JOURNAL OF BACTERIOLOGY, Sept. 2002, p. 5151–5157 Vol. 184, No. 18
0021-9193/02/$04.00+0 DOI: 10.1128/JB.184.18.5151–5157.2002

barS1, a Gene for Biosynthesis of a γ-Butyrolactone Autoregulator, a Microbial Signaling Molecule Eliciting Antibiotic Production in *Streptomyces* Species

Noriyasu Shikura, Junji Yamamura, and Takuya Nihira*

Department of Biotechnology, Graduate School of Engineering, Osaka University, Suita, Osaka 565-0871, Japan

Received 19 April 2002/Accepted 19 June 2002

From *Streptomyces virginiae*, in which production of streptogramin antibiotic virginiamycin M_1 and S is tightly regulated by a low-molecular-weight *Streptomyces* hormone called virginiae butanolide (VB), which is a member of the γ-butyrolactone autoregulators, the hormone biosynthetic gene (*barS1*) was cloned and characterized by heterologous expression in *Escherichia coli* and by gene disruption in *S. virginiae*. The *barS1* gene (a 774-bp open reading frame encoding a 257-amino-acid protein [M_r, 27,095]) is situated in the 10-kb regulator island surrounding the VB-specific receptor gene, *barA*. The deduced BarS1 protein is weakly homologous to β-ketoacyl-acyl carrier protein/coenzyme A reductase and belongs to the superfamily of short-chain alcohol dehydrogenase. The function of the BarS1 protein in VB biosynthesis was confirmed by BarS1-dependent in vitro conversion of 6-dehydro-VB-A to VB-A, the last catalytic step in VB biosynthesis. Of the four possible enantiomeric products from racemic 6-dehydro-VB-A as a substrate, only the natural enantiomer of (2*R*,3*R*,6*S*)-VB-A was produced by the purified recombinant BarS1 (rBarS1), indicating that rBarS1 is the stereospecific reductase recognizing (3*R*)-isomer as a substrate and reducing it stereospecifically to the (6*S*) product. In the Δ*barS1* mutant created by homologous recombination, the production of VB as well as the production of virginiamycin was lost. The production of virginiamycin by the Δ*barS1* mutant was fully recovered by the external addition of VB to the culture, which indicates that the *barS1* gene is essential in the biosynthesis of the autoregulator VBs in *S. virginiae* and that the failure of virginiamycin production was a result of the loss of VB production.

Members of the filamentous, gram-positive bacterial genus *Streptomyces* are versatile producers of many secondary metabolites, including over two thirds of all known antibiotics used in human medicine and in agriculture (4). Many factors, such as nutrient limitations, have been known to affect the antibiotic production in the genus *Streptomyces* (5, 6); however, detailed knowledge of the mechanism and/or hierarchy of the regulatory machinery is generally lacking, which has hampered the rational design of a highly productive industrial strain.

Among factors known to affect the behavior of *Streptomyces* species (10, 11, 41), γ-butyrolactone autoregulators are one of the most studied, and they have been shown for several *Streptomyces* species to trigger the onset of secondary metabolism in general and that of antibiotic production in particular (38, 40). All the known γ-butyrolactone autoregulators, which belong to one of three types (virginiae butanolide [VB] type, possessing a 6-α-hydroxy group [18, 39]; IM-2 type, possessing a 6-β-hydroxy group [30, 36]; and A-factor type, possessing a 6-keto group [14, 20] [Fig. 1A]), possess type-specific receptor proteins that recognize the tiny structural differences among the three types of autoregulators. The effectiveness of the autoregulators at extremely low concentrations, usually at a concentration of a few nanomolars (23), as well as the presence of receptor proteins of high ligand specificity (24, 35, 37), implies that the γ-butyrolactone autoregulators should be regarded as *Streptomyces* hormones.

Streptomyces virginiae is one of the representative strains in which VB-A regulates the production of two structurally different antibiotics, virginiamycin M_1 and virginiamycin S, by binding to the VB-specific receptor protein, BarA. With regard to the signal transduction pathway and/or regulation mechanism after the complex formation between VB-A and BarA, BarA's function as the VB-dependent transcriptional repressor has been clarified (16, 21, 22). However, almost nothing is known about the biosynthesis of VB itself. It is essential to obtain a gene(s) that catalyzes the VB biosynthesis in order to gain insight into how and when VB production is regulated. Furthermore, to clarify the mechanism of why one strain, such as *S. virginiae*, produces only the VB type of γ-butyrolactone autoregulators, and another strain, such as *Streptomyces lavendulae* FRI-5, solely produces the IM-2 type of autoregulators, it is important to biochemically characterize the enzyme that catalyzes the last reduction step of the autoregulator biosynthesis.

Regarding the biosynthetic pathway of the autoregulators, we have previously established a plausible pathway mainly by feeding precursors labeled with radioactive or stable isotopes (27, 28) (Fig. 1B). The estimated pathway suggested the presence of two reduction steps in autoregulator biosynthesis; namely, the NADH-dependent enoyl reductase-type reduction to form the A-factor-type precursor (6-dehydro-VB-A) and the final NADPH-dependent keto-reduction to form either VB-

* Corresponding author. Permanent address: The International Center for Biotechnology, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan. Phone: 81-6-6879-7433. Fax: 81-6-6879-7432. E-mail: nihira@icb.osaka-u.ac.jp.

