Involvement of AfsA in A-factor Biosynthesis as a Key Enzyme

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The *afsA* gene of *Streptomyces griseus* has been postulated to encode a key enzyme for A-factor biosynthesis from primary metabolites commonly present in *Streptomyces* strains. *Escherichia coli* cells harboring *afsA* under the control of the T7 promoter specified distinct A-factor activity in the culture broth, as determined by induction of streptomycin production and aerial mycelium and spore formation in an A-factor-deficient *S. griseus* mutant strain. Production of the substance(s) having A-factor activity was inhibited by cerulenin, an inhibitor of fatty acid biosynthesis. These observations suggest that *afsA* encodes a key enzyme in the A-factor biosynthetic pathway in which a β-keto acid derived from fatty acid biosynthesis and a glycerol derivative serve as precursors.

A-factor, 2-(6′-methylheptanoyl)-3*R*-hydroxymethyl-4-butanolide (see Fig. 4 for the structure of A-factor), is a representative of autoregulatory factors controlling secondary metabolism or cell differentiation, or both, in streptomycetes[1~4]. It is produced in a growth-dependent manner by *Streptomyces griseus* and switches on the expression of several phenotypes, such as streptomycin production, streptomycin resistance, yellow pigment production, and aerial mycelium formation in the same organism at an extremely low concentration, in co-operation with its specific receptor protein, ArpA[5~7]. The ArpA protein with 276 amino acids shows strict ligand specificity and a change in chemical structure of A-factor results in almost complete loss of the activity as a regulator[8]. The repressor-like behavior of ArpA for secondary metabolism and cell differentiation, as implied from genetic studies[9] and *in vitro* experiments[7], has led to the idea that A-factor binds to ArpA as an early event in the A-factor regulatory cascade, resulting in derepression of a still unknown function that is required in secondary metabolism and morphogenesis.

There have been few genetic studies on A-factor biosynthesis, except that the *afsA* gene encoding a protein of 301 amino acids was cloned by complementation of the A-factor-deficiency of a mutant *S. griseus* strain HH1[10,11]. Genetic instability of *afsA*, as phenotypically expressed as streptomycin-nonproduction and bald characteristics, is assumed to be due to its location on the linear chromosome; *afsA* is located very near one end of the chromosome, about 150-kb from the end, and this region is known to be readily deleted[12]. We have postulated that AfsA is a key enzyme for A-factor biosynthesis[13], because (i) a trimmed 1.2-kb fragment encoding only AfsA still conferred A-factor production to strain HH1, (ii) *afsA* showed a strong gene dosage effect on A-factor production, and (iii) *afsA* conferred A-factor production with a marked gene dosage effect on *Streptomyces* strains which were unable to produce A-factor. In this paper, we show that *afsA* directs the synthesis of A-factor or a compound with properties similar to A-factor in *Escherichia coli* containing *afsA*. In addition, the biosynthesis of the compound with A-factor activity in *E. coli* was inhibited by cerulenin, an inhibitor of the fatty acid biosynthesis. Taken together with the biosynthetic studies of virginiae butanolides by SAKUDA *et al.*[14,15], these data show that *afsA* encodes an enzyme essential for A-factor biosynthesis starting from a β-keto acid, probably an intermediate in fatty acid biosynthesis and a glycerol derivative, as we previously suggested[1,13].

