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REVIEW

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Control of Tylosin Biosynthesis in *Streptomyces fradiae*

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Tylosin biosynthesis is controlled in cascade fashion by multiple transcriptional regulators, acting positively or negatively, in conjunction with a signalling ligand that acts as a classical inducer. The roles of regulatory gene products have been characterized by a combination of gene expression analysis and fermentation studies, using engineered strains of *S. fradiae* in which specific genes were inactivated or overexpressed. Among various novel features of the regulatory model, involvement of the signalling ligand is not essential for tylosin biosynthesis.

Keywords: Antibiotic production, gamma-butyrolactone, gene regulation, *Streptomyces*, tylosin

In the genomes of actinomycetes, antibiotic-biosynthetic genes are usually clustered together with one or more resistance determinants that spare the producer from self intoxication. Within such clusters, antibiotic-biosynthetic genes are not commonly expressed constitutively. Their transcription is typically controlled by activators that are likewise encoded within the respective clusters, with intensive usage of operons to minimize (or, at least, reduce) the number of regulated promoters. Parsimony is also evident in the number of activators employed. Without exception to date, there is one activator per cluster, acting in a "pathway-specific" fashion (*i.e.*, one cluster per activator). Regulation of antibiotic biosynthesis also features negative control by transcriptional repressors that control other regulators rather than biosynthetic genes *per se*. In summary, antibiotic-biosynthetic gene clusters are subject to cascade regulation that at the lowest hierarchical level involves positive transcriptional control (for reviews, see [5, 8]).

Tylosin (Fig. 1) is a "macrolide" antibiotic, comprising a polyketide lactone adorned with three deoxyhexose sugars, that binds to bacterial ribosomes and thereby inhibits protein synthesis. The tylosin-biosynthetic (*tyl*) gene cluster of *Streptomyces fradiae* (Fig. 2A) consists of 43 contiguous genes that cover about 1% (~85 kb) of the genome. These include a block of five polyketide synthase megagenes (*tylGI-GV*; ~41 kb), upstream of which are 12 open reading frames (*orfs 1, 1a-11*), and a further 26 genes (*orfs 1*-26**) lie downstream of *tylG* (for details, including gene assignments, see [9]). The *tyl* cluster is flanked at either end by resistance determinants, *tlrB* (*orf26**) and *tlrC* (*orf11*), with yet another resistance gene, *tlrD* (*orf5**), occupying an internal position. Also present are a few nonessential "ancillary" genes that probably enhance the availability of key precursors for secondary metabolism [7], plus a couple of unassigned *orfs*. Perhaps the most remarkable feature of the *tyl* cluster is an island of several regulatory genes (including *tylP*, *tylQ*, *tylS*, *tylU*) positioned downstream of *tylG* and separated by over 65 kb from yet another regulator, *tylR* (*orf7*), that lies on the opposite side of the *tylG* block (Fig. 2B).

Positive Control of Tylosin Biosynthesis

When the *tyl* cluster was first sequenced (for a review, see [9]), three genes were immediately recognized as candidate positive regulators [2]. ("Regulator" and "activator" are used interchangeably herein to mean either a gene or its product.) Two of these genes (*tylS* and *tylT*) were deduced to encode SARPs (*Streptomyces* Antibiotic Regulatory Proteins; [27]), a family of pathway-specific transcriptional activators including ActII-ORF4 [10, 12] and RedD [19, 25] that, respectively, control production of actinorhodin and undecylprodigiosin by *Streptomyces coelicolor* plus DnrI that regulates daunorubicin biosynthesis in *Streptomyces peucetius* [17]. The third candidate regulator, *tylR*, resembled *acyB2* from *Streptomyces thermotolerans*, a producer of carbomycin. The latter gene was needed for expression of its neighbour, *acyB1*, when the pair of them were introduced into *S. fradiae* during a combinatorial biosynthesis project [1]. Accordingly, AcyB2 was posited to be a novel

