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Sporulation Gene *spoIIB* from *Bacillus subtilis*

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We have cloned and characterized the sporulation gene *spoIIB* from *Bacillus subtilis*. In extension of previous nucleotide sequence analysis, our results show that the order of genes in the vicinity of *spoIIB* is *valS folC comC spoIIB orfA orfB mreB mreC mreD minC minD spoIVFA spoIVFB L20 orfX L24 spo0B obg pheB pheA*. All 20 genes have the same orientation; the direction of transcription is from *valS* to *pheA*. We show that *spoIIB* is a 332-codon-long open reading frame whose transcription is under sporulation control. The deduced amino acid sequence of the *spoIIB* gene product, a 36-kDa polypeptide, is highly charged and contains a stretch of uncharged amino acids that could correspond to a transmembrane segment. Surprisingly, mutations in *spoIIB*, including an in vitro-constructed null mutation, cause only a mild impairment of spore formation in certain otherwise wild-type bacteria. However, when combined with mutations in another sporulation gene, *spoVG*, mutations in *spoIIB* cause a severe block in spore formation at the stage (stage II) of septum formation. (As with *spoIIB* mutations, mutations in *spoVG* cause little impairment in sporulation on their own.) The nature of the *spoIIB spoVG* mutant phenotype is discussed in terms of the events involved in the maturation of the sporulation septum and in the activation of sporulation transcription factors σ^F and σ^E.

In response to conditions of nutrient deprivation, cells of the gram-positive bacterium *Bacillus subtilis* enter a developmental program that culminates in the formation of a dormant cell type known as the endospore or spore. Spores are produced according to an ordered sequence of morphological events (52), the earliest of which (stage II) is the formation of an asymmetrically positioned septum that partitions the developing cell (the sporangium) into cellular compartments of unequal sizes known as the forespore and the mother cell. Next, in stage III, the forespore is engulfed by the mother cell, pinching it off as a free protoplast within the sporangium. In subsequent stages (stages IV to VI), layers of cortex and coat material that encase the developing spore are deposited, and upon maturation the spore is released from the sporangium by lysis of the mother cell. Genes whose products are required for the conversion of a growing cell into a spore are known as *spo* genes. Several scores of *spo* genes are known; these are named according to the stage of development beyond which morphogenesis cannot proceed in the absence of their products (44, 52). Thus, the *spoIIB* gene, the subject of the present study, was originally identified by a mutation that blocked sporulation at the stage of septation (7).

Here, we report on the cloning and characterization of *spoIIB*. Our principal discovery is that in certain strains, mutations in *spoIIB*, including an in vitro-constructed deletion mutation, cause little impairment of spore formation. However, when combined with mutant alleles of another sporulation gene, called *spoVG* (58, 62), whose product is itself largely dispensable for sporulation, *spoIIB* mutations cause a severe block in sporulation. We infer that the functions of the *spoIIB* and *spoVG* gene products are largely redundant to each other. We also demonstrate that the sporulation defect resulting from a *spoIIB* mutation alone is enhanced in certain strain backgrounds (or under conditions of low temperature), an observation which suggests allelic variation at the *spoVG* locus of different laboratory strains of *B. subtilis*. Certain aspects of the phenotype of a *spoIIB spoVG* double mutant have significant implications for hypotheses concerning the maturation of the sporulation septum and the activation of the sporulation transcription factors σ^F and σ^E.

MATERIALS AND METHODS

Bacterial strains. The *B. subtilis* strains used are listed in Table 1. Plasmid manipulations were performed in *Escherichia coli* TG1 (laboratory stock [59]). *E. coli* 236c [*valRS* (Ts)] (4) was provided by L. Isaksson (Stockholm University).

General methods. LB medium (59) was used for routine growth of *B. subtilis* and *E. coli*. Sporulation was induced by nutrient exhaustion in DS medium (61), with the start of sporulation (T_0) defined as the end of exponential growth. Selection for prototrophy in *B. subtilis* was performed on TSS medium (12) with the appropriate amino acid supplements at 50 μg/ml each.

Heat resistance and germination assays on sporulated cultures were described previously (10).

Plasmid constructions and transformation in *E. coli* were carried out as described by Sambrook et al. (59). The Geneclean kit (Bio 101) was routinely used to purify DNA fragments from agarose for subcloning. Selection for Ap^r was at 50 μg/ml.

