LuxR-Type SCO6993 Negatively Regulates Antibiotic Production at the Transcriptional Stage by Binding to Promoters of Pathway-Specific Regulatory Genes in *Streptomyces coelicolor*

Maral Tsevelkhoroolo1, Li Xiaoqiang1,2, Xue-Mei Jin1,3, Jung-Ho Shin4, Chang-Ro Lee1, Yup Kang5, and Soon-Kwang Hong1*

1Department of Bioscience and Bioinformatics, Myongji University, Yongin 17058, Republic of Korea
2GeneNet Pharmaceuticals Co. Ltd., Tianjin 300410, P.R. China
3Characteristic Industry Development Center of Yanbian, Jilin Province 133000, P.R. China
4R&D, Health & Bioscience, DuPont-IFF, Wilmington 19898, DE, USA
5Institute for Medical Sciences, Ajou University School of Medicine, Suwon 16499, Republic of Korea

SCO6993 (606 amino acids) in *Streptomyces coelicolor* belongs to the large ATP-binding regulators of the LuxR family regulators having one DNA-binding motif. Our previous findings predicted that SCO6993 may suppress the production of pigmented antibiotics, actinorhodin, and undecylprodigiosin, in *S. coelicolor*, resulting in the characterization of its properties at the molecular level. SCO6993-disruptant, *S. coelicolor ΔSCO6993* produced excess pigments in R2YE plates as early as the third day of culture and showed 9.0-fold and 1.8-fold increased production of actinorhodin and undecylprodigiosin in R2YE broth, respectively, compared with that of the wild strain and *S. coelicolor ΔSCO6993/SCO6993*'. Real-time polymerase chain reaction analysis showed that the transcription of *actA* and *actII-ORF4* in the actinorhodin biosynthetic gene cluster and that of *redD* and *redQ* in the undecylprodigiosin biosynthetic gene cluster were significantly increased by SCO6993-disruptant. Electrophoretic mobility shift assay and DNase footprinting analysis confirmed that SCO6993 protein could bind only to the promoters of pathway-specific transcriptional activator genes, *actII-ORF4* and *redD*, and a specific palindromic sequence is essential for SCO6993 binding. Moreover, SCO6993 bound to two palindromic sequences on its promoter region. These results indicate that SCO6993 suppresses the expression of other biosynthetic genes in the cluster by repressing the transcription of *actII-ORF4* and *redD* and consequently negatively regulating antibiotic production.

Keywords: *Streptomyces coelicolor*, SCO6993, LuxR family, actinorhodin, undecylprodigiosin

**Introduction**

Streptomyces are a group of gram-positive, filamentous soil bacteria that exhibit unique morphological differentiation, developing aerial mycelia and spores. During this morphological differentiation, a secondary metabolism begins which is called physiological differentiation. Numerous scientific findings have revealed that the morphological and physiological differentiations in streptomycetes are closely related and controlled by many regulatory factors [1]. The genes responsible for secondary metabolism and morphological differentiation are clustered in the chromosomal DNA and their expressions are controlled by pathway-specific regulatory gene(s) in the cluster and by global regulatory genes. *Streptomyces coelicolor* produces multiple genetically and structurally distinct antibiotic substances, and the production of pigmented antibiotics, blue-colored actinorhodin and red-colored undecylprodigiosin, is regulated by the pathway-specific transcriptional regulatory genes *actII-ORF4* [2] and *redD* [3] in the biosynthetic gene cluster. ActII-ORF4 and RedD proteins are the transcriptional activators that bind to the operator regions of the biosynthetic genes and positively regulate the transcription of each gene in the clusters. Due to easy discrimination of pigmentations in plate culture, several classes of blocked mutants, such as *abs* [4], *afs* [5], and *bld* [6], which block more than one antibiotic, were isolated to identify the global regulatory genes. For example, *afs* (A-factor synthesis deficient) mutation results in failure to produce A-factor (γ-butyrolactone), a
microbial hormone, and at least four antibiotics, including the two pigmented antibiotics, methylenomycin and Ca²⁺-dependent antibiotic, in S. coelicolor A3(2) [7]. The multiple serine/threonine kinases, including AfsK, AfsL, and PkA phosphorylate AfsR (a central protein that restores afs mutation), and the phosphorylated AfsR bind to the promoter of afs for transcriptional activation. The resulting AfsS protein induces the transcription of pathway-specific transcriptional activators, such as actII-ORF4 and redD, for actinorhodin and undecylprodigiosin production, respectively [5, 8].

However, the regulation of secondary metabolism appears much more complex than speculated. For example, the transcription of actII-ORF4 and redD is controlled by other transcriptional activators, AtrA [3] and RedZ [9], respectively. Moreover, absA1/A2 complementing absA (antibiotic synthesis deficient) mutation that fails to produce four distinct antibiotics encodes the bacterial two-component regulatory proteins, the sensor histidine kinase (AbsA1) and response regulator aspartic acid kinase (AbsA2) [4]. The AbsA2 acts as a negative regulator of antibiotic production in S. coelicolor A3(2) by binding to the promoter regions of actII-ORF4 and redD [10]. Furthermore, tens of activator/repressor proteins have been reported in relation to the transcriptional regulation of antibiotic production in S. coelicolor A3(2), indicating the complexity of the regulatory network for secondary metabolism in Streptomyces [11].