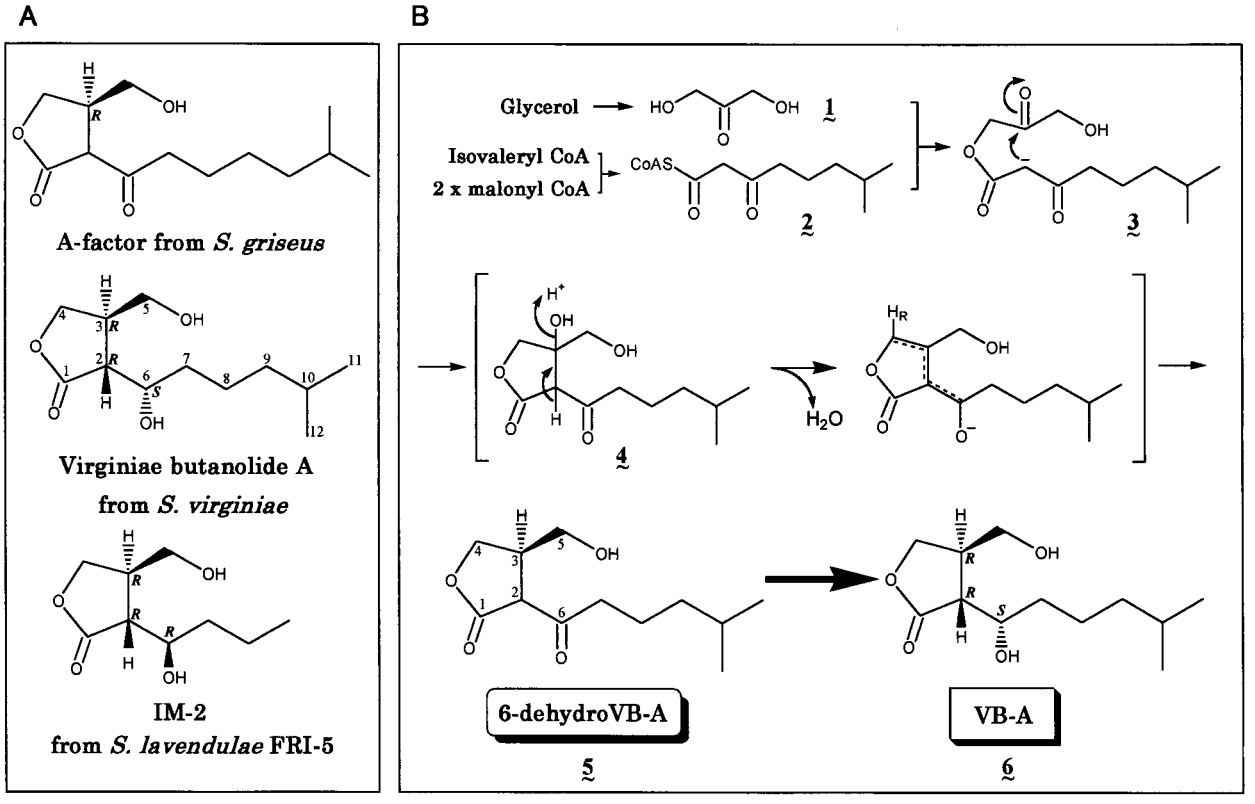


FIG. 1. (A) Three types of γ-butyrolactone autoregulators from *Streptomyces* species. (B) A plausible biosynthetic pathway deduced from precursor feeding. (A) Absolute configurations of A-factor (20), VB-A (38), and IM-2 (38, 40) have been assigned to (3*R*), (2*R*,3*R*,6*S*), and (2*R*,3*R*,6*R*), respectively, as depicted. (B) The condensation to form a β-ketoacyl-CoA (2) occurs between an isovaleryl-CoA and two malonyl-CoAs in a process similar to that of polyketide biosynthesis, and β-ketoacyl-CoA (2) couples with a dihydroxyacetone-type C_3 unit (1) derived from glycerol to create a β-keto ester (3), followed by intramolecular aldol condensation to form a γ-butyrolactone skeleton (4). Successive dehydration and reduction should lead to 6-dehydro-VB-A (5). Finally, reduction of the 6-carbonyl group (5) will result either in (2*R*,3*R*,6*S*)-VB-A (6) or its (6*R*)-epimer.

type compounds of (6*S*) absolute configuration or IM-2-type compounds of (6*R*) absolute configuration. In the course of our searching for genes responsible for the autoregulator biosynthesis a novel open reading frame (ORF) (*orf4*) (13) was identified upstream of the *barA* gene encoding the VB specific receptor, raising the possibility that the gene may participate in the VB biosynthetic pathway. In this study we focused on the *orf4* gene and found that the Orf4 protein is the essential biosynthetic enzyme catalyzing the stereospecific reduction from A-factor-type precursor into VB-type compounds. This is, to our knowledge, the first report describing the isolation and characterization of a gene encoding an actual biosynthetic enzyme for the γ-butyrolactone autoregulators.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and plasmids. *S. virginiae* (strain MAFF 10-06014; National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan) was grown at 28°C as described previously (15, 39). For genetic manipulation in *Escherichia coli*, strain DH5α (7) was used. For expression of the cloned gene in *E. coli*, BL21(DE3)/pLysS (33) was used as the host. pET-3d (34) was used for construction of the expression plasmids. For conjugal transfer of DNA into *S. virginiae*, the methylation-deficient *E. coli* strain ET12567 (*dam-13*::Tn*9 dcm-6 hsdM hsdS*) (19) containing the RP4 derivative pUZ8002 (25) was used as the donor. The plasmid used for conjugal transfer was pKC1132 (3). DNA manipulation in *E. coli* was performed as described by Sambrook et al. (29).

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

Construction of pET-*barS1* and preparation of rBarS1. A *Bam*HI-*Bam*HI (2.0 kb) fragment carrying *barS1* was used as a template in the PCR. PCR was performed with primer 1 (5′-CATGCCATGGCTGATCGTCAGGGCCTTCTG ACAGAC-3′) and primer 2 (5′-CGCGGATCCTGAAATCAGAGGATGGTG AACCCGCC-3′) to generate an *Nco*I site and a *Bam*HI site at the 5′ and 3′ ends of the *barS1* coding sequence, respectively (underlined). The amplified product was digested with *Nco*I and *Bam*HI and was ligated into *Nco*I-*Bam*HI-digested pET-3d, resulting in pET-*barS1*. The nucleotide sequence was confirmed by DNA sequencing. For preparing recombinant BarS1 (rBarS1), *E. coli* BL21 (DE3)/pLysS harboring pET-*barS1* was grown overnight at 37°C in Luria-Bertani medium containing both ampicillin (25 μg/ml) and chloramphenicol (25 μg/ml). Fresh Luria-Bertani medium (250 ml) in a 500-ml Sakaguchi flask was inoculated with 2.5 ml of the preculture and was cultivated at 37°C until the A_{600} reached 0.5, followed by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (final concentration of 1 mM) and 2 to 3 h of induction. Cells were harvested and resuspended (1 g [wet weight] of cells per 10 ml of buffer) in buffer A (0.02 M triethanolamine-HCl, pH 7.0, containing 20% [wt/vol] glycerol, 5 mM EDTA-Na_2, 5 mM 2-mercaptoethanol, 1 mM dithiothreitol, 0.1 mM *p*-[amidinophenyl] methanesulfonyl fluoride hydrochloride, 10 μM leupeptin, and 0.3 μM pepstatin A) containing 0.5 M NaCl and were disrupted by sonication for 3 min at 50% duty cycle (Branson Sonifier 250) in an ice bath. Cell extracts were used for

SDS-PAGE analysis and the assay of 6-dehydro-VB-A reductase activity. 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 the gel was stained with Coomassie brilliant blue G-250.

