Journal of Bacteriology, Nov. 1995, p. 6083–6092 Vol. 177, No. 21
0021-9193/95/$04.00+0
Copyright © 1995, American Society for Microbiology

# Cloning and Characterization of the A-Factor Receptor Gene from *Streptomyces griseus*

HIROYASU ONAKA,[1] NORIKO ANDO,[1] TAKUYA NIHIRA,[2] YASUHIRO YAMADA,[2] TERUHIKO BEPPU,[1]† and SUEHARU HORINOUCHI[1]*

*Department of Biotechnology, Division of Agriculture and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113,[1] and Department of Biotechnology, Faculty of Engineering, Osaka University, Yamada-oka, Suita-shi, Osaka 565,[2] Japan*

Received 10 July 1995/Accepted 18 August 1995

**A-factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone) and its specific receptor protein control streptomycin production, streptomycin resistance, and aerial mycelium formation in *Streptomyces griseus*. The A-factor receptor protein (ArpA) was purified from a cell lysate of *S. griseus* IFO 13350. The $NH_2$-terminal amino acid sequences of ArpA and lysyl endopeptidase-generated fragments were determined for the purpose of preparing oligonucleotide primers for cloning *arpA* by the PCR method. The *arpA* gene cloned in this way directed the synthesis of a protein having A-factor-specific binding activity when expressed in *Escherichia coli* under the control of the T7 promoter. The *arpA* gene was thus concluded to encode a 276-amino-acid protein with a calculated molecular mass of 29.1 kDa, as determined by nucleotide sequencing. The A-factor-binding activity was observed with a homodimer of ArpA. The $NH_2$-terminal portion of ArpA contained an α-helix–turn–α-helix DNA-binding motif that showed great similarity to those of many DNA-binding proteins, which suggests that it exerts its regulatory function for the various phenotypes by directly binding to a certain key gene(s). Although a mutant strain deficient in both the ArpA protein and A-factor production overproduces streptomycin and forms aerial mycelium and spores earlier than the wild-type strain because of repressor-like behavior of ArpA, introduction of *arpA* into this mutant abolished simultaneously its streptomycin production and aerial mycelium formation. All of these data are consistent with the idea that ArpA acts as a repressor-type regulator for secondary metabolite formation and morphogenesis during the early growth phase and A-factor at a certain critical intracellular concentration releases the derepression, thus leading to the onset of secondary metabolism and aerial mycelium formation. The presence of ArpA-like proteins among *Streptomyces* spp., as revealed by PCR, together with the presence of A-factor-like compounds, suggests that a hormonal control similar to the A-factor system exists in many species of this genus.**

The ability to produce a wide variety of secondary metabolites and a mycelial form of growth that develops into spores are two aspects characteristic of the gram-positive bacterial genus *Streptomyces* (5, 6). A-factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone) is a microbial hormone controlling secondary metabolism and cell differentiation in *Streptomyces griseus* (15, 27, 28). It acts as a chemical signal molecule for streptomycin production, streptomycin resistance, yellow pigment production, and aerial mycelium formation at a concentration as low as $10^{-9}$ M (15, 16). Although A-factor itself seems to exert its regulatory function in a limited group including *S. griseus* and *Streptomyces actuosus*, the presence of γ-butyrolactones structurally similar to A-factor in a wide variety of *Streptomyces* spp. suggests that these compounds serve as hormonal regulators for secondary metabolism or morphogenesis, or both, in general in streptomycetes (21–24). Virginiae butanolides controlling virginiamycin production in *Streptomyces virginiae* (31, 51), IM-2 controlling production of a blue pigment in a *Streptomyces* sp. (18, 46), a factor controlling both cytodifferentiation and anthracycline production in *Streptomyces bikiniensis* and *Streptomyces cyaneofuscatus* (12), and an inducer controlling anthracycline production in *Streptomyces viridochromogenes* (13) are examples.

* Corresponding author. Phone: 81 (03)3812-2111, ext. 5123. Fax: 81 (3)5802-2931.

† Present address: Department of Applied Biological Science, College of Agriculture and Veterinary Medicine, Nihon University, Fujisawa-shi, Kanagawa 252, Japan.

Recent identification of specific receptor proteins for A-factor (38), virginiae butanolides (29), and IM-2 (44) has provided a substantial clue to the mechanism by which these γ-butyrolactone-type autoregulators are involved as chemical signal molecules. The experiments with [$^3$H]A-factor and a cell extract of *S. griseus* showed that approximately 40 molecules of the A-factor receptor protein per genome were present in the cytoplasmic fraction (38). The dissociation constant was calculated to be 0.7 nM, in agreement with the extremely low effective concentration of A-factor. The finding that the A-factor receptor protein acts as a repressor-type regulator for streptomycin production and aerial mycelium formation has led to the idea that A-factor binds to the receptor protein as an early event in the A-factor regulatory cascade, resulting in derepression of a still unknown key gene(s) that is required for secondary metabolism and aerial mycelium formation in *S. griseus* (39). The step in which the A-factor receptor is involved is therefore of central importance for transmitting the A-factor signal to the downstream genes leading to streptomycin production and aerial mycelium formation in the regulatory cascade.

In the present study, we purified the receptor protein and cloned the receptor gene by the PCR method. A similar strategy was used for cloning of the virginiae butanolide receptor gene from *S. virginiae* (40). We report here the characterization of the cloned A-factor receptor gene, together with genetic studies with the cloned gene. The phenotypes conferred by the receptor gene on a mutant deficient in the receptor are in agreement with our previous assumption that the A-factor

receptor acts as a repressor-type regulator for secondary metabolism and morphogenesis.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** *S. griseus* IFO 13350 (25) was a source of the A-factor receptor protein. *S. griseus* KM7 (39), derived by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine mutagenesis from an A-factor-deficient mutant strain, HH1, had a defect in the A-factor receptor protein. *Streptomyces lividans* TK21 (20) and *Streptomyces coelicolor* A3(2) M130 (4) were obtained from D. A. Hopwood, John Innes Institute, Norwich, United Kingdom. The other *Streptomyces* strains were obtained from the Institute of Fermentation, Osaka, Japan. *Escherichia coli* JM109 [*recA1 thi-1 endA1 supE44 gyrA96 relA1 hsdR17* Δ(*lac-proAB*) F′ *traD36 proAB lacI*$^q$ *lacZ*Δ*M15*] (52) and DH5α [F$^-$ φ80 *lacZ*Δ*M15* Δ(*lacZY-argF*)*U169 deoR recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1*; Gibco-BRL, Gaithersburg, Md.)] were used in cloning of the A-factor receptor protein gene and in most of the DNA manipulations. *E. coli* CJ236 [*dut1 ung1 thi-1 relA1*/pCJ105 (F′ Cam$^r$)] (36) was used for generation of a uracil-containing, single-stranded DNA for site-directed mutagenesis. Strain JM109 (DE3) [*hsdS gal* (λcI857 *ind1 S*am7 *nin*5 *lac*UV-T7 gene 1)] was purchased from Promega Co. (Madison, Wis.). Ampicillin resistance plasmid pUC19 was used as the cloning vector, and pGEMEX-1 containing the T7 promoter (Promega Co.) was used for expression of the A-factor receptor protein in *E. coli* JM109 (DE3). For expression of the A-factor receptor gene in *Streptomyces* spp., pIJ486 (carrying thiostrepton and neomycin resistance [50]), obtained from D. A. Hopwood, and an *E. coli-Streptomyces* shuttle vector, pKU209 (carrying thiostrepton resistance and the SCP2* replication origin [26]), obtained from H. Ikeda, Kitasato University, Tokyo, Japan, were used. Growth conditions for *E. coli* were as described by Maniatis et al. (35). *S. griseus* strains were grown in YMPG 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).

**General recombinant DNA techniques.** Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, DNA polymerase I (Klenow fragment), and *Taq* DNA polymerase were purchased from Takara Shuzo Co. [α-$^{32}$P]dCTP at 3,000 Ci/mmol for nucleotide sequencing by the M13 dideoxynucleotide method (45) with M13mp18 and M13mp19 (52) and for labeling of DNA with the *Bca*BEST labeling kit (Takara Shuzo) were purchased from Amersham International. Thiostrepton was a gift from Asahi Kasei Co. Ltd. DNA manipulations in *E. coli* were as described by Maniatis et al. (35), and those in *Streptomyces* spp. were as described by Hopwood et al. (19).

**Assay of A-factor receptor protein.** The A-factor binding assay with [$^3$H]A-factor used routinely during purification was performed essentially by the method of Miyake et al. (38). [$^3$H]A-factor had a specific activity of 2.8 Ci/mmol. The assay mixture contained 200 μl of a protein sample and 71.4 nM [$^3$H]A-factor (14.3 pmol) in a 1.5-ml microcentrifuge tube. Specific binding activity was defined as the difference between binding of the radioactive A-factor in the presence and absence of 16.5 nmol of nonlabeled A-factor. After the reaction mixture had been incubated at room temperature for 30 min, it was immediately applied to a disposable Sephadex G-25 column (Pharmacia PD10; 1.2 by 6 cm), previously equilibrated with buffer A (50 mM triethanolamine, 0.5 M KCl [pH 7.0]), for the purpose of rapid separation of receptor-bound from free [$^3$H]A-factor. The column was eluted with buffer A, and 1-ml fractions were collected to measure radioactivity by liquid scintillation counting with a cocktail containing Omnifluor (New England Nuclear). During purification, the sum of the radioactivity of fractions 3 and 4 was measured, because receptor-bound [$^3$H]A-factor was always eluted in these fractions.

