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Structure and regulatory targets of SCO3201, a highly promiscuous TetR-like regulator of *Streptomyces coelicolor* M145

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ABSTRACT

SCO3201, a regulator of the TetR family, is a strong repressor of both morphological differentiation and antibiotic production when overexpressed in *Streptomyces coelicolor*. Here, we report the identification of 14 novel putative regulatory targets of this regulator using *in vitro* formaldehyde cross-linking. Direct binding of purified His_6-SCO3201 was demonstrated for the promoter regions of 5 regulators (SCO1716, SCO1950, SCO3367, SCO4009 and SCO5046), a putative histidine phosphatase (SCO1809), an acetyltransferase (SCO0988) and the polyketide synthase RedX (SCO5878), using EMSA. Reverse transcriptional analysis demonstrated that the expression of the transcriptional regulators SCO1950, SCO4009, SCO5046, as well as of SCO0988 and RedX was down regulated, upon SCO3201 overexpression, whereas the expression of SCO1809 and SCO3367 was up regulated. A consensus binding motif was derived *via* alignment of the promoter regions of the genes negatively regulated. The positions of the predicted operator sites were consistent with a direct repressive effect of SCO3201 on 5 out of 7 of these promoters. Furthermore, the 2.1 Å crystal structure of SCO3201 was solved, which provides a possible explanation for the high promiscuity of this regulator that might account for its dramatic effect on the differentiation process of *S. coelicolor*.

1. Introduction

Streptomyces are Gram-positive filamentous bacteria that live in the superficial layers of the soil where they play fundamental ecological roles [1]. These bacteria have great industrial and economic importance since they produce most of the currently known antibiotics and many other bioactive molecules useful to human health and agriculture [2]. *Streptomyces* are characterized by a complex metabolic and morphological differentiation cycle. The trigger of the differentiation program involves sensing and transmission of extracellular and/or intracellular signals to the transcriptional/translational apparatus or even directly to proteins *via* post-translational modifications [3]. Intracellular metabolic signals are sensed by transcriptional regulators (one-component systems, OCS) able to bind small metabolites [4]. Various families of OCS are present in the genome of *Streptomyces coelicolor* (GntR, AraC/XylR, LacI/DeoR and others) but regulators of the TetR family [5] are the most abundant since approximately 150 of these have been predicted [6]. TetR-type regulators consist of a highly conserved helix-turn-helix (HTH) motif located in the N-terminal part of the protein and of a more variable ligand-binding domain [5]. The DNA binding ability of these regulators is modulated by the binding of chemically diverse metabolites to their ligand pockets [7–9]. TetR regulators are widely known as transcriptional repressors [5], although a small number act as activators [10,11] or have dual repressor/activator roles [12], depending on the position of their operator sites relative to promoter elements.

In a previous study [13], we identified a TetR-like regulator, SCO3201, that was found to strongly repress both antibiotic production and morphological differentiation when overexpressed in *S. coelicolor* M145. Two targets of this regulator were identified: itself and *scbA* (*SCO6266*) whose expression is known to be regulated by the TetR-like regulator ScbR (SCO6265) [14]. In this issue, we report the identification of novel promoter regions recognized by SCO3201 as well as the crystal structure of SCO3201. Since elevated levels of SCO3201 cause major developmental defects, we hypothesized that in condition of over-expression this

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repressor binds regulatory targets that may constitute novel players in the complex differentiation process of *Streptomyces*.

2. Materials and methods

2.1. Bacterial strains, plasmids, media, and culture conditions

The wild-type strain of *S. coelicolor* M145 was used in this study. The *SCO3201* deletion mutant (*S. coelicolor* M145 Δ*sco3201*::*aac(3)IV*) and the strain overexpressing *SCO3201* (*S. coelicolor* M145/pWHM3-*SCO3201*) were constructed as described previously [13]. R2YE solid medium was used to grow *Streptomyces* cultures for total RNA extraction. Where necessary, ampicillin, apramycin and thiostrepton were included in the cultures at 50 μg ml^{-1}. *Escherichia coli* D41 strain was used for protein expression.

