GntR family regulator SCO6256 is involved in antibiotic production and conditionally regulates the transcription of myo-inositol catabolic genes in *Streptomyces coelicolor* A3(2)

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SCO6256 belongs to the GntR family and shows 74% identity with SCO6974, which is the repressor of myo-inositol catabolism in *Streptomyces coelicolor* A3(2). Disruption of SCO6256 significantly enhanced the transcription of myo-inositol catabolic genes in R2YE medium. The purified recombinant SCO6256 directly bound to the upstream regions of SCO2727, SCO6978 and SCO6985, as well as its encoding gene. Footprinting assays demonstrated that SCO6256 bound to the same sites in the myo-inositol catabolic gene cluster as SCO6974. The expression of SCO6256 was repressed by SCO6974 in minimal medium with myo-inositol as the carbon source, but not in R2YE medium. Glutathione-S-transferase pull-down assays demonstrated that SCO6974 and SCO6256 interacted with each other; and both of the proteins controlled the transcription of myo-inositol catabolic genes in R2YE medium. These results indicated SCO6256 regulates the transcription of myo-inositol catabolic genes in coordination with SCO6974 in R2YE medium. In addition, SCO6256 negatively regulated the production of actinorhodin and calcium-dependent antibiotic via control of the transcription of actII-ORF4 and cdaR. SCO6256 bound to the upstream region of cdaR and the binding sequence was proved to be TTTGGCACGAGACAT, which was further confirmed through base substitution. Four putative targets (SCO2652, SCO4034, SCO4237 and SCO6377) of SCO6256 were found by screening the genome sequence of *Strep. coelicolor* A3(2) based on the conserved binding motif, and confirmed by transcriptional analysis and electrophoretic mobility shift assays. These results revealed that SCO6256 is involved in the regulation of myo-inositol catabolic gene transcription and antibiotic production in *Strep. coelicolor* A3(2).

INTRODUCTION

*Streptomyces* are well known for their complex life cycle with morphological differentiation and the production of a wide range of secondary metabolites (Chater, 1998; Gilbert et al., 1995). *Streptomyces coelicolor* A3(2) is the model organism for studying secondary metabolite biosynthesis and its regulation. Twenty-nine gene clusters for such biosynthesis were localized in the genome of *Strep. coelicolor* A3(2) (Nett et al., 2009). Of them, four distinct classes of antibiotics, including the blue-pigmented polyketide actinorhodin (ACT) (Malpartida & Hopwood, 1986), the red oligopyrrole prodigine undecylprodigiosin (RED) (Malpartida et al., 1990), the lipopeptide calcium-dependent antibiotics (CDAs) (Hojati et al., 2002) and coelimycin P1, have been well characterized (Gomez-Escribano et al., 2012).

The ACT biosynthetic gene cluster consists of five transcription units and actII-ORF4 encodes the key activator of ACT production (Wietzorrek & Bibb, 1997). A complex network has been revealed to regulate the biosynthesis of ACT, involving small molecules (ppGpp, N-acetylglucosamine and γ-butyrolactone), two-component systems, RNA regulation and so on (Liu et al., 2013; van Wezel & McDowall, 2011). The factors influence the timing and level of ACT production almost all act via regulating the transcription or translation of actII-ORF4 (Liu et al., 2013). At least eight regulatory proteins could bind to the upstream of actII-ORF4, such as global regulators.
[AdpA (Ohnishi et al., 2005), LexA (Iqbal et al., 2012), DasR (Rigali et al., 2008), AtrA (Uguru et al., 2005; Nothaft et al., 2010) and ROK7B7 (Heo et al., 2008)], and the response regulators of two-component systems [AbsA2 (Uguru et al., 2005; Sheeler et al., 2005), DraR (Yu et al., 2012) and AfsQ1 (Wang et al., 2013)]. The binding sites of these regulators are separated or overlapping in the upstream region of actII-ORF4, indicating the complex regulation of actII-ORF4 transcription. The translation of actII-ORF4 is dependent on bldA due to a rare codon UUA in its mRNA (Fernández-Moreno et al., 1991).

The CDA biosynthesis is activated by a cluster-situated regulator, CdaR (Hojati et al., 2002). The regulation of CDA biosynthesis is poorly understood, except some two-component systems are characterized as its regulators. AbsA2 and PhoP repress the transcription of cdaR (Sheeler et al., 2005; Allenby et al., 2012), while AfsQ1 activates its transcription via binding upstream of cdaR (Wang et al., 2013). Other regulators, such as AbrA2, AbrC3 and NsdA, have been proved to affect CDA production indirectly (Yepes et al., 2011; Li et al., 2006).

The GntR regulators are the most abundant bacterial transcriptional factors (Hoskisson & Rigali, 2009). They generally contain a DNA-binding domain at their N-terminus that is conserved throughout the family, and a domain for ligand binding and protein oligomerization at their C-terminus (Rigali et al., 2002). The GntR regulators play a fundamental role in the modulation of gene expression to respond to a variety of environmental signals. As a result, they regulate many diverse biological processes including bacterial motility (Jaques & McCarter, 2006), development (Hoskisson et al., 2006), antibiotic production (Hillerich & Westpheling, 2006), antibiotic resistance (Truong-Bolduc & Hooper, 2007), plasmid transfer (Reuther et al., 2006) and virulence (Casali et al., 2006; Haine et al., 2005).

