The XRE-DUF397 Protein Pair, Scr1 and Scr2, Acts as a Strong Positive Regulator of Antibiotic Production in Streptomyces

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The xenobiotic response element (XRE) transcription factors belong to a regulator family frequently found in Streptomyces that are often followed by small proteins with a DUF397 domain. In fact, the pair XRE-DUF397 has been proposed to comprise toxin–antitoxin (TA) type II systems. In this work, we demonstrate that one of these putative TA-systems, encoded by the genes SCO4441 and SCO4442 of Streptomyces coelicolor, and denominated Scr1/Scr2 (which stands for S. coelicolor regulator), does not behave as a toxin–antitoxin system under the conditions used as was originally expected. Instead the pair Scr1/Scr2 acts as a strong positive regulator of endogenous antibiotic production in S. coelicolor. The analysis of the 19 Streptomyces strains tested determined that overexpression of the pair Scr1/Scr2 drastically induces the production of antibiotics not only in S. coelicolor, but also in Streptomyces lividans, Streptomyces peucetius, Streptomyces steffisburgensis and Streptomyces sp. CA-240608. Our work also shows that Scr1 needs Scr2 to exert positive regulation on antibiotic production.

Keywords: Streptomyces, positive regulator, antibiotic production, xenobiotic response element, toxin–antitoxin

INTRODUCTION

Streptomyces are Gram-positive bacteria with a complex life cycle that includes the formation of mycelia and spores. In order to compete with other inhabitants of their ecosystems (mainly the soil), these bacteria have developed the capacity to produce a high number of extracellular hydrolytic enzymes and, also, secondary metabolites with antibiotic and antifungal activities among others (Chater, 2016). The production of these metabolites is tightly regulated through a large number of signal transduction proteins, including transcriptional regulators, which confer Streptomyces with the ability to rapidly respond to environmental changes by using available nutrients and producing secondary metabolites. It has been determined that 804 out of the 8300 genes in the genome of Streptomyces coelicolor are associated with this function. Of these, 499 have been classified as transcriptional regulators, 155 as one-component systems, 64 as sigma factors and 9 as DNA-binding proteins1 (Ortet et al., 2012). The xenobiotic response element (XRE) family of transcription factors (TF) is comprised of 70 TFs in S. coelicolor. This XRE family is the second most

1 http://www.p2tf.org
frequently occurring regulator family in bacteria, which control several diverse metabolic functions (Novichkov et al., 2013)\(^2\). Although these TF are abundant in Streptomyces genomes they have been poorly characterized. The most studied member of this group is the master regulator BldD from S. coelicolor. BldD is a small (18 kDa) protein that is a transcriptional regulator essential for morphological development and antibiotic production (den Hengst et al., 2010). WhiJ (SCO4543) is another member that has been studied in this organism, which has been associated with the repression of differentiation (Aínsa et al., 2010). WhiJ has a wide number of uncharacterized paralogous genes that are normally clustered with two additional genes. One of which, in the case of WhiJ, is SCO4542, a small protein belonging to the DNA-binding family that contains a domain of unknown function. This domain has been denominated DUF397 and is thought to interact with WhiJ, preventing it from binding to the operator sequence present in developmental genes (Aínsa et al., 2010). Actually, the DUF397-XRE gene pair encodes proteins that are most abundant in Actinobacteria, which have been assigned the function of class II toxin–antitoxin systems (TAS: TA-systems) among other functions (Makarova et al., 2009). In S. coelicolor, the XRE protein has been predicted to act as an antitoxin, with the associated small DNA-binding protein containing the DUF397 domain acting as a toxin; although its molecular mode of action has not yet been described (Sevin and Barloy-Hubler, 2007; Makarova et al., 2009; Doroghazi and Buckley, 2014).

Toxin–Antitoxin loci systems (TASs) are small genetic elements composed of a stable toxin and its cognate unstable antitoxin. The toxin, when released, prevents or alters cellular processes including translation, DNA replication, and ATP and cell wall synthesis and this activity can lead to cell death or the formation of drug-tolerant persister cells (Schuster and Bertram, 2013). Based on the molecular nature of the antitoxin modules, the TASs are currently grouped into six classes depending on their antitoxin nature: type II, IV, V, and VI are proteins and those in classes I and III are small regulatory RNAs (Lobato-Márquez et al., 2016). Type II class proteins are the most abundant and best described and include both the toxin and antitoxin, small proteins that form a stable complex. The antitoxin blocks the activity of the toxin by hiding the region responsible for toxicity (Goeders and Van Melderen, 2014; Hayes and Kedzierska, 2014).

The use of the TA finder 2.0 http://202.120.12.133/TAfinder/index.php (a TA-systems database web tool) predicted the presence of 42 TAS pairs in the S. coelicolor genome\(^3\), of which 15 are classified as XRE/DFU397 (Shao et al., 2011; Xie et al., 2018). In the present work, the putative TAS functionality of one of these XRE/DFU397 protein pairs from S. coelicolor, encoded by SCO4441/SCO4442 and paralogous to whiJ and its downstream gene (SCO4543/SCO4542, respectively), was studied. Consequently it was found that the overexpression of the putative toxin SCO4442 was neither deleterious in the S. coelicolor wild-type strain or in the deletion mutant obtained in this work. These same results were obtained when Streptomyces lividans wild-type strain was used as the host. Therefore, this gene pair does not function as a toxin–antitoxin system, at least under the conditions assayed, as was originally predicted using bioinformatics.