2.2. Isolation of the putative SCO3201 binding targets

5–10 nM of *S. coelicolor* M145 chromosomal DNA was incubated with 100–200 nM of purified His-SCO3201 [13] for 15 min at room temperature. A negative control reaction containing DNA only was set in parallel. Crosslink buffer (HEPES, NaCl, EDTA, 37% formaldehyde) was added to the reaction mixture and incubated for 10 min at 37 °C and a further 1 h at 4 °C. The reaction mixture was then sonicated to shear the chromosomal DNA to 2–3 kb. The resultant solution was passed through nickel column (Sigma), and the histidine-tagged SCO3201/DNA complexes were isolated. NaCl was added at a final concentration of 200 mM, and the complexes were incubated at 65 °C for 4 h and treated with proteinase K. The recovered DNA was divided into two groups, one digested by *Sma*I and the other by *Sau*3AI. The resulting DNA fragments were cloned into pUC19. The white clones were isolated and the inserted DNA fragments were sequenced.

2.3. Electrophoretic Mobility Shift Assay (EMSA)

DNA fragments containing the different upstream regions of the sequenced genes were amplified from the *S. coelicolor* M145 chromosome by PCR using the primers listed in Supplementary Table 1 and the resultant PCR products were labeled by Cy5′ by PCR using primer P label. EMSA was performed as described previously [13].

2.4. Isolation of total RNA and semi-quantitative RT-PCR

Total RNA of various strains was isolated as described previously [13]. The RNA concentration was adjusted to identical level prior to RT-PCR analysis. cDNA was amplified with random primers by Thermoscript reverse transcription PCR system (Catalogue No. 11146-016), from 2 μl of each RNA template. The resultant cDNA pool was used as template for subsequent individual PCR, using RT-PCR primers listed in Supplementary Table 1. A reaction without adding reverse transcriptase was carried out as negative control.

2.5. X-ray diffraction experiments, phasing and refinement

Details of large-scale purification of SCO3201, crystallization and Se-Met SAD-phasing and refinement are described in Supplementary data. Atomic coordinates and X-ray diffraction data for the crystal structure determination of SCO3201 were deposited in the protein data bank with accession number 4CGR.

3. Results

3.1. Isolation of fourteen putative novel regulatory targets of SCO3201 using in vitro formaldehyde cross-linking

In vitro formaldehyde cross-linking was used to identify putative targets of SCO3201. Sixteen putative targets were found (Table 1) including the promoter regions of *SCO3201* and *scbA* that were previously shown to be regulated by SCO3201 [13], validating the procedure. Among the 14 novel targets identified, 7 were putative transcriptional regulators and the others had various functions. Six of the targets have already been shown to be involved in the regulation of antibiotic production and/or sporulation. These include, besides *scbA* (*SCO6266*) [14], the TetR regulator RrdA (SCO1104) that represses the expression of RedD [15], the kinase CutS (SCO5863) of the two component system CutR/CutS that negatively regulates secondary metabolism in *Streptomyces* [16], the Clp protease (SCO6408) that is playing an important role in the differentiation process [17,18] as well as the regulator WhiA (SCO1950) [19,20] and the small protein WhiB (SCO5046) that are both involved in sporulation [21]. Among the six Transcriptional Regulators (TRs) not previously known to be involved in the regulation of the differentiation process, two belong to the TetR family (SCO1104, located downstream of two putative phosphatases and SCO3367, divergent from a putative exporter) and two others belong to the GntR-family (SCO1417, divergent from an operon of 3 genes encoding a membrane protein, a putative glycerophosphoryl diester phosphodiesterase and a permease of the major facilitator family and SCO1716, the 2nd gene of an operon of 3 genes, located downstream of an homogentisate

Table 1
List of putative SCO3201 regulatory targets.

