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Characterization of *spoIVA*, a Sporulation Gene Involved in Coat Morphogenesis in *Bacillus subtilis*

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We report the cloning and characterization of the *Bacillus subtilis* sporulation locus *spoIVA*, mutations at which cause an unusual defect in spore formation in which the coat misassembles as swirls within the mother cell. We show that *spoIVA* is a single gene of 492 codons that is capable of encoding a polypeptide of 55 kDa. Transcription of *spoIVA* is induced at about the second hour of sporulation by the regulatory protein σ^E from two closely spaced promoters designated P1 and P2. Experiments in which the upstream promoter P1 was removed show that transcription of *spoIVA* from P2 is sufficient for efficient spore formation. Based on these and other findings, we infer that the *spoIVA* gene product is a morphogenetic protein; we discuss its role in the deposition of coat polypeptides around the developing forespore.

Spores of the gram-positive soil bacterium *Bacillus subtilis* are encased in a thick protein shell known as the coat (1, 22, 32, 51). The coat is a complex structure composed of a dozen or more polypeptides that are arranged in an electron-dense outer layer and a lamellar inner layer. These layers protect the spore from certain bacteriocidal agents, such as lysozyme, and also play a role in the responsiveness of the spore to germinants. The coat is produced at a relatively late stage in the process of sporulation when the developing spore or forespore is present as a free protoplast within the mother cell chamber of the sporangium (28, 35). Just before the formation of the coat, a layer of cell wall-like material called the cortex is produced between the membranes that separate the forespore protoplast from the mother cell. Coat biogenesis occurs by the synthesis of the coat polypeptides within the mother cell and their deposition around the mother cell membrane that encases the forespore. Here we describe the characterization of a sporulation gene called *spoIVA*, mutations in which impair cortex formation and cause misassembly of the inner and outer coats as swirls within the mother cell (3, 34, 35) (Fig. 1). Our results show that *spoIVA* is turned on at or just before the onset of coat formation and suggest that its product is a morphogenetic protein that is involved at an early stage of the assembly of the coat. Similar findings are reported in an accompanying paper by Stevens et al. (44).

MATERIALS AND METHODS

Bacterial strains. The *B. subtilis* strains used are listed in Table 1. Except as noted in Table 1, all strains used in this study were derived from the prototrophic wild-type strain PY79 (49).

General methods. Cells were induced to sporulate either by growth and nutrient exhaustion in Difco sporulation (DS) medium as described by Sandman et al. (40) or by suspension in Sterlini-Mandelstam (SM) medium (43). The onset of sporulation was taken as the end of the exponential phase of growth in DS medium or as the time of suspension in SM medium.

Competent *B. subtilis* cells were prepared and transformed by a modification of the method of Dubnau and Davidoff-Abelson (12) as described by Cutting and Vander Horn (8). Chloramphenicol-resistant transformants were selected on agar containing chloramphenicol (5 μg/ml).

Chromosomal DNA was prepared from *B. subtilis* cells as described by Cutting and Vander Horn (8), and all plasmid DNA manipulations were carried out as described by Sambrook et al. (39).

In preparation for sequencing, appropriate fragments were cloned into the phage M13 vector mp18 or mp19. Nucleotide sequence analysis was carried out by the dideoxy-chain termination method of Sanger et al. (42) with single-stranded DNA prepared from the resulting hybrid phages.

Electron microscopy of wild-type and *spoIVA* mutant cells. Cells of strains KS194 and PY79 were induced to sporulate by growing them until the nutrients in DS medium were depleted, and samples were taken 9 and 20 h, respectively, after the onset of sporulation. The cells were then prepared for electron microscopy and examined as described by Cutting et al. (4).

Cloning the *spoIVA* locus. Cloning was carried out by the method of Youngman et al. (49) with the transposon insertion Tn*917*ΩHU194 (41) (herein shown to be located in *spoIVA*) and plasmids pTV20 and pTV21Δ2. The plasmids were linearized and used to transform competent cells of the transposon-bearing strain KS194; the KS194 cells were then subjected to selection for chloramphenicol resistance. Southern hybridization analysis was carried out to confirm that Tn*917*ΩHU194 in the transformants had been replaced by double (marker-replacement) recombination with plasmid-borne derivatives of Tn*917* containing a ColE1 origin of replication and *bla* and *cat* genes. Chromosomal DNA from appropriate transformants was digested with *Sph*I or *Eco*RI, restriction enzymes that were expected to cut once within the Tn*917* derivative and again in flanking DNA, thereby releasing DNA fragments that contained transposon sequences, a ColE1 origin of replication, a *bla* gene, and *B. subtilis* sequences adjacent to one or the other end of the transposon. The resulting restriction fragments were then diluted, circularized with T4 DNA ligase, and used to transform *Escherichia coli*. The *E. coli* were then selected for ampicillin resistance. Plasmids recovered from the resulting transformants were as follows: pSR6 and pSR7, which

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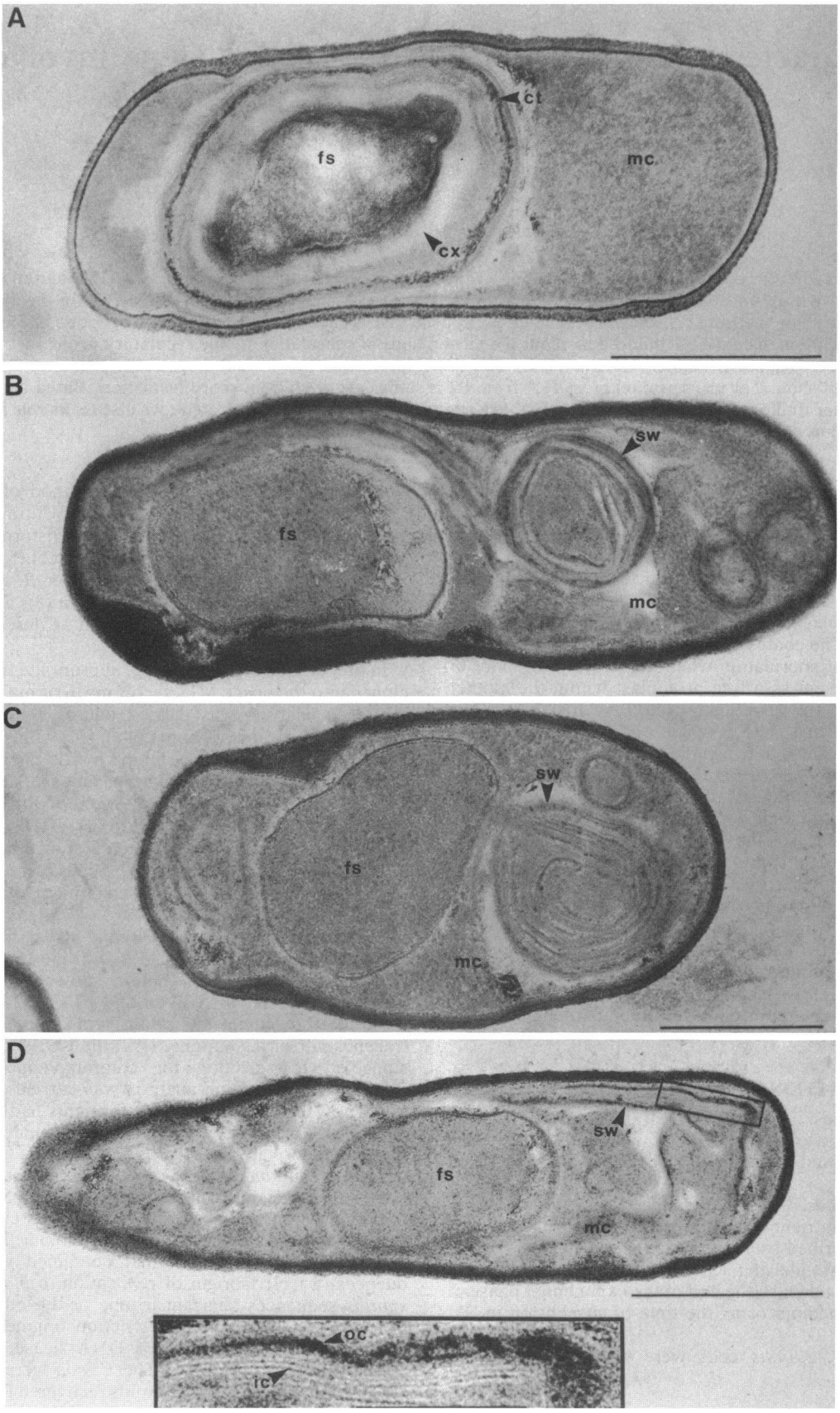
A
ct
fs
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B
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FIG. 1. Electron microscopy of *spoIVA* mutant sporangia. (A) Late-stage wild-type (strain PY79) sporangium in which the forespore (fs) is surrounded by layers of cortex (cx) and coat (ct). Panels B through D are three examples of mutant sporangia from cells (strain KS194) bearing the *spoIVA*::Tn*917*ΩHU194 insertional mutation. The forespores of the mutant cells lack a well-defined cortex, and the coat is present as swirls (sw) in the mother cell (mc) compartment of the sporangia rather than having been deposited around the forespore protoplasts. The inset in panel D is an enlargement of the boxed region in the panel showing the electron-dense outer coat (oc) and lamellar inner coat (ic) characteristic of the coat that normally surrounds the forespore. Bars: A through D, 500 nm; inset of panel D, 100 nm.

