

A Regulatory Gene *SCO2140* is Involved in Antibiotic Production and Morphological Differentiation of *Streptomyces coelicolor* A3(2)

Lingjun Yu¹ · Yuanyuan Pan¹ · Gang Liu¹

Received: 7 January 2016 / Accepted: 8 April 2016 / Published online: 25 April 2016
© Springer Science+Business Media New York 2016

Abstract *Streptomyces coelicolor* is the soil-dwelling bacterium with a complex life cycle and a strong ability to produce plenty of secondary metabolites which are strictly regulated by a variety of regulators. Amino acid alignment shows that the deduced protein of *SCO2140* belongs to the family of Leucine-responsive regulatory proteins (Lrps). Disruption of *SCO2140* significantly decreased the yields of actinorhodin and calcium-dependent antibiotics, and the complemented strain restored the antibiotic productions to the wild-type level. In contrast, overexpression of *SCO2140* increased the actinorhodin production. In agreement with it, the transcriptions of *actII-ORF4* and *cdaR* remarkably reduced in the *SCO2140* disruption mutant. The aerial mycelium formation of the *SCO2140* disruption mutant was clearly delayed in R2YE medium due to the decrease of *ramS* expression while its complemented strain could restore the normal formation of aerial mycelia. These results indicated that *SCO2140* was involved in antibiotic biosynthesis and morphological differentiation of *Streptomyces coelicolor* A3(2).

Introduction

Streptomyces coelicolor A3(2) is the model strain of the *Streptomyces* genus. Twenty-nine biosynthetic gene clusters for secondary metabolites have been discovered in the genome of *S. coelicolor* A3(2) [1]. Up to now, 16 chemicals from the 15 clusters have been identified, which include the four distinct classes of antibiotics, the blue-pigmented polyketide antibiotic actinorhodin (ACT), the red oligopyrrole prodiginine antibiotic undecylprodigiosin (RED), the lipopeptide calcium-dependent antibiotic (CDA), and the yellow-pigmented type I polyketide (coelimycin P1) [2]. The regulation of secondary metabolic biosynthesis is a complex network, involving cluster-situated and global regulators, two-component systems, RNA regulatory elements, and some developmental genes (such as *bldB*, *abaA*, *whiJ*, and *wblA*) [3]. In *S. coelicolor*, *actII-ORF4*, *redD*, *cdaR*, and *cpkO* are the cluster-situated regulatory genes for ACT, RED, CDA, and coelimycin P1 biosynthesis, respectively [3].

The Leucine-responsive regulatory proteins (Lrps), also named FFRP or Lrp/AsnC, are widely distributed among prokaryotes and regulate a large number of genes' expression [4]. Lrp functions as both an activator and a repressor in *Escherichia coli*, and affects the transcriptions of at least 10 % of all *E. coli* genes [5]. The Lrp-regulated genes are involved in amino acid metabolism and transport, the fimbriation, and the formation of virulence [6]. For instance, LrpA is vital to the long-term persistence of *Mycobacterium tuberculosis* and it may be a novel therapeutic target against *M. tuberculosis* [7]. The *S. coelicolor* genome contains 14 putative Lrp/AsnC-like protein encoding genes. Of them, *bkdR* encoding a transcriptional regulator of the branched-chain amino acid dehydrogenase complex affects the morphogenesis and antibiotic

Electronic supplementary material The online version of this article (doi:10.1007/s00284-016-1050-8) contains supplementary material, which is available to authorized users.

✉ Gang Liu
liug@im.ac.cn

¹ State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China

production in *S. coelicolor* [8]. However, little is known about the function of other Lrp/AsnC-like proteins in *S. coelicolor*. In this paper, the Lrp-like protein encoding gene *SCO2140* was shown to be involved in the antibiotic production and morphological differentiation of *S. coelicolor* A3(2).