Target gene	Protein	EMSA	*In vivo* test
SCO0216	Nitrate reductase alpha chain NarG2		
SCO0988	**Putative acetyltransferase**	√	**Down**
SCO1104	RedD regulator RrdA, TetR-family protein		
SCO1417	Putative GntR-family regulatory protein		
SCO1716	**Possible GntR family transcriptional regulator (2nd gene of an operon of 3 genes possibly involved in metabolic degradation of Phe and Tyr)**	√	**NE**
SCO1809	**Putative Phospho histidine phosphatase**	√	**Up**
SCO1950	**Sporulation transcription regulator, WhiA**	√	**Down**
SCO3367	**Putative TetR-family regulator (divergent from exporter)**	√	**Up**
SCO4009	**Putative bifunctional protein (histidine kinase/regulator)**	√	**Down**
SCO5046	**Hypothetical protein, WhiB family**	√	**Down**
SCO5863	Two-component sensor (kinase) CutR/CutS (kinase)		
SCO5878	**Polyketide synthase RedX**	√	**Down**
SCO6129	Putative DNA-binding protein (Upstream of AbaA-like/in divergence His kinase)		
SCO6408	Clp protease ATP binding subunit		

√: promoter regions shifted up in EMSA; up or down: transcription up or down regulated by overexpressed SCO3201; NE: No Effect.

1,2-dioxygenase that might cleave Phe and Tyr aromatic rings). The remaining two TRs are SCO4009, a putative bi-functional protein bearing histidine kinase and regulator domains and SCO6129, located upstream of and likely co-transcribed with an Aba-like regulator [22].

SCO3201 was also shown to be able to bind the promoter regions of *redX* (*SCO5878*), encoding the polyketide synthase of the undecyprodigiosin biosynthetic cluster [23]; of *SCO0216*, the first gene of an operon of 4 genes encoding subunits of a nitrate reductase; of *SCO0988*, encoding an acetyltransferase likely to be co-transcribed with *SCO0987*, encoding a possible iron-regulated membrane protein; and of *SCO1809*, encoding a protein bearing an histidine phosphatase domain.

In order to demonstrate the direct interaction of SCO3201 with its putative novel targets, EMSA were carried out. To do so, the promoter regions of the identified genes (about −370 to +80 relative to the translational start codon), were amplified and Cy5′ labeled for EMSA. The migration of 8 promoters (marked in bold in Table 1), were clearly retarded upon the addition of purified His_6-SCO3201. In all cases, the addition of extra amount of unlabeled probes to the reaction mixture led to the disappearance of the shifted bands, demonstrating the specificity of the protein–DNA interaction (Supplementary Fig. 1). In the case of *SCO0988* and *SCO1809*, only one retarded band was observed, whereas, for the promoter regions of the other five genes, at least two bands were shifted up, suggesting the presence of multiple operator sites binding a dimer of SCO3201 or a unique operator site binding a tetramer of SCO3201.

At last, to confirm the regulation of the targets validated by the EMSA assay by SCO3201, *in vivo*, the transcription levels of the 8 retarded target genes were monitored by RT-PCR in the wild type strain of *S. coelicolor* M145, in the strain deleted for SCO3201 and in the strain overexpressing SCO3201 at 24 h, 48 h and 72 h. As shown in Fig. 1, the overexpression of SCO3201 correlated with reduced expression of the genes encoding the polyketide synthase RedX (SCO5878) at 24 h and 48 h, the sporulation regulatory factor WhiA (SCO1950) and the WhiB-like protein (SCO5046) at 48 h, the acetyltransferase (SCO0988) and the putative bi-functional protein (histidine kinase/regulator, SCO4009) at 72 h.

In contrast, the overexpression of SCO3201 correlated with enhanced expression of genes encoding SCO1809, a protein with a phosphohistidine phosphatase domain at 48 h and SCO3367, a TetR regulator, present in divergence of an efflux protein, at 72 h. Unexpectedly, the expression of *SCO1716* did not vary upon SCO3201 overexpression.

As expected, the inactivation of SCO3201 had no impact on the expression of the tested genes [13]. RT-PCR replicates were performed and similar regulatory patterns were observed (data now shown). These *in vivo* and the EMSA experiments suggested that the control exerted by SCO3201 over the expression of these genes occurs *via* direct interaction with their promoter regions.

