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Regulatory Elements in Tetracycline-Encoding Gene Clusters: the *otcG* Gene Positively Regulates the Production of Oxytetracycline in *Streptomyces rimosus*

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Summary

The expression of bacterial polyketide synthase gene clusters is often controlled by a number of different families of regulatory proteins that can have either a pathway-specific or a pleiotropic mode of action, *e.g.* the SARP family (*Streptomyce*s antibiotic regulatory proteins), ribosome-associated ppGpp synthetase, γ-butyrolactone-binding regulatory proteins, and two-component regulatory proteins. The molecular genetics of such regulatory mechanisms that govern the biosynthesis of tetracyclines is poorly understood. In this work, a comparative bioinformatic analysis of regulatory genes present in three tetracycline antibiotic gene clusters, namely oxytetracycline (OTC), chlortetracycline and recently cloned chelocardin gene clusters of *S. rimosus*, *S. aureofaciens* and *Amycolatopsis sulphurea* has been performed. A SARP family regulatory protein is located in the chlortetracycline gene cluster, but is not detected in the gene cluster encoding OTC biosynthesis. Interestingly, the only regulatory element identified in chelocardin gene cluster was *chdA*, an *otrR* and *ctcR* homologue from the TetR family of regulators that regulates the expression of the *otrB* and *ctc05* exporter genes in the oxytetracycline and chlortetracycline gene clusters. In the oxytetracycline gene cluster, a new LAL (LuxR) family regulatory gene homologue, *otcG*, was identified. This homologue is also present in the *ctc* gene cluster. By gene disruption and overexpression experiments, a 'conditionally positive' role of *otcG* in OTC biosynthesis has been demonstrated. The observation, the bioinformatics data and the previous work on phosphate regulation suggest the presence of a more complex, fine tuning role of the *otcG* gene product in overall expression of genes for OTC biosynthesis.

Key words: type II polyketide synthase, oxytetracycline biosynthesis, *Streptomyces rimosus*, regulatory gene, LAL (LuxR) family regulator, gene cluster, chelocardin, *Amycolatopsis sulphurea*

Introduction

Chlortetracycline (CTC) and oxytetracycline (OTC) produced by *Streptomyces aureofaciens* and *S. rimosus*, respectively, were the first members of the tetracycline antibiotic family to reach the clinic. Historically, several research groups have focused on different aspects of the biosynthesis of tetracyclines (*1–5*). For the extensive ref-

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erence list see the recent review of Petković *et al.* (*6*). Chelocardin (CHD) on the other hand, produced by *Amycolatopsis sulphurea*, is also a powerful tetracycline antibiotic with an unknown mode of action, but it did not reach the clinic (*7*,*8*). We have recently cloned the entire gene cluster encoding CHD biosynthesis from *A. sulphurea* (unpublished results).

The expression of polyketide synthase (PKS) gene cluster elements is often controlled by a number of different families of regulatory proteins that can have either a pathway-specific or a pleiotropic mode of action (*9*). The SARP family (*Streptomyce*s antibiotic regulatory proteins) of positive regulatory proteins encoding genes for pathway-specific transcriptional regulation are the most common positive regulators of type II PKS gene cluster (*10*). These proteins tend to interact with promoters of genes (gene clusters) involved in antibiotic biosynthesis and are generally found in a number of PKS clusters including the type II PKS gene clusters of actinorhodin and daunorubicin (*11*,*12*). Some examples of the more general pleiotropic signals affecting biosynthesis of type II PKSs include the ribosome-associated ppGpp synthetase (*Rel*A) (*9*), γ-butyrolactone-binding regulatory proteins (*13*), and two-component regulators (*14*). Excluding the publication on phosphate regulation by McDowall *et al.* (*15*), any other information on the molecular genetics of the regulatory mechanisms governing the biosynthesis of OTC in *S. rimosus* has not been found.