A large number of GntR-like regulators are found in Streptomyces due to their complex life cycle and inhabitation of a variable environment (Hoskisson & Rigali, 2009). They take part in the morphological differentiation and secondary metabolic biosynthesis in Streptomyces. Fifty-four GntR-like protein-encoding genes are present in Strep coelicolor A3(2). Of them, DasR is the regulator of N-acetylglucosamine catabolism and controls the biosynthesis of ACT and RED directly (Rigali et al., 2008). Defective SCO7168 decreases the production of ACT and delays the formation of aerial mycelium, as well as the sporulation of Streptomyces (Sprouse et al., 2003). In Streptomyces sp. 139, the GntR-like protein Ste1 directly regulates eobisin biosynthesis and influences aerial mycelium formation (Bai et al., 2013).

As a precursor of cellular membrane components, myo-inositol is important for Streptomyces growth and differentiation (Zhang et al., 2012). Although the regulation of myo-inositol catabolism has been extensively studied in some bacteria (Bouette et al., 2008; Yebra et al., 2007; Yoshida et al., 1999), the regulatory mechanism is poorly understood in Streptomyces. In our previous works, the myo-inositol catabolic gene cluster has been identified in Strep. coelicolor A3(2). Among the genes, SCO6984 encodes a myo-inositol dehydrogenase (homologue of IdhA), SCO6982 encodes a 2-keto-myoinositol (2KMI) dehydratase (homologue of IolE), SCO6975 encodes a 3-d- (3,5/4)-trihydroxyxyclohex-ane-1,2-dione (THCDO) hydrolase (homologue of IolD), SCO6976 encodes a 5-deoxy-d-gluconic acid (5DG) isomerase (homologue of IolB), SCO6978 encodes a 2-deoxy-5-keto-d-gluconic acid (DKG) kinase (homologue of IolC), SCO2726 encodes the putative methylmalonate semialdehyde dehydrogenase (homologue of IoIA) and SCO6985 encodes a phytanoyl-CoA dioxygenase (Yu et al., 2015). A cluster-situated regulator SCO6974 controls the transcription of myo-inositol catabolic genes through binding to the upstream regions of SCO2727, SCO6985 and the intergenic region of SCO6978–SCO6979, and the binding sites contain a palindromic sequence (A/T)TGT(A/C)N(G/T)(G/T)ACA (A/T) (Yu et al., 2015). In this study, a GntR-like protein, SCO6256, which showed extensive similarity with SCO6974, was found to regulate myo-inositol catabolic gene transcription as well as antibiotic biosynthesis in Strep. coelicolor A3(2).

**METHODS**

**Bacterial strains, plasmids and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table S1 (available in the online Supplementary Material). R2YE medium was used for the quantitative determination of the production of ACT and RED in Strep. coelicolor M145 (SCP1 SCP2) as the WT strain and its derivatives, as well as for RNA preparation (Kieser et al., 2000). Yeast extract–malt extract liquid medium (YEME) was used for Streptomyces growth (Kieser et al., 2000). Mannitol soya flour (MS) medium and minimal medium with mannitol as the carbon source (MMM) were used for sporulation and intergeneric conjugation (Kieser et al., 2000). Minimal medium with 10 mM myo-inositol as the carbon source (MMI) was used for RNA preparation (Kieser et al., 2000; Yu et al., 2015). Difco nutrient agar (DNA) medium was used for the production of CDAs (Kieser et al., 2000). Staphylococcus aureus was used as the indicator for CDAs (Anderson & Wellington, 2001). For propagating plasmids, Escherichia coli JM109 was cultured at 37 °C in Luria–Bertani (LB) medium supplemented with antibiotics (100 µg ml−1 for ampicillin, 100 µg ml−1 for kanamycin and 100 µg ml−1 for apramycin) when necessary.

**DNA manipulation and conjugal transfer.** Chromosomal DNA and plasmids were extracted from Strep. coelicolor A3(2) or E. coli according to standard techniques (Kieser et al., 2000; Sambrook & Russell, 2001). E. coli ET15256/pUZ8002 was used for intergeneric conjugation as described by Kieser et al. (2000).

**Primers and PCR.** All primers used in this study are listed in Table S2. The PCRs were carried out using EasyTaq DNA polymerase (TransGen Biotech), EasyPfu DNA polymerase (TransGen Biotech), KOD FX (Toyobo) or KOD-Plus (Toyobo). The PCR conditions were as follows: an initial denaturation at 94 °C for 5 min, then 30 cycles of amplification (94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min), and an additional 10 min at 72 °C (68 °C for KOD FX and KOD-Plus). In consideration of the different DNA templates and primers, the annealing temperature and the elongation time were changed in some cases.
Construction of the SCO6256 disruption mutant and its complemented strain, the SCO6256 overexpression strain and the SCO6256–SCO6974 double disruption mutant. To construct the SCO6256 disruption mutant, a DNA fragment corresponding to the downstream region of SCO6256 (extending from position +693 to +2012 with respect to the SCO6256 translation start codon) was amplified by using the primers 6256DF/DR. Then, the amplified DNA fragment was inserted into the BamHI/XbaI sites of pBluescript KS+., giving pD1. A 993 bp DNA fragment containing the kanamycin resistance gene (Kan') was obtained by digesting pUC119 : : Kan' with BamHI/ EcoRI and inserted into the same sites of pD1 to generate pD2. The DNA fragment corresponding to the upstream region of SCO6256 (extending from position −1386 to −4 with respect to the SCO6256 translation start codon) was amplified by using primers 6256U/UR and ligated into the HindIII/EcoRI sites of pD2 to give pD3. A 3.7 kb DNA fragment was isolated by digesting pD3 with HindIII/XbaI, and then it was inserted into the pPKC1132 to generate pD6256. Subsequently, pD6256 was introduced into E. coli ET12567/pUZ8002, and then it was transferred to the WT and the SCO6974 disruption mutant (SCO6974DM) (Yu et al., 2015). After growth on MS agar at 28 °C for 144 h, the transformants were transferred to M9 medium supplemented with ampicillin (10 μg ml−1) or kanamycin (10 μg ml−1). The kanamycin-resistant and ampicillin-sensitive strains were selected as the SCO6256 disruption mutants (SCO6256DM) and the SCO6256–SCO6974 double disruption mutants (SCO6256DM–SCO6974DM). These strains were further confirmed by PCR using the primers 6256YF1/FR1 and 6256YF2/YR2, respectively.

