Identification of a butenolide signaling system that regulates nikkomycin biosynthesis in Streptomyces

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Butenolides are an emerging family of signaling molecules in Streptomyces. They control complex physiological traits, such as morphological differentiation and antibiotic production. However, how butenolides regulate these processes is poorly investigated because of obstacles in obtaining these signaling molecules. This study reports the identification of a butenolide-type signaling system for nikkomycin biosynthesis in Streptomyces anschromogenes with distinct features. We identified a gene cluster, sab, consisting of three genes, sabAPD, for butenolide biosynthesis and two regulator genes, sabR1 and sabR2, and characterized three butenolides (SAB1, -2, and -3) by heterologous expression of sabAPD. sabA disruption abolished nikkomycin production, which could be restored by the addition of SABs or by deletion of sabR1 in ΔsabA. Electrophoretic mobility-shift assays and transcriptional analyses indicated that SabR1 indirectly represses the transcription of nikkomycin biosynthetic genes, but directly represses sabA and sabB1. In the presence of SABs, the SabR1 transcriptional regulator dissociated from its target genes, verifying that SabR1 is the cognate receptor of SABs. Genome-wide scanning with the conserved SabR1-binding sequence revealed another SabR1 target gene, cprC, whose transcription was strongly repressed by SabR1. Intriguingly, CprC positively regulated the pleiotropic regulatory gene adpA by binding to its promoter and, in turn, activated nikkomycin biosynthesis. This is the first report that butenolide-type signaling molecules and their cognate receptor SabR1 can regulate adpA via a newly identified activator, CprC, to control nikkomycin production. These findings pave the way for further studies seeking to unravel the regulatory mechanism and functions of the butenolide signaling system in Streptomyces.

Improper use of antibiotics as well as the horizontal transfer of antibiotic resistance genes has led to the continuous appearance of antibiotic-resistant pathogens and the loss of antibiotic native efficiency. Most commercially important natural antibiotics are produced by Streptomyces. Their biosynthesis is precisely controlled by cluster-situated regulators (CSRs) or global regulators, most of which can respond to small molecules including hormone-like signaling molecules, also known as autoregulators, and some specialized metabolites (1).Defining these signal transmission pathways is desirable to improve the efficiency of antibiotic production yield and to identify new gene clusters to discover previously unknown bioactive products of interest.

Although signaling molecules are widely distributed in Streptomyces, their biosynthesis is usually under stringent control, resulting in low yields in producing strains. This impedes the discovery of new signaling molecules and elucidation of their function. Since the first γ-butyrolactone (GBL) signaling molecule, A-factor, was discovered in Streptomyces griseus, only 33 autoregulators have been identified in Streptomyces (1–3). They are classified into five groups, GBLs, furans, butenolides, PI factor, and N-methylphenylalanine-dehydrobutyrine diketopiperazine, based on their structures. GBL is the largest family of autoregulators, and its regulatory mechanism and biosynthesis have been intensively investigated, whereas studies on other family of autoregulators are very rare (4). Recently, a butenolide signaling molecule, avenolide, was found to be essential for biosynthesis of the clinically important antibiotic avermectin, inspiring more interest in this family of regulators (5). Seven butenolides associated with antibiotic biosynthesis have been discovered so far, including avenolide from Streptomyces avermitilis and Streptomyces albus (3, 5) and SRBs from Streptomyces rochei (6). Butenolide contains a five-member ring backbone, but with an unsaturated bond at C3–C4 and diverse side chains at C3, C4, or C5, which confer unique activities.

Signaling molecules and their receptors can exert considerable impact on the onset and production of antibiotic biosynthesis. A typical autoregulator system has been exemplified in S. griseus and portrayed as a pyramid-like network. A-factor and its receptor ArpA constitute the apex of the pyramid, controlling a series of diverse downstream pathways via a pivot point of control, which may be essential for system efficiency. Further investigation of the biological roles, molecular mechanisms, and utility of butenolide-type signaling systems is desired.

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This article contains Tables S1 and S2 and Figs. S1–S6.

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‡ The abbreviations used are: CSR, cluster-situated regulator; ARE, autoregulator element; qRT-PCR, quantitative real-time PCR; GBL, γ-butyrolactone; HSQC, heteronuclear single-quantum correlation; HMBC, heteronuclear multiple-bond correlation; EMSA, electrophoretic mobility-shift assay; tss, translation start site.

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Figure 1. Effect of sabA disruption on nikkomycin production. A, genetic organization of nik gene cluster and sab gene cluster in S. ansochromogenes 7100. B, HPLC analysis of nikkomycin in different strains. C, nikkomycin bioassays against C. albicans with fermentation filtrates of different strains. WT, S. ansochromogenes 7100. ΔsabA, sabA disruption mutant. ΔsabAc, sabA complementary strain. eΔsabA, the extract of ΔsabA fermentation filtrate. eWT, the extract of WT strain fermentation filtrate. X, nikkomycin X, Z, nikkomycin Z.

 regulator, AdpA. ArpA directly represses the transcription of adpA, whereas binding of A-factor to ArpA results in derepression of adpA transcription and consequently the expression profile switch of numerous target genes of AdpA (7). Subsequently, more autoregulators and their cognate receptors have been characterized. They have intimate association with antibiotic production and morphological differentiation in various Streptomyces species, and some of these are involved in the AdpA regulatory network, a pivotal pleiotropic regulator widespread in Streptomyces. Although the target genes of AdpA and their regulation have been comprehensively illustrated in many Streptomyces species, how AdpA expression itself is maintained under delicate and precise control is poorly investigated (8, 9). Signaling molecules taking part in the AdpA regulatory cascade provide an important mechanism for cells to respond to environmental and physiological changes, and also signal amplification can be achieved via AdpA transmission (10).

