Reciprocal Regulation between SigK and Differentiation Programs in *Streptomyces coelicolor*[∇]

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Received 3 July 2009/Accepted 25 August 2009

Here we reported that deletion of SigK (SCO6520), a sigma factor in *Streptomyces coelicolor*, caused an earlier switch from vegetative mycelia to aerial mycelia and higher expression of *chpE* and *chpH* than that in the wild type. Loss of SigK also resulted in accelerated and enhanced production of antibiotics, actinorhodin, and undecylprodigiosin and increased expression of *actIII-orf4* and *redD*. These results suggested that SigK had a negative role in morphological transition and secondary metabolism. Furthermore, the *sigK* promoter (*sigKp*) activity gradually increased and *sigK* expression was partially dependent on SigK, but this dependence decreased during the developmental course of substrate mycelia. Meanwhile, two potentially nonspecific cleavages occurred between SigK and green fluorescent protein, and the SigK fusion proteins expressed under the constitutive promoter *ermEp** sharply decreased and disappeared when aerial mycelia emerged. If expressed under *sigKp*, 3FLAG-SigK showed similar dynamic patterns but did not decrease as sharply as SigK expressed under *ermEp**. These data suggested that the climbing expression of *sigK* might reduce the prompt degradation of SigK during vegetative hypha development for the proper timing of morphogenesis and that SigK vanished to remove the block for the emergence of aerial mycelia. Thus, we proposed that SigK had inhibitory roles on developmental events and that these inhibitory effects may be released by SigK degradation.

Streptomyces coelicolor is one of the soil-dwelling gram-positive bacteria. It adopts a complex life cycle concomitant with the reiterative morphological development from vegetative mycelia to aerial mycelia and spores, and spores germinate to grow into vegetative mycelia. It is also regarded as a model for antibiotic production, since at least four chemically distinct antibiotics have been discovered in this species, including actinorhodin (Act), undecylprodigiosin (Red), calcium-dependent antibiotic, and methylenomycin (15, 21). The completed genome sequencing project reveals that *S. coelicolor* has 65 sigma factors in total, the highest number in bacteria so far identified, which was presumably consistent with its elaborate transcriptional regulations on morphogenesis, secondary metabolism, and stress responses (2, 16). Of them, 10 group 3 sigma factors of the σ^{70} family were revealed, including SigB (SCO0600), SigL (SCO7278), SigI (SCO3068), SigN (SCO4034), SigF (SCO4035), SigH (SCO5243), SigK (SCO6520), SigM (SCO7314), SigG (SCO7341), and σ^{WhiG} (SCO5621), according to the order similarity to *Bacillus subtilis* SigB, a central regulator of stress responses (24). The typical group 3 sigma factors of the σ^{70} family have three independent domains, σ_2 , σ_3 , and σ_4 , which are essential for the promoter recognition and RNA polymerase (RNAP) binding (16, 29).

In *S. coelicolor*, most of these group 3 sigma factors positively modulate the morphological differentiations at various developmental stages. SigB was proposed as a master pluripotent sigma factor, since *sigB* mutant is bald, shows sensitivity to high levels of osmotic stress, and produces a higher level of Act

but a lower level of Red (24). Interestingly, the mycothiol, which plays a significant role in the detoxification of thiol-reactive substances (28), was synthesized at a lower level in the *sigB* mutant after osmotic stress, thus leading to more oxidized (carbonylated) proteins (24). Microarray analysis suggests the transcriptional control of more than 280 genes by SigB after osmotic induction and a hierarchically transcriptional and posttranscriptional control order from SigB to SigL and SigM. Consistent with hierarchical regulation, SigB positively controls the aerial hyphal development and SigL is required for the sporulation, while SigM is involved in the full formation of spores (11, 24). σ^{WhiG} is required for the trigger of sporulation onset (10), and SigF is proposed to control proper spore formation and integrity (30), while SigH is essential for efficient septation of aerial hyphae into spores (32). Recently, delayed aerial mycelium formation and sporulation in the *sigN* mutant was observed on glucose-containing medium, and higher expression of the *sigN* promoter is restricted to the “subapical stem,” also suggesting the possible involvement of SigN in proper morphogenesis (12).

Meanwhile, these alternative sigma factors are regulated by developmental programs at both transcriptional and posttranslational levels. *sigBp*₁ expression is silenced at the vegetative mycelium phase but persistently increases when morphological differentiation progresses, consistent with the requirement of SigB for development into aerial hyphae and spores (11, 24). The *sigN* promoter is also positively regulated by the developmental programs (12). *sigF* expression is limited only to spores, accordant to its indispensability in proper spore formation (20, 30). However, four promoters of *sigH* are differentially induced during development, since both *sigHp*₁ and *sigHp*₂ are constitutively active, while *sigHp*₃ activity is increased but *sigHp*₄ is decreasingly expressed (23). Interestingly, three isoforms of SigH, SigH- σ^{52} , SigH- σ^{51} , and SigH- σ^{37} , are observed as the

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§ Supplemental material for this article may be found at <http://jb.asm.org/>.

[∇] Published ahead of print on 4 September 2009.

