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Unstable Genetic Determinant of A-Factor Biosynthesis in Streptomycin-Producing Organisms: Cloning and Characterization

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We cloned a DNA fragment directing synthesis of A-factor from the total cellular DNA of streptomycin-producing *Streptomyces bikiniensis* on the plasmid vector pIJ385. Introduction of the recombinant plasmid (pAFB1) into A-factor-deficient *S. bikiniensis* and *Streptomyces griseus* mutants led to A-factor production in the host cells, as a result of which streptomycin production, streptomycin resistance, and spore formation of these mutants were simultaneously restored. The plasmid pAFB1 also complemented both *afsA* and *afsB* mutations of *Streptomyces coelicolor* A3(2). These results indicated that the cloned DNA fragment contained the genetic determinant of A-factor biosynthesis. The cloned fragment, when carried on a multicopy vector plasmid, induced production of a large amount of A-factor in several *Streptomyces* hosts. In Southern blot DNA/DNA hybridization analyses with a trimmed 5-kilobase fragment containing the intact A-factor determinant as probe, total cellular DNA from A-factor-deficient mutants gave no positive hybridization. The DNA blot experiment also showed a wide distribution of sequences homologous to the *S. bikiniensis* A-factor determinant among most, but not all, A-factor-producing actinomycetes with a varying extent of homology and the absence of these sequences from most A-factor nonproducers.

A-factor, 2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone (16), is an autoregulating factor originally found in cultures of *Streptomyces griseus* by Khokhlov et al. (13); it is essential for streptomycin production, streptomycin resistance, and spore formation in *S. griseus* and *Streptomyces bikiniensis* (5–7). Mutants of these organisms that are deficient in A-factor synthesis lose all these characteristics simultaneously, and exogenous addition of A-factor causes their complete recovery. Such mutants are obtainable at high frequency by treatment with acridine dyes or growth at high temperature. Genetic analysis by protoplast fusion showed nonlinkage of the genetic determinant of A-factor biosynthesis to chromosomal markers. Furthermore, its infectious transfer suggested involvement of an extrachromosomal genetic determinant for A-factor synthesis in *S. griseus* (8).

A-factor production has been found to be distributed widely among actinomycetes, including *Streptomyces coelicolor* A3(2). In contrast to the results with *S. griseus*, two fixed loci, *afsA* and *afsB*, on the chromosomal linkage map governed A-factor production in *S. coelicolor* A3(2) (8). Cloning experiments have revealed that *afsB* is a pleiotropic regulatory gene affecting synthesis not only of A-factor but also of several secondary metabolites in *S. coelicolor* A3(2) and *Streptomyces lividans* (10).

In this paper, we describe the cloning of the genetic determinant of A-factor biosynthesis in *S. bikiniensis*. The cloned gene(s), almost identical to the A-factor determinant of *S. griseus*, seemed to be the structural gene(s) for enzyme(s) involved in A-factor biosynthesis. Complete deletion of the gene sequences in A-factor-deficient mutants of *S. bikiniensis* and *S. griseus* was observed by DNA/DNA hybridization experiments, in accordance with the assumption that the A-factor genes are carried on an unstable extrachromosomal element in these streptomycin-producing streptomycetes.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. bikiniensis* IFO 13350, which produces streptomycin, is a stock culture in this laboratory. *S. bikiniensis* HH1 is an A-factor-deficient mutant derived by incubation at 37°C. This strain can neither produce streptomycin nor form spores owing to its lack of A-factor. *S. griseus* FT-1 and IFO 13189 strains and their A-factor-deficient mutants derived by so-called curing treatments have been described (6). Other actinomycete strains (6) and *S. coelicolor* A3(2) strains (8, 10) were previously described. Plasmids pIJ41, pIJ303, pIJ385, and pIJ702 conferring resistances to thiostrepton and neomycin, thiostrepton, thiostrepton and neomycin, and thiostrepton, respectively, were obtained from D. A. Hopwood (2, 12, 14, 19).

Chemicals and enzymes. Thiostrepton was provided by Asahi Chemical Industry. The restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs, Inc., or Nippon Gene Co., Ltd. Conditions for the enzyme reactions used were those recommended by the vendors.

Cellular and plasmid DNA isolation. Mycelium harvested from stationary-phase culture was lysed by the lysozyme-EDTA-sodium dodecyl sulfate method of Horinouchi et al. (11). Plasmid DNA was obtained from the lysate by cesium chloride-ethidium bromide ultracentrifugation. For isolation of total cellular DNA, lysates were heated at 60°C for 10 min, followed by CsCl centrifugation. This heat treatment seemed to enable DNA to separate easily from lysed cells during the centrifugation for obtaining so-called cleared lysates.