contained sequences extending to *Sph*I and *Eco*RI sites located approximately 1.4 and 0.7 kb, respectively, from the *erm*-distal side of the transposon (i.e., to the left of the transposon insertion site in Fig. 2); and pSR8 and pSR9, which contained sequences extending to *Sph*I and *Eco*RI sites located approximately 0.3 and 0.5 kb, respectively, from the *erm*-proximal side of the transposon (to the right of the insertion site in Fig. 2).

An intact wild-type copy of the *spoIVA* gene was then cloned as follows. Chromosomal DNA from PY79 was digested with *Hin*dIII and *Hin*cII. Fragments (2 to 3 kb in length), including one expected to contain *spoIVA* based on Southern analysis, were gel purified and ligated to *Hin*dIII-*Hin*cII-digested pUC19. The mixture was then use to transform *E. coli* TG1. The resulting transformants were screened by colony hybridization (39) with a radioactive fragment from the insert in pSR9 as a probe. The plasmid from one such clone, pSR26, contained a 2.4-kb *Hin*dIII-*Hin*cII fragment with *spoIVA* sequences (Fig. 2) and a second, unrelated *Hin*dIII-*Hin*dIII fragment of 2.5 kb. The additional *Hin*dIII-*Hin*dIII fragment was removed from pSR26 to create pSR27.

Plasmid constructions. To construct pSR11, a fragment from pSR7 extending from the *Eco*RI site at the boundary of *B. subtilis* and vector DNA to a *Bgl*II site located within Tn*917* was subcloned into the integrational vector pSGMU2 (16). pSR23 was constructed by deleting an *Eco*RI-*Eco*RV DNA segment from pSR11. pSR28 was constructed by cloning the entire 2.4-kb *spoIVA*-containing insert from pSR27 into pSGMU2. pSR41 and pSR29 were constructed by deleting *Hin*dIII-*Sph*I and *Hin*dIII-*Nru*I fragments, respectively, from pSR28. Plasmids pSR43 and pSR45 were constructed by deleting inward from the right-hand end of the fragment in pSR41 (Fig. 2) to *Sst*I and *Eco*RV sites, respectively. Finally, to construct plasmids with nested endpoints in the *spoIVA* promoter region, a fragment of pSR7 extending from the *Eco*RI site (near the left-hand end of the map in Fig. 2) to a *Cla*I site within Tn*917* DNA near the junction of the transposon and *B. subtilis* DNA was cloned into phage M13mp19. A series of deletions extending inward from the *Eco*RI site was generated by using the method of Dale et al. (9). Some of the resulting truncated DNA fragments, which had left-hand endpoints at nucleotide positions 248, 332, and 344 of Fig. 3, were subsequently cloned into pSGMU2 to generate pSR24, pSR17, and pSR21, respectively.

TABLE 1. *B. subtilis* strains[a]

Strain	Genotype or description	Source or reference
PY79	Prototrophic	Laboratory stock (50)
KS194	*spoIVA*::Tn*917*ΩHU194	41
67	*spoIVA67 trpC2*	J. Errington (15)
1S46	*spoIVA178 pheA12*	BGSC[b]
SC1159 (RL60)	*spoIIAC1*	4
KS298 (RL64)	*spoIID*::Tn*917*ΩHU298	41
KS440[c]	*spoIIGB41*	Laboratory stock
RL68[d]	*spoIIIA*::Tn*917*ΩHU25	41
KS10[e]	*spoIIIC*::Tn*917*ΩHU10	25, 41
BK541 (RL244)	*spoIIIDΔerm*	24
SC500 (RL78)	*spoIIIGΔ1*	5

[a] All strains except strains 67 and 1S46 are derived from the prototrophic wild-type strain PY79.
[b] *Bacillus* Genetic Stock Center.
[c] Constructed by congression of *spoIIGB41* from strain 1S60 (*Bacillus* Genetic Stock Center) into strain PY78 with selection for Gln^+.
[d] Constructed by congression of the *spoIIIA*::Tn*917*ΩHU25 mutation from strain KS25 (41) into strain PY78 with selection for Gln^+. The mutation was previously designated *spoIIIB*::Tn*917*ΩHU25 but is now known to be allelic to *spoIIIA* (13).
[e] The mutation was previously designated *spoIVD*::Tn*917*ΩHU10 (41) but is now known to be a deletion-mutated allele of *spoIIIC* (25).

Construction of strains harboring a *spoIVA-lacZ* in-frame gene fusion. A *Bam*HI linker was inserted at the *Eco*RV site in pSR6 after the ninth codon of the proposed *spoIVA* open reading frame to create pSR14. The *Hin*dIII-*Bam*HI fragment from pSR14 was then cloned into pLZ206 (52) to generate an in-frame gene fusion between the first nine codons of the *spoIVA* open reading frame and the *E. coli lacZ* gene. The resulting plasmid, pSR15, was used to transform competent cells of *B. subtilis* PY79, which were then selected for resistance to chloramphenicol. Transformants were expected to arise by single, reciprocal (Campbell-like) recombination between the insert in the plasmid and the corresponding region of homology in the chromosome. Strains containing the *spoIVA-lacZ* fusion were created by using chromosomal DNA from one of the transformants to transform (once again) PY79 and the congenic sporulation mutants SC1159, KS298, KS440, RL68, KS10, BK541, and SC500 (Table 1) and then selecting for resistance to chloramphenicol. In the case of mutants in which the introduced fusion appeared to be inactive, chromosomal DNA from those strains was used to reintroduce the fusion into PY79 by transformation to verify that the fusion was functional.

