Chemistry & Biology, Vol. 9, 71–78, January, 2002, ©2002 Elsevier Science Ltd. All rights reserved. PII S1074-5521(01)00095-3

Expression Analysis of the Tylosin-Biosynthetic Gene Cluster: Pivotal Regulatory Role of the *tylQ* Product

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Summary

Expression analysis by RT-PCR, applied to the entire *tyl* cluster, revealed that the pattern of transcription is more complex than expected. For example, the five *tylG* polyketide synthase genes are not necessarily cotranscribed or even coregulated. Among the regulatory genes, *tylQ* has emerged as a key factor. Although several genes (including the positive regulator, *tylS*) were possibly expressed constitutively, only *tylQ* was silent during secondary metabolism. Analysis of engineered strains, in which *tylQ* was disrupted or overexpressed, showed that the TylQ protein is a transcriptional repressor that blocks tylosin biosynthesis by controlling expression of the activator, *tylR*. Before tylosin production can be triggered, *tylQ* must be switched off, or at least downregulated.

Introduction

The tylosin-biosynthetic (*tyl*) gene cluster (Figure 1) contains 43 genes (including the flanking resistance determinants, *tlrB* and *tlrC*) and occupies about 1% (~85 kb) of the *Streptomyces fradiae* genome (for a review, see [1]). This block of DNA probably contains all the structural genes needed for tylosin production, with the sole exception of a gene encoding phosphopantetheinyl transferase, required for posttranslational addition of swinging arms to the TylG polyketide synthase (PKS) complex.

Genes within the *tyl* cluster are, to some extent, grouped according to function. Thus, five *tylG* megagenes (approximately 41 kb) encode synthesis of the PKS enzyme that produces and cyclizes tylactone (protylonolide), a polyketide lactone to which three deoxyhexose sugars are subsequently added, beginning with mycaminose. Synthesis and addition of mycaminose is encoded by three adjacent *tylM* genes plus *tylB*, which, curiously, lies on the opposite side of the *tylG* complex, more than 40 kb distant from the *tylM* genes [2, 3]. The *tylC* "mycarose" genes are also split, with one of them (*tylCVI*) located about 50 kb distant from *tyl[CII-CIV-CIII-CV-CVII]*, which form divergent "arms" [4]. The third tylosin sugar, mycinose, is generated in situ. Deoxyallose is added to the polyketide lactone and is then converted to mycinose via bis-*O*-methylation. The "mycinose/deoxyallose" genes (*tylNEDFJ*) are arranged in divergent blocks and form an intact subcluster together with *tyl[HI-HII]*, required for ring hydroxylation at the site of deoxyallose addition [5]. Most remarkably, the *tyl* cluster contains at least five regulatory genes. One of these, *tylR*, was previously identified as a global activator of the cluster by targeted disruption and bioconversion analysis [6]. This gene is about 60 kb distant from *tylP*, *tylQ*, *tylS*, and *tylT*, which form a regulatory subcluster. The latter were identified by sequence comparisons with the databases and were deduced to encode, respectively, a γ-butyrolactone receptor (TylP, for which *tylQ* was deduced to be a candidate target), and two proteins (TylS and TylT) of the SARP (**S**treptomyces **A**ntibiotic **R**egulatory **P**rotein [7]) family. The rest of the *tyl* cluster includes a miscellaneous collection of recognizable genes of unknown function (such as *orf11**, deduced to encode a GTP binding protein; *aco*, deduced to encode acyl-CoA oxidase[dehydrogenase]; and *orf16**, deduced to encode a cytochrome P450), unassigned genes (e.g., *orf12**, *orf1a*, and *orf9*), and ancillary genes. The latter are similar to genes of primary metabolism (presumably there are paralogs elsewhere in the genome), and they are not essential for tylosin production. Ancillary genes of the *tyl* cluster include *ccr* (encoding crotonyl-CoA reductase), *metK* (encoding *S*-adenosylmethionine synthase), and *metF*, which encodes N^5, N^{10}-methylenetetrahydrofolate reductase [8, 9].

Once the organization of the *tyl* gene cluster was known, we addressed the regulation of tylosin production by analyzing patterns of *tyl* gene expression before and after the onset of tylosin production. The results were unexpected and prompted further studies with engineered strains.

Results

Prelude to Gene Expression Analysis

Total RNA was prepared from *S. fradiae* mycelium after growth for 18 hr and 40 hr (i.e., before and after the onset of tylosin production; see Figure 2) and used as template for gene expression analysis by reverse transcriptase-PCR (RT-PCR). In order to minimize mRNA degradation during isolation, and given that little is known concerning the stability of mRNAs in actinomycetes, we developed a protocol whereby mycelium growing in shake flasks at 28°C could be washed, and then frozen in liquid nitrogen, within 30 s. Thereafter, the mycelium was broken (without thawing) in the Hughes Press, prior to addition of a chaotropic agent and RNA extraction. Primers for RT-PCR were specific to sequences within *tyl* genes (the upstream primer site for *tylGI* was located just outside the coding sequence, but within the transcribed region) and were designed to produce cDNAs of approximately 400 bp. The product from each pair of primers was then authenticated by sequencing. Transcripts were analyzed from 37 of the 43 genes of the *tyl* cluster. The six genes not analyzed were the minigene *tylHII* (deduced to encode a ferredoxin of unknown significance [5], *tylGII*, and four genes