Chromosomal DNA from *B. subtilis* cells was prepared as described by Cutting and Vander Horn (12).

Competent *B. subtilis* cells were prepared and transformed by the two-step method described by Cutting et al. (12, 17). Antibiotic resistance was selected at 5 μg of chloramphenicol per ml for Cm^r or at 25 μg of lincomycin per ml and 1 μg of erythromycin per ml for macrolide-lincosamide-streptogramin B (MLS) resistance.

Amylase activity was assayed by growing putative *amyE* mutants overnight on 1% starch plates and staining the agar with Gram's iodine stain (Sigma) (12).

β-Galactosidase activity in sporulating cultures was determined as previously described (49, 56), except that the cells

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

Strain	Genotype or description	Source or reference
PY79	Prototrophic	78
JH642	*trpC2 pheA1*	5
168	*trpC2*	Laboratory stock
CU267	*trpC2 ilvB2 leuB1*	Laboratory stock (S. Zahler)
131.5[b]	*spoIIB131 trpC2*	22
DZR143[c]	*spo0AΔ::erm trpC2 pheA1*	26
RS169[c,d]	P_{spac}-*spo0H* (*erm*) *trpC2 pheA1*	64
SC1160	*spoIID298*	9
SC1161	*spoIIE48*	9
PY180	*spoIIE*::Tn*917*ΩHU7	62
PM806[e]	*spoIIGAΔ17*	This study
KS265	*spoVG*::Tn*917*ΩHU265	62
LD5[f]	*spoIIAA-lacZ spoIIAC1 trpC2 lys-3*	18
PY415	*spoIIE*::Tn*917*ΩHU181-*lacZ*	27, 60
BZ184[g]	*amyE::spoIID-lacZ*	Laboratory stock
PS900[f,h]	*spoIIG-lacZ trpC2*	72
PS509[f]	*sspB-lacZ trpC2*	48
PM59[i]	SPβ::*cotA-lacZ*	This study
PM694	*spoIIB131 spoVG*::Tn*917*ΩHU265	This study
PM735	*spoIIBΔ::erm*	This study
PM740	*spoIIBΔ::erm spoVG*::Tn*917*ΩHU265	This study
PM760	*amyE::spoIIB*$^+$	This study
PM794	*spoIIB-lacZ*	This study

[a] All strains are congenic with the prototrophic wild-type strain PY79, except as noted.
[b] Congenic with CU267.
[c] Congenic with JH642.
[d] BH19 [P_{spac}-*spo0H* (*cat*) *trpC2 pheA1* (34)] was converted to MLS resistance (64). Strains containing this construct are *spo0H* mutants in the absence of the inducer isopropyl-β-D-thiogalactopyranoside.
[e] The *spoIIGAΔ17* mutation (69) was introduced into PY78 (the auxotrophic parent of PY79) by congression with *glnA*$^+$.
[f] Congenic with 168.
[g] The *amyE::spoIID-lacZ* transcriptional fusion (69) was transferred into PY79 by selection for Cm^r.
[h] A *spoIIIG-lacZ* translational fusion integrated at the *spoIIIG* locus.
[i] PY79 was transduced to Cm^r and MLS resistance with a lysate from SC432 (10).

were permeabilized by the addition of lysozyme (Sigma) to 200 μg/ml in the reaction buffer.

Electron microscopy was performed on sporulating cells harvested at T_4 as previously described (9), except that grids were stained with Reynolds lead exclusively.

Nucleotide sequence analysis. Nucleotide sequence was determined by the dideoxy chain termination method (63) with double-stranded templates and the Sequenase kit (U.S. Biochemicals) according to the strategy diagrammed in Fig. 1 and as described in the legends to Fig. 2 and 3. Oligonucleotide primers were purchased from New England Biolabs or synthesized on an Applied Biosystems 380B DNA Synthesizer. Sequences were assembled and analyzed by the Genetics Computer Group (University of Wisconsin) programs (13). Homology searches were performed with the GenBank data bank (1).

