

afsQ1-Q2-sigQ is a pleiotropic but conditionally required signal transduction system for both secondary metabolism and morphological development in *Streptomyces coelicolor*

Dan Shu · Lei Chen · Weihua Wang · Zhenyu Yu ·
Cong Ren · Weiwen Zhang · Sheng Yang · Yinhua Lu ·
Weihong Jiang

Received: 1 July 2008 / Revised: 25 September 2008 / Accepted: 27 September 2008 / Published online: 24 October 2008
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Abstract Two-component system AfsQ1-Q2 of *Streptomyces coelicolor* was identified previously for its ability to stimulate actinorhodin (ACT) and undecylprodigiosin (RED) production in *Streptomyces lividans*. However, disruption of either *afsQ1* or *afsQ2* in *S. coelicolor* led to no detectable changes in secondary metabolite formation or morphogenesis. In this study, we reported that, when cultivated on defined minimal medium (MM) with glutamate as the sole nitrogen source, the *afsQ* mutant exhibited significantly decreased ACT, RED, and calcium-dependent antibiotic (CDA) production and rapid growth of aerial mycelium. In addition, we also found that deletion of *sigQ*, which is located upstream of *afsQ1-Q2* and encodes a putative sigma factor, led to the precocious hyperproduction of these antibiotics and delayed formation of sporulating

aerial mycelium in the same glutamate-based defined MM. Reverse-transcription polymerase chain reaction and *egfp* fusion analyses showed that the expression of *sigQ* was under control by *afsQ*. In addition, deletion of both *afsQ-sigQ* resulted in the phenotype identical to that of *afsQ* mutant. The results suggested that *afsQ1-Q2* and *sigQ* worked together in the regulation of both antibiotic biosynthesis and morphological development, and *sigQ* might be responsible for antagonizing the function of AfsQ1-Q2 in *S. coelicolor*, however, in a medium-dependent manner. Moreover, the study showed that the medium-dependent regulation of antibiotic biosynthesis by AfsQ1-Q2-SigQ was through pathway-specific activator genes *actII-ORF4*, *redD*, and *cdaR*. The study provides new insights on regulation of antibiotic biosynthesis and morphological development in *S. coelicolor*.

Electronic supplementary material The online version of this article (doi:10.1007/s00253-008-1738-1) contains supplementary material, which is available to authorized users.

D. Shu · L. Chen · W. Wang · Z. Yu · C. Ren · S. Yang ·
Y. Lu (✉) · W. Jiang (✉)
Laboratory of Molecular Microbiology,
Institute of Plant Physiology and Ecology,
Shanghai Institutes for Biological Sciences,
Chinese Academy of Sciences,
300 Fenglin Rd,
Shanghai, 200032, People's Republic of China
e-mail: yhlu@sibs.ac.cn
e-mail: whjiang@sibs.ac.cn

D. Shu · L. Chen · W. Wang · Z. Yu · C. Ren
Graduate University of the Chinese Academy of Sciences,
Beijing, 100080, People's Republic of China

W. Zhang
Center for Ecogenomics, Biodesign Institute,
Arizona State University,
Tempe, AZ 85287-6501, USA

Keywords Morphological differentiation · Sigma factor ·
Secondary metabolism · *Streptomyces coelicolor* ·
Two-component system

Introduction

Microorganisms must modulate their gene expression repertoire in order to adapt to changing environments. One of the predominant signal transduction mechanisms employed by microbes is the phosphotransfer pathway commonly referred to as “two-component” signal transduction systems (TCS), which typically consist of a sensor histidine kinase (HK) and a response regulator (RR) and have been found across all three domains of life, the *Bacteria*, *Archaea*, and *Eukarya* (Hoch 2000). The concept of TCS was initially introduced from extensive studies on

the model prokaryotic bacteria, such as *Escherichia coli* in which 62 two-component proteins have been identified and their regulatory functions are involved in diverse cellular processes, including chemotaxis, osmoregulation, metabolism, and transport (Mizuno 1997, 2005). So far, more than 4,000 TCSs have been detected from 145 sequenced bacterial genomes (Ulrich et al. 2005; Zhang and Shi 2005). Results of recent studies on the model streptomycete, *Streptomyces coelicolor*, have led us to learn a new scenario as to the versatility of TCSs in mycelial prokaryotes, which are involved in stress-induced differentiation, such as sporulation and secondary metabolism.

S. coelicolor is a genetically well-studied strain in streptomycetes, which differ conspicuously from most other bacteria for their fungal-like developmental cycles and synthesis of multiple antibiotics. The complete genome sequence of *S. coelicolor* revealed 84 putative sensor HK genes and 80 putative RR genes, among them 67 HK–RR are located adjacently on the chromosome (Bentley et al. 2002; Hutchings et al. 2004). Currently, the functions and possible downstream regulatory targets of these TCSs are largely unknown, although several have been found involved in regulation of very diverse cellular processes such as osmoregulation, metabolism, cell growth, and differentiation in *S. coelicolor* (Hutchings et al. 2004). Several well-studied TCSs include PhoP-R system mediating phosphate limitation response (Sola-Landa et al. 2003, 2005, 2008; Rodriguez-Garcia et al. 2007), CseB-C and VanR-S systems activating genes involved in integrity of cell envelope when *S. coelicolor* A3 (2) was exposed to vancomycin and some cell-wall-specific antibiotics (Paget et al. 1999b; Hong et al. 2002; Hutchings et al. 2006a), and TCS systems involved in antibiotic production, such as CutR-S (Chang et al. 1996), RapA1-A2 (Lu et al. 2007), AbsA1-A2 (Adamidis et al. 1990; Brian et al. 1996; Aceti and Champness 1998; Anderson et al. 1999, 2001; Ryding et al. 2002; Sheeler et al. 2005; McKenzie and Nodwell 2007), and AfsQ1-Q2 (Ishizuka et al. 1992). Among them, AbsA1-A2 system is the most well studied and has been found to negatively regulate biosynthesis of the four antibiotics synthesized by *S. coelicolor*, actinorhodin (ACT), undecylprodigiosin (RED), methylenomycin, and the calcium-dependent antibiotic (CDA), by directly interfering with the expression of pathway-specific regulatory genes such as *actII-ORF4* (Arias et al. 1999), *cdaR* (Ryding et al. 2002), and *redZ* (White and Bibb 1997).

