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Genetic Characterization of Two *S*-Adenosylmethionine-induced ABC Transporters Reveals Their Roles in Modulations of Secondary Metabolism and Sporulation in *Streptomyces coelicolor* M145

SHIN, SU-KYOUNG, HYUN-SUH PARK, HYUNG-JIN KWON, HYUN-JIN YOON, AND JOO-WON SUH*

Department of Biological Science, Institute of Bioscience and Biotechnology, Myongji University, Yongin 449-728, Korea

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Abstract *S*-Adenosylmethionine (SAM) was previously documented to activate secondary metabolism in a variety of *Streptomyces* spp. and to promote actinorhodin (ACT) and undecylprodigiosin (RED) in *Streptomyces coelicolor*. The SAM-induced proteins in *S. coelicolor* include several ABC transporter components (SCO5260 and SCO5477) including BldKB, the component of a well-known regulatory factor for differentiations. In order to assess the role of these ABC transporter complexes in differentiation of *Streptomyces*, *SCO5260* and *SCO5476*, the first genes from the cognate complex clusters, were individually inactivated by gene replacement. Inactivation of either *SCO5260* or *SCO5476* led to impaired sporulation on agar medium, with the more drastic defect in the *SCO5260* null mutant (Δ*SCO5260*). Δ*SCO5260* displayed growth retardation and reduced yields of ACT and RED in liquid cultures. In addition, SAM supplementation failed in promoting the production of ACT and RED in Δ*SCO5260*. Inactivation of *SCO5476* gave no significant change in growth and production of ACT and RED, but impaired the promoting effect of SAM on ACT production without interfering with the effect on RED production. The present study suggests that SAM induces several ABC transporters to modulate secondary metabolism and morphological development in *S. coelicolor*.

Keywords: *S*-Adenosylmethionine, ABC transporter, gene inactivation, secondary metabolism, sporulation, *Streptomyces coelicolor*

ABC (ATP-binding cassette) transporters, one of the largest protein superfamilies, are responsible for the transport of an enormous range of molecules, from ions to large polypeptides [14]. With its transporting capability, ABC transporters allow bacteria to monitor environmental and physiological changes such as nutrient depletion, and provide the way to cope with those stimuli by evoking various intracellular signaling processes [8]. The *Escherichia coli* genome is predicted to encode 79 ABC transporters, and the coding sequence corresponds to 2.1% of the genome [21]. The complete genome sequence of *Streptomyces coelicolor* also reveals a huge number of ABC transporters including 81 typical ABC permeases and 141 ATP-binding proteins [2].

S. coelicolor is a Gram-positive, soil-inhabiting, filamentous bacterium, which has a complex life cycle to form a sporulating aerial mycelium in response to certain environmental and physiological signals [13]. A series of genetic loci belonging to the "*bld* cascade" are well known in the control of morphological and physiological differentiations in *S. coelicolor* [4, 40]. The *bld* mutants were initially characterized to be blocked in the formation of aerial mycelium at the earliest stage of development, and therefore display a smooth, bald phenotype of colonies [27]. Although the morphological defect is much emphasized, the *bld* mutants are also defective in secondary metabolism such as actinorhodin biosynthesis. The genetic locus of *bldK* was identified as the gene cluster serving as the blueprint of an oligopeptide permease complex that acts as the first receptor of an extracellular oligopeptidyl signal [25, 26].

It is now well documented that *S*-adenosylmethionine (SAM) exerts control of morphological and physiological differentiations in *Streptomyces*. The previous studies demonstrated that SAM inhibits morphological differentiation and enhances secondary metabolism in *S. lividans* [16], *S. griseus* [35], and *S. coelicolor* [29, 42]. It was also demonstrated that structural analogs of SAM retain the ability to promote actinorhodin production in *S. coelicolor* [5, 18, 41]. A proteomic study revealed that exogenous SAM induces several ABC transporter complexes, as indicated with enhancement in the level of soluble components of the cognate complexes [30]. Notably, BldKB is one of those solute-binding proteins induced by SAM. It has immediately been noticed that SAM induces two other

*Corresponding author
Phone: 82-31-330-6190; Fax: 82-31-336-0870;
E-mail: jwsuh@mju.ac.kr

solute-binding proteins, SCO5477 and SCO5260, whose physiological significances have not been characterized. The hypothetical gene cluster including *SCO5477* contains five open-reading frames (ORFs) of *SCO5476* to *SCO5480*, whereas the hypothetical cluster with *SCO5260* contains three ORFs of *SCO5260* to *SCO5258* (complementary sequences).

