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

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Wenxi Wang[‡§1], **Jihui Zhang**[‡1,2], **Xiang Liu**[‡§], **Dong Li**[‡§], **Yue Li**[‡], **Yuqing Tian**[‡§], **and Huarong Tan**[‡§3]

From the [‡]*State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China and the* [§]*College of Life Sciences, University of Chinese Academy of Sciences, Beijing 100049, China*

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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 ansochromogenes* 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 *sabR1*. 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)[4] 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*-methylphenylalanyl-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

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

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[1] Both authors contributed equally to this work.

[2] To whom correspondence may be addressed: Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China. Tel.: 86-10-64807467; E-mail: zhang.jihui@im.ac.cn.

[3] To whom correspondence may be addressed: Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China. Tel.: 86-10-64807461; Fax: 86-10-64807461; E-mail: tanhr@im.ac.cn.

[4] 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.

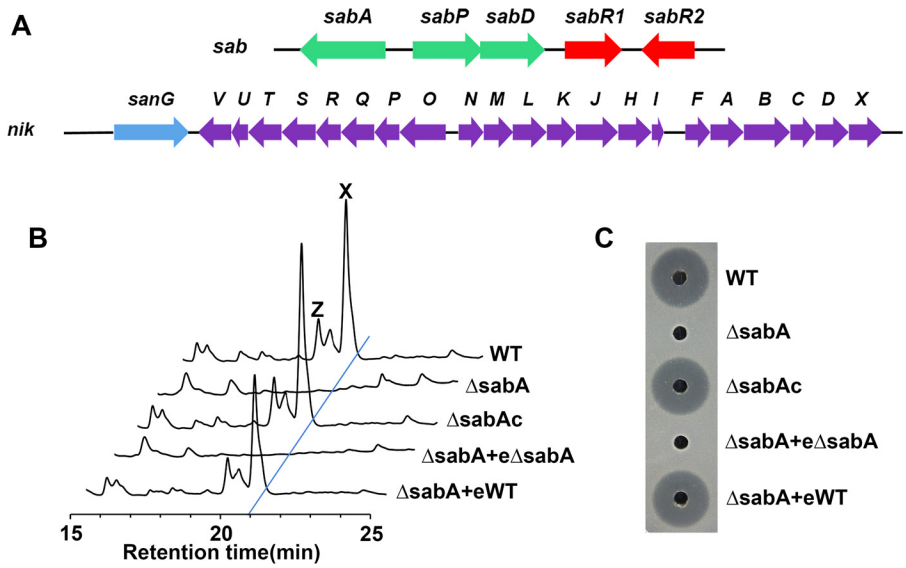

Figure 1. Effect of *sabA* disruption on nikkomycin production. *A*, genetic organization of *nik* gene cluster and *sab* gene cluster in *S. ansochromogenes* 7100. *B*, HPLC analysis of nikkomycin in different strains. *C*, nikkomycin bioassays against *C. albicans* with fermentation filtrates of different strains. *WT*, *S. ansochromogenes* 7100. Δ*sabA*, *sabA* disruption mutant. Δ*sabAc*, *sabA* complementary strain. *e*Δ*sabA*, 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 oviedomycin 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. 1*A*), 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

