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ORIGINAL PAPER

Mass spectrometric screening of transcriptional regulators involved in antibiotic biosynthesis in *Streptomyces coelicolor* A3(2)

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Abstract DNA-affinity capture assay (DACA) coupled with liquid chromatography–tandem mass spectrometry analysis was applied to identify the transcriptional regulators involved in the biosynthesis of actinorhodin (Act) and undecylprodigiosin (Red) in *Streptomyces coelicolor*. The aim of this analysis was to determine the specific transcriptional regulators binding to the promoter region of *actII-ORF4* or *redD*. The results of the DACA, as the first screening tool, identified eight proteins, including AdpA, as candidate regulators binding to those promoter regions. To show the direct physical relationship between the regulators and promoters, we purified four regulators over-expressed in soluble form in *Escherichia coli* and subjected these to an electrophoretic mobility shift assay (EMSA). The results of the EMSA appeared to be compatible with the DACA results for those regulators. A null mutant was also constructed for one of these regulators, SCO6008, which showed early Red production and quite delayed Act production in $R5^{-}$ medium. These observations suggest that DACA can be widely used to find new regulators and that the regulator SCO6008 may be involved in antibiotic production through its binding to the *redD* promoter.

Keywords Actinorhodin · DNA affinity capture assay · *Streptomyces coelicolor* · Transcriptional regulator · Undecylprodigiosin

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Introduction

Streptomycetes are Gram-positive soil bacteria that produce numerous secondary metabolites and undergo unique morphological and physiological differentiation. *Streptomyces coelicolor* A3(2) is the best-characterized streptomycete genetically, and its entire genome has been sequenced, revealing that it has 965 proteins predicted to have regulatory function [1]. This strain produces at least four distinctive antibiotics: actinorhodin (Act, blue-pigmented aromatic polyketide), undecylprodigiosin (Red, red-pigmented tripyrolle), calcium-dependent antibiotic (CDA, non-ribosomal peptide antibiotics), and methylenomycin (cyclopentanone antibiotics). Only the methylenomycin biosynthetic gene cluster is located in the plasmid DNA (SCP1) of *S. coelicolor*. The most frequently studied antibiotics in this strain are Act and Red, whose pathway-specific transcriptional activators are *actII-ORF4* and *redD*, respectively [20]. The transcription levels of these activators increase dramatically during the transition period from exponential to stationary phase in liquid culture, followed by the transcription of their corresponding biosynthetic genes and the production of Red and Act [8].

The production of antibiotics in *Streptomyces* is regulated at multiple levels and is closely related to other cellular events, such as sporulation. The primary level of cell regulation related to antibiotic production is provided by a

number of multifunctional signaling molecules controlling the primary cell metabolism, such as ppGpp [3], S-adenosyl-L-methionine [18], and cyclic AMP [32]. These latter molecules have been reported to be regulators of both antibiotic production and global cellular physiology. However, the signal transduction pathways mediated by these molecules still remain to be determined.

The secondary level of cell regulation related to antibiotic production is governed by genes showing pleiotropic effects on the production of one or more antibiotics. Mutations of *bld* loci pleiotropically block the biosynthesis of antibiotics as well as the production of sporulating aerial hyphae [4]. Several two-component regulatory systems, such as AfsQ1-Q2 [15], AbsA-B [2], and AfsK-R [34], play roles in the regulation of antibiotic production, but the extracellular signals or environmental stresses which trigger these regulatory systems have not been fully investigated. Among the regulatory pathways which have been identified to be active in antibiotic production, the most dramatic and well-known is the signaling by γ-butyrolactone, an autoregulator of *Streptomyces* species. This autoregulator acts as a microbial hormone to control the onset of antibiotic production and complex morphological differentiation. For example, A-factor, which is required for streptomycin production and cell differentiation in *S. griseus*, binds to A-factor receptor protein (ArpA), and the binding results in the dissociation of ArpA from the promoter region of *adpA* [23]. The A-factor-responsive transcriptional activator (AdpA) is subsequently expressed and in turn activates the pathway-specific transcriptional activator for the streptomycin biosynthesis genes and the genes essential for the aerial mycelium formation [37]. In *S. coelicolor*, a γ-butyrolactone, SCB1, and AdpA ortholog have all been studied, but their roles have been reported to be quite different. Although SCB1 elicits precocious Act and Red production, a mutant that does not produce SCB1 is still able to produce Act and Red. And although no obvious Act production was observed in an *adpA* mutant, the mutant was able to produce red pigment on certain media [33]. These reports indicate that complex mechanisms associated with SCB1 and AdpA are involved in the production of antibiotics in *S. coelicolor*. Recently, other A-factor-like signaling molecules, 2-alkyl-4-hydroxymethylfuran-3-carboxylic acids (AHFCAs), were elucidated in *S. coelicolor* [5]. It has been proposed that the *mmfLHP* genes, which are located on the plasmid SCP1 of *S. coelicolor* A3(2), direct the biosynthesis of AHFCAs, which induce methylenomycin production.

The last level of cell regulation related to antibiotic production is represented by pathway-specific regulators affecting one specific antibiotic, such as *actII-ORF4* for Act, *redD* for Red, *strR* for streptomycin [6], *dnrI* for daunorubicin [31], and *ccaR* for clavulanic acid and cephamycin C [13]. These pathway-specific regulators belong to the superfamily of *Streptomyces* antibiotic regulatory proteins (SARPs). To date, the properties of such regulators, which bind to their promoter regions, have not been clearly elucidated.

The study reported here was initiated to identify unknown transcriptional regulators involved in the production of antibiotics, using Act and Red as model systems. To identify the antibiotic transcriptional regulatory mechanism, we recently applied the decoy oligonucleotide technique to the promoter regions of *actII-ORF4* [19]. As a complementary research project, we used the DNA-affinity capture assay (DACA), which we had successfully applied in one of our earlier studies to show that the *lac* promoter binding regulatory proteins can be successfully identified in an *Escherichia coli* system [24]. In order to identify putative transcriptional regulators binding to the promoter region of *actII-ORF4* or *redD*, we used the same DACA approach in this investigation. We expanded our analysis of several regulators to clarify their relationships to antibiotic production by using an electrophoretic mobility shift assay (EMSA) and found that one of them was characterized with a deletion mutant. Our results reveal transcriptional regulators to be novel and interesting candidates for antibiotic biosynthesis, which await investigation in further studies.

Materials and methods

Bacterial strains

Escherichia coli K-12 strains DH5α, BL21 (DE3), JM110 (from KTCC, Korean Collection for Type Cultures, Korea), ET12567, and BW25113 were grown in LB media at 37°C. Strains DH5α and BL21 were used for subcloning and for the target gene expression analyses, respectively, and strains JM110 and ET12567 were used to propagate the unmethylated plasmid and cosmid DNA, respectively. *Streptomyces coelicolor* A3(2) strain M145 [17], which was used for the DACA and for constructing the deletion mutant, was cultured according to the standard procedures of Kieser [17]. Fresh spores of the M145 strain were collected on R5 agar plate and inoculated into R5 or R5$^-$ (R5 lacking KH_2PO_4, $CaCl_2$, and L-proline [10]) liquid medium.

