SsaA, a Member of a Novel Class of Transcriptional Regulators, Controls Sansanmycin Production in *Streptomyces* sp. Strain SS through a Feedback Mechanism

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Sansanmycins, produced by *Streptomyces* sp. strain SS (1, 2), are uridyl peptide antibiotics with activities against *Pseudomonas aeruginosa* and multidrug-resistant *Mycobacterium tuberculosis*. In this work, the biosynthetic gene cluster of sansanmycins, comprised of 25 open reading frames (ORFs) showing considerable amino acid sequence identity to those of the pacidamycin and napsamycin gene cluster, was identified. SsaA, the archetype of a novel class of transcriptional regulators, was characterized in the sansanmycin gene cluster, with an N-terminal fork head-associated (FHA) domain and a C-terminal LuxR-type helix-turn-helix (HTH) motif. The disruption of *ssaA* abolished sansanmycin production, as well as the expression of the structural genes for sansanmycin biosynthesis, indicating that SsaA is a pivotal activator for sansanmycin biosynthesis. SsaA was proved to directly bind several putative promoter regions of biosynthetic genes, and comparison of sequences of the binding sites allowed the identification of a consensus SsaA binding sequence, GTMCTGACAN2TGTCAGKAC. The DNA binding activity of SsaA was directly assessed in vitro. These results indicated that SsaA strictly controls the production of sansanmycins at the transcriptional level in a feedback regulatory mechanism by sensing the accumulation of the end products. As the first characterized regulator of uridyl peptide antibiotic biosynthesis, the understanding of this autoregulatory process involved in sansanmycin biosynthesis will likely provide an effective strategy for rational improvements in the yields of these uridyl peptide antibiotics.

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diversity of the pathway-specific regulators may reflect the variety of regulatory mechanism in the biosynthesis of different secondary metabolites. JadR1, an ARR, was recently characterized to control jadomycin biosynthetic gene expression in an autoregulatory feedback mechanism by binding of the end product, which is the first example of the autoregulation of antibiotic biosynthesis by an end product(s) (19). RedZ, for undecylprodigiosin (19), and Aur1P, for auricin (20), were found to be modulated in a similar way, implying that end product-mediated control of antibiotic pathway-specific ARRs is widespread. However, whether other classes of pathway-specific regulators adopt a similar mechanism remains to be elucidated.

In this work, we report the biosynthetic gene cluster of uridyl peptide antibiotic sansanmycins. The structure homology search of SsaA, the orthologue of PacA and NpsM, revealed that it contained an HTH DNA binding motif at its C terminus. We further provide genetic and biochemical evidence confirming that SsaA is a novel class of pathway-specific transcriptional activators of sansanmycin biosynthesis, and that the end products can bind to and change the activity of SsaA to autoregulate antibiotic production.

MATERIALS AND METHODS

Strains, media, and growth conditions. Streptomyces sp. SS (wild-type strain) and its derivatives were grown at 28°C on SS agar (21) for sporulation, on mannanol soya flour (MS) agar (22) for conjuration, in liquid fermentation medium (1) with addition of 0.1% tyrosine for sansanmycin production, and in liquid phage medium (23) for isolation of genomic DNA. Escherichia coli DH5α (24) was used as the host for cloning purposes. E. coli ET12567/pUZ8002 (25) was used to transfer DNA into Streptomyces from E. coli by conjuration. E. coli XL1-Blue MR (Stratagene, CA) was used as the host strain for the construction of the Streptomyces sp. SS genomic DNA library. E. coli BW25113/pPlT790 was used as the host for Red/ET-mediated recombination (26). E. coli BL21 (DE3) (Novagen, Madison, WI) was used as the host strain to express SsaA protein. They were grown at 37°C in Luria-Bertani medium (LB). For protein expression in E. coli, the strain was grown in liquid ZYM-5052 medium (27). P. aeruginosa 11, the test organism for the antibacterial biosynthesis of sansanmycins (2), was grown on F403 agar (21). When antibiotic selection of bacteria was needed, strains were incubated with 50 μg ml⁻¹ ampicillin (Am), 100 μg ml⁻¹ ampicillin (Ap), 50 μg ml⁻¹ kanamycin (Km), 25 μg ml⁻¹ chloramphenicol (Cm), and 50 μg ml⁻¹ streptomycin (Strep). Strains used and/or constructed in this study are listed in Table 1.

DNA manipulation. Routine DNA manipulations with E. coli were carried out as described by Sambrook and Russell (24). Recombinant DNA techniques in Streptomyces species were performed as described by Kieser et al. (22). The plasmids used and constructed in this study are listed in Table 1. The primers for PCR amplification are shown in Table S1 in the supplemental material.

