Nucleotide Sequence of *afsB*, a Pleiotropic Gene Involved in Secondary Metabolism in *Streptomyces coelicolor* A3(2) and "*Streptomyces lividans*"

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The nucleotide sequence of *afsB* from *Streptomyces coelicolor* A3(2), a pleiotropic gene which positively controls the biosynthesis of A-factor and the pigmented antibiotics actinorhodin and undecylprodigiosin in *S. coelicolor* A3(2) and "*Streptomyces lividans*," was determined. The determinant of the *afsB* gene, which includes the putative AfsB protein consisting of 243 amino acids, was mapped functionally by tests for A-factor and pigment production in "*S. lividans*" and *S. coelicolor* A3(2) after introduction of recombinant plasmids containing various restriction endonuclease fragments on the vector plasmids pIJ41 and pIJ702. The putative AfsB protein contains two regions separated by 167 residues which resemble conserved domains of known DNA-binding proteins. High-resolution nuclease S1 protection mapping revealed that the *afsB* mRNA, approximately 1,300 base pairs (bp) long, which was determined by Northern blot hybridization, had its start point 340 bp upstream of the putative methionine start codon. The Northern hybridization experiment also suggested that the *afsB* gene was constitutively transcribed throughout growth. Also shown by the Northern hybridization was the presence of an unidentified gene with an extraordinary amount of 880-bp mRNA located downstream from *afsB*. Dot hybridization with the brown pigment production genes, possibly involved in polyketide biosynthesis, as the probe suggested that the *afsB* gene did not stimulate transcription of the pigment production genes. In Southern blot DNA-DNA hybridization analysis with the *afsB* sequence as the probe, sequences exhibiting various degrees of homology were found in several *Streptomyces* spp. A DNA sequence showing strong homology to the *afsB* in *Streptomyces griseus* FT-1, a high streptomycin producer, behaved like an extrachromosomal element, homologous to the *afsA* gene, a structural gene for A-factor biosynthesis.

The *afsB* gene cloned from *Streptomyces coelicolor* A3(2) regulates positively the biosynthesis of A-factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone) and the pigmented antibiotics actinorhodin and undecylprodigiosin in this organism (19). Genetic analysis mapped the *afsB* gene to a fixed chromosomal locus between *cysD* and *leuB* and close to *afsA*, possibly a structural gene for A-factor biosynthesis (13). The *S. coelicolor* A3(2) *afsB* gene, when introduced into an A-factor-deficient mutant strain (HH21) of "*Streptomyces lividans*," caused restoration of A-factor production and led to production in large quantity of the pigments actinorhodin and undecylprodigiosin (17, 19). The biosynthetic pathways for these pigments are normally "silent" or expressed at very low levels in "*S. lividans*" under the culture conditions that are usually used. The pleiotropic regulatory function displayed by *afsB* was proved not to be due to the pleiotropic activity of A-factor (19). These observations have suggested the presence of a complex regulatory network which links secondary metabolism with morphological development in *Streptomyces*; *afsB* is involved as a positive regulator of A-factor production and secondary metabolites in a positive manner in *S. coelicolor* A3(2) and "*S. lividans*," whereas in *Streptomyces griseus* and *Streptomyces bikiniensis*, A-factor itself is required to trigger both streptomycin production and sporulation (12).

In this paper, we report the nucleotide sequence of *afsB* together with that of its promoter region. The amino acid sequence deduced from the nucleotide sequence suggests that the putative *afsB* gene product may be a DNA-binding protein.

MATERIALS AND METHODS

Bacterial strains, plasmids, and chemicals. The bacterial strains and plasmids used are listed in Table 1. Other *Streptomyces* strains have been described previously (12). Preparation of plasmid DNA, recombinant DNA work, and protoplast transformation were performed as previously described (19, 21). The restriction endonucleases, T4 DNA ligase, S1 nuclease, T4 polynucleotide kinase, terminal deoxynucleotidyl transferase, and synthetic linkers were purchased from Takara Shuzo Co., Ltd., or New England Biolabs, Inc.

A-factor assay. The amounts of A-factor produced by transformants were measured by the streptomycin cosynthesis method with an A-factor-deficient mutant strain, *S. griseus* FT-1 number 2, and *Bacillus subtilis* ATCC 6633 was used as the indicator organism as described before (12). In the present study, a test strain grown on an agar plug (5 mm in diameter by 3 mm in height) at 28°C for 2 days was transferred to a soft agar layer seeded with the A-factor-deficient *S. griseus* and incubated at 28°C for 2 days. Nutrient soft agar containing spores of the indicator strain *B. subtilis* was overlaid, and the plates were incubated overnight at 37°C. A-factor produced by the test organism diffused from the agar plug into the soft agar and caused the *S. griseus* cells to produce streptomycin, which in turn was detected by growth inhibition of the indicator strain. The amount of A-factor was estimated by measuring the diameter of the inhibition zone and comparing it with a calibration curve obtained with authentic A-factor and paper disks.

DNA sequence studies. A DNA fragment obtained by digestion of pIJ702-AP22 with *Pst*I plus *Sac*I was purified by

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TABLE 1. Bacterial strains and plasmids

Designation	Relevant characteristics	Reference(s)
Streptomyces strains		
"*S. lividans*" TK21	Wild type	15
"*S. lividans*" HH21	A-factor-deficient mutant (possibly *afsB⁻*) derived from TK21	19
S. coelicolor A3(2) BH6	*afsB⁻*; A-factor deficient, low-level production of actinorhodin and undecylprodigiosin	13, 19
S. griseus FT-1	High streptomycin producer, A-factor⁺	12
S. griseus FT-1 number 2	A-factor⁻ (Δ*afsA*), obtained by UV irradiation from FT-1	12, 20
S. griseus FT-1 AO-1	A-factor⁻ (Δ*afsA*), obtained by acridine orange treatment from FT-1	20
S. griseus IFO 13189	Streptomycin producer, A-factor⁺	12, 20
S. bikiniensis IFO 13350	Streptomycin-producer, A-factor⁺	12, 20
Plasmids		
pIJ41	Neomycin and thiostrepton resistance	4
pIJ702	Thiostrepton resistance, melanin⁺	23
pIJ41-AP3	Thiostrepton resistance, containing *afsB*	19
pIJ702-AP20	Thiostrepton resistance, containing *afsB*	19
pARC1	Thiostrepton resistance, containing the brown pigment production gene(s)	18

electroelution after agarose gel electrophoresis and used as the starting material for sequencing. DNA restriction endonuclease fragments were labeled at their 5' ends with [γ-³²P]ATP and T4 polynucleotide kinase or at their 3' ends with [α-³²P]cordycepin triphosphate and terminal deoxynucleotidyl transferase. When necessary, DNA strands were separated by electrophoresis of denatured restriction fragments through a 5% polyacrylamide gel (27). DNA was sequenced with base-specific chemical cleavages (G, G+A, T+C, C, A>C) as described by Maxam and Gilbert (28). The nucleotide sequence obtained by this method was further confirmed by the dideoxynucleoside triphosphate chain termination method with dITP instead of dGTP as described previously (2, 30).

RNA isolation. Total RNA was prepared from "*S. lividans*" HH21 carrying pIJ41-AP3 or pIJ41 grown for 2 to 5 days in YMPG medium (containing [in grams per liter]: yeast extract, 4; malt extract, 10; MgCl₂ · 6H₂O, 2; peptone [Difco Laboratories] 1; and glucose, 10; pH 7.2). Mycelia were washed with 50 mM Tris hydrochloride (pH 8.0)–5 mM EDTA–50 mM NaCl, suspended in 0.3 M sucrose–50 mM Tris hydrochloride (pH 8.0)–5 mM EDTA–2 mg of lysozyme per ml and lysed by incubation at 37°C for 5 to 10 min. Sodium dodecyl sulfate (SDS) was added to give a final concentration of 0.7%, and the mixture was heated at 100°C for 3 min. The lysate was extracted several times with hot phenol and then with ether-chloroform (1:1). After ethanol precipitation, the sample was treated with RNase-free DNase I (Worthington Biochemicals) and phenol extracted. Completion of the DNase treatment was monitored by agarose gel electrophoresis.

S1 nuclease mapping. DNA-RNA hybridization was performed in a sealed glass capillary containing 20 μl of 80% formamide–0.4 M NaCl–40 mM PIPES (pH 6.4)–1 mM EDTA at 54°C for 18 h, as described in the text. In some cases, restriction fragments with ³²P-labeled 5' ends were strand-separated by the method with dimethyl sulfoxide (27). The hybridization mixture was then diluted with 200 μl of 30 mM sodium acetate (pH 4.6)–1 mM ZnSO₄–0.25 M NaCl–20 μg of denatured calf thymus DNA per ml and digested with S1 nuclease at 30°C for 1 h. After ethanol precipitation, protected DNA fragments were analyzed on DNA sequencing gels by the method of Maxam and Gilbert (28).

