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 *tyl*CK 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; Omura 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 deoxyallose 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

Abbreviation: MGT, macrolide glycosyltransferase.

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

**Fig. 1.** Structure of tylosin.
Sequencing of the tyl flanked by the resistance genes tlrB and tlrC. All of the structural genes required for tylacin 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 7*–25* to distinguish them from ORFs on the upstream side of tylG. Only the first 11 downstream genes, proximal to tylG, are shown here.

**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 tylacin production probably lie between tlrB and tlrC. Arrows represent genes of the tyl cluster. The genes numbered 1–11 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 7*–25* to distinguish them from ORFs on the upstream side of tylG. Only the first 11 downstream genes, proximal to tylG, are shown here.

**METHODS**

**Bacterial strains, growth conditions and genetic manipulation.** *St. fradiae* TS9235 (wild-type, also known as C373.1) and its derivatives were maintained and propagated at 37 °C on A-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 *St. fradiae* tyl strains. 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, *ermE*; for expression of the inserted genes, integrates into the chromosomal attB site.

**DNA sequencing and computer analysis.** The sequence of orf6 was obtained by combining that given under accession number U08223 (Merson-Davies & Cundliffe, 1994) and AF145042 (Bate et al., 1999). The *St. fradiae* tyl DNA sequence here was obtained from pMOMT4 (Beckmann et al., 1989). Fragments were subcloned in pIJ2925 (Janssen & Cundliffe, 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 Tag 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 codon preference, BLASTX and 6 frame...
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'-ACTTGAATTCTGACGAGGTCGAGGACG-3' and 5'-GGCGAAGCTTGGCCGGAGTCAGCAGCCGGC-3'. These contained, respectively, EcoRI and HindIII restriction sites (underlined) that were used to ligate the product (1513 bp with a unique central SalI site) into pIJ2925. The hygromycin resistance cassette, \( \Omega \), together with its flanking transcriptional terminators (Blondelet-Rouault et al., 1997) was then inserted at the SalI site, thereby interrupting the orf7-coding sequence 640 bp downstream of the earliest possible translational start.

A 2272 bp KpnI 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 SexAI 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 BamHI 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-disruption fragment (50 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; Gandecha et al., 1997) and subsequently extended (Bate & Cundliffe, 1999; Bate et al., 2006), 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 trID (orf5*) and overlaps by 209 bp the sequence deposited under accession number X97721 (Gandecha & 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’ (Balz & 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-deoxyxgenation. Were epimerization to precede 2-deoxyxgenation, 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-keto-reduction, from that shown in Fig. 3 (compound 2). Alternatively, 2-deoxyxgenation might occur first (Summers et al., 1997), in which case the stereochemistry at C-3 following the enoyl-reductase reaction would dictate the mode of

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

<table>
<thead>
<tr>
<th>ORF</th>
<th>St. fradiae gene name</th>
<th>Sa. erythraea gene name</th>
<th>Gene product</th>
</tr>
</thead>
<tbody>
<tr>
<td>orf6</td>
<td>tylCVII</td>
<td>eryBVII</td>
<td>5- (or 3,5) Epimerase</td>
</tr>
<tr>
<td>orf6</td>
<td>tylCVI</td>
<td>eryBVI</td>
<td>2,3-Dehydratase</td>
</tr>
<tr>
<td>orf10</td>
<td>tylCII</td>
<td>eryBII</td>
<td>2,3-Enoyl-reductase</td>
</tr>
<tr>
<td>orf8</td>
<td>tylCIII</td>
<td>eryBIII</td>
<td>3-C-Methyltransferase</td>
</tr>
<tr>
<td>orf9</td>
<td>tylCVIV</td>
<td>eryBV</td>
<td>4-Ketoreductase</td>
</tr>
<tr>
<td>orf7</td>
<td>tylCV</td>
<td>eryBV</td>
<td>Glycosyltransferase</td>
</tr>
</tbody>
</table>
deoxygenation of dTDP-4-keto-6-deoxy-involving pairs of purified enzymes that catalyse 2-
came from recent
subsequent epimerization. Support for the latter route,
finally converted to mycinose, via bis-
the emerging tylosin molecule by specific glycosyltransferases
1, NDP-4-keto-6-deoxy-D-glucose, represents a branch point in
tylosin production. It remains to be established whether
Alternative biosynthetic routes to mycarose during
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**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
tanglycosylolation.

**Assignment of ORFs**

**orf6** (tyICVII). The deduced product of orf6* is a protein
of 197 amino acid residues maximum (M, 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 (Gaiser et al., 1997;
Summers et al., 1997). In Sä. erythraea, eryBVII encodes
a mycarose-biosynthetic enzyme and similar genes in
other organisms are also associated with the production
of t-sugars, including t-rhamnose (rfBC gene in Shigella;
Macpherson et al., 1994), t-ascarylose (ascE in Yersinia;
Thorson et al., 1994) and t-dihydrostreptose (strM in
Streptomyces griseus; Pissowitzky et al., 1991). Although
direct experimental evidence is lacking, StrM
(Pissowitzky et al., 1991) and EryBVII (Gaiser et al.,
1997; Summers et al., 1997) are believed to be NDP-4-
ket-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 com-
plementation with orf6* restored normal levels of tylosin
production (data not shown). We concluded that Orf6*
(now designated TyICVII) is the 3,5- (or 5-) epimerase
involved in mycarose biosynthesis and noted significant
similarity (41 % sequence identity) between it and TyJ,
the putative 3-epimerase involved in the formation of 6-
doxyallose (Bate & Cundliffe, 1999).

**orf7** (tyICV). In database searches, the deduced product
of orf7* (461 amino acids; M, 50100) displayed greater
than 30 % sequence identity to various macrolide
glycosyltransferases (MGTs), such as OleL and OleD
from St. antibioticus, producer of oleandomycin (Quiros
et al., 1998; Hernandez et al., 1993), GimA from
Streptomyces ambofaciens, producer of spiramycin
(Gournelen et al., 1998) and Mgt from Streptomyces
dihydros (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 glycosyltrans-
ferases involved in antibiotic biosynthesis such as OleG2
(Olano et al., 1998), EryCIII and EryBV (Gaiser 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 fer-
mentation (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

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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 ωhyg 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 macrolytransferases 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; Mr 46600) displays extremely close end-to-end similarity (with 70% sequence identity) to that of ery orf3 (Haydock et al., 1991), now renamed eryBIII (Gaiser 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 (Gaiser 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; Mr 36200) displays 49% sequence identity to that of eryBIV (Gaiser 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
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* (tylClI). The deduced product of orf10* (329 amino acids; M, 36100) is very similar (74% sequence identity) to EryBI 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 EryBI was revealed during incisive analysis of Sa. erythraea mutant BI192 (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 eryBI encodes 2,3-eneoyl reductase activity involved in mycarose biosynthesis and a similar role is proposed here for the orf10* product, designated TylClI.

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-dihydrodesmycosin, together with two minor components consistent with N-demethyl-desmycosin and N-demethyl-20-dihydrodesmycosin (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.

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.
When orf6, together with ermEp, was integrated into the ϕC31 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 (Gaiser 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 product of orf6–dnmT, respectively. Arguing by elimination, it was suggested that EryBVI is the 2,3-dehydratase required for oxygenation at C-2 during mycarose biosynthesis in the erythromycin producer (Gaiser 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 TylCVI 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 and orf9, and since orf7 and orf8 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 mycanose-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).

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