Identification of the *varR* Gene as a Transcriptional Regulator of Virginiamycin S Resistance in *Streptomyces virginiae*

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A gene designated *varR* (for *virginiae* antibiotic resistance regulator) was identified in *Streptomyces virginiae* 89 bp downstream of a *varS* gene encoding a virginiamycin S (VS)-specific transporter. The deduced *varR* product showed high homology to repressors of the TetR family with a conserved helix-turn-helix DNA binding motif. Purified recombinant VarR protein was present as a dimer in vitro and showed clear DNA binding activity toward the *varS* promoter region. This binding was abolished by the presence of VS, suggesting that VarR regulates transcription of *varS* in a VS-dependent manner. Northern blot analysis revealed that *varR* was cotranscribed with upstream *varS* as a 2.4-kb transcript and that VS acted as an inducer of bicistronic transcription. Deletion analysis of the *varS* promoter region clarified two adjacent VarR binding sites in the *varS* promoter.

Streptomyces species are gram-positive filamentous bacteria that are well-known for producing a vast array of bioactive compounds, including more than 70% of the commercially important antibiotics. The production of antibiotics by these organisms is regulated by a variety of physiological and nutritional conditions, often coordinated with processes of morphological differentiation, such as formation of aerial mycelia and spores. A detailed knowledge of the signal cascade and the genetic components involved in antibiotic production will enable the construction of strains that can overproduce these commercially important compounds.

Previous studies have shown that antibiotic production and/or morphological differentiation is controlled in some *Streptomyces* species by low-molecular-weight compounds called γ -butyrolactone autoregulators (28, 34). Because the γ -butyrolactone autoregulators are effective at an extremely low concentration and their signal is transmitted into cells by binding to specific cytoplasmic receptor proteins, the autoregulators are regarded as *Streptomyces* hormones. To date, 10 γ -butyrolactone autoregulators have been isolated and have had their structures chemically identified (33). Among the γ -butyrolactone autoregulators and receptor proteins identified so far, *virginiae* butanolides (VBs) of *Streptomyces virginiae* (18, 26, 32) and the VB receptor protein (BarA) (16, 23) are two of the most frequently studied, and the VB-BarA system has been confirmed to regulate the coordinate production of two structurally different compounds, virginiamycin M₁ (VM₁) and virginiamycin S (VS), a pair of antibiotics that show strong synergistic bactericidal activity (22).

Previous in vitro (16) and in vivo analyses (16, 22) have demonstrated that the VB receptor BarA is a DNA binding

transcriptional repressor. In the absence of VB, BarA binds to the specific DNA sequences in the promoter region of a target gene(s) and/or operon(s), which represses the transcription. When VB is produced, binding of VB to DNA-bound BarA results in the dissociation of BarA from the promoter region, which initiates the transcription of the target gene and/or operon. In our previous study, a target operon designated *barB-varS* was located immediately downstream of the *barA* gene, and the *varS* gene was shown to encode a VS-specific efflux protein taking part in virginiamycin resistance (20). In the course of this earlier study, it was noticed that *varS* was also transcribed with the downstream region, and the transcript was increased by the presence of VS, suggesting the presence of complex regulation on virginiamycin resistance.

In this study, to clarify the overall regulation mechanism governing virginiamycin resistance, the *varS* downstream region was sequenced and an open reading frame (ORF) (*varR*) encoding a TetR-type repressor was identified. A transcriptional analysis of *varR*, as well as an in vitro gel-shift analysis using the recombinant VarR protein, demonstrated that VarR is a DNA binding regulator which mediates the VS-dependent transcription of the *varS* gene.

MATERIALS AND METHODS

Strains, growth 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, 32). pUC18 and pUC19 were used for genetic manipulation in *Escherichia coli*, and pUC19 was used for DNA sequencing. For the genetic manipulation in *E. coli*, strain DH5 α (10) was used. DNA manipulations in *E. coli* were performed as described by Sambrook et al. (27).

Chemicals. All chemicals were of reagent or high-performance liquid chromatography (HPLC) grade and were purchased from either Nacalai Tesque (Osaka, Japan), Takara Shuzo (Shiga, Japan), or Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The RNA ladder was obtained from GIBCO BRL (Gaithersburg, Md.). [α -³²P]dCTP was purchased from ICN Biomedicals Inc. (Costa Mesa, Calif.). VM₁ and VS were purified by a previously described method (19).