Recently, we reported that the introduction of SCO6992 gene encoding a novel β-glucuronidase dramatically increased the biosynthesis of undecylprodigiosin and actinorhodin in absR mutant of S. coelicolor J1501. This antibiotic biosynthesis-promoting effect of SCO6992 was abolised by the presence of SCO6993, which acts in opposition to SCO6992 [12]. Based on this, SCO6993 is expected to be a regulatory gene that suppresses antibiotic biosynthesis. Therefore, we report here the regulatory role of the LuxR-type SCO6993 protein in the antibiotic biosynthesis of S. coelicolor J1501.

**Material and Methods**

**Bacterial Strains and Plasmids**

S. coelicolor J1501 (hisA1 uraA1 strA1 SCP1 SCP2 Pgl), a mutant strain of S. coelicolor A3(2), was kindly provided by Dr. Wendy Champness of Michigan State University [13]. pET28a (+) was used for the expression of SCO6993 in E. coli BL21 (DE3) pLysS (Stratagene, USA). E. coli ET100 (Δacs-3) was used to transform plasmid pUZ8002 plasmid used for conjugal transfer [14]. E. coli BW25113 strain with the λ red recombination plasmid pIJ790 and cosmid SC8F11 was obtained from the John Innes Institute (UK) and used for genetic recombination [15].

**Media and Culture Conditions**

E. coli was maintained on M9 minimal agar and cultured in LB medium [16] at 37°C with agitation. Streptomyces strains were routinely maintained on minimal medium or R2YE medium [17] at 30°C. For cultivation of S. coelicolor J1501, the medium was supplemented with histidine and uracil at final concentrations of 50 μg/ml and 7.5 μg/ml, respectively [12]. Thiosstrepton (25 μg/ml), apramycin (50 μg/ml), and kanamycin (50 μg/ml) were added to the media, when necessary.

**Enzymes and Chemicals**

DNA-modifying enzymes were purchased from DyneBio Inc. (Korea), and other non-specified fine chemicals were purchased from Sigma-Aldrich Corporation (USA). All primer pairs used in this study were synthesized by Xenotech (Korea) and are listed in Table S1 with their respective uses.

**DNA Manipulations and Transformation**

DNA manipulation and transformation were performed by following the methods described by Sambrook and Russel [16] for E. coli and Kieser et al. [17] for Streptomyces. All experimental kits and enzymes were used according to the manufacturer's recommendations.

**Quantitation of Antibiotics**

The production of pigmented antibiotics, actinorhodin and undecylprodigiosin, on plates, was observed by naked eye and identified based on the color change during the cultivation of Streptomyces strains on the R2YE medium. For actinorhodin and undecylprodigiosin quantitation in broth culture, an exponential culture (5 ml) of S. coelicolor was transferred to 100 ml of R2YE broth in a 500 ml baffled flask and incubated at 30°C on a reciprocal shaker. Portions (5.0 ml) of the culture broth were taken out at intervals and the concentrations of actinorhodin and undecylprodigiosin were measured at 633 nm (A₆₃₃) and 530 nm (A₅₃₀), respectively, as previously described [12].

**Construction of ΔSCO6993 Mutant**

The SCO6993-disrupted mutant was constructed by replacing all of the SCO6993 coding sequence with apramycin resistance cassette [aac(3)IV] using the redirect polymerase chain reaction (PCR) targeting method [18]. The upstream primer for ΔSCO6993 contains the SCO6993 sequence up to the start codon (in bold) and is linked to the aac(3)IV sequence (underlined); 5′-GGG GGG TAC TCA AGT CAC CCT GTA TCA GGG ATG ATG ATG ATT TCC GGG ATCC GGT CGA CGA CGA CGA CGA -3′. The downstream reverse primer contains the ΔSCO6993 stop codon (in bold) and is linked to the aac(3)IV sequence (underlined); 5′-GGG CGG CCT GAG CCC GGC TGC CCG GCC GCC GGC GGC TCA TGT ATG CTC GAG CAT GTG CCT TTC CGA CTT G -3′. The resulting PCR product was introduced by electroporation into the E. coli BW25113 strain that harbors the λ red recombination plasmid pIJ790 and cosmid SC8F11 (a gift from K. Chater at John Innes Centre) carrying SCO6993 [15]. The gene structure of the
resulting cosmids (SC8F11 ΔSCO6993::apr) recovered from the selected transformants was verified, and the cosmids were introduced into the E. coli ET12567 carrying pUZ8002 and then transferred into S. coelicolor J1501 by conjugation [18]. The apramycin-resistant and kanamycin-sensitive exconjugants were selected, and the expected gene structure in ΔSCO6993::apr mutant was confirmed by Southern hybridization and reverse transcription (RT)-PCR. For complementing SCO6993 disruption, the recombinant plasmid pHM3-O2 containing the entire SCO6993 with its promoter region as described previously [12] was transformed into ΔSCO6993::apr mutant, yielding S. coelicolor ΔSCO6993/SCO6993.

Southern Hybridization
To confirm the ΔSCO6993::apr mutant, chromosomal DNAs were isolated and digested using BamHI restriction enzyme. The fully digested genomic DNAs were applied to 0.8% TBE-agarose gel. Resolved DNA fragments were depurinated in 0.25 M HCl for 20 min when required, washed with distilled water briefly, denatured in 0.4 M NaOH for 10 min, and transferred to Hybond-N’ membrane (Amersham Life Science, UK) in 0.4 M NaOH. The aac(3)IV probe DNA was amplified with DIG-labeled nucleotides and hybridized overnight. The signal was detected before image scanning following the manufacturer’s instructions.