Construction of strains harboring *spoIVA* at the *amyE* locus. Partially diploid strains harboring a copy of *spoIVA* at the *amyE* locus were generated as described by Cutting and Vander Horn (8). The 2.0-kb *spoIVA*-containing *Hin*dIII-*Ssp*I fragment from pSR27 (Fig. 2) was cloned into pDG364 (a gift of P. Stragier) between the two halves of the *B. subtilis amyE* gene. The resulting plasmid, pSR50, was linearized and used to transform strains KS194, 67, and 1S46 with selection for resistance to chloramphenicol. The *spoIVA*-containing fragment was expected to integrate at the *amyE* locus as a result of double (marker replacement) recombination between the *amyE* sequences of pSR50 and the corresponding homologous sequences in the chromosome. Transformants were checked to verify that they had an Amy^- phenotype.

Measurement of β-galactosidase activity. Samples of sporulating cells were collected by centrifugation, and the resulting cell pellets were stored at −70°C until the samples were

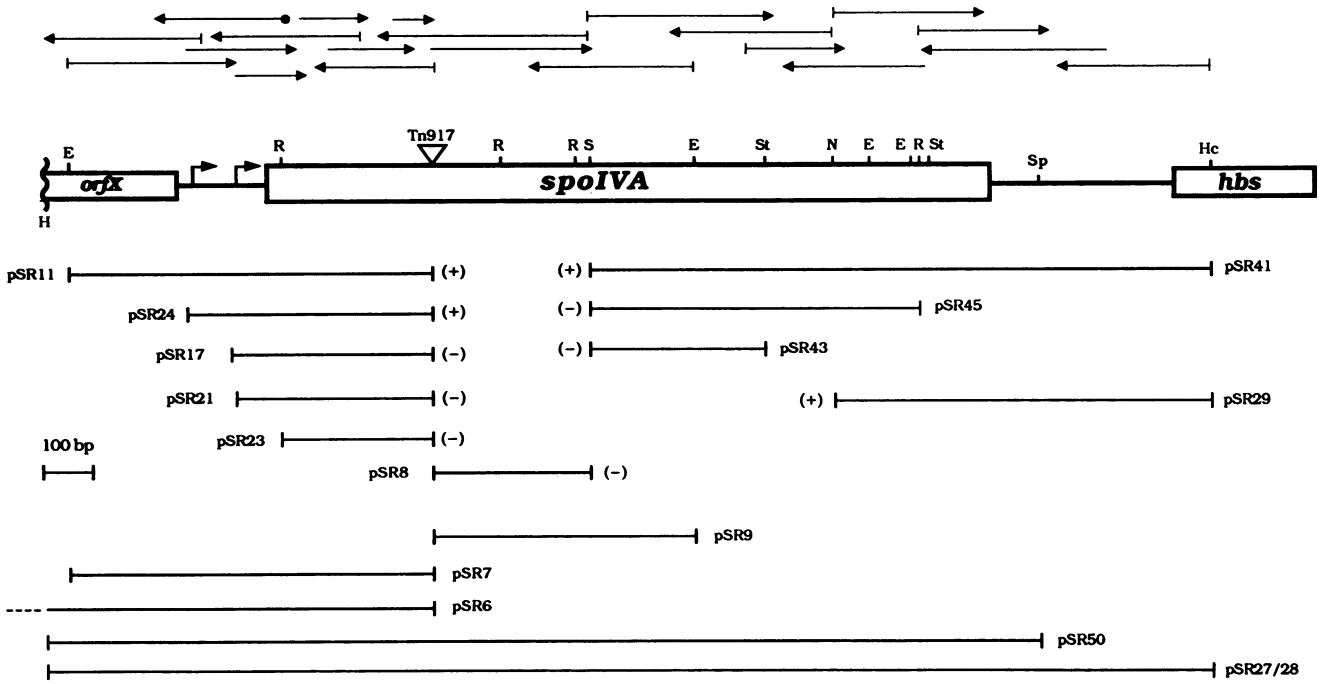


FIG. 2. Physical map of the *spoIVA* region of the chromosome. The thick line is a physical map of the *spoIVA* region of the chromosome showing the positions of selected restriction sites. The positions of the *spoIVA* and *hbs* open reading frames and the 3′ end of the unidentified open reading frame, *orfX*, are indicated by boxes. All three open reading frames are transcribed from left to right. The arrows on the map indicate the positions of the dual *spoIVA* promoters. The lines below the map describe the inserts in the indicated plasmids, some of which are integration plasmids used to map the functional boundaries of *spoIVA*. The + (Spo$^+$) and − (Spo$^-$) symbols indicate whether the insertion of the corresponding integration plasmid into the chromosome of the wild-type strain PY79 caused a mutant sporulation phenotype. The arrows above the map describe the strategy used to sequence the *spoIVA* region of the chromosome. The orientation and length of the arrows indicate the direction and extent of individual sequence determinations. The symbol at the base of each arrow indicates whether the sequence originating at the indicated position corresponds to a restriction site (|), a deletion endpoint (no symbol), or an oligonucleotide (●). Abbreviations: H, *Hin*dIII; E, *Eco*RI; R, *Eco*RV; S, *Sph*I; St, *Sst*I; N, *Nru*I; Sp, *Ssp*I; Hc, *Hin*cII.

