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# In Vivo Expression of the *Bacillus subtilis spoVE* Gene

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**In vivo expression of the *Bacillus subtilis spoVE* gene was studied by S1 nuclease mapping and *spoVE* gene fusion analysis. Transcription of *spoVE* is induced at about the second hour of sporulation from two closely spaced promoters designated P1 and P2. Examination of the precise transcription initiation site by high-resolution primer extension mapping indicated that the nucleotide sequences of the −10 and −35 regions of both P1 and P2 were similar to those of promoters recognized by $E\sigma^E$. Moreover, S1 nuclease mapping and translational *spoVE-lacZ* fusion studies with various *spo* mutants suggest that the expression of *spoVE* P2 requires the *spoIIG* gene product, $\sigma^E$. The sporulation of a wild-type strain was inhibited severely in the presence of a multicopy plasmid, pKBVE, carrying the *spoVE* promoter, indicating the possible titration of a transcriptional regulatory element(s).**

Endospore formation in *Bacillus subtilis* involves an elaborate program of morphological development (13, 19) which requires the expression of at least 50 genetic loci, some of which are operons containing several sporulation-essential genes. Most of these loci, except for *spo0* genes, are not expressed before the initiation of sporulation, which is followed by the sequential expression of the sigma factors (25) and the crisscross regulation of cell-type-specific gene expression (12).

Previously, one of the stage V sporulation genes, *spoVE*, has been cloned (18, 28) and the nucleotide sequence has been determined (2, 22). *spoVE* mutations cause an unusual defect in spore formation in which the forespores are surrounded by well-developed spore coat layers but the cortex is almost entirely absent (19). When the *spoVE* mRNA synthesis was analyzed by dot blot hybridization with a part of the *spoVE* structural gene as a probe, it was found that the *spoVE* mRNA was synthesized from the vegetative-growth phase and that the synthesis was not prevented by early *spo* mutations (16). On the contrary, when the transcriptional regulation was studied by using the *spoVE-lacZ* gene fusion, the expression of the hybrid gene could be detected about 40 min after the onset of sporulation (1). It was also observed that mutations in *spo0H* and *spo0K* but not in *spoIIA* and *spoIIG* impede the expression of *spoVE* (1). For the present paper, we investigated the in vivo expression of the *spoVE* gene by S1 nuclease mapping and *spoVE* gene fusion experiments. Our results show that the *spoVE* gene is turned on just before the onset of cortex formation and suggest that the expression of *spoVE* requires the *spoIIG* gene products.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1. *B. subtilis* strains were grown at 37°C on Schaeffer sporulation medium (SSM) (23). Heat-resistant spores were determined after the cultures at $T_{24}$ ($T_n$ refers to $n$ h after the initiation of sporulation) were treated at 80°C for 10 min. Protoplast transformation was done as described before (3).

**General genetic techniques.** Restriction endonuclease cleavage reactions and ligation were done as described by Sambrook et al. (20). Most of the DNA fragments used for cloning were purified by electrophoresis on low-melting-point agarose. In all instances, the resulting plasmids were checked by restriction site mapping to verify that they had the intended structures.

**Isolation of RNA.** RNA was prepared from *B. subtilis* JH642 and various *spo* mutants grown in SSM. Cells harvested at the indicated stages were used to extract RNA by the method described previously (4) with slight modifications by Okamoto et al. (16).

**S1 nuclease mapping.** The 1,308-bp *Eco*RI-*Nru*I fragment (see Fig. 3) was labeled at the 5′ end with [$\gamma$-$^{32}$P]ATP (Amersham-Japan Co.) and T4 polynucleotide kinase (Toyobo Co.), after removal of the terminal phosphate with alkaline phosphatase (Pharmacia P-L Biochem). The labeled probe (40,000 cpm) was mixed with RNA (50 or 75 μg), dried, and subjected to the hybridization reaction as previously described (4). After the optimal hybridization temperature was first determined, all of the hybridization reactions were carried out at 42°C. The final samples were electrophoresed on a 5 or 6% polyacrylamide gel containing 7 M urea. The bands were visualized by autoradiography with the aid of intensifying screens and preflashing of the film (Kodak X-Omat and XK-5).

**Primer extension mapping.** The procedure for the primer extension analysis has been described by Sambrook et al. (20). The 5′ terminus of the *spoVE* mRNA was mapped by extension of a 25-nucleotide-long synthetic primer (5′-ATGA CGAATAACAAATCAGGCGATG-3′), which was designed to anneal to the part of *spoVE* mRNA. The primer was labeled at the 5′ terminus and incubated under hybridization conditions with total RNA isolated from wild-type (JH642) cells harvested at $T_3$. Reverse transcriptase (Takara Shuzo Co.) was used to generate cDNA primer extension products that were separated by electrophoresis in a 5% polyacrylamide–urea gel alongside the sequencing ladder by the dideoxy chain termination method of Sanger et al. (21) with the 1,308-bp *Eco*RI-*Nru*I fragment and the 25-nucleotide primer.

**Construction of the plasmids pKBVEP1P2, pKBVEP1, and**

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TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Relevant genotype or phenotype | Source or reference |
|---|---|---|
| *B. subtilis* strains | | |
| 4309 | *metB5 nonB recE4* | 27 |
| UOT1285 | *trpC2 lys-1 nprR2 nprE18 ΔaprE* | University of Tokyo |
| UOT0531 | *trpC2 leuA8 metB51 nonB1* | University of Tokyo |
| JH642(= 1A96) | *trpC2 pheA1* | BGSC[a] |
| 1A423(= MI112) | *argA15 leuA8 m(−) 168 r(−) 168 recE4 thr-5* | BGSC |
| UOT0531HUSA1 | *trpC2 leuA8 metB51 nonB1 spo0AΔHB* | Hiroshima University |
| 1S16 | *trpC2 pheA1 spo0B136* | BGSC |
| 1S17 | *trpC2 pheA1 spo0E11* | BGSC |
| 1S19 | *trpC2 pheA1 spo0F221* | BGSC |
| 1S22 | *trpC2 rpoB2 spo0H17* | BGSC |
| 1S27 | *metC3 tal-1 spo0J87* | BGSC |
| 1S28 | *trpC2 spo0K141* | BGSC |
| 1S32 | *trpC2 rpoB2 spoIIA69* | BGSC |
| 1S86 | *trpC2 spoIIA1* | BGSC |
| 1S49 | *trpC2 spoIIB131* | BGSC |
| 1S33 | *trpC2 rpoB2 spoIID66* | BGSC |
| 1S43 | *trpC2 spoIID298* | BGSC |
| 1S35 | *trpC2 rpoB2 spoIIE64* | BGSC |
| 1S59 | *trpC2 spoIIF96* | BGSC |
| 1S60 | *leuA8 tal-1 spoIIG41* | BGSC |
| 1S42 | *metC3 tal-1 spoIIIA53* | BGSC |
| 1S48 | *trpC2 spoIIIB2* | BGSC |
| 1S38 | *trpC2 spoIIIC94* | BGSC |
| 1S39 | *trpC2 spoIIID83* | BGSC |
| 1S63 | *trpC2 spoIIIE36* | BGSC |
| 1S46 | *pheA12 spoIVA178* | BGSC |
| 1S57 | *trp(−) spoIVB165* | BGSC |
| 1S47 | *trpC2 spoIVC133* | BGSC |
| 1S51 | *trpC2 spoVE85* | BGSC |
| Plasmids | | |
| pKB301 | Km[r] subtilisin (*aprE*) | 28 |
| pKZ704 | Km[r] *lacZ* | 28 |
| pKZ724 | Km[r] *lacZ* | 28 |
| pUCVE1 | Amp[r] *spoVE lacZ* | 18 |
| pAV181 | Amp[r] *lacZ* | 28 |
| pAVZ192 | Amp[r] *lacZ* | 28 |