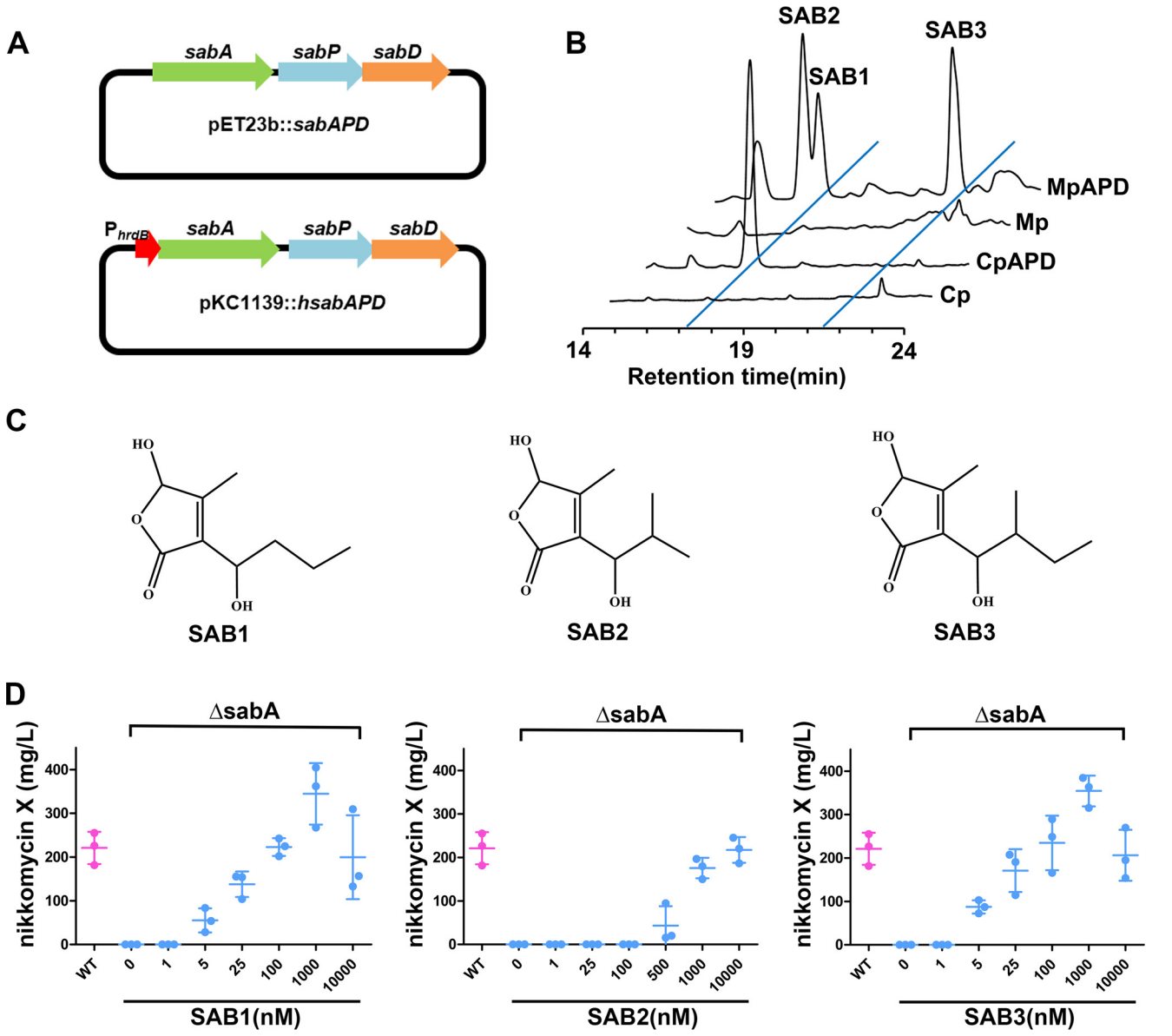

Figure 2. Characterization of structure and bioactivity of SABs. *A*, plasmids used for expression of *sabAPD* in *E. coli* and *Streptomyces*. *B*, HPLC analysis of SABs after expression of *sabAPD* in *S. coelicolor* M1146 and *E. coli* C41. *MpAPD*, *S. coelicolor* M1146 containing *sabAPD*. *Mp*, *S. coelicolor* M1146 containing pKC1139 as control. *CpAPD*, *E. coli* C41 containing *sabAPD*. *Cp*, *E. coli* C41 containing pET23b as control. *C*, structures of SABs. *D*, nikkomycin X production of ΔsabA with the addition of various concentrations of SAB1, SAB2, and SAB3.

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. S1*A*), and the molecular formula was deduced as $C_9H_{14}O_4$. 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 ^{1}H NMR, one methyl group signal (δ_H 0.79; δ_C 15.0) at C2′ in compound **3** was absent, and CH was changed to CH_2 (δ_H 1.8/1.6; δ_C 38.2) (Fig. S1 and Table S1). Further analyses of the ^{1}H-^{1}H 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. 2*C*), a novel member of butenolide autoregulators in *Streptomyces*.

HPLC analysis revealed that two other molecules, SAB2 and -3, were produced by MpAPD in addition to SAB1 (Fig. 2*B*). A

total of 20 mg of purified SAB2 and 10 mg 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 $C_9H_{14}O_4$ and $C_{10}H_{16}O_4$, respectively (Figs. S2 and S3). SAB2 shares the same molecular formula with SAB1, but there is a different retention time on HPLC (Fig. 2*B*), 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. antibioticus* DSM40725 (Figs. S2 and S3) (20). Thus, SAB2 was determined as 5-hydroxy-3-(1′-hydroxy-2′-methylpropyl)-4-methyl-2(5H)-furanone and SAB3 as 5-hydroxy-3-(1′-hydroxy-2′-methylbutyl)-4-methyl-2(5H)-furanone (Fig. 2*C*). 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 *S. ansochromogenes*, and SAB2 and SAB3 are dominant (Fig. S4).

To confirm that purified SABs are functional *in vivo*, SAB1, -2, or -3 was added into ΔsabA at concentrations ranging from 1 nM to 10 μM, and nikkomycin production was detected after incubation for 5 days. HPLC showed that nikkomycin production was elicited in ΔsabA with increasing concentration of SAB1, -2, or -3. SAB1 and SAB3 were effective at 5 nM, and nikkomycin production was fully restored to WT strain levels at 100 nM. In contrast, the minimum effective concentration of SAB2 required for eliciting nikkomycin biosynthesis was 500 nM (Fig. 2*D*), suggesting that the side chain of SABs is key in structure–activity relationships.