TABLE 1. *S. coelicolor* strains used in this study

Strain	Description	Source or reference
M145	Wild type, SCP1 ⁻ , SCP2 ⁻	2
LM1	In-frame deletion of <i>sigK</i> in M145	This work
LM2	LM1/pLM12	This work
LM3	M145/pLM8	This work
LM4	M145/pLM6	This work
LM5	M145/pLM13	This work
LM6	M145/pLM1	This work
LM7	M145/pLM3	This work
LM8	LM1/pLM3	This work
LM9	M145/pLM10	This work
LM10	M145/pLM12	This work

primary translational products synthesized from three distinct translation initiation sites at the early developmental stages, but SigH- σ^{37} is accumulated while SigH- σ^{52} and SigH- σ^{51} are eliminated and proteolytically truncated into SigH- σ^{38} and SigH- σ^{34} , respectively, concurrent with the morphological transitions and suggesting a function in development (36).

Here we characterized the sigma factor SigK (SCO6520) as a negative regulator controlling the vegetative-aerial mycelium switch and secondary metabolism, and we also provide evidence that SigK expression was regulated positively at the transcriptional level and that SigK protein degraded during the development into aerial mycelia.

MATERIALS AND METHODS

Bacterial strains and growth media. The *S. coelicolor* strains used in this study are listed in Table 1. *Escherichia coli* ET12567 (26) containing helper plasmid pUZ8002 was used for routine plasmid transfer into *S. coelicolor* by conjugation, as described previously (21). *E. coli* strain TG1 was used for the usual transformation in plasmid construction. *E. coli* strains were cultured at 37°C in solid or liquid Lennox broth medium. *S. coelicolor* strains were grown at 30°C on MS or R2YE for morphological and secondary metabolism analysis or in 3% tryptic soya broth liquid medium for genomic DNA preparation (21).

Plasmid construction. All primer sequences are provided (see Table S1 in the supplemental material), and the plasmids are listed in Table 2. Promoter *ermEp** was amplified with primers 1 and 2 from plasmid pGH113 (27), digested with BamHI, and inserted into the BamHI site of pIJ8630 (33) to give rise to plasmid pLM1. Primers 3 and 4 were used to generate a PCR product of *sigK* with a linker sequence (18) from the genomic DNA of *S. coelicolor* strain M145. The PCR fragment was added with one deoxyribosyladenine (dA) by *Taq* polymerase (Promega) at 70°C for 30 min and ligated to the T vector of pTA2 (Toyobo) to create plasmid pLM4. Then the NdeI fragment from pLM4 was inserted into the NdeI site of pLM1 after dephosphorylation to generate plasmid pLM6. Plasmid pLM10 was constructed by applying the same procedure except that the forward primer 5 was used instead of primer 3. The *sigK* promoter (*sigKp*) was amplified with primers 6 and 7, digested with BglII, and inserted into the BamHI site of pIJ8630 to create plasmid pLM2. Then the XbaI/NotI fragment in pLM2 was replaced with the XbaI/NotI fragment from pIJ8660 (33) to create pLM3, which contained three stop codons in three reading frames just downstream of the *sigK* promoter. The 3FLAG-*sigK* fragment with a stop codon was amplified with primers 5 and 14 and inserted into pTA2 for plasmid pLM11. The NdeI/NotI fragment from pLM11 was then ligated at the NdeI/NotI site of pLM3 or pLM1 to create plasmid pLM12 or pLM13, respectively. The PCR product with primers 12 and 13 was *atpD*-linker, which was sequentially ligated to pTA2 after one dA addition to give rise to plasmid pLM7, and the NdeI fragment from pLM7 was inserted into the NdeI site of pLM1 to create pLM8. The *sigK* knockout plasmid pLM14 was constructed as follows: two fragments about 0.9 kb and 1.3 kb in size, homologous to regions upstream and downstream of *sigK*, respectively, were the PCR products with primers 8 and 9 and primers 10 and 11, respectively. These fragments were then digested with HindIII/BamHI and BglII/EcoRI, respectively, and sequentially inserted into the HindIII/BamHI and BamHI/EcoRI sites of plasmid pKC1139 with a temperature-sensitive replicon, pSG5 (6). To con-

TABLE 2. Plasmids used in this study

Plasmid	Description	Source or reference
pKC1139	Bifunctional <i>oriT</i> RK2 plasmid for gene deletion	1
pGH113	Conjugal plasmid with <i>ermEp</i> *	3
pIJ8630	Promoter-probing plasmid with <i>egfp</i>	4
pIJ8660	Promoter-probing plasmid with <i>egfp</i>	4
pLM1	<i>ermEp</i> * in pIJ8630	This work
pLM2	<i>sigKp</i> in pIJ8630	This work
pLM3	XbaI/NotI fragment from pLM2 in XbaI/NotI site of pIJ8660	This work
pTA2	T vector	Toyobo
pLM4	<i>sigK</i> -linker in pTA2	This work
pLM6	<i>sigK</i> -linker in pLM1	This work
pLM7	<i>atpD</i> -linker in pTA2	This work
pLM8	<i>atpD</i> -linker in pLM1	This work
pLM9	3FLAG- <i>sigK</i> -linker in pTA2	This work
pLM10	3FLAG- <i>sigK</i> -linker in pLM1	This work
pLM11	3FLAG- <i>sigK</i> in pTA2	This work
pLM12	3FLAG- <i>sigK</i> in pLM3	This work
pLM13	3FLAG- <i>sigK</i> in pLM1	This work
pLM14	<i>sigK</i> in-frame knockout plasmid based on pKC1139	This work
pLM15	<i>hrdB</i> in pTA2	This work
pLM16	<i>redD</i> in pTA2	This work
pLM17	<i>actII-orf4</i> in pTA2	This work
pLM18	<i>chpE</i> in pTA2	This work
pLM19	<i>chpH</i> in pTA2	This work