[a] BGSC, *Bacillus* Genetic Stock Center.

**pKBVEP2 carrying transcriptional *spoVE*-subtilisin gene fusion.** *B. subtilis* promoter probe plasmid pKB301 is a derivative of pSB (26) which was obtained from F. Kawamura with the permission of R. H. Doi. pKB301 was constructed by subcloning a promoterless subtilisin (*aprE*) gene from pSB on an *Escherichia coli-B. subtilis* shuttle vector, pKM1, derived from pUB110 and pUC19 (29). pKB301 contains a promoterless, Shine-Dalgarno (SD) sequence possessing subtilisin gene preceded by a pUC19 polylinker and T4 transcription-translation terminator sequences. The *spoVE* *Hin*dIII C fragment (see Fig. 1) was isolated from pUCVE1 (17) and inserted into the unique *Hin*dIII site of pAV181, which is a recombinant plasmid of pUC18 and pUC19 (29), to create pAVC3. The *Nar*I-*Eco*RI fragment containing P1 and P2 promoters was isolated from pAVC3 and ligated to pKB301 restricted by *Acc*I-*Eco*RI to produce pKBVEP1P2 (Fig. 1). pKBVEP1 was constructed by ligation of the *Nar*I-*Sau*3AI fragment containing the P1 promoter from the *Nar*I-*Eco*RI fragment to *Acc*I-*Bam*HI-digested pKB301. Finally, pKBVEP2 was constructed by inserting the *Sau*3AI-*Eco*RI fragment containing the P2 promoter from the *Nar*I-*Eco*RI fragment into the *Bam*HI-*Eco*RI sites of pKB301 (Fig. 1).

**Construction of the plasmids pKZVE5, pKZVE7, and pKZVE437 carrying translational *spoVE-lacZ* fusion.** *lacZ* fusion vectors, pKZ704 and pKZ724, containing a multiple cloning site upstream to the ninth codon of *lacZ* were used to construct a translational *lacZ* fusion (Fig. 2A). First, pKZVE5 and pKZVE7 were constructed by insertion of the 375-bp *Hin*dIII E fragment and the 850-bp *Hin*dIII C and E fragment (obtained by partial digestion of the 2.8-kb *Eco*RI fragment [Fig. 1]), respectively, into the unique *Hin*dIII site of pKZ704. P1 and P2 promoters were present in the *Hin*dIII C fragment, and two possible initiation codons (TTG) and two putative SD sequences (SD1 and SD2) were present in the *Hin*dIII E fragment (Fig. 1) (25a). pKZVE437 was constructed by using another *lacZ* fusion vector, pKZ724, since it has a *Bgl*II restriction site which can be used for the construction of *spoVE-lacZ* transducing phage (Fig. 2B). First, a 437-bp *Sau*3AI fragment was obtained from *Sau*3AI digestion of the 2.8-kb *Eco*RI fragment (Fig. 1). This fragment, containing the P2 promoter and two initiation codons, was inserted into the *Bam*HI site of pKB301 to produce pKBVE437. The fusion junction of each plasmid was sequenced to verify the construct. Then, pKBVE437 was restricted with *Eco*RI, treated with mung bean nuclease, and digested with *Pst*I. The obtained *Eco*RI (blunt end)-*Pst*I fragment was ligated to *Sma*I-*Pst*I-digested pKZ724 to produce pKZVE437.

**Construction of the *spoVE-lacZ* transducing phage ϕVEZ437**

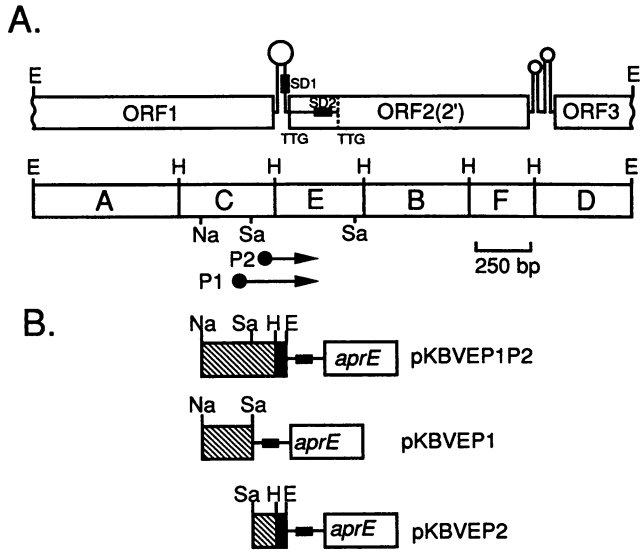



FIG. 1. (A) Schematic representation of the 2.8-kb *Eco*RI fragment carrying the *spoVE* gene DNA. The possible terminator-like structures are indicated. The positions of the promoters, P1 and P2, are shown below the fragment. ORF2 and ORF2′ indicate two possible *spoVE* open reading frames in the same reading frame. ORF2 and ORF2′ encode 40- and 32-kDa proteins, respectively (25a). The small filled boxes (SD1 and SD2) represent the predicted SD sequences for ORF2 and ORF2′, respectively. TTG indicates the initiation codon of *spoVE*. The letters A, C, E, B, F, and D shown in the lower open box represent *Hin*dIII A, C, E, B, F, and D fragments, respectively. (B) Schematic representation of transcriptional *spoVE*-subtilisin fusion. The small filled box indicates the SD sequence of the *aprE* gene. The *Hin*dIII-*Eco*RI region is derived from the multiple cloning site of plasmid DNA. Plasmids pKBVEP1P2, pKBVEP1, and pKBVEP2 carry the P1 and P2, P1, and P2 promoters, respectively. Abbreviations for endonucleases are E, *Eco*RI; H, *Hin*dIII; Na, *Nar*I; and Sa, *Sau*3AI.