3.2. Establishment of a consensus sequence motif for SCO3201 operator site

The putative −10 and −35 boxes of the promoter regions of the validated target genes were determined using the online Promoter Prediction tool (www.fruitfly.org/seq_tools/promoter.html) and putative transcriptional start sites were proposed (Supplementary Fig. 2). The nucleotides conserved between the promoter regions of the isolated targets and the previously identified operator sites in the native SCO3201 promoter were analyzed using the MEME program (http://meme.nbcr.net/meme/). The binding sites of SCO3201 in its native promoter were previously shown to consist of three closely located imperfect or perfect inverted repeats referred to as bs^L, BS^C and bs^R. BS^C contains a perfect 15 bp palindromic sequence (TGGCAGATTCTGCCA) that overlaps the −35 box [13]. The putative operator sequences in the promoter regions of the 5 genes negatively regulated by SCO3201 bear similarity to BS^C and, likewise, overlap the −35 promoter region (Supplementary Fig. 2). This seems consistent with the direct negative regulation of these genes by SCO3201. A consensus sequence motif was thus derived from these 5 promoter sequences using the MEME software and was compared with the perfect palindrome of BS^C (Fig. 2), revealing that the central AT base pair was highly conserved.

The putative operator sequences in the promoter regions of the 2 genes positively regulated by SCO3201 show similarity to bs^R, a site that does not have a perfect palindromic sequence and binds SCO3201 with lower affinity than BS^C. This weak binding might be sufficient to exert a positive effect on the expression of these genes.

3.3. The crystallographic structure of SCO3201

In order to gain further insight into the DNA-binding properties of SCO3201, its crystal structure was determined. The resulting model, refined to a resolution of 2.1 Å (Fig. 3), revealed that the two subunits of the dimeric repressor in the crystal are not

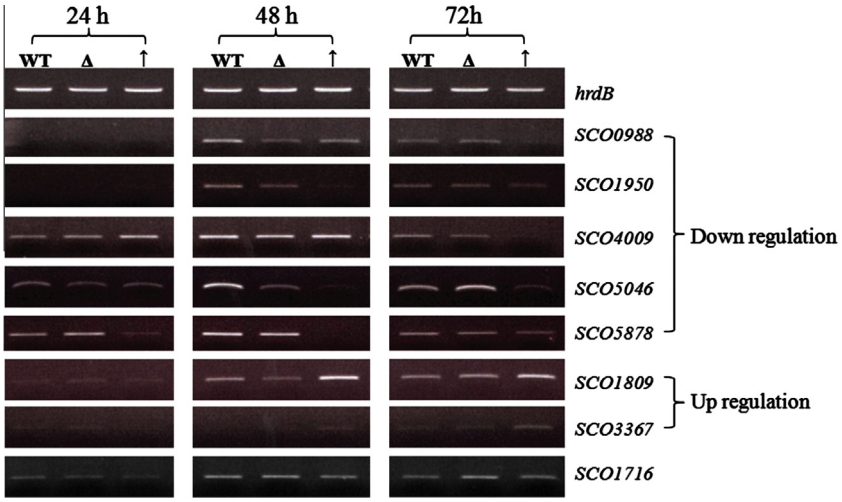

Fig. 1. Assessment of the transcription levels of SCO3201 regulatory targets by RT-PCR in the wild-type (WT), *SCO3201* mutant (Δ) and *SCO3201* overexpression (↑) strains of *S. coelicolor* at 24, 48 and 72 h.

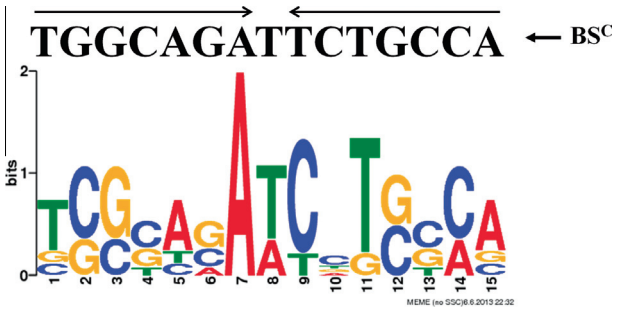

Fig. 2. MEME-derived consensus of SCO3201 operator site in the promoters regions of the negatively regulated targets. This consensus shows similarities to the core binding site BS^C identified in the promoter region of SC3201.