Based on a comparative bioinformatics study of the gene clusters encoding OTC and CTC biosynthesis, we have identified a new LAL (LuxR) family regulatory gene in the *otc* gene cluster. Interestingly, numerous gene clusters encoding type I PKSs contain the so-called LAL (LuxR) family of transcriptional regulators, such as positive regulators RapG from the rapamycin gene cluster, PimM from the pimaricin gene cluster, and PikD from the pikromycin gene cluster (*16*–*18*), which are usually not found in type II PKS gene clusters.

In this work a detailed analysis of the regulatory elements potentially regulating OTC biosynthesis in *S. rimosus* has been carried out. Putative regulatory elements present in the *chd* gene cluster from *A. sulphurea* have also been analysed. Apart from SARP and *tetR*-like regulatory protein, a new LAL (LuxR) gene homologue, *otcG*, has been identified in the *otc* gene cluster. Its 'conditionally positive' role in the biosynthesis of OTC has been demonstrated.

Materials and Methods

Bacterial strains and culture conditions

S. rimosus M4018 (*19*) was used as the donor of DNA and as the host for gene disruption and overexpression experiments. Electroporation of *S. rimosus* was performed as described previously (*20*). For sporulation and selection of morphological variants and resistance phenotypes, *S. rimosus* was grown on soya mannitol medium (*21*). The *S. rimosus* ATCC10970 wild type strain (*4*), and the industrial high-producing strain *S. rimosus* AB04 (Acies Bio Ltd., Ljubljana, Slovenia) were used for DNA isolation and *otcG* sequencing. *S. rimosus* strains were maintained and grown in fermentation medium as described previously (*22*). Thiostrepton (30 μg/mL in solid medium and 5 μg/mL in liquid medium) and erythromycin (50 μg/mL) were added to the media as required. *A. sulphurea* NRRL 2822 strain (*8*) was used as a source of genomic DNA for cloning the *chd* gene cluster. *Escherichia coli* DH10β (*23*) was used for cloning and *E. coli* ET12567 (*24*) for obtaining unmethylated DNA for transformation into *S. rimosus*. *E. coli* cultures were grown on 2TY medium at 37 °C, supplemented with ampicillin (100 μg/mL) and apramycin (50 μg/mL), as required. Plasmids and *Streptomyces rimosus* strains used in this study are described in Table 1.

Table 1. Plasmids and *Streptomyces rimosus* strains used in this study

Plasmid and strain	Key characteristics	Reference
SuperCos1	contains *cos* sites for construction of cosmid library	Stratagene Inc.
pGEM-T easy	cloning vector Amp^R f1-ori *lacZ*	Promega Inc.
pTS55	pSAM2-based integrative plasmid	*28*
pIJ4026	pUC18 carrying *ermE* gene from *Saccharopolyspora erythraea*	*21*
pSGset2	pSET152-derived with an *ermE* promoter	*21*
pTS55*ermE*-*otcG*	pTS55 with a 1.6-kb *Hind*III fragment containing *ermE*-*otcG* construct	this study
pΔ*otcG*	pIJ4026 with a truncated (469 nt) *otcG* cloned in a single *Eco*RI site	this study
S. rimosus ATCC10970	the natural wild type progenitor strain from which most of industrial strains were derived	*4*
S. rimosus M4018	the genetically most characterised strain, the oxytetracycline-producing Pfizer strain	*19*
S. rimosus AB04	industrial high-producing strain	Acies Bio Ltd.

DNA manipulations

Standard methods for isolation and manipulation of DNA were performed as described by Sambrook and Russel (*25*) and Kieser *et al.* (*21*). Southern hybridization was undertaken using the non-radioactive DIG DNA Labelling and Detection Kit from Roche (Mannheim, Germany), according to the manufacturer's instructions. For PCR amplification of the complete *otcG* gene (714 bp) the primers *otc*GF: 5′-GGCATATGGATAACAAAGTCATC-3′ and *otc*GR: 5′-TCAGGTGCCGGCCTGTAC-3′ were used. An *Nde*I restriction site was included in one primer to facilitate subcloning and is indicated by underscoring. For the truncated sequence of the *otcG* gene, the primers partF: 5′-AAGTCATCACCGTCCTCGTC-3′ and partR: 5′-CGCCACGTTCTCCATTATCT-3′ were used, amplifying nucleotides 11 to 480.