Materials and Methods

Bacterial Strains and Plasmids

S. griseus IFO 13350 was the source of *afsA*[10]. *S. griseus* strains HH1[10] and FT-1 no. 2[16], both of which are A-factor-deficient mutants, were used for A-factor assays. *E. coli* JM109 [*recA1 thi-1 endA1 supE44*

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gyrA96 relA1 hsdR17 Δ(lac-proAB) F' traD36 proAB lacIq lacZΔM15][17] and ampicillin resistance plasmid pUC19 (Takara Shuzo, Co., Kyoto) were used for DNA manipulation. *E. coli* JM109 (DE3) [*hsdS gal* (λcI857 *indl Sam7 nin5 lac*UV-T7 gene 1)] used for expression of AfsA was purchased from Promega Co. (Madison, Wisconsin). Plasmid pAFB1 contained *afsA* on pIJ702[11]. Plasmid pET-16b(+) (Novagen, Madison) was used for expression of AfsA in *E. coli* JM109 (DE3). Growth conditions for *E. coli* were as described by MANIATIS *et al.*[18] Ampicillin and chloramphenicol were added at final concentrations of 50 and 34 μg/ml, respectively, when necessary. *S. griseus* strains were grown in YMPD medium containing the following, in grams per liter: yeast extract (Difco Laboratory), 2; meat extract (Wako Pure Chemicals), 2; Bacto-Peptone (Difco), 4; NaCl, 5; $MgSO_4 \cdot 7H_2O$, 2; glycine, 5; and glucose, 10 (pH 7.2).

Recombinant DNA Studies

General techniques were described by MANIATIS *et al.*[18] Restriction endonucleases, T4 DNA ligase, *Taq* polymerase, and DNA polymerase I (Klenow fragment) were purchased from Takara Shuzo, Co. (Kyoto). DNA manipulations in *Streptomyces* were as described by HOPWOOD *et al.*[19]

Expression of *afsA* in *E. coli*

The 1.2-kb *Eco*RI-*Bam*HI fragment containing *afsA* was prepared from pAFB1 and cloned between the *Eco*RI and *Bam*HI sites in pUC19, resulting in pUC-AFSA. Plasmid pET-16b contained the T7 RNA polymerase promoter[20], followed by an ATG translational start codon. The ATG codon was present in a *Nco*I cleavage sequence, CCATGG. To place *afsA* under the control of the T7 promoter, the nucleotide sequence (ACTATG) covering the ATG start codon of *afsA* was changed into CCATGG by the polymerase chain reaction (PCR) mutagenesis using primer I (5'-GCCGAATT*CC*CATG-GACGCGGAGG-3'; the italic letters indicate the bases to be replaced and the underline indicates an *Eco*RI cleavage sequence) and primer II (5'-TAGGCCTTGG-GCGTGCTGAAC-3'; the nucleotide sequence was based on the *afsA*-coding sequence and the underline indicates an *Eco*T14I cleavage sequence), both of which were synthesized on an Applied Biosystems model 380A DNA synthesizer. After amplification by PCR under standard conditions, the *Eco*RI-*Eco*T14I fragment was prepared and cloned between the *Eco*RI and *Eco*T14I sites of pUC-AFSA. The sequence was confirmed by the M13-dideoxynucleotide sequencing[21]. The *afsA* gene thus obtained was excised with *Nco*I and *Sal*I and inserted between the *Nco*I and *Xho*I sites of pET-16b, resulting in pET-AFSA.

A-factor Assay

A portion (0.1 ml) of *E. coli* JM109 (DE3) harboring pET-AFSA grown overnight at 37°C in LB medium containing ampicillin and chloramphenicol was inoculated into 10 ml of the same medium and cultured at 30°C for 4 hours. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added at a final concentration of 1 mM and the cultivation was continued for further 4 hours. When grown in the presence of cerulenin (Sigma), oleate (250 μg/ml), myristate (50 μg/ml), and palmitate (50 μg/ml) were added to ensure cell growth. The culture broth was extracted once with an equal volume of ethyl acetate, concentrated by centrifugal evaporation, and appropriately diluted with ethanol and used for A-factor assays as described below. The recombinant *E. coli* strain was also grown at 28°C for 5 days on an agar plug consisting of YMPD medium supplemented with ampicillin, chloramphenicol, and IPTG. A-factor, when produced by the *E. coli* cells, diffused into the plug.