Shotgun cloning of A-factor gene(s). Plasmid pIJ385 and total cellular DNA of *S. bikiniensis* were digested with *Pst*I and ligated with T4 DNA ligase. The ligated sample was introduced by transformation into protoplasts of *S. bikiniensis* HH1 essentially according to the method of Thompson et al. (20). After protoplast regeneration on R1 medium supplemented with 0.2% yeast extract, transformants were selected by replica plating on Bennet agar medium containing 32 μg of thiostrepton per ml. The proportion of neomycin-sensitive colonies among thiostrepton-resistant transformants obtained in this way was about 20%. More than 20,000 thiostrepton-resistant colonies thus obtained were again transferred onto nutrient agar medium by replica printing and allowed to grow at 28°C for 28 h, after which nutrient soft agar containing *Bacillus subtilis* ATCC 6633 was over-

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laid, and the plates were incubated overnight at 37°C. One presumptive streptomycin-producing colony forming a growth-inhibitory zone on the indicator *B. subtilis* strain was picked and used for further study.

Other methods, including A-factor assay, media, agarose gel electrophoresis, and recombinant DNA work for subcloning the A-factor determinant, were essentially as described (6, 10, 11).

DNA blotting and hybridization. Restriction endonuclease fragments separated in 1.2% agarose-ethidium bromide gel electrophoresis were alkali denatured and then neutralized. The DNA was transferred and fixed to a sheet of nitrocellulose paper by the method of Southern (18). For making ^{32}P-probes, restriction fragments purified from agarose gel segments by using the $NaClO_4$ method (3) were labeled by nick translation with a translation kit (New England Nuclear Corp.) and [^{32}P]dCTP. ^{32}P-probe DNA denatured at 100°C for 10 min was hybridized with nitrocellulose blots in 20 ml of 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50% formamide–0.1% sodium dodecyl sulfate in heat-sealed plastic bags at 42°C overnight. The nitrocellulose sheet was then washed three times in the hybridization buffer at 37°C and twice in 2× SSC–0.1% sodium dodecyl sulfate. After being dried, the hybridized blot was placed against Kodak XRP film for autoradiography.

RESULTS

Cloning of A-factor determinant of *S. bikiniensis*. The transformation efficiencies of streptomycin-producing organisms, i.e., *S. griseus* FT-1, *S. griseus* IFO 13189, and *S. bikiniensis* IFO 13350 were examined by using the thiostrepton resistance plasmid pIJ303. Among these strains, *S. bikiniensis* gave the highest efficiency; 1 μg of pIJ303 DNA gave about 10^7 thiostrepton-resistant transformants. In these experiments, plasmid DNA was propagated in and isolated from the respective strain to avoid possible restriction modification barriers.

The plasmid vector used in cloning was pIJ385, which confers resistances to thiostrepton and neomycin in the host cell. The unique *Pst*I site within the neomycin resistance