In the present study, we inactivated *SCO5260* and *SCO5476*, the utmost upstream genes in two ABC transporter gene clusters, and characterized the resulting mutants, especially in terms of the SAM effect on secondary metabolism.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

S. coelicolor strain M145, a prototrophic, plasmid-free strain of *S. coelicolor* A3(2), was used in this study [15]. *E. coli* DH5α (Stratagene) was used as a host for plasmid constructions. *E. coli* ET12567/pUZ8002 was used for intergeneric conjugation to *S. coelicolor* M145 [10]. The plasmids used in this study are listed in Table 1. *S. coelicolor* M145 wild-type strain (WT) and its derivative mutants were cultivated on R2YE or $R5^-$ agar for the purpose of genetic manipulation and phenotypic characterization. $R5^-$ liquid medium was used for determination of antibiotic production. Ampicillin (50 μg/ml), apramycin (50 μg/ml), chloramphenicol (25 μg/ml), or spectinomycin (200 μg/ml), all from Sigma, added to growth media when required.

Plasmid Construction and Gene Replacement

For disruption of the chromosomal *SCO5260* gene, the upstream and downstream regions of *SCO5260* were amplified by PCR with the following primers. The downstream region of 2,596-bp was amplified with 5'-CATGTC GAATTC-ATGACGAACGCGGACACG-3' (the box for the EcoRI site) and 5'-CATGTC TCTAGA GTGACTGTTGACATCGAC-3' (the box for the XbaI site). The upstream region of 2,151-bp was amplified with 5'-CATGTC CTGCAG AGAGCACA-CACCTTC-3' (the box for the PstI site) and 5'-CATGTC-AAGCTT ATAGACAGCGAACATGAC-3' (the box for the HindIII site). The EcoRI-XbaI fragment of the downstream region was subcloned into the same of pDH5 [12]. Into the PstI and XbaI sites of the resulting plasmid, the 1.5-kb PstI-XbaI fragment of the *acc(3)IV* apramycin resistance gene, which is rescued from pUO9090, was subcloned. The resulting plasmid was digested with PstI plus HindIII and ligated with the PstI-HindIII fragment of the upstream region to generate pJWS-Sc5260. For the purpose of conjugative transfer of pJWS-Sc5260, the *aadA* spectinomycin resistance gene and *oriT* fragment were rescued from pIJ778 as a 1.4-kb EcoRI-HindIII fragment and subcloned into the HindIII site of pJWS-Sc5260 after being blunted with a Klenow fragment. The resulting gene-replacement plasmid was named pJWS-Sco5260.

For disruption of the chromosomal *SCO5476* gene, the upstream and downstream regions of *SCO5476* were amplified by PCR with the following primers. The upstream region of 1,896-bp was amplified with 5'-CATGTC-AAGCTT CCTCGCTGGAGATCGACG-3' (the box for the HindIII site) and 5'-CATGTC CTGCAG TCTCAACCGG-CGGTAGCC-3' (the box for the PstI site). The downstream region of 2,036-bp was amplified with 5'-CATGTC-TCTAGA AACCTCTTCGGTGACGGC-3' (the box for the XbaI site) and 5'-CATGTC GAATTC GATGAGGTAAGC-

Table 1. Strains and plasmids used in this study.