RNA-DNA dot-blot and Northern blot hybridization. For making ³²P-labeled probes for the thiostrepton resistance (thioʳ) gene and the brown pigment production genes, the smallest *Bcl*I fragment (1.05 kilobases [kb]) derived from pIJ702 and the *Bam*HI-*Sph*I fragment (4.5 kb) derived from pARC1, respectively, were purified by agarose gel electrophoresis and nick-translated with a translation kit (New England Nuclear Corp.) and [³²P]dCTP. Glyoxal-denatured RNA (10 μg) separated by 1.2% agarose gel electrophoresis was transferred and baked to a sheet of nitrocellulose paper by the method of Thomas (40). The blot was prehybridized at 42°C for 18 h in the buffer described by Thomas (40) and hybridized at 42°C for 40 h with ³²P-labeled restriction fragments (ca. 10⁷ cpm/μg). The hybridized blot was washed with two changes of 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% SDS for 15 min each at room temperature and then with three changes of 0.1× SSC plus 0.1% SDS for 15 min each at 50°C. *Hind*III-digested λ and *Hinc*II-digested φX174 DNAs as well as *Escherichia coli* 23S and 16S rRNAs denatured with glyoxal were used for molecular weight standards. For dot hybridization, the RNA samples were spotted onto dry nitrocellulose paper that had been pretreated with 20× SSC, baked, prehybridized, hybridized, and washed as described above. After development of the X-ray films, each dot was scanned with a Toyo densitometer, model DMU-33C.

DNA-DNA Southern blot hybridization. A restriction fragment containing the *afsB* gene was obtained by digestion of pIJ702-AP22 with *Sau*3AI and nick translated as described above. The ³²P-labeled probe denatured at 100°C for 5 min was hybridized to a sheet of nitrocellulose paper in which *Bam*HI-digested DNAs had been blotted by the method of Southern (38) in 5× SSC–50% formamide–0.1% SDS at 42°C overnight. The nitrocellulose paper was then washed three times in the hybridization buffer at room temperature and twice in 2× SSC–0.1% SDS.

RESULTS

Determination of *afsB* coding region. The size of the *afsB* gene, including the coding and promoter sequences which caused the A-factor-deficient mutant strain "*S. lividans*" HH21 to produce A-factor, actinorhodin, and undecylprodigiosin, had been reduced to an approximately 2-kb fragment (19). It was also known that the unique *Sph*I site on the 2-kb fragment was located in a region essential for the expression of *afsB*. Based on a fine restriction map of the 2-kb fragment, we subcloned the largest *Sau*3A fragment (1230 bp) of pIJ702-AP20 in two different orientations into the *Bam*HI site of the plasmid vector pIJ41, resulting in plasmids pIJ41-AP25 and pIJ41-AP27 (Fig. 1). Plasmid pIJ41-AP27 conferred A-factor production to "*S. lividans*" HH21, while pIJ41-AP25 did not. This result suggested that the *Sau*3A fragment lacked transcriptional control signals.
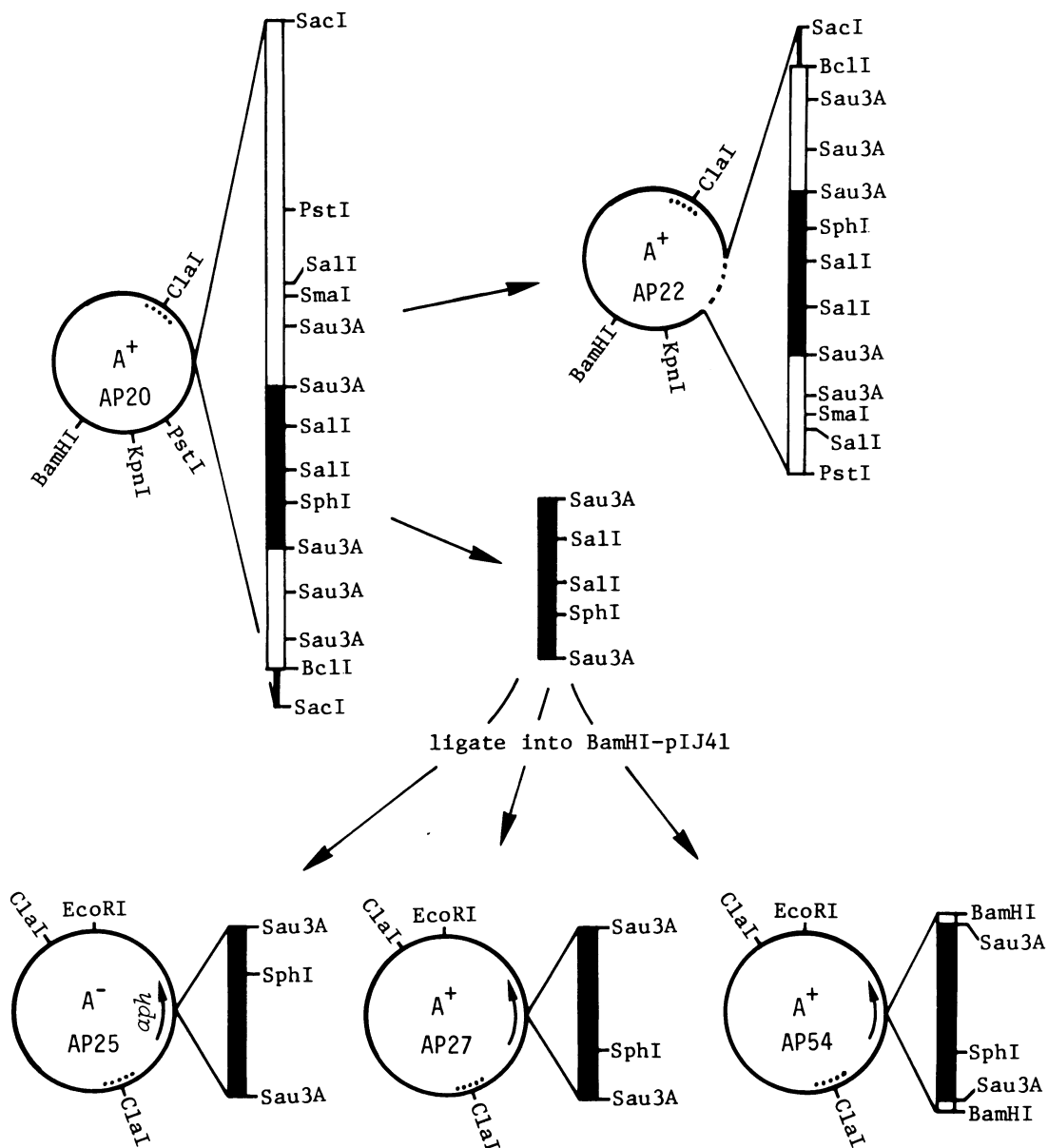
FIG. 1. Schematic representation of plasmids. These include: pIJ702-AP20, containing a cloned *S. coelicolor* A3(2) chromosomal fragment in the *Sac*I site of pIJ702 (19); pIJ702-AP22, obtained by insertion of an *Sac*I-*Pst*I fragment containing the *afsB* gene into pIJ702 digested with *Sac*I plus *Pst*I; pIJ41-AP25, obtained by insertion of the largest *Sau*3A fragment (solid bar) into the *Bam*HI site of pIJ41, resulting in a plasmid which failed to confer A-factor production (A⁻) to "*S. lividans*" HH21; pIJ41-AP27, obtained by insertion of the same *Sau*3A fragment as in pIJ41-AP25 in the opposite orientation in the *Bam*HI site of pIJ41, resulting in a plasmid capable of conferring A-factor production (A⁺) to strain HH21; and pIJ41-AP54, which was obtained by attachment of seven nucleotides (CGGGATC) to the polymerized *Sau*3A ends by using *Bam*HI linkers consisting of 10 nucleotides. Dots stand for the extent of the thiostrepton resistance gene. The nucleotide sequence around the *Bam*HI linker of pIJ41-AP54 was confirmed by determination as part of an overlapping sequence from the closest *Pst*I site.