**by prophage transformation.** ϕCM, a derivative of *B. subtilis* temperate phage ϕ105 containing a chloramphenicol acetyltransferase cassette (24), was used as a transducing phage vector. A 1.2-kb *Bgl*II-*Ban*III fragment isolated from pKZVE 437, a 2.3-kb *Ban*III-*Bam*HI fragment from pAVZ192 (a derivative of pMC1871 [Pharmacia P-L Biochem] and pAV 191 [29]), and *Bam*HI-digested ϕCM DNA were mixed, ligated, and inserted by prophage transformation (11) into the ϕ105 prophage lysogenized in *B. subtilis* UOT0531 cells to construct *spoVE-lacZ* transducing phage ϕVEZ437 (Fig. 2B). Transformants were selected for chloramphenicol-resistant, blue colonies on an SSM plate containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (final concentration, 2 mg/liter) and 5 μg of chloramphenicol per ml. The crude lysates prepared from blue colonies were used to transduce strain UOT0531 (ϕ105 lysogen). Phage DNA was isolated and checked by restriction mapping to verify the phage construction.

**Protease assay.** The serine protease activity was determined at intervals in SSM by the method of Wang and Doi (26) with slight modifications. A total of 1 ml of cell cultures was harvested and centrifuged, and 0.3 ml of the supernatant was mixed with 0.7 ml of 0.1 M Tris-hydrochloride (pH 7.8), containing 5 mg of Hide powder azure. The reaction was carried out at 37°C for 5 to 30 min (until blue color became visible) and stopped by filtration of the reaction mixture through a 0.45-μm-pore-size Millipore filter. The clear filtrate was measured for $A_{595}$. One unit of specific activity was defined as the change of $A_{595}$ per minute per Klett unit (with filter no. 66) of the cell culture × $10^5$.

**β-Galactosidase assay.** β-Galactosidase activity was determined as described by Wang and Doi (27).

**Nucleotide sequence accession number.** The nucleotide sequence data reported here will appear in the DDBJ, EMBL, and GenBank nucleotide sequence data bases under accession no. D14109.

## RESULTS

**Analysis of the *spoVE* transcription.** The nucleotide sequence of the 2.8-kb *Eco*RI fragment containing the *spoVE* gene showed the presence of three open reading frames (Fig. 1). Analysis by deletion, mutation, and subcloning suggested that ORF2 is the *spoVE* gene (2, 16, 17, 22, 28). The nucleotide sequence also revealed the existence of two inverted repeat sequences immediately downstream of the *spoVE* gene and one immediately upstream of this gene.

To identify the transcripts of the *spoVE* gene, S1 nuclease mapping experiments were performed. Strain JH642 was grown in SSM, and total RNA was extracted from vegetative (60 Klett units, filter no. 66) and $T_0$-to-$T_6$ cells and hybridized with the *Eco*RI-*Nru*I fragment as a probe. The S1 nuclease-resistant DNA fragments were analyzed after gel electrophoresis on denatured 5% polyacrylamide gels, and the results are shown in Fig. 3A. The 1,308-bp *Eco*RI-*Nru*I probe, labeled at the *Nru*I site, provided two significant protected bands designated P1 and P2. Both bands could be detected from $T_2$ but were in different quantities. The P1 band appeared clearly at $T_3$ while the P2 band was more abundant at $T_2$, and then they gradually decreased until $T_6$. However, no bands were detected at $T_4$. We think that something must be wrong with this sample, since we could detect both bands with other $T_4$ RNA samples. Approximate sizes of the nuclease-protected DNA fragments were determined by the labeled *Hpa*II-digested M13mp11 DNA as size markers and were calculated to be 330 and 200 bp upstream from the *Nru*I site for P1 and P2, respectively. To determine more precisely the transcription initiation site, high-resolution primer extension mapping with the 25-nucleotide-long synthetic primer was carried out (20) (Fig. 3B). The transcription start site (+1) of the P1 and P2 promoters corresponded to the nucleotide A at positions 335 or 336 and 197 or 198 from the *Nru*I site, respectively (Fig. 4).

It is interesting to note that the transcription start site of P2 was present immediately upstream of the inverted repeat sequences followed by the SD1 sequence (Fig. 4). This promoter contains −10 and −35 regions similar to those of the promoters recognized by $E\sigma^E$. The consensus sequences for −10 and −35 regions are $CATACA^a/_cT$ and $G^a/_t{}^c/_aATA^t/_a{}^t/_a{}^c/_t$ (space, 13 bp), respectively (8). The other promoter, P1, also contains sequences less similar to −10 and −35 regions of the promoters recognized by $E\sigma^E$.

**Expression of transcriptional *spoVE*-subtilisin fusion.** In an attempt to investigate the in vivo expression of the *spoVE* gene, plasmids pKBVEP1P2, pKBVEP1, and pKBVEP2 carrying transcriptional *spoVE*-subtilisin fusions were constructed with a promoter probe vector, pKB301 (29) (an *E. coli-B. subtilis* shuttle vector). This plasmid contains the promoterless subtilisin (*aprE*) gene preceded by a polylinker of pUC19 which was used as a cloning site of the *spoVE* promoter fragments. The construction of these plasmids is illustrated in Fig. 1, and the details of construction are described in Materials and Methods. The promoters were