Streptomyces ansochromogenes 7100 produces nikkomycin under the control of global regulators, such as WblA, AdpA, GBL receptor-like regulator SabR (11–13), and the pathway-specific regulator SanG (14, 15). AdpA positively regulates nikkomycin biosynthesis via binding to the promoter region of sanG, but negatively regulates ovedomycin production by repressing ovmZ/ovmW (16). An autoregulator biosynthetic gene cluster sab (KF170348) was revealed in this strain by genome mining (17). BLAST search suggested that the homolog of sabA, the core gene in sab, is widely distributed in Streptomyces. What autoregulators may be synthesized by sab and how they coordinate with AdpA to regulate the secondary metabolism in S. ansochromogenes are of great interest. In this work, we report the characterization of a novel butenolide signal transduction pathway, in which signal input is transmitted to nikkomycin biosynthesis via a newly discovered activator, CprC, of adpA.

Results

Characterization of butenolide signal molecules (SABs) triggering nikkomycin production

It is well-known that AfsA-like proteins are key enzymes in autoregulator biosynthesis. By genome mining of S. ansochromogenes, an autoregulator biosynthetic gene cluster (sab) was identified (Fig. 1A), and sab is located about 1.87 megabases away from the nikkomycin biosynthetic gene cluster (nik) in the chromosome of S. ansochromogenes. In sab cluster, sabA encodes an AfsA-like enzyme (31% identity with AfsA from S. griseus), whereas sabP and sabD encode phosphatase and dehydrogenase enzymes, respectively, with putative tailoring functions. sabR1 and sabR2 encode TetR family regulators. SabR1 belongs to the GBL receptor family and shows 40% identity with ScbR from S. coelicolor (18), and SabR2 shows 31% identity with pseudo-GBL receptor JadR2 from S. venezuelae (19). sabR1 and sabR2 are situated downstream of sabD in sab cluster, implying their potential correlation with the signal molecules as receptors.

To understand what kind of signal molecules can be synthesized by sab and whether they can affect nikkomycin biosynthesis, a sabA disruption mutant (ΔsabA) was constructed. Nikkomycin was determined by HPLC analysis and bioassays against Candida albicans. No nikkomycin was detected in the culture supernatant of ΔsabA after 5 days’ incubation. When a copy of sabA with its promoter region was introduced into ΔsabA, nikkomycin production was almost restored to the level of the WT strain. Furthermore, nikkomycin in ΔsabA was restored with the addition of ethyl acetate extracts of WT, but not with the extract of ΔsabA as expected (Fig. 1, B and C). These results indicated that compounds synthesized by SabA are closely related to nikkomycin biosynthesis.

Signaling molecules usually work at nanomolar concentrations, and it is difficult to obtain a large enough quantity from
native producer strains for structural determination. To overcome this problem, heterologous expression of sabAPD was carried out in Escherichia coli and in Streptomyces, respectively, and the products were detected by HPLC (Fig. 2, A and B). It was shown that nikkomycin production in ΔsabA was restored by the addition of extracts from E. coli C41 containing sabAPD (CpAPD) or S. coelicolor M1146 containing sabAPD (MpAPD) but not recovered by the addition of extracts from strains E. coli C41/pET23b and S. coelicolor M1146/pKC1139 as controls (data not shown). Clearly, sabAPD were expressed in both E. coli C41 and S. coelicolor M1146, and the resulting products triggered nikkomycin production.

Compounds inducing nikkomycin production were isolated from CpAPD and MpAPD strains and subsequently purified using HPLC by tracking the activity inducing nikkomycin biosynthesis in ΔsabA. A total of 30 mg of purified SAB1 was gained from 3 liters of culture broth of CpAPD. High-resolution electrospray ionization MS showed a molecular ion peak at m/z 187.0964 [M + H]+ (Fig. S1A), and the molecular formula was deduced as C9H14O4. By comparing the NMR spectroscopic data with those of known compounds (compounds 1 and 3 in S. antibioticus) (20), the only difference indicated was in the side chain at C3. On 1H NMR, one methyl group signal (δH 0.79; δC 15.0) at C2 in compound 3 was absent, and CH was changed to CH2 (δH 1.8/1.6; δC 38.2) (Fig. S1 and Table S1). Further analyses of the 1H-1H COSY, HSQC, and HMBC confirmed the side chain at C3 in SAB1 to be hydroxyl-butyl, and the structure of SAB1 was thus determined as 5-hydroxy-3-(1-hydroxyl-butyl)-4-methyl-2(5H)-furanone (Fig. 2C), a novel member of butenolide autoregulators in Streptomyces.

