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Inactivation of a transcriptional repressor during empirical improvement of the tylosin producer, *Streptomyces fradiae*

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Remarkably few changes of significance seem to have occurred within the tylosin-biosynthetic gene cluster of *Streptomyces fradiae* during an extensive portion of the empirical strain improvement programme carried out at Lilly Research Laboratories over many years. None of the promoters for polyketide synthase (PKS) genes or for regulatory elements changed within this part of the lineage, nor were any mutations detected in other *tyl* promoters, although the full set was probably not analysed. Of five regulatory genes within the *tyl* cluster, only *tylQ* was altered, having undergone a single point mutation that inactivated its product (a transcriptional repressor). Also unchanged was a gene with unassigned function. Since point mutations affecting antibiotic-biosynthetic enzymes are unlikely to have played a major role in empirical strain improvement, enhanced tylosin production levels appear to have resulted, in large measure, from uncharacterized mutations occurring outside the *tyl* cluster.
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Introduction

The manner in which the genomes of antibiotic-producing actinomycetes have been modified during industrial strain improvement programmes is poorly understood. Thus, it is not known whether elevated production levels have commonly resulted from mutations occurring within antibiotic-biosynthetic gene clusters as opposed to those inevitably generated elsewhere in the genome. Gross amplification of genes (singular or plural) would be readily detectable, and comparative genomics might reveal disruption of competing metabolic pathways that might otherwise limit secondary metabolism. But even so, assessment of the significance of specific mutations will likely remain intractable. Nor will transcriptome analysis necessarily resolve the situation since DNA microarray analysis cannot detect the consequences of mutations that fail to block transcription. Such considerations argue for a strictly focussed approach to the genetics of antibiotic yield improvement.

The present work centred on tylosin production by *Streptomyces fradiae* and the aim was to determine whether significant changes have occurred within the tylosin-biosynthetic (*tyl*) gene cluster in the lineage of *S. fradiae* strains empirically developed at Lilly Research Laboratories, Indianapolis, IN. Tylosin is a macrolide antibiotic [20] in which three deoxyhexose sugars are attached to a polyketide lactone via a pathway [2] that requires a complex of five giant polyketide synthase (PKS) proteins plus about 20 other enzymes involved in sugar biosynthesis and polyketide ring oxidation. Including the two flanking resistance determinants, *tlrB* and *tlrC*, the *tyl* cluster (Figure 1) contains 43 genes and comprises about 1% (~85 kb) of the *S. fradiae* genome [12]. Given the complexity of tylosin production, it seemed unlikely *a priori* that point mutations affecting the structures of tylosin-biosynthetic enzymes would significantly improve the overall efficiency of the pathway — regardless of where the rate limiting step(s) might normally be. Therefore, we did not expect that empirical strain improvement would have selected such changes. Rather, it seemed more likely that yield enhancement would have been achieved via mutations affecting promoters and/or regulatory genes. Since the *tyl* cluster is unprecedented in harbouring at least five regulators [4], this system seemed well suited to the proposed analysis.

Following this rationale, we did not attempt to generate comparative sequences of the entire *tyl* cluster (~85 kb) from ancestral and developed strains. Instead, we focussed on the candidate regulatory genes, on unassigned genes (i.e., those possessing novel sequences and unknown functions) and on promoter DNA. Although the *tyl* cluster has not been subjected to detailed transcript mapping, various DNA fragments likely to contain promoters were readily identified. For example, in several regions of the cluster [3-5,16,21,27] adjacent genes are divergent (e.g., [*tylGI*-*tylI*], [*tylCIII*-*tylCV*], [*tylHI*-*tylF*]); also, each of the regulatory genes (*tylP,Q,R,S,T*) is preceded by an intergenic gap (these range from ~340 bp to almost 1 kb) strongly indicative of independent expression. Although such prominent gaps are not found within the *tylG* subcluster that encodes the PKS (accession number U78289; B.S. DeHoff, K.L. Sutton and P.R. Rosteck, Jr., Lilly Research Laboratories, Indianapolis, IN), we considered the possibility that these codirectional mega genes might not constitute an operon and, accordingly, DNA upstream of each *tylG* component was included in the present analysis. The full complement of genes and candidate promoters analyzed here is shown in Figure 1.