For complementation analysis, the intact SCO6256 coding region with its putative promoter was amplified by using the primers 6256HF/HR. The 1200 bp amplified DNA fragment was verified by sequencing and inserted into the EcorV site of pSET152 (Kieser et al., 2000). The resulting complementation plasmid pH6256 was introduced into WT and SCO6256DM by conjugation to generate the SCO6256 overexpression strain (SCO6256OE) and the SCO6256 complemented strain (SCO6256CM), respectively. The plasmid pSET152 was also introduced into WT or SCO6256DM for use as the controls.

RNA isolation and real-time reverse transcriptase PCR (RT-PCR). RNA was isolated from WT, SCO6256DM, SCO6974DM (Yu et al., 2015) and SCO6256DM–SCO6974DM grown in R2YE medium or MMI for 24, 48, 72, 96, 120 and 144 h, as described previously (Liu et al., 2005). RNA was treated with DNase I (Promega) to remove the contaminating DNA, and then it was reverse transcribed into the complementary DNA with a PrimeScript RT reagent kit (TaKaRa) using random primers. Real-time PCR was carried out in a RealPlex2 MasterCycler (Eppendorf) by using Ultra SYBR mixture (CWbio) with the primers listed in Table S2. The conditions used were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The transcription of hrdB was used as an internal control. The relative transcriptional levels of tested genes were normalized to the downstream region of SCO6256 were amplified by using the primers 1386 to 693, and the transcription start codon) was introduced into the corresponding sites of pH6256. The plasmid was introduced into E. coli C43 for gene expression (Miron & Walker, 1996). The strains containing pET28a::SCO6256 or pET28a::SCO6256-N were cultured at 37 °C in 200 ml LB supplemented with kanamycin (100 μg ml−1) to an OD600 of 0.6. IPTG was added to a final concentration of 1 mM, and the cultures were incubated at 37 °C for an additional 3 h. After centrifugation (6000 g at 4 °C for 5 min), the cells were collected and washed once with binding buffer (20 mM Tris base, 500 mM NaCl, 5 mM imidazole, pH 7.9), and resuspended in 20 ml of the same buffer. After sonication on ice, the cell suspension was centrifuged (12 000 g at 4 °C for 20 min) and the supernatant was recovered. Then, the supernatant was subjected to Ni-NTA agarose chromatography (Novagen), and the purified His6-SCO6256-His6 or His6-SCO6256–SCO6974DM-N-His6 was separated from the whole-cell lysate. Then, the protein was concentrated and the buffer was changed to imidazole-free buffer (20 mM Tris base, 500 mM NaCl, pH 7.9) using an ultrafiltration device (Millipore).

For expression of SCO6974, the SCO6974 coding region was amplified by using the primers 6974OF/OR. The amplified DNA fragment was verified by sequencing and inserted into the EcoRI/Xhol sites of pGEX-6P-1 (GE Healthcare) to give the plasmid pGEX-6P-1::SCO6974. The plasmid was introduced into E. coli C43 for gene expression (Miron & Walker, 1996). The strain containing pGEX-6P-1::SCO6974 was cultured at 37 °C in 200 ml LB supplemented with ampicillin (100 μg ml−1) to an OD600 of 0.6. IPTG was added to a final concentration of 1 mM, and the cultures were incubated at 37 °C for an additional 3 h. After centrifugation (6000 g at 4 °C for 5 min), the cells were collected and washed once with PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4), and resuspended in 20 ml of the same buffer. After sonication on ice, the cell suspension was centrifuged (12 000 g at 4 °C for 20 min) and the supernatant was recovered. Then, the supernatant was subjected to Glutathione Sepharose (GE Healthcare) chromatography and the purified SCO6256–SCO6974GST (glutathione-S-transferase) was separated from the whole-cell lysate.

The purity of proteins was determined by Coomassie blue staining after SDS-PAGE in a 10 % (w/v) polyacrylamide gel. The purified protein was stored with 5 % (v/v) glycerol at −70 °C until used in the subsequent experiments.