Cloning of the chd gene cluster

A SuperCos1-based genomic library of *A. sulphurea* NRRL 2822 genomic DNA was constructed by using the Gigapack® III Gold Packaging Kit (Stratagene). The library was screened by colony hybridization for the presence of a type II PKS gene cluster using a ketosynthase (KSα) gene from the OTC gene cluster as a probe. The selected clone was sequenced (Macrogen, Korea) and putative open reading frames (ORFs) were determined using the FramePlot beta 4.0. software (*26*). The putative gene functions were determined using the BLASTx algorithm at the NCBI (*27*).

Plasmids

The PCR products of the complete (714 bp) and truncated (470 bp) *otcG* putative regulator gene were cloned into pGEM-T easy plasmid (Promega, Germany) and confirmed by sequencing. The complete *otcG* gene was excised with *Nde*I and cloned into the *Nde*I site of pSGset2 (*21*) downstream of the *ermE* promoter. The *Hind*III fragment carrying the *ermE-otcG* construct was cloned into the *Hind*III site of the pTS55 integrative vector (*28*), resulting in the pTS55*ermE-otcG* plasmid. For the disruption of *otcG* gene, a suicide vector pΔ*otcG* was constructed by cloning the *Eco*RI fragment with the truncated *otcG* sequence from the pGEM-T easy plasmid into the *Eco*RI site of the pIJ4026 suicide plasmid (*21*).

Gene inactivation and overexpression

For the inactivation of the *otcG* gene, a suicide pΔ*otcG* vector was used. The pΔ*otcG* vector contained a 470-bp internal fragment of *otcG*. Single-crossover recombinants were selected by their resistance to erythromycin. For the overexpression of *otcG*, the pTS55*ermE-otcG* plasmid, a pTS55-based integrative vector with an intact *otcG* gene downstream of the *ermE* promoter, was used. The transformants were selected using thiostrepton. Since the OTC-producing strains of *S. rimosus* often display a very high degree of morphological instability which also causes a significant variability in the final OTC yield, only transformants with the correct 'wild-type' colony phenotype were selected. Fermentations using the selected colonies (transformants) of all mutants were undertaken in parallel to ensure reproducibility. Genetic instability, often accompanied with gene deletion and/or DNA amplification (*29*,*30*) of the *otc* gene cluster did not occur, as confirmed by DNA restriction analysis and gel electrophoresis, and finally by Southern hybridization. The OTC production of 18 *S. rimosus* M4018 colonies with a disrupted copy of *otcG* (pΔ*otcG*) and 42 colonies with an additional copy under the *ermE* promoter (pTS55-*ermE-otcG*) were compared to 12 colonies of *S. rimosus* M4018 with pTS55 as a control. The yields were analyzed using the SAS/STAT program and expressed as the least square means with standard errors of the means.

OTC analytical method

Accumulation of oxytetracycline during fermentation was measured by reversed-phase HPLC according to European Pharmacopoeia 5.5 (*31*). The 714-bp sequence for the oxytetracycline regulatory gene, *otcG*, reported here has been deposited in GenBank under accession number FJ503048.

Results

Regulatory elements present in the ctc, otc and chd gene clusters

The gene cluster encoding OTC biosynthesis from the *S. rimosus* strain M4018 was defined as a 34-kb *Eco*RI-fragment that conferred OTC production on the naïve host, *S. lividans* (*32*). Parts of that *otc* gene cluster and the entire gene cluster derived from low-producing strain ATCC 10970 have been sequenced (*4*). Gene organization of both *otc* gene clusters derived from M15883 and ATCC10970 *S. rimosus* strains is identical. On the other hand, comparative analysis of the gene organization between *otc* and *ctc* gene clusters revealed significant differences, including the regulatory genes (Fig. 1).