Construction and screening of the genomic library. Chromosomal DNA isolated from Streptomyces sp. SS was partially digested with Sau3AI to give 40- to 50-kb DNA fragments. These fragments were dephosphorylated by calf intestinal alkaline phosphatase (TaKaRa, Tokyo, Japan) and then ligated into BamHI- and Hpal-digested pOJ446. The ligation products were packaged with Gigapack III XL Gold packaging extract (Stratagene) by following the manufacturer’s instructions, and the resulting recombinant phages were used to transfect E. coli XL1-Blue MR. Approximately 6,000 colonies were screened by colony hybridization with alkaline phosphatase-labeled saqA and saqF probes, which were amplified from Streptomyces sp. SS genomic DNA and labeled using a Gene Images AlkPhos Direct Labeling and Detection System kit (GE Healthcare, Sweden) according to the manufacturer’s instructions. Cosmid 13R-1 was

FIG 1 Structures of sansanmycins and some pacidamycins and napsamycins. m-Tyr, meta-Tyr; TIC, tetrahydro-3-isooquinoline carboxylic acid; MetSO, methionine sulfoxide.
isolated from a positive colony for subsequent complete sequencing using the shotgun method as performed by Majzoribio (Shanghai, China).

**Construction and complementation of Streptomyces sp. SS ssaA mutant.** Gene disruptions were carried out by a PCR targeting method (26). Detailed methods for construction of the *Streptomyces* sp. SS ssaA mutants are provided in the supplemental material. The resulting mutant strain was designated SS/AKO. A 747-bp DNA fragment containing the complete *ssaA* coding region was amplified using *ssaA*-pET16b-F and *ssaA*-pL-sscR (see Table S1 in the supplemental material) as primers and then cloned into the NdeI and BamHI sites of pL646 (28) under the control of a strong constitutive promoter, *ermE*′. The resulting expression vector, pL-ssaA, was introduced into SS/AKO by conjugation to give the complemented strain.

**Analysis of sansanmycin production.** Fermentation, isolation, and high-pressure liquid chromatography (HPLC) analysis of sansanmycins were performed mostly by following Xie et al. (1). Detailed methods are provided in the supplemental material.

**Gene expression analysis by quantitative RT-PCR.** Total RNAs were isolated from different strains after incubation in fermentation medium for 48 h using a PureYield RNA Midiprep system (Promega) according to the manufacturer’s instructions. Samples were treated with DNase I (Promega) and then authenticated by sequencing. Probes were generated by PCR using the primers listed in Table S1 in the supplemental material. All of these DNA fragments were cloned into pGEM-T Easy cloning kit (Promega) and then used for the construction of the genomic library. The resulting plasmids were then used for Red/ET-mediated recombination to give the complemented strain.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td>Streptomyces sp. SS</td>
<td>Wild-type strain (sansanmycin-producing strain)</td>
<td>1</td>
</tr>
<tr>
<td>SS/AKO</td>
<td>Streptomyces sp. SS with disruption of ssaA</td>
<td>This study</td>
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<tr>
<td>SS/AKO/pL-ssaA</td>
<td>SS/AKO with the expression vector pL-ssaA; Amr′</td>
<td>This study</td>
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<tr>
<td>SS/pL-ssaA</td>
<td>Streptomyces sp. SS with the expression vector pL-ssaA; Amr′</td>
<td>This study</td>
</tr>
<tr>
<td>Escherichia coli DH5α</td>
<td>General cloning host</td>
<td>24</td>
</tr>
<tr>
<td>ET12567/pUZ8002</td>
<td>Strain used for <em>E. coli/Streptomyces</em> conjugation</td>
<td>25</td>
</tr>
<tr>
<td>XL1-Blue MR</td>
<td>Host strain for genomic library construction</td>
<td>Stratagene</td>
</tr>
<tr>
<td>BW25113/pIJ790</td>
<td>Strain used for Red/ET-mediated recombination</td>
<td>26</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>Strain used for the expression of SsaA protein</td>
<td>Novagen</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa 11</td>
<td>Strain used for sansanmycin bioassays</td>
<td>2</td>
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**Plasmids**
- pOJ446 Cosmid vector used for the construction of genomic library | 46 |
- 13R-1 Cosmid selected for complete sequencing via shotgun approach | This study |
- 10R-1 Cosmid introduced into *E. coli* BW25113/pIJ790 for disruption of the minimal replicon of SCP2* and then ssaA | This study |
- 10R-1-SCP2KO Cosmid 10R-1 with the minimal replicon of SCP2* replaced by ampicillin resistance marker blac; Ap′, Amr′ | This study |
- 10R-1-SCP2KO-AKO 10R-1-SCP2KO with *ssaA* replaced by streptomyacin resistance gene addA; Ap′, Amr′, Strep′ | This study |
- pIJ779 Vector used as the template for amplifying *saaA* cassette | 26 |
- pSET152 *Streptomyces* integrative vector; Amr′ | 46 |
- pL646 pSET152 derivative containing *ermE*′; Ap′ | 28 |
- pL-ssaA pL646 derivative plasmid containing 747-bp complete coding region of *ssaA* | This study |
- pET-16b *E. coli* expression vector; Ap′ | Novagen |
- pET-16b-A pET-16b containing the coding region of *ssaA* | This study |

