreover, when exogenous tylactone was added to fermentation cultures of the *orf5*-disrupted strain, it was efficiently converted to desmycosin (Figure 5d). When *orf6* was re-introduced into the *orf5*-disrupted strain and tylactone was added to fermentation broths, the resultant strain accumulated significant quantities of tylosin (Figure 5e) and resembled the *orf6*-disrupted strain complemented with the same DNA construct (data not shown). These results proved that disruption of *orf5* exerted a downstream polar effect on the expression of *orf6* and also revealed a specific involvement of *orf5* in polyketide metabolism.

Attempts to determine whether the integral TE domain from the TylGV polyketide synthase component (equivalent to DEBS3) could complement the *orf5*-disrupted strain were negative. When the terminal ACP–TE fragment of *tylGV* was integrated into the genome together with *ermEp**, the levels of desmycosin produced were not significantly elevated and remained within the range otherwise seen with this strain (data not shown). In contrast, macrolide production in the *orf5*-disrupted strain was substantially restored by complementation with a thioesterase gene from another macrolide producer. Thus, when *nbmB* from *Streptomyces narbonensis* (producer of narbomycin) was integrated into the genome together with *ermEp**, desmycosin production was restored to about 35–40% of the level seen following complementation with *orf5* (compare Figure 5f with 5c). In the *S. narbonensis* genome, *nbmB* lies immediately downstream of the *nbmA* (PKS-encoding) genes, that is, immediately adjacent to the DNA sequence encoding the integral TE domain (N.B. and E.C., unpublished observatons).

## Discussion

After completion of this work, we learned that the free thioesterase gene *pikV* has been deleted in *Streptomyces venezuelae*, the producer of pikromycin and methymycin [12]. In agreement with the present data, macrolide production was reduced by 90–95%, again emphasising the importance of free thioesterase activity for polyketide metabolism.