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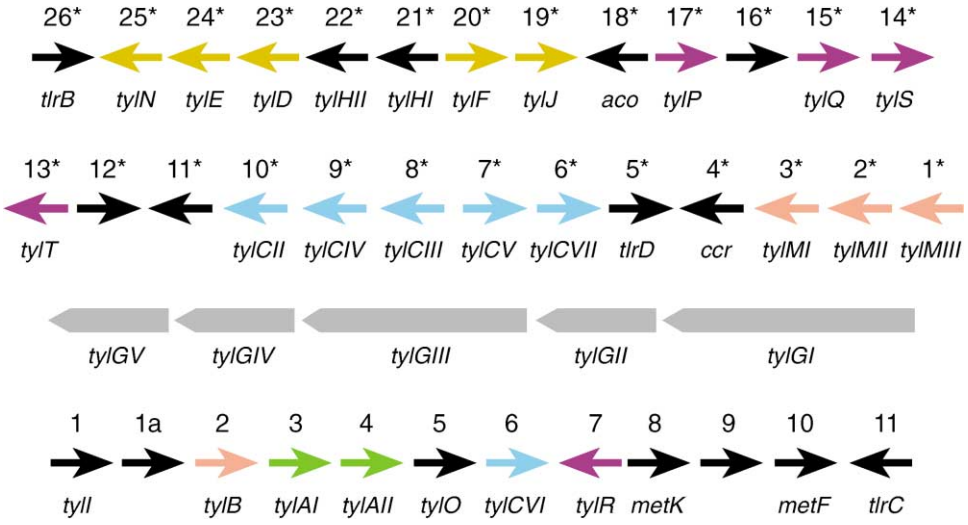


Figure 1. The Tylosin-Biosynthetic Gene Cluster of *S. fradiae*

Not drawn to scale. The cluster of 43 genes occupies a contiguous portion of the genome (approximately 85 kb including the flanking resistance genes *tlrB* and *tlrC)*. The five *tylG* megagenes cover approximately 41 kb. Upstream of *tylG*, 12 genes (*orf1*, *orf1a*, and *orf2*–*orf11*) occupy about 15 kb. Downstream of *tylG*, 26 genes (*orf1**–*orf26**) occupy about 29 kb. Regulatory genes are shown in purple. Other genes shown in color encode the biosynthesis of: polyketide (gray); mycaminose (pink); mycarose (blue); and mycinose (yellow). The products of *tylAI* and *tylAII* (green) catalyze steps common to the synthesis of all three sugars.

from the right-hand end of the cluster in the orientation of Figure 1. Specifically, these four genes were the resistance determinant, *tlrC,* and three genes (*metK*, *orf9*, and *metF*) that are not essential for tylosin production [9]. With each pair of primers, negative controls were carried out in the absence of reverse transcriptase (i.e., with DNA polymerase alone) to confirm that amplified products were derived from mRNA templates and not from chromosomal DNA, which inevitably contaminates RNA preparations, albeit in minute amounts. Having established that contaminating DNA did not yield detectable products after fewer than 30 PCR cycles, we normally used 25 cycles to detect transcripts. Whenever 25 cycles did not yield a product, analysis was repeated at 28 cycles to distinguish low-level transcription (positive at 28 cycles, negative at 25 cycles) from undetectable transcription (negative at 28 cycles). Although we are confident in using these criteria to estimate relative levels of transcripts, we wish to emphasize the dangers inherent in the use of other criteria (such as relative band intensities in Figure 3). This is not quantitative RT-PCR. Different primers cannot be assumed to prime with equal efficiencies, and the conditions for exponential phase have not been rigorously established with each set of primers.

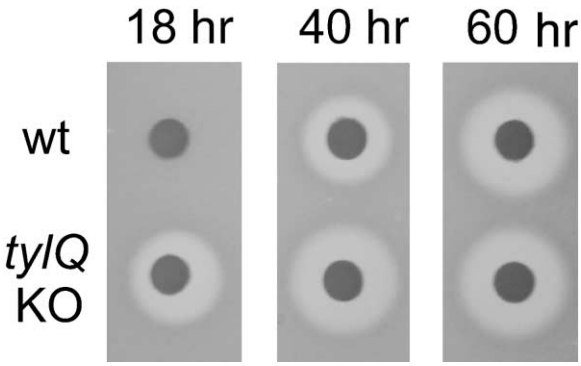
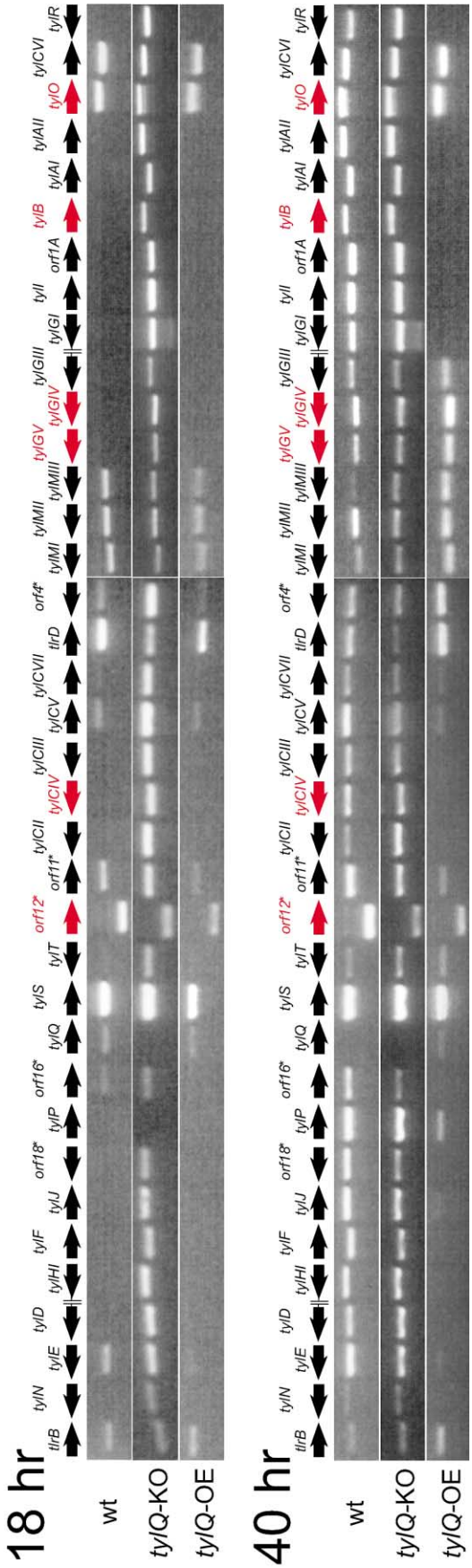