Another TCS, the AfsQ1-Q2 system was identified initially for its ability to stimulate production of ACT and RED in *Streptomyces lividans*. However, disruption of either *afsQ1* or *afsQ2* on the *S. coelicolor* chromosome by use of phage Φ C31KC515 led to no detectable change in secondary metabolite formation or morphogenesis when the mutants were grown under rich YMPG medium. In addition, the

afsQ1 gene on pIJ922 suppressed the *S. coelicolor absA* mutation and caused ACT production, suggesting its possible roles in regulating antibiotic production in *S. coelicolor* (Ishizuka et al. 1992). In this work, to seek further evidence for the function of AfsQ1-Q2 in *S. coelicolor*, we constructed the *afsQ1-Q2* deletion mutant and studied its phenotype under various growth conditions. Consistent with previous report (Ishizuka et al. 1992), no obvious change of any phenotype was observed when cultured on complex media. However, when grown on defined minimal medium (MM) with glutamate as sole nitrogen, the mutant almost lost its ability to generate ACT.

In a previous study, an extracytoplasmic function sigma factor, SigE, that is located upstream of the CseB-CseC two-component system was found to be required for normal cell wall integrity and its expression was controlled by CseB-CseC in response to signals from the cell envelope in *S. coelicolor* (Paget et al. 1999a, b; Hong et al. 2002). Sequence analysis showed that a gene encoding putative sigma factor, *sigQ*, was also located upstream of *afsQ1-Q2* (Bentley et al. 2002). The similar genetic organization tempted us to investigate the possible function of *sigQ* in this study. Similar to the case in SigE-CseB-CseC, the expression of *sigQ* was also under control by *afsQ1-Q2*. However, to our surprise, deletion of *sigQ* led to the precocious hyperproduction of antibiotic and delayed formation of sporulating aerial mycelium in the same glutamate-based defined MM. The results suggested that *afsQ1-Q2* and *sigQ* worked together in the regulation of both antibiotic biosynthesis and morphological development in a medium-dependent manner in *S. coelicolor*.

Materials and methods

Strains, plasmids, and culture conditions Bacterial strains and plasmids are listed in Table 1. MS agar medium (Kieser et al. 2000) was used to make spore suspensions. GYM (Chakraborty et al. 1996), R2YE, and solid glucose-minimal medium (Kieser et al. 2000) in which ammonium was replaced by glutamate were used to determine ACT and RED production. RNA was isolated from cultures grown on MM medium supplemented with 75 mM sodium L-glutamate as the sole nitrogen source. When necessary, the media were supplemented with antibiotics (100 $\mu\text{g ml}^{-1}$ for ampicillin, 50 $\mu\text{g ml}^{-1}$ for kanamycin, 50 $\mu\text{g ml}^{-1}$ for apramycin, 100 $\mu\text{g ml}^{-1}$ for thiostrepton).

Transformation and conjugation Plasmids or cosmids with *oriT* fragment are introduced by transformation into the methylation-deficient *E. coli* host ET12567 containing the RP4 derivative pUZ8002 and then transferred to *S. coeli-*

Table 1 Bacterial strains and plasmids used in this study

Strains/plasmids	Genotype/description	Reference/source
<i>S. coelicolor</i> A3(2)		
M145	Wild type, SCP1 ⁻ SCP2 ⁻	(Kieser et al. 2000)
M145a	M145 with pSET1521	This work
Δ afsQ	M145 Δ afsQ1Q2::aac(3)IV SCP1 ⁻ SCP2 ⁻	This work
Δ afsQa	M145 Δ afsQ1Q2::aac(3)IV SCP1 ⁻ SCP2 ⁻ pSET1521	This work
Δ afsQb	M145 Δ afsQ1Q2::aac(3)IV SCP1 ⁻ SCP2 ⁻ pSET1521-afsQ	This work
Δ afsQc	M145 Δ afsQ1Q2::aac(3)IV SCP1 ⁻ SCP2 ⁻ pSET1521-sigQ	This work
Δ afsQd	M145 Δ afsQ1Q2::aac(3)IV SCP1 ⁻ SCP2 ⁻ pSET1521-afsQ1sigQ	This work
Δ sigQ	M145 Δ sigQ::aac(3)IV SCP1 ⁻ SCP2 ⁻	This work
Δ sigQa	M145 Δ sigQ::aac(3)IV SCP1 ⁻ SCP2 ⁻ pSET1521	This work
Δ sigQb	M145 Δ sigQ::aac(3)IV SCP1 ⁻ SCP2 ⁻ pSET1521-sigQ	This work
Δ afsQsigQ	M145 Δ afsQ1Q2sigQ::aac(3)IV SCP1 ⁻ SCP2 ⁻	This work
Δ afsQsigQa	M145 Δ afsQ1Q2sigQ::aac(3)IV SCP1 ⁻ SCP2 ⁻ pSET1521	This work
Δ afsQsigQb	M145 Δ afsQ1Q2sigQ::aac(3)IV SCP1 ⁻ SCP2 ⁻ pSET1521-afsQsigQ	This work
<i>E. coli</i>		
DH5 α	F-80 Φ dlacZDM15D(lacZYA-argF)U169deoRecA1endA1hsdR17(rk-mk+)supE44 λ -thi-1gyrA96 relA1	GIBCO-BRL
BW25113/ pIJ790	BW25113 containing temperature-sensitive plasmid pIJ790 which encodes the λ RED recombination system	(Gust et al. 2003)
ET12567/ pUZ8002	dam-13::Tn9 dem-6 hsdM; containing the nontransmissible RP4 derivative plasmid pUZ8002	(Gust et al. 2003)
Plasmids		
pIJ773	Plasmid containing the apramycin resistance gene <i>aac(3)IV</i> and the <i>oriT</i> of plasmid RP4, flanked by FRT sites	(Gust et al. 2003)
2StK8	A cosmid from ordered <i>S. coelicolor</i> library which constructed in the pSupercos 1 vector, position from 5,306,803 to 5,345,072 bp of the <i>S. coelicolor</i> genome is included	(Redenbach et al. 1996)
pSET152	pUC19 <i>ori</i> , Φ C31 <i>int/attP</i> , <i>aac(3)IV</i> , <i>lacZα</i> , <i>oriT</i> RK2	(Kieser et al. 2000)
pSET1521	pSET152 with <i>tsr</i> , thiostrepton resistance gene from pWHM3	This work
pSET1521-afsQ	A 3.2-kb PCR fragment with <i>afsQ1Q2</i> and its promoter region in the <i>Bam</i> HI site of pSET1521	This work
pSET1521-sigQ	A 1.9-kb PCR fragment with <i>sigQ</i> and its promoter region in the <i>Bam</i> HI site of pSET1521	This work
pSET1521-afsQ1sigQ	A 3.3-kb PCR fragment with <i>afsQ1</i> and <i>sigQ</i> in the <i>Bam</i> HI site of pSET1521	This work
pSET1521-afsQsigQ	A 3.6-kb PCR fragment with <i>afsQ1Q2sigQ</i> and its promoter region in the <i>Bam</i> HI site of pSET1521	This work
pIJ8660	Promoter probe vector containing the EGFP gene as indicator; <i>oriT</i> , RK2, <i>attP</i> , <i>aac(3)IV</i>	(Sun et al. 1999)
pIJ86601	pIJ8660 with <i>tsr</i> , thiostrepton resistance gene from pWHM3	This work
pIJ86601- <i>hrdBp</i>	A 896-bp PCR fragment of <i>hrdB</i> promoter region inserted into the <i>Kpn</i> I- <i>Bam</i> HI of pIJ86601	This work
pIJ86601-sigQp	A 906-bp PCR fragment of <i>sigQ</i> promoter region inserted into the <i>Kpn</i> I- <i>Bam</i> HI of pIJ86601	This work