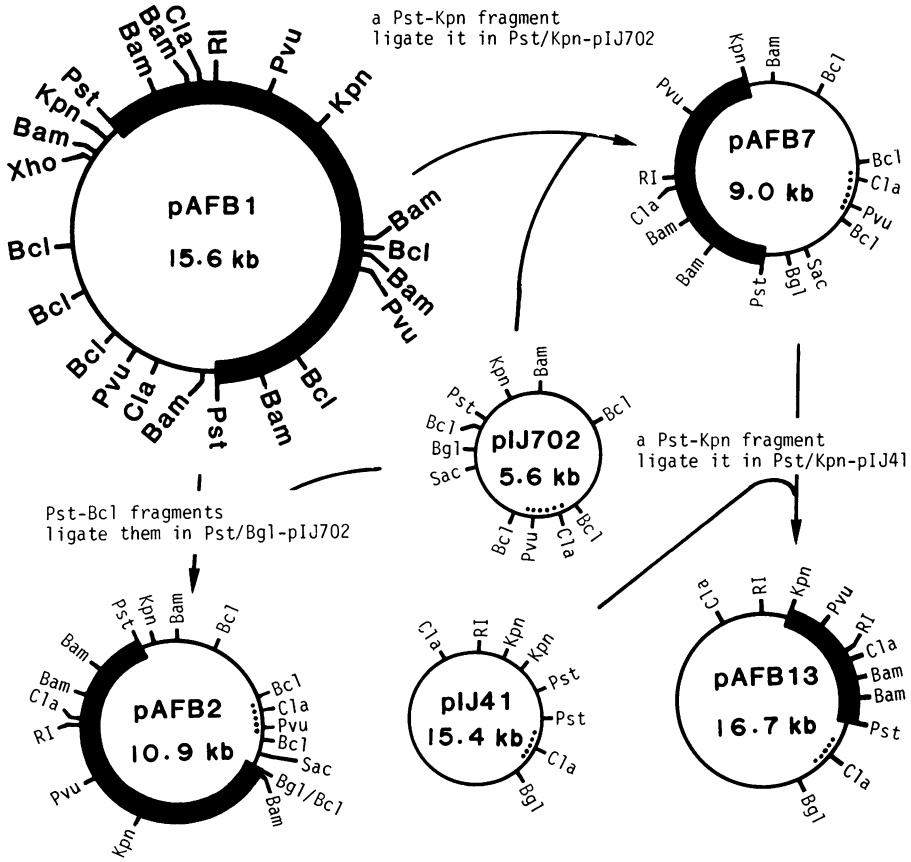

FIG. 1. Restriction endonuclease cleavage map of pAFB1 and schematic diagrams summarizing plasmid constructions. For constructing pAFB2 and pAFB3, the originally cloned 9-kb *Pst*I fragment in pAFB1 was purified by agarose gel electrophoresis and digested partially with *Bcl*I, followed by ligating the digest with pIJ702 cleaved with *Pst*I plus *Bgl*II. Transformants carrying the desired hybrid plasmids were screened by detecting thiostrepton-resistant and melanin-negative colonies, since the *Bgl*II site is located at an essential region of the tyrosinase gene of pIJ702 (12). Plasmid pAFB7 was constructed by ligating the purified *Kpn*I to *Pst*I fragment with the larger fragment of pIJ702 obtained by digestion with *Kpn*I plus *Pst*I, followed by introducing the mixture into *S. bikiniensis* HH1. Transformants were selected as thiostrepton-resistant colonies. Plasmid pAFB13 was constructed by insertion of a 3.8-kb fragment obtained by digesting pAFB7 with *Kpn*I plus *Pst*I into pIJ41 cleaved with *Kpn*I plus *Pst*I, followed by introducing the mixture into *S. lividans* HH21. In this construction, pAFB7 DNA was once propagated in and isolated from *S. lividans* TK21 to avoid restriction modification barriers. Transformants were selected as thiostrepton-resistant and neomycin-sensitive colonies because a cloning event at one of the *Pst*I sites of pIJ41 leads to neomycin sensitivity (2). The structures of the hybrid plasmids were confirmed by digesting the purified DNAs with appropriate restriction endonucleases and analyzing the digests by agarose gel electrophoresis. The restriction enzymes abbreviated are Bam, *Bam*HI; RI, *Eco*RI; Pvu, *Pvu*II; Kpn, *Kpn*I; Bcl, *Bcl*I; Pst, *Pst*I; Xho, *Xho*I; Bgl, *Bgl*II; Cla, *Cla*I; and Sac, *Sac*I. Dots stand for the extent of the thiostrepton resistance gene.

gene allows cloning and screening by insertional inactivation. Total cellular DNA fragments of *S. bikiniensis* cleaved with *Pst*I were ligated with pIJ385 cleaved with *Pst*I, and the ligated mixture was introduced by transformation into an A-factor-deficient mutant strain, *S. bikiniensis* HH1. After the first selection of thiostrepton-resistant transformants, clones carrying the A-factor genes were sought by detecting streptomycin-producing colonies. One colony producing streptomycin was found. The isolate was neomycin sensitive, as was expected for a plasmid with a fragment in the *Pst*I site of the vector. We purified plasmid DNA from this isolate and detected a 9.0-kilobase (kb) fragment in the *Pst*I site by agarose gel electrophoresis. Figure 1 shows the restriction map of this plasmid, named pAFB1; it directed synthesis of A-factor as described below.

Trimming the A-factor determinant by subcloning. To reduce the size of the cloned fragment, we first subcloned the largest *Pst*I-*Bcl*I fragment of pAFB1, consisting of 5.0 kb, into pIJ702 between the *Pst*I and *Bgl*II sites. *Bcl*I and *Bgl*II ends can be readily ligated, since they share the common 4-base sequence GATC and are cleaved at the same position in this sequence. This plasmid, pAFB2 (shown in Fig. 1), conferred A-factor production to strain HH1 when tested as described below, whereas pAFB3, which contained the remainder (4 kb) of the originally cloned fragment, failed to confer A-factor production. Plasmid pAFB7, containing the *Pst*I to *Kpn*I fragment consisting of 3.8 kb, could confer A-factor production. The amount of A-factor produced by the pAFB2- or pAFB7-carrying strain HH1 was equal to that produced by the pAFB1 carrier. From these results, the A-factor determinant capable of conferring A-factor productivity to strain HH1 was narrowed down to this 3.8-kb fragment.