Figure 2. The Onset of Tylosin Production in *S. fradiae* Strains Grown in TSB

Microbiological assay of tylosin production, by the wild-type and by a *tylQ*-disrupted strain, with *Micrococcus luteus* as an indicator strain.

Analysis of *tyl* Transcripts in *S. fradiae* Wild-Type

Transcripts from the *tyl* cluster at 18 hr, before the onset of tylosin production, are represented in Figure 3. Prior to this analysis, we did not know whether any of the *tyl* genes might be constitutively expressed; now there are several candidates, although we have not taken RNA samples back to time zero (i.e., from spores). One example is the resistance determinant, *tlrD*, which was also expressed constitutively when it was first cloned in *S. lividans* [10]. Transcript was also detected at 18 hr from *tlrB* and from several "sugar genes," including *tylE*, the product of which acts together with TylF in bis-*O*-methylation of deoxyallose to generate mycinose [11–13]. It remains to be seen whether the TylE protein is produced ahead of other mycinose-biosynthetic enzymes. Of the mycarose genes, *tyl[CV-CVII]* and *tylCVI* were expressed ahead of *tyl[CIII-CII-CIV]*, and the three *tylM* ("mycaminose") genes were also expressed ahead of their functional (and physically distant) partner, *tylB*. The *tylM* genes were also expressed independently of *tylGV*. This is interesting because *tylGV* and *tylMIII* are terminally overlapping, linked by the GTGA sequence motif that is



commonly (and, evidently, mistakenly!) taken to indicate translational coupling. The cytochrome P450 gene, *orf16**, was also expressed early, as were *orf12** (albeit at a low level, detected only after 28 PCR cycles) and *orf11**. Transcript from the ancillary gene, *ccr*, was also detected at 18 hr along with low levels of the *tylO* transcript. The TylO protein is a type II thioesterase that removes prematurely decarboxylated extender units from the TylG PKS complex [14]. In considering these 18 hr transcripts, however, we attach greatest significance to early expression of the regulatory genes *tylQ* and *tylS.* In contrast, the other regulators *(tylP, tylR*, and *tylT)* were silent at 18 hr. We suppose that TylS is a transcriptional activator, target(s) as yet unknown, but no role for TylQ was obvious prior to this work. Conceivably, TylS is produced constitutively; it soon became apparent that TylQ is not.

After the onset of tylosin production (i.e., at 40 hr; see Figure 2), all genes from the *tyl* cluster revealed transcripts, with one exception; only *tylQ* was silent (Figure 3). Since this gene had been expressed earlier in the fermentation, it was strongly suggested that silencing of *tylQ* might necessarily precede the onset of secondary metabolism, suggesting in turn that the TylQ protein might repress some gene(s) essential for tylosin production. One way to test this hypothesis was to overexpress *tylQ* in *S. fradiae*.

Overexpression and Disruption of *tylQ*

An additional copy of *tylQ* (under control of the strong, constitutive promoter, *ermE*p*) was introduced into the *tyl* cluster via single recombination, and the resultant *tylQ*-OE (overexpression) strain was subjected to gene expression analysis during fermentation (Figure 3). At 18 hr, the pattern of transcription was essentially similar to that of the wild-type, but striking differences were apparent by 40 hr, when tylosin would normally have been produced. Constitutive expression of *tylQ* resulted in a failure to express the *tylIBA* genes and the divergent *tylGI*, but interestingly, transcripts were readily detected from the other PKS-encoding genes, *tyl[GIII-GIV-GV]*. This was the first indication that *tylGI* is expressed independently of its functional partners. Transcripts from several sugar genes were also absent at 40 hr; for example, transcripts of the mycinose genes from the left end of the cluster were missing. However, the most significant finding was that *tylR* was silent throughout the fermentation when *tylQ* was expressed constitutively.

