THE CLONED *STREPTOMYCES BIKINIENSIS* A-FACTOR DETERMINANT

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(Received for publication January 18, 1985)

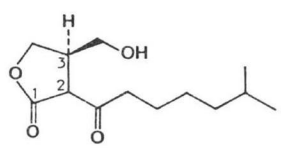
By cleavage with restriction endonucleases and cloning the resultant fragments, the A-factor determinant cloned from streptomycin-producing *Streptomyces bikiniensis* (HORINOUCHI *et al.*, J. Bacteriol. 158: 481~487, 1984) was narrowed down to a 1.1-kilobase (kb) fragment. A hybrid multicopy plasmid (pAFB15) carrying the 1.1-kb fragment conferred A-factor production in a large quantity to A-factor-deficient mutants of *S. bikiniensis* and *Streptomyces griseus* as well as *afsA* mutants of *Streptomyces coelicolor* A3(2). A transcriptional control signal in the 1.1-kb fragment was identified by using a promoter-probe vector pARC 1. Plasmid pAFB15 also caused A-factor production with a marked gene dosage effect in four different *Streptomyces* strains which originally had no ability to produce A-factor and no DNA sequence homologous to the *S. bikiniensis* A-factor determinant, suggesting that precursors of A-factor are common metabolites in streptomycetes and that acquisition of only a single key enzyme encoded by the 1.1-kb fragment is sufficient for *Streptomyces* strains to synthesize A-factor.

A-Factor, 2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone[1] (Fig. 1), is a diffusible and low molecular weight bioregulator essential for streptomycin biosynthesis, streptomycin resistance, and spore formation in *Streptomyces griseus* and *Streptomyces bikiniensis*.[2~5] In these species, A-factor-deficient mutants in which streptomycin production and resistance, and spore forming ability are simultaneously lost are easily obtained by so-called plasmid curing treatments. Our results of genetic mapping together with cloning experiments suggested that the A-factor determinant is carried on an unstable extrachromosomal element, possibly a plasmid.[6,7]

The A-factor determinant cloned from *S. bikiniensis* was capable of conferring A-factor production to A-factor-deficient mutants of streptomycin-producing organisms and to *afsA* mutants of *Streptomyces coelicolor* A3(2) probably having defects in the structural genes for A-factor biosynthesis[7]. In both cases, 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. Also observed was a wide distribution of a sequence homologous to the *S. bikiniensis* A-factor determinant among A-factor-producing actinomycetes. These results had suggested that the cloned *S. bikiniensis* A-factor determinant contained one or more genes encoding A-factor biosynthetic enzymes.

In this communication, we describe further trimming of the cloned fragment and identification of a transcriptional signal on the fragment. In addition, results of introduction of a trimmed DNA into various *Streptomyces* strains suggested that A-factor is synthesized by only a single enzyme from common metabolites present in *Streptomyces* species.

Fig. 1. Chemical structure of A-factor.



Materials and Methods

The restriction endonucleases, T4 DNA ligase, and S1 nuclease were purchased from Takara Shuzo, Co., Ltd. or New England Biolabs., Inc. Thiostrepton was supplied by Asahi Chemical Industry. All the *Streptomyces* strains, plasmids, and growth media used were previously described.[6,7] General methods including recombinant DNA work, ethidium bromide-agarose gel electrophoresis, and A-factor assay were previously described.[7,8]

Results and Discussion

Plasmids pAFB2 and pAFB7 are the recombinant plasmids containing the A-factor determinant of *S. bikiniensis* in the wide host range plasmid pIJ702.[9] The determinant is carried on the cloned fragments with the size of 5.0 and 3.8-kilobase (kb), respectively. Fig. 2 shows the schematic diagram for sub-cloning the A-factor determinant by using these two plasmids as starting materials. The recombinant DNA work was performed essentially as described,[8] using an A-factor-deficient mutant, *S. bikiniensis* HH1 as the host. In this strain, a putative extrachromosomal element carrying the A-factor determinant is completely eliminated.[7] A-Factor assay of transformants carrying the respective plasmids revealed that plasmids pAFB11, pAFB15 and pAFB17 were capable of conferring A-factor production to strain HH1, whereas plasmids pAFB12 and pAFB16 were not. Both pAFB12 lacking a *Bam*H I fragment and pAFB16 lacking a short segment from the *Bst*E II to *Bam*H I site failed to confer A-factor production. Thus, the 1.1-kb fragment from the unique *Eco*R I to the second *Bam*H I site, which was carried by pAFB15, was suggested to be almost the minimum fragment capable of directing A-factor synthesis in strain HH1. Purified plasmid DNAs of pAFB11, pAFB15 and pAFB17 were reintroduced into A-factor-deficient mutants of *S. griseus* FT-1 number 2 and *S. griseus* IFO 13189 AO-1 to check the phenotypic expression. All the transformants were found to produce A-factor with simultaneous recovery of streptomycin production, streptomycin resistance and spore formation, owing to the pleiotropic effects of A-factor.