Strains or plasmids	Relevant genotype	Source and reference
***E. coli* strains**		
DH5α	*supE44 ΔlacU169(Φ80lacZΔM15)hsdR17* recA 1endA1 gyrA96 thi-1 relA1	Stratagene
ET12567/pUZ8002	*dam-13*::Tn*9 dcm cat tet hsd zjj-201*::Tn*10/tra neo* RP4	[10]
***S. coelicolor* strains**		
M145	$SCP1^-$ $SCP2^-$	[15]
Δ*SCO5260*	M145 *SCO5260* is deleted and replaced with *aac(3)IV*	This study
Δ*SCO5476*	M145 *SCO5476* is deleted and replaced with *aac(3)IV*	This study
Plasmids		
pDH5	*E. coli* plasmid pF1 derived from pUC19, carrying *bla*, *lacZα*, MCS, T7 and T3 promoters, and phage f1 intergenic region	[12]
pIJ778	*addA* from Ω-fragment+*oriT*	[10]
pUO0909	pUC-derived cloning vector with apramycin resistance gene and kanamycin resistance gene	Lab. stock
pJWS-Sco5260	pDH5 containing the 2.2 kb fragment of the upstream region of *SCO5260*, the 2.6 kb fragment of the downstream region of *SCO5260*, *addA+oriT*, and *acc(3)IV*	This study
pJWS-Sco5476	pDH5 containing the 1.9 kb fragment of the upstream region of *SCO5476*, the 2.0 kb fragment of the downstream region of *SCO5476*, *addA+oriT*, and *acc(3)IV*	This study

GAGCAC-3' (the box for the EcoRI site). The upstream fragement, the downstream fragment, the *acc(3)IV* gene, the *aadA* gene, and the *oriT* fragment were assembled to give pJWS-Sco5476 with the same procedures as those described for the construction of pJWS-Sco5260.

The gene replacement plasmids were introduced into *E. coli* ET12567/pUZ8002 and then transferred to *S. coelicolor* by conjugation. The exconjugants were selected for their resistance to apramycin, and then screened for those showing spectinomycin-sensitive phenotype. The gene replacement though double-crossover allelic exchange was confirmed by PCR amplification and subsequent restriction digestion analysis. The oligonucleotide primer pairs used in the PCR analysis were 5'-GTCAAGTCTGGCTACA-GATC-3'/5'-GAGAAGTCCCACATGAAGCC-3' and 5'-CTTGTCGATGTCAACAGTCAC-3'/5'-CTCGAAGGT-GTGTGCTCTCG-3' for Δ*SCO5476* and Δ*SCO5260*, respectively.

Assay of Antibiotic Production

Actinorhodin (ACT) and undecylprodigiosin (RED) levels were determined by following the previously documented procedure [15]. Cell growth was assessed by total protein concentration as determined by the Bradford method. Exogenous SAM was administered into liquid R5$^-$ cultures at 12 h after the inoculation.

Scanning Electron Microscopy

Spores and hyphae from WT and its derivative mutants were examined by scanning electron microscopy (SEM) after six days of growth on R2YE agar medium. The agar blocks containing spores and hyphae were fixed with 1% osmium tetroxide for 12 h and then dehydrated by freeze-drying. Each specimen was sputter-coated with platinum-gold and examined with an Hitachi S4000 scanning microscope.

RESULTS

Inactivation of *SCO5260* and *SCO5476*

The previous proteomic study on *S. coelicolor* demonstrates that accumulation of SAM induces extracellular components of ABC transporters such as SCO5260, SCO5477, and SCO5113 (BldKB) [30]. Among them, BldKB is the solute-binding protein component of the BldK complex, which is well known to transport an oligopeptide signal for *S. coelicolor* differentiation control [25, 26]. *SCO5260* and *SCO5477* also belong to the hypothetical gene clusters predicted to encode ABC transporter complexes. The gene cluster with *SCO5477* consists of five ORFs that are predicted to encode two oligopeptide transport integral membrane proteins (SCO5476, SCO5478), an oligopeptide-binding lipoprotein (SCO5477), and two ATP-binding

