The SCO4117 ECF Sigma Factor Pleiotropically Controls Secondary Metabolism and Morphogenesis in Streptomyces coelicolor

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Extracytoplasmic function (ECF) sigma factors are a major type of bacterial signal-transducers whose biological functions remain poorly characterized in streptomycetes. In this work we studied SCO4117, a conserved ECF sigma factor from the ECF52 family overexpressed during substrate and aerial mycelium stages. The ECF52 sigma factors harbor, in addition to the ECF sigma factor domain, a zinc finger domain, a transmembrane region, a proline-rich C-terminal extension, and a carbohydrate-binding domain. This class of ECF sigma factors is exclusive to Actinobacteria. We demonstrate that SCO4117 is an activator of secondary metabolism, aerial mycelium differentiation, and sporulation, in all the culture media (sucrose-free R5A, GYM, MM, and SFM) analyzed. Aerial mycelium formation and sporulation are delayed in a SCO4117 knockout strain. Actinorhodin production is delayed and calcium-dependent antibiotic production is diminished, in the ΔSCO4117 mutant. By contrast, undecylprodigiosin production do not show significant variations. The expression of genes encoding secondary metabolism pathways (deoxysugar synthases, actinorhodin biosynthetic genes) and genes involved in differentiation (rdl, chp, nepA, ssgB) was dramatically reduced (up to 300-fold) in the SCO4117 knockout. A putative motif bound, with the consensus "CŠGYN-17bps-SRHA" sequence, was identified in the promoter region of 29 genes showing affected transcription in the SCO4117 mutant, including one of the SCO4117 promoters. SCO4117 is a conserved gene with complex regulation at the transcriptional and post-translational levels and the first member of the ECF52 family characterized.

Keywords: Streptomyces, ECF, sigma factor, differentiation, secondary metabolism, antibiotic

INTRODUCTION

Extracytoplasmic function (ECF) sigma factors, together with one- and two-component systems, are a major type of bacterial signal-transducing proteins (Huang et al., 2015). The ECF sigma factors belong to the σ70 family, but harbor only two of the four conserved regions of this group (σ2 and σ4 regions), which is sufficient for promoter recognition and RNA
polymerase recruitment (reviewed in Mascher, 2013). The ECF sigma factors activate genes that confer resistance to agents that threaten the integrity of the envelope or cellular homeostasis (Kormanec et al., 2016) and are tightly regulated by diverse and complex mechanisms (Mascher, 2013). Most ECF sigma factors are negatively regulated by anti-sigma factors (ASF), usually co-expressed with its target ECF sigma factor. The correct stimulus leads to the inactivation of the ASF and allows the ECF sigma factors to bind to their target promoters and RNA polymerase (Mascher, 2013). However, several ECF sigma factors lack a known ASF and are therefore differently regulated (Staron et al., 2009; Huang et al., 2015). Members of the ECF41 and ECF42 families possess C-terminal extensions that regulate their activities, thereby acting like ASFs (Gómez-Santos et al., 2011; Wecke et al., 2012), while the activity of other ECF sigma factors was proposed to be regulated by ASF-independent transcriptional regulators and post-translational modifications (Ser/Thr/Tyr phosphorylation) (Mascher, 2013).

*Streptomyces* is a genus of Gram-positive soil bacteria of great importance for biotechnology given their ability to produce a large array of bioactive compounds, including antibiotics, anticancer agents, immunosuppressants, and industrial enzymes (Hopwood, 2007). *Streptomyces* has a complex morphogenesis that includes hyphal differentiation and sporulation. In high density laboratory cultures, after spore germination, a fully compartmentalized mycelium (MI) initiates the development until it undergoes an ordered process of programmed cell death (PCD) and develops into a second multinucleated mycelium (substrate mycelium, early MI). This mycelium further develops into aerial hyphae and makes the hydrophobic proteins necessary for growth into the air (reviewed in Yagüe et al., 2013a). Secondary metabolism and differentiation are largely controlled by specific sigma factors that enable the recognition of specific promoters, directing the expression of specific genes (reviewed in Kormanec et al., 2016). *Streptomyces coelicolor*, the best-characterized *Streptomyces* strain, harbors 65 σ factors, including principal σ factors (σA, σC, and σE), general stress response σ factors, and ECF sigma factors (Kormanec et al., 2016). The S. coelicolor genome encodes for 51 ECF sigma factors, of which only four have been characterized so far: SigE, required for normal cell wall structure (Hutchings et al., 2006); SigR, a global regulator of redox homeostasis (Feeney et al., 2017); SigT, regulating actinorhodin production in response to nitrogen stress (Feng et al., 2011); and σBldN, an ECF sigma factor required for aerial mycelium formation (Bibb et al., 2012).

