Identification and In Vivo Functional Analysis of a Virginiamycin S Resistance Gene (*varS*) from *Streptomyces virginiae*

CHANG-KWON LEE, YUKA KAMITANI, TAKUYA NIHIRA,* AND YASUHIRO YAMADA

Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

BarA of *Streptomyces virginiae* is a specific receptor protein for virginiae butanolide (VB), one of the γ-butyrolactone autoregulators of the *Streptomyces* species, and acts as a transcriptional regulator controlling both virginiamycin production and VB biosynthesis. The downstream gene *barB*, the transcription of which is under the tight control of the VB-BarA system, was found to be transcribed as a polycistronic mRNA with its downstream region, and DNA sequencing revealed a 1,554-bp open reading frame (ORF) beginning at 161 bp downstream of the *barB* termination codon. The ORF product showed high homology (68 to 73%) to drug efflux proteins having 14 transmembrane segments and was named *varS* (for *S. virginiae* antibiotic resistance). Heterologous expression of *varS* with *S. lividans* as a host resulted in virginiamycin S-specific resistance, suggesting that *varS* encoded a virginiamycin S-specific transport protein. Northern blot analysis indicated that the bicistronic transcript of *barB-varS* appeared 1 to 2 h before the onset of virginiamycin M_1 and S production, at which time VB was produced, while exogenously added virginiamycin S apparently induced the monocistronic *varS* transcript.

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 the long 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 the construction of strains that can overproduce these commercially important compounds.

Antibiotic production and/or morphological differentiation are controlled in some *Streptomyces* species by low-molecular-weight compounds called butyrolactone autoregulators (21). Their effectiveness at extremely low concentrations, as well as the presence in these species of specific receptor proteins, implies that they should be regarded as *Streptomyces* hormones. To date, 10 butyrolactone autoregulators have been isolated and their structures have been elucidated chemically (25). Virginiae butanolide (VB) (11, 19, 24) and the corresponding receptor protein (BarA) (14) of *Streptomyces virginiae* have been among the most frequently studied. In *S. virginiae*, the VB-BarA system regulates the coordinate production of two structurally different compounds (15), virginiamycin M_1 (VM_1) and virginiamycin S (VS), a pair of antibiotics showing strong synergistic bactericidal activity.

In our previous in vitro (9) and in vivo (9, 13) analyses to clarify how the VB signal is transmitted into the cell to result, ultimately, in virginiamycin production, we demonstrated that the VB-specific receptor BarA is a DNA-binding protein acting as a transcriptional repressor; the binding of VB to DNA-bound BarA caused the dissociation of BarA from the promoter region of a target gene(s), enabling the transcription of the target gene(s) to occur. One of the target genes, designated *barB*, was located immediately downstream of the *barA* gene. However, transcriptional analysis suggested that *barB* and its downstream region were of a polycistronic nature, indicating that the *barB* downstream region also contains the target gene of BarA. To obtain clues to the overall signal-transmitting pathway governing virginiamycin production, the *barB* downstream region containing the plausible target gene of BarA was analyzed in detail in this study.

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 (8, 24). *Streptomyces* strains were grown at 28°C in yeast extract-malt extract liquid medium for preparation of protoplasts (7), in tryptic soy broth (Oxoid, Hampshire, United Kingdom) for preparation of plasmid DNA, on agar medium R5 (7) for spore formation, and on agar medium NE (12) for determination of sensitivity to several antibiotics. *S. lividans* TK21 (7) was used as a host for cloning with *Streptomyces* plasmid pIJ486 (23) or pIJ4083. *S. lividans* TK21, pIJ486, and pIJ4083 were kindly provided by D. A. Hopwood (John Innes Centre, Norwich, United Kingdom). DNA manipulations in *Escherichia coli* and *Streptomyces* were performed as described by Sambrook et al. (20) and Hopwood et al. (7), respectively.