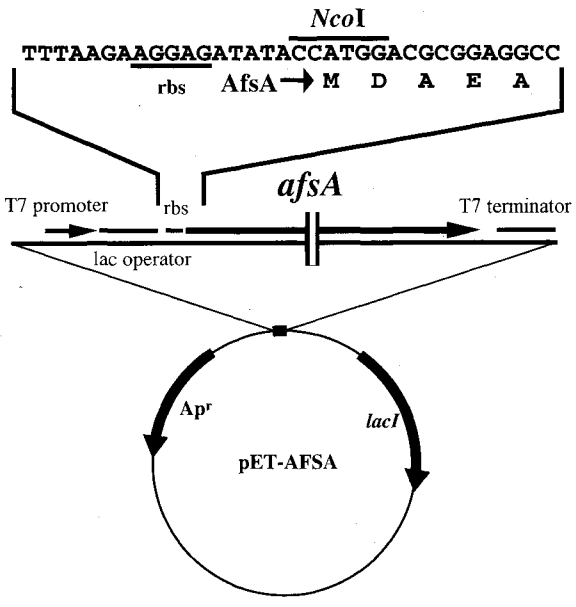
A-factor activity was detected by the streptomycin-cosynthesis method described previously[10]. Briefly, a test strain grown on an agar plug was transferred to a soft agar layer seeded with an A-factor-deficient mutant strain, *S. griseus* FT-1 no. 2, and incubated at 28°C for a few days. Nutrient soft agar containing spores of *B. subtilis* as the indicator strain was then overlaid, and the plate was further incubated overnight at 37°C. The principle of this method is that A-factor diffused from the agar plug into the soft agar causes the mutant *S. griseus* strain to produce streptomycin, which in turn is detected by growth inhibition of the indicator. A-factor activity of culture broths was similarly measured by applying them onto paper discs after appropriate dilution or concentration. Restoration of spore formation of *S. griseus* HH1 was also examined by adjacent growth of the recombinant *E. coli* cells and strain HH1 on YMPD medium supplemented with IPTG.

Results and Discussion

Production by *E. coli* Harboring *afsA* of Autoregulatory Substances Conferring Streptomycin Production and Aerial Mycelium Formation on A-factor-deficient Mutants of *S. griseus*

Since *afsA* had appeared to encode a key enzyme for

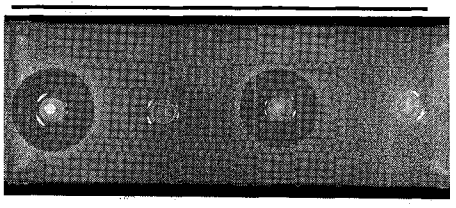
Fig. 1. Structure of pET-AFSA used for expression of *afsA* in *E. coli*.



The *afsA*-coding sequence introduced between a ribosome-binding sequence (rbs) and the T7 terminator was placed under the control of the T7 promoter and the *lac* operator, as described in Materials and Methods. Ap^r, ampicillin resistance determinant; *lacI*, the repressor gene in the lactose operon.

Fig. 2. A-factor activity of *E. coli* harboring pET-AFSA, as determined by the streptomycin-cosynthesis method.

	Strains			
	S. griseus IFO13350	no strain	pET-AFSA /*E. coli*	pET-16b /*E. coli*
Ap (50 μg/ml)	-	+	+	+
Cm (34 μg/ml)	-	+	+	+
IPTG (1 mM)	-	+	+	+



E. coli strains were grown on an agar plug (diameter, 5 mm) punched from YMPD agar medium and subjected to A-factor assay by the streptomycin-cosynthesis method, as described in Materials and Methods. A growth inhibition zone around the plug containing *E. coli* harboring pET-AFSA, indicative of A-factor activity, is seen, whereas no growth inhibition zone around the plug containing *E. coli* harboring the vector plasmid pET-16b is seen. As controls, the wild-type strain, *S. griseus* IFO 13350, and an agar plug similarly prepared were also subjected to the assay. Ampicillin and chloramphenicol cause no detectable effect in this assay, because no growth inhibition zone is seen around the bacteria-free plug.

