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Self-Cloning in *Streptomyces griseus* of an *str* Gene Cluster for Streptomycin Biosynthesis and Streptomycin Resistance

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An *str* gene cluster containing at least four genes (*strR*, *strA*, *strB*, and *strC*) involved in streptomycin biosynthesis or streptomycin resistance or both was self-cloned in *Streptomyces griseus* by using plasmid pOA154. The *strA* gene was verified to encode streptomycin 6-phosphotransferase, a streptomycin resistance factor in *S. griseus*, by examining the gene product expressed in *Escherichia coli*. The other three genes were determined by complementation tests with streptomycin-nonproducing mutants whose biochemical lesions were clearly identified. *strR* complemented streptomycin-sensitive mutant SM196 which exhibited impaired activity of both steptomycin 6-phosphotransferase and amidinotransferase (one of the streptomycin biosynthetic enzymes) due to a regulatory mutation; *strB* complemented strain SD141, which was specifically deficient in amidinotransferase; and *strC* complemented strain SD245, which was deficient in linkage between streptidine 6-phosphate and dihydrostreptose. By deletion analysis of plasmids with appropriate restriction endonucleases, the order of the four genes was determined to be *strR-strA-strB-strC*. Transformation of *S. griseus* with plasmids carrying both *strR* and *strB* genes enhanced amidinotransferase activity in the transformed cells. Based on the gene dosage effect and the biological characteristics of the mutants complemented by *strR* and *strB*, it was concluded that *strB* encodes amidinotransferase and *strR* encodes a positive effector required for the full expression of *strA* and *strB* genes. Furthermore, it was found that amplification of a specific 0.7-kilobase region of the cloned DNA on a plasmid inhibited streptomycin biosynthesis of the transformants. This DNA region might contain a regulatory apparatus that participates in the control of streptomycin biosynthesis.

Streptomycin, *N*-methyl-α-L-glucosamine(1″→2′)-α-L-streptose(1′→4)streptidine, is produced by certain strains of *Steptomyces* spp. In batch cultures, streptomycin is accumulated only after cell growth has virtually ceased; this type of nongrowth association is a well-known feature of antibiotic synthesis (5, 19). A majority of the enzymatic reactions dedicated to streptomycin biosynthesis have been identified through extensive studies by a number of investigators (11, 12, 16, 34, 35; Fig. 1). The activities of these streptomycin-biosynthetic enzymes are coordinately expressed just before the onset of streptomycin accumulation (35).

In addition to the biosynthetic enzymes, the streptomycin-producing strains synthesize streptomycin 6-phosphotransferase, which catalyzes the reaction step N in Fig. 1, to protect themselves from the suicidal action of the drug (24, 31). Streptomycin 6-phosphotransferase activity appears together with the streptomycin-biosynthetic enzymes and increases in parallel with streptomycin accumulation (24).

Cloning of the *str* genes involved in streptomycin biosynthesis and streptomycin resistance would provide an approach to understanding their genetic structure and the regulatory mechanisms. We have already developed a host-vector system in *Steptomyces griseus* ATCC 10137, a streptomycin producer (21, 23), and have isolated a series of well-characterized streptomycin-nonproducing mutants (22). In this report we describe the cloning and analysis of four contiguous *str* genes, including a regulatory gene, by using these mutants as recipients.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *S. griseus* strains used in this study are listed in Table 1. pOA154 (13.7 kilobases [kb]), carrying a tetracycline resistance gene of *Streptomyces rimosus* (23), was used as a vector plasmid in *S. griseus*. The copy number of pOA154 was assessed to be about 20 copies per chromosome by referring to the copy number of its parental plasmid, pOA15 (21). For the construction of recombinant plasmids in *Escherichia coli*, *E. coli* C600-1 (10) and pBR322 (3) were used.

Bacillus subtilis ATCC 6633 and *B. subtilis* MI113 harboring pTB90 (which confers tetracycline resistance to the host) (9) were used as indicator bacteria in the bioassay of streptomycin.

Media. Nutrient agar and antibiotic medium no. 5 were purchased from Difco Laboratories, Detroit, Mich. Trypticase soy broth was from BBL Microbiology Systems, Cockeysville, Md. Glycerol-asparagine medium (28), GMP medium (6), R2YE medium (4), and LB medium (20) have been described previously.

Preparation of plasmid and chromosomal DNA. For the isolation of *Streptomyces* plasmids, *S. griseus* strains harboring plasmids were grown in Trypticase soy broth containing 0.8% glycine and 20 μg of tetracycline per ml, and the rapid alkaline extraction method was performed as described previously (23). Chromosomal DNA of *S. griseus* was isolated by lysozyme-pronase-RNase treatment and phenol extraction as described elsewhere (23). *E. coli* plasmids were isolated by the modified alkaline lysis method after chloramphenicol amplification (18).