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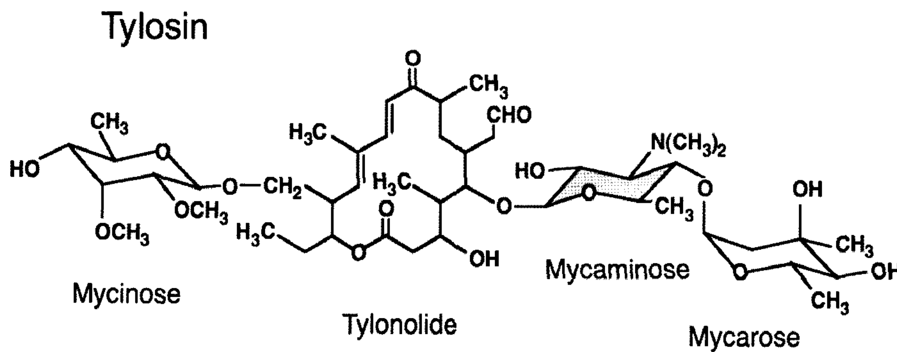


Fig. 1. Structure of tylosin.

transcriptional activator (the first to be implicated in macrolide production), although its likely involvement in carbomycin biosynthesis was not pursued in detail.

Studies involving various actinomycetes, extending back to the 1980s, first revealed that increased expression or self-cloning of pathway-specific activator genes typically enhances antibiotic production (implying that the activator proteins are not normally present in saturating amounts in wild-type strains), whereas deletion or inactivation of such genes invariably has the opposite effect. When gene knockout ("KO") analysis was applied to the candidate *tyl* activators, it soon became clear that *tylT* is not essential for tylosin production, whereas disruption of *tylS* or *tylR* completely abolished the accumulation of tylosin or any of its biochemical precursors [2, 3]. The functional relationship between the two latter genes was clarified when gene expression analysis, by RT-PCR, revealed that the *tylR* transcript was lost in *tylS*-KO strains, but not *vice versa* (Fig. 3). Evidently, *tylR* is controlled by *tylS*. Such analysis also revealed a regulated expression of *tylT*. This enigmatic gene was expressed in the wild type during tylosin production and, at least to that extent, appears not to be defective, although its function remains obscure. The ascendancy of TylR was confirmed when tylosin production was restored in a *tylS*-KO strain by forced expression of *tylR* using a heterologous promoter, but not when expression of *tylS* was engineered in *tylR*-KO strains [3, 23]. Under these conditions, TylR appeared necessary and sufficient for activation of *tyl* biosynthetic genes.

Orthologs of *tylR* from several actinomycetes have recently been characterized *in silico*, although activities of their products have not been reported. In contrast, SARPs are widespread among actinomycetes and are well established as important regulators of antibiotic production although, hitherto, this had been attributed to direct control of antibiotic-biosynthetic genes and not to regulation of other regulators.

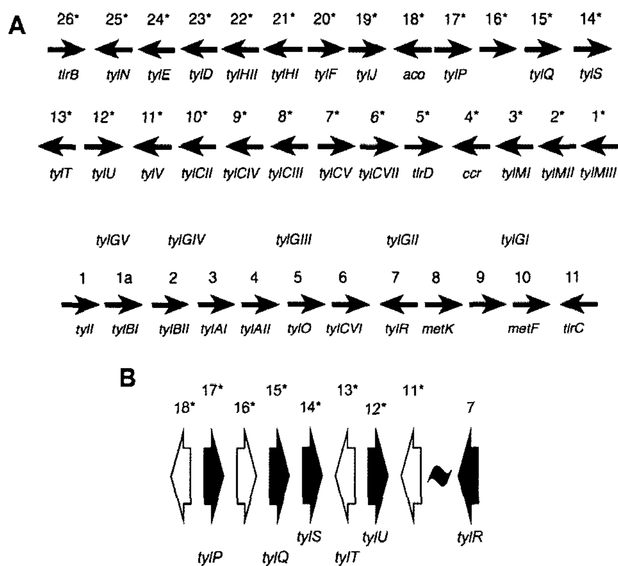


Fig. 2. A. The tylosin biosynthetic (*tyl*) gene cluster of *S. fradiae*. Not drawn to scale. The cluster of 43 contiguous genes (~85 kb) occupies about 1% of the genome. For gene assignments, see [9]. **B.** Regulatory genes within the *tyl* cluster. Not drawn to scale. Genes that regulate tylosin production are represented by BLACK arrows. An island of regulators (*orfs 12*-18**) is separated by about 65 kb from *tylR* (*orf7*).