S. bikiniensis HH1 carrying pAFB15 or pAFB17 produced large amounts of A-factor (3.0 μg/ml), due to the gene-dosage effect by the multicopy vector plasmid pIJ702 (40 to 300 copies per chromosome), in comparison with the parental strain which produced about 0.3 μg/ml of A-factor. The result also suggested that the 1.1-kb fragment contained its own promoter, because pAFB15 and pAFB17 containing the 1.1-kb fragment in the opposite orientation with respect to the vector sequence directed synthesis of almost the same amount of A-factor. The presence of promoter signals in the 1.1-kb fragment was confirmed as described below.

Presence of a long open reading frame deduced from partial nucleotide sequencing of this region suggested the orientation of the *afsA* was from the *Eco*R I site to the *Bam*H I site (unpublished results). A promoter-probe plasmid pARC1 with a unique *Bam*H I cloning site contains a pigment production gene(s) on the vector plasmid pIJ41 and allows chromogenic identification of transcriptional signals.[10] Plasmid pARC1, when a DNA fragment with transcriptional control signals is inserted in the correct orientation in the *Bam*H I site, directs production of a brown pigment, presumably a shunt product in the actinorhodin biosynthetic pathway. For cloning a transcriptional signal in the 1.1-kb fragment into the promoter-probe plasmid pARC1 in a convenient way, a *Hin*d III linker (octanucleotide) was attached to the *Eco*R I site in pAFB1[7] by filling in *Eco*R I-created sticky ends with the Klenow fragment of DNA polymerase I, followed by ligating *Hin*d III linkers to the polymerized ends. Plasmid pAFB1 which was originally isolated is the parent of pAFB7. After the liga-

Fig. 2. Schematic diagram summarizing plasmid constructions.

The thick and light lines stand for the cloned *S. bikiniensis* sequence and the vector pIJ702 sequence, respectively. For clarity, the thiostrepton resistance gene is shown by dots.

The abbreviations are for restriction endonuclease recognition sites; *Bam*, *Bam*H I; *Bst*, *Bst*E II; *Bgl*, *Bgl* II; *Kpn*, *Kpn* I; *Pst*, *Pst* I; *Pvu*, *Pvu* II; RI, *Eco*R I; and *Sac*, *Sac* I.

Plasmids pAFB11 and pAFB12 were constructed by first cleaving pAFB7 DNA completely with *Bgl* II and then cleaving it partially with *Bam*H I. The digest was separated by 1% agarose-ethidium bromide gel electrophoresis and the resultant bands with the molecular sizes corresponding to the two plasmids were extracted. The extracts were ligated through the common four base ends, GATC, of *Bam*H I and *Bgl* II and introduced by transformation into *S. bikiniensis* HH1. Transformants were selected as thiostrepton (32 μg/ml) resistant colonies as previously described.[7]

For construction of pAFB15, the larger fragment obtained by digestion of pAFB11 with *Kpn* I plus *Eco*R I was purified by agarose gel electrophoresis, trimmed at the both ends with S1 nuclease to create flush ends, ligated with T4 DNA ligase, and introduced into strain HH1. Plasmid pAFB17 was constructed using pAFB2 DNA prepared from *S. lividans* as the starting material and restriction enzymes, *Eco*R I plus *Sac* I, in a similar manner as for pAFB15. The reason we used pAFB2 DNA propagated once in *S. lividans* was that DNA propagated in *S. bikiniensis* was not cleaved with *Sac* I, probably because of DNA modification present in this strain. To construct plasmid pAFB16, a restriction fragment obtained by digestion with *Pvu* II plus *Pst* I was cut with *Bst*E II and the *Bst*E II end was filled in with the Klenow fragment of DNA polymerase I. *Pst* I linkers (8 nucleotides) were attached to the both ends and cut with *Pst* I plus *Eco*R I to create the small segment with an *Eco*R I terminus at one end and a *Pst* I terminus at the other end. The segment was purified by agarose gel electrophoresis and ligated with the larger part of pAFB7 DNA cleaved with *Eco*R I plus *Pst* I, resulting in pAFB16. The structures of all the plasmids were confirmed by digesting the DNAs with appropriate restriction enzymes and analyzing the digests by agarose gel electrophoresis.