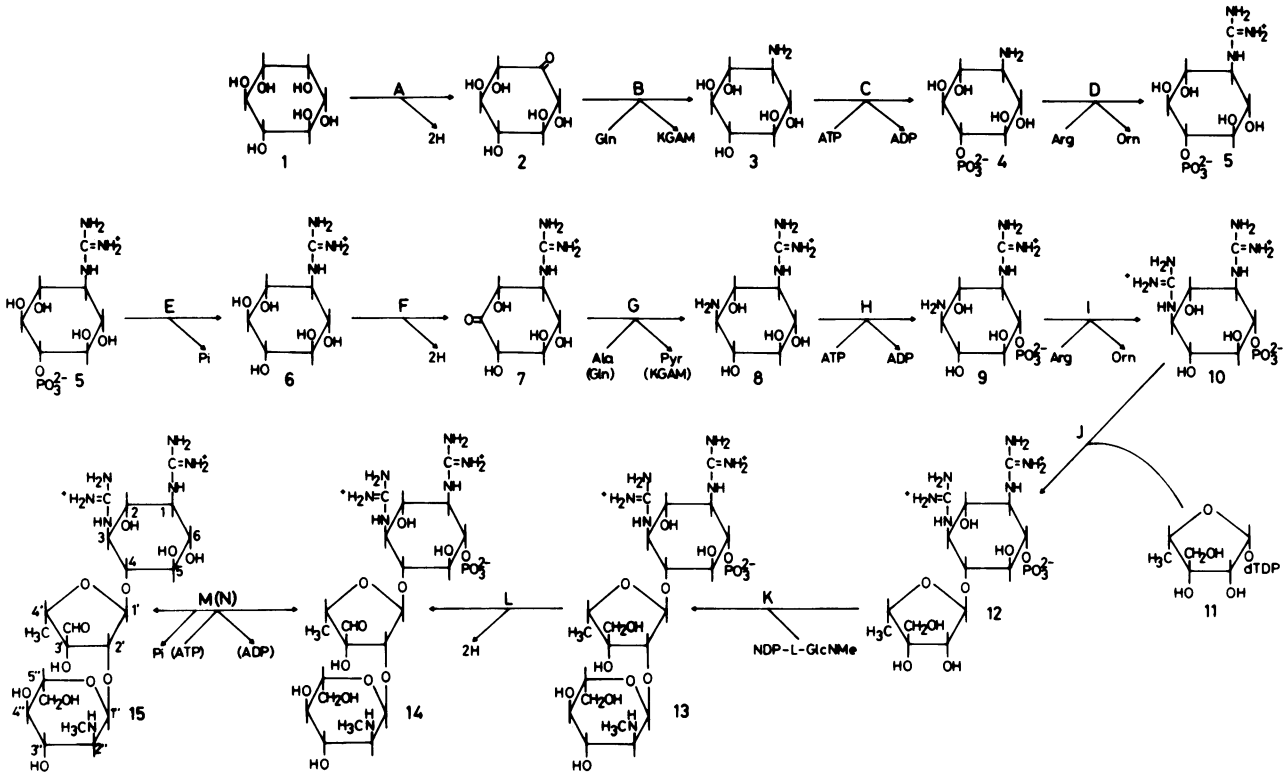

FIG. 1. Biosynthetic pathway of streptomycin (11, 12, 16, 34, 35). Compounds: 1, *myo*-inositol; 2, *scyllo*-inosose; 3, *scyllo*-inosamine; 4, *scyllo*-inosamine 4-phosphate; 5, amidino-*scyllo*-inosamine 4-phosphate; 6, amidino-*scyllo*-inosamine; 7, amidino-3-keto-*scyllo*-inosamine; 8, amidinostreptamine; 9, amidinostreptamine 6-phosphate; 10, streptidine 6-phosphate; 11, dTDP-L-dihydrostreptose; 12, *O*-α-L-dihydrostreptose(1′→4)streptidine 6-phosphate; 13, dihydrostreptomycin 6-phosphate; 14, streptomycin 6-phosphate; 15, streptomycin; KGAM, α-ketoglutaramate; Orn, ornithine; Pyr, pyruvate; NDP-L-GlcNMe, NDP-*N*-methyl-L-glucosamine.

Both plasmid and chromosomal DNA were further purified by cesium chloride-ethidium bromide equilibrium density gradient centrifugation.

DNA manipulation. Treatment of DNA with restriction endonucleases and ligation of DNA with T4 DNA ligase were performed by the protocol recommended by the suppliers. To prevent recircularization of the vector plasmid, bacterial alkaline phosphatase treatment was done at 53°C overnight in 10 mM Tris-hydrochloride buffer (pH 8.0) containing 1 mM $MgCl_2$. *Bgl*II linkers were phosphorylated with T4 polynucleotide kinase at 22°C overnight in 66 mM Tris-hydrochloride buffer (pH 7.6) containing 1 mM ATP, 1 mM spermidine, 10 mM $MgCl_2$, 15 mM dithiothreitol, and 1.2 mg of bovine serum albumin per ml. Purification of DNA fragments cleaved with restriction endonuclease was performed by using low-melting-temperature agarose (36). Agarose gel electrophoresis was carried out in Tris-acetate buffer (9) with *Hin*dIII digests of phage λ DNA used as size standards.

TABLE 1. *S. griseus* strains used in this study

Strain	Phenotype (biochemical lesion steps)[a]	Reference or source
ATCC 10137	Wild type	
SD20	Str⁻ (B, G)	22
SD81	Str⁻ (C, H)	22
SD141	Str⁻ (D, I)	22
SD189	Str⁻ (F)	22
SD245	Str⁻ (J)	22
SD261	Str⁻ (C, H)	22
SD263	Str⁻ (B, G)	22
SD274	Str⁻ (C, H)	22
SM196	Str⁻ Str^s (D, I, N)	This study

[a] Str⁻, unable to produce streptomycin; Str^s, sensitive to streptomycin. Defective step(s) in the biosynthetic pathway to streptomycin are shown in parentheses (see Fig. 1). The two steps of transamination (B, G), phosphorylation (C, H), and transamidination (D, I) seem to be catalyzed by single enzymes, respectively (22).

Transformation. Protoplasts of *S. griseus* were prepared from mycelia grown in Trypticase soy broth containing 0.8% glycine as described elsewhere (23) and were transformed with plasmid DNAs by the method of Thompson et al. (32). After protoplast regeneration on R2YE plates, transformants resistant to tetracycline were selected by replica plating onto nutrient agar containing tetracycline (25 μg/ml) and incubated at 28°C for 4 days. Clones producing steptomycin were identified by overlaying antibiotic medium no. 5 seeded with spores of *B. subtilis* carrying pTB90.

For the transformation of *E. coli*, competent cells were prepared by the calcium chloride procedure (14), and transformants were selected on LB plates containing ampicillin (35 μg/ml) or tetracycline (12.5 μg/ml).

DNA hybridization. Southern hybridization analysis (29), using a ^{32}P-labeled probe made by nick translation with [α-^{32}P]dCTP (26) was performed in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C, and filters were washed four times with 3× SSC at 65°C.

Enzyme assays. Spores of *S. griseus* strains were inoculated into 20 ml of GMP medium and incubated at 28°C on a rotary shaker. The fully grown preculture (5 ml) was transferred to 100 ml of GMP medium and incubated at 28°C to the early stationary phase. With strains carrying plasmids, tetracycline (20 μg/ml) was added to the medium to ensure stable maintenance of the plasmid. Mycelia were harvested by filtration and washed with distilled water. To the washed pad (0.5 to 1.0 g) was added 1.5 ml of distilled water, and cell extracts were prepared by sonication (33). For the preparation of cell extracts of *E. coli* strains, cells grown to an optical density at 550 nm of about 1.0 in 100 ml of LB medium supplemented with appropriate antibiotics were centrifuged, suspended in 1 ml of distilled water, and sonicated.

Assays of streptomycin-inactivating activity and streptomycin 6-phosphotransferase activity were carried out essentially as described by Piwowarski and Shaw (24). The reaction mixture for the inactivation of streptomycin consisted of 50 μl each of cell extract (about 30 mg of protein per ml), 1 mM streptomycin sulfate solution, 40 mM ATP solution (pH 7.6), and 10 mM Tris-hydrochloride buffer (pH 7.8) containing 100 mM $MgCl_2$. After a 1-h incubation at 30°C, the mixture was heated at 100°C for 5 min. On a paper disk (8 mm in diameter) was spotted 50 μl of the mixture, and the remaining active streptomycin was biologically assayed with *B. subtilis* ATCC 6633.

The reaction mixture for the streptomycin 6-phosphotransferase assay contained 5 μl each of 4 mM streptidine sulfate solution, 2.6 mM [γ-^{32}P]ATP solution (38 Ci/mol), cell extract (about 30 mg of protein per ml), and 0.5 M glycyl-glycine buffer (pH 8.0) containing 40 mM $MgCl_2$; and the reaction was carried out at 30°C for 1 h. After heating at 100°C for 5 min, 10 μl of the reaction mixture was subjected to paper chromatography with a solvent system of 80% (vol/vol) phenol and an NH_3 atmosphere for the separation of reaction products (33). The paper strips were cut at 1-cm intervals, and radioactivity was counted with a liquid scintillation system. One unit of streptomycin 6-phosphotransferase is defined as the amount that catalyzes the formation of 1 nmol of streptidine 6-phosphate per h at 30°C.

For assay of amidinotransferase activity, transamidination from L-arginine to hydroxylamine, yielding hydroxyguanidine, was measured by the method of Walker (33). This assay works well with enzyme(s)-catalyzing reaction steps D and I in Fig. 1 (33). One unit of amidinotransferase is defined as the amount that catalyzes the formation of 1 μmol of hydroxyguanidine per h at 37°C. The protein concentration was determined by the method of Lowry et al. (15), using bovine serum albumin as the standard.

Purification of amidinotransferase. Spores of *S. griseus* ATCC 10137 were inoculated into 100 ml of GMP medium and incubated at 28°C for 24 h. The preculture (25 ml) was transferred to 500 ml of GMP medium and incubated for 21 h. Mycelia were collected by filtration, washed with 150 ml of a 1 M KCl solution and then with 150 ml of a 0.85% NaCl solution, and stored frozen at −70°C. By the repeated runs of cultivation, 265 g of frozen mycelia were obtained.