The ECF52 family of sigma factors is characterized by long C-terminal extensions that contain a zinc finger domain, a variable number of transmembrane helices and a long proline-rich C-terminal extension, which includes a carbohydrate-binding domain (Huang et al., 2015; Figure 1). Members of this family are only present in Actinobacteria (Huang et al., 2015). In this work, we studied SCO4117, a conserved ECF52 sigma factor that was detected as overexpressed during the substrate and aerial mycelia stages of *Streptomyces* development (Yagüe et al., 2013b), suggesting a role in the regulation of secondary metabolism and differentiation. To our knowledge, SCO4117 is the first member of the ECF52 sigma factors that has been characterized.

### MATERIALS AND METHODS

#### Bacterial Strains and Culture Conditions

All *Streptomyces* and *Escherichia coli* strains used in this work are listed in Table 1. Spores were harvested from SFM solid plates (Kieser, 2000) after growth at 30°C for 7 days. The differentiation analyses were carried out on GYM plates covered with cellophane and on minimal medium (MM) plates supplemented with glucose (10 g/l), both inoculated with 10^7 spores from a fresh water suspension and cultured at 30°C. The samples for quantification of actinorhodin and undecylprodigiosin production were obtained from 100-ml sucrose-free R5A (Fernández et al., 1998) cultures grown at 30°C and 200 rpm in 500-ml flasks. Calcium-dependent antibiotic (CDA) production was measured on nutritive agar from Oxoid. *E. coli* strains were cultured in LB and 2xTY media at 37°C. The following antibiotics were added to select plasmid-bearing and mutant strains: ampicillin (100 µg/ml), apramycin (100 µg/ml for *E. coli*, 25 µg/ml for *S. coelicolor*), chloramphenicol (25 µg/ml), hygromycin (100 µg/ml for *E. coli*, 200 µg/ml for *S. coelicolor*), kanamycin (50 µg/ml), and nalidixic acid (25 µg/ml).

#### Nucleic Acid Manipulations

Genomic DNA isolation and conjugation were performed following standard methods (Kieser, 2000; Sambrook and Russell, 2001).

Total RNA samples were isolated as previously described (Rioseras et al., 2016) using RNeasy Mini spin columns and treated with DNase I (Qiagen). The quantity and integrity of the RNA samples were measured with Nanodrop 2000 (Thermo Scientific) and 2100 Bioanalyzer (Agilent).

#### SCO4117 Mutagenesis

The SCO4117 ORF was replaced with the apramycin resistance cassette. The fragments upstream (UP-1944pb) and downstream (DOWN-2234pb) of SCO4117 were amplified by PCR from *S. coelicolor* DNA using the primers SCI59-SCO160 and SCO163-SCO164, respectively. The PCR products were cloned and sequenced in pCR™-Blunt II-TOPO® obtaining pTOPO-UP and pTOPO-DW. The DW fragment was liberated from pTOPO-DW by EcoRV/PstI digestion and subcloned in pTOPO-DW digested with the same restriction enzymes. The resulting plasmid, pTOPO-UPDW, was linearized with PstI and ligated with a PstI-fragment from pIJ773 containing an apramycin resistance cassette with an oriT, obtaining pΔSCO4117. This vector was introduced in the wild-type strain by conjugation and double recombinants were selected as kanamycin-sensitive and apramycin-resistant. Southern hybridization and PCR amplification with SCI59 and 4117R (Table 1) primers were performed to check the ΔSCO4117 mutant.

**Abbreviations:** ECF, extracytoplasmic function; ASF, anti-sigma factor; CDA, calcium dependent antibiotic; PCD, programmed cell death; ORF, open reading frame; NGS, next generation sequencing; RT-PCR, reverse transcription PCR.
To discard polar effects in the expression of the SCO4117 neighbor genes due to the apramycin gene insertion, we recreated the SCO4117 knockout by CRISPR-Cas9. We used the system designed by Tong et al. (2015). The 20-nt target sequence was selected inside the SCO4117 and amplified by PCR with the primers SCO215 and sgRNA-R. The product of 110 bps was digested with NcoI/SnaBI and cloned in NcoI/SnaBI-digested pCRISPR-Cas9, obtaining pCRISPR-SgSCO4117. The SCO4117-surrounding regions were amplified by PCR with SCO216/SCO217 and SCO220/SCO221 primers. The couple of DNA fragments were combined by overlap extension PCR (Lee et al., 2010) with the primers SCO216/SCO217 and SCO220/SCO221. The PCR product was cloned and sequenced in pCR™-Blunt II-TOPO®. The insert was released with EcoRV and cloned into pCRISPR-SgSCO4117 digested with StuI. The final vector pCRISPR-SCO4117A was introduced into the S. coelicolor chromosome using the primers SCO134 and 4117R. The 2171 bp-fragment was cloned and sequenced in pCR™-Blunt II-TOPO® and cloned in pMS82 digested with SpeI and filled with the Klenow enzyme and amplified by PCR with the primers SCO220/SCO221 and SCO222/SCO219 primers were used (primer SCO222 contain the SCO4117 stop codon in frame with the first 305 amino acids) to amplify the SCO4117 sigma factor-surrounding regions. DNA fragments were combined by overlap extension PCR with the primers SCO220/SCO221. The amplicon was cloned and sequenced in pCR™-Blunt II-TOPO®. The insert was released with EcoRV and cloned into pCRISPR-SgSCO4117 digested with StuI, obtaining vector pCRISPR-SgSCO4117B. Conjugation, mutagenesis, and mutant confirmation was performed as described above (Table 1).