Preparation of protein extracts

Production of the respective antibiotic by the *S. coelicolor* culture was recognized by a color change in the culture medium—i.e. the medium became red due to the presence of the red-pigmented undecylprodigiosin and then purple due to the blue-pigmented actinorhodin. The cells were harvested at this time, and the production of the antibiotics confirmed by Spectrophotometry of the extracted Red and Act.

In the case of the *redD* promoter, the sample for DACA was taken at the time when the culture broth started to become red, which occurred approximately 2 days after the inoculation of M145 spores to $R5^-$ medium. In the case of the *actII-ORF4* promoter, the sample for DACA was taken at the time when the culture broth started to become purple, which was about a half day after the production of Red was detected. The harvested cells were washed twice with phosphate buffered saline [PBS: 137 m*M* NaCl, 10 m*M* phosphate, 2.7 m*M* KCl, pH 7.4), suspended in Buffer II (20 m*M* HEPES, pH 7.8, 10% w/v glycerol, 100 m*M* KCl, 0.05 m*M* EDTA, 0.5 m*M* dithiothreitol (DTT), 0.01% Nonidet P-40, 1 m*M* PMSF, 1 m*M* benzamidine, 1 μg/ml leupeptin, and 1 μg/ml pepstatin] with 0.5 *M* NaCl, and then subjected to ultrasonic disruption for 20 min (10-s pulse at 10-s intervals duration with 10 s interval). Cell debris was removed by centrifugation at 13,000 *g*. Protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA), and samples were stored at −20°C.

DNA-affinity capture assay

The DACA procedure follwed is outlined in Fig. 1. The promoter region of *actII-ORF4* or *redD* (Fig. 2) was generated by PCR using biotinylated primers and their pairs (Pa-s and Pa-as for *actII-ORF4*; Pr-s and Pr-as for *redD*; Table 1). The PCR products were purified using PCR and gel purification spin kits (Cosmo, Korea), and the concentrations of the purified DNA were determined using a UV spectrophotometer. A detailed description of the DACA procedure is given in was described in Park et al. [24]. Streptavidin Dynabeads (Dynal Biotech, Oslo, Norway) were washed three times in Buffer I (20 m*M* Tris pH 8.0, 20 m*M* $MgCl_2$, 200 m*M* KCl). Annealed oligonucleotides were incubated with the washed beads (100 pmol/mg of beads) for 30 min at room temperature in Buffer I, followed by the addition of biotin (100 μg/ml) and another 15 min of incubation. One milliliter of a pre-mixed solution of cell extract (500 μg/ml) and sheared salmon sperm DNA (0.1 mg/ml) was incubated for 15 min on ice and then added to 100 μl of the bead solution (0.5 mg beads/100 μl Buffer II). After a 40-min incubation at room temperature, the beads were washed four times with Buffer II and once with 100 m*M* NH_4HCO_3 pH 8.0 and then resuspended in 100 m*M* NH_4HCO_3. The protein mixtures captured on the beads were digested with trypsin.

Tryptic digestion

The suspended beads were incubated in 10 m*M* DTT for 30 min at 56°C. After cooling to room temperature, the bead solution was treated for 30 min at room temperature

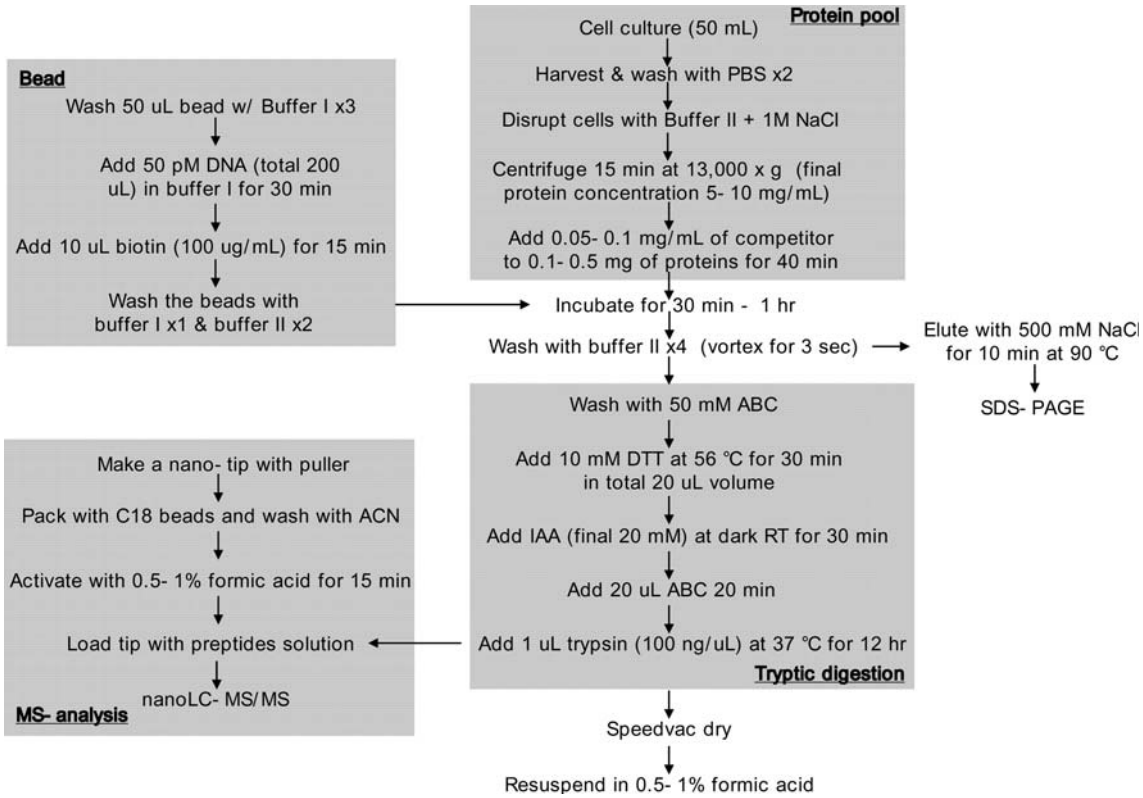

Fig. 1 Scheme of DNA-affinity capture assay for *Streptomyces*. *DTT* Dithiothreitol, *IAA* iodoacetamide, *PBS* phosphate buffer saline, *ABC* ammonium bicarbonate, *RT* room temperature, *LC–MS/MS* liquid chromatography–tandem mass spectrophotometry