Materials and methods

Strains, media and genetic tools

S. fradiae T59235 (also known as C373.1; referred to here as wild-type or ancestral strain) and its derivatives were grown at 28°C in tryptic soy broth (TSB Difco, Sparks, MD). Spores were

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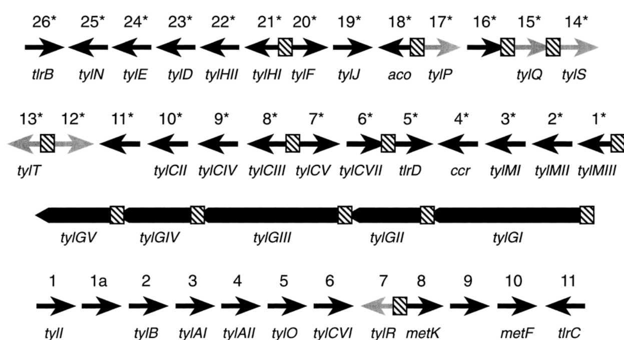

Figure 1 The tylosin-biosynthetic gene cluster of *S. fradiae*. Not drawn to scale. The contiguous cluster of 43 genes occupies ~85 kb. The five *tylG* mega genes cover ~41 kb. Upstream of *tylG*, 12 genes (orfs 1,1a,2–11) occupy about 15 kb. Downstream of *tylG*, 26 genes (designated 1*–26*) occupy about 29 kb. DNA regions subjected to sequence comparisons between ancestral and derived strains are shown as grey arrows (genes) and cross-hatched boxes (promoters).

harvested from AS-1 agar plates [27] following incubation at 37°C for 3 days and were filtered, washed and pregerminated as described elsewhere [17] prior to use. Cultures for RNA isolation (150 ml of TSB per 250-ml flask) were inoculated with approximately 10^8 spores (1 ml of suspension at OD_{600} ~0.1) and grown at 28°C with orbital shaking at 250 rpm. DNA was inserted into the *S. fradiae* genome, via single recombination, using pOJ260 [9], a suicide vector that cannot replicate in *Streptomyces* spp. This was introduced into *S. fradiae* via transconjugation from *Escherichia coli* S17-1 as described elsewhere [14]. Plasmids were constructed and manipulated in *E. coli* DH5α using standard protocols [23].

Gene expression analysis via reverse transcriptase–PCR

Total RNA was extracted from *S. fradiae* mycelium and used as template in reverse transcriptase–PCR (RT-PCR) as described elsewhere [26]. cDNAs were amplified from 37 *tyl* gene transcripts using the primers previously described [26].

Overexpression of ancestral and derived tylQ

Ancestral *tylQ* was PCR amplified from wild-type spores as a 725 bp DNA fragment including the putative Shine–Dalgarno sequence but lacking the native promoter. The PCR primers were: 5′-GGGG**GGATCC**TCAGCCAGGAGACAA-3′ and 5′-GGTT**AAGCTT**ATCCCAAAGGACCGC-3′. The same set of primers was used to amplify *tylQ** from cosmid pMOMT4 [7], which contains DNA from an advanced production strain of *S. fradiae*. Both amplicons were cloned in pIJ2925 [18], utilising the *Bam*HI and *Hin*dIII sites purposely introduced by the primers (shown in bold), and sequenced to confirm that mutations had not been introduced during PCR. To ensure efficient expression of these genes in *S. fradiae*, the powerful, constitutive promoter, *ermE*p* [8], was introduced into the upstream *Bam*HI site. Each overexpression (OE) cassette was then ligated into pOJ260 and integrated into the *S. fradiae* genome via a single crossover within the *tylQ* region. The apramycin resistance gene carried by pOJ260 allowed selection for this event. Finally, a PCR product from the *tylQ*-OE strain was sequenced to confirm that the resident copy of *tylQ*, together with its native promoter, remained intact.

Targeted disruption of tylQ

This was done as described elsewhere [26].