Throughout the following operation, temperatures of buffers, samples, etc., were maintained below 5°C. The mycelia (265 g) were suspended in 530 ml of 50 mM phosphate buffer (50 mM potassium phosphate buffer [pH 7.4] containing 1 mM EDTA and 5 mM β-mercaptoethanol) supplemented with 2 mM phenylmethanesulfonyl fluoride and were disrupted twice through a French press at about 500 kg/cm^2. The homogenate was clarified by centrifugation (27,000 × *g*, 30 min). To the supernatant, a 10% solution of polyethyleneimine was added at a final concentration of 0.3% to remove the nucleic acid. After centrifugation, fractionation with ammonium sulfate between 40 and 70% of saturation was carried out. The precipitate was dissolved in 20 mM phosphate buffer (20 mM potassium phosphate buffer [pH 7.2] containing 1 mM EDTA and 5 mM β-mercaptoethanol) and dialyzed against the same buffer.

The dialyzed sample was loaded onto a DEAE-cellulose column (3.2 by 62 cm). Initially, 1 liter of 20 mM phosphate buffer was passed, and the activity was eluted with 500 ml of 0.3 M NaCl in 20 mM phosphate buffer. Active fractions were pooled and precipitated with ammonium sulfate (70% of saturation). After dialysis against 50 mM phosphate buffer, the sample was loaded onto a Sephadex G-100 column (3.2 by 45 cm) and eluted with 50 mM phosphate buffer. Active fractions were adsorbed onto a DEAE-Toyopearl 650M column (2.0 by 50 cm). The column was washed with 200 ml of 20 mM phosphate buffer, and then the activity was eluted with a linear gradient of NaCl (0 to 0.3 M) in 20 mM phosphate buffer (total 1.5 liters). Amidinotransferase was pooled and stored at −20°C with 10% (vol/vol) glycerol.

SDS-polyacrylamide gel electrophoresis and gel filtration. Analytical polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was carried out by the method of Laemmli (13). Protein in the gel was stained with Coomassie brilliant blue R and destained in methanol-acetate-water (2:3:35 [vol/vol]). The molecular weight of a native amidinotransferase was estimated by gel filtration on a Sephadex G-200 column (1.6 by 90 cm). The mixture of 10 U of the purified amidinotransferase, 2 mg of blue dextran, 4 μmol of dinitrophenylalanine, and 1 mg each of the marker proteins was applied to the column that had been equilibrated with 50 mM phosphate buffer, and was eluted with the same buffer.

Chemicals and enzymes. Reagents were obtained from the following sources: [α-^{32}P]dCTP and [γ-^{32}P]ATP were from New England Nuclear Co., Boston, Mass.; *Aat*I (an isoschizomer of *Stu*I) was from Toyobo Biochemicals, Osaka, Japan; restriction endonucleases other than *Aat*I, T4 DNA ligase, T4 polynucleotide kinase, and *Bgl*II octanucleotide linkers were from Takara Shuzo Co. Ltd., Kyoto, Japan; marker proteins for SDS-gel electrophoresis and gel filtration were from Boehringer GmbH, Mannheim, Federal Republic of Germany; Sephadex G-100 and G-200 were from Pharmacia Fine Chemicals, Uppsala, Sweden; DEAE-cellulose was from Wako Chemical Industries, Osaka, Japan; DEAE-Toyopearl 650M was from Toyo Soda Co., Tokyo, Japan. The sources of other reagents have been described previously (22, 23).

RESULTS

Characterization of a streptomycin-nonproducing, streptomycin-sensitive mutant. Str$^-$ strains with clearly defined mutations would be useful as host cells for the cloning of *str* genes. Nine Str$^-$ mutants used in this study were derived from *S. griseus* ATCC 10137 by UV irradiation. Eight mutants (SD20, SD81, SD141, SD189, SD245, SD261, SD263, and SD274) were streptidine idiotrophs which required streptidine to produce streptomycin, and their biochemical lesions in the biosynthetic pathway to streptomycin are shown in Table 1 (22). An Str^s mutant, SM196, was selected among Str$^-$ mutants that retained the ability to

TABLE 2. Biochemical characterization of strains SM196 and SD141

Strain	Addition of streptomycin to the culture[a]	Streptomycin 6-phospho-transferase activity (U/mg of protein)[b]	Amidinotransferase activity (U/mg of protein)
ATCC 10137	−	13	0.081
ATCC 10137	+	10	0.074
SM196	−	0.0	0.004
SM196	+	3.0	0.004
SD141	−	0.9	0.002
SD141	+	20	0.002

[a] The presence (+) or absence (−) of added streptomycin (10 μg/ml) in GMP medium is shown.

[b] The specific activity of streptomycin 6-phosphotransferase was calculated from a ratio of radioactivity of streptidine 6-phosphate formed (R_f, 0.4) to total radioactivity (for the product plus unreacted ATP).

sporulate (22) and was used in this study because of its intriguing phenotype.

Strain SM196 was Str$^-$ on nutrient agar and in GMP medium and could not grow on glycerol-asparagine medium containing 20 μg of streptomycin per ml, which allowed the growth of the wild-type strain. Its sensitivity to streptomycin was also observed in a liquid culture; when a fully grown preculture of SM196 was transferred to GMP medium containing 20 μg of streptomycin per ml at an inoculum size of 5% (vol/vol), further growth of SM196 was severely inhibited, whereas a comparably grown culture of the wild-type strain exhibited normal growth.

Simultaneous loss of the capability to produce streptomycin and streptomycin resistance inferred a possibility that SM196 might be deficient in biosynthesis of an autoregulating factor, A-factor (2*S*-isocapryloyl-3*S*-hydroxymethyl-γ-butyrolactone), which is essential for streptomycin synthesis, streptomycin resistance, and spore formation in *S. griseus* (6). However, A-factor secreted from a colony of the wild type could restore the capability of producing streptomycin to putative A-factor-defective mutants (characterized as Str$^-$, asporogenic mutants in a previous study [22]) but not to SM196. This, and the ability of SM196 to sporulate, make it unlikely that SM196 is A-factor defective.

It has been reported that streptomycin resistance in streptomycin-producing *Streptomyces* spp. is mediated by inactivation of the drug with streptomycin 6-phosphotransferase (24, 31). Assays of streptomycin 6-phosphotransferase activity were carried out with cell extracts from stationary-phase GMP cultures of the wild-type, SM196, and SD141 strains (Table 2). The activity was streptomycin inducible in Str$^-$ mutant SD141. The possession by the wild-type strain of significant activity even in the absence of added streptomycin may reflect autoinduction by endogenously produced streptomycin (the cultures did indeed produce 10 to 20 μg of streptomycin per ml). Activity was also inducible in the Str$^-$ Str^s mutant SM196, but levels were lower than in SD141 or the wild type. Comparable results were obtained in assays of streptomycin inactivation (data not shown).

Since streptomycin 6-phosphotransferase is not involved in streptomycin biosynthesis, SM196 may well contain biochemical lesions in the streptomycin biosynthetic reactions. We measured amidinotransferase activity as a representative of streptomycin biosynthetic enzymes (Table 2). The Str$^-$ mutants, except for SM196 and SD141, synthesized nearly the same amount of amidinotransferase as the parental strain (data not shown). This fact indicates that amidinotransferase activity is not induced with streptomycin, in contrast to streptomycin 6-phosphotransferase activity. On the other hand, very little amidinotransferase activity was found in SM196 and SD141.