struct plasmids for single-stranded DNA (ssDNA) preparation in an S1 nuclease protection assay, *hrdB*, *redD*, *actII-orf4*, *chpE*, and *chpH* were amplified with primers 15 and 16, primers 17 and 18, primers 19 and 20, primers 21 and 22, and primers 23 and 24, respectively, and ligated to pTA2 after one dA addition. All the PCRs were demonstrated with KOD-Plus polymerase (Toyobo), and the cloned fragments were verified by DNA sequencing.

***sigK* knockout and complementation.** *sigK* was knocked out in the wild-type strain M145 genome by an in-frame deletion strategy via double-crossover homologous recombination (21). Knockout plasmid pLM14 was introduced into M145 by conjugation, and the transformants were subinoculated on apramycin-containing MS medium at 37°C for integration of pLM14 at the *sigK* locus, as verified by Southern blot analysis (data not shown). After three rounds of relaxed growth at 37°C on MS without apramycin, the recombinants were streaked into single colonies and the *sigK* mutant was screened out from apramycin-sensitive strains by PCR analysis (data not shown) and Southern blot analysis (see Fig. S1 in the supplemental material). Plasmid pLM12 was transformed into the *sigK* mutant for complementation.

RNA preparation. About 50 mg of mycelia collected from MS or R2YE plates overlaid with cellophane was resuspended in 500 μ l of ice-cold buffer (1% sodium dodecyl sulfate, 4% β -mercaptoethanol, 5 mM EDTA in diethyl pyrocarbonate [DEPC]-treated H₂O) and homogenized by ultrasonication. The lysate was immediately extracted thoroughly with 500 μ l of phenol-chloroform-isoamyl alcohol (pH 5.3) twice and once with 500 μ l of chloroform. After centrifugation at 13,000 $\times$ g for 10 min at 4°C, the nucleic acids were precipitated with 500 μ l of isopropanol in the presence of 50 μ l of Na-acetyl (pH 5.3) and washed with 70% ethanol. The genomic DNA was removed after treatment with RNase-free DNase I (Takara). Total RNA was precipitated after extraction with phenol-chloroform-isoamyl alcohol (pH 5.3) and resuspended in DEPC-treated H₂O. The concentration of RNA was determined by spectrometry.

Low-resolution S1 nuclease protection assay. About 30 μ g of total RNA was subjected to S1 nuclease mapping as described previously (31), with some modifications. Briefly, RNA was hybridized to about 10 ng of ssDNA probe(s) in aqueous buffer (0.4 M NaCl, 40 mM piperazine-*N,N'*-bis[2-ethanesulfonic acid] [PIPES], pH 6.4, 1 mM EDTA) at 65°C for 16 h after denaturation at 90°C for 10 min. The single-stranded nucleic acids were removed with 100 U of S1 nuclease at 37°C for 1 h, and the hybrids were precipitated and subjected to Southern blot analysis by neutral transfer to nylon membrane (31). The biotin-labeled probes for Southern blot hybridization were prepared by PCR with

FIG. 1. Involvement of SigK in morphological development. (A) Wild-type strain LM7 (WT+V), the *sigK* mutant LM8 ($\Delta sigK+V$), and the *sigK* complementated strain LM2 ($\Delta sigK+sigK$) were streaked on the R2YE plate simultaneously, incubated at 30°C for 40 h, and photographed from the top of the plates. Single-colony (B) and cell (C) morphology of wild-type strain M145 (WT) and the *sigK* mutant LM1 ($\Delta sigK$) grown on R2YE medium at 30°C for 4 days. Cells were photographed by the coverslip impression method in panel C. Scale bars are indicated. (D) RNA was extracted from *sigK* mutant LM1 ($\Delta sigK$) and wild-type M145 (WT) after grown on R2YE medium overlaid with cellophane for 24, 36, and 48 h, respectively. S1 nuclease protection assay was demonstrated with an ssDNA probe of *chpE* and *chpH*, respectively. About 1 μg of total RNA was loaded on 1% Tris-borate-EDTA agarose gel and photographed. 16S and 23S rRNA were shown for loading control and RNA integrity. V and A indicated the presence of vegetative mycelia and aerial hyphae, respectively.

universal primers 26 and 27 from pTA2 vector with cloned fragments in the presence of biotin-11-dUTP (Fermentas) (25).

The ssDNA probes for RNA hybridization were prepared with λ exonuclease (Fermentas). The 5' phosphorylated primer 25 and unphosphorylated primer 27 were used to amplify the double-stranded DNA from pTA2 vector with cloned fragments. The PCR products were purified and digested with λ exonuclease to remove the 5' phosphorylated sense strand DNA. The antisense ssDNA was extracted once with phenol-chloroform-isoamyl alcohol (pH 8.0), precipitated with isopropanol, and resuspended in DEPC-treated H₂O.