color A3(2) M145 and its derivatives by intergeneric conjugation as described previously (Kieser et al. 2000).

Construction of Δ afsQ, Δ sigQ, and Δ afsQsigQ mutants The mutants were constructed by replacing the entire coding region of the target genes with an apramycin resistance *oriT* cassette using the polymerase chain reaction (PCR)-targeting system described by Datsenko and Wanner (2000) and Gust et al. (2003). Primers used for construction of the deletion mutants in this study are listed in Supplementary Table 1. All mutants generated were

confirmed by PCR, reverse-transcription (RT)-PCR, and DNA sequencing.

Complementation of the deletion mutants The integrative plasmid pSET1521 was modified from pSET152 (Kieser et al. 2000) by adding a thiostrepton resistance gene (*tsr*) and constructed as follows. Plasmid pSET152 was digested with *Sph*I and treated with Klenow polymerase fragment first and then alkaline phosphatase. The *Bc*III fragment containing the *tsr* gene from pWHM3 was cloned in pSET152, generating pSET1521. The primers AQc1-c2,

A1SQc1-c2, SQc1-c2, and ASQc1-c2 (listed in Supplementary Table 1) were used to amplify the fragments containing the open reading frames and the putative promoter regions of *afsQ1-Q2*, *afsQ1-sigQ*, *sigQ*, and *afsQ1-Q2-sigQ*, respectively, from the genomic DNA of *S. coelicolor* M145. The fragments were first cloned on pMD18-T vector and then moved into pSET1521. The resulting complemented vectors were introduced into *S. coelicolor* mutant strains by conjugation. The plasmid pSET1521 was used as a negative control in complementation assay.

Assay of the calcium-dependent antibiotic CDA were assayed according to the method described by Kieser et al. (2000). Briefly, the tested strains were cultured on MM agar with glutamate for 24–48 h and then overlaid with soft LB agar containing *Staphylococcus aureus* and $\text{Ca}(\text{NO}_3)_2$ at a final concentration of 12 mM throughout the plate. A zone of inhibition, which is absent when $\text{Ca}(\text{NO}_3)_2$ is omitted, is diagnostic for CDA.

RNA preparation and RT-PCR analysis RNA preparation and RT-PCR assays were performed as described previously (Lu et al. 2007). RT-PCR primers used in this study are listed in Supplementary Table 2. MM with 75 mM glutamate covered with cellophane disks was used for RNA isolations. For each RT reaction, 1 μg of RNA was denatured together with 500 ng of random hexamers and 10 mM deoxynucleotide triphosphates at 75°C for 5 min and quick-chilled on ice. 5 \times RT buffer, RNase inhibitor (Takara), and ReverTra Ace[®] (Toyobo) were added to each RNA–primer mixture to give a total volume of 20 μl . The reaction mixtures were incubated at 37°C for 10 min, followed by 42°C for 60 min. The reactions were stopped at 85°C for 5 min and chilled on ice. The PCR program was as follows: 30 s at 94°C (denaturation), 30 s at 62°C (annealing), and 30 s at 72°C (elongation). The reaction was completed by incubating for 5 min at 72°C. Samples were separated on 1.5% agarose gels in Tris–borate–ethylenediaminetetraacetic acid (TBE) buffer and stained with ethidium bromide. 16S rRNA gene in *S. coelicolor* was used as internal control. To exclude the possibility of DNA contamination in the RNA isolations, PCR amplification was performed on all RNA samples without prior reverse transcription.

egfp fusions and expression Plasmid pIJ8660 (Sun et al. 1999) was digested with *NheI* and treated with Klenow polymerase fragment and alkaline phosphatase, respectively. The *BclI* fragment containing the *tsr* gene from pWHM3 was cloned in pIJ8660, generating pIJ86601. For *egfp* fusions, the upstream regions of *hrdB* (896 bp) and *sigQ* (906 bp) were amplified by PCR with the primers PHrdB1-2 and PSQ1-2, respectively (listed in Supplementary Table 1). Both fragments were sequenced in pMD18-

T vector, and the correct clones were digested with *KpnI* and *BglII* and transferred to vector pIJ86601, which was digested with *KpnI* and *BamHI* generating pIJ86601-*hrdBp* and pIJ86601-*sigQp*, respectively. The constructs were introduced into *S. coelicolor* M145 by conjugation from *E. coli*. Microscopic images were obtained by a Zeiss LSM 510 META confocal microscope (argon laser at 488 nm, objective 40 \times 0.75 NA dry PL Fluotar).

