Influence of Ancillary Genes, Encoding Aspects of Methionine Metabolism, on Tylosin Biosynthesis in *Streptomyces fradiae*

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The tylosin-biosynthetic (tyl) gene cluster of *Streptomyces fradiae* contains ancillary genes that encode functions normally associated with primary metabolism. These can be disrupted without loss of viability, since equivalent genes (presumably used for ‘housekeeping’ purposes) are also present elsewhere in the genome. The tyl cluster also contains two genes that encode products unlike any proteins in the databases. Two ancillary genes, metF (encoding N5,N10-methylenetetrahydrofolate reductase) and metK, encoding S-adenosylmethionine synthase, flank one of the ‘unknown’ genes (orf9) in the tyl cluster. In a strain of *S. fradiae* in which all three of these genes were disrupted, tylosin production was reduced, although this effect was obscured in media supplemented with glycine betaine which can donate methyl groups to the tetrahydrofolate pool. Apparently, one consequence of the recruitment of ancillary genes into the tyl cluster is enhanced capacity for transmethylation during secondary metabolism.

Tylosin, produced by *Streptomyces fradiae*, is a macrolide antibiotic consisting of a polyketide lactone substituted with three 6-deoxyhexose sugars. The biosynthetic route to tylosin was revealed by tracer incorporation plus bioconversion analysis and depended heavily on studies involving mutants of *S. fradiae* blocked in tylosin production1~5). Thus, the TylG polyketide synthase (PKS) produces and cyclizes the aglycone (tylactone, also known as protylonolide), which is subsequently oxidised at C20 and C23 to generate tylonolide. Concurrently with ring oxidation, the polyketide lactone is substituted with sugars (D-mycaminose, 6-deoxy-D-allose and L-mycarose) in a preferred, but not obligatory, order although mycaminose is always added first. Finally, the deoxyallose moiety is converted to D-mycinose via stepwise bis-O-methylation, thereby generating tylosin.

When thirteen tyl loci were mapped by complementation of blocked mutants with cloned DNA6~7), they all lay between two resistance genes, *tlrB* and *tlrC*, that were separated in the genome by about 85 kb (Fig. 1). It therefore seemed plausible that most, or all, of the tylosin-biosynthetic genes might be clustered within that region of the *S. fradiae* genome (reviewed in Ref. 8). That notion has since been confirmed by sequence analysis. Ten or more years ago, DNA adjacent to *tlrB* and *tlrC* was sequenced9,10) at Lilly Research Laboratories, Indianapolis, although not all the data were made public (B. S. DeHoff & P. R. Rosteck, Jr., personal communication). Those workers also sequenced a contiguous piece of DNA (about 41 kb: accession number U782890), located between *tlrB* and *tlrC*, encoding the 5 giant multimodular proteins that comprise the TylG PKS complex. More recently, the rest of the DNA between *tlrB* and *tlrC* has been sequenced in this laboratory11~18) revealing that the tylG PKS genes are flanked by sugar biosynthetic genes, ancillary genes, regulatory elements and, finally, the resistance determinants (for a review, see Ref. 20).

The work presented here completes the sequence of the tyl cluster upstream of tylG (Fig. 1), filling the gap between tylR and tlrC, and describes three open reading frames (orfs 8~10) two of which represent genes involved in methionine metabolism. These genes were first sequenced privately at Lilly Research Laboratories (B. S. DeHoff and P. R. Rosteck, Jr., personal communication). In the present...
Fig. 1. The 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 trhB and trhC. The tylG locus represents 5 mega genes (~41 kb) encoding the polyketide synthase complex. Upstream of tylG lie 12 orfs (including trhC) labelled 1, la~11. Genes analysed here are shown as GREY arrows and their position within the cluster is indicated on the bar.

work, orfs 8~10 have been specifically disrupted and the consequences for tylosin production have been analysed.

Materials and Methods

Bacterial Strains, Plasmids and Growth Conditions

*S. fradiae* T59235 (also known as C373.1, and referred to here as wild type) was maintained and propagated at 37°C on AS-1 agar (14) or at 30°C in tryptic soy broth (Difco). Plasmids were manipulated in *Escherichia coli* using standard protocols (21). DNA was introduced into *S. fradiae* via conjugal transfer from *E. coli* as described elsewhere (22) using pOJ260. The latter (23) is a suicide vector, unable to replicate in *Streptomyces* spp., and was used for targeted gene disruption.