Phenotypic expression of the cloned fragment. Purified pAFB1 DNA was reintroduced by transformation into *S. bikiniensis* HH1 to test for phenotypic expression in the host cell. All the thiostrepton-resistant transformants obtained in this way were confirmed to produce streptomycin (Fig. 2), as well as to form spores (Fig. 3). In addition, colonies of these transformants restored streptomycin production to an adjacent colony of an A-factor-deficient *S. griseus* (Fig. 2). In addition to streptomycin production and spore formation, streptomycin resistance was also restored by introduction of the plasmid pAFB1. The resistance level of the transformants was the same as that of the parental *S. bikiniensis* strain (100 μg of streptomycin per ml), whereas that of the host strain HH1 was 40 μg/ml. These results indicated that pAFB1 conferred A-factor production to the host cell.

In a bioautogram of chloroform extracts of culture filtrates of *S. bikiniensis* (pAFB1), which was performed as previously described (6), a strongly positive spot with an R_f value of 0.3 that was identical to that of chemically synthesized A-factor (17) was detected. The amount of A-factor produced by the plasmid-carrying strain HH1 was estimated to be 3.4 μg/ml by the bioassay, whereas the parental *S. bikiniensis* strain produced 0.2 μg/ml under the same conditions. Thus, the plasmid-carrying strain HH1 produced about 18 times as much A-factor as the parental strain, perhaps because of gene dosage. Plasmid pAFB7 conferred A-factor production to strain HH1 to the same extent as pAFB1 did. This finding that an increased amount of A-factor did not lead to an enhancement of streptomycin production or an increase of streptomycin resistance confirms the regulatory or "switching" function of A-factor.

Expression of pAFB1 in *S. griseus*. *S. griseus* FT-1 is a high-level streptomycin producer with a high resistance to streptomycin (more than 10 mg/ml). The A-factor-deficient mutant number 2 of FT-1 lost both streptomycin productivity and spore-forming ability with a marked reduction of streptomycin resistance (to 1.2 mg/ml) (6, 7). No intermediate in the streptomycin biosynthetic pathway, rather only A-factor, is capable of restoring streptomycin production to the A-factor-deficient mutant. Introduction of pAFB1 or pAFB7 into this mutant caused A-factor production in a large quantity, perhaps owing to gene dosage, and restored all these defects to the level of the parental FT-1 strain. A similar reversal was also observed with A-factor-deficient mutants derived from *S. griseus* IFO 13189. These results indicated that the cloned A-factor determinant of *S. bikiniensis* could complement the A-factor deficiency of *S. griseus* mutants.

