

Expression of *tylM* genes during tylosin production: Phantom promoters and enigmatic translational coupling motifs

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In the genome of *Streptomyces fradiae*, the three *tylM* genes are codirectional with the upstream gene, *tylGV*. Although the introduction of transcriptional blocks into the *tylM* genes revealed that they are normally cotranscribed, expression of *tylMI* still persisted (albeit at a very low level) when either of the upstream genes, *tylMII* or *tylMIII*, was disrupted. Such expression apparently resulted from transcriptional initiation at spurious sites that probably contribute insignificantly, if at all, to promote activity in the wild type. Prior to the onset of tylosin production, *tylMIII* is transcribed independently of *tylGV* from an authentic promoter buried within *tylGV*. This latter observation is interesting given that the TGA stop codon of *tylGV* overlaps the GTG start codon of *tylMIII*. Evidently, terminally overlapping genes are not always translationally coupled.

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Introduction

The structural genes for tylosin biosynthesis (*tyl* genes) are clustered within about 1% (~85 kb) of the *Streptomyces fradiae* genome (Figure 1) together with resistance determinants, regulatory elements and also ancillary genes that have paralogues elsewhere in the genome (for a review, see Ref. [11]). Tylosin is a macrolide antibiotic (Figure 2) comprising a polyketide lactone substituted with three deoxyhexose sugars [16]. Deoxyhexose biosynthetic genes lie on either side of a group of five mega genes (*tylGI–GV*; 41 kb in total) encoding the TyIG polyketide synthase (PKS) that produces the aglycone, tylactone (also known as protylonolide). Tylosin production begins with synthesis and cyclization of the aglycone and continues *via* concurrent ring oxidation (converting the polyketide moiety to tylonolide) plus glycosylation, during which mycaminose is always added first [1,2,4,18–20].

The generation of glycosylated intermediates, such as *O*-mycaminosyl-tylonolide (OMT), is necessary to stimulate bulk tylactone production in *S. fradiae*. The existence of that uncharacterized regulatory mechanism was revealed when targeted gene disruption was first applied to the mycaminose biosynthetic gene, *tylMII* [12]. Whereas disruption of genes involved in the synthesis or addition of mycaminose or mycarose resulted in production of the predicted products, demycinosyl-tylosin [25] or demycarosyl-tylosin [3], respectively, the *tylMII*-disrupted strain (at the time designated “SF01”) did not accumulate any macrolide at all, unless a glycosylated tylosin precursor (such as OMT) was added exogenously to the fermentation broth. Under the latter conditions, strain SF01 accumulated copious quantities of tylactone. Since then, strains specifically disrupted in the other three mycaminose biosynthetic genes, *tylMI*, *tylMIII* and *tylB*, have been generated and shown to behave similarly to strain SF01 with

respect to tylactone production, or lack of same in the absence of glycosylated macrolides [9,13]. The *tylM* genes respectively encode 3-*N*-methyltransferase (TylMI), mycaminosyltransferase (TylMII) and putative 3,4-isomerase (tylMIII) activities involved in the formation of NDP-mycaminose and subsequent glycosylation of tylactone [14]. The fourth “mycaminose gene” (*tylB*) encodes 3-aminotransferase activity [17]. That gene is located at least 45 kb distant from *tyl[MIII–MII–MI]* (Figure 1) and was not manipulated in the present work. For ease and conformity of nomenclature, the various disrupted (“knockout”) strains are referred to here as *tylMI*-KO, *tylMII*-KO and *tylMIII*-KO, and the present work describes results obtained when the disrupted strains were complemented with wild type *tylM* genes (singly or in combination) and subjected to fermentation analysis. Also, transcript analysis has been used to study the expression of *tylM* and *tylG* genes.

Materials and methods

Bacterial strains, growth conditions and genetic manipulation

S. fradiae T59235 (synonym C373.1) was maintained and propagated at 37°C on AS-1 agar [25] or at 30°C in tryptic soy broth (TSB; Difco, Sparks, MD). Plasmids were manipulated in *Escherichia coli* DH5 α using standard protocols [23]. DNA was introduced into *S. fradiae* *via* conjugal transfer from *E. coli* as described elsewhere [12] using pOJ260 [6] and pLST9828 [8]. The former is a suicide vector, unable to replicate in *Streptomyces* spp., and was used for targeted gene disruption. pLST9828, used for complementation analysis, integrates into the chromosomal Φ C31 *attB* site and contains a powerful constitutive promoter, *ermEp** [5], to ensure expression of cloned genes.

Targeted gene disruption via gene transplacement

The generation of strains specifically disrupted in *tylMIII*, *tylMII* or *tylMI* has been described elsewhere [9]. In each case, *S. fradiae*

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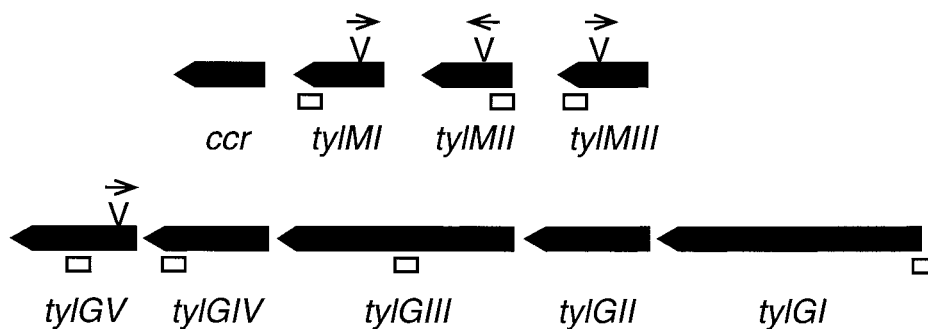


Figure 1 Contiguous portion of the tylosin biosynthetic gene cluster of *S. fradiae*. Not drawn to scale. The five *tylG* mega genes (~41 kb) lie immediately upstream of the other four genes (~4.5 kb in total). Sites at which the respective genes were disrupted are marked (V), above which the orientation of the disruption cassette is shown (arrows). White boxes, not drawn to scale relative to gene sizes, indicate the approximate positions of PCR-amplified sequences (~400 bp each).