Figure 3. Gene Expression Analysis by RT-PCR Applied to the *tyl* Cluster

Three strains of *S. fradiae* were analyzed: wild-type (wt); a *tylQ*-disrupted strain (*tylQ*-KO); and a strain overexpressing *tylQ* (*tylQ*-OE) under control of the strong, constitutive promoter, *ermE*p*. Total mycelial RNA that was extracted before the onset of tylosin production (after 18 hr fermentation) and during production (after 40 hr fermentation) was used as a template, and products amplified with each pair of primers were authenticated by sequencing. Twenty-five cycles of PCR were routinely employed (black); whenever this generated no product, analysis was repeated at 28 cycles (red) to detect low-level transcripts. In negative controls, containing DNA polymerase but lacking reverse transcriptase, amplified products were not detected.

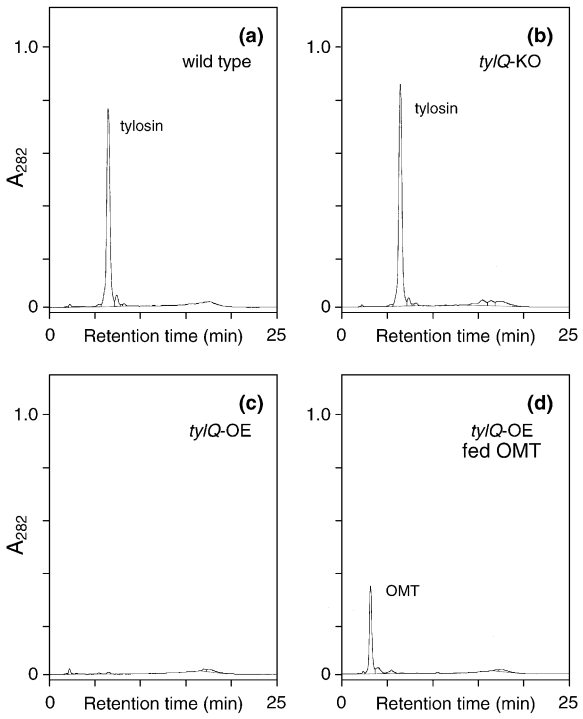


Figure 4. Fermentation of *S. fradiae* Strains

HPLC analysis of material produced during fermentation of (a) wild-type, (b) a *tylQ*-KO strain, (c) a *tylQ*-OE strain constitutively overexpressing an additional copy of *tylQ*, and (d) the *tylQ*-OE strain fed exogenously with *O*-mycaminosyl-tylonolide (OMT) at day 3 of the 7 day fermentation. Addition of OMT (an early biosynthetic precursor of tylosin) was previously shown to stimulate tylactone production in mycaminose-deficient strains [23].

The TylR protein was previously shown to be a global activator of the tylosin-biosynthetic pathway by a combination of targeted gene disruption and fermentation analysis [6]. In short, disruption of *tylR* prevented tylactone synthesis in *S. fradiae* and also blocked bioconversion of exogenously added tylosin precursors. Since gene expression analysis has not yet been applied to a *tylR*-KO strain, it is not clear to what extent the transcriptional pattern that was revealed in the [*tylQ*-OE] strain reflected failure to express *tylR*. However, examination of that pattern suggested that the fermentation phenotype of the [*tylQ*-OE] strain should closely resemble that of a *tylR*-KO strain. Given that transcripts from *tylAI* and *tylAII* (genes required for synthesis of all three tylosin sugars) were not detected in the [*tylQ*-OE] strain, and given the absence of *tylGI* transcript, it was predicted that the [*tylQ*-OE] strain would fail to produce tylosin and would also be unable to synthesize tylactone or bioconvert exogenously added tylosin precursors. These predictions were confirmed by fermentation analysis (Figure 4).

The conclusion that *tylQ* encodes a repressor was further strengthened when a *tylQ*-KO strain was found to initiate tylosin production ahead of the wild-type. Production in the *tylQ*-KO strain began prior to 18 hr (Figure 2), at which time transcripts were detected from the entire *tyl* cluster, except for *tylP* and (of course) *tylQ*. At later times (e.g., at 40 hr), *tylP* was expressed, and only the *tylQ* transcript was absent. The early onset of tylosin production in this strain raised the additional possibility that disruption of *tylQ* might lead directly to elevated tylosin yields. However, in various high-production media, the *tylQ*-KO strain did not differ significantly from the wild-type in this regard (Figure 4).

tylQ upstream seq. ..TACAAACCGCGT-CAGCGGTTTGTA..

BARE-like consensus aCa(AA/TG)cCGGC(GAAA)CGgtc-ggTTTG

Figure 5. A Candidate Regulatory Site Upstream of *tylQ*

Comparison of intergenic sequence approximately 220 bp upstream of *tylQ* with the BARE-like consensus sequence (BarA-responsive elements [19]) derived from target sequences for BarA and other γ-butyrolactone receptor proteins. Inverted repeat sequences are indicated by arrows.

Discussion

The present work revealed, for the first time, that *tyl[GIII-GIV-GV]* are expressed independently of *tylGI*, although not necessarily as an operon. Since cotranscription of all five *tylG* genes would generate an extremely long (approximately 41 kilobase) product, independent expression of *tylG* genes might not be surprising, but differential regulation of their expression was certainly unexpected. These various observations set *tylG* apart from *eryA*, the only other type I polyketide system for which transcripts have been analyzed. In the latter case, not only were the three *eryA* mega genes cotranscribed, but the transcript also covered downstream "sugar" genes [15]. Evidently, one size does not fit all!