Electrophoretic mobility shift assays (EMSAs). The EMSAs were performed as described previously (Yang et al., 2007). The primers were labelled with T4 polynucleotide kinase (Promega) and the DNA fragments used for 32P-labelled probes were amplified by PCR. DNA probes covering the upstream regions of SCO2727, SCO6256, SCO6985 and cdaR, and the intergenic region of SCO6978–SCO6979, were generated by PCR with the primers listed in Table S2. The hrdB probe was obtained by PCR with the primers hrdBPF/hrdBPR and was used as a negative control. During the EMSAs, the 32P-labelled DNA probes (1000 c.p.m.) were incubated individually with varying quantities of His6-SCO6256-His6 at 4 °C for 20 min in 20 μl buffer containing 1 μg poly(dI–dC) (Sigma), 20 mM Tris base (pH 7.5), 1 mM DTT, 10 mM MgCl2, 54 mM KCl, 0.5 mg calf BSA ml−1 and 5 % (v/v) glycerol. After incubation, the protein-bound DNA complex and the free DNA probe were separated by electrophoresis in non-denaturing 4.5 % (w/v) polyacrylamide gels, with a running buffer containing 45 mM Tris/HCl (pH 8.0), 45 mM boric acid and 1 mM EDTA, at 150 V for 1 h. After drying, the gels were exposed to BioMax radiographic film (Kodak).

DNase I footprinting assay with the fluororescence-labelled primers. DNase I footprinting assays were performed as described by Ziani et al. (2006). The forward primers were labelled with 6-carboxyfluorescein (FAM) and the reverse primers were labelled with hexachlorofluorescein (HEX). The upstream regions of SCO2727, SCO6256, SCO6985 and cdaR, and the intergenic region of SCO6978–SCO6979, were amplified using the relevant fluororescence-labelled primers listed in Table S2. The FAM–HEX-labelled probes were purified by using a Cycle Pure kit (Omega) and quantified with a...
Nanodrop 2000 (Thermo Scientific). For this assay, 100 ng probes were incubated in a total volume of 50 μl individually with varying quantities of His6-SCO6256-His6 in the same buffer as used in EMSAs. After incubation at 25 °C for 20 min, 1 μl of a solution containing 0.08 U DNAse I (Promega) and 5.5 μl 10 × DNAse I reaction buffer [400 mM Tris/HCl (pH 8.0), 100 mM MgSO4, 10 mM CaCl2] were added, followed by further incubation at 37 °C for 1 h. The reaction was stopped by adding 50 μl DNAse I stop solution (20 mM EGTA, pH 8.0). Samples were extracted with phenol/ chloroform and precipitated with ethanol, and the pellets were dissolved in 10 μl Milli-Q water. Then, the samples were sequenced by a company (Genolab), and the results were analysed with GeneMarker V1.80 (http://www.softgenetics.com).

Alterations of the SCO6256-binding sequence. To evaluate the importance of the identified SCO6256-binding sites, the conserved sequence (A/T)TG(T/AC)N(T/G)(G/T)ACA(A/T) in the upstream regions of SCO6256, SCO6277, and SCO6985, and the intergenic region of SCO6978–SCO6979, was changed into (A/T)TG(T/AC)N (G/T)/G(T)GCA(A/T) using the Easy Mutagenesis system (TransGen Biotech). The binding sequence AGTTTCGccAGCAGACAT in the upstream region of cdaR was also changed into AGCTAGGTTAAGTTACGACAT by using the Easy Mutagenesis system. The binding capacity of His6-SCO6256-His6 to the mutagenized probes was measured using EMSAs as described above.

Mutational analysis of the cdaR-binding site of SCO6256. The WT and the mutant promoter regions of cdaR were obtained using primers cda-PF1/PR1. Then, the fragments were digested with EcoRI/BamHI and inserted into the same sites of pSET152: : xyE (Pan et al., 2009). The resulting plasmids were introduced into WT and SCO6256DM by conjugation. The constructed strains were cultured in M9 medium, and incubated at 28 °C for 24, 48, and 72 h. The activity of catechol dioxygenase was detected as described previously (Pan et al., 2013).

Determination of antibiotic production. In order to assess the production of ACT and RED, 1 × 1020 spores of the variant strains were plated on cellophane discs laid on the surface of R2YE solid medium, and incubated at 28 °C for 24, 48, 72, 96, 120 and 144 h. Results were evaluated as described by Kieser et al. (2000). The CDA production in DNA medium was assayed as described by Anderson & Wellington (2001).

GST pull-down assay of SCO6256 and SCO6974. To detect the interaction of SCO6256 and SCO6974, the GST pull-down assay was performed as described elsewhere (Sambrook & Russell, 2001), with some modifications. A mixture containing 180 μM SCO6256-GST, 20 μl Glutathione Sepharose, 800 μl pull-down buffer [50 mM Tris, 150 mM NaCl, 1 mM Na2EDTA, 1 mM DTT, 0.5 % (v/v) Nonidet P-40, pH 7.4] and 100 μl BSA (100 × ) was incubated at 4 °C for 2 h. After centrifugation (3000 g at 4 °C for 1 min), the Sepharose was collected and washed once with pull-down buffer. Then, 3.6 μM His6-SCO6256-His6 was added into reaction buffer containing 20 μl Sepharose-SCO6256-GST, 800 μl pull-down buffer and 100 μl BSA (100 × ), which was incubated at 4 °C for 2 h. After centrifugation (3000 g at 4 °C for 1 min), the Sepharose was collected and washed five times with pull-down buffer. After adding 50 μl 1 × SDS loading buffer to the Sepharose and boiling for 5 min, the supernatant was collected through centrifugation (12 000 g for 1 min). Then the supernatant was used for Western blot analysis. The GST protein was used as a negative control.