Replacement of tylQ by tylQ*

A 1309-bp fragment was generated by PCR with pMOMT4 as template using the following primers: 5′-CCTG**AAGCTT**CTGCGCGCCGGCCGCGACAA-3′ and 5′-GGTT**TCTAGA**ATCCCAAAGGACCGC-3′. The amplified DNA contained 222 bp from *orf16**, 400 bp of intergenic DNA, and the whole of *tylQ**. The fragment was ligated into pOJ260 using the *Hin*dIII–*Xba*I sites (in bold; deliberately introduced by the primers), sequenced to confirm that mutations had not been introduced by PCR, and integrated into the *tylQ* disrupted region of the *tylQ*-KO strain as a single crossover. The mutational change in *tylQ** (T147A) had occurred 15 bp downstream of the corresponding site in the wild-type allele at which Ωhyg had been inserted to generate the *tylQ*-KO strain. Therefore, if the vector containing *tylQ** had recombined into the *tylQ*-KO genome between those respective sites, the undesired consequence would have been generation of a doubly modified version of *tylQ* (i.e., an insertionally inactivated version of *tylQ**) together with a wild-type copy of *tylQ*. Confirmation that this had not happened (and also that *orf16** had not been altered during recombination) was provided when a PCR product from the resultant strain was sequenced. As intended, an intact version of *tylQ** had been introduced into the *tylQ*-KO genome.

Isolation and sequencing of genes and promoter DNA from the ancestral strain

DNA fragments containing putative promoter regions and/or open-reading frames (orfs) from the *tyl* cluster of the ancestral strain (for details, see Table 1) were generated by Genomic PCR using proofreading *Pfu* DNA polymerase (Promega, Southhampton, UK) and ligated into pIJ2925 via restriction sites purposely introduced by the primers. DNA sequencing was carried out using an ABI 377 automated sequencer, using Taq FS polymerase in conjunction with dye terminator chemistry (Perkin-Elmer, Cambridge, UK). For each amplified region, two independent PCR products were sequenced. DNA sequences were generated from both strands, edited independently using Seq Ed version 1.0.3 and aligned in overlapping fashion using AUTO ASSEMBLER.

Fermentation and metabolite analysis

Fermentation of *S. fradiae* strains, extraction of products and HPLC analysis were performed as described elsewhere [11] with the exception that MM-1 production medium was replaced by GRF medium. The latter (which also supports high-level production of tylosin) contained per litre: 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% methyl oleate, plus 4 ml trace elements solution [17]. The pH was adjusted to 7.0 with KOH.

Results

The starting material for the present work was a cosmid library containing DNA from an advanced production strain and PCR-amplified products derived from the genome of the ancestral *S. fradiae* strain. In the latter case, authenticity was ensured by sequencing multiple independent PCR products. No attempt was made to identify translational start codons within orfs, which were

Table 1 Primers for PCR amplification

Primers (5′–3′)[a]	Target	PCR product size (bp)
	Orfs	
GCAT**GGATCC**ATTCCGGGGTCACACATCACCG	*tylP*	1011
GCTC**TCTAGA**CGGCCAGGTCAGTCCCCGGC		
GGGG**TCTAGA**TCAGCCAGGAGACAA	*tylQ*	725
GGTT**TCTAGA**ATCCCAAAGGACCGC		
CTCG**AAGCTT**TCTGCAAGCGGGCGGGAGTC	*tylS*	2016
GGAG**GAATTC**TACCCCGCGGCGGAGAACCG		
CCGC**AAGCTT**GTGGCCGGTCGCCGCCACCG	*tylT*	1235
CGGT**GAATTC**TCAGAAGGTCGGCGGTGTAC		
GGCG**TCTAGA**ACGGGAGGAGACCCA	*orf12**	694
GGTG**TCTAGA**TCTTCCGGGTCCGCG		
GGGG**TCTAGA**TCATGCCGTCGCTCT	*tylR*	1319
CCTC**TCTAGA**GGTGTCATGAGCGCC		
	Promoters	
GGTC**TCTAGA**TCATCACCGGGGACGCCGTCCT	*tylHp-tylFp*	594
TCGG**TCTAGA**TCATCAGTGCTGCGGGCAACA		
GCAA**TCTAGA**GACTTCCCCCTCCGT	*tylPp*	427
CCCG**TCTAGA**TCATCACCTGCCTCCACCTCCGAG		
GGCG**TCTAGA**CGTCCCGCCGCACCG	*tylQp*	420
GCTT**TCTAGA**CGGTTGTCTCCTGGC		
GGGC**TCTAGA**GCATCGGAGCCGGCG	*tylSp*	447
GGG**TCTAGA**TCATCACGAGAGGCCGTTCTC		
TGCC**TCTAGA**TCTACACGCGGCTCCGTTCCG	*tylTp-orf12*p*	365
GCGT**TCTAGA**GGGTCTCCTCCCGTA		
AGGC**TCTAGA**TCATCACTGGTCCTCTCGGGG	*tylCIIIp-tylCVp*	442
ATGC**TCTAGA**TCATCAGCGGCAGGACCCCTTC		
ACGA**TCTAGA**CATGAACTACCTCTA	*tlrDp*	485
CGGC**TCTAGA**CGGGCGCGCGTCCCTAA		
CACT**TCTAGA**GCGCCAACATGGCCC	*tylMIIIp*	493
GTGT**TCTAGA**CCTCTCCTCTCTCCC		
AACA**TCTAGA**GGACCTGCTGACACC	*tylGVp*	530
ATGG**TCTAGA**TCAGCTTCATGACTCGTGTGGG		
ATCC**TCTAGA**ACCGGGCCTTCAAGG	*tylGIVp*	497
GTTG**TCTAGA**TCATTTCACCTGTGCTGTCGCTG		
AGCT**TCTAGA**AAGCGGGGCTTCGACT	*tylGIIIp*	503
TGGA**TCTAGA**TTCACTCAGCTTTCCC		
AGGA**TCTAGA**CTTCGACTCGCTGAC	*tylGIIp*	534
CGCG**TCTAGA**TCAGCGGTTCACCCTTCAGATACT		
GTGC**TCTAGA**TTCGCGGTCCGCTCG	*tylGIp-tylIp*	1038
CCGC**TCTAGA**TCATCAGCAGTTCCCAGAATT		
GACG**TCTAGA**GGCTCTCCAGTGGTG	*tylRp-orf8p*	1001
CGGC**GAATTC**GACACCTCTGGTGAG		