Preparation of Total RNA and cDNA
The signal was detected before image scanning following the manufacturer’s instructions.

Southern Hybridization
To confirm the ΔSCO6993::apr mutant, chromosomal DNAs were isolated and digested using BamHI restriction enzyme. The fully digested genomic DNAs were applied to 0.8% TBE-agarose gel. Resolved DNA fragments were depurinated in 0.25 M HCl for 20 min when required, washed with distilled water briefly, denatured in 0.4 M NaOH for 10 min, and transferred to Hybond-N’ membrane (Amersham Life Science, UK) in 0.4 M NaOH. The aac(3)IV probe DNA was amplified with DIG-labeled nucleotides and hybridized overnight. The signal was detected before image scanning following the manufacturer’s instructions.

Preparation of Total RNA and cDNA
The fresh spores of S. coelicolor strains were inoculated into 25 ml R2YE broth in 250 ml Erlenmeyer flasks or R2YE plates and incubated at 28°C. The cultured cells were quickly harvested, flash frozen in liquid nitrogen, and then ground into powder. Total RNA was purified using an RNeasy Mini Kit in combination with RNAprotect Bacteria Reagent (Qiagen, Netherlands). The DNA in the RNA sample was removed through on-column DNAse digestion using a RNase-Free DNAse Set (Qiagen). Then, cDNA was synthesized from the total RNA sample using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, USA). Annealing reaction was performed at 25°C for 10 min, and then RT was performed at 50°C for 50 min. Reverse transcriptase was inactivated by heat treatment (85°C, 5 min). After cooling at 4°C, the remaining RNA was removed by RNase H treatment (37°C, 20 min). The synthesized cDNA was stored at -80°C.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)
To check the transcription level of the genes related to actinorhodin and undecylprodigiosin biosynthesis, real-time PCR analysis was performed using the synthesized cDNA, primers (Table S1), and Taq Plus 5× PCR Master Mix (ELPIS Biotech, Korea). After denaturation at 95°C for 3 min, the PCR reaction (denaturation at 95°C for 10 s; annealing at 68°C for 10 s; and extension at 72°C for 10 s) was repeated 28 times. The housekeeping hrdB gene encoding RNA polymerase sigma factor was used as control.

Real-Time PCR Analysis
To check the transcription level of the genes related to actinorhodin and undecylprodigiosin biosynthesis, real-time PCR analysis was performed using the synthesized cDNA and 2x Real-Time PCR Premix with Evagreen (SolGent, Korea). actA (encoding an efflux pump) and actII-ORF4 (encoding a transcriptional activator) were chosen among the actinorhodin biosynthetic genes, and redD (encoding a transcriptional activator) and redQ (encoding an acyl carrier protein) were chosen from the undecylprodigiosin biosynthetic gene clusters. The reaction was performed at 95°C for 10 min followed by 40 cycles of 95°C for 20 s, 58°C for 20 s, and 72°C for 20 s in a 20 μl volume mix containing 1 μl of 20x EvaGreen, 0.25 μM primers (Table S1), and 40 ng cDNA. Thermocycling and fluorescence detection were performed in triplicates for each cDNA sample using the Stratagene Mx3000p Real-Time PCR System (Stratagene). Following amplification, the experiment was converted to a comparative quantification (calibrator) experiment type and analysis was performed using the Mx3000P software v2.02 (Stratagene). The housekeeping hrdB gene was used as a reference.

Heterologous Expression and Purification of SCO6993
The SCO6993 gene was amplified by PCR from the S. coelicolor J1501 genomic DNA using primer sets (Table S1). The Ndel-Xhol-digested PCR product was subcloned into pET28a (+) yielding pET28a-SCO6993. E. coli BL21 (DE3)/pET28a-SCO6993 was cultured in 200 ml of LB broth to an optical density at 600 nm (OD$_{600}$) of 0.5. The overexpression of SCO6993 was induced by adding IPTG (0.5 mM), and there was further cultivation at 16°C for 12 h. From the cells, the recombinant SCO6993 (rSCO6993) protein was purified using His60 Ni Superflow Resin & Gravity Columns (Takara Bio Company, Japan). Protein concentration was measured using a Bradford Protein Microassay Kit (Bio-Rad, USA) with bovine serum albumin as the standard protein.

The molecular mass of the purified rSCO6993 was determined by gel filtration chromatography. The ÄKTA-FPLC System (GE Healthcare Life Sciences, USA) equipped with a Superose 12 10/300 GL column was used at 25°C. The mobile phase (20 mM Tris-HCl (pH 7.9) + 100 mM NaCl) was applied at a flow rate of 0.7 ml/min and protein concentration was monitored at 280 nm.