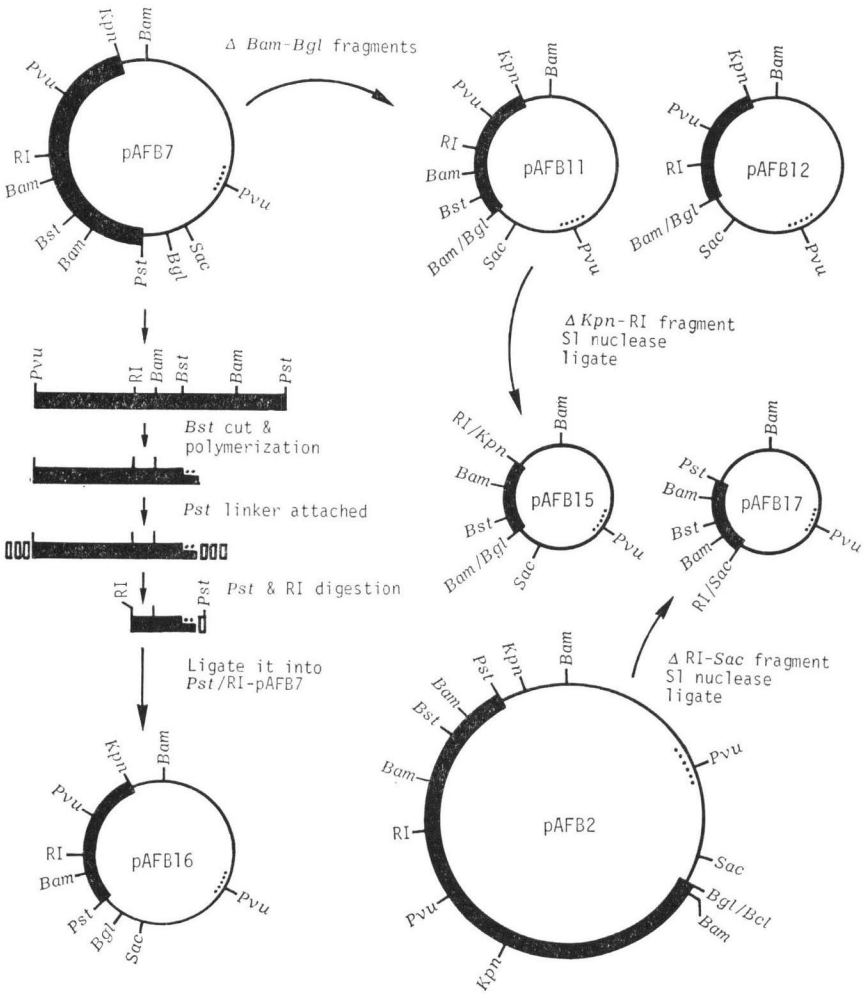
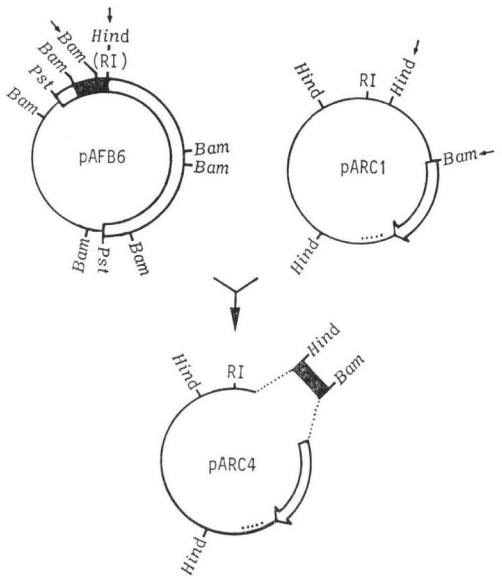


Fig. 3. Construction of pARC4 in which a 450-bp *Hin*d III-*Bam*H I fragment is inserted in pARC1 digested with *Bam*H I plus *Hin*d III.