[a]Restriction sites are shown in bold.

sequenced in their entirety. Equally, the locations of transcriptional start points were not sought within promoter DNA fragments, each of which extended at least 340 bp upstream of the respective orf (see Table 1).

The results of sequence analysis were startling in their simplicity. None of the promoter DNA fragments differed between ancestral and derived sources. The unassigned gene (*orf12**) was unchanged throughout this part of the lineage and, of the regulatory genes (*tylP,Q,R,S,T*), only *tylQ* had been altered (a T to A transversion at position 147 had occurred during strain development). The location of this mutation was highly suggestive, changing histidine to glutamine (TylQ to TylQ* sequence) within the DNA recognition element of a helix–turn–helix motif in the N-terminal region of the deduced protein (Figure 2). Since TylQ is a repressor that controls *tylR* (a global activator of the *tyl* cluster) and must be switched off before tylosin production can begin [26], the mutational change observed here was potentially significant and prompted further analysis aimed at establishing whether the function of TylQ* (present in the advanced production strain) had been affected.

Elsewhere, we have shown that *tylQ* is expressed in ancestral *S. fradiae* prior to the onset of tylosin production, but is the only silent gene in the cluster once production gets underway [26]. When an additional copy of *tylQ* was introduced and overexpressed under control of the strong, constitutive promoter, *ermE*p*, the ancestral strain failed to produce tylosin (Figure 3, see also Ref. [26]) and several *tyl* genes remained silent throughout the fermentation (Figure 4, see also Ref. [26]). In contrast, when *tylQ** from the advanced production strain (controlled by *ermE*p*) was integrated into the genome of the ancestral strain via single homologous recombination, the resultant strain still produced tylosin (Figure 3) and gene expression analysis involving RT-PCR revealed that all genes of the *tyl* cluster were expressed in the *tylQ**-OE strain in a manner indistinguishable from the wild-type pattern (Figure 4). Evidently, the product of *tylQ** is inactive as a repressor.

The latter conclusion prompted investigation of the effect of *tylQ* disruption, and/or its replacement with *tylQ**, on tylosin production in the ancestral strain. Having generated a *tylQ*-KO strain via gene transplacement, the additional effect of introducing a

