Feedback control of polyketide metabolism during tylosin production

Andrew R. Butler,[†] Simon A. Flint[†] 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

Tylosin is produced by *Streptomyces fradiae* via a combination of polyketide metabolism and synthesis of three deoxyhexose sugars, of which mycaminose is the first to be added to the polyketide aglycone, tylactone (protylonolide). Previously, disruption of the gene (*tylMI*) encoding attachment of mycaminose to the aglycone unexpectedly abolished accumulation of the latter, raising the possibility of a link between polyketide metabolism and deoxyhexose biosynthesis in *S. fradiae*. However, at that time, it was not possible to eliminate an alternative explanation, namely, that downstream effects on the expression of other genes, not involved in mycaminose metabolism, might have contributed to this phenomenon. Here, it is shown that disruption of any of the four genes (*tylMI-III* and *tylB*) specifically involved in mycaminose biosynthesis elicits a similar response, confirming that production of mycaminosyl-tylactone directly influences polyketide metabolism in *S. fradiae*. Under similar conditions, when mycaminose biosynthesis was specifically blocked by gene disruption, accumulation of tylactone could be restored by exogenous addition of glycosylated tylosin precursors. Moreover, certain other macrolides, not of the tylosin pathway, were also found to elicit qualitatively similar effects. Comparison of the structures of stimulatory macrolides will facilitate studies of the stimulatory mechanism.

Keywords: mycaminose biosynthesis, polyketide, *Streptomyces fradiae*, tylactone, tylosin production

INTRODUCTION

Tylosin, a macrolide antibiotic produced by *Streptomyces fradiae*, consists of a polyketide lactone substituted with three 6-deoxyhexose sugars. The TylG polyketide synthase (PKS) produces and cyclizes the aglycone (tylactone, also known as protylonolide), which is subsequently oxidized at C-20 and C-23 to generate tylonolide and concurrently substituted with three deoxyhexose sugars (D-mycaminose, 6-deoxy-D-allose and L-mycarose; Fig. 1). These are added in a preferred but not obligatory order, although (importantly, in the present context) mycaminose always goes on first. Finally, the deoxyallose moiety is converted to D-mycinose via stepwise bis-O-methylation, thereby generating tylosin (for a review, see Baltz & Seno, 1988).

[†] These authors made equal contributions to this work.

Abbreviations: OMT, O-mycaminosyltylonolide; PKS, polyketide synthase.

Elucidation of the biochemical route to tylosin relied heavily on blocked *S. fradiae* mutants, which displayed nine distinct phenotypes in cross-feeding analysis and eventually allowed 13 genetic loci (*tylA-M*) to be mapped by complementation with cloned fragments of *tyl* DNA (Fishman *et al.*, 1987; Beckmann *et al.*, 1989).

More recently, the entire *tyl* gene cluster (~85 kb) has been sequenced (Szoke *et al.*, 1989; Rosteck *et al.*, 1991; Merson-Davies & Cundliffe, 1994; Gandechea & Cundliffe, 1996; Gandechea *et al.*, 1997; Wilson & Cundliffe, 1998; Bate & Cundliffe, 1999; Bate *et al.*, 1999, 2000; Fouces *et al.*, 1999; Wilson & Cundliffe, 1999; see also GenBank accession no. U78289), revealing five mega genes (total ~41 kb) that encode the TylG PKS, flanked by sugar biosynthetic genes, ancillary genes, regulatory elements and resistance determinants (for a review, see Cundliffe, 1999). The present work is focused on the four genes involved in mycaminose biosynthesis, three of which (*tylMI-III*) are located immediately downstream of the *tylG* group, about 45 kb

Fig. 1. Structures of tylactone (protylonolide), OMT and tylosin.