DNA containing the target gene flanked by DNA arms of approximately equal size (~1 kb) was introduced into pIJ2925 [15] and disrupted before introduction into *S. fradiae*. The *tylM* genes were disrupted using the hygromycin B resistance cassette, Ω hyg [7], in which the *hyg* gene is flanked by transcriptional and translational terminators. A similar strategy was used to disrupt *tylGV* using the Ω interposon, in which a streptomycin–spectinomycin resistance gene (*aadA*) is again flanked by transcriptional and translational terminators [21]. This was introduced into *tylGV* at a *HincII* site located about 4611 bp upstream of *tylMIII*, within the β -keto acyl synthase (KS) domain near the start of *tylGV* (total size ~5.5 kb). A 2.1-kb *NotI*–*KpnI* fragment from *tylGV*, end-filled at the *NotI* terminus and containing an approximately central *HincII* site, was ligated into *KpnI*–*HincII* prepared pIJ2925, thereby destroying the *HincII* site in the polylinker. Then, the Ω interposon was ligated into the central *HincII* site within the insert and the disrupted fragment of *tylGV* was transferred as a 4.2-kb *BglII* fragment into pOJ260 for

conjugal transfer into *S. fradiae*. Following initial selection on hygromycin B (75 μ g/ml) or on spectinomycin (100 μ g/ml) plus streptomycin (1 μ g/ml), transconjugants were screened for sensitivity to apramycin (25 μ g/ml) to identify double recombinants (i.e., lacking the resistance marker carried by derivatives of pOJ260) in which specific chromosomal genes had been replaced with the disrupted constructs.

Complementation of disrupted strains

pLST9828 was used to integrate DNA fragments, containing *tylM* genes (singly or in combination) together with flanking DNA, into the chromosomal Φ C31 *attB* site of each of the three *tylM*-KO strains. The integrated genes were positioned downstream of the strong constitutive promoter, *ermEp**, and were oriented favourably for expression. The respective DNA fragments were: *tylMIII*, a 1495-bp *BssSI* fragment with 83 bp of upstream DNA, i.e., separating *tylMIII* from *ermEp**; *tylMII*, a 1677-bp *BstEII*–*MscI*

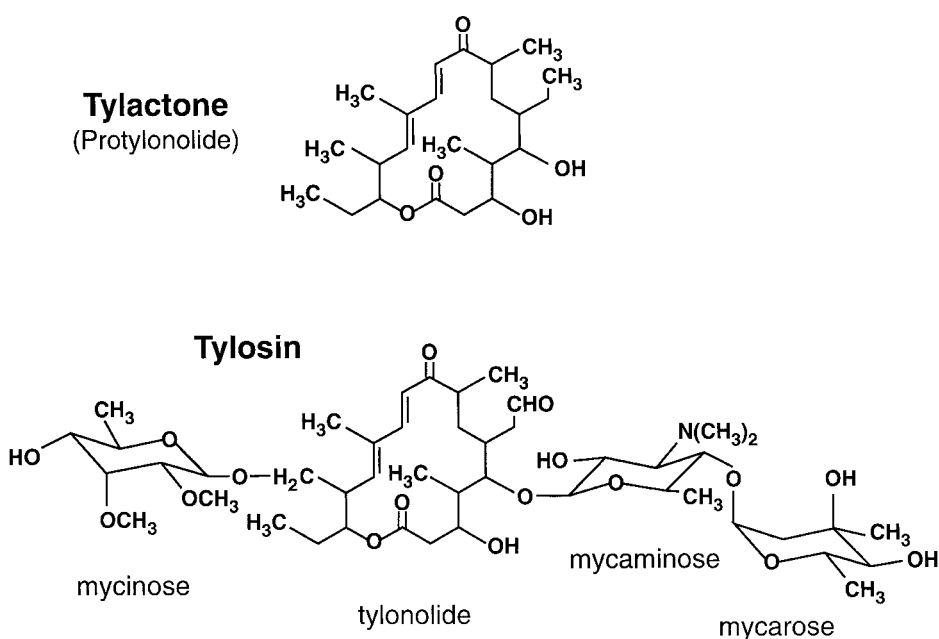


Figure 2 Structures of tyractone and tylosin.

fragment containing 54 bp of upstream DNA; *tyIMI*, a 1381-bp *SaII-SstI* fragment with 385 bp upstream DNA; *tyl[MIII-MII-*

MI], a 3992-bp *MscI-HincII* fragment with 194 bp of upstream DNA; *tyl[MIII-MII]*, a 3018-bp *MscI-SphI* fragment again with

