JOURNAL OF BACTERIOLOGY, Nov. 2000, p. 6259–6263 Vol. 182, No. 21
0021-9193/00/$04.00+0

Identification by Gene Deletion Analysis of a Regulator, VmsR, That Controls Virginiamycin Biosynthesis in *Streptomyces virginiae*

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Received 13 March 2000/Accepted 9 August 2000

Virginiae butanolide (VB)-BarA of *Streptomyces virginiae* is one of the newly discovered pairs of a butyrolactone autoregulator and a corresponding receptor protein of *Streptomyces* species and regulates the production of the antibiotic virginiamycin (VM) in *S. virginiae*. The gene *vmsR* was found to be situated 4.7 kbp upstream of the *barA* gene, which encodes the VB-specific receptor. The *vmsR* product was predicted to be a regulator of VM biosynthesis based on its high homology to some *Streptomyces* pathway-specific transcriptional regulators for the biosynthetic gene clusters of polyketide antibiotics, such as *Streptomyces peucetius* DnrI (47.5% identity, 84.3% similarity), which controls daunorubicin biosynthesis. A *vmsR* deletion mutant was created by homologous recombination. Neither virginiamycin M_1 nor virginiamycin S was produced in the *vmsR* mutant, while amounts of VB and BarA similar to those produced in the wild-type strain were detected. Reverse transcription-PCR analyses confirmed that the *vmsR* deletion had no deleterious effects on the transcription of the *vmsR*-surrounding genes, indicating that VmsR is a positive regulator of VM biosynthesis in *S. virginiae*.

Streptomycetes are gram-positive filamentous bacteria that are well-known for producing a vast array of bioactive compounds, including more than 70% of commercially important antibiotics. The production of antibiotics by these organisms is regulated by a variety of physiological and nutritional conditions and is coordinated with processes of morphological differentiation, such as the formation of aerial mycelia and spores. Despite many years of research on antibiotics driven by their commercial importance, the overall regulatory pathway governing antibiotic production is still poorly understood. A detailed knowledge of the signal cascade and the genetic components involved in antibiotic production should permit construction of strains that can overproduce these commercially important compounds.

Antibiotic production and/or morphological differentiation is controlled in several *Streptomyces* species by low-molecular-weight compounds called butyrolactone autoregulators (32). To date, 11 butyrolactone autoregulators have been chemically identified and classified into three types based on minor structural differences in their C-2 side chains: (i) the virginiae butanolide (VB) type, containing a 6-α-hydroxy group (13, 31); (ii) the IM-2 type, containing a 6-β-hydroxy group (25, 27); and (iii) the A-factor type, containing a 6-keto group (16). Their effectiveness at nanomolar concentrations, as well as the presence in these species of specific receptor proteins (BarA as a VB-specific receptor in *Streptomyces virginiae* [9, 10, 21], FarA as an IM-2-specific receptor in *Streptomyces lavendulae* FRI-5 [23, 28], and ArpA as an A-factor-specific receptor in *Streptomyces griseus* [22]) as mediators of autoregulator signaling, implies that they should be regarded as *Streptomyces* hormones.

VB-BarA of *S. virginiae* has been among the most frequently studied pairs and is known to regulate the coordinated production of two structurally different compounds, virginiamycin M_1 (VM_1) and virginiamycin S (VS), a pair of antibiotics showing strong synergistic bactericidal activity. In vitro (11, 12) and in vivo (11, 17, 18) analyses have demonstrated that BarA in the absence of VB is a DNA-binding transcriptional repressor and that the release of DNA-bound BarA in the presence of VB from the promoter region of a target gene(s) will result, ultimately, in virginiamycin (VM) production. However, the VB signaling cascade beyond BarA is not clear at present.

In our previous study on a pleiotropic regulatory gene (*barX*) situated 259 bp upstream of the *barA* gene (8), a phenotypic defect in VM and VB production in the *barX* deletion mutant was correlated with the lack of transcription of a plausible pathway-specific regulatory gene (*vmsR*; formerly called *orf2*), which raised the possibility that the *vmsR* product might be responsible for the initiation of VM and/or VB production. To clarify the in vivo function of the VmsR protein, we created a *vmsR* deletion mutant of *S. virginiae* by homologous recombination and report here the results from phenotypic and transcriptional analyses on the *vmsR* mutant, which clearly indicate that VmsR is the regulator positively controlling the biosynthesis of both VM_1 and VS in *S. virginiae*.