**Expression and purification of His<sub>10</sub>-tagged SsaA.** Expression and purification of His<sub>10</sub>-tagged SsaA were performed mostly by following Wang et al. (21), with detailed protocols provided in the supplemental material. Protein purity was determined by Coomassie brilliant blue staining after SDS-PAGE on a 12% polyacrylamide gel. The concentration of the purified SsaA was determined by the bicinchoninic acid (BCA) assay (Applygen, Beijing, China) using bovine serum albumin (BSA) as a standard.

**EMSA.** Electrophoretic mobility shift assays (EMSAs) were performed mostly by following Wang et al. (21), with detailed protocols provided in the supplemental material.

**DNase I footprinting.** DNA fragments used in DNase I footprinting were generated by PCR using the primers listed in Table S1 in the supplemental material. All of these DNA fragments were cloned into pGEM-T (Promega) and then authenticated by sequencing. Probes were generated by PCR with the universal T7 and SP6 promoter primers, with one of them being labeled with 5′-Alexa-647 (Invitrogen, CA). The PCR products were purified by agarose gel electrophoresis and quantified with a NanoDrop 2000 spectrophotometer (Thermo Scientific). His<sub>10</sub>-SsaA (2.67 μM) was incubated with labeled DNA fragment (50 ng) in a total volume of 90 μl at 25°C for 20 min. Following several optimization experiments, DNase I digestions were performed with 0.1 U DNase I (grade I; Roche, Mannheim, Germany) at 30°C for 60 s. The reactions were stopped by adding 10 μl of 500 mM EDTA (pH 8.0) and then heating at 75°C for 10 min. After phenol-chloroform extraction and ethanol precipitation, digested DNA was resuspended in 20 μl of sample loading solution (containing 0.3 μl of DNA size standard marker 600; Beckman Coulter, Brea, CA) and loaded in a GenomeLab GeXP genetic analysis system (Beckman Coulter). The results were analyzed with the GenomeLab eXpress Profiler program (Beckman Coulter).

**SPR analysis.** For SsaA-sansanmycin binding experiments, surface plasmon resonance (SPR) measurements were performed on a BIACore
Identification and analysis of the sansanmycin gene cluster (ssa). Sansanmycins share a structural scaffold with pacidamycins and napsamycins (Fig. 1); therefore, we initially attempted to locate the sansanmycin gene cluster from the producer, *Streptomyces* sp. SS, through genome mining by using pacidamycin biosynthetic genes as probes. Nucleotide sequencing of the *Streptomyces* sp. SS genome was undertaken by using an Illumina/Hiseq 2000 sequencing kit (32). The size of the *Streptomyces* sp. SS draft genome was predicted to be approximately 8.1 Mb, distributed over 69 scaffolds containing 87 contigs (GenBank accession no. AKXV000000000). For the local BLASTP, each open reading frame (ORF) of the pacidamycin gene cluster was used as a probe to query against the database consisting of all of the scaffolds. The bioinformatic search identified 14 ORFs (designated SsaH-N, SsaP-T, and SsaX-Y) on scaffold 7 and 10 ORFs (designated SsaA-G, SsaO, and SsaV) on scaffold 36 that are putatively responsible for sansanmycin biosynthesis (see Fig. S1 in the supplemental material). In order to obtain the complete *ssa* cluster, we subsequently constructed a cosmid library of genomic DNA from *Streptomyces* sp. SS. The alkaline phosphate-labeled fragments of coding sequence of *ssaQ* on scaffold 7 and that of *ssaF* on scaffold 36 were used as probes to screen the library. Of ~6,000 cosmids, 5 gave positive signals (see Fig. S1). Three cosmids were positive for both probes, and cosmid 13R-1 was selected for complete sequencing via the shotgun approach. Assembly of the sequences of scaffold 7, scaffold 36, and cosmid 13R-1 resulted in an approximately 440-kb contiguous sequence in which a 33.1-kb segment carrying 25 ORFs is assigned to the *ssa* cluster (Fig. 2). *ssaH* and *ssaW* are predicted to be the left and right boundaries of the *ssa* cluster, respectively, based on the BLAST analysis. The deduced function of the ORFs within the *ssa* cluster and five ORFs upstream or downstream of the *ssa* cluster is presented in Table S2. Interestingly, the upstream and coding sequence of *ssaW* is almost identical to that of *ssaU* (99% identity between bp 23677 and 25320 and bp 36823 and 38465 in the *ssa* cluster).

