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Cloning and Characterization of the Gene (*farA*) Encoding the Receptor for an Extracellular Regulatory Factor (IM-2) from *Streptomyces* sp. Strain FRI-5

MIYOKO WAKI, TAKUYA NIHIRA,* AND YASUHIRO YAMADA

Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565, Japan

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IM-2 is a butyrolactone autoregulator that controls production of blue pigment and nucleoside antibiotics in *Streptomyces* sp. strain FRI-5. An IM-2-specific receptor gene, *farA*, was cloned from strain FRI-5, and nucleotide sequencing revealed that the *farA* gene consists of 666 bp encoding a 221-amino-acid protein of 24.3 kDa with an NH_2-terminal amino acid sequence identical to that of purified native receptor. Another gene, *farX*, encoding a homolog of AfsA of *Streptomyces griseus*, was present upstream of *farA*. The monocistronic nature of the *farA* transcript was shown by Northern blot hybridization, and the transcript level increased upon addition of IM-2. Recombinant FarA expressed in and purified from *E. coli* showed clear ligand specificity toward IM-2, with a dissociation constant (K_d) for [^{3}H]IM-2-C_5 of 18.2 nM. FarA showed high overall homology to BarA (virginiae butanolide receptor from *S. virginiae*) and ArpA (A-factor receptor from *S. griseus*). Sequence alignment of the three receptor proteins revealed that the NH_2-terminal region containing a helix-turn-helix DNA binding motif was highly conserved. The DNA binding motif is common in procaryotic repressors of the TetR family, suggesting that all the *Streptomyces* autoregulator receptors may act as transcriptional repressors.

IM-2 [(2*R*,3*R*,1′*R*)-2-(1′-hydroxybutyl)-3-(hydroxymethyl) butanolide] of *Streptomyces* sp. strain FRI-5 is one of the butyrolactone autoregulators of *Streptomyces* species and triggers production of blue pigment as well as the nucleoside antibiotics showdomycin and minimycin at a concentration of 0.6 ng/ml (6, 17, 27, 34). Butyrolactone autoregulators have been regarded as a kind of *Streptomyces* hormone which switches on morphological differentiation, such as aerial mycelium formation, and/or physiological differentiation, such as the production of antibiotics. They have a 2,3-disubstituted γ-butyrolactone skeleton in common, and the 10 butyrolactone autoregulators identified to date are classified into the following three groups, on the basis of their minor structural differences: (i) A-factor type, possessing the 1'-keto group, to which only A-factor of *S. griseus* belongs (13, 18); (ii) virginiae butanolide (VB) type, possessing the 1'-α-hydroxyl group, to which VB-A through VB-E (14, 24, 33) and Gräfe's three factors (3) belong; and (iii) IM-2 type, possessing the 1'-β-hydroxyl group, to which IM-2 and factor I (4) belong (Fig. 1).

Although the three groups of butyrolactone autoregulators differ in only minor structural detail, almost no cross-reactivity was observed among the responsive strains; i.e., induction by VB of aerial mycelium formation or streptomycin production by A-factor in *Streptomyces griseus* was not observed (16), while virginiamycin production in *Streptomyces virginiae* triggered by VBs requires a 1.7×10^4-fold higher concentration of A-factor or IM-2 (7, 19). Blue pigment production in strain FRI-5 triggered by IM-2 requires 170- and 1.7×10^5-fold-higher concentrations of the corresponding VB- and A-factor-type compounds, respectively (our unpublished data). In addition to the strict requirement for IM-2 in strain FRI-5, IM-2 is unique in that it can modify the antibiotic production profile. While *Streptomyces* sp. strain FRI-5 produces D-cycloserine in the absence of IM-2, addition of IM-2 at 5 h of cultivation results in termination of D-cycloserine production and instead triggers production of blue pigment (17, 34) and the nucleoside antibiotics showdomycin and minimycin (6). The ability to switch from production of one kind of antibiotic to that of another was observed only in the case of IM-2 in *Streptomyces* sp. strain FRI-5.

The strict ligand specificity and the unique antibiotic-switching ability of IM-2 likely reflect the characteristics of the corresponding receptor protein. Previously we purified an IM-2-specific receptor protein from *Streptomyces* sp. FRI-5 (23) and showed that this IM-2 receptor is a homodimer of 27-kDa subunits, although its detailed characterization was impeded due to the very small amount of the purified protein available. To clarify the basis of the strict ligand specificity among autoregulator receptors and the unique signal-transducing mechanism of IM-2 leading to antibiotic production switching, we have cloned and sequenced the gene for the IM-2 receptor. The recombinant receptor was overexpressed in *Escherichia coli*, purified, and characterized in more detail.

MATERIALS AND METHODS

Bacteria and plasmids. *Streptomyces* sp. strain FRI-5 (MAFF10-06015; National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan) (23) was used as a source of native IM-2 receptor, genomic DNA, and total RNA. The strain was grown at 28°C as described previously (6) in a medium containing, per liter, 7.5 g of yeast extract (Difco), 7.5 g of glycerol, and 1.25 g of NaCl (pH 6.5). For genetic manipulation in *E. coli*, strain DH5α (5) was used. For expression of the cloned genes in *E. coli* and *Streptomyces*, *E. coli* BL21(DE3)/pLysS (29) and *Streptomyces lividans* TK21 (9), respectively, were used as hosts.

pUC19 was used for the construction of a genomic library and for DNA sequencing. pET-3d (30) was used for construction of the expression plasmids. *Streptomyces* plasmid pIJ486 (31) was kindly provided by D. A. Hopwood (John Innes Institute, Norwich, England). DNA manipulations in *E. coli* and in *Streptomyces* were performed as described by Sambrook et al. (25) and Hopwood et al. (8), respectively.

