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Transcriptional Control by A-Factor of *strR*, the Pathway-Specific Transcriptional Activator for Streptomycin Biosynthesis in *Streptomyces griseus*

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A-factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone) triggers streptomycin production by inducing the transcription of *strR*, encoding the pathway-specific transcriptional activator, through signal transduction in the A-factor regulatory cascade in *Streptomyces griseus*. AdpA, one of the key transcriptional activators in the cascade, bound two upstream activation sites, approximately at nucleotide positions −270 and −50 with respect to the transcriptional start point of *strR*, as determined by gel mobility shift assays and DNase I footprinting. Transcriptional analysis of the *strR* promoter with mutated AdpA-binding sites showed that both sites were required for full transcriptional activation of *strR* by AdpA. Potassium permanganate footprinting showed that AdpA assisted RNA polymerase in forming an open complex at an appropriate position for transcriptional initiation of *strR*. Nine transcriptional units within the streptomycin biosynthesis gene cluster, including the *strR-aphD* operon, depended on StrR, indicating that StrR is the pathway-specific transcriptional activator for the whole gene cluster. Consistent with this, expression of *strR* under the control of a constitutively expressed promoter in an *adpA* null mutant caused the host to produce streptomycin.

The ability to produce a wide variety of secondary metabolites and a mycelial growth that develops into chains of spores are two aspects characteristic of the gram-positive, filamentous, soil-dwelling bacterial genus *Streptomyces*. A-factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone) is a representative of the γ-butyrolactone autoregulators that control secondary metabolism, morphological development, or both in actinomycetes, mainly including members of the genus *Streptomyces* (5, 21). It acts as a chemical signaling molecule, or a microbial hormone, for secondary metabolism and morphological differentiation at a concentration as low as 10^{-9} M in streptomycin-producing *Streptomyces griseus* (6). A-factor was originally discovered by Khokhlov et al. (14) in the 1960s as an autoregulatory factor that restored streptomycin production and spore formation in a mutant of *S. griseus*. In the 1980s, we confirmed the pioneer work of Khokhlov et al. and have studied A-factor regulation since then.

We have so far revealed the A-factor regulatory cascade as follows (5, 21). A-factor is gradually accumulated in a growth-dependent manner by the action of AfsA, probably condensing a glycerol derivative and a β-keto acid. When the concentration of A-factor reaches a critical level at or just before the decision point, it binds an A-factor-specific receptor, ArpA, which has bound and repressed the promoter of *adpA*, and dissociates ArpA from the promoter, thus leading to transcription and translation of *adpA*. *adpA* is a sole significant target of ArpA (11). The transcriptional activator AdpA then activates a variety of genes that are required for secondary metabolite formation and morphological differentiation (22). Members of the AdpA regulon so far identified are *strR*, the pathway-specific transcriptional activator for streptomycin biosynthesis (20, 24); an open reading frame encoding a probable pathway-specific regulator of a polyketide compound (29); *adsA*, encoding an extracytoplasmic function sigma factor of RNA polymerase essential for aerial mycelium formation (26); *ssgA*, encoding a small acidic protein essential for spore septum formation (27); *amfR*, encoding a regulatory protein essential for aerial mycelium formation (28); *sgmA*, encoding a metalloendopeptidase probably involved in apoptosis of substrate mycelium during aerial mycelium development (12); two trypsin genes (10); and three chymotrypsin genes (our unpublished data). Although the phenotype of streptomycin production was a start of the A-factor study by Khokhlov et al. and our group, no detailed study as to the molecular mechanism of *strR* regulation by AdpA has been accomplished.

We previously detected an A-factor-responsive protein that bound an upstream activation sequence (UAS) of *strR* (24) and later purified the protein and named it AdpA (20). AdpA bound the region approximately at nucleotide position −270, with respect to the transcriptional start point of *strR*, as determined by gel mobility shift assay (20, 24). In the present study, we determined the exact AdpA-binding site by DNase I footprinting in order to reveal how AdpA activates the transcription of *strR*. During these studies, we have identified an additional AdpA-binding site at nucleotide position −50 in front of the *strR* promoter and found that both AdpA-binding sites are necessary for full transcriptional activation of *strR* by AdpA. Concerning the regulation of the streptomycin biosynthesis genes by StrR, Retzlaff and Distler (23) showed strict dependence of *strB1* on StrR and predicted the dependence of some

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transcriptional units because of StrR binding to their upstream regions (see Fig. 5 for the organization of the streptomycin biosynthesis gene cluster). We therefore determined the dependence of nine transcriptional units within the streptomycin biosynthesis gene cluster on StrR and showed that all the streptomycin biosynthesis genes are under the control of StrR.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *S. griseus* IFO13350 was obtained from the Institute of Fermentation, Osaka (IFO), Japan. The *S. griseus* Δ*adpA* mutant was described previously (20). *Streptomyces* strains were grown in YMPD medium (20). YMPD agar contained 2% agar. Bennett agar medium (yeast extract, 0.1%; meat extract, 0.1%; NZ amine, 0.2%; agar, 2%; pH 7.2) was used for streptomycin production. R2YE medium (15) was used for the regeneration of protoplasts. Neomycin (5 μg/ml) was added when necessary. *Escherichia coli* JM109 and pUC19 for DNA manipulation were purchased from Takara Biochemicals. *E. coli* JM110 containing *dam* and *dcm* mutations was used for preparing nonmethylated *Streptomyces* DNA for gene disruption. Histidine-tagged AdpA (AdpA-H) was purified from *E. coli* BL21(DE3) harboring pET-*adpA* as described previously (26). Media and growth conditions for *E. coli* were described earlier (1). Ampicillin (50 μg/ml) and kanamycin (50 μg/ml) were used when necessary.

General recombinant DNA studies. Restriction enzymes, T4 DNA ligase, and other DNA-modifying enzymes were purchased from Takara Biochemicals. [γ-^{32}P]ATP (220 TBq/mmol) for end labeling at 5′ ends with T4 polynucleotide kinase was purchased from Amersham Pharmacia Biotech. Digoxigenin-labeled probes for Southern hybridization were prepared with the *Bca*Best DIG labeling kit (Takara). DNA was manipulated in *Streptomyces* (15) and in *E. coli* (1, 17) as described earlier. Nucleotide sequences were determined by the dideoxy chain termination method with a Thermo Sequenase fluorescence-labeled primer cycle sequencing kit (Amersham). Chromosomal DNA of *S. griseus* or an appropriate plasmid was used for a template in the PCR.

Cloning of a part of the streptomycin biosynthesis gene cluster. A 0.4-kb fragment (nucleotide positions −225 to +218 with respect to the transcriptional start point of *strR*) was amplified by PCR with primers pb-F and gs-R (Table 1) and labeled with digoxigenin. By standard DNA manipulation including colony hybridization using this DNA fragment as a probe, a 5.4-kb KpnI-PvuII fragment containing five complete genes (*strD*, *strR*, *aphD*, *strB1*, and *strF*) and two truncated genes (*strE* and *strG*) was cloned between the KpnI and HincII sites of pUC19.

Gel mobility shift assay. Purification of AdpA-H from *E. coli* BL21(DE3) and gel mobility shift assays were described previously (26). For gel mobility shift assays with AdpA-H and various regions in front of *strR*, a 700-bp DNA fragment containing the intervening region between *strR* and *strD* was amplified by PCR with primers gs-F and gs-R (Table 1). Six DNA fragments, *a* to *f* (see Fig. 1), were prepared by digestion of the amplified fragment with appropriate restriction enzymes and ^{32}P labeled with T4 polynucleotide kinase.

