The mycarose-biosynthetic genes of *Streptomyces fradiae*, producer of tylosin

Neil Bate, Andrew R. Butler, Ian P. Smith 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

The *tylCK* region of the *Streptomyces fradiae* genome was sequenced, revealing an incomplete set of five *tylC* genes encoding all-but-one of the enzymes involved in the biosynthesis of mycarose. The latter is a 6-deoxyhexose sugar required during production of the macrolide antibiotic, tylosin. The missing mycarose-biosynthetic gene, *tylCVI*, was found about 50 kb distant from its functional partners, on the other side of the *tylG* (polyketide synthase) gene complex. Mutational analysis, involving targeted gene transplacement, was employed to confirm the functions of specific genes, including *tylCVI*. Particularly interesting was the similarity between the tylosin-biosynthetic mycarosyltransferase enzyme, TylCV, and proteins of the macrolide glycosyltransferase (MGT) family that inactivate macrolides via glycosylation of attached sugar residues and are involved in resistance and/or antibiotic efflux. The arrangement of genes within the 'mycarose cluster' would allow their expression as two short operons with divergent, and perhaps co-regulated, promoters. Whether displacement of *tylCVI* relative to the other *tylC* genes provides additional regulatory opportunities remains to be established.

Keywords: tylosin production, *Streptomyces fradiae*, mycarose biosynthesis, deoxyhexose metabolism

INTRODUCTION

Tylosin, a macrolide antibiotic produced by *Streptomyces fradiae*, comprises a polyketide lactone, protylonolide (also known as tylactone), to which are attached three 6-deoxyhexose sugars (Fig. 1). The biosynthetic route to tylosin was determined by analysis of compounds accumulated by blocked mutants of *St. fradiae*, together with co-synthesis and bioconversion studies (Baltz & Seno, 1981; Ōmura *et al.*, 1982a, b; Baltz *et al.*, 1983). Thus, tylactone is first substituted with D-mycaminose followed, after ring hydroxylation at C-20 and C-23, by 6-deoxy-D-allose and then L-mycarose, in a preferred but not obligatory order. The last two steps of tylosin biosynthesis involve conversion of the deoxy-allose moiety to D-mycinose via bis-O-methylation. Complementation of blocked mutants using cloned DNA fragments (Beckmann *et al.*, 1989; Fishman *et al.*,

1987), allowed 13 genetic loci (*tylA–M*) to be mapped within the *St. fradiae* genome (Fig. 2). Most of the mutants displayed the TylG phenotype and were defective in polyketide biosynthesis. The TylA phenotype was initially characterized as inability to produce or add any of the three tylosin sugars, but complementation analysis later divided such mutants into two groups (*tylA* and *tylL*) with the respective loci located on either side of *tylG*. Similarly, mycarose-minus mutants (originally designated *tylC*) were subdivided into *tylC* and

Fig. 1. Structure of tylosin.

Abbreviation: MGT, macrolide glycosyltransferase.

The GenBank accession number for the sequence determined in this work is AF147704.

Fig. 2. Tylosin-biosynthetic structural gene cluster of *St. fradiae* (not drawn to scale). The box represents the entire cluster (~85 kb) showing 13 *tyl* loci flanked by the resistance genes *tlrB* and *tlrC*. All of the structural genes required for tylosin production probably lie between *tlrB* and *tlrC*. Arrows represent genes of the *tyl* cluster. Those analysed here are shown in grey and their locations within the cluster are indicated on the box. The *tylG* locus represents 5 mega genes (~41 kb) encoding the polyketide synthase complex. Immediately upstream of *tylG* are 12 genes including *tlrC* (*orf1*, 1a and 2–11), which occupy about 15 kb. On the downstream side, *tylG* is separated from *tlrB* by 25 ORFs that occupy about 29 kb. Beginning immediately downstream of *tylG*, these are numbered 1*–25* to distinguish them from ORFs on the upstream side of *tylG*. Only the first 11 downstream genes, proximal to *tylG*, are shown here.

tylK strains, while others defective in deoxyallose metabolism were sorted into *tylD* and *tylJ* strains. Polyketide ring hydroxylation at C-20 and C-23 was associated with the *tylI* and *tylH* loci, respectively, and bis-O-methylation of the deoxyallose moiety was shown to involve sequential action of the TylE and TylF proteins (Baltz & Seno, 1981). The various *tyl* loci are flanked by the resistance genes *tlrB* and *tlrC*, which lie about 85 kb apart in the *St. fradiae* genome (Baltz & Seno, 1988; Beckmann *et al.*, 1989).