As determined by BLASTP analysis, the ORFs of the *ssa* cluster show high amino acid sequence similarity to those of the pacidamycin gene cluster (7, 8) and napsamycin gene cluster (9) and a gene cluster found in *S. roseosporus* NRRL 15998, a known dapto-mycin producer (7). The genetic organization of the *ssa* cluster resembles that of the napsamycin gene cluster and the similar cluster in *S. roseosporus* NRRL 15998, while in the pacidamycin gene cluster, *pacA-G* is located upstream of *paci-U*. The functions of most of the genes of the pacidamycin gene cluster have been well studied, especially those responsible for the peptide scaffold assembly, and a biosynthetic pathway has been proposed based on *in vivo* and *in vitro* experiments (7, 10). All of the defined pacidamycin biosynthetic genes, including 22 uninterrupted genes (*pacA-V*) and a digene cassette (*paciW* and *paciX*) identified elsewhere, have a homologue in the proposed *ssa* cluster, with only
one ORF (ssaY) showing homology to hypothetical genes with no known function in the biosynthesis of a uridyl peptide compound.

**SsaA is a putative pathway-specific transcriptional regulator.**

For the several ORFs with no information on the putative functions in sansanmycin biosynthesis, a structural homology search was performed using the online program HHpred (33). Similar to PacA (10), SsaA was predicted to contain an N-terminal fork head-associated (FHA) domain and a C-terminal helix-turn-helix (HTH) DNA binding domain (DBD) (Fig. 3A). The FHA domain of SsaA displays the 11-stranded β-sandwich seen in other eukaryotic or prokaryotic FHA domains (Fig. 3B), which mediates the protein-protein interaction with threonine-phosphorylated peptides in a sequence-specific manner (34, 35). The alignment of FHA domains of orthologues of SsaA and the other 4 closest FHA structural neighbors shows overall homology, but the well-known amino acid residues important for the binding of phosphopeptide are not conserved in SsaA orthologues. The DBD of SsaA displays the domain architecture of the tetrahelical version of the HTH, with α3 as the recognition helix (Fig. 3C). The closest DBD structural neighbors defined in HHpred were the LuxR family of transcription factors, whereas the orthologues of SsaA have longer coils between the helices.

To investigate the contribution of *ssaA* to the regulation of sansanmycin biosynthesis, the coding region of *ssaA* was cloned into a pSET152-derived expression plasmid, pL646 (28), under the control of a strong constitutive promoter, *ermE* (28), to give pL-ssaA. The plasmid was introduced into the wild-type strain by conjugation, and the resulting *ssaA* overexpression strain was designated SS/pL-ssaA. The antibacterial bioassay against *P. aeruginosa* showed that the overexpression of *ssaA* increased sansanmycin production in SS/pL-ssaA (Fig. 4A), which was further confirmed by HPLC analysis (Fig. 4B). The presence of an extra

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**FIG 3** Domain structure of SsaA and structure-based alignment of SsaA with its structurally homologous proteins. (A) Predicted domain structure of SsaA. (B) Structure-based alignment of the FHA domain of SsaA with its structurally homologous proteins. Three orthologues of SsaA, PacA (GenBank accession number ADN26237.1), NpsM (ADY76675.1), and SrosN15 (ZP_04694256.1), and the 4 closest FHA structural neighbors of SsaA, EmbR (PDB entry code 2FF4), yeast Rad53 FHA1 (1QU5), yeast Rad53 FHA2 (1QU5), and human Ki67 (1R21), were aligned. The known conserved amino acid residues important for the binding of phosphopeptide are shaded gray. Numbers inserted in the sequences count residues omitted from the nonconserved regions. (C) Structure-based alignment of the DNA binding domain (DBD) of SsaA with its structurally homologous proteins. Three orthologues of SsaA, PacA (GenBank accession number ADN26237.1), NpsM (ADY76675.1), and SrosN15 (ZP_04694256.1), and the 5 closest DBD structural neighbors of SsaA, TraR (PDB entry code 1L3L), QcsR (1P4W), CviR (3QP6), RcsB (3SZT), and ComA (3ULQ), were aligned.
copy of ssaA under ermE' p in the chromosome led to an approximately 100% increase in the production of sansanmycin A (SS-A) and sansanmycin H (SS-H) compared to that in the wild-type strain containing pSET152. The results suggested that ssaA is a positive regulator of sansanmycin biosynthesis.