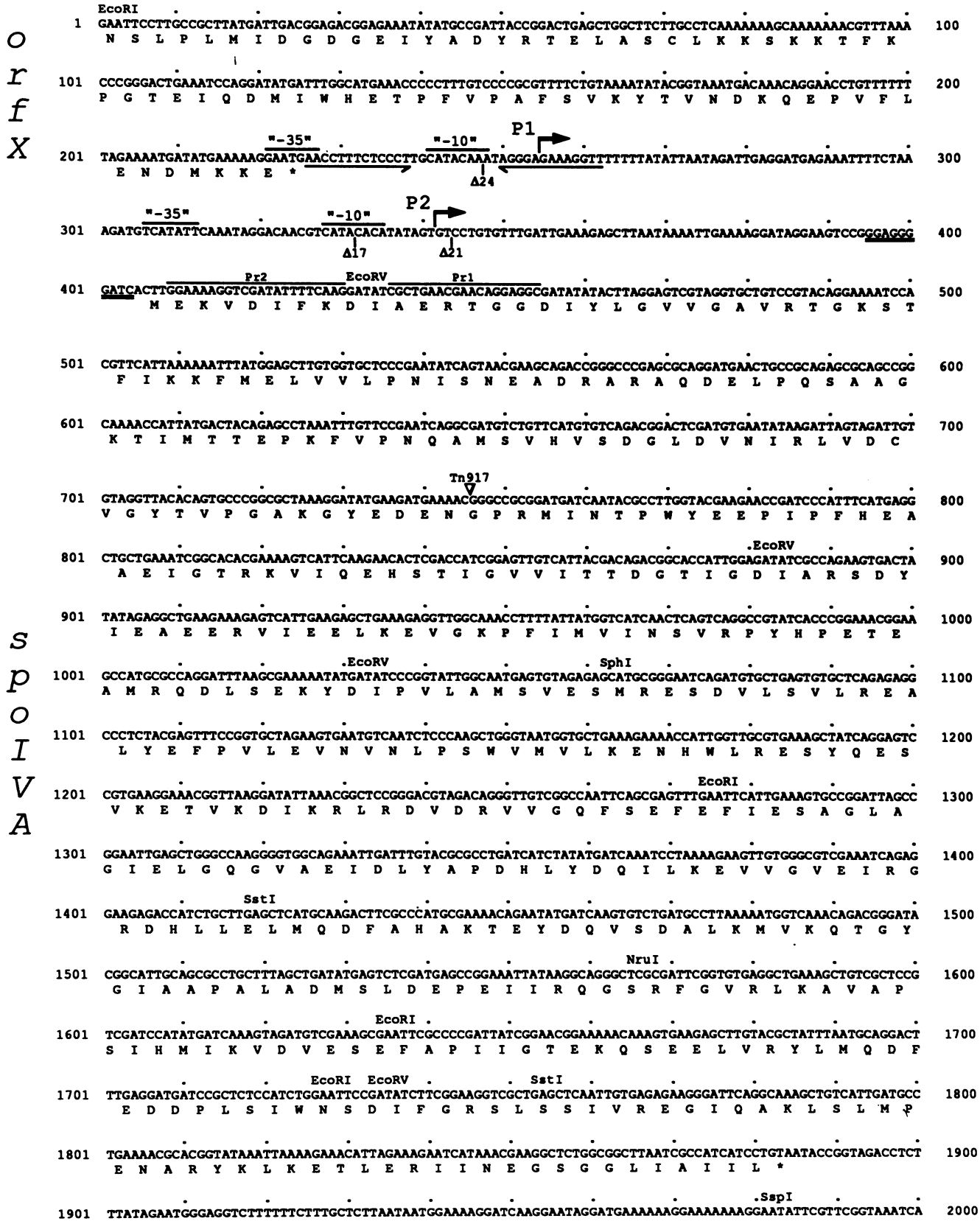
assayed. The specific activity of β-galactosidase was determined as described by Miller (31) with the substrate *o*-nitrophenol-β-D-galactoside after permeabilization by treatment with toluene (31) or lysozyme (29).

Isolation of RNA. RNA for the Northern hybridization experiments was prepared as follows. Strains PY79 (*spo*$^+$) and KS194 (*spoIVA*::Tn*917*ΩHU194) were sporulated in DS medium, and 5-ml samples were harvested at intervals after the end of the exponential phase of growth (the onset of sporulation). The cells were collected by centrifugation and suspended in a mixture of 2 ml of buffer (10 mM Tris-HCl [pH 8.0], 50 mM LiCl, 10 mM EDTA [pH 8.0], 1% sodium dodecyl sulfate), 1 ml of phenol (pH 5.5), 1 ml of chloroform, and 1 ml of baked glass beads. The samples were then vortexed vigorously for 2 min to lyse the cells. After centrifugation at 7,000 × *g* for 10 min at 4°C, the aqueous phase was extracted with a 1:1 (vol/vol) mixture of phenol (pH 5.5) and chloroform. Finally, the RNA was precipitated with ethanol and suspended in diethylpyrocarbonate-treated water.

RNA for the primer extension analysis was prepared by a modification of the above procedure as described by Cutting et al. (7).

Northern hybridization analysis. Northern hybridization analysis was carried out as modified from Sambrook et al. (39). Samples of 10 μg of RNA were loaded onto the lanes of a 1.0% agarose gel containing 2.2 M formaldehyde. After electrophoresis, the RNA was transferred to a nylon membrane (Hybond-N; Amersham). Prehybridization and hybridization were carried out at 65°C in hybridization buffer (6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 2× Denhardt reagent, 0.1% sodium dodecyl sulfate, 100 μg of salmon sperm DNA per ml). A nick-translated fragment of pSR9 extending from an *Ava*I site at the end of

FIG. 3. Nucleotide sequence of the *spoIVA* region of the chromosome. The figure shows the nucleotide sequence of the nontranscribed strand of the *spoIVA* region of the chromosome. The predicted amino acid sequences of the *spoIVA* protein, the COOH-terminal portion of the *orfX* protein, and the NH_2-terminal portion of the *hbs* protein are shown below the nucleotide sequence. The putative ribosome binding sites preceding the *spoIVA* and *hbs* start codons are double underlined. The positions of the proposed *spoIVA* transcription start sites are indicated by arrows and regions corresponding to the −10 and −35 sequences of the *spoIVA* promoters are indicated by lines above the sequence. The symbols Δ17, Δ21, and Δ24 denote the upstream endpoints of the fragments present in pSR17, pSR21, and pSR24, respectively (Fig. 2). The figure also shows sequences complementary to the Pr1 and Pr2 oligonucleotide primers used for primer extension analysis, the site of the Tn*917*ΩHU194 insertion, and the position of selected restriction sites. A region of dyad symmetry that may serve as a transcription terminator for the *orfX* transcription unit is underlined. The open diamond marks a T nucleotide that was reported to be a C in the sequence of Micka et al. (30).


orfX
spoIVA
EcoRI
"-35"
"-10"
P1
P2
Δ24
Δ17
Δ21
Pr2
EcoRV
Pr1
Tn917
.EcoRV
.EcoRV
SphI
EcoRI
NruI
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SstI
EcoRI EcoRV
SstI
.SspI

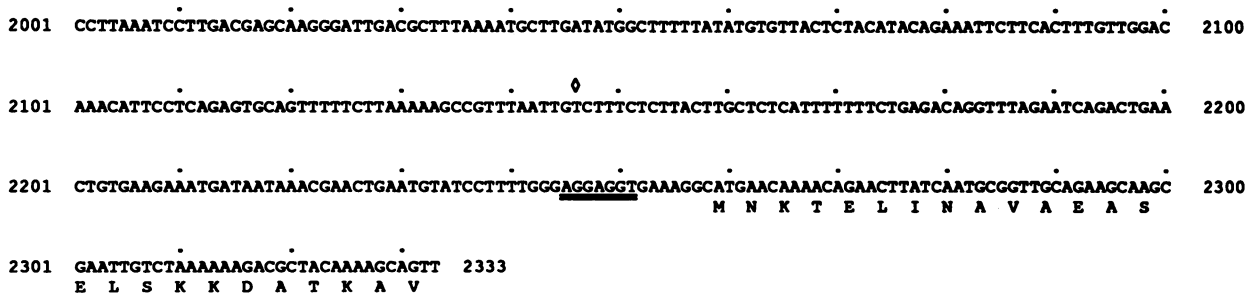

FIG. 3—*Continued.*

the Tn*917* sequence to the downstream *Eco*RI site was used as a probe. A 0.24-9.5 kb RNA ladder (BRL) was used for molecular weight standards.