Bacterial strains, plasmids, growth conditions, and transformation. *S. virginiae* (strain MAFF 10-06014; National Food Research Institute, Ministry of Agriculture, Forestry, and Fisheries, Tsukuba, Japan) was grown at 28°C in modified yeast extract-malt extract liquid medium (7) for protoplast formation, in tryptic soy broth (Oxoid, Basingstoke, Hampshire, United Kingdom) containing thiostrepton (5 μg ml^{-1}) for plasmid preparation, on ISP2 agar medium (Difco, Detroit, Mich.) for spore formation, and in liquid f medium (33) for VM and VB production. *S. virginiae* was transformed as described previously (7). A plasmid, pSVR1, containing a 3.5-kbp *Eco*RI-*Bam*HI fragment carrying *barZ-vmsR-varM* (8) (DDBJ accession no. AB035547) in the *Eco*RI-*Bam*HI site of pUC18 was used as a template for PCR. DNA manipulations in *Escherichia coli* and *S. virginiae* were performed as described by Sambrook et al. (24) and Hopwood et al. (6), respectively.

Construction and identification of a *vmsR* deletion mutant. The *vmsR* gene product was predicted to be a regulator of VM

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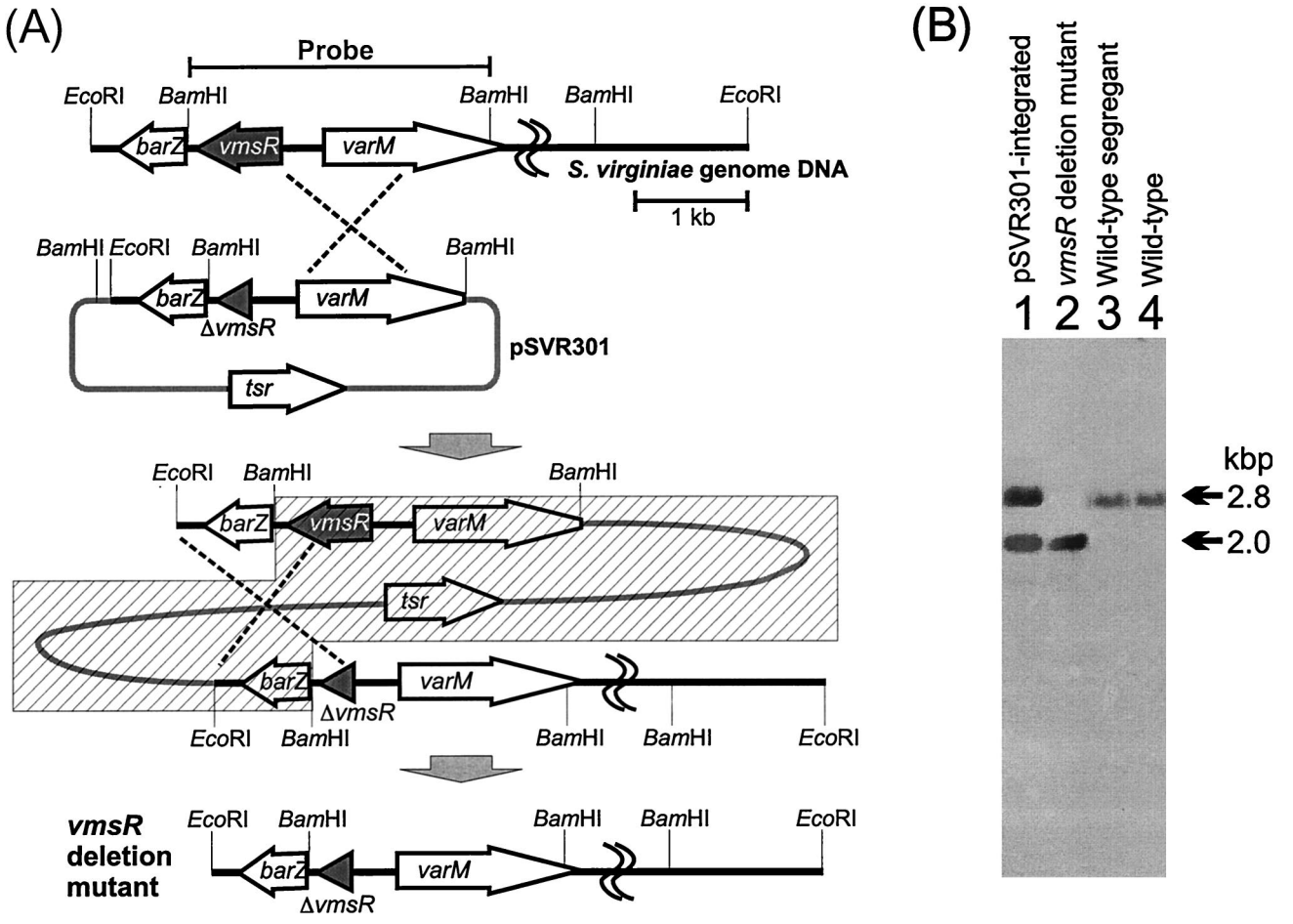