Sequencing of the *tyl* gene cluster, defined for present purposes as the region of the *St. fradiae* genome flanked by *tlrB* and *tlrC*, began at Lilly Research Laboratories, Indianapolis, USA, in the late 1980s and has been completed in this laboratory. The sequence (41 kb) of *tylG*, encoding the polyketide synthase that produces tyactone, has been released but not formally published (GenBank accession number U78289) and the genes (*tylB*, *tylMI*, *tylMII* and *tylMIII*) encoding mycaminos biosynthesis have also been sequenced (Merson-Davies & Cundliffe 1994; Gandechea *et al.*, 1997). More recently, a contiguous DNA 'cassette' encoding the full complement of genes involved in mycinose biosynthesis, together with hydroxylation of the polyketide ring at C-23 to allow attachment of the sugar, has been analysed (Bate & Cundliffe, 1999). In the present work, the genes involved in mycarose biosynthesis were sequenced and functions were assigned to specific gene products by a combination of database comparisons, targeted gene disruptions and complementation of specifically disrupted strains.

METHODS

Bacterial strains, growth conditions and genetic manipulation. *St. fradiae* T59235 (wild-type, also known as C373.1) and its derivatives were maintained and propagated at 37 °C on AS-1 agar (Wilson & Cundliffe, 1998) or at 30 °C in tryptic soy broth (Difco). Plasmids were manipulated in *Escherichia coli* using standard protocols (Sambrook *et al.*, 1989). DNA was introduced into *St. fradiae* via conjugal transfer from *E. coli* as described elsewhere (Fish & Cundliffe, 1997). The vector used for targeted gene disruption/transplacement was pOJ260 (Bierman *et al.*, 1992); this plasmid, which carries an apramycin resistance marker, does not replicate in *Streptomyces* spp. Cloned fragments of the *tyl* gene cluster were inserted into the vector pLST9828 and tested for their ability to complement specific *tyl* gene disruptions. This vector, which uses the strong constitutive promoter, *ermEp**, for expression of the inserted genes, integrates into the chromosomal ϕ C31 *attB* site.

DNA sequencing and computer analysis. The sequence of *orf6* was obtained by combining that given under accession numbers U08223 (Merson-Davies & Cundliffe, 1994) and AF145042 (Bate *et al.*, 1999). The *St. fradiae* *tyl* DNA sequenced here was obtained from pMOMT4 (Beckmann *et al.*, 1989). Fragments were subcloned in pIJ2925 (Janssen & Bibb, 1993) and both strands of the DNA were sequenced independently in overlapping fashion by a combination of nested deletion analysis and primer walking. Sequencing was done on an ABI 377 automated DNA sequencer using *Taq* FS polymerase with dye terminator chemistry (Perkin Elmer). DNA sequences together with the corresponding chromatograms were imported into Seq Ed version 1.0.3 and aligned using AUTO ASSEMBLER. Sequences were analysed using the University of Wisconsin GCG software programmes. ORFs were identified using CODONPREFERENCE, BLASTX and 6 frame

translation with DNA STRIDER. Deduced products were analysed using BLASTP.

Targeted gene disruption through gene transplacement. The DNA fragment used to disrupt *orf7** was generated by PCR using pMOMT4 as template (Beckmann *et al.*, 1989). The primers used to create the *orf7**-disruption fragment were 5'-ACTTGAATTCATGACGACGGGTCCGGGACG-3' and 5'-GGCGAAGCTTGGCCGGAGGTCAAGGCCGCGG-3'. These contained, respectively, *Eco*RI and *Hind*III restriction sites (underlined) that were used to ligate the product (1513 bp with a unique central *Sal*I site) into pIJ2925. The hygromycin resistance cassette, Ω hyg, together with its flanking transcriptional terminators (Blondelet-Rouault *et al.*, 1997) was then inserted at the *Sal*I site, thereby interrupting the *orf7** coding sequence 640 bp downstream of the earliest possible translational start.

A 2272 bp *Kpn*I fragment containing *orf6* together with flanking DNA was excised from pSET552 (Beckmann *et al.*, 1989) and inserted into pIJ2925. The hygromycin resistance cassette was inserted at the unique *Sex*AI site, 584 bp downstream from the start of *orf6* and 784 bp upstream of the translational stop.

Disrupted ORFs plus flanking DNA were ligated into the *Bam*HI site of pOJ260 and introduced into *St. fradiae*. Transconjugants that had undergone gene transplacement were resistant to hygromycin and sensitive to apramycin.

Complementation of disrupted strains. Three different DNA fragments were reintroduced into the *orf7**-disrupted strain. These were: (i) a 2167 bp fragment containing *orf7** plus *orf6** selected from a nested deletion library; (ii) an 825 bp fragment containing *orf6** together with flanking DNA (227 bp upstream and 4 bp downstream) also selected from a nested deletion library; and (iii) the 1513 bp PCR product containing *orf7** plus flanking DNA (118 bp downstream and 9 bp upstream). The *orf6*-disrupted strain was complemented using a 1630 bp *Nco*I-*Sma*I fragment generated from pSET552. This contained the whole of *orf6* together with flanking DNA (150 bp upstream and 112 bp downstream). DNA fragments for complementation analysis were ligated into pLST9828 downstream of *ermEp**.

Antibiotic-production fermentation and metabolite analysis. Fermentation of *St. fradiae* strains and the analysis of products by HPLC was done as described elsewhere (Butler *et al.*, 1999).