When analysing the ORFs of the *ctc* gene cluster (*33*), regulatory elements can be identified at both extremities of the gene cluster. On the right extremity of the *ctc* gene cluster (Fig. 1a), a *tetR*-family homologue of the repressor regulatory element *ctcR* can be identified, adjacent to the exporter gene *ctc05*. Analogously to the *ctc* gene cluster, the counterparts of the *ctc05*/*ctcR*, the *otrB*/*otrR* homologues from the *otc* (2), are located at the right extremity of the *otc* gene cluster (Fig. 1b), albeit in the opposite orientation with respect to the biosynthetic genes.

The *tetR*-like repressor from the *otc* gene cluster encodes the MarR (multiple antibiotic resistance regulator) protein, which was originally characterized as the repressor of the multiple antibiotic resistance operon *marRAB* in *Escherichia* (*34*). The TetA system of the transposon Tn10, which is a paradigm of this class of resistance, has been studied extensively (*35*). The TetR homologue in OTC biosynthesis regulates the expression of the exporter (*36*). However, it is not clear whether the *otrR* gene product is solely involved in the regulation of *otrB* or also in the regulation of other genes in the cluster. Two ORFs encoding transcriptional regulators can be identified on the left extremity of the *ctc* gene cluster in *S. aureofaciens*, just adjacent to the ribosomal tetracycline resistance gene *ctc14*, as reported earlier (Fig. 1a; *33*). Both gene homologues belong to the putative positive regulatory genes according to BLAST homology searches. The *ctc11* gene homologue is a typical SARP family, positive pathway specific transcriptional regulator, such as the *act*II-ORF4 in the actinorhodin gene cluster in *S. coelicolor* (*37*). The ORF *ctc13*, which is located at the extreme left fringe of the *ctc* cluster, adjacent to the SARP family homologue and transcribed in the opposite direction of the *ctc11* gene, is a homologue of two component transcriptional regulators of the LAL (LuxR) family (Fig. 1a). We have identified a new ORF designated as *otcG* in the *otc* cluster, with a high homology to the *ctc13* gene in the *ctc* gene cluster, designated according to Hunter and Hill (*2*). The *otcG* gene lies adjacent to the *otrA* gene (encoding ribosomal resistance) and it is expressed in the opposite orientation. Surprisingly, no *ctc11* gene homologue encoding a SARP pathway specific regulator could be identified in the OTC gene cluster (Fig. 1b). Interestingly, only *chdA* and *chdR*, the *otrR* and *otrB* homologues from oxytetracycline gene cluster, can be identified in the chelocardin gene cluster (Fig. 1c).