Additionally, we found that the proteins encoded by SCO4441/4442 act as a positive regulator of endogenous antibiotic production in S. coelicolor and were named Scr1 and Scr2, respectively. The overexpression of Scr1, in combination with Scr2, drastically induces the production of antibiotics not only in S. coelicolor, but also in S. lividans, Streptomyces

\(^2\)http://regprecise.lbl.gov/RegPrecise/collection_tffam.jsp?ttfamily_id=70

\(^3\)http://202.120.12.133/TAfinder/report.php?job_id=XZiXXD6mm

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Plasmids used.</th>
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<tr>
<td><strong>Plasmid</strong></td>
<td><strong>Characteristics</strong></td>
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<tr>
<td>pUL773</td>
<td>pBlueScript SK derivative conjugative plasmid containing the Apramycin resistance cassette.</td>
</tr>
<tr>
<td>pXHis1</td>
<td>pBlueScript SK derivative. Ampicillin resistance. The xysA promoter from S. halstedii controls xysA expression.</td>
</tr>
<tr>
<td>pN702Gem3</td>
<td>High-copy number E. coli/Streptomyces shuttle vector. Neomycin resistance.</td>
</tr>
<tr>
<td>pN702Gem3c</td>
<td>Conjugal pN702Gem3 derivative. A BamHI fragment of 1.380 KB, from plU773, containing the oriT and the apramycin resistance was cloned in the BigI.</td>
</tr>
<tr>
<td>pNX4441</td>
<td>pN702Gem3 derivative. The xysA promoter from S. halstedii controls SCO4441 expression. The protein SCO4441(Scr1) has His Tag at the carboxy terminal.</td>
</tr>
<tr>
<td>pNX4442</td>
<td>pN702Gem3 derivative. The xysA promoter from S. halstedii controls SCO4441 expression. The protein SCO4442 (Scr2) has His Tag at the carboxy terminal.</td>
</tr>
<tr>
<td>pNX4441/42</td>
<td>pN702Gem3 derivative. The xysA promoter from S. halstedii controls SCO4441 expression and the SCO4442 is expressed under its own promoter control. The protein SCO4442 (Scr2) has His Tag at the carboxy terminal.</td>
</tr>
<tr>
<td>pNX4441/42c</td>
<td>Conjugal pN702Gem3 derivative. A BamHI fragment of 1.38 kb, from pUL773, containing the oriT and the apramycin resistance was cloned in the BigI site of the plasmids pNX4441/42.</td>
</tr>
<tr>
<td>pHJL401</td>
<td>Low-copy number E. coli/Streptomyces shuttle vector. Ampicillin and thiostrepton resistances.</td>
</tr>
<tr>
<td>pHJL401c</td>
<td>Conjugal pHJL401 derivative obtained by insertion of a HindIII-NheI band of 1.6 Kb from pN702Gem3c in the plasmid pHJL401 digested with HindIII-XbaI.</td>
</tr>
<tr>
<td>pHAX41/42c</td>
<td>Conjugal pHJL401 derivative. Obtained by insertion of a HindIII-NheI band of 3.8 kb from pNX4441/42 conj in the plasmid pHJL401 digested with HindIII-XbaI.</td>
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FIGURE 1 | Overexpression of the putative SCO4441/SCO4442 TAS (Scr1/Scr2). (A) S. coelicolor M145 (wt); (B) S. coelicolor Δscr1/scr2. In each panel: colonies obtained after transformation with the different multicopy plasmids overexpressing the putative antitoxin Scr1 (pNX4441), the putative toxin Scr2 (pNX4442), both proteins Scr1/Scr2 (pNX4441/42) and the empty plasmid (pN702Gem3) in R2YE medium (Top); Detail of the morphology of the colonies obtained after 10 days (Bottom).

peeceuties, Streptomyces steffisburgensis, and Streptomyces sp. CA-240608, as determined from the 19 strains tested. Analysis of the chromatographic peaks of the molecules induced in each case was performed, and an increment in some endogenous compounds and the appearance of new induced metabolites were detected. In conclusion, this protein pair seems to function as a positive regulator in the complex regulatory network of antibiotic production. These results open new doors to the application of Scr1/Scr2 in biotechnology, with the possibility of discovering new and natural products.