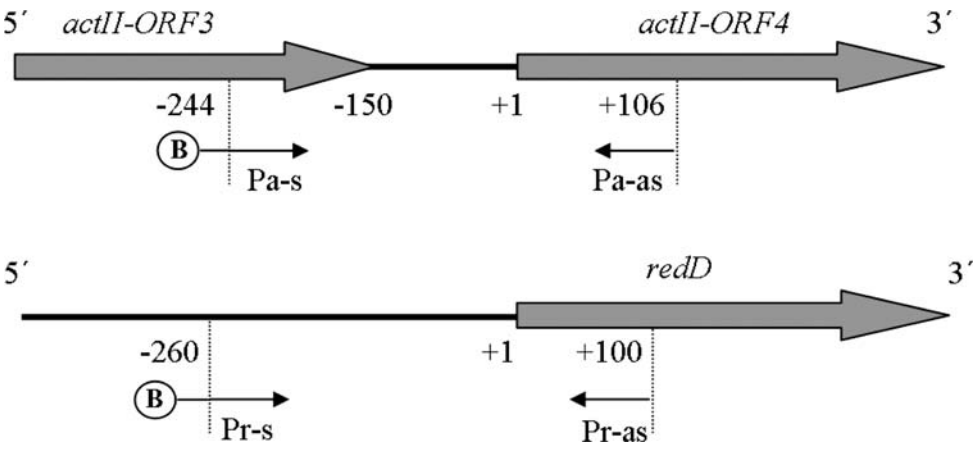

Fig. 2 The promoter regions of *actII-ORF4* and *redD* used in this study. The position of the nucleotides was counted from the start codon of the target gene (+1). *Small arrows* Primers for PCR amplification, *Encircled character B* biotin modifications of primers. The reported transcriptional start point of each promoter is located at −32 of *actII-ORF4* and +23 of *redD*

Table 1 Primer sequences used in this study

Primer name	Sequence (5′ → 3′)
Pa-s	Biotin-CGCTCGCCCGGCGCGAGGACCCTTC
Pa-as	TGAGGAGCAGCAGCACCAGGAGCTG
Pr-s	Biotin-ACCACTCTTCTTCTCTGCCCTCTGA
Pr-as	CGTCCTGACCGGCCGCGACGGGGTG
PadpA-s	CGTggatcc**ATG**AGCCACGACTCCACCGC
PadpA-as	ACGaagctt**TCA**GTGGTGGTGGTGGTGGTGCGGCGCGCTGCGCTGGC
P1480-s	CGTggatcc**GTG**GCTCTTCCGCCCCTTAC
P1480-as	ACGaagctt**TCA**GTGGTGGTGGTGGTGGTGGCTGCCGGTGCTGCCGA
P5405-s	CGTggatcc**ATG**GAGACCGAGACGGCCAC
P5405-as	ACGaagctt**TCA**GTGGTGGTGGTGGTGGTGGGGTCGTCCCCGCTGCC
P6008-s	CGTggatcc**ATG**GAGACTCCCGGGTCGCA
P6008-as	ACGaagctt**CTA**GTGGTGGTGGTGGTGGTGAGTGAAGGCAGGCGTGG
Pd6008-s	GGGTGACGCGGACCCAGGGGATGAGGGTGAGAGTCAATGATTCCGGGGATCCGTCGACC
Pd6008-as	AATCCTTAACGAGATGACAACGGTGCCATCCGTGCTCTATGTAGGCTGGAGCTGCTTC

The lowercase letters indicate *Bam*HI and *Hind*III sites; the bold letters indicate the start and termination codons of each gene; the underlined letters indicate the codons for 6× His-tag

with additional iodoacetamide (final 20 m*M*) for carboxyamidomethylation. For in-solution digestion, 100 ng/0.5 mg beads of sequencing grade trypsin (Promega, Madison, WI) in 100 m*M* NH_4HCO_3 was added, and proteins were digested overnight at 37°C. Tryptic peptides were collected, dried under a vacuum drier, and stored at −20°C for analysis by liquid chromatography–tandem mass spectrophotometry (LC-MS/MS).

Mass spectrometry

Peptides were pressure-loaded onto 11 cm × 100-μm fused silica capillary needles packed with 5 μm C18 beads (Vydac 218TP51; Grace, Deerfield, IL) and equilibrated with 0.5% formic acid. The peptides were eluted in a 0–90% acetonitrile (ACN; 0.5% formic acid) gradient solution for 60 min at 240 nl/min and analyzed by LC-MS/MS using an LCQ Deca XP ion trap mass spectrometer (Thermo Finnigan; Thermo Fisher, Waltham, MA) as described. Peptide ions were detected in a full scan mode from 400 to 1700 *m/z* followed by three data-dependent MS/MS scans (isolation width: 2.5 *m/z*, 35% normalized collision energy, dynamic exclusion for 5 min) in a completely automated fashion.

Data analysis

Proteins were identified by searching MS/MS spectra against a protein database of *S. coelicolor* using SEQUEST, where the following criteria were used to sort out proteins from MS spectra: First, the cross-correlation score (Xcorr) should be >1.7 for +1 charged tryptic peptides, >2.5 for +2 charged peptides, or >3.0 for +3 charged peptides. Second, the delta correlation value (ΔCn) should be at least 0.15,

regardless of the charge state. Third, two or more peptides from a protein should be identified in order to be regarded as an accepted SEQUEST result.

Protein over-expression in *E. coli*

The 6× His-tagged fusion proteins for the four putative regulators (i.e., AdpA, SCO1480, SCO5405, and SCO6008) were constructed by amplifying the coding region by PCR using *S. coelicolor* chromosomal DNA as a template and the following primers: PadpA-s and PadpA-as for *adpA*; P1480-s and P1480-as for SCO1480; P5405-s and P5405-as for SCO5405; P6008-s and P6008-as for SCO6008 (Table 1). The amplified fragments were digested with *Bam*HI plus *Hin*dIII and cloned into pET24ma. After no errors in PCR had been found by nucleotide sequencing, the constructed plasmids were transformed into *E. coli* BL21. Cells harboring each plasmid were grown at 37°C in 50 ml LB broth supplemented with 50 μg/ml kanamycin. When the culture reached an absorbance of about O.D. 0.3, protein expression was induced with 0.5 m*M* isopropyl thio-*β*-D-galactopyranoside for 6 h, following which the cells were washed twice with 20 ml ice-cold PBS, resuspended in 5 mL Buffer II supplemented with 20 m*M* imidazole, and then sonicated for 20 min in an ice bath. Cell debris was removed by centrifugation. The resultant supernatant was applied to an in-house-made mini-column packed with Ni-NTA agarose resin (Qiagen, Valencia, CA) that had been equilibrated with 20 m*M* imidazole buffer (50 m*M* phosphate buffer pH 8.0, 0.01% Tween 20, and 100 m*M* NaCl). His-tagged proteins were eluted with 1 ml of Buffer II supplemented with 250 m*M* imidazole. The concentration of the purified protein was measured using the Bradford assay, and the samples were stored at −20°C.