Chromosomal walking. The *spoIIB* gene was cloned by a chromosomal walk from the previously cloned *spoIVF* operon with pSR3 as a starting point (42, 55), which was created during the cloning of *spoIVF* (11). The insert in pSR3 extends from *spoIVF* to an *Eco*RI site located 4 kb upstream of the operon. A 1.1-kb *Pst*I-ended fragment from pSR3, which contained 0.8 kb of DNA from upstream of *spoIVF* and 0.3 kb of vector DNA, was subcloned into *Pst*I-cut pSGMU2 (24) to create pPM53. pPM53 was inserted into the *B. subtilis* chromosome by transforming competent PY79 and selecting for the *cat* gene on the plasmid. Chromosomal DNA from one such transformant (designated PM545) was digested with restriction endonucleases and circularized by ligation at a low DNA concentration; plasmid-associated sequences were then recovered by transforming *E. coli* TG1 to ampicillin resistance, which selects for the vector's *bla* gene. Endonucleases that cut once in the plasmid polylinker and once in the flanking *B. subtilis* DNA permitted recovery of upstream or downstream DNA, depending on the specific enzyme (20). Digestion with *Sma*I, *Bam*HI, or *Xba*I followed by circularization and transformation into *E. coli* yielded plasmids pPM56, pPM54, and pPM57, respectively (Fig. 1).

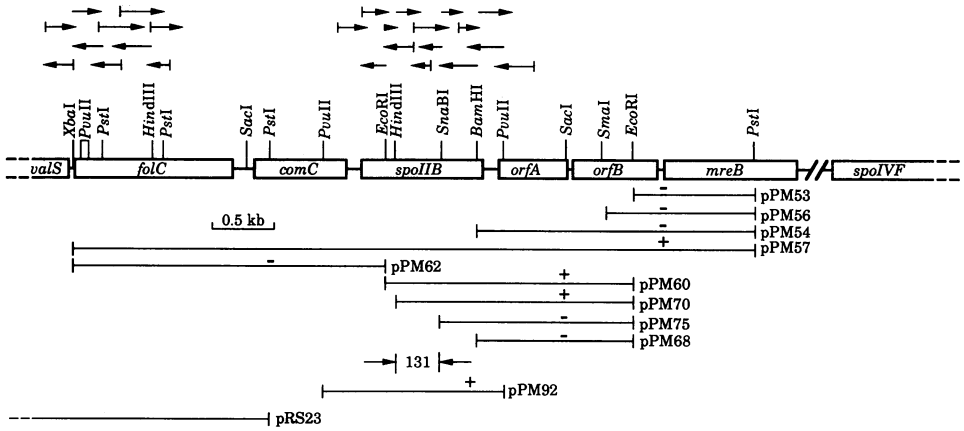

FIG. 1. Physical map of the *spoIIB* region of the chromosome. The figure shows a restriction map of the *spoIIB* region of the chromosome, with the ORFs predicted by nucleotide sequence analysis indicated in boxes. The direction of transcription is left to right in all cases. The broken line indicates a 3-kb gap between *mreB* and *spoIVF* which has been omitted for simplicity. The arrows above the restriction map indicate the directions and extents of the individual nucleotide sequence determinations. The symbols at the base of each arrow indicate whether the sequence was generated by use of a restriction site endpoint (no symbol) or an oligonucleotide primer (|). The names of recombinant plasmids and the interval of chromosomal DNA that each bears are indicated by the lines below the restriction map. + or −, ability or failure, respectively, of each plasmid to rescue the *spoIIB131* mutation upon integration into the chromosome; 131, the interval within which the *spoIIB131* mutation is expected to lie (see text). The insert in plasmid pRS23 (64) extends approximately 4 kb upstream of the leftmost restriction site (*Xba*I) shown in the figure.

pPM53 was also used to transform the *spoIIB131* mutant strain 131.5 to Cm^r, yielding strain PM544. Chromosomal DNA from PM544 was used to repeat the chromosomal walk described above via *Xba*I digestion, providing a plasmid (pPM66) that was expected to be identical to pPM57 except for the presence of the *spoIIB131* mutation.

pRS23 (64) was obtained in a chromosomal walk designed to retrieve DNA sequences upstream of the *Xba*I site that marked the upstream endpoint of the insert in pPM57. The construction of pRS23 will be described elsewhere.