MATERIALS AND METHODS

Strains, Media, and Growth Conditions

Streptomyces strains used in this study are: S. albus J1074, S. argillaceus ATCC 12596, S. coelicolor M145, S. glaucescens Tü49, S. griseus ATCC13273, S. lividans 1326, S. parvulus JJ12283, S. peucetius ATCC 27952, S. rochei CECT 3329, S. steffisburgensis NRRL3193, S. vinaceus JJ2838, and 8 Streptomyces sp. strains isolated from different soil samples (Supplementary Table S1). These strains were grown on R2YE, MS, PGA, and NA solid media for transformation, sporulation, conjugation, and phenotypic assays, respectively (Coco et al., 1991; Kieser et al., 2000). YES xylose (Sevillano et al., 2016) or NMMP (Kieser et al., 2000) containing 1% of xylose were used in the overexpression assays. Routine plasmid construction and plasmid isolation was done in Escherichia coli DH5α, and E. coli ET12567, a non-methylating strain, was used to obtain the plasmids to be transformed into S. coelicolor. E. coli strain BW25113 (pIJ790) (containing the λ Red system) (Datsenko and Wanner, 2000) and ET12567 (pUZ8002) (harboring the tra genes in the non-transmissible RP4-derivative plasmid pUZ8002) (MacNeil et al., 1992) were used for PCR-targeted mutagenesis
of *S. coelicolor* M145 and conjugation plasmid transfer to the different *Streptomyces* species. *Staphylococcus aureus* MB5393, *E. coli* ATCC25922 and *Candida albicans* ATCC64124 were used in the antibiotic analysis. Antibiotics were used when needed for plasmid selection (*E. coli*: 100 µg mL⁻¹ for ampicillin; 50 µg mL⁻¹ for apramycin; 50 µg mL⁻¹ for kanamycin; 34 µg mL⁻¹ for chloramphenicol, and 25 µg mL⁻¹ for nalidixic acid. *S. coelicolor*: 20 µg mL⁻¹ for neomycin, 10 µg mL⁻¹ for apramycin and 10 µg mL⁻¹ for thiostrepton).

### DNA Manipulation

DNA transformation and manipulation of *E. coli* and *S. coelicolor* were carried out using the methods by Green and Sambrook (2012) and Kieser et al. (2000), respectively. The plasmids used in this work are listed in Table 1.

### Mutant Generation

The coding regions of SCO4441, SCO4442 or both genes were replaced by an apramycin resistance cassette (aac(3)IV gene) by using REDIRECT PCR-targeting technology (Gust et al., 2003). The primers LS-090 and LS-091 (Supplementary Table S2) were used to generate the mutation cassette from the plasmid pIJ773 (Gust et al., 2003), which was used as the template. The mutated genes were obtained using cosmid SCD6 (Redenbach et al., 1996) and transferred by conjugation from ET12567 (pUZ8002) to *S. coelicolor* M145. The desired mutants were selected by apramycin resistance and sensitivity to kanamycin. PCR assays confirmed the deletion of the SCO4441, SCO4442 or both genes in *S. coelicolor* M145.

### Antibiotic Determination

Qualitative actinorhodin (ACT) production of the *S. coelicolor* strains was observed on different solid media. Approximately 10⁵ spores were deposited in 5 µL drops onto plates that were incubated at 30°C. ACT production was detected after three to 10 days of growth as a blue halo around the colonies.

Colorimetric quantification of prodiginines (RED) and ACT production was determined by the spectrophotometric method described in Yepes et al. (2011). All experiments were performed in triplicate.

### LC-HRMS-Analyses

Liquid cultures (10 mL) of *S. coelicolor* or *S. lividans* in YES+Xylose, or NMMP+Xylose, containing 20 µg mL⁻¹ for neomycin or 10 µg mL⁻¹ for apramycin, depending on the plasmid used, were incubated at 28°C for 8 days at 200 rpm. Then, 1 mL of the culture was extracted using 0.7 volume of 1% formic acid acidified ethyl acetate and the organic layer was dried in vacuo. The dry extracts were finally resuspended in methanol (100 µL). LC-HRMS-analyses were performed as previously described using a Bruker maXis QTOF mass spectrometer coupled to an Agilent 1200 LC (Martin et al., 2014; Perez-Victoria et al., 2016). Differential peaks were selected by direct comparison of the DAD signal base peak chromatograms.

### Plasmid Construction

The multicopy overexpression vectors pNX4441, pNX4442, and pNX4441-42 were derivatives of the pN703GEM3 plasmid (Fernández-Ábalos et al., 2003) and carry the neomycin resistance gene as selective marker. They were obtained by cloning the corresponding coding sequences SCO4441 (*scr1*), SCO4442 (*scr2*) or both, previously amplified by PCR using the corresponding oligonucleotides (see Supplementary Table S1), into the NdeI and XhoI sites of the intermediate plasmid pXHis1 (Adham et al., 2001b), yielding pX4441His, pX4442His, and pX4441/42His, respectively. In a second step, the corresponding BglII/HindIII fragments of these three plasmids were cloned into the same sites of pN702GEM3, obtaining pNX4441, pNX4442, and pNX4441-42. In these constructs, the strong xylanase promoter *xysAp* (Rodriguez et al., 2005) controls the ORFs of *scr1*, *scr2* and *scr1* (*scr2* was under the control of its own promoter in pNX4441-42), respectively. In these plasmids the corresponding Scr1, Scr2, and Scr2 were tagged, respectively, with a six His tag at the carboxy terminus.