**S. coelicolor ΔSCO4117 Complementation**

The complementation of S. coelicolor ΔSCO4117 was performed via the integration of plasmid pMS82 (Gregory et al., 2003), harboring the SCO4117 ORF and an upstream region large enough to include the two promoter regions identified by Jeong et al. (2016). The SCO4117 was amplified from the S. coelicolor chromosome using the primers SCO134 and 4117R. The 2171 bp-fragment was cloned in pCR™-Blunt II-TOPO® and sequenced to check the absence of mutations. A SpeI fragment obtained from TOPO-4117 was ligated into pMS82 digested with SpeI resulting in pMS82-SCO4117. This plasmid was transferred by conjugation to the S. coelicolor ΔSCO4117 mutant. The conjugants harboring the plasmid were selected by hygromycin resistance and the correct plasmid insertion at the SCO4848 attB site was verified by PCR using the primers SCO4848F and pMS82R (Table 1).

Restoration of the wild-type genotype of the ΔSCO4117 mutant was achieved using the CRISPR-Cas9 system for Streptomyces designed by Tong et al. (2015). The 20-nt target sequence was selected inside the apramycin resistance cassette, replacing SCO4117 and amplified by PCR with the primers SCO197 and sgRNA-R. The 120-bps product was digested with NcoI/SnaBI and cloned in NcoI/SnaBI-digested pCRISPR-Cas9 obtaining pCRISPR-120. A single fragment of 3.7 kb, containing the complete SCO4117 sequence and the surrounding regions, was amplified by PCR from S. coelicolor chromosome using SCO4117-FA and SCO4117-R primers. The product was then cloned and sequenced in pCR™-Blunt II-TOPO®. The resulting plasmid was digested with EcoRV/SpeI to liberate a 3.7 kb fragment. The SpeI-end was filled with the Klenow enzyme and the product was cloned into pCRISPR-120 linearized with StuI. The final vector pCRISPR-4117, was introduced in S. coelicolor ΔSCO4117 by conjugation. Conjugant selection and plasmid clearance were performed as described above. Restoration of the wild-type genotype was checked by the loss of apramycin resistance (Table 1).

**Viability Staining**

Samples were obtained from GYM plates covered with cellobiophane at different developmental stages. The bacteria were stained with SYTO 9 and propidium iodide (LIVE/DEAD BacLight Bacterial Viability Kit, Invitrogen, L-13152) and observed under a Leica TCS-SP8 confocal laser-scanning microscope at wavelengths of 488 and 568 nm excitation and at 530 (green) or 640 nm (red) emissions.

**Antibiotic Production and Protein Quantification**

Undecylprodigiosin and actinorhodin were quantified spectrophotometrically according to Tsao et al. (1985) and Bystrek et al. (1996). For actinorhodin quantification, KOH was added to the culture samples at a final concentration of 1N. Cellular pellets were discarded by centrifugation...
TABLE 1 | Bacterial strains, plasmids, cosmids, and primers used in this study.