Electrophoretic Mobility Shift Assay (EMSA)

The binding ability of rSCO6993 to the promoter regions of SCO6993, actA, actII-ORF4, redQ, and redD was evaluated using the EMSA. For the SCO6993, two kinds of DNA probes, SCO6993-P1 (206 bp) and SCO6993-P2 (192 bp), were amplified from the S. coelicolor J1501 genomic DNA PCR using primer sets (Table S1). Other probes for actA, actII-ORF4, redQ, and redD were also prepared in the same way. Binding reactions (20 μl) were performed in binding buffer (20 mM Tris-HCl (pH 8.0), 1 mM MgCl$_2$, 0.1 mM dithiothreitol, 12.5% glycerol,
50 mM NaCl, and 0.4 mM EDTA) with a 0.3 mM DNA probe at 28°C for 30 min. The sonicated salmon sperm DNA (100 mg/ml) was used as a competitor and the binding of the SCO6993 to the DNA fragments was analyzed using 1.5% TBE agarose gel.

Mapping of the SCO6993-Binding Sites
DNase I footprinting analysis was performed with capillary DNA sequencer using a fluorescent-labeled PCR fragment as previously described [19]. Primers for analysis of each promoter region were used by labeling the same primers as those used in the EMSA experiment with 5’-6-FAM (6-carboxyfluorescein) (Table S1). The labeled DNA probes (540 ng) were incubated with the SCO6993 protein (25 μg) at 28°C for 20 min in the binding buffer (20 mM Tris-HCl (pH 8.0), 1 mM MgCl₂, 0.1 mM DTT, 12.5% glycerol, 50 mM NaCl, and 0.4 mM EDTA). The digestion of the DNA probe was performed at 37°C using one unit of DNase I (Promega, USA) per 50 μl reaction solution. After 5 min, a stop buffer (130 mM NaCl, 20 mM EDTA, and 0.6% SDS) was added, and the digested DNA probe was purified using a PCR purification kit (Qiagen). The fluorescence patterns of the sample were analyzed using a 3730 Automated DNA Sequencer (Applied Biosystems, USA), and electropherograms were used for determining the protected patterns of each promoter after digestion with DNase I following incubation with SCO6993 protein.

Results
SCO6993 Belongs to the Large ATP-Binding Regulators of the LuxR (LAL) Family
The SCO6993 protein (606 amino acids, 65.7 kDa, GenBank AAF19104.1) showed significant similarity with numerous proteins annotated as LuxR-type, DNA-binding regulators in the genus *Streptomyces*. BlastP analysis (BLAST: Basic Local Alignment Search Tool (nih.gov)) identified more than 100 proteins showing more than 98% homology to SCO6993 in the amino acid sequence in *Streptomyces*, implying that the SCO6993 homologous gene is widely distributed in genus *Streptomyces*. SCO6993 has one ATP/GTP-binding P-loop motif conserved in ATPase and one helix-turn-helix LuxR-type DNA-binding motif in the C-terminal region, which is a characteristic of the LAL family protein (Fig. 1). In this context, SCO6993 was expected to be a DNA-binding regulatory protein.

SCO6993 Gene Deletion Results in Increased Production of Pigmented Antibiotics
In a previous study [12], SCO6993 was expected to be a suppressor for pigmented antibiotic production. Thus, SCO6993-disrupted strain (ΔSCO6993) was constructed using a PCR targeting system (Fig. 2A). For the apramycin-resistant and kanamycin-sensitive exconjugants, ΔSCO6993::apr mutant was confirmed using Southern blotting and hybridization with a DIG-labeled apramycin-cassette probe (Fig. 2B). According to the RT-PCR analysis, the transcript of SCO6993 in the *S. coelicolor* J1501 on R2YE plate was maximally detected on the third day of cultivation, and a significant amount was also detected on the fifth day. However, no transcript of

![Fig. 1. Characteristics of SCO6993 gene in *S. coelicolor* J1501.](image-url)

(A) Chromosomal gene organization (upper) and distribution of conserved domains (lower). The arrows indicate individual ORFs, with the stop codon marked by the arrowhead. The SCO6992 and SCO6993 genes are in opposite directions, and the two genes are 78 bp apart from each putative transcription initiation point. SCO6993 (646 amino acids) contains ATP/GTP-binding P-loop domain (Phe-23 – Leu-135, E-value of 2.36e-03) and one LuxR-type helix-turn-helix DNA binding domain (Leu-543 – Arg-590, E-value of 6.58e-14). (B) Alignment of amino acids sequence of C-terminal DNA-binding domain of LuxR-like proteins (cd06170). Highly conserved amino acids for DNA binding are indicated by asterisks.

September 2022 | Vol. 32 | No. 9
SCO6993 was detected in the ΔSCO6993 strain during cultivation (Fig. 2C), indicating that the SCO6993 gene in the ΔSCO6993::apr mutant completely lost its function.

The antibiotic production ability of the ΔSCO6993 mutant was compared to that of the parent strain by culturing in solid and liquid media. On the R2YE plates, little pigment production was observed in the J1501 parental strain, while the ΔSCO6993 mutant continuously produced excess pigments from the third day of culture (Fig. 3A).

In the R2YE liquid culture, the ΔSCO6993 strain began producing large amounts of pigment from the third day of culture. On the seventh day, the ΔSCO6993 strain showed 9.0-fold and 1.8-fold increased production of actinorhodin and undecylprodigiosin, respectively, compared to that by the S. coelicolor J1501 strain (Fig. 3B).