Protein analysis. For assays of *sigK* promoter activity and SigK protein regulation, spores were spread on cellophanes overlaid on MS plates (21). After incubation for the indicated time, cells were collected, resuspended in lysis buffer (100 mM NaH₂PO₄, 10 mM Tris, pH 8.0, 1 mM phenylmethylsulfonyl fluoride), and destroyed by ultrasonication. Total protein concentration was determined by the Bradford method (Invitrogen) (7). About 20 μg of total protein was loaded for Western blot analysis (24) with anti-green fluorescent protein (GFP) antibody (Proteintech Group) or anti-FLAG M2 antibody (Sigma) and Coomassie brilliant blue R250 staining of total protein for loading control (Beyotime, Haimen, China).

Antibiotic assay. Antibiotic assays for cell-associated Act and Red were performed as described previously (8, 21, 35, 39). Briefly, about 10 mg of mycelia cultured on R2YE covered with cellophanes at various developmental stages was collected, extracted with KOH (1 M final concentration), and centrifuged at 5,000 $\times g$ for 5 min. The A_{640} of the supernatants was measured for quantitative analysis of total Act. For Red measurement, the pellet described above was treated with methanol acidified with HCl (final concentration, 0.5 M) overnight after vacuum freeze-drying, and the A_{530} of the supernatants was determined after centrifugation at 5,000 $\times g$ for 5 min.

RESULTS

Loss of *sigK* results in the earlier appearance of aerial mycelia and spores. The genome sequencing project has revealed that SCO6520 encoded a putative sigma factor, SigK (2). Domain anatomy by BLAST indicated that SigK contained three independent domains, σ_2 , σ_3 , and σ_4 (data not shown). Moreover, SigK could interact with the RNAP core enzyme in vivo and initiate transcription from the *Bacillus subtilis* *ctc* promoter in vitro (data not shown), suggesting that SigK was also an alternative group 3 sigma factor of the σ^{70} family (16, 29).

In *S. coelicolor*, all group 3 sigma factors showed high levels of similarity to *Bacillus subtilis* SigB, which is the central regulator for various stress responses and expression of more than 200 genes (24). However, SigK exhibited 46% identity and 63% similarity to *S. coelicolor* SigB. BLAST analysis (<http://www.genedb.org/genedb/scoelicolor/blast.jsp>) also revealed

that SigK was more related to SigG, with 71% identity and 84% similarity, and second-most related to SigM, with 69% identity and 80% similarity (24). However, the *sigG* mutant has phenotypes indistinguishable from those of the wild type (22), while SigM is required for efficient sporulation (24), suggesting the diversity of potential functions of SigK-related sigma factors.

To examine the functions of SigK in *S. coelicolor*, the *sigK* gene was knocked out by the in-frame deletion method (21), which left 39 bp and 123 bp intact at 5' and 3' of the *sigK* open reading frame, respectively, thus eliminating the coding regions for σ_2 , σ_3 , and σ_4 domains. Successful deletion of *sigK* in the genome of strain M145 was verified by PCR analysis (data not shown) and Southern blotting (see Fig. S1 in the supplemental material).

Previous global microarray measurement has revealed decreased or increased expression of *sigK* after a high-temperature shift or a high osmotic shift, respectively, and *sigI*, *sigJ* and/or *sigB*, *sigK*, *sigM*, *sigH*, and *sigL* were expressed in a sequential order upon the sucrose upshift (19). This implied additional possible roles of SigK on heat shock adaptation and osmoprotection by elaborate cooperative regulation with other sigma factors. However, we did not observe any significant growth difference between the wild type and the *sigK* mutant after temperature elevation and osmotic increase with KCl or sucrose (data not shown).

On R2YE medium for 40 h, the *sigK* mutant showed precocious aerial mycelium formation, as demonstrated in Fig. 1A, where the mutant is covered with aerial hyphae while the wild-type strain M145 has just begun developing into the aerial mycelium stage (Fig. 1A). After incubation for 4 days, the gray appearance of *sigK* mutant colonies indicated matured spores with pigment deposition, but the wild-type strain still stayed at the aerial mycelium stage (Fig. 1B and C). Reintroduction of *sigK* under its native promoter *sigKp* (5, 27) into the *sigK* mutant could restore the phenotype of the *sigK* mutant (Fig. 1A). These phenomena indicated that *sigK* deletion accelerated the morphological development.

FIG. 2. Antibiotic production assay. (A) Wild-type LM7 (WT+V), the *sigK* mutant LM8 (Δ *sigK*+V), and the *sigK* complementated strain LM2 (Δ *sigK*+*sigK*) were streaked on R2YE plates, incubated at 30°C for the indicated hours and photographed from the bottom of the plates. (B and C) M145 (WT; ▲) and *sigK* mutant LM1 (Δ *sigK*; ■) cells were collected from R2YE covered with cellophane and were sequentially extracted with 1 M KOH and 0.5 M HCl-acidified methanol for Red (B) and Act (C) and quantitatively measured by A_{530} and A_{640} , respectively. The ratios of absorbance to mycelium dry weight were calculated, and numbers in the graphs are the mean values of three independent experiments. Standard deviation bars are shown on graphs. (D) RNA was extracted from the *sigK* mutant LM1 (Δ *sigK*) and wild-type strain M145 (WT) after grown on R2YE medium overlaid with cellophane for the indicated hours, respectively. An S1 nuclease protection assay was demonstrated with the ssDNA probe of *actII-orf4*, *redD* and *hrdB*, respectively.