HPLC analysis revealed that two other molecules, SAB2 and SAB3, were produced by MpAPD in addition to SAB1 (Fig. 2B). A
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total of 20 µg of purified SAB2 and 10 µg of SAB3 was gained from MpAPD extract. [M + H]+ ions m/z 187.0968 for SAB2 and 201.1124 for SAB3 were observed on high-resolution electrospray ionization MS, corresponding to molecular formulae of C₉H₁₄O₄ and C₁₀H₁₆O₄, respectively (Figs. S2 and S3). SAB2 shares the same molecular formula with SAB1, but there is a different retention time on HPLC (Fig. 2B), suggesting that it is an isomer of SAB1. NMR spectroscopic data of SAB2 and SAB3 were consistent with those of compounds 1 and 3 isolated from S. antimicrobicus DSM40725 (Figs. S2 and S3) (20). Thus, SAB2 was determined as 5-hydroxy-3-(1′-hydroxy-2′-methylpropyl)-4-methyl-5(5H)-furanone and SAB3 as 5-hydroxy-3-(1′-hydroxy-2′-methylbutyl)-4-methyl-5(5H)-furanone (Fig. 2C). Compounds SAB1, -2, and -3 all contain a 4-methyl-5-hydroxybutenolide ring as the core structure but attached with a different side chain at the C3 position. To characterize the native SABs in S. ansochromogenes, the extract of S. ansochromogenes was analyzed by LC-electrospray ionization-MS. It was shown that native SAB1, -2, and -3 were all present in WT strain, where SABs are present (Fig. 4/A). Similarly, in the absence of SABs, SabR1 repressed the transcription of sabA, sabR2, and sabA in ΔsabA but not in the WT strain, where SABs are present (Fig. S5). Taken together, it was suggested that SABs are associated with SabR1 to regulate nikkomycin biosynthesis.

To verify the recognition of SABs by SabR1, electrophoretic mobility-shift assays (EMSAs) were performed using SabR1-His₆ and potential target gene promoter regions as probes. Complexes of SabR1 with the upstream regions of sabA, sabR1, or sabR2 were formed in a concentration-dependent manner (Fig. 4A), whereas binding of SabR1 to the upstream regions of sanF, sanG, or adpA was not observed (data not shown), implying that SabR1 regulates nikkomycin biosynthesis indirectly. When SABs were added into the EMSA reaction mixture, the complex could not be formed, demonstrating that sabA, sabR1, and sabR2 are the direct targets of SabR1, and SABs can interact with SabR1 to cause its dissociation from the target DNA (Fig. 4B). Thus, SabR1 was determined as the cognate receptor of SABs in vitro.

To identify the precise binding sequences of SabR1 on its target genes, DNase I footprinting experiments were performed. The results showed that SabR1 protected regions from −137 to −111 bp relative to the sabA translation start site (tss), −118 to −88 bp relative to the sabR1 tss, and −282 to −254 bp relative to the sabR2 tss (Fig. 4, C–E). Analysis of the three binding sites using the MEME program (21) revealed a conserved consensus sequence (5′-AAAWAAACCCGDBRD-TYSGTWY-3′) (Fig. 4F). In agreement with EMSAs, this consensus sequence was not found in the promoters of sanF, sanG, and adpA, confirming that SabR1 regulates nikkomycin biosynthesis indirectly.

cprC is directly repressed by SabR1 and positively regulates nikkomycin biosynthesis

As mentioned above, SabR1 regulates nikkomycin production indirectly via unknown intermediates. One candidate is sabR2, a target gene of SabR1, encoding a TetR family regulator, which is potentially an intermediate protein between SabR1 and nikkomycin biosynthetic genes. However, sabR2 showed no impact on nikkomycin production after its disruption (Fig. S6), implying that SabR1 exerts its regulatory function on nikkomycin biosynthesis through other means. To explore more SabR1 potential target genes, S. ansochromogenes genome scanning was undertaken using the conserved 23-bp SabR1 binding consensus. This revealed another TetR-family regulator gene, cprC, whose encoded protein CprC shows 68% identity with CprA and 70% identity with CprB from S. coelicolor (22). The promoter region of cprC (PcprC) contains a SabR1 conserved binding motif 5′-AAACACAAACCCGATGTCTC-TYCTTTCGTG-3′ (Fig. 5A). EMSA experiments confirmed that P_cprC could be recognized by SabR1 to form a complex. When P_cprC was mutated to 5′-GGGCGCCGCGATGTCTC-TYCTTTCGTG-3′ at the specific sites shown in boldface type, SabR1 no longer bound the mutated P_cprC (Fig. 5, B and C). Thus, cprC is confirmed as a direct target gene of SabR1.

Analyses by qRT-PCR showed that the transcription of cprC was dramatically reduced in ΔsabA but restored in ΔsabA/ΔsabR1, suggesting that SabR1 is a repressor of cprC (Fig. 5D). Along with the EMSA results, it was verified that SabR1 represses the transcription of cprC by directly binding to the promoter region, which was also further illustrated by the
expression of gusA encoding a β-glucuronidase reporter system (Fig. 5E). It can be hypothesized that the decreased transcription of cprC may lead to abolition of nikkomycin production in ΔsabA.