DNA Manipulation and Sequencing

The *S. fradiae* tyl DNA sequenced here was obtained from pSET504 (27). An 8kb *PstI* fragment from pSET504 was subcloned in pJ2925 (24) to create pLST98253 from which ~3.75 kb of sequence was generated (see Fig. 2). Both strands of the DNA were sequenced independently in overlapping fashion by primer walking. This was done on an ABI 377 automated DNA sequencer using fluorescent dye-labelled dideoxynucleotide chain terminators and *Taq* or *Taq* FS polymerase. DNA sequences together with the corresponding chromatograms were imported into Seq Ed v 1.0.3, edited and then aligned using AUTO ASSEMBLER (Applied Biosystems). Sequences were analysed using the University of Wisconsin GCG software programmes. Open reading frames were identified using CODONPREFERENCE, BLASTX and 6 frame translation with DNA STRIDER. Deduced products were matched against databases using BLASTP and Peptide Mass was used to calculate Mr values. Sequence motifs indicative of function were sought within the deduced protein sequences using ProfileScan (Swiss Institute for Experimental Cancer Research).

Targeted Gene Disruption via Gene Transplacement

A 3882 bp *BglII-StuI* fragment containing tyl orfs 8, 9 and 10 together with flanking DNA was excised from pLST98253 and inserted into pJ2925 (Fig. 2). A *MscI-NotI* fragment (containing orf9 plus portions orfs 8 and 10) was then released and replaced with the 2.3 kb hygromycin B-resistance cassette, Ωhyg (25) via blunt-end ligation. The 2029 bp deletion thus generated removed the whole of orf9 together with significant portions of the flanking orfs, 8 and 10 (Fig. 2), leaving only the first 913 bp of orf8 (normally 1224 bp) and the terminal 328 bp of orf10 (intact length, 918 bp). The disrupted block of tyl DNA was then excised as a *BglII* fragment (using a *BglII* site located in the multicloning region to the right of the *StuI* site in Fig. 2) and ligated into the *BamHI* site of pOJ260, before the resultant plasmid was introduced into *S. fradiae* via conjugal transfer from *E. coli*. 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 orfs 8~10 had been replaced in the chromosome by the deleted and disrupted sequence. Since Ωhyg is flanked by transcriptional terminators, the generation of
A 3882 bp BglII-StuI fragment of tyl DNA, originally from pSET504, was inserted into pIJ2925. A 2029 bp Mscl-NotI fragment was then excised and replaced with the 2.3 kb hygromycin B-resistance cassette, Ωhyg, via blunt-end ligation leaving flanking tyl DNA arms of approximately equal length. The disrupted block of tyl DNA was then excised as a BglII fragment (using a BglII site located in the pIJ2925 multi-cloning region to the right of the StuI site) and ligated into the BamHI site of pOJ260. The resultant plasmid was introduced into S. fradiae via conjugal transfer from E. coli.

Fermentation Analysis
Growth of S. fradiae in tylosin production medium MM-1 and HPLC analysis of products are described elsewhere. Gene transplacement is a stable event and this eliminated the need for antibiotic selection during fermentation.

Results and Discussion
DNA Sequence Analysis
The tyl DNA analysed here (approximately 3.75 kb) overlapped previously published sequences. At the left hand end in the orientation of Fig. 2, the present sequence extended beyond the BamHI site that lies between the divergent tylR and orf8, and overlapped by 50 bp sequence previously deposited (accession number U08223)\(^{17}\). At the right hand end, the present sequence extended into the resistance gene tlrC\(^{10}\) and overlapped by 50 bp sequence generated elsewhere (accession number M57437). Between these regions of known sequence, were found three codirectional open reading frames (accession number AY045759), divergent from tylR and convergent with tlrC (Fig. 2). These were designated orfs 8~10 in accordance with the numbering scheme previously used for genes located on this side of tylG (see Fig. 1). Each of these three orfs demonstrates the biased codon usage typical of actinomycete genes, with G or C occupying the third position in ≈90% of the codons.