Western blot analysis. Approximately 2 mg purified His6-SCO6256-His6, mixed with Freund’s complete adjuvant was injected into one healthy mouse three times (once per week). Antisera were collected from the mouse after 1 month and used as polyclonal antibodies against SCO6256. For Western blot analysis, the supernatant samples from GST pull-down experiments were separated by 10 % (w/v) SDS-PAGE and transferred to a PVDF membrane (Roche). Then, the proteins on the membrane were incubated with anti-SCO6256, which was hybridized with suitable horseradish peroxidase-conjugated secondary antibodies (Jackson), followed by detection with enhanced chemiluminescence (Pierce).

EMSA combined with Western blotting to detect the interaction of SCO6256 and SCO6974. A 100 μl mixture containing 35 μM SCO6974-GST, 2 μl PreScission protease (7escalpharmtech) and 10 μl cleavage buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM DTT) was incubated at 4 °C for 16 h to cleave the GST tag. His6-SCO6256-His6 was mixed with the DNA probe covering the intergenic region of SCO6978–SCO6979 in EMSA reaction buffer, and then various amounts of SCO6974 were added to perform the EMSA. After that, one gel was treated with SYBR dye to see the binding activity, and another identical gel was used for Western blotting analysis with anti-His-tag antibody to observe the band shift of SCO6256. A sample with BSA instead of SCO6974 and a sample without DNA probe were used as negative controls.

RESULTS

SCO6256 encodes a GntR-like protein

Based on sequence analysis, the deduced protein of SCO6256 (GenBank accession no. CAB66175.1) belongs to the metabolite-responsive GntR family of proteins, which contain a DNA-binding domain (WHtt_GntR) at the N-terminus, and a ligand-binding and oligomerization domain (UTRA domain) at the C-terminus. Sequence alignment showed that SCO6256 is highly conserved through Streptomyces species (Fig. S1). In Strept. coelicolor A3(2), SCO6256 showed 74 % identity with SCO6974 (GenBank accession no. CAB89305.1), which is the key regulator of myo-inositol catabolism, and 37 % identity with DasR (GenBank accession no. Q9K492.1) (Fig. S2), but its biological function remains unknown.

Disruption of SCO6256 increased the transcription of myo-inositol catabolic genes conditionally

As SCO6256 shows extensive similarity to SCO6974, SCO6256 could be involved with myo-inositol catabolism. To confirm this, SCO6256 was disrupted through homologous recombination (Fig. S3a). The expected mutants were selected randomly and verified by PCR. As shown in Fig. S3(b), a distinctive band of 1119 bp was amplified in the SCO6256 disruption mutant (SCO6256DM) and an 822 bp band appeared in the WT strain as predicted. A 132 bp DNA fragment from the coding region of SCO6256 was amplified in WT but not in SCO6256DM. These results confirmed that SCO6256 was replaced by the kanamycin-resistance cassette (Kan’). In R2YE medium, disruption of SCO6256 resulted in the transcriptional increase of the following myo-inositol catabolic genes, SCO2726, SCO6975, SCO6976, SCO6978 and SCO6698, while the transcription of SCO6984 and SCO69985 was not affected (Fig. 1).
A similar result has also been obtained in the SCO6974 disruption mutant (Yu et al., 2015). However, the transcription of these genes in SCO6256DM was almost the same as that in WT when MMI was used (Fig. S4). The data implied that SCO6256 regulates the transcription of myo-inositol catabolic genes in R2YE medium, but not in MMI.

**SCO6256 directly bound to the upstream regions of myo-inositol catabolic genes**

To determine whether SCO6256 plays a direct role in the regulation of myo-inositol catabolic gene transcription, EMSAs were performed as described previously (Yang et al., 2007). In our previous work, we demonstrated that the myo-inositol catabolism cluster contains four transcription units, SCO2727–SCO2726, SCO6978–SCO6974, SCO6979–SCO6983 and SCO6985–SCO6984 (Yu et al., 2015). As a result, probes covering the upstream regions of SCO2727, SCO6256 and SCO6985, and the intergenic region of SCO6978–SCO6979, were used in EMSAs (Fig. 2a). Binding activity was enhanced when the amounts of SCO6256 were increased (up to 120 nM) (Fig. 2b–e). SCO6256 bound to the intergenic region of SCO6978–SCO6979 to form four stable complexes, while it bound to the upstream region of SCO2727 to form three stable complexes and bound to the other regions to form one or two stable complexes (Fig. 2b–e). The specificity of the SCO6256 binding ability was examined with the addition of excess unlabelled probes. The addition of the excess unlabelled specific probes clearly abolished the binding ability of SCO6256 (120 nM) to the corresponding labelled fragments, while addition of the excess unlabelled non-specific probe (hrdB) could not abolish the binding ability of SCO6256 (Fig. 2b–e). Combined with the transcriptional analysis, this is indicative that SCO6256 could repress myo-inositol catabolic gene transcription through interaction

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**Fig. 1.** Transcriptional analysis of the myo-inositol catabolic genes in WT and SCO6256DM. The transcription levels of myo-inositol catabolic genes were detected in WT (white bars) or SCO6256DM (grey bars) by real-time RT-PCR after growth in R2YE medium for 24, 48, 72, 96 and 120 h. All real-time RT-PCR data were normalized according to the abundance of hrdB in each sample. Results represent the mean ± SD of three independent experiments.
with the upstream regions of myo-inositol catabolic genes directly in R2YE medium.