SABs and their receptor SabR1 coordinately regulate nikkomycin biosynthesis

Autoregulators usually perform their regulatory functions on secondary metabolism and morphological differentiation through specific receptor proteins. By multiple-sequence alignment and phylogeny evolution analysis, SabR1 appears to belong to GBL receptor family.

To verify this hypothesis, *sabR1* was disrupted in WT *S. ansochromogenes* to generate ΔsabR1 and also in SABs-defective mutant (ΔsabA) to generate ΔsabA/ΔsabR1. Nikkomycin production was not affected in ΔsabR1, where SABs are present. However, nikkomycin production was abolished in ΔsabA due to the absence of SABs, which could be reversed by further disruption of *sabR1* in ΔsabA. The complementation of *sabR1* in ΔsabA/ΔsabR1 (ΔsabA/ΔsabR1c) completely repressed nikkomycin biosynthesis (Fig. 3, *A* and *B*). Accordingly, qRT-PCR revealed that the transcription of key regulatory genes *adpA* and *sanG* for nikkomycin biosynthesis as well as the structural genes *sanF*, *sanN*, and *sanO* in *nik* cluster was reduced in ΔsabA, but restored in ΔsabA/ΔsabR1 (Fig. 3*C*). These experiments clearly demonstrated that the inhibition of nikkomycin production in ΔsabA resulted from SabR1-dependent repression of the transcription of nikkomycin biosynthetic genes. Similarly, in the absence of SABs, SabR1 repressed the transcription of *sabR1*, *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-His_6 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. 4*A*), 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. 4*B*). 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′-AAWAVAAACCRGDBRDTYSGTWY-3′) (Fig. 4*F*). 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* (P_{cprC}) contains a SabR1 conserved binding motif 5′-**AAAACAAA**CCGCATGTCCTGTTCTTTTG-3′ (Fig. 5*A*). EMSA experiments confirmed that P_{cprC} could be recognized by SabR1 to form a complex. When P_{cprC} was mutated to 5′-**GCGGCCGC**CCGCATGTCCTGTTCTTTTG-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. 5*D*). 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