| Strain, plasmid, cosmid | Description | Reference |
|-------------------------|-------------|-----------|
| S. coelicolor M145 SCP1<sup>−</sup> SCP2<sup>−</sup>, reference strain | | Kieser, 2000 |
| S. coelicolor ΔSCO4117 SCO4117 replaced with acc(3)IV, Apr<sup>R</sup> | | This study |
| E. coli TOP10 F<sup>−</sup> mcr<sup>A</sup> Δmrr-hsdRMS-mcrBQ α3&lambdach21 Δ(aac-6Ⅰ)7697 galU galK rpsL endA1 nupG | | Invitrogen |
| E. coli ET12567 dam-13::Tn9, dcm-6, hsdM, hsdR | | MacNeil et al., 1992 |
| E. coli ET12567/pUZ8002 | E. coli ET12567 harboring pUZ8002, a non self-transmissible plasmid which can mobilize oriT-containing plasmids by conjugation | Flett et al., 1997 |
| Bacillus subtilis Indicator microorganism for CDA bioassay | | |
| PLASMIDS AND COSMIDS | | |
| pMS82 | Integrative and conjugative vector, Hyg<sup>R</sup> | Gregory et al., 2003 |
| PCR<sup>TM</sup>-Blunt II-TOPO® | Zero Blunt<sup>®</sup> TOPO<sup>®</sup> PCR Cloning Kit, Kan<sup>R</sup> | Invitrogen |
| pU773 | Apr cassette in pU699 | Gust et al., 2003 |
| pΔSCO4117 | SCO4117 deletion construction, a Streptomyces non-replicative plasmid transmissible by conjugation, Apr<sup>R</sup> Kan<sup>R</sup> | This study |
| pMS82-SCO4117 | Integrative and conjugative plasmid derived from pMS82 with the completed SCO4117 gene under its own promoter control. Hyg<sup>R</sup> | This study |
| pCRISPR-Cas9 | Conjugative and thermosensitive plasmid harboring Cas9 | Tong et al., 2015 |
| pCRISPR-120 | pCRISPR-Cas9 harboring the apramycin resistance target sequence | This study |
| pCRISPR-SGSCO4117 | pCRISPR-Cas9 harboring the target SCO4117 sequence | This study |
| pCRISPR-SgSCO4117 | pCRISPR-Sg9CO4117 harboring a 2.5 kb fragment used to create the SCO4117 knockout | This study |
| pCRISPR-SCO4117 | pCRISPR-Sg9CO4117 harboring a 2.2 kb fragment used to create the truncated SCO4117 gene harboring the sigma factor domain | This study |
| PRIMERS | | |
| SCO134 | GACGTGCTGCTGGTCATAGC | This study |
| SCO4117R | GGAGCTAGTGTAGCGCCCGCAAGTTG | This study |
| SCO159 | GGAGCCAGATCCTCAGTGG | This study |
| SCO160 | TCAACGCTCATCGCCGAAGAG | This study |
| SCO163 | CTGACGCGCAAGTCCGCTTCACA | This study |
| SCO164 | QATATCGACTCTCGTCCTCGACAAGCT | This study |
| SCO4845F | CGTGCTATCCCTCGGTGG | This study |
| pMS82R | GACGCCGGAAGCTCATTCA | Gonzalez-Quiñonez et al., 2016 |
| SCO197 | CATGCCATGTTCCGCGTAAAGCGCTGGAATAGC | Gonzalez-Quiñonez et al., 2016 |
| sgRNA-R | ACGCCTACGTTAAAAAAACGCCCGACTGCCGCTGCAAG | This study |
| SCO4117-FA | GGAGGATACGACCGGCGTACGCTCGGCC | This study |
| RT-SCO4117-F | CGACGACCGATCCGCTCGCA | This study |
| RT-SCO4117-R | CGACCGACGCGCTCAAGAT | This study |
| hrdB-F | CGGCGCATGCTGCTTTCT | Kurt et al., 2013 |
| hrdB-R | AGGTTGGGCGTACGTGGAGAAC | Kurt et al., 2013 |
| bldN-F | CTCAACCAGCAGCGACCTTGC | This study |
| bldN-R | TCGTTGCGTGCGATCATCT | This study |
| SCO1178-F | TCGTCTCTCTGTGCTTTGCA | This study |
| SCO1178-R | GGCATCCGCTGCTAGCGAT | This study |
| chpA-F | CTGGTGTGCTGCTCGAATCT | This study |
| chpA-R | GTGCTGCTGCTCCGCTCGG | This study |
| chpH-F | CACCGGGTGGTGCTGCTCCTCT | This study |
| chpH-R | ATCGGCTGCGTGAGGGAT | This study |
| SCO2748-F | GAGATCACCCCGAAACTGG | This study |
| SCO2748-R | AAGTGCCAGTCGATGACGTT | This study |
| actVA2-F | ACTACGCCTCCCAGAAACTG | This study |
| actVA2-R | TTGTGCCGCCGATGTC | This study |

(Continued)
CDA production was determined via a bioassay against Bacillus subtilis. Oxid nutritive agar (ONA) plates (90 mm in diameter) were inoculated with 5 µl of a Streptomyces spore suspension at 1 × 10^5 spores/ml and incubated at 30°C. After 2 days, the plates were overlayered with 5 ml of soft ONA (0.75% agar), inoculated with B. subtilis (OD = 0.25) and supplemented with Ca(NO_3)_2 (60 mM). Negative controls were performed in parallel without adding calcium. Inhibitory halos were measured after 15 h at 30°C.

Growth was determined by measuring the protein concentration with the Bradford assay (Biorad) and a bovine serum albumin standard (Sigma). Total protein extracts were obtained mixing a volume of culture with a volume of 1 M NaOH, boiling for 5 min, and removing cell debris by centrifugation at 7740 g.

**RNA-Seq and Bioinformatic Analysis**

Next-generation sequencing (NGS) was performed by Stab Vida (Caparica, Portugal) from two biological replicates using the ΔSCO4117 mutant and the S. coelicolor wild-type strain. Ribosomal RNA was depleted with the Ribo-Zero Bacteria Kit (Illumina), and the cDNA library construction was carried out using the TruSeq Stranded mRNA Library Preparation Kit (Illumina). The DNA was sequenced in the Illumina HiSeq 2500 platform using 100-bp paired-end sequencing reads (at least 20 M reads per sample). Raw data are available via the Gene Expression Omnibus database (accession GSE107661).