* Corresponding author. Mailing address: Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565, Japan. Phone: 81-6-879-7433. Fax: 81-6-879-7432. E-mail: nihira@biochem.bio.eng.osaka-u.ac.jp.

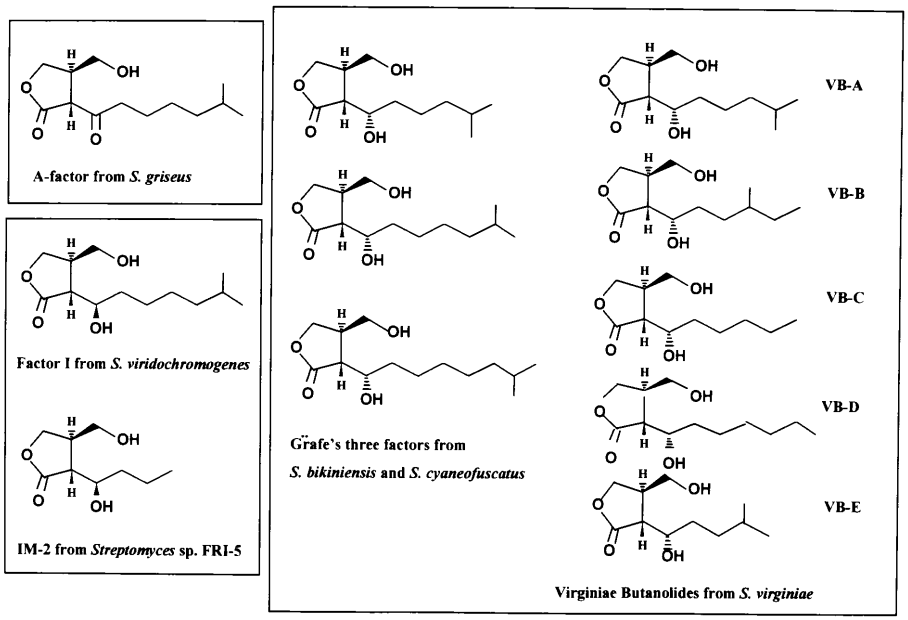


FIG. 1. Structures of butyrolactone autoregulators from *Streptomyces* species. Absolute configurations of A-factor (13, 18), VBs (14, 24), and IM-2 (17) have been assigned to (3*R*), (2*R*,3*R*,6*S*), and (2*R*,3*R*,6*R*), respectively, as shown. Absolute configurations of factor I and three factors from *S. bikiniensis* and *S. cyaneofuscatus* are not yet determined (3, 4).

Chemicals. All chemicals were of reagent or high-performance liquid chromatography (HPLC) grade and were purchased from either Nacali Tesque, Inc. (Osaka, Japan), Takara Shuzo Co., Ltd. (Shiga, Japan), or Wako Pure Chemical Industrial, Ltd. (Osaka, Japan). Marker proteins for molecular sieve HPLC and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were purchased from Oriental Yeast Co., Ltd. (Osaka, Japan), and Pharmacia Biotech K.K. (Osaka, Japan), respectively.

Southern blot hybridization and molecular cloning of *farA*. Total DNA of *Streptomyces* sp. strain FRI-5 was obtained by the method of Rao et al. (22). Genomic DNA (5 μg/lane) was digested to completion with restriction endonucleases and electrophoresed on a 1.0% agarose gel prior to blotting onto a nylon membrane (Hybond-N; Amersham). The membrane was hybridized with an *S. virginiae barX* probe (1.2-kbp *Bam*HI-*Eco*T22I fragment [20]) ^{32}P labeled with [α-^{32}P]dCTP (>3,000 Ci/mmol; ICN Biomedical Inc.) and a random primer labeling kit (Takara Shuzo). Hybridization was performed in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]) containing 0.5% SDS, 5× Denhardt's solution, and 0.02 mg of salmon sperm DNA per ml for 18 h at 65°C, followed by washing twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% SDS for 5 min each time at room temperature, with 1× SSC containing 0.1% SDS for 10 min once at room temperature and once at 65°C, with 0.5× SSC containing 0.1% SDS for 10 min at 65°C, and finally with 0.1× SSC containing 0.1% SDS for 10 min at 65°C. The blot was exposed to X-ray film (Fuji RX, −80°C, 3.5 h).

A partial genomic library was constructed with size-fractionated *Pst*I fragments (ca. 8.0 kbp) and pUC19, using *E. coli* DH5α as a host, and screened by colony hybridization with the ^{32}P-labeled *barX* probe. The DNA sequence was determined by the dideoxy-chain termination method (26) for both strands, using double-stranded templates of pUC19 clones with a Thermo Sequenase cycle sequencing kit (Amersham) and Cy5-labeled primers on a fluorescence DNA sequencer (ALFred; Pharmacia Biotech).

Sequence analyses and homology comparisons were done on a personal computer with the GENETYX software package (Software Development Co., Ltd., Tokyo, Japan).