The major finding from the present work was unexpected. TylQ is a repressor that directly or indirectly controls expression of the activator gene, *tylR*. When the *tyl* gene cluster was first sequenced, BLASTP analysis immediately suggested that *tylQ* might be the target for a γ-butyrolactone receptor protein—perhaps the product of *tylP*, given the proximity of the two genes within the cluster [6]. Respectively, *tylP* and *tylQ* are orthologs of *barA* and *barB* from *Streptomyces virginiae*, in which production of virginiamycin is triggered by γ-butyrolactones ("butanolides") that bind to BarA and de-repress its target genes, including *barB* [16, 17]. Similarly, the γ-butyrolactone, A-factor, induces streptomycin production in *S. griseus* by binding to ArpA and thereby derepressing a transcriptional activator gene [18]. Since these two systems have powerfully influenced current thinking in this context, we suspected a priori that TylQ might be a positive regulator of transcription, subject to negative control by TylP. Now, however, unless TylP also violates the paradigm, we are potentially faced with a repressor that represses a repressor. Although there is no direct evidence for involvement of TylP in the regulation of *tylQ*, there is indirect evidence consistent with this hypothesis. Thus, target sequences for BarA and other γ-butyrolactone receptor proteins have recently been characterized, leading to a consensus "BARE-like" sequence (BarA-responsive elements; [19]) to which there is a good match in the intergenic DNA upstream of *tylQ* (Figure 5). If TylP does, indeed, control *tylQ*, it

will be important to discover how *tylP* is activated since this gene is apparently silent during the early stages of fermentation.

Significance

In the model that emerges from this work, the TylQ protein acts as a transcriptional repressor that controls (directly or indirectly) the expression of *tylR*. The latter gene was previously shown to encode a transcriptional activator required for all aspects of tylosin production, including polyketide metabolism, synthesis and/or attachment of all three of the tylosin sugars, and bis-*O*-methylation of one of those sugars in the final steps of the biosynthetic pathway. Since *tylQ* was expressed prior to the onset of tylosin production, silencing of this gene is revealed as an essential switch that enables such production. However, throwing that switch by inactivating *tylQ* did not increase tylosin yields. To achieve that goal, it will probably be necessary to manipulate other gene(s) that operate below *tylQ* in the regulatory hierarchy that governs tylosin production.

Experimental Procedures

Bacterial Strains, Growth Conditions, and Genetic Manipulations

S. fradiae T59235 (also known as C373.1; referred to here as wild-type) and its derivatives were grown at 28°C in tryptic soy broth (TSB; Difco). Spores were harvested from AS-1 agar plates [20] after incubation at 37°C for 3 days and were filtered, washed, and pregerminated as described elsewhere [21] prior to use. Cultures for RNA isolation (150 ml of TSB per 250 ml flask) were inoculated with approximately 10^8 spores (i.e., 1 ml of suspension at an OD_{600} of approximately 0.1) and grown with orbital shaking at 250 rpm. pOJ260 [22], a suicide vector that cannot replicate in *Streptomyces* spp., was used to insert DNA into the *S. fradiae* genome, via single or double recombination. This shuttle plasmid was propagated in *Escherichia coli* DH5α and introduced into *S. fradiae* via transconjugation from *E. coli* S17-1, as described elsewhere [23]. Other plasmids were also manipulated in *E. coli* DH5α.

Isolation of Total RNA from *S. fradiae* Mycelium

Mycelium was separated from fermentation broth by filtration through Miracloth (CALBIOCHEM) and frozen by immersion in liquid N_2. The total elapsed time from growth at 28°C to freezing was less than 30 s. Frozen mycelium was then broken by shearing (without thawing) in a Hughes Press (Type A1, APEX Constructions), and the frozen lysate was added to Buffer RLT (QIAGEN) in the presence of 1% v/v 2-mercaptoethanol (SIGMA). Buffer RLT contains the chaotropic agent, guanidinium thiocyanate. The RNeasy Midi Kit (QIAGEN) was used for nucleic acid precipitation and isolation, according to the manufacturer's instructions. Nucleic acid preparations were treated with DNase I (DNA-free kit; Ambion), again according to the manufacturer's instructions.

RT-PCR

Transcript detection analysis was carried out by SUPERSCRIPT One-Step RT-PCR with PLATINUM Taq (GIBCOBRL) plus 0.5 μg of total RNA as template. Dimethyl sulfoxide (5% v/v, final) was added to all PCR reactions along with RNAguard RNase Inhibitor (Amersham Pharmacia; 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 (19–23mers; average Tm value 60°C; Table 1) were designed with the aid of the software PRIMER v. 1.0 (Ashland, MA) to generate PCR products of approximately 400 bp, except for the *orf12**-specific primers that amplified a fragment of 241 bp. With each set of primers, negative controls were carried out with PLATINUM Taq DNA Polymerase (GIBCOBRL) in the absence of reverse transcriptase to confirm that amplified products were not derived from chromosomal DNA that inevitably contaminates RNA preparations. Finally, each amplified product was authenticated by single-strand sequence analysis primed by one of the amplification primers.