identical. In monomer A, residual electron density is observed in a funnel-like cavity that runs deep into the protein core. The F_o–F_c map for this region, contoured at 2.5σ, is shown in Fig. 4A and B. Since none of the buffer components from the crystallization set-up fits the observed shape, the electron density is likely to correspond to an organic molecule sequestered from the expression host. The unidentified ligand in subunit A appears to have a strong influence on the over-all repressor structure, as the SCO3201 homo-dimer is remarkably asymmetric. Least-squares superposition of the complete dimer onto a copy of itself, with subunits inverted, yields a C_α-RMSD of 3.0 Å. This is almost entirely due to structural differences between the ligand-binding domains (helices α4–α10), which when superimposed alone show a C_α-RMSD of 1.7 Å. On the other hand, the DNA-binding domains (helices α1–α3) of the two monomers are nearly identical and can be superposed with a C_α-RMSD of 0.42 Å. Perhaps the most striking difference between the two subunits is the presence of a pronounced kink in helix α4 of the unliganded B-monomer, *i.e.* the N-terminal helix of the ligand-binding domain that connects it to the DNA-binding domain. This kink is not observed in subunit A, where helix α4 is almost perfectly straight (Fig. 4C). This leads to a markedly different positioning of the DNA-binding domains in the two subunits.

An interesting and unique feature of SCO3201 is the length of its α1-helix, which is two full turns longer than that of other TetR family members. One of the most closely related structures that we identified is PDB entry 1JT0, corresponding to the multidrug-binding protein QacR in complex with its cognate promoter DNA [24]. Superposition of the DNA-binding domain of SCO3201 onto the equivalent in 1JT0 (C_α-RMSD 0.84 Å for most similar chains) suggests that the elongated α1-helix of SCO3201 is in a position to interact with the minor groove located beyond the major groove that accommodates the actual HTH motif (Fig. 3C). Interestingly, the additional N-terminal turns of the helix 1 contain four positively charged residues (Arg28, Arg29, Lys30 and Arg34), all of which seem ideally positioned for sequence-independent interaction with the phosphate backbone. Together, these observations suggest an extended binding site for SCO3201 as compared to other TetR-like repressors. Such additional sequence-independent contacts between helix α1 and DNA phosphate groups could explain SCO3201's relaxed specificity.

4. Discussion

In silico analysis of *S. coelicolor* genome predicts 151 TetR family transcriptional regulators [5,6]. A few of these have already been shown to play a role in the regulation of antibiotic biosynthesis/