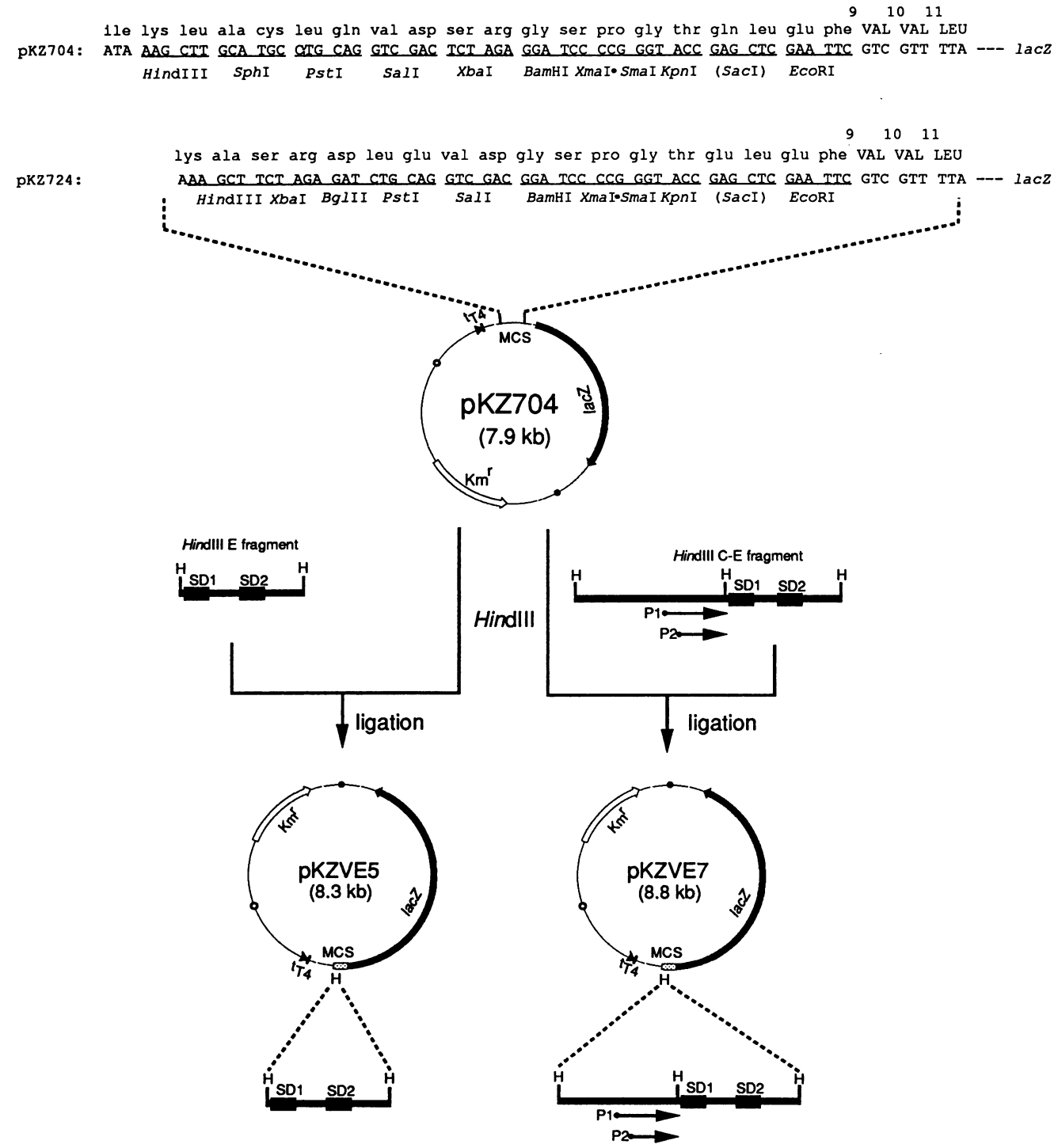
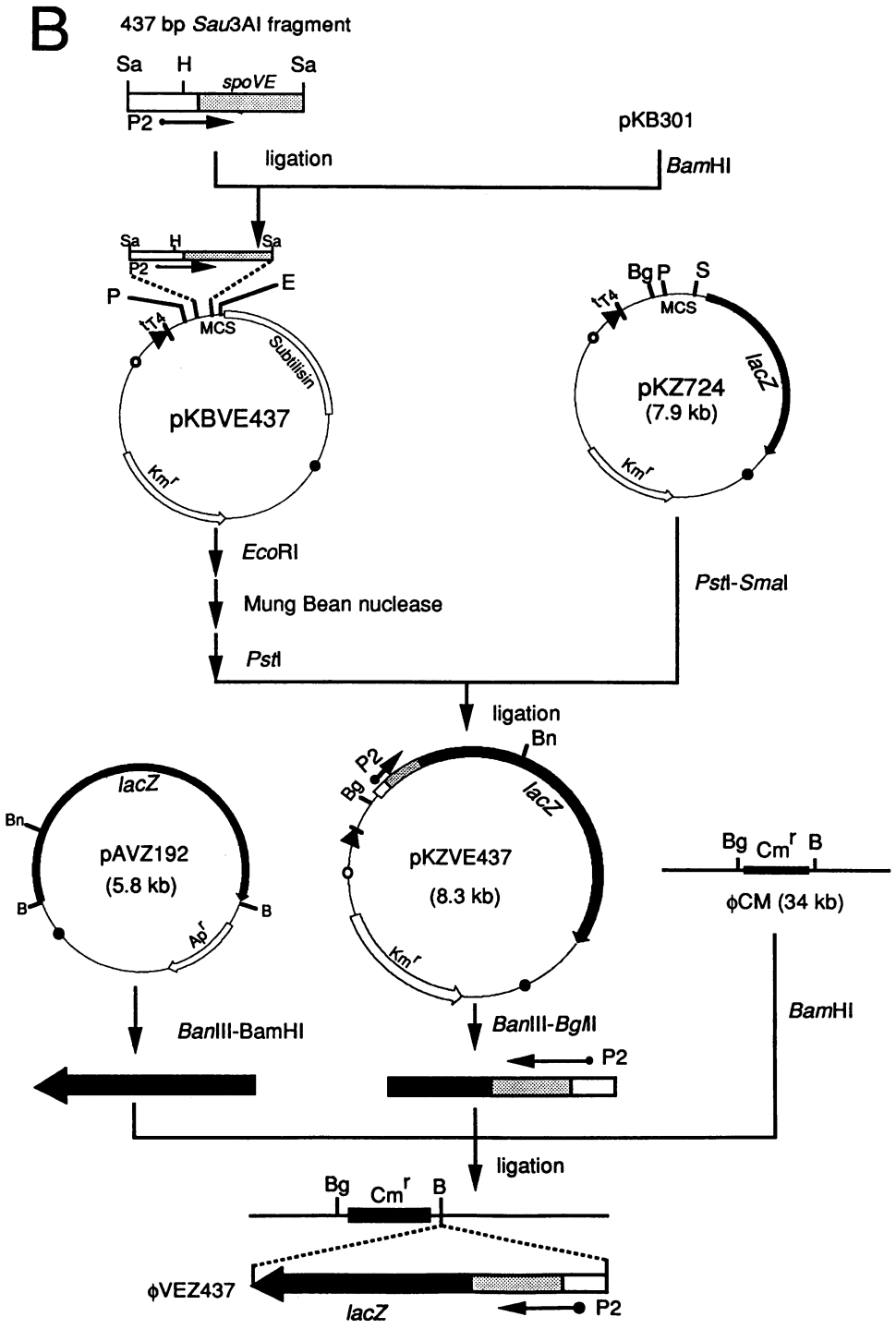