To ascertain the effect of CprC on nikkomycin production, disruption of cprC was performed to generate ΔsabA cprC. The yield of nikkomycin in ΔsabA cprC was notably decreased compared with that in WT, suggesting that CprC is an important activator for nikkomycin production. In addition, cprC was complementarily expressed in ΔsabA sabR1 complementary strain of ΔsabA/ΔsabR1 ΔsabR1, disruption mutant of sabR1. C, qRT-PCR transcriptional analyses of adpA, sanG, sanO, sanN, and sanF in WT, ΔsabA, and ΔsabA/ΔsabR1 strains. Error bars, S.D. calculated from three independent experiments.

CprC directly activates the transcription of adpA

To understand how CprC activates nikkomycin biosynthesis, EMSAs were performed using purified CprC-His6 and the promoters of potential target genes, adpA, sanG, sanO, sanN, and sanF. It was shown that CprC binds to the upstream region of adpA (Fig. 7A), but not to the promoter regions of sanG and sanF (data not shown). DNase I footprinting experiments revealed that the protected sequence on adpA promoter by CprC is 5'-CACCCGGGGCCAGACACCGGTCGACTGACCTGTTTTC-3', from −406 to −369 bp relative to the tss of adpA (Fig. 7, B and C). Therefore, adpA is verified to be a target gene of CprC.

Analyses by qRT-PCR revealed that the transcription of adpA was significantly decreased in ΔcprC (Fig. 7D), confirming that CprC is an activator of adpA in S. ansochromogenes.
Remarkably, upon adpA overexpression under the control of P_{hrdB} in ΔcprC (ΔcprC/adpAoe), nikkomycin production was restored compared with ΔcprC (ΔcprC/adpA). Each lane contains 50 ng of labeled probes and 1 μg of poly(dI-dC). S, unlabeled specific probe (30-fold) was added; N, unlabeled nonspecific probe P_{hrdB} (30-fold) was added. B, EMSAs of SabR1 binding to unlabeled PsabR1, PsabR2, and PsabA in the absence or presence of SAB1, -2, and -3. Each lane contains 20-ng probes. The lanes marked with M were added with methanol as control. The lanes marked with B1, B2, and B3 were added with SAB1 (5 μM), SAB2 (25 μM), and SAB3 (5 μM), respectively. C, determination of SabR1-binding site on P_{sabR1} (ARE-R1) and the nucleotide sequences of P_{sabR1}. D, determination of SabR1-binding site on P_{sabR2} (ARE-R2) and the nucleotide sequences of P_{sabR2}. E, determination of SabR1-binding site on P_{sabA} (ARE-sA) and the nucleotide sequences of P_{sabA}. The blue letters represent the translational start sites. The underlined red letters are binding sites of SabR1. F, alignment of SabR1-binding sequences and the sequence logo of conserved bases. The sequences in the black box are consensus nucleotides, and 6-bp inverted repeats are indicated by arrows. The sequence logo was created using the WebLogo program (version 2.8.2; Department of Plant and Microbial Biology, University of California, Berkeley (http://weblogo.berkeley.edu/)). (Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site.) The height of each letter is proportional to the frequency of the base appearance.

Discussion

The dramatic rise in drug-resistant pathogens creates an urgent need for effective alternatives. Bacteria of the genus *Streptomyces* are a particularly abundant source of antibiotics, and their signaling systems are appealing for their critical roles in the regulation of secondary metabolism, particularly in antibiotic biosynthesis. The discovery of new signaling molecules and elucidation of their regulatory roles would have great significance for their use. In this work, a novel member of the butenolide autoregulators, SAB1, along with two analogues, SAB2 and SAB3, were characterized. A cascade regulation of nikkomycin biosynthesis mediated by SABs and its receptor SabR1 via a newly discovered adpA activator CprC was revealed for the first time, which may be applicable for other *Streptomyces*, as SabA and CprC homologs are widely distributed. Moreover, butenolide regulation of global regulator AdpA indicates their potential pleiotro-
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These findings provide new insights into the butenolide signaling system and also facilitate the application of signaling molecules in natural product discovery. Genome sequencing has revealed the presence of a large number of cryptic secondary metabolic gene clusters in *Streptomyces*. Such pathways are usually under stringent control by a variety of different regulatory mechanisms, in which signaling molecules might play important roles. A lack of specific signaling molecules, such as γ-butyrolactones and butenolides, may be a factor causing the silence of gene clusters (23). Characterization of signaling molecules or autoregulators is essential to evaluate their significance in antibiotic production and morphological differentiation. However, this task is hindered by the low yield of autoregulators in producing strains, which makes their study by large-scale fermentation rather burdensome and inefficient (1). It has been evidenced that AfsA and its homologs as well as tailoring enzymes are responsible for the biosynthesis of various signaling molecules, such as GBLs, furans, and some butenolides (4, 6, 24). In this study, heterologous expression of *afsA* homologues and two tailoring enzyme genes, *sabP* and *sabD*, of *S. ansochromogenes* significantly improved the signal molecule yield, and three different butenolides were characterized. SAB1, SAB2, and SAB3 share a common ring skeleton but with different side chains at the C-3 position. In addition, more than one configurational isomer of SABs was visible on NMR spectra. SAB1 and SAB3 are more effective than SAB2 in nikkomycin production, reflecting the structure–activity relationship. Thus, heterologous expression of the butenolide biosynthetic genes proved to be an effective and feasible approach for producing higher yield and diverse structures of signal molecules, which can confer on them different receptor binding affinity and regulatory activity on antibiotic biosynthesis.