RESULTS AND DISCUSSION

DNA sequence analysis

The sequence of *tylCK* DNA (5908 bp, GenBank accession number AF147704) contains five complete ORFs, each of which displays the biased codon usage typical of

actinomycete genes, plus flanking DNA. Following the numbering scheme introduced previously (Merson-Davies & Cundliffe, 1994; Gandechea *et al.*, 1997) and subsequently extended (Bate & Cundliffe, 1999; Bate *et al.*, 1999), the 'new' ORFs are designated *orf6**-10* (Fig. 2). At the left-hand end in the orientation of Fig. 2, the sequence presented here extends 50 bp into the co-directional *orf11** and overlaps by 100 bp the sequence given under accession number AF145049 (Bate *et al.*, 1999). At the right-hand end, the sequence terminates 50 bp inside the resistance determinant *tlrD* (*orf5**) and overlaps by 209 bp the sequence deposited under accession number X97721 (Gandechea & Cundliffe, 1996). Database comparisons and mutational analysis (below) revealed that *orf6**-10* comprise an incomplete set of mycarose-biosynthetic genes (lacking one functional member). The 'missing' mycarose-biosynthetic gene (*orf6*) was unexpectedly encountered over 50 kb distant from the *tylCK* region on the other side of the *tylG* cluster (accession number AF145042; Bate *et al.*, 1999) and its function has been confirmed here via targeted disruption analysis. Since the mycarose-minus phenotype was originally termed 'TylC' (Baltz & Seno, 1981), the mycarose-biosynthetic genes of *St. fradiae* have been assigned 'tylC' designations. To facilitate comparison with the mycarose-biosynthetic (*eryB*) genes of *Saccharopolyspora erythraea*, the numbering system already in use for the *eryB* genes has been adopted here (Table 1).

The biosynthetic route to mycarose

The biosynthetic pathway leading to mycarose is not known with certainty (Fig. 3). Epimerization at C-5 must occur at some stage since mycarose is an L-sugar derived ultimately from D-glucose. *A priori*, this could be achieved via 5-epimerization or 3,5-epimerization and might occur before or after 2-deoxygenation. Were epimerization to precede 2-deoxygenation, the stereo-specificity at C-3 would be lost in the dehydratase step and would ultimately be determined by the 2,3-enoyl-reductase (here, TylCII). Consistent with such a model, an *eryBII* mutant of *Sa. erythraea* produced a derivative of erythromycin C (Salah-Bey *et al.*, 1998) containing a neutral sugar ostensibly derived, via 4-ketoreduction, from that shown in Fig. 3 (compound 2). Alternatively, 2-deoxygenation might occur first (Summers *et al.*, 1997), in which case the stereochemistry at C-3 following the enoylreductase reaction would dictate the mode of

Table 1. Mycarose-biosynthetic genes of *St. fradiae* and *Sa. erythraea*

ORF	<i>St. fradiae</i> gene name	<i>Sa. erythraea</i> gene name	Gene product
<i>orf6*</i>	<i>tylCVII</i>	<i>eryBVII</i>	5- (or 3,5) Epimerase
<i>orf6</i>	<i>tylCVI</i>	<i>eryBVI</i>	2,3-Dehydratase
<i>orf10*</i>	<i>tylCII</i>	<i>eryBII</i>	2,3-Enoylreductase
<i>orf8*</i>	<i>tylCIII</i>	<i>eryBIII</i>	3-C-Methyltransferase
<i>orf9*</i>	<i>tylCIV</i>	<i>eryBIV</i>	4-Ketoreductase
<i>orf7*</i>	<i>tylCV</i>	<i>eryBV</i>	Glycosyltransferase

Fig. 3. Alternative biosynthetic routes to mycarose during tylosin production. It remains to be established whether epimerization precedes or follows 2-deoxygenation. Compound 1, NDP-4-keto-6-deoxy-D-glucose, represents a branch point in biosynthesis of the three tylosin sugars. These are produced as their respective NDP-adducts (i.e. NDP-D-mycaminose, NDP-L-mycarose and NDP-D-deoxyallose) and are transferred into the emerging tylosin molecule by specific glycosyltransferases (in the case of mycarose, TyICV). The deoxyallose moiety is finally converted to mycinose, via bis-O-methylation, following transglycosylation.

subsequent epimerization. Support for the latter route, at least in the biosynthesis of other 2,6-dideoxyhexoses, came from recent *in vitro* studies (Draeger *et al.*, 1999) involving pairs of purified enzymes that catalyse 2-deoxygenation of dTDP-4-keto-6-deoxy-D-glucose (Fig. 3, compound 1) during the synthesis of L-rhodinose in *Streptomyces violaceoruber* Tü22, and of L-oleandrose in *Streptomyces antibioticus* Tü99. In either case, dTDP-4-keto-2,6-dideoxy-D-glucose (Fig. 3, compound 3) was

characterized as the product. From our data, we cannot discriminate between these alternative models.