The SCO6993-complemented S. coelicolor ΔSCO6993/SCO6993+ strain had its normal phenotype restored as in J1501 in solid (Fig. 3A) and liquid media (Fig. 3B).
Fig. 3. Effect of SCO6993 disruption on pigmented antibiotic production in S. coelicolor J1501. (A) Photographs of S. coelicolor strains cultured on R2YE agar plate for 5 days. S. coelicolor ΔSCO6993 showed a large amount of pigment production from the third day of culture, but the parent strain J1501 and the mutant complemented with SCO6993 (S. coelicolor ΔSCO6992/SCO6992+) produced a small amount of pigment even on the fifth day. (B) Pigmented antibiotic production in R2YE broth. The culture samples were taken on the seventh day of cultivation and processed. The quantification of actinorhodin (left) and undecylprodigiosin (right) was performed by measuring the absorbance at 633 nm and 530 nm, respectively. (C) Transcript analysis by real-time polymerase chain reaction for actinorhodin (upper) and undecylprodigiosin (lower) biosynthetic genes of S. coelicolor strains cultured on R2YE agar plate. The culture samples were taken at indicated time intervals and processed as described in the Materials and Methods section. Then, the experiment was converted to a comparative quantification (calibrator) experiment and analyzed with the Mx3000P software v2.02. The housekeeping hrdB gene was used as a reference. In (B) and (C), all the experiments were repeated at least three times, and their average values were calculated. J1501, S. coelicolor J1501; ΔSCO6993, S. coelicolor ΔSCO6993; ΔSCO6993/SCO6993+, S. coelicolor ΔSCO6993 complemented with native SCO6993 gene.
Transcriptions of Pigmented Antibiotic Biosynthetic Genes Are Enhanced in SCO6993-Deleted Strain

To understand the reason for the increased production of pigmented antibiotics in ΔSCO6993 strain, real-time PCR analysis was performed on the two genes (actA and actII-ORF4) in the actinorhodin biosynthetic gene cluster and the two genes (redD and redQ) in the undecylprodigiosin biosynthetic gene cluster. The real-time PCR analysis showed that the transcription of all the tested genes was significantly enhanced in the ΔSCO6993 strain from the beginning of the culture in R2YE broth, compared with that in J1501 and S. coelicolor ΔSCO6993/SCO6993+ (Fig. 3C).

The transcription of actII-ORF4 increased in proportion to the cultivation time, but that of redD showed a maximum level on the first day of culture. When observing the R2YE plate culture of these strains, the production of red-colored undecylprodigiosin was visually confirmed from the first day of culture, and the production of blue-colored actinorhodin was observed from the third day of culture (Fig. 3A). These results strongly suggest that undecylprodigiosin production begins temporally faster than actinorhodin production.

SCO6993 Binds to the Promoter Regions of actII-ORF4 and redD Regulatory Genes

To determine the DNA-binding ability of the LuxR-type regulator, the recombinant protein rSCO6993 was overexpressed with 20 additional amino acids including 6xHis-tag, which originated from the vector at the N-terminal. The purified rSCO6993 protein showed a single band by SDS-PAGE analysis, which is consistent with a calculated molecular weight of 66.8 kDa (Fig. 4A). The gel filtration chromatography analysis revealed that the molecular weight of native SCO6993 was 129.6 kDa, indicating that it forms a dimer (Fig. 4B).

Next, a DNA fragment containing the upstream regions of actA, actII-ORF4, redD, and redQ served as the binding partner of rSCO6993 in the EMSAs. DNA fragments of 327 bp (pactII-ORF4; between positions -207 and +120 with respect to the actII-ORF4 translation start site), 383 bp (pactA; between positions -273 and +110 with respect to the actA translation start site), 400 bp (predD; between positions -350 and +50 with respect to the redD translation start site), and 350 bp (predQ; between positions -300 and +27 with respect to the redQ translation start site) were used in the EMSAs.
start site) were used as DNA probes for actII-ORF4, actA, redD, and redQ, respectively (Table S1). As a result, the retardation of DNA mobility for the two pathway-specific genes, actII-ORF4 and redD, was confirmed, but not for biosynthetic genes actA and redQ (Fig. 5A). The intensity of the retardation signal increased in proportion to the concentration of added SCO6993. The addition of salmon sperm DNA (100 mg/ml) to the reaction inhibited complex formation between SCO6993 and the DNA substrate.