Electrophoretic mobility shift assay

The double-stranded oligonucleotides of the *actII-ORF4* or *redD* promoter region (the same PCR product used in DACA) were radiolabeled with T4 polynucleotide kinase in the presence of [γ-^{32}P]-dATP and the unreacted γ-[^{32}P]-dATP was removed using ProbeQuant G-50 Micro Columns (Amersham Biosciences, Piscataway, NJ). The labeled probes were incubated with purified His-tagged proteins for 30 min at room temperature in a total volume of 20 μl of 20 m*M* HEPES buffer (pH 7.8) containing 10% w/v glycerol, 100 m*M* KCl, 0.05 m*M* EDTA, 5 m*M* $MgCl_2$, 0.5 m*M* DTT, 0.01% Nonidet P-40, and 2 μg sheared salmon sperm DNA (ssDNA). Protein-bound and -free DNAs were resolved on 5% acrylamide gels in 0.5× TBE buffer at room temperature. The gels were dried and analyzed with a Typhoon 8600 multimode scanner (Molecular Dynamics, Sunnyvale, CA).

Construction of deletion mutants

A mutant *SC7B7.05* allele, in which the complete coding region of SCO6008 excluding start and termination codons was deleted and replaced with an apramycin resistance gene, was constructed by PCR targeting using oligonucleotide primers (Pd6008-s and Pd6008-as in Table 1) with the 5′ ends overlapping the upstream (36 bp) and downstream (36 bp) of the SCO6008 coding sequence, and 3′ (priming) ends designed to amplify the apramycin resistance disruption cassette of pIJ773. The mutant was constructed as described in Gust et al. [9]. Deletions of SCO6008 were confirmed by sequencing the corresponding PCR products. One colony was isolated, which was further confirmed by complementation with the SCO6008 overexpression vector under the *ermE* promoter (pWHM3 derivative pSE34; a gift from Prof. Yeo Joon Yoon).

Results and discussion

Proteins identified using the DACA

Biotinylated probes of the *actII-ORF4* or *redD* promoter regions were generated by PCR (Fig. 2) and immobilized to Dynabeads streptavidin. These beads were used to capture DNA-specific proteins from protein extracts that were prepared using the cells starting to produce each antibiotic. All of the binding proteins on beads were digested with trypsin, and the resulting total peptides were analyzed by LC-MS/MS. Several putative transcriptional regulators as well as RNA polymerase (Table 2) were detected. However, several bound proteins appeared to be nonspecific since their annotated functions included DNA replication and recombination and cellular metabolism (Table 3). SCO0200 and SCO2599 are known to be conserved hypothetical proteins, and a BLAST search was performed to obtain additional information on these proteins. SCO2599 shows a similarity to the center region of CafA and ribonuclease G and E (score 419; E-value 1e-177) and SCO0200 to the N-terminal region of the universal stress protein (Usp) family (score 72.1; E-value 8e-14). In the case of putative dihydrolipoamide succinyltransferase (SCO2181) and the putative acyl-CoA carboxylase complex A subunit (SCO4921), there is biotin–lipoyl attachment domain based on information on its *E. coli* homologs [26, 28]. These may bind to empty biotin-binding sites of streptavidin on the beads. SCO5748, putative sensory histidine kinase, had some potential to be involved in gene regulation. However, this protein was commonly detected in other DACA experiments we performed; therefore, we also regarded these as nonspecific bindings. These nonspecific proteins were checked further using PCR products that were obtained

Table 2 Identified sequence of RNA polymerase

Protein	Protein ID	%C	Sequence of identified peptides	Score
DNA-directed RNA polymerase beta chain	SCO4654	8.8	VFDREEGDELPPGVNQLVR	110.2
			IGAEVVAGDILVGK	
			DIPNVSEEVLADLDER	
			INPIEDMPFLEDGTPVDIILNPLAVPSR	
			AGVVQEVSADYITTTNDDGTYITYR	
DNA-directed RNA polymerase beta′ chain	SCO4655	6.5	KLETDLAELEAEGAK	160.4
			GFEPPAREEGEPEWQQGDTFTLK	
			LGIQAFEPQLVEGK	
			LLDLGAPEIIVNNEK	
			TFHTGGVAGDDITQGLPR	
RNA polymerase alpha subunit	SCO4729	15.0	FVIEPLEPGFGYTLGNSLR	40.3
			QGPGLVTAADIAPPAGVEVHNPDLVLATLNGK	

Table 3 Nonspecific binding proteins in *Streptomyces coelicolor*

Protein	Protein ID	%C	Score
Conserved hypothetical protein	SCO0200	9.7	20.2
DNA polymerase I	SCO2003	5.6	90.4
Putative dihydrolipoamide succinyltransferase	SCO2181	6.8	40.3
Conserved hypothetical protein	SCO2599	2.2	20.2
DNA-binding protein Hu	SCO2950	46.2	60.7
DNA topoisomerase I	SCO3543	3.1	40.4
Putative endonuclease	SCO3569	7.6	40.3
Putative decarboxylase	SCO4490	10.9	40.2
Putative acyl-CoA carboxylase complex A subunit	SCO4921	7.1	50.2
Putative sensory histidine kinase	SCO5748	1.3	20.2
DNA gyrase subunit B	SCO5822	12.4	80.4
DNA gyrase-like protein	SCO5836	14.2	90.2

from the *S. coelicolor glnA* promoter and *E. coli lac* promoter (data not shown). Although the number of nonspecific proteins varied between five and ten, repeated experiments showed that all of the proteins in Table 3 showed nonspecific binding. The candidate specific proteins were selected only when they were identified in three independent analyses.

Although DACA is a powerful tool by which to identify the proteins binding to specific DNA fragments, it has an innate problem of nonspecific binding to the DNA fragments as well as to the streptavidin beads. Although most of nonspecific-binding proteins were removed with the addition of an excessive amount of random chromosomal DNA to the reaction mixture, it is still unclear whether or not the remaining binding proteins were specific. Therefore, bioinformatic techniques and a literature survey were used to confirm the possibilities as the first sorting criteria.

Proteins binding to the *actII-ORF4* promoter

Four proteins, i.e., SCO0310, SCO3932, SCO5405, and AdpA, were analyzed as binding proteins to *actII-ORF4* promoter region. A summary of the identified peptides based on the LC-MS/MS analysis is provided in Table 4, including all of the amino acid sequences, SEQUEST scores, and percentage of the protein sequence covered by all the identified peptides. As a rough prediction of the putative regulators, a homology search was introduced using BLAST. While homology searches cannot give absolute confidence that a putative protein is a transcriptional regulator, the presence of features such as helix-turn-helix (HTH) structures are common features of DNA-binding proteins. According to the following bioinformatic analogy, the four proteins have high probabilities of playing roles as transcriptional regulators for Act biosynthesis.

SCO0310 showed significant similarity both to the N-terminal of FarA (score 41.2; E-value 0.0017; 38% identity in amino acids 27–76) and to the members of the TetR/AcrR family of transcriptional regulators. FarA is an IM-2 receptor protein from *Streptomyces* sp. strain FRI-5 [36], and IM-2 is a butyrolactone autoregulator controlling the production of blue pigment and nucleoside antibiotics. However, the similarity of SCO0310 to FarA was low and limited to the N-terminal domain. The possibility of relationship to FarA needs to be further examined.