Mapping the 5′ terminus of *spoIVA* mRNA. Primer extension was carried out by use of the synthetic oligonucleotides Pr1 (5′-GCCTCCTGTTCGTTCAGCG) and Pr2 (5′-CTTGA AAATATCGACCTTTTCC). The oligonucleotides were 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (BRL) as described by Sambrook et al. (39). One picomole of 5′-end-labeled oligonucleotide was incubated with 10 μg of RNA in 10 μl of annealing buffer (50 mM Tris-HCl [pH 7.6], 100 mM KCl) at 90°C for 2 min and then at 47°C for 30 min. The primer-RNA hybrids were precipitated by the addition of 90 μl of 300 mM sodium acetate (pH 5.2) and 250 μl of ethanol followed by incubation at −20°C for 1 h. The hybrids were then suspended in 5 μl of reverse transcriptase buffer (50 mM Tris-HCl [pH 7.6], 60 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 2.5 mM [each] deoxynucleoside triphosphate, 1 U of placental RNase inhibitor [Pharmacia] per μl) and warmed to 47°C. Then 1 μl (5 U) of Moloney murine leukemia virus reverse transcriptase (Pharmacia) was added, and the reaction mixture was incubated at 47°C for 1 h; 4 μl of 95% formamide loading dye was then added. The 5′-end-labeled Pr1 oligonucleotide was also used to generate a sequence ladder by the dideoxy-chain termination method of Sanger et al. (42). The products of primer extension were then subjected to electrophoresis in 5% polyacryamide slab gels containing 8 M urea.

Nucleotide sequence accession number. The sequence of *spoIVA* reported herein has been deposited with the GenBank data base under accession number M80926.

RESULTS

Cloning of the *spoIVA* locus. The *spoIVA* locus was cloned by taking advantage of a previously identified insertion of transposon Tn*917* called Tn*917*ΩHU194 (41) and a previously described method for the rapid cloning of chromosomal DNA adjacent to the site of insertion of Tn*917* (49). Tn*917*ΩHU194 was anticipated to be an insertion in a new *spo* gene designated *spoVP* (41). However, the close proximity on the chromosome of the insertion to *spoIVA* (41) and the discovery that the insertional mutation causes the misassembly of the spore coat as swirls in the mother cell (Fig. 1), a phenotype that is characteristic of *spoIVA* mutants (3, 35), suggested that Tn*917*ΩHU194 is allelic to the previously known sporulation locus, an inference that is confirmed below. The insertion is hereafter designated *spoIVA*::Tn*917* ΩHU194.

DNA adjacent to the site of the transposon insertion was cloned as described in Materials and Methods. The cloned DNAs were then used as hybridization probes to screen a plasmid library generated with *Hin*dIII- and *Hin*cII-digested chromosomal DNA from *spoIVA*$^+$ cells to identify clones harboring an intact copy of the *spoIVA* region. One such clone contained a *Hin*dIII-*Hin*cII fragment of 2.4 kb. Figure 2 shows a restriction map of the 2.4-kb region of the chromosome and the site of the *spoIVA*::Tn*917*ΩHU194 insertion.

To locate the functional boundaries of *spoIVA*, fragments of the cloned DNAs were subcloned in *E. coli* into integrational plasmids containing a chloramphenicol resistance gene (*cat*). The resulting plasmids were then used to transform wild-type cells of *B. subtilis*, which were then selected for the plasmid-conferred resistance to chloramphenicol. Since the integrational plasmids were unable to replicate autonomously in *B. subtilis*, drug-resistant transformants were expected to arise by single reciprocal (Campbell-like) recombination between the insert of *B. subtilis* DNA in each plasmid and the corresponding region of homologous DNA in the chromosome. If a given plasmid insert was entirely internal to *spoIVA*, then integration of the plasmid into the chromosome would be expected to disrupt the gene and hence cause a Spo$^-$ phenotype. In contrast, if at least one end of the insert lay outside a functional boundary of *spoIVA*, then the resulting transformants would be expected to be Spo$^+$. The results of this analysis are summarized in Fig. 2, in which it can be seen that the left-hand boundary of *spoIVA* lies between the left-hand ends of the inserts in pSR17 and pSR24 and that the right-hand boundary lies between the right-hand ends of the inserts in pSR45 and pSR41 (and pSR29). Thus, the entire locus was evidently contained within the 2.4-kb *Hin*dIII-*Hin*cII fragment.

This conclusion was verified by a complementation experiment in which a 2-kb segment of DNA extending from the *Hin*dIII site at the left-hand end of the *spoIVA* region to an *Ssp*I site near the right-hand boundary of the locus (Fig. 2) was cloned in *E. coli* between the two halves of the *B. subtilis amyE* gene contained in pDG364. The disrupted *amyE* gene contained in the plasmid was then used to replace the wild-type *amyE* gene in the chromosome by marker replacement recombination as described in Materials and Methods. Introduction of the disrupted *amyE* gene into various *spoIVA* mutants demonstrated that the 2-kb *Hin*dIII-*Ssp*I DNA segment was capable of restoring sporulation proficiency to *spoIVA*::Tn*917*ΩHU194 mutant cells and to cells bearing the traditional and defining *spoIVA* mutations *spoIVA67* and *spoIVA178*. We infer the following from these results: that the entire *spoIVA* locus is contained within the 2-kb *Hin*dIII-*Ssp*I DNA insert in *amyE*; that the right-hand boundary of *spoIVA* must lie in the 248-bp interval between the *Eco*RV site used in constructing pSR45 and the *Ssp*I site

used in constructing the *amyE* insert (Fig. 2); and that *spoIVA*::Tn*917*ΩHU194, *spoIVA67*, and *spoIVA178* are closely clustered mutations.

Nucleotide sequence of the *spoIVA* region of the chromosome. Figure 3 shows the nucleotide sequence of DNA extending from the *Eco*RI site near the left-hand end of the restriction map of Fig. 2 to the *Hin*cII site near the right-hand boundary of the map. The sequence reveals an open reading frame of 492 codons that spans the site of the transposon insertion. Because the ends of the open reading frame closely correspond to the functional boundaries of *spoIVA*, we conclude that the open reading frame is *spoIVA* and that the *spoIVA* locus consists of a single gene. Nucleotide sequence analysis of the junctions of transposon and *B. subtilis* DNA showed that Tn*917* in the *spoIVA*::Tn*917*Ω HU194 mutant was inserted just after codon 113 (AAC), causing a duplication of the sequence AAAAC. Just upstream of the proposed initiation codon is a putative ribosome binding site (GGAGGgGATC) that exhibits high sequence complementarity to the 3′ end of *B. subtilis* 16S rRNA (33). The *spoIVA* open reading frame is capable of encoding a polypeptide of 55,174 Da. The predicted amino acid sequence of this protein showed no significant similarity to those of proteins represented in the GenBank, EMBL, SwissProt, and PIR data bases.

Part of a second open reading frame, designated *orfX*, the 3′ end of which is shown in Fig. 2 and 3, was identified upstream of *spoIVA*. The insertion of a neomycin resistance gene at the *Hin*dIII site in *orfX* (Fig. 2) does not impair viability or sporulation (37). Also, the 90-amino-acid sequence encoded by this portion of the open reading frame showed no similarity to proteins represented in the GenBank, EMBL, SwissProt, and PIR data bases.

A third open reading frame identified downstream of *spoIVA* was found to be the *hbs* gene described by Micka et al. (30); this gene encodes the histonelike HBsu protein. In combination with the results of Gollnick et al. (19) and Henner et al. (21), this finding extends the physical map of the tryptophan operon region of the chromosome to include about 20 kb of DNA. The order of genes in this region is *spoIVA*, *hbs*, *mtrAB*, *gerC1-3*, *ndk*, *cheR*, *aroFBH*, *trpEDCFBA*, *hisH*, *tyrA*, and *aroE*; all of the genes have the same orientation.