The *Bam*HI site of pIJ41 is located in the structural gene for neomycin phosphotransferase (*aph*), and the direction of transcription is as indicated by an arrow in Fig. 1 (41). To exclude the possibility that an *aph*-*afsB* fused protein causes the host strain to produce A-factor, we attached *Bam*HI linkers 10 nucleotides long to the ends of the *Sau*3A fragment, which had been filled in with the Klenow fragment of DNA polymerase I, resulting in plasmid pIJ41-AP54. Plasmid pIJ41-AP54, containing seven additional nucleotides at the *Sau*3A ends, whose structure was confirmed by nucleotide sequence determination, still conferred A-factor produc-

tion on "*S. lividans*" HH21. This result suggested that the *Sau*3A fragment contained the whole coding sequence, including the start and possibly the termination codons but not a promoter signal.

Determination of region essential for *afsB* expression. To determine the region essential for the *afsB* promoter function, we constructed various hybrid plasmids containing the *Sau*3A fragment with upstream regions of different lengths, according to the strategy illustrated in Fig. 2. All the plasmids contained the *Sau*3A fragment in such an orientation that the transcriptional direction of the *afsB* gene, as
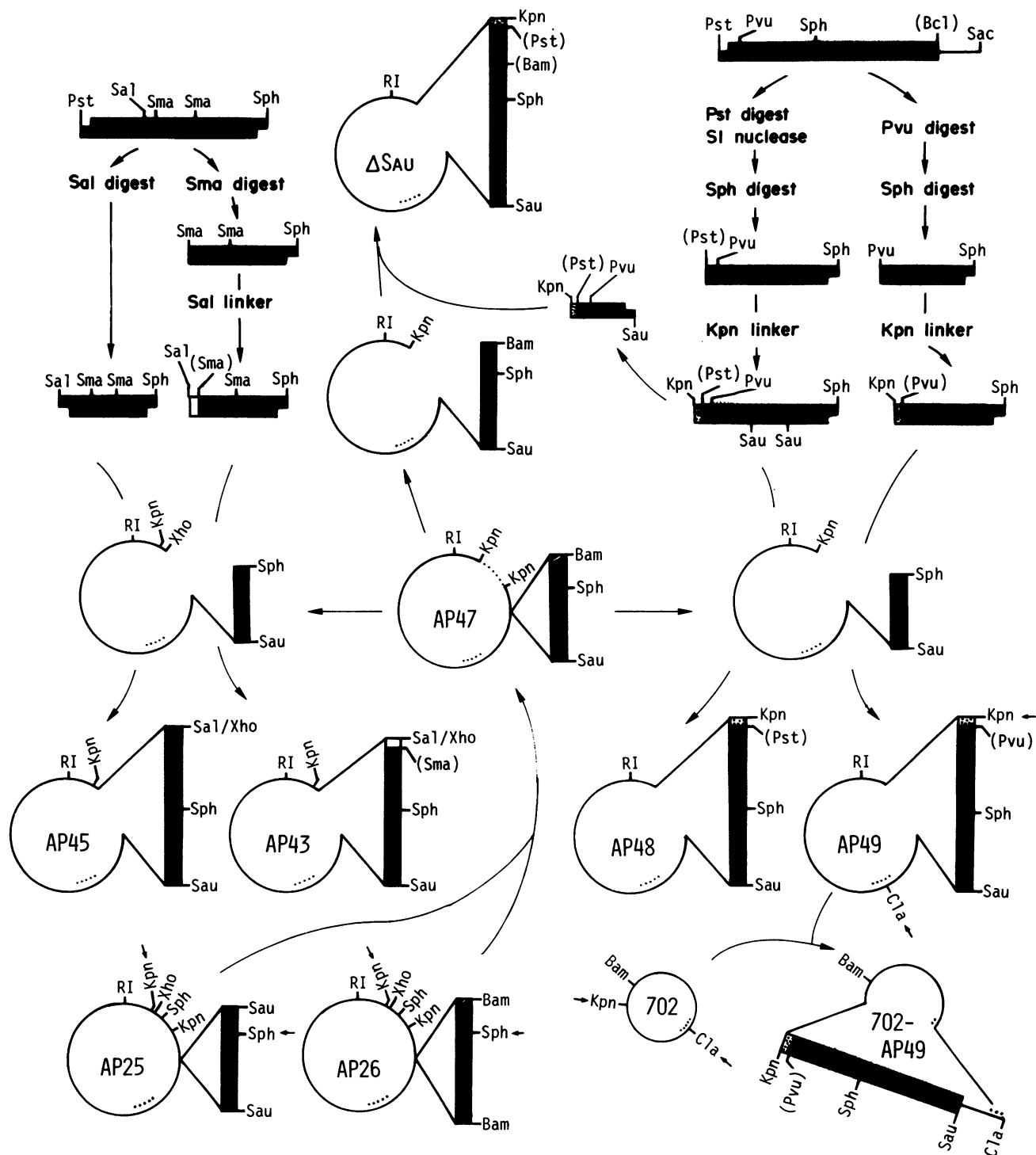
FIG. 2. Construction of pIJ41 and pIJ702 series plasmids for determination of the *afsB* promoter region. Plasmid pIJ41-AP47 was constructed by ligation of the largest *Kpn*I-*Sph*I fragment of pIJ41-AP25 with a small *Kpn*I-*Sph*I fragment of pIJ41-AP26. By using pIJ41-AP47 as the starting material for cloning various lengths of 5' region and two restriction fragments (the *Pst*I-*Sph*I and *Pst*I-*Sac*I fragments obtained from pIJ702-AP22) as sources of the 5' region, five composite plasmids, pIJ41-AP49, -AP48, -AP45, -AP43, and -ΔSau, were constructed. All the linkers used consisted of eight nucleotides. For construction of the pIJ702 series plasmids, the small *Kpn*I-*Cla*I fragment from each of the composite plasmids was inserted into pIJ702 digested with *Kpn*I plus *Cla*I. In this scheme, an example of the construction of pIJ702-AP49 is shown.

expected from the above experiments, was opposite that of the *aph* promoter to avoid a readthrough transcription into the *Sau*3A fragment. The structures of all the constructed plasmids were confirmed by purifying the plasmid DNAs, followed by analysis of the cleavage patterns with appropriate restriction enzymes.

Plasmid pIJ41-AP47, containing only the *Sau*3A fragment with the whole coding sequence, and plasmid pIJ41-ΔSau, lacking a 169-bp segment upstream of the *Sau*3A fragment, failed to confer A-factor production on "*S. lividans*" HH21 (Fig. 3A). Neither pIJ41-AP43 nor pIJ41-AP45, both of which contained relatively long sequences upstream of the *Sau*3A fragment, conferred A-factor production. Plasmid pIJ41-AP48 and pIJ41-AP49 conferred A-factor production on strain HH21. These results suggested that *afsB* required a relatively long upstream sequence for expression in "*S. lividans.*"

To examine whether a similar conclusion could be drawn for the *afsB* mutant strain BH6 of *S. coelicolor* A3(2), which has lost the ability to produce A-factor as well as the pigments actinorhodin and undecylprodigiosin owing to a deficiency of the *afsB* gene, we next constructed a similar series of plasmids by using the high-copy-number plasmid pIJ702, because pIJ41 cannot replicate in *S. coelicolor* A3(2). Construction of pIJ702 series plasmids and the structures are shown in Fig. 2 and 3B. *S. coelicolor* A3(2) BH6 transformants carrying pIJ702-AP48 or pIJ702-AP49 produced the pigments on Bennett agar medium containing 40 μg of thiostrepton per ml in 3 days of growth at 30°C. After 3 days, the pigment turned blue, as in the case of the parental *S. coelicolor* A3(2) strain. On the other hand, transformants carrying pIJ702-AP47A, -AP47B, -ΔSau, -AP45, or -AP43 failed to produce the pigments even after 5 days of growth. With the latter group of transformants, slight pigment production was observed by cultivation for more than 6 to 7 days. As to A-factor production, the transformants carrying pIJ702-AP48 or pIJ702-AP49 produced 4 to 6 ng of A-factor per colony, almost the same amount as produced by the parental strain. The transformants carrying the latter group of plasmids produced detectable amounts of A-factor (about 1 to 2 ng per colony). We assume that the low productivity of A-factor as well as of the pigments caused by the latter group of plasmids is ascribable to readthrough transcription from the high-copy-number vector pIJ702 sequence, with 40 to 300 copies (23). These results, suggesting that a considerably long sequence upstream of the *afsB* coding region is necessary for *afsB* expression, are consistent with those obtained from the experiments with "*S. lividans*" and pIJ41 as the host-vector system.