FIG. 1. Replacement of the *S. virginiae vmsR* gene with plasmid pSVR301 to form truncated *vmsR* by homologous recombination. (A) Restriction maps of pSVR301 and the genomic DNA of the *S. virginiae* wild-type strain. The *varM* promoter region was amplified by PCR with primers 5′-GGGGCCACTCGCGACTCCAACCG CCGC-3′ (a newly created recognition site for *Nru*I is indicated by underlining) and 5′-GCAAGCGGCTCGCCGTACGGGAGCGCTG-3′ (a *Bsi*WI recognition site is indicated by underlining) using pSVR1 as a template. The *Nru*I-*Bsi*WI-digested PCR fragment was then used to replace the original 1.0-kbp *Nru*I-*Bsi*WI fragment of pSVR1, resulting in the deletion of a 145-bp 5′ untranslated region and the N-terminal 698 bp of *vmsR*. From the resulting plasmid (pSVR201), a 2.8-kbp *Eco*RI-*Hin*dIII fragment containing *barZ*, the C-terminal 118 bp of *vmsR*, and the entire *varM* gene was cloned into the *Eco*RI-*Hin*dIII site of pUWL-KS to create plasmid pSVR301. A single crossover between pSVR301 and a homologous DNA in the genome gave the pSVR301-integrated strain, and a second crossover generated a *vmsR*-disrupted strain. Only one of the two possible first crossover events is shown. The hatched box indicates the region deleted by the second crossover. (B) Hybridization patterns of *Bam*HI-digested genomic DNA of the pSVR301-integrated strain (lane 1), *vmsR*-disrupted strain (lane 2), wild-type segregant (lane 3), and the *S. virginiae* wild-type strain (lane 4). A 2.8-kbp *Bam*HI fragment containing the *vmsR* gene was used as a probe. When hybridized with the probe, the *vmsR*-disrupted strain (lane 2) gave a 2.0-kbp fragment, as a result of the deletion of the *vmsR* gene.

biosynthesis based on its high homology to some *Streptomyces* pathway-specific transcriptional regulators of the biosynthetic gene clusters of polyketide antibiotics, such as *Streptomyces peucetius* DnrI (47.5% identity, 84.3% similarity), which controls daunorubicin biosynthesis (26); *Streptomyces coelicolor* A3(2) ActII-ORF4 (36.1% identity, 76.6% similarity), which controls actinorhodin biosynthesis (3); and *S. coelicolor* A3(2) RedD (28.9% identity, 67.3% similarity), which controls undecylprodigiosin biosynthesis (19). Alternatively, the VmsR protein might be responsible for the initiation of VB biosynthesis, because the phenotypic defect of VB production in the *barX* mutant correlated with the lack of *vmsR* transcription (8). In order to investigate these possibilities, a *vmsR* deletion mutant was created by homologous recombination within the *S. virginiae* genome (Fig. 1A).

Plasmid pSVR301 was constructed by ligating a 2.8-kbp *Eco*RI-*Hin*dIII fragment containing *barZ*-Δ*vmsR*-*varM* into the *Eco*RI-*Hin*dIII site of pUWL-KS (29). The *S. virginiae* wild-type strain was transformed with pSVR301, and a single crossover between pSVR301 and a homologous DNA in the *S. virginiae* genome gave the pSVR301-integrated strain. When we cultivated the pSVR301-integrated strain in liquid tryptic soy broth in the absence of thiostrepton, we obtained two types of strains: one is a *vmsR* deletion mutant, and the other is a regenerated wild-type strain (Fig. 1B). The integration of pSVR301 in the first crossover strain and its deletion in the second crossover strain were confirmed by Southern blot hybridization using a 2.8-kbp *Bam*HI fragment encoding *vmsR* as a probe (Fig. 1B). We also confirmed the 802-bp deletion in the *vmsR* mutant by DNA sequence analysis. One of the resulting *vmsR* deletion mutants (strain DR1) was used for further investigation.