The 1.1-kb fragment carrying the intact *afsA* gene is shown in black. The open arrow indicates the extent and direction of the pigment production gene(s).



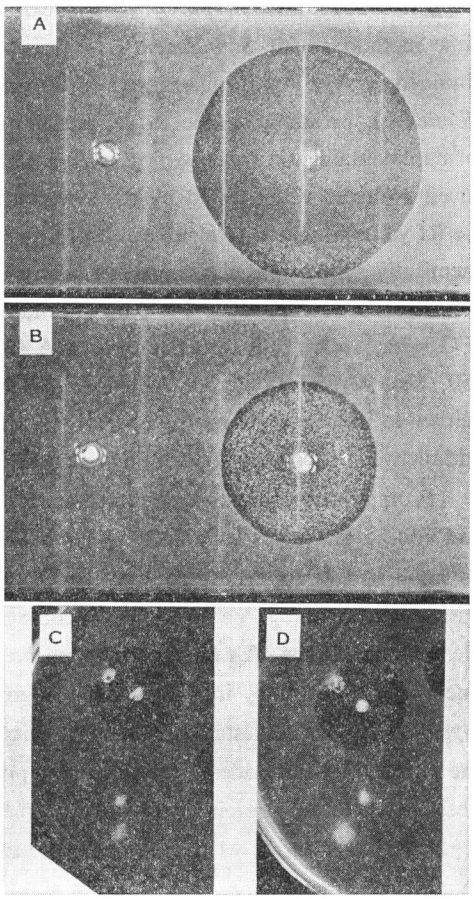
tion mixture was introduced by transformation into *S. lividans* TK21, thiostrepton resistant transformants were selected. Plasmid DNA was purified from one of the transformants and analyzed by agarose gel electrophoresis. By restriction digest, we identified a plasmid, named pAFB6, having a *Hin*d III cleavage site at the original *Eco*R I site (Fig. 3). A *Hin*d III-*Bam*H I fragment consisting of 450-bp was excised from pAFB6 and ligated into pARC1 digested with *Bam*H I completely and *Hin*d III partially, resulting in pARC4, as shown in Fig. 3. Deletion of the *Bam*H I to *Hin*d III fragment of pARC1 does not increase read-through transcription from the vector sequence, if any, to such an extent that pigment production can be detected.[10] Transformants carrying pARC4 produced a brown pigment in a large quantity, which revealed the presence of a promoter signal whose direction is from the original *Eco*R I site to the *Bam*H I site. In another experiment in which the 650-bp

Fig. 4. A-Factor assay of transformants carrying pAFB15 by the streptomycin-cosynthesis method.[5]

(A) *S. albus* IFO 3422, (B) *S. coelicolor* A3(2) IFO 3114, (C) *S. albus* IFO 3710, and (D) *S. viridochromogenes* IFO 12376.

In panels (A) and (B), the left and right colonies are the plasmid-free and plasmid-carrying strains, respectively. A-Factor produced by the transformants diffuses into agar medium seeded with an A-factor-deficient mutant strain, *S. griseus* FT-1 number 2, and triggers the streptomycin biosynthesis of the A-factor-deficient *S. griseus*, which in turn is detected by an inhibitory zone of indicator organisms, *B. subtilis* ATCC 6633.

In panels (C) and (D), the upper pairs consist of the plasmid-carrying strain and the A-factor-deficient mutant strain, *S. griseus* FT-1 number 2, and the lower pairs consist of the plasmid-free strain and *S. griseus* FT-1 number 2. A-Factor produced by the transformants carrying pAFB15 triggers the streptomycin synthesis of the A-factor-deficient *S. griseus* strain which grows close to the transformants.



*Bam*H I-*Bam*H I (the remainder) fragment in the 1.1-kb fragment was inserted into the *Bam*H I site of pARC1 in two orientations, none of the transformants showed a pigment-producing phenotype.