SCO3932 is a putative GntR-family transcriptional regulator that has one helix–turn–helix (HTH) DNA-binding motif and shows a similarity to KorSA of *Streptomyces* species (score 359.5; E-value 1.1e-12; 30.4% identity in 253 amino acids), a transcriptional repressor [30]. SCO5405 belongs to the MarR-family of transcriptional regulators involved in intrinsic resistance systems to various antibiotics and shows high similarities to both MmcW from *Streptomyces lavendulae* (score: 284, *E*-value: 7e-25,

Table 4 Identified proteins binding to promoter regions

Protein	Protein ID	%C[a]	Sequence of identified peptides	Score[b]
Promoter of *actII-ORF4*				
AdpA, AraC-family transcriptional regulator	SCO2792	10.1	LLETSDYSVDEVAGR	60.2
			AGTVVVPAWR	
			SITSPPPEEALDALR	
Putative TetR-family transcriptional regulator	SCO0310	12.5	ALVELVLDK	40.1
			GFQAVTVEEITERADIGR	
Putative GntR-family transcriptional regulator	SCO3932	12.4	MLIPFEVAESVPALAK	30.2
			ATLQLPDATPILHLSR	
Putative MarR-family transcriptional regulator	SCO5405	27.2	MSDLATATMQSK	28.2
			GLFAVLTEHGLETMR	
			HFIDLLAPEDLTELDK	
Promoter of *redD*				
Conserved hypothetical protein	SCO1480	31.8	HSGASLHEVIK	30.2
			QGQENDVIGK	
			VSALLESLPGVGK	
AdpA, AraC-family transcriptional regulator	SCO2792	31.0	RQLGSSPAAYR	170.3
			LLETSDYSVDEVAGR	
			IVGL**C**TGAFVLAAAGLLDGR	
			ELFVDDGDVLTSAGTAAGIDL**C**LHIVR	
Putative regulator	SCO3606	15.8	IHQLTGANASDPVHR	30.2
			AADMLVYPVLAR	
			GATVSGADATGTVLAAVAQAVDAFAEGYER	
Putative DNA-binding protein	SCO3859	62.6	NWYGEPLGALFR	240.4
			KSQGGSVLSNTTTTTSSSGAPTVK	
			SVAAAGDIIDAADSLSSSHPELAEFLR	
			LAGVLGLSAPMLSQLMSGQR	
Promoter of *redD*				
Putative MarR-family transcriptional regulator	SCO5405	39.9	MSDLATATMQSK	40.2
			WLTDTEQ**C**AWR	
			GLFAVLTEHGLETMR	
			DLQPFGLTMNDYEILVNLSESEGDR	
Putative transcriptional repressor	SCO6008	44.4	METPGSQSSLHR	90.3
			GPGGTAGEIGHITLDEAGPV**C**R	
			VAVGNLAHQVLAEESEPLDVDASSDQGFDR	
			YVLPLLQPSHGTDLTMEGVVR	

The C in bold represents the alkylated cysteine

[a] Percentage of the protein sequence covered by all of the identified peptides

[b] The preliminary scores obtained during the database search using SEQUEST. The score is calculated based on the number of ions from the MS/MS spectrum that matched the candidate peptide

and 43% identities in amino acids 1–151) and PecS from *Erwinia chrysanthemi* (score 131; *E*-value 4e-07; 28.7% identity in amino acids 24–155). Although SCO3205 and SCO3133 among *S. coelicolor* proteins are the most similar to MmcW and PecS, respectively, it is SCO5405 that shows high similarities to both MmcW and PecS. One of the two regulatory genes in the biosynthesis gene cluster of mitomycin C in *S. lavendulae* is *mmcW* [21]. PecS is one of the major regulators involved in the expression of virulent enzymes from plant pathogens [27]. *Erwina chrysanthemi* is responsible for the soft-rot disease of many plants. During its infection, the plant cell wall and the middle lamella are macerated by large amounts of bacterial extracellular hydrolases, such as proteases, cellulases, and pectinases, the production of which is finely tuned by complex regulatory networks and dependent on the growth phase of the

cell [12]. It is thought that their production profiles share common features with that of antibiotic production by *Streptomyces*.

AdpA has been reported to be an A-factor-dependent protein in *S. griseus* [35]. In *S. coelicolor*, the *adpA*-deletion mutant was found to be unable to produce Act, but to overproduce Red. However, AdpA is not restricted to being an acceptor of the signal of butyrolactone; it is also considered to be a global regulator affecting morphological development and antibiotic production through an unknown signal cascade [33].

Proteins binding to the *redD* promoter

Using the *redD* promoter, we were able to identify the following seven putative transcriptional regulators: AdpA, SCO5405, SCO6008, SCO3859, SCO3606, and SCO1480 (Table 4). SCO5405 was discussed in the previous section. SCO6008 contains an HTH motif of the NagC/XylR family and shows a high homology to XylR, a xylose repressor from *Anaerocellum thermophilum* (score 542; E-value 5e-54; 32% identity in amino acids 5–381). It is very interesting that a protein with a high homology to the xylose repressor binds to the *redD* promoter region because antibiotic production is closely related to primary metabolism. SCO3859 shows a high homology to Gra-orf8 from *Streptomyces violaceoruber* Tu22 (score 615 *E*-value 4e-63; 69% identity in amino acids 18–182). This granaticin is a member of the aromatic polyketides, whose best-known member is actinorhodin. Within the granaticin biosynthetic gene cluster of *S. violaceoruber* Tu22, Gra-orf8 has been annotated as a putative regulator [14]. SCO3606 shows a high homology to SrmR, the regulatory protein for spiramycin biosynthesis in *Streptomyces ambofaciens* (score 143; *E*-value 8e-08; 24% identity in amino acids 112–359). Geistlich et al. [7] found that SrmR was required for the accumulation of the transcripts of *srmG* (polyketide synthase), *srmB* (spiramycin resistance gene), and itself in *S. ambofaciens*. Since no genes related to secondary metabolism were found in the proximity of either SCO3859 or SCO3606 on the *S. coelicolor* chromosome, these three regulators are likely to function other than as pathway-specific regulators. SCO1480 shows a high similarity to MihF, an integration host factor (IHF) from *Mycobacterium smegmatis* (score 206; E-value 5e-16; 43% identity in amino acids 1–102). A thorough examination revealed that there are no IHF-like proteins in *S. coelicolor*, with the exception of SCO1480. Therefore, it may be possible that SCO1480 functions as an integration host factor. *Escherichia coli* IHF is known to be not only required for various cellular processes, but it is also involved in the transcription of at least 59 operons through direct interactions with DNA [16]. Interestingly, one of the five identified candidates for decoy oligonucleotides had a match to the promoter region of *redD* whose motif was a predicted binding site for the IHF [19]. This result is a supplementary result in our study.