Results

AfsQ1-Q2 system regulates antibiotic production and morphological development in a medium-dependent manner in *S. coelicolor*

The *afsQ* genes of *S. coelicolor* were identified for their ability to stimulate ACT and RED production in *S. lividans* when *afsQ1-Q2* or just *afsQ1* was introduced on a plasmid (Ishizuka et al. 1992). However, disruption of *afsQ1* or *afsQ2* on the *S. coelicolor* genome had no effect on either antibiotic production or morphological differentiation when grown in rich media, such as YEME and YMPG liquid medium (Ishizuka et al. 1992). In this study, we constructed a mutant with *afsQ1-Q2* gene cluster deleted by PCR-targeting system. When compared to wild-type M145, the *afsQ* mutant established no visible change on pigmented antibiotic synthesis and morphogenesis when grown on MS, R2YE, and GYM media (data not shown), consistent with early study (Ishizuka et al. 1992). We then tested the mutant in an MM medium supplemented with various nutritional factors in a plate assay. It was found that deletion of *afsQ1-Q2* genes impairs the production of ACT, RED, and CDA when grown on MM with 75 mM sodium L-glutamate as the sole nitrogen source (Fig. 1a–c). Even cultivated more than 4 days, the *afsQ* mutant showed almost no extracellular ACT production. Meanwhile, growth of aerial mycelium in the mutant was found to be faster than M145 (Fig. 2). To exclude the possibility that the effects were caused by sodium, we also replaced sodium L-glutamate by several other sodium salts; in those cases, no phenotype change between the mutant and M145 was observed (data not shown), indicating that the effects were indeed caused by glutamate. In addition, to exclude the possibility that the phenotype changes in the *afsQ* mutant were caused by polar effect on downstream genes, *afsQ1* and *afsQ2* with their possible promoter region were introduced into the mutant by using the integrative vector pSET1521 (modified from pSET152 with a thiostrepton-resistant gene; Kieser et al. 2000). Introduction of the complementary constructs into this mutant restored antibiotic production, and the aerial mycelium formation

Fig. 1 Phenotypes of *S. coelicolor* M145 and its derivatives. **a** ACT production by the wild type (M145), the *afsQ* mutant (Δ *afsQ*), *sigQ* mutant (Δ *sigQ*), and *afsQ-sigQ* double mutant (Δ *afsQsigQ*) on MM supplemented with 75 mM glutamate was observed at day 2. **b** RED production by the wild type (M145), the *afsQ*, and *sigQ* and *afsQsigQ* mutants on MM with 10 mM glutamate at day 2. **c** CDA production by the wild type (M145) and the *afsQ*, *sigQ*, and *afsQsigQ* mutants and their complementary transformants on MM with 75 mM glutamate for 31 h. **d** The phenotype of *S. coelicolor* M145a (M145 with pSET1521), *afsQa* (Δ *afsQ* with pSET1521), *sigQa* (Δ *sigQ* with pSET1521), *afsQsigQa* (Δ *afsQsigQ* with pSET1521), and their complementary transformants *afsQb* (Δ *afsQ* with pSET1521-*afsQ*), *afsQd* (Δ *afsQ* with pSET1521-*afsQ1-sigQ*), *sigQb* (Δ *sigQ* with pSET1521-*sigQ*), and *afsQsigQb* (Δ *afsQsigQ* with pSET1521-*afsQsigQ*) on MM with 75 mM glutamate

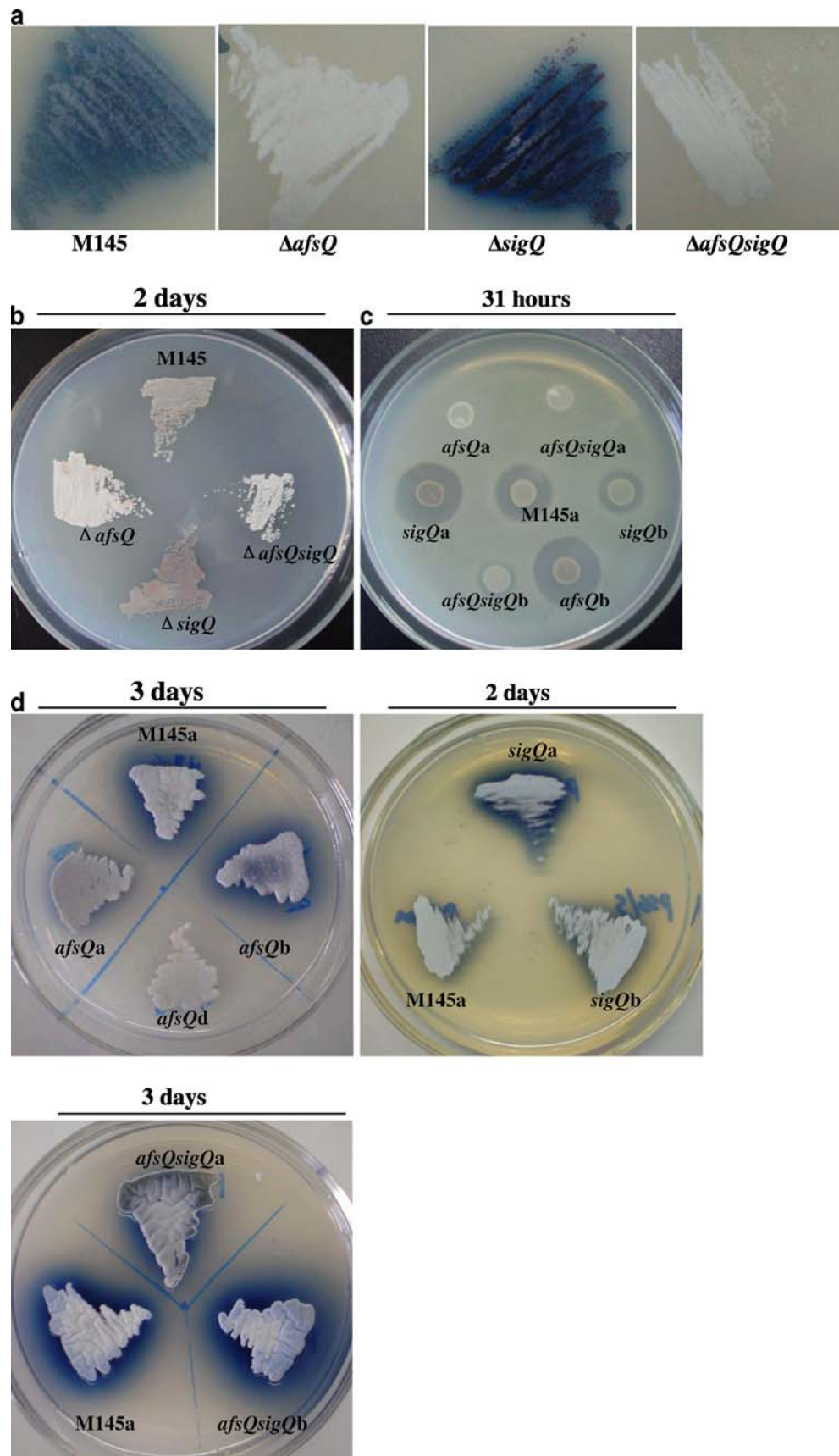
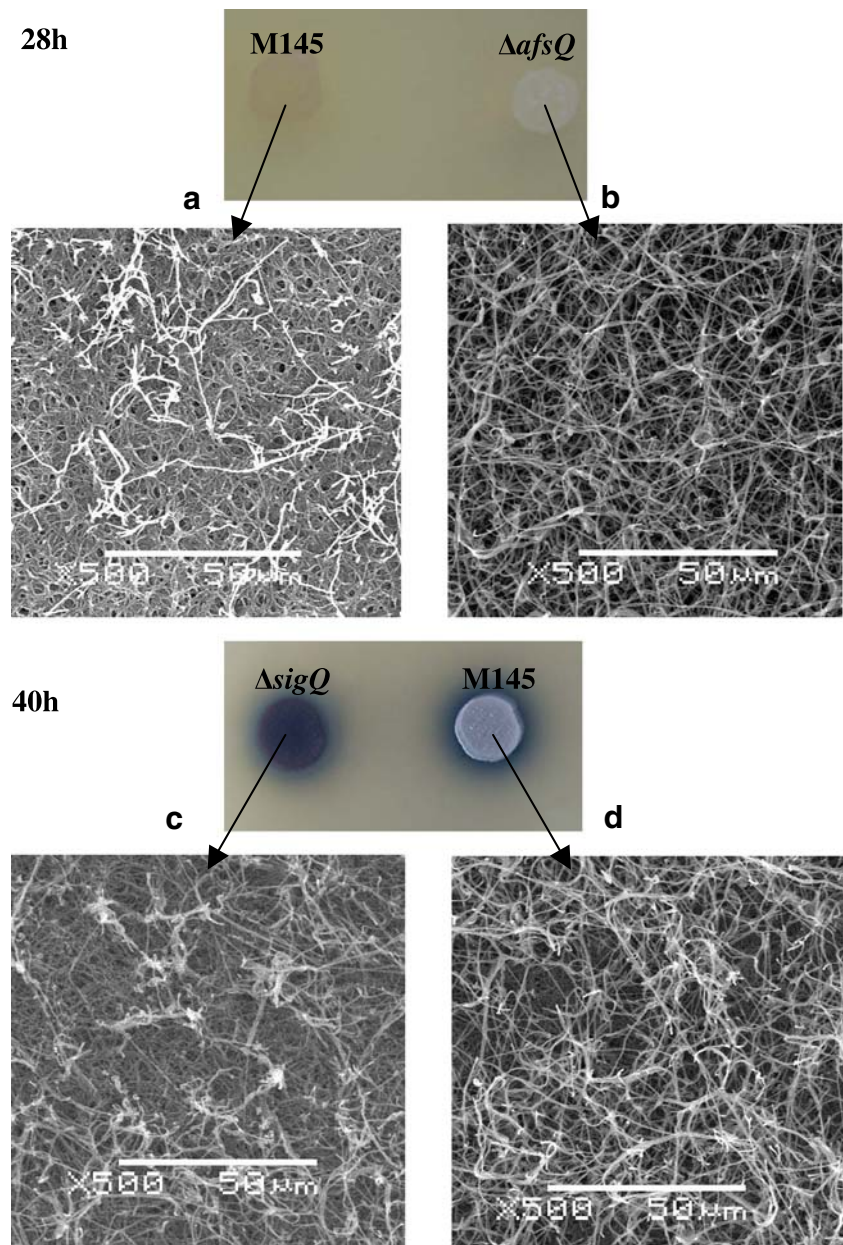


