JOURNAL OF BACTERIOLOGY, Apr. 1994, p. 2003–2012
0021-9193/94/$04.00+0

Vol. 176, No. 7

Cloning and Characterization of *spoVR*, a Gene from *Bacillus subtilis* Involved in Spore Cortex Formation

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Received 17 December 1993/Accepted 28 January 1994

Screening for σ^E-dependent promoters led to the isolation of a gene from *Bacillus subtilis*, designated *spoVR*, which appears to be involved in spore cortex formation. Cultures of strains carrying mutations in *spoVR* had an increased proportion of phase-dark spores, which correlated with an increased proportion of cortexless spores seen by electron microscopy. The numbers of heat- and chloroform-resistant phase-bright spores produced by these mutants were decreased by about 3- to 10-fold, and accumulation of dipicolinate was decreased by more than 3-fold. The *spoVR* gene was located on the *B. subtilis* chromosome immediately upstream from, and in the opposite orientation of, the *phoAIV* gene. Expression of *spoVR* was initiated at the second hour of sporulation from a σ^E-dependent promoter, and this expression did not require any of the other known mother-cell-specific transcriptional regulators. The *spoVR* gene was predicted to encode a product of 468 residues.

During endospore formation in *Bacillus subtilis*, the cell undergoes a series of complex morphological alterations. After an asymmetrical cell division that produces a small cell and a large cell, three remarkable morphological changes occur (reviewed in references 11, 12, and 26). In the first of these, the larger cell (mother cell) engulfs the smaller cell. This process produces a small protoplast (the forespore) surrounded by two bilayer membranes which separate it from the cytoplasm of the mother cell. The next most conspicuous structure to form is a thick peptidoglycan layer that is deposited between the two membranes of the forespore protoplast, forming the spore cortex. In the final stages of differentiation, a complex coat consisting of more than a dozen proteins is layered around the outside of the forespore membranes.

Little is known about the enzymes and structural proteins that produce these morphological changes during endospore formation. Genetic studies have identified genes whose products may be directly involved in these morphogenetic processes. For example, mutations in *spoIID* prevent the engulfment step (16). It is not known whether the *spoIID* product is directly involved; however, since its amino acid sequence is similar to the sequence of the N-terminal portion of a modifier of an *N*-acetylmuramoyl-L-alanine amidase (21), the *spoIID* product may affect the hydrolysis of the spore septum peptidoglycan that precedes forespore engulfment. Several proteins that may play direct roles in cortex formation have been identified (e.g., the products of *spoVB* [27], *spoVD* [6], *spoVE* [14, 33], and *spoVM* [22]), and several morphogenetic proteins, the products of *cotE*, *spoIVA*, and *spoVID*, have recently been shown to play a role in the assembly of the spore coat (9). The exact role of each of these proteins is unknown, and it is likely that these complex morphogenetic processes require a number of proteins and enzymes that have not yet been identified.

Although all the genes encoding the morphogenetic functions required for cortex and coat formation have not been identified, it is likely that many of these (probably all for coat synthesis) are expressed exclusively after the onset of sporulation. Moreover, the expression of these genes may be controlled by σ^E, one of the four sporulation-induced sigma factors that control gene expression during sporulation (reviewed in references 11 and 13), since σ^E is active during the period of development in which the dramatic morphological changes occur, and σ^E-deficient mutants fail to complete the engulfment step and fail to express components of the cortex and spore coat. Furthermore, transcription of all the genes listed above, which encode morphogenetic proteins, is directed by σ^E. For these reasons, we have been isolating new genes that are transcribed during sporulation by RNA polymerase containing σ^E in order to identify genes that may control these morphogenetic processes. In this report, we describe a new gene, *spoVR*, that appears to affect spore cortex formation.

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MATERIALS AND METHODS

Strains. All strains are listed with relevant markers in Table 1. EU8702 is a strain in which the chromosomal *sigE* gene has been deleted and which carries plasmid pDG180 (2). EU101 has a single wild-type *sigE* allele under the control of the space promoter (32). SPβ phages containing transcriptional fusions of *lacZ* to *cotB* and *gerE* were provided by R. Losick, and those containing fusions to *sspE* were provided by P. Setlow. A transcriptional *lacZ* fusion to *spoVF* (within the *spoVFA* structural gene) was isolated during this work.

Plasmids. The DNA fragments within integrational plasmids used for determining the functional boundaries of the *spoVR* gene are depicted in Fig. 1. pTV17 is a plasmid that replicates in *B. subtilis* and contains Tn*917* (35). Plasmid pUS5V5 and the other plasmids shown in Fig. 1 are derivatives of the integrational plasmid pUS19, which has a spectinomycin resistance determinant selectable in *B. subtilis* in single copy (9). To obtain the 3′ end of the *spoVR* gene and further downstream DNA, pUS5V5 was used to transform strain 168 to spectinomycin resistance, and the chromosomal DNA was then cleaved with *Hin*dIII. The DNA was ligated and used to transform *Escherichia coli* JM109 to ampicillin and spectinomycin resistance, resulting in plasmid pUS5H. Plasmid pUS5sph was obtained in the same way except that *Sph*I was used instead of *Hin*dIII. Plasmid p5BgK carries a 1.5-kb *Bam*HI fragment containing a kanamycin resistance gene from pDG102 (9) cloned into the *Bgl*II site of pUS5V3 within the *spoVR* gene

TABLE 1. Bacterial strains

Strain	Relevant genotype	Source or reference
B. subtilis		
PY79	Spo$^+$	Laboratory stock
JH642	Spo$^+$, *trpC2 pheA1*	Laboratory stock
CU1050	*leu8 metB5 thrA5 sup-3 pla-1* SPβ^s	Laboratory stock
EU8701	JH642 *spoIIGBdel::erm*	19
EU101	*Pspac-sigE*	32
EU8702	EU8701 (pDG180)	
M01027	JH642 *spoIVCB::erm*	Patrick Stragier
BK541	PY79 *spoIIIDdel::erm*	20
E. coli		
JM101	del (*lac-proAB*) F′ *traD36 proAB* del (*lacZ*) M15 *lacI*^{q}	23
JM109	del (*lac proAB*) *hsdR17 recA1* F′ *traD36 proAB* del (*lacZ*)M15 *lacI*^{q}	23

(Fig. 1). Plasmid p5Bclac contains the *lac-cat* cassette from pSGMU38 (10) cloned into the *BclI* site of pUS5V3 in the same orientation as orf1 (Fig. 1).

Cloning the *spoVR* promoter and structural gene. The procedure for isolating and cloning the *spoVR-lacZ* fusion present in SPβE5 was similar to the method described previously (2), except that in the current screen the wild-type *sigE* allele encoding pro-σ^E was placed under the control of the inducible space promoter in strain EU101 (32). This procedure included transducing strain EU101 to chloramphenicol resistance with an SPβ library (provided by P. Zuber) containing random 1- to 5-kb chromosomal *Sau*3A fragments of the *B. subtilis* chromosome cloned in front of the *lacZ* structural gene. Transductants were selected on DSM agar containing 10 mM X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and 5 μg of chloramphenicol per ml.