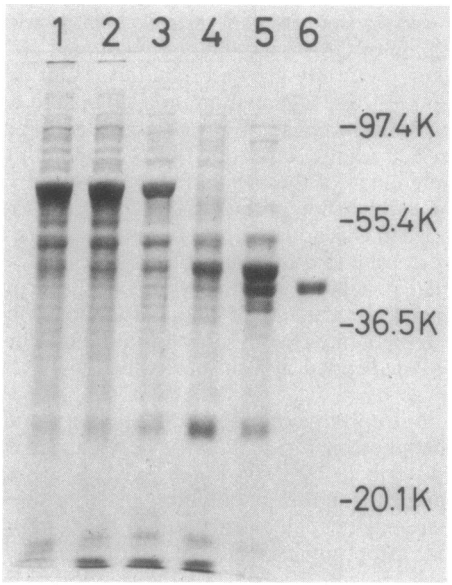

FIG. 2. Purification of amidinotransferase from *S. griseus* ATCC 10137. Protein samples from each step of the purification were subjected to SDS-12.5% polyacrylamide gel electrophoresis. Lanes: 1, crude extract; 2, polyethyleneimine supernatant; 3, salting-out fractions between 40 and 70% ammonium sulfate; 4, DEAE-cellulose column pooled fractions; 5, Sephadex G-100 column pooled fractions; 6, DEAE-Toyopearl 650M column pooled fractions. Marker proteins used are trypsin inhibitor from soybean (molecular weight [MW], 20,100), lactate dehydrogenase (MW, 36,500), glutamate dehydrogenase (MW, 55,400), and phosphorylase *b* (MW, 97,400).

Purification of amidinotransferase. Amidinotransferase was the first target enzyme of the following cloning experiment. Since nothing was known of the characteristics of amidinotransferase, we attempted to purify the enzyme. Figure 2 shows the purification steps of amidinotransferase from *S. griseus* ATCC 10137 by SDS-polyacrylamide gel

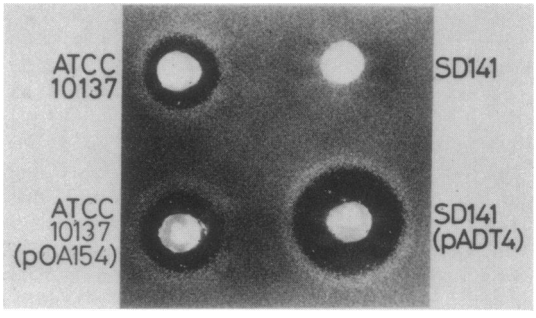

FIG. 3. Restoration of the Str$^+$ phenotype to SD141 by transformation with pADT4. Strains ATCC 10137 and SD141 were cultured on cylindrical plugs (5 mm in diameter by 4 mm in height) of nutrient agar at 28°C for 4 days. For ATCC 10137(pOA154) and SD141(pADT4), tetracycline (25 μg/ml) was added to the agar medium. Biological activity of the streptomycin formed was assayed with *B. subtilis*(pTB90). Note that SD141 which carried pADT4 produced more streptomycin than did the wild-type strain.

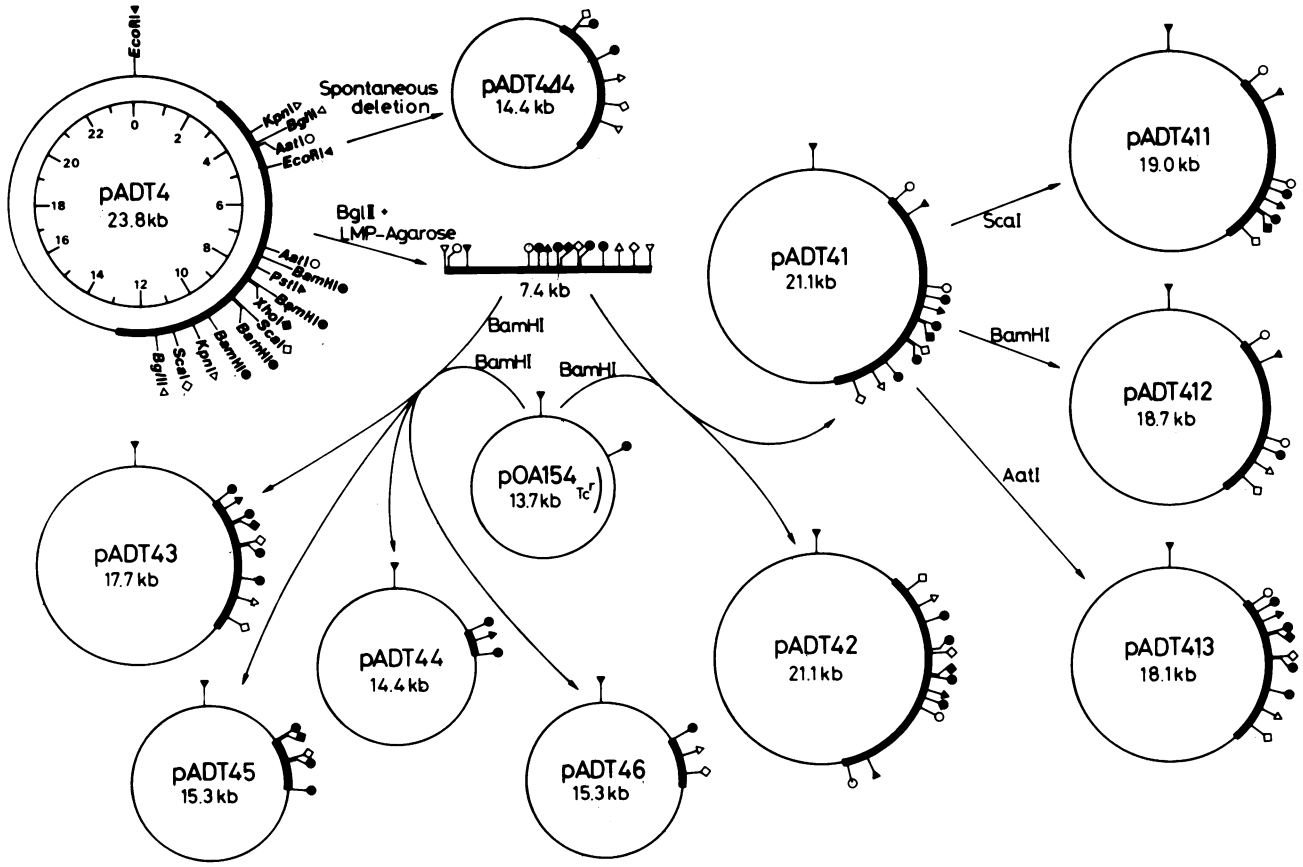

FIG. 4. Restriction endonuclease cleavage map of pADT4 and the construction of its derivatives in *S. griseus*. Heavy lines indicate the DNA inserts derived from the cloned DNA of pADT4. Symbols for the restriction sites are as shown in the restriction map of pADT4. The 7.4-kb *Bgl*II fragment of pADT4 was purified by low-melting-temperature agarose gel electrophoresis and ligated with *Bam*HI-digested pOA154, resulting in pADT41 and pADT42. pADT43, pADT44, pADT45, and pADT46 were constructed by partial digestion of the purified 7.4-kb fragment with *Bam*HI, followed by ligation with *Bam*HI-digested pOA154 at a low DNA concentration (about 5 μg/ml). pADT411, pADT412, and pADT413 were constructed by digestion of pADT41 with the indicated restriction endonucleases, followed by religation of the mixtures. pADT4Δ4 was obtained by an in vivo deletion of about 9.4 kb of DNA encompassing the vector and cloned DNA regions of pADT4.

electrophoresis. We did not observe multiple activities of amidinotransferase during enzyme purification and thus conclude that the single amidinotransferase catalyzed both transamidination steps (steps D and I in Fig. 1) in streptomycin biosynthesis. The final preparation had a specific activity of 11.5 U per mg of protein, which corresponded to about a 70-fold purification from the crude cell extract and a final recovery of about 5%. The apparent molecular weight of the purified amidinotransferase was about 42,000, as determined by SDS-polyacrylamide gel electrophoresis. On Sephadex G-200 gel filtration, the enzyme was eluted at the fraction corresponding to an estimated molecular weight of about 50,000 (data not shown). Hence, the amidinotransferase of *S. griseus* ATCC 10137 appeared to be a monomer.