Fig. 5. DNA-binding ability of SCO6993 to the promoter regions of actinorhodin (act) and undecylprodigiosin biosynthetic genes. (A) Electrophoretic mobility shift assays (EMSAs) were performed with purified His6-SCO6993 on the promoter regions of the genes involved in actinorhodin (act) and undecylprodigiosin (red) biosynthesis. The DNA probes used were the upstream regions of actII-ORF4 (pactII-ORF4), actA (pactA), redD (predD), and redQ (predQ). Each 20 μl binding reaction mixture containing the DNA probe (0.3 nM) and the SCO6993 protein at various concentrations (0–30 μg), was subjected to electrophoresis on 1.5% TBE agarose gel. sDNA, salmon sperm DNA. (B, C) DNase 1 footprinting assays. Electropherograms showing the protection pattern of each promoter of the fluorescent dye-labeled upstream regions of actII-ORF4 (B) and redD (C) after digestion with DNase 1 following incubation in the absence (upper) or presence (lower) of SCO6993 protein. The y-axis represents fluorescence intensity (i.e., fragment abundance), while the x-axis represents elution position, which is proportional to size. At the bottom is a scale that shows nucleotide position relative to each gene. The protected regions are indicated with a red box, and the protected sequences (SCO6993-binding sites) with palindrome sequences are shown below. The numbers refer to the distance from the translation start site of each gene.
Fig. 6. DNA-binding ability of SCO6993 to its promoter region. (A) Electrophoretic mobility shift assays (EMSAs) were performed with purified His$_6$-SCO6993 on the promoter regions of SCO6993. The DNA probes were SCO6993-P1 (-240 ~ -34) and SCO6993-P2 (-55 ~ +114). Each 20 μl binding reaction mixture containing the DNA probe (0.3 nM) and the SCO6993 protein at various concentrations (0–30 μg) was subjected to electrophoresis on 1.5% TBE agarose gel. sDNA, salmon sperm DNA. (B, C) DNase 1 footprinting assays using Probe-P1 (B) and Probe-P2 (C). Electropherograms showing the protection pattern of each promoter of fluorescent dye-labeled upstream region of SCO6993 after digestion with DNase 1 following incubation in the absence (upper) or presence (lower) of SCO6993 protein. The y-axis represents fluorescence intensity (i.e., fragment abundance), while the x-axis represents elution position, which is proportional to size. At the bottom is a scale that shows the nucleotide position relative to each gene. Two protected regions are indicated with a red box, and the protected sequences (SCO6993-binding sites) with palindrome sequences are shown below. The numbers refer to the distance from the translation start site of each gene.
SCO6993 Binds to Palindromic Sequences in the Promoter Regions of actII-ORF4 and redD Genes

SCO6993-binding sites within the promoter regions of actII-ORF4 and redD were validated using DNase 1 footprinting analysis. The electropherograms showed the protected patterns of each promoter after digestion with DNase 1 following incubation with SCO6993 protein. The results showed that the region of actII-ORF4 promoter protected by SCO6993 was from -190 to -174 nt (ATCTGAATTGATTCGGAA)⁻¹, palindromic sequence is underlined) (Fig. 5B), whereas the region of redD promoter protected by SCO6993 was -22 to -1 nt (CCGGATTCGGTTGCG⁻¹, palindromic sequence is underlined) (Fig. 5C). The binding motif of most LuxR family transcriptional regulators is palindromic, but highly degenerate due to sequence variations in each promoter [20]. Similarly, the binding sequences in the promoter regions of actII-ORF4 and redD comprise palindromes; however, no homology was found between the two.

In summary, SCO6993 appears to suppress the transcription of the pathway-specific regulatory genes actII-ORF4 and redD by binding directly to their promoter regions. Accordingly, the transcription of the antibiotic biosynthesis genes actA and redQ will be regulated in succession by the pathway-specific transcriptional activators ActII-ORF4 and RedD, respectively.

SCO6993 Binds to Its Own Promoter Region

Many transcriptional regulatory proteins can bind to their respective promoter for self-regulation and thus EMSA was performed on the promoter region of SCO6993 gene. The shifted bands by retarded mobility were detected in all the reactions using probes SCO6993-P1 (207 bp; between positions -240 and -34 with respect to the SCO6993 translation start site) and SCO6993-P2 (192 bp; between positions -78 and +114 with respect to the SCO6993 translation start site), and the amount of shifted DNA was proportional to that of protein added (Fig. 6A). The addition of excess salmon sperm DNA abolished the band shift of DNA. Consistent with the EMSA results, DNase 1 footprinting analysis confirmed the two SCO6993-binding sites. The regions of SCO6993 promoter protected by SCO6993 were from -144 to -131 nt (GGCGCCAGCCGCCG⁻¹, palindromic sequence is underlined) and from -54 to -37 nt (GGTGCCCGGGCGGCTGAGTC, palindromic sequence is underlined) as we expected (Figs. 6B and 6C). These results strongly suggest that SCO6993 can self-regulate the expression of its encoding genes by binding to its promoter region.

Discussion

In this study, we demonstrated that SCO6993, a LAL-type global regulator, suppressed actinorhodin and undecylenylprodigiosin production by binding to the promoter regions of the pathway-specific regulatory genes actII-ORF4 and redD, respectively, in S. coelicolor J1501 and repressing their transcription.

LuxR family proteins were identified as transcriptional regulators for quorum sensing-related genes [21, 22] in Vibrio fischeri. They have a DNA-binding domain (helix-turn-helix motif) at the C-terminal module for binding specific nucleotide sequences near target promoters and the N-terminal domain for binding effector molecules, thereby regulating the transcription of target genes [23, 24]. In the quorum-sensing regulation, the LuxR family recognizes and binds to specific target binding sites with inverted repeats. For instance, LuxR dimers involved in quorum sensing binds to a 20-bp inverted repeat [22], and GerE dimers controlling respiratory-related operons in E. coli bind to two 12-bp inverted repeats having a central four-base overlap [28]. Our results indicate that SCO6993 can recognize specific nucleotide sequences containing short, inverted repeats, but the consensus binding site could not be predicted.