Sporulation, germination, and β-galactosidase assays. Sporulation was carried out by growth and exhaustion in DS medium as described by Schaefer et al. (30). Resistance of cultures and purified spores to heat, chloroform, and lysozyme was tested as described in reference 24. Dipicolinate (DPA) assays were also performed as described in reference 24. Germination was assayed in 10 mM D-alanine–AGFK (30 mM L-asparagine, 10 mM glucose, 10 mM fructose, 10 mM KCl)–Difco PAB as previously described with Renografin-purified spores (34). Spore germination was also assessed qualitatively by the tetrazolium overlay method (24). β-Galactosidase assays were performed as previously described (8).

Primer extension of mRNA. RNA was prepared from cells grown in L broth or DS medium as previously described (2). The primer GTAATTTCCTCAATTGCCCG, which anneals to the noncoding strand of the *spoVR* gene at positions 869 to 888, was end labelled with [γ-^{32}P]ATP and annealed to 40 μg of total RNA from *B. subtilis* cultures, and primer extensions were carried out with avian myeloblastosis virus reverse transcriptase as previously described (28).

DNA sequencing. Plasmid DNA was prepared from small-scale cultures of both *E. coli* and *B. subtilis* cultures by the same method as described in reference 3. Plasmid pTVE5 prepared from small-scale *B. subtilis* PY222 (pTVE5) was used to transform *E. coli* HB101 to ampicillin resistance, from which

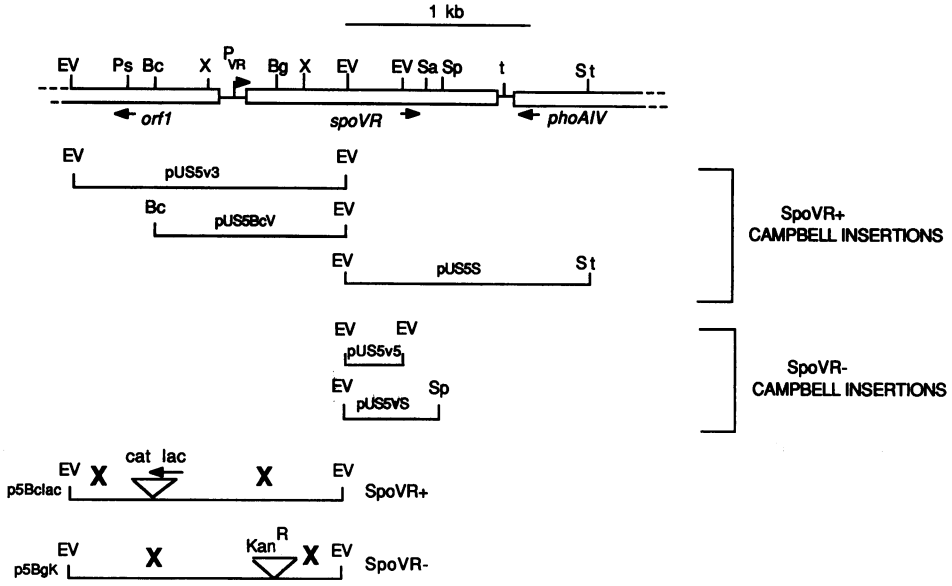

FIG. 1. Chromosomal region and integrational analysis of the *spoVR* region. The figure represents a map of the DNA sequenced in the *spoVR* region. Just upstream of *phoAIV* is a potential transcriptional terminator (t). On the figure, the *spoVR* stop codon is located to the left of this putative terminator, which could function to terminate transcripts originating from the convergent *phoAIV* and *spoVR* promoters. Just upstream of *spoVR* is another open reading frame, orf1, which lies in the opposite orientation. The restriction fragments shown were cloned into the integrational vector pUS19. The names of the resultant plasmids are shown above each restriction fragment. These plasmids were integrated into the chromosome by transformation of strain PY79. The sporulation phenotypes of strains containing the integrated plasmids are indicated at the right of the figure. Linearized plasmid p5Bclac was used to introduce a *lacZ* fusion cassette within the *BclI* site of orf1 by the double-crossover event depicted. Similarly, p5BgK was used to replace the wild-type chromosomal *spoVR* gene with a disrupted allele containing the Kan^r cassette within the *BglII* site of *spoVR*. The *Sau*IIIA site (Sa) shown within *spoVR* is the boundary of the chromosomal sequence transcriptionally fused to *lacZ* in phage SPβE5. Abbreviations: Bc, *BclI*; X, *Xmn*I; Sa, *Sau*IIIA; EV, *Eco*RV; Ps, *Pst*I; Bg, *Bgl*II; Sp, *Sph*I; St, *Stu*I.