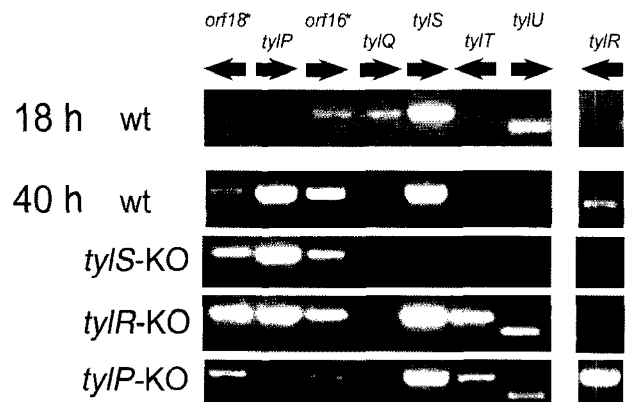


Fig. 3. Expression analysis by RT-PCR applied to *tyl* regulatory genes. RNA was extracted from *S. fradiae* wild type before and after commencement of tylosin biosynthesis (*i.e.*, after 18 h and 40 h of fermentation, respectively) and from various "KO" strains after 40 h only. Primers for RT-PCR and other conditions are described elsewhere [21]. Data adapted from [22, 23].

Further embellishment of the "SARP model" came with the realization that activation of *tylR* is not the sole function of TylS and that TylS does not always act alone. When RT-PCR analysis was applied to the entire *tyl* cluster [23], and not just to candidate activators, a gene from the regulatory island (*tylU*; formerly *orf12**) was found to be silent as well as *tylR* in a *tylS*-KO strain (Fig. 3). Until then, *orf12** had been unassigned and attracted little interest. However, when *tylU (orf12*)* was inactivated, tylosin production was reduced by 80% and was restored to wild-type levels by forced expression of *tylR* but not *tylS* [4]. It was concluded that efficient synthesis of TylR somehow depends on the combined action of TylS plus TylU (the first "SARP-helper" to be identified) and that, in the absence of TylU, lowered levels of TylR resulted in lowered levels of tylosin production. The validity of this interpretation was confirmed by Western analysis using anti-His-tag antibody. The levels of TylR-His produced in engineered derivatives of *tylU*-KO strains were much lower than in otherwise isogenic *tylU*$^{+}$ strains [4]. In summary: synthesis of TylU is driven by TylS; TylS-dependent synthesis of TylR is enhanced by TylU; TylS is essential for TylR synthesis but TylU is not (Fig. 4).