To identify the specific binding sites of SCO6256, DNase I footprinting assays were carried out with the upstream regions of myo-inositol catabolic genes. Protected regions were observed in the upstream regions of SCO2727, SCO6256 and SCO6985, and the intergenic region of SCO6978–SCO6979 (Fig. 3). The SCO6256-binding sequence in the intergenic region of SCO6978–SCO6979 included two palindromic sequences, while the binding sequence contained one palindromic sequence in the other three upstream regions (Fig. 3). Sequence analysis showed that the SCO6256-binding sites contain the palindromic sequence (A/T)TGT(A/C)N(G/T)(G/T)ACA(A/T) (Fig. S5). To evaluate the importance of the identified SCO6256-binding sites, the conserved binding sequence of SCO6256 was mutagenized to (A/T)TAG(A/C)N(G/T)(G/T)GCT(A/T). The base substitution analysis demonstrated that the binding capacity of SCO6256 to the mutagenized probes was abolished completely in comparison with their corresponding WT targets (Fig. S6). These results are indicative that the conserved sequence was essential for SCO6256-binding activity.
Disruption of SCO6256 enhanced ACT and CDA production through increasing the transcription of actII-ORF4 and cdaR in Strep. coelicolor A3(2)

In R2YE medium, disruption of SCO6256 did not affect the growth of Strep. coelicolor A3(2) (Fig. 4a). However, disruption of SCO6256 significantly increased the production of ACT, while the complemented strain could restore the ACT production to the WT level and the control strain SCO6256DM/pSET152 produced the same amount of ACT as that in SCO6256DM (Fig. 4b). Moreover, ACT production in the SCO6256 overexpression strain (SCO6256OE) was reduced to half of the WT level (Fig. 4b). In contrast, RED production in SCO6256DM remained at the same level as that of WT (Fig. S7). In DNA medium, the production of CDA in SCO6256DM was also clearly enhanced compared with that in WT (Fig. 4c). In agreement with antibiotic production, the transcription of actII-ORF4 and cdaR, which encode the cluster-situated regulators of ACT and CDA biosynthesis, were significantly increased in SCO6256DM (Fig. 4d, e). These results indicated that SCO6256 repressed the biosynthesis of ACT and CDA via regulation of the transcription of actII-ORF4 and cdaR.

SCO6256 directly controlled CDA biosynthesis but regulated ACT production indirectly

Since the transcription of actII-ORF4 and cdaR was repressed by SCO6256, these genes could be the targets of SCO6256. EMSAs demonstrated that SCO6256 directly bound to the upstream region of cdaR (Fig. 5a, b), suggesting SCO6256 controlled CDA biosynthesis through cdaR directly. DNase I footprinting assays revealed SCO6256 bound to a palindromic sequence (AGTTTCGG-CACGCAGACAT) in the upstream region of cdaR (Fig. 5c). To confirm this, the binding sequence of SCO6256 was changed into AGCTAGGGTACTTAGACCT. SCO6256 could not bind to the mutagenized probe (Fig. 5d), suggesting this palindromic sequence was essential for SCO6256 binding to the upstream of cdaR. To test whether this binding was essential for the transcriptional repression in vivo, the catechol 2,3-dioxygenase-encoding gene xylE was used as a reporter and was placed under the control of the promoter region of cdaR. 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Fig. 4. Disruption of SCO6256 enhanced the production of ACT and CDA in Strep. coelicolor A3(2). (a) Growth curves of WT, WT/pSET152, SCO6256DM, SCO6256DM/pSET152, SCO6256CM and SCO6256OE. Biomass was calculated as the mycelium dry weight in R2YE medium. (b) ACT production of WT, WT/pSET152, SCO6256DM, SCO6256DM/pSET152, SCO6256CM and SCO6256OE in R2YE medium. Cell cultures at each time point were treated with KOH (final concentration 1 M) and the $A_{640}$ corresponding to 10 mg mycelium was determined. (c) CDA production of WT and SCO6256DM at 48 h and 72 h in DNA medium. Staph. aureus was used as the indicator strain in the presence or absence of Ca(NO$_3$)$_2$. (d) Transcription of actII-ORF4. The transcription level of actII-ORF4 in WT (white bars) and SCO6256DM (grey bars) was detected by real-time RT-PCR after growth in R2YE medium for 24, 48, 72, 96, 120 and 144 h. (e) Transcription of cdaR. The transcription level of cdaR in WT (white bars) and SCO6256DM (grey bars) was detected by real-time RT-PCR after growth in DNA medium for 24, 48 and 72 h. All real-time RT-PCR values were normalized according to the abundance of hrdB in each sample. Results represent the mean ± SD of three independent experiments.
SCO6256, as expected. This indicated that the SCO6256 binding to the promoter of cdaR was essential for the transcriptional repression in vivo.