Sequence of the *varS* gene. To identify a gene cotranscribed with *barB*, 1.95 kbp of the *barB* downstream region was sequenced on both strands by the dideoxy chain termination method with a BcaBEST dideoxy sequencing kit (Takara Shuzo Co.) or a Thermo sequencing kit (Amersham Pharmacia Biotech, Tokyo, Japan) and an ALF DNA sequencer (Amersham Pharmacia Biotech, Tokyo, Japan) (Fig. 1). Frame analysis (3) of the nucleotide sequence revealed a 1,554-bp open reading frame (ORF) transcribed in the same direction as *barB* and flanked by a typical Shine-Dalgarno sequence (GGGA GG) 6 bp upstream of the TTG initiation codon and a perfectly matched inverted repeat sequence 10 bp downstream of the

* Corresponding author. Mailing address: Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan. Phone: 81-6-6879-7433. Fax: 81-6-6879-7432. E-mail: nihira@biochem.bio.eng.osaka-u.ac.jp.

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GGATCCCTCTCCGGGTTCTGGAAGCTGATGCTCCCCATGATCGCGGCCGGTCCGGTCGAGCGCGGCGAACTGGACTGCCGGCCCGCCGTG  90
 G  S  L  S  G  F  W  K  L  M  L  P  M  I  A  A  G  P  V  E  R  G  E  L  D  C  R  P  A  V
CCGGCCGACGTCCGGCGCGCGCCCGCCGTCTGAGCACCGGCCCTCGCTCCTTGCCGTCCCACCGGACGGCTCCTGTCCGTGTCCGGGTCG   180
 P  A  D  V  R  R  A  P  A  V  *  (end of barB)                              +1
CACGTCACGCGCCCCGGGCAGGCTTCACCCGCATGTCACTTGTACATCGTATAACTCTCATATACGTTGTAGAACAGTTCCTCACTCACT   270
                                       ----                     -------- 
                                        -35                       -10
CCTGGGAGGTCTCCTTGCAAGACACACAGATCACGGCGGCGCCTGTACCGGGCCCGCAACCCCAGGCGCTGGGTGATCCTCGGCGTCATCT   360
   SD          M  Q  D  T  Q  I  T  A  A  P  V  P  A  R  N  P  R  R  W  V  I  L  G  V  I
GCCTCGCCCAACTCGTCGTCCTCCTGGACAACACGGTCCTGAACCTCGCGATCCCCGCGCTCACCGAGGACCTGGGCGCGAGCACGACGG   450
  C  L  A  Q  L  V  V  L  L  D  N  T  V  L  N  L  A  I  P  A  L  T  E  D  L  G  A  S  T