Targeted Disruption of *tylQ*

The disruption construct consisted of the hygromycin-resistance cassette (Ωhyg, which includes flanking transcriptional and translational terminators [24]), sandwiched between two PCR products ("arms") that each contain sequence from *tylQ* plus flanking DNA. The arms (which were authenticated by sequence analysis) were of approximately equal size (750–800 bp) to facilitate insertion of Ωhyg into the *tylQ* region of the *S. fradiae* genome via double recombination. The PCR primers were as follows: 5′-CCTG**AAGCTT**CTGCGCG CCGGCCGCGACAA-3′ plus 5′-GAGC**GGATCC**CCCGCTGCTGACT CCCGCCC-3′; and 5′-GCTC**GGATCC**TTCATTTCCAATTCGAGAGC-3′ plus 5′-GTCT**GAATTC**CGACCCTGCCGCTCAGGTAT-3′. The template was pLST962, a derivative of pHJL311 [25]. The primers introduced restriction sites (shown in bold) into the arms (EcoRI and BamHI in the upstream arm, BamHI and HindIII in the downstream arm), and thus allowed fusion at the BamHI sites by ligation into pIJ2925 [26]. Then, Ωhyg (a 2.3 kb BglII fragment) was introduced into the BamHI site and thereby disrupted *tylQ* 132 bp downstream of the start (within the helix-turn-helix motif that is conserved among TylQ and its orthologs in other actinomycetes [6]). Disrupted *tylQ* plus flanking DNA (approximately 3.84 kb in total) was excised from pIJ2925 as a BglII fragment, ligated into the BamHI site of pOJ260, and introduced into *S. fradiae*. Transconjugants that had undergone gene transplacement via double recombination into the genome were resistant to hygromycin and sensitive to apramycin (a marker carried by pOJ260).

Confirmation of *tylQ* Disruption

Hemi-nested PCR was used to confirm that the Ωhyg cassette had disrupted the expected target gene. This technique is more definitive and convenient than Southern analysis. In order to combat higher order structure(s) in DNA containing the Ωhyg cassette, unusually high temperatures had to be employed in conjunction with long (30mer) primers in Step Down (or Touch Down) PCR. Conditions were as follows: primary DNA melting at 96°C for 5 min followed by five cycles of [96°C for 1 min, 65°C for 1.5 min, 73°C for 3.5 min], ten cycles of [96°C for 1 min, 63°C for 1.5 min, 73°C for 3.5 min], ten cycles of [96°C for 1 min, 62°C for 1.5 min, 73°C for 3.5 min] and, finally, ten cycles of [96°C for 1 min, 61°C for 1.5 min, 73°C for 3.5 min].

Primers used for the first round of amplification were (1) 5′-TGTGA TCGCCGAGACGATGCGGACCAAGAA-3′, (2) 5′-TCACAGCCAAAC TATCAGGTCAAGTCTGCT-3′, (3) 5′-CCCACAGTTCCTCGATCAGC GCGCTGACGG-3′, and (4) 5′-CACAATTCCACACAACATACGAGCC GGAAG-3′.

Primer sites (1) and (3) were located in sequences flanking *tylQ*, whereas primer sites (2) and (4) lay within the Ωhyg cassette. The second round of amplification involved two additional primers internal to the previously amplified sequences. Thus, internal primer 5′-GTCTCCTTCGCCGCTCCCGGGCTCGACCCC-3′ used together with primer (2) amplified a definitive 1137 bp upstream sequence, and internal primer 5′-CGTGCGTGAGTGAGTCGCGAGTCGTCC GAC-3′ together with primer (4) amplified a 1199 bp downstream sequence (Figure 6). The latter two products each included one end of the Ωhyg cassette plus DNA sequence extending through one of the "arms" used for recombination and out into untouched DNA. Authentication of these products by single-strand sequence analysis confirmed successful integration of the disruption cassette at the desired locus.

Overexpression of *tylQ*

A DNA fragment (725 bp) carrying *tylQ* together with the candidate Shine-Dalgarno sequence, GGAG (but lacking the native promoter),