Cloned fragment complements *afs* mutations of *S. coelicolor*

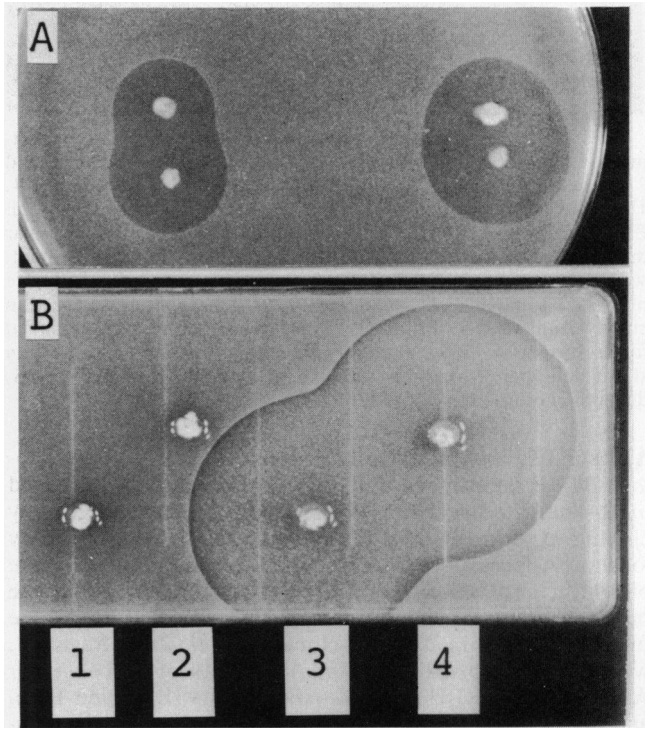

FIG. 2. A-factor assay of the transformants of *S. bikiniensis* (A) and *S. coelicolor* A3(2) (B). (A) Colonies were grown on nutrient agar medium for 2 days, after which nutrient soft agar containing *B. subtilis* ATCC 6633 was then overlaid and incubated overnight at 37°C. Streptomycin production by *S. bikiniensis* HH1 carrying pAFB1 (upper colony), as well as the A-factor-deficient mutant number 2 of *S. griseus* FT-1 (lower colony), was restored. The difference between the left and right pairs was the distances between the two colonies (1 and 0.5 cm, respectively). (B) A-factor production of *S. coelicolor* A3(2) strains was assayed by detecting streptomycin production of an A-factor-deficient *S. griseus* of which streptomycin production depends on A-factor feeding by the test organisms, as described (10). Test strains were grown on an agar plug (5 mm [diameter] by 3 mm [height]) at 28°C for 2 days and was transferred onto 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 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* to produce streptomycin, which in turn was detected by the growth inhibition of the indicator. Colony 1, BH2; colony 2, BH10; colony 3, BH2 carrying pAFB1; and colony 4, BH10 carrying pAFB1.

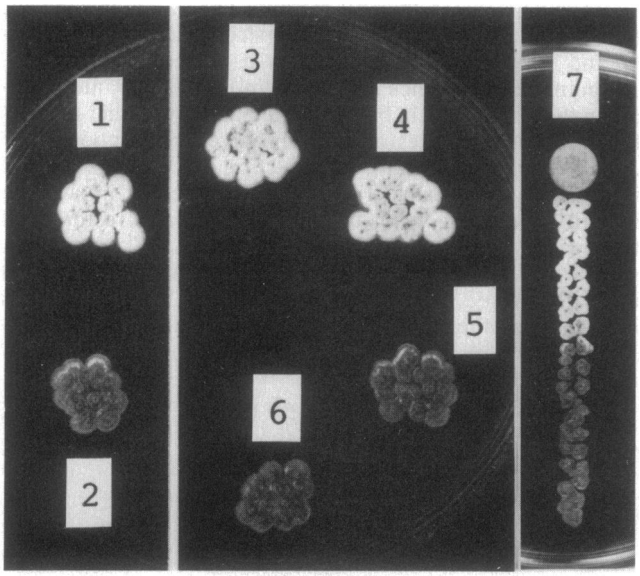

FIG. 3. Spore formation stimulated by the cloned DNA fragment. Colony 1, wild-type *S. bikiniensis* IFO 13350; colonies 2 and 7, *S. bikiniensis* HH1; colony 3, HH1(pAFB1); colony 4, HH1(pAFB2); colony 5, HH1(pAFB3), and colony 6, HH1(pIJ702). Each colony was formed by using a tooth pick on Bennet agar medium with cultivation at 28°C for 6 days. Thiostrepton (32 μg/ml) was added when the test strain harbored thiostrepton resistance plasmids. A paper disk containing 10 ng of A-factor was placed in colony 7 after 2 days of growth, and the incubation was continued for a further 4 days; A-factor diffusing into the agar medium stimulated spore formation of the A-factor-deficient *S. bikiniensis*, depending on the concentration.

A3(2) and *S. lividans*. Biosynthesis of A-factor in *S. coelicolor* A3(2) is determined by two chromosomal loci, *afsA* and *afsB*. The latter has been deduced to be a regulatory gene by cloning experiments (8, 10). Mutations at *afsA* cause only loss of A-factor production, whereas *afsB* mutants lose biosynthetic activities not only of A-factor but also of the pigments actinorhodin and undecylprodigiosin. To reveal the functions determined by the cloned *S. bikiniensis* fragment, we introduced pAFB1 into *afsA* mutants of *S. coelicolor* A3(2) (BH2 and BH10) and *afsB* mutants (BH5 and BH6) and tested for A-factor production by the cosynthesis method as described (10). Transformants of both *afsA* and *afsB* mutants carrying pAFB1 produced A-factor. On the other hand, production of the pigments was not restored in the *afsB* transformants. The amount of A-factor produced by both the *afsA* and *afsB* transformants was estimated by the (bioassay to be 100 ng per colony, an amount which was about 20 times higher than that produced by the parental *S. coelicolor* A3(2). Plasmid pAFB7 also caused A-factor production to the same extent in both the *afsA* and *afsB* mutants. The increased production of A-factor was probably due to a gene dosage effect of the multicopy vector plasmid.