Phenotypes of the *vmsR* deletion mutant. The amounts of VM produced were determined by reverse-phase high-pressure liquid chromatography (HPLC) using purified VM_1 and VS as standards under the following conditions: column, Cos-

TABLE 1. Phenotypic comparison of wild-type *S. virginiae*, a *vmsR* disruptant, and a wild-type segregant

Strain	VM production[a] (μg/ml)	Resistance (MIC, μg/ml)		VB production[b] (nM)	VB binding activity[c] (pmol/mg of protein)	Sporulation
		VM_1	VS			
Wild type	86	300	300	390	0.44	+
Δ*vmsR*	0	200	200	390	0.44	+
Wild-type segregant	87	300	300	370	0.46	+

[a] VM production was determined after 24 h of cultivation.
[b] VB production was determined after 12 h of cultivation.
[c] VB binding activity was determined for 12-h mycelia.

mosil $5C_{18}$ (4.6 by 100 mm; Nacalai Tesque, Kyoto, Japan); flow rate, 0.75 ml/min; temperature, 40°C; detection, UV radiation at 305 nm; elution, 5 min with 20% CH_3CN containing 0.1% trifluoroacetic acid followed by the linear increase of CH_3CN from 20 to 80% for 15 min. The amounts of VB were determined by measuring the VB-dependent production of VM (20). VB binding activity was assayed by the ammonium sulfate precipitation method (10) with ^{3}H-labeled VB-C_7 (54.6 Ci/mmol) in the presence and absence of 2,000-fold cold VB-C. When traits relating to VB-BarA in *S. virginiae* were measured (Table 1), all of them (VB production, VB binding activity, VM production, VM resistance, and morphology) were found to be identical, with high reproducibility, in the wild-type strain and the wild-type segregant (Table 1), indicating that no unexpected mutations relating to the VB-BarA signal transduction pathway took place during the pSVR301 integration into the wild-type strain or during the second crossover event generating either the wild-type segregant or the *vmsR* disruptant. The *vmsR* mutant, however, did not produce any VM, even after 48 h of cultivation, and was slightly more sensitive to VM_1 and VS (Table 1). No differences in VB production, VB binding activity, growth rate, or morphology on either solid or liquid media were observed between the *vmsR* mutant and the wild-type strain (Table 1). The phenotypic defect of VM production in the *vmsR* mutant was complemented by introducing intact *vmsR* on pUWL-KS into the *vmsR* mutant (data not shown). These results suggested that VmsR does not participate in morphological control or VB biosynthesis in *S. virginiae* but does participate, directly or indirectly, in the regulation of VM biosynthesis.

Analysis of antibiotic products. A further experiment was designed to determine the amounts of two components of VM, namely VM_1 and VS, because the loss of either VM_1 or VS production in the *vmsR* mutant would lead to great loss of antibiotic activity from the synergistic action of VM_1 and VS. The 24-h culture supernatant was recovered after centrifugation, and the amounts of VM_1 and VS were analyzed by HPLC. Under the HPLC conditions employed, VM_1 and VS produced by the wild-type strain were detected easily at retention times of approximately 18 and 21 min, respectively (Fig. 2A), while the *vmsR* mutant did not show any signs of VM_1 or VS (Fig. 2B), indicating clearly that the VmsR protein is essential for the production of both VM_1 and VS.

Transcriptional patterns of the *vmsR*-surrounding genes in the *vmsR* mutant and the wild-type strain. Reverse transcription (RT)-PCR of two genes immediately adjacent to *vmsR*, namely *barZ* transcribed in the same direction as *vmsR* and *varM* transcribed divergently from *vmsR*, revealed that transcription of these genes in the *vmsR* mutant is similar to that in the wild-type strain, indicating that the deletion in *vmsR* did not have a polar effect on *barZ* or a deleterious effect on *varM*. Therefore, the defect in VM biosynthesis in the *vmsR* mutant can be concluded to be due to the lack of functional VmsR protein rather than any undesired effect on the surrounding genes. Thus far, six other genes (*orf4*, *orf5*, *barX*, *barA*, *barB*, and *varS*) have been sequenced, and none of them showed any changes in transcription in the *vmsR* deletion mutant (Fig. 3), which suggests that they are not under the control of *vmsR*.