Construction of plasmids used to localize the *spoIIB131* mutation. Digestion of pPM57 with *Eco*RI yielded two nonvector fragments of 2.0 and 2.4 kb (Fig. 1; the upstream *Eco*RI site of the 2.4-kb fragment was derived from the plasmid polylinker). The 2.0-kb fragment was subcloned into *Eco*RI-cut pSGMU2 in either orientation to give pPM60 and pPM61 (not shown in Fig. 1). The 2.4-kb fragment was inserted into *Eco*RI-cut pSGMU2 in a single orientation (pPM62).

pPM61 was cleaved with *Bam*HI (which cuts once within the plasmid polylinker and once within the insert), ligated, and recovered by transformation into *E. coli*. The resulting plasmid (pPM68) was deleted for part of the insert DNA. This strategy was used in the construction of pPM70 (by digesting pPM61 with *Hin*dIII). The 1.6-kb *Sna*BI-to-*Eco*RI fragment of pPM60 was ligated into pSGMU2 double cut with *Sma*I-*Eco*RI to generate pPM75.

Construction of a strain harboring *spoIIB* at the *amyE* locus. Partially diploid strains harboring a copy of *spoIIB* at the *amyE* locus were generated as described by Cutting and Vander Horn (12) with a 1.5-kb *Pvu*II-ended fragment from pPM57 that spans the complete *spoIIB* open reading frame (ORF) and flanking DNA. Several sequential subcloning steps resulted in the placement of this 1.5-kb fragment between the *Hin*cII and *Sma*I restriction sites of the pUC18 polylinker (77), generating pPM91. A 1.7-kb, *spoIIB*-containing *Pvu*II-*Sph*I fragment from pPM91 was subsequently subcloned into the *amyE* vector pDG364 (12) that had been cut with both *Eco*RV and *Sph*I. The resulting plasmid, designated pPM92, was linearized by cleavage with both *Xho*I and *Pvu*II and transformed directly into PY79, selecting for Cm^r. Since the linearized plasmid should recombine into the *amyE* locus by a double crossover ("marker replacement") event, *amyE* will be disrupted. The resulting transformants will be Cm^r and unable to produce amylase. One such *amyE*::*spoIIB*$^+$ derivative was designated PM760. Chromosomal DNA from PM760 was used to correct known *spoIIB* mutants by selection for Cm^r.

Construction of a strain harboring a *spoIIB* deletion mutation. The Tn*917*-derived *erm* cassette of pUC18-*erm* (37) was isolated as a *Bam*HI-to-*Pvu*II 2.4-kb fragment and ligated into *Bam*HI-*Sna*BI-cut pPM70 to create pPM86. In pPM86, the *Bam*HI-to-*Sna*BI interval of the *spoIIB* gene has been replaced with the *erm* gene oriented in the direction opposite to that of *spoIIB*. pPM86 was then linearized by digestion with *Eco*RI and used to transform competent PY79 to MLS resistance. Transformants, which were MLS resistant and Cm^s, were expected to have arisen by replacement of the endogenous *spoIIB* gene by the *spoIIB*Δ::*erm* mutant gene by means of double-crossover recombination. One such transformant was designated PM735. To construct a *spoIIB*Δ::*erm* strain congenic to JH642, chromosomal DNA from PM735 was used to transform competent cells of strain JH642 to MLS resistance; the resulting strain was designated PM797. A similar strategy was used to construct PM799 (*spoVG*::Tn*917*ΩHU265), which is congenic with JH642, by transforming competent JH642 to MLS resistance with chromosomal DNA from the *spoVG*::Tn*917* insertion mutant strain KS265 (Table 1).

Construction of the *spoIIB spoVG* double mutants. To construct the *spoIIB131 spoVG*::Tn*917*ΩHU265 double mutant, we transformed competent PY79 simultaneously with chromosomal DNA from strains KS265 and 131.5 and selected for the Tn*917*-conferred MLS resistance. These transformants were screened for the reduced sporulation efficiency indicative of the introduction of the *spoIIB131* mutation by congression (12). One such rare congressant was designated PM694. The presence of *spoIIB131* was confirmed by the ability of *spoIIB*$^+$-containing plasmids to correct the asporogenous phenotype of PM694. PM694 was subsequently transformed or transduced to Cm^r to introduce various *spo-lacZ* fusions that were marked with a *cat* gene (see Table 3). Transformation of PM694 with chromosomal DNA from LD5 (*spoIIAA-lacZ*), PY415 (*spoIIE-lacZ*), BZ184 (*spoIID-lacZ*), PS900 (*spoIIIG-lacZ*), or PS509 (*sspB-lacZ*) generated strains PM709, PM710, PM711, PM712, or PM800, respectively. Transduction of PM694 with a lysate from PM59 (*cotA-lacZ*) generated strain PM801. Similarly, we transformed competent PY79 cells to Cm^r with chromosomal DNA from LD5, PY415, PS900, or PS509 to generate the congenic wild-type strains PM706, PM707, PM708, or PM8, respectively.