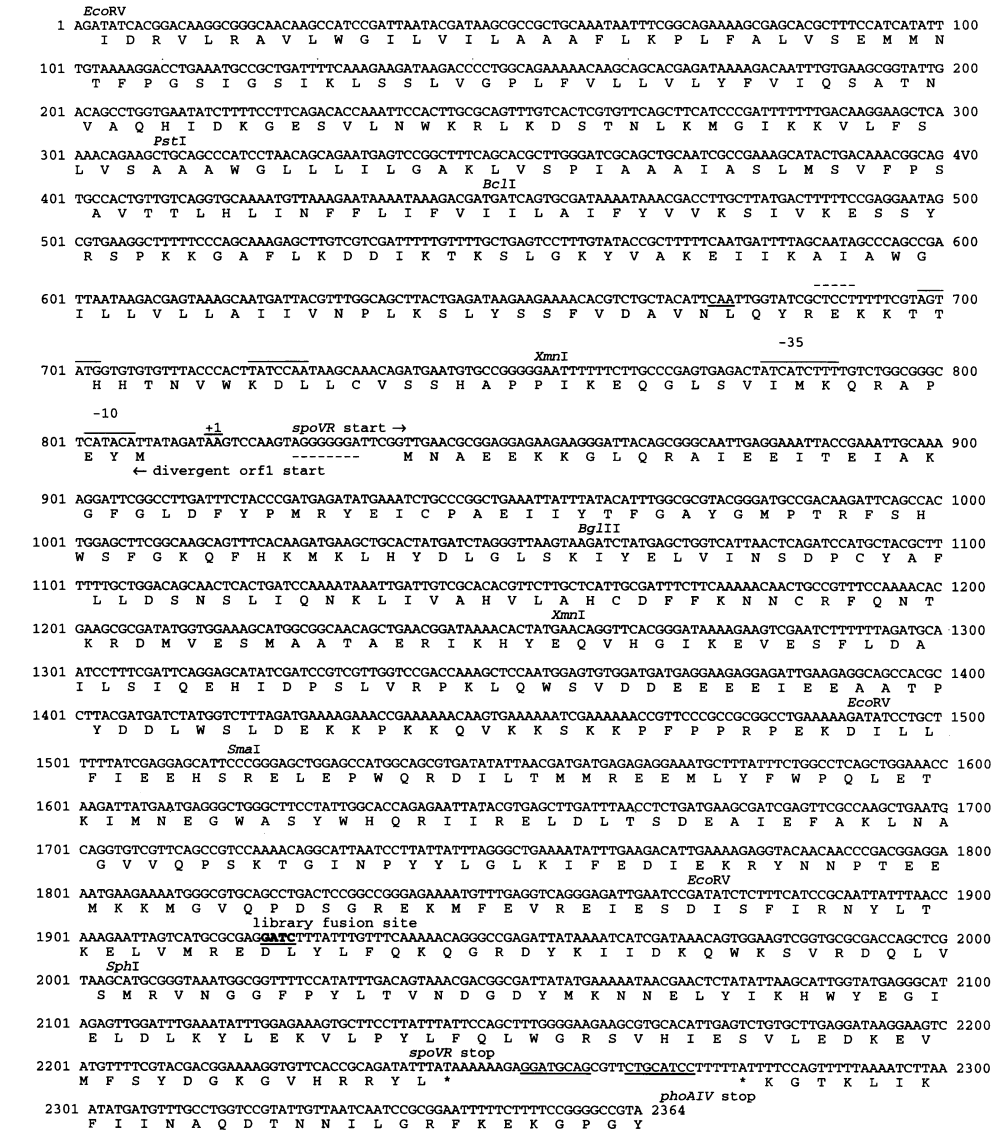

FIG. 2. Nucleotide sequence and translation of the *spoVR* region. The −35 and −10 sequences of the *spoVR* promoter are indicated by a solid overline, as are the potential promoter sequences for orf1. Potential ribosome binding sites are indicated by a dashed underline (*spoVR*) or dashed overline (orf1). Inverted repeats of a potential transcriptional terminator downstream of *spoVR* are underlined. The sequences of divergent open reading frames, orf1 and *phoAIV*, immediately upstream and downstream, respectively, of *spoVR* are also shown. Note that, although both strands were fully sequenced, the DNA sequence shown represents the nontranscribed strand of *spoVR* and the transcribed strand for the divergent open reading frames. Asterisks indicate stop codons.

plasmid was prepared. Plasmid DNA was sequenced according to the Sequenase protocol (U.S. Biochemical Corp.) for double-stranded DNA with a primer to the 5′ end of the *lacZ* gene. Additionally, appropriate M13 subclones of the *spoVR* region were sequenced by the Sequenase protocol.

Nucleotide sequence accession number. The DNA sequence reported here has been deposited in GenBank with the accession number L26337.

RESULTS

Cloning *spoVR*. To identify genes from *B. subtilis* that are transcribed by σ^E-RNA polymerase, we used an approach similar to one that we described previously (2) in which we screened for σ^E-dependent promoters in a phage SPβ-borne library of random *B. subtilis* DNA fragments fused to *lacZ*. In the previous study, the collection of transducing phage was used to lysogenize *B. subtilis* cells that had been engineered to produce an active form of σ^E (that is, lacking the pro-amino acid sequence) in response to IPTG. Those lysogens were screened for transductants that produced β-galactosidase in response to IPTG and hence contained *lacZ* fused to a gene whose transcription is directed by σ^E. For reasons that remain obscure, strains containing the engineered active form of σ^E fail to complete sporulation in spite of their ability to express σ^E-dependent promoters. This sporulation defect offers the advantage that the LacZ-positive transductants probably contain promoter fusions that are directly dependent on σ^E, rather than promoters that are dependent on other sigma factors that

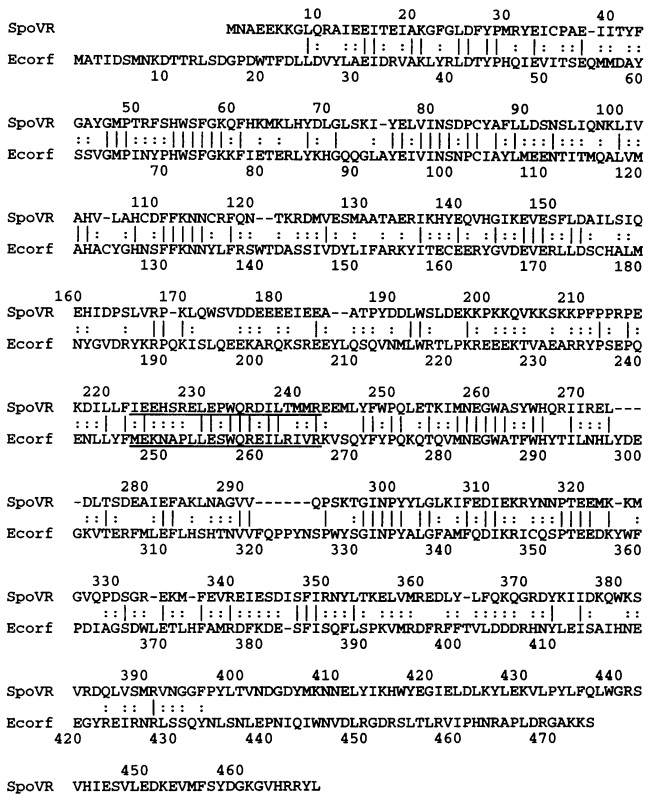

FIG. 3. Comparison of SpoVR with a deduced product of an open reading frame in *E. coli* with unknown function. The two sequences (SpoVR and Ecorf) were compared by using the method of Pearson and Lipman (25). Identical residues are indicated by a line, and conservative substitutions are shown by two dots. A possible DNA binding region shared by the two proteins is underlined at positions 223 to 242 of SpoVR and 247 to 266 of the *E. coli* protein.

are produced during the later stages of sporulation after the period in which σ^E is active. In the present study, however, we chose to use a strain in which expression of a wild-type allele of *sigE* (the σ^E structural gene) was dependent on an IPTG-inducible promoter. Induction of this *sigE* allele not only results in expression of σ^E-dependent genes but also enables the strain to complete endospore formation. By screening the phage library in this strain (EU101), we expected to find genes that were directly dependent on σ^E, as well as genes whose expression was indirectly dependent on σ^E, for example, those that are transcribed by σ^E-RNA polymerase.

We used the library of SPβ phage to transduce strain EU101 and screened 8,000 transductants on DS agar plates for transductants that produced β-galactosidase when IPTG was present but not in its absence. We identified 26 IPTG-dependent Lac$^+$ transductants. Preliminary characterization of most of these transductants showed that *lacZ* was induced about 2 h after the end of the exponential growth phase in DS medium, about the time that pro-σ^E is processed to the active form. However, β-galactosidase production was induced 4 h after the onset of sporulation in three transductants, indicating that these strains may contain promoter fusions that are activated by factors other than σ^E. The promoter-*lacZ* fusion from each transducing phage was crossed onto plasmid pTV17 as described previously (2) in order to move the fusion into *E. coli*, in which the DNA was amplified for purification, subcloning, and sequence analysis.