Northern hybridization. Total RNA was isolated by a modification of the procedure of Chomczynski and Sacchi (1), and the amount was calculated from the A_{260}. Sixteen micrograms of RNA was loaded on each lane, electrophoresed on a 1.0% agarose gel, and then transferred to Hybond-N (Amersham) as recommended by the manufacturer. The membrane was then hybridized with a 967-bp *Sac*I-*Nae*I fragment (see Fig. 3) labeled with [α-^{32}P]dCTP by using a random primer labeling kit (Takara Shuzo). Hybridization was performed in 5× SSPE containing 0.5% SDS, 5× Denhardt's solution, 50% formamide, and 0.02 mg of salmon sperm DNA per ml for 19 h at 65°C, followed by two washes with 2× SSC containing 0.1% (wt/vol) SDS for 10 min each at 50°C.

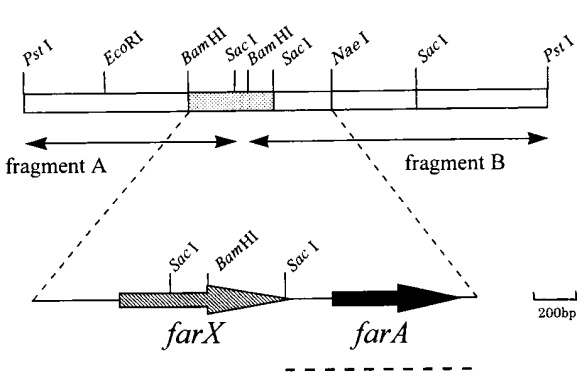
Construction of pET-*farA* and preparation of recombinant FarA (rFarA). A *Sac*I-*Nae*I (967 bp) fragment carrying *farA* was used as a template in the PCR. PCR was performed with primer 1 (5′-AACCATGGCCGAACAAGTCCGAG CCATCCGCACGCGCC-3′) and primer 2 (5′-AAGGATCCTGGTGCCGGG GCCGCTCAG-3′) to generate an *Nco*I site and a *Bam*HI site at the 5′ and 3′ ends of the *farA* coding sequence, respectively (underlined). After partial digestion with *Nco*I due to the presence of another *Nco*I site in the middle of *farA*, a 683-bp *Nco*I-*Bam*HI fragment was recovered and cloned into *Nco*I- and *Bam*HI-digested pET-3d, resulting in pET-*farA*. The nucleotide sequence around the *Nco*I junction was confirmed by DNA sequencing.

For preparing rFarA, *E. coli* BL21(DE3)/pLysS harboring pET-*farA* was grown overnight at 37°C in LB medium containing 0.2% glucose, 25 μg of ampicillin per ml, and 2 μg of chloramphenicol per ml. Two hundred fifty milliliters of fresh medium in a 500-ml Sakaguchi flask was inoculated with 5 ml of the preculture and cultivated at 27°C for 3 to 4 h until the A_{600} reached 0.4, followed by addition of 0.1 mM isopropyl-β-D-(−)-thiogalactopyranoside and 1 h of induction at 27°C. Cells were harvested and resuspended (1 g [wet weight] of cells per 10 ml of buffer) in buffer A [0.02 M triethanolamine (TEA)-HCl (pH 7) containing 20% glycerol, 0.5 mM dithiothreitol (DTT), and 0.1 mM (*p*-amidinophenyl)methanesulfonylfluoride hydrochloride (pAPMSF)] plus 0.1 M KCl and disrupted by sonication. Cell extracts were used for SDS-PAGE analysis and the assay of IM-2 binding activity. For purification of rFarA, the supernatant after centrifugation was absorbed on a DEAE HPLC column (TSKgel DEAE-5PW, 0.75 by 7.5 cm; Tosho) preequilibrated with buffer A containing 0.1 M KCl. After being washed with the same buffer, the bound proteins were eluted with a linear gradient of 0.1 to 0.35 M KCl (10 mM/min) in buffer A, and fractions showing a single band on SDS-PAGE eluting at around 0.23 M KCl were stored at −80°C until use.

IM-2 binding assay. IM-2 binding activity was assayed as described elsewhere (23) by measuring the difference between binding of [^{3}H]IM-2-C_5 (10 pmol, 40 Ci/mmol) in the absence and presence of nonlabeled IM-2-C_5 (15 nmol, 1,500-fold molar excess) in a total volume of 100 μl by equilibrium dialysis against 1 ml of buffer A containing 0.1 M KCl for 4 h. When dilution was necessary, hemoglobin was added to a final concentration of 100 μg/ml to prevent rapid inactivation of rFarA due to a low protein concentration. For determining ligand specificity, nonlabeled IM-2-C_5 was replaced with the ligand specified at different molar excesses. More effective ligand can give a larger difference between the extent of [^{3}H]IM-2-C_5 binding in the absence and that in the presence of the nonlabeled ligand. All of the nonlabeled ligands were chemically synthesized as previously described (11). For determining pH dependence of the IM-2 binding activity, the following buffers containing 0.1 M KCl and 20% glycerol were used for diluting the protein sample and for equilibrium dialysis: pH 5 to 9, 0.2 M TEA-HCl; pH 7 to 9, 0.02 M TEA-HCl; and pH 8 to 11, 0.02 M glycine-KOH. The radioactivity in the solution was measured with a liquid scintillation counter (LS6000; Beckman).