Materials and Methods

Bacterial Strains and Growth Conditions

Bacterial strains and plasmids used in this study are listed in Table S1. All media used for *S. coelicolor* M145 (the wild-type strain) and its derivatives were prepared according to Practical *Streptomyces* Genetics [9]. R5 liquid medium was used for the quantitative determination of antibiotic production as well as for RNA preparation of *S. coelicolor*. R2YE medium was used for the growth of *S. coelicolor* and observation of the aerial mycelium formation. Yeast extract–malt extract liquid medium (YEME) and 2× YT liquid medium were used for the growth of *S. coelicolor*. Mannitol soya flour medium (MS) was used for the sporulation and intergeneric conjugation of *S. coelicolor*. Difco Nutrient Agar (DNA) medium supplemented with 0.5 % (w/v) NaCl was used for the production of calcium-dependent antibiotic (CDA) and *Staphylococcus aureus* was used as the indicator of CDA production [10]. The *Escherichia coli* strains were cultured at 37 °C in Luria–Bertani (LB) medium supplemented with antibiotics (100 µg ml⁻¹ for ampicillin, 100 µg ml⁻¹ for kanamycin, 100 µg ml⁻¹ for apramycin, 25 µg ml⁻¹ for chloromycetin, and 15 µg ml⁻¹ for tetracycline) when necessary for propagating plasmids.

Construction of the *SCO2140* Disruption Mutant and Its Complementation

To construct the *SCO2140* disruption mutant (SCO2140DM), a DNA fragment corresponding to the upstream region of *SCO2140* (extending from the positions -655 to +39 with respect to the *SCO2140* translation start codon) was amplified by using primers 2140UF/UR (Table S2). Then the amplified DNA fragment was inserted into the *HindIII/BamHI* sites of pUC119::Kan^r [11], giving pD1. After digesting pD1 with *HindIII* and *EcoRI*, the fragment containing the upstream region of *SCO2140* and the kanamycin resistance gene (*Kan^r*) was inserted into the corresponding sites of pBluescript KS+ to generate pD2. The DNA fragment corresponding to the downstream region of *SCO2140* (extending from the positions +226 to +1258 with respect to the *SCO2140* translation start codon) was amplified by using primers 2140DF/DR

(Table S2) and ligated into the *EcoRI/XbaI* sites of pD2 to give pD3. A 2.7 kb DNA fragment was isolated after digesting pD3 with *HindIII* and *XbaI*, and then it was inserted into the corresponding sites of pKC1132, giving pD2140. Subsequently, pD2140 was introduced into *E. coli* ET12567/pUZ8002 [9] and then it was transferred into the *S. coelicolor* wild-type strain (WT) by intergeneric conjugation. After growing on MS agar at 28 °C for 144 h, colonies were selected and transferred to the minimal medium with mannitol as carbon source (MMM) and supplemented with apramycin (10 µg ml⁻¹) or kanamycin (10 µg ml⁻¹). The kanamycin-resistant and apramycin sensitive strains were selected as SCO2140DM and confirmed by PCR using the primers 2140YF1/YR1 and 2140YF2/YR2 (Table S2).

To construct the complemented strain, the entire *SCO2140* coding region was amplified using the primers 2140HF/HR (Table S2). Then the amplified DNA fragment was verified by DNA sequencing and inserted into the *NdeI/BamHI* sites of pIJ8600. The resulting plasmid pH2140 was introduced into SCO2140DM by conjugation to generate the *SCO2140* complemented strain (SCO2140CM). To over-express *SCO2140*, the entire *SCO2140* coding region was amplified using the primers 2140HF1/HR1 (Table S2) and verified by DNA sequencing. Then the amplified DNA fragment was inserted into the *EcoRV* site of pIMEP [12]. The resulting plasmid pIMEP::SCO2140 was introduced into WT by conjugation to generate the *SCO2140* overexpressed strain (SCO2140OE).

Determination of Antibiotic Productions

In order to assess the productions of ACT and RED, 1 × 10⁹ spores of the *S. coelicolor* wild-type strain and its derivatives were cultured in 50 ml 2× YT liquid medium at 28 °C and shaken at 200 r/min for 10 h. Mycelia were collected and inoculated into 50 ml of R5 liquid medium at 28 °C and shaken at 200 r/min for 24, 48, 72, 96, 120, 144, and 168 h, respectively. Then the production of ACT was measured as described previously [9]. CDA production in DNA medium was assayed as described previously [10].