DNase I footprinting. The method of DNase I footprinting was described previously (26). For analysis of AdpA-binding sites, a ^{32}P-labeled fragment was prepared by PCR with a pair of ^{32}P-labeled (indicated with an asterisk) and unlabeled primers listed in Table 1: fAa-F and fAa-R* for the antisense strand of site A, fAs-F* and fAs-R for the sense strand of site A, fBa-F and fBa-R* for the antisense strand of site B, and fBs-F* and fBs-R for the sense strand of site B.

Alterations of the AdpA-binding sequences by PCR. Mutations were introduced by PCR into the AdpA-binding sites in front of *strR*. The TTTCGGTC sequence in the consensus AdpA-binding sequence at site A (see Fig. 3A) was changed to CCTCTAGA, containing an XbaI recognition sequence, as follows. A DNA fragment upstream of the mutation point was amplified by PCR with primers 5′-CGggatccATCCCGGCGGCACGTATGGCC-3′ (primer bs-F; positions −488 to −468; the lowercase letters indicate a BamHI site) and 5′-Ggaattc*<u>TCTAGA</u>*<u>GG</u>AGCGACCTCGAAATAGGAGGGC-3′ (positions −64 to −85; the lowercase and italic letters indicate EcoRI and XbaI sites, respectively, and the underlining indicates the nucleotides to be changed). Similarly, a DNA fragment downstream of the mutation point was amplified with 5′-CGggatcc*<u>TCTAGA</u>*ATGCGGACAGCTTTACTTGGCCG-3′ (positions −55 to −33; the lowercase and italic letters indicate BamHI and XbaI sites, respectively) and 5′-GgaattcTGCATGCCGTCGATGACGCGC-3′ (primer bs-R; positions +306 to +286; the lowercase letters indicate an EcoRI site). After the absence of PCR errors had been checked by nucleotide sequencing, the two amplified fragments were connected via the common XbaI site and placed between the BamHI and EcoRI sites of pUC19, resulting in pUC19-RmA. The ^{32}P-labeled probe containing the XbaI mutation at site A was prepared by PCR with primers fAa-F and fAs-R (Table 1) with pUC19-RmA as the template.

For introduction of a mutation into the two AdpA-binding sequences at site B, we first created an NdeI mutation (see Fig. 3A) in the same way as for the XbaI mutation at site A. The GCGCGAT sequence in the consensus AdpA-binding sequence at site B (see Fig. 3A) was changed to CATATGG, containing an NdeI recognition sequence, as follows. The upstream region was amplified by PCR with primers bs-F and 5′-Ggaattc*<u>CATATG</u>*CACGGCGGCTGATTCTCTTCGA-3′ (positions −267 to −288; the lowercase and italic letters indicate EcoRI and NdeI sites, respectively; the underlining indicates the nucleotides to be changed). The downstream region was amplified with primers 5′-CGggatcc*<u>CATATG</u>*<u>G</u>CCTGTGCATCCGTGTAAGGGGC-3′ (positions −259 to −238; the lowercase

TABLE 1. Primers used in this study

Gene and primer	Positions[a]	Sequence (5′ to 3′)
strR		
gs-F	−482 to −463	GCGGCACGTATGGCCTCCAG
gs-R	+218 to +199	CGACATCCTCGCCGGCACTG
fAa-F	−184 to −162	AATGAAATCGCTGACAGGCGGTG
fAa-R	+19 to −4	AGAGCAATGCTTTCGCACTTCGC
fAs-F	−127 to −107	GTTCCGGTCCTCTCCGCCCTG
fAs-R	+77 to +53	TGTTCCCTGAAATATGCTCCATTAC
fBa-F	−394 to −373	TGAGGCGGGTTCCTGTGCCGCC
fBa-R	−200 to −223	AACCGCAGTTTGATTGCCGAATAC
fBs-F	−344 to −324	TCCTCGCGTGGTCTTGGGCCG
fBs-R	−138 to −158	AGCCAGCGCCTGCGCCCGATC
pb-F	−225 to −202	ATGTATTCGGCAATCAAACTGCGG
pf-R	+68 to +44	AAATATGCTCCATTACACACCCTTC
hrdB		
hrdB-F	−121 to −98	TCGGCCCATTTCGTCACGTATGAG
hrdB-R	+193 to +170	TCGATGAGCGCCATCACAGACTCG
adpA		
adpA-F	−223 to −200	AGCCCCCGCATCCCTCCGCGGCGA
adpA-R	+54 to +31	ACTCGCGAAGCGCACAGGGAAGTG
strB1		
sB1-F	−198 to −176	AGCCCTGAACTCCTGAAGCACTG
sB1-R	+177 to +156	AAGGCTCGACTCGGCAGTGCTC
strD		
sD-F	−428 to −404	TGTTCCCTGAAATATGCTCCATTAC
sD-R	−8 to −28	TCCTCGCGTGGTCTTGGGCCG
strON		
sON-F	−252 to −231	ACGGTACGACCAACTGTCCTCG
sON-R	+158 to +138	AACAGGGCCTTCTCCACGGCG
stsB		
sB-F	−244 to −220	ATCCATGATTTCCCTCGATTTCGAG
sB-R	+148 to +138	TCGGCTCCCTGTTCGGCCAGC
stsC		
sC-F	−250 to −230	ACTGCCGGGCATCGCTGACTC
sC-R	+158 to +137	TACGAATCCCGGCCCTGATAGG
strV		
sV-F	−250 to −230	AAGCGCGAGGCTAGGTCGGTC
sV-R	+155 to +135	ACCACCGGGATGAGCGCCTGC
strU		
sU-F	−450 to −431	AAGCGGCCAACCCGGCCGTC
sU-R	−34 to −54	AATGCGACCCGTCGCAGCGGG

[a] The nucleotide positions for *strR*, *hrdB*, *adpA*, and *strB1* are given taking their transcriptional start points as +1. Those for *strD*, *stsB*, *stsC*, *strV*, and *strU* are given taking the first letters of their start codons as +1. The nucleotide positions for *strO* and *strN* are given taking the first letter of the start codon of *strO* as +1.

and italic letters indicate BamHI and NdeI sites, respectively) and bs-R. The two amplified fragments were connected via the common NdeI site and placed between the BamHI and EcoRI sites of pUC19, resulting in pUC19-RmB1. Next, we further introduced a PstI mutation (see Fig. 3A) into the other AdpA-binding sequence at site B. The AATCAGC sequence in the other AdpA-binding sequence at site B (see Fig. 3A) was changed to CCTGCAG, containing a PstI recognition sequence, as follows. The upstream region was amplified with bs-F and 5′-Ggaattc*CTGCAG*GCTCTTCGATAACTGTTCGGAAGAAC-3′ (positions −281 to −305; the lowercase and italic letters indicate EcoRI and PstI sites, respectively; the underlining indicates the nucleotides to be changed). The downstream region was amplified with 5′-CGggatcc*CTGCAG*CGCCGTGCATATGGCCTGTGC-3′ (positions −273 to −254; the lowercase and italic letters indicate BamHI and PstI sites, respectively; the mutated nucleotides are indicated by underlining) and bs-R using pUC19-RmB1 as the template. The two amplified fragments were connected via the common PstI site placed between the BamHI and EcoRI sites of pUC19, resulting in pUC19-RmB. Similarly, using pUC19-RmA as the template, pUC19-RmAB containing XbaI, NdeI, and PstI mutations was constructed. The ^{32}P-labeled probe containing the NdeI and PstI mutations at site B was prepared by PCR with primers fBa-F and fBs-R (Table 1) with pUC19-RmB as the template.