FIG. 2. Construction of translational *spoVE-lacZ* fusion plasmids pKZVE5, -7, and -437 and a transducing phage, ϕVEZ437. (A) Construction of pKZVE5 and pKZVE7. The multiple cloning sites (MCS) of the *lacZ* fusion vectors pKZ704 and pKZ724 are shown at the top. *Hin*dIII E and *Hin*dIII C-E fragments were inserted into the *Hin*dIII site of pKZ704. (B) Construction of pKZVE437 and a specialized transducing phage, ϕVEZ437. Details of the construction are described in Materials and Methods. Open circle indicates replication origin in *B. subtilis*; filled circle indicates replication origin in *E. coli*. Abbreviations for endonucleases are B, *Bam*HI; Bg, *Bgl*II; Bn, *Ban*III; H, *Hin*dIII; and Sa, *Sau*3AI.




cloned separately by taking advantage of a *Sau*3AI site that lies between them. These plasmids were introduced into protoplasts of *B. subtilis* UOT1285, a protease mutant, to determine the promoter activity of the inserted fragments. The protease-positive transformants were selected on an SSM plate containing 1% skim milk and 5 μg of kanamycin per ml and grown in SSM containing 5 μg of kanamycin per ml. The samples were withdrawn at intervals and assayed for protease activity.

As shown in Fig. 5, protease activities of strains harboring pKBVEP1P2, pKBVEP1, or pKBVEP2 plasmid increase dramatically at $T_2$. In addition, expression of both promoters was prevented by the addition of 1% glucose to SSM, which also suppressed sporulation. These results coincided very well with the S1 nuclease mapping and revealed that both of the *spoVE* promoters are expressed after the cells enter the sporulation phase. These results also suggest that a sporulation-specific regulatory factor(s) may be involved in the

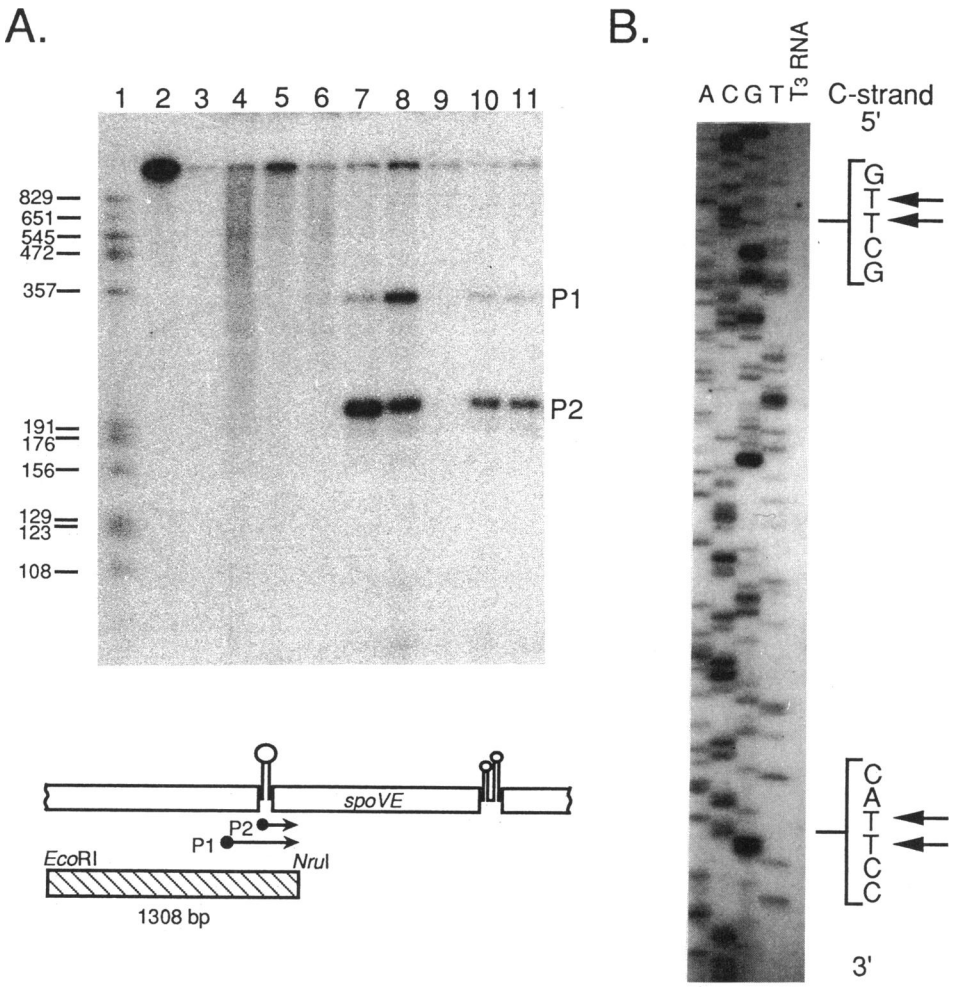



FIG. 3. (A) Low-resolution S1 nuclease mapping of the *spoVE* transcripts. The 1,308-bp *Eco*RI-*Nru*I probe (labeled at the *Nru*I site) was hybridized with 50 μg of RNA as described in Materials and Methods. Lane 1 is M13mp11-*Hpa*II fragments used as size markers, and lanes 2 and 3 are the probe alone and the probe hybridized with RNA isolated from *E. coli* HB101, respectively. Lanes 4 through 11 show S1 nuclease-protected fragments of the probe hybridized with RNA isolated from *B. subtilis* JH642 at vegetative-growth phase, at the end of exponential growth ($T_0$), and at hourly intervals until $T_6$. (B) High-resolution primer extension mapping of the *spoVE* gene transcripts from P1 and P2. The 25-nucleotide primer was hybridized with 20 μg of RNA isolated from JH642 at $T_3$. Dideoxynucleotide sequencing reactions were carried out with the same primer, to map the 5′ end of *spoVE*. The samples were analyzed on a 5% polyacrylamide–urea gel. The complementary bases of the start sites are indicated by arrows.

initiation of transcription of the *spoVE* promoters. The weak activities detected during the vegetative phase of growth may be due to the artifacts of nonspecific protease activities.

**Expression of translational *spoVE-lacZ* fusion.** To further investigate whether *spoVE* promoter and SD sequences can function in vivo, plasmids pKZVE5, pKZVE7, and pKZVE437, carrying translational *spoVE-lacZ* fusions, were constructed (Fig. 2). Plasmid constructions were described in Materials and Methods. These plasmids were introduced into *B. subtilis* 1A423. Transformants harboring pKZVE437 and pKZVE7 formed dark blue colonies on SSM plates containing X-Gal (final concentration, 2 mg/liter) and 5 μg of kanamycin per ml, whereas pKZVE5 transformants produced white colonies on the same medium. Determination of β-galactosidase activity of these transformants in SSM showed that the synthesis of β-galactosidase starts at $T_2$ and reaches its maximum at $T_6$ (Fig. 6). These results (Fig. 5 and 6) suggest that P2 is a major promoter for the in vivo expression of the *spoVE* gene.