RNA Isolation and Real-Time RT-PCR

Total RNA was isolated from the *S. coelicolor* wild-type strain and SCO2140DM grown in R5 liquid medium or the R2YE medium for 24, 48, 72, 96, 120, and 144 h. The isolated RNA was further treated with DNase I (Promega) to remove the contaminated DNA, and then it was reverse transcribed into the complementary DNA by using PrimeScript RT reagent kit (TaKaRa). Real-time PCR was carried out in realplex2 Master cycler (Eppendorf, Germany) using Ultra SYBR Mixture (CWBIO). The

condition is used as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min. Three independent experiments were performed and three PCR replicates were used in parallel for each transcript. The *hrdB* was used as an internal control. The relative transcriptional levels of tested genes were normalized to *hrdB* and determined by using the $-2^{\Delta\Delta C_T}$ method [13]. The values were presented as fold change in comparison with the relative expression levels for each gene at the first test time point in the wild-type strain.

Expression and Purification of *SCO2140* from *E. coli*

The *SCO2140* coding region was amplified by using primers 2140OF/OR (Table S2) and verified by DNA sequencing. The amplified DNA fragment was inserted into the *NdeI/XhoI* sites of pET23b to give the plasmid pET23b::*SCO2140*. The plasmid was introduced into *E. coli* C43 [14] for gene expression. The *E. coli* C43 containing pET23b::*SCO2140* was cultured at 37 °C in 200 ml of LB supplemented with kanamycin (100 µg ml⁻¹) to an OD₆₀₀ of 0.6. After isopropyl β-D-thiogalactopyranoside (IPTG) was added up to 0.1 mM, and the cultures were incubated at 37 °C for an additional 3 h. After centrifugation (6000×g for 5 min at 4 °C), the cells were collected and washed with binding buffer [20 mM Tris base, 500 mM NaCl, 5 mM imidazole, 5 % (v/v) glycerol (pH 7.9)], and re-suspended in 20 ml of the same buffer. After sonication on ice, the cell suspension was centrifugated (12,000×g for 20 min at 4 °C) and the supernatant was recovered. The supernatant was treated with Ni-NTA agarose chromatography (Novagen), and the purified *SCO2140*-His₆ was separated from the whole-cell lysate. Protein purity was determined by Coomassie blue staining after electrophoresis on a 15 % (w/v) SDS-polyacrylamide gel. The purified protein was stored with 5 % (v/v) glycerol at -70 °C until used in the subsequent experiments.

Preparation of the Antibody and Western Blot Analysis

Approximately 2 mg of the purified *SCO2140*-His₆ was mixed with Freund's complete adjuvant and injected into two healthy mice for four times (once per week). Antisera were collected from the mice after 1 month and used as the polyclonal antibody against *SCO2140*-His₆.

To detect the expression of *SCO2140*, WT, *SCO2140DM*, *SCO2140CM*, and *SCO2140OE* were cultured in R5 liquid medium at 28 °C for 24, 48, 72, 96, and 120 h. After collected, mycelia were ground in liquid nitrogen and sonicated on ice. The supernatant samples containing 50 µg proteins were separated by 15 % (w/v) SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Roche). Then the proteins on the

membrane were probed with anti-*SCO2140* which were hybridized with the horseradish peroxidase-conjugated suitable secondary antibodies (Jackson), followed by detection with enhanced chemiluminescence (Pierce).

Electrophoretic Mobility Shift Assays (EMSAs)

DNA probes containing the promoter regions of *actII-ORF4* and *cdaR* were generated by PCR using the primers act-PF/act-PR and *cda*-PF/*cda*-PR (Table S2). A mixture of the probes and varying quantities of *SCO2140*-His₆ (0, 45, 180, 360, 450, 1800, 3600, 4500, and 6750 nM protein) was incubated at 25 °C for 20 min in the reaction buffer which contained 1 µg of poly-(dI-dC) (Sigma), 20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol (DTT), 10 mM MgCl₂, 54 mM KCl, 0.5 mg ml⁻¹ calf BSA, and 5 % (v/v) glycerol in a total volume of 20 µl. Then the mixture was separated on a 5 % (w/v) native polyacrylamide gel 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. Then the gel was stained with SYBR Gold Nucleic Acid Gel Stain (Invitrogen) for 30 min in 0.5× TBE buffer, and photographed under ultraviolet transillumination using Bio-Rad GelDoc XR.