***spoIVA* specifies a 1.6-kb transcript that appears during the third hour of sporulation.** To investigate *spoIVA* transcription, we first carried out Northern hybridization analysis to determine the size and approximate time of appearance of its RNA product. *spoIVA* specified a transcript of approximately 1.6 kb that appeared about 2.5 h after the onset of sporulation (Fig. 4). The length of the transcript is consistent with that expected from the size (1,476 bp) of the *spoIVA* open reading frame, and the observation that the transcript does not appear until a few hours into sporulation is consistent with the expectation that *spoIVA* is subject to developmental regulation.

Use of a *lacZ* fusion to study the dependence of *spoIVA* expression on the products of other *spo* genes. To study the regulation of *spoIVA* expression, *spoIVA* was joined in frame at its 9th codon to the *lacZ* gene of *E. coli* as described in Materials and Methods. *spoIVA*-directed β-galactosidase production during sporulation was monitored after introduction of the fusion into a collection of congenic sporulation mutants. A representative time course experiment is shown in Fig. 5, and the results obtained for the entire collection of mutants are summarized in Table 2. In agreement with the results of Northern hybridization analysis, expression of the *spoIVA-lacZ* fusion commenced about 2 h after the onset of sporulation in Spo$^+$ cells.

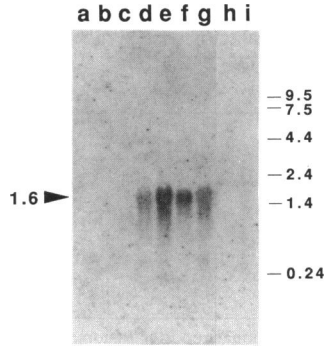

FIG. 4. Northern hybridization analysis of *spoIVA* mRNA. Total RNA was purified from cells of KS194 (*spoIVA*::Tn*917*ΩHU194) and its congenic parent PY79 (*spo*$^+$) that had been harvested just before and at intervals after the onset of sporulation (time zero [T_0]) in DS medium. The RNAs were subjected to electrophoresis in a formaldehyde-agarose gel, transferred to a nylon membrane, and hybridized with a radiolabeled fragment of *spoIVA* DNA (containing a sequence located downstream of the site of the transposon insertion) as described in Materials and Methods. The figure is an autoradiograph of the filter taken after the hybridization reactions. The filter contained RNAs from PY79 cells collected at $T_{-0.7}$ (lane a), $T_{0.3}$ (lane b), $T_{1.4}$ (lane c), $T_{2.5}$ (lane d), $T_{3.5}$ (lane e), $T_{3.9}$ (lane f), and $T_{4.6}$ (lane g) and RNA from KS194 cells collected at $T_{2.5}$ (lane h) and $T_{4.6}$ (lane i). The position and estimated size (in kilobases) of the *spoIVA* mRNA are indicated on the left side of the figure. Also shown are the positions of RNA size markers (BRL).

Its time of induction and its function in coat morphogenesis suggested that *spoIVA* might be transcribed under the control of sporulation transcription factor σ^E, which controls an early stage of gene expression in the mother cell (11, 14, 52). This expectation was borne out by the results of the

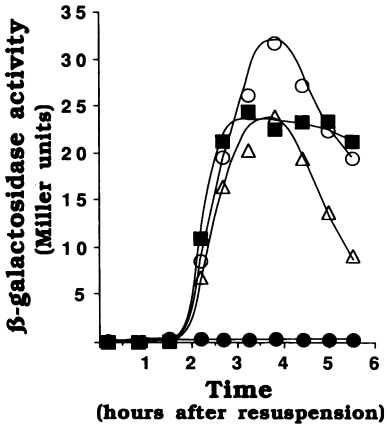

FIG. 5. Effect of *spo* mutations on *spoIVA*-directed β-galactosidase synthesis during sporulation. Wild-type and mutant strains harboring a *spoIVA-lacZ* fusion were induced to sporulate by resuspension in SM medium. Samples were taken at the indicated times relative to the onset of sporulation (T_0) and assayed for β-galactosidase as described in Materials and Methods. The strains used were congenic derivatives of the *spo*$^+$ strain PY79 (○) and contained the mutations *spoIIGB41* (●), *spoIIIC*::Tn*917*ΩHU10 (■), and *spoIIIDΔerm* (△). The endogenous background (1 to 4 Miller units) present in cells of each parent strain lacking the fusion was subtracted.

TABLE 2. Effect of *spo* mutations on *spoIVA*-directed β-galactosidase synthesis

Relevant mutation[a]	Synthesis of β-galactosidase[b]
None	100
spoIIAC1	<5
spoIIGB41	<5
spoIID::Tn*917*ΩHU298	66
spoIIIA::Tn*917*ΩHU25	139
spoIIIDΔerm	75
spoIIIC::Tn*917*ΩHU10	98
spoIIIGΔ1	98

[a] A *spoIVA-lacZ* translational reporter fusion was introduced into a congenic collection of strains containing the indicated mutations as described in Materials and Methods.

[b] Numbers indicate the maximum level of β-galactosidase activity expressed as a percentage of that observed (about 30 Miller units) for wild-type cells harboring the *spoIVA-lacZ* fusion. Values shown represent the averages of at least two independent trials.

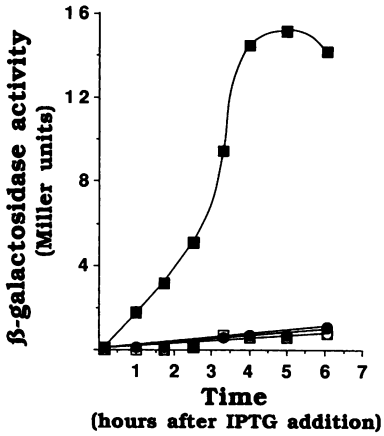

FIG. 6. Induction of *spoIVA-lacZ* in cells engineered to produce σ^E in response to IPTG. PY79 cells harboring *spoIVA-lacZ* and the σ^E-encoding plasmid pDG180 (□, ■) or the parent vector pDG148 (○, ●) were grown in 2× YT medium (39). At an optical density at 600 nm of about 0.3, the cultures were each split into two parts; IPTG (1 mM) was added to one part (■, ●). Samples were removed at the indicated times and assayed for β-galactosidase activity.

dependence studies, which showed that *spoIVA*-directed β-galactosidase synthesis was prevented by mutations in *spoIIGB*, which encodes the proprotein precursor to σ^E, and *spoIIAC*, whose product is required for pro-σ^E processing. In contrast, *spoIVA-lacZ* expression was not significantly impaired by mutations in *spoIID* and *spoIIIA*, which depend on σ^E for their transcription. Also, *spoIVA-lacZ* expression was not prevented by mutations in later-acting genes that specify other known sporulation regulatory proteins, such as *spoIIID*, which encodes the DNA-binding protein SpoIIID; *spoIIIC*, which encodes the COOH-terminal region of the mother-cell transcription factor σ^K; and *spoIIIG*, which encodes the forespore transcription factor σ^G.

