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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 N^5,N^{10}-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 production[1~5]. 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 DNA[6,7], 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 sequenced[9,10] 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 laboratory[11~18] and elsewhere[19] 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*[17] and *tlrC*[10], 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

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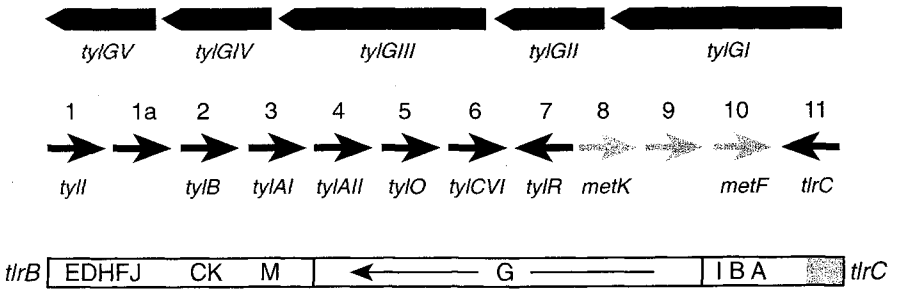
Fig. 1. The tylosin-biosynthetic gene cluster (not drawn to scale).



The bar represents the entire *tyl* cluster (~85 kb) showing the various *tyl* loci flanked by the resistance genes *tlrB* and *tlrC*. The *tylG* locus represents 5 mega genes (~41 kb) encoding the polyketide synthase complex. Upstream of *tylG* lie 12 orfs (including *tlrC*) labelled 1, 1a~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.

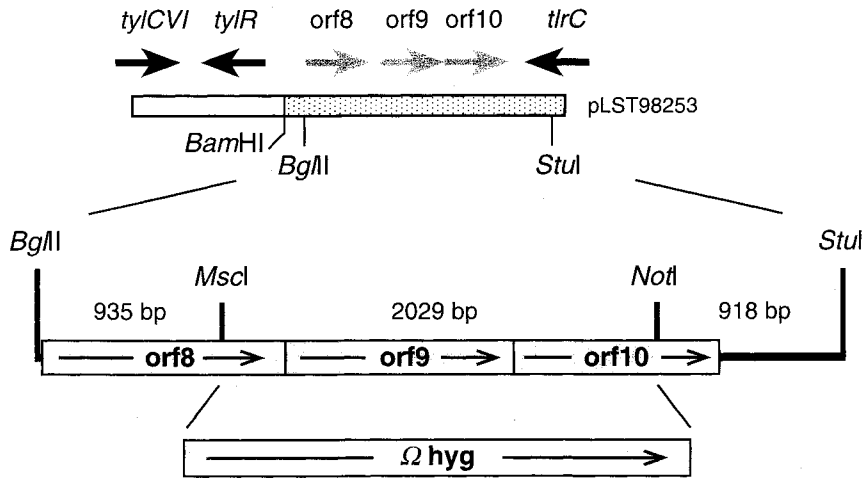
DNA Manipulation and Sequencing

The *S. fradiae tyl* DNA sequenced here was obtained from pSET504[7]. An 8kb *PstI* fragment from pSET504 was subcloned in pIJ2925[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 *Bgl*II-*Stu*I fragment containing *tyl* orfs 8, 9 and 10 together with flanking DNA was excised from pLST98253 and inserted into pIJ2925 (Fig. 2). A *Msc*I-*Not*I 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 *Bgl*II fragment (using a *Bgl*II site located in the multi-cloning region to the right of the *Stu*I site in Fig. 2) and ligated into the *Bam*HI 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

Fig. 2. Disruption of orfs 8~10.



A 3882 bp *Bgl*II–*Stu*I fragment of *tyl* DNA, originally from pSET504, was inserted into pIJ2925. A 2029 bp *Msc*I-*Not*I 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 *Bgl*II fragment (using a *Bgl*II site located in the pIJ2925 multi-cloning region to the right of the *Stu*I site) and ligated into the *Bam*HI site of pOJ260. The resultant plasmid was introduced into *S. fradiae via* conjugal transfer from *E. coli*.

fusion protein(s) from sequences spanning the ends of the disruption was considered unlikely. Also, since the proximal downstream gene, orf11 (*tlrC*), is convergent with orfs 8~10, the gene disruptions generated here should not have been accompanied by polar effects on downstream gene expression.

Fermentation Analysis

Growth of *S. fradiae* in tylosin production medium MM-1 and HPLC analysis of products are described elsewhere[26]. Gene transplacement is a stable event and this eliminated the need for antibiotic selection during fermentation.