Fig. 2 Morphology of mycelium formation by M145 and *afsQ* and *sigQ* mutants. Cells were incubated at 30°C for 28 and 40 h on MM plates with 75 mM glutamate. In contrast to M145 (**a**), Δ *afsQ* (**b**) was covered with abundant aerial mycelia after 28-h growth. However, the *sigQ*-null mutant (**c**) exhibited a severe delay in formation of aerial hyphae even incubated for 40 h when M145 (**d**) was covered with abundant aerial hyphae



was back to the same level as wild-type M145, indicating that the phenotype changes in the mutant resulted solely from the *afsQ* mutation (Fig. 1c,d). However, pSET1521 carrying the *afsQ1* without *afsQ2* did not restore ACT production (Fig. 1d), suggesting that kinase AfsQ2 might be the sole phosphorus group donor of response regulator AfsQ1.

SigQ regulates antibiotic production and morphological development in a medium-dependent manner in *S. coelicolor*

Right upstream of *afsQ1-Q2* genes on the *S. coelicolor* chromosome is a *sigQ* gene which encodes a putative sigma

factor (Bentley et al. 2002). The *sigQ* gene is transcribed in the opposite direction from *afsQ1-Q2*, so they were not in the same operon. The similar genetic organization was also found previously for the TCS *cseB-cseC* system and sigma factor *sigE*, which worked together for normal cell wall integrity (Paget et al. 1999b; Hong et al. 2002). The similarity in terms of putative functions (i.e., AfsQ2 has ~40% identity to CseC at the amino acid level; Hutchings et al. 2006b) and genetic organization between the *sigQ-afsQ1-Q2* and *sigE-CseB-CseC* systems prompted us to study whether SigQ is also involved in regulating antibiotic production and morphological differentiation in *S. coelicolor*. The *sigQ* gene was disrupted by replacing the *sigQ* coding sequence with the apramycin resistance gene and

the phenotype of the mutant was studied. Although the *sigQ* mutant appeared identical to the wild-type strain on all rich media tested, it produced ACT and RED earlier and in greater amounts than M145 on an MM medium supplemented with glutamate (Fig. 1a,b). In addition, formation of aerial mycelium of the mutant was also obviously delayed (Fig. 2). The overproduction of antibiotic and the poor sporulation could be complemented in *trans* by integrating a functional copy of *sigQ* gene into M145 strain by using pSET1521 (Fig. 1c,d). The results demonstrated that SigQ also regulates antibiotic production and morphological development in a medium-dependent manner, and its function is antagonizing from that of AfsQ1-Q2 in *S. coelicolor*.