Disruption of ssaA completely abolished sansanmycin production. To confirm the role of ssaA in sansanmycin biosynthesis, a part of the coding region of ssaA (547 bp) was replaced with a streptomycin-resistant gene, aadA, to create the ssaA-disrupted strain SS/AKO (see Fig. S2A in the supplemental material) according to standard methods (26). The disruption of the intended target was confirmed by PCR with primers priming about 100 bp outside the region affected by homologous recombination (see Fig. S2B) and further confirmed by Southern blotting using the streptomycin-resistant gene aadA as the probe (see Fig. S2C). Antibacterial activity results (Fig. 5A) and HPLC analysis (Fig. 5B) showed that disruption of ssaA totally blocked sansanmycin production. When pL-ssaA was introduced into the SS/AKO mutant, the complemented transconjugant was found to resume sansanmycin production (Fig. 5A) at an early stage and at a higher level than that of the wild-type strain, which might be ascribed to the use of the constitutive and strong promoter. These results demonstrated that the gene cluster identified is indeed responsible for
sansanmycin biosynthesis, and that ssaA is a pivotal positive regulator of sansanmycin biosynthesis.

**Disruption of ssaA decreased the transcription of biosynthetic genes.** To examine further the involvement of the ssaA gene in transcriptional regulation of the ssa cluster, gene expression analysis was conducted using quantitative RT-PCR. The relative levels of the transcripts of five structural genes, ssaH, ssaN, ssaP, ssaX, and ssaC, located on the possible cluster boundary or as one of the two divergent ORFs, were analyzed together with ssaA. Total RNAs from the wild-type, ssaA knockout mutant SS/AKO, and complemented SS/AKO/pL-ssaA strains were isolated under conditions in which the wild-type strain commenced sansanmycin production at about 48 h (Fig. 5A). As expected, the transcripts of ssaA were completely undetectable in the knockout mutant, while they were readily detectable in the wild-type strain and the complemented strain. The results showed that the transcripts of the structural genes tested were almost undetectable in the knockout strain. In addition, the transcripts in the complemented strain were restored to a higher level than that in the wild-type strain (Fig. 5C), consistent with the sansanmycin production at about 48 h (Fig. 5A). These results indicated that ssaA could positively regulate the sansanmycin biosynthesis by controlling the expression of the structural genes of the ssa cluster.

**SsaA bound to the promoter regions of the ssa cluster.** To be a positive regulator of sansanmycin biosynthesis, SsaA was speculated to promote sansanmycin production by binding at the promoter regions of biosynthetic genes and thereby activating their transcription. Therefore, a series of EMSAs were performed to verify whether SsaA indeed interacts with the promoter regions. SsaA was overexpressed in _E. coli_ BL21(DE3) as a His$_{10}$-tagged protein with a predicted molecular mass of 28,380 Da, and it was purified to a purity of >90% by nickel affinity chromatography (see Fig. S3A in the supplemental material). Twelve DNA fragments of intergenic regions containing possible promoters of interest were chosen as probes (Fig. 2), including ssaHp, ssaKp, ssaN-Pp, ssaQp, ssaX-Yp, ssaU-A-1p, ssaU-A-2p, ssaU-A-3p, ssaBp, ssaC-Dp, ssaVp, and ssaWp (the long intergenic region between the divergent ssaU and ssaA was separated into three overlapping fragments). The EMSA results showed that the purified His$_{10}$-tagged SsaA bound to the divergent intergenic region fragments ssaN-Pp, ssaU-A-1p, ssaU-A-2p, and ssaC-Dp and upstream region fragments of two designated boundary genes, ssaH and ssaW (Fig. 6), but it did not bind to the other six fragments detected (see Fig. S3B), suggesting that most of the sansanmycin biosynthetic genes were transcribed as an operon. The bindings were enhanced by increasing the amount of SsaA (up to 1.75 μM). Shifting of the probes was decreased when the unlabeled specific competitor DNA fragments were added in excess to binding reactions. Notably, only a single retarded band was observed for ssaHp, ssaU-A-1p, ssaU-A-2p, and ssaC-Dp probes with all concentrations of SsaA tested, whereas a second retarded band was observed for the ssaN-Pp probe with higher concentrations of SsaA (0.7, 1.4, and 1.75 μM), suggesting the presence of two binding sites for SsaA in this region. To restrict the binding regions to shorter segments, several subfragments were generated by PCR for ssaN-Pp, ssaC-Dp, ssaU-A-1p, and ssaU-A-2p. The results showed that two subfragments within ssaN-Pp, subfragment 3 within ssaC-Dp and subfragment B, C, and E within ssaU-Ap, were each shifted by SsaA (see Fig. S3C). Specific binding of SsaA to several promoter regions of the ssa cluster in _vitro_, together with the results of overexpression and disruption of ssaA, indicated that SsaA controlled sansanmycin biosynthesis by directly binding to the promoter regions of biosynthetic genes.