Multiple LAL family regulators have been reported to be present in the biosynthetic gene clusters of Streptomyces. PikD [29], RapH [30], NysRI [31], TmcN [32], Sng [33], TrdH [34], and AmphiRI [35] were identified as LAL family pathway-specific regulators in pikromycin, rapamycin, nystatin, tautomycetin, nikkomycin, tirandamycin, and amphotericin biosynthetic pathways, respectively. Furthermore, LAL regulators play a global regulatory role at higher levels of the regulatory cascade. For example, LAL regulator PmrB activates PmrM expression in Streptomyces natalensis and LuxR-type pathway-specific regulator PmrM directly stimulates the transcription of pimaricin biosynthetic genes [36], which is very similar to the relationship between SCO6993 and pathway-specific regulatory genes actII-ORF4 and redD.

Most LAL regulators function as positive or negative regulators, but some of them act simultaneously as positive and negative regulators depending on the regulatory pathway. In S. coelicolor, the LAL regulators SCO0877 and SCO7173 function as repressors of the expression of the two-component PhoRP system in phosphate starvation response and as activators of actinorhodin biosynthesis [37]. The pathway-specific regulator AveR was also reported to be a positive regulator of avermectin production but a negative regulator of oligomycin biosynthesis in Streptomyces avermitilis [38]. Interestingly, we found that the ability of aerial mycelium formation was completely abolished by deleting the SCO6993 in S. coelicolor (Figs. S2 and S3), indicating that SCO6993 regulates morphological...
differentiation positively like the bld genes [6, 39]. Thus, SCO6992 can be defined as a global regulatory protein acting negatively or positively in controlling the physiological and morphological differentiations of S. coelicolor J1501.

Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (NRF-2020R1F1A1060789).

Conflict of Interest

The authors have no financial conflicts of interest to declare.