ACATCCAGTGGATGATCAACGCCTATGCGTTGGTCCAGTCCGGCCTGCTGCTCACCGCCGGAAGCCTCAGCGACCGGTACGGCCGCAAGA   540
 D  I  Q  W  M  I  N  A  Y  A  L  V  Q  S  G  L  L  L  T  A  G  S  L  S  D  R  Y  G  R  K
AGGCGCTGATCACGGGTCTGGTCGCATTCGGCCTGTGCTCGACGGCCGCGGCCTTCAGCGCGTCGTCCGGCCAGCTGATCGCCGCCCGTG   630
 K  A  L  I  T  G  L  V  A  F  G  L  C  S  T  A  A  A  F  S  A  S  S  G  Q  L  I  A  A  R
CGGGCATGGGCATCGGCGGCGCCCTGCTCATGACCACCACCCTCGCGGTGATCATGCAGGTGTTCGACGGCGAGGAACTGCCGAAGGCGA   720
 A  G  M  G  I  G  G  A  L  L  M  T  T  T  L  A  V  I  M  Q  V  F  D  G  E  E  L  P  K  A
TCGGTATGTGGGGTGCCGTCAGCTCGCTCGGCTTCGCCGGCGGCCCCCTGGTGGGCGGCGTTCTGCTGGCCCACTTCTGGTGGGGCTCGA   810
 I  G  M  W  G  A  V  S  S  L  G  F  A  G  G  P  L  V  G  G  V  L  L  A  H  F  W  W  G  S
TCTTCCTGATCAACATCCCGGTGGCGCTGATCGGCGGGCTGGCCGTCGCCAAGCTCGTCCCGGAGAGCAAGAACCCGCTGGGCAAGCGCC   900
 I  F  L  I  N  I  P  V  A  L  I  G  G  L  A  V  A  K  L  V  P  E  S  K  N  P  L  G  K  R
CGGACCTGGTCGGCGCCGTACTTTCCACGGTCGGCATGGTCGGCGTGGTCTTCGCGATCATCTCCGGCCCTGAGCACGGCTGGACGTCCG   990
 P  D  L  V  G  A  V  L  S  T  V  G  M  V  G  V  V  F  A  I  I  S  G  P  E  H  G  W  T  S
TCAGCGTGCTGGTATCGGCCCTGGTCGGCCTGGCAAGCCTCACCGCCTTCGTCCGCTGGGAGTTGCACGTCCCGGCGCCGATGCTCGACA  1080
 V  S  V  L  V  S  A  L  V  G  L  A  S  L  T  A  F  V  R  W  E  L  H  V  P  A  P  M  L  D
TGAGCTTCTTCCGCAACCGGCGGTTCAACGGCGCCGTCGGCGCAGGCATCCTCGTCGCGTTCGGCATGGCCGGCAGCCTGCTCCTGCTCA  1170
 M  S  F  F  R  N  R  R  F  N  G  A  V  A  G  G  I  L  V  A  F  G  M  A  G  S  L  F  L  L
CGCAGCACCTGCAGCTCGTCCTGAACTACAGCCCGTTGGAAGCCGGCCTGCGCATGGCTCCGCTCGCCTTGACCATCGTCGGACTCAACC  1260
 T  Q  H  L  Q  L  V  L  N  Y  S  P  L  E  A  G  L  R  M  A  P  L  A  L  T  I  V  G  L  N
TGGCGGGGTGTCGGCGCGAAGGTGCTGCCGAAGCTCGGTGCCGCCGGAGCCATCACCTCCGGCATGACCCTGCTCGCCGGCGGGCTCGCGG  1350
 L  A  G  V  G  A  K  V  L  P  K  L  G  A  A  G  T  I  T  S  G  M  T  L  L  A  G  G  L  A