**Identification of SsaA binding sites in ssa promoter regions.** To identify the specific binding sites of SsaA in _ssA_ promoter regions, the promoter region fragments shown to be retarded in EMSAs (ssaHp, ssaC-Dp, ssaN-Pp, and ssaU-Ap) were studied by DNase I footprinting analysis. Footprinting experiments were...
performed on both the coding strand and the complementary strand of the DNA fragments (Fig. 7A; also see Fig. S4 in the supplemental material).

Footprinting analysis of the \(ssaH\) region revealed a 31-nucleotide protected sequence in the coding strand (positions \(11002\) to \(11035\) relative to the translation start site of \(ssaH\)). In the complementary strand, the protected sequence was 13 nucleotides long, at positions \(11002\) to \(11014\). These two protected regions overlapped by 12 nucleotides (see Fig. S4A in the supplemental material).

Results of the analysis of the \(ssaC-D\) region revealed that SsaA protected a region of 28 nucleotides, from bp \(238\) to \(211\) relative to the \(ssaD\) translation start site in the coding strand. In the complementary strand, SsaA protected a 21-bp sequence, located at nucleotide positions \(-228\) to \(-208\) relative to the \(ssaD\) translation start site (see Fig. S4B in the supplemental material).

In the case of the \(ssaU-A\) fragment, the footprint on the coding strand covered a region of 25 nucleotides, from bp \(-411\) to \(-387\) relative to the \(ssaU\) translation start site. A 16-nucleotide footprint of SsaA binding at the complementary strand was obtained, spanning from positions \(-385\) to \(-370\) relative to the \(ssaU\) translation start site. The two protected regions did not overlap at all (see Fig. S4C in the supplemental material).

Footprinting analysis with the \(ssaN-P\) region revealed two protected areas in each strand, in accordance with the appearance of two retarded bands in EMSA experiments. In the coding strand,
SsaA protected two regions, stretching from positions −320 to −295 and from −193 to −173 with respect to the ssaP translation start site. In the complementary strand, two protected regions, stretching from positions +286 to +271 and from +175 to +156 relative to the ssaP translation start site, were observed (Fig. 7A).

It is interesting that the SsaA footprints on the coding strand are not accompanied by totally equivalent footprints on the complementary strand for all four promoter regions detected, but by aligning the five sequences within the protected regions of both strands and the homologous sequence in ssaWp, an SsaA consensus binding sequence of GTMCTGACAN2TGTCAGKAC (where M represents A or C and K is G or T) was identified (Fig. 7B), which consisted of two 9-bp inverted repeats (IRs; indicated by underlining) separated by a 2-bp linker. A sequence logo (36) that depicts the binding sites is shown in Fig. 7B.

To validate the identified SsaA consensus binding site, we designed three duplexes, one containing the canonical binding site (GTCCTGACAGCTGTCAGGAC; D1), a second one in which CTGAC of one of the IRs was replaced by ATTCA (GTCATTCAAGCTGTCAGGAC; D2), and a third one with GTCAG of one of the IRs deleted (GTCCTGACAGCT-----GAC; D3) (underlining indicates the nucleotides that were mutated or deleted in D2 and D3). We examined the binding of SsaA to these three duplexes in EMSA experiments. The results showed that SsaA interacts with the D1 duplex (Fig. 7C). These results validated the identified SsaA consensus binding site and implied that the two IRs both are important for the binding activity of SsaA.

Sansanmycins modulate the DNA binding activity of SsaA by interacting with SsaA. To test whether the sansanmycin end products have an effect on the DNA binding activity of SsaA, EMSAs were performed using the promoter probes ssaC-Dp or ssaU-A-1p in the presence of different amounts of SS-A or SS-H (Fig. 8). The results showed that SS-A inhibited the band shifting of ssaC-Dp and ssaU-A-1p, which was caused by SsaA in a concen-
tion-dependent manner (Fig. 8A), while SS-H did it in a similar manner (Fig. 8B). These results were further confirmed by SPR analysis, as shown in Fig. 8C. A concentration-dependent inhibition of SsaA binding to the ssaC-Dp-3 fragment immobilized on an SA sensor chip was readily observed when the amount of SS-A was increased. All of these results suggested that sansanmycins could modulate the binding activity of SsaA for its target DNA.