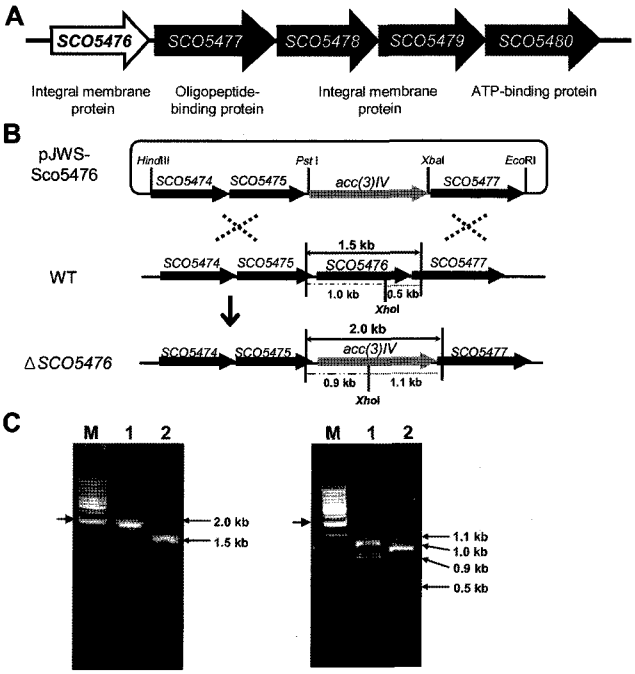

Fig. 1. Inactivation of *SCO5476* by gene replacement *via* double-crossover homologous recombination.

A. Genetic organization from *SCO5476* to *SCO5480* with the deduced protein functions shown below. **B.** Construction of the *SCO5476* null mutant (Δ*SCO5476*) and restriction maps of the replacement plasmid pJWS-Sco5476, *S. coelicolor* M145 wild-type, and Δ*SCO5476* showing predicted sizes of PCR product before and after XhoI digestion. **C.** XhoI restriction digestion and electrophoretic analysis of the PCR product amplified from the chromosome of Δ*SCO5476* (1) or wild-type (2). M indicates the DNA molecular weight marker with the 2.0 kb fragment emphasized with an arrow (0.25, 0.5, 0.75, 1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 8.0, and 10.0 kb from the bottom).

proteins (SCO5479, SCO5480) (Fig. 1A). SCO5477 is a member of the OppA (oligopeptide permease component A) family (COG4166; a periplasmic component of the ABC-type oligopepetide transport system) and displays the highest homology to SAV2768 from *S. avermitilis*. It must be noted that BldKB is also a member of the OppA family. The gene cluster harboring *SCO5260* consists of three ORFs that are predicted to encode an ATP-binding protein (SCO5258), an inner-membrane protein (SCO5259), and an extracellular solute-binding protein (SCO5260) (Fig. 2A). SCO5260 bears a conserved domain of bacterial periplasmic solute-binding protein (pfam00497) and displays 79% amino acid identities to a putative arginine-binding protein of *S. avermitilis* (SAV2982).

Bacterial Opp complexes belong to the ABC transporter family and have been found to be involved in transporting specific regulatory molecules [7, 20] and regulating cell physiology, including competence development [11, 23], sporulation [32, 36], DNA transfer by conjugation [6, 19], protein secretion [31], chemotaxis [1], and virulence development [7]. In general, Opp complexes are also