In *S. lividans*, biosynthesis of A-factor is positively regulated by *afsB* (10). Plasmids pAFB1 and pAFB7 conferred A-factor production to the same extent to *S. lividans* HH21, which seems to be a spontaneous *afsB* mutant. To examine a gene dosage effect, we subcloned the same *Pst*I to *Kpn*I fragment carried by pAFB7 onto pIJ41, resulting in pAFB13 (Fig. 1), and introduced it into strain HH21. The vectors used for pAFB7 and pAFB13 were pIJ702 with the copy number of 40 to 300 and pIJ41 with the copy number of 3 to 4, respectively (2). Although the actual copy numbers of the hybrid plasmids were not determined, they were almost the same as those of the respective vectors, judging from the intensity of the plasmid bands on agarose gels. The amount of A-factor produced by strain HH21 carrying pAFB7 and pAFB13 was determined to be 130 and 100 ng per colony, respectively.

Detection of A-factor determinants by DNA blotting analysis. To determine the presence or absence of A-factor determinants in A-factor-deficient mutants of *S. griseus* and *S. bikiniensis*, we performed Southern blot experiments with the ^{32}P-labeled 5.0-kb fragment (the *Pst*I to *Bcl*I fragment, carried by pAFB2) containing the intact A-factor gene(s), with probe and total cellular DNA from A-factor-deficient mutants of *S. griseus* and *S. bikiniensis*, as well as their parental strains, as targets. All the A-factor-positive strains showed positive hybridization; however, their A-factor-deficient mutants gave no hybridization (Fig. 4 and 5).

A-factor-producing *S. griseus* FT-1 had a corresponding DNA sequence with the same intensity and the same *Bam*HI restriction pattern as that of *S. bikiniensis*. These results suggested that the nucleotide sequences of the A-factor genes of the two strains were well conserved. *Bam*HI-digested total DNA of *S. griseus* IFO 13189 gave hybridizing bands at slightly different positions. All the A-factor-deficient mutants, such as *S. griseus* FT-1 number 2 (obtained by UV irradiation), *S. griseus* IFO 13189 AO-1 (obtained by acridine orange treatment), and *S. bikiniensis* HH1 (obtained by growth at 37°C), gave no hybridization. Neither was positive hybridization observed with other A-factor-deficient mutants, such as *S. griseus* FT-1 AO-1 and *S. bikiniensis* AO-1 (both obtained by acridine orange treatment) (data not shown). These results indicate that the entire DNA sequences homologous to the cloned A-factor determinant of *S. bikiniensis* are easily lost as a unit in these organisms.

To analyze distribution of the A-factor genes in actinomycetes, we hybridized the above 5.0-kb fragment with *Bam*HI-digested total cellular DNA extracted from various other A-factor-producing and nonproducing strains. Table 1 summarizes the A-factor productivity of each strain tested by the cosynthesis method, together with the hybridization results (data not shown). Most of the A-factor producers carried a sequence homologous to the probe, with a varying degree of homology with different *Bam*HI restriction patterns, whereas most of the nonproducers did not. However, a few contradictory cases were also observed; several A-factor-negative strains contained homologous sequences and several A-factor producers gave no positive hybridization.

DISCUSSION

A 3.8-kb DNA fragment of *S. bikiniensis* cloned on multicopy plasmid vector pIJ702 conferred A-factor production with simultaneous recovery of streptomycin production, streptomycin resistance, and spore formation in A-factor-deficient mutants of *S. bikiniensis* and *S. griseus*. A marked gene dosage effect, depending on the copy number of the vector plasmid, was observed on the amount of A-factor produced by the transformants carrying the hybrid plasmids. These results strongly suggest that the cloned fragment contains a structural gene(s) encoding one or more enzymes for A-factor biosynthesis. This conclusion is also supported by the finding that the cloned fragment is able to complement mutations in the *afsA* gene of *S. coelicolor* A3(2), which we presume are in a structural gene(s) for A-factor biosynthesis (8, 10).

As we already reported (6), A-factor-deficient mutants of *S. griseus* and *S. bikiniensis* are easily obtained by so-called