Results and Discussion

SCO2140 Encodes a Novel Lrp/AsnC Family Protein

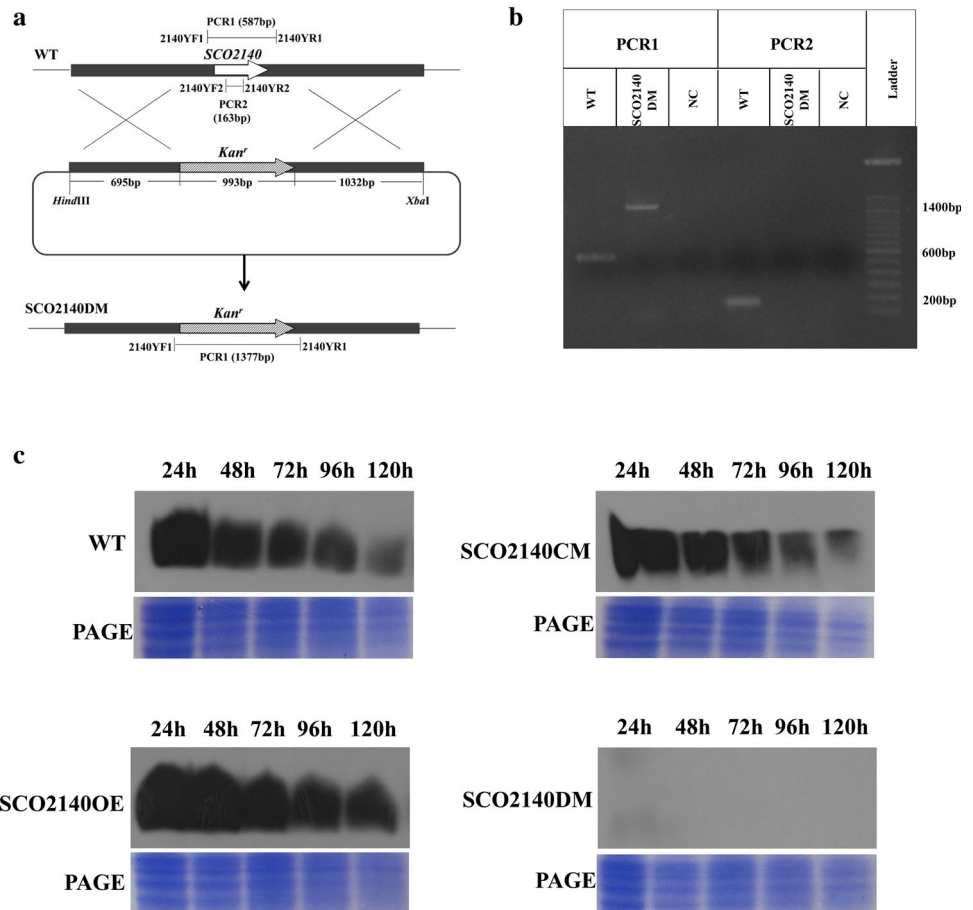
SCO2140 encodes a protein of 93 amino acids. In search of database, the deduced protein of *SCO2140* is well conserved through *Streptomyces* species (Fig. S1a). *SCO2140* shows 25 % identity and 48 % similarity over 84-aa region with Lrp from *E. coli* [15], 31 % identity and 44 % similarity over the 59-aa region with LrpA from *M. tuberculosis* [16]. However, sequence alignment showed that *SCO2140* lacks the typical DNA binding domain (Fig. S1b), suggesting *SCO2140* could not directly interact with DNA as other Lrps. As *SCO2140* contains the AsnC-type C-terminal domain which is responsible for the ligand-response and protein-protein interaction, it is possible that *SCO2140* plays a cooperative role via forming heterodimers with other transcriptional regulators.