The three transductants in which *lacZ* was induced after the fourth hour of sporulation were found to contain *lacZ* fusions to promoters that are very similar to those utilized by σ^K-RNA polymerase (data not shown). One of these contained a fusion to the *spoVF* operon, which is known to be transcribed by σ^K-RNA polymerase (7). Primer extension analysis combined with genetic analysis strongly suggests that the other two promoters are also used by σ^K-RNA polymerase, and these will be the subject of future studies (data not shown).

Those transductants in which *lacZ* was induced about 2 h after the onset of sporulation were thought likely to contain fusions to σ^E-dependent promoters. Subsequent analysis showed that several of these contained fusions to previously described σ^E-dependent promoters (data not shown). These included three independent isolates containing fusions to *spoIID* (29), one isolate containing a fusion to *dacB* (4), and one containing a fusion to *cotE* (36, 37). We also found six different σ^E-dependent *lacZ* fusions that appear to be in genes that have not been described previously, since searches of the GenBank bacterial sequence base did not reveal that these sequences had been deposited. The characterization of one of these genes, *spoVR*, is described below.

Strains containing fusions to *spoVR* were isolated independently twice. To clone the DNA containing the *spoVR-lacZ* transcriptional fusion contained in one transductant, chromosomal DNA from the transductant, which carried the *spoVR-lacZ* fusion in the prophage designated SPβE5, was used to transform *B. subtilis* PY222 (pTV17) to chloramphenicol resistance and LacZ$^+$, resulting in a strain harboring recombinant plasmid pTVE5. Sequence analysis of pTVE5 with a primer to the *lacZ* gene revealed that the *lacZ* proximal *Sau*IIIA site was within an open reading frame (Fig. 2, position 1923). Plasmid pUS5V5 (Fig. 1) was then constructed and used to transform strain 168 to spectinomycin resistance, which resulted in Campbell-type insertion of the entire plasmid into the chromosome. DNA from one transformant was cleaved with *Hin*dIII, ligated, and used to transform *E. coli* JM109 to ampicillin and spectinomycin resistance. The plasmid obtained from these transformants, pUS5H, contained the rest of the *spoVR* gene and about 2.7 kb of sequence downstream of *spoVR*.

Nucleotide sequence of the *spoVR* region. Restriction fragments from plasmid pTVE5 were subcloned into M13 phages mp18 and mp19 as well as plasmid pUS19 and used for DNA sequence analysis (as described in Materials and Methods). The DNA sequence of the pTVE5-derived subclones revealed an open reading frame that is preceded by a potential ribosome binding site (Fig. 2, positions 826 to 833) and extends 359 codons to the junction of the *lacZ* fusion (Fig. 2, position 1924). The sequence obtained with subclones of pUS5H, which overlapped and extended downstream of the library junction site, extended this open reading frame to 468 codons and also revealed the 3′ end of the *phoAIV* gene, which encodes a vegetatively expressed alkaline phosphatase (15) (Fig. 2). The *phoAIV* gene is located at about 73° on the genetic map (18), in the opposite orientation from *spoVR*. Between the 468-codon open reading frame designated *spoVR* (see below) and *phoAIV* lies a DNA sequence characteristic of a transcriptional terminator, which may terminate transcripts from the *spoVR* promoter, as well as the converging transcripts from the *phoAIV* promoter (Fig. 1 and 2, positions 2251 to 2276).

A data base (GenBank) search revealed a high degree of similarity of the deduced *spoVR* gene product to a deduced gene product of unknown function from *E. coli* (accession no.

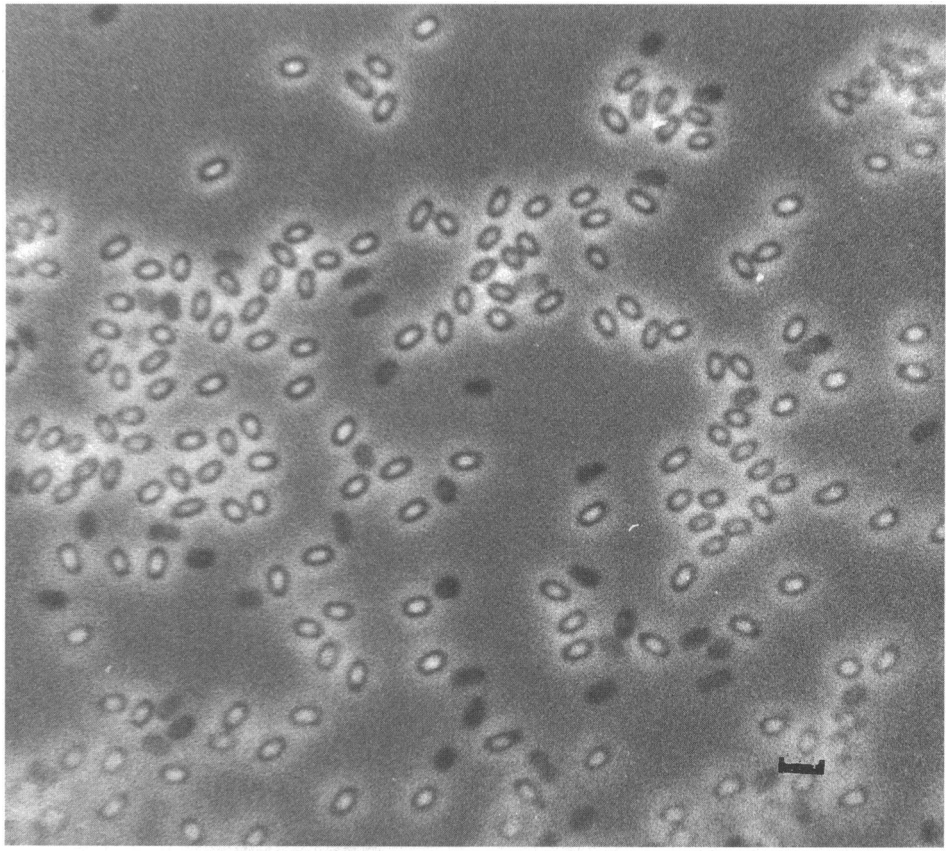

FIG. 4. Phase-contrast microscopy of *spoVR* mutant spores. Spores were purified by centrifugation through Renografin. The size bar indicates 1,000 nm.