**Purification of A-factor receptor protein.** *S. griseus* IFO 13350 was used as a source of the A-factor receptor protein. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on slab gels was used to monitor protein purification and to estimate molecular weights under denaturating conditions. The concentrations of polyacrylamide were 10 or 12.5% in separating gels and 4% in stacking gels. The gels were stained with 0.1% Coomassie brilliant blue R-250. During purification, protein concentrations were measured with a Bio-Rad protein assay kit, using bovine serum albumin as the standard.

**(i) Preparation of cell extract.** *S. griseus* IFO 13350 was cultured in 100 ml of YMPG medium at 30°C for 48 h. The mycelium was homogenized with a glass homogenizer and transferred to 15 liters of YMPG medium in a 30-liter jar fermentor. After cultivation at 30°C for 42 h, the mycelium was harvested by centrifugation. Of the 320 g (wet weight) of mycelium obtained, 160 g was suspended in 600 ml of buffer A and disrupted by three times passages at 700 kg/cm$^2$ through a Manton-Gaulin apparatus (model 15M8TA; Gaurin Corp., Everett, Mass.). The supernatant obtained by centrifugation of the disrupted mycelium at 15,000 × *g* for 30 min at 4°C was used as the cell extract.

**(ii) Ammonium sulfate fractionation.** Solid ammonium sulfate was added to the cell extract to 32% saturation, and the mixture was gently stirred at 4°C for 1 h. The precipitate was removed by centrifugation at 15,000 × *g* for 30 min, and then the ammonium sulfate concentration was increased to 43.5% saturation. The mixture was gently stirred at 4°C for 1 h, and the precipitate was collected by similar centrifugation. The precipitate dissolved in 54 ml of buffer A was dialyzed overnight against buffer B (50 mM triethanolamine, 0.1 M KCl, 2 mM dithiothreitol, 0.1 mM *p*-aminophenylmethylsulfonyl fluoride [pH 7.0]).

**(iii) DEAE-Sephacel column chromatography.** The dialyzed sample was applied to a DEAE-Sephacel column (4.6 by 50 cm; Pharmacia Biotech) previously equilibrated with buffer B. After the column had been washed with 750 ml of buffer B, proteins were eluted with a linear gradient of KCl from 0.1 to 0.5 M in a total volume of 1.5 liters at a flow rate of 2.5 ml/min. Fractions (340 ml) containing A-factor-binding activity were pooled and dialyzed overnight against buffer C (50 mM triethanolamine, 0.2 M KCl, 2 mM dithiothreitol, 0.1 mM *p*-aminophenylmethylsulfonyl fluoride [pH 7.0]).

**(iv) Mono Q column chromatography.** The dialyzed sample was divided into six portions, and each was applied to a Mono Q HR 10/10 FPLC column (Pharmacia) equilibrated with buffer C, because of the small capacity of the column. Proteins were eluted with 120 ml of a linear gradient of 0.2 to 0.4 M KCl in buffer C at a flow rate of 3 ml/min. One cycle of chromatography gave fractions (54 ml) containing activity. These fractions obtained from six cycles of chromatography were collected and concentrated to 9.5 ml by ultrafiltration through a Diaflo YM19 membrane (Amicon Corp., Lexington, Mass.).

**(v) Gel filtration column chromatography.** The concentrated sample was divided into two portions, and each (50 mg of protein) was applied to a 75-pg Superdex column (Pharmacia) previously equilibrated with buffer A. Proteins were eluted with buffer A at a flow rate of 2.6 ml/min, and protein activity was detected in fractions (14 ml) as a single ArpA peak. The fractions containing activity in a total of 28 ml were pooled and dialyzed against 1 liter of buffer B.

**(vi) Heparin affinity column chromatography.** The dialyzed sample was divided into nine portions, and each was applied to a HiTrap heparin fast protein liquid chromatography (FPLC) column (Pharmacia) equilibrated with buffer B. Proteins were eluted with two different linear gradients continuously, first 0.1 to 0.3 M KCl in 5 ml of buffer B and then 0.3 to 0.6 M KCl in 15 ml of buffer B, both at a constant flow rate of 0.5 ml/min. Protein activity was eluted in fractions (5 ml) as a single peak at 0.35 M KCl. Fractions (45 ml) containing activity were collected from nine cycles of chromatography and dialyzed overnight against 1 liter of buffer D (50 mM triethanolamine, 0.1 M KCl, 1 M ammonium sulfate, 2 mM dithiothreitol, 0.1 mM *p*-aminophenylmethylsulfonyl fluoride [pH 7.0]).

**(vii) Hydrophobic column chromatography.** The sample obtained by the procedure described above was divided into two portions, and each was applied to a phenyl-Superose HR 5/5 FPLC column (Pharmacia) equilibrated with buffer D. Proteins were eluted with two continuous linear gradients, first 1.0 to 0.3 M ammonium sulfate in 15 ml of buffer D and then 0.3 to 0 M ammonium sulfate in buffer D, both at a constant flow rate of 0.5 ml/min. The A-factor-binding activity was eluted as a single peak at about 0.15 M ammonium sulfate. Fractions containing activity were pooled, dialyzed against buffer A, and stored at −80°C.

**Amino acid sequence determination.** For determination of the $NH_2$-terminal amino acid sequence of the A-factor receptor protein, the purified sample (110 μg of protein) was precipitated with 0.4 M trichloroacetic acid, washed with ethanol-ether (1:1), dried, and solubilized in 62.5 mM Tris-HCl (pH 6.8) containing 2% SDS and 1% 2-mercaptoethanol. The protein was electrophoresed in a SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Immobilon Transfer; 0.45-μm pore size; Millipore). The membrane was stained with 0.1% amide black in 40% methanol–10% acetic acid and destained with 10% isopropanol–10% acetic acid. The protein band to be examined was cut out and washed with high-pressure liquid chromatography (HPLC)-grade water (Wako Pure Chemicals). Amino acid sequences were determined with an Applied Biosystems model 447A pulsed liquid-phase sequencer equipped with a model 120A on-line phenylthiohydantoin amino acid analyzer. All reagents for sequence analysis were purchased from Applied Biosystems. For determination of inner amino acid sequences, 205 μg of the purified protein obtained by precipitation with trichloroacetic acid was dissolved in 200 μl of 50 mM Tris-HCl (pH 9.0) containing 8 M urea and 0.5 μl of 2-mercaptoethanol and then incubated at 37°C for 1 h. After the concentration of urea had been adjusted to 2.7 M by addition of 400 μl of 50 mM Tris-HCl (pH 9.0), lysyl endopeptidase (9 mU; Wako Pure Chemicals) was added and the mixture was incubated at 37°C for 12 h. The lysyl endopeptidase-generated fragments were directly applied to an HPLC column equipped with a reverse-phase VP-304-1251 column (Senshu Science Co., Ltd., Tokyo, Japan) and eluted with a linear gradient of acetonitrile (0 to 60%) at a decreasing rate of 1%/min. Three peaks with retention times of 34.8, 48.0, and 50.1 min were chosen for amino acid sequence analysis using the above-described amino acid sequencer.

**Cloning of the A-factor receptor gene.** Chromosomal DNA was purified from *S. griseus* IFO 13350 as described previously (25). Two oligonucleotide primers were synthesized on an Applied Biosystems model 380A DNA synthesizer, each with additional restriction sites (underlined in the following sequences) added to the 5′ end to facilitate cloning of the amplified product: primer I, 5′-CCGAAGCTT-CGC-GCC-GT[C, G, A, T]-CA[G, A]-AC-3′ (23 nucleotides with eight variants); and primer II, 5′-GCCGAATTC-TT-GGA-GGC-GAA-GTG-[G, A]AA-[G, A]TA-3′ (27 nucleotides with four variants). Primers I and II were based on the amino acid sequences of the $NH_2$ terminus and the lysyl endopeptidase-generated fragment eluted by HPLC at 34.8 min, respectively. PCR was carried out in 100 μl of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM each deoxynucleotide, 200 pmol of each primer, 1 μg of *S. griseus* chromosomal DNA, and 5 U of *Taq* DNA polymerase at 94°C for 1 min, 43°C for 1.5 min, and 72°C for 1.5 min in a total of 35 cycles. The amplified product was

digested with *Hin*dIII and *Eco*RI, separated by agarose gel electrophoresis, and purified with a GeneClean kit (Bio101 Inc., Vista, Calif.). The amplified DNA of 150 bp was cloned into pUC19. Southern hybridization (48) with the 150-bp DNA fragment as the probe gave a single positive signal of 4.0 kb in the *S. griseus* chromosomal DNA digested with *Sal*I. From an agarose gel slice which contained *Sal*I fragments in the size range 3.0 to 4.3 kb, DNA was extracted and ligated into pUC19. Colony hybridization (14) with the same probe identified an *E. coli* DH5α transformant giving a positive signal. The plasmid, named pARPA1, in the transformant contained a 4.0-kb *Sal*I fragment. Restriction endonuclease analysis together with Southern hybridization experiments with the same probe indicated the location of the A-factor receptor gene in the 4.0-kb fragment.