Disruption of *SCO2140* Reduced the Productions of ACT and CDA in *S. coelicolor*

To study its function, *SCO2140* was disrupted through homologous recombination (Fig. 1a). The expected mutants were selected randomly and confirmed by PCR. A distinctive band of 1377 bp was amplified from the *SCO2140* disruption mutant (*SCO2140DM*) and a 587 bp

Fig. 1 Construction of the *SCO2140* disruption mutant (SCO2140DM) and Western blot analysis of *SCO2140*.

a Strategy for construction of *SCO2140*DM via homologous recombination. WT, *S. coelicolor* M145; *Kan^r*, kanamycin resistance gene. **b** Verification of *SCO2140*DM by PCR. PCR1 and PCR2 were performed with the gene-outside primers (2140YF1/YR1) and the gene-inside primers (2140YF2/YR2), respectively. NC negative control, *Ladder* 100 bp DNA ladder. **c** Expression of *SCO2140*. About 50 μ g of total protein from WT, *SCO2140OE*, *SCO2140CM*, and *SCO2140DM* cultured in R5 medium was loaded for Western blot analysis with the anti-*SCO2140* antibodies or the total protein shown by SDS-PAGE as the loading control. *Data* were representative of three independent experiments



band was obtained from WT as predicted while the 163 bp DNA fragment from the coding region of *SCO2140* was amplified only from WT (Fig. 1b). These confirmed that *SCO2140* was replaced by the kanamycin resistance cassette (*Kan^r*). To further investigate its function, the complemented (*SCO2140CM*) and overexpressed (*SCO2140OE*) strains of *SCO2140* were constructed. Western blotting showed the expression of *SCO2140* significantly enhanced in *SCO2140OE*, compared with that of WT and *SCO2140CM* (Fig. 1c). Expectably, the expression of *SCO2140* did not detect in *SCO2140DM* (Fig. 1c).

In R5 liquid medium, ACT extracted from the mycelia of *SCO2140DM* was only about 40 % of that in WT (Fig. 2a). In agreement with it, ACT extracted from the medium of *SCO2140DM* also decreased (Fig. 2b). Combined with that *SCO2140CM* produced the same amount of ACT as that in WT, these results suggested that ACT decrease in *SCO2140DM* is due to the disruption of *SCO2140*. Meanwhile, the ACT extracted from the medium of *SCO2140OE* was nearly twofold of the wild-type level (Fig. 2b).

When DNA medium was used, CDA production in *SCO2140DM* was also decreased. In *SCO2140CM*, the CDA production restored to the wild-type level (Fig. 2c). These results revealed that *SCO2140* positively regulates

the production of ACT and CDA in *S. coelicolor*. Unlike ACT production, the RED production was not affected by the disruption of *SCO2140* (Fig. S2).

Disruption of *SCO2140* Decreased the Transcriptions of *actII-ORF4* and *cdar*

As the cluster-situated regulatory genes, *actII-ORF4* and *cdar* positively regulate the production of ACT and CDA. The transcriptions of *actII-ORF4* and *cdar* were measured in *SCO2140DM* by real-time PCR. The transcriptional level of *actII-ORF4* in *SCO2140DM* was only about 30 % of that in WT when its transcriptional level reached the highest level at 96 h (Fig. 3a). Like *actII-ORF4*, the transcriptional level of *cdar* in *SCO2140DM* was only about 20 % of that in WT and it decreased rapidly after 24 h in both WT and *SCO2140DM* (Fig. 3b). These results suggested that disruption of *SCO2140* reduced the production of ACT and CDA at least partially through decreasing the transcriptions of *actII-ORF4* and *cdar*. Since *SCO2140* could not bind to the upstream regions of *actII-ORF4* and *cdar* (Fig. S3), *SCO2140* may indirectly regulate antibiotic production or cooperate with other transcriptional regulators which involved in ACT and CDA production.