Nucleotide sequence of *afsB*. To obtain a DNA fragment for sequencing in large quantity, we constructed pIJ702-AP22 (Fig. 1) by deleting two small *Pst*I-*Sac*I segments from pIJ702-AP20. Plasmid pIJ702-AP22 conferred production of A-factor, actinorhodin, and undecylprodigiosin to "*S. lividans*" HH21 as well as to *S. coelicolor* A3(2) BH6. A *Pst*I-*Sac*I fragment purified by agarose gel electrophoresis was used for the starting material for nucleotide sequence determination. The nucleotide sequence of the *Sau*3A fragment and the upstream region as far as the *Pst*I site was determined by the strategy shown in Fig. 4, first by the chemical cleavage method. Because of the high G+C composition, we also used the chain termination method, using dITP instead of dGTP to confirm the nucleotide sequence obtained by the above method. All restriction sites used for end labeling and cloning into M13 phages were verified by determination as part of an overlapping sequence. The

complete nucleotide sequence of the *afsB* gene, including a potential polypeptide and inverted complementary repeat sequences, is shown in Fig. 5.

The putative AfsB protein deduced from the nucleotide sequence presumably has the ATG initiation codon at nucleotide (nt) 678, as shown in Fig. 5. One can predict the reading frame of experimentally determined DNA sequences, especially in *Streptomyces* genes of extreme G+C content, as described by Bibb et al. (6). An overall average G+C content of the coding sequence of the putative AfsB protein is 73 mol%, and those for codon positions 1, 2, and 3 are 74.9, 53.1, and 91.0 mol%, respectively. The distribution of GC pairs at each of the three positions within the codons is in good agreement with a feature of most *Streptomyces* genes, as described by Bibb et al. (4). Other GTG (nt 543), ATG (nt 610), and GTG (nt 612) are potential initiation codons, but they do not follow the prediction rule. At present we cannot exclude the possibility that ATG (nt 735) or GTG (nt 771), both of which are in frame with the presumptive AfsB protein, serves as the initiation codon.

It is known that a degree of complementarity between the 3' end of the 16S rRNA of the procaryotes and a nucleotide sequence (the Shine-Dalgarno sequence) situated several nucleotides upstream from the translational initiation codon of mRNA is necessary for the initiation of translation (10, 37). However, as in the case of the *aph* gene (41), there is no stretch of nucleotides complementary to the 3' end of the 16S rRNA of "*S. lividans*," 5'-GAUCACCUCCUUUCU$_{OH}$-3' (5).

Mapping of the 5' end of *afsB* mRNA. To map the 5' end of *afsB* mRNA by S1 nuclease mapping, we first determined the hybridization temperature by a simple agarose gel assay (32). Each restriction fragment described below was dissolved in the hybridization buffer, heated at various temperatures, and separated on a 1% agarose-ethidium bromide gel. At 54°C but not at 51°C, every fragment produced additional bands corresponding to single-stranded DNAs, which indicated that the thermal denaturation temperature of the fragments under the conditions used was between 51 and 54°C. Therefore, we performed the RNA-DNA hybridization at 54°C.

Three restriction fragments, *Pst*I-*Sal*I (nt 1–188), *Ava*I-*Ava*I (nt 215–420), and *Sau*3A-*Sau*3A (nt 366–538), were end-labeled at the 5' ends with T4 polynucleotide kinase, hybridized under the conditions described above with total RNA preparations from a 3-day culture of "*S. lividans*" (pIJ41-AP3), digested with S1 nuclease, and analyzed by polyacrylamide gel electrophoresis. Similarly, two restriction fragments, *Sal*I-*Dde*I (nt 185–674) with ^{32}P at the 5' end of the *Dde*I site and *Taq*I-*Hinf*I (nt 369–568) with ^{32}P at the 5' end of the *Hinf*I site, were analyzed. Among the five fragments tested, the *Sau*3A-*Sau*3A and *Taq*I-*Hinf*I fragments were fully protected and the *Ava*I-*Ava*I and *Sal*I-*Dde*I fragments were partially protected. The lengths of the protected DNAs suggested that an mRNA species with a definite 5' end protected these fragments from S1 digestion. We next performed a similar protection experiment using separated strands of the *Ava*I-*Ava*I fragment of 206 bp. The slow-moving strand but not the fast-moving strand produced the protected DNA bands shown in Fig. 6. These results clearly indicated that the 5' end of *afsB* mRNA, whose direction was toward the presumptive AfsB coding sequence, was G at nt 342.

The nucleotide sequence upstream of the mRNA start point did not contain "consensus" sequences for promoters found in other procaryotes (33). This also appears to be true of the *aph* gene (41), and alignment of the *afsB* and *aph*
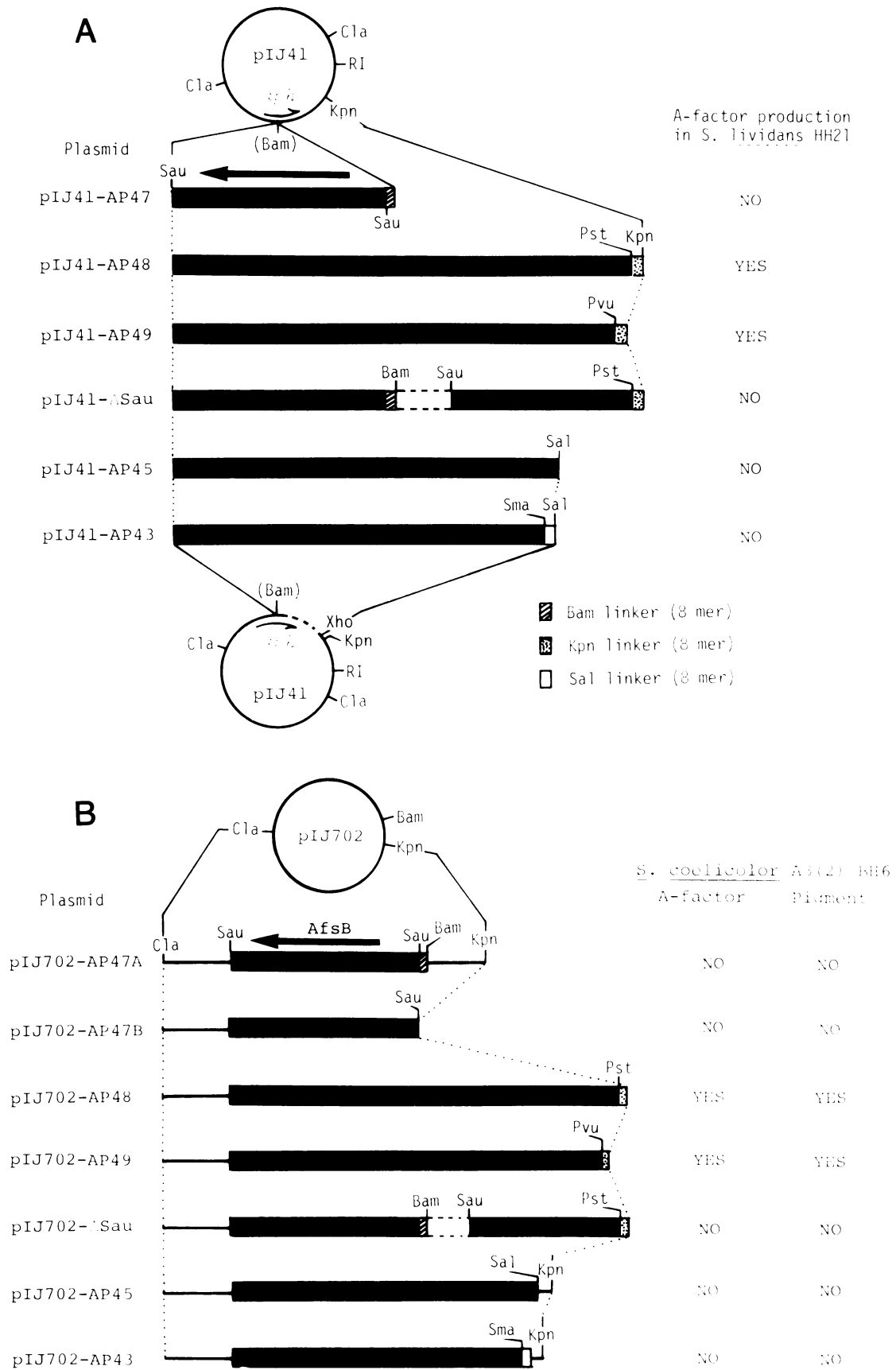
FIG. 3. Schematic representation of (A) pIJ41 and (B) pIJ702 series plasmids and their phenotypes determined by tests for A-factor and pigment production in "*S. lividans*" HH21 and *S. coelicolor* A3(2) BH6.
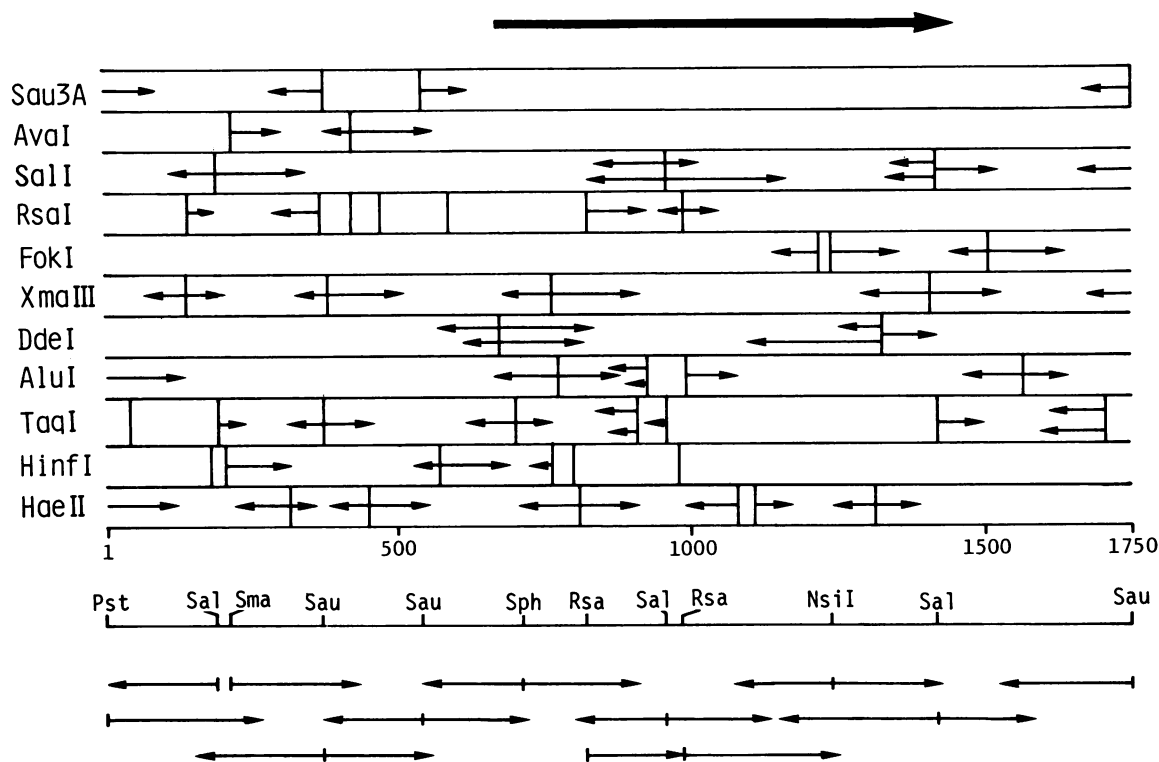
FIG. 4. Sequencing strategy for the *afsB* gene. The top and bottom sections show the strategy by the Maxam-Gilbert method and by the M13-dideoxy method, respectively. The sites used for end-labeling or cloning of DNA fragments obtained by digestion with restriction enzymes are indicated together with arrows whose length and direction indicate the extent of sequence determination from these sites. Numbers below the map indicate the distance (in base pairs) from the *Pst*I site. The thick arrow at the top shows the coding region of the putative AfsB gene product.