Results and Discussion

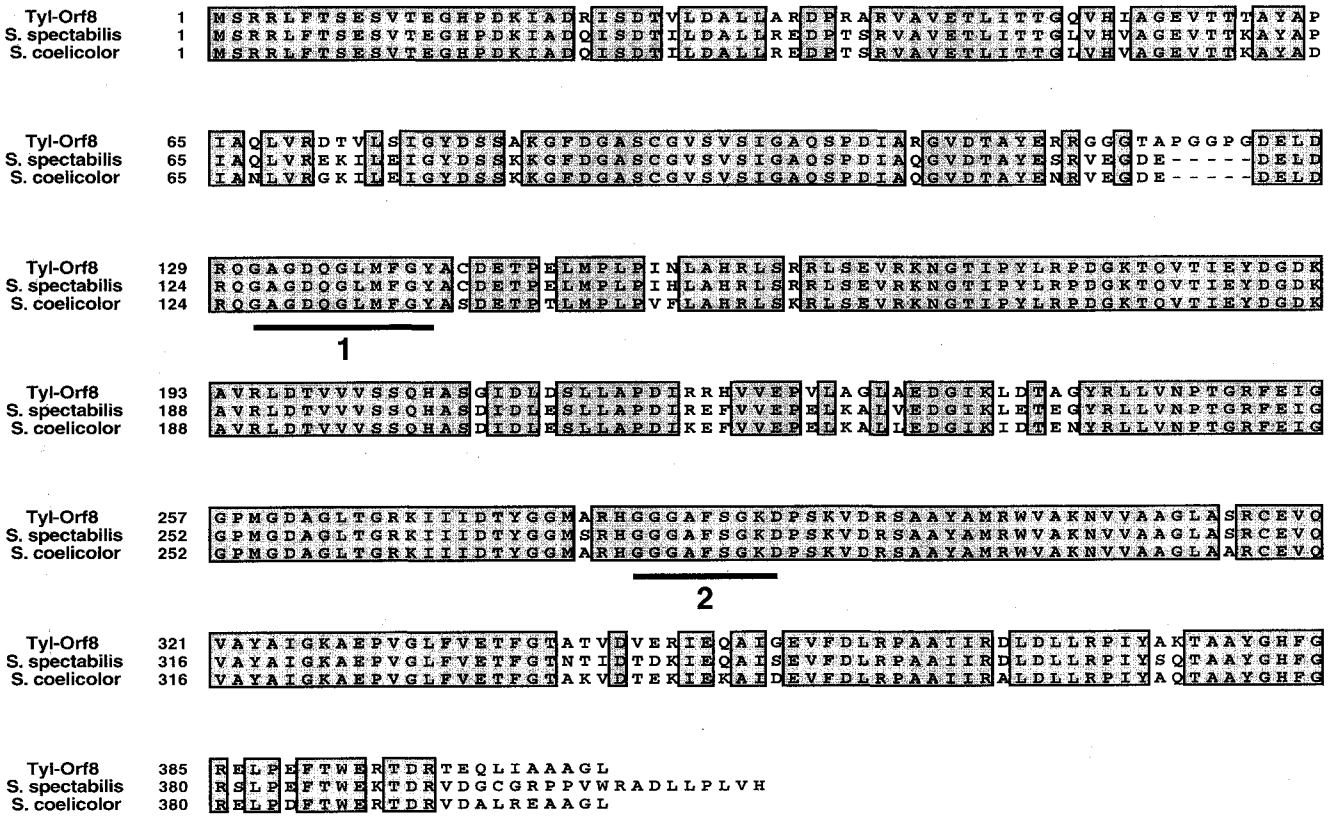
DNA Sequence Analysis

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 *Bam*HI 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

Fig. 3. Alignment of the deduced Orf8 sequence with MetK sequences from *S. spectabilis* (AAD22464.1) and *S. coelicolor* (CAB76898.1).



Conserved residues are boxed and highlighted in GREY. The *S*-adenosylmethionine synthase signatures are underlined and labelled 1~2.

involved in ATP-binding[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*.

