*J. Microbiol. Biotechnol.* (2006), **16**(1), 77–83

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY



# Cloning and Characterization of a Gene Encoding γ-Butyrolactone Autoregulator Receptor from *Saccharopolyspora erythraea*

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Received: April 14, 2005
Accepted: May 24, 2005

**Abstract** A gene encoding a γ-butyrolactone autoregulator receptor was cloned from *Saccharopolyspora erythraea*, and the biochemical characteristics, including the autoregulator specificity, were determined with the purified recombinant protein. Using primers designed for the conserved amino acid sequence of *Streptomyces* γ-butyrolactone autoregulator receptors, a 120 bp *S. erythraea* DNA fragment was obtained by PCR. Southern and colony hybridization with the 120 bp fragment as a probe allowed to select a genomic clone of *S. erythraea*, pESG, harboring a 3.2 kb SacI fragment. Nucleotide sequencing analysis revealed a 615 bp open reading frame (ORF), showing moderate homology (identity, 31–34%; similarity, 45–47%) with the γ-butyrolactone autoregulator receptors from *Streptomyces* sp., and this ORF was named *seaR* (*Saccharopolyspora erythraea* autoregulator receptor). The *seaR*/pET-3d plasmid was constructed to overexpress the recombinant SeaR protein (rSeaR) in *Escherichia coli*, and the rSeaR protein was purified to homogeneity by DEAE-Sephacel column chromatography, followed by DEAE-ion-exchange HPLC. The molecular mass of the purified rSeaR protein was 52 kDa by HPLC gel-filtration chromatography and 27 kDa by SDS-polyacrylamide gel electrophoresis, indicating that the rSeaR protein is present as a dimer. A binding assay with tritium-labeled autoregulators revealed that rSeaR has clear binding activity with a VB-C-type autoregulator as the most effective ligand, demonstrating for the first time that the erythromycin producer *S. erythraea* possesses a gene for the γ-butyrolactone autoregulator receptor.

**Key words:** *Saccharopolyspora erythraea*, autoregulator, purification, receptor

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Streptomycetes are Gram-positive bacteria characterized by their versatile ability to produce many different kinds of secondary metabolites, in addition to their morphological complexity. One of their unique properties is the presence of low-molecular-weight compounds called "γ-butyrolactone autoregulators" [10, 17, 30], which in general switch on several phenotypes, such as antibiotic production and/or aerial mycelium formation [3, 4, 6–9, 13, 26, 36–38], and they are regarded as a *Streptomyces* hormone. Although

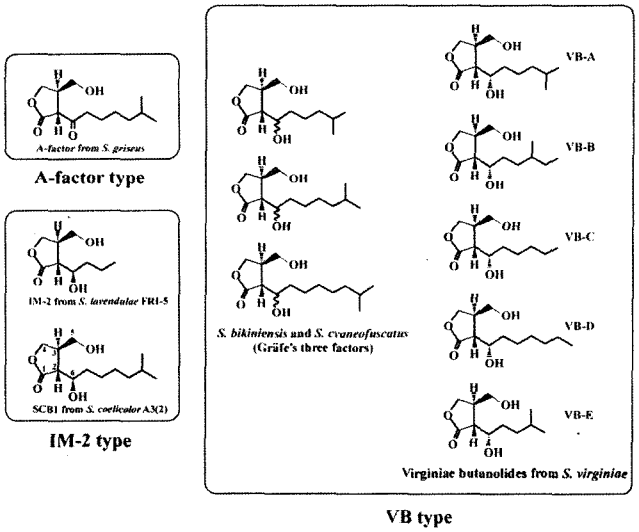


**Fig. 1.** Structures of butyrolactone autoregulators isolated from *Streptomyces* species.
Absolute configurations of A-factor [18], VBs [15, 23], and IM-2 [17] have been assigned as (3R), (2R, 3R, 6S), and (2R, 3R, 6R), respectively. Although the absolute configurations of three factors from *S. bikiniensis* and *S. cyaneofuscatus* have not yet been determined, the most probable forms based on spectroscopic data [3, 4] are depicted.