sequences indeed revealed significant homology, especially around the −45 to −25 region, as shown in Fig. 7.

An imperfect inverted complementary repeat region spanning 59 nucleotides ($\Delta G = -57.2$ kcal/mol) between the transcriptional start site and the putative start codon at nt 472 to 530 is present (Fig. 5). The role of this structure is not clear.

Analysis of mRNA complementary to *afsB* by dot-blot and Northern blot hybridization. Since *afsB* positively regulates the formation of secondary metabolites in *S. coelicolor* A3(2) and "*S. lividans*," it was of interest to examine at what stage of growth this gene is expressed. The amount of mRNA complementary to the *afsB* sequence was measured by dot-blot hybridization by using the ^{32}P-labeled *Sau*3A fragment (nt 538-1750) and total RNA prepared from "*S. lividans*"(pIJ41-AP3). The thiostrepton resistance gene was used as a control. Radioactivity hybridized to each dot suggested that the amount of mRNA complementary to the probe did not vary with growth phase, in comparison with the thiostrepton resistance gene (Fig. 8). Under the culture conditions used when the RNAs were prepared, "*S. lividans*"(pIJ41-AP3) began to produce actinorhodin in 3 days, as judged by the blue color of the culture medium, which reached the maximum level in 5 days.

We also analyzed the mRNA complementary to the *afsB* gene by Northern blot hybridization using the probe described above (Fig. 9). Nonspecific hybridization, probably owing to the high GC content of *Streptomyces* genes, interfered with identification of the exact band corresponding to the *afsB* mRNA. However, two distinct mRNA species of 1,300 and 880 bp were seen when the pattern of

nonspecific hybridization was compared with that observed in the control experiment with the thiostrepton resistance gene. The smaller mRNA, which was transcribed in a very large amount, was not long enough to cover the *afsB* gene. In fact, as described below, this small mRNA was found to be transcribed from the region downstream of *afsB*. Therefore, we concluded that the *afsB* mRNA corresponded to the hybridizing band of 1,300 bp. This size is in agreement with that expected on the basis of the nucleotide sequence. If one assumes that an inverted complementary repeat sequence ($\Delta G = -36.4$ kcal/mol) at nt 1547 to 1586 serves as a transcriptional termination signal, the size of the *afsB* mRNA with the start point at nt 342 would be approximately 1,250 bp. It is reasonable to assume that inverted repeat sequences followed by a series of T's on the noncoding DNA strand serve as transcriptional terminators in "*S. lividans*" too, since Jaurin and Cohen (22) showed that *rho*-independent transcriptional termination signals of *E. coli* could function in "*S. lividans*."

The concentration of *afsB* mRNA was highest in the total RNA prepared from a 2-day culture and then gradually decreased with cultivation time. This finding was not consistent with that obtained by the dot-blot hybridization experiment described above. We assume that this might be caused by nonspecific hybridization as observed in the Northern blot. The mRNA species specific to the thiostrepton resistance gene also decreased in quantity, as detected by the dot-blot analysis (Fig. 8) and by the Northern blot analysis (Fig. 9, lanes 6 and 7), although the mRNA was prepared from the mycelium grown in the presence of 40 μg of thiostrepton per ml, and therefore the thio^r gene was

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Pst                                              Pvu                                                        
CTGCAGGCCGGCGACCAGGCGGTCGAGGCCACCTTCGAACTGGGCTACGGCCAGCTGGAACCGGCCCAGGCCCGCGCCTTCCGCCTGCTGGGCCTGGCCG  100

                                                                                          Sal
ACGGCCCGGACATCTCCCTCGCGGCGGCGGCGGCCGTACTGGACCTCCCGGCGCAGGACACGGAGGACCTCCTGGAGTCCCTGGTCGACACCTCGCTGCT  200

         Sma
CGAATCGGCGGCCCCGGGCCGCTACCGCTTCCACGACCTGGTCCGCCTCTACGCGCGTGCCTGCGCGGAACGGACGGAACGGGACGGGAACGCGCCGAGC  300

                                              ┌──►                            Sau
GAGCGGGGGGCGGCGCTGTCGCGGCTGCTGGACTTCTACCTGGCGACGGCGGCGGGGGTGTACGCGATCGAGCGGCCGGGGGACCGGCTGGTGGACGGCC  400

         Sma
TGGAGCCGACGGAGTACCCGGGGCTGACGTTCACCGAGGGCTCGGCGGCGCTGGACTGGCTGTACACGGAGGCCGCGCCGCTGCTGGCGTGCGTGCGGCA  500
                                                                         ──────────────────────────►

                           Sau
GTCCGCCGGGACGGCCAGGCTGCGGCGGGCGGTGGATCTGCTGTGGGCGGCCAAGGACCTGACGGAGTCCGGCGCGAACTCACACCAGTACGAGGCGACG  600
──────◄

GCCAGGGCGATGTGCGACGCCACCGGTTCCGCTGCCGGACACCCGCGCCGAGGGAAGGGCTCGCACCGTTCTCAGCGATGTGCTCCTGGTGTCCGGTCGC  700
                                                                                MetCysSerTrpCysProValAla

       Sph
ATCGAGCATGCCGAGGAAGAGGCACCGGCTGGCCATGCGGCTGGCGGGCTCCGCCGAGGACTCGGCCGCCGTGAGCTGGGTCGCCAACAACCGAGGACTC  800
     SerSerMetProArgLysArgHisArgLeuAlaMetArgLeuAlaGlySerAlaGluAspSerAlaAlaValSerTrpValAlaAsnAsnArgGlyLeu