Construction of expression plasmids for *strR* and *aphD* with a mutated AdpA-binding site(s). The *strR* coding sequence, together with *aphD*, was amplified by PCR with primers 5′-GCtctaga*CATATG*GAGCATATTTCAGGGAACAGC-3′ (positions +56 to +79; the lowercase and italic letters indicate an XbaI and NdeI sites, respectively; the ATG in the NdeI site is the start codon of *strR*) and 5′-GgaattcATGCGTTCGACTGCGTGTGATCG-3′ (positions +2381 to +2359; the lowercase letters indicate an EcoRI site) and placed between the XbaI and EcoRI sites of pUC19. After the absence of PCR errors had been checked by nucleotide sequencing, the SphI-EcoRI fragment (positions +300 to +2381; an SphI site is present at position +305) was excised. This SphI-EcoRI fragment and the BamHI-SphI fragment from pUC19-RmA were placed between the BamHI and EcoRI sites of pUC19 by three-fragment ligation. The HindIII-EcoRI fragment containing the XbaI mutation at site A and the *strR-aphD* region was then excised from the pUC19 plasmid and placed between the HindIII and EcoRI sites of pKUM20 with its copy number of one to two per chromosome (28), resulting in pRmA (see Fig. 4C). pRmB containing the PstI and NdeI mutations at site B and pRmAB containing the mutations at both site A and site B were similarly constructed using the BamHI-SphI fragment from pUC19-RmB and pUC19-RmAB, respectively.

Disruption of *strR*. A 2.0-kb KpnI-SphI (position +305) fragment containing a 5′ portion of *strR* was cloned between the KpnI and SphI sites of pUC19 (plasmid pRU). A 2.6-kb AviII (position +942)-SacI fragment containing a 3′ portion of *strR* was cloned between the HincII and SacI sites of pUC19 (plasmid pRD). The SacI-SphI fragment excised from pRU by use of the restriction sites in the multicloning site, the SphI-EcoRI fragment from pRD, and a HindIII-SacI fragment carrying the kanamycin (neomycin) resistance gene from Tn*5* were placed between the EcoRI and HindIII sites by four-fragment ligation. This plasmid was introduced into *S. griseus* IFO13350, and neomycin (5 μg/ml)-resistant transformants containing the whole plasmid sequence as a result of a single crossover were selected. Neomycin-sensitive colonies as candidates of the Δ*strR* mutant were then isolated after a neomycin-resistant transformant had been cultured in the absence of neomycin. Correct *strR*-disrupted mutants were selected by Southern hybridization with the kanamycin (neomycin) resistance gene and a 0.4-kb fragment amplified by PCR with pb-F and gs-R (Table 1) as digoxigenin-labeled probes (data not shown).

S1 nuclease mapping. Total RNA was isolated with ISOGEN (Nippon Gene) from cells grown on cellophane on the surface of Bennett agar medium. The method of S1 nuclease mapping was described previously (13). Hybridization probes were prepared by PCR with a pair of ^{32}P-labeled and nonlabeled primers. Table 1 lists the forward (F) and reverse (R) primer sequences for preparing these probes: hrdB-F and hrdB-R* for *hrdB*; adpA-F and adpA-R* for *adpA*; fAa-F and gs-R* for *strR*; sB1-F and sB1-R* for *strB1*; sD-F and sD-R* for *strD*; sON-F and sON-R* for *strO*; sON-F* and sON-R for *strN*; sB-F and sB-R* for *stsB*; sC-F and sC-R* for *stsC*; sV-F and sV-R* for *strV*; and sU-F and sU-R* for *strU*. The primers with an asterisk were ^{32}P labeled at the 5′ end with T4 polynucleotide kinase before PCR.

For detection of the transcription of *strR* on plasmids pRW, pRmA, pRmB, and pRmAB, the probe with ^{32}P at the 5′ end which was deleted from the chromosome of the Δ*strR* mutant was prepared as follows. pRA containing the DNA fragment (positions +56 to +2381) between the XbaI and EcoRI sites of pUC19 was constructed, and the SphI-EcoRI fragment (positions +300 to +2381) was inserted into pUC19, generating pRA-SE. By using pRA-SE as the template, a DNA fragment containing a portion of the pUC19 sequence (230 bp) and a portion of the *strR* coding sequence (positions +300 to +470) was amplified by PCR with 5′-TACGCAAACCGCCTCTCCCCG-3′ (positions 225 to 205 upstream of the HindIII site of pUC19) and *5′-TCTCCAGGACACGGGTCGCCG-3′ (positions +470 to +450). It was used as the probe to detect the *strR* transcript from +300 to +470.

Potassium permanganate footprinting. The methods of potassium permanganate footprinting and RNA polymerase preparation were described previously (28). The ^{32}P-labeled DNA fragment containing the *strR* promoter and the two AdpA-binding sites was prepared by PCR with primers gs-F and pf-R* (Table 1).

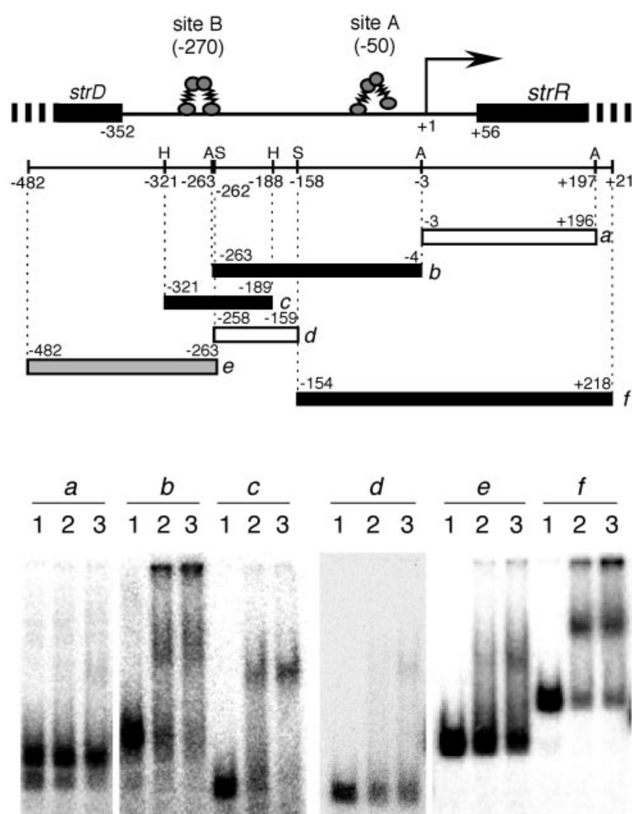

FIG. 1. Gel mobility shift assays for determination of AdpA-binding sites upstream of *strR*. Six different probes, *a* to *f*, were used. The restriction enzymes are abbreviated as follows: A, AccII; H, HaeIII; S, Sau3AI. The numbers below the restriction map indicate the positions, taking the transcriptional start point of *strR* as +1. The amounts of AdpA-H used in the gel mobility shift assays were 0 μg (lane 1), 0.1 μg (lane 2), and 0.4 μg (lane 3). Approximate AdpA-binding sites A and B are shown above the restriction map.