### Figure 5

**SabR1 repressed the transcription of cprC by directly binding to its promoter region.**

*Panel A*: sequence of *cprC* promoter region (*P_cprC*). The underlined red letters are the predicted binding site of SabR1 on *P_cprC* (*ARE-C*). *Panel B*: the underlined ARE-C site in *P_cprC* was mutated to generate the mutant promoter region (*P_cprCM*) of *cprC*. *Panel C*: EMSA of SabR1 binding to fluorescently labeled *P_cprC* and *P_cprCM*.

### Figure 6

**Effect of cprC disruption and overexpression on nikkomycin production.**

*Panel A*: HPLC analysis of nikkomycin production in different strains. *Panel B*: nikkomycin bioassays of fermentation filtrates from different strains. WT, *S. ansochromogenes* 7100. ΔcprC, disruption mutant of cprC. ΔcprCc, cprC complementary strain of ΔcprC. ΔsabA, sabA disruption mutant. ΔsabA/cprCoe, cprC overexpression strain in ΔsabA.

Figure 5. SabR1 repressed the transcription of cprC by directly binding to its promoter region. A, sequence of cprC promoter region (*P_cprC*). The underlined red letters are the predicted binding site of SabR1 on *P_cprC* (*ARE-C*). B, the underlined ARE-C site in *P_cprC* was mutated to generate the mutant promoter region (*P_cprCM*) of cprC. C, EMSA of SabR1 binding to fluorescently labeled *P_cprC* and *P_cprCM*. Each lane contains 50 ng of labeled probes and 1 μg of poly(dI-dC). S, unlabelled specific probe *P_cprC* (30-fold) was added; N, unlabelled nonspecific probe *P_nudb* (30-fold) was added. D, qRT-PCR transcriptional analysis of cprC in WT, ΔsabA, and ΔsabA/ΔsabR1. Error bars, S.D. calculated from three independent experiments. E, GusA activity in derivatives of *S. coelicolor* M1146 containing various promoters fused with *gusA*, *P_G* and *P_cmB*, *S. coelicolor* M1146 containing *P_cprC* or *P_cprCM* fused with *gusA*, *P_G-BR1* and *P_cmB-BR1*, strains *P_G* and *P_cmB* containing *P_nudb* fused with *sabrB*, *P_G-R1* and *P_cmB-R1*, strains *P_G* and *P_cmB* containing *sabrB* with its own promoter.
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Figure 7. CprC activates the transcription of adpA by directly binding to its promoter region. A, EMSA of CprC binding to the fluorescently labeled promoter region of adpA (PadpA). Each lane contains 50 ng of labeled probes and 1 μg of poly(dI-dC). S, unlabeled specific probe PadpA (30-fold) was added; N, unlabeled nonspecific probe P_hyp (30-fold) was added. B, DNase I footprinting of CprC-binding site on PadpA (site-aA). C, the nucleotide sequence of PadpA. Blue letters, translational start sites. Underlined red letters, binding sites of CprC. D, qRT-PCR transcriptional analysis of adpA in WT and ΔcprC. Error bars, S.D. calculated from three independent experiments. E, nikkomycin bioassays of fermentation filtrates from different strains. F, HPLC analyses of nikkomycin in different strains. WT, S. ansochromogenes 7100. ΔcprC, disruption mutant of cprC. ΔcprC/AdpAoe, adpA overexpressed in ΔcprC.

Figure 8. A plausible model for the roles of SabR1 and its ligands (SABs) in regulation of nikkomycin biosynthesis. Signal molecules SABs synthesized by sab exert regulatory functions via the cognate receptor SabR1. SabR1 can repress the transcription of cprC and other target genes (sabr1, sabr2, and sabA). CprC is a new activator of adpA, and AdpA positively regulates nikkomycin production by activating the cluster-situated regulatory gene sanG. In WT strain, binding of SABs to SabR1 causes the dissociation of SabR1 from cprC promoter and increases cprC transcription, which in turn activates adpA transcription to trigger nikkomycin biosynthesis. When sabA is disrupted or SABs are absent, binding of SabR1 to cprC would result in the repression of cprC and consequently cause the transcriptional reduction of adpA and nikkomycin biosynthetic genes.