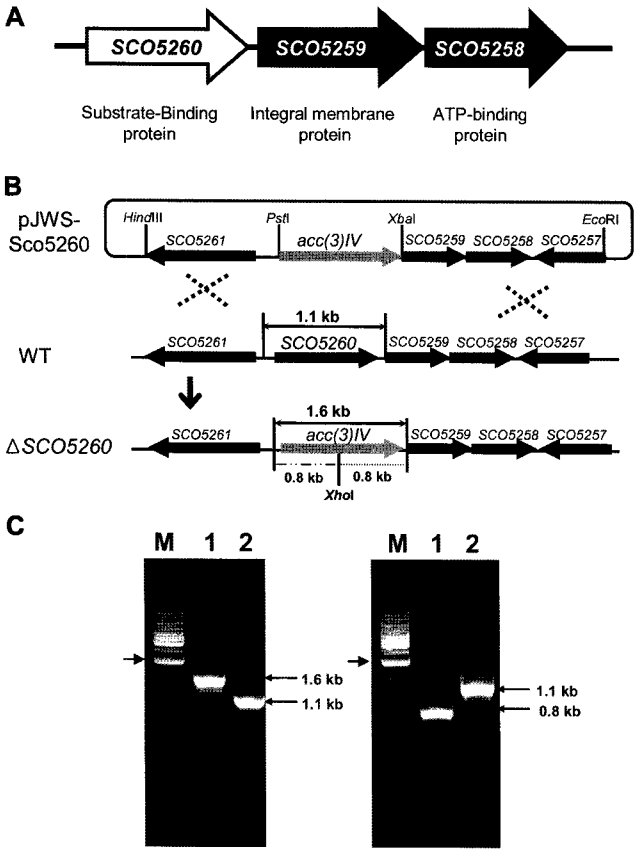

Fig. 2. Inactivation of *SCO5260* by gene replacement *via* double-crossover homologous recombination.

A. Genetic organization from *SCO5260* to *SCO5258* with the deduced protein functions shown below. **B.** Construction of the *SCO5260* null mutant (Δ*SCO5260*) and restriction maps of the replacement plasmid pJWS-Sco5260, *S. coelicolor* M145 wild-type, and Δ*SCO5260* showing predicted sizes of PCR product before and after XhoI digestion. **C.** XhoI restriction digestion and electrophoretic analysis of the PCR product amplified from the chromosome of Δ*SCO5260* (1) or wild-type (2). M indicates the DNA molecular weight marker with the 2.0 kb fragment emphasized with an arrow (0.25, 0.5, 0.75, 1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 8.0, and 10.0 kb from the bottom).

envisioned to play a role in salvaging extracellular peptides as nutrients. Besides the BldK complex, some ABC transporter complexes such as RamAB of *S. coelicolor* [22, 24] and AmfAB of *S. griseus* [38] are known to be involved in the control of aerial mycelium formation. Here, we investigated the roles of two ABC transporter systems harboring SCO5260 and SCO5477 on differentiations of *S. coelicolor*, *i.e.*, production of secondary metabolites for the physiological differentiation, and sporulation for the morphological differentiation. To achieve this goal, we prepared the two null mutants of Δ*SCO5260* and Δ*SCO5476* by replacing the respective coding region with the apramycin resistance gene (Figs. 1B and 2B). In this study, *SCO5476* was chosen for the inactivation target, instead of *SCO5477*; a simple logic is that the utmost upstream gene was inactivated. The PCR analysis assisted with XhoI digestion confirmed that *SCO5476* (XhoI digestion liberates 1.0 and 0.5 kb fragments from the 1.5 kb PCR product) and *SCO5260* (1.1 kb PCR product with no XhoI site) were replaced with *aac(3)IV* in Δ*SCO5476* (XhoI digestion liberates 0.9 and 1.1 kb fragments from the 2.0 kb PCR product) and Δ*SCO5260* (XhoI digestion liberates two 0.8 kb fragments from the 1.6 kb PCR product), respectively (Figs. 1C and 2C).

Inactivation of *SCO5260* and *SCO5476* Impaired Morphological Differentiation

Phenotypes of Δ*SCO5260* and Δ*SCO5476* were compared with WT on R2YE plates after incubation at 28°C for 6 days. WT formed fluffy and grey colonies and produced a large amount of blue-pigmented antibiotic actinorhodin (Fig. 3A). WT showed abundant regular and circular chains of spores when examined by scanning electron microscopy (Fig. 3A). Δ*SCO5476* displayed a pale grey color on the agar medium and was dominated with undifferentiated aerial hyphae formation with spores remarkably longer than those of WT (Fig. 3B). Δ*SCO5260* showed an apparent retardation in sporulation and its colonies exhibited cracks over their surface on the agar medium. Scanning electron microscopy revealed that Δ*SCO5260* also produced undifferentiated aerial hyphae with immature, aberrantly sized spore chains showing occasionally irregular and atypical septum placement (Fig. 3C). Therefore, it can be conceived that two respective ABC transporters containing each of SCO5477 and SCO5260 contribute to development of normal aerial hyphae and sporulation either by transporting of extracellular signals or by acquiring nutrients.