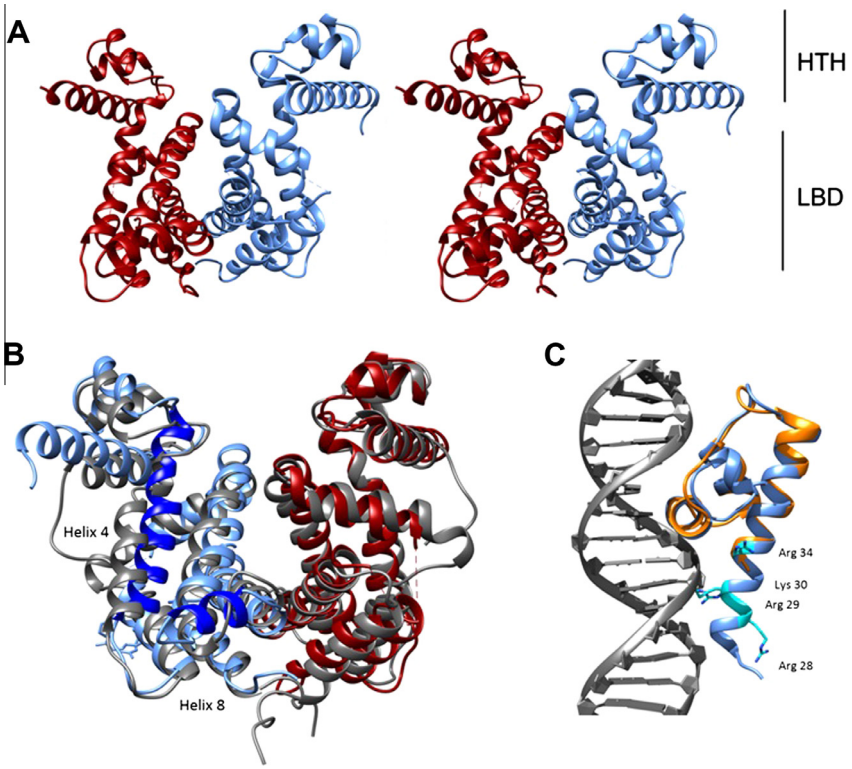

Fig. 3. The crystal structure of SCO3201. (A) Stereo view of the SCO3201 dimer that constitutes the asymmetric unit. Monomer A is shown in blue, monomer B in red. HTH: helix-turn-helix DNA-binding domain; LBD: ligand-binding domain. (B) Superposition of SCO3201 onto a TetR-like regulator from *Rhodococcus sp. RHA1* (PDB entry 2RAE, shown in grey). Helices α1 and α8 are labeled and shown in darker blue. (C) Superposition of the HTH motifs of SCO3201 and that of QacR in complex with DNA (PDB entry 1JT0). Positively charged residues in helix α1 of SCO3201 that are in a position to interact with the DNA backbone have been indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