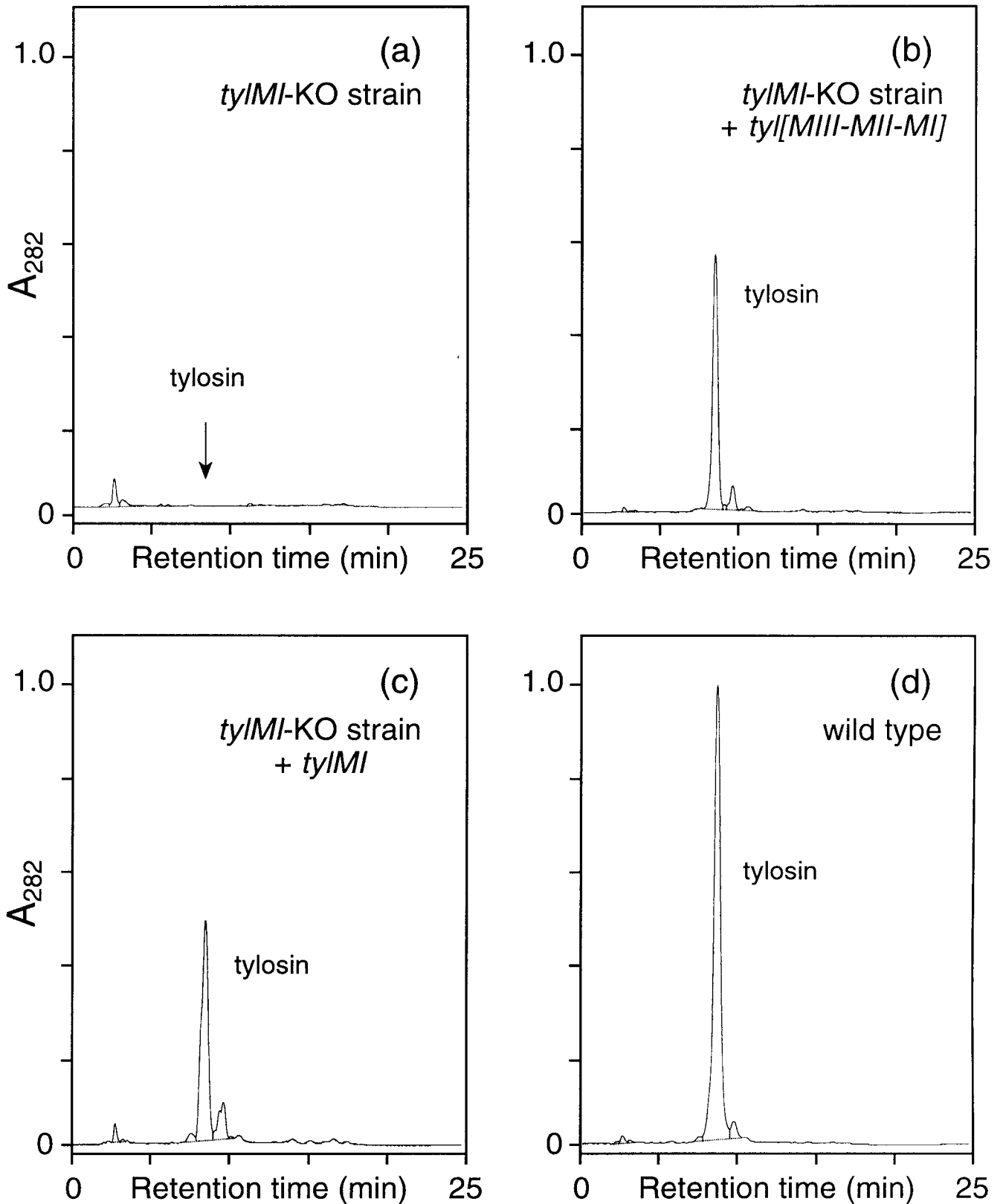


Figure 3 Fermentation products from *S. fradiae* strains. HPLC analysis of material produced by: (a) *tyIMI*-disrupted strain; (b) *tyIMI*-disrupted strain complemented with *tyl[MIII-MII-MI]*; (c) *tyIMI*-disrupted strain complemented with *tyIMI*; (d) wild type.

194 bp of upstream DNA; *tyl[MII-MI]*, a 2280-bp *Bst*EI fragment with 54 bp of upstream DNA.

Fermentation analysis

Fermentation of *S. fradiae*, bioconversion of exogenously added ty lactone and high-performance liquid chromatography (HPLC) analysis of products, with internal standards, are described elsewhere [8]. Gene transplacement is a stable event and this, together with the use of integrative plasmids for complementation, obviated the need for antibiotic selection during fermentation.

RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

Transcript analysis was carried out on *S. fradiae* wild type grown in TSB at 30°C. Cultures for RNA isolation (50 ml of TSB per 250-ml flask) were inoculated with approximately 4×10^7 spores and grown with orbital shaking at 250 rpm. The protocol for rapid extraction of total RNA is described elsewhere [24]. RNA samples were treated with DNase I using DNA-free[™] kit (Ambion, Abington, UK) according to the manufacturer's instructions. RT-PCR was carried out with 1 µg of total RNA as template using SUPERScript[™] One-Step RT-PCR with PLATINUM[®] Taq DNA polymerase (GIBCO-BRL, Parsley, UK). Dimethyl sulfoxide (5% vol/vol, final) was added to all PCR reactions together with RNAGuard[™] RNase Inhibitor (Amersham Pharmacia, Amersham, UK; 29.4 U per reaction). Conditions were as follows: first strand cDNA synthesis, 50°C for 30 min followed by 94°C for 2 min; amplification, 1 cycle of 94°C for 1 min, 52°C for 1 min and 72°C for 1 min followed by 24 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. Primers were designed using the software PRIMER v 1.0 Ashland MA 1996 to generate amplified products of approximately 400 bp from template mRNA. The authenticity of each amplified product was confirmed by single strand sequence analysis using one of the amplification primers. For each set of primers, negative controls, carried out using PLATINUM[®] Taq DNA Polymerase in the absence of RT, confirmed that amplified products were derived from mRNA and not from