A-factor biosynthesis from precursors present commonly as primary metabolites in *Streptomyces* strains[13], we attempted to express *afsA* in *E. coli* and synthesize A-factor or its derivatives on the assumption that the putative precursors were also present in a wide variety of bacteria. For this purpose, we placed *afsA* under the control of the T7 promoter in vector pET-16b (plasmid pET-AFSA), as shown in Fig. 1. Expression was induced by IPTG[20].

The culture broth from the recombinant *E. coli* cells was extracted with ethyl acetate, concentrated, and assayed for the A-factor activity by the streptomycin-cosynthesis method. We first examined A-factor activity of the concentrated culture broth by applying it on a paper disc for the A-factor assay. The culture broth from the *E. coli* cells was found to contain a distinct amount of a substance(s) having A-factor activity, whereas that from *E. coli* harboring only the vector was not (data not shown). The quantity calibrated to an A-factor standard was approximately 1 ng per ml. We next examined the A-factor activity of pET-AFSA-containing *E. coli* cells grown on an agar plug (Fig. 2); this strain produced a substance(s) that induced streptomycin production in the A-factor-deficient mutant strain, FT-1 no. 2, whereas *E. coli* containing the vector plasmid pET-16b was inactive in this respect. The activity in one plug containing the *E. coli* cells grown at 28°C for 5 days corresponded to approximately 5 ng A-factor, when calculated with a calibration curve[10].

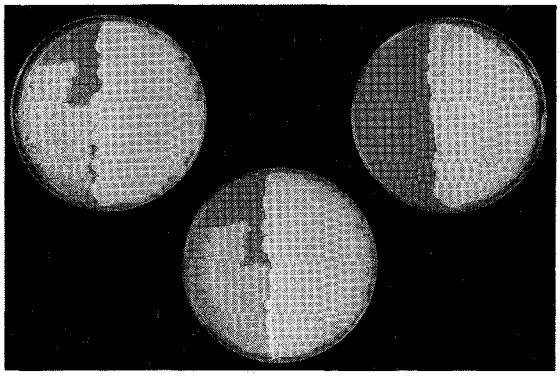
We next determined whether the recombinant *E. coli* cells made products that induced sporulation of the A-factor-deficient mutant *S. griseus* strain. As shown in Fig. 3, the A-factor-deficient mutant strain adjacent to the recombinant *E. coli* formed aerial mycelium and spores, whereas that grown apart from the *E. coli* cells did not. This demonstrates that a diffusible substance(s) produced by pET-AFSA-containing *E. coli* caused aerial mycelium and spore formation. These results suggest that the substance(s) produced by the recombinant *E. coli* was A-factor or an A-factor homologue very similar to A-factor, because of the known ligand specificity of ArpA[5].

HPLC Analysis of the Substance Produced by Recombinant *E. coli* Cells

We analyzed the substance(s) produced by the recombinant *E. coli* by HPLC. The concentrated sample

prepared from 500 ml of the culture broth was applied to a SEP-PAK column and eluted step-wise with chloroform/methanol. Each fraction was assayed for A-factor activity by the streptomycin-cosynthesis method (Fig. 4). A major A-factor activity was eluted with chloroform/methanol (99.5 : 0.5) and a very weak activity was eluted with chloroform/methanol (97 : 3). This suggested that two substances having A-factor activity were produced; the minor activity could be due to a modification of the major substance. An authentic sample of A-factor eluted in the major activity peak. Taking into consideration the strict ligand specificity of ArpA, we assume that the major activity is A-factor.

Fig. 3. A-factor activity of *E. coli* harboring pET-AFSA, as determined in terms of their ability to induce aerial mycelium and spore formation in an A-factor-deficient mutant *S. griseus* strain HH1.