Secondary Metabolism was Conditionally Impaired in Δ*SCO5260* and Δ*SCO5476*

It is widely known that mutants defective in aerial hyphae development occasionally exhibit defects in ACT and RED production in *S. coelicolor*, and the antibiotic production and aerial hyphae development share regulatory links. The observation that Δ*SCO5476* and Δ*SCO5260* are defective in spore development, together with the fact that the cognate transporter complexes are overexpressed by SAM accumulation [30], allowed us to hypothesize that Δ*SCO5476* and Δ*SCO5260* may also be defective in the control of ACT and RED production, especially relating to the SAM-mediated promotion. To test this idea, the levels of ACT and RED were determined in R5$^-$ liquid cultures of Δ*SCO5476* and Δ*SCO5260* with or without 2 μM of SAM.

The growth of Δ*SCO5260* was obviously retarded at the early phase of culture, whereas that of Δ*SCO5476* appeared promoted when compared with WT (Fig. 4A). The growth of Δ*SCO5260* was recovered after 2 days incubation and the growth rate went beyond the WT level at 3 days after inoculation. For Δ*SCO5476*, the growth profile was comparable

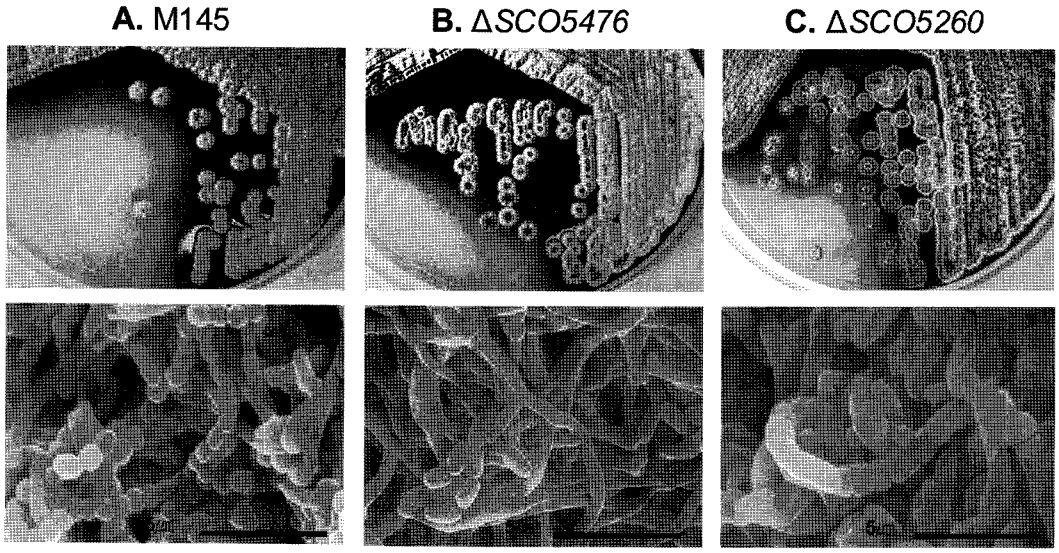

Fig. 3. Phenotypes and scanning electron micrographs showing the colony surfaces of *S. coelicolor* M145 wild-type (**A**), Δ*SCO5476* (**B**), and Δ*SCO5260* (**C**).