Cloning of *str* genes. Antibiotic production genes are often clustered (8, 17, 25). Our strategy for cloning *str* genes was to search for a DNA segment capable of complementing the amidinotransferase-deficient mutant SD141 and then to examine the capacity of cloned DNA to complement the other Str^- mutants.

The chromosomal DNA (10 μg) of *S. griseus* ATCC 10137 was partially digested with *Sau*3A into fragments of about 2 to 25 kb in length and mixed with pOA154 (1.0 μg) treated with *Bam*HI and thereafter with bacterial alkaline phosphatase. After phenol extraction and ethanol precipitation, the DNA sample was ligated with T4 DNA ligase in 100 μl of ligation buffer. The ligated DNA was used to transform 4×10^9 protoplasts of SD141. After regeneration of protoplasts and sporulation, spores were replica plated onto nutrient agar containing tetracycline. About 16,000 primary transformants could grow on the plates.

When overlaid with antibiotic medium no. 5 containing spores of *B. subtilis*(pTB90), six clones exhibited an inhibitory zone against the *B. subtilis* strain. Plasmids purified from the six clones were used to transform SD141 again. In all cases, most of the tetracycline-resistant transformants produced antibiotics. Therefore, it was concluded that some genes conferring the ability to synthesize an antibiotic on SD141 were cloned into these recombinant plasmids, which were named as pADT1 to pADT6, respectively. For example, Fig. 3 demonstrates that SD141 recovered the ability to produce an antibiotic when transformed with pADT4.

The restriction endonuclease cleavage maps of these plasmids were constructed (pADT4 is shown in Fig. 4; the maps of the other plasmids are not shown). pADT1, pADT2, pADT3, pADT4, pADT5, and pADT6 had DNA inserts of 14, 2.8, 6.2, 10, 9.0, and 5.4 kb, respectively. As far as their cleavage maps were concerned, none of the DNA inserts shared a common DNA region.

TABLE 3. Streptomycin productivity and specific activities of amidinotransferase and streptomycin 6-phosphotransferase in the transformants of *S. griseus* strains

Plasmid	Streptomycin productivity (μg/colony on agar plug) in:				Amidinotransferase sp act (U/mg of protein) in:			Streptomycin 6-phosphotransferase sp act (U/mg of protein) in:	
	ATCC 10137	SD141	SD245	SM196	ATCC 10137	SD141	SM196	ATCC 10137	SM196
None	0.5	0	0	0	0.081	0.002	0.004	13	0.0
pOA154	0.6	0	0	0	0.066	0.001	0.002	6.4	0.0
pADT4	2.5	2.4	2.4	1.8	ND[a]	0.27	ND	ND	ND
pADT41	2.8	3.2	0	2.3	0.26	0.46	0.38	14	21
pADT42	2.8	3.0	0	2.3	ND	0.36	0.45	ND	28
pADT411	3.4	0	0	1.9	0.084	0.003	0.038	15	13
pADT412	3.3	0	0	2.3	0.12	0.001	0.059	12	13
pADT413	0.3	0.2	0	0	0.081	0.070	0.002	5.8	0.0
pADT43	±[b]	±	0	0	0.020	0.032	0.004	0.8	0.0
pADT4Δ4	0.6	±	0.3	0	0.062	0.032	0.004	ND	ND
pADT44	±	0	0	0	0.014	ND	ND	0.7	ND
pADT45	0.7	0	0	0	0.057	0.002	ND	8.8	ND
pADT46	0.7	0	0	0	ND	0.002	ND	ND	ND

[a] ND, Not determined.

[b] ±, Poor streptomycin production that was below 0.1 μg per colony on an agar plug (a limit of detection by the agar plug method); when tested for streptomycin production by directly overlaying antibiotic medium no. 5 containing spores of *B. subtilis*(pTB90), those transformants (selected by replica plating on nutrient agar with tetracycline) gave rise to a narrow inhibitory zone against the indicator strain.

The activity of amidinotransferase synthesized by the SD141 strains carrying each of the six plasmids was determined by using crude extracts of liquid-grown cultures as the enzyme source. Only in the clone carrying pADT4 was the activity detected, whereas the remaining clones did not contain a detectable amount of the activity, as observed in SD141. This finding means that the antibiotic produced by the clone carrying pADT4 was streptomycin and that the antibiotics produced by the other clones were not streptomycin. Therefore, further investigation was focused exclusively on pADT4.

To examine whether the cloned DNA carried some other streptomycin biosynthesis genes, pADT4 was introduced by transformation into the other streptidine-idiotrophic mutants (SD20, SD81, SD189, SD245, SD261, SD263, and SD274) and the Str^- Str^s mutant (SM196). Tetracycline-resistant transformants of SD245 and SM196 gave rise to an inhibitory zone against *B. subtilis*(pTB90), whereas those of the other mutants did not. This result suggests that pADT4 encodes genes that complement SD245 and SM196 as well as SD141.

Southern analysis of *Bgl*II-digested total DNA samples of *S. griseus* ATCC 10137, SD141, SD245, and SM196, using pSMP1 (constructed by subcloning the 7.4-kb *Bgl*II fragment of pADT4 into a unique *Sca*I site of pBR322 [Fig. 5]) as a probe, showed hybridization solely to a 7.4-kb band in every case (data not shown). Therefore, at least for this 7.4-kb fragment, there are no major deletions or rearrangements in the cloned DNA or in the three mutants.

Subcloning of *str* genes. The cloned genes that complemented SD141, SD245, and SM196 were designated *strB*, *strC*, and *strR*, respectively; *strA* was for a structural gene of streptomycin 6-phosphotransferase (see below). To localize these *str* genes on the cloned DNA and also to assess the effect of amplification of each *str* gene on streptomycin productivity, amidinotransferase activity, and streptomycin 6-phosphotransferase activity, we subcloned various segments of the cloned *str* DNA into the *Bam*HI site of pOA154 (Fig. 4). The resulting derivative plasmids and a spontaneously deleted pADT4 derivative pADT4Δ4 (Fig. 4) were introduced by transformation into strains SD141, SD245, and SM196 as well as into the wild-type strain (ATCC 10137). Streptomycin productivities of the transformants were estimated by the agar-plug method as exemplified in Fig. 3, and activities of amidinotransferase and streptomycin 6-phosphotransferase were assayed (Table 3 and Fig. 6).