For purification of rBarS1, the dialyzed supernatant after centrifugation (18,000 × *g*, 20 min) was adsorbed on a DEAE-Sephacel column (bed volume of 140 ml) preequilibrated with buffer A containing 0.05 M NaCl and 100 μM NADPH. After being washed with 560 ml of the same buffer the protein was successively eluted with 910 ml of buffer A containing 0.1 M NaCl and 100 μM NADPH, 700 ml of buffer A containing 0.15 M NaCl and 100 μM NADPH, and finally 840 ml of buffer A containing 0.2 M NaCl and 100 μM NADPH. The column was eluted at a flow rate of 45 ml/min, and 14-ml fractions were collected. Fractions 169 to 214 eluted at 0.2 M NaCl were pooled and concentrated to 61.5 ml by ultrafiltration (UK-20; Advantech Toyo). Fractions showing a single band on SDS-PAGE were stored at −80°C until use.

Determination of molecular weight. The molecular weight of purified rBarS1 under nondenaturing conditions was estimated by gel filtration HPLC with a Superose 12 column on a SMART system (Amersham Pharmacia Biotech) with buffer A (without 2-mercaptoethanol) containing 0.3 M NaCl and 10 μM NADPH at a flow rate of 50 μl/min. A calibration curve was prepared with glutamate dehydrogenase (M_r, 290,000), lactate dehydrogenase (M_r, 142,000), yeast enolase (M_r, 67,000), yeast adenylate kinase (M_r, 32,000), and cytochrome *c* (M_r, 12,400).

Assay of 6-dehydro-VB-A reductase activity. 6-Dehydro-VB-A reductase activity was assayed at 25°C by using buffer A (without EDTA-Na_2 [pH 7.5]) containing 0.5 M NaCl as described previously (31, 32). One unit of enzyme activity is defined as the amount of enzyme necessary to produce 1 μmol of VB-A per min at 25°C. Protein content was determined either by a Bio-Rad protein assay kit or by comparison of peak areas on HPLC charts with bovine serum albumin as a standard. (±)-6-Dehydro-VB-A was added to a final concentration of 766 μM. NADPH or NADH was added to a final concentration of 5 mM.

Enzyme reaction with commercial dehydrogenases was performed at 24°C in buffer A (pH 7.0) (without EDTA-Na_2 [pH 7.5]) containing 0.5 M NaCl in the presence of 5 mM NADH and 5 mM NADPH. The reaction with 3-hydroxybutyrate dehydrogenase was carried out at 37°C. Glycerol was omitted from the buffer for assay with glycerol dehydrogenase.

For measuring the activity of a series of A-factor-type substrates, the amount of the resulting VB product was determined as the amount of dibenzoyl derivative on C_{18} reverse-phase HPLC as described below. Reaction progress was followed intermittently, and the catalytic rate was calculated from the linear part of each reaction. The enzyme reaction was initiated as described previously (31, 32) in a total volume of 575 μl and was terminated by adding 200 μl of 1% trifluoroacetic acid and 1,225 μl of cold water. Five micrograms of racemic VB-D was added as an internal standard. The mixture was diluted with water (9 ml) and applied to an OASIS HLB extraction cartridge (1 ml; Waters) followed by washing with 5 ml of water. The absorbed VB together with VB-D was eluted with 8 ml of 75% (vol/vol) CH_3CN. The 75% CH_3CN fraction was evaporated to dryness. The lyophilized sample was benzoylated with benzoyl cyanide and tri-*n*-butylamine as described previously (31, 32). The benzoylation mixture was extracted with 4 ml of hexane-methanol-water (3 ml–250 μl–750 μl), and the organic layer containing both VB dibenzoate and VB-D dibenzoate was evaporated to dryness. The amount of VB dibenzoate was measured by a method similar to that used for VB-A by comparing the peak area with an authentic VB dibenzoate standard. Dibenzoyl derivatives of VB-C_4, VB-C_5, VB-C_6, VB-A, VB-D (VB-C_7), VB-type isomer of SCB1, VB-C_8, and VB-C_9 were eluted at 9.78, 12.5, 16.5, 21.0, 23.0, 27.2, 30.4, and 44.1 min, respectively. (±)-6-Dehydro-VB-A, racemic A-factor C_4∼C_9, racemic A-factor, racemic VB-A, racemic VB-C_4∼C_9, and racemic VB-type isomer of SCB1 were chemically synthesized as described elsewhere (23, 27, 36). Racemic standards of VB dibenzoates were prepared similarly by benzoylation of corresponding racemic VBs, and their identities were confirmed by chemical ionization-mass spectrometry (CI-MS).

Isolation and chiral HPLC analysis of the rBarS1-catalyzed product. VB-A produced by rBarS1 was purified as dibenzoate by C_{18} reverse-phase HPLC (32), and the chemical structure was verified by 600 MHz ^{1}H-nuclear magnetic resonance and CI-MS with the data reported previously (27). The product was then analyzed by chiral HPLC to determine the optical purity. Authentic chiral standards of VB-A dibenzoate isomers were synthesized as described previously (32).

Preparation of crude cell extract from *S. virginiae*. The *S. virginiae* culture was initiated by inoculating 2.1 ml of preculture into 70 ml of f medium (15) in a 500-ml baffled flask. After cultivation at 28°C on a reciprocating shaker (120 strokes per min), cells were harvested by centrifugation (3,000 × *g*, 10 min, 4°C). The cells suspended in a fivefold volume of buffer A (without EDTA-Na_2 [pH 7.5]) containing 0.5 M NaCl and 100 μM NADPH were disrupted by sonication for 5 min as described above. After centifugation (18,000 × *g*, 20 min, 4°C), the dialyzed supernatant was stored at −80°C and was used as the source of native 6-dehydro-VB-A reductase of *S. virginiae*.

The Δ*barS1* mutant of *S. virginiae*. A 5.0-kb fragment of *Bst*1107I-*Eco*T22I (*varM*, *orf4*, *orf5*, and *barX*; Fig. 2) was blunt ended and subcloned into the *Sma*I site of pUC19. From the resulting plasmid a 297-bp *Psp*1406I-*Bsm*I fragment internal to the *orf4* coding region was removed to create disruption plasmid pJK101, by which an in-frame deletion of 99 amino acids from ^{84}Phe to ^{192}Gly was introduced. The status of the in-frame deletion in the *orf4* (*barS1*) gene on pJK101 was confirmed by DNA sequencing, and the deletion mutation was designated Δ*barS1*.