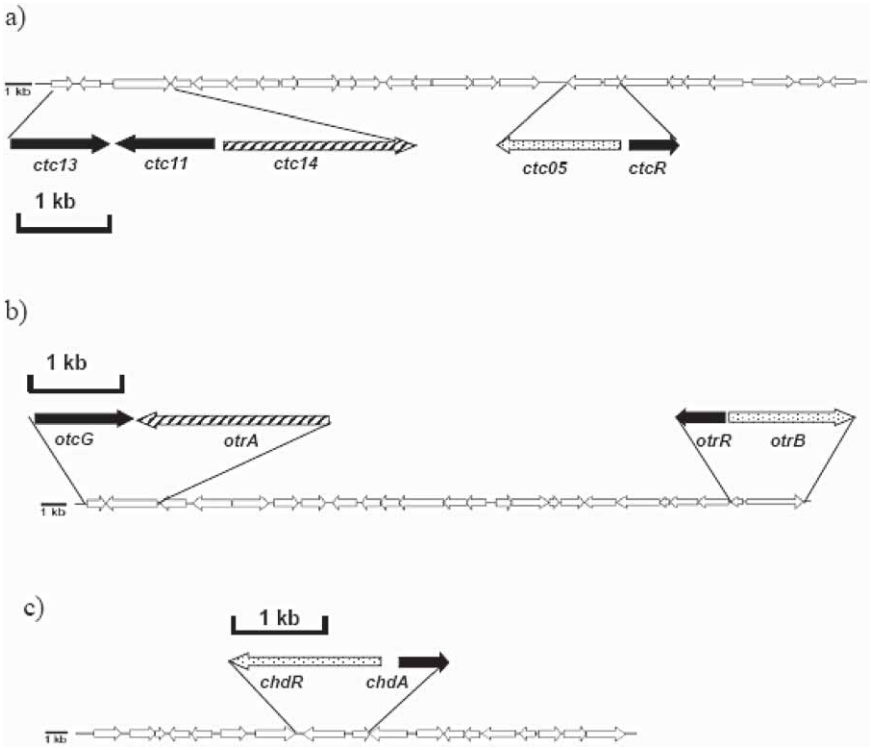

Fig. 1. The clusters encoding tetracycline antibiotics: (a) chlortetracycline, (b) oxytetracycline and (c) chelocardin from *S. aureofaciens*, *S. rimosus* and *A. sulphurea*, respectively. The magnified black arrows represent the putative regulatory genes *ctc13*, *ctc11*, and *ctcR* from chlortetracycline gene cluster, their homologues *otcG* and *otrR* from oxytetracycline gene cluster, and *chdA* from chelocardin gene cluster. The dashed (*ctc14* and *otrA*) homologues at the left extremities of the clusters, and the dotted (*ctc05, otrB* and *chdR*) homologues are the ribosomal and exporter resistance genes, respectively

Database comparisons revealed that the *otcG* gene product (237 aa) has amino acid sequence homology with the LAL (LuxR) family of bacterial regulatory proteins, a putative two-component system response transcriptional regulatory protein. The characteristic architecture of the LAL (LuxR) family of DNA binding proteins, containing a nucleotide triphosphate (NTP) binding motif at the N--terminus and a C-terminally-located helix-turn-helix (HTH) motif, can be clearly assigned to the *otcG* gene product (Fig. 2).

Interestingly, there is a rare TTA codon within the coding region of the *otcG* gene (Fig. 2), which is characteristic of many SARP genes and can have a profound influence on secondary metabolism and morphological differentiation, as demonstrated in *S. coelicolor* (*9*) where the appearance of the tRNA associated with the TTA codon is developmentally-regulated. Considering the interesting features of the *otcG* gene and its putative role in the regulation of OTC biosynthesis in *S. rimosus*, we have also sequenced this gene homologue in the two independent strains, namely *S. rimosus* ATCC10970 wild type strain (*4*), and the industrial high-producing strain *S. rimosus* AB04 (DNA provided by Acies Bio Ltd., Slovenia). Sequencing of the *otcG* gene from all three strains revealed 100 % identity at the DNA level (data not shown); therefore, no mutation was introduced in the *otcG* gene during the intensive strain improvement processes carried out over the years.

When a putative gene cluster encoding CHD was compared to the *ctc* and *otc* gene clusters (Fig. 1), no SARP or LAL (LuxR) family of bacterial regulatory proteins were detected in the *chd* gene cluster. Adjacent to the *chdR* exporter gene homologue, we have identified the *chdA* gene homologue, a putative *tetR*-like repressor gene, analogously to the *otc* and *ctc* gene clusters (Fig. 1). Although *chdA* gene homologue is clearly a putative *tetR* gene homologue (*34*), it shows little similarity to the *otc* and *ctc* gene cluster counterparts *otrR* and *ctcR*, which share 46 % identity and 57 % similarity (Fig. 3). Unexpectedly, the *chdA* gene shows the highest similarity to the *tetR* gene homologues from Gram-negative bacteria such as *Klebsiella pneumoniae*, *Burkholderia* spp. and *E. coli* (*38*,*39*).

Inactivation of the otcG gene

Gene inactivation and overexpression was adopted as the fastest way to examine a possible role of *otcG* gene product in the regulation of OTC biosynthesis in *S. rimosus*. As *otcG* is transcribed convergently with the *otrA* ribosomal resistance gene, a simple and straight--forward single-cross over, suicide-disruption experiment could be undertaken using a plasmid carrying a truncated version of the *otcG* gene without influencing the expression of the surrounding ORFs, particularly the *otrA* gene (Fig. 1b). For this purpose, the truncated DNA fragment of the *otcG* gene was inserted into the suicide vector pIJ4026 (*21*).