References

1. McCormick JR, Flåtz K. 2012. Signals and regulators that govern Streptomyces development. FEMS Microbiol. Rev. 36: 206-231.
2. Arias P, Fernández-Moreno MA, Malpartida F. 1999. Characterization of the pathway-specific positive transcriptional regulator for actinorhodin biosynthesis in Streptomyces coelicolor A3(2) as a DNA-binding protein. J. Bacteriol. 181: 6958-6968.
3. Ugaru GC, Stephens KE, Stead JA, Towle JE, Baumberg S, McDowell KJ. 2005. Transcriptional activation of the pathway-specific regulator of the actinorhodin biosynthetic genes in Streptomyces coelicolor. Mol. Microbiol. 58: 131-150.
4. Ryding NJ, Anderson TB, Champness WC. 2002. Regulation of the Streptomyces coelicolor calcium-dependent antibiotic by absA, encoding a cluster-linked two-component system. J. Bacteriol. 184: 794-805.
5. Hong SK, Kito M, Beppu T, Horinouchi S. 1991. Phosphorylation of the AfsR product, a global regulatory protein for secondary-metabolite formation in Streptomyces coelicolor A3(2). J. Bacteriol. 173: 2311-2318.
6. Chater KE. 2013. Curbing baldness activates antibiotic production. Chem. Biol. 20: 1199-1200.
7. Horinouchi S, Hara O, Beppu T. 1983. Cloning of a pleiotropic gene that positively controls biosynthesis of A-factor, actinorhodin, and prodigiosin in Streptomyces coelicolor A3(2) and Streptomyces lividans. J. Bacteriol. 155: 1238-1248.
8. Horinouchi S. 2003. AfsR as an integrator of signals that are sensed by multiple serine/threonine kinases in Streptomyces coelicolor A3(2). J. Ind. Microbiol. Biotechnol. 30: 462-467.
9. White J, Bibb M. 1997. bldA dependence of undecylprodigiosin production in Streptomyces coelicolor A3(2) involves a pathway-specific regulatory cascade. J. Bacteriol. 179: 627-653.
10. McKenzie NL, Nodwell JR. 2007. Phosphorylated AbsA2 negatively regulates antibiotic production in Streptomyces coelicolor through interactions with pathway-specific regulatory gene promoters. J. Bacteriol. 189: 5284-5292.
11. van der Heul HU, Blyck BL, McDowell KJ, Seipek RE, van Welzel GP. 2018. Regulation of antibiotic production in actinobacteria: new perspectives from the post-genomic era. Nat. Prod. Rep. 35: 575-604.
12. Kim JM, Choi SS, Tsevelkhoroloo M, Park U, Shu JW, Hong SK. 2021. SCO6992, a protein with β-glucuronidase activity, complements a mutation at the absIR locus and promotes antibiotic biosynthesis in Streptomyces coelicolor. J. Microbiol. Biotechnol. 31: 1591-1600.
13. Anderson TB, Brian P, Champness WC. 2001. Genetic and transcriptional analysis of absA, an antibiotic gene cluster-linked two-component system that regulates multiple antibiotics. Mol. Microbiol. 39: 553-566.
14. Mazodier P, Petter R, Thompson C. 1989. Intergeneric conjugation between Escherichia coli and Streptomyces species. J. Bacteriol. 71: 3583-3585.
15. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc. Natl. Acad. Sci. USA 97: 6640-6645.
16. Sambrook J, Russell DW. 2001. Molecular cloning: a laboratory manual. 3rd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
17. Kieser H, Bibb MJ, Buttner MJ, Chater FK, Hopwood DA. 2000. Practical Streptomyces Genetics. The John Innes Foundation, Norwich, UK.
18. Gust B, Challis GL, Fowler K, Kieser T, Chater KE. 2003. PCR-targeted Streptomyces gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc. Natl. Acad. Sci. USA 100: 1541-1546.
19. Yin-de-he-wen, Schell MA, 2000. Footprinting with an automated capillary DNA sequencer. Biotechniques 29: 1034-1036.
20. Newman JD, Russell MM, Fan L, Wang YX, Gonzalez-Gutierrez G, van Kessel JC. 2021. The DNA binding domain of the Vibrio vulnificus SmcR transcription factor is flexible and binds diverse DNA sequences. Nucleic Acids Res. 49: 5967-5984.
21. Engerbeck J, Nealsom K, Silverman M. 1983. Bacterial bioluminescence: isolation and genetic function of enzymes from Vibrio Fischeri. Cell 32: 773-781.
22. Fuqua C, Greenberg EP. 2002. Listening in on bacteria: acyl-homoserine lactone signalling. Annu. Rev. Microbiol. 56: 727-751.
23. Fuqua C, Greenberg EP. 2002. Listening in on bacteria: acyl-homoserine lactone signalling. Nat. Rev. Mol. Cell Biol. 3: 685-695.
24. Fuqua C, Winans SC, Greenberg EP. 1996. Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. Annu. Rev. Microbiol. 50: 727-751.
25. Patautker AT, Gonzalez JE. 2009. Orphan LuxR regulators of quorum sensing. FEMS Microbiol. Rev. 33: 739-756.
26. Boos W, Shuman H. 1998. Malto/1-mannose-dependent system of Escherichia coli transport, metabolism, and regulation. Microbiol. Mol. Biol. Rev. 62: 204-229.
27. Walker ME, Saraste M, Runswick MJ, Gay NJ. 1982. Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. 1: 945-951.
28. Raibaud O, Vidal-Ingigliardi D, Richet EA. 1989. Complex nucleoprotein structure involved in activation of transcription of two divergent Escherichia coli promoters. J. Mol. Biol. 205: 471-485.
29. Maris AE, Sawaya MR, Kaczerz-Gorszykwa M, Jarvis MR, Beasnon SM, Kopka MI, et al. 2022. Dimerization allows DNA target site recognition by the NarL response regulator. Nat. Struct. Biol. 9: 771-778.
30. Wilson DJ, Xue Y, Reynolds KE, Sherman DH. 2001. Characterization and analysis of the PkD regulatory factor in the pikromycin biosynthetic pathway of Streptomyces venezuelae. J. Bacteriol. 183: 3468-3475.
31. Apicario JF, Molinari I, Schwecke T, König A, Haydock SF, Khaw LE, et al. 1996. Organisation of the biosynthetic gene cluster for rapamycin in Streptomyces hygroscopicus: analysis of the enzymatic domains in the modular polypeptide synthase. Gene 169: 9-16.
32. Sekurova ON, Beuttardet T, Stutta H, Boros J, Jakobsen OM, Ellingsen TE, et al. 2004. In vivo analysis of the regulatory genes in the nystatin biosynthetic gene cluster of Streptomyces noursei ATCC 11455 reveals their differential control over antibiotic biosynthesis. J. Bacteriol. 186: 1345-1354.
33. Hur YA, Choo SS, Sherman DH, Kim ES. 2008. Identification of TmRN as a pathway-specific positive regulator of tautomycin biosynthesis in Streptomyces sp. CK4412. Microbiology 54: 2912-2919.
33. He X, Li R, Pan Y, Liu G, Tan H. 2010. SanG, a transcriptional activator, controls nikkomycin biosynthesis through binding to the sanN-sanO intergenic region in *Streptomyces ansochromogenes*. *Microbiology* 156: 828-837.
34. Mo X, Wang Z, Wang B, Ma J, Huang H, Tian X, Zhang S, et al. 2011. Cloning and characterization of the biosynthetic gene cluster of the bacterial RNA polymerase inhibitor tirandamycin from marine-derived *Streptomyces* sp. SC570666. *Biochem. Biophys. Res. Commun.* 406: 341-347.
35. Carmody M, Byrne B, Murphy B, Breen C, Lynch S, Flood E, et al. 2004. Analysis and manipulation of amphotericin biosynthetic genes by means of modified phage KCS15 transduction techniques. *Gene* 343: 107-115.
36. Santos-Aberturas J, Vicente CM, Payero TD, Martín-Sánchez L, Cañibano C, Martín JF, et al. 2012. Hierarchical control on polyene macrolide biosynthesis: PimR modulates pimaricin production via the PAS-LuxR transcriptional activator PimM. *PLoS One* 7: e38536.
37. Guerra SM, Rodríguez-García A, Santos-Aberturas J, Vicente CM, Payero TD, Martín JF, et al. 2012. LAL regulators SCO0877 and SCO7173 as pleiotropic modulators of phosphate starvation response and actinorhodin biosynthesis in *Streptomyces coelicolor*. *PLoS One* 7: e31475.
38. Guo J, Zhao J, Li L, Chen Z, Wen Y, Li J. 2010. The pathway-specific regulator AveR from *Streptomyces avermitilis* positively regulates avermectin production while it negatively affects oligomycin biosynthesis. *Mol. Genet. Genomics* 283: 123-133.
39. Hackl S, Bechthold A. 2015. The Gene bldA, a regulator of morphological differentiation and antibiotic production in *Streptomyces*. *Arch. Pharm. (Weinheim)* 348: 455-462.