Plasmid pAFB15 also conferred A-factor production in a large quantity to A-factor-deficient mutants of *S. griseus*, the strains FT-1 number 2 and IFO 13189 AO-1. In addition, pAFB15 complemented the *afsA* mutations of *S. coelicolor* A3(2) strains BH2 and BH10 with a marked gene-dosage effect as observed in *S. bikiniensis* and *S. griseus*. These results indicate that the function specified by the 1.1-kb fragment corresponds to the *afsA* which we have presumed to be the structural gene(s) for A-factor biosynthetic enzyme(s). In *S. coelicolor* A3(2), a putative regulatory gene, *afsB*, is required for the expression of *afsA* leading to A-factor production.[6,8] In the previous paper,[7] however, we concluded that the cloned *S. bikiniensis* A-factor determinant did not require such a positive regulatory gene for the expression. This conclusion was supported by using pAFB15. Introduction of pAFB15 into *S. coelicolor* A3(2) strains BH5 and BH6 which have mutations in *afsB* led to A-factor production at almost the same amount as those for *afsA* mutants of *S. coelicolor* A3(2) carrying pAFB15.

To determine whether plasmid pAFB15 causes A-factor production to other various *Streptomyces* strains, we introduced the plasmid by transformation into *Streptomyces* strains which originally had no ability to produce A-factor and no DNA sequence homologous to the *S. bikiniensis* A-factor determinant.[7] Four strains, namely, *Streptomyces albus* IFO 3422, *S. albus* IFO 3710, *S. coelicolor* A3(2) IFO 3114, and *Streptomyces viridochromogenes* IFO 12376, were transformed by essentially the same method as for *S. bikiniensis*. Transformants resistant to 30 μg of thiostrepton per ml were obtained at very low frequencies (3 to 50 transformants per μg of pAFB15 DNA prepared from *S. bikiniensis*), presumably due to restriction-modification barriers. The thiostrepton resistant transformants obtained in this way possessed a plasmid with the same molecular size as pAFB15 and produced A-factor when tested by the streptomycin-cosynthesis method (Fig. 4). In a bioautogram,[2] the Rf values of the active substance produced by each of the transformants were the same as that of chemically synthesized A-factor.[1] Usually, A-factor-producing *Streptomyces* strains obtained from culture collections produce 2 to 5 ng of A-factor per colony, while the above transformants of *S. albus* IFO 3422, *S. albus* IFO 3710, *S. coelicolor* A3(2) IFO 3114, and *S. viridochromogenes* IFO 12376 produced 150, 120, 90 and 120 ng of A-factor, respectively. Acquisition of A-factor productivity appeared neither to influence the morphological features of the transformants nor to cause production of any antimicrobial substance when *Bacillus subtilis* ATCC 6633 was used as the indicator strain.

From all the results described above, it is evident that the 1.1-kb fragment contains both promoter and coding sequences capable of A-factor biosynthesis in a wide variety of streptomycetes. From its small size, it appears likely that the fragment contains only one gene. It is most likely that the putative gene is a structural gene encoding an enzyme involved in A-factor biosynthesis. Complementation of the *afsA* function of *S. coelicolor* A3(2) by the cloned fragment and a marked overproduction of A-factor by introducing the fragment on the multicopy vector plasmid in all the *Streptomyces* strains so far tested also support this assumption. Although there remains a possibility that the 1.1-kb fragment codes for a regulatory protein, it is not likely that an increase in the amount of a positive regulatory protein leads to such a remarkable overproduction of A-factor. In fact, a positive regulatory gene, *afsB*, when carried on the same multicopy plasmid pIJ702 and introduced into *afsB* mutants of *S. coelicolor* A3(2), did not show a gene-dosage effect on A-factor production but led to restoration of A-factor production only to the level of the parental strain.[8] If A-factor is synthesized

by a single key enzyme encoded by the 1.1-kb fragment, then the precursors which this enzyme works are commonly present in *Streptomyces* strains. We speculate that A-factor may be synthesized from a 3 carbon unit, probably a glycerol derivative, and a 10 carbon unit, a β-keto acid.

Acknowledgments

This work was supported in part by research grants from the Ministry of Education, Culture and Science of Japan, from the Waksman Foundation of Japan, and from the Araki Memorial Foundation for Medical and Biochemical Researchs.

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