However, no band shift was found in EMSAs when using the upstream region of actII-ORF4 as a probe (Fig. 5f), indicating that SCO6256 repressed ACT biosynthesis indirectly. In agreement with this, no conserved sequence similar to the SCO6256-binding site was found upstream of actII-ORF4. To look for the targets of SCO6256, the genome database of Strep. coelicolor A3(2) was checked based on the conserved sequence of the SCO6256-binding sequence. The upstream regions of 40 genes other than myo-inositol catabolic genes contained the conserved sequence:

SCO6256 regulates myo-inositol catabolic genes

Fig. 5. SCO6256 directly regulates CDA production in Strep. coelicolor A3(2). (a) Organization of cdaR. The probe used in EMSAs is indicated by a black bar. (b) SCO6256 bound to the upstream region of cdaR. The upstream region of cdaR was incubated with increasing amounts of SCO6256 (lanes 1–6 contained 0, 30, 60, 150, 300 and 600 nM, respectively). Lanes 7–9, EMSA of 150 nM SCO6256 with 50-, 100- and 200-fold excess of labelled probe and unlabelled specific competitor probe. Lanes 10 and 11, EMSA of 150 nM SCO6256 with 100- and 200-fold excess of labelled probe and unlabelled non-specific competitor hrdB. The arrows indicate the free probe and SCO6256–DNA complex. (c) Identification of the SCO6256-binding sequence by DNase I footprint analysis of the upstream region of cdaR. The control reaction is shown in the upper electropherogram and the protected nucleotide sequence is underlined. (d) Mutational analysis of the SCO6256-binding site. BS, Probe without mutagenesis as a control – the sequence is AGTTTCGGCACGCAGACAT; M, probe with base substitution – the sequence is AGCTAGGGTACTTAGACCT. (e) Promoter activity of cdaR with the mutant SCO6256-binding site. Error bars represent the SD from three independent experiments. (f) EMSA of SCO6256 with the upstream region of actII-ORF4.
sequence, but only 4 of them could be bound by SCO6256 (Fig. 6a). These genes were SCO2652, SCO4034, SCO4237 and SCO6377. Consistent with these results, the transcription of all these genes was increased in the SCO6256 disruption mutant (Fig. 6b). SCO2652 encodes a putative BadM/Rrf2 family transcriptional regulator. SCO6377 encodes a lipoprotein with unknown function. SCO4237 encodes a possible integral membrane protein. SCO4034 encodes a sigma factor that is involved in morphological differentiation and positively regulates the production of ACT (Wang et al., 2010). Therefore, disruption of SCO6256 might enhance ACT production through increasing the transcription of these genes.

**SCO6974 negatively regulated the transcription of SCO6256**

Since both SCO6256 and SCO6974 bind to the same sites in the myo-inositol catabolic gene cluster, it was of interest to check the relationship between SCO6256 and SCO6974. The transcriptional levels of SCO6256 and SCO6974 were compared (Fig. 7a, b). In MMI, the transcriptional level of SCO6256 was only one-eighth of the SCO6974 transcriptional level (Fig. 7a), while in R2YE medium, the transcriptional levels of SCO6256 and SCO6974 were similar (Fig. 7b). Disruption of SCO6974 significantly increased the transcription of SCO6256 in MMI (Fig. 7c), indicating that SCO6974 represses the expression of SCO6256. In contrast, disruption of SCO6256 did not affect the transcription of SCO6974 in MMI (Fig. 7d). Considering that the transcription of SCO6974 was eight times more than that of SCO6256, it is possible that SCO6974 plays the key role in repression of myo-inositol catabolism in MMI. However, SCO6256 and SCO6974 did not affect the transcription of each other in R2YE medium (Fig. 7e, f).

EMSAs demonstrated that SCO6974 bound to the upstream region of SCO6256 (Fig. 8a), suggesting that SCO6974 directly regulates the transcription of SCO6256. DNase I footprinting assays revealed that the SCO6974-binding sequence includes a palindromic sequence (ATGT-CCGGACAA) which is similar to the conserved binding sequence of SCO6974 in the myo-inositol catabolic gene cluster (Fig. 8b). Base substitution analysis further confirmed that the sequence was essential for the binding activity of SCO6974 to the upstream region of SCO6256 (Fig. 8c). Like GntR proteins, the C-terminus of both SCO6974 and SCO6256 contains the ligand-binding and oligomerization domain. To detect whether SCO6974 and SCO6256 interact with each other, a GST pull-down assay was carried out (Fig. 8d). The pull-down assay showed that SCO2652 could interact with SCO6974 directly. EMSAs combined with Western blotting further indicated that SCO6256 and SCO6974 could interact with each other (Fig. S8). These results suggested that SCO6256 directly interacts with SCO6974, and the regulation by SCO6974 and SCO6256 on the transcription of myo-inositol catabolic genes is complex.

![Fig. 6. The putative targets of SCO6256. (a) EMSAs of SCO6256 with the upstream regions of the putative target genes. The amounts of SCO6256 (nM) used in this experiment are indicated. (b) Transcriptional analysis of the target genes. All real-time RT-PCR values were normalized according to the abundance of hrdB in each sample. The white bars represent the gene transcription in WT and the grey bars represent the gene transcription in SCO6256DM. Results represent the mean ± SD of three independent experiments.](image-url)
**DISCUSSION**

SCO6256 was found to repress the transcription of *myo*-inositol catabolic genes in R2YE medium, but not in MMI. Our previous work demonstrated that SCO6974 is the key repressor of *myo*-inositol catabolism in MMI (Yu et al., 2015). SCO6974 also represses the transcription of SCO6256 through binding to its upstream region.