To obtain conjugative plasmids, a BamHI fragment of 1380 bp containing the *oriT* and the apramycin resistance, from pIJ773 (Gust et al., 2003), was cloned in the BglII site of the plasmids pNX4441/42 and pN702Gem3, respectively, obtaining the plasmids pNX4441/42c and pN702Gem3c. Later a 3900-bp band was obtained from the plasmid pNX4441/42c and a 1600-bp band was obtained from the plasmid pN702Gem3c using the enzymes.
HindIII and NheI. These fragments were individually cloned into the low copy plasmid pHJL401 (Larson and Hershberger, 1986) digested with HindIII and XbaI to obtain the final plasmids pHAX4441/42c and its control pHJL401c, respectively. These plasmids carry resistance genes to thiostrepton and apramycin.

RESULTS

Is Scr1/Scr2 a Functional TAS?

The SCO4441 gene (scr1) encodes a 295 aa protein with a 7.18 isoelectric point, has a molecular weight of 33.59 kDa and has been predicted to be a putative antitoxin. This protein contains two regions, the HTH-XRE domain (aas 23 to 80) and the HipB domain (aas 24 to 62) (NCBI database). The gene SCO4442 (scr2) encodes a 63 aa protein with a 6.49 isoelectric point, has a molecular weight of 6.48 kDa and has been predicted to be a putative toxin. This protein contains a DUF397 region (aas 11 to 62).

As Scr1 and Scr2 are proposed to form part of a putative toxin–antitoxin system (TAS) (Shao et al., 2011; Xie et al., 2018), here we carried out a study to analyze the effect of its putative toxicity using the over-expression of Scr1, and/or Scr2 in S. coelicolor M145 and in S. lividans 1326. Three multicopy plasmids, pNX4441, pNX4442, and pNX4441/42 (see section “Materials and Methods”) were generated, where the expression of these genes was controlled by the xylanase strong promoter xysAp (Rodriguez et al., 2005). The plasmids and the control, the empty plasmid pN703Gem3, were introduced into both Streptomyces species to assess the possibility of Scr2 acting as a toxin.

The S. coelicolor cells overexpressing the putative toxin in plasmid pNX4442 were viable, generating colonies which were able to differentiate like those obtained with both the control.
Santamaría et al. Scr1/Scr2 Positive Regulator Antibiotic Production

FIGURE 4 | Metabolites induced by Scr1/2 in S. coelicolor. (A) Metabolite production induced by the overexpression of Scr1/Scr2 (pNX4441/42) in S. coelicolor M145 in YES+Xyl and detected by UV-Vis absorbance (200–900 nm) base peak chromatography. (B) Amplified region of the four most different peaks produced and their predicted compounds (1) SEK 4/SEK 4B; (2) ε-actinorhodin; (3) C_{32}H_{24}O_{14}; (4) γ-actinorhodin.

plasmid and the putative antitoxin (pNX4441). A slight delay in differentiation was observed when both genes of the putative TAS were overexpressed (pNX4441/42) (Figure 1A). The same results were obtained when the plasmids were transformed into S. lividans where no toxic effect was observed when the putative toxin encoded in the plasmid pNX4442 was expressed (data not shown).

These results indicated that the protein encoded by scr2 was not acting as a conventional toxin, inducing lethality when overexpressed in S. coelicolor and S. lividans, at least under the conditions used (Figure 1A), as was the case of YefM/YoeBsl, isolated from S. lividans, which was previously characterized in our laboratory (Sevillano et al., 2012).

To exclude the possibility that a single gene copy of scr1, present in the S. coelicolor genome (wild-type), could be sufficient to counteract the toxicity of Scr2 overexpression, a deletion mutant strain lacking both putative TAS genes, ΔSCO4441/42 (Δscr1/scr2), was generated (see section “Materials and Methods”) and used as a host for plasmids pNX4441, pNX4442, and pNX4441/42. The results obtained when overexpressing these plasmids in the new mutant strain were similar to those described for the wild-type (Figure 1B). Hence, the function of these two genes did not correspond to a TAS under these particular conditions. Interestingly, the mutant Δscr1/scr2 did not show any phenotype divergent from that of the wild-type in the conditions used (see section “Discussion”).

Involvement of Scr1/Scr2 in a Positive Regulation of Antibiotic Production in S. coelicolor and in S. lividans

Furthermore, a clear phenotype of colored antibiotic induction was observed when the strains of S. coelicolor wild-type and Δscr1/scr2, carrying the overexpression plasmid pNX4441/42, were grown on several solid media such as NMMP+Xyl. A high overproduction of the blue-red antibiotic ACT was observed when scr1 was overexpressed either alone or with scr2 in the wild-type strain. Nevertheless, when the S. coelicolor Δscr1/scr2 strain was used as a host, this high overproduction of ACT (blue color) was only observed with the overexpression of both genes at the same time (pNX4441/42) (Figure 2). These results indicated that Scr1 was a positive regulator of ACT production and required the presence of Scr2 to function.

To further delve into the functionality of these genes, two new mutants lacking only one of the two genes, S. coelicolor Δscr1 and S. coelicolor Δscr2, were generated (see section “Materials
These two new single mutant strains, the double Δscr1/scr2 mutant and the wild-type were transformed with four plasmids, pNX4441, pNX4442, pNX4441/42, and pN702Gem3, and the production in liquid cultures was analyzed. The increase of the production of colored antibiotics was observed in all of the liquid media assayed [R2YE, NMMP + Xyl (data not shown) and YES + Xyl] when pNX4441/42 was used. Since the highest production was obtained in YES + Xyl, this medium was used to carry out the rest of the experiments.