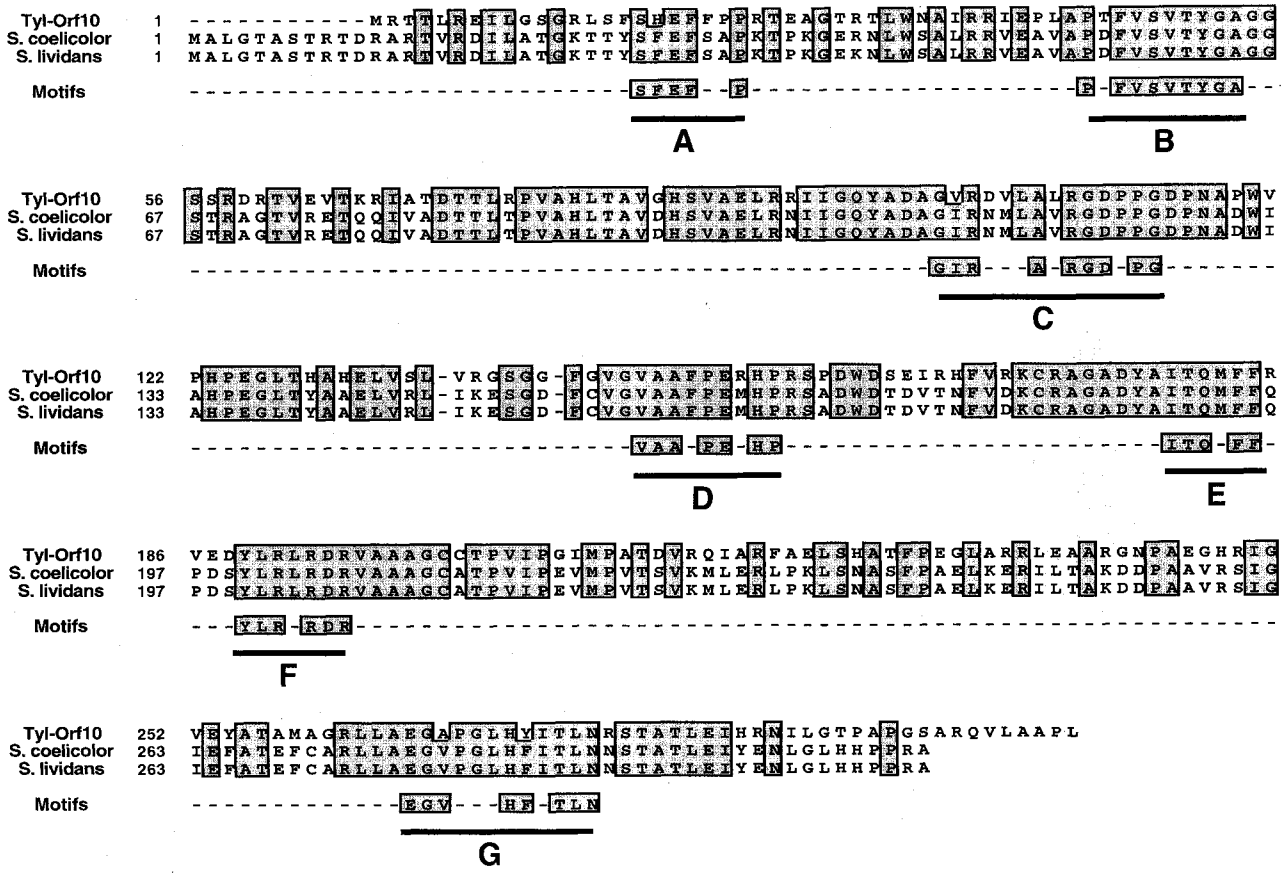
orf9

This gene is separated from orf8 by a gap of 31 bp and is deduced to encode a protein of 366 amino acid residues (Mr 38,150), for which no clear function was apparent following a BLASTP search of the databases. Only two sequences showed significant matches (54% and 49% identity, respectively), to the Orf9 protein. These were a hypothetical kinase from *S. coelicolor* (CAB39885.1) and a hypothetical sugar kinase, CbhK, from *Mycobacterium tuberculosis* (CAA94245.1). However, the Orf9 sequence did not display convincing matches to motifs characteristic of carbohydrate kinases.

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^5,N^{10}-methylenetetrahydrofolate reductases in a BLASTP search, including MetF from *Streptomyces lividans*[28] (63% identity), the first such gene to be identified in a Gram-positive bacterium (Fig. 4). MetF

Fig. 4. Alignment of the deduced Orf10 sequence with MetF sequences from *S. coelicolor* (CAB52012.1) and *S. lividans* (CAA04885.1).