Confirmation of protein binding to the promoters

To further confirm whether the regulator proteins that we screened are physically able to bind to the corresponding promoter regions, we performed EMSA. Each gene was cloned into pET24ma with a C-terminal His-tag and expressed in *E. coli*, and the expressed proteins were purified using affinity chromatography. The four putative regulator proteins, i.e., AdpA, SCO5405, SCO6008, and SCO1480, were obtained in soluble form, and their binding specificities were examined using [^{32}P]-labeled probes. AdpA, SCO5405, and SCO1480 showed binding activities to both the *actII-ORF4* ($P_{actII\text{-}ORF4}$) and *redD* (P_{redD}) promoter regions. Although the shifted bands with $P_{actII\text{-}ORF4}$ appeared to be weak, the presence of AdpA resulted in DNA probes shifting to two bands with P_{redD} (Fig. 3a). As *S. griseus* orthologous protein binds to DNA as a dimer form [37], *S. coelicolor* AdpA is thought to be highly probable to show the same pattern. SCO5405 and SCO1480 shifted DNA probes to one band, which moved upward slightly at higher concentrations of the proteins (Fig. 3b, d), suggesting the oligomerization of DNA-bound proteins. Such oligomerization results can be also found in the case of PecS from *E. chrysanthemi* where PecS–DNA complexes are formed [25]. However, the binding of SCO1480 to $P_{actII\text{-}ORF4}$ was not detected in DACA. The results obtained with the DACA may be different from those obtained with the EMSA because a cell extract was used in the former and purified recombinant proteins were used in the latter; the use of purified recombinant proteins may facilitate SCO1480 binding to the *actII-ORF4* promoter. SCO6008 seemed to discriminate the two promoter regions, binding to only one of the two promoter regions, *redD* (Fig. 3c).

The results obtained with EMSA were in agreement with those obtained with DACA except for the binding of SCO1480 to the *actII-ORF4* promoter. As mentioned, although EMSA is an experimental tool that can be used to investigate the property of DNA-binding in protein, such experiments cannot absolutely prove its binding because of variations in the conditions from in vitro to in vivo, binding affinity, or unknown cofactors. Therefore, the evaluation of a deletion mutant in vivo was also required to reconfirm the regulatory function of the screened proteins.

Deletion mutant of SCO6008

Based on the results obtained from the two assays, we focused on SCO6008, whose two promoter regions show

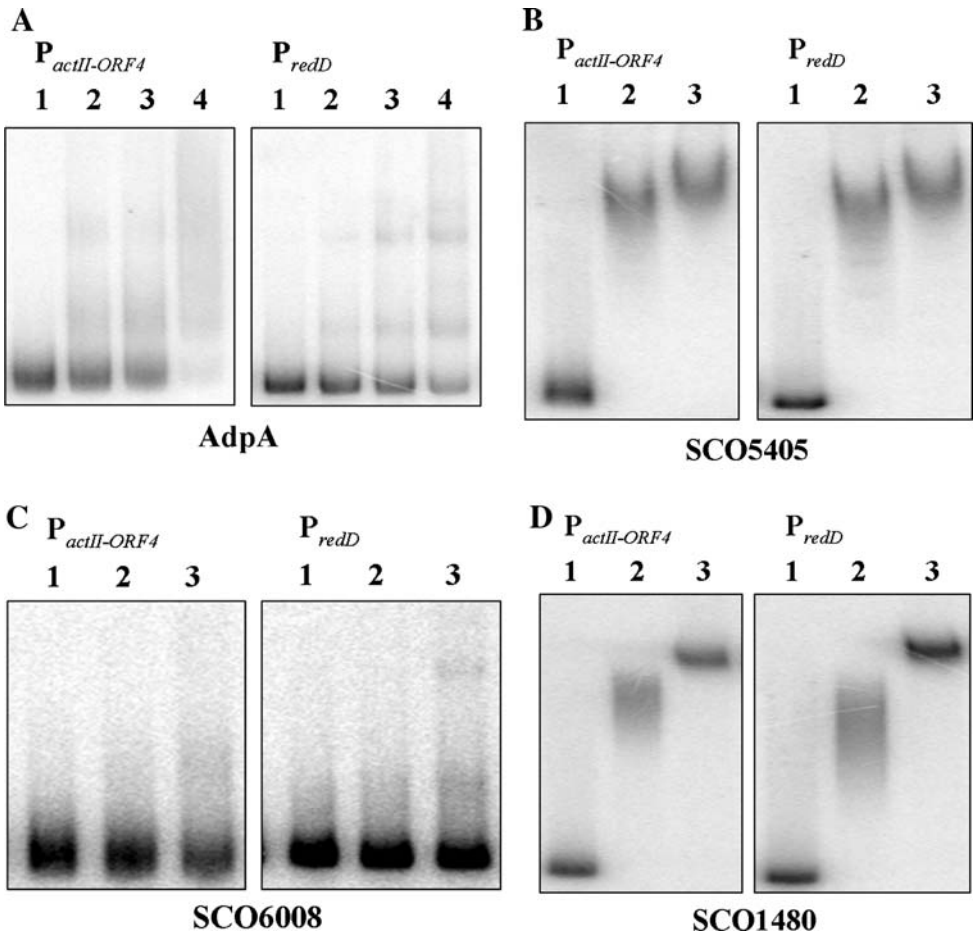

Fig. 3 Electrophoretic mobility shift assay of the promoter regions of *actII-ORF4* and *redD*. Four putative binding proteins (AdpA, SCO5405, SCO6008, and SCO1480) were expressed in *Escherichia coli*, purified using affinity chromatography and obtained in soluble form. Their binding to the promoter regions of *redD* and *actII-ORF4* were examined using [^{32}P]-labeled probes. (**a**) *Lanes*: *1* 2 fmol of free probes; complete reaction with 10 ng (*lane 2*), 40 ng (*lane 3*) and 100 ng (*lane 4*) of purified protein. **b**, **c**, **d** *Lanes*: *1* 2 fmol of free probes; complete reaction with 10 ng (*lane 2*) and 100 ng (*lane 3*) of purified proteins

different binding properties. The deletion mutant of SCO6008 was constructed by the PCR targeting method [9]. The constructed mutant (B101) showed quite different properties from the wild type and overproduced Red while showing quite delayed Act production in R5$^-$ medium. During 3 days of cell growth on R5$^-$ plates, mutant B101 was unable to produce Act at all, but its morphological phenotype was almost the same as that of the wild type, M145 (Fig. 4). This result agrees well with the earlier result that the *rep* gene from the environmental DNA library, which showed 68% homology to SCO6008, conferred early and increased sporulation and antibiotic production in *Streptomyces* species [22]. However, our EMSA experiments showed the binding of SCO6008 onto the promoter of *redD*, not *actII-ORF4*, and that the direct and/or indirect regulation of SCO6008 on Act biosynthesis was quite possible. There are several regulators that cross-regulate between the Act and Red biosynthetic pathways [11]. In addition, even the deletion of *actII*-ORF4 causes the overproduction of Red, and the deletion of *redD* also causes the overproduction of Act [29]. In that regard, SCO6008 most certainly is involved in the regulation of multiple secondary metabolites. Interestingly, similar to the wild type, mutant B101 produced Act on R2YE agar plates. The effect of each component of the R5$^-$ medium lacking $CaCl_2$, KH_2PO_4, and proline on Act production was examined, and proline was found to be responsible for Act production in B101. However, during the later stage of cell growth, the addition of other amino acids, such as aspartate, glutamate, and arginine, was able to recover the Act production (data not shown). We are currently studying the function of SCO6008 in order to gain an understanding of the exact effect of the amino acids on Act production.