Bioinformatic analysis of the sequenced data was performed under the Linux operative system using the following software: FastQC to check the quality of the sequences, Cutadapt for trimming sequences, Bowtie2 for mapping with the Streptomyces coelicolor genome and Cuffdiff for differential expression test analysis (Trapnell et al., 2012). Variations in transcript abundances were considered significant if they had a p-value < 0.03 (Supplementary Table 1). Transcript abundances without significant variations (p-values higher than 0.03) are shown in Supplementary Table 2.

The transmembrane topology of the SCO4117 gene was analyzed using the Phobius software (http://phobius.sbc.us.se/). The ΔSCO4117 orthologs were obtained from the Strepdb database (http://strepdb.streptomyces.org.uk/): SLI_4349 (S. lividans), SAV_3491 (S. avermitilis), SVEN15_3779 (S. venezuelae), SGR_3904 (S. griseus), and SCLAV_3143/ SCLAV_3144 (S. clavuligerus). Amino acid similarities were estimated using the software package Lalign (http://www.ch.embnet.org/software/LALIGN_form.html).

**SCO4117 Motif Bound Search**

The 46 genes significantly down-regulated in the ΔSCO4117 mutant were grouped into 33 putative operons (Supplementary Table 1). A library of putative regulatory sequences has compiled from the 250 nucleotide-long sequences located upstream of the putative operons. BioProspector (Liu et al., 2001) was used to search for bipartite overrepresented motifs in these sequences. Searches were performed only in the forward strand, and the following parameters were varied iteratively: the lengths of the −35 and −10 motifs were varied between 5 and 7 nucleotides; the spacer length was varied between 15 and 20 nucleotides in 1-nucleotide intervals. From all generated motifs, the highest scoring bipartite motif was selected. The motif logo was created using Weblogo (Crooks et al., 2004).

**Quantitative RT-PCR (qRT-PCR)**

A High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used to synthetize cDNA from 0.5 µg of RNA from two biological replicates. Real-Time PCRs of the SCO4117

| Strain, plasmid, cosmid | Description | Reference |
|-------------------------|-------------|----------|
| redF-F                  | CCGAGAACAAGGCGAACG | This study |
| redF-R                  | CAGGGGATGGCGGAAG | This study |
| actII4-F                | GCGGCTTTTTGGAATGC | This study |
| actII4-R                | GCAGGCTGTCTGGTACG | This study |
| SCO215                  | CATGCCATGGAAGAGACTGGTTGTAAGTTTAGAGCTGGAAAAATAGC | This study |
| SCO216                  | GATATCGGGAACCAAGTCGGCACGG | This study |
| SCO217                  | CGGTTGGCGAGGTCACTGAGGAAAGCCCAGCCCGCAAC | This study |
| SCO218                  | GTTGGCGGGGGCTTTCCGCGTAGGAGCCTGCGGAACCG | This study |
| SCO219                  | GATATCGGCGAGGTCACTGAGGACAGGGACGACCG | This study |
| SCO220                  | GATACCGGTACTCTCTCCGCGCAAG | This study |
| SCO221                  | CGGTTGGCGAGGGCTCTCTCTGGTAGAGGACGCTGCGGAACCG | This study |
| SCO222                  | CAAAGAGGGGCGCTACGCATGGAAGCCTGCGGAACCG | This study |
| Mut4117F                | CCGGGGACACTGCTGCG | This study |
| Mut4117R                | AGCACGACCATGCG | This study |
gene were carried out on an ABI PRISM 7900 HT thermocycler (Applied Biosystems). The reactions were performed in triplicate, containing 2 µl of 2-fold diluted cDNA, 10 µl of SYBR Green PCR Master Mix (Applied Biosystems), and 300 nM of specific primers (RT-SCO4117-F/R) (listed in Table 1) in a final volume of 20 µl. The hdrB (SCO5820, amplified using primers hdrB-F/R) was used as reference since its expression showed no variation between strains in our RNA-seq results (Supplementary Table 2). The DNA contamination and primer dimer amplification were tested in negative controls replacing cDNA by RNA or water. Amplification conditions were as follows: 2 min at 50°C, 10 min at 90°C, 40 repetitions of 15 s at 95°C, and 1 min at 60°C. Primer efficiencies were measured using serial dilutions of genomic DNA as template and the relative quantification of gene expression was performed by the ∆∆Ct method (Livak and Schmittgen, 2001).

The expression of the most differentially expressed genes between the SCO4147 mutant and the wild strain was validated by qRT-PCR: SCO5077, actVA2; SCO2478, reductase activated by actinorhodin; and SCO1178 epimerase. We also analyzed key genes from the actinorhodin (actII-4, SCO5085) and undecylprodigiosin (redF, SCO5898) clusters; one of the bald genes (bldN, SCO3323); two of the genes participating in the aerial hyphae formation (chpA, SCO2716 / chpH, SCO1675); and SCO0761, a hypothetical protein (primers are listed in Table 1). The correlation between transcript abundances quantified by RNA-seq and qRT-PCR was adequate (regression coefficient of 0.71) (Supplementary Figure 1).