these autoregulators share a characteristic 2,3-disubstituted-γ-butyrolactone skeleton, 11 butyrolactone autoregulators chemically identified to date can be classified into three types, based on minor structural differences in the C-2 side chain (Fig. 1): (i) the virginiae butanolide (VB) type, exemplified by VB-A to VB-E of *Streptomyces virginiae*, which contains a 6-α-hydroxy group [18, 27, 36]; (ii) the IM-2 type, exemplified by IM-2 of *Streptomyces lavendulae* FRI-5 and SCB1 of *Streptomyces coelicolor* A3(2), which contains a 6-β-hydroxy group [20, 30, 33]; and (iii) the A-factor type, which contains a 6-keto group [10, 19, 21]. Evidently, autoregulators possessing a 6-hydroxy group constitute the majority of autoregulators. As the mediator of the autoregulator's signal inside the cell, a receptor protein highly specific to VB was purified from *Streptomyces virginiae* [14, 15, 22]. Since then, a receptor protein that was similar but specific to A-factor was isolated from *S. griseus* [23], and other similar receptor proteins specific for IM-2 and SCB1 were isolated from *S. lavendulae* FRI-5 [16, 25, 35] and *S. coelicolor* A3(2) [34], respectively, demonstrating that the receptor-mediated signal transduction is common to *Streptomyces* species that possess the γ-butyrolactone autoregulators.

*Saccharopolyspora erythraea* has been used industrially for the production of erythromycin. In recent years, 6-deoxyerythronolide B synthases (DEBS), a multifunctional polyketide synthase (PKS) responsible for biosynthesis of the erythromycin aglycon in *S. erythraea*, has served as a model for genetically manipulating the biosynthesis to generate a diverse array of novel macrolide antibiotics [2]. During our search for autoregulator receptors from different *Streptomyces* species, we have identified a putative receptor gene (*seaR*) from *S. erythraea*. Through the overexpression of the *seaR* gene in *Escherichia coli* and purification of the recombinant SeaR protein (rSeaR), we confirmed that rSeaR is the autoregulator receptor, and demonstrated that autoregulator-mediated signaling cascades may operate in *S. erythraea*. In the present study, the relation of *SeaR* to the regulatory mechanism of erythromycin production was clarified by gene disruption of *SeaR* and phenotypic analyses.

## MATERIALS AND METHODS

### Chemicals

All chemicals were of reagent or high-performance liquid chromatography (HPLC) grade and were purchased from Nacali Tesque, Inc. (Osaka, Japan), Takara Bio, Inc. (Osaka, Japan), and Wako Pure Chemical Industrial, Ltd. (Osaka, Japan). Marker proteins for gel filtration HPLC and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Oriental Yeast (Osaka, Japan) and Amersham Biosciences (Osaka, Japan), respectively.

### Bacteria and Plasmids

*Saccharopolyspora erythraea* IFO 13426 was grown at 28°C on ISP no. 2 medium (Difco, Detroit, MI, U.S.A.) for spore formation, and in tryptic soy broth (TSB; Oxoid, Hampshire, U.K.) for preparation of total DNA. For genetic manipulation, *Escherichia coli* DH5α [5] was used. For expression of cloned gene, *E. coli* BL21 (DE3)/pLysS [31] was used as a host. The vector pUC19 was used for genetic manipulation and cloning. The vector pET-3d [32] was used for expression of cloned gene. DNA manipulations in *E. coli* and *Saccharopolyspora* sp. were performed as described by Sambrook and Russell [28] and Kieser *et al.* [11], respectively.

### Southern Blot Hybridization and Molecular Cloning of *seaR*

In order to clone a gene encoding a γ-butyrolactone autoregulator receptor protein from *S. erythraea*, the AF-V primer (5'-CGCGGATCC-GCS-GCS-GCS-NNN-GTS-TTC-GA-3') and AR-1 primer (5'-CGCGGATCC-GAA-GTG-GAA-GTA-SAG-SGC-SCC-3') were used for PCR to amplify an internal segment of the putative receptor gene from the total DNA of *S. erythraea* (the underlined nucleotides were added to introduce a BamHI site for cloning). The PCR products were analyzed by 2% (w/v) agarose gel electrophoresis and DNA sequencing, and were used as a probe to clone a gene encoding the γ-butyrolactone autoregulator receptor protein from *S. erythraea*.