contaminating chromosomal DNA. Primers used were (all 5'-3'): for *thrD* (internal control), [cgtgtatggggccagaattt and ctgcccgtgtactgtctt]; for *tylGI*, [agaacgaaccgacggcac and cccagaattcctcgacaccg]; for *tylGIII*, [gagatgctggtagtctcc and atgttgacttcaggagacc]; for *tylGIV*, [acggtaggaggtcctcagc and aactgaaccgactccttg]; for *tylGV*, [caccgacgactggatgacc and ctctccatagctgcatca]; for *tylMI*, [cctgcatgttcagctcgatc and gcatgaactccacggacagt]; for *tylMII*, [tgagaaggcactggatgac and atgtggagctcgagttgaa]; for *tylMIII*, [gcgatcaccgaaactgct and tcaactcggggacatacggg].

Results and discussion

Complementation of *tylM*-KO strains with *tylM* DNA

None of the three *tylM*-KO strains accumulated macrolide material when fermented in tylosin production medium (Figures 3a, 4a and 5a) although, as expected, each could produce tylosin when complemented with a block of wild type DNA containing *tyl[MIII-MII-MI]* under control of the strong, constitutive promoter, *ermEp** (see Figures 3b and 5b). Note, however, that wild type levels of tylosin production were not achieved by the complemented strains (compare Figure 3b and d), since the Φ C31 *attB* site, used for integration of complementing DNA, is not a neutral site in the context of tylosin production. Thus, integration of the "empty" vector, pLST9828, into that site reduces tylosin yields by up to 50% (unpublished data; this laboratory). Note also that it was not necessary to include the *ccr* gene (Figure 1), downstream of and codirectional with the *tylM* genes, in the blocks of complementing DNA, since strains lacking *ccr* can still produce tylosin [9].

Evidence for cotranscription of *tylM* genes

Tylosin production was restored in the *tylMI*-KO strain by reintroduction of wild type DNA containing only *tylMI* (Figure 3c). However, production was not restored in the *tylMII*-KO strain by *tylMII* (Figure 4c), or in the *tylMIII*-KO strain by *tylMIII* alone

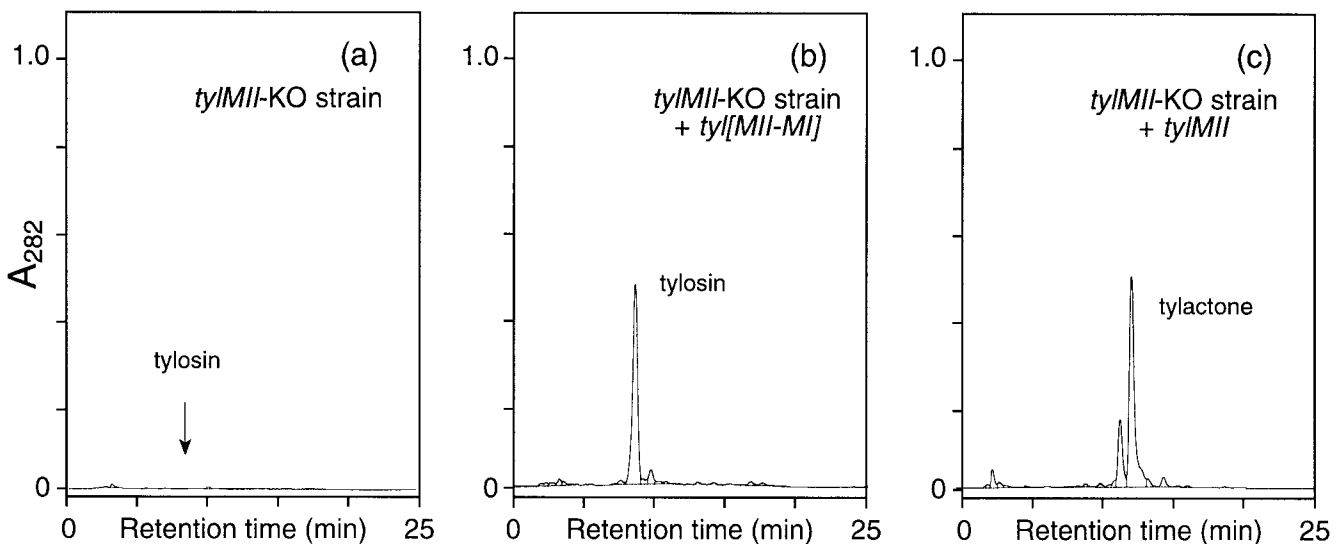


Figure 4 Fermentation products from an *S. fradiae* strain disrupted in *tylMII*. HPLC analysis of material produced by: (a) *tylMII*-disrupted strain; (b) *tylMII*-disrupted strain complemented with *tyl[MII-MI]*; (c) *tylMII*-disrupted strain complemented with *tylMII* alone.