**Effect of *spo* mutations on expression of *spoVE*.** To investigate the effect of *spo* mutations on expression of the *spoVE* gene, *spo* mutant strains were grown in SSM at 37°C and RNA was isolated from the cells harvested at $T_3$. S1 nuclease mapping was carried out as described previously, and the results are shown in Fig. 7. Transcripts from P1 and P2 promoters could not be detected in any of the *spo0* mutants, except mutant *spo0J87*, or in most of the *spoII* mutants. However, it should be noted that the transcript from the P1 promoter could be detected in a *spoIIG* mutant. Further analysis of β-galactosidase activity in various *spo* mutants lysogenized with a transducing phage, φVEZ437, carrying a *spoVE-lacZ* translational fusion (Fig. 8 and Table 2) indicated that all mutations that blocked the expression of the *spoIIG* gene (9) also blocked the expression of the *spoVE* gene.

**Sporulation inhibition by multiple copies of *spoVE* promoters.** It has been suggested that promoters which require a regulatory factor(s) essential for sporulation may inhibit

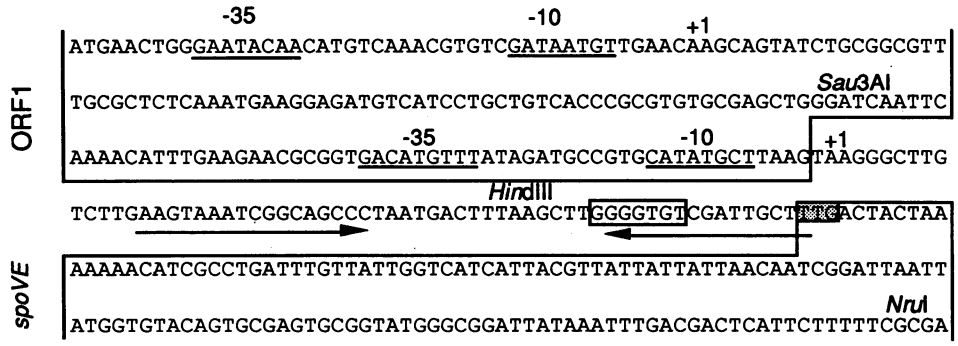



FIG. 4. Nucleotide sequence of the regulatory region of *spoVE*. The nucleotide sequence (2, 22) is given in the 5'-to-3' direction. Transcription initiation sites (+1) of the promoters are indicated above the sequences. The tentative −10 and −35 regions of two promoters are underlined, and the inverted repeat sequences are shown by arrows. The predicted SD1 sequence and the first initiation codon (TTG) are boxed and shaded, respectively.

sporulation, when present in multiple copies, by titration of the factor(s) (30). To investigate whether the *spoVE* promoter titrates such a factor, the following experiments were performed. Transcriptional *spoVE*-subtilisin fusion plasmids pKBVEP1P2, pKBVEP1, and pKBVEP2 were introduced into *B. subtilis* 4309 (*spo*$^+$ *recE4*). The transformants were grown in SSM, and viable cells were counted at $T_5$ whereas heat-resistant spores were counted at $T_{24}$ after 10 min of treatment at 80°C.

The sporulation frequencies of strains harboring pKBVE plasmids are shown in Table 3. All of them inhibit severely the sporulation of wild-type strain 4309 (*spo*$^+$ *recE4*), giving rise to about $10^3$ to $10^4$ spores per ml, whereas $10^8$ spores per ml were produced when the strains carried pKB301.

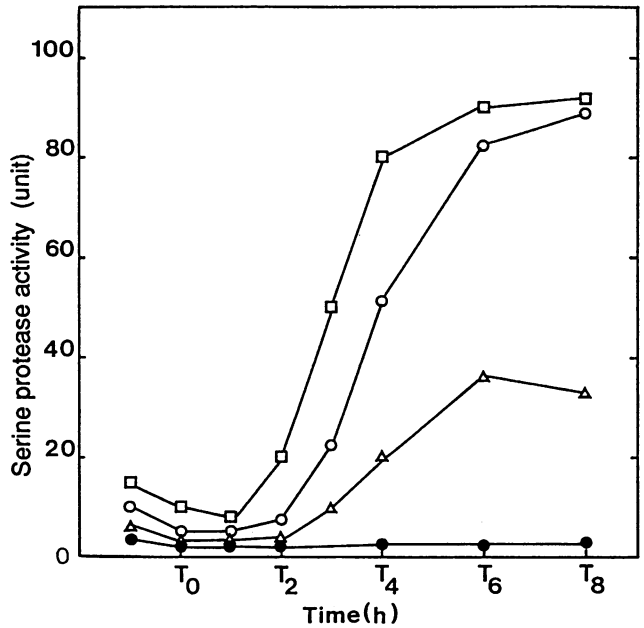



FIG. 5. In vivo expression of the *spoVE* promoters. Recombinant plasmids pKBVEP1P2, pKBVEP1, and pKBVEP2 were introduced to *B. subtilis* UOT1285 (a double protease mutant strain). Selected transformants were grown in SSM with or without 1% glucose. Samples were withdrawn at intervals during growth, and their protease activity was determined as described in Materials and Methods. Shown are pKBVEP1P2 (□), pKBVEP1 (△), pKBVEP2 (○), and pKB301 and all of the above plasmids in the presence of 1% glucose (●).