Once again, a high level of ACT was induced by Scr1 (pNX4441) in the wild-type strain. This induction was also observed in the S. coelicolor Δscr1 strain, but not in the two strains lacking the SCO4442 gene (Δscr2 and Δscr1/scr2) (Figure 3A). Overproduction of the colored antibiotics was also observed in strains Δscr2 and Δscr1/scr2, but only when the plasmid pNX4441/42 was used (Figures 3A,B). Antibiotic production in these cultures was quantitated, and the yield of ACT was between 450 and 550 µM after expressing Scr1 alone or together with Scr2 in the wild-type M145 strain. The production of prodigines (RED) was also induced under the same conditions reaching concentrations of 35–45 µM in this strain (Figure 3C). The quantification of these antibiotics in the Δscr2 and Δscr1/scr2 transformant strains reinforced the hypothesis that Scr1 acts as a positive regulator of antibiotic production and requires the presence of the protein encoded by scr2. Therefore, these two genes must act in conjunction (Figures 3B,C).

Additionally, the analysis of the S. coelicolor M145 cultures overexpressing Scr1 and Scr2 by LC-HRMS was performed for identifying new putative compounds, originating from silent pathways that may be promoted by Scr1/Scr2. Several compounds were produced de novo by the action of Scr1/2 and were putatively identified as SEK 4, SEK 4b, ε-actinorhodin and γ-actinorhodin, and one putative new compound with the molecular formula C32H28O14 and an UV absorbance similar to actinorhodin (Figure 4). SEK 4 and SEK 4b have been previously described as shunt polyketide intermediates, non-enzymatically cyclized products from the biosynthesis of actinorhodin (Fu et al., 1994; Jetter et al., 2013).

The ability of Scr1, Scr2, and Scr1/Scr2 from S. coelicolor to induce antibiotics was also tested in S. lividans 1326 in the same way as above. The plasmids pN702Gem3, pNX4441, pNX4442, and pNX4441/42 were introduced in this strain, cultured in liquid YES + Xyl and compared. As in S. coelicolor, production of ACT (59 µM) and RED (430 µM) was detected when this strain was transformed with the plasmids pNX4441 and pNX4441/42, but not observed when transformed with pNX4442. Interestingly the production of RED was almost ten times higher than the production reached for this antibiotic in S. coelicolor (Figures 5A,B). LC-HRMS analysis of the cultures of S. lividans 1326 overexpressing scr1 and scr2 detected the production of SEK 4, SEK 4b, fogacin, a putative new compound with the molecular formula C25H33N3O with six isomeric prodigiosins found in the Dictionary of Natural Products (DNP) (Figure 6). Fogacin has been previously described as a cyclic octaketide obtained from a Streptomyces sp. (strain Tü 6319) isolated from a contaminated soil in Romania (Radzom et al., 2006).
Scr1/Scr2, a New Genetic Tool to Activate Secondary Metabolite Production in *Streptomyces* Species

A potential strategy for improving or inducing cryptic metabolites of different species of *Streptomyces* producers is the cloning of a positive regulator of antibiotic production. Based on the results obtained from the overexpression of Scr1/2 in *S. coelicolor* and *S. lividans*, these genes appeared to be good candidates for obtaining new natural products using this type of strategy. Therefore, we generated a plasmid that could be used to transfer these regulators into other *Streptomyces* species. To do so, a conjugative plasmid derived from pHJL401 was generated (pHJL401c) and used to introduce *scr1* and *scr2* in the final plasmid pHAX4441/42c. These two new plasmids were transferred from *E. coli* to nine *Streptomyces* species known to produce different antibiotics: *S. albus*, *S. argillaceus*, *S. coelicolor*, *S. albicyaneus*, *S. aurantiacus*, *S. avermitilis*, *S. averantinicus*, *S. chartreusis*, and *S. griseus*. The plasmid pHAX4441/42c was introduced into these species to study the effects of the overexpression of Scr1/2 on antibiotic production. The results obtained showed that the overexpression of Scr1/2 in these species led to the production of new metabolites, which were identified using UV-Vis absorbance and base peak chromatography. The amplified region of the four most different peaks produced and their predicted compounds are shown in Figure 6. The four most different peaks are: (1) SEK 4/SEK 4b; (2) Fogacin; (3) C$_{32}$H$_{28}$O$_{14}$; (4) Prodigiosin 25b.
**S. glaucescens**, **S. griseus**, **S. parvulus**, **S. peucetius**, **S. rochei**, **S. steffisburgensis** and **S. vinaceus**. The conjugants obtained were grown in liquid YES+Xyl containing 10 µg mL⁻¹ of apramycin for 8 days and the metabolites produced and extracted with acidified ethyl acetate were analyzed using LC-HRMS to look for the increased production of known or new compounds. This strategy was successful in **S. peucetius** and **S. steffisburgensis**. In **S. peucetius**, two putative new compounds were produced (peaks B and C) and an additional one (peak A) was greatly induced by Scr1/Scr2 (Figure 7). The analysis of the differentially detected peaks did not correspond with those of known compounds. This may have occurred in two of the cases (peaks A and B) due to the lack of ionization in both positive and negative ESI. In the case of peak C, the only component found in the DNP with the predicted molecular formula C₁₈H₁₄N₂O₄ (Supplementary Figure S1) corresponded to Flazine methyl ester, which had an UV spectrum that did not coincide with the spectrum determined experimentally (Buckingham, 2017). In **S. steffisburgensis**, an induced compound, N-[1-Hydroxy-2-(1H-indol-3-yl)-2-oxoethyl]acetamide, was highly produced and putatively identified (Figure 8). No changes were observed in the peaks obtained in the other studied strains (Supplementary Figures S2–S5).