GTCTGCCTGCACCAGCGCCGGTACGCCGAGGGCAAGGGCCTCTTCCACCAGGCCATCGCGGGTTTCCGGGCAACGGACAACCGCGCGGGCGAGGCGTCCG  900
ValCysLeuHisGlnArgArgTyrAlaGluGlyLysGlyLeuPheHisGlnAlaIleAlaGlyPheArgAlaThrAspAsnArgAlaGlyValuAlaSerAla

                                                              Sal
CGTTGTCGAACCTCTCCCGTGCCCAGCTCGGCATGGGCAACGTCGCGGAGGCCGTCGACATCGCCCGGCAAGGACTCGCGGTGTACGCCGAGCTGGGCCG  1000
  LeuSerAsnLeuSerArgAlaGlnLeuGlyMetGlyAsnValAlaGluAlaValAspIleAlaArgGlnGlyLeuAlaValTyrAlaGluLeuGlyArg

CACCATGCGCCTCGCCAACGGCCACTTCGCCCTGGGAGTGGCACTGACCAGGGCAGGCCGGCACGAAGAGGCACTGGGCGAGTTCGCCGAGGCGCTGGAC  1100
  ThrMetArgLeuAlaAsnGlyHisPheAlaLeuGlyValAlaLeuThrArgAlaGlyValArgHisGlyGluGluAlaLeuGluGlyValuPheAlaGluAlaLeuAsp

CTCTTCGGGGACCACCGGCAGCGCCTGTGGGAGGGCGCCACCAACTTCCGCCTCGCCGAGGTGCACCTGGCCGCCGGACGCCCCTCCTCGGCGGCCCAGC  1200
  LeuPheGlyAspHisArgGlnArgLeuTrpGluGlyValAlaThrAsnPheArgLeuAlaGlyLeuValHisLeuAlaAlaGlyValArgProSerSerAlaAlaGlnHis

              Nsi
ACGCCGAGCAGGCACTCGCCCTGGGATGCATCGGGGGCGACCGGATGCGGGGCAACGTCCTGGCTCTGCTGGGACGCGCCCTGTCGGCCCTGGGCCAGGC  1300
  AlaGluGlnAlaLeuAlaLeuGluGlyCysIleGlyGlyAspArgMetArgGlyAsnValLeuAlaLeuLeuGlyValArgAlaLeuSerAlaLeuGlyGlnAla

GGACCGCGCGCGAGCGTGCTGGCGCGAGGCGCTCAGCCTCTACGAGCAGCACGACGCCCAGGAGGTCGGCGAGGTAAGGGCGTTGCTGGCTCGCAGTGTG  1400
  AspArgAlaArgAlaCysTrpArgGluAlaLeuSerLeuTyrGluGlnHisAspAlaGlnGluValGlyGluValArgAlaLeuLeuLeuAlaArgSerVal

              Sal
GCGCGGTGAACCCGGCCGTCGACCGGCGGTAGCCGGAGCGTTCAGCGTTCGTTTATCTCCCCCTGGCACTGTCATCTCCGTCAGACCGTCGCATCGGGGG  1500
AlaArgTRM

GCAGACGGATGACGACCGAGCCCACCAGTTAGTGTGCGGCTCCGCGCGCCCGTCCGGCAGCCTACGGGGGGAGCTACCGGACGGGCGTTGCTACCTCGTCA  1600
                              ────────────────────────────►◄───────────────────