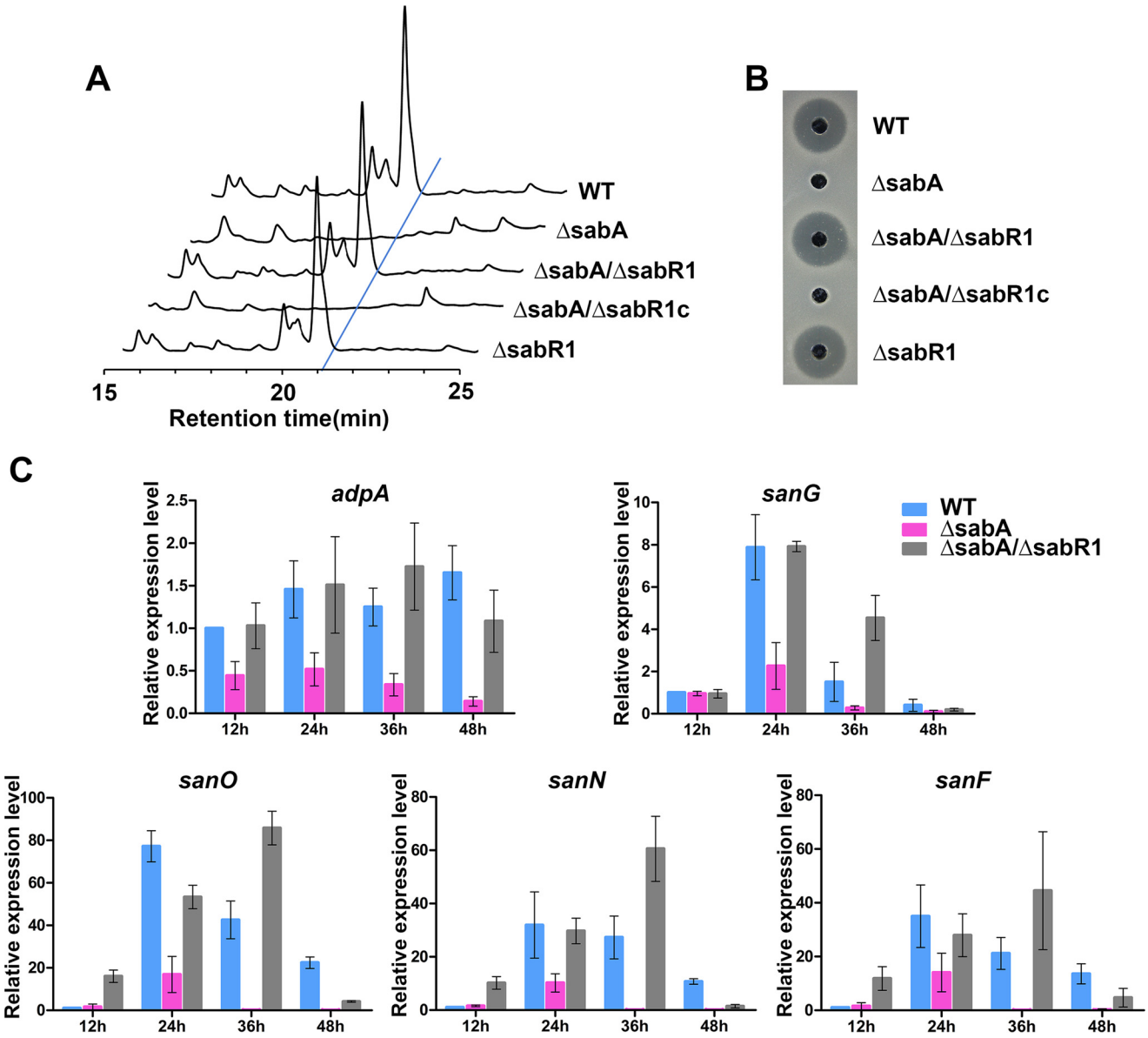

Figure 3. Effect of *sabR1* disruption on nikkomycin production. *A*, HPLC analysis of fermentation filtrates in different strains. *B*, nikkomycin bioassays against *C. albicans* with fermentation filtrates of different strains. *WT*, *S. ansochromogenes* 7100. Δ*sabA*, *sabA* disruption mutant. Δ*sabA*/Δ*sabR1*, disruption mutant of *sabA* and *sabR1*. Δ*sabA*/Δ*sabR1c*, *sabR1* complementary strain of ΔsabA/ΔsabR1. Δ*sabR1*, 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.

expression of *gusA* encoding a β-glucuronidase reporter system (Fig. 5*E*). 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 ΔcprC. The yield of nikkomycin in ΔcprC 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 under the control of *hrdB* promoter (P_{hrdB}), and the resulting strain (ΔsabA/cprCoe) efficiently restored nikkomycin production (Fig. 6). Thus, CprC was confirmed to be an activator for nikkomycin biosynthesis as the target of SabR1.

CprC directly activates the transcription of adpA

To understand how CprC activates nikkomycin biosynthesis, EMSAs were performed using purified CprC-His_6 and the promoters of potential target genes, *adpA*, *sanG*, and *sanF*. It was shown that CprC binds to the upstream region of *adpA* (Fig. 7*A*), 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′-CACCC-GGGGCCAGACACCGGTCGACTGACCTGTTTTC-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. 7*D*), confirming that CprC is an activator of *adpA* in *S. ansochromogenes*.