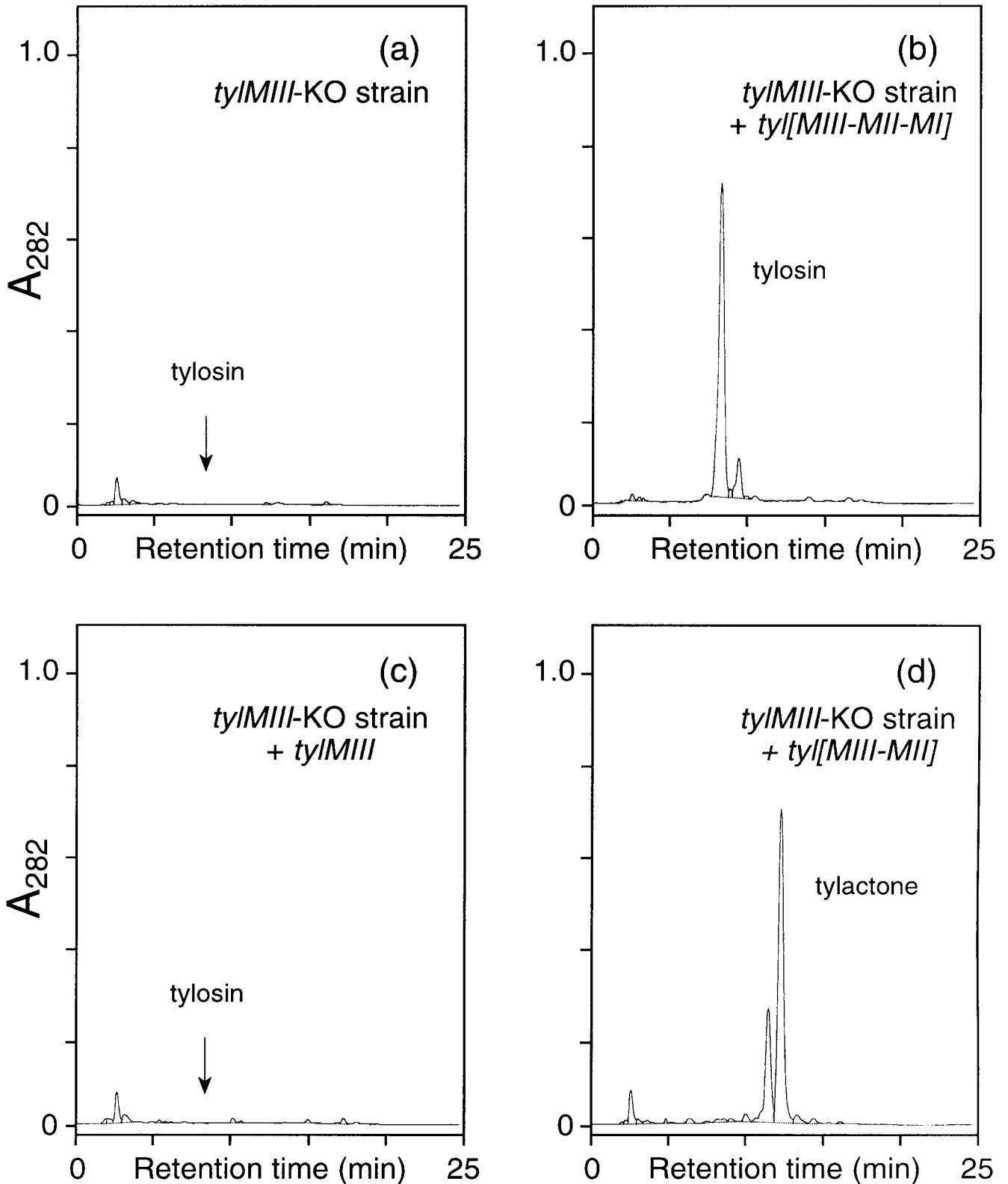


Figure 5 Fermentation products from an *S. fradiae* strain disrupted in *tylMIII*. HPLC analysis of material produced by: (a) *tylMIII*-disrupted strain; (b) *tylMIII*-disrupted strain complemented with *tyl[MIII-MII-MI]*; (c) *tylMIII*-disrupted strain complemented with *tylMIII*; (d) *tylMIII*-disrupted strain complemented with *tyl[MIII-MII]*.

(Figure 5c) or by *tylMIII* plus *tylMII* (Figure 5d). Evidently, the three *tylM* genes are normally cotranscribed so that disruption of

tylMII or *tylMIII* prevented the expression of downstream genes. Consistent with this conclusion, the *tylMII*-KO strain was

successfully complemented by a DNA fragment containing *tylMIII* plus *tylMI* (Figure 4b), whereas *tyl[MIII-MII-MI]* was necessary for restoration of tylosin production in the *tylMIII*-KO strain (Figure 5b).

When is a “promoter” not a promoter?

Although reintroduction of intact *tylMII* into the *tylMII*-KO strain did not restore production of tylosin or other glycosylated macrolide(s), tylactone readily accumulated in the “complemented” strain (Figure 4c). In the light of earlier observations [9,12] that glycosylated macrolides are required to stimulate bulk synthesis of tylactone in *S. fradiae*, this result suggested that *tylMI* must still have been expressed in the *tylMII*-KO strain, albeit at a very low level. This was unlikely to have resulted from transcription initiating within the favourably oriented Ω hyg cassette with which *tylMII* had been disrupted, given the presence of transcriptional terminators flanking that cassette. More likely, low level transcription had originated from a spurious “promoter” located downstream of the site of disruption of *tylMII*. Similarly, tylactone accumulated in the *tylMIII*-KO strain following reintroduction of *tyl[MIII-MII]* (Figure 5d), although not when *tylMIII* alone was reintroduced (Figure 5c), suggesting that *tylMI* could be expressed at a low level in the *tylMIII*-KO strain. Since the Ω hyg cassette had been inserted into *tylMIII* in the reverse orientation relative to the gene, transcription of *tylMI* must have been initiated downstream of the site of disruption of *tylMIII*, perhaps from same site as in the *tylMII*-KO strain. According to this rationalization, the fortuitous level of *tylMI* expression in either strain was sufficient to