to WT, except for a promotion at entering a stationary phase, and the total protein content was found as 135% that of WT at the final culture time. SAM supplementation did not affect growth profiles of all the strains used in this study (Fig. 4A). Levels of ACT and RED in Δ*SCO5260* were remarkably lower than those of WT (Figs. 4B and 4C), which may associate with the early growth retardation. In the case of Δ*SCO5476*, no significant difference was seen in levels of ACT and RED when compared with WT (Fig. 4). Marginal differences, an increase for ACT and a decrease for RED, were characteristics to secondary metabolism of Δ*SCO5476*. Supplementation of SAM clearly activated ACT production in WT, but no change was seen in both mutants. Obviously, the ACT level was not enhanced by SAM supplementation in Δ*SCO5476*, indicating that an intracellular message generated by SAM accumulation necessitates *SCO5476*, and possibly the cognate complex, to achieve a promotion on ACT biosynthesis. We therefore propose that the SCO5476-bearing ABC transporter complex plays a regulatory role in differentiation of *S. coelicolor* as does the BldK complex. Contrary to the case of ACT production, the presence of SAM led both WT and Δ*SCO5476* to overproduce RED, thus indicating that the proposed regulatory role of the SCO5476-bearing complex is specific to ACT biosynthesis. It must be noted that ACT, but not RED, was abolished in a *bldK* mutant, and the phenotypic defect is dependent on carbon source in the culture medium [26]; supplementation of mannitol as a carbon source suppresses a defect in ACT biosynthesis of *bldK* mutant. Exogenous SAM had no effect on the production of ACT and RED in Δ*SCO5260*. It is likely that the SCO5260-bearing complex may also control secondary metabolism in terms of the SAM signal, but an apparent retardation on growth does not allow us to propose that the SCO5260 complex plays a specific regulatory role in secondary metabolism.

DISCUSSION

The present study reveals that *SCO5260* and *SCO5476*, the genes located within distinctive ABC transporter clusters, are required for normal sporulation (Fig. 3), which allows us to propose that these two ABC transporter complexes play regulatory roles in *S. coelicolor* sporulation. Furthermore, it is proposed that the SCO5476-bearing complex is conditionally implicated in the control of ACT biosynthesis, as grounded on the fact that Δ*SCO5476* showed no response to SAM supplementation in ACT production (Fig. 4). Inactivation of *SCO5260* retarded early growth of cells and impaired production of ACT and RED. SAM supplementation failed in promoting ACT and RED production in Δ*SCO5260* (Fig. 4). In *S. coelicolor*, multiple signals have been implicated in morphogenesis and there involves the extracellular oligopeptide signaling cascade related to a series of *bld* loci [3]. Specifically, *bldK*, the gene encoding an ABC transporter to import extracellular oligopeptide(s), was documented to control the signaling cascade for aerial mycelium formation [25, 26]. The present genetic characterization of *SCO5476* and *SCO5260* substantiates that two additional ABC transporter complexes are involved in differentiation of *S. coelicolor* and play roles in coupling the accumulation of SAM to a consecutive differentiation process.

Streptomyces undergoes a complex life cycle, and the metabolic transition occurs to support secondary metabolism and morphological development such as aerial hyphae