To construct a conjugation plasmid for gene replacement in *S. virginiae*, a *Hin*dIII-*Eco*RI fragment (4.7 kb; *varM*-Δ*barS1-orf5-barX*) from pJK101 was subcloned at the corresponding sites of pKC1132 (3), a conjugation plasmid containing *oriT* and an apramycin resistance cassette, resulting in pJK102. The conjugation protocol between *E. coli* ET12567/pUZ8002 containing pJK102 and *S. virginiae* spores was essentially similar to those for *S. lavendulae* FRI-5 (17) on ISP-2 solid medium with selection by overlaying nalidixic acid and apramycin.

Analyses on the production of virginiamycin and VB. Spores of *S. virginiae* (10^8) were inoculated in 70 ml of f broth (7.5 g of Bacto Casitone [Difco Laboratories] per liter, 7.5 g of yeast extract [Difco Laboratories] per liter, 15 g of glycerol per liter, and 2.5 g of NaCl per liter [pH 6.5] [23]) and were reciprocally incubated for 24 h (140 strokes per min). Cells were harvested and resuspended in the same volume of f broth. The cell suspension was immediately frozen and kept at −80°C until use. The frozen culture was thawed at room temperature and was added to a 70-ml f broth, diluted to an A_{600} of 0.075. For conventional analysis of virginiamycin production, the culture was withdrawn periodically and supernatant (after centrifugation) was used for bioassay with *Bacillus subtilis* PCI219 as an indicator strain (23). Purified virginiamycins obtained from C.-K. Lee (Osaka University) were used as the standard.

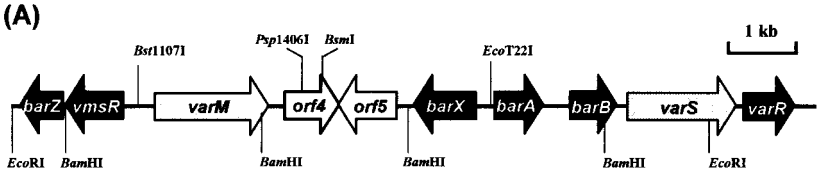
The amount of VB produced during the growth in f broth was estimated by measuring the VB-dependent production of virginiamycin essentially according to the previously described method (23). Chemically synthesized VB-C_6 (23) was used as the standard. One unit of VB activity is defined as the minimum amount of VB-A needed to induce virginiamycin production (0.6 ng/ml or 2.6 nM) (39).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper has been submitted to the DDBJ/EMBL/GenBank data bank as accession number AB035548.

RESULTS AND DISCUSSION

Nucleotide sequence of the *orf4* gene. Nucleotide sequencing of the 10-kb *Eco*RI-*Eco*RI fragment containing *barA* revealed the *orf4* gene starting 2,986 bp upstream of the *barA* start codon in the same direction as *barA* (Fig. 2A). The resulting ORF is predicted to encode a 257-amino-acid protein of 27,095 Da which showed moderate similarity to several β-ketoacyl-acyl carrier protein/coenzyme A (CoA) reductases belonging to the short-chain alcohol dehydrogenase superfamily, such as DpsE from *S. peucetius* (36.0% identity), functioning as a reductase in the biosynthesis of daunorubicin (8); FrnP from *S. roseofulvus* (36.0% identity), participating in the biosynthesis of frenolicin (2); and ActIII from *S. coelicolor* A3 (2) (34.8% identity), functioning as a reductase of a β-keto group during the assembly of the actinorhodin polyketide chain (9). Multiple alignment of the deduced Orf4 product with these short-chain alcohol dehydrogenase proteins (Fig. 2B) revealed that the most significant identity exists at an amino-terminal region containing a typical βαβ fold as an NAD(P)H-binding motif, Gly-X-X-X-Gly-X-Gly, as well as at a middle region that includes Ser^{148}, Tyr^{161}, and Lys^{165} assigned as active-site residues (26).

Overexpression and purification of rOrf4. To examine the *orf4* product in more detail, we expressed recombinant Orf4 (rOrf4) protein in *E. coli* by means of the T7 expression vector pET-3d. The coding region was amplified by PCR and placed under the control of the T7 RNA polymerase promoter. SDS-



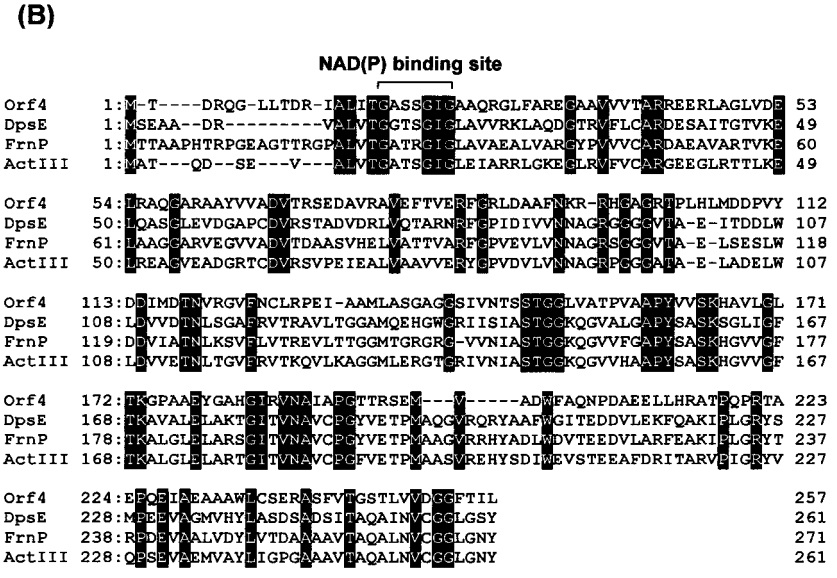


FIG. 2. (A) Location of the *orf4* gene in the 10-kb *Eco*RI-*Eco*RI fragment containing *barA*. (B) Amino acid alignment of the *orf4* product with several β-ketoacyl-acyl carrier protein/CoA reductases. (A) Solid arrows, shaded arrows, and open arrows indicate plausible regulatory genes, resistance genes for virginiamycin, and genes for catalytic enzymes, respectively. (B) Identical residues are indicated by white letters in black boxes. Orf4, *S. virginiae* (this study); DpsE, *S. peucetius* (8); FrnP, *S. roseofulvus* (2); ActIII, *S. coelicolor* A3(2) (9).

PAGE analysis (Fig. 3, lane 2) indicated that IPTG-induced *E. coli* BL21(DE3)/pLysS harboring pET-*orf4* significantly overexpressed a 27-kDa protein, whose identity to the *orf4* product was confirmed by analysis of its N-terminal amino acid sequence (data not shown).