SigQ is secondary to AfsQ1-Q2 in the signal transduction cascade

Although the *afsQ1-Q2* and *sigQ* seemed to function differently in regulating antibiotic production and morphological differentiation in *S. coelicolor*, our next question is whether they are functionally coupled, like in the case of SigE-CseB-CseC (Paget et al. 1999a; Hong et al. 2002). We then determined the transcript level of *afsQ* and *sigQ* in the wild-type M145 strain and the *afsQ* or *sigQ* mutant using semiquantitative RT-PCR. RNA samples were isolated from cells grown on cellophane on the solid MM containing 75 mM glutamate at 36, 48, and 60 h, respectively. 16S rRNA was used as a negative control for this assay. The results showed a significant reduction of the *sigQ* transcript in the *afsQ* mutant (Fig. 3a), while deletion of *sigQ* had no effect on *afsQ1-Q2* transcription (data not shown). The reduction can be restored by complementing the *afsQ* mutant with the *afsQ1-Q2* cluster (Fig. 3b). In addition, introduction of plasmid pSET1521-*sigQ* and pSET1521-*afsQ1sigQ* in the *afsQ* mutant could not restore the transcription of *sigQ* (Fig. 3c), suggesting that the *afsQ1-Q2* genes are required for the transcription of *sigQ* under the condition we tested. To further confirm the regulation of *sigQ* by *afsQ1-Q2*, in vivo transcription level of *sigQ* was compared between M145 and the *afsQ* mutant using an enhanced green fluorescence reporter (*egfp*) system. The promoter region of the *sigQ* gene (*sigQp*) was fused to the *egfp* gene in the integrative plasmid pIJ86601 (modified from pIJ8660 with a thiostrepton-resistant gene; Sun et al. 1999). The resulting plasmid was introduced into the wild-type M145 and the *afsQ* mutant, respectively, and the expression of EGFP was investigated using a confocal microscopy. The results showed that in M145, EGFP was clearly induced from the *sigQ* promoter on the MM supplemented with glutamate, whereas the expression of EGFP was not observed in the *afsQ* mutant (Fig. 4). In a control *egfp* experiment, the promoter of the housekeeping

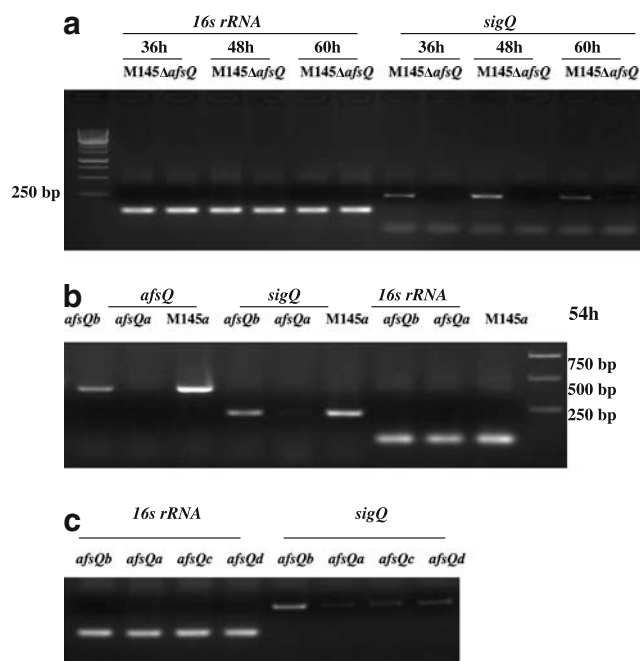


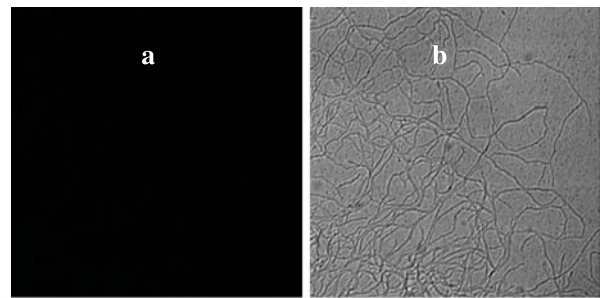
Fig. 3 Transcription analysis of *sigQ* and *afsQ* gene in *S. coelicolor* M145 and its derivatives grown on MM with 75 mM glutamate. RNA was extracted from mycelium harvested at different time points. PCR products were separated on 1.5% gel in TBE 1× buffer. 16S rRNA gene was used as a control. **a** RT-PCR analysis of the *sigQ* gene in *S. coelicolor* M145 and the *afsQ* mutant (Δ *afsQ*). RNA was extracted from mycelium harvested 36, 48, and 60 h. **b** RT-PCR analysis of the *afsQ* and *sigQ* gene in *S. coelicolor* M145 (M145a), the *afsQ* mutant (Δ *afsQa*), and the complemented strain (Δ *afsQb*). RNA was extracted from mycelium harvested at 54 h. **c** RT-PCR analysis of the *sigQ* gene in *afsQa* (Δ *afsQ* with pSET1521), *afsQb* (Δ *afsQ* with pSET1521-*afsQ*), *afsQc* (Δ *afsQ* with pSET1521-*sigQ*), and *afsQd* (Δ *afsQ* with pSET1521-*afsQ1sigQ*). RNA was extracted from mycelium harvested at 36 h

gene *hrdB* was shown to be insensitive to disruption of *afsQ* gene in M145 (Fig. 4). The results demonstrated that the transcription of *sigQ* is controlled by AfsQ1-Q2.

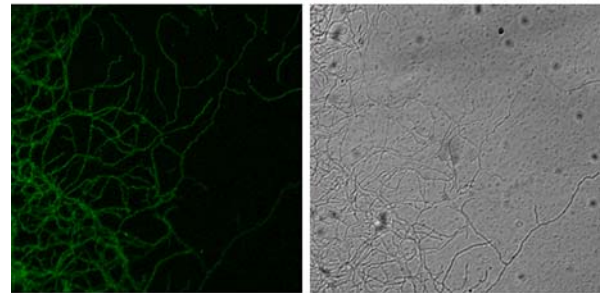
We also checked the dependence of SigQ on AfsQ1-Q2 by genetic studies. The results showed that, although the deletion of *afsQ* or *sigQ* caused different phenotypes in the conditions we tested, the *afsQ-sigQ* double mutant had the same phenotypes as the *afsQ* mutant (Fig. 1a), suggesting that the disruption of *sigQ* in *afsQ* mutant cannot reverse the decreased antibiotic production resulting from the deletion of *afsQ1-Q2*. Moreover, introduction of both *afsQ1-Q2* and *sigQ* genes (pSET1521-*afsQsigQ*) into the *afsQ-sigQ* double mutant resulted in the phenotype similar to M145 (Fig. 1d), and introduction of only *afsQ1-Q2* genes (plasmid pSET1521-*afsQ*) generated a phenotype similar to the *sigQ* mutant (i.e., overproduction of ACT in the *afsQ-sigQ* mutant; Fig. 1d). The results further suggested that, while AfsQ1-Q2 and SigQ may function