Conclusion

Putative transcriptional regulators binding to the promoter region of *actII-ORF4* or *redD* were identified using DACA coupled with LC-MS/MS. Protein functions were further investigated by EMSA and a gene deletion study. The EMSA experiments showed that the identified proteins are characterized by a physical binding activity to *actII-ORF4* or *redD* promoter, confirming that DACA is a quite useful technique for screening candidates of transcriptional regulators. The DACA was introduced in the mass spectrometry scientific community using in vitro model system, but has not been applied to practical research work nor widely used

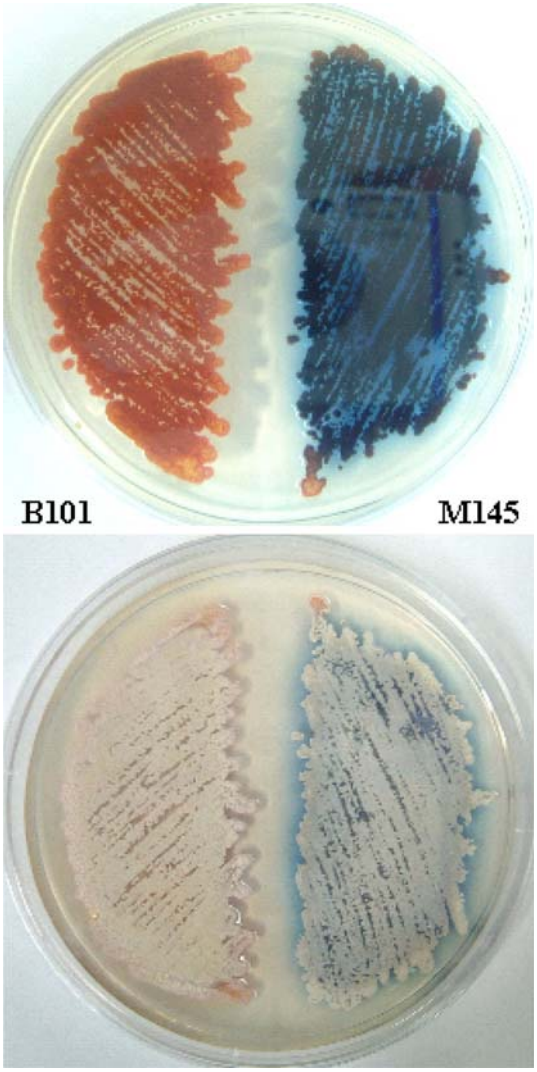

Fig. 4 The deletion mutant (B101) of SCO6008 produces no actinorhodin on R5$^-$ medium agar plate. The cultures were grown on R5$^-$ medium for 3 days. *Upper panel* Mutant B101 produces only *red* pigment and the wild type, M145, produces *blue* pigment with *red* pigment (see the *red edge* of the culture). *Bottom panel* Both strains show the same phenotype of gray spore formation (Color figure online)

since time-consuming optimization techniques are essential for its direct application to screening transcriptional regulators. Although this study has not completed a detailed characterization of the functions of all of the screened putative transcriptional regulators, it is a good example of a screening study of putative transcriptional regulators in bacteria using DACA and a good follow-up to our *E. coli* model system [24]. This study will expedite a systematic biological approach by helping to draw the map of transcriptional networks of *S. coelicolor*.

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References

1. Bentley SD, Chater KF, Cerdeno-Tarraga AM, Challis GL, Thomson NR et al (2002) Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). Nature 417:141–147. doi:10.1038/417141a
2. Brian P, Riggle PJ, Santos RA, Champness WC (1996) Global negative regulation of *Streptomyces coelicolor* antibiotic synthesis mediated by an *absA*-encoded putative signal transduction system. J Bacteriol 178:3221–3231
3. Chakraburtty R, Bibb M (1997) The ppGpp synthetase gene (*relA*) of *Streptomyces coelicolor* A3(2) plays a conditional role in antibiotic production and morphological differentiation. J Bacteriol 179:5854–5861
4. Chater KF (2001) Regulation of sporulation in *Streptomyces coelicolor* A3(2): a checkpoint multiplex? Curr Opin Microbiol 4:667–673. doi:10.1016/S1369-5274(01)00267-3
5. Corre C, Song L, O'Rourke S, Chater KF, Challis GL (2008) 2-Alkyl-4-hydroxymethylfuran-3-carboxylic acids, antibiotic production inducers discovered by *Streptomyces coelicolor* genome mining. Proc Natl Acad Sci USA 105:17510–17515. doi:10.1073/pnas.0805530105
6. Distler J, Ebert A, Mansouri K, Pissowotzki K, Stockmann M, Piepersberg W (1987) Gene cluster for streptomycin biosynthesis in *Streptomyces griseus*: nucleotide sequence of three genes and analysis of transcriptional activity. Nucleic Acids Res 15:8041–8056. doi:10.1093/nar/15.19.8041
7. Geistlich M, Losick R, Turner JR, Rao RN (1992) Characterization of a novel regulatory gene governing the expression of a polyketide synthase gene in *Streptomyces ambofaciens*. Mol Microbiol 6:2019–2029. doi:10.1111/j.1365-2958.1992.tb01374.x
8. Gramajo HC, Takano E, Bibb MJ (1993) Stationary-phase production of the antibiotic actinorhodin in *Streptomyces coelicolor* A3(2) is transcriptionally regulated. Mol Microbiol 7:837–845. doi:10.1111/j.1365-2958.1993.tb01174.x
9. Gust B, Challis GL, Fowler K, Kieser T, Chater KF (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. doi:10.1073/pnas.0337542100
10. Huang J, Lih CJ, Pan KH, Cohen SN (2001) Global analysis of growth phase responsive gene expression and regulation of antibiotic biosynthetic pathways in *Streptomyces coelicolor* using DNA microarrays. Genes Dev 15:3183–3192. doi:10.1101/gad.943401
11. Huang J, Shi J, Molle V, Sohlberg B, Weaver D, Bibb MJ, Karoonuthaisiri N, Lih CJ, Kao CM, Buttner MJ, Cohen SN (2005) Cross-regulation among disparate antibiotic biosynthetic pathways of *Streptomyces coelicolor*. Mol Microbiol 58:1276–1287
12. Hugouvieux-Cotte-Pattat N, Condemine G, Nasser W, Reverchon S (1996) Regulation of pectinolysis in *Erwinia chrysanthemi*. Annu Rev Microbiol 50:213–257. doi:10.1146/annurev.micro.50.1.213
13. Hung TV, Ishida K, Parajuli N, Liou K, Lee HC, Sohng JK (2006) Enhanced clavulanic acid production in *Streptomyces clavuligerus* NRRL3585 by overexpression of regulatory genes. Biotechnol Bioprocess Eng 11:116–120. doi:10.1007/BF02931894
14. Ichinose K, Bedford DJ, Tornus D, Bechthold A, Bibb MJ et al (1998) The granaticin biosynthetic gene cluster of *Streptomyces violaceoruber* Tu22: sequence analysis and expression in a heterologous host. Chem Biol 5:647–659. doi:10.1016/S1074-5521(98)90292-7