The emergence of aerial hyphae requires the hydrophobic surfactants, such as chaplins and SapB protein, which facilitate the vegetative mycelium to overcome the surface tension of the mycelium-air interface (9, 13). When the expression levels of two of the chaplin genes, *chpE* and *chpH* (13), were examined, both the *sigK* mutant and the wild-type strain showed relatively low and similar expression levels of *chpE* and *chpH* at the vegetative hypha stage. After entering the aerial mycelium phase, the *sigK* mutant exhibited higher expression levels of *chpE* and *chpH* than the wild type (Fig. 1D), which might account for the accelerated aerial hypha formation after deletion of *sigK*. These data suggested that SigK had a repressive role for morphological development.

Similar phenotypes were also observed on MS medium (data not shown). All these data suggested that loss of SigK accelerated the developmental processes by quickening the transition into aerial mycelia and spores and that SigK was a repressive regulator of the developmental program of *S. coelicolor*.

***sigK* deletion accelerates production of antibiotics.** The secondary metabolism profiles were also checked qualitatively and quantitatively, in both the *sigK* mutant and the wild type. On the R2YE plate after 24 h, the *sigK* null mutant appeared redder than the wild type and the complemented strain, suggesting that *sigK* also inhibits secondary metabolism (Fig. 2A). After a further 1 day of incubation, the *sigK* mutant lawn was already deep purple, indicating the production of blue antibiotic Act, while the other two strains had just begun to turn purple (Fig. 2A). The *sigK* mutant was much purpler after further incubation, indicating the higher production of Act (data not shown). Consistent with the observation on plates, quantitative measurement of the Act and Red amounts, respectively, also showed that the *sigK* mutant had earlier and higher production levels of antibiotics than the wild type on R2YE medium (Fig. 2B and C). In particular, Act production

was more than 10 times higher in the *sigK* mutant after 6 days (Fig. 2C). The overproduction of Red and Act in the *sigK* mutant coincided with higher expression of *redD* and *actII-orf4* in the *sigK* mutant (Fig. 2D), which encode two positive transcription activators of gene clusters for Red and Act production, respectively (14, 34). These phenomena suggested that SigK also had a repressive role in secondary metabolism.

***sigK* expression increases before transition into aerial mycelia.** Our results suggested that SigK was a negative regulator of morphogenesis and secondary metabolism of *S. coelicolor*. However, when we checked the expression profile of *sigK* by the S1 nuclease protection assay during morphological development, *sigK* expression gradually increased on MS plates before 22 h when the cells were at the vegetative mycelium stage but were consistent after development into aerial mycelia and spores (Fig. 3A). The *sigK* expression pattern was further validated with a promoter-probing plasmid, in which GFP was under the control of the *sigK* promoter (*sigKp*). After incubation on MS plates for 14 h, the *sigKp* activity was comparatively low, but it increased after a further 8-h incubation when the cells were still in the vegetative mycelium phase (Fig. 3B). At the aerial mycelium (24 and 29 h) (Fig. 3B) and spore (41 and 48 h) (Fig. 4) stages, the *sigKp* activity was consistent with that at the 22-h stage. As a control, the band was not observed in the strain bearing the plasmid without a promoter, indicating the specificity of GFP expression driven from *sigKp* activity (data not shown). Since the GFP protein is stable in bacteria (1), the increase of GFP protein driven from *sigKp* might result from the accumulation of GFP at vegetative hypha stage. To exclude this possibility, GFP was then expressed under the control of a constitutive promoter *ermEp** and found to exhibit a steady protein level during the vegetative mycelium development (Fig. 3C), even after development into aerial hyphae and spores (Fig. 5A). This excluded the interference of GFP sta-

FIG. 3. Transcriptional profile of *sigK*. (A) An S1 nuclease protection assay was performed with total RNA from wild-type strain M145 grown on MS overlaid with cellophane at 30°C. RNA were prepared at 14, 16, 18, 20, 22, 29, 41, and 48 h at different morphological stages of vegetative mycelium (V), aerial mycelium (A), and spores (S). The *sigK* probe was the single-strand antisense DNA generated by PCR and λ exonuclease digestion. For loading control, about 1 μ g of total RNA was loaded on 1% Tris-borate-EDTA agarose gel and photographed. 16S and 23S rRNA are shown. (B) Mycelia of strain LM7 containing *egfp* under *sigK* promoter (*sigKp*) were collected from MS plates overlaid with cellophane after incubation for the indicated time when cells developed into the vegetative mycelia (V) and aerial hyphae (A). Samples were extracted by sonication, and about 20 μ g of total protein was loaded for 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis with an anti-GFP antibody or Coomassie brilliant blue staining as the loading control. (C) Strain LM6 with *ermEp*-egfp* was inoculated on MS plates overlaid with cellophane and incubated at 30°C after the indicated hours. Mycelia were collected and treated in the same way as described for panel B.

bility with the monitoring of promoter activity with GFP as a reporter and also suggested the specific augmentation of *sigKp* activity at the vegetative hypha stage. All of the data described above suggested that *sigK* expression or the *sigKp* activity was positively regulated earlier before development into the aerial mycelium stage but was almost invariable at the late stage of vegetative mycelia and throughout the aerial mycelium and spore phases.