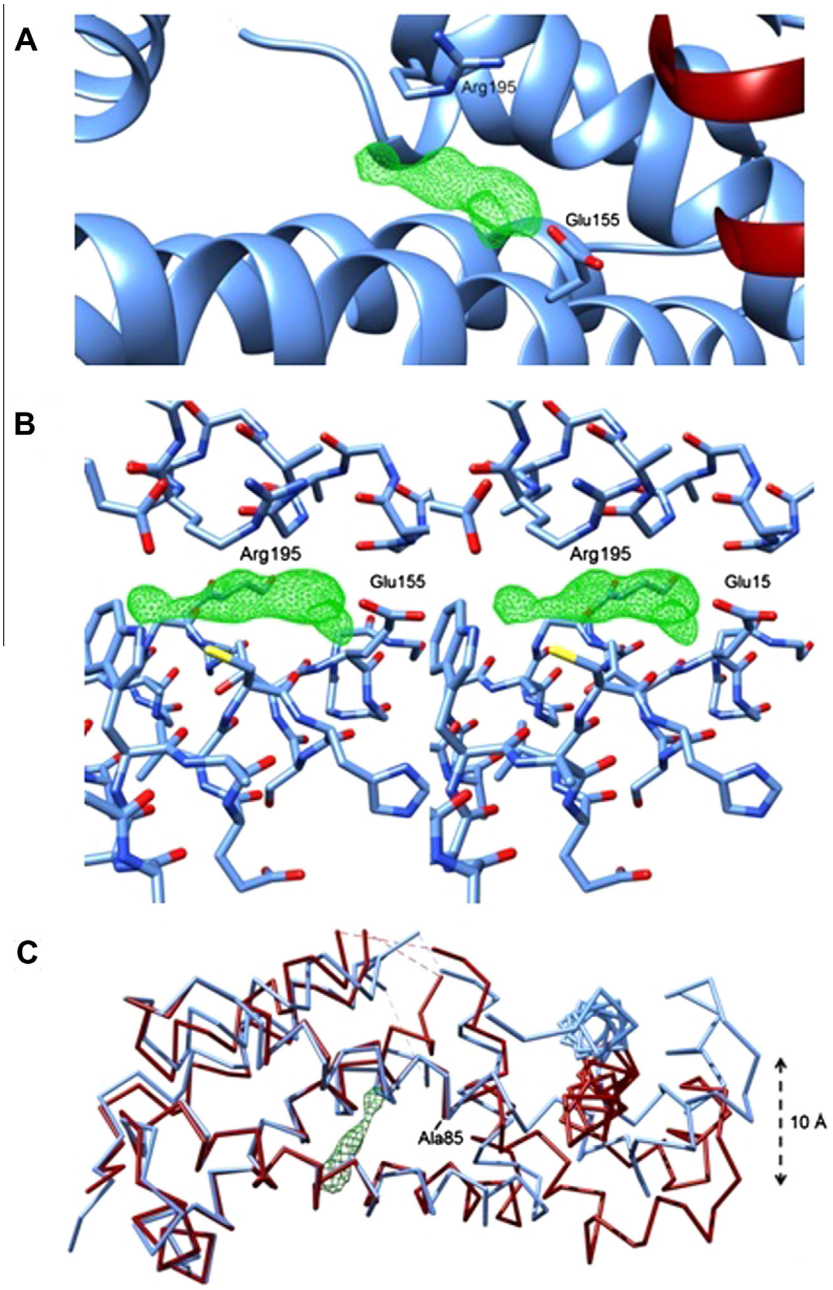

Fig. 4. Ligand binding and structural differences between SCO3201 dimer subunits. (A) The ligand-binding site in monomer A. Electron density from the F_o–F_c map, contoured at 2.5σ, is shown in green. Charged residues in the vicinity of the putative inducer have been indicated. (B) Detailed stereo view of the ligand density and the surrounding protein chains. (C) Superposition of the A and B subunits. Straightening of the kink at Ala85 in helix α4 leads to a 26° rotation of the DNA-binding domain and, consequently, a shift of its outermost atoms by 10 Å. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

export and/or morphological differentiation [15,25–28]. However, for the vast majority of TetR regulators, the biological functions of their regulatory targets in *Streptomyces* remain to be characterized.

In this study, regulatory targets of overexpressed SCO3201 were identified and characterized. Some of the validated (WhiA, WhiB-like, RedX and ScbA) and unvalidated targets (RedD, CutS, Clp protease) of this regulator were previously shown to be involved in the regulation of morphological differentiation and/or secondary metabolites biosynthesis. The down regulation of the expression of sporulation factors *whiA* (*SCO1950*) [19] and *whiB*-like (*SCO5046*) [29] is likely to be at least partly responsible for the delay in sporulation observed upon SCO3201 over-expression. Similarly, the up regulation of *scbA* (*SCO6266*) [14] and the down regulation of *redX* (*SCO5878*) [23] might be at least partly responsible for the inhibition of undecylprodigiosin production observed upon SCO3201 overexpression. Interestingly, novel targets of SCO3201 that might constitute new players in the complex regulation of the differentiation process of *S. coelicolor* were identified in the course of this study. SCO4009, SCO1809 and SCO3367 might play signaling and/or regulatory roles whereas such role is unlikely for the putative acetyltransferase encoded by *SCO0988*. SCO4009 is annotated as a bi-functional histidine kinase/regulator [30]. SCO1809 bears similarity to the phosphohistidine phosphatase SixA from *E. coli* [31] and might thus have a regulatory role *via* its ability to dephosphorylate a phosphorylated sensory kinase. Finally, SCO3367 is a classical transcriptional regulator of the TetR family located in divergence of an efflux protein/drug resistance

exporter, a classical genetic context for TetR regulators in various bacteria [7]. In conditions of overexpression and because of its lack of DNA-binding specificity, SCO3201 thus behaves as a master regulator at the top of a regulatory cascade. It likely governs the expression of genes encoding proteins with putative signaling/regulatory functions that might have a great impact on the metabolic and morphological differentiation of the bacteria.

In an attempt to explain the broad DNA-binding specificity of this regulator, its structure was analyzed by X-ray crystallography. The structure confirms that the protein belongs to the TetR family repressors and reveals a number of intriguing novel features. In the first place, a detailed comparison to the structurally related repressor QacR from *Staphylococcus aureus*, whose co-crystal structure with operator DNA is available, suggests that the binding site of SCO3201 is extended *via* its exceptionally long α1-helix. This helix might provide sequence-independent salt bridges with the DNA backbone. This specific feature may contribute to SCO3201's ability to bind rather diverse operator sequences. Moreover, pairs of QacR homodimers are known to bind to overlapping sites on opposite faces of the double helix, which leads to cooperativity *via* joint distortion of the DNA in the absence of direct contacts between the homodimers [24]. A similar process may also play a role in the rather loose DNA-binding specificity of SCO3201.

This study that describes the interaction of a broad range DNA-binding TetR regulator with illegitimate targets and the dramatic consequences of this binding on the metabolic and morphological differentiation of the bacteria, illustrates the importance of TetR regulators in the control of the complex regulatory cascade that governs the *Streptomyces* developmental cycle. The disruption/overexpression of the four validated novel SCO3201 regulatory targets will establish whether these genes are novel players in the regulation of the morphological and metabolic differentiation process of *S. coelicolor*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.003.

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