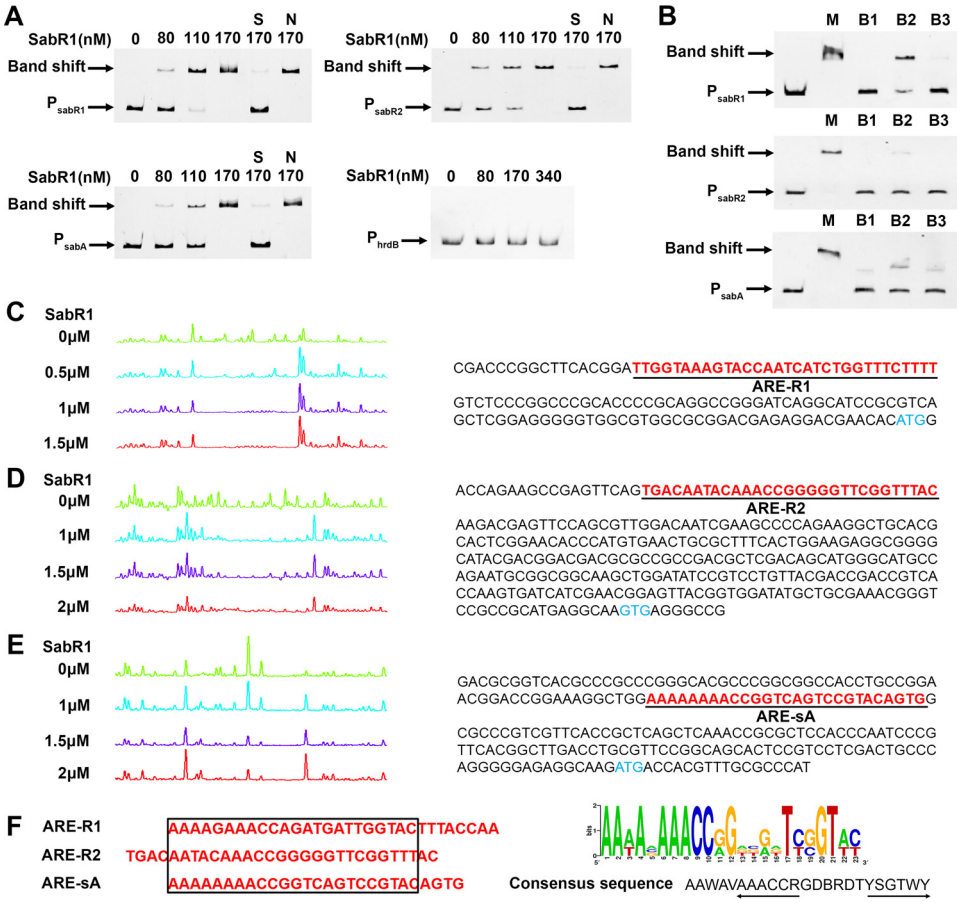

Figure 4. EMSAs and DNase I footprinting of SabR1 binding to the promoter regions of *sabR1*, *sabR2*, and *sabA*. *A*, EMSAs of different concentrations of SabR1 binding to the fluorescently labeled promoter regions of *sabR1* (P_{sabR1}), *sabR2* (P_{sabR2}) and *sabA* (P_{sabA}). Each *lane* contains 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 (110 nM) binding to unlabeled P_{sabR1}, P_{sabR2}, and P_{sabA} 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.

Remarkably, upon *adpA* overexpression under the control of P_{hrdB} in ΔcprC (ΔcprC/adpAoe), nikkomycin production was restored compared with ΔcprC (Fig. 7, *E* and *F*), demonstrating that CprC activation on *adpA* transcription is an important route in regulating nikkomycin biosynthesis.

Taken together, these findings enable us to propose a plausible regulatory model of the SABs-SabR1 signaling system in *S. ansochromogens*. *sabR1*, encoding a receptor of signaling molecule, is situated in a minicluster *sab*, in which *sabAPD* are signaling molecule biosynthetic genes. SabR1 can repress the transcription of *cprC*, which can be relieved by signaling molecules synthesized by SabAPD. CprC is newly identified as an activator of *adpA*, whereas AdpA positively regulates nikkomycin production by activating the cluster-situated regulatory gene *sanG*. In this complicated regulatory system, CprC and AdpA are key factors that transmit butenolide signals to secondary metabolism and thereby regulate nikkomycin biosynthesis (Fig. 8).

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-

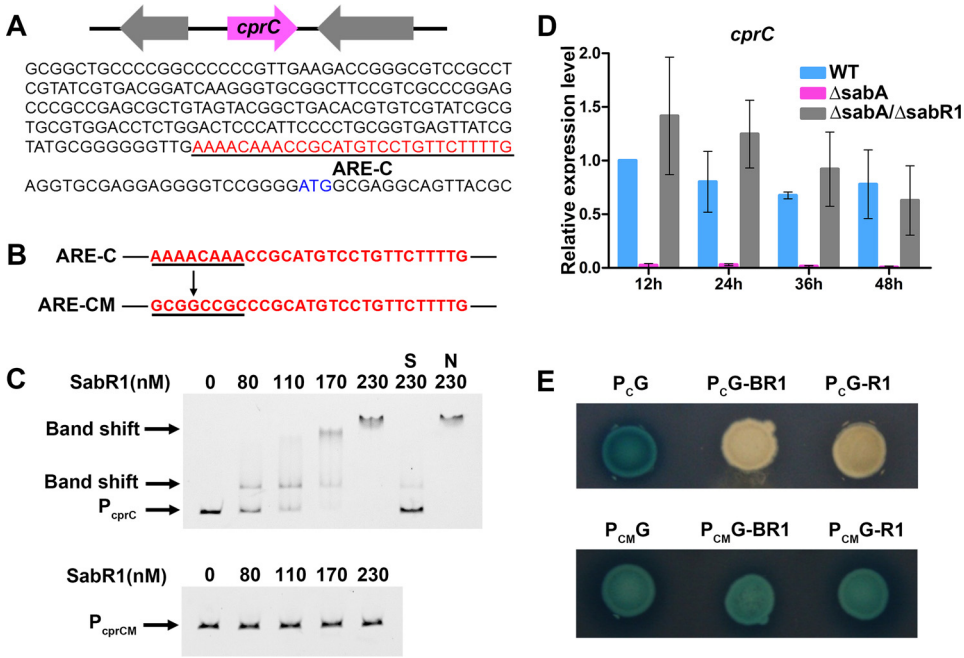

Figure 5. SabR1 repressed the transcription of *cprC* by directly binding to its promoter region. *A*, sequence of *cprC* promoter region (P_{cprC}). The *underlined red letters* are the predicted binding site of SabR1 on P_{cprC} (*ARE-C*). *B*, the *underlined* ARE-C site in P_{cprC} was mutated to 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*, unlabeled specific probe P_{cprC} (30-fold) was added; *N*, unlabeled nonspecific probe P_{hrdB} (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_CG and $P_{CM}G$, *S. coelicolor* M1146 containing P_{cprC} or P_{cprCM} fused with *gusA*. P_CG-*BR1* and $P_{CM}G$-*BR1*, strains P_CG and P_{CM}G containing P_{hrdB} fused with *sabR1*. P_CG-*R1* and $P_{CM}G$-*R1*, strains P_CG and P_{CM}G containing *sabR1* with its own promoter.