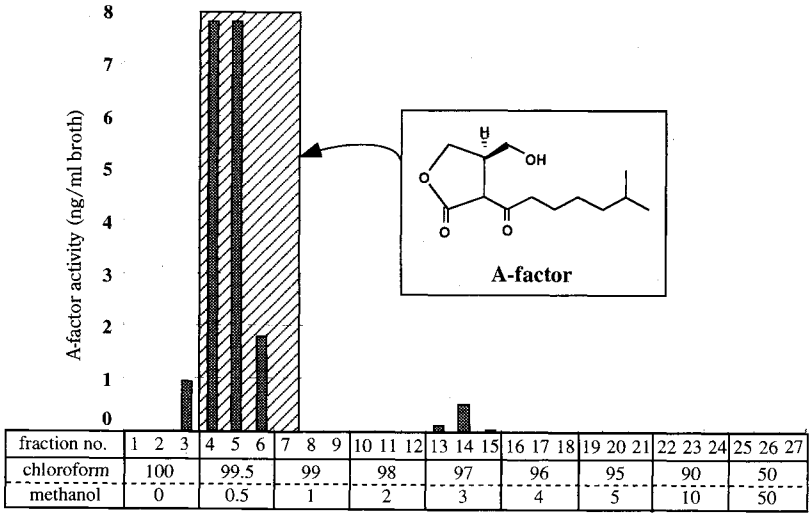


(*Top left*) *E. coli* harboring pET-16b (left) was grown close to *S. griseus* HH1 at 28°C for 5 days on YMPD medium supplemented with 1 mM IPTG. No sporulation area is seen. (*Top right*) As a negative control, *S. griseus* HH1 was grown similarly. (*Bottom*) Distinct sporulation of *S. griseus* HH1 in a zone close to *E. coli* harboring pET-AFSA.

Inhibition of Production of the Substance with A-factor Activity by Cerulenin

The above observations suggested a similarity between biosynthesis of the presumed A-factor with that of homoserine lactones which control various biological functions in Gram-negative bacteria[22~24]. The homoserine lactones are produced from *S*-adenosylmethionine and a β-keto acid derived from the fatty acid biosynthetic pathway[23,24]. It was likely that the substance having A-factor activity was derived from a β-keto acid, possibly derived from the fatty acid biosynthetic pathway. We therefore examined the possible effect of a fatty acid synthetase inhibitor, cerulenin, on production of the substance produced by the recombinant *E. coli* cells. Cerulenin irreversibly inhibits β-ketoacyl-acyl carrier protein synthetase catalyzing the condensation reaction of fatty acid biosynthesis[25]. Cerulenin inhibited production of the A-factor activity in a concentration-dependent manner (Fig. 5), suggesting that a precursor is derived from an intermediate in the fatty acid biosynthetic pathway. The growth of *E. coli* in the medium supplemented with the fatty acids was not significantly inhibited by cerulenin at the concentrations tested.

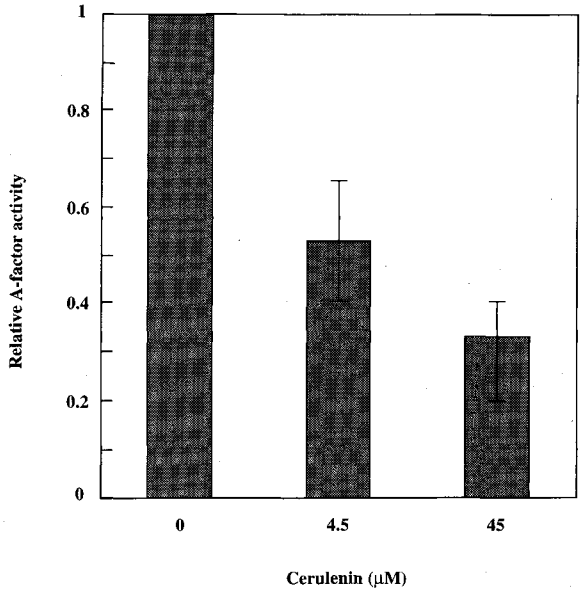
Fig. 4. Separation of A-factor activity in the culture broth by HPLC.