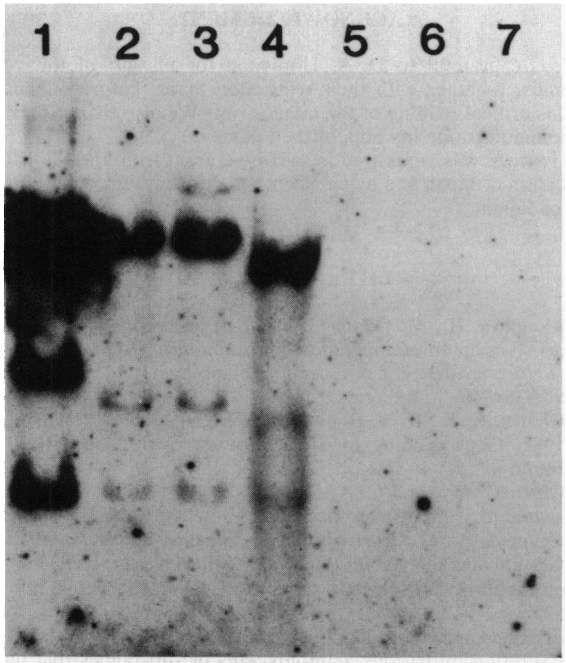

FIG. 4. Autoradiogram of DNA/DNA hybridizations between the cloned 5.0-kb A-factor determinant of *S. bikiniensis* and *Bam*HI-digested total cellular DNAs of A-factor-producing and nonproducing strains. Lane 1, *Bam*HI-digested plasmid pAFB1 DNA as a positive control; lane 2, wild-type *S. bikiniensis* IFO 13350, A-factor$^+$; lane 3, *S. griseus* FT-1, A-factor$^+$; lane 4, *S. griseus* IFO 13189, A-factor$^+$; lane 5, *S. bikiniensis* HH1, A-factor$^-$; lane 6, *S. griseus* FT-1 number 2, A-factor$^-$; and lane 7, *S. griseus* IFO 13189 AO-1, A-factor$^-$.

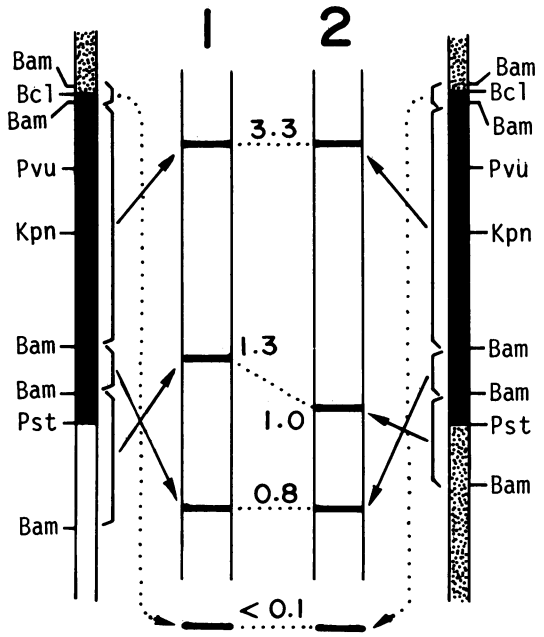

FIG. 5. Schematic representation showing the identity of the hybridized bands in Fig. 4. Restriction maps are for relevant regions of pAFB1 (left) and the intact DNA sequence carrying the cloned A-factor determinant of *S. bikiniensis* (right). The probe sequence used consists of 5.0 kb from the *Bcl*I to *Pst*I site, shown in black. The smallest bands (less than 100 base pairs) in lane 1 (*Bam*HI-digested pAFB1 DNA) and lane 2 (*Bam*HI-digested total cellular DNA of *S. bikiniensis* IFO 13350) ran out of the gel in this experiment. From the size of the second largest band (1.0 kb) in lane 2, the location of a *Bam*HI site (at the bottom) outside the cloned sequence can be determined. Abbreviations are the same as those described in the legend to Fig. 1.

plasmid-curing treatments, such as treatment with acridine dyes or growth at high temperature. Progeny from genetic crosses of A-factor-deficient *S. griseus* with the parental strain by protoplast fusion were almost all A-factor positive, irrespective of their chromosomal markers. The extrachromosomal and highly transmissible characteristics of the A-factor determinant which were suggested by these experiments are consistent with the results of the DNA/DNA hybridization analysis reported here, with the cloned A-factor determinant as probe. Absence of sequences complementary to the cloned A-factor determinant in the deficient mutants also suggests that this determinant is carried as a unit, possibly on an unstable plasmid. This suggests that the A-factor gene(s) has spread on a highly movable genetic element. We have failed to detect a plasmid DNA in *S. bikiniensis* and *S. griseus* so far, but similar failures have been reported, with *Streptomyces* plasmids such as SCP1, which determines fertility and methylenomycin biosynthesis in *S. coelicolor* A3(2) (15), and the plasmid for chloramphenicol production in *Streptomyces venezuelae* (1).