P29013) (Fig. 3). Both proteins have a predicted isoelectric point of about 5.5 to 5.9. The hydropathy profiles of the two proteins are similar along most of their lengths (not shown), with neither of the two proteins having hydrophobic sequences typical of integral membrane proteins. Analyses of the two proteins with the Regulat program of PC/Gene, release 6.01, from IntelliGenetics, Inc., revealed potential helix-turn-helix DNA binding motifs typical of transcriptional regulatory proteins (residues 223 to 242 for *spoVR* and 247 to 266 for the *E. coli* hypothetical protein) (Fig. 3).

The open reading frame encoding the *spoVR* homolog lies adjacent to the 5′ end of the *E. coli dadA* operon (accession no. L02948), although in the opposite orientation. No function has been deduced for this open reading frame found adjacent to the *dadA* operon in *E. coli*. It is interesting that there is also a long open reading frame adjacent to the 5′ end of *spoVR* that is in the opposite orientation from *spoVR* (Fig. 1). The deduced product of this open reading frame has no obvious significant homology to the *dadA* gene product which encodes D-alanine dehydrogenase. However, the *dadA* gene product has two potential membrane-spanning regions characteristic of integral membrane proteins, and it may be worth noting that this deduced product encoded upstream of *spoVR* also has several potential membrane-spanning sequences. Also notable is a sequence similar to promoters directed by σ^E-RNA polymerase just upstream of a potential translation start site for this open reading frame (Fig. 2).

Insertional mutagenesis of the *spoVR* region. To determine

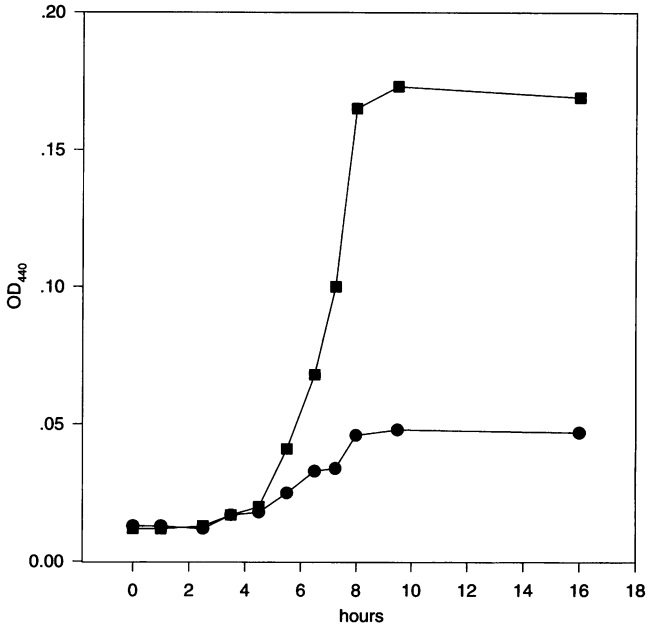

FIG. 5. DPA accumulation in a *spoVR* mutant. Cultures of the *spoVR* mutant (●) and the wild-type strain (■) were allowed to sporulate in DS medium, and aliquots were harvested at the indicated time points. Time zero (0) corresponds to the onset of sporulation. DPA was assayed by the calorimetric assay described in reference 24 and is expressed as units of optical density at 440 nm, which are directly related to DPA concentration.

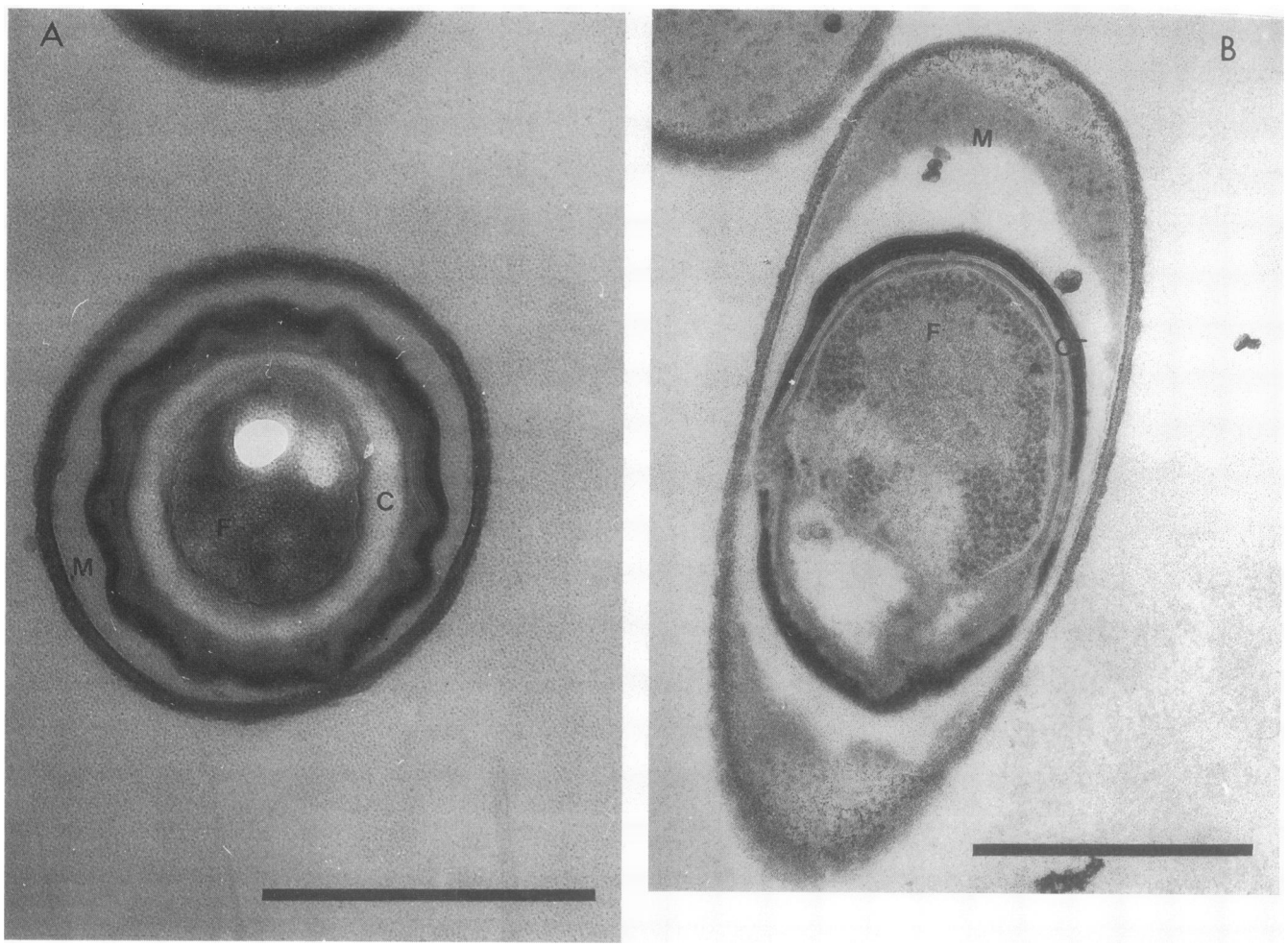

FIG. 6. Electron microscopy of a *spoVR* mutant. Strain PY79ΩpUS5v5 was allowed to sporulate, and samples were examined at hour 16 of sporulation. (A) Wild-type sporangia with the forespore (F) surrounded by the cortex (C) and the coat (CT); M indicates the mother cell compartment. (B and C) *spoVR* mutant sporangia in which little or no cortex is seen surrounding the forespore. (D) *spoVR* mutant sporangium in which cortex appears more diffuse than normal. Size bars indicate 500 nm.