15. Ishizuka H, Horinouchi S, Kieser HM, Hopwood DA, Beppu T (1992) A putative two-component regulatory system involved in secondary metabolism in *Streptomyces* spp. J Bacteriol 174:7585–7594
16. Karp PD, Riley M, Saier M, Paulsen IT, Collado-Vides J et al (2002) The EcoCyc database. Nucleic Acids Res 30:56–58. doi:10.1093/nar/30.1.56
17. Kieser T, Bibb MJ, Buttner MJ, Chater K, Hopwood DA (2000) Practical *Streptomyces* genetics. John Innes Centre, Norwich
18. Kim DJ, Huh JH, Yang YY, Kang CM, Lee IH et al (2003) Accumulation of S-adenosyl-L-methionine enhances production of actinorhodin but inhibits sporulation in *Streptomyces lividans* TK23. J Bacteriol 185:592–600. doi:10.1128/JB.185.2.592-600.2003
19. McArthur M, Bibb MJ (2008) Manipulating and understanding antibiotic production in *Streptomyces coelicolor* A3(2) with decoy oligonucleotides. Proc Natl Acad Sci USA 105:1020–1025. doi:10.1073/pnas.0710724105
20. Malpartida F, Niemi J, Navarrete R, Hopwood DA (1990) Cloning and expression in a heterologous host of the complete set of genes for biosynthesis of the *Streptomyces coelicolor* antibiotic undecylprodigiosin. Gene 93:91–99. doi:10.1016/0378-1119(90)90141-D
21. Mao Y, Varoglu M, Sherman DH (1999) Molecular characterization and analysis of the biosynthetic gene cluster for the antitumor antibiotic mitomycin C from *Streptomyces lavendulae* NRRL 2564. Chem Biol 6:251–263. doi:10.1016/S1074-5521(99)80040-4
22. Martinez A, Kolvek SJ, Hopke J, Yip CL, Osburne MS (2005) Environmental DNA fragment conferring early and increased sporulation and antibiotic production in *Streptomyces* species. Appl Environ Microbiol 71:1638–1641. doi:10.1128/AEM.71.3.1638-1641.2005
23. Ohnishi Y, Kameyama S, Onaka H, Horinouchi S (1999) The A-factor regulatory cascade leading to streptomycin biosynthesis in *Streptomyces griseus*: identification of a target gene of the A-factor receptor. Mol Microbiol 34:102–111. doi:10.1046/j.1365-2958.1999.01579.x
24. Park SS, Ko BJ, Kim BG (2005) Mass spectrometric screening of transcriptional regulators using DNA affinity capture assay. Anal Biochem 344:152–154. doi:10.1016/j.ab.2005.05.019
25. Praillet T, Reverchon S, Nasser W (1997) Mutual control of the PecS/PecM couple, two proteins regulating virulence-factor synthesis in *Erwinia chrysanthemi*. Mol Microbiol 24:803–814. doi:10.1046/j.1365-2958.1997.3901755.x
26. Reche PA (2000) Lipoylating and biotinylating enzymes contain a homologous catalytic module. Protein Sci 9:1922–1929. doi:10.1110/ps.9.10.1922
27. Reverchon S, Nasser W, Robert-Baudouy J (1994) *pecS*: a locus controlling pectinase, cellulase and blue pigment production in *Erwinia chrysanthemi*. Mol Microbiol 11:1127–1139. doi:10.1111/j.1365-2958.1994.tb00389.x
28. Ricaud PM, Howard MJ, Roberts EL, Broadhurst RW, Perham RN (1996) Three-dimensional structure of the lipoyl domain from the dihydrolipoyl succinyltransferase component of the 2-oxoglutarate dehydrogenase multienzyme complex of *Escherichia coli*. J Mol Biol 264(1):179–190. doi:10.1006/jmbi.1996.0632
29. Rigali S, Titgemeyer F, Barends S, Mulder S, Thomae AW, Hopwood DA, van Wezel GP (2008) Feast or famine: the global regulator DasR links nutrient stress to antibiotic production by *Streptomyces*. EMBO Rep 9:670–675. doi:10.1038/embor.2008.83
30. Sezonov G, Possoz C, Friedmann A, Pernodet JL, Guerineau M (2000) KorSA from the *Streptomyces* integrative element pSAM2 is a central transcriptional repressor: target genes and binding sites. J Bacteriol 182:1243–1250. doi:10.1128/JB.182.5.1243-1250.2000
31. Stutzman-Engwall KJ, Otten SL, Hutchinson CR (1992) Regulation of secondary metabolism in *Streptomyces* spp. and overproduction of daunorubicin in *Streptomyces peucetius*. J Bacteriol 174:144–154
32. Susstrunk U, Pidoux J, Taubert S, Ullmann A, Thompson CJ (1998) Pleiotropic effects of cAMP on germination, antibiotic biosynthesis and morphological development in *Streptomyces coelicolor*. Mol Microbiol 30:33–46. doi:10.1046/j.1365-2958.1998.01033.x
33. Takano E, Tao M, Long F, Bibb MJ, Wang L et al (2003) A rare leucine codon in *adpA* is implicated in the morphological defect of *bldA* mutants of *Streptomyces coelicolor*. Mol Microbiol 50:475–486. doi:10.1046/j.1365-2958.2003.03728.x
34. Umeyama T, Lee PC, Ueda K, Horinouchi S (1999) An AfsK/AfsR system involved in the response of aerial mycelium formation to glucose in *Streptomyces griseus*. Microbiology 145(Pt 9):2281–2292
35. Vujaklija D, Horinouchi S, Beppu T (1993) Detection of an A-factor-responsive protein that binds to the upstream activation sequence of *strR*, a regulatory gene for streptomycin biosynthesis in *Streptomyces griseus*. J Bacteriol 175:2652–2661
36. Waki M, Nihira T, Yamada Y (1997) Cloning and characterization of the gene (*farA*) encoding the receptor for an extracellular regulatory factor (IM-2) from *Streptomyces* sp. strain FRI-5. J Bacteriol 179:5131–5137
37. Yamazaki H, Tomono A, Ohnishi Y, Horinouchi S (2004) DNA-binding specificity of AdpA, a transcriptional activator in the A-factor regulatory cascade in *Streptomyces griseus*. Mol Microbiol 53:555–572. doi:10.1111/j.1365-2958.2004.04153.x