To clarify the actual enzymatic function of rOrf4, we tested its activity by using 6-dehydro-VB-A as a substrate (23, 24). Cell extracts prepared from IPTG-induced cells harboring pET-*orf4* showed a high 6-dehydro-VB-A reductase activity for forming VB-A (268 mU/mg of protein) in the presence of NADPH, while cell extracts from the control cells harboring pET-3d showed no activity (Table 1). The overexpressed rOrf4 was purified to homogeneity in an activity yield of 32.8% on a DEAE-Sephacel column (Fig. 3, lane 3). The apparent molecular weight of 27,000 on SDS-PAGE agreed well with that calculated from the nucleotide sequence (M_r, 27,095). Under native conditions by molecular sieve HPLC, purified rOrf4 showed an apparent M_r of 54,000, indicating the dimeric nature of the enzyme. Enzymatic reaction of this purified enzyme with 6-dehydro-VB-A was carried out, and the product after benzoylation was purified by reverse-phase C_{18} HPLC. All the 600 MHz ^{1}H-nuclear magnetic resonance and CI-MS spectra of the isolated product agreed well with those of synthetic VB-A dibenzoate (27), confirming that the catalytic product of the rOrf4 protein is VB-A.

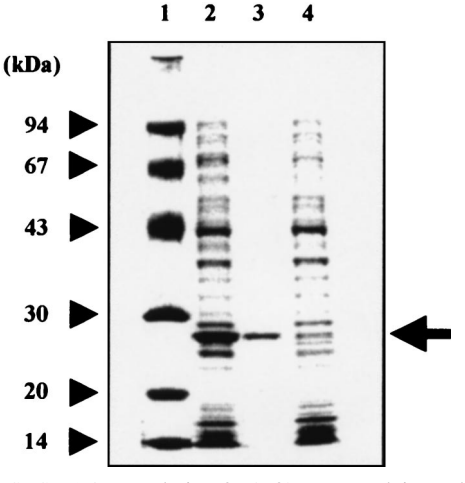


FIG. 3. SDS-PAGE analysis of rOrf4 expressed in and purified from *E. coli*. The rOrf4 protein is indicated by an arrow. Lane 1, molecular size markers; lane 2, crude extract from IPTG-induced *E. coli* BL21/pLysS harboring pET-*orf4*; lane 3, purified rOrf4 after DEAE-Sephacel anion-exchange chromatography; lane 4, crude extract from IPTG-induced *E. coli* BL21/pLysS harboring pET-3d. A sample containing 7 μg was separated on a 10 to 20% linear gradient gel, and the gel was stained with Coomassie brilliant blue G-250.

TABLE 1. Assay of 6-dehydro-VB-A reductase activity for crude and purified rOrf4 protein

Protein	Results with the following substrates:			Amt of VB-A produced (pmol/min)[c]
	6-Dehydro-VB-A[a]	NADPH[b]	NADH[b]	
Crude (pET-*orf4*)	+	+	−	3.51×10^2
Crude (pET-3d)	+	+	−	0
Purified rOrf4	+	+	−	3.34×10^2
Purified rOrf4	+	−	+	0
Purified rOrf4	+	−	−	0
Purified rOrf4	−	+	−	0
No protein	+	+	−	0

[a] (±)-6-Dehydro-VB-A was added to a final concentration of 766 μM.
[b] NADPH or NADH was added to a final concentration of 5 mM.
[c] For assays with crude extracts, 1.31 μg of pET-*orf4* protein and 14.6 μg of pET-3d protein were used. For assays with purified rOrf4, 3.0 μg of protein was used. The enzyme activity was measured within the linear range of activity after 0.5 to 20 h of incubation, with a 3-pmol detection limit per assay (31, 32).

In order to verify that the 6-dehydro-VB-A reductase activity is a specific feature of rOrf4, we tested several commercial dehydrogenases for their 6-dehydro-VB-A reductase activity. No detectable activity was found in any of the five dehydrogenases tested (alcohol dehydrogenase from baker's yeast, glycerol dehydrogenase from *Bacillus megaterium*, 3-hydroxybutyrate dehydrogenase from *Rhodopseudomonas sphaeroides*, 3α-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni*, and β-hydroxysteroid dehydrogenase from *P. testosteroni*), even in the presence of 2,000 times the amount of rORF4 (1 U per assay) during a 20-h incubation (data not shown). From these results we concluded that the *orf4* gene encodes the 6-dehydro-VB-A reductase catalyzing the reduction of 6-dehydro-VB-A to VB-A, and we designated it *barS1* (for butyrolactone autoregulator synthesis).

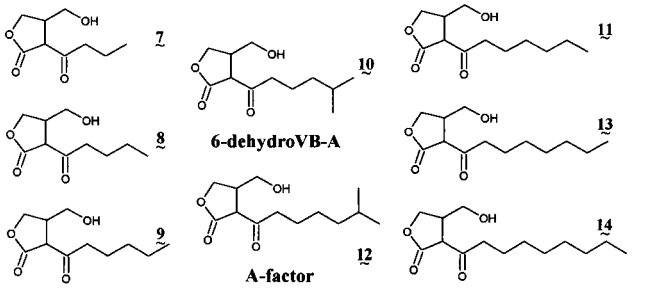
Characterization of rBarS1. To characterize the coenzyme specificity, purified rBarS1 was assayed in the presence of 5 mM NADH or NADPH. Clear activity was detected with 5 mM NADPH but none was detected with NADH, indicating that rBarS1 was NADPH specific (Table 1). The K_m value for (±)-6-dehydro-VB-A and the V_{max} value were determined to be 11.1 μM and 269 mU/mg of protein in the presence of 10 mM NADPH, respectively. The optimum pH was determined to be 7.5. The optimum temperature was narrow, with a maximum at 25°C, together with a narrow range of stability, as is evident from the 80% loss and the complete loss of activity after 30 min of incubation at 35°C and at 40 to 50°C, respectively.

To determine the substrate specificity of the enzyme, especially the influence of the C-2 side chain structure, we performed a 6-dehydro-VB reductase assay in the presence of a series of synthetic A-factor-type analogues, i.e., A-factor-C_4 to A-factor-C_9 (linear side chain), A-factor (natural form), and 6-dehydro-VB-A (Table 2). The activity increased sharply with a chain length of 4 to 6 carbons, but any further increase in the side chain length only resulted in a moderate decrease in activity. This substrate specificity agreed well with the profiles of natural autoregulators produced by *S. virginiae*, namely, VBs having a linear or branched 6- to 7-carbon C-2 side chain (VB-A, -B, -C, and -D) are predominant, while the amount of a VB with a shorter side chain (VB-E) is scarce (18).