allow production of glycosylated macrolide(s) in amounts great enough to trigger bulk production of tylactone, but not great enough to allow detection of such material(s) by HPLC. This interpretation was supported when the fermentation extract analysed in Figure 4c was examined by electrospray mass spectrometry. Material consistent with the presence of OMT (m/z value for $[M+H]^+ = 598$) was detected (data not shown). Such “fortuitous” or “spurious” transcription, originating from phantom promoters (i.e., sites that normally contribute insignificantly, if at all, to promoter activity in the wild type), will presumably be encountered in other situations when normal patterns of transcription are disrupted.

The significance of terminally overlapping genes

Immediately upstream of the three *tylM* genes lies *tylGV* (~5.5 kb), the most downstream component of the *tylG* complex (Figure 1). This gene terminally overlaps *tylMIII*, affording possible translational and/or transcriptional coupling of the orfs. Moreover, since it is not yet known how many promoters are used to express the *tylG* genes in *S. fradiae*, it remained possible that the four similarly oriented genes downstream of *tylGV* might even be cotranscribed with the five *tylG* mega genes. Such an arrangement operates in *Saccharopolyspora erythraea*, where a single promoter drives transcription of the entire *eryA* complex of PKS genes (~30 kb) together with four downstream orfs, giving a giant transcript of about 35 kb [22]. It was therefore interesting to ascertain whether the *tyl* cluster is equally parsimonious in promoter usage and, in particular, whether *tylGV* and the *tylM* genes might be cotran-

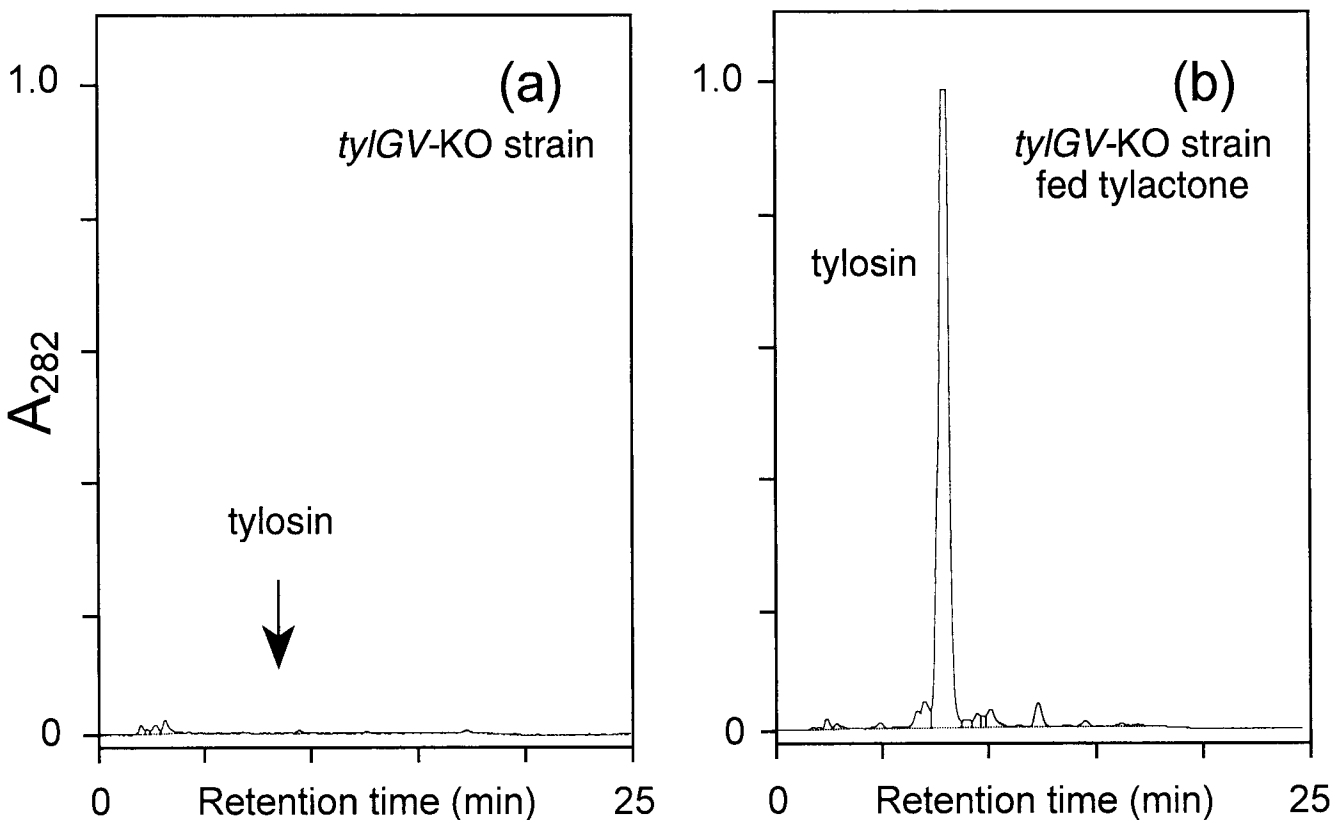


Figure 6 Fermentation products from an *S. fradiae* strain disrupted in *tylGV*. HPLC analysis of material produced after 7 days of fermentation, supplemented as follows: (a) control, not supplemented; (b) fed 25 mg of tylactone (per 50-ml culture) after 48 h.