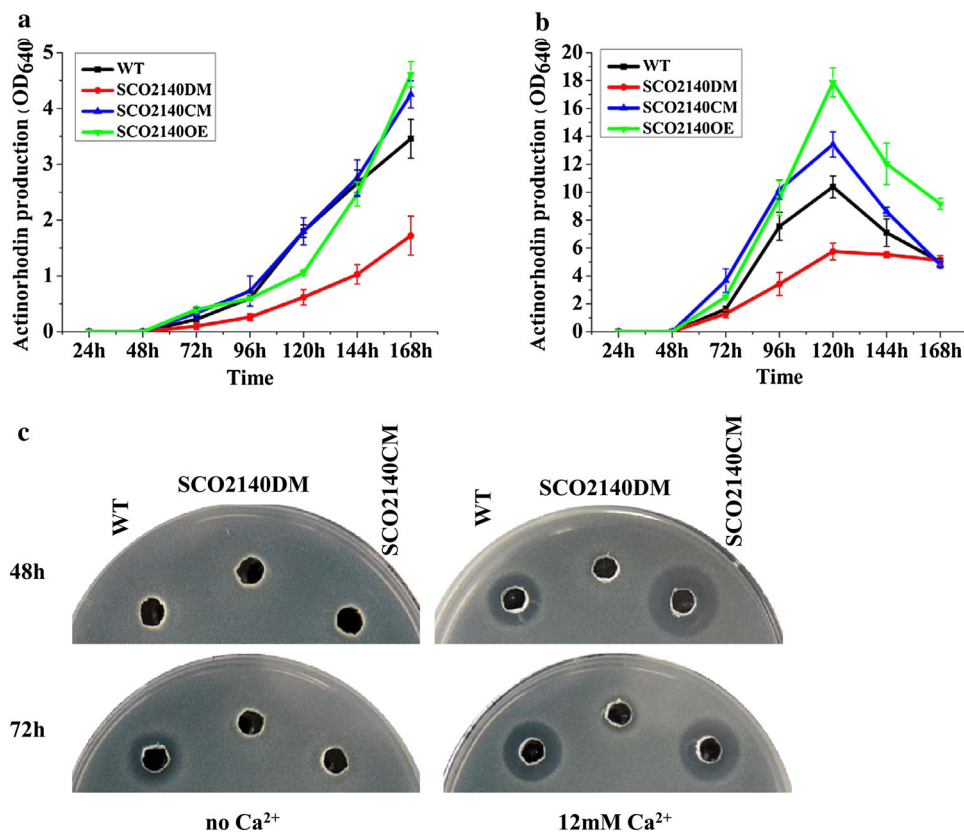


Fig. 2 The productions of ACT and CDA. **a** ACT extracted from the mycelia of WT, SCO2140DM, SCO2140CM, and SCO2140OE, respectively. **b** ACT extracted from the cultured media of WT, SCO2140DM, SCO2140CM, and SCO2140OE, respectively. **c** The

CDA productions of WT, SCO2140DM, and SCO2140CM. *Staphylococcus aureus* was used as the indicator strain in the presence or absence of Ca(NO₃)₂. Error bars represent the standard deviations from three independent experiments