As a further test of whether *spoIVA* is under the control of σ^E, we tested for the expression of *spoIVA-lacZ* in cells that had been engineered to produce the sporulation sigma factor during vegetative growth in response to the inducer isopropyl-β-D-thiogalactopyranoside (IPTG). Plasmid pDG180 (a gift of P. Stragier) was introduced by transformation into cells containing a *spoIVA-lacZ* fusion. pDG180 contains a truncated copy of the σ^E structural gene *spoIIGB* under the control of the IPTG-inducible *spac* promoter (48). The truncated *spoIIGB* gene lacks the first 16 codons of the proprotein-coding sequence; as a consequence, its direct product is an active transcription factor that does not require processing. The addition of IPTG to the plasmid-bearing cells caused an induction of *spoIVA*-directed β-galactosidase synthesis (Fig. 6). Expression of the bona fide σ^E-controlled gene fusion *spoIID-lacZ* (38, 46) was similarly induced in parallel experiments in response to the addition of IPTG (37).

Mapping the 5′ terminus of *spoIVA* mRNA. Figure 7 shows the results of primer extension experiments to map the 5′ terminus of *spoIVA* mRNA. Two 5′ termini designated P1 and P2 were detected that were present in RNA from sporulating cells but not in RNA from vegetative cells. P1 and P2 were located 152 and 65 bp, respectively, upstream of the initiation codon for the *spoIVA* open reading frame (Fig. 3). Sequences centered about 10 and 32 bases preceding each of the 5′ termini closely resemble the corresponding sequences in the −10 and −35 regions of known σ^E-controlled promoters (Fig. 8) (see Discussion), a finding in further support of the view that *spoIVA* is under the control of σ^E.

Relevant to the discovery that *spoIVA* transcripts originate from two 5′ termini are the results of the plasmid integration experiments of Fig. 2. These results suggest that both the P1 and P2 transcripts are the products of independent transcription initiation events (that is, that P2 transcripts are not nucleolytic products of transcripts originating from P1) and that the P2 promoter is sufficient for sporulation. This follows from the observation that integration of pSR24, the upstream boundary of whose insert precedes the P1 start site by only 7 bp, did not cause a Spo$^-$ phenotype, whereas integration of pSR17, the upstream boundary of whose insert precedes the P2 start site by only 10 bp, did cause a Spo$^-$ phenotype (Fig. 2 and 3). It is not known whether transcription from P1 alone would be sufficient to support sporulation.

DISCUSSION

We have located *spoIVA* on the physical map of the *B. subtilis* chromosome in the region of the tryptophan operon and have shown that it consists of a single gene. The gene is 492 codons in length and is capable of encoding a polypeptide of 55,174 Da. Transcription of *spoIVA* is controlled from two closely spaced promoters located just upstream of the open reading frame. The promoters are activated about 2 h into sporulation and are under the control of the sporulation transcription factor σ^E. Interestingly, the upstream promoter P1 is redundant to the downstream promoter P2 in that transcription from P2 alone is sufficient to support spore formation.

The characterization of the *spoIVA* promoters P1 and P2 brings to nine the number of promoters that appear to be directly under the control of σ^E. An alignment of these promoters is shown in Fig. 8, revealing an updated consensus for the −35 (kmATATT, where k is G or T and m is A or C) and −10 (CATACA-T) recognition sequences for σ^E-controlled promoters, in extension of the recent analysis of Foulger and Errington (17). In determining the above consensus, we excluded three other σ^E-controlled promoters (*cotE*-P2, *spoVJ*-P1, and the *sigK* [*spoIVCB*] promoter) (Fig. 8) from which transcription is known to be additionally dependent upon the DNA-binding protein SpoIIID (17, 26, 52). Expression from the *spoIIID* promoter is also dependent

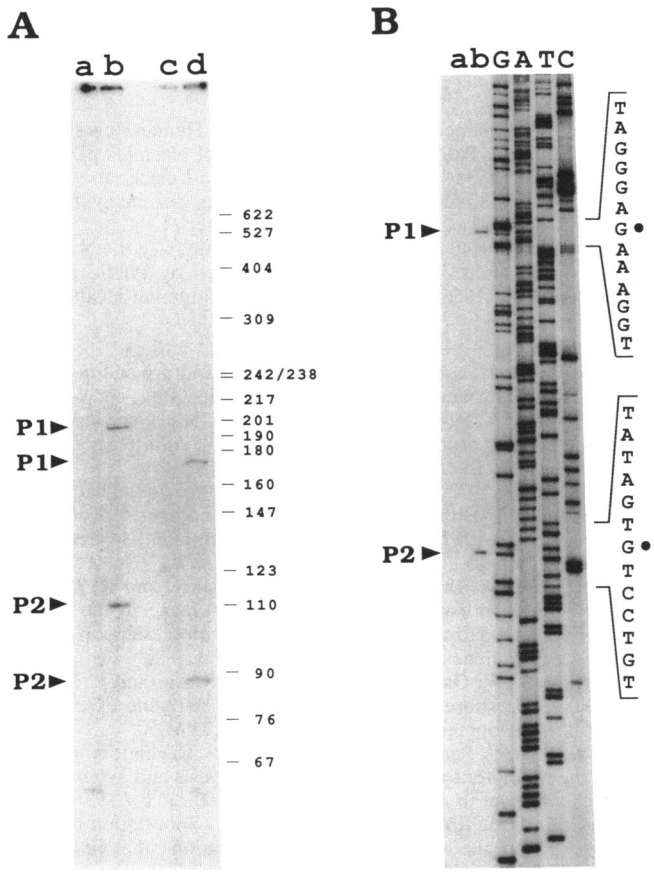

FIG. 7. Mapping the 5′ termini of *spoIVA* mRNA. (A) Low-resolution mapping of the 5′ termini of *spoIVA* mRNA. Oligonucleotides (Fig. 3) Pr1 (lanes a and b) and Pr2 (lanes c and d) were used to prime cDNA synthesis from total RNA from PY79 (*spo*$^+$) cells harvested at $T_{-0.2}$ (lanes a and c) and at $T_{3.5}$ (lanes b and d). The products of the primer extension reactions were subjected to electrophoresis in a 4% polyacrylamide–8 M urea gel alongside radioactive *Msp*I fragments of pBR322 DNA, whose positions are indicated on the right-hand side of the panel. The arrows indicate the positions of the two extension products, P1 and P2, obtained with each oligonucleotide primer. (B) High-resolution mapping of the 5′ termini of *spoIVA* mRNA. The products of primer extension reactions with the Pr1 primer and RNA from $T_{-0.2}$ cells (lane a) and $T_{3.5}$ cells (lane b) (from the same reactions used for lanes a and b of panel A) were subjected to electrophoresis alongside a nucleotide sequencing ladder generated with the Pr1 primer. The sequences shown in the vicinity of the 5′ termini, P1 and P2, correspond to the nontranscribed DNA strand. ●, Nucleotide corresponding to each terminus.