Fig. 4 Analysis of *sigQp-egfp* and *hrdBp-egfp* transcriptional fusions in whole colonies of M145 and the *afsQ* mutant grown on minimal agar in the presence of 75 mM glutamate. EGFP fluorescence was visualized using a Zeiss LSM 510 META confocal microscope after 42-h incubation at 30°C as described in experimental procedures. **a** Fluorescence microscope, **b** light microscope

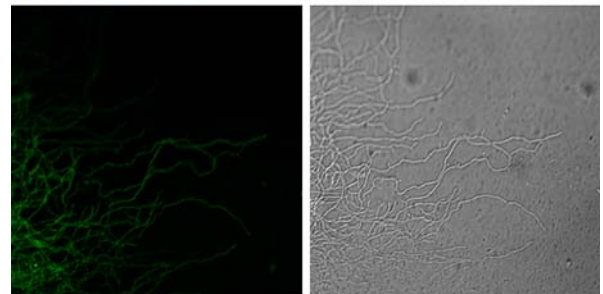
M145 (pIJ86601)



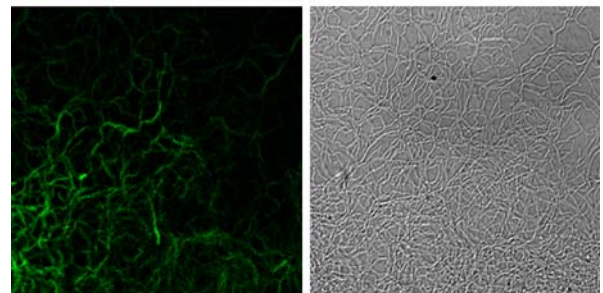
M145 (*hrdBp-egfp*)



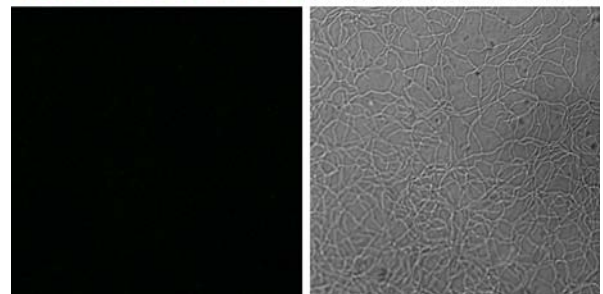
Δ *afsQ* (*hrdBp-egfp*)



M145 (*sigQp-egfp*)



Δ *afsQ* (*sigQp-egfp*)



together in the regulation of antibiotic biosynthesis and morphological development, AfsQ-Q2 may be located at a higher level than SigQ in the signal transduction cascade and *sigQ* may be involved in antagonizing the function of *afsQ1-Q2*.

AfsQ-SigQ system regulates antibiotic production through pathway-specific activators

To obtain a better understanding of the possible regulatory mechanism of the AfsQ1-Q2-SigQ on antibiotic production,

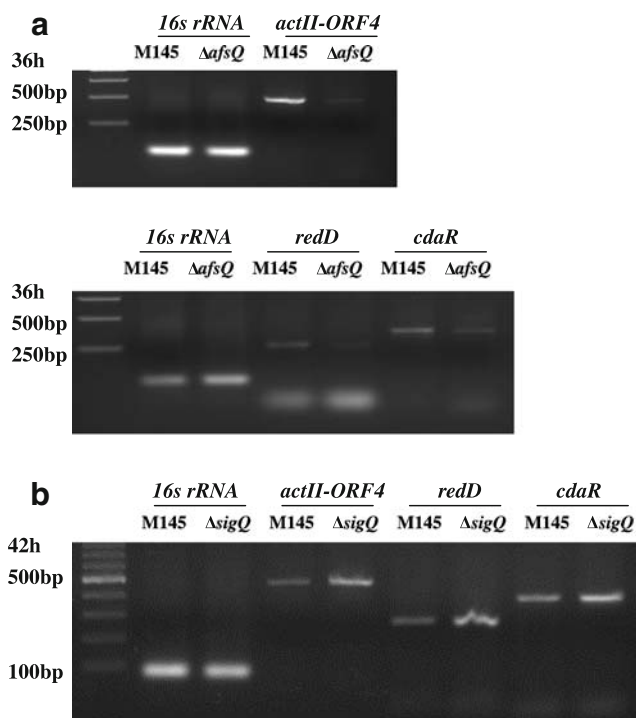


Fig. 5 Transcription analysis of pathway-specific regulatory genes in *S. coelicolor* M145 and its derivatives grown on MM with 75 mM glutamate. Expression of *actII-ORF4*, *redD*, and *cdaR* in Δ *afsQ* **a**, **b** and Δ *sigQ* **c**. RNA was extracted from mycelium harvested 36 or 42 h. PCR products were separated on 1.5% gel in TBE 1 \times buffer. 16S rRNA gene was used as a control

we determined the effects of *afsQ1-Q2-sigQ* gene expression on the transcription of pathway-specific factors for ACT, RED, and CDA biosynthesis, including *actII-ORF4* (Arias et al. 1999), *redD* (Takano et al. 1992; White and Bibb 1997), and *cdaR* (Ryding et al. 2002). RT-PCR studies were conducted using RNA samples isolated from M145, Δ *afsQ*, and Δ *sigQ* at 36 or 42 h in solid MM media supplemented with 75 mM glutamate as the sole nitrogen. The results showed that transcription of *actII-ORF4*, *redD*, and *cdaR* were significantly reduced in the *afsQ* mutant (Fig. 5a), while it increased in the *sigQ* mutant (Fig. 5b). The results were consistent with the physiological measurements and suggested that AfsQ and SigQ regulate antibiotic production of *S. coelicolor* by affecting the transcription of pathway-specific activators.