the effects of inactivation of *spoVR*, and to determine its functional boundaries, fragments of the gene were subcloned into the vector pUS19, which cannot replicate in *B. subtilis* but contains a spectinomycin resistance gene that is selectable in this host. Integration of plasmids pUS5V5 and pUS5VS, which contain 375- and 570-bp internal *spoVR* gene fragments, respectively (Fig. 1), resulted in about a 3- to 10-fold reduction of lysozyme-, heat-, and chloroform-resistant spores, presumably from disruption of the *spoVR* gene (data not shown). Insertion of these plasmids resulted in the formation of free phase-dark spores which appeared to outnumber the phase-bright spores by at least a 3:1 ratio after overnight incubation in DS medium (not shown). The defective sporulation phenotype of this strain was easily detectable upon examination of DS agar plates after 1 to 2 days of incubation at 37°C, since colonies of the *spoVR* mutant were more translucent than colonies of the parental strain.

We also wanted to isolate a mutant containing an insertion in *spoVR* that resulted from a double crossover. Therefore, a 1.5-kb kanamycin resistance cassette was inserted into the *spoVR* coding region in vitro as described in Materials and Methods. The plasmid carrying this allele of *spoVR*, p5BgK (Fig. 1), was linearized with *Bam*HI and used to transform strain PY79 to kanamycin resistance. Resulting transformants were spectinomycin sensitive, indicating the absence of vector sequence, and indicating that a double- rather than single-crossover recombinational event had occurred. These transformants, in which *spoVR* was replaced with the *spoVR* allele containing a 1.5-kb kanamycin resistance cassette at the *Bgl*II site within *spoVR* (Fig. 1), had phenotypes identical to those of strains containing *spoVR* alleles that were disrupted by the Campbell-type insertions of plasmids pUS5V5 and pUS5VS.

Campbell-type integration of plasmid pUS5BcV, which contained a fragment that included the 5′ end of *spoVR* and its σ^E-dependent promoter, and integration of plasmid pUS5S, which contained a fragment that included the 3′ end of *spoVR* and extended into the *phoAIV* gene, conferred no detectable sporulation or germination phenotypes (Fig. 1). We also inactivated the divergent open reading frame directly upstream of *spoVR* by replacement with a fragment carried on plasmid p5BcK that contained an insertion of a *lacZ-cat* cassette in the *Bcl*I site at position 458 (Fig. 1 and 2). This insertion also conferred no detectable growth or sporulation phenotypes. However, it should be noted that expression of *lacZ* from this