The *spoIIB*Δ::*erm spoVG*::Tn*917*ΩHU265 double mutant was constructed in two steps. First, we performed a congression, this time cotransforming KS265 and JH642 chromosomal DNAs into competent PY79 and screening the MLS-resistant transformants for a Phe auxotrophy. Competent cells of one such congressant (named PM737) were then transformed to Phe$^+$ with PM735 chromosomal DNA. Since *pheA*$^+$ and *spoIIB*Δ::*erm* cotransform at low frequency, Phe$^+$ transformants could be screened for the asporogeny indicative of the *spoIIB spoVG* double-mutant genotype. One transformant with reduced sporulation efficiency was called PM740; the presence of a *spoIIB* mutation in this strain was confirmed by the ability of pPM57 to correct PM740's sporulation defect.

Construction of strains harboring a *spoIIB-lacZ* transcriptional fusion. Plasmid pPM89 was constructed by ligating the *Pvu*II-to-*Bam*HI fragment that includes the upstream end of the *spoIIB* ORF between the *Sma*I and *Bam*HI sites of the pUC18 polylinker. pSGMU32 (19) was cleaved with *Pst*I and *Sma*I to release an approximately 5-kb fragment that contains the *spoIIAA* ribosome-binding site upstream of *E. coli lacZ* as well as a *cat* gene. This *lacZ cat* cassette was ligated into the 3.7-kb *Pst*I-to-*Sna*BI fragment of pPM89 to generate pPM95-3, which carries a *spoIIB-lacZ* transcriptional fusion. pPM95-3 was transformed into competent cells of PY79 by selecting for Cm^r. The resulting strain (designated PM794) contains the plasmid integrated by single reciprocal (Campbell-like) recombination at the *spoIIB* locus; this strain contains a complete *spoIIB* gene as well as a second copy of the *spoIIB* promoter region upstream of and transcribing through *lacZ*. Competent cells of strain PM794 were transformed to MLS resistance with chromosomal DNA from strains (Table 1) RS169, DZR143, or PY180 to create PM809, PM853, or PM857, respectively. Other strains (PM855 and PM854, respectively) that are congenic with PM809 and PM853 but lack the *spoIIB-lacZ* fusion were constructed by transforming PY79 to MLS resistance with chromosomal DNA from strains RS169 and DZR143.

Similar manipulations permitted us to introduce a *spoIIB-lacZ* transcriptional fusion into the prophage of SPβ (36, 82).