To determine whether sansanmycins modulate the DNA binding activity of SsaA through direct interaction with SsaA, SPR experiments were conducted using a CM5 sensor chip. Over a concentration range of 3.125 to 200 |X|S-A, Ssa sensorgrams showed an increasing binding of SS-A to the immobilized SsaA (Fig. 8D). A similar result was obtained for the SPR analysis of the interaction of SS-H with immobilized SsaA (Fig. 8E). The best fits for both SS-A and SS-H were obtained with the two-stage reaction model, yielding a $K_d$ (equilibrium dissociation constant) value of 318.1 and 781.7 |X|S for SS-A and SS-H, respectively, suggesting that the binding of a first molecule allows, by inducing a conformational change, the binding of a second molecule.

**DISCUSSION**

The sansanmycin biosynthetic gene cluster was identified in *Streptomyces* sp. SS by a combination of genome mining and conventional probing and cosmid sequencing. Consistent with the high similarity of the structures of sansanmycins to those of pacidamycins and napsamycins, the three clusters share a vast majority of homologues (ssaA-V). The ssa and nps clusters present almost the same genetic organization, except for several unidentified small ORFs. They both contain the homologue of pacX responsible for the synthesis of m-Tyr in the pseudopeptide, which was identified elsewhere in a pacWX digene cassette in *S. coeruleorubidus* NRRL 18370 (10). However, the homologue of NpsU, responsible for the reduced uracil moiety in napsamycin (9), was not found in ssa and pac clusters. Interestingly, the ssa cluster contains an extra ORF (SSaW) at the right boundary of the cluster. SsaW is predicted to encode a freestanding A domain of NRPSs, showing 74% identity to PacU and 77% identity to PacW from *S. coeruleorubidus* NRRL 18370. PacU was demonstrated to specifically activate the N-terminal Ala found in pacamycin D/S (7), and PacW was found to activate the N-terminal m-Tyr found in pacamycin A/C/S/ST (10). However, SsaW matches almost perfectly with the amino acid sequence of SsaU (with only one alteration of Lys to Arg), suggesting that SsaW is the early duplicate of SsaU in the cluster and is evolutionarily viable for incorporation of a new kind of amino acid. Unlike the nps cluster, which has an ArsR-type regulator around the cluster boundary, there is no homologue to any kind of transcriptional regulator in the near upstream or downstream of the defined ssa cluster.

Our studies have identified a new class of transcriptional factor, SsaA, which functions as a pivotal activator of the sansanmycin production in *Streptomyces* sp. SS. This was established by targeted ssaA gene disruption and complementation together with transcript analysis. It was confirmed when the overexpression of SsaA in the wild-type strain and complementation of SsaA in the ssaA disruption strain under a strong promoter both significantly increased the sansanmycin production level. This indicates that the endogenous SsaA is not present in saturating amounts, and elevating the expression level of ssaA could be a practical strategy to improve uridyl peptide antibiotic production. To the best of our knowledge, SsaA is the first-described example of a regulator that combines an N-terminal fork head-associated (FHA) domain with a C-terminal helix-turn-helix (HTH) motif of the LuxR type for DNA binding. SsaA bears no significant homology to any characterized gene products in the NCBI database according to BLASTP searches. In addition to there being orthologues in reported uridyl peptide antibiotic clusters with >80% identity, there are some hypothetical proteins from actinomycetes, mainly streptomycetes, showing considerable similarity (>30% overall identity), suggesting that this class of regulators is generally involved in cellular physiology, including secondary metabolite biosynthesis in actinomycetes.

EMSA were used here to prove the direct binding of SsaA to certain promoters of the ssa genes. Binding of the SsaA protein was demonstrated at 6 out of 12 postulated intergenic fragments. More than one shift band was obtained with ssaN-Pp at increasing protein concentrations, indicating that more than one binding site is present in this region, a result that was confirmed by EMSA of subfragments of this region. The lack of binding to the upstream region of ssaKp, ssaQp, ssaBp, and ssaVp, which is in the same direction as its upstream gene and has a relatively long intergenic region, suggests that most, if not all, blocks of codirectional genes are controlled as operons. Thus, it appears plausible that transcription from most of the sansanmycin biosynthetic genes can be controlled by SsaA. This is a notable feature of some pathway-specific regulators of antibiotic biosynthesis, minimizing the number of promoters by extensive usage of operon control, as exemplified by control of streptomycin production by StrR in *S. griseus* (37).