RESULTS AND DISCUSSION

Identification of two AdpA-binding sites in front of the *strR* promoter. Because AdpA binds multiple sites of some target genes (29), we performed gel mobility shift assays by using various probes covering a long region at and around the transcriptional start point of *strR* (Fig. 1). In addition, it was hard to predict the actual AdpA-binding sites in silico because the consensus AdpA-binding sequence is rather generous (29). For convenience, we used histidine-tagged AdpA with the structure AdpA-Leu-Glu-His$_6$. AdpA gave a strong shift signal for probe *b* (nucleotide positions −263 to −4) and probe *f* (positions −154 to +218), in addition to probe *c* (positions −321 to −189). Probe *c* contained the originally identified AdpA-binding site at position −270. No significant shift signal for probe *d*

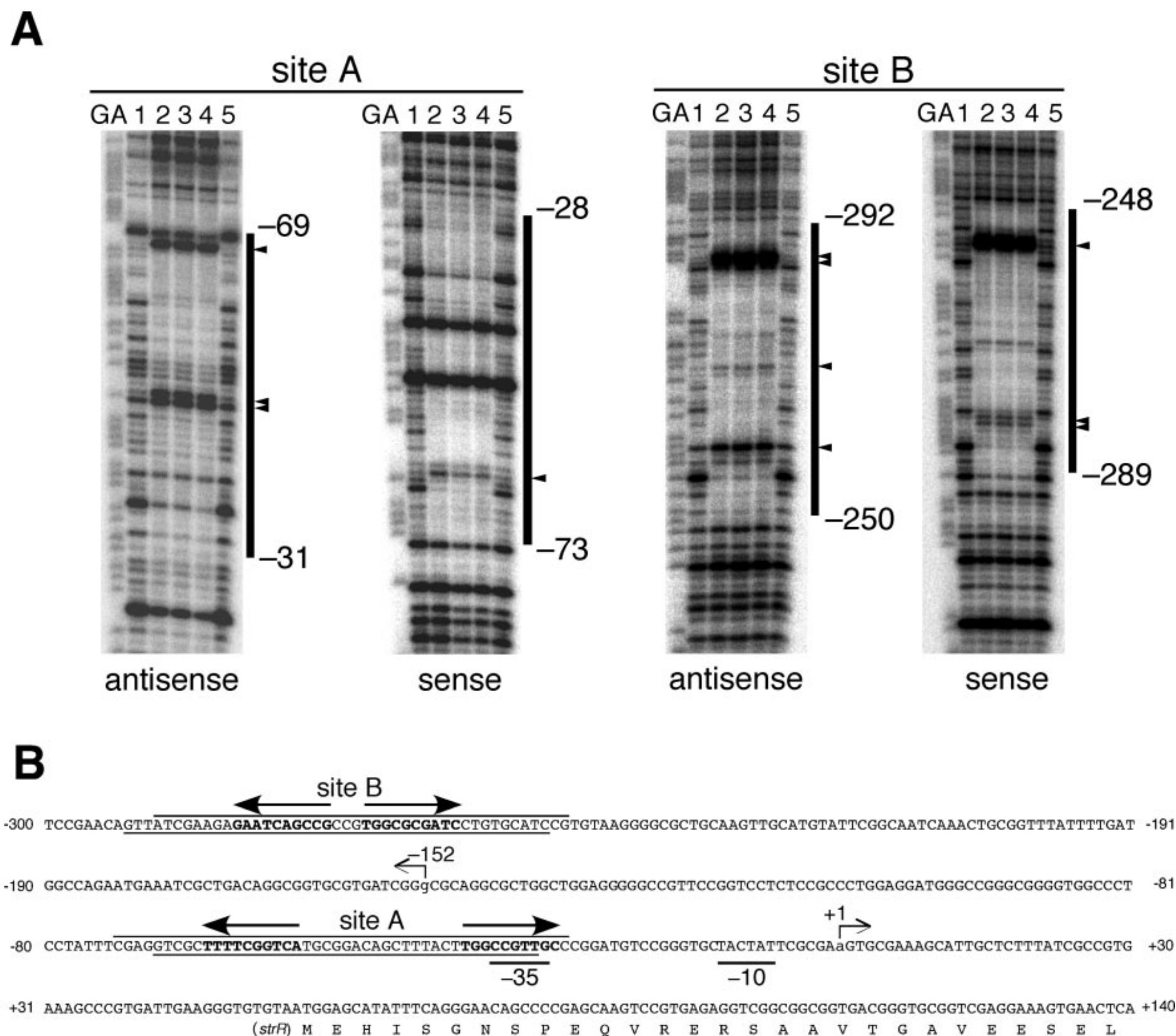

FIG. 2. DNase I footprinting for determination of the two AdpA-binding sites upstream of *strR*. (A) DNase I footprinting assays were performed on the antisense and sense strands of sites A and B. The amounts of AdpA-H used in lanes 1 to 5 were 0, 0.6, 1.2, 2.4, and 0 μg, respectively. The DNase I digests were run with the same probes that were chemically cleaved (GA lanes). The nucleotide sequences protected from DNase I digestion, together with the nucleotide positions of their boundaries, are indicated. The nucleotides that are hypersensitive to DNase I are indicated by triangles. (B) Nucleotide sequence of the *strR* promoter and AdpA-binding sites. The arrow at position +1 indicates the transcriptional start point of *strR*. The arrow at position −152 indicates the transcriptional start point of *strD*. The DNA sequences protected from DNase I digestion are also shown, together with the AdpA-binding sequences (in boldface letters and with arrows) at sites A and B. The arrows indicate the AdpA-binding sequences (5′-TGGCSNGWWY-3′) that go from the 5′ to the 3′ side.

(positions −258 to −159) and probe *a* (positions −3 to +196) was observed, which indicated that AdpA bound an additional site located between positions −158 and −4. Probe *e* (positions −482 to −263) gave a faint signal, probably because the right end of this probe lacked a portion of the AdpA-binding site at the originally identified site at −270 in probe *c*. The originally detected site was named site B, and the newly detected site was named site A.

DNase I footprinting analysis of the two AdpA-binding sites. The two AdpA-binding sites upstream of *strR* were determined by DNase I footprinting with ^{32}P-labeled probes (Fig. 2). AdpA-binding site A was protected from DNase I digestion at nucleotide positions −31 to −69 of the antisense strand and positions −73 to −28 of the sense strand (Fig. 2A). Several positions on the antisense strand at which enhanced DNase I digestion was observed, −46, −47, and −68, were probably exposed on the surface of the AdpA-DNA complex. On the sense strand, position −67 was also sensitive to DNase I. AdpA-binding site B was determined to be at positions −250 to −292 of the antisense strand and positions −289 to −248 of the sense strand. Positions −257, −268, −285, and −286 on the antisense strand and −255, −283, and −284 on the sense strand were hypersensitive to DNase I.