	"−35"		"−10"	+1
Consensus	**kmATATT**	**− 14 bp −**	**CATACA-T**	**r**
	98766		689967 7	
spoIID	agagTCATATTagcttgt		ccctgccCATAgAcTagactagagt	
spoIIID	tttaGCATATTcccaaaa		gaatgctaATACAcTgttacaaacc	
G4	gcctGAATATTtctttga		gctaatgaATACAaTaaatcgatag	
spoIVF	aaccGAATATTtgccatg		gacaagaCATAtgaTgtacaaaccc	
spoIIIA	cttgTCATAaagtctgc		ctcacatCATACAtTttaaagaagc	
spoIVA P1	aaagGAATgaacctttc		tcccttgCATACAaatagggagaaa	
spoIVA P2	gatgTCATATTcaaataggacaacgtCATACAcatatagtgtcc			
cotE P1	taagTAAagTTctaggc		acccctgCATACAaTggaacagaaa	
bvx	cttcGAATAaatactata		aatcaaaacTAtgaTgtcagaaa	
spoVJ P1	catgGttTgTccaccccatgtccgtgaATACAaTaagaaatAaaa			
sigK	acagaCAcAgacagcctc		ccggtcaCATACAtTtacatatagg	
cotE P2	aaatGCAcAcTagacaaa		tgcccagCATAagaTaacacgaaga	

FIG. 8. Alignment of σ^E-controlled promoters. Shown are the sequences near the proposed transcription start sites for genes that are under the control of σ^E. The first nine sequences were used to determine the consensus shown above the sequences. A base was included in the consensus if it was found in at least six of the nine promoters; the numbers below the consensus indicate the number of times a given base occurred. Also shown are positions at which one of two bases occurred in each of the nine promoters (k = G or T; m = C or A; r = G or A). Bases that match the consensus are set in uppercase letters, and the positions of the 5′ terminus of the transcripts for each gene are underlined. The promoter regions of three other σ^E-controlled genes (*spoVJ* P1, *sigK*, *cotE* P2) are shown for comparison, but since expression from these promoters is known to additionally require the regulatory protein SpoIIID they were not used to determine the consensus. The promoter sequence information was obtained from the following sources: *spoIID* (38), *G4* (36), *spoIIID* (24, 47), *spoIVF* (7), *spoIIIA* (17), *cotE* (52), *bvx* (20), *sigK* (26), *spoVJ* (17).

upon SpoIIID but only to a modest extent (24), and thus it was included in determining the consensus. The *cotE*-P2, *spoVJ*-P1, and *sigK* (*spoIVCB*) promoters conform well to the above consensus in their −10 regions but less well in general, relative to the other σ^E-controlled promoters, in their −35 regions.

The motivation for the present work and the primary reason for interest in *spoIVA* is that its protein product is required for proper morphogenesis of the spore coat. Although *spoIVA* mutant cells synthesize a normal-looking coat, consisting of an electron-dense (outer) layer and a lamellar (inner) layer, the coat misassembles as swirls in the cytoplasm of the mother cell instead of being deposited around the forespore (3, 34, 35) (Fig. 1). *spoIVA* mutant cells are also defective in formation of the cortex, the layer of peptidoglycan that is produced underneath the coat between the two membranes that delimit the forespore protoplast (3, 35) (Fig. 1).

Previous work has shown that mutations in *spoIVA* do not prevent the expression of genes (*cotA*, *cotC*, *cotD*, *cotE*) that encode structural components of the spore coat (40, 52). Thus, although it is possible that the *spoIVA* product is required for the expression of an as yet unidentified or uncharacterized coat protein gene, we propose that SpoIVA is a morphogenetic protein that is involved in spore coat assembly. For example, SpoIVA could be a structural component of the spore coat or a basement protein that is deposited around the forespore and upon which the inner and outer layers of the coat assemble. If *spoIVA* does encode a structural component of the coat, it would be unique among the coat protein genes identified thus far, none of which is required for sporulation. Indeed, with the exception of mutations in *cotE*, mutations in known coat protein genes do not prevent the formation of normal-looking spores with normal resistance properties (2, 10, 51).

An alternative possibility is that SpoIVA is involved in cortex formation and that proper deposition of the coat around the forespore depends on some aspect of cortex formation. However, a properly formed cortex is not necessarily required for deposition of coat material, based on the observation that *spoVE* mutant sporangia are defective in cortex formation but are evidently unimpaired in coat assembly (35, 45). Conversely, if cortex formation is dependent upon coat morphogenesis, then the defect in cortex formation observed in *spoIVA* mutant sporangia could be due to the failure of the coat to assemble arround the forespore. Although cortex formation reaches an advanced stage before

the initiation of coat assembly, it is possible that in the absence of coat assembly the cortex is not completed or is unstable.

Whatever the possible interrelationship between coat and cortex formation, the timing of *spoIVA* expression and the finding that *spoIVA* is a σ^E-controlled gene is consistent with the view that SpoIVA participates at an early stage of coat morphogenesis. Synthesis of SpoIVA is presumably restricted to the mother cell compartment, the site of coat protein synthesis and assembly; recent work (11) indicates that in at least one case σ^E-directed gene expression is restricted to the mother cell. Moreover, *spoIVA* is expressed relatively early in the mother cell line of gene expression, earlier than all previously identified coat protein genes with the exception of *cotE*, whose P1 promoter is switched on at about the same time as those of *spoIVA* (52).

Although *spoIVA* is not absolutely required for the expression of any coat protein gene examined, mutations in *spoIVA* do dramatically affect the level of expression of the late-activated coat protein gene *cotC* under certain nutritional conditions (52). *cotC* is unique among the coat protein genes that have been examined in that it is expressed at very different levels depending on the medium in which sporulation is carried out. When wild-type cells are induced to sporulate by exhaustion in rich sporulation (DS) medium, *cotC* expression, as monitored by a *cotC-lacZ* translational fusion, is approximately 20-fold higher than when cells are sporulated by suspension in nutrient-deficient minimal (SM) medium. High-level expression of *cotC* is *spoIVA* dependent because the level of *cotC* expression in *spoIVA* mutant cells in DS medium is reduced to that seen for wild-type (or *spoIVA* mutant) cells sporulating in SM medium (52). This dependence of the level of *cotC* expression on SpoIVA does not reflect an influence of medium on *spoIVA* expression, because *spoIVA-lacZ* is expressed at similar levels in DS and SM media (37).

The basis for the effect of medium on *cotC* expression is not known, but the dependence on SpoIVA could indicate that the expression of *cotC* (and perhaps other late-activated coat protein genes) is coupled to an earlier event in the morphogenesis of the coat. Thus, by disrupting coat assembly, *spoIVA* mutations impair late-activated coat gene expression. Precedents for the coupling of gene expression to morphogenesis are the dependence of the transcription of class III (late) flagellar genes in *E. coli* and *Salmonella typhimurium* on the assembly of the basal body structure of the flagellum (18, 23) and the dependence of the processing of the proprotein precursors to sporulation transcription factors σ^E and σ^K in *B. subtilis* on the formation of the sporulation septum (27, 46) and the forespore protoplast (5), respectively.

In summary, we have shown that *spoIVA*, which is required for proper morphogenesis of the *B. subtilis* spore coat, consists of a single gene that is transcribed from two closely spaced promoters under the control of the sporulation transcription factor σ^E. The time of *spoIVA* expression and the dependence of this expression on σ^E are consistent with the view that the *spoIVA* gene product is involved at an early stage in coat assembly. Characterization of the SpoIVA protein and the relationship between *spoIVA* and other genes (for example, *spoVB* and *spoVD* [35]) that affect coat morphogenesis should reveal important details of the coat assembly process. Last, to explain the effect of *spoIVA* mutations on the level of expression of *cotC*, we have proposed a model in which the level of expression of late-activated coat protein genes is coupled to morphogenesis of the coat.

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