Assignment of ORFs

orf6* (tylCVII). The deduced product of *orf6** is a protein of 197 amino acid residues maximum (M_r 21400) that shows convincing end-to-end similarities to the products of numerous putative NDP-4-keto-6-deoxyglucose 3,5-epimerases including (with 52% and 49% sequence identity, respectively) the products of *gra-orf25* from *Streptomyces violaceoruber* Tü22 (Ichinose *et al.*, 1998) and *lanZ1* from *Streptomyces cyanogenus* (Westrich *et al.*, 1999). In particular, the Orf6* protein shares 44% sequence identity with EryBVII (Gaisser *et al.*, 1997; Summers *et al.*, 1997). In *Sa. erythraea*, *eryBVII* encodes a mycarose-biosynthetic enzyme and similar genes in other organisms are also associated with the production of L-sugars, including L-rhamnose (*rbc* gene in *Shigella*; Macpherson *et al.*, 1994), L-ascarylose (*ascE* in *Yersinia*; Thorson *et al.*, 1994) and L-dihydrostreptose (*strM* in *Streptomyces griseus*; Pissowatzky *et al.*, 1991). Although direct experimental evidence is lacking, StrM (Pissowatzky *et al.*, 1991) and EryBVII (Gaisser *et al.*, 1997; Summers *et al.*, 1997) are believed to be NDP-4-keto-6-deoxyglucose 3,5- (or 5-) epimerases and a similar role is proposed here for the *orf6** product. Proof that Orf6* is involved in the biosynthesis of mycarose was obtained by fermentation analysis of a strain in which *orf6** was specifically disrupted. The resultant strain produced desmycosin (demycarosyl-tylosin) and complementation with *orf6** restored normal levels of tylosin production (data not shown). We concluded that Orf6* (now designated TylCVII) is the 3,5- (or 5-) epimerase involved in mycarose biosynthesis and noted significant similarity (41% sequence identity) between it and TylJ, the putative 3-epimerase involved in the formation of 6-deoxyallose (Bate & Cundliffe, 1999).

orf7* (tylCV). In database searches, the deduced product of *orf7** (461 amino acids; M_r 50100) displayed greater than 30% sequence identity to various macrolide glycosyltransferases (MGTs), such as OleI and OleD from *St. antibioticus*, producer of oleandomycin (Quirós *et al.*, 1998; Hernandez *et al.*, 1993), GimA from *Streptomyces ambofaciens*, producer of spiramycin (Gourmelen *et al.*, 1998) and Mgt from *Streptomyces lividans* (Jenkins & Cundliffe, 1991). Such enzymes inactivate their substrates and are involved in antibiotic efflux and/or resistance. Less close matches (20–25% sequence identity) were also seen with glycosyltransferases involved in antibiotic biosynthesis such as OleG2 (Olano *et al.*, 1998), EryCIII and EryBV (Gaisser *et al.*, 1997; Summers *et al.*, 1997; Salah-Bey *et al.*, 1998). Nevertheless, when the role of *orf7** was addressed by targeted gene disruption it proved to be a mycarose-biosynthetic gene and not an *mgt*. Thus, the *orf7**-disrupted strain accumulated desmycosin during fermentation (Fig. 4a) and tylosin production was restored following complementation with cloned DNA, but only when *orf6** was added together with *orf7** (Fig. 4b), in this case on a single DNA fragment. Reintroduction of

Fig. 4. Fermentation products from *orf7**-disrupted *St. fradiae*. HPLC analysis of material produced by (a) *orf7**-disrupted strain, (b) *orf7**-disrupted strain complemented with *orf7** and 6*, (c) *orf7**-disrupted strain complemented with *orf7**, and (d) *orf7**-disrupted strain complemented with *orf6**. The arrows indicate where tylosin would be expected to elute.

either gene alone did not restore tylosin production (Fig. 4c, d) although unidentified material, which was clearly separable from both tylosin and desmycosin, accumulated together with the latter when complementation was attempted with *orf7** alone (Fig. 4c). These data established that *orf7**, like *orf6**, must be a mycarose-biosynthetic gene and that normally the two genes are probably co-transcribed. Disruption of *orf7** using the Ω hyg cassette (which is flanked by transcriptional terminators) evidently caused a downstream polar effect on the expression of *orf6**.

We concluded that the *orf7** product (designated TylCV) is the mycarosyl transferase involved in tylosin biosynthesis and we noted its closer resemblance to macrolide glycosyltransferases than to enzymes involved in antibiotic production. Unlike EryBV, which attaches mycarose directly to the polyketide during erythromycin production, TylCV acts on a glycosylated macrolide and forms a disaccharidic moiety. In this sense, TylCV more closely resembles MGT enzymes, although the latter modify their substrates at the 2'-OH rather than the 4'-OH that is targeted by TylCV.