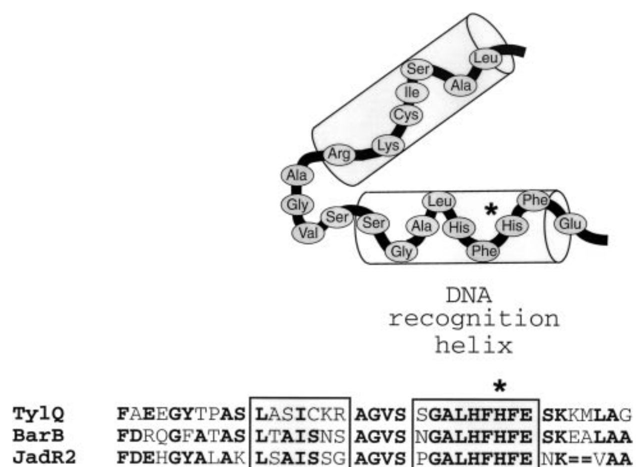

Figure 2 Amino-terminal sequences of TylQ and orthologues in the databases. Helix–turn–helix motifs are indicated. The downstream "DNA recognition" helix that binds into the major groove of target DNA is especially well conserved. The T147A change in *tylQ* that occurred during strain improvement caused a histidine to glutamine substitution in TylQ at the position marked with an asterisk. The ancestral sequence of TylQ is shown. Accession numbers: *tylQ*, AF145049 [4]; *barB*, AB001609 [19]; *jadR*$_2$, U24659 [28].

single copy of *tylQ** (with its native promoter) was also examined. Although disruption of *tylQ* was shown previously to cause earlier onset of tylosin production in TSB [26] this did not translate into elevated tylosin yields in high level production media (Figure 5). Nor did replacement of *tylQ* by *tylQ** significantly affect tylosin production.

Although we did not have access to the advanced production strain from which *tylQ** was originally obtained, we did possess *S. fradiae* C4 — an earlier strain in the lineage. Strain C4 is the product of several rounds of mutagenesis (involving UV irradiation, nitrous acid and nitrosoguanidine) and produces several times more tylosin than does the wild type [24]. PCR amplification of *tylQ* from strain C4 revealed the *tylQ** sequence, implying that the T147A mutation had been selected quite early in the lineage.

Discussion

Very little is known about genetic changes associated with enhanced production in industrial antibiotic-producing actinomycetes. In contrast, penicillin production in empirically improved strains of *Penicillium chrysogenum* has been studied in detail and shown to involve amplification of the entire biosynthetic gene cluster [25] resulting in tandem repeats [13]. This apparently happened on multiple occasions during strain selection and studies of a lineage of industrial producers demonstrated a general correlation between copy number and penicillin titre [22]. In such strains, no changes were discovered in the promoters of penicillin-biosynthetic genes, although it was suggested that regulatory genes might have been mutated. In the present case, inactivation of TylQ, a key transcriptional repressor, was observed in the Lilly lineage of tylosin-producing *S. fradiae* strains with no evidence of gene amplification, at least in strain C4. Thus, a collection of nonproducing mutants was readily generated from strain C4 and used to map *tyl* loci within the *S. fradiae* genome [1]. Also, we have generated various strains with null fermentation phenotypes following targeted gene disruptions in strain C4 [15]. In such studies, the possibility of concurrent mutation, or disruption, of multiple gene copies would have been remote. Here, as in *P. chrysogenum*, mutations were not encountered in the promoters of antibiotic-biosynthetic genes although, admittedly, we probably did not analyse the full set. Especially significant was the observation that sequences upstream of all five *tylG* genes remained unaltered in *tylQ** strains. Regardless of how many *tylG* promoters there might be [26], they have apparently not changed among the *S. fradiae* strains featured here. Since tylosin production is normally limited by the rate of polyketide metabolism [10], enhanced tylosin production by strains such as C4 must therefore reflect enhanced availability of polyketide precursors rather than elevated levels of the TylG PKS enzyme.