**Site-directed mutagenesis to construct pT7ARPA.** Plasmid pGEMEX-1 carried a cassette containing the T7 RNA polymerase promoter, followed by an ATG translational start codon of T7 gene 10 (49). The ATG codon was included in a *Nde*I cleavage sequence, CATATG. For placing the A-factor receptor gene under the control of the T7 promoter, the nucleotide sequence (GAAATG) covering the ATG start codon of the receptor gene was first changed into CATATG by site-directed mutagenesis (32). For this purpose, a 41-bp-long nucleotide (5′-CGGAGGCAG*CATA*TGGCGAAGCAGGCTCGCGCAGTC CAGTCCAGAC-3′; italic letters indicate the bases to be replaced) was synthesized and used as the primer. As the target DNA, a 1,328-bp *Bam*HI-*Sal*I fragment covering the whole receptor gene was cloned into the polylinker of M13mp19. The phage DNA was propagated once in *E. coli* CJ236 to prepare uracil-containing single-stranded DNA, and the complementary strand was synthesized with DNA polymerase and ligase. *E. coli* JM109 was then transfected with the reaction mixture (53). The mutation thus generated was checked by nucleotide sequencing, and the *Nde*I-*Hin*dIII fragment containing the mutated DNA sequence was then inserted between the *Nde*I and *Hin*dIII sites of pGEMEX-1*, resulting in pT7ARPA. The original pGEMEX-1 plasmid contained another *Nde*I site, in addition to that covering the ATG start codon connected to the T7 promoter. For experimental convenience, this additional *Nde*I site was deleted by fill-in with Klenow fragment, resulting in pGEMEX-1*.

**Expression of the A-factor receptor gene in *E. coli*.** *E. coli* JM109 (DE3) containing pT7ARPA was grown overnight at 37°C in Luria broth (37) containing 50 μg of ampicillin per ml. The culture was diluted by 1:100 into the same fresh medium, and cultivation was continued until the $A_{600}$ reached 0.3. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added at a final concentration of 0.5 mM, and cultivation was continued for 5 h. The cells were harvested by centrifugation and washed with buffer A. The washed cells were suspended in buffer A and disrupted with a sonicator (Branson Sonifier cell disruptor model 200). Cell debris was removed by centrifugation at 10,000 × *g* for 30 min at 4°C. Solid ammonium sulfate was added to 45% saturation, and the precipitate was collected by centrifugation. The precipitate was dissolved in buffer B and dialyzed overnight against 1 liter of buffer B. The dialysate was used as the protein sample for A-factor binding assays.

**Molecular mass determination of the receptor produced in *E. coli*.** Starting with the ammonium sulfate-fractionated sample, we further purified ArpA by three steps of column chromatography (DEAE-Sephacel, Mono Q, and heparin columns) essentially as described above. For molecular mass determination of the receptor, the final preparation (250 μg of protein) was applied to an FPLC system equipped with a Superose 12 HR 10/30 column (1 by 30 cm; Pharmacia) and eluted with 0.1 M KCl at a flow rate of 0.5 ml/min. One-half milliliter of each was collected and analyzed by the A-factor binding assay and SDS-polyacrylamide gel electrophoresis. The molecular mass standards used were aldolase (158 kDa), albumin (68 kDa), ovalbumin (45 kDa), and chymotrypsinogen A (25 kDa).

**Construction of pARPH1 and pARPL1.** For expression of *arpA* in *Streptomyces* spp., pUC19 containing the 4-kb *Sal*I fragment (see Fig. 2) in the *Sal*I site was used as the starting material. From this recombinant pUC19 DNA, a 1.4-kb *Bam*HI fragment containing *arpA* was excised by partial digestion and then cloned in the *Bam*HI site of pUC19. Then, the *arpA* gene was excised as an *Eco*RI-*Hin*dIII fragment and ligated with pIJ486 digested with *Eco*RI and *Hin*dIII, resulting in pARPH1. Plasmid pARPH1 was introduced by transformation into *S. lividans* TK21 and *S. griseus* KM7. For construction of a low-copy-number plasmid containing *arpA*, an *E. coli-Streptomyces* shuttle vector, pKU209 (26), was used. Plasmid pKU209 contains the replication origins derived from SCP2* and pMB9 for the replication in *Streptomyces* spp. and *E. coli*, respectively. The *arpA* gene was recovered as an *Eco*RI-*Sal*I fragment from the recombinant pUC19 DNA, ligated with pKU209 digested with *Eco*RI and *Xho*I, and introduced into *E. coli* JM109. The plasmid, pARPL1, constructed in this way was then introduced into *S. griseus* strains.

**Nucleotide sequence accession number.** The nucleotide sequence of the A-factor receptor gene has been submitted to the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number D49782.

## RESULTS

**Purification of the A-factor receptor protein.** Starting with the mycelium of *S. griseus* IFO 13350, we purified the A-factor receptor protein about 2,160-fold by ammonium sulfate fractionation followed by five steps of column chromatography. The phenyl-Superose column profile at the final step is illustrated in Fig. 1A, and the overall purification results are summarized in Table 1. The specific activity is expressed as the difference between binding of the radioactive A-factor in the presence and absence of an excess of nonlabeled A-factor. We suppose that the specific activities and the fold purification shown in Table 1 are underestimated, because the A-factor receptor protein was unstable and gradually decreased its activity during the operations and because the final preparation contained truncated receptor proteins (see below). The ammonium sulfate fractionation between 32 and 43.5% was very useful, enriching the receptor protein sixfold and reducing the volume of cell extract with almost no loss in yield. The affinity chromatography on heparin also enriched the receptor 24-fold and was essential to remove contaminating proteins through the overall operations in the present scheme. We observed no protein which tightly bound the receptor throughout the puri-

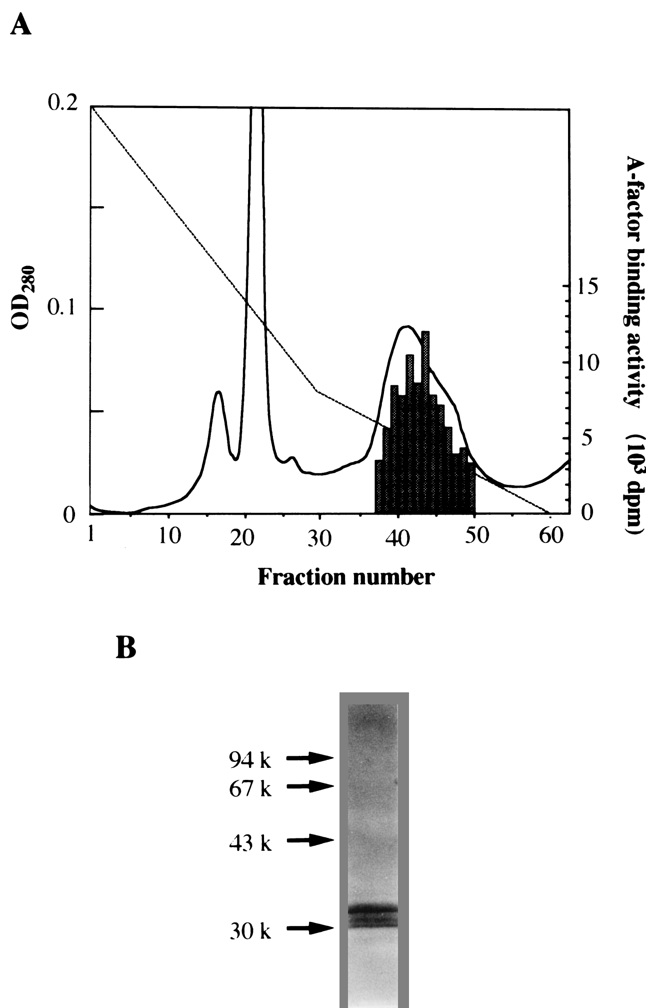


FIG. 1. Phenyl-Superose chromatography of the A-factor receptor protein isolated from *S. griseus* IFO 13350 (A) and SDS-polyacrylamide gel electrophoresis of the purified receptor protein (B). (A) Chromatography conditions are described in Materials and Methods. The A-factor-binding activity of each fraction (solid bars) is expressed as the difference between binding of [$^3$H]A-factor in the presence and absence of an excess of nonlabeled A-factor. (B) The purified receptor sample consists of three major proteins whose $NH_2$ termini are resistant to Edman degradation as a result of some modification. $OD_{280}$, optical density at 280 nm.

TABLE 1. Purification of the A-factor receptor protein from *S. griseus* IFO 13350

| Purification step | Total protein (mg) | Total activity ($10^6$ dpm) | Sp act (dpm/μg) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude protein | 12,262.0 | 61.8 | 5.04 | 1.0 | 100 |
| $(NH_4)_2SO_4$ fractionation | 2,022.6 | 61.1 | 30.2 | 6.0 | 99 |
| DEAE-Sephacel | 849.8 | 58 | 68.1 | 13.5 | 94 |
| Mono Q | 129.8 | 29.8 | 229.4 | 45.5 | 48 |
| Superdex 75 | 37.25 | 15.7 | 422.6 | 83.8 | 25 |
| Heparin | 0.95 | 9.8 | 10,356 | 2,054.8 | 16 |
| Phenyl-Superose | 0.34 | 3.6 | 10,863 | 2,155.4 | 6 |

fication procedure, in contrast to the case for the virginiae butanolide receptor, in which case NusG of 36 kDa was copurified with the receptor (40). Another point to be noted is that the receptor was eluted from the gel filtration column at the fractions corresponding to a molecular mass of more than 150 kDa, calculated by reference to the molecular mass standards, which will be discussed in relation to the characteristics of the A-factor receptor.

The final sample gave three protein bands on SDS-polyacrylamide gel electrophoresis (Fig. 1B). The amino acid sequence Arg-Ala-Val-Gln-Thr-Trp was deduced from the HPLC pattern of each of these proteins, although the area of each peak in HPLC was very small by comparison with the amounts of proteins applied to Edman degradation. This was true for all three proteins. We therefore assumed that the $NH_2$ termini of the three proteins have some modification resistant to Edman degradation and that the amino acid sequence was derived from an $NH_2$ terminus-truncated protein species contained in a small amount in the purified sample. As will be described later, the determined amino acid sequence was found to start from the sixth residue of the putative primary translation product of the A-factor receptor gene. Because the three proteins turned out to be the A-factor receptor, as determined by internal amino acid sequencing with these proteins (see below), we concluded that the final sample was mainly a mixture of the A-factor receptor protein with different COOH termini, probably as a result of the proteolysis by proteases in the cell extract during purification. The apparent molecular mass of the largest protein is calculated to be 33 kDa by SDS-polyacrylamide gel electrophoresis.