CCGTGGCCACCGTCGGCGGCAGCCTCGGCTACAGCGGCATGCTGGTCGGCCTGGTGCTCATGGGCTCCGGCATCGCCCTGGCGATGCGGCG  1440
 A  V  A  T  V  G  G  S  L  G  Y  S  G  M  L  V  G  L  V  L  M  G  S  G  I  A  L  A  M  R
CGGAATGGGCCAACGCCATCATGGGCTCCATCCCGCCGGAGAAGGCCGGTGTAGGAGCGGGCGTGCCAGGGAACGATCACCGAATTCGGCG  1530
 P  E  W  A  N  A  I  M  G  S  I  P  P  E  K  A  G  V  G  A  G  V  Q  G  T  I  T  E  F  G
GCGGGCCTCGGCGTCGCGGTCCTCGGTGCCGTGCTGAACTCCCGCTTCGCGGCGCTGCTCCCGGCCACCGTGGGGGGCCGGCACGGCCGGG  1620
 G  G  L  G  V  A  V  L  G  A  V  L  N  S  R  F  A  A  L  L  P  A  T  V  G  G  R  H  G  R
GTTCGCTGCACGAGGCGCTCGCCGCCGACCCCTCAGAGGACGTGCAGGAACAGGTCCGGGACGCGTTCGCTTCCGGGGTCGGCACCAGCG  1710
 G  S  L  H  E  A  L  A  A  D  P  S  E  D  V  Q  E  Q  V  R  D  A  F  A  S  G  V  G  T  S
AGCTGATCGGCGCCGCGGCAGTGTTCGCGGGCGGCCTGCTGGCCGGCCTGCTGCTCCACCGGCGGGCGGCGGTGCCGAGGCCGCCGGCCGG  1800
 Q  L  I  G  A  A  A  V  F  A  G  G  L  L  A  G  L  L  H  R  A  A  R  A  E  A  A  A  A
AGACCTCCGCGGTCCCCGCCGGGGACAAGGCTGCCGCCTGATCCCCACGCGCCGGGTGCGAGCACGCTCGCCACCCGGCCGCGTACCTTTG  1890
 E  T  S  A  V  P  A  G  D  K  A  A  A  *
TACAGCCCGTAGCCGAAGGACGACCCCAGGGAGGACGAACGTGGCCGCCAAGCGCGGCTC
                                                             1950
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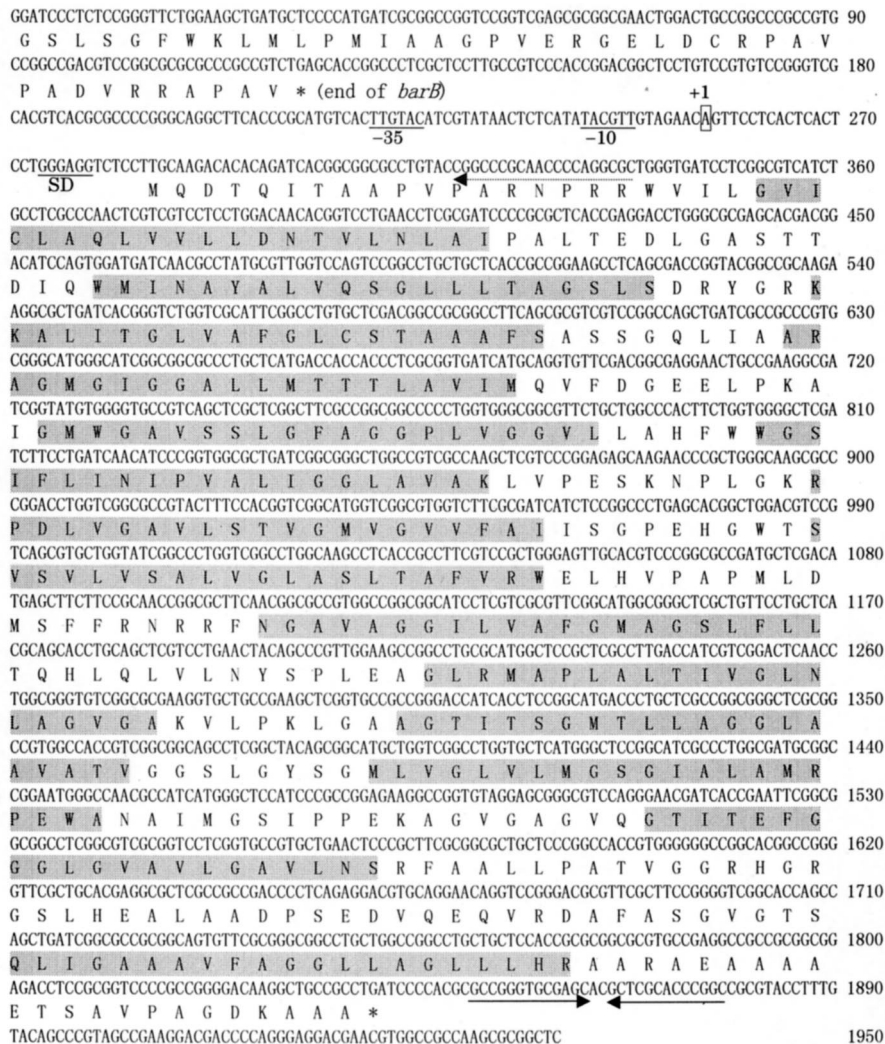
FIG. 1. Nucleotide and deduced amino acid sequences for the *varS* locus. *varS* spans nucleotides 285 to 1838. The deduced amino acids are shown below the nucleotides as one-letter notations. The putative ribosome-binding site and the −35 and −10 sequences are underlined and marked as SD, −35, and −10, respectively. The transcriptional start site is boxed and marked as +1. Inverted repeat sequences in the *varS* 3′ region are indicated by arrows. The oligonucleotide used for primer extension analysis is indicated by a broken arrow. The 14 TMS of VarS are indicated by shading.