DNase I footprinting experiments revealed protected areas of approximately 20 to 30 nucleotides in the target promoters. Typically, the protected region in the sense strand of the activated gene is accompanied by a slightly displaced protection in the complementary strand, suggesting one monomer of the protein binds one face of the DNA helix. A comparison of the protected sequences led to the identification of a conserved palindromic sequence spanning 20 nucleotides. The relevance of the IRs as the cis regulatory element of SsaA was demonstrated by mutation or deletion of the most conserved CTGAC sequence in the IRs. The dyad symmetry of the binding site of SsaA is in agreement with the binding sites of regulators of the LuxR type, such as LuxR (38) and TraR (39). The presence of highly conserved SsaA orthologues in all of the reported uridyl peptide antibiotic biosynthetic clusters suggests the relevance of this new class of transcriptional regulators in the regulation of their production. Interestingly, the other reported uridyl peptide antibiotic clusters also contain the highly conserved SsaA binding sites in the divergent intergenic regions or the upstream region of genes on the cluster boundary (see Table S3 in the supplemental material), proving the general applicability of the consensus binding site of these transcriptional regulators.

Gene expression analysis in the wild-type strain and SS/AKO mutant by quantitative RT-PCR revealed that the transcription of ssaX was dependent on SsaA. However, EMSA results showed that SsaA cannot bind to the intergenic region between ssaX and ssaY. Sequence search of this region and further upstream region to the end of ssaU did not find the conserved SsaA binding site either. One explanation for these results is that the transcription of ssaX is controlled by another hierarchical regulator, which would be activated by SsaA. Another possibility is that ssaX is cotranscribed with ssaU, which is located in the far upstream of ssaX. All of these hypotheses required further experimental tests.
In more detailed EMSA analyses, the major end products of sansanmycins were found to markedly inhibit the DNA binding activity of SsaA to the two promoter regions tested. This result, further confirmed by an SPR analysis, raised the possibility that the end products of sansanmycins modulate SsaA activity. However, sansanmycins overproduced in a pL-ssaA overexpression strain did not reduce the transcription of sansanmycin biosynthesis genes. It is possible that the elevated SsaA protein levels in the pL-ssaA-bearing strain are much higher than the concentration required for the titration of the sansanmycin end products. The direct interaction of SS-A and SS-H with SsaA was then demonstrated by SPR analysis, indicating that the DNA binding activity of SsaA could be directly modulated by the end products of sansanmycins. It is well known that the FHA domain is responsible for the interaction of phosphoprotein containing a phosphothreonine (pThr), which is involved in diverse biological pathways (34). The typical FHA domain comprises approximately 75 amino acids for pThr peptide recognition involving the highly conserved residues Arg, Ser, and Asn. However, SsaA resembles some FHA domains functioning as a non-pThr binding module (40), with the only invariant residue, Gly, following strand B3. As each uridy1 peptide antibiotic consists of a pseudopeptide, it seems reasonable to speculate that the end products can bind to the FHA domain to change the conformation of SsaA, which influence its DNA binding activity.

Low-molecular-weight compounds, including intermediates or end products of the respective biosynthetic pathways, were previously reported to influence antibiotic production and export. For example, in tylosin biosynthesis, glycosylated macrolides were proposed to exert a pronounced positive effect on polyketide metabolism in S. fradiae (41). Rhodomycin D, the first glycosylated intermediate in donurubicin biosynthetic pathways, can interact with the TetR-like pathway-specific regulator DnrO to relieve its self-repression, thereby positively enhancing end production (42). A feed-forward mechanism has been proposed by Tahlan et al. (43) in which an antibiologically inactive precursor relieves repression caused by the TetR-type regulator ActR, ensuring the expression of the efflux pump ActA to couple the antibiotic biosynthesis and export. A similar regulation of efflux of simocyclinone was reported by Le et al. (44). Here, we demonstrated that the novel class of FHA-LuxR-type regulator, SsaA, adopted a negative autoregulation mechanism for antibiotic biosynthesis in streptomycetes. The negative autoregulation by end products may be a widespread regulatory mechanism for antibiotic biosynthesis in streptomycetes.

In summary, we have identified and characterized a member of a novel class of transcriptional regulators with the FHA domain in the global regulation of the sansanmycin biosynthesis. Knowledge of this regulator may set the stage for an understanding of the genetic control of uridy1 peptide antibiotic biosynthesis and its regulation and provide an effective strategy to improve the yields of these antibiotics.

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