## DISCUSSION

The results presented in this paper revealed the existence of tandem promoters, P1 and P2, immediately upstream of

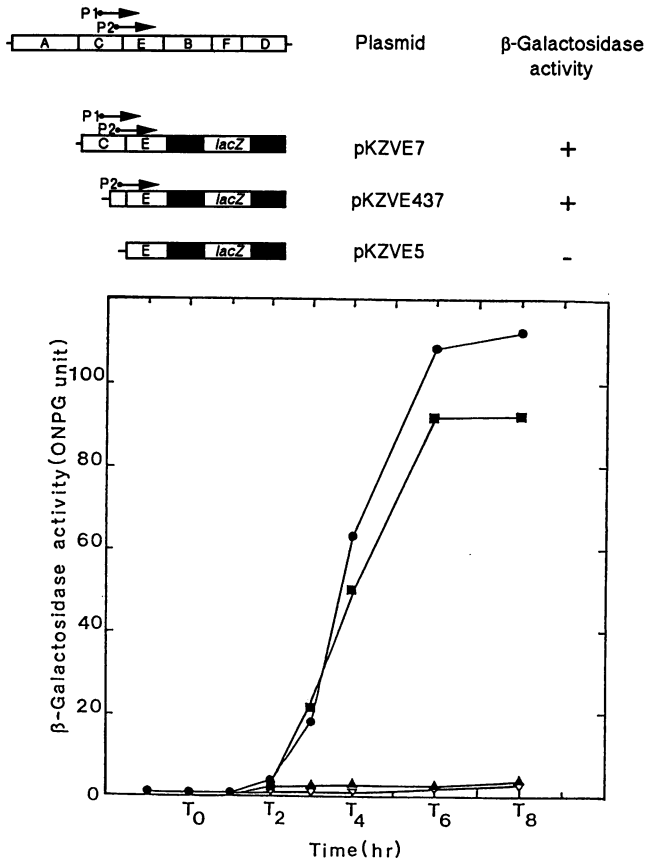



FIG. 6. β-Galactosidase activities (in SSM) of *B. subtilis* MI112 carrying *spoVE-lacZ* fusion plasmids. pKZ704 (▽), pKZVE5 (▲), pKZVE7 (●), and pKZVE437 (■) are shown. β-Galactosidase activities were determined as described in the text. ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

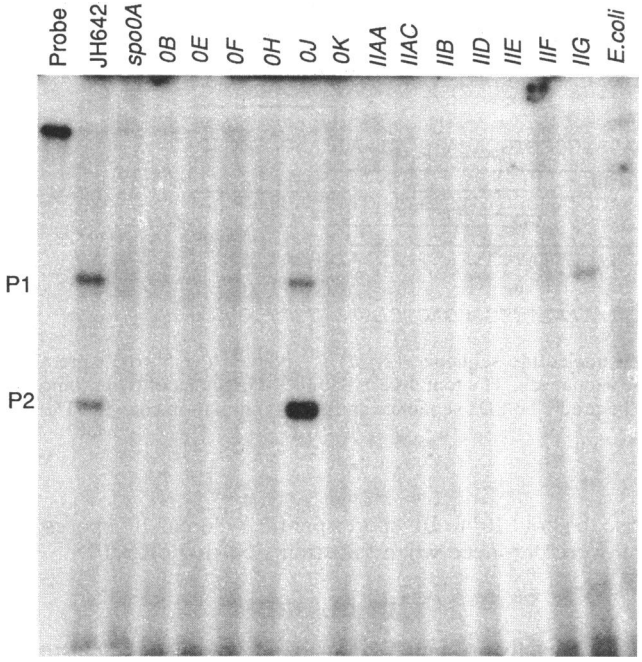



FIG. 7. Transcription of the *spoVE* gene in *spo* mutants. RNAs isolated from *spo* mutants indicated at the top of the figure and JH642 and *E. coli* were hybridized with the same probe as described in the legend to Fig. 3. Final samples were electrophoresed on a 5% polyacrylamide gel containing 7 M urea.

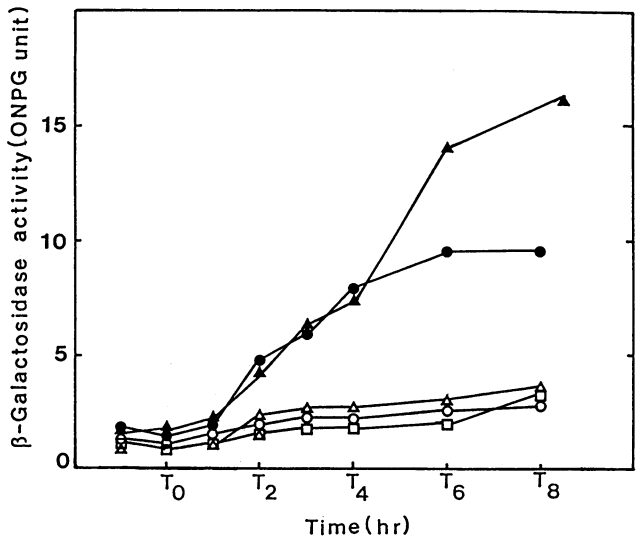



FIG. 8. Expression of the translational *spoVE-lacZ* fusion in *spo* mutants lysogenized with a specialized transducing phage, ϕVEZ437. *spo* mutants were lysogenized with ϕVEZ437 in the presence of ϕ105 (helper phage). Transductants were selected on Schaeffer agar containing X-Gal (final concentration, 2 mg/liter) and 5 μg of chloramphenicol per ml. Obtained transductants were grown in SSM, harvested at intervals, and assayed for β-galactosidase activities as described in the text. UOT0531(ϕCM) (○), UOT0531 (ϕVEZ437) (●), *spo0AΔHB*(ϕVEZ437) (△), *spo0J87*(ϕVEZ437) (▲), and *spoIIG41*(ϕVEZ437) (□) are shown. ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

TABLE 2. Effects of various *spo* mutations on the expression of *spoVE*[a]

| Mutation | β-Galactosidase activity (ONPG[b] unit) at: | | |
|---|---|---|---|
| | $T_3$ | $T_4$ | $T_6$ |
| ϕCM[c] | 2.4 | 2.3 | 2.6 |
| UOT0531 (wild type) | 6.0 | 8.0 | 9.4 |
| *spo0AΔHB* | 3.2 | 3.0 | 3.2 |
| *spo0B136* | 3.7 | 3.9 | 4.5 |
| *spo0E11* | 3.0 | 3.4 | 4.2 |
| *spo0F221* | 3.6 | 3.4 | 4.7 |
| *spo0H17* | 1.8 | 1.7 | 2.0 |
| *spo0H81* | 1.3 | 1.4 | 1.6 |
| *spo0J87* | 6.2 | 7.0 | 13.7 |
| *spo0K141* | 1.8 | 1.7 | 2.3 |
| *spoIIA69(IIAA)* | 1.8 | 1.4 | 3.0 |
| *spoIIA1(IIAC)* | 2.0 | 5.0 | 8.0 |
| *spoIIB131* | 1.5 | 1.2 | 1.2 |
| *spoIID66*[d] | 2.4 | 2.7 | 4.4 |
| *spoIID298* | 4.2 | 6.0 | 9.4 |
| *spoIIE64* | 1.9 | 2.0 | 2.2 |
| *spoIIG41* | 1.9 | 2.1 | 2.5 |
| *spoIIIA53* | 3.0 | 4.0 | 26.0 |
| *spoIIIB2*[d] | 2.0 | 3.0 | 3.8 |
| *spoIIIC94*[d] | 2.0 | 2.5 | 3.0 |
| *spoIIID83*[d] | 2.5 | 3.0 | 5.0 |
| *spoIIIE36* | 2.5 | 3.8 | 15.5 |
| *spoIVA178* | 5.6 | 12.2 | 23.6 |
| *spoIVB165* | 15.5 | 19.4 | 27.0 |
| *spoIVC133* | 2.0 | 3.0 | 16.5 |
| *spoVE85* | 7.0 | 11.0 | 13.2 |