Based on the phenotypic analysis, the level of resistance to VM_1 and VS was slightly reduced in the *vmsR* mutant compared to that in the wild-type strain (Table 1), although the transcription of the resistance genes (*varS*, which encodes the VS transporter and therefore confers VS resistance [14], and

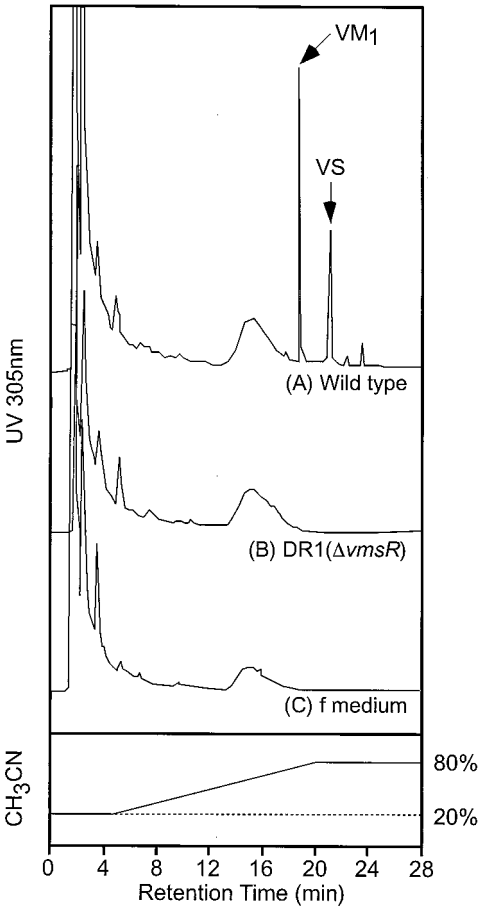

FIG. 2. Detection of two components of antibiotics, VM_1 and VS, by C_{18} reverse-phase HPLC analysis. Authentic VM_1 and VS were purified from the commercial animal-feed additive STAFAC 500 (Smith Kline-RIT, Rixensart, Belgium) by C_{18} reverse-phase HPLC (14).