CGACCATGAGGAGCGAAGTCCATGAGCGACAAGATGAAGGACGCGGACGCCACACCGCAGGACAACCACATGCCCACTCCGCCGGCGACGGAAGAGCCCG  1700

                                           Sau
TCACGACGCTCGACAACCACATGCCGAGCGGGCCCGCGAACGAAGCGATC                                                1750
```

FIG. 5. Nucleotide sequence of afsB, numbered from the unique PstI site, is shown together with the putative AfsB protein deduced from the nucleotide sequence as well as inverted complementary repeat sequences. The thick arrow indicates the 5' end of the mRNA as determined by S1 mapping. The wavy lines indicate amino acid sequences having a resemblance to DNA-binding domains in known DNA-binding proteins.

transcribed throughout growth. This may be due to the experimental condition that the amounts of RNA used as the targets were adjusted without taking notice of the ratio of rRNA and tRNA in the RNA preparation. It therefore seems reasonable to conclude that the afsB gene is transcribed throughout growth; i.e., constitutively, on the basis of the results of the Northern blot experiment, because in estimating the quantity of afsB mRNA by this method we can exclude most of the nonspecific hybridization.

As the smaller mRNA species of 880 bp had been expected to be derived from a region downstream of the presumptive

termination codon, we carried out a similar Northern blot experiment using a ^{32}P-labeled Sau3A fragment (ca. 180 bp) which is located about 85 bp away from the Sau3A site at nt 1750. Again, a strongly hybridized band with the same molecular size as that detected in the former experiment was observed (Fig. 9, lane 8). These results clearly showed that another unknown gene with a transcriptional unit different from afsB existed downstream of afsB.

afsB does not affect transcription of the brown pigment production genes. The afsB gene regulates the production of actinorhodin and undecylprodigiosin, both of which are
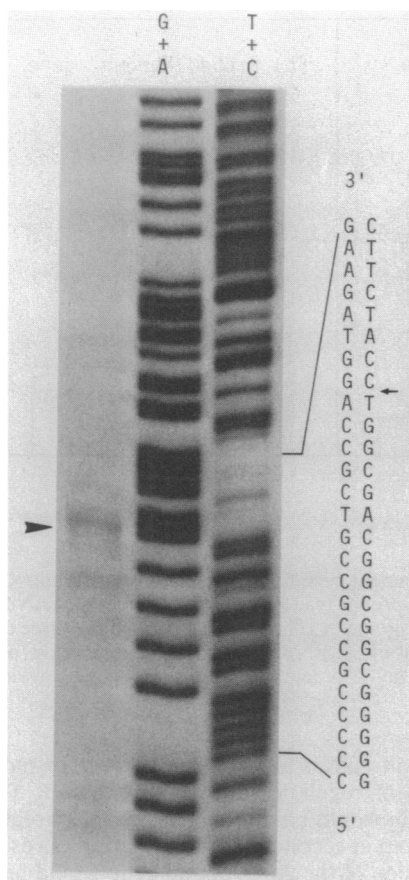
FIG. 6. Position of the 5' terminus of the *afsB* mRNA as based on S1 nuclease protection mapping with total RNA prepared from a 3-day-old culture of "*S. lividans*"(pIJ41-AP3) and the slow-moving strand of the 206-bp *Ava*I fragment. The S1-protected fragment (left lane) was analyzed in parallel with the sequencing ladders (G+A, T+C) of the 5'-end-labeled coding strand. Arrows indicate the position of the mainly protected band 80 bp in length.

supposedly biosynthesized via a polyketide (11). The brown pigment genes on pARC1, cloned from *S. coelicolor* A3(2), had been thought to code for biosynthetic enzymes for polyketides on the basis of the structure of the brown pigment (18), although it was not clear to what extent the pigment genes actually participate in the biosynthesis of actinorhodin and undecylprodigiosin. "*S. lividans*" possesses a DNA sequence significantly homologous to the pigment genes of *S. coelicolor* A3(2) (18). To test for the possibility that the *afsB* gene product stimulates transcription of polyketide biosynthetic genes in "*S. lividans*," we performed a dot-blot hybridization experiment using the brown pigment production genes on pARC1 as the probe. Contrary to our expectation, the intensity of hybridization

was not influenced by the presence of the *afsB* gene on the vector pIJ41 (Fig. 8c).

Distribution of *afsB* sequence among *Streptomyces* spp. We examined the distribution of sequences homologous to *afsB* among *Streptomyces* spp. by Southern blot DNA-DNA hybridization. The *Sau*3A fragment (nt 538–1750) was used as the ^{32}P-labeled probe, and chromosomal DNAs digested with *Bam*HI were used as the targets. Sequences homologous to the *afsB* gene with various extents of homology were found in *Streptomyces albus* IFO 3195, *Streptomyces flaveolus* IFO 3408, *Streptomyces antibioticus* IFO 3126, and *S. antibioticus* IFO 12838, as well as strains of "*S. lividans*" and *S. coelicolor* (Table 2).

Among the streptomycin-producing organisms, namely, *S. bikiniensis* IFO 13350, *S. griseus* IFO 13189, and *S. griseus* FT-1, only *S. griseus* FT-1, which is a high streptomycin producer, was found to contain a sequence considerably homologous to the *afsB* gene. On the other hand, the sequence homologous to the probe in this strain was completely eliminated in the A-factor-deficient mutants *S. griseus* FT-1 number 2, obtained by UV irradiation, and *S. griseus* FT-1 AO-1, obtained by an acridine orange treatment (Fig. 10). These results strongly suggest that the entire sequence homologous to *afsB* in *S. griseus* FT-1 is easily lost by so-called "curing" treatments. Such behavior is similar to that of the *afsA* gene, which may encode a biosynthetic enzyme for A-factor (20) in these organisms.

DISCUSSION

The cloned *S. coelicolor* A3(2) *afsB* gene regulates in a positive manner the biosynthesis of A-factor, actinorhodin, and undecylprodigiosin. In an *afsB* mutant of *S. coelicolor* A3(2), production of a calcium-dependent antibiotic (25) and methylenomycin (24), in addition to the above metabolites, appears to be significantly reduced (D. A. Hopwood, personal communication). These observations suggest a stimulatory function of *afsB* in the formation of secondary metabolites. The biosynthetic genes for these metabolites, except for methylenomycin, are dispersed on the chromosomal linkage map (13, 16, 34, 35). The whole pathway for methylenomycin biosynthesis is located on a fertility plasmid (1, 43). In addition, the chemical structures of these substances are totally different. Therefore, we have assumed that a cytoplasmic regulator, possibly a protein, encoded by the cloned *afsB* gene plays a regulatory role in the biosynthesis of these secondary metabolites. In agreement with the assumption, the nucleotide sequence of *afsB* predicted the presence of a protein consisting of 243 amino acids. Analysis of the *afsB* coding sequence revealed a highly biased codon usage feature characteristic of the high G+C composition (73 mol%). The high G+C composition is reflected in the third codon position and somewhat in the first codon position, consistent with the observations on the *aph* and *vph* genes (4, 6).

The most striking feature of the AfsB protein, inferred from the amino acid sequence, is the presence of two regions
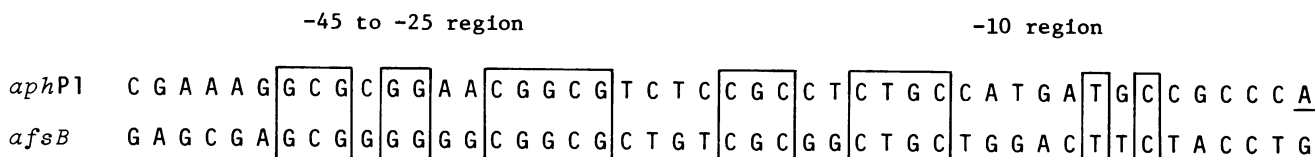

FIG. 7. Alignment of promoter sequences of the *afsB* and *aph* genes. The underlined nucleotides indicate transcriptional start points. Identical nucleotides are enclosed by boxes.
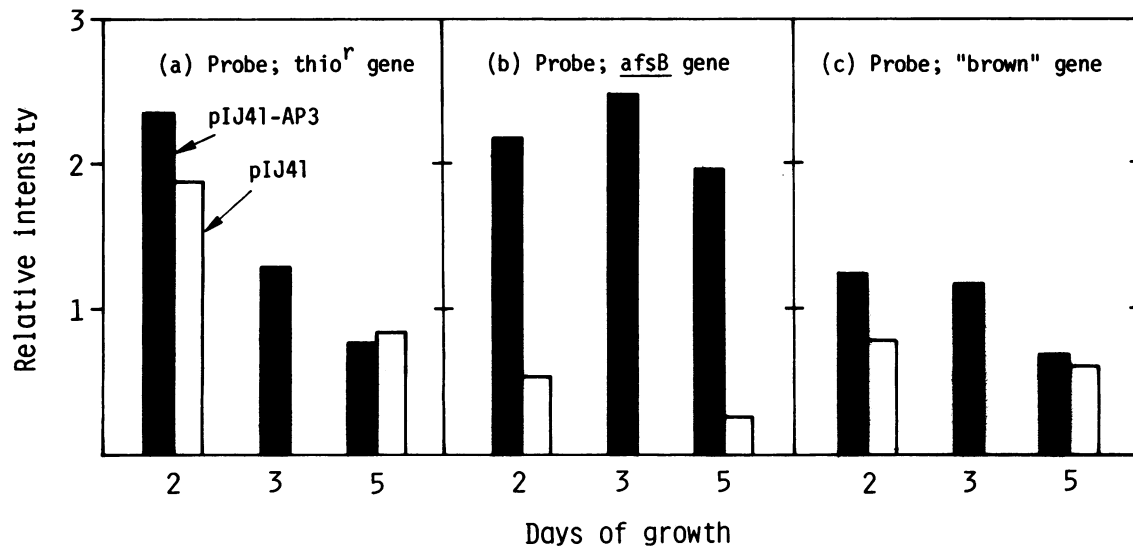
FIG. 8. Dot-blot analysis of mRNA specific to *tsr* (a), *afsB* (b), and the brown pigment gene (c). Three different amounts of RNA (2, 1, and 0.2 μg) baked onto a sheet of nitrocellulose paper were hybridized with the three probes (ca. 5×10^6 cpm). The radioactivity hybridized to each dot was calculated from the area obtained by integration of the peak with a densitometer. The radioactivities of the three dots, in which different amounts of RNA are contained, showed linearity with respect to the amount of the RNA spotted. Solid and hollow bars represent relative radioactivities obtained by hybridization with the respective probes with the RNAs prepared from "*S. lividans*" carrying pIJ41-AP3 and pIJ41, respectively, grown for the indicated number of days.

that resemble the consensus sequence for the two α-helices containing the main DNA-binding proteins, such as the repressors and *cro* proteins of phages λ and P22 (36). Both regions of AfsB contain the pattern Ala-$(N)_3$-Gly-$(N)_5$-Leu(Ile) common to DNA-binding proteins. The presence of two regions bearing a resemblance to known DNA-binding

domains leads us to postulate an attractive model for the mechanism of regulation displayed by *afsB*. The putative AfsB protein would bind to some regulatory region of a key gene involved in secondary metabolism, as a result of which the expression of the AfsB-bound gene would be stimulated or decreased, leading to the "switching on" of several genes
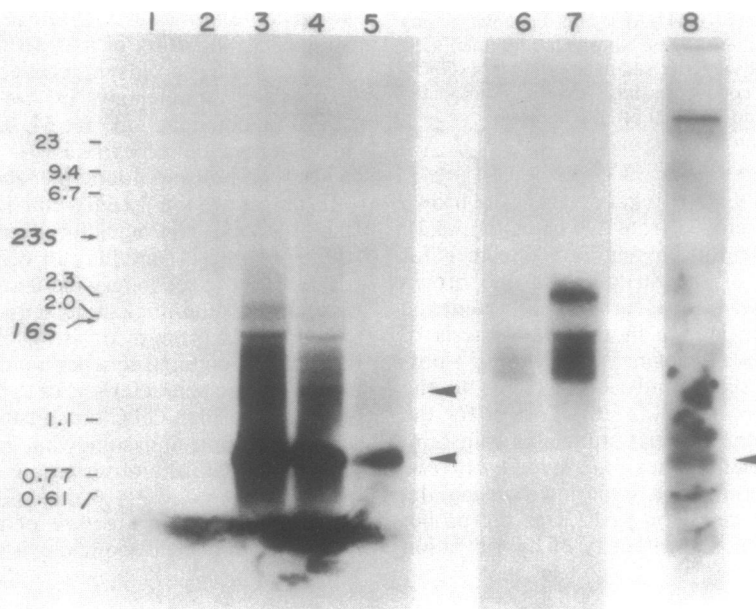