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FIG. 1. Gene organization in the 6.8-kb *Bam*HI-*Pst*I region containing *varS* in *S. virginiae*. Probes used for the Northern blot analysis of *barB* (*Stu*I-*Bam*HI fragment) and *varR* (*Nor*I-*Sph*I fragment) are indicated by solid boxes. Intergenic regions between *barB* and *varS* (*Eco*52I-*Bsa*I fragment; SVK14.2 in Fig. 5A) and between *varS* and *varR* (*Nae*I-*Bss*HII fragment; SVK13.1 in Fig. 5A) used for the gel-shift assay are indicated by shaded boxes. These fragments were cloned in pUC19 and were FITC labeled as described in Materials and Methods.

DNA sequencing and sequence analysis. The nucleotide sequence was determined for both strands using an ALF red DNA sequencer (Amersham Pharmacia Biotech, Tokyo, Japan). Sequencing reactions were carried out with a Thermo sequencing kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Homology searches were carried out using the programs BLAST (1) and FASTA (25).

RNA preparation and Northern blot analysis. Total RNA was isolated by the method of Kirby et al. (17), with modifications by Hopwood et al. (13), and was quantified by absorbance at 260 nm. RNA (10 µg) was loaded on each lane, electrophoresed on a 1.2% agarose gel, and transferred to Hybond-N+ (Amersham Pharmacia Biotech) according to the manufacturer's recommendations. Hybridization was carried out at 65°C for 1 h in Rapid-hyb buffer (Amersham Pharmacia Biotech) followed by washing of the blot three times at 50°C for 10 min with 2× SSC (1× SSC contains 0.015 M sodium citrate and 0.15 M NaCl [pH 7.7]) containing 0.1% sodium dodecyl sulfate (SDS). The *Nor*I-*Sph*I and *Stu*I-*Bam*HI fragments were used as specific probes against *varR* and *barB*, respectively (Fig. 1). The DNA fragments were labeled with [α -³²P]dCTP by using the random primer DNA labeling kit (version 2) (Takara Shuzo Co.).

Overexpression of *varR* and purification of recombinant VarR protein (rVarR). *varR* was amplified by PCR using oligonucleotides 5'-ATACATATGT

GGCCGCCAAGCGCGC-3' and 5'-TATGGATCCCAGTCACACATGCCGG C-3' containing artificial *Nde*I and *Bam*HI recognition sites, respectively. The amplified product was digested with *Nde*I and *Bam*HI and was ligated into *Nde*I-*Bam*HI-digested pET32a(+) (Novagen, Madison, Wis.), resulting in six surplus His codons at the 3' end of *varR*. This plasmid (pVN303) was introduced into *E. coli* BL21(DE3)/pLysS, and the transformed cells were cultured in Luria broth containing ampicillin (25 µg/ml) and chloramphenicol (25 µg/ml). When growth reached an absorbance of 0.6 at 600 nm, isopropyl- β -D-thiogalactopyranoside (IPTG) (final concentration of 1 mM) was added to the culture, followed by a further 2 h of cultivation. Cells were harvested by centrifugation at 7,000 × g for 15 min at 4°C, washed with 0.05 M triethanolamine-HCl (pH 7), and resuspended in buffer A [0.05 M triethanolamine-HCl (pH 7) containing 0.2 M KCl, 20% (vol/vol) glycerol, 5 mM dithiothreitol (DTT), and 0.5 mM *p*-amidino-phenylmethanesulfonyl fluoride]. The cells were disrupted by sonication for 2 min at 25% duty cycle (Branson Sonifier 250) in an ice bath, followed by centrifugation at 10,000 × g for 15 min at 4°C to obtain a supernatant as crude lysate.

The crude lysate was applied to a DEAE-Sephacel column, and the adsorbed proteins were eluted with a linear gradient of KCl concentrations from 0.1 to 0.5 M in 50 mM triethanolamine-HCl containing 20% (vol/vol) glycerol (pH 7). Fractions containing rVarR were mixed and loaded on a Ni-NTA column (Qia-gen, Tokyo, Japan). Unbound proteins were washed out with buffer A (minus DTT) containing 0.1 M imidazole, and rVarR was eluted with buffer A (minus DTT) containing 10 mM imidazole.