***sigK* expression is partially dependent on SigK during the vegetative mycelium phase.** Some SigB-like sigma factors in *S. coelicolor* can regulate transcription on its own promoter, such as SigB on *sigBp*₁ (11), SigH on *sigHp*₂ (32), and SigN on *sigNp* (12). We then checked whether *sigK* expression could also be autoregulated. With GFP as a reporter protein, about five-times-lower *sigKp* activity was observed in the *sigK* mutant than

FIG. 4. Positive autoregulation of *sigK* expression. Cells of wild-type strain LM7 (M145+*sigKp-egfp*) or the *sigK* mutant strain LM8 (Δ *sigK*+*sigKp-egfp*) were grown on MS plates covered with cellophane at 30°C and analyzed in the same manner as described in legend to Fig. 3B at different developmental stages, including vegetative mycelia (V), aerial hyphae (A), and spores (S), as indicated.

in the wild type at the 16-h incubation time point, when the cells were developing into vegetative hyphae. But wild-type cells showed only about 1.5-times-higher yields of GFP protein than the *sigK* mutant at the 22-h point, when the wild type was still at the vegetative mycelium stage, while aerial mycelia had begun to erect in the *sigK* mutant (Fig. 4). These results suggested partial positive autoregulation of *sigKp* activity from SigK and decreased dependence of *sigK* expression on SigK during the development of vegetative mycelia. However, no apparent difference in *sigKp* activity was observed after 24 h between the *sigK* mutant and the wild type (Fig. 4), suggesting independence of *sigKp* activity on SigK after development into aerial mycelia.

Posttranscriptional regulation of SigK. If SigK somehow inhibits the developmental stages of the normal *S. coelicolor* life cycle, how is this repression relieved given that *sigK* expression is persistent throughout the developmental growth? To address this question, we further investigated the stability of the SigK protein along with the developmental progression. SigK was C-terminally tagged with GFP and was expressed under *ermEp**. As a control, GFP was also expressed under *ermEp**, and consistent expression of GFP protein during the developmental courses was observed (Fig. 5A), suggesting that the *ermEp** activity and posttranscription of GFP were independent of morphological transitions. Interestingly, the SigK-GFP fusion protein, with an estimated molecular mass of about 58 kDa, was detected to be at about 70 kDa after 22 h of incubation, when the cells were developing into vegetative mycelia, but vanished after an incubation of 29 h or longer, when cells were developing into aerial mycelia and spores (Fig. 5A). The SigK-GFP protein could restore the *sigK* mutant phenotypes (data not shown), suggesting that this fusion protein was stable and functional. Moreover, two small fragments, one about 28 kDa (Fig. 5A, arrow b) and the other about 30 kDa (Fig. 5A, arrow a) could also be detected at the vegetative mycelium stage but disappeared after further morphological development. But all of these three bands were not detected in the GFP control sample (Fig. 5A), suggesting that the two smaller fragments were derived from SigK-GFP. However, another smaller band, with molecular mass similar to that of GFP, was constitutively detected throughout the developmental phases even with the disappearance of SigK-GFP and its derivatives (Fig. 5A). If a truncated isoform of SigK, which

FIG. 5. Posttranscriptional regulation of SigK. (A) Wild-type strain M145 containing plasmid pLM1 or pLM6 for expression of GFP or SigK-GFP, respectively, was harvested after being cultured on MS plates covered with cellophane at 30°C at different time points for vegetative mycelia (V), aerial hyphae (A), and spores (S) and treated as described in legend to Fig. 3B. Two small fragments are indicated by arrows a and b. Protein molecular mass markers are shown to the left as kilodaltons. (B) Wild-type strain M145 containing plasmid pLM8 expressing AtpD-GFP was inoculated on MS plates covered with cellophane at 30°C, and mycelia were gathered at 22, 29, 41, 48, 72, and 144 h after incubation for immunoblot analysis and Coomassie brilliant blue staining as described in legend to Fig. 3B. Different morphological phases of vegetative mycelia (V), aerial hyphae (A), and spores (S) are indicated.

contained a stop codon at the C terminus of SigK, was expressed at the same condition, this GFP band was still constitutively observed (data not shown), suggesting another translation initiation for GFP, which had the start codon ATG within the NdeI site.

We then checked whether the two smaller bands (Fig. 5A, arrows a and b) were specific for SigK-GFP. AtpD, the ATP synthase β subunit of *S. coelicolor* in catalysis of ATP production (37), was expressed with fusion to GFP in the same way as SigK-GFP, and the two bands, smaller than AtpD-GFP but slightly larger than GFP, were repeatedly observed (Fig. 5B). This suggested that these were nonspecific fragments arising only in the vegetative hypha stage. Moreover, though the AtpD-GFP protein level decreased gradually, it was detected throughout all the developmental stages (Fig. 5B), which was consistent with its biological activity as the housekeeping protein for ATP production (17, 38), and also suggested the specific disappearance of SigK-GFP in aerial hyphae and spores.