In this study) of this family of signaling molecules from five Streptomyces species have been discovered to date, but their signal transduction system has been the subject of little investigation. In S. avermitilis, avenolide receptors AvaR1 and AvaR2 were verified, and AvaR2 directly repressed the transcription of aveR, a CSR activator gene for avermectin production (25–27). Recently, four compounds structurally resembling avenolide were found in Streptomyces albus strain J1074 with different avenolide-like activity (3). In S. rochei, SRB1 and SRB2 activated the production of lankacidin and lankamycin but showed a negative effect on morphological differentiation. Their receptor, SrrA, controls two SARP regulators, SrrY and SrrZ, but the exact molecular mechanisms of these regulators activating antibiotic production and morphological differentiation still remain obscure (28, 29). Moreover, four butenolide compounds were isolated from S. antibioticus DSM40725, but their activities on antibiotic biosynthesis have not yet been reported in detail (20). It is noteworthy that the regulation mediated by the above butenolides, avenolide or SRBs, is AdpA-independent. Interestingly, our work indicated that SabR1 does not target to the CSRs, but can regulate adpA via an activator CprC, and the established signaling system might be significant for elucidating complicated regulatory network in antibiotic biosynthesis.

CprC, a new activator of adpA discovered in this work, is a member of the TetR-family regulators. BLAST searches revealed that its homologs are widespread in Streptomyces, but only CprA and CprB have been studied. CprA and CprB are two ArpA-like proteins in S. coelicolor and possess regulatory func-
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Table 1

| Strains/plasmids | Relevant characteristics | Source/references |
|------------------|--------------------------|-------------------|
| **S. ansochromogenes** |
| 7100 WT strain | ref.16 |
| ΔsabA sabA disruption mutant | This work |
| ΔsabAc sabA complementary strain of ΔsabA | This work |
| ΔsabRΔsabR1 sabR1 deletion mutant | This work |
| ΔsabRΔsabR1c sabR1 deletion mutant | This work |
| ΔsabR2 sabR2 disruption mutant | This work |
| ΔsabR/sabR2 CprC overexpression strain of ΔsabR | This work |
| ΔcprC CprC complementary strain of ΔcprC | This work |
| ΔsabA/cprCoe CprC overexpression strain of ΔsabA | This work |
| ΔcprC/adpAoe adpA overexpression strain of ΔcprC | This work |
| **S. coelicolor** |
| M1146 act-, red-, cpk-, cda-, SCP1-, SCP2- | ref.33 |
| M6APD M1146 derivative carrying P_{sabAPD} fusion plasmid for SABs production | This work |
| P_{cpr} G M1146 derivative carrying P_{cpr}-gusA fusion plasmid | This work |
| P_{cpr}G-R1 M1146 derivative carrying P_{cpr}-gusA fusion plasmid and plasmid containing sabR1 | This work |
| P_{cpr}-G-ΔsabR1 M1146 derivative carrying P_{cpr}-gusA fusion plasmid and plasmid containing sabR1 with its own promoter | This work |
| P_{cpr}-ΔsabR1 M1146 derivative carrying P_{cpr}-gusA fusion plasmid and plasmid containing sabR1 | This work |
| **E. coli** |
| JM109 F', proA^B', lacI, Δ(lacZ)M15/Δ(lac-proAB), gyrA96, recA1, relA1, endA1, hsdR17 | Invitrogen |
| C41(DE3) F', ompT, gal dcm hsd S_{B}^{F-}(r_{B-}, m_{B-}) (DE3) | Lucigen |
| ET15267/pUZ8002 dam-- dcm-- hsdM-- pUZ8002 | ref.34 & 52 |
| C41 derivative containing PET22b-sabAPD for SABs production | This work |
| **Candida albicans** CGMCC2.4159 Indicator strain for nikkomycin bioactivity | CGMCC |

**Plasmids**

| Plasmids | Relevant characteristics | Source/references |
|-----------|--------------------------|-------------------|
| pSET152 | aac(3)IV, lacZ, rep^{mlll} attC31, oriT | ref.32 |
| pKC1139 | aac(3)IV, E. coli-Streptomyces shuttle plasmid contains a Streptomyces temperature-sensitive origin of replication | ref.35 |
| pJ10500 |权利*, a derivative of pMS2 containing dfr11 integrase gene | ref.32 |
| pET23b | Expression vector | Novagen |
| pGUS | Plasmid containing gusA | ref.36 |
| pKC1139 AD | pKC1139 derivative used for disruption of sabA | This work |
| pSET152:sabA | pSET152 carrying intact sabA with its putative promoter used for complement of sabA | This work |
| pET23b:sabAPD | pET23b containing intact sabAPD | This work |
| pKC1139-sabBAPD | pKC1139 carrying sabAPD and the promoter P_{sabB} | This work |
| pKC1139R1D | pKC1139 derivative used for disruption of sabR1 | This work |
| pSET152:sabR1 | pSET152 carrying intact sabR1 with its putative promoter used for complement of sabR1 | This work |
| pET23b:sabR1 | pET23b containing SabR1 coding region | This work |
| pKC1139R2D | pKC1139 derivative used for disruption of sabR2 | This work |
| pSET152:sabR2 | pSET152 carrying sabR2 and the promoter P_{sabB} | This work |
| pKC1139 RD | pKC1139 derivative used for disruption of cprC | This work |
| pSET152:cprC | pSET152 carrying intact cprC with its putative promoter used for complement of cprC | This work |
| pET23b:cprC | pET23b containing cprC coding region | This work |
| pJ10500:cmgusA | pJ10500 containing gusA and the promoter P_{cprC} | This work |
| pJ10500:cmgusA | pJ10500 containing gusA and the promoter P_{cprC} | This work |
| pSET152:sabBAPD | pSET152 carrying sabBAPD and the promoter P_{sabB} | This work |
| pET23b:sabBAPD | pET23b containing sabBAPD and the promoter P_{sabB} | This work |
| pJ10500:cmgusA | pJ10500 containing gusA and the promoter P_{cprC} | This work |
| pSET152:sabBAPD | pSET152 carrying sabBAPD and the promoter P_{sabB} | This work |
| M13 | Cloning vector | Stratagene |
| pPsab1 P_{sab1} was inserted into plasmid M13 | This work |
| pPsab2 P_{sab2} was inserted into plasmid M13 | This work |
| pPsab3 P_{sab3} was inserted into plasmid M13 | This work |
| pPsabA P_{sabA} was inserted into plasmid M13 | This work |
| pPsabA P_{sabA} was inserted into plasmid M13 | This work |
| pSET152:sabBAPD | pSET152 carrying sabBAPD and the promoter P_{sabB} | This work |