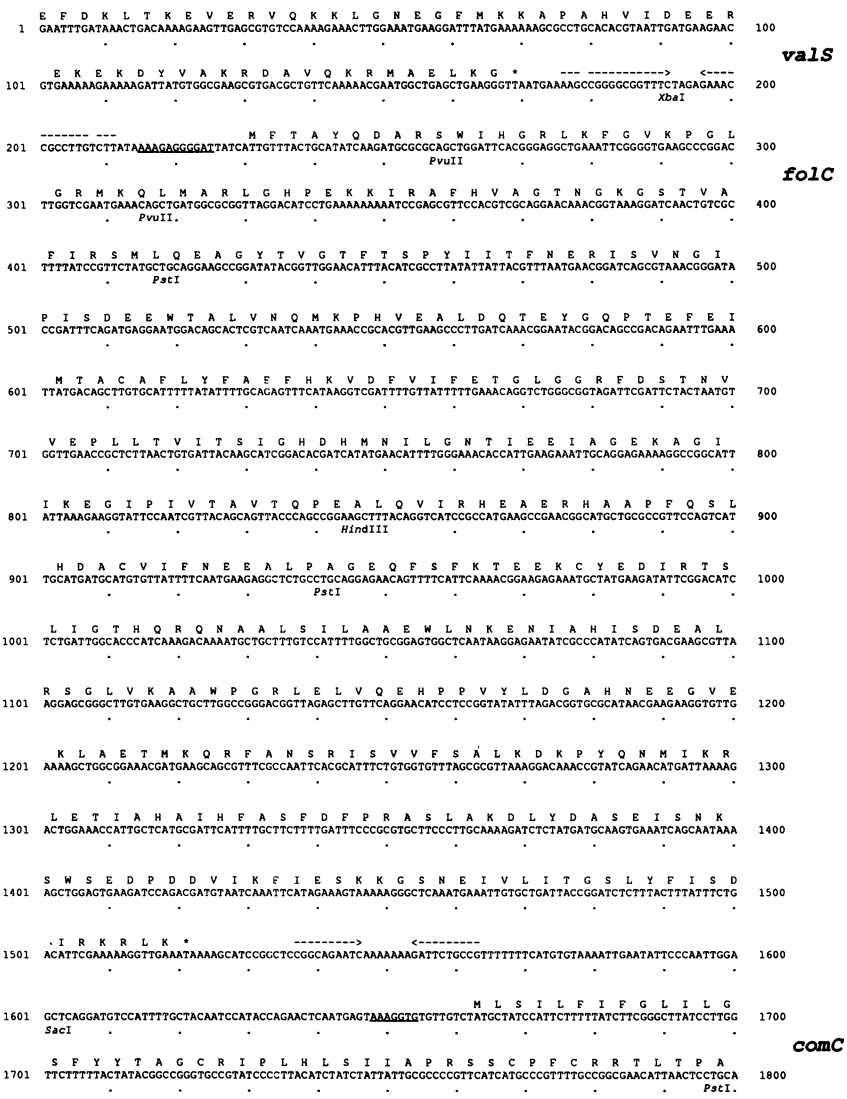

FIG. 2. Nucleotide sequence of the *folC* region of the chromosome. This figure shows the nucleotide sequence of the *valS*, *folC*, and *comC* ORFs and their predicted amino acid sequences. Mohan et al. (50) (GenBank accession number M30805) previously reported the sequence of nucleotides 844 through 2830 (Fig. 3); we have determined the sequence of bases 1 through 1020 on both strands. Within the region of overlapping nucleotide sequence determination, the two sequences were found to be in agreement, except at position 933 (see text). Amino acid sequence is shown in one-letter codes positioned above the first base pair of each codon. *, a stop codon. Proposed ribosome-binding sites are underlined. Horizontal arrows indicate inverted repeats that could serve as rho-independent transcriptional terminators.

The regulation of expression of this single-copy reporter fusion was indistinguishable from that observed for PM794 and its derivatives (47).

Nucleotide sequence accession numbers. The nucleotide sequences of the *valS*, *folC*, and *comC* genes (Fig. 2) and of the *spoIIB* gene (Fig. 3) have been deposited in the GenBank data base under accession numbers L04520 and L04519, respectively.

RESULTS

Nucleotide sequence of the *spoIIB* region of the chromosome. Previous genetic studies placed the *spoIIB* locus at 248° on the genetic map, within transforming distance of the *spoIVF* and *pheA* loci; the gene order was *spoIIB spoIVF pheA* (8, 41). To clone *spoIIB*, we took advantage of the availability from previous work (11) of cloned DNA adjacent to and upstream of *spoIVF*. This DNA was used as a starting point in a chromosomal walk (see Materials and Methods) that extended 9 kb upstream of *spoIVF* (Fig. 1). The previously determined linkage (60% cotransformation [41]) between *spoIIB* and *spoIVF* indicated that the *spoIIB* locus should be found within this 9-kb interval.

Nucleotide sequence analysis showed that the most-distal end of the cloned DNA upstream of *spoIVF* contained the 3′ portion of an ORF whose predicted product strongly resembles the carboxy terminus of the valyl-tRNA synthetases of *Bacillus stearothermophilus* (67% identical, 85% similar) and *E. coli* (35% identical, 57% similar) (3, 28). A plasmid, which was generated in other work (64) and which contains the sequenced portion of this ORF and an additional 3 kb of upstream DNA, was able to partially rescue the growth defect of an *E. coli valS* mutant (47). Therefore, structural