TGA stop codon. The inverted repeat sequence was judged to form a strong secondary structure ($\Delta G = -37.4$ kcal/mol), as evident from the complete termination of further DNA sequencing. Only by using a minimized template of 100 bp on an M13 phage and an extension reaction with BcaBEST DNA polymerase at 65°C were we able to determine the nucleotide sequence of the corresponding region. The ORF started 161 bp downstream of the *barB* stop codon, and the intergenic region contained several pairs of hexanucleotides that resembled typical −10 sequences for *Streptomyces* promoters (4), suggesting that the ORF may be transcribed monocistronically in addition to the bicistronic transcription with *barB* (described in more detail below).

Characterization of the deduced ORF product. From the nucleotide sequence, the ORF was deduced to encode a hydrophobic 518-amino-acid protein (M_r, 52,191) containing multiple potential transmembrane domains. Database searches revealed that the ORF product likely belongs to a superfamily of integral membrane proteins that act as drug resistance proteins by exporting toxic compounds from cells with the aid of transmembrane electrochemical gradients (data not shown). Very high homology (68 to 73% identity and 78 to 84% similarity) was observed with RifP (1) of *Amycolatopsis mediterranei* and Ptr (22) of *S. pristinaespiralis*, while moderate homology (31 to 37%) was observed with several proteins, such as ActVA.1 (5) of *S. coelicolor*, QacA (18) of *Staphylococcus aureus*, and TcmA (6) of *S. glaucescens*. All the homologous proteins were found to belong to family 1 of Paulsen et al. (16) and were classified as drug (resistance) transporters having 14 transmembrane segments (TMS). Because both the hydropathy plot and the sequence alignment (data not shown) indicated the probable presence of TMS in the ORF product, the ORF was named *varS* (for *S. virginiae* antibiotic resistance).

Transcriptional analysis of the varS gene. To elucidate the transcriptional pattern of *varS*, we carried out a Northern blot analysis by using a *varS* probe (*Van*91I-*Eco*RI fragment [Fig. 2A]) against mRNA samples collected from an 8- to 21-h culture of *S. virginiae* by the method of Kirby et al. (10) with modifications by Hopwood et al. (7) (Fig. 3A). Two different *varS* transcripts (1.6 and 2.5 kb) were detected at 12 h of
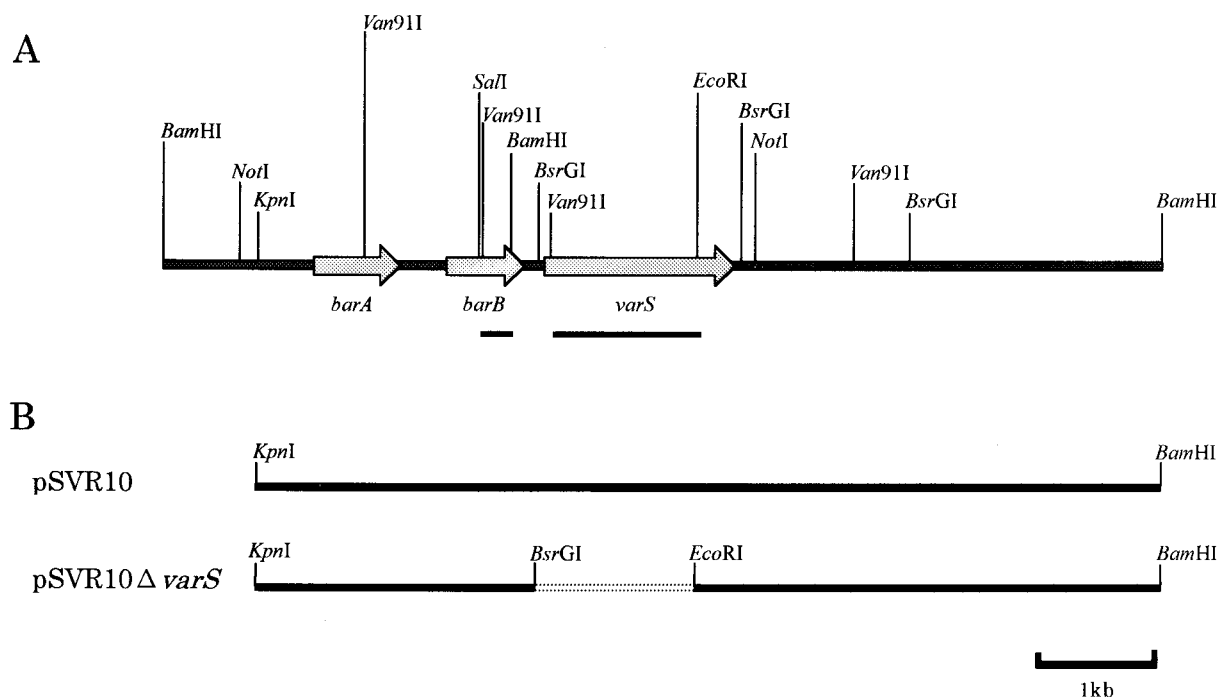
FIG. 2. (A) Restriction map of an 8.2-kb BamHI fragment containing varS and the upstream and downstream regions. ORFs corresponding to barA, barB, and varS are indicated by shaded arrows. Probes (1.19-kb Van91I-EcoRI fragment for varS and 262-bp SalI-BamHI fragment for barB) used for Northern blot hybridization are indicated by filled boxes below the arrows. (B) Schematic representation of the inserts in pSVR10 and pSVR10ΔvarS used for the in vivo functional analysis of varS. Inserts were first constructed in pUC18, recovered as HindIII-XbaI fragments by use of the corresponding flanking restriction sites of pUC18, and then ligated into HindIII-XbaI-digested pIJ486. Broken lines indicate the deletion of varS.