Preparation of crude lysate 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 for 14 h on a reciprocating shaker (120 strokes per min), cells were harvested by centrifugation (3,000 × g, 10 min, 4°C). The cells were suspended in buffer A and were disrupted by sonication as described above. After centrifugation, the supernatant was stored at -20°C and was used as the source of native VarR of *S. virginiae*.

Gel-shift assay. The binding mixture consisted of a protein sample (100 µg of crude lysate or 20 µg of purified rVarR) and 0.5 nM fluorescein isothiocyanate (FITC)-labeled fragment in 1× binding buffer [50 mM triethanolamine-HCl (pH 7.0) containing 0.2 M KCl, 10% (vol/vol) glycerol, and 1.5 µg of poly(dI-dC) · poly(dI-dC)] in a total volume of 20 µl. After incubation at 25°C for 10 min, the reaction mixture was separated at 4°C by electrophoresis on a high-ionic-strength gel containing 5% acrylamide and 0.167% *N*, *N'*-methylenebisacrylamide with a running buffer of 50 mM Tris-HCl (pH 8.5) containing 380 mM glycine and 2 mM EDTA. The DNA fragments in the gel were detected by a fluorometry scanner (FMBIO; Hitachi). The FITC labeling was performed by PCR on fragments subcloned in pUC19 as templates, using a FITC-labeled forward primer (5' FITC-CGCCAGGGTTTCCAGTCACGAC-3') and a reverse primer (5' FITC-TTTCACACAGGAACAGCTATGAC-3').

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 AB046994.

FIG. 2. Multiple alignment of VarR and several repressors of the TetR family, including a repressor of rifamycin resistance from *A. mediterranei* (RifQ) (2, 14), a TetR homolog from *S. coelicolor* (8), a transcriptional regulator of *Deinococcus radiodurans* (Deinococcus) (31), a class D tetracycline repressor of *E. coli* (TetR class D) (30), TetR of *Salmonella enterica* serovar Typhimurium DT104 (Salmonella) (5), and TetR of *Pseudomonas* sp. (Pseudomonas) (29). Identical residues are indicated by white letters in black boxes.

FIG. 3. Northern blot hybridization analysis of *varR* transcription. (A) *varR* transcription during cultivation of *S. virginiae*. Total RNA was extracted from cells cultivated for the indicated period at 28°C. Under the experimental conditions, the production of VB and virginiamycin started at 10 and 14 h of cultivation, respectively, as shown by arrows above the lanes. (B) The effect of virginiamycin addition on the transcription of *varR*. VM₁ (10 µg/ml) or VS (10 µg/ml) was added to the culture at 8 h of cultivation. The term noninduced indicates that virginiamycin was not added. (C and D) Effect of VB addition on the transcription of *barB* (C) and *varR* (D). RNA was prepared from cells with VB (VB-C₆, 300 nM) added at 8 h of cultivation. A *varR*-specific probe (*NotI*-*SphI* fragment, shown in Fig. 1) was used in panels A, B, and D to detect the 2.4-kb *varS*-*varR* transcript, and a *barB*-specific probe (*StuI*-*Bam*HI fragment, shown in Fig. 1) was used in panel C to detect the 2.5-kb *barB*-*varS* transcript.

RESULTS AND DISCUSSION

Sequence of the *varS* downstream region and identification of the *varR* gene. To search for regulatory genes responsible for the VS-dependent increase in the *varS* transcript, the 0.9-kb region downstream of *varS* was sequenced. FRAME analysis (3) of the region identified a 744-bp ORF starting 89 bp downstream of the *varS* stop codon in the same direction as *varS* (Fig. 1). The ORF product (247 amino acids, $M_r = 27,296$) showed significant homology with *Amycolatopsis mediterranei* RifQ (63% identity, 78% similarity), a transcriptional repressor for the rifamycin efflux protein RifP (2). Moderate homology of 33 to 38% identity was also observed with several transcriptional repressors, such as a class D tetracycline repressor of *E. coli* (TetR) or a TetR homolog of *Streptomyces coelicolor*. Multiple alignment of the deduced ORF product with the member repressors of the TetR family revealed that the most significant identity exists at an amino-terminal region contain-