To study the stereospecificity of the reaction, we analyzed

TABLE 2. VB synthesizing activity of rBarS1 for a series of A-factor-type substrates

Substrate[a]		Relative activity[b] (%)
n = 4	7	0
n = 5	8	65.7
n = 6	9	102
n = 7*	10	100
n = 7	11	88.9
n = 8*	12	83.3
n = 8	13	82.8
n = 9	14	75.3



[a] Total carbon number in the C-2 side chain is indicated by n, with an asterisk indicating the substrate having terminal isopropyl moiety.
[b] Each reaction was performed with purified rBarS1 (1.54 μg) at a substrate concentration of 1 mM and with 10 mM NADPH at 25°C in buffer A (without EDTA-Na_2 [pH 7.5]) containing 0.5 M NaCl and 100 μM NADPH.

purified enzymatic VB-A dibenzoate by chiral HPLC (Fig. 4) (32) and found it to be an optically pure (2*R*,3*R*,6*S*)-form. Considering that no IM-2-type compound [(2*R*,3*R*,6*R*)- or (2*S*,3*S*,6*S*)-form] was detected by our highly sensitive procedure (data not shown), we concluded that rBarS1 accepts only (3*R*)-6-dehydro-VB-A as a substrate and catalyzes the stereospecific reduction to form natural (2*R*,3*R*,6*S*)-VB-A. To deduce the in vivo function of *barS1* further, we investigated the time course of the 6-dehydro-VB-A reductase activity in the cell during the cultivation of *S. virginiae* for 6 to 16 h. Specific activity was almost constant (521 μU/mg of protein) during the entire cultivation, which agreed well with the constitutive expression of *barS1* by reverse transcription-PCR (13).

Disruption of *barS1* by homologous recombination. To know the in vivo function of the *barS1* in either the production of VB and/or virginiamycin, the *barS1* gene was disrupted by in-frame deletion of a 297-bp fragment internal to the *barS1* gene. The disruption plasmid (pJK102; Fig. 5A) derived from a conjugation vector, pKC1132, was introduced into *S. virginiae* via conjugation from *E. coli* ET12567/pUZ8002. After selection of the single-crossover strain on solid ISP-2 containing apramycin (50 μg/ml), Δ*barS1* mutant strains of *S. virginiae* were obtained by three rounds of cultivation from spore to spore on solid ISP-2 without apramycin. The integration of pJK102 in the single-crossover strain and the replacement of the wild-type allele

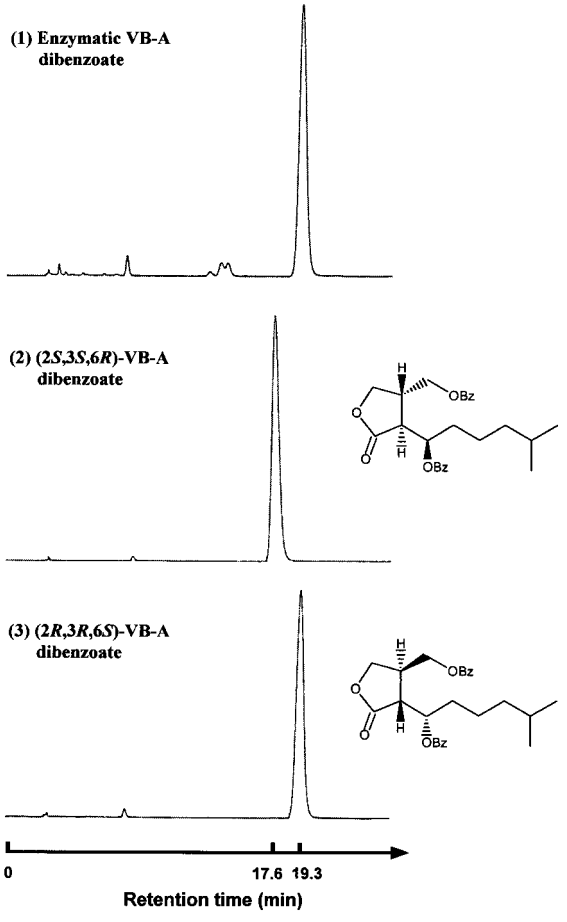


FIG. 4. Chiral HPLC profiles of synthetic VB-A dibenzoates and the benzoylated reaction product with rBarS1. HPLC was performed with a Chiralpak AD column (4.6 mm [inside diameter] by 25 cm) at 22°C, with hexane and isopropanol (90:10) as the mobile phase at a flow rate of 1.0 ml/min. (1) Enzymatic product dibenzoate; (2) synthetic (2*S*,3*S*,6*R*)-enantiomer dibenzoate; and (3) synthetic (2*R*,3*R*,6*S*)-enantiomer dibenzoate.

TABLE 3. Concentration of VB and virginiamycin produced by two independent Δ*barS1* disruptants and by the wild-type strain in the absence of external VB addition

Strain	Cultivation time and concn of:							
	VB (U/ml)				Virginiamycin (μg/ml)			
	10 h	13 h	16 h	24 h	10 h	13 h	16 h	24 h
Wild type	<0.2	≥150	≥150	≥150	0	94	92	94
Δ*barS1* disruptant no. 1	<0.2	<0.2	<0.2	<0.2	0	0	0	0
Δ*barS1* disruptant no. 2	<0.2	<0.2	<0.2	<0.2	0	0	0	0

with the Δ*barS1*-mutated allele in the Δ*barS1* mutant were confirmed by PCR (data not shown) and Southern blot hybridization (Fig. 5). Two of the representative double-crossover strains were selected for further study. No defect of growth either in liquid media or on solid media was observed for the *barS1* disruptants compared to that with wild-type *S. virginiae*. In contrast, the *barS1* disruption caused complete loss of the production of VB and virginiamycin (Tables 3 and 4). Usually, *S. virginiae* starts producing VB after 10.5 h of cultivation and the concentration of VB reaches up to 150 U per ml at around 12 h of cultivation, which in turn induces the production of virginiamycin after 13 h of cultivation. In the *orf4* disruptant, no VB production was detected even after 24 h of cultivation and no virginiamycin production was observed. To clarify whether this defect in virginiamycin production was due to the loss of VB production, external VB was added to the culture of the *barS1* disruptants at 8 h of cultivation, which restored the virginiamycin production to a level identical to that of the wild-type strain. These results indicate that the *barS1* gene is the essential and only gene encoding the 6-dehydro-VB-A reductase catalyzing the last biosynthetic step of the autoregulator VB.