**Figure 4**

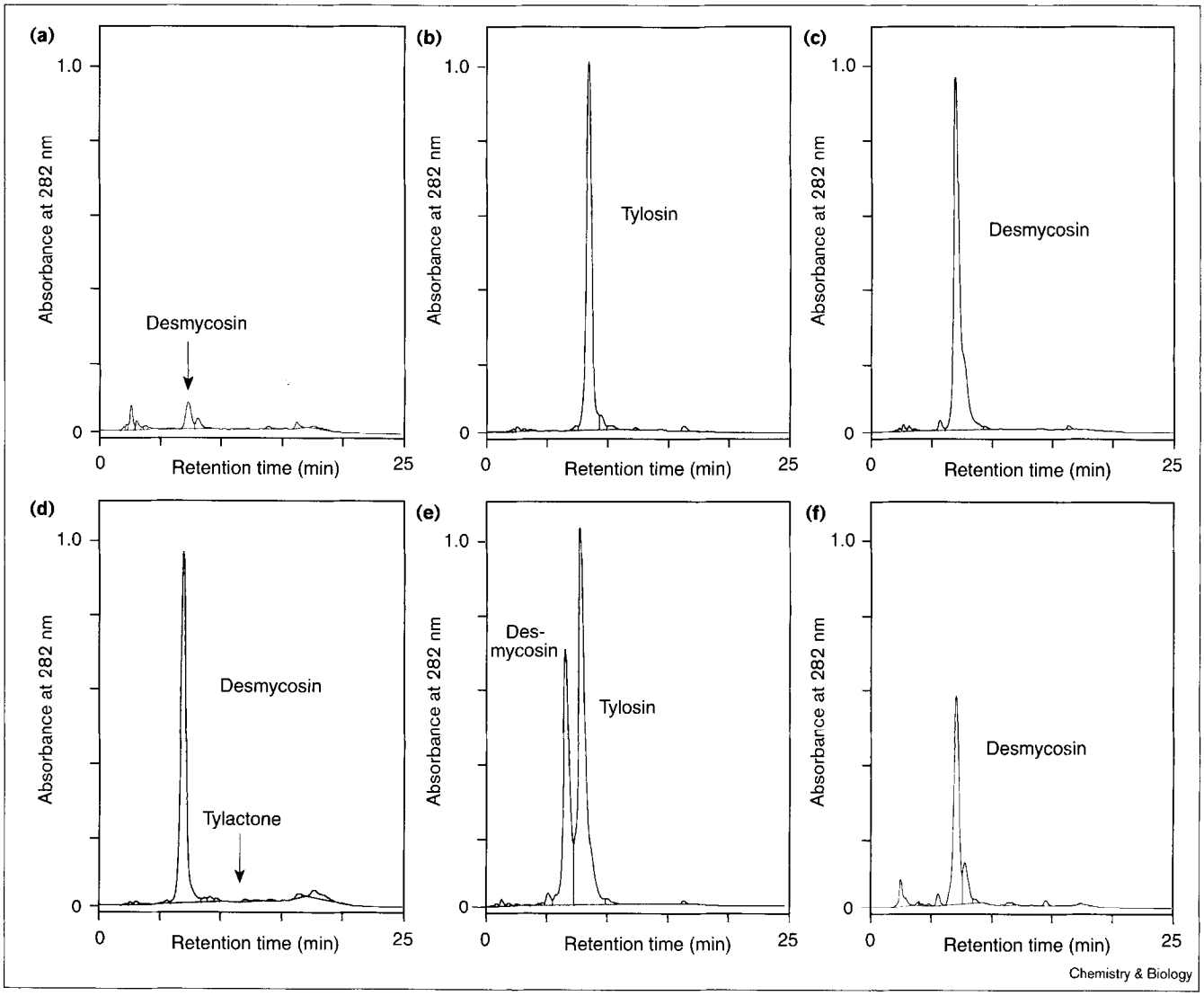



Confirmation of the targeted disruption of chromosomal *orf5*. *Bam*HI digests of *S. fradiae* genomic DNA were probed at high stringency with a 625 bp *Not*I–*Nco*I fragment derived from *tyl orf5* (Figure 3c). Genomic DNA fragments were from **(a)** wild type and **(b)** *orf5*-disrupted strains.

In attempting to rationalise the role of the *orf5* product, TylO, we call to mind a suggestion made to us several years ago (PF Leadlay, personal communication) that free thioesterase activity might fulfil an editing function during macrolide biosynthesis, by purging the polyketide synthase of aberrant materials that might otherwise block the expensive enzyme complex. Such putative editing enzymes might ignore normal polyketide intermediates and, if so, would be unlikely to substitute for the normal termination/cyclisation thioesterases in specifically deleted or engineered strains [6,7]. Editing enzymes might also lack chain-length specificity, so it was interesting that NbmB, which is normally associated with the synthesis of a 14-atom ring (narbonolide) in *S. narbonensis*, could function quite well in *S. fradiae* in which a 16-atom ring (octaketide) is produced. The observed variation in the levels of desmycosin produced by *orf5*-disrupted *S. fradiae* strains is also compatible with the editing hypothesis, and would even be demanded by it if errors in polyketide assembly were known to occur randomly. Although there is little direct evidence to support the editing hypothesis, we note that abberant polyketide precursors of tylactone have been observed (together with normal precursors) in certain mutants of *S. fradiae* [13] and could represent the products of editing.

Very recently, we learned that others have examined the role(s) of thioesterases during nonribosomal peptide biosynthesis in *Bacillus subtilis*. Like modular PKS enzymes, the synthetase complexes that produce peptide antibiotics also

**Figure 5**




Fermentation products from *S. fradiae* wild type and the *orf5*-disrupted strain. HPLC analysis of material produced by **(a)** *orf5*-disrupted strain, **(b)** wild-type, **(c)** *orf5*-disrupted strain complemented with intact *orf5*, **(d)** *orf5*-disrupted strain fed tylactone, **(e)** *orf5*-disrupted strain fed tylactone and complemented with an extra copy of *orf6* and **(f)** *orf5*-disrupted strain complemented with *nbmB* from *S. narbonensis*. Complementation involved the integration of DNA fragments (each accompanied by *ermEp**) into the chromosomal ϕC31 *attB* site, using pLST9828. The panels illustrate equivalent proportions of fermentation yields and are therefore directly comparable. Fermentation products represented by single component elution peaks were identified by 'spiking' samples with standard reference compounds prior to HPLC analysis.