Recently, reiteration of a special class of DNA sequences accompanying chromosomal deletion of the adjacent region at high frequency has been suggested in unstable tyrosinase-negative and hydroxystreptomycin-sensitive mutants of *Streptomyces glaucescens*, both of which are mapped at unique positions on the chromosome (4; R. Hütter, personal communication). However, nonlinkage of the A-factor gene with chromosomal markers and its highly transmissible property in *S. griseus* show marked differences from the properties of the unstable tyrosinase and hydroxystreptomycin resistance genes in *S. glaucescens*. Ethidium bromide-stained agarose gel that was used for the blot experiment in Fig. 4 showed the absence of a markedly amplifiable unit in the A-factor-deficient mutants. This observation also indicates a difference from the unstable genes, suggesting that occurrence at high frequency of A-factor-deficient mutants by plasmid-curing treatments may not be associated with reiteration of DNA sequences.

A-factor is determined chromosomally in *S. coelicolor* A3(2), in contrast to the above case in streptomycin-producing organisms. Of the two chromosomal loci, *afsA* and *afsB*, for A-factor biosynthesis in *S. coelicolor* A3(2), *afsA* seems to be for the structural gene(s) for A-factor synthesizing enzymes, whereas *afsB* appears to be a positive regulatory gene with markedly pleiotropic characteristics (8, 10). The cloned *afsB* gene on a 2.0-kb DNA fragment caused biosynthesis not only of A-factor but also of the pigments actinorhodin and undecylprodigiosin, which have no structural and biosynthetic relationships with each other or with A-factor. In the present work, the cloned A-factor gene(s) of *S. bikiniensis* restored A-factor production to both *afsA* and *afsB* mutants of *S. coelicolor* A3(2) but did not restore production of the pigments to the *afsB* mutants. The latter fact implies that the cloned *S. bikiniensis* DNA fragment does not contain a regulatory gene complementary to *afsB*. A DNA blot experiment with the *afsB* gene sequence as probe revealed that there was no homologous sequence to the probe in total cellular DNA of *S. bikiniensis* (unpublished data). It seems probable that A-factor genes of *S. bikiniensis* and also *S. griseus* do not require the positive regulatory function of *afsB* to express their activities, even in *S. coelicolor* A3(2).

TABLE 1. A-factor productivity and cross-homology with the *S. bikiniensis* A-factor gene of various actinomycetes

Organism and strain	A-factor production	Hybridization	Size (kb) of hybridized bands[a]
Streptomyces albus IFO 3195	+	+	5.0, 1.9
S. albus IFO 3422	−	−	
S. albus IFO 3710	−	−	
S. antibioticus IFO 3126	+	+	8.1, 4.4
S. antibioticus IFO 12652	−	+	3.8
S. antibioticus IFO 12838	−	+	7.4 (weak), 5.8
S. blastmyceticus IFO 12747	−	−	
S. coelicolor A3(2) IFO 3114	−	−	
S. coelicolor A3(2) A700	+	−	
S. flaveolus IAM 0117	+	+	>24
S. flaveolus IFO 3408	−	−	
S. fradiae ATCC 21096	+	−	
S. globisporum IFO 12208	+	+	6.0
S. globisporum IFO 12209	−	−	
S. griseoflavus IFO 12372	+	+	4.8
S. sindenensis IFO 12915	+	+	6.0
S. viridochromogenes IFO 12337	−	−	
S. viridochromogenes IFO 12338	+	+	20
S. viridochromogenes IFO 12376	−	−	
S. viridochromogenes IFO 12377	+	+	>24 (weak), 15
Actinomyces fluorescens IFO 12861	+	−	
A. citreofluorescens IFO 12853	+	+	4.6, 2.8
Nocardia brasiliensis	+	−	

[a] The molecular weights of hybridized bands were determined by using *Hin*dIII-digested λ DNA as standard markers.

The presence of the A-factor genes detected by hybridization showed a considerable correlation with A-factor productivity in a wide variety of actinomycetes. Exceptional cases in which a homologous sequence was found in a few A-factor nonproducers might be attributable to the quantitative limit for detecting A-factor production by the method used or to naturally occurring point mutations. Most of the A-factor-producing strains were found to share homologous sequences with the *S. bikiniensis* A-factor gene(s); however, their homology varied from strain to strain. Observed lack of homology in some of the A-factor producers can most likely be ascribed to decreased homology among the wide divergency of actinomycetes. An example representing a similar situation was shown by Horinouchi et al. (9), in which macrolide-lincosamide-streptogramin B resistance genes from staphylococcal and streptococcal strains failed to show relatedness by nucleic acid hybridization but still possessed similar amino acid sequences. These findings suggest that the A-factor determinant of *S. bikiniensis* and those of other actinomycetes are related and that they have diverged from a common ancestral sequence.

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