cultivation, 1 h before the production of virginiamycin. As previously reported (9), a barB probe (SalI-BamHI fragment [Fig. 2A]) also hybridized to the large varS transcript (data not shown), confirming that varS was cotranscribed with the upstream barB gene. This fact indicates that both barB and varS are under the transcriptional control of the BarA-VB system. Because the presence of VB (at 11 h of cultivation [Fig. 3A]) leads to virginiamycin production in S. virginiae at 13 h of cultivation, although via a still-unknown pathway, the occurrence of varS transcription prior to virginiamycin production is rational if VarS participates in antibiotic resistance.

In addition to the bicistronic transcription with barB, varS seemed to be transcribed independently from barB, as evident from the presence of the 1.6-kb transcript, which agreed well with the size of varS alone (1,554 bp).

To confirm that varS has its own promoter, primer extension analysis was performed. A 26-mer primer (5′-GCGCCTGGG GTTGCGGGCCGGTACAG-3′) complementary to positions +54 to +28 relative to the putative varS start codon was 5′ end labeled with [γ-^{32}P]ATP and hybridized with RNA from a 14-h culture. The hybrid was extended with reverse transcriptase as described by Sambrook et al. (20). The extended product suggested that varS has a single transcriptional start site at an A situated 29 bp upstream from the TTG initiation codon (Fig. 4). Furthermore, the presence of a functional promoter was confirmed in S. lividans with the aid of promoter-probe vector pIJ4083 (data not shown). The transcriptional start site was consistent with the presence of typical −35 (TTGTAC) and −10 (TACGTT) sequences that showed a high degree of similarity to the consensus sequence of the Streptomyces G2 promoter (4). However, the presence of the promoter raised the possibility that an additional mechanism regulates the monocistronic promoter. For the barB-varS bicistronic operon, BarA

bound to the barB promoter sequence and repressed transcription (9). For the varS promoter region, no BarA-binding sequence was present, nor was any binding of BarA detected by surface plasmon resonance analysis (unpublished data), suggesting that a factor(s) other than BarA is involved.