Total DNA was prepared by the method of Rao *et al.* [24]. Five μg of the genomic DNA digested with SacI was resolved by 1% agarose gel and was blotted onto a nylon membrane (Hybond-N; Amersham Biosciences, Piscataway, NJ, U.S.A.). The membrane was hybridized with a PCR fragment of *S. erythraea*, which was $^{32}$P-labeled with [α-$^{32}$P] dCTP (3,000 Ci/mM; ICN Biomedical, Tokyo, Japan) and a random primer labeling kit (Takara Bio, Tokyo, Japan). Hybridization was performed in rapid-hybrid buffer (Amersham Biosciences, Piscataway, NJ, U.S.A.) at 65°C for 4 h, 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, 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-U; Fuji Photo Film, Tokyo, Japan, −80°C, 4 h). A partial genomic library of *S. erythraea* IFO 13426 was constructed by cloning the size-fractionated SacI fragments (ca. 3.2 kb) of the total DNA into pUC19, using *E. coli* DH5α as a host. The library was screened to select clones containing a gene encoding γ-butyrolactone autoregulator receptor protein by colony hybridization with the $^{32}$P-labeled PCR fragment prepared as described above as a probe.

**DNA Sequencing and Sequence Analysis**

The nucleotide sequence was determined by the dideoxy-chain termination method [29] for both strands, using double-stranded templates of pUC19 clones with a thermo sequenase cycle sequencing kit (Amersham Biosciences, Piscataway, NJ, U.S.A.) and Cy5-labeled primers on a fluorescence DNA sequencer (ALF DNA sequencer; Pharmacia Biotech., Tokyo, Japan). The nucleotide data and deduced amino acid sequence were analyzed with GENETYX-WIN (ver. 2) software (Software Development, Tokyo, Japan). Homology searches in GenBank, SwissProt, and EMBL were carried out using BLAST and FASTA.

**Construction of pET-*seaR* and Preparation of Recombinant SeaR (rSeaR)**

A SacI (3.2 kb) fragment carrying *seaR*, which encodes the putative γ-butyrolactone autoregulator receptor protein from *S. erythraea* (Fig. 3), was used as a template in the PCR to amplify the coding region of *seaR*. The PCR was performed with primer A (5'-ACGCGCCATGGATGCC-GCAGCAGCGTCG-3') and primer B (5'-ATCGGATCCT-GAAAGCAGGCCTCGGCG-3') to generate an NcoI site and a BamHI site at the 5' and 3' ends of the seaR coding sequence, respectively (underlined). After partial digestion of *seaR*, a 615 bp NcoI-BamHI fragment was recovered and cloned into NcoI- and BamHI-digested pET-3d, resulting in pET-*seaR*. The nucleotide sequence around the NcoI and BamHI junction was confirmed by DNA sequencing. For preparing rSeaR, *E. coli* BL21 (DE3)/pLysS harboring pET-*seaR* was grown overnight at 37°C in LB medium containing 25 μg of ampicillin per ml and 25 μg of chloramphenicol per ml. Two-hundred milliliters of fresh medium in a 500-ml Sakaguchi flask was used for inoculating 2 ml of the preculture and cultivated at 37°C for 2 h until the $OD_{600}$ reached 0.4. Isopropyl-β-D-thiogalactopyranoside (IPTG; final concentration of 0.1 mM) was added, and the mixture was incubated for 3 h at 37°C. The 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 7.0) containing 20% glycerol, 0.5 mM dithiothreitol (DTT), and 0.1 mM *p*-amidinophenyl methanesulfonyl fluoride hydrochloride (pAPMSF)] plus 0.1 M KCl and disrupted by sonication. The supernatant was prepared as crude cell-free extract by centrifugation at 4°C and used for SDS-PAGE analysis and binding activity assay. For purification of rSeaR, the crude cell-free extract was applied onto a DEAE-Sephacel (3.3×43 cm; Amersham Biosciences, Piscataway, NJ, U.S.A.) column preequilibrated with buffer A. After washing with buffer A, bound proteins were eluted with a linear gradient of 0.1 to 0.3 M KCl in buffer A. Fractions containing rSeaR were combined and concentrated by ultrafiltration (UHP-62K; Advantec, Tokyo, Japan). The concentrated fractions were applied onto a DEAE-HPLC column (TSK gel DEAE-5PW, 0.75×7.5 cm; Tosho, Tokyo, Japan) preequilibrated with buffer A containing 0.1 M KCl. After being washed with the same buffer, bound proteins were eluted with a linear gradient of KCl formed from 0.1 to 0.6 M (10 mM/min) in buffer A, and fractions showing a single band on SDS-PAGE, which was eluted at around 0.3 M KCl, were stored at −80°C until use. The protein concentration was determined by a dye binding assay (Bio-Rad protein assay kit; Bio-Rad, Hercules, CA, U.S.A.), using bovine serum albumin as a standard.