Table 1. Primers for RT-PCR

Primer	Sequence 5′-3′	Description
TLRB-RT-5′	CCGACGTATGGGACACACTT	Forward primer for *tlrB*
TLRB-RT-3′	GAGAGCGTGACCTCGTGGAT	Reverse primer for *tlrB*
TYLN-RT-5′	AGAACCAGTACCCGGTCACA	Forward primer for *tylN*
TYLN-RT-3′	GTACGACCAGGAGGTCCTGG	Reverse primer for *tylN*
TYLE-RT-5′	GCTATCTCACTCCCAAGTGGG	Forward primer for *tylE*
TYLE-RT-3′	TCCTCGATCACATACAGCCC	Reverse primer for *tylE*
TYLD-RT-5′	AGCTCCACCGAGGTGTACTG	Forward primer for *tylD*
TYLD-RT-3′	GAGGATGGACACCTCTTCGG	Reverse primer for *tylD*
TYLHI-RT-5′	GCTCGTTCTGCTACAGCACC	Forward primer for *tylHI*
TYLHI-RT-3′	AGCTCGTACACCCCGAAGAC	Reverse primer for *tylHI*
TYLF-RT-5′	GATCTCTACATCGAGCTGCTGAA	Forward primer for *tylF*
TYLF-RT-3′	GTTGTACTGGTGGAGGTCGATCT	Reverse primer for *tylF*
TYLJ-RT-5′	CAGGTCAATATCGCGGTGTC	Forward primer for *tylJ*
TYLJ-RT-3′	CTGGTAGGAGGGCAGAATCC	Reverse primer for *tylJ*
ORF18*-RT-5′	TGACCACAGAAATGTTCCGG	Forward primer for *orf18**
ORF18*-RT-3′	TGGTGTTGGGCATGAACTTC	Reverse primer for *orf18**
TYLP-RT-5′	ATCGCGGAGATCCTCAAGAG	Forward primer for *tylP*
TYLP-RT-3′	AGGATGTTCGACAGGATCTGG	Reverse primer for *tylP*
ORF16*-RT-5′	AATGACCAACGGGACCTTGT	Forward primer for *orf16**
ORF16*-RT-3′	GTTGACGATGAGGGAGGACA	Reverse primer for *orf16**
TYLQ-RT-5′	AGAGCAAGAAGATGCTGGCC	Forward primer for *tylQ*
TYLQ-RT-3′	GATTCCAGAATCCGGTGACC	Reverse primer for *tylQ*
TYLS-RT-5′	TGCAGTTACGGGAGCTGATC	Forward primer for *tylS*
TYLS-RT-3′	GTGCAGATTCTCATGGGTGC	Reverse primer for *tylS*
TYLT-RT-5′	GCTGGAGATCCTGTACGAGATG	Forward primer for *tylT*
TYLT-RT-3′	AGAGCTATCTGACGGCGGTT	Reverse primer for *tylT*
ORF12*-RT-5′	CCCACGGCTGTACGAGGACT	Forward primer for *orf12**
ORF12*-RT-3′	GAGACGCCGTACCAGGAAGG	Reverse primer for *orf12**
ORF11*-RT-5′	TGTCCTCTACCGAACGAGTGAT	Forward primer for *orf11**
ORF11*-RT-3′	GGCATCATGTACTGCATCTGC	Reverse primer for *orf11**
TYLCII-RT-5′	GAACTTCAGCGGGAGAGTCC	Forward primer for *tylCII*
TYLCII-RT-3′	GTTCGAGGACCCGATGTAGC	Reverse primer for *tylCII*
TYLCIV-RT-5′	GGTGAGAGGTGTCGTGCTGC	Forward primer for *tylCIV*
TYLCIV-RT-3′	GAGTCGGGGGTACGGAAGTC	Reverse primer for *tylCIV*
TYLCIII-RT-5′	GTGATGGAGCAGAGCTATCTGC	Forward primer for *tylCIII*
TYLCIII-RT-3′	AGGATGACATTGCCCTTGGT	Reverse primer for *tylCIII*
TYLCV-RT-5′	GAAACTGCTGAAGGAGCACG	Forward primer for *tylCV*
TYLCV-RT-3′	GTTGACGAGCAGATCGGTGT	Reverse primer for *tylCV*
TYLCVII-RT-5′	ATCATCACCGAGACCAGGGT	Forward primer for *tylCVII*
TYLCVII-RT-3′	CCCGGGTGTAGAGGTAGTTCA	Reverse primer for *tylCVII*
TLRD-RT-5′	CGTGTATGGGGCCAGAATTT	Forward primer for *tlrD*
TLRD-RT-3′	CTTGCGCGTGTACTGCTTCT	Reverse primer for *tlrD*
ORF4*-RT-5′	CAAGTCCAACCAGCTGATGC	Forward primer for *orf4**
ORF4*-RT-3′	CTCGAAGACGATGTCCACGT	Reverse primer for *orf4**
TYLMI-RT-5′	CCTGCATGTTCAGCTCGATC	Forward primer for *tylMI*
TYLMI-RT-3′	GCATGAACTCCACGGACAGT	Reverse primer for *tylMI*
TYLMII-RT-5′	TGAGAAGGGCACTGGATGAC	Forward primer for *tylMII*
TYLMII-RT-3′	ATGGTGGAGTCGCAGTTGAA	Reverse primer for *tylMII*
TYLMIII-RT-5′	GCGATCACCGAAACACTGCT	Forward primer for *tylMIII*
TYLMIII-RT-3′	TCACTCGGGGACATACGGG	Reverse primer for *tylMIII*
TYLGV-RT-5′	CACCGACGACTGGATGTACC	Forward primer for *tylGV*
TYLGV-RT-3′	CTCCTCCATAGCCTGCATCA	Reverse primer for *tylGV*
TYLGIV-RT-5′	ACGGTGAGGAGTTCCTCAGC	Forward primer for *tylGIV*
TYLGIV-RT-3′	AACTGAACCCCAGCTCCTTG	Reverse primer for *tylGIV*
TYLGIII-RT-5′	GAGATGCTGGTGGAGTTCTCC	Forward primer for *tylGIII*
TYLGIII-RT-3′	ATGTTGGACTTCAGGGAGCC	Reverse primer for *tylGIII*
TYLGI-RT-5′	AGAACGAACCCGAACGGCAC	Forward primer for *tylGI*
TYLGI-RT-3′	CCCAGAATTCCTCGACACCG	Reverse primer for *tylGI*
TYLI-RT-5′	AGCAATCCGCGGGTGATCTC	Forward primer for *tylI*
TYLI-RT-3′	ATGACGACACAGACGCTCGA	Reverse primer for *tylI*
TYL1A-RT-5′	GGAATGTCGGCAGGATCAAG	Forward primer for *orf1a*
TYL1A-RT-3′	CTCAGGAACTCGTCGTAGTCCC	Reverse primer for *orf1a*
TYLB-RT-5′	TGATGCCCGTGCATCTCTAC	Forward primer for *tylB*
TYLB-RT-3′	GTACTGATGCCAGACCGGCT	Reverse primer for *tylB*
TYLAI-RT-5′	GGCTGAGCATCAGTTATGCC	Forward primer for *tylAI*
TYLAI-RT-3′	GCCCTGCTCCAGATAGACCTT	Reverse primer for *tylAI*
TYLAII-RT-5′	ATCTCCACGGACGAGGTCTAC	Forward primer for *tylAII*