Preparation of crude cell extracts from *S. lividans*. *S. lividans* TK21 harboring the indicated plasmid was grown for 70 h in YEME as described by Hopwood et al. (8). One gram of mycelia was suspended in 4 ml of 0.05 M TEA-HCl (pH 7) containing 0.5 M KCl, 5 mM DTT, and 0.1 mM pAPMSF and disrupted by

a



b

plasmid		IM-2 binding activity *pmol/100 μl cell extract*	Specific activity *pmol/mg protein*
*Pst*I 8k fragment	clone 13	1.9	1.5
	clone 16	1.5	1.3
fragment A	clone 4	0	0
	clone 5	0	0
fragment B	clone 7	0.28	0.23
	clone 8	0.51	0.35
pIJ486		0	0
None		0.01	0

FIG. 2. Restriction endonuclease maps of the 8.0-kbp *Pst*I-*Pst*I fragment and 2.2-kbp *Bam*HI-*Nae*I fragment containing the *farX* and *farA* genes (a), and IM-2 binding activities of cell extracts prepared from *S. lividans* TK21 harboring plasmids carrying each fragment (b). The region homologous to *barX* localized by Southern hybridization is indicated as a dotted bar. Arrows indicate the coding regions of *farX* and *farA*. Fragment A (*Pst*I-*Sac*I) and fragment B (*Bam*HI-*Pst*I) indicate regions used to transform *S. lividans* TK21.

sonication. Cell debris was removed by centrifugation (28,000 × *g*, 20 min, 4°C), and the supernatant was stored at −80°C until use.

Determination of molecular weight. SDS-PAGE was performed with a precast 10 to 20% linear gradient gel (Daiichi Pure Chemical Co. Ltd., Tokyo, Japan) by using a minigel apparatus (Daiichi Pure Chemical Co.) and stained with Coomassie brilliant blue G-250.

The molecular weight of purified rFarA under nondenaturing conditions was estimated as described elsewhere (23) by gel filtration HPLC (TSK-G2000SW$_{XL}$, M_r, <160,000; Tosoh) with buffer A containing 0.3 M KCl and 5 mM DTT.

Protein assay. Protein concentration was determined by a dye binding assay (Bio-Rad protein assay kit), using bovine serum albumin as a standard.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL data bank with accession no. AB001683.

RESULTS AND DISCUSSION

Southern hybridization analyses. To clone a gene for the IM-2 receptor protein, we first performed a series of Southern blot hybridizations against genomic DNA of strain FRI-5. However, with *barA* encoding a VB-specific receptor of *S. virginiae* as a probe, no clear signal was obtained; with synthetic degenerate oligonucleotides designed from the N-terminal Ala-Glu-Gln-Val-Arg-Ala-Ile-Arg determined from the purified native IM-2 receptor, many ambiguous signals were detected, due probably to the high redundancy of the oligonucleotide probe (data not shown). Then we used *barX* (accession no. AB001608), which is present 259 bp upstream of *barA* oriented in the opposite transcriptional direction and seems to encode a homolog of AfsA postulated to catalyze A-factor synthesis in *S. griseus* (10), in the hope that the gene arrangement of the *afsA* homolog-receptor gene is conserved in strain FRI-5. Among the several clear signals detected by the *barX* probe, we selected an 8.0-kbp *Pst*I fragment, constructed a partial genomic library from size-fractionated *Pst*I fragments, and screened it by colony hybridization using the *barX* probe. After isolating the 8.0-kbp *Pst*I fragment, we localized the *barX* homolog to a 1.3-kbp *Bam*HI-*Sac*I fragment by subsequent Southern hybridizations (Fig. 2a).

To confirm the existence of and further localize the region containing the IM-2 receptor gene, we transformed *S. lividans* TK21 with the multicopy vector pIJ486 carrying the 8.0-kbp *Pst*I-*Pst*I fragment, 3.4-kbp *Pst*I-*Sac*I fragment A, or 4.5-kbp *Bam*HI-*Pst*I fragment B. In IM-2 binding assays of cell extracts prepared from the corresponding transformants, although the one containing fragment B reproducibly showed lower IM-2 binding activity than the one containing the 8.0-kbp fragment, due probably to a lower level of expression, both the entire 8.0-kbp *Pst*I fragment and the right-hand *Bam*HI-*Pst*I fragment B were suggested to encode a protein with strong IM-2 binding activity, which in turn suggested that the IM-2 receptor gene is located on fragment B (Fig. 2b).

Nucleotide sequence of the IM-2 receptor gene and *barX* homolog. Nucleotide sequencing of the 1.3-kbp *Bam*HI-*Sac*I fragment and the adjacent 967 bp of fragment B revealed two open reading frames (ORFs) transcribed in the same direction