Moreover, eight additional strains of **Streptomyces** sp., with no clear antibiotic activity against the microorganisms tested (**S. aureus**, **E. coli**, and **C. albicans**) when grown in seven different types of media, were also used in the experiment. Conjugants were only obtained for two of the strains: **Streptomyces** sp. CA-240608 and **Streptomyces** sp. CA-258987. LC-HRMS-analysis of liquid culture extracts of **Streptomyces** sp. CA-240608 detected the overproduction of one component with the molecular formula of C₂₅H₁₆N₄O₆ (Supplementary Figure S1) that could correspond to any of the isomers Izumiphenazine A, Izumiphenazine B, Phenazinoline D or Phenazinoline E (Figure 9). These were the only compounds described in the DNP with this predicted molecular formula. Interestingly, the antibiogram activity of the clones of this strain overexpressing Scr1/Scr2 exhibited slight antibiotic activity against **E. coli**. By contrast, the control strain did not show any antibiotic activity (Figure 9). No changes in the production pattern of the strain **Streptomyces** sp. CA-258987 were detected (Supplementary Figure S5).

Therefore, these results validate the use of Scr1/Scr2 as a biotechnological tool for increasing or promoting different metabolite pathways in some **Streptomyces** species. The overexpression of Scr1/Scr2 strongly activates antibiotic production in **S. coelicolor** and **S. lividans**, and may be useful...
as a means for searching for new natural products in some Streptomyces species.

DISCUSSION

In this work, the positive role of the Scr1/Scr2 proteins in the regulation of antibiotic production has been established. This role has not been described previously and their function as a putative toxin–antitoxin system has only been inferred by bioinformatic comparison (Shao et al., 2011; Xie et al., 2018). One orthologous gene of scr1, with 99% identity, was previously cloned from S. lividans and denominated cpb1. The protein encoded for this gene was identified as a DNA binding protein that specifically binds to the promoter of the chitinase encoding gene chiA. Deletion of the gene cpb1 partially relieves the glucose repression of chitinase production and chitinases are produced up to wild-type levels when grown in a medium containing colloidal chitin without glucose (Fujii et al., 2005). A qualitative experiment has been conducted, comparing the chitinase halo of the S. coelicolor wild-type strain and the Δscr1 in the presence of 0.15% colloidal chitin with or without glucose. No clear differences were observed for both strains (Supplementary Figure S6A).

Based on sequence homology, Aínsa et al. (2010) classified the gene scr1 (SCO4441) as one of the 26 whiJ (SCO4543) paralogs on the S. coelicolor chromosome. These whiJ genes vary considerably with respect to their conservation in other organisms, being widespread in streptomycetes but generally absent from any other

FIGURE 8 | Metabolites induced by Scr1/2 in S. steffisburgensis. (A) Metabolite production induced by overexpression of Scr1/Scr2 (pHAX4441/42c) in S. steffisburgensis in YES+Xyl for 8 days and detected by UV-Vis absorbance (200–900 nm) base peak chromatography. (B) Amplified region of the peak produced and their predicted compound: N-[1-Hydroxy-2-(1H-indol-3-yl)-2-oxoethyl]acetamide.
bacterial genomes. The *scr1* gene is present in all *Streptomycineae* and also in other complex actinomycetes such as *Kitasatospora, Microtetraspora, Streptosporangium* (Chandra and Chater, 2014). In *S. coelicolor*, most of these *whiJ* paralogs (22 out of 26 members) are neighbors of genes that encode either a very small binding protein with a DUF397 domain, like in the case of Scr2 (*SCO4442*), or by an antisigma factor or by both (Chandra and Chater, 2014). A model in which WhiJ binds to some operator sequences repressing developmental genes has been described. Interestingly, this repression might be released by its interaction with the small WhiJ-associated protein, SCO4542 (scr2 paralog), whose activity is prevented by an unknown signal (Aínsa et al., 2010). These authors demonstrated that the total deletion of *whiJ* (scr1 paralog) does not lead to a clear effect. However, the deletion of *SCO4542* causes colonies to appear bald (*bld*) in MSA medium and results in the *whi* phenotype in R2YE. Both of these phenotypes were suppressed by the simultaneous deletion of *whiJ*. The authors also proposed that the deletion of *SCO4542* would result in the constitutive binding of WhiJ to its target(s), repressing development and leading to a *bld* colony phenotype. In other words, *SCO4542* prevents WhiJ from binding to target DNA and represses development (Aínsa et al., 2010).