FIG. 9. Northern blot analysis of *afsB* and *tsr* mRNAs. The left panel shows hybridization patterns between the nick-translated 1,230-bp *Sau*3A fragment (nt 538–1750) containing the whole coding sequence and various RNAs (lanes 1 and 2, RNAs prepared from "*S. lividans*" carrying pIJ41 grown for 2 and 5 days, respectively; lanes 3 through 5, RNAs from "*S. lividans*" carrying pIJ41-AP3 grown for 2, 3, and 5 days, respectively). The middle panel shows hybridization patterns, as a control, between the nick-translated thio^r gene sequence and the RNAs (lanes 6 and 7, RNAs from "*S. lividans*" carrying pIJ41 grown for 5 and 2 days, respectively). The right panel shows the hybridization pattern between the 180-bp *Sau*3A fragment and RNA prepared from "*S. lividans*" carrying pIJ41-AP3 grown for 3 days. RNA separation by agarose gel electrophoresis in each panel was done at different times. The numbers beside the left panel indicate the size (in kilobases) of internal standards. Two distinct bands of 1300 and 880 bp are indicated by arrowheads.

to let the host cell begin the formation of secondary metabolites. Or more directly, the AfsB protein would bind to part of the biosynthetic genes for the secondary metabolites and stimulate expression. To reveal the mechanism of regulation exerted by the *afsB*, further investigation, such as isolation of the hypothesized *afsB*-dependent genes and estimation of mRNA species complementary to the actinorhodin and undecylprodigiosin biosynthetic genes in the presence and absence of *afsB*, is necessary. The dot hybridization experiment with the brown pigment production genes as the probe, which was easy to carry out with materials we had obtained at that time, was one such approach, although the results were contrary to our expectation. Also done was a dot matrix analysis at the protein level (31) between the putative AfsB protein and other pleiotropic proteins with features similar to AfsB, such as *Bacillus subtilis* Spo0A and Spo0F, involved in sporulation (42), and *E. coli* OmpR, involved in osmoregulation (44). However, we could not find any similarity between AfsB and the others.

An additional point we would like to make is that, as Merrick and Gibbins pointed out (29), several functionally related proteins, i.e., *E. coli* RpoD (7) and HtpR (26, 45), *B. subtilis* RpoD (9) and SpoIIG (39), *B. subtilis* bacteriophage SPO1 gene product 34 (8), and *Klebsiella pneumoniae* NtrA (14, 29), all of which are RNA polymerase sigma factors, contain two potential DNA-binding sites separated by 40 to 100 residues. There is no significant similarity at the protein

TABLE 2. Cross-homology with the *S. coelicolor* A3(2) *afsB* gene of various actinomycetes

Organism and strain	Hybridization	Size (kb) of hybridized bands[a]
Streptomyces albus IFO 3195	+	15
S. albus IFO 3422	−	
S. albus IFO 3710	−	
S. antibioticus IFO 3126	+	>24, 9.0
S. antibioticus IFO 12652	−	
S. antibioticus IFO 12838	+	>24, 9.0
S. bikiniensis IFO 13350	−	
"*S. blastmyceticus*" IFO 12747	−	
S. coelicolor IFO 3114	+	15
S. coelicolor A3(2) A700	+	23
S. coelicolor A3(2) BH2	+	23
S. coelicolor A3(2) BH6	+	23
S. flaveolus IAM 0117	−	
S. flaveolus IFO 3408	+	12
S. fradiae ATCC 21096	−	
S. globisporus IFO 12208	+	7.5
S. globisporus IFO 12209	−	
S. griseoflavus IFO 12372	−	
S. griseus IFO 13189	−	
S. griseus FT-1	+	20
S. griseus FT-1 number 2	−	
S. griseus FT-1 AO-1	−	
"*S. lividans*" TK21	+	23
"*S. lividans*" HH21	+	23
S. sindenensis IFO 12915	−	
S. viridochromogenes IFO 12337	−	
S. viridochromogenes IFO 12338	−	
S. viridochromogenes IFO 12376	−	
S. viridochromogenes IFO 12377	−	
"*Actinomyces fluorescens*" IFO 12861	−	
"*A. citreofluorescens*" IFO 12853	−	
Nocardia brasiliensis	−	

[a] The molecular weights of the hybridized bands were determined by using *Hind*III digested λ DNA as standard markers.
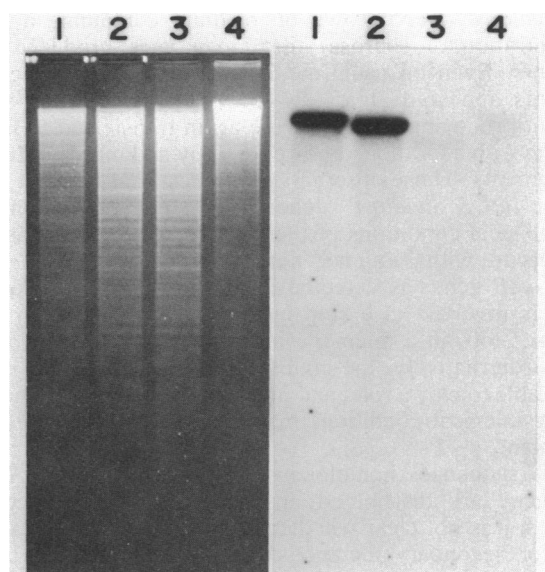

FIG. 10. Agarose gel electrophoresis of *Bam*HI-digested chromosomal DNAs (left panel) and autoradiogram of DNA-DNA hybridizations between the *afsB* coding sequence and chromosomal DNAs (right panel). Lanes 1, *S. coelicolor* A3(2) M130 as a positive control; lanes 2, *S. griseus* FT-1, A-factor⁺ (*afsA*⁺); lanes 3, *S. griseus* FT-1 number 2, A-factor⁻ (Δ*afsA*); and lanes 4, *S. griseus* FT-1 AO-1, A-factor⁻ (Δ*afsA*).

level between the AfsB protein and the above sigmas. The sigma proteins are relatively acidic, but AfsB is not. The sigma factors have a role in promoter selection during formation of the RNA polymerase-promoter complex. It is tempting to speculate that the two possible DNA-binding domains of the AfsB protein may play a role in recognizing a specific DNA sequence where it is bound.

The mRNA start point of *afsB* is far from the putative start codon, which explains why a long nucleotide sequence upstream of the *afsB* coding region is required. A puzzling finding is that pIJ41-ΔSau and pIJ702-ΔSau lacking a 169-bp *Sau*3A fragment located between the mRNA start point and the presumptive start codon failed to confer the AfsB⁺ phenotype to the host strains. There is no open reading frame which has ATG or GTG codons at the N-terminal end and the translational termination codons at the C-terminal end on both strands within the region from the *Pst*I to the *Sph*I sites (nt 1–710). The inverted repeat with a very low ΔG value present in the 169-bp *Sau*3A fragment may have some function in transcription or in translation. If so, either transcription of the *afsB* gene or translation of the *afsB* mRNA would occur in a way characteristic of *afsB*, since the whole *afsB* coding sequence exhibited the AfsB⁺ phenotype when it was connected downstream from the *aph* promoter. At any rate, a fine deletion analysis of this region seems to be necessary to make clear the puzzling finding.

The identification of a second gene with an apparently different transcriptional unit located downstream of the *afsB* gene raised the question of its function. The amount of the mRNA specific to the unknown gene was extraordinarily high throughout growth. Nucleotide sequencing and determination of the phenotype of mutations in this region will provide information on the gene.

In "*S. lividans*," the biosynthetic pathways for actinorhodin and undecylprodigiosin are normally silent or expressed at very low levels. On the other hand, when both wild-type strain TK21 and A-factor-deficient mutant strain HH21 of

"*S. lividans*" were grown on medium containing a high concentration of sucrose, they sometimes produced the pigments. Even on usual medium, colonies producing the pigments appeared at a very low frequency. When such pigment-producing colonies were again transferred to fresh medium with a toothpick, however, they no longer produced the pigments. These observations suggest that pigment production in "*S. lividans*" depends, to some extent, on the physiological conditions of the cells. It is possible that the counterpart with sequence homology to the *S. coelicolor* A3(2) *afsB* gene, as detected in the Southern blot experiment, is involved in a step in pigment production in "*S. lividans*" too, in a manner similar to that in *S. coelicolor* A3(2). Alternatively, the counterpart in "*S. lividans*" may not be able to play a role, and pigment production dependent on physiological conditions may be caused without its involvement.

DNA sequences homologous to the *S. coelicolor* A3(2) *afsB* gene are distributed among *Streptomyces* species, although it is not clear whether they represent a regulatory gene for secondary metabolism in those strains. It is of particular interest that a DNA sequence strongly homologous to *afsB* is present in a high streptomycin-producing strain, *S. griseus* FT-1, and that the sequence is completely eliminated by plasmid curing treatments, such as treatment with acridine orange and growth at high temperatures. The *afsA* gene of this strain, which we assume codes for an A-factor biosynthetic enzyme, also behaved in a similar way; the *afsA* gene, possibly carried by a plasmid, is easily eliminated by curing treatments, which leads to loss of the ability to produce streptomycin and to form spores (13, 20). We infer that the sequence homologous to *afsB* is also carried either on the same plasmid carrying *afsA* or on a different plasmid. In contrast to A-factor biosynthesis in *S. coelicolor* A3(2), the *afsA* gene cloned from *S. bikiniensis* did not require the function of *afsB* for its expression (20). Possible regulatory roles of the *afsB*-like sequence in *S. griseus* FT-1 should be clarified by further experiments.

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