Developmental phase dependence of SigK degradation. The two smaller derivatives (Fig. 5A, arrows a and b) of SigK-GFP could have resulted from the posttranslational modification of SigK-GFP by two independent cleavages or two alternative translation initiations, as proposed for GFP in Fig. 5A.

To discriminate these two possibilities, SigK-GFP was further tagged at its N terminus with 3FLAG. Examination of the 3FLAG-SigK-GFP protein (apparently about 72 kDa) dynamics by immunoblot analysis with anti-GFP with narrower time intervals showed that the SigK fusion protein level dropped sharply at the vegetative hypha stage and was very faint at 24 h but undetectable after 26 h, when cells were developing into aerial hyphae (Fig. 6A). Meanwhile, immunoblot analysis with anti-FLAG antibody also showed that 3FLAG-SigK-GFP protein decreased sharply in the vegetative hypha phase, but this fusion protein was detected at the 26-h time point and was absent after further incubation (Fig. 6A). These results suggested that the SigK protein was comparatively abundant at the vegetative stage, although it was in continuing decline and

FIG. 6. Developmental phase dependence of degradation of SigK. (A) Mycelia of strain LM9 (M145+*ermEp**-3flag-sigK-egfp) were collected from MS plates with cellophane at 30°C after incubation for different hours and treated as described in legend to Fig. 3B but with an additional anti-FLAG antibody for immunoblot analysis. Arrows a, b, a', and b' indicate the proteolytically cleaved fragments when detected with anti-GFP and anti-FLAG antibodies, respectively. Protein molecular mass markers are shown on the left as kilodaltons. The presence of vegetative mycelia (V), aerial hyphae (A), and spores (S) are shown. (B) Mycelia of strain LM5 (M145+*ermEp**-3flag-sigK) were treated in the same way as described for strain LM9 in panel A, but the immunoblot experiment was demonstrated only with the anti-FLAG antibody.

FIG. 7. Endogenous dynamics of SigK. Immunoblot analysis of about 20 μ g total protein from LM9 (M145+*ermEp**-3*flag-sigK-egfp*) (lane 1) and LM10 (M145+*sigKp-3flag-sigK*) (lanes 2 to 7) grown on MS medium at 30°C for the indicated hours with the anti-FLAG antibody. Arrows a' and b' indicate the proteolytically cleaved fragments, as described in legend to Fig. 6A. Protein molecular mass marker are shown to the left as kilodaltons. Vegetative mycelia (V), aerial hyphae (A), and spores (S), indicating different morphological phases, are shown. Coomassie brilliant blue staining of total protein is shown for the loading control.

fell below a detectable threshold at the early phase of aerial mycelium formation.

After immunoblot analysis with anti-GFP antibody, the two smaller 3FLAG-SigK-GFP derivatives repeatedly appeared (Fig. 6A, arrows a and b). Correspondingly, two smaller bands (Fig. 6A, arrows a' and b'), with apparent molecular masses of about 42 kDa and 44 kDa, respectively, were also detected after immunoblot analysis with anti-FLAG antibody (Fig. 6A). These data suggested that these four bands (fragments a, b, a', and b') resulted from the two independent cleavages of the 3FLAG-SigK-GFP protein. Moreover, the two 3FLAG-containing fragments (Fig. 6A, arrows a' and b') vanished rapidly at the late stage of vegetative mycelium development (Fig. 6A). The similarity of these dynamics also supported that they were the proteolytical derivatives of 3FLAG-SigK-GFP protein.

The 3FLAG-SigK protein without GFP was also expressed under *ermEp**. We found that this protein was abundant at the vegetative mycelium stage but also fell dramatically when cells were developing into aerial mycelia and disappeared after 26 h of incubation (Fig. 6B). Thus, the 3FLAG-SigK protein exhibited a similar dynamic with SigK-GFP and 3FLAG-SigK-GFP proteins, ensuring that the disappearance of SigK fusion proteins was the intrinsic characteristic of SigK protein but was not because of the structural instability of the SigK-GFP proteins.

Endogenous dynamics of SigK protein. The SigK fusion proteins described above were all expressed under promoter *ermEp**. To examine the native dynamics of SigK during morphological development, the 3FLAG-SigK fusion protein was expressed under the *sigK* native promoter *sigKp*. 3FLAG-SigK migrated in the same position as the smaller fragment (Fig. 6A, arrow a') split from 3FLAG-SigK-GFP (Fig. 6A and 7), further supporting the hypothesis described above of two cleavages in the linker sequence. In addition, though the protein level of 3FLAG-SigK also dropped (coinciding with the development

of vegetative mycelia) and was almost undetectable after 26 h of incubation, the amount of 3FLAG-SigK driven from *sigKp* was much lower than 3FLAG-SigK-GFP expressed from *ermEp**. Also, the 3FLAG-SigK protein level did not decrease as rapidly as SigK fusion proteins under *ermEp** (Fig. 6 and 7), which was consistent with observations that *ermEp** was one of the strong constitutive promoters and that *sigKp* activity increased during vegetative mycelium growth. These data suggested that the endogenous SigK protein level fell concomitantly with vegetative hypha development and faded away during the vegetative-aerial mycelium transition.