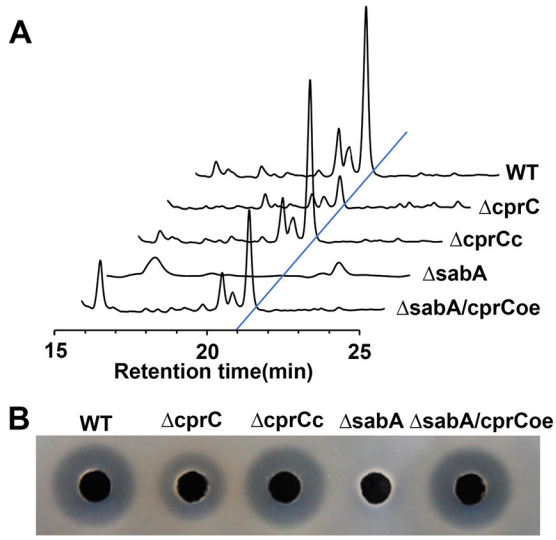

Figure 6. Effect of *cprC* disruption and overexpression on nikkomycin production. *A*, HPLC analysis of nikkomycin production in different strains. *B*, nikkomycin bioassays of fermentation filtrates from different strains. *WT*, *S. ansochromogenes* 7100. Δ*cprC*, disruption mutant of *cprC*. Δ*cprCc*, *cprC* complementary strain of ΔcprC. Δ*sabA*, *sabA* disruption mutant. Δ*sabA/cprCoe*, *cprC* overexpression strain in ΔsabA.

pic effect. 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* homologue *sabA* 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. Interestingly, genome mining revealed that the homologs of SabA are present in *Streptomyces lavenduligriseus*, *Streptomyces achromogenes*, *Streptomyces* sp. NRRL S-31, and so on, suggesting that SAB-like autoregulators may exist widely in *Streptomyces*.

Autoregulators usually function via binding to cognate receptor proteins, and their effects can be transmitted in cascade

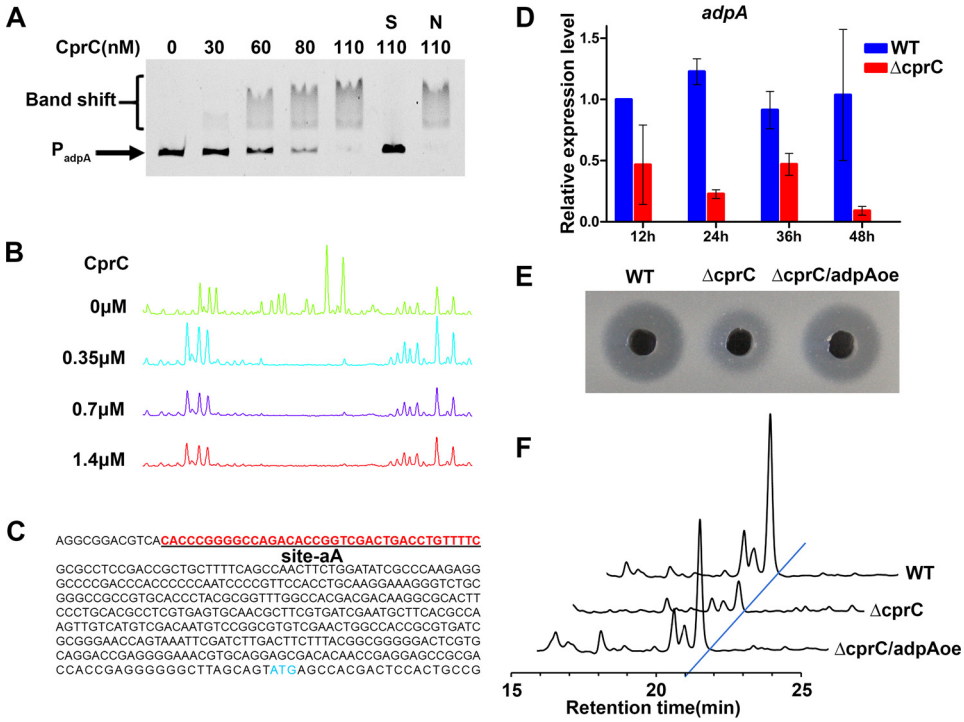

Figure 7. CprC activates the transcription of *adpA* by directly binding to its promoter region. *A*, EMSA of CprC binding to the fluorescently labeled promoter region of *adpA* (P_{adpA}). Each *lane* contains 50 ng of labeled probes and 1 μg of poly(dI-dC). *S*, unlabeled specific probe P_{adpA} (30-fold) was added; *N*, unlabeled nonspecific probe P_{hrdB} (30-fold) was added. *B*, DNase I footprinting of CprC-binding site on P_{adpA} (*site-aA*). *C*, the nucleotide sequence of P_{adpA}. *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. Δ*cprC*, disruption mutant of *cprC*. Δ*cprC/adpAoe*, *adpA* overexpressed in ΔcprC.