**Amino acid sequence determination.** We tried to determine the amino acid sequence of each of the three major proteins in Fig. 1B by Edman degradation after the transfer of the proteins from the polyacrylamide gel to a polyvinylidene difluoride membrane. Although the amino acid sequence Arg-Ala-Val-Gln-Thr-Trp-Arg-Ser-Ile-Val-Asp-$Ala_3$-Ser-Val was determined for all three proteins, the area of each peak in HPLC was very small. As described above, we supposed that this sequence was derived from a protein that was contained in a small amount in the purified sample. The determined amino acid sequence was found to correspond to the sixth amino acid residue of the A-factor receptor protein, as described below. Since it is unclear whether all three truncated proteins show the same activity and whether one of them corresponds to the native form of the receptor in *S. griseus*, the fold purification presented in Table 1 is underestimated.

We also determined the $NH_2$-terminal amino acid sequences of lysyl endopeptidase-generated fragments. The sequence of the fragment eluted at 34.8 min by HPLC was Gly-Ala-Leu-Tyr-Phe-His-Phe-Ala-Ser-Lys, and that eluted at 48 min was $Ile_2$-Val-Ala-Ser-Phe-Thr-Gly-Ile-Gln-Leu-Val-Ser-Glu-Ala-Asp-Ser-Gly-Arg-Ala. The $NH_2$ terminus of the fragment eluted at 50.1 min was the same as those of the purified sample.

**Cloning of the A-factor receptor gene.** The primers used for cloning the A-factor receptor gene by PCR were based on the $NH_2$-terminal amino acid sequence of the purified sample and a lysyl endopeptidase-generated fragment eluted at 34.8 min by HPLC as described in Materials and Methods. PCR with the primers and the *S. griseus* chromosomal DNA as the template yielded an amplified band of 150 bp. Only this band was constantly amplified under different PCR conditions; a few bands were differently amplified depending on the PCR conditions (see Fig. 9). The amplified DNA was then cloned into pUC19 in *E. coli*. Direct nucleotide sequencing of the 150-bp fragment showed the presence of an open reading frame (ORF) continuing over the entire sequence. We then performed Southern hybridization with this 150-bp fragment as the probe against the *S. griseus* chromosomal DNA digested with various restriction endonucleases. Among positive signals with different sizes depending on the restriction enzymes used, we chose a 4.0-kb signal in the *Sal*I digest and cloned the corresponding DNA sequence into pUC19. The restriction map of the cloned 4.0-kb fragment is shown in Fig. 2. Subsequent Southern hybridization indicated the location of the originally cloned 150 bp within the indicated 1.0-kb *Bam*HI fragment.

**Nucleotide sequence of the A-factor receptor gene.** Nucleotide sequencing of the 1.0-kb *Bam*HI fragment by the M13 dideoxynucleotide method showed that the above-described ORF encompassed one of the *Bam*HI ends, and we further determined the nucleotide sequence as far as the nearest *Sal*I site. The complete ORF deduced from the nucleotide sequence is shown in Fig. 3. This ORF is in agreement with the codon usage pattern characteristic of *Streptomyces* genes with an extremely high G+C content, as determined by the FRAME analysis developed by Bibb et al. (3). A probable ribosome-binding sequence, GGAGG, is present six nucleotides upstream of the putative translational start codon. The Arg residue at the $NH_2$ terminus of the purified A-factor receptor protein corresponds to the sixth residue of this ORF. The two inner amino acid sequences determined with the lysyl endopeptidase-generated fragments are also contained in this ORF. We named this gene *arpA* (A-factor receptor protein). The calculated molecular mass of the *arpA* product with 276 amino acids is 29.1 kDa.

Southern blot analysis at relatively high stringency with the DNA sequence encoding the virginiae butanolide-binding protein (BarA) as a probe showed the absence of DNA sequence homologous to the probe in *S. griseus* IFO 13350 from which

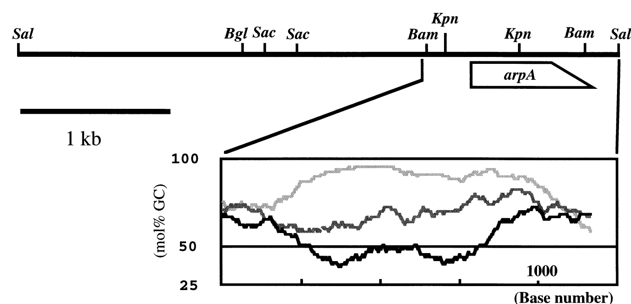


FIG. 2. Restriction map of the cloned fragment and location of the ORF corresponding to the A-factor receptor protein. The nucleotide sequence of 1,328 bp from the indicated *Bam*HI-to-*Sal*I sites was determined by FRAME analysis (3), using a sliding window of 80 codons.

```
BamHI                                                                                                            KpnI
GGATCCGGAGCGGAGGTCCCGGAGTTGTTCCGGGGTCCGATGCCGGGTATCCGGGCGTTCCTCCCCCTCCGCGTTCCGGCCGGTCCGTGTTTTCGAGGGGCGGGTGGGGTACCAACAGGC   120

GGGTGAGGAGGAGGTGCGGCGGGTGTCCGCGGGCCGTGATCCACCGGGTGCGGGCACGGTTTCCGGACAGACCGATGGCCCGTGCAACGGCACACTTGCATACGCTGGCCGCAGAGGTTG   240

GATCGACGGCGACTCGGCGGTGAAGGGCTGAGCGCACAGAACGTCCCGGAGGCAGGAAATGGCGAAGCAGGCTCGCGCAGTCCAGACGTGGCGGTCGATCGTGGATGCCGCGGCGAGTGT   360
                                                           M  A  K  Q  A  R  A  V  Q  T  W  R  S  I  V  D  A  A  A  S  V

CTTCGACGACTACGGCTACGAGCGTGCCGCCATCTCGGAGATTCTGCGCCGCGCCAAGGTCACCAAGGGGGCCTTGTACTTCCACTTCGCCTCCAAGGAGGCCATCGCCCAGGCGATCAT   480
 F  D  D  Y  G  Y  E  R  A  A  I  S  E  I  L  R  R  A  K  V  T  K  G  A  L  Y  F  H  F  A  S  K  E  A  I  A  Q  A  I  M

                                                                                                             SmaI    KpnI
GGACGAGCAGACGTCCACGGTGGAGTTCGAGCAGGAGGGCTCGCCGCTTCAGTCCCTGGTGGACGGGGGCCAGCAGTTCGCTTTCGCCCTGCGCCACAACTCGATGGCCCGGGCCGGTAC   600
 D  E  Q  T  S  T  V  E  F  E  Q  E  G  S  P  L  Q  S  L  V  D  G  G  Q  Q  F  A  F  A  L  R  H  N  S  M  A  R  A  G  T

CAGGCTCTCCATCGCGGGCGTCTTCCTCGGCGGGCCGCACCCCTGGGGCGACTGGATCGACGCGACGGCCCGGATGCTGGAGCTGGGCCAGGAGCGCGGCGAGGTGTTCCCGCAGATCGA   720
 R  L  S  I  A  G  V  F  L  G  G  P  H  P  W  G  D  W  I  D  A  T  A  R  M  L  E  L  G  Q  E  R  G  E  V  F  P  Q  I  D

CCCGATGGTGTCAGCCAAAATCATCGTCGCTTCGTTCACCGGTATCCAGCTGGTCTCGGAGGCCGATTCCGGCCGGGCCGATCTCCGCGAGCAGGTGGCGGAGATGTGGCGGCACATCCT   840
 P  M  V  S  A  K  I  I  V  A  S  F  T  G  I  Q  L  V  S  E  A  D  S  G  R  A  D  L  R  E  Q  V  A  E  M  W  R  H  I  L

                SmaI
GCCGTCGATCGCCCACCCCGGGGTCATCGCCCACATCAAACCGGAGGGCCGGGTGGATCTGGCGGCCCAGGCGCGCGAGAAGGCCGAGCGTGAGGAGCAGGAGGCCAGGATCGCCGCGGA   960
 P  S  I  A  H  P  G  V  I  A  H  I  K  P  E  G  R  V  D  L  A  A  Q  A  R  E  K  A  E  R  E  E  Q  E  A  R  I  A  A  E

                                                                            BamHI
GGCCAAGGGGGCGGGCTCCGATCCCACGAGCGAGGGCGGCACCCGGTCGGGCGGGTCGGGGCTGCGGGGCGGGGGATCCGGCCGTGGTCCGCGCGCCGGGGTGACCGGCGACGAGGGCGA  1080
 A  K  G  A  G  S  D  P  T  S  E  G  G  T  R  S  G  G  S  G  L  R  G  G  G  S  G  R  G  P  R  A  G  V  T  G  D  E  G  D

CGAGGAGCCCGCAGGTGCCGGGGTGGCGGCCGGGGGGATCGTCGCCTGAGTCGCTCCGGGCATGCTGTGTTTCACGTGAAACGTCCGATTCTTCATGATCATCATACGACAGGGTCGCGG  1200
 E  E  P  A  G  A  G  V  A  A  G  G  I  V  A  *

TGCTCCCGGAGCCTCCCGGCGGGGTCGGAGTTCACCGGGCGGTCGCTCACCTTGAGTGAGCCGCCAAGTGCGGCCCGTACTCTGAGGACATGGCGGATCACGGCGAGCAGCCCTGTCGGC  1320
  SalI
GGGTCGAC                                                                                                                  1328
```

FIG. 3. Nucleotide sequence of the 1,328-bp *Bam*HI-*Sal*I fragment and deduced amino acid sequence in single-letter code. An asterisk denotes a translational stop codon. A possible ribosome-binding sequence is underlined. The three amino acid sequences determined by using the purified protein are indicated by broken lines, all of which completely match those deduced from the nucleotide sequence.