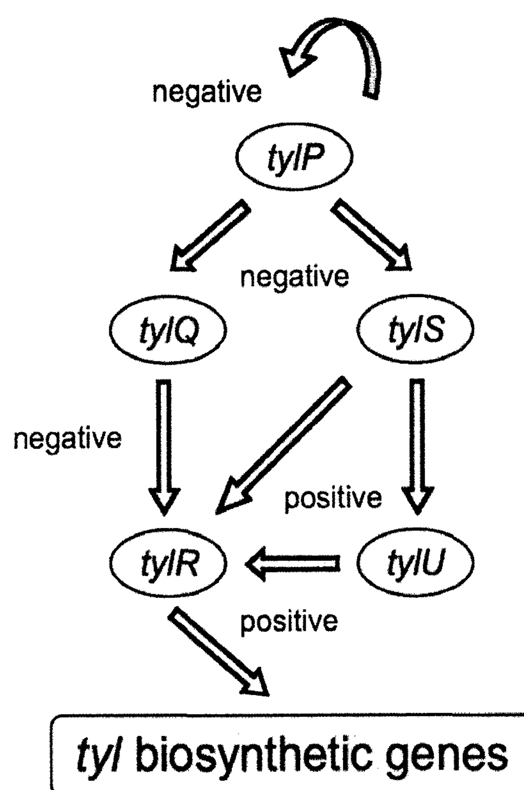


Fig. 4. Model for regulation of tylosin production in *S. fradiae*. Transcription of tylosin-biosynthetic genes is activated by the TylR protein. Expression of *tylR* is controlled in acomplementary fashion by interplay between the activators [TylS plus TylU] and the repressor, TylQ. With differential sensitivities, *tylS* and *tylQ* are repressed by TylP that also targets its "own" gene, *tylP*. At early stages of fermentation, *tylP* is silent owing to autorepression, and TylQ silences *tylR*. Derepression of *tylP* due to TylP-ligand material normally accompanies the onset of tylosin production. Refer to text for consideration of tylosin production by *tylP*-disrupted strains or those lacking TylP ligand.

NEGATIVE CONTROL OF TYLOSIN BIOSYNTHESIS

Control of the *tylR* Promoter

In *S. fradiae* wild type, expression of *tylR* is regulated *via* interplay between regulatory proteins of opposite persuasion: the transcriptional activator, TylS, (aided and abetted by TylU) versus a transcriptional repressor, TylQ. The pivotal role of TylQ became apparent when gene-expression analysis by RT-PCR was applied to the entire *tyl* cluster [21]. Although *tylQ* was active at early stages of fermentation, it was the only gene within the cluster that was silent during tylosin production. Similar analysis was also applied to engineered strains in which *tylQ* was disrupted ("*tylQ*-KO") or in which an extra copy of *tylQ* was constitutively overexpressed ("*tylQ*-OE") from a heterologous promoter. The latter produced no tylosin and failed to express many of the *tyl* genes, even after lengthy fermentation. In contrast, tylosin appeared early in *tylQ*-KO strains and accumulated at enhanced levels relative to wild type. Significantly, transcripts from *tylQ* and *tylR* were never observed concurrently in any of these strains. These various findings generated a model (Fig. 4) that invokes negative control of the *tylR* promoter by TylQ, so that *tylQ* must be switched off (or, at least, turned down) before expression of *tylR* can be activated by [TylS+TylU] with resultant production of tylosin.

Gamma-Butyrolactones and Their Receptor Proteins

Gamma-butyrolactones (GBLs) are small diffusible molecules ("microbial hormones"; [13]) involved in the regulation of morphological differentiation and/or secondary metabolism in actinomycetes. This was first shown in the 1960s by Kokhlov and colleagues working with mutants of *Streptomyces griseus* defective in streptomycin production and sporulation (for a review of GBLs, see [28]). Such strains were restored to wild-type behavior by GBL material ("A-factor") exogenously added at nanomolar concentrations, and later proved to be defective in A-factor biosynthesis. Since then, GBLs have been detected in various other actinomycetes, including "non-*Streptomyces*" spp., where they act as classical inducers (*i.e.*, derepressors) of antibiotic production; sometimes, but not always, in concert with similar effects on sporulation. In *S. griseus*, the cluster of streptomycin-biosynthetic (*str-sts*) genes is activated in a pathway-specific fashion by StrR, produced when its determinant, *strR*, is activated by AdpA. The latter controls a regulon of genes whose products separately trigger various aspects of morphological differentiation and secondary metabolism in *S. griseus*. Operation of this regulatory cascade is ultimately induced by A-factor, which displaces its receptor protein, ArpA, from the promoter of *adpA*, where it otherwise functions as a

transcriptional repressor [20, 26]. In other organisms, including the virginiamycin producer *Streptomyces virginiae*, GBLs known as "virginiae butanolides" (VBs) induce secondary metabolism without affecting sporulation. Thus, VB(s) trigger virginiamycin production by binding to a repressor, BarA, and thereby displacing it from multiple target promoters. Those targets include the promoter of *barA* (which is therefore negatively autoregulated) and that of its downstream neighbour, *barB* [14, 18].