The $strB^+$ plasmids that complemented SD141 were pADT41, pADT42, pADT413, pADT43, and pADT4Δ4, which shared a common region from the left end of the insert of pADT4Δ4 to the *Bgl*II(2) site (Fig. 6) (restriction sites appearing more than once on the DNA insert are distinguished by the numbers in paretheses). The molecular size of the *strB* region was about 3.0 kb, which is large enough to encode amidinotransferase (molecular weight, about 42,000). Neither pADT45 nor pADT46 could confer the ability to synthesize amidinotransferase on SD141, suggesting that the *Bam*HI(4) site is located in an essential region of the *strB* gene. Only pADT4 and pADT4Δ4 conferred the ability to produce streptomycin (Str^+ phenotype) on SD245, indicating that part or all of *strC* is to the right of the *Bgl*II(2) site (Fig. 6). pADT41, pADT42, pADT411, and pADT412 could transform SM196 for the Str^+ phenotype (Table 3). It was confirmed by enzyme assays that these $strR^+$ plasmids restored the ability of SM196 to synthesize both amidinotransferase and streptomycin 6-phosphotransferase (Table 3). The *strR* region spanned the restriction fragment (about 3.3 kb) from *Bgl*II(1) to *Bam*HI(1) (Fig. 6).

Since the recovery of the pleiotropic defects in SM196 by *strR* implied that *strR* might be a regulatory gene, it was important to determine whether *strR* encoded a gene product. SM196 carrying pADT412 ($strR^+$ *strA strB strC*) (see below for *strA* of the plasmid) was grown on nutrient agar without tetracycline, and after sporulation spores were suspended in distilled water and plated on nutrient agar. A total of 30 tetracycline-sensitive segregants (obtained at a frequency of 30%) were Str^-. This result indicates that restoration of the Str^+ phenotype to SM196 with pADT412 does not require homologous recombination between the cloned DNA on the plasmid and the chromosome of the mutant. Therefore, we conclude that the *strR* gene encodes a *trans*-acting substance.

The transformants harboring either pADT41 or pADT42, irrespective of the host strains, expressed much more (three- to sixfold) amidinotransferase activity than the wild-type strain (Table 3). This result suggests that a structural gene of

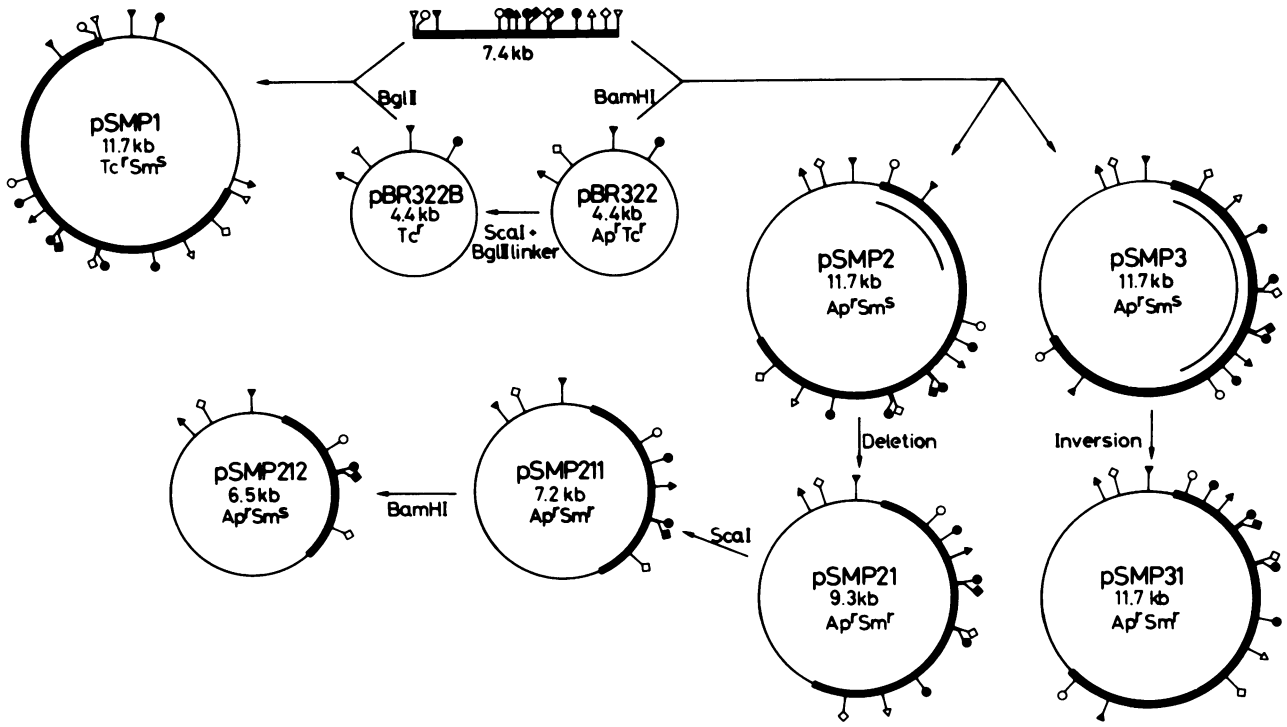

FIG. 5. Construction of pSMP plasmids in *E. coli*. Heavy lines indicate the DNA inserts derived from the cloned DNA of pADT4. Symbols for the restriction sites are the same as in Fig. 4. The details of the procedures for plasmid constructions are given in the text. Boundaries of the deletion (indicated by a thin line inside the map of pSMP2) and the inversion (indicated inside the map of pSMP3) were roughly estimated; an *Eco*RV site on pSMP21 and pSMP31 was mapped at the same position as on pBR322, i.e., about 0.2 kb apart counterclockwise from the *Bam*HI site of pBR322.

amidinotransferase is carried on the 7.4-kb *Bgl*II fragment. However, none of the other plasmids enhanced the expression of amidinotransferase activity in *S. griseus* ATCC 10137 even if they carried either *strR* or *strB*; pADT411 and pADT412 were *strR*$^+$ *strB* plasmids, whereas pADT413 was a *strR strB*$^+$ plasmid (Table 3). Streptomycin 6-phosphotransferase activity was slightly enhanced in SM196 carrying pADT41 or pADT42, but not for SM196 carrying any other plasmids (Table 3).

Streptomycin productivity of *S. griseus* ATCC 10137 was increased five- to sevenfold by transformation with each of pADT41, pADT42, pADT411, and pADT412 (Table 3). The DNA region responsible for the enhancement of streptomycin production ranged from *Bgl*II(1) to *Bam*HI(1), in which the *strR* gene was coded (Fig. 6).

We unexpectedly discovered that pADT43 and pADT44 markedly inhibited streptomycin production of *S. griseus* ATCC 10137 (Table 3). In addition, pADT43 and pADT44 were found to impair activities of both amidinotransferase and streptomycin 6-phosphotransferase (Table 3). Tetracycline-sensitive segregants were derived from *S. griseus* ATCC 10137(pADT44) by cultivation on nutrient agar without tetracycline. All the segregants (obtained at a frequency of 20%) produced the same amount of streptomycin as the wild-type strain. This result indicates that the suppression of streptomycin production is due to amplification of the specific 0.7-kb DNA region from *Bam*HI(1) to *Bam*HI(2) (Fig. 6).

Expression of streptomycin 6-phosphotransferase gene in *E. coli*. We expected that a structural gene of streptomycin 6-phosphotransferase might be located near the probable regulatory gene *strR*. To examine whether the cloned DNA carried the streptomycin 6-phosphotransferase gene, we studied expression in *E. coli* of streptomycin 6-phosphotransferase activity from the cloned DNA. The 7.4-kb *Bgl*II fragment of pADT4 was inserted into a *Bam*HI site of pBR322 in two possible orientations, and the resulting plasmids were named pSMP2 and pSMP3 (Fig. 5). *E. coli* cells harboring pSMP2 or pSMP3 were resistant to ampicillin but sensitive to streptomycin; they could not grow on LB plates containing 20 μg of streptomycin per ml, and their cell extracts contained no streptomycin-inactivating activity.