**Assay of Autoregulator-Binding Activity and Protein**

Autoregulator-binding activity was assayed in the presence of one of the three tritium-labeled autoregulators {73 nM of [$^3$H]VB-$C_7$ (54.6 Ci/mM), 100 nM of [$^3$H]IM-2-$C_5$ (40 Ci/mM), and 86 nM of [$^3$H]SCB1 (46.3 Ci/mM), respectively}. The autoregulator-binding assay was performed as described by Kim *et al.* [14]. The activity was measured as the specific binding of each [$^3$H]-ligand, calculated from the difference of protein-bound [$^3$H]-ligand in the absence and presence of nonlabeled autoregulators [0.125 mM of VB-C (1,710-fold excess against the $^3$H-derivative), IM-2 (1,250-fold excess), and SCB1 (1,450-fold excess), respectively]. The protein-ligand complex was precipitated by the addition of saturated ammonium sulfate in 0.05 M triethanolamine-HCl containing 0.5 M KCl to remove unbound [$^3$H]-ligand, and recollected by centrifugation at 10,000 ×*g* for 5 min. The precipitate was dissolved in 100 μl of distilled water. The radioactivity in the solution was measured with a liquid scintillation counter (LS 6000; Beckman, CA, U.S.A.).

**Determination of Molecular Weight**

SDS-PAGE was performed with a precast 14% gel (Daiichi Pure Chemical, Japan) by using a minigel apparatus (Daiichi Pure Chemical) and stained with Coomassie Brilliant Blue G-250. The molecular weight of purified rSeaR under nondenaturing conditions was estimated as described elsewhere [25] by gel filtration HPLC (TSK-G3000$SW_{XL}$, $M_r$ 500,000; Tosoh, Japan) with buffer B (0.1 M potassium phosphate, pH 7.0) containing 0.2 M NaCl and 5 mM DTT.

**Nucleotide Sequence Accession Number**

The nucleotide sequence reported in this paper has been deposited to the DDBJ databank under accession no. AB188088.

## RESULTS AND DISCUSSION

**Cloning of the *seaR* Gene**

In the genus *Streptomyces*, there are three types of γ-butyrolactone autoregulator receptors, corresponding to three types of autoregulators: (i) BarA, as a VB-type receptor from *S. virginiae* [14, 15, 22]; (ii) FarA and ScbR, as IM-2-type receptors from *S. lavendulae* FRI-5 and

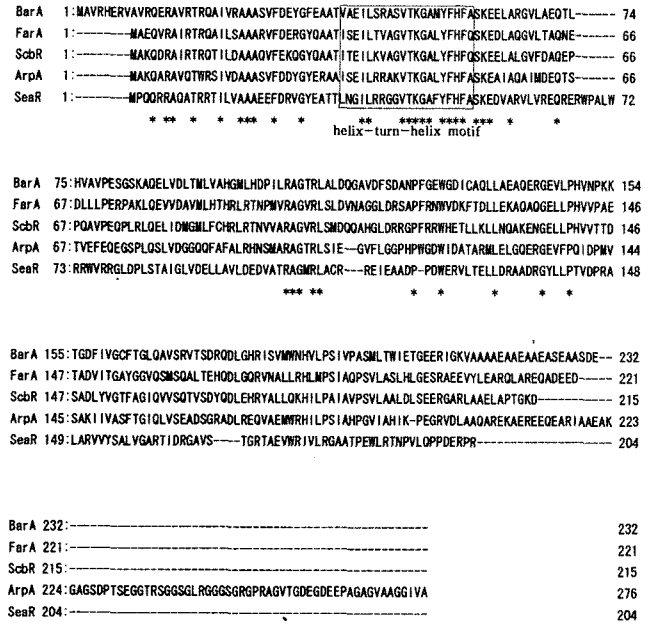


**Fig. 2.** Multiple alignment of overall amino acid sequences of BarA (*S. virginiae*), FarA (*S. lavendulae*), ScbR (*S. coelicolor* A3(2)), and ArpA (*S. griseus*), encoding VB receptor, IM-2 receptor, SCB1 receptor, and A-factor receptor, respectively, together with SeaR from *S. erythraea.*