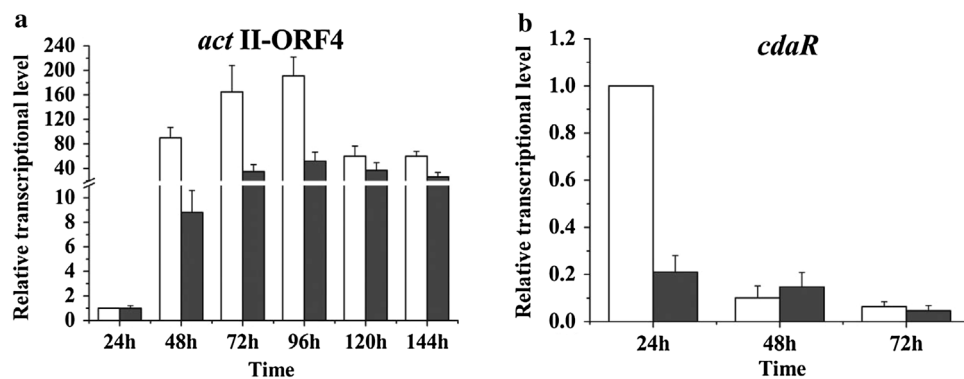


Fig. 3 The transcriptions of *actII-ORF4* and *cdaR*. **a** The transcriptional level of *actII-ORF4* was detected in WT and SCO2140DM which were cultured in R5 medium. **b** The transcriptional level of *cdaR* was detected in WT and SCO2140DM which were cultured in

DNA medium. The *open columns* represent the gene transcriptions in WT and the *solid columns* represent the gene transcriptions in SCO2140DM. Error bars represent the standard deviations from three independent experiments

Disruption of *SCO2140* Affected the Morphological Differentiation of *S. coelicolor*

In R2YE medium, SCO2140DM showed rare aerial mycelium. Introduction of the entire wild-type *SCO2140* in SCO2140DM restored the aerial mycelium formation, suggesting *SCO2140* is required for the aerial mycelium

formation in R2YE medium (Fig. 4a). Glucose is the main carbon source in R2YE medium, and the formation of aerial mycelium in this medium was related with the protein SapB [17]. Based on this consideration, the transcription of *ramS* which encodes SapB was detected in both WT and SCO2140DM. The transcriptional level of *ramS* decreased remarkably when *SCO2140* was disrupted (Fig. 4b),

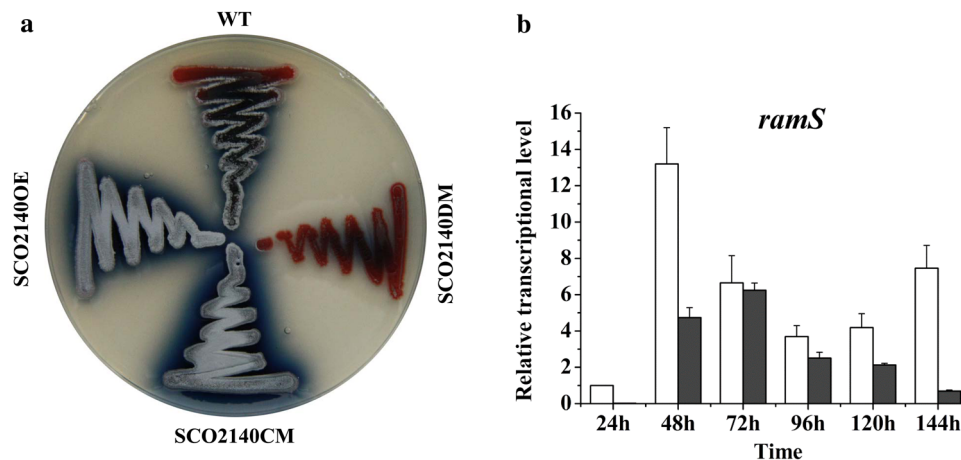


Fig. 4 Morphology analysis of the *S. coelicolor* M145 and its derivatives in R2YE media. **a** The phenotypes of WT, SCO2140DM, SCO2140CM, and SCO2140OE grown in R2YE medium for 3 days. **b** The transcriptional level of *ramS* was detected in WT and

SCO2140DM which were cultured in R2YE medium. The *open columns* represent the gene transcriptions in WT and the *solid columns* represent the gene transcriptions in SCO2140DM. *Error bars* represent the standard deviations from three independent experiments