*arpA* was cloned (40). However, ArpA showed 39.1% identity in amino acid sequence to BarA (Fig. 4). The similarity in the $NH_2$-terminal portions probably serving as DNA-binding domains (see below) was especially high. We assume that Southern hybridization at low stringency might have revealed the presence of *arpA* as a *barA* homolog.

No protein significantly homologous to the entire sequence of ArpA was predicted by a computer-aided homology search in the databases. However, it predicted that ArpA contained a region near the $NH_2$ terminus (amino acids 1 to 70) which resembled the α-helix–turn–α-helix DNA-binding motifs of a number of transcriptional regulators (7, 41). The helixy score of this region calculated by the method of Dodd and Egan (7) was not very high but similar to those of the possible DNA-binding domains of *E. coli* DnaA and phage 186 B. The proteins predicted by the computer are EnvR (30), an *E. coli* potential repressor for the *acrEF*/*envCD* operon; AcrR (34), an *E. coli* potential repressor for *acrAB* operon; BetI (33), an *E. coli* repressor involved in choline regulation of the osmoregulatory choline-glycine betaine pathway; and ORF188 (43), an *S. aureus* repressor for *qacA* encoding an efflux-mediated antiseptic resistance determinant. Alignments with BetI and ORF188 as examples are shown in Fig. 5. The presence of a region resembling α-helix–turn–α-helix DNA-binding motifs in ArpA suggests that it modulates transcription of a certain gene(s) by binding directly to its promoter region in response to A-factor.

**Expression of the *arpA* protein in *E. coli*.** We next produced the ArpA protein in *E. coli* by use of an expression vector, pGEMEX-1, in order to examine A-factor-binding activity of ArpA. The *arpA* gene was placed under the control of the T7 RNA polymerase promoter controllable with IPTG in such a way that the second GCG codon of *arpA* was fused to the ATG start codon of T7 gene 10 by means of site-directed mutagenesis. The resulting plasmid, pT7ARPA, was introduced in *E. coli* JM109 (DE3). When the recombinant *E. coli* cells were cultured at 37°C in the presence of IPTG, growth was severely inhibited, and very little A-factor-binding activity was detected in the cell extract. Consistent with this finding, no protein responsive to IPTG was detected by SDS-polyacrylamide gel

```
ArpA □         MAKQARAVQTWRS-IVDAAASVFDDYGYERAAIS-EILRRAK   40
                  * *** * *  ** ******* ** * **   *** **
BarA ● MAVRHERVAVRQERAVRT-RQAIVRAAASVFDEYGFE-AATVAEILSRAS   48

     □ VTKGALYFHFASKEAIAQAIMDEQTSTVEFEQE-GSPLQSLVDGGQQFAF   89
       **************  *     ***  *    * **  * ***     *
     ● VTKGAMYFHFASKEELARGVLAEQTLHVAVP-ESGSKAQELVDLTMLVAH   97

     □ A-LRHNSMARAGTRLSIA---G-V-FLGG-PHP-WGDWIDATARML-ELG  130
         * *    ******  *   * * *    *   *** * *    * *
     ● GML-HDPILRAGTRL--ALDQGAVDFSDANPFGEWGD-ICAQL--LAEA-  140

     □ QERGEVFPQIDPMVSAKIIVASFTGIQLVSEADSGRADL--REQVAEMWR  178
       ****** *   *      **  *** * **     * **  *  *  **
     ● QERGEVLPHVNPKKTGDFIVGCFTGLQAVSRVTSDRQDLGHRISV--MWN  188

     □ HILPSIAHPGVIAHIKPEGRVDLAAQAREKAEREEQEARIAAEAKGAGSD  228
       * ****  *         *       *      *  **  **
     ● HVLPSIV-PASMLTWIETGEERIGKVAAAAEAAEAAEASEAASDE       232

     □ PTSEGGTRSGGSGLRGGGSGRGPRAGVTGDEGDEEPAGAGVAAGGIVA    276
```

FIG. 4. Amino acid sequence alignment between the A-factor and virginiae butanolide receptor proteins. Identical amino acids are marked by asterisks.

FIG. 5. Alignment of the helix-turn-helix sequences of ArpA, BetI, and ORF188. Identical and similar amino acids are marked by asterisks and dots, respectively. BetI, a repressor involved in choline regulation of the osmoregulatory pathway (33); ORF188, a repressor for *qacA* encoding a drug resistance determinant (43).

electrophoretic analysis of the cell extract. These observations suggested that ArpA had some deleterious effect on *E. coli* and that ArpA produced, if any, formed inactive inclusion bodies as a result of abrupt and rapid production of the protein by induction of the T7 promoter with IPTG. We then cultured the recombinant *E. coli* cells at a lower temperature to avoid the formation of possible inclusion bodies, because Schein and Noteborn (47) observed that *E. coli* cells grown at lower temperatures produced significant amounts of recombinant proteins in a soluble form. When *E. coli* containing pT7ARPA was cultured at 30°C, a distinct A-factor-binding activity was detected in the cell extract. Although a protein responsive to IPTG was not detected by SDS-polyacrylamide gel electrophoresis of the crude lysate, the activity expressed in disintegrations per minute per microgram of protein in the *E. coli* cell extract was more than 10 times as much as that detected in the crude lysate of *S. griseus* (Table 1). Even after cultivation at 30°C, however, the $A_{600}$ value of the culture broth at 5 h after induction with IPTG was 0.55, whereas that without IPTG was 1.48. Figure 6 illustrates the A-factor-binding activity of the proteins precipitated with 40% ammonium sulfate from the crude lysate of the recombinant *E. coli* cells. The A-factor-binding activity was chased by an excess of nonlabeled A-factor. No A-factor-binding activity was present in *E. coli* containing pGEMEX-1 (data not shown). It was thus concluded that the recombinant ArpA protein possessed A-factor-binding activity.

FIG. 6. A-factor-binding activity in the crude lysate prepared from *E. coli* containing pT7ARPA. The recombinant *E. coli* cells were grown at 30°C for 5 h in the presence of 0.5 mM IPTG, and the cells were harvested by centrifugation. The cells were washed and disrupted by sonication. Solid ammonium sulfate was added to 45% saturation to the supernatant obtained by centrifugation of the sonic extract. The precipitate was then dissolved in and dialyzed against buffer B. The dialysate (26 μg of protein) was incubated with [$^3$H]A-factor in the absence (○) or presence (●) of an excess of nonlabeled A-factor and fractionated on a Sephadex G-25 column as described in Materials and Methods.

FIG. 7. Molecular mass determination of ArpA. (A) The recombinant ArpA protein (250 μg) purified from *E. coli* JM109 (DE3) containing pT7ARPA was applied to a Superose gel filtration column equipped in an FPLC system and eluted as described in Materials and Methods. The positions of the molecular mass standards are indicated by the arrows at the top. The A-factor-binding activity of each fraction is shown by histograms. The protein concentration (optical density at 280 nm [$OD_{280}$]) monitored automatically and the SDS-polyacrylamide gel electrophoretic analysis (the gel was stained with Coomassie brilliant blue) are also shown. The three distinct protein bands at about 30 kDa corresponding to ArpA, as determined by $NH_2$-terminal amino acid sequencing, are eluted at the position corresponding to more than 450 kDa. (B) The recombinant ArpA protein (250 μg) was first incubated with [$^3$H]A-factor (17.9 pmol) and then applied to the Superose column. A single peak of radioactivity is eluted in the fraction corresponding to about 60 kDa, whereas most of ArpA is eluted in the same fraction as in panel A. The proteins separated by SDS-polyacrylamide gel electrophoresis were stained with a silver-staining kit (Daiichi Pure Chemicals Co., Tokyo, Japan) to reveal a protein in a small amount. (C) An ammonium sulfate (45%) precipitate (2.6 mg of protein) obtained from a crude extract of *S. griseus* IFO 13350 was dissolved in a buffer, incubated with [$^3$H]A-factor (17.9 pmol), and then applied to the Superose column. A single peak of radioactivity is eluted in the fraction corresponding to about 60 kDa.

**Characterization of ArpA produced in *E. coli*.** The overpro-

duction of ArpA in *E. coli* facilitated the purification of recombinant ArpA. It was purified by ammonium sulfate fractionation and three steps of chromatography on DEAE-Sephacel, Mono Q, and heparin columns, giving a major 33-kDa protein band, in addition to two smaller protein bands, on SDS-polyacrylamide gel electrophoresis. (The degree of purity can be assessed by the protein profiles shown in Fig. 7A.) The $NH_2$-terminal amino acid sequence of the 33-kDa protein was determined to be Ala-Lys-Gln-Ala-Arg, which corresponds to the sequence from the second Ala of ArpA. The Edman degradation of the two proteins with sizes slightly smaller than 33 kDa gave the same $NH_2$-terminal amino acid sequences, indicating that these were truncated ArpA proteins whose COOH-terminal portions were degraded by an *E. coli* protease(s). As described above, the truncation at the COOH terminus of ArpA occurred during the ArpA purification from *S. griseus.*

As observed during the purification of ArpA from *S. griseus*, the recombinant ArpA protein rapidly lost its A-factor-binding activity during the purification, and only a very little activity was detected with the final preparation. Rapid inactivation hampered the calculation of the dissociation constant, $K_d$, with the purified protein. A similar situation was also observed for BarA (40).