RESULTS
SCO4117 Gene Structure and Conservation in the Streptomyces Genus
Gene SCO4117, the only member of the ECF52 family present in S. coelicolor, encodes a conserved multidomain protein (average protein similarity of 68.4% among S. griseus, S. avermitilis, S. lividans, S. clavuligerus, and S. venezuelae). The SCO4117 harbors a putative ECF sigma factor domain (conserved domain database accession TRIGR02937, 86% average similarity), a putative zinc finger domain (pfam13490, 86% average similarity), a putative transmembrane domain (79% average similarity), a putative carbohydrate-binding module (smart00776, 74% average similarity), and a proline-rich region separating the transmembrane and the carbohydrate module (Figure 1). Surprisingly, in the case of the S. clavuligerus ortholog, there is a stop codon separating the carbohydrate-binding domain module ORF (SCLAV_3143) from the other domain ORFs (SCLAV_3144) (http://strepdb.streptomyces.org.uk/), perhaps due to an error in the sequence at the proline-rich region. Gene SCO4117 is localized downstream the actinorhodin positive regulator atrA (SCO4118), but both genes were described as being expressed independently (Uguru et al., 2005). This synteny is maintained in all Streptomyces species analyzed, with the exception of Streptomyces avermitilis, in which the SCO4117 and atrA orthologs (SAV_3491 and SAV_4110 respectively) are separated.

Mutation of SCO4117 Affects Antibiotic Production and Morphogenesis in Different Culture Conditions (Liquid and Solid) and Media (Sucrose-Free R5A, GYM, MM, SFM)
The S. coelicolor SCO4117 knockout mutant (∆SCO4117) do not show a significant variation in growth (Figure 2A), or in the maximum amount of actinorhodin and undecylprodigiosin production (Figures 2B,C), in sucrose-free R5A liquid cultures. However, the mutant shows a delay in actinorhodin production compared to the wild-type strain (Figure 2B). The SCO4117 knockout mutant showed lower CDA production (Figure 2D) and a delay in MII differentiation (notice the discontinuities characterizing the MI hyphae in the mutant at 24 h) (Figure 2F) (Manteca et al., 2006) and sporulation (Figures 2E,F) in solid GYM solid cultures. The delay in development was not complemented when SCO4117 and an upstream region large enough to include the two promoter regions identified by Jeong et al. (2016) were introduced into the mutant using the integrative plasmid pMS82-SCO4117 (Figure 2G). As discussed below, the SCO4117 gene expression was not restored in the complemented mutant (the SCO4117 transcript abundance was 4-fold less than in the wild-type strain) (Figure 2H). Interestingly, the ∆SCO4117 phenotype was only restored when a copy of the SCO4117 ORF was introduced at the native position in the ∆SCO4117 mutant, using the CRISPR-Cas9 methodology (Figure 2I). This result discards the possibility of artificial mutations at chromosomal positions different to SCO4117, generating the phenotypes observed.

As discussed below, the above data suggest a complex regulation of the expression of the SCO4117 gene. Polar effects in the expression of SCO4117 neighbor genes due to the apramycin-resistance gene transcription, are unlikely, because, as detailed below, we did not detect significant variations in the expression of the SCO4116 and SCO4118 genes in the ∆SCO4117 mutant compared to the wild-type strain (Supplementary Table 2). In order to further discard a possible effect of the apramycin-resistance gene in the phenotypes observed, we recreated the knockout mutant by CRISPR/Cas9. We eliminated the SCO4117 ORF, obtaining the CRISPR-Cas9-∆SCO4117 mutant (strain “1” in Figures 3A,B). This mutant, shows a phenotype similar to the ∆SCO4117 mutant (strain “2” in Figures 3A,B).

SCO4117 Gene Expression during S. coelicolor Development
The expression of SCO4117 was analyzed at three key developmental stages (MI, 17 h; aerial mycelia, 48 h; sporulating mycelia, 72 h) by qRT-PCR, in solid GYM cultures of the wild-type strain. As reported previously in our transcriptomic work (Yاغية et al., 2013b), SCO4117 is overexpressed during the sporulating stage in the S. coelicolor wild-type strain (Figure 3C).

The SCO4117 Sigma Factor Regulates the Expression of Its Own Gene
A mutant expressing the sigma factor domain from the SCO4117 ORF was created by CRISPR/Cas9 (CRISPR/Cas9-σ mutant) (Figure 1). The CRISPR/Cas9-σ mutant shows a
delayed actinorhodin production and lower CDA production than the wild-type strain (Supplementary Figure 2). Sporulation is also delayed, but it is faster than in the CRISPR/Cas9-ΔSCO4117 knockout mutant (Figure 4A). Interestingly, the expression of SCO4117 is slightly up-regulated (1.3-fold) in the CRISPR/Cas9-σ mutant compared to the wild strain (Figure 4B), suggesting that the sigma factor domain regulates the expression of its own gene.