***orf8** (tylCIII).** The deduced product of *orf8** (418 amino acids maximum; M_r 46600) displays extremely close end-to-end similarity (with 70% sequence identity) to

that of *ery orf3* (Haydock *et al.*, 1991), now renamed *eryBIII* (Gaisser *et al.*, 1998), and is much more closely related to that protein than to any other in the database. Moreover, Orf8* and EryBIII each possess multiple sequence motifs characteristic of S-adenosylmethionine-dependent methyltransferases (Kagan & Clarke, 1994). Since a strain of *Sa. erythraea* with a point mutation in *eryBIII* accumulated 3"-C-desmethyl-erythromycin A during fermentation (Gaisser *et al.*, 1998), the unequivocal conclusion was that *eryBIII* encodes a 3-C-methyltransferase activity required during mycarose biosynthesis. An equivalent role is proposed for the *orf8** product, designated TylCIII.

***orf9** (tylCIV).** The deduced product of *orf9** (maximum size, 347 amino acids; M_r 36200) displays 49% sequence identity to that of *eryBIV* (Gaisser *et al.*, 1997; Summers *et al.*, 1997). The only other significant end-to-end sequence match (46% identity) was to an uncharacterized protein, PCZA 361.4, from vancomycin-producing *Amycolatopsis orientalis* (van Wageningen *et al.*, 1998). Mutants of *Sa. erythraea*, altered in *eryBIV*, accumulated modified erythromycins in minor amounts and mass spectrometric analysis (Salah-Bey *et al.*, 1998) revealed that the neutral sugar (mycarose) appeared to lack two hydrogen atoms. From these data, it was

Fig. 5. Fermentation products from *St. fradiae* wild-type and the *orf6*-disrupted strain. HPLC analysis of material produced by (a) wild-type, (b) *orf6*-disrupted strain, and (c) *orf6*-disrupted strain complemented with *orf6*.

concluded that *eryBIV* encodes 4-ketoreductase activity involved in mycarose metabolism. An equivalent role is proposed here for the *orf9** product, TylCIV. Consistent with this proposal, TylCIV and EryBIV are distantly related to UDP-glucose 4-epimerases and to various oxidoreductases that manipulate sugars at the 4-position, including NDP-glucose 4,6 dehydratases such as TylAII (formerly TylA2; Merson-Davies & Cundliffe, 1994) and StrE (Pissowotzki *et al.*, 1991). Moreover, TylCIV contains a candidate NADPH-binding sequence (GRGVIAVTAG) with a downstream arginine cluster (RDLLDVR) positioned suitably to form a binding site for the 2'-phosphate of NADPH (N. S. Scrutton, personal communication).

***orf10** (tylCII).** The deduced product of *orf10** (329 amino acids; M_r 36 100) is very similar (74% sequence identity) to EryBII from *Sa. erythraea* (Summers *et al.*, 1997; Salah-Bey *et al.*, 1998). Less close matches (41% and 30% sequence identity, respectively) were found with MocA from *Agrobacterium tumefaciens* (Kim & Farrand, 1996), which is 'weakly related' to glucose-6-phosphate dehydrogenase, and with an authentic aryl alcohol dehydrogenase from the white rot fungus, *Phanerochaete chrysosporium* (Reiser *et al.*, 1994). Despite the absence of any obvious NAD(P)H-binding motif in the amino acid sequence, these data suggest that Orf10* might be one of the two reductases involved in mycarose biosynthesis. The function of EryBII was revealed during incisive analysis of *Sa. erythraea* mutant BII92 (Salah-Bey *et al.*, 1998), a specifically deleted strain that produced a 2'',3''-unsaturated derivative of 3''-C-desmethyl-erythromycin C. Those authors suggested that *eryBII* encodes 2,3-enoyl reductase activity involved in mycarose biosynthesis and a similar role is proposed here for the *orf10** product, designated TylCII.

***orf6* (tylCVI).** The 'missing' gene of the mycarose-biosynthetic pathway (*tylCVI*) is not present in the

tylCK region of the *St. fradiae* genome. Somewhat surprisingly, it is located about 50 kb distant from *orf6*-10**, on the opposite side of the *tylG* gene cluster adjacent to the regulatory gene *tylR* (Bate *et al.*, 1999). The maximum length of the *orf6* product is 455 amino acid residues, which would give a 50 kDa protein. The involvement of *orf6* in mycarose metabolism was first suspected when the upstream gene (*orf5*) was specifically disrupted and then complemented with intact *orf5*. The resultant strain produced desmycosin (demycarosyl-tylosin) rather than tylosin, suggesting that disruption of *orf5* had caused a downstream polar effect on the expression of a mycarose-biosynthetic gene (Butler *et al.*, 1999). Validation of that interpretation involved targeted disruption of *orf6* via gene transplacement (confirmed by Southern analysis) followed by HPLC screening of fermentation products. This revealed a major peak of UV-absorbing material that co-eluted with desmycosin and was clearly resolved from tylosin (Fig. 5b). The yield of desmycosin approximated that of tylosin seen in control fermentations with the wild-type strain (Fig. 5a). More detailed analysis, involving HPLC-MS, revealed that the single peak in Fig. 5(b) represented a mixture of desmycosin plus 20-dihydro-desmycosin, together with two minor components consistent with *N*-demethyl-desmycosin and *N*-demethyl-20-dihydro-desmycosin (D. E. Kiehl & H. A. Kirst, personal communication). Macrolide reductase activity, which converts tylosin to relomycin (20-dihydrotylosin) and also acts on the corresponding aldehyde moieties of other macrolides, was purified from tylosin-producing strains of *St. fradiae* and was also detected in other *Streptomyces* spp. (such as *St. lividans*) that do not produce macrolides (Huang *et al.*, 1993). Therefore, the presence of 20-dihydrodesmycosin in the *orf6*-disrupted strain is readily rationalized, although the detection of *N*-demethyl compounds, albeit at low levels, was unexpected.