orf8 (metK)
This gene is divergent from tylR and is separated from it by 981 bp. The deduced product of orf8 is a protein of 407 amino acid residues (Mr 43,400) with convincing end-to-end sequence similarity to a large number of S-adenosylmethionine (SAM) synthases, both authentic and hypothetical. The closest matches (88% and 87% identity respectively), were to the deduced MetK proteins from Streptomyces spectabilis (AF117274) and Streptomyces coelicolor (AL159139). The SAM synthases comprise a family of highly conserved proteins which all share a pair of signature sequences: a hexapeptide believed to be
involved in ATP-binding\cite{27} and a glycine rich nonapeptide of unknown significance, both of which are present in the deduced sequence of Orf8 (Fig. 3). These enzymes catalyse ATP-dependent synthesis of SAM from methionine and afford the only route to the principal methyl donor in intermediary metabolism. The presence of a metK gene in the tylosin biosynthetic cluster is readily rationalised since all 3 of the tylosin sugars are methylated and methyltransferase activities are encoded by 3 of the 4 tylosin resistance (tlr) genes thus far characterized in S. fradiae.

orf10 (metF)

This gene, linked to orf9 in terminally overlapping fashion by the sequence GTGA, is deduced to encode a protein of 305 amino acid residues (Mr 33,100). The product showed convincing end-to-end similarity to a large number of $N^2,N^6$-methyleneetrahydrofolate reductases in a BLASTP search, including MetF from Streptomyces lividans\cite{28} (63% identity), the first such gene to be identified in a Gram-positive bacterium (Fig. 4). MetF
converts N\(^5\),N\(^{10}\)-methylenetetrahydrofolate to N\(^5\)-methyltetrahydrofolate, the triglutamyl derivative of which acts as methyl donor during the MetE-catalysed conversion of homocysteine to methionine (Fig. 5). In Gram-negative bacteria, there is a second route from homocysteine to methionine, involving cobalamin (vitamin B\(12\))-dependent methylation catalysed by MetH, and this also involves N\(^5\)-methyltetrahydrofolate as methyl donor. Since disruption of metF resulted in methionine auxotrophy in S. lividans\(^{22}\), the involvement of folate derivatives in the methionine biosynthetic pathway of this actinomycete was confirmed, whether or not actinomycetes utilise two forms of methionine synthase. The Orfl0 protein contains all 7 of the sequence motifs\(^{28}\) that are conserved among the MetF family of enzymes (Fig. 4).

Conserved residues are boxed and highlighted in GREY. Motifs conserved in all tetrahydrofolate reductases are underlined and labelled A-G.

Targeted Disruption of orfs 8-10

All three orfs were disrupted in a single event involving complete removal of orf9 plus parts of the flanking genes and introduction of the hygromycin-resistance gene cassette (Fig. 2). Resultant strains were then subjected to fermentation analysis and shown to produce tylosin at levels within the range of variability seen in comparable fermentations with the parental strain (Fig. 6a, b). Although these data were initially somewhat surprising, they were not without conceptual precedent.

The presence of ancillary genes within the tyl cluster is a novel feature not (yet?) commonly associated with other antibiotic-biosynthetic gene clusters. The central importance of SAM in transmethylation reactions implies that metK must be an essential cellular gene, in which case the
Fig. 5. One-carbon metabolism and transmethylation.

The active methyl cycle (RIGHT) and the tetrahydrofolate cycle (LEFT). Abbreviations: FH4, tetrahydrofolate; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; Pi, inorganic phosphate; PPI, inorganic pyrophosphate.

Fig. 6. Fermentation of S. fradiae strains.

HPLC analysis of material produced during fermentation of: (a) wild type; (b) a strain in which orfs 8–10 had been disrupted; (c) the disrupted strain in medium lacking glycine betaine. Identification of products was confirmed by the use of internal standards.
Modification of the Fermentation Medium

The tylosin-production medium (MM-1) routinely used in this laboratory contains a significant amount (0.5% w/v) of glycine betaine which acts as a methyl donor to the tetrahydrofolate pool. It was therefore decided to carry out comparative fermentations in media lacking betaine. Interestingly, omission of betaine had no discernable effect on tylosin production by wild type S. fradiae but the level was reduced by about one third in strains disrupted in orf4*, reduced, but did not abolish, tylosin production under conditions similar to those employed here.

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