*S. coelicolor* A3(2), respectively [16, 25, 34, 35]; and (iii) ArpA, as an A-factor-type receptor from *S. griseus* [23]. Alignment of the amino acid sequences of the above four receptors revealed two highly conserved regions (corresponding to amino acid residues 25 to 30 and 52 to 58 of BarA) (Fig. 2). Based on these conserved sequences, and codon usage bias derived from 64 *Streptomyces* genes [39], degenerate oligonucleotide primers were designed for use in PCR and applied to *S. erythraea.* PCR was performed using *S. erythraea* DNA as a template, and a 120 bp PCR product was cloned in the pUC19 to yield pHS5284. Sequencing and database analyses revealed its similarity to the segment of genes encoding γ-butyrolactone autoregulator receptors of known function (data not shown). Subsequently, the insert of pHS5284 was used as a probe of Southern and colony hybridization with total DNA of *S. erythraea* to obtain the entire region of the plausible autoregulator receptor. A 3.2 kb SacI fragment was selected as a positive signal, and a positive clone harboring the fragment was screened by colony hybridization, resulting in plasmid pESG harboring a 3.2 kb SacI fragment. The restriction and genetic map of the insert is shown in Fig. 3.

## Sequence of the *seaR* Gene

The 3.2 kb SacI fragment was sequenced on both strands by the dideoxy chain termination method to identify a gene encoding the autoregulator receptor protein. Frame analysis [1] of the determined nucleotide sequence revealed a

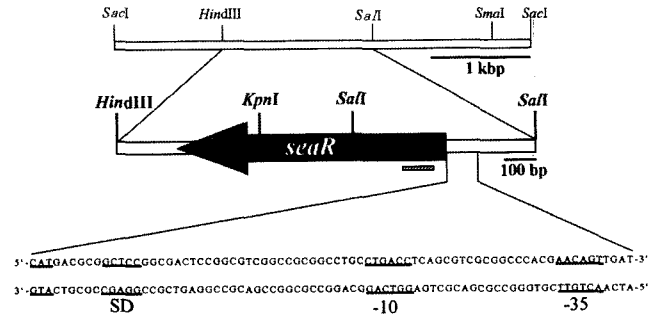


**Fig. 3.** Restriction endonuclease maps of the 3.2 kb SacI fragment containing the *seaR* gene.
Solid arrow, ORF of *seaR*; shaded box, amplified fragment used as a probe in Southern and colony hybridization.

615 bp ORF. A probable Shine-Dalgarno sequence, CGAGG, is present 6 nucleotides upstream of the putative ATG initiation codon, and the putative −10 (GACTGG) and −35 sequences (TTGTCA) were predicted to be present at 39 bp and 63 bp, respectively, upstream of the initiation codon. From the nucleotide sequence, the ORF was deduced to encode a 203 amino acid protein of 23 kDa, which shows significant homology to FarA (34% identity and 47% similarity), ScbR (32% identity and 47% similarity), BarA (32% identity and 45% similarity), and ArpA (31% identity and 46% similarity). Furthermore, multiple alignment of the deduced ORF product with autoregulator receptor proteins revealed that the most significant identity exists at an amino-terminal region containing a helix-turn-helix DNA-binding motif (Fig. 2). Thus, the ORF can be assumed to encode a DNA-binding regulator from *S. erythraea* and was designated *seaR* (*S. erythraea* autoregulator receptor).

## Expression of Recombinant SeaR in *E. coli* and its Characterization

In order to analyze the function of the *seaR* product in more detail, we expressed *seaR* in *E. coli* by means of the T7 expression vector pET-3d. The coding region of *seaR* was amplified by PCR and placed under the control of the T7 RNA polymerase promoter, as described in Materials and Methods. SDS-PAGE analysis indicated that isopropylthiogalactopyranoside (IPTG)-induced *E. coli* BL21 (DE3)/pLysS harboring pET-*seaR* significantly overproduced a 27 kDa protein (Fig. 4, lane 2). To investigate whether the recombinant SeaR protein (rSeaR) possessed any autoregulator-binding activity, autoregulator-binding assays were carried out against three types of ligands, namely, VB, IM-2, and SCB1. Only the crude cell-free extract prepared from IPTG-induced cells harboring pET-*seaR* showed binding activity against all three ligands (0.051, 0.031, and 0.023 pM binding/mg protein for VB, SCB1, and IM-2 binding activity, respectively), whereas cell extracts from the control cells harboring pET-3d showed