When *orf6*, together with *ermEp**, was integrated into the ϕ C31 *attB* site of the genome, tylosin production was restored in the *orf6*-disrupted strain and HPLC revealed two main fermentation products (Fig. 5c). One of these co-eluted with desmycosin whereas the major product was inseparable from tylosin. These data clearly established a role for *orf6* in mycarose biosynthesis despite its isolated location with respect to its functional partners.

The *orf6* product, TylCVI, displays end-to-end similarity (with 51% sequence identity) to the deduced product of *eryBVI* from *Sa. erythraea* (Gaisser *et al.*, 1997; Summers *et al.*, 1997) and to the products of *dnmT* from *Streptomyces peucetius* (Scotti & Hutchinson, 1996) and *orf3* from *Streptomyces* sp. C5 (Dickens *et al.*, 1996), producers of doxorubicin and daunomycin, respectively. Arguing by elimination, it was suggested that EryBVI is the 2,3-dehydratase required for deoxygenation at C-2 during mycarose biosynthesis in the erythromycin producer (Gaisser *et al.*, 1997; Summers *et al.*, 1997) and a similar role is invoked for TylCVI during tylosin production in *St. fradiae*.

Concluding remarks

It will be interesting to study regulation of the *tylC* genes during tylosin production. The physical organization of *orf6**–*10** would permit possible co-regulation from a pair of divergent (perhaps equivalent) promoters located in the non-coding gap between *orf7** and *orf8**. Although the minimum size of this gap is 212 bp, we suspect that the TylCV coding sequence is considerably shorter than *orf7** and that the intergenic region is accordingly greater than 400 bp. Gaps of this size are rare in the *tyl* cluster. Since there are no spaces between *orf8**, *9** and *10**, and since *orf7** and *6** are separated by only 43 bp, this group of genes might constitute two short operons. But what, if anything, is gained by having one mycarose gene displaced so far from the others? And is it significant that the mycaminose-biosynthetic genes are similarly split, with three co-directional *tylM* genes (*orf1**–*3**) located near the ‘mycarose-gene cluster’ whereas *tylB* lies on the other side of *tylG*, near *tylCVI* (Merson-Davies & Cundliffe, 1994).

ACKNOWLEDGEMENTS

This work was supported by an MRC research studentship awarded to I.P.S., by project grant 91/T08195 from the BBSRC, and by Eli Lilly and Co., Indianapolis, USA. We thank Douglas E. Kiehl and Herbert A. Kirst for mass spectrometric analysis of fermentation products and Nigel S. Scrutton for evaluation of protein sequence motifs.

REFERENCES

Baltz, R. H. & Seno, E. T. (1981). Properties of *Streptomyces fradiae* mutants blocked in biosynthesis of the macrolide antibiotic tylosin. *Antimicrob Agents Chemother* **20**, 214–225.