consist of giant multifunctional proteins and, again, the components that catalyse incorporation of the final extender units have integral TE domains [14]. Moreover, as in macrolide-producing organisms, additional thioesterase genes are located adjacent to the peptide synthetase genes in the respective genomes [15–17]. Interestingly, deletion of DNA encoding the integral TE domain of the peptide synthetase, or the additional thioesterase gene, reduced the production of surfactin in *B. subtilis* by 97% or 84%, respectively [18]. Those results complement the present findings in emphasising the importance of free thioesterase activity for polyketide or nonribosomal peptide synthesis and, together with earlier observations [6,7], imply that free thioesterase activity cannot compensate for loss of the activity integral to PKS or peptide synthetase complexes. In accounting for their data, the authors [18] found it 'tempting to speculate' that the free thioesterase might carry out an editing function equivalent to that proposed here.

In conclusion, we suggest that the TylG polyketide synthase complex operates with a significant error frequency and that macrolide accumulation in *S. fradiae* depends

heavily on editing activity associated with the *orf5* product, TylO.

## Significance

Type I polyketide synthase (PKS) complexes, such as those involved in the synthesis of macrolide antibiotics, consist of multifunctional enzymes within which separate catalytic domains are sequentially responsible for each step *en route* to the end product. The enzyme that catalyses addition of the final extender unit usually possesses a carboxy-terminal thioesterase domain that terminates polyketide synthesis and cyclises the product. Macrolide biosynthetic gene clusters usually contain additional thioesterase genes of hitherto unknown function, however. Here, it is shown that free thioesterase activity is necessary for bulk accumulation of tylosin in *Streptomyces fradiae* and that at least 85% of antibiotic production is lost when the corresponding gene (*tyl orf5*; now designated '*tylO*') is inactivated. The equivalent thioesterase gene, *nbmB*, from *Streptomyces narbonensis* (producer of narbomycin) was able to substitute for *tylO*, but the terminal thioesterase fragment of the *tylGV* polyketide synthase gene could not. We propose that the TylO and NbmB proteins carry out an editing function, whereby aberrant polyketide precursors blocking the synthase complex can be removed to allow continued synthesis of normal products. It is concluded that antibiotic accumulation depends on a putative editing activity associated with TylO that is responsible for unblocking the polyketide synthase complex.

## Materials and methods

### *Bacterial strains and media*

*S. fradiae* T59235 (wild type; also known as C373.1) and its derivatives were maintained and propagated at 37°C on AS-1 agar [19] or at 30°C in tryptic soy broth (Difco). Plasmids were manipulated in *Escherichia coli* using standard protocols [20].

### *Genetic manipulation of* S. fradiae

To obviate restriction problems [21], DNA was introduced into *S. fradiae* in single-stranded form via conjugal transfer from *E. coli*, using vectors described elsewhere [22]. Targeted gene disruption was carried out using derivatives of pOJ260, a suicide plasmid that is unable to replicate in *Streptomyces* spp. Complementation analysis with cloned DNA involved a derivative of pSET152, which integrates into the ϕC31 *attB* site of the *Streptomyces* chromosome. The powerful constitutive promoter, *ermEp** (derived from *ermEp1* of *S. erythraea* by deletion of TGG from the putative −35 region [11]), was introduced into the multiple cloning region of pSET152, thereby generating pLST9828. DNA fragments for use in complementation analysis were introduced into pLST9828 downstream of *ermEp** to ensure the expression of possibly promotorless genes. Both pOJ260 and pLST9828 carry a gene that confers resistance to apramycin and gentamicin. For convenience, gentamicin was used for selection of *E. coli* transformants, whereas apramycin was used to select *Streptomyces* transconjugants. Plasmids were introduced into *S. fradiae* via conjugative transfer from *E. coli* S17-1 [23] essentially as described elsewhere [24].