When replica plated onto LB plates containing both ampicillin (35 μg/ml) and streptomycin (20 μg/ml), ampicillin-resistant colonies of *E. coli* carrying either pSMP2 or pSMP3 gave rise to streptomycin-resistant clones with a frequency of 0.7 or 0.02%, respectively. In control experiments, no streptomycin-resistant clone was obtained from *E. coli* carrying only vector plasmid pBR322.

pSMP21 and pSMP31 were isolated from these streptomycin-resistant clones; pSMP21 was derived from pSMP2, while pSMP31 was derived from pSMP3. All ampicillin-resistant colonies obtained after retransformation with each of the two plasmids could grow on LB plates containing 20 μg of streptomycin per ml, indicating that a streptomycin resistance gene was encoded on these plasmids. Restriction endonuclease cleavage maps of the streptomycin resistance plasmids were constructed. pSMP21 had a deletion of about 2.4 kb, losing the *Aat*I(1) and *Eco*RI sites in the insert of pSMP2, whereas pSMP31 resulted from an inversion of about 4-kb segment encompassing the fragment from *Aat*I(2) to *Sca*I(2) (Fig. 5 and 6).

Cell extracts of the transformants with pSMP21 or pSMP31 were capable of inactivating streptomycin, and the inactivated product was reversely activated by treatment with *E. coli* alkaline phosphatase (Fig. 7). To confirm that

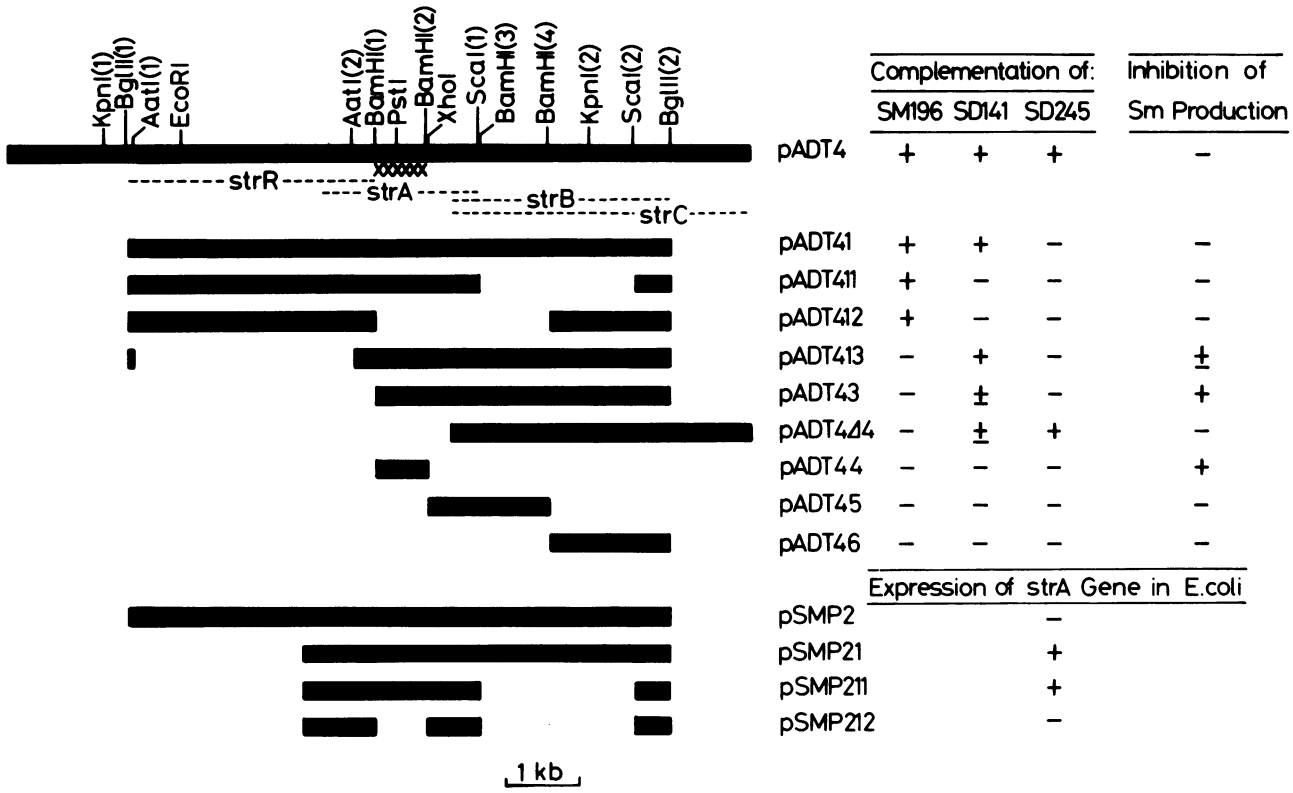

FIG. 6. Localization of the *str* genes on the cloned DNA. Solid bars represent the DNA insert of the indicated plasmids. The pADT plasmids were constructed in *S. griseus* as shown in Fig. 4, whereas the pSMP plasmids were constructed in *E. coli* as shown in Fig. 5. Restriction sites appearing more than once on the DNA insert of pADT4 are distinguished by the numbers in parentheses. Genes responsible for complementation of SM196, SD141, and SD245 were designated as *strR*, *strB*, and *strC*, respectively. A structural gene of streptomycin 6-phosphotransferase was designated as *strA*. Broken lines indicate the approximate location of the *str* genes. A set of crosses denotes the DNA region responsible for inhibition of streptomycin biosynthesis by pADT43 and pADT44, i.e., the 0.7-kb DNA fragment from *Bam*HI(1) to *Bam*HI(2).

the streptomycin phosphotransferase in *E. coli* carrying pSMP21 or pSMP31 phosphorylated the streptidine moiety of streptomycin, we performed a radiochemical assay using streptidine and [^{32}P]ATP as substrates. Both cell extracts gave a radioactive compound with an R_f of 0.4, which corresponds to that of streptidine 6-phosphate (33), and their specific activities of streptomycin 6-phosphotransferase were about 4 U per mg of protein, regardless of the plasmids.

The results described thus far indicate that streptomycin 6-phosphotransferase is expressed from the DNA inserts of pSMP21 and pSMP31. Therefore, we conclude that a structural gene of streptomycin 6-phosphotransferase is cloned together with the other *str* genes, and we designate the gene as *strA*.

To localize the *strA* gene on the cloned DNA, derivative plasmids of pSMP21 were constructed as shown in Fig. 5. pSMP211, which lacked the 2.1-kb *Sca*I fragment of pSMP21, conferred streptomycin resistance to *E. coli*, whereas pSMP212, which lacked the 0.7-kb segment from *Bam*HI(1) to *Bam*HI(2), did not (Fig. 5 and 6). Streptomycin-inactivating activity was detected in *E. coli* cells carrying pSMP211 but not in those carrying pSMP212. These results indicate that the *strA* gene is located on an approximately 2-kb DNA region from the left end of the DNA insert of pSMP211 to the *Sca*I(1) site, and that the 0.7-kb *Bam*HI fragment contains an essential region of the *strA* gene (Fig. 6).

DISCUSSION

We cloned an *str* gene cluster including *strR*, *strA*, *strB*, and *strC* by using an *S. griseus* host-vector system. By deletion analysis, the order of the four genes was determined to be *strR-strA-strB-strC* (Fig. 6).