The consensus AdpA-binding sequence is 5′-TGGCSNGW WY-3′ (S is G or C, W is A or T, Y is T or C, and N is any nucleotide) (29). Site A contains two consensus AdpA-binding sequences, 5′-TGACCGAAAA-3′ and 5′-TGGCCGTTGC-3′, as a divergent repeat with a space of 16 bp (Fig. 2B). The

AdpA-binding sites of this type are called type I (29). The AdpA-binding site for *adsA* (26), site 1 and site 3 of *ssgA* (27), and site 1 of *amfR* (28) are examples. According to our speculative model (29), a dimer of AdpA supposedly binds type I sites by anchoring one AdpA-binding sequence with the two helix-turn-helix DNA-binding motifs in one subunit and the other AdpA-binding sequence with the other DNA-binding motifs in the other subunit. Because of a very flexible linker between the N-terminal dimerization domain and the C-terminal DNA-binding domain, AdpA binds type I sites with various spaces, with its optimum spaces of 13 to 14 bp and 2 bp (29). The manner of AdpA binding to site A will be discussed below because the AdpA-binding sequence near the transcriptional start point completely overlaps the −35 element of the *strR* promoter.

Site B contains two AdpA-binding sequences, 5′-CGGCTGATTC-3′ and 5′-TGGCGCGATC-3′, as a divergent repeat with a space of 3 bp. AdpA binds sites of type I with optimal spaces of 13 to 14 bp and 2 bp. Since the AdpA-binding site of type I with a space of 2 bp has been named type I′, as is found for site 1 for *ssgA* (29), we can also group site B in type I′ and predict the manner of AdpA binding to this site, as shown in Fig. 1.

Alterations of the two AdpA-binding sequences. We generated nucleotide changes at sites A and B to determine the importance of the AdpA-binding sequences at these sites in AdpA binding. An XbaI site was created in one of the AdpA-binding sequences at site A so that the number of nucleotides was unchanged (Fig. 3A). Gel mobility shift assays with the ^{32}P-labeled probe containing the XbaI mutation at site A showed that AdpA-H no longer bound this mutated site A (Fig. 3B).

For introduction of a mutation into the two AdpA-binding sequences at site B, we first created an NdeI mutation (Fig. 3A) in one of the AdpA-binding sequences at site B. Because site B containing only the NdeI mutation still showed significant affinity for AdpA (data not shown), a PstI mutation (Fig. 3A) was further introduced into the other AdpA-binding sequence at site B. The ^{32}P-labeled probe containing both the NdeI and PstI mutations showed very weak affinity for AdpA-H (Fig. 3B).

Disruption of one of the two AdpA-binding sequences at site A abolished the affinity for AdpA, whereas disruption of both AdpA-binding sequences at site B was required for abolishment of the affinity. We assume that two AdpA-binding sequences are required, in principle, for AdpA to bind type I and type I′ sites. However, it is apparent that one of the two AdpA-binding sequences in some type I and type I′ sites has enough affinity for AdpA to bind, like a single AdpA-binding sequence in type II sites (29).

Importance of the two AdpA-binding sites for transcriptional activation of *strR*. We constructed an *strR* null mutant (the Δ*strR* mutant) by double crossover to use this mutant as a host for determining the importance of the two AdpA-binding sites in the transcriptional activation of *strR* (Fig. 4A). The Δ*strR* mutant constructed in this way contained an in-frame deletion from His-84 to Val-295 of StrR (nucleotide positions +305 to +940). As expected, the Δ*strR* mutant grew normally and formed spores in the same time course as the wild-type strain. We constructed *strR* expression plasmids containing the

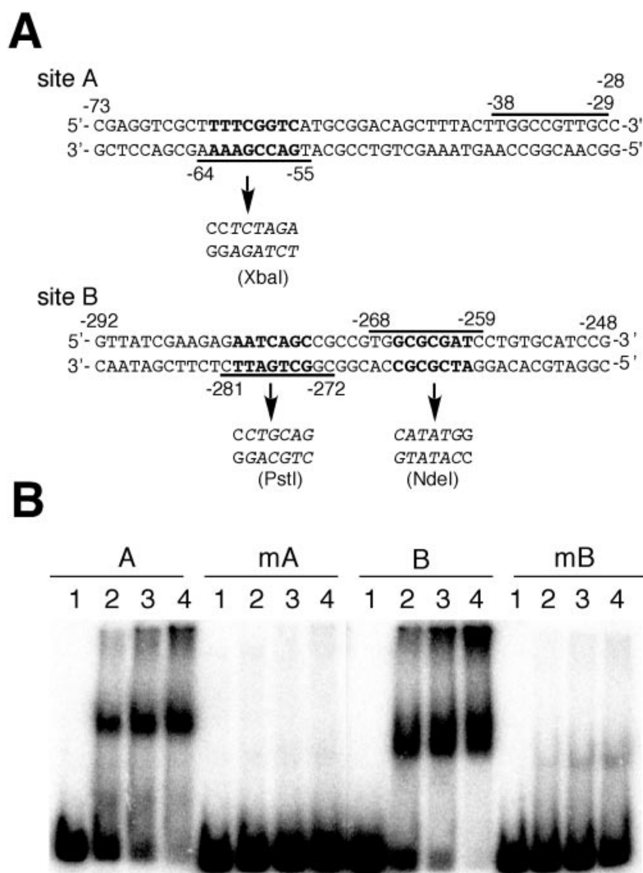

FIG. 3. Mutational analysis of the two AdpA-binding sites. (A) Mutations introduced in the AdpA-binding sites. An XbaI site generated at site A and a PstI and an NdeI site generated at site B are shown by italic letters. The nucleotides changed are indicated by boldface letters. The AdpA-binding sequences, positions −64 to −55 and positions −38 to −29 at site A and positions −281 to −272 and positions −268 to −259 at site B, are shown. (B) Gel mobility shift assays for determination of AdpA binding to the mutated sites. Probe A (positions −184 to +77) contained the intact AdpA-binding site, and probe mA contained mutated site A. Probe B (positions −394 to −138) contained the intact AdpA-binding sites, and probe mB contained mutated site B. The amounts of AdpA-H used in lanes 1 to 4 were 0, 0.06, 0.12, and 0.24 μg.

mutations at site A and/or site B to determine whether one or both of the AdpA-binding sites are required for transcriptional activation of the *strR* promoter (Fig. 4C). *aphD*, encoding a streptomycin resistance determinant (streptomycin-6-phosphotransferase), was included in this construction because *aphD* is transcribed mainly by readthrough from the *strR* promoter (25). The DNA fragment containing the *strR-aphD* region together with the upstream region was cloned into pKUM20 with its copy number of one to two per chromosome (28), resulting in pR series plasmids. pRmA had an XbaI mutation at site A. pRmB had NdeI and PstI mutations at site B. pRmAB had all mutations at both sites. pRW was a control plasmid with no mutations. These four plasmids were introduced by transformation into the Δ*strR* mutant.