Another protein containing a DUF397 conserved domain, which is critical for the correct progression of the developmental program and antibiotic production, is BldB (*SCO5723*) (Eccleston et al., 2006; Akpe San Román et al., 2010). It has been suggested that a partner, included in the Hpb (for helix-turn-helix partner of BldB) family, modulates its activity (Eccleston et al., 2006; Doroghazi and Buckley, 2014). Hpb is predicted to have an XRE-class helix-turn-helix domain, and diversification of the *bldb/hpb* family gene pairs suggests that a mutation in one gene encourages a compensatory change in its partner at the same locus. Based on different aspects
of the relationship between this gene pair, some authors have proposed that the proteins containing the XRE domain function as antitoxins whereas DUF397 family proteins are novel toxins (Makarova et al., 2009; Doroghazi and Buckley, 2014).

As stated previously, *S. coelicolor* has 15 XRE-DUF397 pairs that have been predicted to act as toxin–antitoxin systems (Shao et al., 2011; Xie et al., 2018). Until now, this group of genes has not been studied experimentally. In this work, we chose to begin by studying the gene pair comprised of *scr1/scr2* in an attempt to describe the mode of action of a new group of TAS. However, under the conditions used in this work, this system did not behave as a typical TAS on which overexpression of the putative toxin (*Scr2* in this case), in the absence of adequate levels of the putative antitoxin, (*Scr1* in our study) produces a lethal effect. In our experiments, the number of colonies obtained in the transformation with the plasmids containing the antitoxin, the toxin or both genes was similar suggesting the no toxic effect of the toxin.

The mutation of these genes did not have a drastic phenotype when grown on several of the media assayed (Supplementary Figure S6B). Only the Δ*scr1* strain showed an accelerated differentiation when grown in NA solid media. No other clear differences were observed for the different strains in R2YE, RS5, or in MSA, as observed by Aínsa et al. (2010) with WhiJ/SCO4542 pair.

According to our results it is possible that Scr1 and Scr2 interact, although evidence of this interaction could not be obtained using purified proteins due to the insolubility of Scr2. Nevertheless, an indirect relationship was observed in *S. coelicolor* using strains Δ*scr2* and Δ*scr1/scr2* overexpressing Scr1. Induction of colored antibiotics was only observed when overexpression of Scr1 and Scr2 was carried out in these strains, but not with Scr1 on its own.

It is worth noticing that several additional products of ACT and RED were induced in *S. coelicolor* by the overexpression of the regulator Scr1 (Figure 4). For example, production of the compounds Sek4 and Sek4b were detected in this strain. These two products were also detected in *S. lividans*. In this species, fogaclin was also overproduced due to the overexpression of Scr1 and Scr2. This compound differs from all known intermediates of actinorhodin described and is a product of stereospecific cyclization and miscellaneous tailoring steps (Radzom et al., 2006). To our knowledge, the production of these three compounds has not been previously reported in *S. lividans*. In addition, the induction of other compounds was observed under the culture conditions used. This was the case for *S. steffisburgensis* in which the production of the putative N-[1-Hydroxy-2-(1H-indol-3-yl)-2-oxoethyl] acetamide was detected. This compound was previously described in *Streptomyces ramulosus* Tü 34 (Chen et al., 1983). Here, the overexpression of Scr1/Scr2 in a strain that did not present any clear antibiotic activity against Gram positive, Gram negative bacteria or yeasts, under different culture conditions, was also analyzed. The overexpression of Scr1/Scr2 in this strain permitted us to detect a weak antibiotic activity against *E. coli* that was absent in the control strain. Under these conditions, this strain, *Streptomyces* sp. CA-240608, produces izumiphenazines A/B or Phenazinoline (Figure 9). The production of izumiphenazines A/B was previously reported from *Streptomyces* sp. IFM 11204, a strain isolated from a soil sample collected from Izumi forest in Chiba city, Japan. These compounds exert moderate activity over TRAIL-resistant AGS cells, a cell line typically used for evaluating cancer cell apoptosis (Abdelfattah et al., 2010). The compound Phenazinoline was previously reported from *S. diastaticus* YIM DT26, isolated from a soil sample collected in Yunnan, China (Ding et al., 2011), and from the strain of *S. fradiae* A196 that was isolated from hailstone in Gijón, Spain (Sarmiento-Vizcaino et al., 2018). These results suggest that this strategy may be useful for some *Streptomyces* strains for increasing the level of production of some compounds. However, the regulators analyzed do not present a general activating action in all of the strains studied, as was initially expected.