Comparison of ΔSCO4117 and S. coelicolor Wild-Type Transcriptomes

The differences between the ΔSCO4117 mutant and the wild-type transcriptomes were analyzed at 44 h on solid GYM cultures, the developmental conditions preceding the differences observed in sporulation (Figures 2E,F). Fifty-six genes showed altered expression in the ΔSCO4117 mutant compared to the wild-type strain (Supplementary Table 1). As expected, the SCO4117 transcript was absent in the ΔSCO4117 knockout mutant (Supplementary Table 1). The expression of the SCO4117 neighbor genes (SCO4116 and SCO4118) was not significantly affected (Supplementary Table 2). Particularly interesting is that 46 of 56 transcripts, 82% of the total, are down-regulated in the ΔSCO4117 mutant compared to the wild strain (green bars in Figure 5, Supplementary Table 1), suggesting a pleiotropic activator effect of the SCO4117 ECF which is absent in the knockout mutant. 7,679 transcripts did not show significant variations (p-values higher than 0.03) (Figure 5).

Several key developmental and physiological genes were differentially expressed in the ΔSCO4117 mutant compared to the wild-type strain (Figure 5, Table 2). The expression of genes involved in secondary metabolism (deoxysugar synthases and...
actinorhodin biosynthetic genes) was highly down-regulated (up to 0.003, i.e., up-regulated 333-fold in the wild-type strain). The expression of genes involved in aerial mycelium formation and sporulation (chaplins, rodlins, nepA, ssgB, SCO7449) was also highly down-regulated in the mutant (up to 0.005, i.e., up-regulated up to 200-fold in the wild-type strain) (Figure 5; Table 2). SCO4684 (encoding the ScoF3 cold shock protein) was down-regulated in the mutant. The other 22 transcripts down-regulated in the ΔSCO4117 mutant included, in addition to SCO4117, transcripts of genes encoding enzymes, stress and secreted proteins, as well as uncharacterized proteins (Supplementary Table 1). The 10 transcripts up-regulated in the ΔSCO4117 mutant included: SCO0194, encoding a putative sigma factor; SCO2162, encoding a quinolinate synthetase; SCO6102, encoding a nitrite/sulphite reductase; and 6 transcripts from uncharacterized genes (Figure 5; Table 2).

**SCO4117 DNA Motif Bound**

The 46 genes encoding transcripts down-regulated in the ΔSCO4117 mutant (Figure 5) (Supplementary Table 1) might harbor SCO4117 motif bounds in their promoters. These genes were grouped into 33 putative operons (Supplementary Table 1) whose promoter regions were analyzed (see Materials and Methods). Twenty-nine promoters harbored the putative “C$\text{SGYN-17bps-SRHA}$” SCO4117 motif bound at their promoter regions (Figure 6) (Supplementary Table 1).

**DISCUSSION**

The study of the *Streptomyces coelicolor* SCO4117 knockout mutant revealed that the SCO4117 ECF sigma factor is a pleiotropic activator of antibiotic production (actinorhodin and CDA) in solid and liquid cultures (Figures 2B–D). Aerial mycelium differentiation and sporulation are also enhanced by SCO4117 in solid sporulating cultures (Figures 2E,F). The effect of SCO4117 activating the expression of secondary metabolism and differentiation was corroborated by transcriptomics. The expression of secondary metabolism (deoxysugar synthases, actinorhodin) and differentiation genes (bld, rdl, chp, nepA, ssgB) was dramatically reduced (up to 300-fold) in the knockout strain compared to the *S. coelicolor* wild strain. The effect of SCO4117 in secondary metabolism was not universal, since the expression of some secondary metabolite genes (for instance the undecylprodigiosin genes) did not appear to be affected (Supplementary Table 2).