Because VarS seemed to be involved in antibiotic resistance, we used Northern blot analysis to investigate the possibility that either VM$_1$ or VS influences the synthesis of the monocistronic transcript (Fig. 3B). RNA was prepared from cells with added VM$_1$ or VS at 8 h of culturing, at which time neither internal virginiamycin nor internal VB was present. The 1.6-kb monocistronic varS transcript was detected only in the RNA sample with added VS, indicating that VS, not VM$_1$, induced the synthesis of the monocistronic varS transcript. A 2.4-kb transcript was also observed. Because the barB probe did not show any sign of the corresponding signal on the same membrane (data not shown), we conclude that the 2.4-kb transcript is a minor transcript covering varS and the downstream region, rather than a barB-varS bicistronic transcript, although the transcript sizes are similar (2.5 kb for the barB-varS transcript and 2.4 kb for the varS-downstream region transcript). Therefore, the large varS transcript in Fig. 3A, especially from 14 h of cultivation, should be considered to contain both the barB-varS bicistronic transcript and the varS-downstream region transcript.

In vivo functional analysis of the varS gene. To confirm the function of VarS in vivo, we first attempted to introduce a 2.0-kbp BamHI-NotI fragment containing varS alone into S. lividans by using pIJ486. However, no S. lividans transformant harboring intact varS was obtained, suggesting that the overexpression of VarS is toxic to the cells, probably because of the very hydrophobic nature of the VarS protein. Next, a 7.5-kbp fragment containing both a 2.2-kbp fragment from the up-
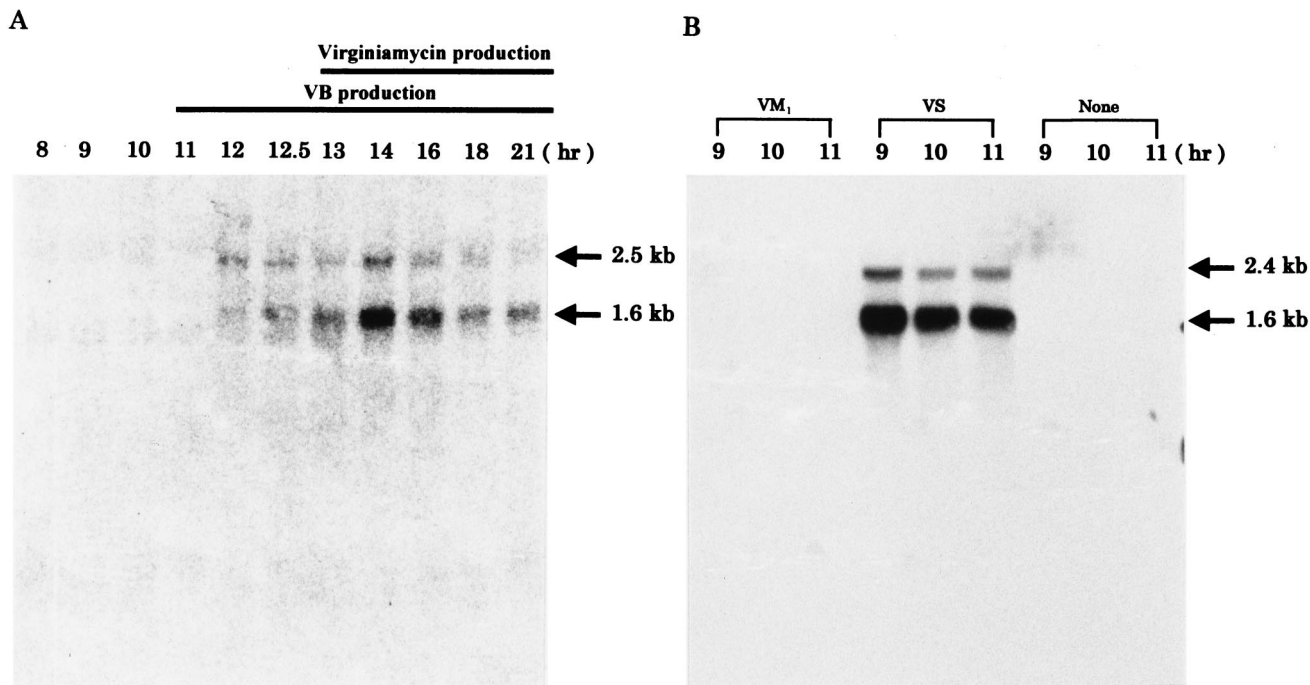
A