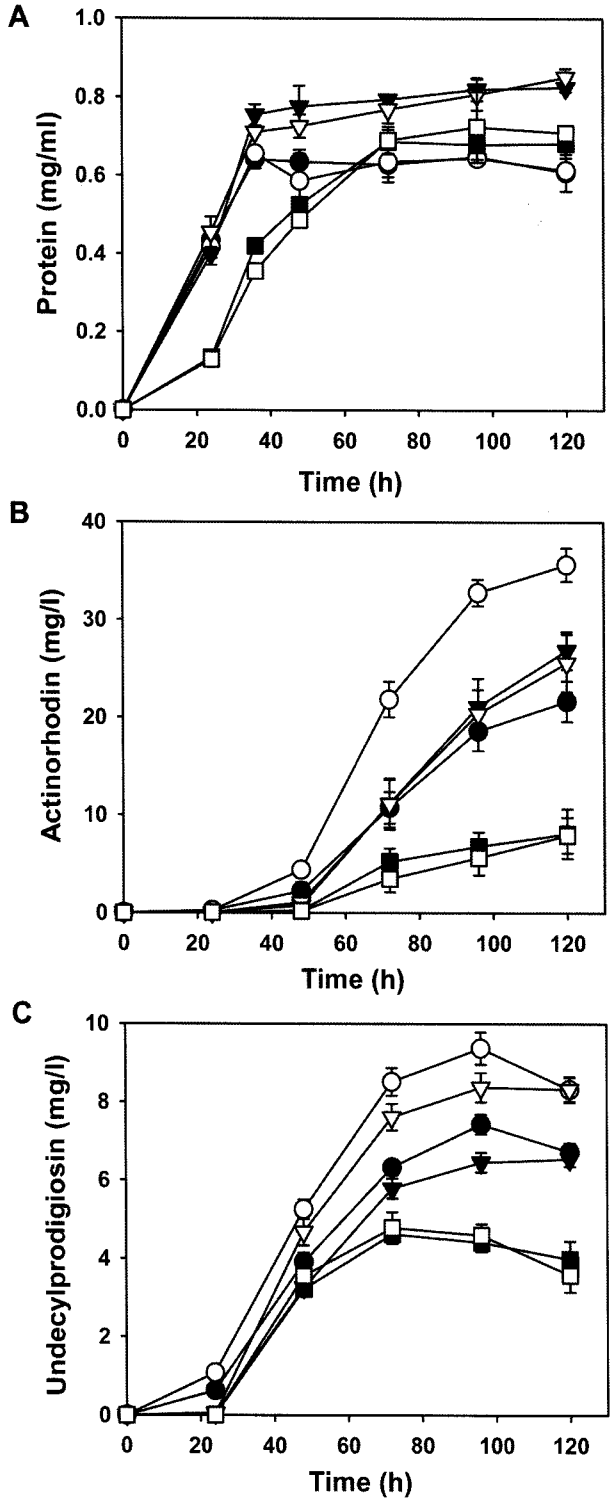

Fig. 4. Production of antibiotics in Δ*SCO*5260 and Δ*SCO5476* and their growths. The growth was expressed by protein concentration (**A**). Levels of actinorhodin (**B**) and undecylprodigiosin (**C**) were determined over time after feeding 2 μM of SAM at 12 h after the initiation of cultures.

Symbols: closed types, non-treated culture; open types, SAM-treated culture; circle, *S. coelicolor* M145 wild-type strain; triangle, Δ*SCO5476*; square, Δ*SCO*5260.

formation that leads to sporulation [37]. Therefore, specialized regulatory systems monitor a series of unique physiological stages and control the expression of numerous genes to induce developmental processes. Previous proteomic study documented that SCO5260 and SCO5477 were induced at different developmental stages; SCO5260 tends to decrease in abundance as growth goes, whereas SCO5477 and BldKB remain abundant throughout the growth [17]. Differentiated phenotypes were observed between Δ*SCO5260* and Δ*SCO5476* in the present study. The growth of Δ*SCO5260* was significantly repressed at the early stage of the culture, but its growth was recovered to the level of WT after 3 days (Fig. 4A). Therefore, together with the proteome data [17], the SCO5260-bearing complex is proposed to support mycelial growth at an early stage, which is associated with physiological and morphological developments.

Novotna *et al.* [28] revealed that some proteins involved in the control of developmental programs are commonly induced by stress conditions. For example, BldKB was induced by salt shock, environmental stress, and starvation. SCO5477 was also induced as two different isoforms by heat (65 kDa) and cold (74 kDa) shock [28]. Interestingly, two spots for SAM synthetase in proteome analysis belong to respective developmental expression clusters that are shared by either BldKB or SCO5477 (http://proteom.biomed.cas.cz/). It was reported that the SAM synthetase gene is overexpressed in response to various environmental stress conditions in several plants [9, 33, 34, 39]. It will therefore be interesting to see whether there is any relationship between the intracellular SAM level and stress responses, especially regarding their respective interplay with these stress-induced ABC transporters. We anticipate that further physiological and molecular studies on Δ*SCO5476* and Δ*SCO5260* will provide a keen insight into the relationship of SAM and stress responses in *Streptomyces*.

Acknowledgments

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