scribed. Accordingly, *tylGV* was disrupted close to the start of the gene to reduce the likelihood that *tylMIII* would be separated from its natural promoter (should such exist) within *tylGV*, and the resultant strain was subjected to fermentation analysis. As expected (Figure 6a), the *tylGV*-KO strain produced no detectable macro-lide, but when the fermentation medium was supplemented with ty lactone, the latter was converted quantitatively to tylosin (Figure 6b). In experiments designed to determine the efficiency of such conversion (and with it, the efficiency of expression of the *tylM* genes), it became clear that the *tylGV*-KO strain could produce wild type levels of tylosin by bioconversion of exogenously added ty lactone (Figure 6b). Given that the Ω interposon had been introduced into *tylGV* in the reverse orientation relative to *tylGV*, transcription of *tylMIII* in the *tylGV*-KO strain must have been initiated within *tylGV* downstream of the disruption site. In a similar vein, and after completion of the present paper, we became aware of the suggestion [10] that transcription of three genes, *des[VIII–VII–VI]*, within the *pikB* locus of *S. venezuelae* is dependent on the integrity of a “transcription unit,” with a potential promoter sequence located inside an upstream gene (*pikAV*) which terminally overlaps *desVIII*. By any criteria, the initiation site for transcription within *tylGV* resembled a genuine promoter and we wondered whether it might be the natural promoter for *tylMIII* in the wild type.

Transcript analysis by RT-PCR

The complexity of the three-stage fermentation protocol, routinely used in this laboratory for analysing bulk production of macro-lide(s) by *S. fradiae*, made it unsuitable for transcript analysis. For this purpose, *S. fradiae* was grown in TSB, under which conditions

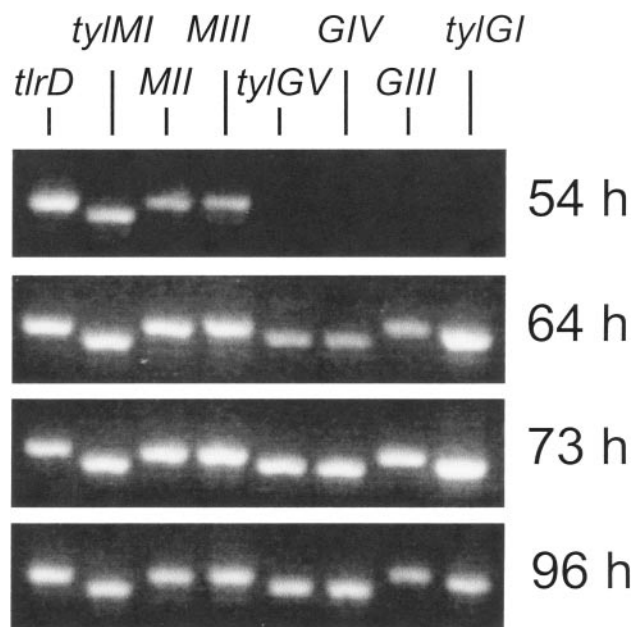


Figure 7 Transcriptional analysis of *tylM* and *tyG* genes by RT-PCR. Total RNA was extracted from *S. fradiae* wild type grown in TSB and used as template to generate amplified products of approximately 400 bp derived from the upstream ends of the respective genes. Amplified product was also generated from the constitutively expressed resistance determinant, *tlrD*, as an internal control. Production of tylosin, not detectable after 64 h, was first observed by mass spectrometry after 73 h and was maximal at 96 h.

tylosin production could not be detected (even by mass spectrometry) until around 73 h. RT-PCR was carried out with primers specific to sequences internal to *tylM* and *tyG* genes, using as template total RNA extracted from wild type mycelium before and after the onset of tylosin production. Negative controls lacking RT did not reveal amplified products. The results were unequivocal: prior to the onset of tylosin production, *tylM* gene transcripts were present in the absence of *tylG* expression (Figure 7).

Concluding comments

In *S. fradiae* wild type prior to the onset of tylosin production, *tylMIII* is expressed independently of *tylGV* from a promoter located within *tylGV*. The *tylMIII* promoter might well remain active throughout the duration of tylosin production and, given its apparent strength, it might even be the only promoter that contributes significantly to expression of the *tylM* genes. If so, *tylGV* and *tylMIII* would never be cotranscribed, let alone translationally coupled, even though they are terminally overlapping. Equally plausibly, the *tylM* genes might be cotranscribed with *tylGV* (or even with the entire *tylG* complex) once expression of the latter begins. This could occur in parallel with, or instead of, expression from the *tylMIII* promoter. The bottom line, however, is clear. The mere fact that two genes are terminally overlapping does not necessarily imply that they are always, if ever, translationally coupled. We conclude that the fairly common occurrence of GTGA or ATGA sequences linking gene pairs in actinomycetes reflects the almost exclusive usage (~95% frequency) of TGA as the translational stop codon in these organisms. Such linker sequences are much more reliable as indicators of translational coupling when encountered in organisms, such as *E. coli*, that use TGA only sparingly.

Actinomycetes presumably do utilise translational coupling, but its operation in any given context cannot be established by sophistry or downright wishful thinking.