Prior to the present work, GBLs and their receptor proteins from *S. griseus*, *S. virginiae*, and other *Streptomyces* spp. were subjected to detailed physical and functional analysie, which yielded molecular structures of the GBLs, authentication of their interactions with specific receptors, characterization of DNA sequences targeted by the latter, and confirmation that GBL receptors are displaced from their targets upon binding of the cognate ligands. In consequence, deduced sequences of authentic GBL receptors were already available for comparison when the *tyl* genes were sequenced [2] so that TylP was immediately recognizable *in silico* as a likely GBL receptor. Moreover, although no specific function could be ascribed at that time to TylQ or to BarB, deduced similarities between them, plus the prior observation that BarA regulates *barB*, suggested by analogy that TylP might control *tylQ* [2]; a And so it transpired.

Role of TylP, a Putative GBL Receptor, in Regulation of Tylosin Production

Consonant with the hypothesis that TylP might be a GBL receptor that controls *tylQ*, inspection of the *tylQ* promoter region revealed a partially palindromic sequence resembling the "ARE" (AutoRegulatory Element) consensus sequence [11] characteristic of those targeted by authentic GBL receptors [15, 16, 29]. When similar sequences were also observed in the *tylP* and *tylS* promoters (Fig. 5), it was germane to enquire whether TylP might regulate any or all of these promoters. Accordingly, reporter plasmids were constructed [22] in which promoter DNA fragments containing the three respective "PARE" sequences (implying possible recognition by TylP) were separately fused to promoterless copies of the aminoglycoside resistance gene, *aphII*. These plasmids were then integrated into the genome of *Streptomyces lividans* where they separately conferred resistance to kanamycin. Subsequent expression of *tylP* in the engineered strains revealed powerful inhibition of the *tylP* and *tylQ* promoters, with lesser inhibition of *tylS*p [22]. These data clearly implied that, in *S. fradiae*, TylP likely controls not only *tylQ* (a repressor represses a repressor) but also exerts negative control of *tylS* and, in powerful autoregulatory fashion, *tylP*. In other words, genes directly involved in the regulation of *tylR* (the global activator of tylosin-biosynthetic genes) are controlled by a candidate GBL receptor protein (Fig. 4).

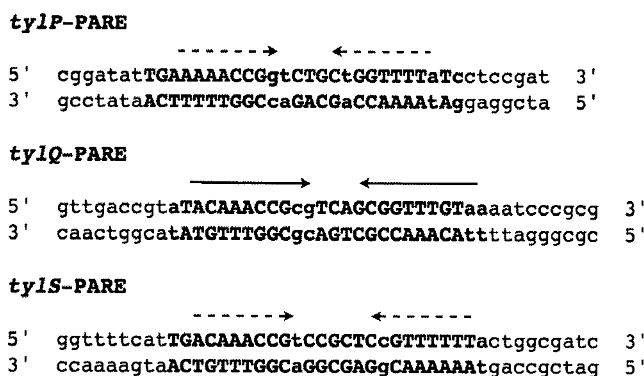


Fig. 5. Target sequences for TylP located upstream of *tylP*, *tylQ*, and *tylS* in the *S. fradiae* genome. Bases in bold correspond to the 26 bp "ARE" consensus sequence targeted by GBL receptor proteins [11]. ("PARE" denotes candidate target for TylP.) Bases in upper case match the consensus. Perfect and imperfect inverted repeats are indicated by solid and broken arrows, respectively. Synthetic deoxyoligonucleotides having the 40–44 bp PARE sequences given here were used as probes in gel electrophoretic mobility band-shift assays involving TylP-His (Fig. 7).

The influence of TylP regulatory activity on tylosin production can be rationalized by comparing predictions from Fig. 4 with the performance of engineered strains in which *tylP* was either overexpressed ("*tylP*-OE" strains) or disrupted ("*tylP*-KO"). In essence, TylP regulates tylosin production mainly by influencing the mycelial content of TylS and, consequently, of TylR. Mycelial levels of the latter directly influence the levels of tylosin produced, as shown with strains engineered to express *tylR* with differing efficiencies [4]. Thus (Fig. 6), enhanced production of TylP, predicted to result in downregulation of both *tylQ*