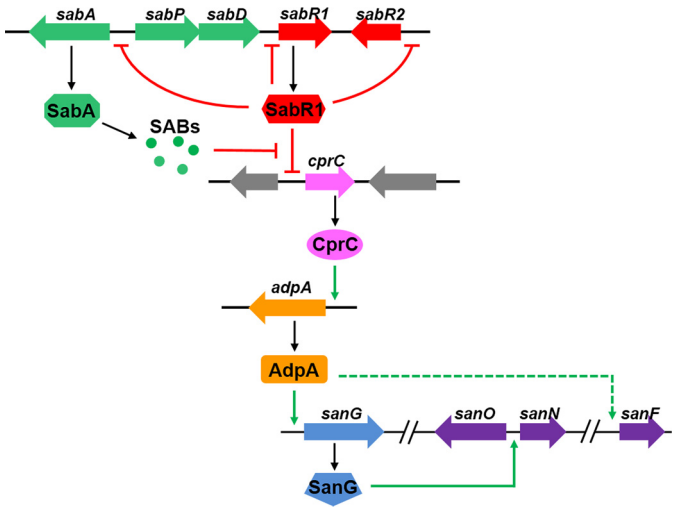

Figure 8. A plausible model for the roles of SabR1 and its ligands (SABs) in regulation of nikkomycin biosynthesis. Signal molecules SABs synthesized by *sab* exert regulatory functions via the cognate receptor SabR1. SabR1 can repress the transcription of *cprC* and other target genes (*sabR1*, *sabR2*, 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 *crpC* 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.

fashion to specific pathways by a series of regulators. The well-exemplified representative signaling system is GBLs. For butenolides, a total of 10 members (including SAB1, -2, and -3 identified 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-

Table 1
Bacterial strains and plasmids used in this study

Strains/plasmids	Relevant characteristics	Source/references
Strains		
S. ansochromogenes		
7100	WT strain	Ref. 16
ΔsabA	*sabA* disruption mutant	This work
ΔsabAc	*sabA* complementary strain of ΔsabA	This work
ΔsabR1	*sabR1* disruption mutant	This work
ΔsabA/ΔsabR1	*sabA* and *sabR1* disruption mutant	This work
ΔsabA/ΔsabR1c	*sabR1* complementary strain of ΔsabA/ΔsabR1	This work
ΔsabR2	*sabR2* disruption mutant	This work
ΔsabA/sabR2oe	*sabR2* overexpression strain of ΔsabA	This work
ΔcprC	*cprC* disruption mutant	This work
ΔcprCc	*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
MpAPD	M1146 derivative containing P_{hrdB}-*sabAPD* fusion plasmid for SABs production	This work
P_CG	M1146 derivative containing P_{cprC}-*gusA* fusion plasmid	This work
P_{CM}G	M1146 derivative containing P_{cprCM}-*gusA* fusion plasmid	This work
P_CG-R1	M1146 derivative containing P_{cprC}-*gusA* fusion plasmid and plasmid containing *sabR1* with its own promoter	This work
P_CG-BR1	M1146 derivative containing P_{cprC}-*gusA* fusion plasmid and P_{hrdB}-*sabR1* fusion plasmid	This work
P_{CM}G-R1	M1146 derivative containing P_{cprCM}-*gusA* fusion plasmid and plasmid containing *sabR1* with its own promoter	This work
P_{CM}G-BR1	M1146 derivative containing P_{cprCM}-*gusA* fusion plasmid and P_{hrdB}-*sabR1* fusion plasmid	This work
E. coli		
JM109	F′, *proA*$^+$*B*$^+$, *lacI*^{q}, Δ*(lacZ)M15/*Δ*(lac-proAB), gyrA96, recA1, relA1, endA1, hsdR17*	Invitrogen
C41(DE3)	F$^-$, *ompT, gal dcm hsd* S_B (r_B^-, m_B^-) (DE3)	Lucigen
ET12567/pUZ8002	*dam*− *dcm*− *hsdM*− pUZ8002	Refs. 34 and 52
CpAPD	C41 derivative containing pET23b::*sabAPD* for SABs production	This work
Candida albicans CGMCC2.4159	Indicator strain for nikkomycin bioactivity	CGMCC
Plasmids		
pSET152	*aac (3)IV, lacZ,* rep^{pMB1*}*attϕC31, oriT*	Ref. 32
pKC1139	*aac (3)IV, E. coli-Streptomyces* shuttle plasmid contains a *Streptomyces* temperature-sensitive origin of replication *eptomyces* temperature-sensitive origin of replication	Ref. 35
pIJ10500	Hyg^R, a derivative of pMS82 containing ϕBT1 integrase gene	Ref. 32
pET23b	Expression vector	Novagen
pGUS	Plasmid containing *gusA*	Ref. 36
pKC1139AD	pKC1139 derivative used for disruption of *sabA*	This work
pSET152::*sabA*	pSET152 containing intact *sabA* with its putative promoter used for complement of *sabA*	This work
pET23b::*sabAPD*	pET23b containing intact *sabAPD*	This work
pKC1139::*hsabAPD*	pKC1139 containing *sabAPD* and the promoter P_{hrdB}	This work
pKC1139R1D	pKC1139 derivative used for disruption of *sabR1*	This work
pSET152::*sabR1*	pSET152 containing 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::*hsabR2*	pSET152 containing *sabR2* and the promoter P_{hrdB}	This work
pKC1139CD	pKC1139 derivative used for disruption of *cprC*	This work
pSET152::*cprC*	pSET152 containing intact *cprC* with its putative promoter used for complement of *cprC*	This work
pSET152::*hcprC*	pSET152 containing intact *cprC* and P_{hrdB}	This work
pET23b::*cprC*	pET23b containing *cprC* coding region	This work
pIJ10500::*cgusA*	pIJ10500 containing *gusA* and the promoter P_{cprC}	This work
pIJ10500::*cmgusA*	pIJ10500 containing *gusA* and the promoter P_{cprCM}	This work
pSET152::*hsabR1*	pSET152 containing *sabR1* and P_{hrdB}	This work
M13	Cloning vector	Stratagene
pPsabR1	P_{sabR1} was inserted into plasmid M13	This work
pPsabR2	P_{sabR2} was inserted into plasmid M13	This work
pPsabA	P_{sabA} was inserted into plasmid M13	This work
pPadpA	P_{adpA} was inserted into plasmid M13	This work
pSET152::*hadpA*	pSET152 containing *adpA and* P_{hrdB}	This work