An explanation of the instability of ArpA was obtained from the analysis for determination of the apparent molecular mass of ArpA. As mentioned above, during the purification from *S. griseus* ArpA was eluted from the Superdex gel filtration column in the fractions corresponding to a molecular mass of more than 150 kDa. To determine the molecular mass of ArpA in detail, we applied the recombinant ArpA protein to an FPLC system equipped with a Superose 12 gel filtration column, eluted the proteins, and measured A-factor-binding activity for each fraction. A single peak of A-factor-binding activity and ArpA were both eluted in the fractions corresponding to a molecular mass of more than 450 kDa (Fig. 7A). This result indicates that ArpA has a multimeric form. Because of the extremely low A-factor-binding activity of the multimeric form, we assume that ArpA aggregates to form a multimer with little or no A-factor-binding activity.

We next mixed the ArpA sample with [$^3$H]A-factor before applying it to the column. As shown in Fig. 7B, a single peak of radioactivity was eluted in the fraction corresponding to a molecular mass of about 60 kDa, whereas most of ArpA was again eluted as the large multimeric or aggregated form. Addition of a large amount of nonlabeled A-factor to the protein sample before separation did not increase the population of the protein species eluted at the 60-kDa position (data not shown). It appears that upon binding A-factor, a very small number of ArpA molecules are released from the aggregates to form an active dimeric conformation and elute as a dimer, since no binding activity is detected at this position after the protein has been separated without A-factor (Fig. 7A). The radioactivity detected with the multimeric form (Fig. 7A) is probably due to the A-factor-binding activity of a dimeric form which has been formed from a very small number of ArpA molecules contained in the aggregate. The lack of detectable binding activity at the position corresponding to a dimer, when separated in the absence of A-factor, can be explained in terms of the absence of the active dimeric form of ArpA in an amount detectable by the assay method used. Speculatively, A-factor may accelerate the formation of a dimer from an ArpA molecule released from the aggregated form and enable the dimeric form to be stable. As mentioned above, the rapid loss of binding activity of ArpA caused by aggregation leads to underestimation of the fold purification shown in Table 1.

We previously observed that A-factor-bound ArpA was eluted from a gel filtration column in the fractions corresponding to a molecular mass of about 26 kDa (38). We therefore repeated the experiment by using the FPLC system. An ammonium sulfate (45%) precipitate obtained from a crude lysate of *S. griseus* IFO 13350 was dissolved in a buffer, mixed with [$^3$H]A-factor, applied to the Superose 12 gel filtration column, and fractionated. Careful fractionation with respect to the positions of molecular mass standards identified a single peak of radioactivity corresponding to about 60 kDa (Fig. 7C). It is thus concluded that A-factor-bound ArpA is a dimeric form. Our previous mistake in measuring the molecular mass of ArpA may be due to incorrect assignment of the fraction with respect to the positions of molecular mass standards which were assigned with a hand-made gel filtration column.

**Expression of *arpA* in *S. lividans* and *S. coelicolor* A3(2).** As described above, *arpA* directed the synthesis of a protein with A-factor-binding activity. We next introduced pARPH1 containing *arpA* on high-copy-number plasmid pIJ486 into *S. lividans* TK21 and *S. coelicolor* A3(2) M130, both of which were previously shown to produce an A-factor-like compound but to contain no A-factor-binding activity. One of the A-factor-like compounds among several A-factor homologs so far reported (1, 8) is likely to be A-factor itself because it gives the same $R_f$ values on bioautography by thin-layer chromatography with different solvent systems (15), although the chemical structure of the compound having A-factor activity on *S. griseus* has not yet been determined. A crude extract prepared by sonication from *S. lividans* containing pARPH1 was fractionated with 33 to 43% ammonium sulfate, and the A-factor-binding activity was similarly examined. The A-factor-binding activity expressed in disintegrations per minute per milligram of protein of the recombinant *S. lividans* strain was determined to be 10.2-fold higher than that of the wild-type *S. griseus* strain. Expression of ArpA in *S. lividans*, however, did not result in any detectable effect on the growth or morphogenesis of this strain, nor did introduction of pARPH1 into *S. coelicolor* A3(2). In addition, blue pigment (actinorhodin) production by *S. coelicolor* A3(2) was not affected by this plasmid. This finding is consistent with the observation that an A-factor-negative mutation in this strain caused no detectable effect on the growth, morphogenesis, or secondary metabolism (17).

**Reversal of the phenotype of an *S. griseus* mutant strain defective in A-factor-binding activity by the *arpA* gene.** We also introduced pARPH1 into the wild-type *S. griseus* strain IFO 13350 to determine possible effects of the overexpression of ArpA on streptomycin production and morphogenesis. The A-factor binding assay with a crude lysate prepared from this recombinant *S. griseus* strain revealed about 3.2-fold-greater activity than strain IFO 13350, as expected. However, the timing and abundance of sporulation of the two strains were the same when the strains were grown on YMPG medium or nutrient agar broth (data not shown). In addition, the amounts of streptomycin produced by the recombinant strain and the wild-type strain containing the vector plasmid pIJ486 were the same (data not shown). This can be explained in terms of the extreme difference in the molecular numbers of A-factor as the ligand and its receptor with an extremely small dissociation constant in the cell; because of an overwhelmingly large number of A-factor molecules, the regulatory role of ArpA in response to A-factor is not affected when the A-factor binding activity and probably the number of receptors increase 3.2-fold at most. The wild-type *S. griseus* strain produced 0.2 μg of A-factor per ml (25), which corresponds to $5 \times 10^{14}$ molecules per ml.

We next introduced pARPH1 into *S. griseus* KM7, which was derived from A-factor-deficient mutant strain HH1 and defi-

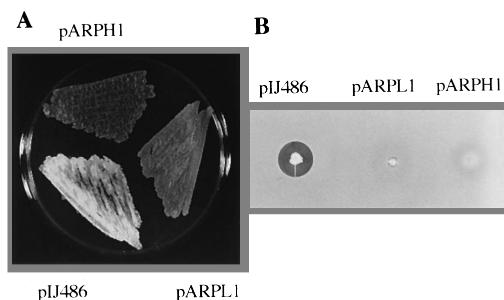


FIG. 8. Repression by *arpA* of aerial mycelium and spore formation (A) and of streptomycin production (B) of *S. griseus* KM7 deficient in ArpA. (A) Strain KM7 containing only the vector plasmid pIJ486 and grown for 7 days on YMPG medium produces spores, whereas that containing *arpA* on pIJ487 (plasmid pARPH1) or on pKU209 (plasmid pARPL1) does not. (B) Strain KM7 containing *arpA* on pARPH1 and grown for 3 days on nutrient agar medium produces no streptomycin, whereas that containing pIJ486 produces a large amount of streptomycin. Strain KM7 containing pARPL1 produces a very small amount of streptomycin.

cient in ArpA (39). This mutant strain overproduced streptomycin and formed spores earlier than the wild-type strain. The phenotypes of strain KM7 led to the idea that ArpA acted as a repressor-type regulator for secondary metabolism and morphogenesis (39). We therefore expected that endowing strain KM7 with A-factor receptor activity would result in the repression of streptomycin production and sporulation. As shown in Fig. 8, strain KM7 containing *arpA* on high-copy-number plasmid pIJ486 did not form spores, as expected. In addition, strain KM7 containing multiple copies of *arpA* produced no streptomycin when assayed by bioautography with *Bacillus subtilis* as the indicator. Plasmid pARPL1, whose copy number was supposedly 1 to 2 in *S. griseus* as in *S. lividans*, also repressed sporulation completely and streptomycin production severely (Fig. 8). Since the copy number of *arpA* supplemented in *trans* by pARPL1 in strain KM7 is supposedly 1 to 2, the genotype of this recombinant strain is almost the same as that of strain HH1, which shows Bld and streptomycin-negative phenotypes. These observations are in agreement with the idea that ArpA acts as a repressor-type regulator for these phenotypes.

**Amplification of a DNA fragment by PCR with the primers designed for ArpA and the chromosomal DNAs from various *Streptomyces* spp.** A wide distribution of A-factor-like compounds in *Streptomyces* spp. has been suggested by the fact that many *Streptomyces* strains produce active compounds triggering streptomycin production and aerial mycelium formation in an A-factor-deficient mutant strain of *S. griseus* (15). Similar observations were reported by Efremenkova et al. (9) and Eritt et al. (10, 11). In addition to these compounds having A-factor activity, several other A-factor-like compounds as signaling molecules have been also reported (21, 22). These observations led us to suppose that receptor proteins for these compounds were distributed among these *Streptomyces* strains. Because of the failure to detect DNA sequences homologous to the *barA* gene sequence in various *Streptomyces* strains, except for *S. virginiae*, as determined by Southern hybridization under relatively high stringent conditions (40), we used a PCR method with the same primers as those used for the cloning of *arpA* from *S. griseus* to search for an *arpA* homolog among the chromosomal DNAs from various *Streptomyces* spp. We tested four strains that were shown to produce a compound(s) having A-factor activity (15). As shown in Fig. 9, a 150-bp fragment, together with several other fragments, was amplified with the chromosomal DNAs from all the four *Streptomyces* strains examined. This size was the same as that of the fragment encoding part of ArpA of *S. griseus*. The presence of amplified fragments with the same size as that for *arpA* suggests that almost all *Streptomyces* strains contain a receptor protein similar to ArpA and BarA. In fact, nucleotide sequencing of the region covering the 150-bp fragment amplified with the *S. coelicolor* A3(2) chromosomal DNA showed that it encoded a protein homologous with ArpA (unpublished data).