The OTC-producing *S. rimosus* strains are well known for their morphological instability (*40*). This instability is promoted even further by gene manipulation, resulting

Fig. 2. Alignment of the LuxR homologues from the *Streptomyces rimosus*, *S. aureofaciens*, and *S. avermitilis* strains. The Sigma-70 (region 4) domain involved in binding to the -35 promoter element *via* a helix-turn-helix motif is marked with *, the signal receiver domain is marked with ——, the TTA codon is highlighted in bold, the phosphorylation site is marked with ■, and the dimerization interface is marked with +++

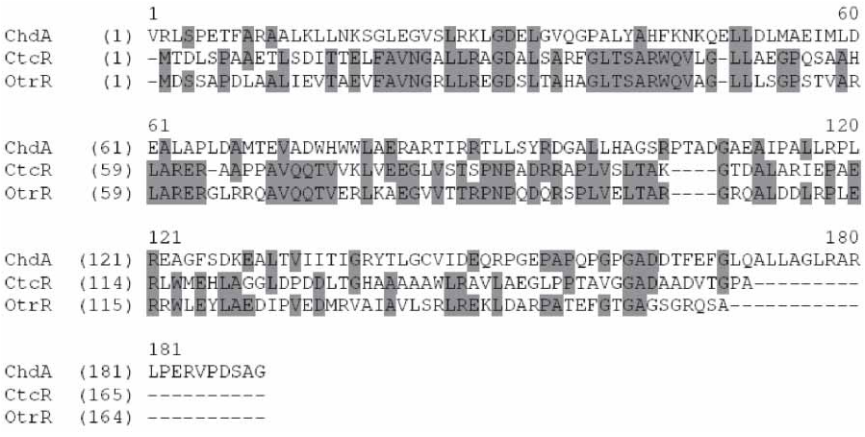

Fig. 3. Alignment of the TetR family of the regulators from the chelocardin (ChdA), chlortetracycline (CtcR) and oxytetracycline (OtrR) gene clusters. Gray shadows indicate the conserved regions of the proteins

in a great proportion of the colonies having a range of morphological deformations, most often causing a significant decrease of the OTC yield (*29*,*30*). Therefore, only morphologically stable, well-sporulated colonies were selected for further testing in order to reduce the variability in OTC production caused by morphological instability. Eighteen independent transformants carrying the disrupted *otcG* gene were then tested in industrial production medium, as described in Materials and Methods. The inactivation of the *otcG* gene did not abolish the production of OTC. However, the production of OTC in the wt:Δ*otcG* mutants was statistically significantly reduced by more than 40 % in comparison with the wt:pTS55 as demonstrated by the p-value of 0.0034 (Fig. 4). Inactivation of the *otcG* gene carried out under the conditions applied during the experiments presented in this work did not affect any of the morphological characteristics of the 4018 strain.

Overexpression of the otcG gene

The entire *otcG* gene was amplified by PCR and subcloned into the pTS55-based integrative vector (*28*) to generate pTS55*ermE-otcG*. Expression of the *otcG* gene was under the control of the *ermE* promoter (*21*), proven to be functional in the *S. rimosus* 4018 strain. Forty-two

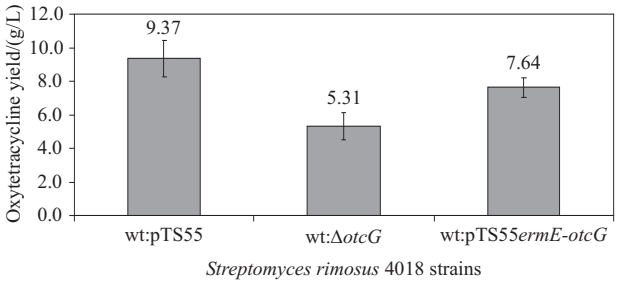

Fig. 4. Comparison of the oxytetracycline yields in *Streptomyces rimosus* 4018 strains within a single fermentation run. The strain with the truncated *otcG* gene (wt:Δ*otcG*; 9 independent transformants) and the strain with the additional copy of the *otcG* gene expressed under the *ermE* promoter (wt:pTS55*ermE-otcG;* 21 independent transformants) were compared to the *S. rimosus* 4018 with the pTS55 plasmid as a control