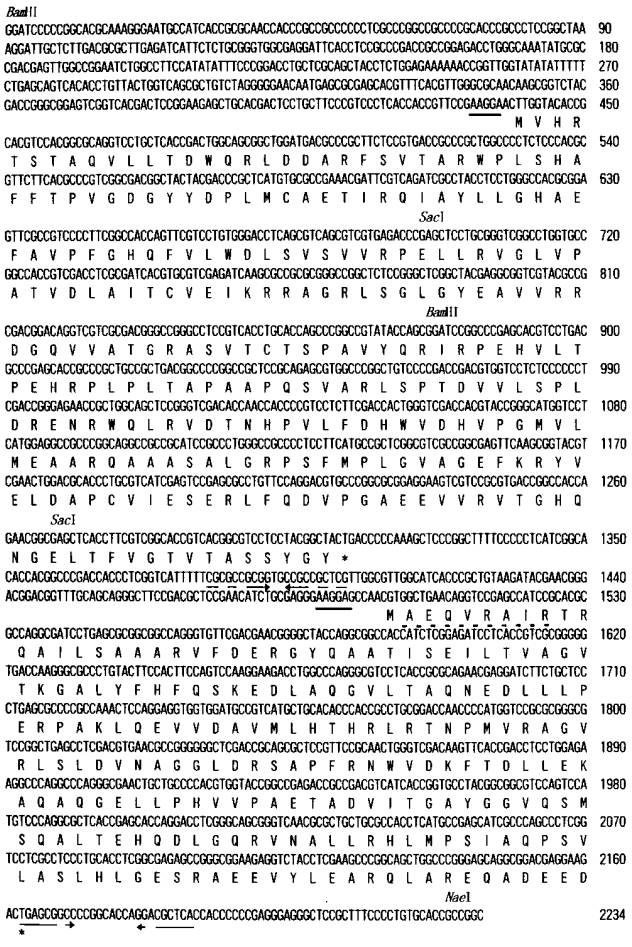


FIG. 3. Nucleotide and deduced amino acid (one-letter notation) sequences of *farA* and *farX*. The nucleotide numbering begins at the 5′-most *Bam*HI site of the 2,234-bp *Bam*HI-*Nae*I fragment. An asterisk denotes a translational stop codon. Possible ribosome binding sequences are underlined, and inverted arrows show potential transcriptional terminator sequences. The N-terminal sequence determined from the purified IM-2 receptor is indicated by broken lines.

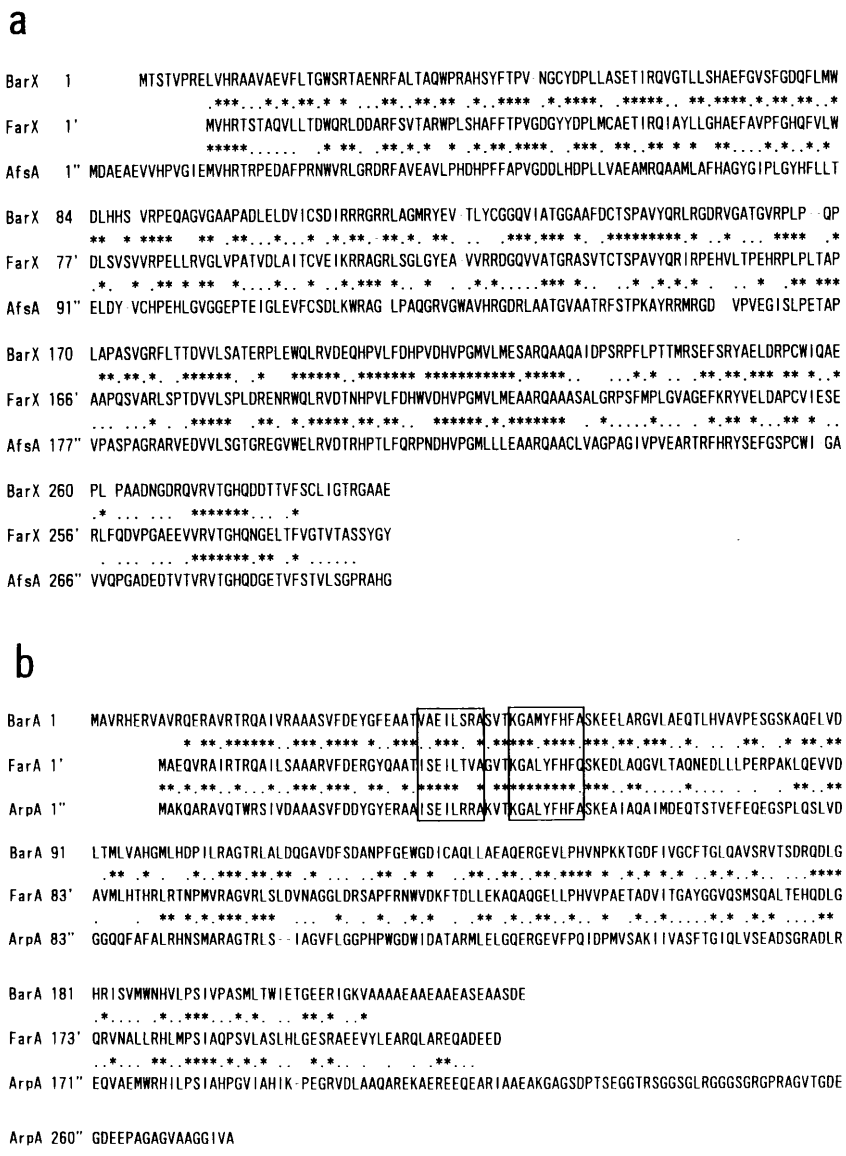


FIG. 4. Alignment of overall amino acid sequences of FarX, BarX and AfsA (a) and FarA, BarA and ArpA (b). Homology among the sequences was identified by a computer search using the algorithm of Lipman and Pearson (15). Dashes are introduced to optimize alignments. Identical and similar amino acids as determined by the method of Dayhoff (2) are indicated by asterisks and dots, respectively. The sequences corresponding to helix 2 and helix 3 in the helix-turn-helix DNA binding motif are boxed.