The *strA* gene was verified to encode streptomycin 6-phosphotransferase by testing expression of the enzyme activity from the cloned DNA in *E. coli*. Bibb and Cohen (2) have reported that many, if not all, *Streptomyces* promoters cannot be recognized by *E. coli* RNA polymerase. In this study, the indigenous promoter of *strA* seems to be inert in *E. coli*, since *E. coli* cells carrying pSMP2 or pSMP3 expressed no streptomycin 6-phosphotransferase activity, despite the presence of the entire *strA* gene and the flanking regions on the plasmids. Only after deletion (resulting in pSMP21) or inversion (in pSMP31) was the detectable expression of *strA* recognized in *E. coli*. In these streptomycin resistance plasmids, the *strA* gene might be transcribed by readthrough from the P2 promoter for the *tet* gene on pBR322 (30), judging from the orientation and location of *strA* to the promoter. Accordingly, it is inferred that the *strA* gene might be transcribed toward the right in Fig. 6.

Streptomycin 6-phosphotransferase genes of *S. griseus* HUT 6037 and *Streptomyces glaucescens* have been cloned in *Streptomyces lividans* (7, 27). The restriction maps of the streptomycin 6-phosphotransferase genes and the adjacent

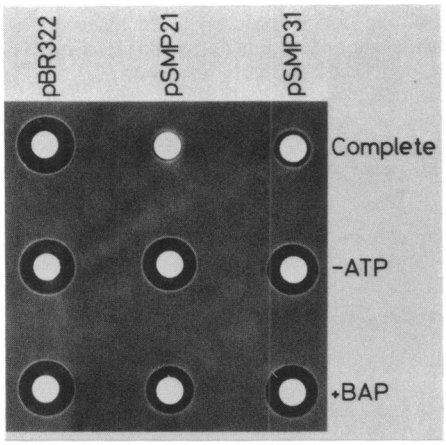

FIG. 7. Inactivation of streptomycin by cell extracts of *E. coli* carrying the streptomycin resistance plasmids. *E. coli* cells carrying pSMP21 or pSMP31 were grown in the presence of ampicillin (35 μg/ml) and streptomycin (20 μg/ml), whereas those carrying pBR322 were grown in the presence of ampicillin (35 μg/ml). The inactivation reaction, using cell extracts prepared from the cultures described above, was carried out in the mixture with (complete) and without (−ATP) ATP. For the reactivation of inactivated streptomycin, a sample (100 μl) was treated with 0.2 U of *E. coli* alkaline phosphatase type III (Sigma Chemical Co., St. Louis, Mo.) at 37°C for 2 h (+BAP).

regions of *S. griseus* ATCC 10137, *S. griseus* HUT 6037, and *S. glaucescens* show no similarity among them. Streptomycin 6-phosphotransferase genes would be suitable to investigate the divergency of *Streptomyces* genes, since a number of wild-type strains of wide geographical distribution that produce streptomycin are available.

The *strR* gene complemented the Str$^-$ Str^s strain SM196 that suffered a mutation impairing both amidinotransferase and streptomycin 6-phosphotransferase activities, whereas the *strB* gene complemented the Str$^-$ strain SD141 specifically deficient in amidinotransferase. The *strR*$^+$ *strA strB* plasmid (pADT412) did indeed restore the ability of SM196 to express both enzyme activities (Table 3). It was also demonstrated by segregation of pADT412 that *strR* encoded a *trans*-acting gene product. The *strR*$^+$ *strA*$^+$ *strB*$^+$ plasmids (pADT41 and pADT42) led to a high expression of amidinotransferase in the transformed cells (Table 3). Based on these results, we conclude that the *strB* gene encodes amidinotransferase and that the *strR* gene encodes a positive effector required for the full expression of the *strA* and *strB* genes.

The *strR* gene was localized on the 3.3-kb DNA region from *Bgl*II(1) to *Bam*HI(1) (Fig. 6). The plasmids containing this region induced the overproduction of streptomycin in *S. griseus* ATCC 10137. It seems strange that the wild-type strain carrying the *strR*$^+$ *strB* plasmids (pADT411 and pADT412) exhibited the enhanced streptomycin productivity, despite only a little increase of amidinotransferase activity (Table 3). This fact could be explained by the assumption that the *strR* gene might positively control the expression of some other *str* genes in addition to the *strA* and *strB* genes. Another possible explanation is that the 3.3-kb region of DNA might carry an unidentified gene of a key enzyme for streptomycin biosynthesis, if it exists.

The *strC* gene was defined as a gene conferring the Str$^+$ phenotype on SD245. The Str$^-$ strain SD245 had a defect in the linkage step J (Fig. 1) due to a change of substrate specificity of dihydrostreptosyltransferase, which catalyzes the reaction; the mutated enzyme in SD245 was altered to utilize streptidine but not streptidine 6-phosphate, a normal intermediate, as an acceptor (22). Therefore, the *strC* gene probably encodes dihydrostreptosyltransferase. However, there is an alternative possibility that the *strC* gene may encode streptomycin 6-phosphohydrolase, which catalyzes reaction step M in Fig. 1 (33); if a structural gene for that enzyme were carried on a plasmid and the enzyme were substantially synthesized in the transformed cells, the streptidine 6-phosphate accumulated in SD245 would be dephosphorylated to streptidine, which could be converted to streptomycin by SD245 (22). The gene product of *strC* deserves further biochemical studies with the transformant that carries an *strC* plasmid.

An interesting finding in this study was that amplification of the specific 0.7-kb DNA region on plasmids inhibited both streptomycin biosynthesis (enzymologically, amidinotransferase activity) and streptomycin resistance (streptomycin 6-phosphotransferase activity) (Table 3 and Fig. 6). A similar phenomenon has been reported in studies concerning spore formation in *B. subtilis*. Banner et al. (1) have discovered that propagation of the *SpoVG* promoter region on a high-copy-number plasmid interferes with the process of sporulation in *B. subtilis* and have proposed that the interference is due to titration of a sporulation-specific regulatory protein that binds at the promoter region. By analogy, the 0.7-kb fragment might titrate a gene product of *strR*; pADT43 inhibited streptomycin production of the transformed cells, whereas pADT41 did not. Therefore, the 0.7-kb region might carry a regulatory apparatus that participates in the control mediated by an *strR* gene product. Alternatively, the region might encode a toxic or a repressible substance that inhibits both streptomycin synthesis and streptomycin resistance when overproduced from the plasmids.

As described above, the streptomycin resistance gene (*strA*) and the streptomycin biosynthesis gene(s) share a common regulatory system directed by the *strR* gene. In addition to the *strR* system, the regulation of *strA* expression involves an induction system with streptomycin, which was demonstrated by a streptomycin 6-phosphotransferase assay with the Str$^-$ mutants (Table 2). This induction system might permit the continuance of *strA* expression in parallel with streptomycin accumulation even after the cessation of the expression of streptomycin biosynthesis genes.

Nucleotide sequencing and further analysis of regulatory sequences of the cloned *str* genes would provide a new insight on the regulatory mechanisms involved in streptomycin biosynthesis and streptomycin resistance at the molecular level.

In the course of shotgun cloning, the DNA segments that conferred on SD141 the ability to produce unidentified antibiotics were cloned in pADT1, pADT2, pADT3, pADT5, and pADT6. The other Str$^-$ mutants carrying each of these plasmids also produced the antibiotics. We suppose that amplification of the specific DNA regions might elicit the potential of *S. griseus* ATCC 10137 to produce some antibiotics other than streptomycin. Identification of genetic functions carried on the cloned DNAs merits further studies.

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