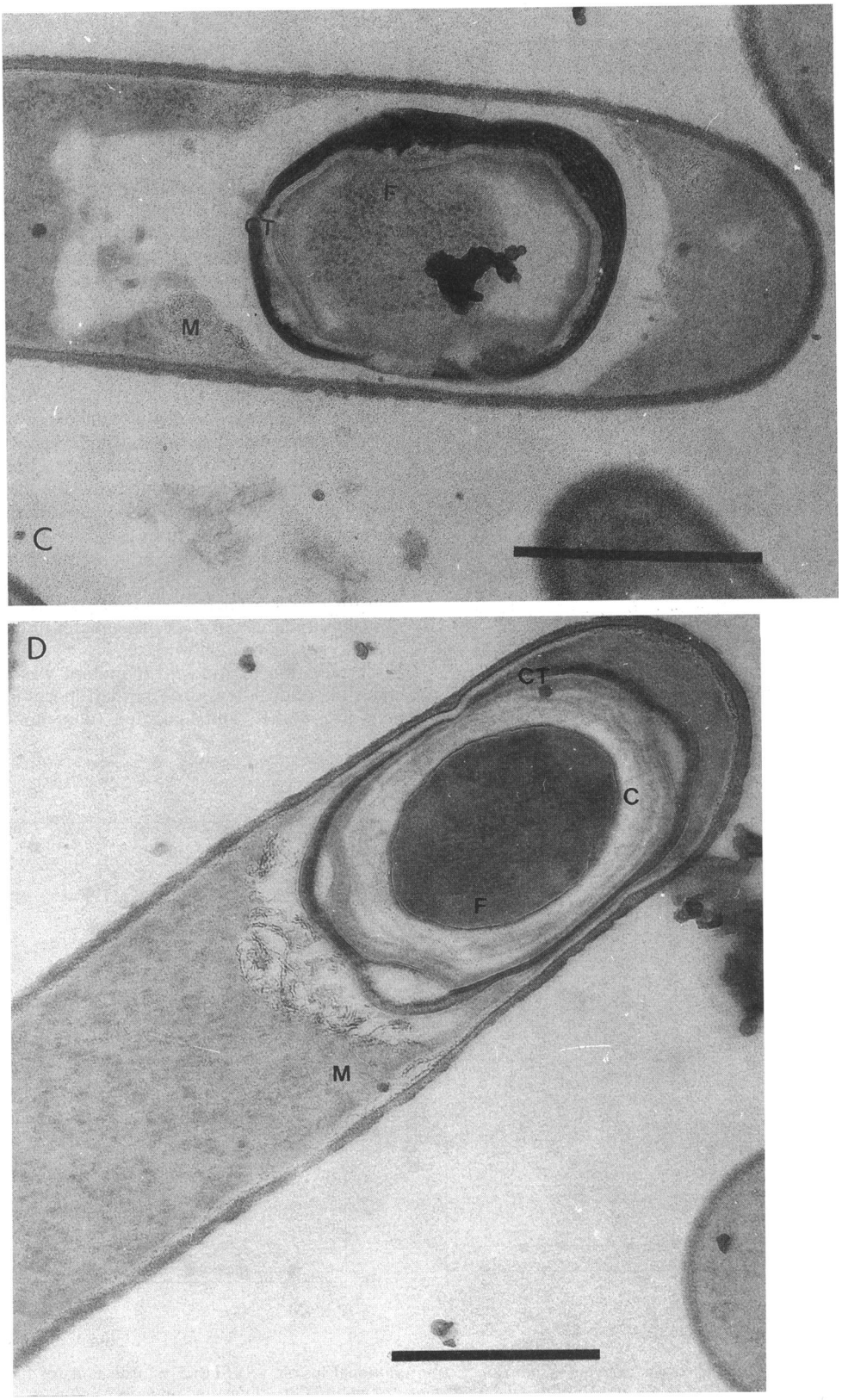

FIG. 6—*Continued.*

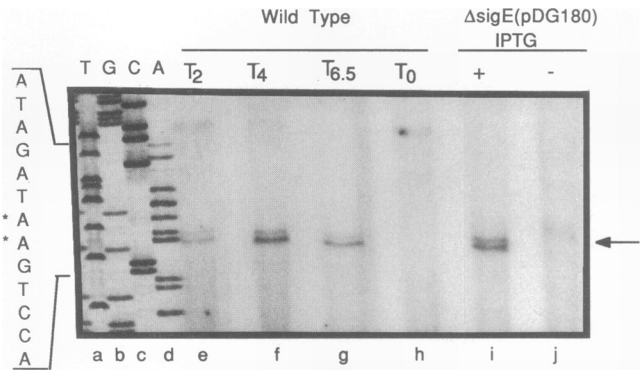

FIG. 7. Mapping the transcription start site of the *spoVR* gene. An oligonucleotide complementary to the 5′ sequence of the nontranscribed strand of the *spoVR* structural gene was used to prime cDNA synthesis from total RNA prepared from sporulating DS cultures of strain 168 (lanes e to h) at the indicated times relative to the onset of sporulation. Also, total RNA was used from mid-log-phase cultures of strain EU8701 (pDG180) in the presence and absence of 1 mM IPTG (lanes i and j). Plasmid pDG180 contains an allele of *sigE* that encodes an active form of σ^E under spac promoter control. The primer extensions shown were generated with equal amounts of total RNA. The asterisks correspond to the transcription start site of *spoVR* and the start of each extension product (arrow). The DNA sequence in lanes a to d was generated on the nontranscribed strand in an M13 subclone with the same oligonucleotide used for the primer extensions.

transcriptional fusion, albeit weak, increased by hour 2 to 6 of sporulation. This transcription was not investigated further.

Disruption of *spoVR* affects cortex formation. Examination of *spoVR* mutants by phase-contrast microscopy of sporulated cultures (not shown) and Renografin-purified spores (Fig. 4) revealed that, unlike wild-type strains, these mutants accumulated a large fraction of phase-dark spores that did not become phase bright. We also found that the mutant spores accumulated about threefold less DPA than did wild-type strains (Fig. 5). We found that expression of *spoVF*, which encodes DPA synthetase, was normal in the *spoVR* mutant (not shown); therefore, it appears likely that DPA is synthesized in the *spoVR* mutant but does not accumulate to normal levels. Furthermore, addition of DPA at a level known to rescue the sporulation defect of a DPA^- mutant (1) had no effect on the *spoVR* mutant phenotype (data not shown).

Electron microscopy of sporulated cultures revealed that many *spoVR* mutant sporangia were entirely or almost entirely lacking cortex, although spore coat layers were evident (Fig. 6b and c). Additionally, many *spoVR* mutant spores contained cortex that appeared more diffuse than that in wild-type spores (Fig. 6d and a).

Free phase-dark spores of the *spoVR* mutant copurified through Renografin gradients with phase-bright spores (Fig. 4), although, after pelleting through Renografin, the ratio of phase-dark to phase-bright spores had shifted from a 3:1 ratio in 24-h DSM cultures to about a 1:3 ratio, indicating that many of the phase-dark spores may be less dense. Electron microscopy of purified spores from a *spoVR* mutant revealed many spore ghosts which consisted of empty coat structures, indicating that these defective spores were damaged during preparation for electron microscopy (not shown). These ghosts were not seen upon electron microscopic examination of wild-type purified spores (not shown).

Germination of the *spoVR* mutant was monitored by the change in optical density of purified spores in response to PAB medium, AGFK, and L-alanine (data not shown). In these

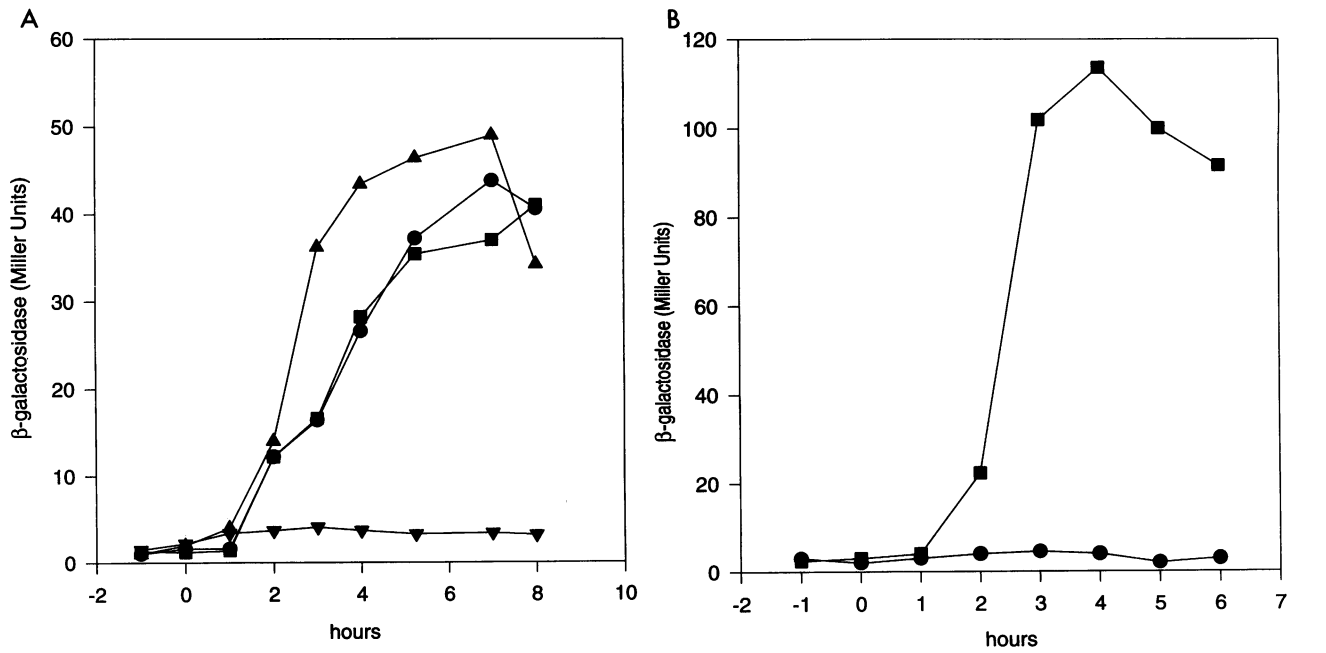

FIG. 8. β-Galactosidase activity of strains carrying a *spoVR-lacZ* transcriptional fusion. (A) SPβE5 transductants of PY79 (wild type) (●), BK541 (*spoIIID*) (■), MO1027 (*spoIVC*) (▲), and EU8701 (*sigE*) (▼) were grown in DS medium, and aliquots were assayed for β-galactosidase activity at the indicated time points. (B) A SPβE5 transductant of EU101 was grown to mid-log phase in DS medium and divided into two cultures. IPTG (1 mM) was added to one of the cultures (■), and no IPTG was added to the other (●). Cells were harvested and assayed for β-galactosidase activity at the indicated time points. Time zero corresponds to the onset of sporulation.

assays, the mutant spores appeared to germinate as efficiently as those of the wild-type parental strain. The *spoVR* mutant spores were also lysozyme and chloroform resistant (data not shown).