## DISCUSSION

PCR with oligonucleotide primers designed on the basis of the amino acid sequences of the purified ArpA sample led to the cloning of *arpA*. One of the primers happened to be apparently based on the $NH_2$-terminal amino acid sequence of the $NH_2$ terminus-truncated ArpA contained in a small amount in the sample. The ability of the recombinant ArpA protein produced in *E. coli* and *S. lividans* to bind A-factor confirms that *arpA* actually encodes the A-factor receptor protein. We observed that ArpA purified from both *S. griseus* and *E. coli* was very unstable and gradually lost its A-factor-binding activity. The instability of ArpA appears to be due to its characteristic feature of forming an inactive aggregate. It is well conceivable that a higher concentration of ArpA or a high degree of purity of ArpA accelerates the aggregation. The instability of ArpA hampered the biochemical characterization of ArpA. The dissociation constant, 0.7 nM, and the number of ArpA molecules per genome, 40, which were previously obtained with a crude lysate of *S. griseus* (38) will be calculated when we purify the fully active ArpA protein through preventing the inactivation. If some of the ArpA molecules are present in an inactivated aggregated form in the cell, the molecular number of ArpA is far larger than the original estimate, 40 per genome.

ArpA contains a glycine-rich COOH-terminal region which is absent from BarA. Glycines frequently found in hinge regions in many proteins are known to favor the unfolded state and decrease stability of the folded proteins (42). BarA containing no such glycine-rich region is a dimeric form irrespective of the presence and absence of its ligand, virginiae butanolide (40). We therefore imagine that ArpA is easy to aggregate via this glycine-rich COOH-terminal region because of a high degree of flexibility. The truncation at the COOH-terminal portion that occurred during purification from *S. griseus* and *E. coli* may be due to this high flexibility.

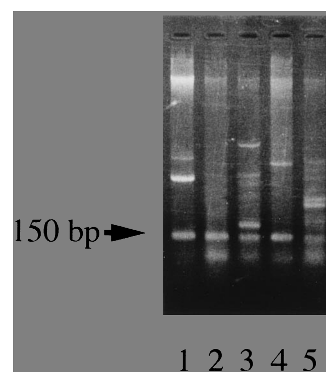


FIG. 9. Amplification of DNAs by PCR with the primers designed for ArpA and chromosomal DNAs of various *Streptomyces* strains. The primers and PCR conditions are described in Materials and Methods. The chromosomal DNAs used were prepared from *S. griseus* IFO 13350 (control; lane 1), *S. coelicolor* A3(2) M130 (lane 2), *S. lividans* TK21 (lane 3), *S. antibioticus* IFO 12838 (lane 4), and *S. collinus* IFO 12759 (lane 5). A 150-bp DNA, in addition to several other fragments, is amplified in all cases.

The molecular mass determined for ArpA in the presence and absence of A-factor (Fig. 7) provides a complex situation. Almost all ArpA molecules were found to exist as an inactive aggregated form in the absence of A-factor. The addition of A-factor to the aggregated ArpA molecules seems to release a very small number of ArpA molecules to form a dimer having A-factor-binding activity. It is possible that A-factor binding to an ArpA molecule in the aggregate or a free ArpA molecule released spontaneously in a very small amount enhances the formation of a dimer, since a high A-factor-binding activity at the position corresponding to a dimer appears only when A-factor has been added before separation by gel filtration. It is unclear whether similar aggregation of ArpA and similar recovery of A-factor-binding activity by A-factor occur in vivo, since the concentration of ArpA in the cells is apparently low.

ArpA is suggested to be a DNA-binding protein, because its $NH_2$-terminal portion contains an α-helix–turn–α-helix DNA-binding motif that is highly similar to those of other DNA-binding proteins. In connection with the finding that ArpA behaves as a repressor-type regulator for streptomycin production and aerial mycelium formation in the absence of A-factor, we assume that ArpA controls transcription of a certain key gene(s) by directly binding to its promoter region. Although it is still unclear whether ArpA itself and A-factor-bound ArpA in a dimeric form can bind to its target gene(s), we at present imagine the following models to explain its repressor-type behavior. Our speculative model is that ArpA, either in the aggregated form or in a free form, represses the expression of the putative key gene at an early stage of growth when A-factor is not yet produced, and at a specific stage of growth, it dissociates from the gene on binding A-factor, leading to transcription of the gene. The intracellular concentration of A-factor thus determines the timing of derepression of the key gene whose expression is repressed by ArpA. It is conceivable that the binding of A-factor to ArpA causes a subtle conformational change of the receptor and affects its DNA-binding activity. The gene product synthesized in this way leads to the expression of many genes required for secondary metabolism and morphogenesis. In fact, A-factor is produced just before streptomycin production and aerial mycelium formation (15). Furthermore, exogenous supplementation of A-factor to both the wild type and an A-factor-deficient mutant strain at the beginning of cultivation causes streptomycin production earlier by 1 day (2).

Comparison of the amino acid sequences of ArpA and BarA reveals end-to-end similarity. This is conceivable because the chemical structures of their ligands, A-factor and virginiae butanolide, are very similar. Notwithstanding the similarity between ArpA and BarA, their ligand specificities greatly differ; virginiae butanolide did not compete for the binding of A-factor to ArpA (38), and vice versa (40). In addition, virginiae butanolide had no A-factor activity in vivo (38). We imagine that the COOH-terminal portions of the two receptor proteins recognize and bind their respective ligands, since the $NH_2$-terminal portions of these receptors containing an α-helix–turn–α-helix DNA-binding motif seem to serve as DNA-binding domains. Because of the great similarity between ArpA and BarA in both amino acid sequence and autoregulatory role, we assume that the regulatory steps in which these receptors and ligands are involved are controlled in the same way.

DNA sequences homologous with *arpA* seem to be distributed widely among *Streptomyces* spp. Our success in detection of the homologous sequences was owing to the PCR method. Southern hybridization with the *arpA* sequence as the probe under routine conditions of relatively high stringency failed to show the presence of an *arpA* homolog in *S. coelicolor* A3(2) and several other strains (data not shown), although the PCR method predicted the presence of an amplified 150-bp fragment in *S. coelicolor* A3(2) which was later found to encode an ArpA-like protein (unpublished results). Southern hybridization with the *barA* sequence also failed to detect any homologous sequence in various *Streptomyces* spp. (40). In addition to the wide distribution of A-factor-like compounds in a variety of *Streptomyces* spp. (21, 22), the presence of a 150-bp fragment amplified by PCR with the chromosomal DNAs from various *Streptomyces* spp. supports the idea that a hormonal regulatory system, like the A-factor system in *S. griseus*, controls morphogenesis and/or secondary metabolism in *Streptomyces* spp. in general.

In *S. coelicolor* A3(2) and *S. lividans*, the defect in A-factor production, or, more precisely, in the production of a compound with A-factor activity able to restore sporulation and antibiotic production of the A-factor-deficient *S. griseus* mutant strain, had no effect on their morphogenesis or secondary metabolism (17). In addition, introduction of *arpA* into their parental strains did not have any detectable effect. However, this does not rule out a possible regulatory role of the *S. coelicolor* A3(2) ArpA homolog, as detected by the PCR method, because *S. coelicolor* A3(2) produces a series of compounds structurally very similar to A-factor (1, 8). One or some of these compounds, which do not show A-factor activity, may exert a regulatory role in cooperation with their own receptor proteins in this strain. Experiments are now in progress to test for the regulatory role of the ArpA homolog in *S. coelicolor* A3(2) by determining the ligand specificity of the ArpA homolog and the phenotype of the strain having an *arpA* mutation.

## ACKNOWLEDGMENTS

We thank Hideo Takahashi, Institute of Molecular and Cellular Biosciences, The University of Tokyo, for help in computer-aided analysis of the amino acid sequence of ArpA. Thiostrepton was a gift from Asahi Chemical Industry Co., Ltd., Shizuoka, Japan.

This work was supported in part by the Ciba-Geigy Foundation (Japan) for the Promotion of Science and the Ministry of Agriculture, Forestry, and Fisheries of Japan [BMP 95-V-1-(3)-4].

## REFERENCES

1. **Anisova, L. N., I. N. Blinova, O. V. Efremenkova, Y. P. Koz'min, V. V. Onoprienko, G. M. Smirnova, and A. S. Khokhlov.** 1984. Regulators of the development of *Streptomyces coelicolor* A3(2). Izv. Akad. Nauk SSSR Ser. Biol. **1:**98–108.
2. **Beppu, T.** 1992. Secondary metabolites as chemical signals for cellular differentiation. Gene **115:**159–165.
3. **Bibb, M. J., P. R. Findlay, and M. W. Johnson.** 1984. The relationship between base composition and codon usage in bacterial genes and its use for the simple and reliable identification of protein-coding sequences. Gene **30:**157–166.
4. **Bibb, M. J., and D. A. Hopwood.** 1981. Genetic studies of the fertility plasmid SCP2 and its SCP2* variants in *Streptomyces coelicolor* A3(2). J. Gen. Microbiol. **126:**427–442.
5. **Chater, K. F.** 1984. Morphological and physiological differentiation in *Streptomyces*, p. 89–115. *In* R. Losick and L. Shapiro (ed.), Microbial development. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
6. **Chater, K. F.** 1989. Sporulation in *Streptomyces*, p. 277–299. *In* I. Smith, R. A. Slepecky, and P. Setlow (ed.), Regulation of procaryotic development: structural and functional analysis of bacterial sporulation and germination. American Society for Microbiology, Washington, D.C.
7. **Dodd, I. B., and J. B. Egan.** 1987. Systematic method for the detection of potential λ Cro-like DNA-binding regions in proteins. J. Mol. Biol. **194:**557–564.
8. **Efremenkova, O. V., L. N. Anisova, and Y. E. Bartoshevich.** 1985. Regulators of cytodifferentiation in actinomycetes. Antibiot. Med. Biotekhnol. **9:**687–707.
9. **Efremenkova, O. V., L. N. Anisova, and A. S. Khokhlov.** 1979. Excretion by various actinomycetes of substances that induce sporulation in an asporogenic mutant of *Streptomyces griseus*. Mikrobiologiya **48:**999–1003.
10. **Eritt, I., U. Gräfe, and W. F. Fleck.** 1982. A screening method for autoregu-