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References

- Baltz RH and ET Seno. 1988. Genetics of *Streptomyces fradiae* and tylosin biosynthesis. *Annu Rev Microbiol* 42: 547–574.
- Baltz RH, ET Seno, J Stonesifer and GM Wild. 1983. Biosynthesis of the macrolide antibiotic tylosin. A preferred pathway from ty lactone to tylosin. *J Antibiot* 36: 131–141.
- Bate N, AR Butler, IP Smith and E Cundliffe. 2000. The mycarose-biosynthetic genes of *Streptomyces fradiae*, producer of tylosin. *Microbiology* 146: 139–146.
- Beckmann RJ, K Cox and ET Seno. 1989. A cluster of tylosin biosynthetic genes is interrupted by a structurally unstable segment containing four repeated sequences. In: Hershberger CL, SW Queener and G Hegeman (Eds.), *Genetics and Molecular Biology of Industrial Microorganisms*. American Society for Microbiology, Washington, DC, pp. 176–186.
- Bibb MJ, J White, JM Ward and GR Janssen. 1994. The mRNA for the 23S rRNA methylase encoded by the *ermE* gene of *Saccharopolyspora erythraea* is translated in the absence of a conventional ribosome-

- binding site. *Mol Microbiol* 14: 533–545.
- 6 Bierman M, R Logan, K O'Brien, ET Seno, RN Rao and BE Schoner. 1992. Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. *Gene* 116: 43–49.
 - 7 Blondelet-Rouault M-H, J Weiser, A Lebrihi, P Branny and J-L Pernodet. 1997. Antibiotic resistance cassettes derived from the Ω interposon for use in *E. coli* and *Streptomyces*. *Gene* 190: 315–317.
 - 8 Butler AR, N Bate and E Cundliffe. 1999. Impact of thioesterase activity on tylosin biosynthesis in *Streptomyces fradiae*. *Chem Biol* 6: 287–292.
 - 9 Butler AR, SA Flint and E Cundliffe. 2001. Feedback control of polyketide metabolism during tylosin production. *Microbiology* 147: 795–801.
 - 10 Chen S, JB Roberts, Y Xue, DH Sherman and KA Reynolds. 2001. The *Streptomyces venezuelae pikAV* gene contains a transcription unit essential for expression of enzymes involved in glycosylation of narbonolide and 10-deoxymethynolide. *Gene* 263: 255–264.
 - 11 Cundliffe E. 1999. Organization and control of the tylosin-biosynthetic genes of *Streptomyces fradiae*. *Actinomycetologica* 13: 68–75.
 - 12 Fish SA and E Cundliffe. 1997. Stimulation of polyketide metabolism in *Streptomyces fradiae* by tylosin and its glycosylated precursors. *Microbiology* 143: 3871–3876.
 - 13 Flint SA. 2000. Mycaminose metabolism and control of tylosin synthesis in *Streptomyces fradiae*. PhD Thesis, University of Leicester.
 - 14 Gandecha AR, SL Large and E Cundliffe. 1997. Analysis of four tylosin biosynthetic genes from the *tylLM* region of the *Streptomyces fradiae* genome. *Gene* 184: 197–203.
 - 15 Janssen GR and MJ Bibb. 1993. Derivatives of pUC18 that have *Bgl*III 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.
 - 16 Kirst HA. 1992. Antibiotics (macrolides). In: Howe-Grant M (Ed.), Kirk — Othmer Encyclopedia of Chemical Technology, 4th edition Vol. 3. Wiley, New York, pp. 169–213.
 - 17 Merson-Davies LA and E Cundliffe. 1994. Analysis of five tylosin biosynthetic genes from the *tylIBA* region of the *Streptomyces fradiae* genome. *Mol Microbiol* 13: 349–355.
 - 18 Omura S, H Takeshima, A Nagakawa, J Miyazawa, F Piriou and G Lukacs. 1977. Studies on the biosynthesis of 16-membered macrolide antibiotics using carbon-13 nuclear magnetic resonance spectroscopy. *Biochemistry* 16: 2860–2866.
 - 19 Omura S, N Sadakane and H Matsubara. 1982. Bioconversion and biosynthesis of 16-membered macrolide antibiotics: XXII. Biosynthesis of tylosin after protylonolide formation. *Chem Pharm Bull* 30: 223–229.
 - 20 Omura S, H Tanaka and M Tsukui. 1982. Biosynthesis of tylosin: oxidations of 5-*O*-mycaminosylprotylonolide at C-20 and C-23 with a cell-free extract of *Streptomyces fradiae*. *Biochem Biophys Res Commun* 107: 554–560.
 - 21 Prentki P and HM Krisch. 1984. *In vitro* insertional mutagenesis with a selectable DNA fragment. *Gene* 29: 303–313.
 - 22 Reeves AR, RS English, JS Lampel, DA Post and TJ Vanden Boom. 1999. Transcriptional organization of the erythromycin biosynthetic gene cluster of *Saccharopolyspora erythraea*. *J Bacteriol* 181: 7098–7106.
 - 23 Sambrook J, EF Fritsch and T Maniatis. 1989. Molecular Cloning: A Laboratory Manual, 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
 - 24 Stratigopoulos G and E Cundliffe. 2002. Expression analysis of the tylosin-biosynthetic gene cluster: pivotal regulatory role of the *tylQ* product. *Chem Biol* 9.
 - 25 Wilson VTW and E Cundliffe. 1998. Characterization and targeted disruption of a glycosyltransferase gene in the tylosin producer, *Streptomyces fradiae*. *Gene* 214: 95–100.

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1. Ref. [24]: Please provide update on status of publication.