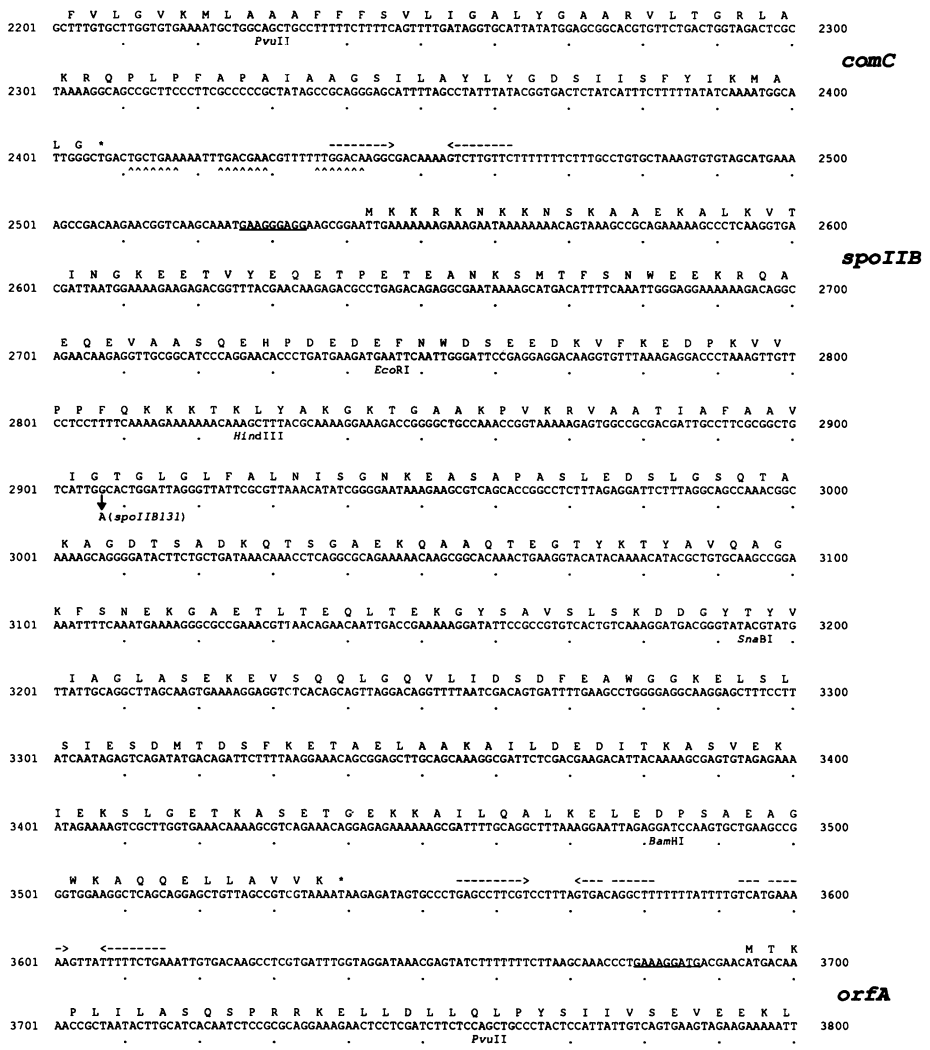

FIG. 3. Nucleotide sequence of the *spoIIB* region of the chromosome. This figure presents the nucleotide sequence of the *comC*, *spoIIB*, and *orfA* ORFs and their predicted amino acid sequences. Mohan et al. (50) (GenBank accession number M30805) previously reported the sequence of nucleotides 844 (Fig. 2) through 2830. We have determined the sequence of one strand between bases 2345 and 2540 and of both strands downstream of base 2540. The region of overlapping nucleotide sequence determination was found to be in agreement, except at position 2717 (see text). Levin et al. (42) (GenBank accession number M96343) presented the sequence downstream of the *spoIIB* gene (base 3539 and following). Stewart (67) has independently confirmed the nucleotide sequence downstream of base 2744. The numbering and annotation of the sequence in this figure are consistent with those of Fig. 2. ∧, similarity to the proposed SpoOA binding site [TG(A/T)CGAA; 71]. The vertical arrow indicates the site of the G-to-A nucleotide substitution corresponding to the *spoIIB131* mutation.

and functional analysis indicates that this ORF is the *valS* homolog of *B. subtilis*. The *valS* ORF is followed by an inverted repeat, which could serve as a transcriptional terminator.

Nucleotide sequence analysis extending downstream of *valS* revealed an ORF whose predicted product is similar to the N terminus of the folyl-polyglutamate synthetases (FolC) of *E. coli* and *Lactobacillus casei* (2, 73). The sequence that we obtained overlapped with the beginning of a previously reported ORF (ORF1 [50]) that could encode a protein with similarity to the carboxy terminus of the *E. coli folC* gene product. In the sequence reported by Mohan et al. (50), ORF1 is preceded by an in-frame stop codon and thus would not be part of the upstream ORF. However, our nucleotide sequence analysis revealed a single base (nucleotide 933; Fig. 2) that was missing in the analysis of Mohan et al. (50). As a result of this correction, we infer the existence of a single ORF extending from nucleotides 231 through 1520 (Fig. 2), whose product is highly similar to *E. coli* FolC along its entire length (30% identical, 53% conserved). In confirmation of this correction, the added base pair disrupts a potential *Sac*I restriction site predicted by the sequence reported by Mohan et al. (50) but which is absent from our clones (47). In addition, disruptions of the 5′ end of our full-length ORF displayed the slow-growth phenotype (47) described for ORF1 mutations (50), a finding consistent with the view that *folC* extends upstream of ORF1.