(Fig. 3). The first ORF has three possible initiation sites, ATG at nucleotide (nt) 317, TTG at nt 440, and GTG at nt 512, of which ATG at nt 317 and TTG at nt 440 were preceded by probable ribosome binding sites. From the alignment with BarX and AfsA as described below, TTG at nt 440 was chosen as the most probable initiation site. The resulting ORF consisting of 876 bp is predicted to encode a 291-amino-acid protein of 32.1 kDa which shows significant sequence homology to AfsA (40.6% identity and 78.8% similarity) and BarX (53.2% identity and 84.3% similarity) (Fig. 4a), and the ORF was designated *farX* (FRI-5 *barX* homolog). No other proteins with significant sequence homology were found in database searches. The second ORF was concluded to start from GTG at nt 1500 due to the presence of the preceding ribosome binding sequence (AAGGAG) and to the complete match of the amino acid sequence deduced from the ORF to the N-terminal sequence of the purified IM-2 receptor (AEQVRAIR) except for the initiation methionine residue. This ORF was named *farA* (FRI-5 autoregulator receptor) and predicted to encode a 221-amino-acid protein of 24,250 Da, which agrees moderately well with the estimated molecular weight of the purified native IM-2 receptor (27,000) (23).

In a computer-aided homology search, FarA was found to have high overall homology to only BarA, specifying a VB-specific receptor from *S. virginiae* (20), with 48.5% identity and 86.1% similarity, and ArpA, specifying an A-factor-specific receptor from *S. griseus* (21), with 38.5% identity and 80.7% similarity (Fig. 4b). The NH_2-terminal one-fourth of FarA containing a helix-turn-helix DNA binding motif was highly conserved among them, but the rest of the sequences showed much less homology. Because a very similar DNA binding motif is conserved among procaryotic repressor-type transcrip-

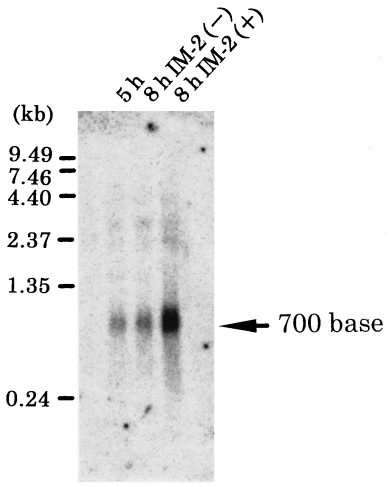


FIG. 5. Northern blot analysis of the *farA* transcript. Total RNA from mycelia of *Streptomyces* sp. strain FRI-5 grown for 5 and 8 h (with or without IM-2 added at 5 h) was electrophoresed on a 1.0% agarose–formaldehyde gel together with size markers. After transfer to a nylon membrane (Hybond-N), the blot was hybridized with a ^{32}P-labeled *farA* probe as described in Materials and Methods and exposed to X-ray film (Kodak X-Omat AR, −80°C, 70 h). Migration positions of size markers are shown at the left.

tional factors such as TcmR, regulating the tetracenomycin resistance gene in *Streptomyces glaucescens*, and TetR, controlling the tetracycline resistance gene of Tn*1721* in *E. coli* (32), FarA may act as a transcriptional repressor by binding directly to a specific DNA sequence(s) with the conserved helix-turn-helix sequence. Although regions other than the N-terminal one-fourth should participate in recognition and discrimination of the corresponding ligand, we are at present unable to delimit the region. Creation of several chimeric receptors is under way in our laboratory, and their analysis will clarify the regions essential for ligand specificity.

Northern hybridization analysis of *farA*. To study transcription of *farA* during cultivation, we performed Northern blot hybridization with total RNA of *Streptomyces* sp. strain FRI-5, using a *Sac*I-*Nae*I fragment as a *farA* probe (Fig. 5). We analyzed the transcripts in the mycelia after a 5-h cultivation and an 8-h cultivation with or without addition of IM-2 (20 ng/ml) at 5 h, because exogenous addition of IM-2 at 5 h caused the production of blue pigment at 8 h (6, 34). In all samples, only a 0.7-kbp band was observed, suggesting the monocistronic nature of *farA*. The addition of IM-2 caused a slight but reproducible increase (1.5- to 1.7-fold) in the level of *farA* transcription. Although the significance and the mechanism of this phenomenon are unclear at present, gel shift analyses using recombinant FarA toward possible target sequences, including those in the *farA* promoter region, are under way in our laboratory and may clarify the basis of this phenomenon.

Expression of recombinant FarA in *E. coli* and its characterization. To examine the *farA* product in more detail, we expressed *farA* in *E. coli* by means of the T7 expression vector pET-3d. The coding region of *farA* was amplified by PCR and placed under the control of the T7 RNA polymerase promoter as described in Materials and Methods. SDS-PAGE analysis (Fig. 6a) indicated that isopropylthiogalactopyranoside (IPTG)-induced *E. coli* BL21(DE3)/pLysS harboring pET-*farA* significantly overproduced a 27-kDa protein, the identity of