To assess whether *spoVR* mutants were defective in expression of genes expressed during the later stages of sporulation, for example, those controlled by σ^K-RNA polymerase in the mother cell, *lacZ* fusions to *cotD*, *gerE*, and *spoVF* carried on SPβ specialized transducing phages were introduced into the *spoVR* mutant. Expression of these fusions in the *spoVR* mutant was not significantly different from expression of these fusions in the sporulation-proficient strain PY79 (data not shown). Similar results were found with an SPβ-borne *sspE-lacZ* fusion, suggesting that genes transcribed by σ^G-RNA polymerase are expressed normally in a *spoVR* mutant (data not shown).

Expression of the *spoVR* gene in *B. subtilis*. Primer extension of total RNA from sporulating cultures of wild-type *B. subtilis* with an end-labelled primer revealed a transcript with a 5′ terminus mapping to position 816 (Fig. 7, lanes a to g). The transcript appeared by about 2 h after the start of sporulation, increased by hour 4, and was still abundant at hour 6.5 of sporulation. The same 5′ terminus was observed by using RNA from vegetatively growing cultures of strain EU8702 that had been induced with IPTG (Fig. 7, lane i). The 5′ terminus of this transcript maps downstream of a sequence (ATCATCTTT-13 bp-CATACA) with high similarity to a consensus sequence for σ^E-directed promoters (Fig. 2) (2). These results taken together with the results of the genetic analysis described below lead us to conclude that this region is used as a promoter by σ^E-RNA polymerase. A faint extension product that mapped upstream of the *spoVR* promoter that was visible at T_0, T_2, and T_4 was also seen (Fig. 7, lanes e, f, and h).

To examine the genetic requirements for *spoVR* expression during sporulation, the *spoVR-lacZ* fusion contained in SPβE5 was examined in *sigE*, *spoIIID*, and *spoIVCB* mutant backgrounds. There was no detectable fusion activity in a *sigE* null mutant (Fig. 8a). Similarly, there was no measurable fusion activity in a strain with *sigE* under space promoter control, unless the inducer IPTG was added to the culture (Fig. 8b). These results show that transcription of *spoVR* is dependent on σ^E. Therefore, the σ^E-dependent transcript identified in the primer extension experiments probably accounts for most *spoVR* transcription. The role, if any, of the faint primer extension product seen before σ^E becomes active is unknown.

There was no apparent effect on *spoVR-lacZ* fusion activity when expressed in a strain with a null mutation in *spoIIID* (Fig. 8a), a gene which is involved in the regulation of some σ^E-dependent promoters (20, 31). Likewise, there was no decrease of expression in a *spoIVCB* mutant, which indicates that σ^K is not required for *spoVR* expression (Fig. 8a).

DISCUSSION

The spore cortex consists of a thick peptidoglycan layer that is deposited on the inner surface of the outer prespore membrane. The cortex is critical for providing spore resistance properties since defective cortex structure has been correlated with sensitivity to heat and organic solvents (1, 11, 26). DPA probably also plays an important role in the heat resistance. DPA accumulates to high levels in wild-type spores, and most mutants defective in its synthesis or accumulation produce temperature-sensitive spores (11). Spores do not attain heat resistance until they become refractile under the phase-contrast microscope, and refractility appears to be largely attributable to the completion of cortex synthesis and dehydration of the spore (17). Spores with completed cortex that fully lack the spore coat are refractile, and several known mutants that are defective in cortex synthesis are either phase dark or only partially refractile (11, 26).

The results presented here suggest that mutations in the *spoVR* gene result in spores that lack a typical spore cortex. Electron microscopy of a *spoVR* mutant revealed that a significant portion of the spores completely or nearly completely lacked cortex. They also appeared to have less than a normal content of cortex because of their phase-dark appearance and decreased accumulation of DPA.

The amount of cortex synthesis can be controlled by limiting concentrations of diaminopimelic acid in sporulating cultures of *Bacillus sphaericus* mutants defective in its synthesis (17). In these experiments, it was shown that refractility and DPA accumulation in the spores required about 20% of the maximum cortex content (17). We found reduced DPA accumulation by a *spoVR* mutant. Since transcription of the *spoVF* operon encoding DPA synthetase is normal in this strain, it is unlikely that DPA synthesis is defective in *spoVR* mutants. Therefore, the reduced accumulation of DPA in the *spoVR* mutant probably results from leakage through the defective, or in some spores completely absent, cortex. In this way, *spoVR* mutants resemble *spoVD* mutants, which lose 50% of synthesized DPA to the surrounding medium, apparently because of an altered cortex structure (5).

Although *spoVR* mutants produce spores that lack typical cortex structures, it seems somewhat unlikely that the *spoVR* product plays a direct role in the enzymatic synthesis of cortex peptidoglycan. The amino acid sequence of the *spoVR* product is not similar to those of other enzymes that are known to be involved in peptidoglycan synthesis. Moreover, 10 to 30% of the spores produced by *spoVR* mutants probably contain nearly wild-type levels of cortex since more than 90% of the wild-type cortex content is thought to be required for the heat resistance exhibited by these spores (17). In our view, it seems more likely that *spoVR* may be involved in regulating cortex synthesis, possibly by interacting with the enzymes that synthesize cortex peptidoglycan. In this regard, it may be interesting to study the effects of inactivation of the *E. coli* homolog of *spoVR*. The function of this homolog is unknown, but given its similarity to SpoVR, it could play a role in regulating peptidoglycan synthesis. SpoVR and its *E. coli* homolog both have a conserved sequence that is similar to those found in helix-turn-helix domains involved in binding to DNA. However, currently there is no evidence that supports a regulatory role at the transcriptional level for these proteins. Instead, the similarity between these proteins may reflect conserved interactions with other proteins involved in peptidoglycan synthesis or structure.

ACKNOWLEDGMENTS

We thank L. Melson for preparing samples for electron microscopy; P. Zuber for the gift of the SPβ phage library; R. Losick, W. Haldenwang, P. Stagier, and P. Setlow for strains; Shashi Pankhaniya for technical assistance; and A. O. Henriques for helpful discussions.

This work was supported by PHS grant AI20319 to C.P.M. and by postdoctoral fellowship AI08490 to B.B. from the National Institutes of Health.

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