Gene SCO4117 encodes a multidomain ECF sigma factor belonging to the ECF52 family (Figure 1). A putative SCO4117 motif bound ("C$\text{SGYN-17bps-SRHA}$") was identified...
| Function       | SCO no<sup>a</sup> | Gene               | Log<sub>2</sub> fold-change (<ΔSCO4117/Wt) | Fold-change (<ΔSCO4117/Wt) |
|---------------|-----------------|--------------------|------------------------------------|-------------------------|
| Secondary metabolism | Deoxysugar synthases | SCO0381 Glycosyl transferase | −5.2                          | 0.03                     |
|                |                 | SCO0382 Dehydrogenase     | −5.2                          | 0.03                     |
|                |                 | SCO0383 Glycosyl transferase | −3.8                          | 0.07                     |
|                |                 | SCO0386 Asparagine synthetase | −3.3                          | 0.1                      |
|                |                 | SCO0387 Oxidoreductase     | −3.1                          | 0.1                      |
|                |                 | SCO0389 Lipoprotein       | −3.8                          | 0.07                     |
|                | ACT             | SCO5071 Dehydrogenase     | −8                            | 0.003                    |
|                |                 | SCO5072 Dehydrogenase     | −4.5                          | 0.04                     |
|                |                 | SCO5074 Dehydratase       | −5.6                          | 0.02                     |
|                |                 | SCO5077 actVA2            | −3.3                          | 0.1                      |
|                |                 | SCO5078 actVA3            | −2.8                          | 0.1                      |
|                |                 | SCO5085 actI-4            | −4                            | 0.06                     |
|                |                 | SCO5086 actIII           | −5.7                          | 0.02                     |
|                |                 | SCO5087 actORF1           | −3                            | 0.1                      |
|                |                 | SCO5092 actVB             | −3                            | 0.1                      |
| Differentiation | SCO1541 ssgB | −3.4                  | 0.09                          |
|                | SCO2705 chpF   | −5.1                  | 0.03                          |
|                | SCO2716 chpA   | −3.8                  | 0.07                          |
|                | SCO2717 chpD   | −3.8                  | 0.07                          |
|                | SCO2718 rdlA   | −7.5                  | 0.005                         |
|                | SCO2719 rdlB   | −5.5                  | 0.02                          |
|                | SCO4002 nepA   | −4.5                  | 0.04                          |
|                | SCO7449 Spore pigmentation<sup>a</sup> | −2.6                  | 0.2                          |
| Stress         | SCO4684 scoF3  | −4.5                  | 0.05                          |
| Regulators     | SCO0194 Sigma factor | 2.4                  | 5.2                          |
| Catabolic enzymes | SCO1236 Urease | 2.7                  | 6.5                          |
| Anabolic enzymes | SCO2162 Quinolinate synthetase | 2.2                  | 4.6                          |
|                | SCO6102 Nitrite/sulphite reductase | 2.5                  | 5.7                          |
| Unknown        | SCO4256 Hypothetical protein | 2.2                  | 4.6                          |
|                | SCO1293 Putative acetyltransferase | 2.3                  | 4.9                          |
|                | SCO4258 Putative hydrolytic protein | 2.8                  | 6.9                          |
|                | SCO0195 Putative lipoprotein | 2.9                  | 7.5                          |
|                | SCO2788 Hypothetical protein | 2.9                  | 7.5                          |
|                | SCO7643 Hypothetical protein | ∞<sup>b</sup>                  | ∞<sup>b</sup>                          |

The genes discussed in the text are indicated.
<sup>a</sup> Secreted protein related to spore pigmentation (Salerno et al., 2013).
<sup>b</sup> Transcripts not detected in the wild-type strain.

(Figure 6). This motif differs from the theoretical ECF52 sigma factor promoter signature predicted by Pinto and Mascher (2016). Both motif bounds are theoretical, but the promoter signature identified in this work was present in the promoter regions of 29 putative operons down-regulated in the SCO4117 knockout mutant. Further experimentation will be necessary to unequivocally identify the ECF52 sigma factor promoter signature. Unfortunately, our attempts to overproduce the SCO4117 protein to study its interaction with the identified motif, were unsuccessful (data not shown).

SCO4117 gene expression has a complex regulation. Two promoters were identified controlling the expression of this gene (Jeong et al., 2016), one of them harboring the putative SCO4117 motif bound identified in this work. However, further uncharacterized regulation should exist, as the two promoters together with the SCO4117 ORF, did not complement the wild-type phenotype (Figure 2G), or the SCO4117 gene expression
crooks et al., 2004). The putative SCO4117 motif bound found in
proposed this kind of regulation for ECF52
further work will be necessary to fully
described a putative transcription terminator
amount of actinorhodin production) is complemented by a
that the
variations in the
between
SCO4117 was lesser than in the absence of the whole gene in the
mutant (delay in differentiation/sporulation)
SCO4117 and SCO4118 genes from the atrA promoter. However,
Uguru et al. (2005) described a putative transcription terminator
between
SCO4118 and
SCO4117. They also demonstrated
putative regulation of the SCO4117 activity by phosphorylation
(Manteca et al., 2011). Further work will be necessary to fully
understand the post-translational regulation of the
SCO4117 activity.

Overall, in this work, we demonstrated pleiotropic effects on the regulation of secondary metabolism and differentiation of
SCO4117, the first member of the ECF52 family characterized. Gene
SCO4117 is a conserved gene overexpressed during substrate and aerial mycelium stages, with complex regulation at the transcriptional and post-translational levels.

**AUTHOR CONTRIBUTIONS**

ML-G, PY, NG-Q, and BR performed the experiments. ML-G and AM planned the experiments and wrote the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00312/full#supplementary-material
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**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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