The transcription of *myo*-inositol catabolic genes in SCO6256DM–SCO6974DM was similar to that in SCO6974DM in MMI (Figs S9 and S10a), confirming that SCO6974 is the key regulator of *myo*-inositol catabolism in MMI. SCO6256 showed extensive similarity with SCO6974 (Fig. S2), and the binding sequence for SCO6256 was the same as that for SCO6974 in the *myo*-inositol catabolic
gene cluster (Figs 2 and 3). GST pull-down assays and EMSAs combined with Western blotting demonstrated SCO6256 directly interacted with SCO6974 (Fig. 8d), and the transcription of myo-inositol catabolic genes (except for SCO6984) in SCO6256DM–SCO6974DM significantly increased when compared to that in SCO6256DM or SCO6974DM in R2YE medium (Figs 1 and S10b), indicating that SCO6256 interacts with SCO6974 to regulate the transcription of myo-inositol catabolic genes in R2YE medium.

The transcription of SCO6984 and SCO6985 in SCO6256 DM, SCO6974DM and SCO6256DM–SCO6974DM was the same as that in WT (Figs 1, S4 and S10b), indicating that SCO6256 interacts with SCO6974 to regulate the transcription of myo-inositol catabolic genes in R2YE medium.

SCO6256 contains the UTRA domain, which is responsible for the first step of myo-inositol catabolism, it is easy to understand that expression of SCO6984 is highly controlled to keep the myo-inositol balance in Streptomyces. SCO6256 was shown to bind to its own upstream region, which means it might be regulated by itself since many members of the GntR regulator family have a negative autoregulatory nature (Hoskisson & Rigali, 2009).

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**Fig. 8.** SCO6974 directly regulates the transcription of SCO6256. (a) SCO6974 bound to the upstream region of SCO6256. The probe containing the upstream region of SCO6256 was incubated with increasing amounts of SCO6974 (lanes 1–6 contained 0, 40, 80, 200, 400 and 2000 nM protein). EMSAs of 200 nM SCO6974 with 10- and 100-fold excess of unlabelled specific probe are shown in lanes 7 and 8, and with 100-fold excess of non-specific competitor hrdB in lane 9. The arrows indicate the free probes and the brace shows SCO6974–DNA complexes. (b) Identification of the SCO6974-binding site by DNase I footprint assays of the upstream region of SCO6256. The control reaction is shown in the upper electropherogram and the protected nucleotide sequence is underlined. (c) Mutational analysis of the SCO6974-binding site. BS, Probe without mutagenesis as a control; M, probe with base substitution. (d) GST pull-down assay of SCO6256 and SCO6974. 1, SCO6256 protein used as positive control; 2, test sample; 3, sample from a pull-down assay of GST protein and SCO6256 used as negative control.
solution (Kataoka et al., 2008). SCO6256 may also form these polymers when binding to its targets. As a result, although there is only one binding site, multiple protein–DNA complexes could be detected in EMSAs.

The promoter sequences and transcriptional start points of SCO2727, SCO6256, SCO6978–SCO6979 and SCO6985 were predicted using an online tool (http://www.fruitfly.org/seq_tools/promoter.html). However, the promoter sequences of SCO6256 and SCO6985 were predicted at low scores, which may be unreliable. The promoter sequences of SCO2727, SCO6978 and SCO6979 were predicted at high scores. The binding sequences in the upstream regions of SCO2727 and SCO6978 overlapped with their predicted promoter sequences. The binding sequence in the upstream regions of SCO6979 overlapped with its predicted promoter sequence and stretched into its 5′-untranslated region (Fig. S12). Based on the analysis, SCO6256 regulated the transcription of these genes mainly through affecting the recruitment of RNA polymerase to their predicted promoter regions.

The SCO6256-binding sequences in the upstream regions of myo-inositol catabolic genes contain the palindromic sequence (A/T)TGT(A/C)N(G/T)(G/T)ACA (A/T), and this is consistent with the sequence 5′-(N)9 GT(N)9 AC(N)9-3′, which is the consensus sequence of the GntR regulator family for binding (Rigali et al., 2002). The SCO2727-binding sequence in the cddlR promoter region is AGTTTCGGCACG-9543–9556, which also conforms to the sequence 5′-(N)9 GT(N)9 AC(N)9-3′. So SCO6256 may recognize variable sequences for different groups of its target genes.

According to the conserved binding sequence of SCO6256, four putative targets have been found by genome screening. Of them, SCO4034 encodes SigN, which is involved in morphological development, secondary metabolism and stress responses of Strep. coelicolor (Wang et al., 2010). Disruption of sigN affects the sporulation, and decreases the production of ACT and RED (Wang et al., 2010). We do not know whether other targets are related to ACT production, but it is possible that SCO6256 controls ACT biosynthesis via regulating the transcription of these regulatory genes including sigN. As disruption of SCO6256 also increased the transcription of myo-inositol catabolic genes in R2YE medium, the possibility that the enhanced myo-inositol catabolism produced more acetyl-CoA, which is the precursor of ACT biosynthesis (Ryu et al., 2006), could not be excluded.

In conclusion, both SCO6256 and SCO6974 are involved in the regulation of myo-inositol catabolic gene transcription, and they may regulate their transcription cooperatively. SCO6256 is also involved in the biosynthesis of antibiotics of Strep. coelicolor A3(2).

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