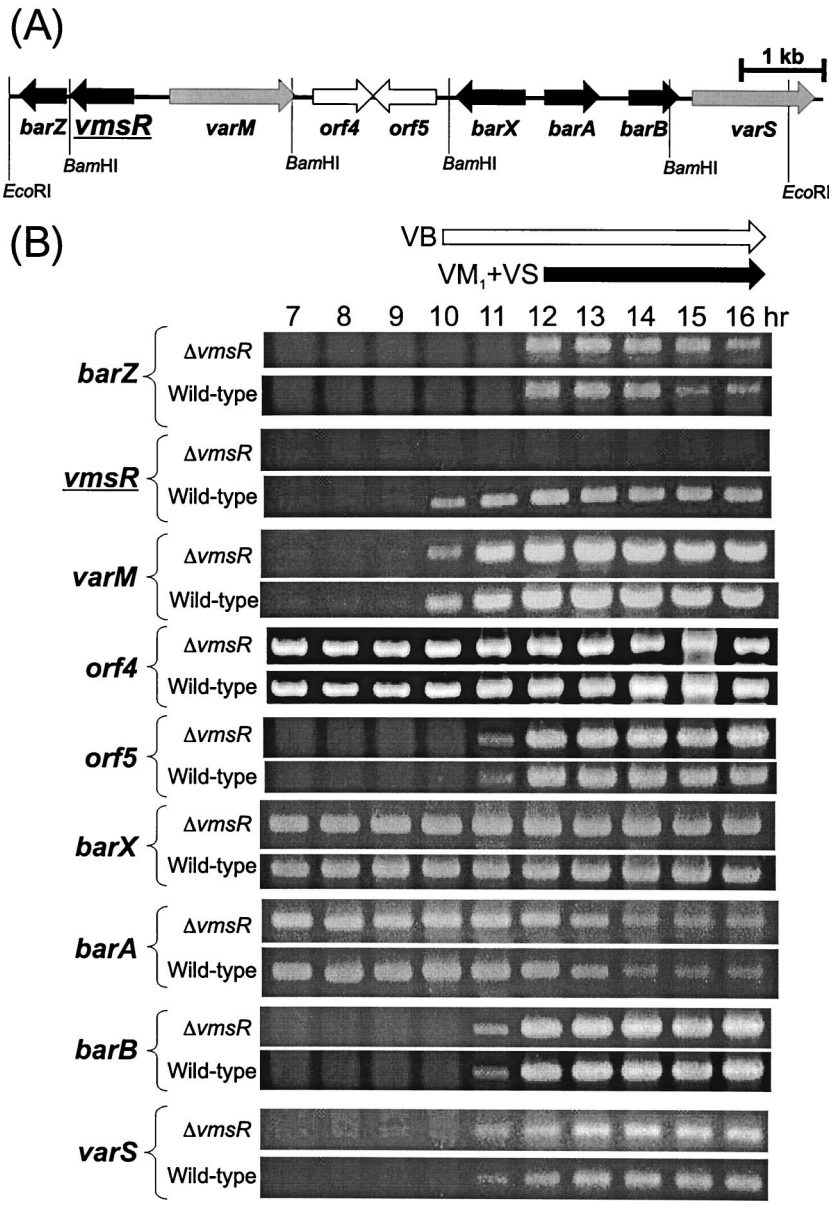

FIG. 3. (A) Gene organization in the 10-kbp region containing the *vmsR* gene in *S. virginiae*. Solid arrows, gray arrows, and open arrows indicate regulator genes, resistance genes for VM, and genes of unknown function, respectively. (B) Transcriptional comparison of *vmsR*-surrounding genes in the *vmsR* disruptant and the wild-type strain by RT-PCR. RT-PCR conditions were previously described (8). Production of VB and VM_1 plus VS is indicated by open and solid arrows, respectively. With the entire sample, RT-PCR was carried out without reverse transcriptase to confirm that the signals shown were derived from mRNA and not from contaminating genomic DNA. Δ*vmsR*, *vmsR* disruptant; Wild-type, *S. virginiae* MAFF 10-06014.

varM, which likely encodes the VM_1 transporter [8]) was not impaired in the *vmsR* mutant (Fig. 3). In the case of *varS*, in addition to the VB-dependent derepression of *barB-varS* bicistronic transcription, *varS* monocistronic transcription was induced by the presence of VS (14), which should function to strengthen the VS resistance level. Similar regulation to enhance the VM_1 resistance may be operative in the presence of VM_1; the absence of VM_1 and VS (i.e., not the absence of the VmsR protein per se) in the *vmsR* mutant seems to be the main reason for the slightly lowered resistance.

The amino acid sequence of VmsR is very similar to those of DnrI, ActII-ORF4, and RedD, all of which belong to an expanding family of *Streptomyces* antibiotic regulatory proteins (SARPs). SARPs are predicted to have a similar mechanism of transcriptional activation through binding to specific nucleotide sequences, and probable DNA recognition sites (helix α3 and a loop connecting two C-terminal β-strands [β6 and β7]) have been estimated on the basis of the crystal structure of OmpR (30). Alignment of the DNA-binding domains of OmpR with homologous regions of VmsR and SARPs reveals a number of highly conserved amino acids. Recently, as predicted by Wietzorrek and Bibb (30), the ActII-ORF4 protein has been confirmed to be a DNA-binding transcriptional activator by gel shift assays and DNase I footprinting (1). Thus, it seems highly probable that VmsR is a DNA-binding protein which acts as a transcriptional activator. It is currently unclear whether VmsR activates VM production by directly activating the transcription of the biosynthetic gene cluster for VM or via another regulator, be-

cause no biosynthetic genes for VM have been cloned from *S. virginiae*, although biosynthetic genes for the closely related antibiotic pristinamycin have been found in *Streptomyces pristinaespiralis* (2).

In this work, we obtained in vivo evidence that VmsR is a positive regulator of the biosynthesis of both VM_1 and VS by constructing and analyzing the *vmsR* deletion mutant. However, it is not clear at present whether the transcription of *vmsR* is regulated directly by VB-BarA or indirectly via another regulator(s), such as the BarB protein. We are currently constructing a *barB* disruptant, the phenotypic and transcriptional analyses of which will clarify the transcriptional cascades among several regulators, such as BarA, VmsR, and BarB.

This work was supported in part by a grant from the Research for the Future Program of the Japan Society for the Promotion of Science (JSPS).

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