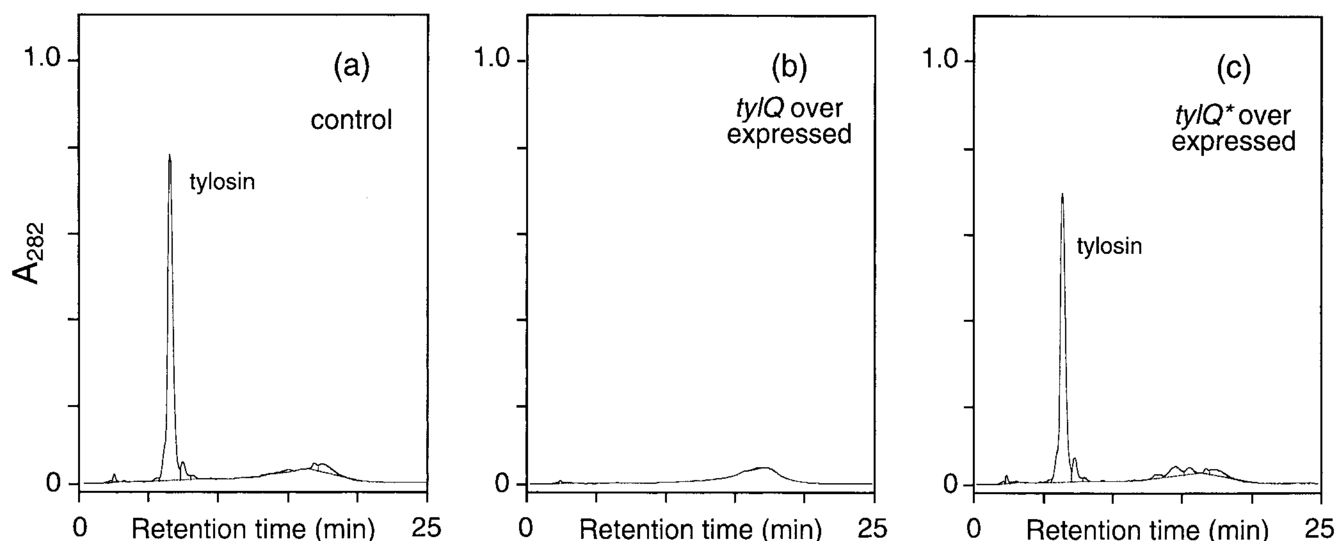

Figure 3 Fermentation products from *S. fradiae* strains. HPLC analysis of material produced by: (a) ancestral strain; (b) ancestral strain with an extra copy of ancestral *tylQ* driven by the strong, constitutive *ermE** promoter; (c) ancestral strain with an added copy of *tylQ** from an advanced strain, driven by *ermE*p*.

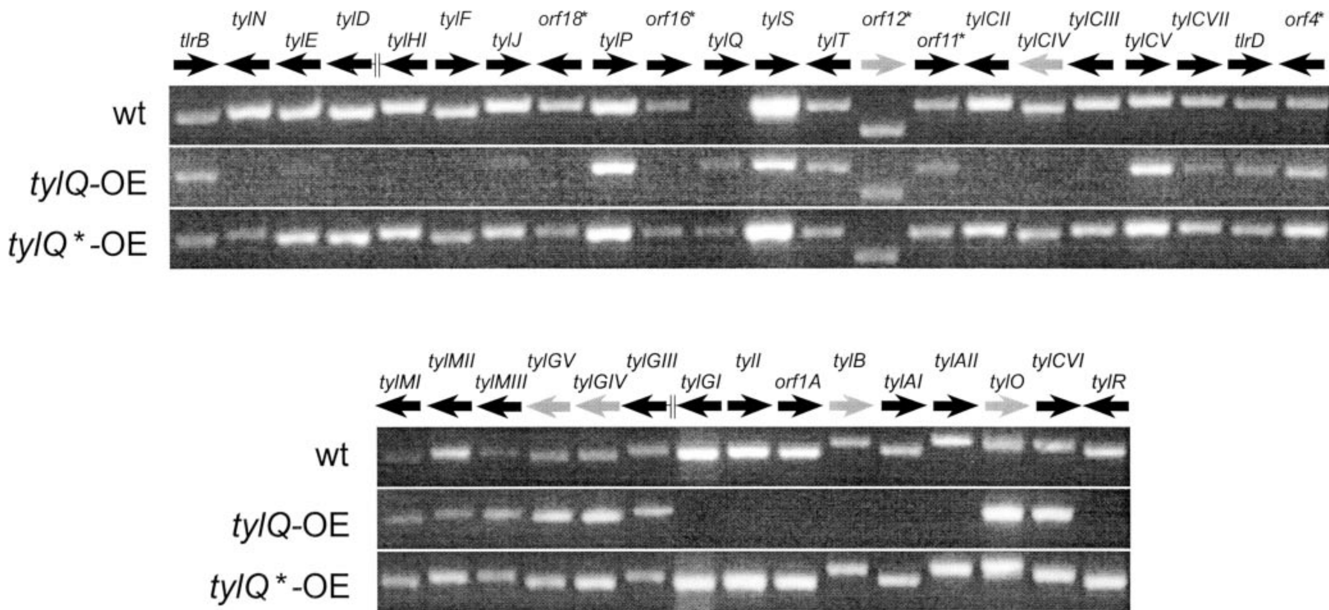

Figure 4 Gene expression analysis by RT-PCR applied to the *tyl* cluster. Three strains of *S. fradiae* were analysed: wild type (wt); a strain (*tylQ*-OE) overexpressing ancestral *tylQ* under control of *ermE*p*; a strain (*tylQ**-OE) overexpressing *tylQ** under control of *ermE*p*. Total mycelial RNA was extracted after 40 h fermentation and used as template for RT-PCR. Products amplified with each set of primers were confirmed by sequencing. Twenty-five cycles of PCR was routinely employed (black) to detect transcripts but, as necessary, analysis was repeated at 28 cycles (grey) to detect low-level transcripts. In negative controls, containing DNA polymerase but lacking reverse transcriptase, amplified products were not detected after 28 cycles.