- Baltz, R. H. & Seno, E. T. (1988). Genetics of *Streptomyces fradiae* and tylosin biosynthesis. *Annu Rev Microbiol* **42**, 547–574.
- Baltz, R. H., Seno, E. T., Stonesifer, J. & Wild, G. M. (1983). Biosynthesis of the macrolide antibiotic tylosin. A preferred pathway from tylactone to tylosin. *J Antibiot* **36**, 131–141.
- Bate, N. & Cundliffe, E. (1999). The mycinose-biosynthetic genes of *Streptomyces fradiae*, producer of tylosin. *J Ind Microbiol Biotechnol* **23**, 118–122.
- Bate, N., Butler, A. R., Gandechea, A. R. & Cundliffe, E. (1999). Multiple regulatory genes in the tylosin biosynthetic cluster of *Streptomyces fradiae*. *Chem Biol* **6**, 617–624.
- Beckmann, R. J., Cox, K. & Seno, E. T. (1989). A cluster of tylosin biosynthetic genes is interrupted by a structurally unstable segment containing four repeated sequences. In *Genetics and Molecular Biology of Industrial Microorganisms*, pp. 176–186. Edited by C. L. Hershberger, S. W. Queener & P. L. Skatrud. Washington, DC: American Society for Microbiology.
- Bierman, M., Logan, R., O'Brien, K., Seno, E. T., Rao, R. N. & Schoner, B. E. (1992). Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. *Gene* **116**, 43–49.
- Blondelet-Rouault, M.-H., Weiser, J., Lebrhi, A., Branny, P. & Pernodet, J.-L. (1997). Antibiotic resistance cassettes derived from the Ω interposon for use in *E. coli* and *Streptomyces*. *Gene* **190**, 315–317.
- Butler, A. R., Bate, N. & Cundliffe, E. (1999). Impact of thioesterase activity on tylosin biosynthesis in *Streptomyces fradiae*. *Chem Biol* **6**, 287–292.
- Dickens, M. L., Ye, J. & Strohl, W. R. (1996). Cloning, sequencing, and analysis of aklaviketone reductase from *Streptomyces* sp. strain C5. *J Bacteriol* **178**, 3384–3388.
- Draeger, G., Park, S.-H. & Floss, H. G. (1999). Mechanism of the 2-deoxygenation step in the biosynthesis of the deoxyhexose moieties of the antibiotics granaticin and oleandomycin. *J Am Chem Soc* **121**, 2611–2612.
- Fish, S. A. & Cundliffe, E. (1997). Stimulation of polyketide metabolism in *Streptomyces fradiae* by tylosin and its glycosylated precursors. *Microbiology* **143**, 3871–3876.
- Fishman, S. E., Cox, K., Larson, J. L., Reynolds, P. A., Seno, E. T., Yeh, W.-K., Van Frank, R. & Hershberger, C. L. (1987). Cloning genes for the biosynthesis of a macrolide antibiotic. *Proc Natl Acad Sci USA* **84**, 8248–8252.
- Gaisser, S., Böhm, G. A., Cortés, J. & Leadlay, P. F. (1997). Analysis of seven genes from the *eryAI*–*eryK* region of the erythromycin biosynthetic gene cluster in *Saccharopolyspora erythraea*. *Mol Gen Genet* **256**, 239–251.
- Gaisser, S., Böhm, G. A., Doumith, M., Raynal, M.-C., Dhillon, N., Cortés, J. & Leadlay, P. F. (1998). Analysis of *eryBI*, *eryBIII* and *eryBVII* from the erythromycin biosynthetic gene cluster in *Saccharopolyspora erythraea*. *Mol Gen Genet* **258**, 78–88.
- Gandechea, A. R. & Cundliffe, E. (1996). Molecular analysis of *tlrD*, an MLS resistance determinant from the tylosin producer, *Streptomyces fradiae*. *Gene* **180**, 173–176.
- Gandechea, A. R., Large, S. L. & Cundliffe, E. (1997). Analysis of four tylosin biosynthetic genes from the *tylLM* region of the *Streptomyces fradiae* genome. *Gene* **184**, 197–203.
- Gourmelen, A., Blondelet-Rouault, M.-H. & Pernodet, J.-L. (1998). Characterization of a glycosyl transferase inactivating macrolides, encoded by *gimA* from *Streptomyces ambofaciens*. *Antimicrob Agents Chemother* **42**, 2612–2619.
- Haydock, S. F., Dowson, J. A., Dhillon, N., Roberts, G. A., Cortés, J. & Leadlay, P. F. (1991). Cloning and sequence analysis of genes

involved in erythromycin biosynthesis in *Saccharopolyspora erythraea*, sequence similarities between EryG and a family of S-adenosylmethionine-dependent methyltransferases. *Mol Gen Genet* **230**, 120–128.

Hernandez, C., Olano, C., Méndez, C. & Salas, J. A. (1993). Characterization of a *Streptomyces antibioticus* gene cluster encoding a glycosyltransferase involved in oleandomycin inactivation. *Gene* **134**, 139–140.

Huang, S.-L., Hassell, T. C. & Yeh, W.-K. (1993). Purification and properties of NADPH-dependent tylosin reductase from *Streptomyces fradiae*. *J Biol Chem* **268**, 18987–18993.

Ichinose, K., Bedford, D. J., Tornus, D., Bechtold, A., Bibb, M. J., Revill, W. P., Floss, H. G. & Hopwood, D. A. (1998). The granaticin biosynthetic gene cluster of *Streptomyces violaceoruber* Tü22: sequence analysis and expression in a heterologous host. *Chem Biol* **5**, 647–659.

Janssen, G. R. & Bibb, M. J. (1993). Derivatives of pUC18 that have BglII sites flanking a modified multiple cloning site and that retain the ability to identify recombinant clones by visual screening of *Escherichia coli* colonies. *Gene* **124**, 133–134.

Jenkins, G. & Cundliffe, E. (1991). Cloning and characterization of two genes from *Streptomyces lividans* that confer inducible resistance to lincomycin and macrolide antibiotics. *Gene* **108**, 55–62.