Discussion

One well-established mechanism to conduct signal transduction in streptomycetes is the two-component system (Horinouchi and Beppu 1992), through which environmental signal recognition results in the autophosphorylation of

sensor histidine protein kinase, followed by transfer of the phosphoryl group to the second component protein, a response regulator (Parkinson and Kofoed 1992). Analysis of the *S. coelicolor* genome has revealed evidences of abundant two-component regulatory systems (Bentley et al. 2002). Although the functions of these regulators are still largely unknown, the existence of so many two-component regulatory systems in the *S. coelicolor* genome clearly implies their important physiological roles. Thus, the elucidation of their functions will certainly benefit the understanding of complex regulatory networks involved in the production of secondary metabolites in these organisms. In the past several years, our laboratory has been studying functions of various TCS systems in *Amycolatopsis mediterranei* and *S. coelicolor* using molecular genetics approach (Wang et al. 2004; Wei et al. 2004; Lu et al. 2007). As part of these continuous efforts, we reported here the functional characterization of *afsQ1-Q2* and its upstream *sigQ* gene. Instead of using the rich media in which disruption of either *afsQ1* or *afsQ2* in *S. coelicolor* led to no detectable change in secondary metabolite formation or morphogenesis (Horinouchi and Beppu 1992), in this study, we checked the effects of *afsQ1-Q2* gene deletion on antibiotic biosynthesis and morphological development in an MM medium supplemented with various nitrogen sources in a plate assay. The results showed that, when cultivated on defined MM with glutamate as the sole nitrogen source, the *afsQ* mutant exhibited significantly decreased production of antibiotic and rapid growth of aerial mycelium, suggesting that *afsQ1-Q2* is a pleiotropic TCS system involved in both antibiotic production and morphological differentiation in *S. coelicolor*, and its regulatory effects are in a medium (glutamate)-dependent manner. The detailed mechanism of this medium (glutamate)-dependent regulation is still yet to be discovered in *Streptomyces*; however, it has been reported in *E. coli* that the P (II) signal transduction proteins GlnB and GlnK are uridylylated–deuridylylated in response to the intracellular glutamine level, and the cellular glutamine signal is transduced by uridylyltransferase and GlnB to modulate TCS NtrB–NtrC-dependent gene expression (Maheswaran and Forchhammer 2003).

Two-component signal system genes characterized so far in *Streptomyces* fall into two classes according to their pleiotropic effects on secondary metabolism and morphological differentiation: (1) those that affect only antibiotic production, such as *absA-absB* (Anderson et al. 2001) and *cutR-cutS* (Chang et al. 1996), and (2) those that affect only morphological differentiation, such as *ramR* (Ma and Kendall 1994). We found here that AfsQ1-Q2 system not only regulates antibiotic production but also is involved in the formation of aerial mycelium in *S. coelicolor*. These

two phenotypes are closely related. For example, it has been reported that during carbon- and phosphate-limited cultures, *Bacillus subtilis* attempts a number of adaptive approaches (motility, chemotaxis, and synthesis of extracellular degradative enzymes and antibiotics), any of which, if successful, would allow vegetative growth to resume before it begins sporulation (Fisher and Sonenshein 1991; Fisher 1999). If it is also the case for *S. coelicolor*, it may explain the early formation of aerial mycelium formation in the *afsQ* mutant that has stopped producing antibiotic.

In early studies, a CseB-CseC two-component system has been found activating its upstream *sigE* gene in response to signals from the cell envelope in *S. coelicolor* (Paget et al. 1999b). Consistently, a constructed *sigE*-null mutant has the same phenotype as a *cseB*-null mutant. In addition, analysis of the expression of 12 putative target genes controlled by *sigE* (the *cwg* operon) showed a medium-dependent manner: in low-Mg²⁺ medium, transcription of the *cwg* operon was induced by vancomycin in a *sigE*-dependent manner but, in high-Mg²⁺ medium, there was substantial *cwg* transcription even in a *sigE*-null mutant (Paget et al. 1999b; Hong et al. 2002). A putative sigma factor, *sigQ* is also located upstream of *afsQ1-Q2* genes (Bentley et al. 2002). We thus wonder whether the set of them behaves the same way as SigE-CseB-CseC. Consistent with our expectation, RT-PCR and *egfp* analyses showed that *sigQ* expression is under control by AfsQ1-Q2; however, to our surprise, deletion of *sigQ* led to the precocious hyperproduction of antibiotic and delayed formation of sporulating aerial mycelium in a medium (glutamate)-dependent manner, contrary to the decreased antibiotic production and early formation of aerial mycelium by *afsQ1-Q2* deletion, and the double mutant $\Delta\text{afsQsigQ}$ has the same altered phenotypes as the *afsQ* mutant. The results provided evidence for a novel medium-dependent regulatory mechanism in which a sigma factor SigQ, under the transcriptional regulation of by *afsQ1-Q2*, might be responsible for antagonizing the function of AfsQ1-Q2. In addition, we also showed that ACT, RED, and CDA pathway-specific activator genes, *actII-ORF4*, *redD*, and *cdar*, are among the possible target genes regulated by *afsQ1-Q2-sigQ*.

Although, to our best knowledge, no similar type of antagonizing mechanism has been reported for *Streptomyces*, it was found that KbpA, which is located just upstream of a Ser/Thr protein kinase *afsK*, inhibited autophosphorylation of AfsK by binding the catalytic domain of the unphosphorylated form of AfsK and put a brake on the unlimited production of the pigments under the control of the AfsK-AfsR system, thus playing a role in a negative-feedback system (Umeyama and Horinouchi 2001). Although more proof is still needed, it is speculative that a

similar mechanism may be present that σ^Q is recruited by core RNA polymerase to transcribe some genes encoding regulatory protein (s) as KbpA, which may antagonize the function of AfsQ1-Q2 by interacting directly with the sensor domain of AfsQ2 to modulate its activity and then control the level of phospho-AfsQ1. When *sigQ* is disrupted, this repression disappears and the function of phospho-AfsQ1 was enhanced, resulting in precocious overproduction of antibiotic. However, it needs further investigations.

Taken together, we can conclude that *afsQ1-Q2-sigQ* is a pleiotropic but conditionally required signal transduction system for both secondary metabolism and morphological development in *S. coelicolor*. This highly regulated process, in which different regulatory mechanisms are involved, could provide benefits in fine-tuning the timing and strength of the expression of the genes involved in antibiotic biosynthesis and morphological development in *S. coelicolor*. Finally, as early Southern blot hybridization experiments showed that sequences homologous to *afsQ1-Q2* are present in almost all of the actinomycetes examined (Ishizuka et al. 1992) and our recent examination of the *Streptomyces avermitilis* and *Streptomyces griseus* genome has revealed that the *sigQ* and *afsQ1-Q2* homologs (SAV_3351, SAV-3352, and SAV_3353; SGR_2634, SGR_2635, and SGR_2636, respectively) were organized in an identical way (Ikeda et al. 2003; Ohnishi et al. 2008), it is worth further investigation whether the novel regulatory mechanism reported here for *afsQ1-Q2-sigQ* represents a commonly used regulatory strategy in other actinomycetes.

Acknowledgement This work was supported by the National Natural Science Foundation of China (30770023, 30700022), the Knowledge Innovation Program of the Chinese Academy of Sciences (KSCX2-YW-G-007), and the National Basic Research Program of China (2007CB707803).

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