The observation that disruption of *tylQ* in the ancestral strain did not enhance the yield of tylosin is curious (Figure 3). If indeed the *tylQ** mutation was selected by Lilly on the basis of enhanced yield, rather than some other characteristic, this might imply that the phenotype is expressed only under specific fermentation conditions or that it might have depended on other mutation(s) acquired earlier in the lineage. Control of tylosin production centres on dual control of the gobal transcriptional activator, *tylR*. Derepression of *tylR* when *tylQ* is switched off does not, by itself, galvanize the *tyl* cluster. That depends on activation of *tylR* (plus at least one other, unidentified, gene involved in polyketide metabolism) by the TylS protein [6]. In consequence, tylosin yields might well be determined by the extent to which the system is saturated with transcriptional activators, in which case inactivation of *tylQ* (at least in the ancestral strain) would not be a panacea for enhanced tylosin production. On the other hand, the consequences of mutation and insertional inactivation might differ. It would therefore be particularly interesting to apply site-directed mutagenesis to the genome of *S. fradiae* wild type and generate the point mutation seen in *tylQ**.

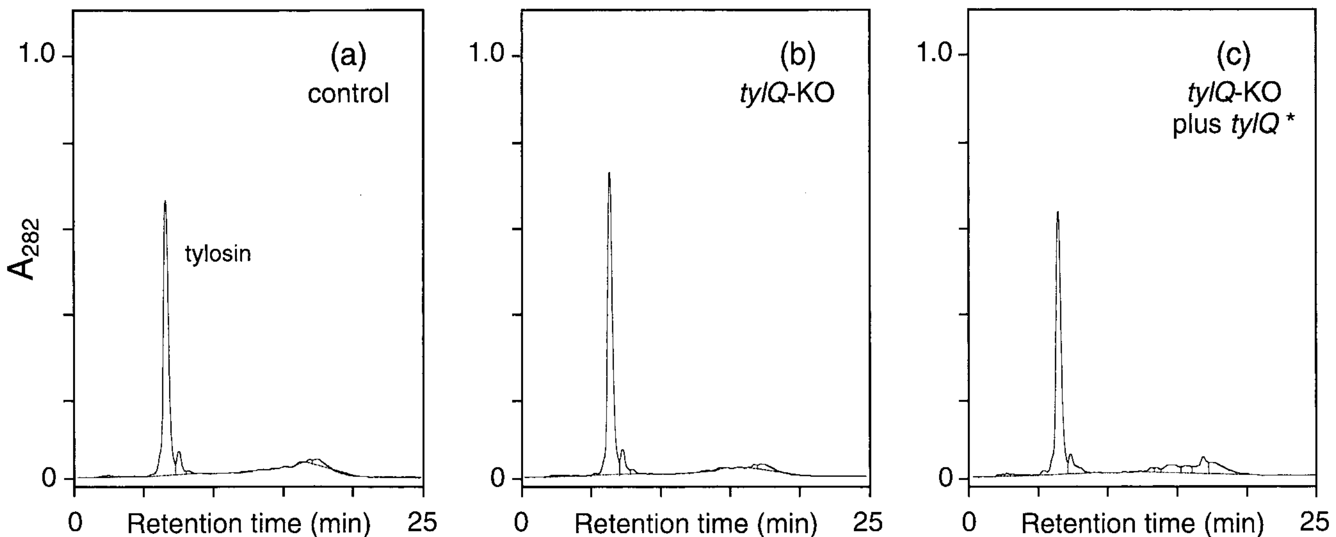

Figure 5 Fermentation products from *S. fradiae* strains disrupted in *tylQ*. HPLC analysis of material produced by: (a) control, ancestral strain not disrupted (b) *tylQ*-disrupted strain; (c) *tylQ*-disrupted strain with an added copy of *tylQ**, driven by the native promoter.

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