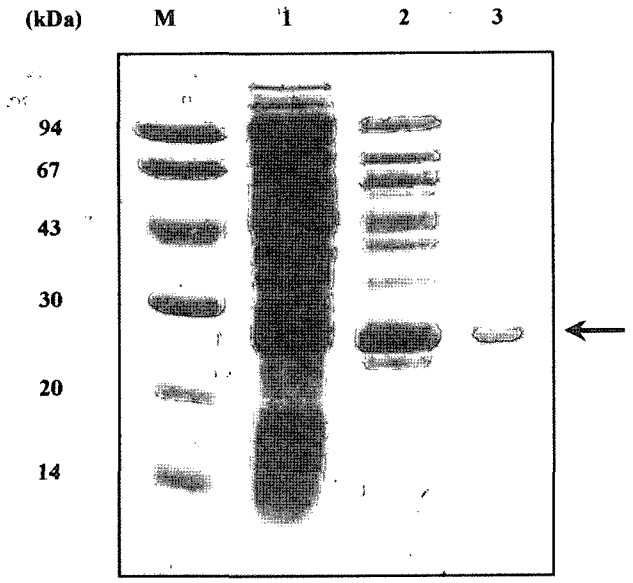


**Fig. 4.** SDS-PAGE analysis of rSeaR expressed in *E. coli* and purified.

Each sample was subjected to 14% SDS-PAGE, and the gel was stained with Coomassie brilliant blue G-250. Lane M, molecular mass markers; lane 1, crude cell-free extract from *E. coli* BL21/pLysS harboring pET-*seaR* without induction by IPTG; lane 2, crude cell-free extract from IPTG-induced *E. coli* BL21/pLysS harboring pET-*seaR*; lane 3, purified rSeaR after DEAE-5PW HPLC. Arrow indicates the position of purified rSeaR.

no activity, suggesting that SeaR functions as an autoregulator receptor in *S. erythraea* (Table 1). The overexpressed rSeaR was purified to homogeneity by DEAE-5PW HPLC (Fig. 4, lane 3), with an apparent molecular weight of 27,000 daltons on SDS-PAGE. Under native molecular sieve HPLC, purified rSeaR was eluted at the region of 52 kDa, indicating that rSeaR is present as a dimer under native conditions. To determine whether rSeaR is responsible for the autoregulator-binding activity in the crude cell-free extracts, autoregulator-binding assays were carried out with the purified rSeaR. As shown in Table 1, the purified rSeaR was found to exhibit high binding activity toward γ-butyrolactone autoregulators, especially toward [$^3$H]VB-$C_7$ and [$^3$H]SCB1, whereas [$^3$H]IM-2 was a poor ligand. Both [$^3$H]VB-$C_7$ and [$^3$H]SCB1 have a C2-side-chain length of 7 carbons, whereas [$^3$H]IM-2 has a shorter side-chain length of 5 carbons, suggesting that rSeaR should recognize the longer side-chain length of 7 carbons. Based on the fact that the binding of [$^3$H]VB-$C_7$ was 2.4-fold higher than that of [$^3$H]SCB1, rSeaR appears to prefer VB-type stereoconfiguraton at C-6 (viz., 6-α orientation of the hydroxyl group) suggesting that SeaR is a receptor for VB-type γ-butyrolactone autoregulators.

So far, more than 10 butyrolactone autoregulators have been isolated from *Streptomyces* species and comprise a family of *Streptomyces* hormones that regulate secondary metabolism and/or cytodifferentiation [12]. Since corresponding receptors for γ-butyrolactone autoregulators have been identified from *Streptomyces* species and the receptors function as key mediators on the signaling cascade, the pair of a butyrolactone autoregulator and the corresponding receptor should be regarded as an essential regulatory element in *Streptomyces* species. In this study, a gene *seaR* encoding a VB-type autoregulator receptor from the erythromycin producer *Sacharopolyspora erythraea* was cloned and characterized. Successful cloning and analysis of SeaR will facilitate the clarification of its own regulatory roles on the production of secondary metabolites.

## Acknowledgments

This work (R12-2001-044-02001-0) was supported by the Ministry of Science & Technology (MOST) and the Korea Science and Engineering Foundation (KOSEF) through the Center for Traditional Microorganism Resources (TMR) at Keimyung University.

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**Table 1.** Autoregulator-binding activity of the purified rSeaR with tritium-labeled autoregulator analogues.

| | Specific binding activity (pM of protein-bound tritium ligand/mg protein) | | |
|---|---|---|---|
| | [$^3$H]VB-$C_7$ | [$^3$H]SCB1 | [$^3$H]IM-2-$C_5$ |
| pET-3d/*E. coli* BL21 (DE3)/pLysS | 0 | 0 | 0 |
| pET-*seaR*/*E. coli* BL21 (DE3)/pLysS | 0.051 | 0.023 | 0.031 |
| Purified rSeaR | 0.116 | 0.047 | 0.06 |

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