indicating that *SCO2140* positively regulates aerial mycelium formation through controlling the transcription of *ramS*.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Nett M, Ikeda H, Moore BS (2009) Genomic basis for natural product biosynthesis diversity in the actinomycetes. *Nat Prod Rep* 26(11):1362–1384. doi:10.1039/b817069j
2. Gomez-Escribano JP, Bibb MJ (2012) *Streptomyces coelicolor* as an expression host for heterologous gene clusters. *Methods Enzymol* 517:279–300. doi:10.1016/B978-0-12-404634-4.00014-0
3. Liu G, Chater KF, Chandra G, Niu G, Tan H (2013) Molecular regulation of antibiotic biosynthesis in *Streptomyces*. *Microbiol Mol Biol Rev* 77(1):112–143. doi:10.1128/MMBR.00054-12
4. Pérez-Rueda E, Janga SC (2010) Identification and genomic analysis of transcription factors in archaeal genomes exemplifies their functional architecture and evolutionary origin. *Mol Biol Evol* 27(6):1449–1459. doi:10.1093/molbev/msq033
5. Tani TH, Khordusky A, Blumenthal RM, Brown PO, Matthews RG (2002) Adaptation to famine: a family of stationary-phase genes revealed by microarray analysis. *Proc Natl Acad Sci USA* 99(21):13471–13476. doi:10.1073/pnas.212510999
6. Peterson SN, Reich NO (2010) LRP: a nucleoid-associated protein with gene regulatory functions. In: Dame RT, Dorman CJ (eds) *Bacterial Chromatin*, 1st edn. Springer-Verlag, Berlin, pp 353–364
7. Parti R, Shrivastava R, Srivastava S, Subramanian A, Roy R, Srivastava B, Shrivastava R (2008) A transposon insertion mutant of *Mycobacterium fortuitum* attenuated in virulence and persistence in a murine infection model that is complemented by Rv3291c of *Mycobacterium tuberculosis*. *Microb Pathog* 45(5–6):370–376. doi:10.1016/j.micpath
8. Sprusansky O, Stirrett K, Skinner D, Denoya C, Westpheling J (2005) The *bkdR* gene of *Streptomyces coelicolor* is required for morphogenesis and antibiotic production and encodes a transcriptional regulator of a branched-chain amino acid dehydrogenase complex. *J Bacteriol* 187(2):664–671. doi:10.1128/JB.187.2.664-671.2005
9. Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (2000) *Practical Streptomyces genetics*. The John Innes Foundation, Norwich
10. 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 in *Streptomyces coelicolor*. *Mol Microbiol* 39(3):553–566. doi:10.1046/j.1365-2958.2001.02240.x
11. Yang H, Wang L, Xie Z, Tian Y, Liu G, Tan H (2007) The tyrosine degradation gene *hppD* is transcriptionally activated by HpdA and repressed by HpdR in *Streptomyces coelicolor*, while *hppA* is negatively autoregulated and repressed by HpdR. *Mol Microbiol* 65(4):1064–1077. doi:10.1111/j.1365-2958.2007.05848.x
12. Wang SL, Fan KQ, Yang X, Lin ZX, Xu XP, Yang KQ (2008) CabC, an EF-hand calcium-binding protein, is involved in Ca²⁺-mediated regulation of spore germination and aerial hypha formation in *Streptomyces coelicolor*. *J Bacteriol* 190(11):4061–4068. doi:10.1128/JB.01954-07
13. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(−ΔΔC(T)) method. *Methods* 25(4):402–408. doi:10.1006/meth.2001.1262
14. Miroux B, Walker JE (1996) Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *J Mol Biol* 260(3):289–298. doi:10.1006/jmbi.1996.0399
15. Calvo JM, Matthews RG (1994) The Leucine-responsive regulatory protein (Lrp), a global regulator of metabolism in *Escherichia coli*. *Microbiol Rev* 58(3):466–490
16. Reddy MC, Palaninathan SK, Shetty ND, Owen JL, Watson MD, Sacchettini JC (2007) High resolution crystal structures of *Mycobacterium tuberculosis* adenosine kinase—insights into the mechanism and specificity of this novel prokaryotic enzyme. *J Biol Chem* 282(37):27334–27342. doi:10.1074/jbc.M703290200
17. Capstick DS, Willey JM, Buttner MJ, Elliot MA (2007) SapB and the chaplins: connections between morphogenetic proteins in *Streptomyces coelicolor*. *Mol Microbiol* 64(3):602–613. doi:10.1111/j.1365-2958.2007.05674.x