[a] Wild-type strain UOT0531 and *spo* mutants were lysogenized with the specialized transducing phage ϕVEZ437. The obtained transductants were grown in SSM, harvested at intervals, and assayed for β-galactosidase activities as described in the text.
[b] ONPG, *o*-nitrophenyl-β-D-galactopyranoside.
[c] Strain UOT0531 lysogenized with ϕCM.
[d] The appearance of β-galactosidase was delayed. The activity at $T_8$ was 6.5, 11.2, 5.2, 21.2, and 2.6 for *spoIID66*, *spoIIIB2*, *spoIIIC94*, *spoIIID83*, and ϕCM, respectively.

the *spoVE* structural gene. S1 nuclease mapping showed the appearance of two nuclease-protected bands which could be detected about 2 h after the onset of sporulation. Further analysis by high-resolution primer extension mapping showed the precise transcription initiation sites and the −10 and −35 regions. It is interesting that these two consensus regions of the P2 promoter are remarkably similar to those recognized by $\sigma^E$.

Expression of the transcriptional *spoVE*-subtilisin fusions demonstrates that both P1 and P2 are functional in vivo. Expression is induced at about 2 h after the onset of sporulation. Use of a translational *lacZ* fusion also indicated that the P2 promoter and SD sequences are functional in *B. subtilis* 1A423. Results obtained from the transcriptional

TABLE 3. Sporulation frequencies of the 4309 (*spo*$^+$ *recE4*) strains harboring pKBVE derivatives

| Plasmid | Heat-resistant spores (no./ml) |
|---|---|
| None | $1.30 \times 10^8$ |
| pKB301 | $1.05 \times 10^8$ |
| pKBVEP1 | $1.46 \times 10^4$ |
| pKBVEP2 | $1.00 \times 10^4$ |
| pKBVEP1P2 | $7.85 \times 10^3$ |

*spoVE*-subtilisin and translational *spoVE-lacZ* fusions coincided well with results obtained from the runoff transcription and in vitro SpoVE protein synthesis experiments (14). In addition, the expression of the *spoVE*-subtilisin gene was shown to be prevented by glucose, which also prevents sporulation, suggesting that the induction is under the control of catabolite repression. Moreover, the inhibition of sporulation caused by multiple copies of the *spoVE* promoter-containing fragments might indicate the possible titration of a specific transcription factor(s) by *spoVE* promoters. A similar phenomenon was previously observed for the *spoVG* promoter (30).

Expression of *spoVE* in various *spo* mutants, investigated by both S1 nuclease mapping and use of a *lacZ* translational fusion, indicated that all mutations that block the expression of *spoIIG*, which encodes $\sigma^E$, also block the expression of *spoVE*. The following results also support the idea that the *spoVE* promoter is recognized by $E\sigma^E$: (i) coincidence of the timing of expression ($T_2$) of *spoVE* with the appearance of $E\sigma^E$ and (ii) similarity of the −10 and −35 sequences to the consensus $\sigma^E$ recognition sequences. In the accompanying paper, Miyao et al. (14) show that the *spoVE* gene is transcribed by purified $E\sigma^E$.

Previously, Okamoto et al. (16) investigated *spoVE* mRNA synthesis by dot blot analysis with a part of the *spoVE* structural gene (*Hin*dIII B fragment [Fig. 1]) as a probe. The results indicated that *spoVE* mRNA is synthesized during vegetative-growth phase and that synthesis is not prevented by early *spo* mutations. However, SpoVE protein was synthesized specifically by $T_2$ cell extracts when pUCVE1, a derivative of pUC18 carrying the 2.8-kb *Eco*RI fragment inserted in the opposite orientation to the *lac* promoter, was used as a template (16). Bugaichuk (1) also investigated the transcriptional regulation of the *spoVE* gene with a *spoVE-lacZ* transcriptional fusion which was integrated into the chromosome. He found that the expression of β-galactosidase could be detected about 40 min after the onset of sporulation and that mutations in *spo0H* or *spo0K* but not *spoIIA* or *spoIIG* impeded the expression of *spoVE*. These results are contradictory to the data presented in this paper but would be understandable if an additional promoter(s) located far upstream of the *spoVE* gene were functional during vegetative-growth phase and were not dependent on *spo0* or *spoII* gene products. This is likely because the *spoVE* gene may be located in a large operon concerned with peptidoglycan synthesis (7, 15). The transcript(s) from the upstream promoter(s) may read through the terminator-like structures located at the ends of ORF1 and ORF2 (*spoVE*) and terminate at the end of ORF3 (on the basis of studies of integrative plasmids [unpublished data]). The transcriptional *spoVE-lacZ* fusion constructed by Bugaichuk (1) contained both P1 and P2 promoters. This may be the reason why β-galactosidase activity could be detected in a *spoIIG* mutant since our result also showed that transcript from P1 promoter could be detected in a *spoIIG41* mutant. It should be noted that in the expression experiments reported here, the *spoVE* gene was carried on a plasmid or integrated at a phage locus. As a result, transcription from promoters far upstream would not have been measured.

The *spoVE* gene may be located in an operon that plays an important role in cell division of *B. subtilis* (7, 15). Analysis of the *spoVE* transcriptional unit with integrational plasmids has been carried out many times, but very few normal transformants were obtained whereas many small colonies showing abnormal phenotypes could be observed. Furthermore, analysis of the DNA isolated from the normal transformants indicated that the integrative plasmids were not integrated via a Campbell-type mechanism (unpublished data). A similar result was previously observed by P. J. Piggot et al. (18). Further analysis of plasmid integration suggested that *spoVE* is located in an operon which causes an abnormal phenotype when it is disrupted. A similar result was obtained for the *B. subtilis rodC* operon (6). Recently, it was found that the *spoVE* gene product is homologous to the newly identified *E. coli ftsW* gene product, in which mutations induce filamentous cell growth at the nonpermissive temperature, and to the newly sequenced *E. coli rodA* gene product (7, 10). These results suggest that SpoVE or the succeeding gene product MurG (15) plays an essential role not only during sporulation but also during vegetative growth. In fact, Henriques et al. (5) have recently reported that *murG* is essential for normal growth, although *spoVE* is only required for sporulation.

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