**AUTHOR CONTRIBUTIONS**

RS and LS conducted most of the experiments. JM performed the LC-HRMS-analysis. LS, JM, IG, OG, MD, and RS analyzed the results. MD, LS, and RS conceived the experiments and wrote the manuscript. All authors have read and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplemental Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02791/full#supplementary-material
REFERENCES


**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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<table>
<thead>
<tr>
<th>Strain</th>
<th>Taxonomy</th>
<th>Country</th>
<th>Sample type</th>
<th>Ecology</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA-240608</td>
<td>Streptomyces sp.</td>
<td>Central African Republic</td>
<td>Soil</td>
<td>Tropical humid forest</td>
</tr>
<tr>
<td>CA-243829</td>
<td>Streptomyces sp.</td>
<td>Central African Republic</td>
<td>Soil</td>
<td>Waterlogged forest</td>
</tr>
<tr>
<td>CA-135535</td>
<td>Streptomyces sp.</td>
<td>South Africa</td>
<td>Soil</td>
<td>Soil from Olive (Olea africana)</td>
</tr>
<tr>
<td>CA-248979</td>
<td>Streptomyces sp.</td>
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<td>Rhizosphere soil</td>
<td>Rhizosphere soil of Neotorullaria eldarica</td>
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<tr>
<td>CA-256884</td>
<td>Streptomyces sp.</td>
<td>Georgia</td>
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<td>Streptomyces sp.</td>
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<tr>
<td>CA-243318</td>
<td>Streptomyces sp.</td>
<td>New Zealand</td>
<td>Soil</td>
<td>Canopy Beilschmiedia tawa, Prumnopitys taxifolia, Melicytus sp.</td>
</tr>
</tbody>
</table>
## Table S2: oligonucleotides used

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’-3’)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS-048</td>
<td>tttttcatATGAGTCACGCAGCCGAGGTG</td>
<td>Forward oligonucleotide for amplifying ( SCO4441 (scr1) ). The sequence recognized by NdeI is underlined.</td>
</tr>
<tr>
<td>LS-049</td>
<td>ttttctegacCGTGCGTACTCCTTCGCCAC</td>
<td>Reverse oligonucleotide for amplifying ( scr1 ). The sequence recognized by XhoI is underlined.</td>
</tr>
<tr>
<td>LS-050</td>
<td>tttttcatATGGCAATTCTTCAGGGCGCCC</td>
<td>Forward oligonucleotide for amplifying ( SCO4442 (scr2) ). The sequence recognized by NdeI is underlined.</td>
</tr>
<tr>
<td>LS-051</td>
<td>ttttctegacCTCCTTGACCAGGTCACGAAG</td>
<td>Reverse oligonucleotide for amplifying ( scr2 ). The sequence recognized by XhoI is underlined.</td>
</tr>
<tr>
<td>LS-090</td>
<td>CACCTTGGTGCCAGTCTCTACAGCACGCA GATACACAAAGTGGAAGTA(_{\text{TGATTCCG}}) GGGATCCGTCGACC</td>
<td>Forward oligonucleotide to obtain the ( scr1 ) mutation. The initiation codon is highlighted.</td>
</tr>
<tr>
<td>LS-091</td>
<td>GGCGAGTCTTCCAGTACATCGACGCAGG GGTAAGATGCCGACCGCTCATGTAGG CTGGAGCTGGCTTC</td>
<td>Reverse oligonucleotide to obtain the mutant ( scr1 ). The termination codon is highlighted.</td>
</tr>
<tr>
<td>LS-092</td>
<td>GCGTGTCGACCAGGCGCATATCG</td>
<td>External forward oligonucleotide to check ( scr1 ) mutation.</td>
</tr>
<tr>
<td>LS-093</td>
<td>CGTACGGAGCAGCCATTCACTC</td>
<td>External forward oligonucleotide to obtain ( scr1 ) mutation.</td>
</tr>
<tr>
<td>LS 109</td>
<td>AACGTCATTGGCGTAAACACACTCGCG ACTCAGGCTACGAGCAGC(_{\text{TGATTCCG}}) GGGATCCGTCGACC</td>
<td>Forward oligonucleotide to obtain the mutant ( scr2 ). The initiation codon is highlighted.</td>
</tr>
</tbody>
</table>
Reverse oligonucleotide to obtain the mutant \textit{scr2}. The termination codon is highlighted.

External forward oligonucleotide to check \textit{SCO4442}.

External reverse oligonucleotide to check \textit{SCO4442} mutation.
Figure S1: Mass spectrometry of peak C from *S. peucetius* (C_{18}H_{14}N_{2}O_{4}+H^{+}=323.10263). Experimental value m/z=323.1021. Error= -1.6ppm) and of the compound with formula C_{25}H_{16}N_{4}O_{6} from *Streptomyces* sp. CA-240608 (C_{25}H_{16}N_{4}O_{6}+H^{+}= 469.114261. Experimental value m/z=469.1140. Error= 0.6ppm).
Figure S2: Chromatograms of S. albus and S. argillaceus grown in liquid YES+Xyl for 8 days.
Figure S3: Chromatograms of *S. glaucescens* and *S. griseus* grown in liquid YES+Xyl for 8 days.
Figure S4: Chromatograms of *S. parvulus* and *S. rochei* grown in liquid YES+Xyl for 8 days.
Figure S5: Chromatograms of *S. vinaceus* and *Streptomyces sp.* CA258987 grown in liquid YES+Xyl for 8 days.
Figure S6: A) Chitinase production in *S. coelicolor* and in *S. coelicolor* Δscr1 in ISP4+0.15 % colloidal chitin without or with 1 % glucose. B) Antibiotic production of *S. coelicolor* strains in the indicated solid media after 7 days of culture.