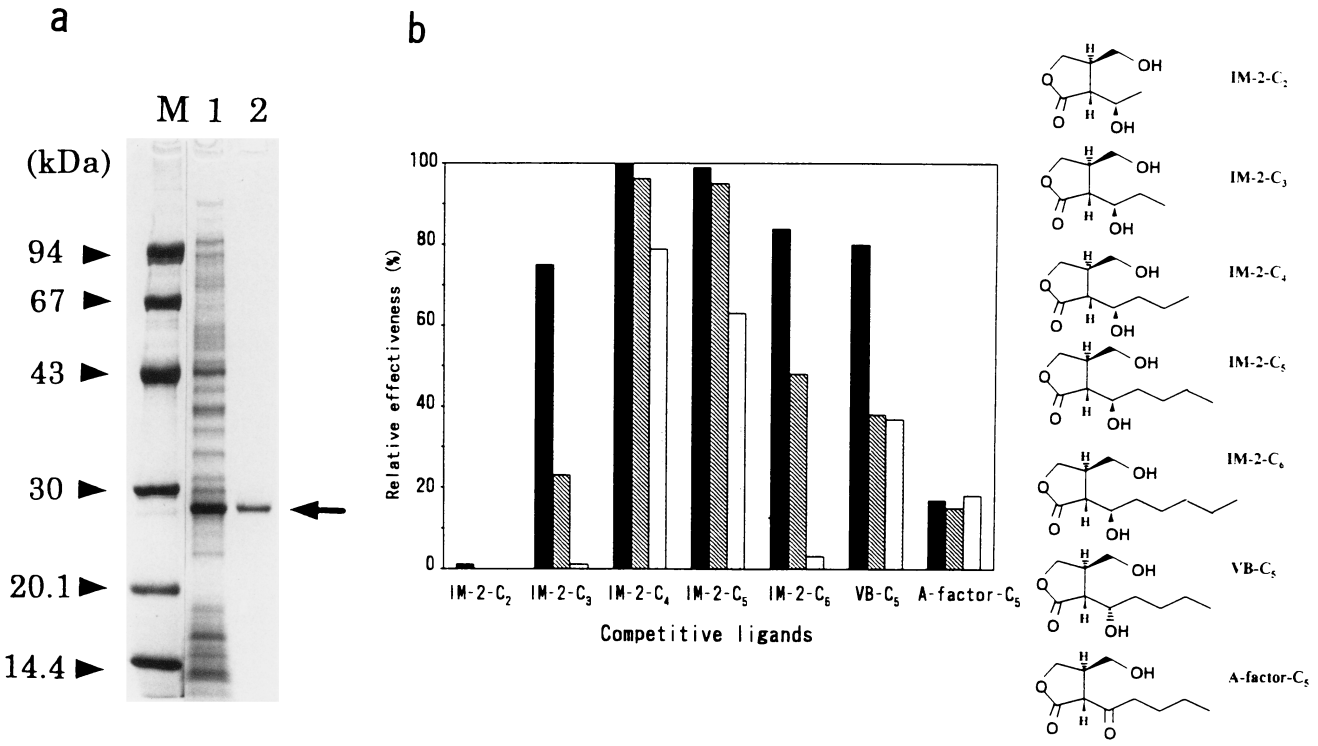


FIG. 6. SDS-PAGE analysis of rFarA expressed in and purified from *E. coli* (a) and effectiveness of several autoregulators as competitive ligands in the binding of [^{3}H]IM-2-C_5 to rFarA (b). (a) Lane M, molecular weight markers; lane 1, crude extract from *E. coli* BL21/pLysS harboring pET-*farA*; lane 2, purified rFarA after DEAE-5PW HPLC. A sample containing 7 μg of protein was separated on a 10 to 20% linear gradient gel, and the gel was stained with Coomassie brilliant blue G-250. (b) Nonlabeled ligands are present at 1.5-fold (dotted bars), 15-fold (stripe bars), and 150-fold (black bars) molar excesses over [^{3}H]IM-2-C_5. The value for 150-fold IM-2-C_4 was taken as 100%. The structures of the competitive ligands are indicated on the right.

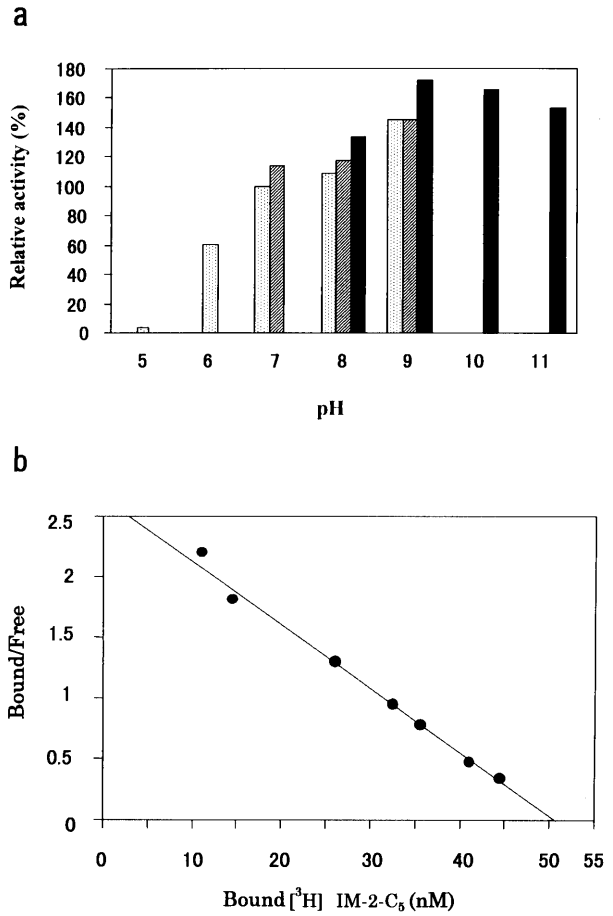


FIG. 7. pH dependence of IM-2 binding activity (a) and Scatchard analysis of purified rFarA (b). (a) IM-2 binding activity was assayed by equilibrium dialysis as described previously (12), using buffers of the indicated pHs: pH 5 to 9, 0.2 M TEA-HCl (striped bars); pH 7 to 9, 0.02 M TEA-HCl (dotted bars); and pH 8 to 11, 0.2 M glycine-NaOH buffer (black bars). The IM-2 binding activity at pH 7 was taken as 100%. (b) Purified rFarA (12.4 pmol, 0.3 μg) in 100 μl of buffer C containing 0.1 M KCl, 20% glycerol, 0.5 mM DTT, and 0.1 mM pAPMSF was incubated with various amounts of [^{3}H]IM-2-C_5 for 4 h at 25°C in the presence or absence of nonlabeled IM-2-C_5 and then subjected to equilibrium dialysis.