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

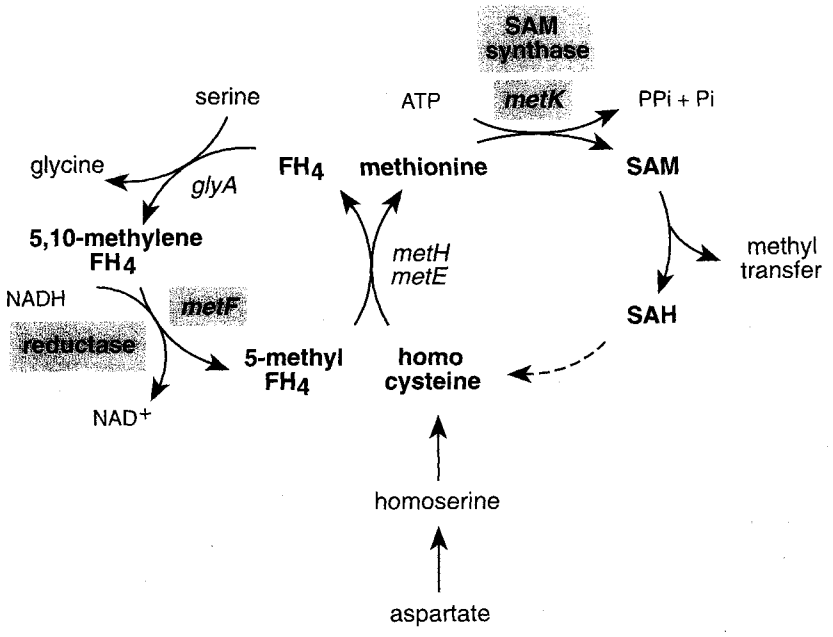
converts N^5,N^{10}-methylenetetrahydrofolate to N^5-methyltetrahydrofolate, the triglutamyl derivative of which acts as methyl donor during the MetE-catalysed conversion of homocysteine to methionine (Fig. 5). In Gram-negative bacteria, there is a second route from homocysteine to methionine, involving cobalamin (vitamin B12)-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*[28], 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 Orf10 protein contains all 7 of the sequence motifs[28] that are conserved among the MetF family of enzymes (Fig. 4).

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.

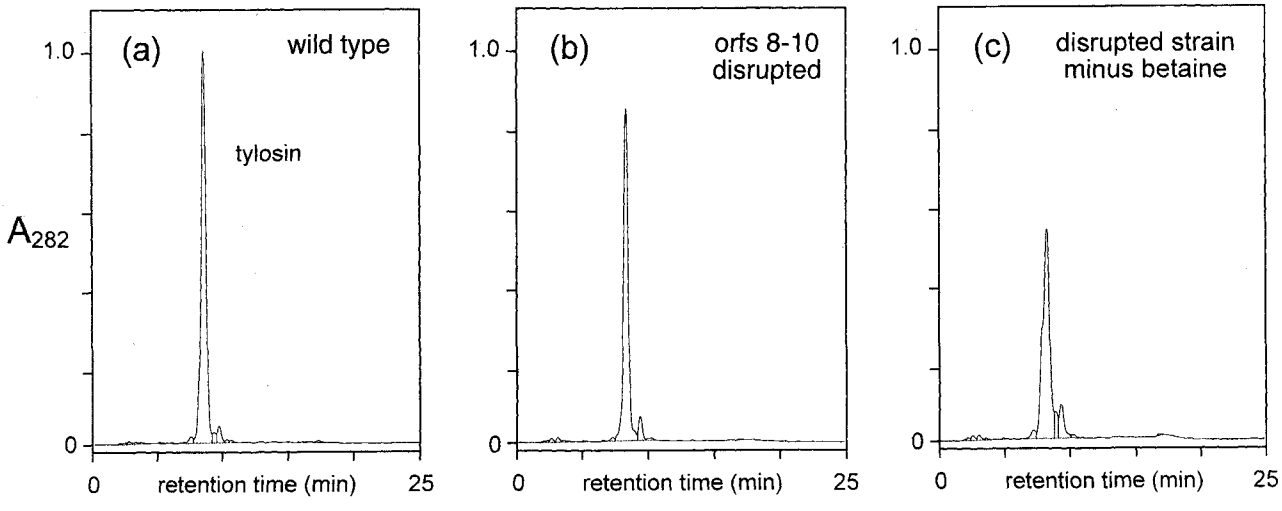
The presence of ancillary genes within the *tyl* cluster is a novel feature not (yet?) commonly associated with other antibiotic-biosynthetic gene clusters. The central importance of SAM in transmethylation reactions implies that *metK* must be an essential cellular gene, in which case the

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.

viability of *S. fradiae* strains disrupted in orfs 8~10 further implies that an additional *metK* gene must be present elsewhere in the genome. Similar considerations also apply to *metF*. Presumably, the *met* genes were recruited into the *tyl* cluster to fulfil an ancillary role during tylosin production, although not an essential one under the fermentation conditions pertaining here. Another ancillary gene in the *tyl* cluster is *ccr* (orf4*), encoding crotonyl-CoA reductase. This gene lies on the other side of *tylG*, distal to orfs 8~10, and is thought to provide 4C extender units for the synthesis of tylactone, the polyketide aglycone of tylosin[13]. The presence of an additional *ccr* gene elsewhere in the *S. fradiae* genome has recently been confirmed by hybridization analysis and disruption of orf4* reduced, but did not abolish, tylosin production under conditions similar to those employed here[29].

Modification of the Fermentation Medium

The tylosin-production medium (MM-1) routinely used in this laboratory[26] 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 orfs 8~10 (Fig. 6c). Evidently, the presence of betaine in MM-1 medium had earlier masked the disruption phenotype so that tylosin-specific function(s) of orfs 8~10 could only be revealed in its absence.

Acknowledgements

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