**Experimental procedures**

**Bacterial strains, plasmids, and growth conditions**

Bacteria strains and plasmids used in this study are listed in Table 1. *S. ansochromogenes* and its derivative strains were grown at 28 °C on MS medium agar for sporulation and in SP medium for nikkomycin production (11). *E. coli* strains were grown in lysogeny broth medium at 37 °C containing corresponding antibiotics (16).
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Construction of disruption and complementation mutants

Gene disruption mutants were constructed via homologous recombination. The plasmids for disruption, complementation, and overexpression were first constructed in E. coli JM109 with appropriate primers for individual genes as shown in Table S2 and then conjugally transferred into Streptomyces through ET12567/pUZ8002.

To inactivate sabA, the fragment corresponding to the upstream region of sabA was amplified using the primer pairs sabALF/R, and the resulting product was digested with EcoRI/XbaI. The DNA fragment corresponding to the downstream region of sabA was amplified using primer pair sabARF/R and followed by XbaI/HindIII digestion. The two fragments were then ligated into the EcoRI/HindIII-digested sites of pKC1139 to generate plasmid pKC1139::sabA. Primers sabACF/R were used to verify the mutant ΔsabA. To complement sabA, a 1.55-kb XbaI/BamHI-digested fragment containing the intact sabA and its own promoter region was inserted into pSET152 to give plasmid pSET152::sabA. Primers sabACF/R were used to confirm the complementary strain ΔsabAc. The construction of disruption strains of sabR1, sabR2, and cprC and the complementation strains of sabR1 and cprC was performed as mentioned above using appropriate primer pairs as shown in Table S2.

To overexpress cprC in ΔsabA, a 714-bp fragment containing the coding sequence of cprC was amplified using primers cprCoF/R, and the constitutive promoter P\textsubscript{hrd} of S. coelicolor was amplified using primer pair PhrdBF/R. After digestion with NdeI/EcoRI and XbaI/NdeI, respectively, the two fragments were inserted into the EcoRI/XbaI-digested sites of pKC1139AD. Primers PhrdBF and cprCoR were used to confirm the strain ΔsabAc/cprCoE. The overexpression strains of sabR2 in ΔsabA and adpA in ΔcprC were constructed as mentioned above using appropriate primer pairs as shown in Table S2.

Expression and purification of SabR1 and CprC

SabR1 coding region was amplified by PCR using primers SabR1F/R (Table S2). After digestion with Ndel and Xhol, the amplified fragments were inserted into the same sites of pET-23b to give plasmid pET23b::sabR1. The plasmid pET23b::cprC for expression and purification of CprC-His\textsubscript{6} was constructed as mentioned above with suitable primers as shown in Table S2. Plasmids pET23b::sabR1 and pET23b::cprC were transferred into E. coli C41 for expression of SabR1-His\textsubscript{6} and CprC-His\textsubscript{6}. Protein purification was performed with a nickel/nitritolriacetic acid–agarose column as described previously (30).

Construction of plasmids pET23b::sabAPD and pKC1139::hsabAPD

To construct the plasmids for heterologous expression of sabAPD, a 0.96-kb fragment containing sabA was amplified using the primer pairs sabAF/R, and a 1.56-kb fragment containing sabPD was amplified using the primer pairs sabDF/R. The two fragments were digested with Ndel/XbaI and Xbal/EcoRI, respectively, and then cloned into the Ndel/EcoRI-digested sites of pET23b to generate plasmid pET23b::sabAPD. It was transferred into E. coli C41 to generate CpAPD. A 2.5-kb fragment containing sabAPD digested with NdeI/EcoRI from pET23b::sabAPD and PhrdF of S. coelicolor digested with XbaI/NdeI were inserted into the EcoRI/XbaI-digested sites of pKC1139 to generate plasmid pKC1139::hsabAPD, which was transferred into S. coelicolor M1146 to generate MpAPD.