which was confirmed by analysis of its N-terminal amino acid sequence. Furthermore, cell extracts prepared from IPTG-induced cells harboring pET-*farA* showed an extremely high IM-2 binding activity (7.1×10^7 dpm/mg of protein), while cell extracts from the control cells harboring pET-3d showed no activity (data not shown). The overexpressed rFarA was purified to homogeneity by DEAE-5PW HPLC (Fig. 6a), with an apparent molecular weight of 27,000 on SDS-PAGE. Under native conditions by molecular sieve HPLC, purified rFarA was eluted as a trimer at a high protein concentration (3.9 mg/ml), but the elution position shifted to that of a dimer at a lower protein concentration (0.98 mg/ml), indicating that rFarA tends to aggregate at high protein concentrations. This phenomenon agrees well with the instability of rFarA in a buffer containing no glycerol, in which rFarA lost its IM-2 binding activity rapidly upon forming high-molecular-weight aggregates (data not shown). Because the native IM-2 receptor exists solely as a cytoplasmic soluble protein (23) and we have observed no sign of its association with membrane under several conditions, this aggregation of rFarA should be an artificial event at high protein concentrations. IM-2, a rather hydrophobic and noncharged ligand, seems to diffuse freely across the membrane and bind with the IM-2 receptor in the cytoplasm.

To study the ligand specificity of rFarA, we performed a series of competitive binding assays in which we used several autoregulators, i.e., IM-2-C_2 to IM-2-C_6, VB-C_5, and A-factor-C_5, as competitive nonlabeled ligands against [^{3}H]IM-2-C_5. The experiments were conducted at 1.5-, 15-, and 150-fold molar excesses of unlabeled ligands (Fig. 6b). IM-2-type compounds were shown to be the most effective ligands at each concentration, with IM-2-C_4 and IM-2-C_5 being the most effective. As the chain length at C-2 deviated from the optimum, the effectiveness decreased sharply. VB-C_5 showed less affinity than did IM-2-C_5, and A-factor-C_5 showed very poor binding affinity, confirming that FarA is actually the IM-2-specific receptor. The concentration dependence of IM-2 binding activity was studied by Scatchard plotting (Fig. 7b). The plot gave a straight line, and the slope of the line indicates a dissociation constant (K_d) of 18.2 nM and B_{max} (binding maximum) of 0.38 mol of [^{3}H]IM-2-C_5/mol of rFarA monomer. The K_d value was 14-fold higher than that for a partially purified native IM-2 receptor from *Streptomyces* sp. strain FRI-5 (1.3 nM) (23). A similar discrepancy of K_d values between the native receptor in the crude state and a recombinant receptor in the purified state is observed for the VB receptor (20), the K_d of which is 8.6 nM for the crude native receptor and 30 to 130 nM for the purified recombinant receptor, due mainly to the highly unstable nature of the purified receptor, although we cannot exclude the possibility of the existence of some kind of an accessory protein which stabilizes the corresponding receptor in *Streptomyces*.

Figure 7a shows the pH dependence of the IM-2 binding activity of rFarA, demonstrating clearly the pH optimum at 9. Although this alkaline optimum might suggest that the true ligand is in the free-acid form rather than the lactone form, based on the well-known fact that an ester bond is unstable at alkaline pH, this possibility is unlikely for the following two reasons. (i) In the reverse-phase HPLC, less than 0.5%, if not at all, of the IM-2-C_5 was found to exist in the free-acid form when the pH of the running buffer was 7, while 28% was in the free-acid form when the pH of the running buffer was 9. Despite this large increase in the amount in the free-acid form, the IM-2 binding activity of the rFarA increased only 1.4-fold from pH 7 to 9. (ii) When the free-acid form of [^{3}H]IM-2-C_5, instead of the lactone form, was used in the standard assay at pH 7, at which a small fraction of the free-acid form was instantaneously converted to the lactone form but the majority remained in the free-acid form, the IM-2 binding activity was 1.7% of that with the lactone form of [^{3}H]IM-2-C_5, excluding the possibility that the free-acid form of IM-2 is the true ligand form.

More than 10 butyrolactone autoregulators have been identified in *Streptomyces* species, and they comprise a family of *Streptomyces* hormones which regulate cytodifferentiation and/or secondary metabolism. Since corresponding receptors have been identified for all three classes of butyrolactone autoregulators from *Streptomyces* species—the IM-2, VB, and A-factor receptors—the receptor proteins most likely participate in autoregulator-dependent signal transduction. Successful cloning and analysis of the gene encoding the IM-2 receptor, together with already cloned genes for VB and A-factor receptors, will facilitate the identification of specific regulatory roles at the molecular level.

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