FIG. 4. (A) Overexpression and purification of rVarR. A sample from each purification step was separated on an SDS-10% polyacrylamide gel and was stained with Coomassie brilliant blue R-250 (27). Lane 1, molecular mass protein standard; lane 2, crude lysate (10 µg) of *E. coli* BL21(DE3)/pLysS containing pET32a as a negative control; lane 3, crude lysate (10 µg) of *E. coli* BL21(DE3)/pLysS containing pVN303; lane 4, eluate (7 µg) from DEAE-Sephacel chromatography; lane 5, eluate (7 µg) from Ni-NTA affinity chromatography. The arrow indicates the position of rVarR. (B) The elution profile of the purified rVarR from the molecular-sieve HPLC on a TSK gel G3000SW_{XL} (TOSO) column. MW indicates the elution profiles of the standard proteins having the molecular masses shown above the figure. OD₂₈₀, optical density at 280 nm.

ing a helix-turn-helix DNA binding motif (Fig. 2). The presence of the helix-turn-helix structure was also supported by the high score (SD score of 5.18) for the prediction of the motif (7). Thus, the ORF can be assumed to encode a DNA binding regulator and was designated *varR* (for *virginiae* antibiotic resistance regulator) due to its regulatory function on *varS* transcription (described in detail below).

Transcriptional analysis of the *varR* gene. To deduce the in vivo function of *varR*, the time course of the transcription was investigated by Northern blot analysis using the *varR*-coding region as a probe against RNA samples from 8- to 16-h cultures of *S. virginiae* (Fig. 3A). Although the signals were faint, *varR* transcript was detected as a 2.4-kb band in the 10- and 12-h samples, and the signal intensity reached a maximum at 14 h of cultivation, which agreed well with the onset of virginiamycin production in *S. virginiae* (16). Because the size of the transcript (2.4 kb) was far larger than that of *varR* alone (0.7 kb), and because the upstream *varS*-specific probe hybridized to the same band (20) while the probe covering the downstream region did not (data not shown), we concluded that *varR* formed an operon with the upstream *varS* gene. This conclusion agreed well with the lack of typical promoter sequences in the 5' untranslated region of *varR* and the presence of an inverted repeat in the 3' region of *varR* ($\Delta G = -9.1$ kcal) as a plausible transcriptional terminator.

Our previous analysis of the *barB*-*varS* operon (20) and the present data on the *varS*-*varR* operon indicated that the *varS*

FIG. 5. Gel-shift analyses on the DNA binding activity of VarR. (A) DNA binding activity of purified rVarR to the FITC-labeled SVK14.2 (lanes 2 through 5), to SVK13.1 (lanes 9 and 10), and to the coding region of the gene *rplk* (24), which encodes the 50S ribosomal protein L11, as a negative control (lanes 11 and 12, designated negative). See Fig. 1 for the regions covered by SVK14.2 and SVK13.1. A nonlabeled *barB-varS* intergenic fragment (0.5 nM) was added in lane 4 as competitor DNA. Lanes 5 and 8 contained 0.5 mM VS. The activity of native VarR in the crude lysate from *S. virginiae* (VarR) to SVK14.2 was tested in lanes 6, 7, and 8. The crude protein from *S. virginiae* was obtained from a 14-h culture, at which time the transcription of *varR* reached a maximum (Fig. 3A). FITC-labeled probe was omitted in lanes 1 and 6. The lowest band and the top band in lanes 6, 7, and 8 are nonspecific fluorescence bands due to unknown contaminants in the crude lysate. (B) The effect of several antibiotics on the DNA binding activity of rVarR. VS was added at a concentration of 0.5 mM. Antibiotics other than VS were added at a concentration of 50 mM.

gene is transcribed in three different ways: as a monocistronic transcript (1.6 kb) (20), as a bicistronic *barB-varS* transcript (2.5 kb) (20), and as a bicistronic *varS-varR* transcript (2.4 kb). The presence of a perfectly matched inverted repeat sequence 10 bp downstream of the *varS* stop codon seems to dictate that the transcriptional termination is after *varS*. Although the *barB-varS* transcript and the *varS-varR* transcript are very similar in size, they can be easily distinguished by use of specific probes, such as those internal to *barB* and *varR*. Just as in a previous study, where the use of the *varS*-specific probe confirmed that the 2.4-kb bicistronic transcription was induced by the presence of VS (20), in the present study the *varR*-specific probe confirmed that the transcription of the *varS-varR* operon was induced by the addition of VS at 8 h of cultivation but not by VM₁ (a synergistic counterpart of VS in the virginiamycin mixture) (Fig. 3B).