(i) Effects of the mutations at sites A and B on in vivo transcription. We first examined the transcription of *strR* in

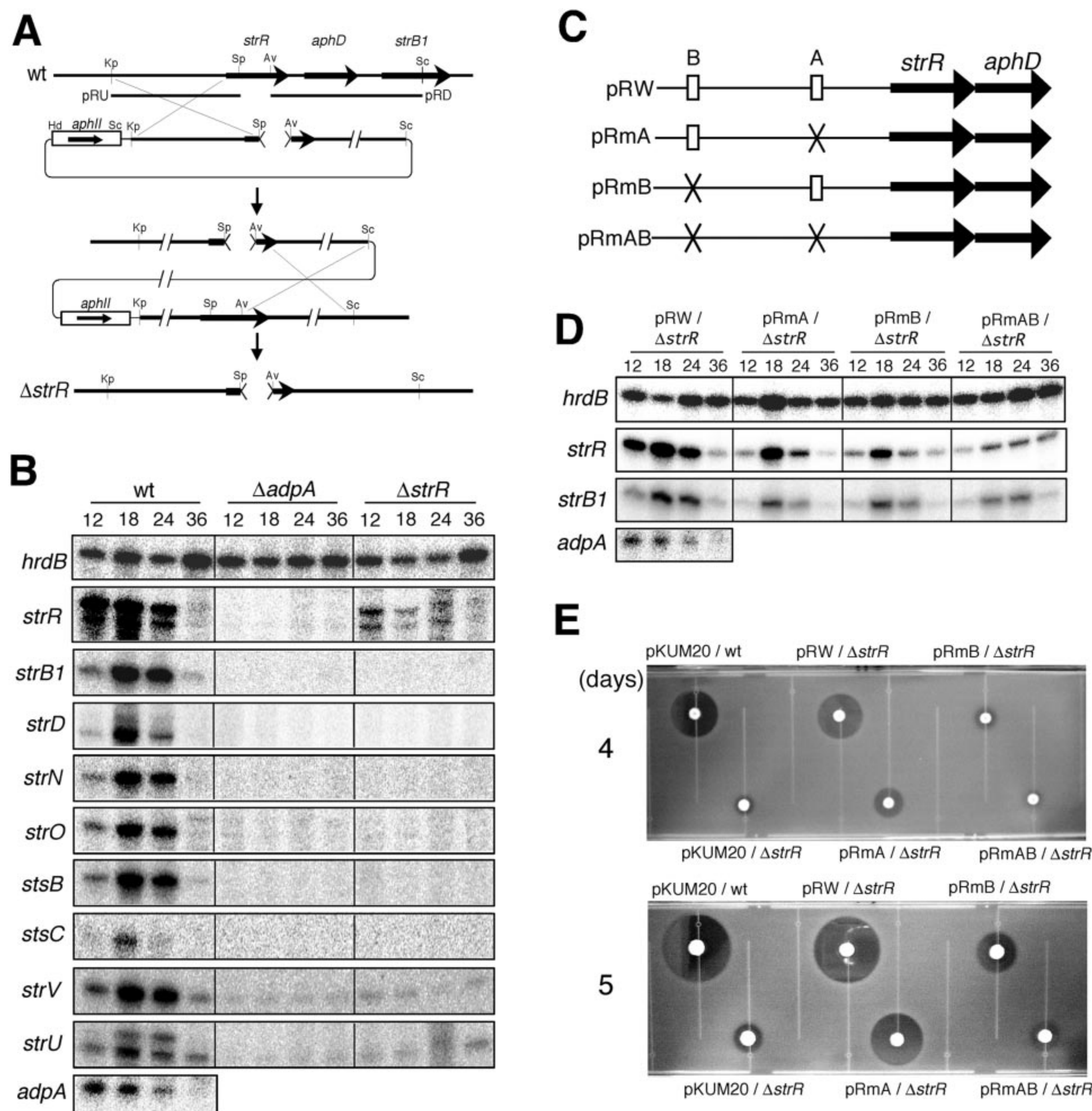

FIG. 4. Requirement of both site A and site B for full activation of *strR* by AdpA. (A) Schematic representation of the procedure used for construction of the Δ*strR* mutant. The restriction enzymes are abbreviated as follows: Av, AviII; Kp, KpnI; Pv, PvuII; Sc, SacI; Sp, SphI. (B) Low-resolution S1 nuclease mapping of *strR* and other promoters within the streptomycin biosynthetic gene cluster (*strB1*, *strD*, *strN*, *strO*, *stsB*, *stsC*, *strV*, and *strU*) in *S. griseus* Δ*adpA* and Δ*strR* mutants. RNA was prepared from cells grown on Bennett agar medium at 28°C for the indicated number of hours. (C) Schematic representation of the plasmids used for promoter assays. (D) Low-resolution S1 mapping of *strR* and *strB1* in the Δ*strR* mutant harboring pRW, pRmA, pRmB, or pRmAB. (E) Streptomycin production by the Δ*strR* mutant harboring pRW, pRmA, pRmB, or pRmAB. As controls, the wild-type (wt) strain harboring the vector pKUM20 and the Δ*strR* mutant harboring pKUM20 were also assayed. These strains were grown on Bennett agar medium at 28°C for 4 or 5 days, and soft agar containing spores of *B. subtilis* was overlaid. The plates were incubated at 37°C overnight, and streptomycin production was detected by inhibition of the growth of the indicator.

the Δ*adpA* and Δ*strR* mutants by S1 nuclease mapping (Fig. 4B). RNA was isolated with Isogen (Nippon Gene) from cells grown on cellophane on the surface of Bennett agar medium. *hrdB* encoding a principal σ factor of RNA polymerase was used to check the purity and amount of the RNA used, as described previously (26). In wild-type strain IFO13350, *strR* was transcribed in the same time course as *adpA*. Of the two transcripts for *strR*, the large transcript corresponds to that reported previously (25), and therefore the small one is a degraded transcript. On the other hand, no *strR* transcript was detected in the Δ*adpA* mutant, as observed previously (20). An unexpected finding was that the *strR* transcription was greatly reduced in the Δ*strR* mutant. This suggests that StrR acts as an activator of its own transcription, although there is no plausible

StrR-binding sequence, 5′-GTTCGActG$(N)_{11}$CagTcGAAc-3′ (highly conserved nucleotides are in capitals, and conserved nucleotides are in lowercase letters; N is any nucleotide) (23), in front of its transcriptional start point.

Under the culture conditions used, strain IFO13350 and the Δ*strR* mutant grew as substrate mycelium until 24 h and as aerial mycelium thereafter. An additional unexpected observation was that transcription of *strR* occurred rather early in the cells from which the RNA was isolated. As described below, streptomycin production was observed at 3 days of growth. This may be ascribed to the difference in the inoculation of the cells; the RNA was prepared from the cells grown on agar medium by spreading a lump of mycelium, which supposedly contains A-factor in a larger amount than in the cells grown by inoculating a small amount of mycelium with a toothpick for streptomycin assays.

We next determined the transcription of *strR* on plasmids pRW, pRmA, pRmB, and pRmAB by using the probe with ^{32}P at the 5′ end which was deleted from the chromosome of the Δ*strR* mutant. This means that the *strR* transcripts in Fig. 4D originated from the *strR* promoters on these plasmids. pRW containing native sites A and B and the *strR-aphD* region (Fig. 4C) allowed the transcription of plasmid-borne *strR* in the Δ*strR* mutant (Fig. 4D). However, the transcription of *strR* in the Δ*strR* mutant harboring pRmA or pRmB was much less active than that from the intact *strR* promoter on pRW. The *strR* transcription in the Δ*strR* mutant harboring pRmAB was severely impaired. The detectable transcription of *strR* in the Δ*strR* mutant harboring pRmA or pRmB may be ascribed to the ability of AdpA to bind to the mutated sites with very low affinity. These findings suggest that both site A and site B are required for full activation of *strR* by AdpA.