B

FIG. 3. Northern blot hybridization analysis of the *varS* transcripts during cultivation of *S. virginiae* (A) and for virginiamycin-induced mRNAs (B). (A) Total RNA was extracted from cells cultivated for the indicated times (hours) at 28°C. RNA (10 μg) was loaded in 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 20 h with the *varS* probe (Fig. 2A). VB and virginiamycin production under the experimental conditions started at 11 and 13 h of cultivation, respectively. (B) RNAs from cells without any addition or with either VM₁ (10 μg/ml) or VS (10 μg/ml) added at 8 h and harvested at 9, 10, or 11 h of cultivation were analyzed. Probe and hybridization conditions were the same as those used for panel A.
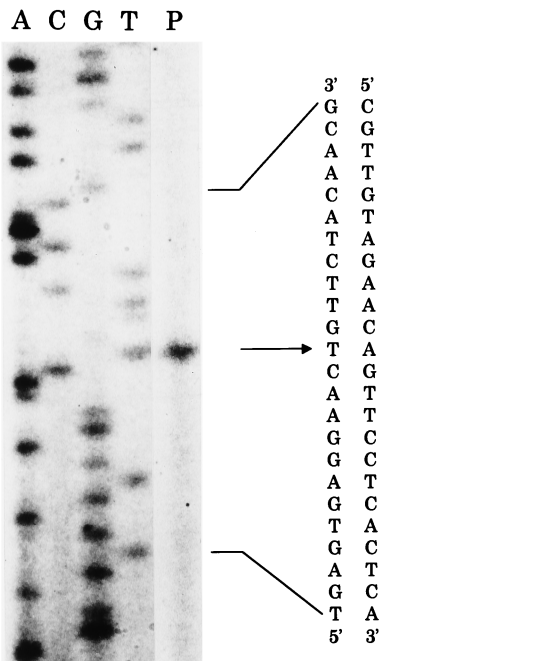
FIG. 4. Primer extension analysis for the *varS* transcriptional start site. The primer extension reaction was carried out with total RNA prepared from a 14-h culture of *S. virginiae*. Lanes A, C, G, and T, DNA sequencing ladder obtained with the same primer; lane P, primer extension reaction. The *varS* transcriptional start site is indicated by an arrow.

stream region and a 3.8-kbp fragment from the region downstream of *varS* (pSVR10 [Fig. 2B]) was introduced into *S. lividans* TK21. Transformants were readily available, but the reason for this result is unknown. As a control, pSVR10Δ*varS* lacking only *varS* was used. Both constructs were used to determine susceptibility to several antibiotics. *S. lividans* harboring pSVR10 was 16 times more resistant to VS (Table 1) than *S. lividans* harboring pSVR10Δ*varS*, while no difference between the strains was observed with VM₁, erythromycin, tylosin, gramicidin, polymyxin, streptomycin, kanamycin, gentamicin, rifampin, lincomycin, chloramphenicol, or tetracycline. Because VM₁ is known to enhance synergistically the antibacterial activity of VS (2), the susceptibility of both strains to the synergistic mixture of VM₁ plus VS (VM₁/VS ratio, 7:3) was measured. *S. lividans* containing pSVR10 was 3.3 times more resistant than *S. lividans* harboring pSVR10Δ*varS* (Table 1). These results, together with the VS-dependent increase of *varS* transcription, indicated that *varS* encodes a VS-specific resistance protein which presumably transports VS from *S. virginiae* cells.

TABLE 1. Virginiamycin resistance conferred by *varS* in *S. lividans* TK21

Plasmid	MIC[a] (μg/ml) of:		
	VM₁	VS	VM₁ + VS
pIJ486	40	5	3
pSVR10Δ*varS*	40	5	3
pSVR10	40	80	10

[a] The MIC was determined as the concentration lethal for cell growth after incubation for 3 days at 28°C.

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