continued

Table 1. Continued

Primer	Sequence 5′-3′	Description
TYLAII-RT-3′	TGATCTTGGTGGTGTCGACC	Reverse primer for *tylAII*
TYLO-RT-5′	AGACGCACGCCGTCCAGTAC	Forward primer for *tylO*
TYLO-RT-3′	GAACACGGTCAGCGGACAGC	Reverse primer for *tylO*
TYLCVI-RT-5′	CAACCTGGTCAACATGGACG	Forward primer for *tylCVI*
TYLCVI-RT-3′	GAGTTCCGCCACGTTGAGAT	Reverse primer for *tylCVI*
TYLR-RT-5′	ACCTCTTCCTGGTGCTGGAC	Forward primer for *tylR*
TYLR-RT-3′	GGTCAGCTCATCCTCCTCGT	Reverse primer for *tylR*

was generated from wild-type spores by colony PCR and authenticated by sequence analysis. The primers were 5′-GGGG**GGATCC** TCAGCCAGGAGACAA-3′ and 5′-GGTT**AAGCTT**ATCCCAAAGGAC CGC-3′. These introduced restriction sites (shown in bold) for BamHI (upstream) and HindIII (downstream) and facilitated ligation of the product into pIJ2925. The powerful, constitutive promoter, *ermE*p* [27], was then introduced into the upstream BamHI site, as an EcoRI-BamHI fragment, to ensure efficient expression of *tylQ* after integration into the *S. fradiae* genome. To do this, we excised [*ermE*p*-*tylQ*] from pIJ2925 as a BglII-HindIII fragment, ligated it into pOJ260, and introduced the resultant vector into *S. fradiae*. Transconjugants in which the entire pOJ260 derivative had integrated into the *S. fradiae* genome, via a single crossover within the *tylQ* region, were obtained by selection for apramycin resistance (a marker carried by pOJ260).

DNA Sequencing

DNA sequencing was performed on an ABI 377 automated sequencer with Taq FS polymerase and dye terminator chemistry (Perkin Elmer). DNA sequences were generated for both strands in overlapping fashion, independently edited with Seq Ed v1.0.3, and aligned in overlapping fashion with AUTO ASSEMBLER.

Tylosin Production Fermentation and Metabolite Analysis

Fermentation of *S. fradiae* strains, bioconversion of tylosin precursors, and HPLC analysis of the products were performed as described elsewhere [28], with the exception that GRF medium replaced MM-1 medium (used previously). Like MM-1, GRF medium also sustained high-level tylosin production. GRF medium contained, per liter, 17.5 g monosodium glutamate, 5 g glucose, 5 g betaine, 5 g $MgSO_4$, 2.3 g K_2HPO_4, 2 g NaCl, 10 mg $ZnSO_4$, 1 mg $CoCl_2$, 20 ml 70% v/v methyl oleate, and 4 ml trace elements [21]; pH was adjusted to 7.0 with KOH.

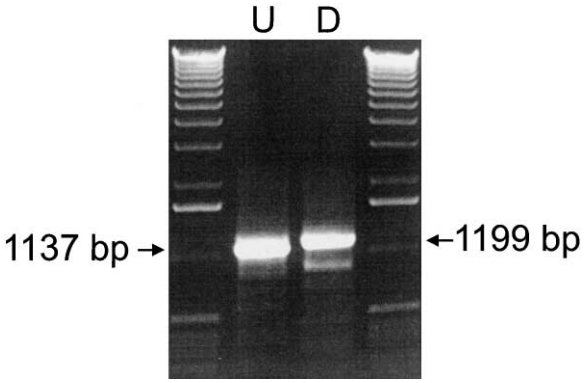


Figure 6. Confirmation of *tylQ* Disruption by Hemi-Nested PCR

As a preferred alternative to Southern analysis, successful disruption of *tylQ* within the *S. fradiae* genome was confirmed by the amplification of definitive sequences spanning either end (U, upstream; D, downstream) of the disruption construct. Pairs of primers were complementary to sequences within the Ωhyg cassette used to disrupt *tylQ* and within untouched DNA flanking the *tylQ* region of the genome. Amplified products were authenticated by sequencing. Flanking tracks contain size markers (1 kb ladder; GIBCOBRL).

Acknowledgments

This work was supported by a scholarship awarded to G.S. by the Alexander S. Onassis Public Benefit Foundation and by a research studentship awarded to G.S. by the Biotechnology and Biological Sciences Research Council.

Received: August 6, 2001
Accepted: October 2, 2001

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