### *Targeted gene disruption*

An 1825 bp *Hin*fI fragment containing *orf5* (Figure 3a) together with flanking DNA was ligated into pIJ2925 [25]. This DNA fragment possessed a unique *Not*I site close to the start of *orf5*, flanked on either side by approximately equal-sized arms of *tyl* DNA to facilitate double recombination into the *S. fradiae* genome. The plasmid was then linearised with *Not*I, 'end-filled' to generate blunt ends and ligated with the (blunt-ended) hygromycin B-resistance cassette, Ωhyg [10]. Disrupted *orf5* was then excised as a *Bgl*II fragment, ligated into the *Bam*HI site of pOJ260 and introduced into the tylosin-producing strain, *S. fradiae* C373.1, via conjugal transfer from *E. coli* S17-1. Following initial selection on hygromycin B (75 μg $ml^{-1}$), transconjugants were screened for sensitivity to apramycin (25 μg $ml^{-1}$) to identify double recombinants in which gene transplacement had replaced chromosomal *orf5* with the disrupted gene copy.

### *Hybridisation analysis*

To confirm the disruption of chromosomal *orf5* in *S. fradiae*, Southern blot hybridisation analysis was performed on *Bam*HI digests of genomic DNA isolated from candidate disrupted strains and from the wild-type strain. Restriction fragments were resolved on 0.8% agarose gels and transferred to Hybond N membranes (Amersham International, UK), which were then probed at high stringency (0.5 × SSC; 65°C) using a 625 bp *Not*I–*Nco*I fragment (Figure 3c). The probe was [$^{32}$P]-labelled by random-hexamer priming.

### *Complementation of the* orf5*-disrupted strains*

DNA fragments containing *orf5* or *orf6* (Figure 3b) were ligated into the integrating vector pLST9828, downstream of *ermEp**, and introduced into *S. fradiae* by conjugal transfer from *E. coli* S17-1. Attempts were also made to rescue the *orf5*-disrupted strain with the thioesterase gene (*nbmB*) from *S. narbonensis*, a 1.5 kb *Nco*I–*Not*I fragment, (N.B., unpublished observations) and with a 1.9 kb *Mlu* I fragment encoding the terminal acyl carrier protein (ACP) and thioesterase (TE) domains derived from the polyketide synthase gene *tylGV* (accession number U78289). Again, these DNA fragments were introduced into *S. fradiae* using pLST9828.

### *Tylosin production fermentation*

Conical flasks (100 ml capacity) containing 30 ml of prefermentation medium were inoculated with 100 μl of *S. fradiae* mycelial fragments and incubated at 28°C with rotary shaking at 300 rpm for 3 days. Prefermentation medium (pH adjusted to 7.8 with NaOH) contained per litre: 10 g corn steep liquor, 5 g yeast extract, 5 g soya bean meal, 3 g calcium carbonate and 5 ml 70% methyl oleate (Aldrich). Then, 5 ml portions of each culture were used to inoculate 50 ml batches of tylosin-production medium MM-1 [26], in 250 ml conical flasks, and incubation was continued at 28°C with rotary shaking for a further 7 days. MM-1 medium contained per litre: 17.5 g monosodium glutamate, 5 g glucose, 5 g betaine HCl, 5 g $MgSO_4$, 3 g $CaCl_2$, 3 g ferric ammonium citrate, 2.3 g $K_2HPO_4$, 2 g NaCl, 10 mg $ZnSO_4$, 1 mg $CoCl_2$, 20 ml 70% methyl oleate, pH adjusted to 7.0 with KOH. 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 redissolved in 1 ml of HPLC grade chloroform. Gene transplacement is a stable event and this, together with the use of integrative plasmids for complementation, eliminated the need for antibiotic selection during fermentation.

### *Bioconversion of tylosin precursors*

Fermentation cultures were supplemented with intermediates of the tylosin biosynthetic pathway (10 mg each) following 2 days incubation in MM-1 medium. Incubation was then continued for a further 5 days before the cultures were extracted and the products analysed by HPLC.

### *Analysis of fermentation products*

Fermentation products were analysed using reverse-phase HPLC, essentially as described previously [13], using a 3.9 × 300 mm C18 μBondapak column protected by a C18 μBondapak guard column (Waters Associates, Inc., Milford, Mass.). Chloroform extracts were applied to the column in 0.3% (w/v) ammonium formate (pH 4.0 with HCl) containing 50% (v/v) methanol and eluted using 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. Identification of fermentation products was facilitated by the use of tylosin and desmycosin as internal standards; these were added to selected extracts immediately prior to HPLC analysis.

## Acknowledgements

This work was supported by project grants GR/J43219 and 91/T08195 from BBSRC, and by Eli Lilly and Co., Indianapolis.

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