independent well-sporulated transformants from SM agar medium were then tested for the production of OTC, as described in the Materials and Methods. The introduction of a second copy of *otcG*, expressed under the constitutive promoter *ermE*, did not yield any statistically significant change in OTC production (p=0.1602) compared to the *S. rimosus* 4018 strain containing pTS55 plasmid alone (Fig. 4).

Discussion

The aim of this work was to gain a fresh look into the putative regulatory elements that may govern the biosynthesis of OTC, through a comparative bioinformatics study of the entire *ctc*, *otc* and *chd* gene clusters from *S. aureofaciens*, *S. rimosus* and *A. sulphurea*, respectively. The DNA sequence of part of the gene cluster encoding OTC biosynthesis in *S. rimosus* ATCC10970 was recently published by Zhang *et al.* (*4*), thus allowing us to compare the entire gene clusters encoding CTC and OTC biosynthesis. Importantly, gene clusters encoding OTC and CTC biosynthesis were expressed heterologously in *S. lividans*, confirming that all the necessary genes for the biosynthesis of CTC and OTC were encoded by the published DNA sequences (*32*,*33*).

Although the structures of the *otc* and *ctc* gene clusters do differ significantly, the expected DNA homologues with high identity/similarity can be identified in both gene clusters (Figs. 1a and b). The annotation of the *otc* gene clusters, which was carried out in both strains of *S. rimosus*, the ATCC10970 and the M15883 strains, presumed that all the necessary genes encoding OTC biosynthesis were flanked by the *otrA* (ribosomal) resistance gene at one end, and the *otrB* (exporter) gene on the other side of the gene cluster (*2*). However, led by the annotation of the putative ORFs from the *ctc* cluster, we have identified an additional ORF in the *otc* gene cluster from a putative LAL (LuxR) family of transcriptional regulator, *otcG* (Fig. 1b), with a characteristic nucleotide triphosphate (NTP) binding motif at the N--terminus and a helix-turn-helix (HTH) motif at its C--terminal side (Fig. 2). This gene lies within the *Eco*RI fragment used by Binnie *et al.* (*32*) for the heterologous production of OTC in *S. lividans.* Another objective of this work was to determine whether the *otcG* gene product is actually involved in OTC biosynthesis. To our knowledge, the LAL (LuxR) family of regulatory proteins has not been found previously in type II PKS gene clusters. Little is understood of the overall role of these proteins. Given the location of the *otcG* gene, flanking externally the *otrA* gene (Fig. 1b), it was not unreasonable to question whether this protein is actually involved in the regulation of OTC biosynthesis. Simple and straightforward gene disruption and overexpression experiments were carried out to answer this question.

Inactivation of the *otcG* gene did not entirely abolish, but instead significantly reduced OTC biosynthesis. Overexpression of the *otcG* gene, through the ectopic expression of a second copy of the *otcG* gene did not influence the final OTC yield, thus demonstrating an only 'conditionally-positive' regulatory involvement of the *otcG* gene product.