tions in both secondary metabolism and morphogenesis in *S. coelicolor* A3 (2). CprA stimulates actinorhodin and undecylprodigiosin biosyntheses and sporulation, whereas CprB represses actinorhodin biosynthesis and sporulation (22). However, their mechanism of action has not been revealed. Here we demonstrated that CprC directly binds to the *adpA* promoter region to activate its transcription, which may be also applicable for CprC homologs in other *Streptomyces* species. Interestingly, a signal binding domain at the C terminus of these proteins is present, but how they coordinate with small molecules to influence the expression of target genes is unclear. It will be of interest to verify the ligand of CprC and dissect the coordinated regulatory network in future studies.

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).

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 pKC1139AD. 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 complement 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_{hrdB} 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 pSET152 to generate pSET152::*hcprC*. Primers PhrdBF and cprCoR were used to confirm the strain ΔsabA/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 NdeI and XhoI, 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_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_6 and CprC-His_6. Protein purification was performed with a nickel-nitrilotriacetic 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 sabPDF/R. The two fragments were digested with NdeI/XbaI and XbaI/EcoRI, respectively, and then cloned into the NdeI/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 P_{hrdB} 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 1-thio-β-D-galactopyranoside was added at a final concentration of 0.1 mM and further incubated at 28 °C for 4 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 AlaMM 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 processed 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 1260/6460) in positive mode with an Agilent ZORBAX SB-C18 column (3.5 μm, 2.1 × 100 mm). ^{1}H and ^{13}C NMR spectra as well as ^{1}H-^{1}H COSY, ^{1}H-^{13}C HMBC, and ^{1}H-^{13}C HSQC were recorded on a 500-MHz Bruker spectrometer using $CDCl_3$ 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-

mids were used as templates for probe amplification using fluorescently labeled primers FAM-F/HEX-R. 50-ng labeled probes were incubated with SabR1 in a 20-μl reaction mixture containing 1 μg of poly(dI-dC) at 25 °C for 30 min. The fluorescently labeled DNA was detected by Tanon-5200 Multi. For competition assays, 1.5 μg of unlabeled specific probe or the nonspecific probe P_{hrdB} was added into the binding reaction mixture. The EMSAs of CprC binding to DNA probes were performed similarly as mentioned above with appropriate probes.

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 SabR1 to P_{cprC}, two reporter plasmids containing P_{cprC} or P_{cprCM}-*gusA* fusions were constructed. P_{cprC} was first amplified using the primers PGcprCF/PGcprCR. To obtain the template of P_{cprCM} (mutant of P_{cprC} at the binding motif), two fragments amplified by PCR using PcprCF/PcprCMR and PcprCMF/PcprCR were digested with NotI and then ligated by T4-ligase. The resulting mixture was diluted 10 times and then used as the template of P_{cprCM}. P_{cprCM} was amplified using the primers PGcprCF/PGcprCR. *gusA* was amplified from pGUS using primer pair gusA-F/R. Then the P_{cprC} or P_{cprCM} digested with SpeI/NdeI and *gusA* with NdeI/XhoI were cloned into the SpeI/XhoI-digested sites of pIJ10500 to generate plasmid pIJ10500::*cgusA* and pIJ10500::*cmgusA*. Then the two constructs were respectively introduced and integrated into the ΦBT1 *attB* site of *S. coelicolor* M1146. Subsequently, pSET152::*sabR1* and pSET152::*hsabR1* were respectively introduced into the two *S. coelicolor* M1146 derivatives containing different reporter constructs and integrated into the ΦC31 *attB* site. GusA activity was detected as described previously (32).

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