Kagan, R. M. & Clarke, S. (1994). Widespread occurrence of three sequence motifs in diverse S-adenosylmethionine-dependent methyltransferases suggests a common structure for these enzymes. *Arch Biochem Biophys* **310**, 417–427.

Kim, K.-S. & Farrand, S. K. (1996). Ti plasmid-encoded genes responsible for catabolism of the crown gall opine mannopine by *Agrobacterium tumefaciens* are homologs of the T-region genes responsible for synthesis of this opine by the plant tumor. *J Bacteriol* **178**, 3275–3284.

Macpherson, D. F., Manning, P. A. & Morona, R. (1994). Characterization of the dTDP-rhamnose biosynthetic genes encoded in the *rfb* locus of *Shigella flexneri*. *Mol Microbiol* **11**, 281–292.

Merson-Davies, L. A. & Cundliffe, E. (1994). Analysis of five tylosin biosynthetic genes from the *tylIBA* region of the *Streptomyces fradiae* genome. *Mol Microbiol* **13**, 349–355.

Olano, C., Rodriguez, A. M., Michel, J.-M., Méndez, C., Raynal, M.-C. & Salas, J. A. (1998). Analysis of a *Streptomyces antibioticus* chromosomal region involved in oleandomycin biosynthesis, which encodes two glycosyltransferases responsible for glycosylation of the macrolactone ring. *Mol Gen Genet* **259**, 299–308.

Omura, S., Sadakane, N. & Matsubara, H. (1982a). Bioconversion and biosynthesis of 16-membered macrolide antibiotics. XXII. Biosynthesis of tylosin after protylonolide formation. *Chem Pharm Bull* **30**, 223–229.

Omura, S., Tanaka, H. & Tsukui, M. (1982b). Biosynthesis of tylosin: oxidations of 5-O-mycaminosylprotylonolide at C-20

and C-23 with a cell-free extract from *Streptomyces fradiae*. *Biochem Biophys Res Commun* **107**, 554–560.

Pissowotzki, K., Mansouri, K. & Piepersberg, W. (1991). Genetics of streptomycin production in *Streptomyces griseus*: molecular structure and putative function of genes *strELMB2N*. *Mol Gen Genet* **231**, 113–123.

Quirós, L. M., Aguirrezabalaga, I., Olano, C., Méndez, C. & Salas, J. A. (1998). Two glycosyltransferases and a glycosidase are involved in oleandomycin modification during its biosynthesis by *Streptomyces antibioticus*. *Mol Microbiol* **28**, 1177–1185.

Reiser, J., Muheim, A., Hardegger, M., Frank, G. & Fiechter, A. (1994). Aryl-alcohol dehydrogenase from the white rot fungus *Phanerochaete chrysosporium*. *J Biol Chem* **269**, 28152–28159.

Salah-Bey, K., Doumith, M., Michel, J.-M., Haydock, S., Cortés, J., Leadlay, P. F. & Raynal, M.-C. (1998). Targeted gene inactivation for the elucidation of deoxysugar biosynthesis in the erythromycin producer *Saccharopolyspora erythraea*. *Mol Gen Genet* **257**, 542–553.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Scotti, C. & Hutchinson, C. R. (1996). Enhanced production by manipulation of the *Streptomyces peucetius dnrH* and *dnmT* genes involved in doxorubicin (adriamycin) biosynthesis. *J Bacteriol* **178**, 7316–7321.

Summers, R. G., Donadio, S., Staver, M. J., Wendt-Pienkowski, E., Hutchinson, C. R. & Katz, L. (1997). Sequencing and mutagenesis of genes from the erythromycin biosynthetic gene cluster of *Saccharopolyspora erythraea* that are involved in L-mycarose and D-desosamine production. *Microbiology* **143**, 3251–3262.

Thorson, J. S., Lo, S. F., Ploux, O., He, X. & Liu, H. W. (1994). Studies of the biosynthesis of 3,6-dideoxyhexoses: molecular cloning and characterization of the *asc* (ascarylose) region from *Yersinia pseudotuberculosis* serogroup VA. *J Bacteriol* **176**, 5483–5493.

van Wageningen, A. M. A., Kirkpatrick, P. N., Williams, D. H., Harris, B. R., Kershaw, J. K., Lennard, N. J., Jones, M., Jones, S. J. M. & Solenberg, P. J. (1998). Sequencing and analysis of genes involved in the biosynthesis of a vancomycin group antibiotic. *Chem Biol* **5**, 155–162.

Westrich, L., Domann, S., Faust, B., Bedford, D., Hopwood, D. A. & Bechthold, A. (1999). Cloning and characterization of a gene cluster from *Streptomyces cyanogenus* S136 probably involved in landomycin biosynthesis. *FEMS Microbiol Lett* **170**, 381–387.

Wilson, V. T. W. & Cundliffe, E. (1998). Characterization and targeted disruption of a glycosyltransferase gene in the tylosin producer, *Streptomyces fradiae*. *Gene* **214**, 95–100.

Received 18 June 1999; revised 29 September 1999; accepted 11 October 1999.