Fig. 2. Tylosin-biosynthetic gene cluster. Not drawn to scale. The bar represents the entire *tyl* cluster (~85 kb) showing the various *tyl* loci flanked by the resistance genes *tlrB* and *tlrC*. The *tylG* locus represents five mega genes (~41 kb) encoding the PKS complex. Upstream of *tylG* lie 12 ORFs (including *tlrC*), seven of which are shown here. Downstream of *tylG* lie 26 ORFs including *tlrB*, five of which are shown here. These are designated 1*, 2*, etc. to distinguish them from ORFs on the other side of *tylG*. Genes analysed here are shown as grey arrows and their locations within the cluster are indicated on the bar.

distant from their functional partner (*tylB*), which curiously lies on the other side of *tylG* (Fig. 2). Although the *tylL* locus was originally mapped to the region occupied by *tylMI–III* (cited in Fishman *et al.*, 1987) and although a *tylL* mutant was found to harbour an opal mutation in *tylMII* (Clark, 1997), the TyL phenotype (i.e. failure to synthesize or add any of the three tylosin sugars) cannot readily be explained by impairment of the function of any one of the *tylM* genes and, therefore, probably results from multiple mutations. For that reason, the ‘*tylL*’ locus is not represented in Fig. 2.

The present work arises from earlier studies (Fish & Cundliffe, 1997) in which *tylMII* (inappropriately referred to as ‘*tylM2*’ at that time) was disrupted in the genome of *S. fradiae*. This gene (*orf2** in the systematic

nomenclature, see Fig. 2) encodes mycaminosyl-transferase (Gandecha *et al.*, 1997), the enzyme that normally adds the first sugar during tylosin production. It was therefore expected that the *tylMII*-disrupted strain, SF01, would be unable to produce tylosin or any of its glycosylated precursors, but would still retain the ability to bioconvert such precursors to tylosin. Such proved to be the case but, surprisingly, strain SF01 did not accumulate tylactone, except when fed exogenously with glycosylated macrolides such as O-mycaminosyltylonolide (OMT). In contrast, when genes involved in the biosynthesis or addition of mycinose or mycarose were disrupted (Wilson & Cundliffe, 1998; Bate *et al.*, 2000), the predicted products (demycinosyl-tylosin and demycarosyl-tylosin, respectively) accumulated. Although no unequivocal explanation was offered for

the results obtained with strain SF01, it seemed possible that tylactone production was somehow stimulated (mechanism unspecified) by glycosylated macrolides. As an added complication, however, impairment of polyketide metabolism in strain SF01 in the absence of glycosylated compounds might not have resulted exclusively from disruption of *tylMII* per se, but might have involved possible downstream effects – particularly on expression of *orf4** (*ccr*). This gene encodes crotonyl-CoA reductase with a likely role in production of butyryl-CoA, the 4-carbon extender substrate that provides carbons 5, 6, 19 and 20 of tylactone (Gandecha *et al.*, 1997). The aim of the present work was to resolve this matter and to examine further the apparent influence of glycosylated macrolides on polyketide metabolism in *S. fradiae*.

METHODS

Bacterial strains and genetic manipulations. *S. fradiae* T59235 (also known as C373.1; referred to here as wild-type) and derivatives thereof were propagated at 30 °C in tryptic soy broth (TSB; Difco) in sprung flasks or at 37 °C on AS-1 agar plates (Wilson & Cundliffe, 1998) and maintained at –70 °C as mycelial fragments following addition of DMSO (5%, v/v; final concentration) to TSB-grown cultures. Plasmids were manipulated in *Escherichia coli* DH5 α using standard protocols (Sambrook *et al.*, 1989). DNA was introduced into *S. fradiae* by conjugal transfer from *E. coli* S17-1 as described elsewhere (Fish & Cundliffe, 1997) using pOJ260 (Bierman *et al.*, 1992) and pLST9828 (Butler *et al.*, 1999). pOJ260 is incapable of replication in *Streptomyces* spp. and was used for targeted gene disruption. pLST9828, used for complementation analysis, integrates with high efficiency into the chromosomal Φ C31 *attB* site and contains a powerful constitutive promoter, *ermEp** (Bibb *et al.*, 1994), for expression of inserted DNA. Both vectors carry apramycin-resistance markers.