DISCUSSION

SigK, a repressive sigma factor in the group 3 subfamily. SigK (SCO6520) was previously supposed to be one of 10 SigB-like sigma factors as determined by genome mining (2). Of the other nine sigma factors in the group 3 subfamily characterized to date, seven (SigB, SigL, SigN, SigF, SigH, SigM, and σ^{WhiG}) were positive regulators in morphological development (10, 12, 24, 30, 32). Meanwhile, the *sigL* mutant showed a complete absence of Act (24), and the *sigB* mutant showed enhanced Act but decreased Red production (11, 24). We reported the dual repressive roles of SigK on morphological development and secondary metabolism, suggesting that this alternative sigma factor (SigK) had unique functions in the group 3 σ^{70} subfamily. It was also suggested that the functional diversity and hierarchy of these sigma factors in this group might play a vital role in the precise transcription controlling for the proper subsistence of *S. coelicolor* under various environmental stresses.

How does SigK control the developmental processes? We presumed that SigK, as a negative regulator for development, antagonistically binds to the RNAP core enzyme against other sigma factors as positive regulators, such as seven group 3 members or one of the extracellular function sigma factors, BldN, as mentioned above. The gradual removal of SigK during morphological development causes the andante occupancy of other sigma factors on the promoters of genes for the proper development. Another possibility is that although SigK is a transcriptional activator, it positively controls the expression of some genes encoding the repressor proteins. Loss of SigK in the *sigK* mutant results in the advanced shutdown of these gene expressions, thus leading to the precocious maturation of *Streptomyces* cells.

Developmental phase dependence of SigK protein degradation. The SigK protein, under the control of *ermEp**, showed a sharp decline during morphological development. It was relatively abundant and decreased rapidly in the vegetative mycelium phase, was faint during the vegetative-aerial mycelium transition, and was absent at the early phase of aerial hyphae. Moreover, two independent cleavages at the linker sequence between SigK and GFP were proposed, and the proteolytical products vanished rapidly before developing into aerial mycelia. Further expression of 3FLAG-SigK under *sigKp* showed endogenous dynamics of SigK during morphological development. 3FLAG-SigK displayed a dynamics pattern similar to that of SigK fusion proteins under *ermEp**, except that they decreased much more gradually during vegetative hypha growth. This set of dynamics of SigK was consistent with the

repressive roles of SigK on morphological development and secondary metabolism. Since SigK disappeared when cells were developing into aerial mycelia, the removal of SigK as a block might account for the initiation of aerial hypha formation, and the accelerated sporulation in the *sigK* mutant might result from advanced aerial hypha formation, not from *sigK* deletion.

Several sigma factors have been reported proteolytically processed in *S. coelicolor*, for example, SigH as introduced above and one of the extracellular function sigma factors, BldN. BldN is required for the formation of aerial mycelia (4), but BldN is translated into a pro- σ^{BldN} and subsequently proteolytically processed into mature BldN by removal of about 86 residues at its N terminus when aerial mycelia emerge (3). Here we initially reported the specific degradation of another sigma factor, SigK, concomitant with the morphological developmental phases.

Possible correlations between *sigK* expression and level of SigK protein. One of the interesting observations was that *sigK* expression gradually enhanced before the aerial mycelium stage and that this elevated expression partially resulted from the auto-positive feedback, since loss of SigK reduced the *sigK* promoter activity before aerial mycelium development and that the gradually diminished endogenous SigK was also consistent with the decreased dependence of *sigKp* activity on SigK during vegetative mycelium development.

Since SigK functioned as a repressive regulator for the vegetative-aerial mycelium switch as discussed above, the activated *sigK* expression in substrate hyphae should not be expected. However, based on our observations, it was speculated that the enhanced *sigK* expression in substrate mycelia might slow down the rapidly decreased concentration of SigK protein resulting from fast degradation of SigK to an appropriate protein level, thus optimizing the timing of aerial hyphal emergence. SigK driven from the strong constitutive promoter *ermEp**, which excluded the possible transcriptional effects on the protein level, exhibited an acute drop, while the protein level change of SigK expressed under *sigKp* was much milder, suggesting that the endogenous decreased degradation rate of SigK protein was most probably because of the increased *sigK* expression. Moreover, the amount of SigK protein from *ermEp** was much greater than that from *sigKp*. If the *sigKp* activity was not elevated, the SigK protein would vanish more quickly during vegetative mycelium development, thus causing the advanced disappearance of SigK and accelerated aerial hyphal formation as in the *sigK* mutant. Nevertheless, further efforts would be required to explain the noneconomical expression from the *sigK* promoter after development into aerial hyphae, when the cells lacked the SigK protein because of its rapid degradation.

ACKNOWLEDGMENTS

We gratefully thank Mark J. Buttner at the John Innes Centre, Norwich, United Kingdom, for his kind gifts of *S. coelicolor* strain J1981, pIJ8630, and pIJ8660. We also thank Ben Shen at University of Wisconsin—Madison for his critical reading and constructive suggestions for the improvement of the manuscript.

This work was supported by the National Sciences Foundation of China (30700006) and the Zijin Project of Zhejiang University to Xu-Ming Mao.

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