Although *S. griseus afsA* has been proposed to encode one of the biosynthetic enzymes for A-factor (1, 12), the actual cata-

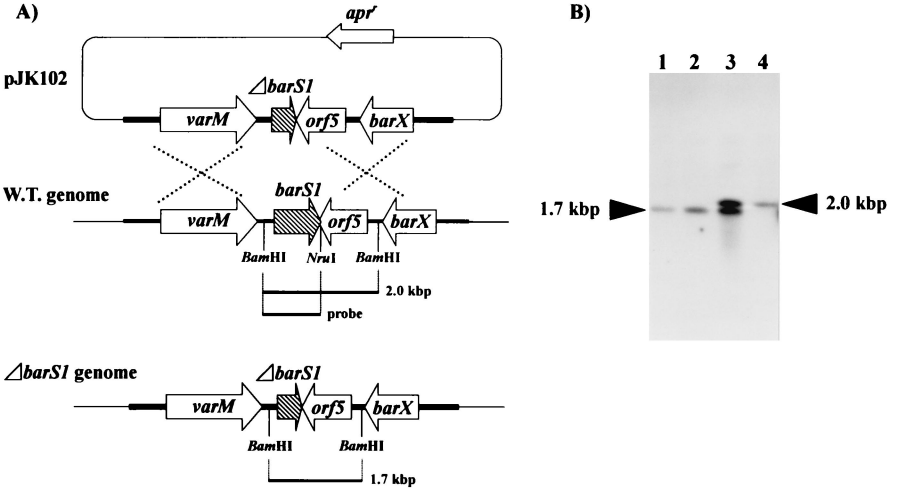


FIG. 5. Construction of a *barS1* disruption mutant. (A) Schematic representation of the strategy used for the disruption of *barS1*. The shaded arrow represents the *barS1* gene, and open arrows represent the apramycin resistance gene (*apr*) and the *varM*, *orf5*, and *barX* genes. W.T., wild type. (B) Southern hybridization analysis of chromosomal DNA from the wild-type strain (lane 4), Δ*barS1* strains (lanes 1 and 2), and a single-crossover strain (lane 3) digested with *Bam*HI. The probe used was the *Bam*HI-*Nru*I fragment shown as the probe in panel A. *Bam*HI digestion results in a 2.0-kb fragment and a 1.7-kb fragment in the wild-type chromosome and the Δ*barS1* chromosome, respectively.

TABLE 4. Concentration of virginiamycin produced by the Δ*barSI* disruptants with external VB addition (VB-C_6; 60 ng/ml) at 8 h of cultivation

Strain	Cultivation time and virginiamycin concn (μg/ml)			
	8 h	10 h	12 h	14 h
Δ*barSI* disruptant no. 1 plus VB	0	43	81	94
Δ*barSI* disruptant no. 2 plus VB	0	40	76	81

lytic step or enzymatic function in the biosynthetic pathway has been left unexplained until now. Furthermore, contradictory information regarding the function of AfsA has become apparent recently in the findings that two close homologues of *afsA*, namely, *S. virginiae barX* and *S. coelicolor scbA*, act as pleiotropic regulatory proteins rather than as catalytic enzymes (13, 35). Successful cloning and analysis of *barS1* as one of the genes in the autoregulator biosynthesis will promote the elucidation and understanding of the actual biosynthetic mechanism of γ-butyrolactone autoregulators at the molecular level.