Targeted gene disruption via gene transplacement. Fragments of *tyl* DNA, each containing a specific mycaminosynthetase gene, were ligated into pIJ2925 (Janssen & Bibb, 1993). Each of these DNA fragments contained a central, unique restriction site located within the cloned gene, into which the hygromycin B resistance cassette, Ω hyg (2.3 kb, with flanking transcriptional terminators; Blondelet-Rouault *et al.*, 1997), was inserted via blunt-end ligation. The complete insert was then excised using *Bgl*III sites which flank the pIJ2925 polylinker and ligated into the *Bam*HI site of pOJ260. Following conjugal transfer into *S. fradiae*, transconjugants were selected on hygromycin B and then screened for apramycin sensitivity (Fish & Cundliffe, 1997) to identify those in which chromosomal target genes had been replaced with disrupted constructs via double recombination. The gene disruption constructs were assembled as follows.

Disruption of *tylMIII* (*orf1).** A 2126 bp *Age*I fragment from cosmid pMOMT4 (Beckmann *et al.*, 1989) was cloned in pIJ2925. Insertion of Ω hyg at the unique *Mlu*I site interrupted the sequence encoding TylMIII (normally 423 amino acids) 291 bp downstream of the proposed translational start.

Disruption of *tylMII* (*orf2).** A strain disrupted in *tylMII* and designated SF01 was already available for analysis (Fish & Cundliffe, 1997).

Disruption of *tylMI* (*orf3).** A 1546 bp *Hinc*II fragment from pMOMT4 was ligated into pIJ2925 and the unique *Msc*I site

provided a disruption site for *tylMI*, 256 bp downstream of the proposed translational start. The deduced length of intact TylMI is 254 amino acid residues.

Disruption of *ccr* (*orf4).** A 1911 bp *Sty*I fragment from pMOMT4 was ligated into pIJ2925 and Ω hyg was inserted into the *Bsa*AI site. This interrupted the *ccr* coding sequence 168 bp downstream of the translational start. The deduced length of intact Ccr is 449 amino acid residues.

Disruption of *tylB* (*orf2*). A 2251 bp *Hinc*II fragment from pSET552 (Beckmann *et al.*, 1989) was ligated into pIJ2925 and Ω hyg was inserted at a unique *Bst*EII site, thereby disrupting the *tylB* coding sequence 638 bp downstream from its putative translational start. The truncated gene product was predicted to be 212 amino acids in length compared with 388 amino acid residues for the intact protein.

Authentication of disrupted strains. Southern blot hybridization analysis, using the Boehringer Mannheim DIG High Prime DNA Labelling and Detection Starter Kit II, was used to confirm each of the gene disruptions using probes specific to the respective target genes.

Complementation of disrupted strains. To compensate for possible effects on the expression of downstream genes in the *tylB*-disrupted strain, a 3118 bp *Mlu*I–*Eco*RV fragment of *tyl* DNA from pSET552 (together with *ermEp**) was inserted into the chromosomal Φ C31 *attB* site using pLST9828. The complementing DNA fragment contained the co-directional genes *tylAI*, *tylAII* and *tylO* together with flanking sequences (139 bp upstream of *tylAI* and 144 bp downstream of *tylO*) and was oriented favourably for control by *ermEp**.

Tylosin production fermentation and metabolite analysis. Fermentation and HPLC analysis of the products was carried out as described elsewhere (Butler *et al.*, 1999) except that, for convenience, 0.5% (w/v) corn steep solids (Sigma) replaced 1.0% (v/v) corn steep liquor in pre-fermentation media used for some later fermentations. Control experiments revealed no detectable impact of this change on extracted fermentation products. In bioconversion studies, tylosin precursors or other macrolides (10 mg each, dissolved in 100 μ l DMSO) were added to fermentations (50 ml cultures) after 2 d and incubation was continued for a further 5 d before analysis. DMSO was added to control fermentations.