The effect of VB on the transcription of the *varS-varR* operon was also investigated by adding VB at 8 h of cultivation (Fig. 3C and D). Just as in our previous study (20), we observed a large increase of the 2.5-kb *barB-varS* bicistronic transcript 1 h after the VB addition (Fig. 3C), which is the typical induction pattern under the direct control of the VB-BarA system. The 0.8-kb band seemed to be the degradation product from the 2.5-kb transcript by posttranscriptional processing. With

regard to the 2.4-kb *varS-varR* bicistronic transcript, however, induction was observed only 2 h after the VB addition (10 h of cultivation) (Fig. 3D), which suggests that the VB-BarA system does not directly control the *varS-varR* transcription.

Overproduction and purification of rVarR. To evaluate the actual function of VarR, we overexpressed rVarR in *E. coli*. rVarR with six surplus histidine residues at the C terminus was purified with successive chromatographies on a DEAE-Sepharose and a Ni-NTA column, and the purity was confirmed by SDS-polyacrylamide gel electrophoresis (Fig. 4A). A protein of 27 kDa was observed in the extracts of IPTG-induced cells carrying the *varR* gene in pET32a(+) (pVN303; see Materials and Methods for its construction), whereas the corresponding band was absent in the extracts of cells carrying vector pET32a(+). The molecular mass of 27 kDa agreed well with that calculated from the nucleotide sequence ($M_r = 27,296$), and N-terminal amino sequencing of the purified sample confirmed that the 27-kDa protein is rVarR (data not shown). The native molecular size of the purified rVarR was determined to be 67 kDa by molecular-sieve HPLC on a TSK gel G3000SW_{XL} (TOSO) column (Fig. 4B). Therefore, we concluded that rVarR existed as a dimer consisting of two 27-kDa subunits, similar to the cases of other member repressors of the TetR family (5, 12, 29, 30).

FIG. 6. Determination of VarR binding sites. (A) Fragment map of the *barB*-*varS* intergenic region used for the gel-shift assay. The *Eco*52I-*Bsa*I fragment was designated SVK14.2. The fragment for nt -39 to +2 (SVK14.5) was PCR-amplified by primers 5'-TCGGAATTCT CACTTGTACATCGTAT-3' and 5'-TTAGCATGCACTGTTCTACAACGTAT-3'. The fragment for nt -163 to -19 (SVK14.6) was amplified by primers 5'-ATAGAATTCACGTCCGGCGCGCGCC-3' and 5'-GCCGCATGCGAGTTATACGATGTACAAG-3', and the fragment for nt -19 to +23 (SVK14.7) was amplified by primers 5'-CGCGAATTCCTCATATACGTTGTAGAAC-3' and 5'-TATGCATGCGAGACCTCCCA GGAGTG-3'. The fragment for nt -163 to -40 (SVK14.8) was amplified by primers 5'-ATAGAATTCACGTCCGGCGCGCGCC-3' and 5'-ATACTGCAGACATGCGGGTGAAGCCTG-3', and the fragment for nt +3 to +23 (SVK14.9) was amplified by primers 5'-GCGGAATT CTTCCTCACTCACTCCTGG-3' and 5'-TATGCATGCGAGACCTCCAGGAGTG-3'. All fragments were cloned into *Sph*I-*Eco*RI sites of pUC19 and then were PCR-amplified with FITC-labeled primers as described in Materials and Methods. (B) Binding of purified rVarR to the fragments. FITC-labeled fragments used for the gel-shift assay are shown above the lanes. (C) Promoter sequence of *varS* containing two dyad symmetry sequences. The residues showing dyad symmetry are indicated by white letters in black boxes or by double underlining. The open triangles indicate the axis of symmetry for each dyad symmetry sequence.