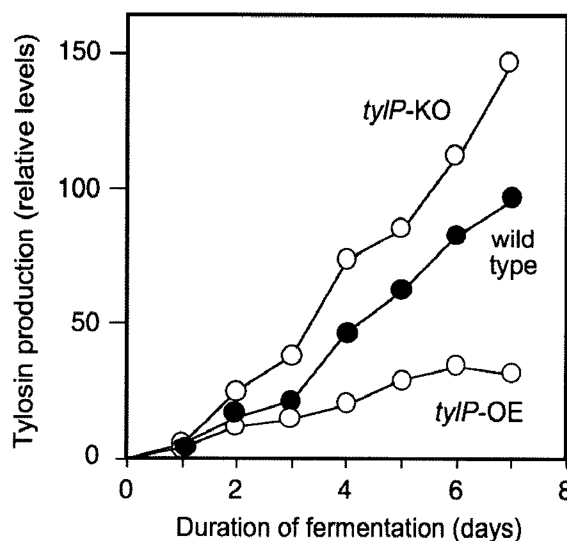


Fig. 6. Tylosin production by *S. fradiae* strains. HPLC analysis of tylosin production by *S. fradiae* wild type; a "KO" in which *tylP* had been disrupted; and a strain in which an additional copy of *tylP*, integrated into the genome, was overexpressed ("OE") under control of the *ermE*p* promoter. For details see [22].

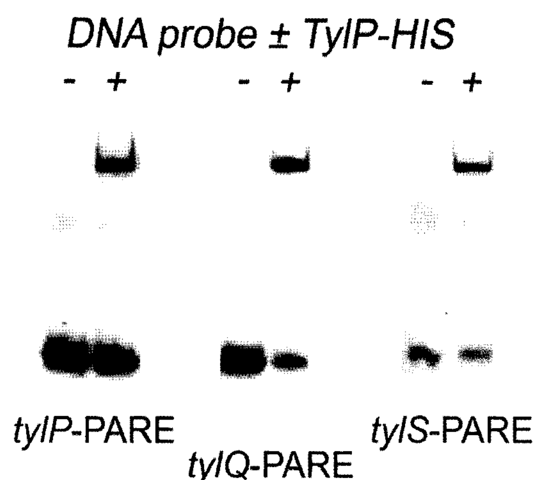


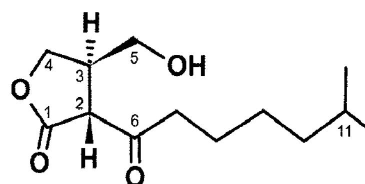
Fig. 7. Gel electrophoretic mobility band-shift assays of TylP binding to DNA. Synthetic deoxyoligonucleotides (40–44 bp each; DIG-labeled) having the three PARE sequences shown in Fig. 5 were used as probes together with TylP-His. For details, see [6].

and *tylS*, was associated with reduced yields of tylosin in *tylP*-OE strains, whereas yields were enhanced in *tylP*-KO strains [22]. Note that as transcription of *tylQ* was still shut down under the latter circumstances (Fig. 3), TylP cannot be the sole agent capable of silencing *tylQ*, and the model represented in Fig. 4 must, even yet, be incomplete.

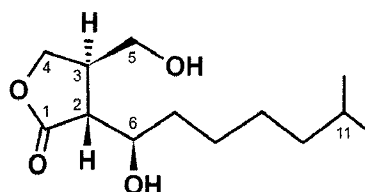
Role of TylP Ligand(s) in Regulation of Tylosin Production

When TylP binds to its three target promoters, it does so *via* specific recognition of the respective "PARE" sequences described above. This was demonstrated [6] in gel electrophoretic mobility band-shift assays (Fig. 7) using His-tagged TylP together with synthetic double-stranded deoxyoligonucleotide probes (40–44 bp) comprising little more than the palindromic PARE sequences (Fig. 5). Moreover, such binding was disrupted or prevented by material produced by *S. fradiae* and extractable from cultures by organic solvent. The TylP-ligand material was readily detectable in fermentation media shortly before the onset of tylosin production and persisted for an undetermined period thereafter, albeit in diminished amounts [6]. Thus, extracts obtained from stationary phase cultures late in fermentation still prevented DNA-protein complex formation, but only when concentrated 10-fold. That active material was also produced by a mutant of *S. fradiae* specifically disrupted in polyketide synthase activity that normally produces the tylosin aglycone. Evidently, the TylP ligand was not derived from the tylosin-biosynthetic pathway. Rather, given marked similarities between the binding sites for TylP within the *tylP*, *tylQ*, and *tylS* promoters of *S. fradiae* and the target sequences for authentic GBL receptors in other genomes, it seemed plausible (but still not secure [6]) that the "TylP ligand(s)" might be one or more GBL species.