lators of anthracycline-producing streptomycetes. Z. Allg. Mikrobiol. **22:**91–96.
11. **Eritt, I., U. Gräfe, and W. F. Fleck.** 1984. Inducers of both cytodifferentiation and anthracycline biosynthesis of *Streptomyces griseus* and their occurrence in actinomycetes and other microorganisms. Z. Allg. Mikrobiol. **24:**3–12.
12. **Gräfe, U., G. Reinhardt, W. Schade, I. Eritt, W. F. Fleck, and L. Radics.** 1983. Interspecific inducers of cytodifferentiation and anthracycline biosynthesis in *Streptomyces bikiniensis* and *S. cyaneofuscatus*. Biotechnol. Lett. **5:**591–596.
13. **Gräfe, U., W. Schade, I. Eritt, and W. F. Fleck.** 1982. A new inducer of anthracycline biosynthesis from *Streptomyces viridochromogenes*. J. Antibiot. **35:**1722–1723.
14. **Grunstein, M., and D. S. Hogness.** 1975. Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. USA **72:**3961–3965.
15. **Hara, O., and T. Beppu.** 1982. Mutants blocked in streptomycin production in *Streptomyces griseus*—the role of A-factor. J. Antibiot. **35:**349–358.
16. **Hara, O., and T. Beppu.** 1982. Induction of streptomycin inactivating enzyme by A-factor in *Streptomyces griseus*. J. Antibiot. **35:**1208–1215.
17. **Hara, O., S. Horinouchi, T. Uozumi, and T. Beppu.** 1983. Genetic analysis of A-factor synthesis in *Streptomyces coelicolor* A3(2) and *Streptomyces griseus*. J. Gen. Microbiol. **129:**2939–2944.
18. **Hashimoto, K., T. Nihira, S. Sakuda, and Y. Yamada.** 1992. IM-2, a butyrolactone autoregulator, induces production of several nucleoside antibiotics in *Streptomyces* sp. FRI-5. J. Ferment. Bioeng. **73:**449–455.
19. **Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf.** 1985. Genetic manipulation of *Streptomyces*: a laboratory manual. The John Innes Foundation, Norwich, United Kingdom.
20. **Hopwood, D. A., T. Kieser, H. M. Wright, and M. J. Bibb.** 1983. Plasmids, recombination and chromosome mapping in *Streptomyces lividans* 66. J. Gen. Microbiol. **129:**2257–2269.
21. **Horinouchi, S., and T. Beppu.** 1990. Autoregulatory factors of secondary metabolism and morphogenesis in actinomycetes. Crit. Rev. Biotechnol. **10:**191–204.
22. **Horinouchi, S., and T. Beppu.** 1992. Autoregulatory factors and communication in actinomycetes. Annu. Rev. Microbiol. **46:**377–398.
23. **Horinouchi, S., and T. Beppu.** 1992. Regulation of secondary metabolism and cell differentiation in *Streptomyces*: A-factor as a microbial hormone and the AfsR protein as a component of a two-component regulatory system. Gene **115:**167–172.
24. **Horinouchi, S., and T. Beppu.** 1994. A-factor as a microbial hormone that controls cellular differentiation and secondary metabolism in *Streptomyces griseus*. Mol. Microbiol. **12:**859–864.
25. **Horinouchi, S., Y. Kumada, and T. Beppu.** 1984. Unstable genetic determinant of A-factor biosynthesis in streptomycin-producing organisms: cloning and characterization. J. Bacteriol. **158:**481–487.
26. **Kakinuma, S., Y. Takada, H. Ikeda, H. Tanaka, and S. Omura.** 1991. Cloning of large DNA fragments, which hybridize with actinorhodin biosynthesis genes, from kalafungin and nanaomycin A methyl ester producers and identification of genes for kalafungin biosynthesis of the kalafungin producer. J. Antibiot. **44:**995–1005.
27. **Khokhlov, A. S.** 1988. Results and perspectives of actinomycete autoregulators studies, p. 338–345. *In* Y. Okami, T. Beppu, and H. Ogawara (ed.), Biology of actinomycetes '88. Japan Scientific Societies Press, Tokyo.
28. **Khokhlov, A. S., I. I. Tovarova, L. N. Borisova, S. A. Pliner, L. A. Schevchenko, E. Y. Kornitskaya, N. S. Ivkina, and I. A. Rapoport.** 1967. A-factor responsible for the biosynthesis of streptomycin by a mutant strain of *Actinomyces streptomycini*. Dokl. Akad. Nauk SSSR **177:**232–235.
29. **Kim, H. S., T. Nihira, H. Tada, M. Yanagimoto, and Y. Yamada.** 1989. Identification of binding protein of virginiae butanolide C, an autoregulator in virginiamycin production from *Streptomyces virginiae.* J. Antibiot. **42:**769–778.
30. **Klein, J. R., B. Henrich, and R. Plapp.** 1991. Molecular analysis and nucleotide sequence of the *envCD* operon of *Escherichia coli*. Mol. Gen. Genet. **230:**230–240.
31. **Kondo, K., Y. Higuchi, S. Sakuda, T. Nihira, and Y. Yamada.** 1989. New virginiae butanolides from *Streptomyces virginiae*. J. Antibiot. **42:**1873–1876.
32. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.
33. **Lamark, T., I. Kaasen, M. W. Eshoo, P. Falkenberg, J. McDougall, and A. R. Strom.** 1991. DNA sequence and analysis of the *bet* genes encoding the osmoregulatory choline-glycine betaine pathway of *Escherichia coli*. Mol. Microbiol. **5:**1049–1064.
34. **Ma, D., D. N. Cook, M. Alberti, N. G. Pon, H. Nikaido, and J. E. Hearst.** 1991. Molecular cloning and characterization of *acrA* and *acrE* genes of *Escherichia coli*. J. Bacteriol. **175:**6299–6313.
35. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101:**20–78.
37. **Miller, J.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Miyake, K., S. Horinouchi, M. Yoshida, N. Chiba, K. Mori, N. Nogawa, N. Morikawa, and T. Beppu.** 1989. Detection and properties of A-factor-binding protein from *Streptomyces griseus*. J. Bacteriol. **171:**4298–4302.
39. **Miyake, K., T. Kuzuyama, S. Horinouchi, and T. Beppu.** 1990. The A-factor-binding protein of *Streptomyces griseus* negatively controls streptomycin production and sporulation. J. Bacteriol. **172:**3003–3008.
40. **Okamoto, S., K. Nakamura, T. Nihira, and Y. Yamada.** 1995. Virginiae butanolide binding protein from *Streptomyces virginiae*: evidence that VbrA is not the virginiae butanolide binding protein and reidentification of the true binding protein. J. Biol. Chem. **270:**12319–12326.
41. **Pabo, C. O., and R. T. Sauer.** 1984. Protein-DNA recognition. Annu. Rev. Biochem. **53:**293–321.
42. **Richardson, J. S., and D. C. Richardson.** 1989. Principles and patterns of protein conformation, p. 1–98. *In* G. D. Fasman (ed.), Prediction of protein structure and the principles of protein conformation. Plenum Press, New York.
43. **Rouch, D. A., D. S. Cram, D. DiBerardino, T. G. Littlejohn, and R. A. Skurray.** 1990. Efflux-mediated antiseptic resistance gene *qacA* from *Staphylococcus aureus*: common ancestry with tetracycline- and sugar-transport proteins. Mol. Microbiol. **4:**2051–2062.
44. **Ruengjitchatchawalya, M., T. Nihira, and Y. Yamada.** 1995. Purification and characterization of the IM-2-binding protein from *Streptomyces* sp. strain FRI-5. J. Bacteriol. **177:**551–557.
45. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
46. **Sato, K., T. Nihira, S. Sakuda, M. Yanagimoto, and Y. Yamada.** 1989. Isolation and structure of a new butyrolactone autoregulator from *Streptomyces* FRI-5. J. Ferment. Bioeng. **68:**170–173.
47. **Schein, C. H., and M. H. M. Noteborn.** 1988. Formation of soluble recombinant proteins in *Escherichia coli* is favored by lower growth temperature. Bio/Technology **6:**291–294.
48. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
49. **Studier, F. W., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Biol. Chem. **189:**113–130.
50. **Ward, J. M., G. R. Janssen, T. Kieser, M. J. Bibb, M. J. Buttner, and M. J. Bibb.** 1986. Construction and characterization of a series of multi-copy plasmid vectors for *Streptomyces* using the aminoglycoside phosphotransferase gene from Tn*5* as indicator. Mol. Gen. Genet. **203:**468–478.
51. **Yamada, Y., K. Sugamura, K. Kondo, M. Yanagimoto, and H. Okada.** 1987. The structure of inducing factors for virginiamycin production in *Streptomyces virginiae*. J. Antibiot. **40:**496–504.
52. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of M13mp18 and pUC19 vectors. Gene **33:**103–119.
53. **Zoller, M. J., and M. Smith.** 1983. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. Methods Enzymol. **100:**468–500.