RESULTS AND DISCUSSION

Disruption of *ccr* (*orf4**)

This was achieved via targeted gene replacement (involving double recombination) using the hygromycin B resistance cassette, Ω hyg (Blondelet-Rouault *et al.*, 1997). Having confirmed the disruption ('knockout') by Southern analysis, the resultant '*ccr*-KO' strain was fermented and the products were examined by HPLC (Fig. 3). Since the disrupted strain still produced significant, although reduced, amounts of tylosin, the *ccr* gene product was evidently not essential for tylosin production under these conditions. Interestingly, the *ccr*-specific probe that was used to confirm the authenticity of the knockout strain also found a second hybridization target at high stringency (presumably, another *ccr* gene) elsewhere in the *S. fradiae* genome. We do not know whether any requirement for Ccr activity during tylosin production under these conditions can be satisfied (partially or otherwise) by the product of the

Fig. 3. Fermentation products from *S. fradiae* wild-type and the *ccr*-disrupted strain. HPLC analysis of material produced by (a) wild-type and (b) *ccr* (*orf4**)-disrupted strain.

Fig. 4. Biosynthetic route to mycaminosyl. The pathway represented here begins with the two reactions common to the synthesis of all three sugar moieties of tylosin.

'other' gene but, in any event, impaired expression of *orf4** could not have accounted for the failure of strain SF01 to accumulate ty lactone in earlier studies.

Disruption of mycaminosyl-biosynthetic genes

Targeted gene replacement, again using Ω hyg, was employed to disrupt (separately) *tylMI*, *tylMIII* and *tylB* in the genome of *S. fradiae* and the respective disruptions were confirmed by hybridization analysis using probes specific to each of the target genes (data not shown). The latter have been proposed (see Cundliffe, 1999) to encode methyltransferase (product of *tylMI*), putative 3,4-

isomerase (product of *tylMIII*) and aminotransferase (TylB) activities, which, together with mycaminosyl-transferase (encoded by *tylMII*), constitute the entire complement of enzymes specifically required for the biosynthesis and addition of mycaminosyl during tylosin production (Fig. 4). When fermentation products from the three disrupted strains (designated *tylMI*-KO, *tylMIII*-KO and *tylB*-KO) were analysed by HPLC, the results were similar and resembled those previously observed with strain SF01 (*tylMII*-KO). None of these strains produced tylosin (confirming for the first time that *orf1** is indeed a 'tyl' gene), nor did they accumulate ty lactone unless a glycosylated macrolide (here, OMT)

Fig. 5. OMT-induced tylactone biosynthesis in the *tylB*-disrupted strain. HPLC analysis of material produced during fermentation of: (a) *tylB*-disrupted strain; (b) *tylB*-disrupted strain complemented with *tylAI*+*tylAII*+*tylO*; (c) *tylB*-disrupted strain complemented with *tylAI*+*tylAII*+*tylO* and fed 10 mg OMT.

was added exogenously to the fermentation medium. Only data obtained with the *tylB*-KO strain are shown here (Fig. 5). Again, it was necessary to control for possible downstream effects resulting from disruption of the target gene. Thus, *tylAI* and *tylAII* (inappropriately designated *tylA1* and *tylA2* earlier; Merson-Davies & Cundliffe, 1994) lie immediately downstream of *tylB* (Fig. 2) and encode enzymes common to the biosynthesis of all three tylosin sugars (Fig. 4). Also, and more importantly in the present context, *tylO* encodes an 'editing' thioesterase that greatly affects the level of tylactone production (Butler *et al.*, 1999). However, when a DNA fragment containing [*tylAI*–*tylAII*–*tylO*] under control of the strong constitutive promoter *ermEp** was integrated into the genome of the *tylB*-KO strain, the resultant organism remained unable to accumulate tylactone except in the presence of exogenous OMT (Fig. 5b, c). Under the latter conditions, the added OMT was converted to desmycosin (demycarosyl-tylosin) and not to tylosin. This was not unexpected since *orf6* (downstream of, and co-directional with, *tylB*) had previously been characterized as a mycarose-biosynthetic gene, i.e. *tylCVI* (Bate *et al.*, 2000). Evidently, *orf2* (*tylB*) and *orf6* (*tylCVI*) are normally co-transcribed.