In vitro functional analysis of VarR. To clarify the function of VarR, the DNA binding ability of both the purified rVarR and the natural VarR of *S. virginiae* was assessed by gel-shift assay (Fig. 5) against an FITC-labeled *Eco*52I-*Bsa*I fragment (SVK14.2) (Fig. 1). This fragment covers the intergenic region between *barB* and *varS* and corresponds to nucleotides (nt) -163 to +23 relative to the transcription start site of *varS*. rVarR showed a clear band shift (Fig. 5A, lane 3), and the specific nature of the binding was confirmed by competition with the unlabeled fragment (Fig. 5A, lane 4). An identical specific binding was observed when crude lysate of *S. virginiae* was used as a source of native VarR (Fig. 5A, lane 7), thereby confirming that the C-terminal six surplus His residues are not detrimental to the function of rVarR. When the FITC-labeled *Nae*I-*Bss*HII fragment (SVK13.1) covering the *varS*-*varR* intergenic region was used, no band shift was observed (Fig. 5A, lane 10). These results indicated that rVarR specifically binds to the 5' upstream region of *varS*, not to the 5' upstream region of *varR*. This result seems to corroborate the bicistronic nature of the *varS*-*varR* operon.

When VS was present in the reaction mixture of the gel-shift assay, the binding of VarR to the *varS* upstream region was clearly inhibited (Fig. 5A, lanes 5 and 8, and B, lane 3). This inhibition was specific by VS, as evident from the lack of signs

of inhibition by the other antibiotics tested (two peptidic antibiotics [valinomycin and gramicidin S] and three polyunsaturated macrolactone antibiotics [VM₁, erythromycin, and avermectin B₁]), even at a concentration 100 times higher than that of VS (Fig. 5B). From these results, it can be proposed that VarR plays a central role in the VS-dependent induction of the *varS*-*varR* operon. VarR seems to repress the transcription of the *varS*-*varR* operon by binding to the *varS* promoter region until the onset of VS production. When VS production starts, the repression is relieved through the dissociation of VarR from the promoter region of *varS*.

To localize more precisely the VarR binding region, a gel-shift assay was conducted with various shorter fragments from the *barB*-*varS* intergenic region (Fig. 6A). Gel-shift assays against SVK14.3 and SVK14.4 revealed that VarR bound to the right half of the region corresponding to nt -60 to +23 relative to the transcriptional start site of *varS* (Fig. 6B). Because the target sequence of a DNA binding protein often shows a dyad symmetry reflecting the symmetrical structure of the dimeric protein (21), dyad sequences were surveyed in SVK14.4, and two sequences (nt -39 to -20 and -19 to +2) were found to be plausible VarR binding sites (Fig. 6C). The region SVK14.5 containing the two dyad sequences (nt -39 to +2) showed clear binding with VarR, whereas the rest of

FIG. 7. Transcriptional regulation mechanism of the *varS-varR* operon by VB and VS.

SVK14.2 (SVK14.8 for nt +1 to +23 and SVK14.9 for nt -163 to -40) did not, indicating that the VarR binding sites should be in the region of nt -39 to +2 (Fig. 6B). Because the VarR binding sites overlapped with the predicted binding sites for RNA polymerase (both the nt -35 and -10 elements), it seems likely that the transcription of *varS* is repressed when VarR remains bound at these sites.

The two dyad sequences shared 12 consensus nucleotides (5'-NCNTNTACNTNGTANAACN-3' [boldface indicates consensus nucleotide]) (Fig. 6C), but together they can form a large dyad structure, with C at nt -19 as a center of symmetry. To assess whether or not one of the two dyad sequences was sufficient for VarR binding, each of the two dyad sequences was tested for VarR binding (SVK14.6 for nt -163 to -20 and SVK14.7 for nt -19 to +23). Both fragments showed clear retardation by VarR, indicating that there are two adjacent VarR binding sites on the *varS* promoter. It should be noted that the shift level observed for SVK14.7 was much smaller than that observed for SVK14.5, although these fragments are similar in size. Such a difference in shift level was also observed between SVK14.6 and SVK14.2. These results suggested that fragments containing the two dyad sequences (SVK14.2 and SVK14.5) are bound with a greater number of VarR molecules than fragments containing only one dyad sequence (SVK14.6 and SVK14.7), resulting in greater retardation.