A-factor (*S. griseus*)



SCB1 (*S. coelicolor)*



VB-C7 synonym VB-D (*S. virginiae*)

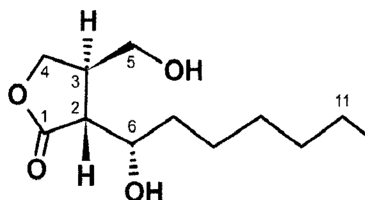


Fig. 8. Gamma-butyrolactones representing the three chemical classes characterized from actinomycetes.

The GBLs characterized from actinomycetes have been classified [28] into three groups (6-keto; 6-α-OH; 6-β-OH; see Fig. 8), although they also display minor differences in the length and branching of the acyl side chain. However, synthetic GBLs representative of the three chemotypes (materials kindly provided by S. Kitani, Osaka University) failed to prevent complex formation between TylP-His and any of the three *tyl*-PARE probes. At present, these negative (but intriguing) data are not conclusive, given that some GBL receptors display strict ligand specificity [24] that might not have been satisfied by the limited range of compounds tested. Accordingly, the possible GBL status of the TylP ligand(s) remains unresolved. This material might even be novel!

As *tylP* is silent at early stages of fermentation (Fig. 3), presumably due to negative autoregulation, the cognate ligand(s) must first displace TylP from the *tylP* promoter. This might happen as a result of constitutive, growth-dependent accumulation of the ligand(s). Alternatively, production of this material might be regulated, in which case additional detail would need to be added at the "top" of the *tyl* regulatory cascade represented in Fig. 4.

Synthesis of the TylP-Interactive Ligand(S)

In various *Streptomyces* spp., genes associated with GBL biosynthesis have been found in proximity to those

encoding GBL receptors [24] and a similar arrangement pertains to genes that flank *tylP* within the regulatory island of *S. fradiae* (Fig. 2). Thus, *orf18** and *orf16**, deduced to encode acyl-CoA oxidase (dehydrogenase) activity and a cytochrome P450, respectively, are somehow involved in synthesis of the ligand material that abrogates binding of TylP to target DNA. Although *orf18**-KO and *orf16**-KO mutants grow and sporulate normally, TylP ligand activity could not be detected at all in fermentation extracts from the former strain and only at very low levels with the latter [6]. However, both strains still produced tylosin, albeit in reduced amounts (~50% compared with wild type). This is a salient observation given, that null mutants of various other actinomycetes lacking GBLs do not produce their respective antibiotics at all. The role of the TylP-interactive ligand(s) during tylosin production is one of fine tuning.

Speculative Finale

According to the model presented in Fig. 4, strains unable to produce the TylP ligand should be defective in derepression of *tylP*, and hence, their phenotypes should to some extent resemble those of *tylP*-minus strains. But they do not! The *orf18**-KO mutant produces less tylosin than wild type, whereas *tylP*-KO strains produce more. From this, it follows that derepression of *tylP* cannot be the sole function of the "TylP ligand", for which there must be another receptor yet to be identified. Database sequence comparisons reveal TylQ to be an interesting candidate, as it too has features characteristic of GBL receptors. According to Fig. 4, derepression of *tylR* should depend upon repression of *tylQ*, followed by time-dependent dilution of TylQ activity allowing progressive activation of *tylR* by [TylS plus TylU]. However, were TylQ to be an additional receptor for the "TylP ligand", derepression of *tylR* and the onset of tylosin production might occur much sooner following first appearance of the ligand.

Conclusion

The model proposed here for the regulation of tylosin production differs in many respects from those in the literature pertaining to other antibiotic-biosynthetic systems, with no obvious convergence on a common pattern. One size does not fit all!

Acknowledgments

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