We have identified a rare TTA codon within the coding region of the *otcG* gene, which is characteristic of many SARP genes (*9*). In the context of actinorhodin biosynthesis, it has been demonstrated that the expression of an *act*II-ORF4 carrying a mutated TTA codon leads to *bld*A-independent actinorhodin production (*41*). However, the inactivation of the *otcG* gene did not lead to any obvious morphological changes. Interestingly, the *ctc* gene cluster counterpart of *otcG*, *ctc13*, does not contain the rare leucine TTA codon, nor does the SARP homologue from the *ctc* cluster contain the rare TTA codon. The OTC biosynthesis in *S. rimosus* is not regulated by the SARP family of regulatory elements located in the *otc* gene cluster. A SARP family regulator is clearly identified in the *ctc* gene cluster in *S. aureofaciens*. The regulatory activity of the SARP-like regulatory protein located outside the *otc* gene cluster, which positively regulates OTC biosynthesis, is also a feasible option, as demonstrated in *S. coelicolor.* It has been shown that the *act*II-ORF4 gene product of the SARP family, which is located in the actinorhodin gene cluster also influences the expression of the spore pigment-encoding *whi*E locus and the gene clusters encoding the biosynthesis of coelichenin and calcium-dependent (CDA) antibiotics, and the genes governing the biosynthesis of undecylprodigiosin (*9*). McDowall *et al.* (*15*) predicted the existence of a regulatory element involved in OTC biosynthesis, when studying the phosphate-mediated control of OTC production by *S. rimosus*. In this study they showed that the transcription of the OTC biosynthetic genes *otcC* and *otcX* is triggered by phosphate starvation, indicating that the synthesis of OTC is controlled, at least in part, at the level of transcription. Interestingly, promoters of OTC biosynthesis overlap by tandem repeats, whose spacing resembles that of the activation sites of the OmpR family of transcriptional regulators (*18*). Based on these findings, McDowall *et al.* (*15*) suggested that the activator of the *otc* genes may be a member of the OmpR family, most likely a member of the SARP group. However, no such type of regulatory element has been identified in the gene cluster encoding OTC biosynthesis (*2*). On the other hand, the *otcG* gene product belongs to the LAL (LuxR) family of transcriptional regulators, which is classified as a member of the bigger group of the

OmpR family of transcriptional regulators with a typical conserved gene architecture consisting of a helix-turn--helix DNA binding motif, a signal receiver domain (Fig. 2). The *otcG* gene product may likely interfere with the promoters of the genes involved in OTC biosynthesis, a regulatory element which was predicted by McDowall *et al.* (*15*). Interestingly, the putative phosphorylation site in the *ctc3* gene in *S. aureofaciens* seems to be mutated from aspartic acid to glycine residue; thus, *ctc13* might even be inactive in the *ctc* gene cluster (Fig. 2).

Finally, no regulatory genes were identified in the chelocardin gene cluster, with the exception of *chdA* gene, a putative *tetR* gene homologues located beside the *chdR* exporter gene homologue (Fig. 1c). It is reasonable to believe that the *chdR* exporter gene is the only resistance determinant present in the *chd* gene cluster. We could not identify the *otrA* ribosomal resistance gene homologue in the *chd* cluster. However, this is not surprising, considering that the chelocardine does not target the ribosome (*42*). However, it was very surprising that the putative *tetR* homologue *chdA* shows very high similarity to the *tetR* gene homologues present in the Gram--negative bacteria, considering that *A. sulphurea* taxonomically belongs to the order of Actinomycetales, *i.e.* a Gram-positive and the DNA GC-rich bacteria.

Conclusion

Regulatory elements from three gene clusters encoding tetracycline biosynthesis have been compared. Significant overall differences have been identified in the regulatory elements that are likely governing the corresponding biosynthetic pathways. When comparing the *tetR* homologue (*chdA*) from the chelocardin-producing strain *A. sulphurea* to the *otc* and *ctc* counterparts, unexpected evolutionary distance was identified. In this work, a new regulatory element has been identified, not previously identified in the *otc* gene cluster, and clearly demonstrated that the *otcG* gene, a member of the LAL (LuxR) family of regulatory proteins, exerts a positive role in the regulation of OTC biosynthesis. However, it has only a 'conditionally positive' regulatory role, since inactivation of the *otcG* gene does not completely abolish OTC production. On the other hand, overexpression of the *otcG* gene did not cause an increase of the OTC yield, as it has often been demonstrated with, for example, the SARP family of regulatory elements. Coupled with the bioinformatics data and the work on phosphate regulation carried out by McDowall *et al.* (*15*), it is possible to envisage a more complex, 'fine tuning' role of the *otcG* gene product, which may likely be interfering with the rather complex promoter regions of the genes in the OTC gene cluster.

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