(ii) Effects of the mutations at sites A and B on streptomycin production. The wild-type strain *S. griseus* IFO13350 harboring the vector pKUM20 produced 9.0 ± 2.9 μg and 200 ± 46 μg of streptomycin per colony on day 4 and day 5, respectively (Fig. 4E), as determined by a bioassay using *Bacillus subtilis* as an indicator (7). The amount of the streptomycin produced was calculated by using a calibration curve, obtained with authentic streptomycin. Consistent with the idea that StrR is the pathway-specific activator for streptomycin biosynthesis, almost no streptomycin was produced by the Δ*strR* mutant harboring pKUM20. A tiny halo around the colony was probably due to some other anti-*B. subtilis* substance produced by *S. griseus*. This is in agreement with the observation that in the Δ*strR* mutant no transcription of streptomycin biosynthesis genes was detected (see below). The Δ*strR* mutant harboring pRW produced 4.7 ± 2.1 μg and 100 ± 61 μg of streptomycin on day 4 and day 5, respectively, showing that *strR* transcription occurred to almost the same extent as in the wild-type strain. On the other hand, the Δ*strR* mutant harboring pRmA produced 1.6 ± 0.4 μg and 26.5 ± 11.2 μg of streptomycin per colony on days 4 and 5, respectively. Likewise, the Δ*strR* mutant harboring pRmB produced 0.6 ± 0.2 μg and 2.8 ± 0.5 μg of streptomycin per colony on days 4 and 5, respectively. In addition, the Δ*strR* mutant harboring pRmAB produced almost no streptomycin, just like the Δ*strR* mutant harboring the vector pKUM20. The amounts of streptomycin produced by these mutants reflected the *strR* promoter activities on the plasmids (Fig. 4D). Furthermore, the transcriptional assays and streptomycin assays showed that site B contributed more to the transcriptional activation by AdpA than site A.

(iii) Effects of the mutations at sites A and B on the transcription of *strB1*, one of the streptomycin biosynthesis genes. Retzlaff and Distler (23) reported that *strB1*, encoding aminocyclitol amidinotransferase, one of the streptomycin biosynthesis enzymes, possesses an StrR-binding sequence, 5′-GTTCG ActG$(N)_{11}$CagTcGAAc-3′, and is activated by StrR. We confirmed their observation; in the wild-type strain, *strB1* was transcribed actively at 18 and 24 h whereas no *strB1* transcription occurred in the Δ*strR* mutant or in the Δ*adpA* mutant (Fig. 4B). We next determined the *strB1* transcription in the Δ*strR* mutant harboring pRmA, pRmB, and pRmAB (Fig. 4D). *strB1* transcription was restored by *strR* on pRW containing the intact *strR* gene to a significant level. The degrees of restoration of *strB1* transcription by pRmA, pRmB, and pRmAB reflected, in principle, those of *strR* transcription (Fig. 4D). The large difference in the yields of streptomycin does not reflect the rather small difference in *strB1* transcription, which is an example to show that the level of transcription does not always correspond to the level of translation.

Transcription of all the streptomycin biosynthesis genes is dependent on StrR. Retzlaff and Distler (23) showed that StrR binds the intervening region between *stsC* and *stsB* and predicted the presence of an StrR-binding sequence between *strU* and *strV* (see Fig. 5), although no transcriptional analysis other than *strB1* and *strR* has yet been performed. We determined the dependence of the transcription of these genes on StrR by low-resolution S1 mapping (Fig. 4B). In the wild-type strain, these four genes were transcribed in the same time course as *strB1*. The approximate transcriptional start points of *strU*, *stsC*, and *stsB* were 215, 25, and 70 nucleotides upstream of the respective start codons. The transcriptional start point of *strV* was near the first nucleotide of the ATG start codon. However, no transcription of these genes occurred in the Δ*adpA* or Δ*strR* mutant, indicating that these four transcriptional units depend on StrR. Since introduction of pRW into the Δ*strR* mutant restored streptomycin production, the transcription of all these units was supposedly restored by *strR*.

There is no apparent StrR-binding sequence upstream of *strO*, *strN*, or *strD* (23). However, the transcription of these genes also depended on StrR; no transcription occurred in the Δ*strR* or Δ*adpA* mutant, whereas these genes were transcribed in the wild-type strain in the same time course as *strB1* (Fig. 4B). The transcriptional start point of *strO* was near the first nucleotide of the ATG start codon. The transcriptional start points of *strN* and *strD* were approximately 20 and 200 nucleotides upstream from the respective start codons. These findings showed that these three transcriptional units were also dependent on StrR, either directly or indirectly. We assume that StrR binds to the upstream regions of the respective promoters, despite the absence of an apparent StrR-binding sequence, because no DNA-binding proteins other than StrR are encoded within the streptomycin biosynthetic gene cluster, as described below. Further analysis of the StrR-controlled transcription of these genes is required.

***strR* as a unique regulator in the streptomycin biosynthesis gene cluster.** Synteny analysis of the streptomycin biosynthesis gene cluster, in a total of 32.6 kb (Fig. 5), shows that this gene cluster has been inserted into the genome of *S. griseus* by

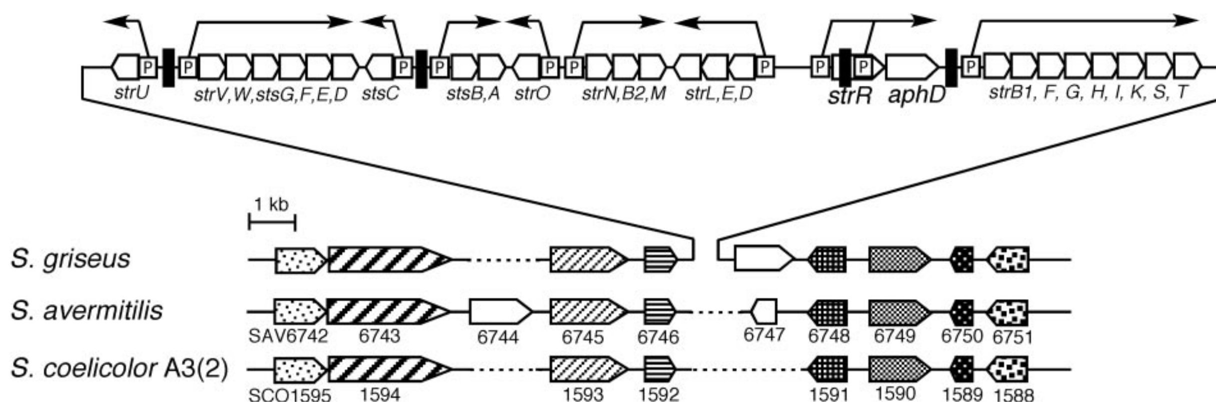

FIG. 5. Synteny analysis of the streptomycin biosynthesis gene cluster in *S. griseus*. The orthologues in *S. griseus*, *S. avermitilis*, and *S. coelicolor* A3(2) are discriminated by different markings. The StrR-binding sequences predicted by Retzlaff and Distler (23) are indicated by black bars. Promoters (P) and transcriptional units (arrows) are also indicated.

horizontal transfer, as suggested previously by Egan et al. (4). Comparison of the genome structures of *Streptomyces coelicolor* A3(2) (3) and *S. griseus* (our unpublished data) reveals that the streptomycin biosynthesis gene cluster, together with a gene for a hypothetical membrane protein, was inserted into the *S. griseus* genome between the genes corresponding to *SCO1592* and *SCO1591*. At this site in *Streptomyces avermitilis* (8), a functionally unknown gene, *SAV6747*, is inserted. An important implication of the synteny analysis is that the streptomycin biosynthesis gene cluster contains *strR* as a unique transcriptional regulator; no transcriptional regulator-like proteins other than StrR are encoded within the horizontally transferred DNA fragment.