Purification and structural analyses of SABs

Seed broth (60 ml) of CpAPD grown in lysogeny broth medium at 37 °C for 12 h was inoculated into 3 liters of M9 medium. After incubation at 37 °C for 4 h, isopropyl-β-D-galactopyranoside was added at a final concentration of 0.1 mM and further incubated at 37 °C for 24 h, followed by another 12 h at 37 °C with an airflow rate of 3 liters/min and agitation speed of 200 rpm. The culture filtrate was extracted twice with equal volumes of ethyl acetate, and the organic phase was dried and redissolved in methanol. SAB1 was isolated from the extract by three rounds of HPLC separation (Zorbax, SB-C18, 9.4 × 250 mm, 5 μm) with a flow rate of 3 ml/min at 210-nm detection wavelength. The elution gradient was set as follows: 31% methanol for 15 min on first HPLC, followed by a linear gradient of 5–20% acetonitrile in 40-min elution on second HPLC, and finally 5–100% methanol in a 30-min linear gradient elution.

Seed broth (60 ml) of MpAPD grown in YEME medium at 28 °C for 48 h was inoculated into 3 liters of modified AlapMM medium (24) (supplemented with 10 g/liter mannitol and 3 g/liter casaminoacids, pH 6.0) with an airflow rate of 0.8 liter/min and agitation speed of 200 rpm. After incubation at 28 °C for 5 days, the culture filtrate was processes with the same procedure as that of CpAPD. To separate SAB1, -2, and -3, the elution profile was set as follows: 10–40% methanol for 30 min and then 40–60% methanol for 10 min. SAB1, -2, and -3 were eluted at 25.7–27 min, 24.2–25.7 min, and 34.7–36.2 min, respectively. The collected fractions of SAB1 were further purified with the elution profile of 14% acetonitrile in 20 min, SAB2 with a linear gradient of 14–16% acetonitrile in 20 min, and SAB3 with a linear gradient of 22–23% acetonitrile in 20 min.

Mass spectral analysis was performed on a triple quadrupole LC/MS/MS system (Agilent 1206/6460) in positive mode with an Agilent ZORBAX SB-C18 column (3.5 μm, 2 × 100 mm). 1H and 13C NMR spectra as well as 1H-1H COSY, 1H-13C HMBC, and 1H-13C HSQC were recorded on a 500-MHz Bruker spectrometer using CDCl\textsubscript{3} as solvent.

EMSAs and DNase I footprinting

The EMSAs and DNase I footprinting assays were performed as described (16, 31). All probes for EMSAs were amplified by PCR using the corresponding primer pairs listed in Table S2. For EMSAs with unlabeled probes, SabR1 was incubated with 20-ng probes in a 20-μl reaction mixture at 25 °C for 30 min, and then the samples were loaded on 4% (w/v) native polyacrylamide gels for electrophoresis. The gel was stained with SYBR Gold nucleic acid gel stain for 30 min and photographed under UV transillumination using Quantity One. EMSAs with fluorescently labeled probes were carried out as follows. To obtain the fluorescently labeled DNA, the promoter regions of target genes were individually inserted into the EcoRV site of plasmid M13. The resulting plas-
RNA isolation and real-time quantitative PCR

Total RNA was isolated from cultures of *S. ansochromogenes* and its derivative strains at various time points. RNA isolation, genomic DNA removal, reverse transcription, and qRT-PCR were performed as described previously (30). All of the primers used were listed in Table S2. 23S rRNA of *S. ansochromogenes* was used as internal control.

Bioassay and HPLC analysis of nikkomycin

The bioassay and HPLC of nikkomycin in culture filtrates of *S. ansochromogenes* and its derivative strains was carried out as described previously (12).

Bioassays of SABs

The activity of SABs in vivo was determined by the ability to restore nikkomycin production in ΔsabA. Different concentrations of SABs were added into 50-ml cultures of ΔsabA at the beginning of fermentation. After incubation for 5 days, nikkomycin production was detected by HPLC as described above.

**gusA transcriptional fusion assays**

To confirm the binding activity of Sbr1 to P<sub>cprC</sub>, two reporter plasmids containing P<sub>cprC</sub> or P<sub>cprCM-gusA</sub> fusions were constructed. P<sub>cprC</sub> was first amplified using the primers PGcprCF/PGcprCR. To obtain the template of P<sub>cprCM</sub> (mutant of P<sub>cprC</sub> at the binding motif), two fragments amplified by PCR using PcpCR/ PcpCRM and PcpCRM/PcppCR were digested with NotI and then ligated by T4-ligase. The resulting mixture was diluted 10<sup>3</sup> times and then used as the template of P<sub>cprCM</sub>. P<sub>cprCM</sub> was amplified using the primers PGcprCF/PcppCR. gusA was amplified from pGUS using primer pair gusA-F/R. Then the P<sub>cprC</sub> or P<sub>cprCM</sub> digested with SpeI/NdeI and gusA with NdeI/Xhol were cloned into the SpeI/Xhol-digested sites of pJ10500 to generate plasmid pJ10500:cgusA and pJ10500:cmgusA. Then the two constructs were respectively introduced and integrated into the FB1T1 a<sub>ttb</sub> site of *S. coelicolor* M1146. Subsequently, pSET152:ΔsabR1 and pSET152:ΔsabR1 were respectively introduced into the two *S. coelicolor* M1146 derivatives containing different reporter constructs and integrated into the F361 a<sub>ttb</sub> site. GusA activity was detected as described previously (32).

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