Combining the data presented here with that from previous studies (16, 20), a plausible model for the transcriptional regulation of the *varS-varR* operon can be deduced as follows (Fig. 7). Before the onset of VB production—and hence before VS production—transcription of *varS-varR* is likely to be repressed by a small but sufficient amount of VarR derived from basal level transcription of *varR*. Alternatively, vegetative sigma factor might not work for transcription from the *varS* promoter. When VB production starts, VB-bound BarA dissociates from the *barB* promoter and hence induces bicistronic *barB-varS* transcription, which confers VS resistance before the onset of virginiamycin production. The weak *varS-varR* transcript detected in this cultivation period (from 10 to 12 h in Fig. 3A) seems to reflect the read-through of RNA polymerase from the *barB-varS* region into the *varS-varR* region. When the produc-

tion of virginiamycin starts, the binding of VS to VarR causes VarR to dissociate from the *varS* promoter region, thereby leading to derepression of *varS-varR* transcription.

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REFERENCES

- Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**:403–410.
- August, P. R., L. Tang, Y. J. Yoon, S. Ning, R. Muller, T. W. Yu, M. Taylor, D. Hoffmann, C. G. Kim, X. Zhang, C. R. Hutchinson, and H. G. Floss. 1998. Biosynthesis of ansamycin antibiotic rifamycin: deductions from the molecular analysis of the *rif* biosynthetic gene cluster of *Amycolatopsis mediterranei* S699. *Chem. Biol.* **5**:69–78.
- Bibb, M. J., P. R. Findlay, and M. W. Johnson. 1984. The relationship between base composition and codon usage in bacterial genes and its use for the simple and reliable identification of protein-coding sequences. *Gene* **30**:157–166.
- Bourn, W. R., and B. Babb. 1995. Computer assisted identification and classification of Streptomyces promoters. *Nucleic Acids Res.* **23**:3696–3703.
- Briggs, C. E., and P. M. Fratamico. 1999. Molecular characterization of an antibiotic resistance gene cluster of *Salmonella typhimurium* DT104. *Antimicrob. Agents Chemother.* **43**:846–849.
- Caballero, J. L., F. Malpartida, and D. A. Hopwood. 1991. Transcriptional organization and regulation of an antibiotic export complex in the producing *Streptomyces* culture. *Mol. Gen. Genet.* **228**:372–380.
- Dodd, I. B., and J. B. Egan. 1990. Improve detection of helix-turn-helix DNA-binding motif in protein sequences. *Nucleic Acids Res.* **18**:5019–5026.
- Fernandez-Moreno, M. A., J. L. Caballero, D. A. Hopwood, and F. Malpartida. 1991. The *act* cluster contains regulatory and antibiotic export genes, direct targets for translation control by the *bldA* tRNA gene of *Streptomyces coelicolor*. *Cell* **66**:769–780.
- Guilfoile, P. G., and C. R. Hutchinson. 1992. The *Streptomyces glaucescens* TcmR protein represses transcription of the divergently oriented *tcmR* and *tcmA* genes by binding to an intergenic operator region. *J. Bacteriol.* **174**:3659–3666.
- Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166**:557–580.
- Hillen, W., and C. Berens. 1994. Mechanisms underlying expression of TN10 encoded tetracycline resistance. *Annu. Rev. Microbiol.* **48**:345–369.
- Hinrichs, W., C. Kisker, M. Duvel, A. Muller, K. Tover, W. Hillen, and W. Saenger. 1994. Structure of the Tet repressor-tetracycline complex and regulation of antibiotic resistance. *Science* **264**:418–420.
- Hopwood, D. A., M. J. Bibb, K. F. Chater, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrepf. 1985. Genetic manipulation of *Streptomyces*: a laboratory manual. The John Innes Foundation, Norwich, United Kingdom.
- Kim, C. G., T. W. Yu., C. Fryhle, S. Handa, and H. G. Floss. 1998. 3-Amino-5-hydroxybenzoic acid synthase, the terminal enzyme in the formation of the precursor of mC7N units in rifamycin and related antibiotics. *J. Biol. Chem.* **273**:6030–6040.
- Kim, H. S., T. Nihira, H. Tada, M. Yanagimoto, and Y. Yamada. 1989. Identification of binding protein of virginiac butanolide C, an autoregulator in virginiac production, from *Streptomyces virginiae*. *J. Antibiot.* **42**:769–778.
- Kinosita, H., H. Ipposhi, S. Okamoto, H. Nakano, T. Nihira, and Y. Yamada. 1997. Butyrolactone autoregulator receptor protein (BarA) as a transcriptional regulator in *Streptomyces virginiae*. *J. Bacteriol.* **179**:6986–6993.
- Kirby, T., E. Fox-Carter, and M. Guest. 1967. Isolation of deoxyribonucleic acid and ribosomal ribonucleic acid from bacteria. *Biochem. J.* **104**:258–262.
- Kondo, K., Y. Higuchi, S. Sakuda, T. Nihira, and Y. Yamada. 1989. New virginiac butanolides from *Streptomyces virginiae*. *J. Antibiot.* **42**:1873–1876.
- Lee, C. K., M. Minami, S. Sakuda, T. Nihira, and Y. Yamada. 1996. Stereospecific reduction of virginiac M1 as the virginiac resistance pathway in *Streptomyces virginiae*. *Antimicrob. Agents Chemother.* **40**:595–601.
- Lee, C. K., Y. Kamitani, T. Nihira, and Y. Yamada. 1998. Identification and in vivo functional analysis of virginiac resistance gene (*varS*) from *Streptomyces virginiae*. *J. Bacteriol.* **181**:3293–3297.
- Lewin, B. 1997. *Genes VI*, p. 348–353. Oxford University Press, New York, N.Y.
- 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 virginiac biosynthesis. *J. Bacteriol.* **180**:3317–3322.
- Okamoto, S., K. Nakajima, T. Nihira, and Y. Yamada. 1995. Virginiac