Two conclusions follow from these data. Firstly, failure of these various disrupted strains to accumulate tylactone was a direct consequence of the impairment of mycaminosyl biosynthesis and did not result indirectly from effects on the expression of genes downstream of the respective knockout targets. Secondly, tylosin precursors such as OMT (and, to a lesser extent, tylosin itself; see Fish & Cundliffe, 1997) exert a positive influence on tylactone production in the various knockout strains. Presumably, in wild-type *S. fradiae*,

tylactone biosynthesis proceeds at only minimal levels unless the product gets glycosylated, in which case a trickle becomes a flood.

Effects of macrolides other than tylosin precursors

It was argued previously (Fish & Cundliffe, 1997) that accumulation of tylactone in strain SF01 after feeding with OMT could not be due to degradation of OMT, since cleavage of the latter would release tylosin – not tylactone. This point was reinforced in the present work when tylactone accumulation in the *tylMII*-disrupted strain, SF01, was triggered by OMT at inputs too low to be detected by HPLC following re-extraction from fermentation cultures (data not shown). It was also observed that accumulation of tylactone in this strain could be provoked, albeit with differing efficiencies, by glycosylated macrolides other than tylosin precursors, including rosaramicin and spiramycin, but not by chalcomycin, erythromycin or carbomycin (Fig. 6). Although these data do not reveal the structural features needed to elicit this effect, it is clear that the accumulated tylactone could not have been derived from the added compounds.

Summary and conclusions

The present data establish that tylactone biosynthesis in *S. fradiae* is controlled by glycosylated macrolides, although it is not clear whether stimulation of polyketide metabolism by OMT, etc. involves activation or derepression of *tylG* expression. In other work, tylactone production was shown to require expression of the regulatory gene *tylR*, disruption of which prevented *S. fradiae* from accumulating tylactone even in the presence

Fig. 6. Stimulation of ty lactone synthesis in the *tylMII*-disrupted strain (SF01) by exogenous glycosylated macrolides other than tylosin precursors. HPLC analysis of material produced during fermentation of the *tylMII*-disrupted strain fed with 10 mg of: (a) rosamycin; (b) chalomycin; (c) erythromycin; (d) carbomycin; (e) spiramycin.

of exogenously added OMT (Bate *et al.*, 1999). Since *tylR* appears to encode a positive regulator, it is possible that efficient transcription of *tylG* requires the TylR protein together with glycosylated macrolides as cofactors. Moreover, expression of *tylR* is also needed for deoxyhexose metabolism (and, in particular, for mycaminose biosynthesis) in *S. fradiae*, since ty lactone was not glycosylated when fed exogenously to a *tylR*-disrupted strain (Bate *et al.*, 1999). These data could be reconciled if TylR were to regulate the synthesis of mycaminose (and the other tylosin sugars?) in concert with ty lactone production, in which case it would be instructive to know whether OMT also influences deoxyhexose metabolism in *S. fradiae*, perhaps by binding to TylR.

In conclusion, the manner in which glycosylated macrolides stimulate ty lactone production in *S. fradiae* is not yet understood. Future studies will centre on the (presumed) DNA-binding properties of the TylR protein and on attempts to identify OMT-binding regulatory protein(s).

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