REFERENCES

1. **Ando, N., N. Matsumori, S. Sakuda, T. Beppu, and S. Horinouchi.** 1997. Involvement of AfsA in A-factor biosynthesis as a key enzyme. J. Antibiot. **50:**847–852.
2. **Bibb, M. J., D. H. Sherman, S. Ômura, and D. A. Hopwood.** 1994. Cloning, sequencing and deduced functions of a cluster of *Streptomyces* genes probably encoding biosynthesis of the polyketide antibiotic frenolicin. Gene **142:** 31–39.
3. **Bierman, M., R. Logan, K. O'Brien, E. T. Seno, R. N. Rao, and B. E. Schoner.** 1992. Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyce*s spp. Gene **116:**43–49.
4. **Champness, W.** 2000. Actinomycete development, antibiotic production, and phylogeny: questions and challenges, p. 11–31. *In* Y. V. Brun and L. J. Shimkets (ed.), Prokaryotic development. ASM Press, Washington, D.C.
5. **Chater, K. F., and M. J. Bibb.** 1997. Regulation of bacterial antibiotic production, p. 57–105. *In* H. Kleinkauf and H. V. Döhren (ed.), Bio/Technology, vol. 7. VCH Press, Weinheim, Germany.
6. **Demain, A. L., and A. Fang.** 1995. Emerging concepts of secondary metabolism in actinomycetes. Actinomycetology **9:**98–117.
7. **Grant, S. G. N., J. Jessee, F. R. Bloom, and D. Hanahan.** 1990. Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. Proc. Natl. Acad. Sci. USA **87:**4645–4649.
8. **Grimm, A., K. Madduri, A. Ali, and C. R. Hutchinson.** 1994. Characterization of the *Streptomyces peucetius* ATCC 29050 genes encoding doxorubicin polyketide synthase. Gene **151:**1–10.
9. **Hallam, S. E., F. Malpartida, and D. A. Hopwood.** 1988. Nucleotide sequence, transcription and deduced function of a gene involved in polyketide antibiotic synthesis in *Streptomyces coelicolor*. Gene **74:**305–320.
10. **Hobbs, G., A. I. C. Obanye, J. Petty, J. C. Mason, E. Barratt, D. C. J. Gardener, F. Flett, C. P. Smith, P. Broda, and S. G. Oliver.** 1992. An integrated approach to studying regulation of production of the antibiotic methylenomycin by *Streptomyces coelicolor* A3(2). J. Bacteriol. **174:**1487–1494.
11. **Hood, D. W., R. Heidstra, U. K. Swoboda, and D. A. Hodgson.** 1992. Molecular genetic analysis of proline and tryptophan biosynthesis in *Streptomyces coelicolor* A3(2): interaction between primary and secondary metabolism—a review. Gene **115:**5–12.
12. **Horinouchi, S., H. Suzuki, M. Nishiyama, and T. Beppu.** 1989. Nucleotide sequence and transcriptional analysis of the *Streptomyces griseus* gene (*afsA*) responsible for A-factor biosynthesis. J. Bacteriol. **171:**1206–1210.
13. **Kawachi, R., T. Akashi, Y. Kamitani, A. Sy, U. Wangchaisoonthorn, T. Nihira, and Y. Yamada.** 2000. Identification of an AfsA homologue (BarX) from *Streptomyces virginiae* as a pleiotropic regulator controlling autoregulator biosynthesis, virginiamycin biosynthesis and virginiamycin M_1 resistance. Mol. Microbiol. **36:**302–313.
14. **Khokhlov, A. S.** 1980. Problems of studies of specific cell autoregulators (on the example of substances produced by some actinomycetes), p. 201–210. *In* S. N. Ananchenko (ed.), Frontiers of bioorganic chemistry and molecular biology. Pergamon Press, Oxford, United Kingdom.
15. **Kim, H. S., T. Nihira, T. Tada, H. 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.
16. **Kinoshita, H., T. Tsuji, H. Ipposhi, T. Nihira, and Y. Yamada.** 1999. Characterization of binding sequences for butyrolactone autoregulator receptors in streptomycetes. J. Bacteriol. **181:**5075–5080.
17. **Kitani, S., Y. Yamada, and T. Nihira.** 2001. Gene replacement analysis of the butyrolactone autoregulator receptor (FarA) reveals that FarA acts as a novel regulator in secondary metabolism of *Streptomyces lavendulae* FRI-5. J. Bacteriol. **183:**4357–4363.
18. **Kondo, K., Y. Higuchi, S. Sakuda, T. Nihira, and Y. Yamada.** 1989. New virginiae butanolide from *Streptomyces virginiae*. J. Antibiot. **42:**1873–1876.
19. **MacNeil, D. J., J. L. Occi, K. M. Gewain, T. MacNeil, P. H. Gibbons, C. L. Ruby, and S. J. Danis.** 1992. Complex organization of the *Streptomyces avermetilis* genes encoding the avermectin polyketide synthase. Gene **115:**119–125.
20. **Mori, K.** 1983. Revision of the absolute configuration of A-factor. Tetrahedron **39:**3107–3109.
21. **Nakano, H., E. Takehara, T. Nihira, and Y. Yamada.** 1998. Gene replacement analysis of the *Streptomyces virginiae barA* gene encoding the butyrolactone autoregulator receptor reveals that BarA acts as a repressor in virginiamycin biosynthesis. J. Bacteriol. **180:**3317–3322.
22. **Nakano, H., C. K. Lee, T. Nihira, and Y. Yamada.** 2000. A null mutant of the *Streptomyces virginiae barA* gene encoding the butyrolactone autoregulator receptor, and its pleiotropic and transcriptional analyses. J. Biosci. Biotechnol. **90:**204–207.
23. **Nihira, T., Y. Shimizu, H. S. Kim, and Y. Yamada.** 1988. Structure-activity relationships of virginiae butanolide C, an inducer of virginiamycin production in *Streptomyces virginiae*. J. Antibiot. **41:**1828–1837.
24. **Okamoto, S., K. Nakamura, T. Nihira, and Y. Yamada.** 1995. Virginiae butanolide binding protein from *Streptomyces virginiae*. J. Biol. Chem. **270:** 12319–12326.
25. **Paget, M. S. B., L. Chamberlin, A. Atrih, S. J. Foster, and M. J. Buttner.** 1999. Evidence that the extracytoplasmic function sigma factor σ^E is required for normal cell wall structure in *Streptomyces coelicolor* A3(2). J. Bacteriol. **181:**204–211.
26. **Persson, B., M. Krook, and H. Jörnvall.** 1991. Characteristics of short-chain alcohol dehydrogenases and related enzymes. Eur. J. Biochem. **200:**537–543.
27. **Sakuda, S., A. Higashi, S. Tanaka, T. Nihira, and Y. Yamada.** 1992. Biosynthesis of virginiae butanolide A, a butyrolactone autoregulator from *Streptomyces*. J. Am. Chem. Soc. **114:**663–668.
28. **Sakuda, S., and Y. Yamada.** 1998. Biosynthesis of butyrolactone and cyclopentanoid skeltons formed by aldol condensation, p. 139–158. *In* D. H. I. Barton, K. Nakanishi, and O. Meth-Cohn (ed.), Comprehensive natural products chemistry, vol. 1. Elsevier Science Ltd., Oxford, United Kingdom.
29. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
30. **Sato, K., T. Nihira, S. Sakuda, M. Yanagimoto, and Y. Yamada.** 1989. Isolation and structure of a new butyrolactone autoregulator from *Streptomyces* sp. FRI-5. J. Ferment. Biotechnol. **68:**170–173.
31. **Shikura, N., T. Nihira, and Y. Yamada.** 1999. Identification and characterization of 6-dehydroVB-A reductase from *Streptomyces antibioticus*. FEMS Microbiol. Lett. **171:**183–189.
32. **Shikura, N., T. Nihira, and Y. Yamada.** 2000. Identification of a plausible biosynthetic enzyme for the IM-2-type autoregulator in *Streptomyces antibioticus*. Biochim. Biophys. Acta **1475:**329–336.
33. **Studier, F. W., and B. A. Moffat.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189:**113.
34. **Studier, F. W., and B. A. Moffat.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185:**60–89.
35. **Takano, E., R. Chakraburtty, T. Nihira, Y. Yamada, and M. J. Bibb.** 2001. A complex role for the γ-butyrolactone SCB1 in regulating antibiotic production in *Streptomyces coelicolor* A3(2). Mol. Microbiol. **41:**1015–1028.
36. **Takano, E., T. Nihira, Y. Hara, J. J. Jones, C. J. L. Gershater, Y. Yamada, and M. J. Bibb.** 2000. Purification and structural determination of SCB1, a γ-butyrolactone that elicits antibiotic production in *Streptomyces coelicolor* A3(2). J. Biol. Chem. **275:**11010–11016.
37. **Waki, M., T. Nihira, and Y. Yamada.** 1997. Cloning and characterization of the gene (*farA*) encoding the receptor for an extracellular regulatory factor (IM-2) from *Streptomyces* sp. strain FRI-5. J. Bacteriol. **179**:5131–5137.
38. **Yamada, Y.** 1999. Autoregulatory factors and regulation of antibiotic production in *Streptomyces*, p. 177–196. *In* R. England, G. Hobbs, N. Bainton, and D. L. McRoberts (ed.), Microbial signalling and communication. Society for General Microbiology, Cambridge University Press, Cambridge, United Kingdom.
39. **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.
40. **Yamada, Y., and T. Nihira.** 1998. Microbial hormones and microbial chemical ecology, p. 377–413. *In* D. H. R. Barton and K. Nakanishi (ed.), Comprehensive natural products chemistry, vol. 8. Elsevier Science Ltd., Oxford, United Kingdom.
41. **Yang, K., L. Han, and L. C. Vining.** 1995. Regulation of jadomycin B producing in *Streptomyces venezuelae* ISP5230: involvement of a repressor gene, $jadR_2$. J. Bacteriol. **177:**6111–6117.