- butanolide binding protein from *Streptomyces virginiae*. J. Biol. Chem. **270**:12319–12326.
24. Okamoto, S., T. Nihira, H. Kataoka, A. Suzuki, and Y. Yamada. 1992. Purification and molecular cloning of a butyrolactone autoregulator receptor from *Streptomyces virginiae*. J. Biol. Chem. **267**:1093–1098.
 25. Pearson, W. R., D. J. Lipman. 1988. Imported tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85**:2444–2446.
 26. Sakuda, S., and Y. Yamada. 1991. Stereochemistry of butyrolactone autoregulators from *Streptomyces*. Tetrahedron Lett. **32**:1817–1820.
 27. 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.
 28. 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. Bioeng. **68**:170–173.
 29. Schnabel, E. L., and A. L. Jones. 1999. Distribution of tetracycline resistance genes and transposons among phylloplane bacteria in Michigan apple orchards. Appl. Environ. Microbiol. **65**:4898–4907.
 30. Tovar, K., A. Ernst, and W. Hillen. 1988. Identification and nucleotide sequence of the class E tet regulatory elements and operator and inducer binding of the encoded purified Tet repressor. Mol. Gen. Genet. **215**:76–80.
 31. White, O., J. A. Eisen, J. F. Heidelberg, E. K. Hickey, J. D. Peterson, R. J. Dodson, D. H. Haft, M. L. Gwinn, W. C. Nelson, D. L. Richardson, K. S. Moffat, H. Qin, L. Jiang, W. Pamphile, M. Crosby, M. Shen, J. J. Vamathevan, P. Lam, L. McDonald, T. Utterback, C. Zalewski, K. S. Makarova, L. Aravind, M. J. Daly, K. W. Minton, R. D. Fleischmann, K. A. Ketchum, K. E. Nelson, S. Salzberg, H. O. Smith, J. C. Venter, and C. M. Fraser. 1999. Genome sequence of the radioresistant bacterium *Deinococcus radiodurans* R1. Science **286**:1571–1577.
 32. 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.
 33. Yamada, Y., T. Nihira, and S. Sakuda. 1997. Butyrolactone autoregulators, inducers of virginiamycin in *Streptomyces virginiae*: their structures, biosynthesis, receptor proteins, and induction of virginiamycin biosynthesis, p. 63–79. In W. R. Strolhl (ed.), Biotechnology of antibiotics. Marcel Dekker, Inc., New York, N.Y.
 34. Yanagimoto, Y. K., and T. Enatsu. 1983. Regulation of blue pigment production by g-nanolactone in *Streptomyces* sp. J. Ferment. Technol. **61**:545–550.