fraction no.	1 2 3	4 5 6	7 8 9	10 11 12	13 14 15	16 17 18	19 20 21	22 23 24	25 26 27
chloroform	100	99.5	99	98	97	96	95	90	50
methanol	0	0.5	1	2	3	4	5	10	50

The culture broth of *E. coli* harboring pET-AFSA was ethyl acetate-extracted, concentrated by evaporation, and applied to HPLC equipped with a SEP-PAK column. A-factor activity was eluted step-wise with chloroform/methanol as indicated, and assayed by the streptomycin-cosynthesis method. A-factor activity of each fraction is expressed in ng/ml broth. Authentic A-factor eluted in fractions 4 to 7, as indicated by oblique lines.

Fig. 5. Inhibition of production of substances with A-factor activity by cerulenin.



E. coli harboring pET-AFSA was grown in the presence and absence of cerulenin in LB broth and A-factor activity in the culture broth was measured by the streptomycin-cosynthesis method. Relative activities are shown.

Possible Route for Biosynthesis of A-factor: A Hypothesis

Since introduction of *afsA* into *Streptomyces* strains causes A-factor production with a marked gene dosage effect, we have proposed that A-factor is biosynthesized by the action of AfsA from precursors commonly present in streptomycetes, probably a glycerol derivative (C3 carbon unit) and a β-keto acid (C10 carbon unit). This is in agreement with the observation that *afsA* directs the biosynthesis of compounds having A-factor activity from precursors in *E. coli*. Thus the required precursors are present widely in Gram-positive and Gram-negative bacteria. One of the precursors appears to be derived from the fatty acid biosynthetic pathway, and it is likely that this is a β-keto acid. The other precursor is then possibly a glycerol derivative, as was postulated previously[13]. We speculate that AfsA catalyzes some modification of a substrate resulted from condensation of the putative C10 β-keto acid and the C3 glycerol derivative. We need further data to reveal the exact biosynthetic step catalyzed by AfsA.

HPLC analysis showed the presence of two compounds having A-factor activity (Fig. 4). The A-factor receptor protein, ArpA, shows a very strict ligand specificity[5,26] and alterations in the structure of A-factor results in severe reduction of A-factor activity[8,27]. In addition, the keto group at the 6-position is essential for A-factor activity. The strict ligand specificity of ArpA suggests that the putative β-keto acid has the same or similar carbon chain length as A-factor without a branched carbon, with a keto group at the position corresponding to the 6-position of A-factor. This may explain the presence of two compounds with A-factor activity.

Together with the virginiae butanolide biosynthetic pathway proposed by SAKUDA *et al.*[14,15], the present study suggests that the γ-butyrolactone-type regulators with a keto group at the 6-position, such as A-factor, are synthesized from primary metabolites, a glycerol derivative and a β-keto acid derived from the fatty acid biosynthetic pathway. AfsA may catalyze the condensation of the two precursors to produce A-factor or some modification of a compound resulted from condensation of the precursors. The γ-butyrolactones with a hydroxyl group at the 6-position, such as virginiae butanolides in *Streptomyces virginiae*[28], IM-2 in a *Streptomyces* sp.[29], an autoregulator in *S. coelicolor* A3(2)[30,31], a regulator in *Streptomyces bikiniensis* and *Streptomyces cyaneofuscatus*[32], and an inducer in *Streptomyces viridochromogenes*[33], are presumably synthesized by reduction of the keto group at the 6-position of the A-factor-type compounds. The present study of A-factor synthesis in *E. coli* provides additional information on the biosynthesis of γ-butyrolactone-type autoregulators.

Acknowledgments

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