Open-complex formation facilitated by AdpA. The AraC/XylS family proteins increase the affinity of RNA polymerase for their target genes and facilitate the formation of an open complex (18, 19). As demonstrated for AraC (9), MelR (2), SoxS (16), and Rns (19), we previously showed that AdpA recruits RNA polymerase to the promoter region of *amfR*, *adsA*, and *ssgA* and facilitates the isomerization of the RNA polymerase-DNA complex into an open complex competent for transcriptional initiation (28, 29). We examined whether AdpA facilitates open-complex formation at the *strR* promoter by potassium permanganate footprinting (Fig. 6). This method is based on the characteristic of potassium permanganate to react preferentially with thymidines on unpaired nucleotides. Briefly, $KMnO_4$-reacted nucleotides on a single strand, formed as a result of open-complex formation, were chemically cleaved with piperidine and detected on a sequencing gel. RNA polymerase was prepared from exponentially growing cells of *S. griseus* IFO13350 as described previously (28). Incubation of the DNA fragment (positions −482 to +68) containing the *strR* promoter in the presence of RNA polymerase alone gave no open complex (data not shown), as was found for *amfR* (28), *ssgA* (29), and *adsA* (29). However, addition of both RNA polymerase and AdpA-H revealed several thymidine residues, from position −11 to +1 on the antisense strand, that were modified and detected by the footprinting. When the concentration of AdpA-H was fixed at 20 nM, open-complex formation was detected depending on the concentration of RNA polymerase. These results show that AdpA facilitates open-complex formation at an appropriate position for transcriptional initiation of *strR*.

Signal transduction from A-factor to the streptomycin biosynthesis gene cluster. Some target genes of AdpA, such as

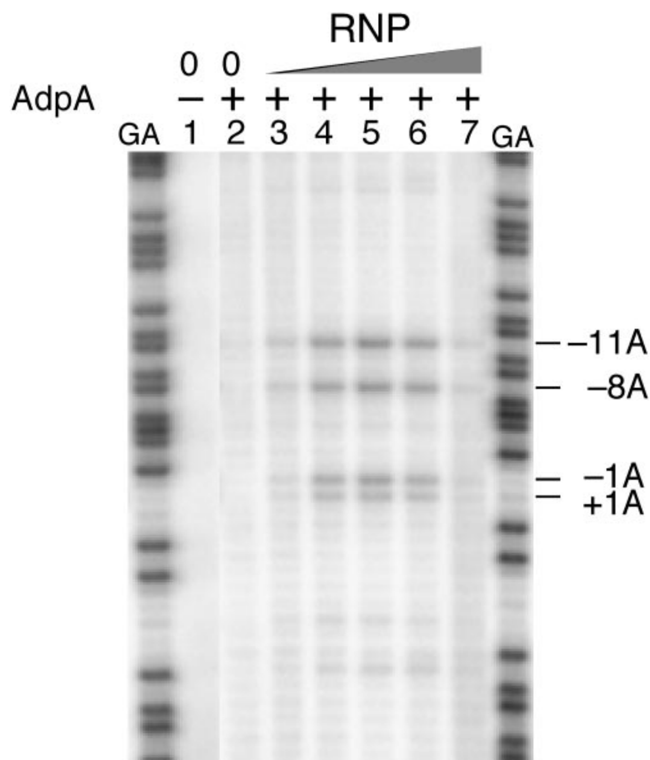

FIG. 6. Dependence of open-complex formation at the *strR* promoter on AdpA, as determined by potassium permanganate footprinting. The ^{32}P-labeled antisense strand of the *strR* promoter region (positions −482 to +68) was used. The concentration of AdpA-H in lanes 2 to 7 was 20 nM. Ten nanomolar AdpA equals 8.8 ng of AdpA in a total volume of 20 μl. The concentrations of RNA polymerase (RNP) in lanes 3 to 7 were 5, 15, 50, 150, and 300 nM, respectively. The positions of the thymidines reacted with $KMnO_4$, corresponding to adenines of the sense strand, were determined using the G+A sequencing ladder (lane GA) as a reference. The nucleotide numbers are shown taking the transcriptional start point as +1.

amfR (28), *sgmA* (12), and *sprA* (our unpublished data), contain two UASs to which AdpA binds. The two UASs are both necessary for transcriptional activation by AdpA, the molecular mechanism of which still remains to be elucidated. It should be noted that one of the AdpA-binding sequences at site A overlaps with the −35 element of the *strR* promoter. If the DNA-binding domain in one subunit of the AdpA dimer sat at this position, it would prevent RNA polymerase from binding to the *strR* promoter properly. Since the AdpA dimer bound near the promoter supposedly interacts with RNA polymerase, we speculate that, on binding RNA polymerase, AdpA binds site A by anchoring the AdpA-binding sequence of the 5′ side with only one of the DNA-binding domains so as to make room for RNA polymerase to bind the promoter. This is consistent with the observation that only the XbaI mutation at site A was sufficient to avoid the affinity of AdpA (Fig. 3B).

We show here that the pathway-specific transcriptional activator gene for streptomycin biosynthesis, *strR*, is activated by two molecules of AdpA that separately bind two UASs located at nucleotide positions −270 and −50. In addition, *strR* appears to activate its own transcription because the *strR* transcription in the Δ*strR* mutant was greatly repressed. Due to this autoactivation system, the A-factor signal starting from A-factor to *strR*, via ArpA and AdpA, to trigger streptomycin production must lead to a rapid increase in the amount of StrR, which in turn leads to rapid and simultaneous transcription of other streptomycin biosynthetic genes within the cluster. Concerning the streptomycin biosynthesis genes, *strB1* was under the control of StrR, as reported by Retzlaff and Distler (23). They also showed that *stsB* and *stsC*, having an *strR*-binding sequence upstream of their promoters, are bound by StrR. Their prediction that StrR activates the transcription of these genes is true since the present study shows that all four of these genes are activated by StrR in the same manner as *strB1*. We have also shown that the remaining three promoters for *strO*, *strN*, and *strD* in the cluster are similarly activated by StrR although these promoters do not contain an apparent StrR-binding sequence (23). All these results suggest that StrR, as a sole regulator within the gene cluster, serves as a transcriptional activator for all the streptomycin biosynthesis genes. Consistent with this idea, *strR* (together with *aphD*) under the control of the *hrdB* promoter on pKUM20 caused the Δ*adpA* mutant to produce streptomycin, although its growth was significantly impaired, probably due to some inhibitory effect of *strR* overexpression (data not shown).

Biosynthesis of secondary metabolites is controlled by a variety of external signals, such as nutrient conditions, including carbon, nitrogen, and phosphate, and physiological conditions. Since *strR* is a unique regulator in the streptomycin biosynthesis gene cluster, these signals must be gathered to the promoter of *strR*. We previously detected several different proteins that bind the upstream region of the *strR* promoter by gel mobility shift assay (24). These proteins may represent a certain signal and regulate *strR* in either a positive or a negative way, thus controlling the whole streptomycin biosynthesis genes. The structure of the streptomycin biosynthesis gene cluster is simple and makes a simple target to study secondary metabolite formation in response to external signals.

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