Downstream of *folC* is the competence gene *comC*, whose nucleotide sequence was previously reported by Mohan et al. (50). Two-factor crosses placed *comC* in the 240 to 250° region of the chromosome (29). In extension of the previous mapping results, our molecular characterization shows that *comC* is on the *spoIVF*-proximal side of *pheA*; the gene order is *comC spoIVF pheA*.

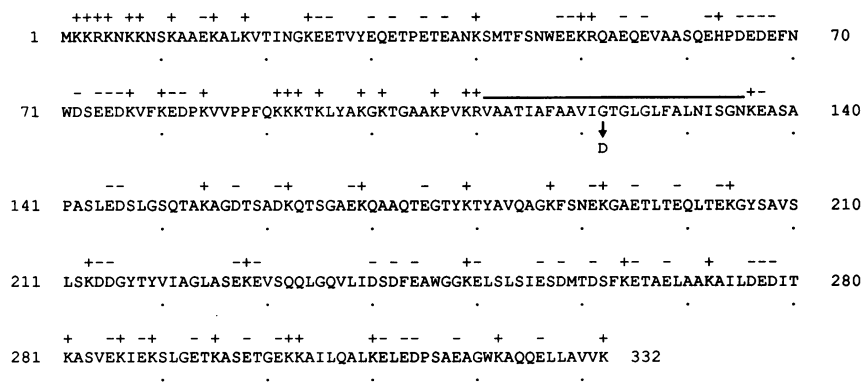

FIG. 4. The predicted *spoIIB* gene product. The predicted amino acid sequence of the *spoIIB* gene is presented in single-letter amino acid code. + and −, basic and acidic residues, respectively. A heavy bar overlies a stretch of 25 hydrophobic or uncharged amino acids that could serve as a transmembrane domain. The *spoIIB131* mutation results in the indicated Gly-to-Asp substitution (G122D), which introduces a charged residue into this uncharged region.

Downstream of *comC* is a large ORF, which, as demonstrated below, is the *spoIIB* gene. Our nucleotide sequence analysis, which incorporates the sequence published by Mohan et al. (50), initially revealed a 255-codon-long ORF beginning at position 2774 (Fig. 3). However, as pointed out to us by P. Stragier (68), an error in the sequence reported by Mohan et al. (50) would extend the 5′ end of the ORF to position 2543 (Fig. 3). Indeed, additional sequence analysis confirmed that the sequence reported by Mohan et al. (50) was missing a base pair at position 2717 (Fig. 3). In confirmation of this correction, the added base pair disrupts a potential *Fsp*I restriction site predicted by the sequence reported by Mohan et al. (50) but which is absent from our clones (47). The resulting extended ORF contains several possible in-frame start codons, but the most upstream start codon, the TTG at position 2543 (Fig. 3 [68]), is preceded at an appropriate distance by an excellent potential ribosome-binding site (GAAgGGAGG [capital letters indicate complementarity to the 3′ end of the *Bacillus* 16S rRNA] [51]). If this TTG is the start codon, then the *spoIIB* ORF would encode a polypeptide of 332 amino acids (M_r = 35,922). The predicted protein (Fig. 4) is rich in alanines (15%) but also includes a large number of charged amino acids, including lysines and glutamines (13% each). The distribution of charged and hydrophobic residues along this polypeptide suggests the following three protein domains (13): an amino terminus with alternating patches of positive and negative charge (110 residues, net charge of +4), a stretch of 25 nonpolar amino acids, and an acidic carboxy terminus (197 residues, net charge of −15). Immediately downstream of this ORF, we observe two different inverted repeats, which could serve as transcriptional terminators (Fig. 3).

Immediately downstream of the 332-codon ORF is a previously discovered ORF called *orfA* and six other ORFs in the interval extending to *spoIVF*, the nucleotide sequences of which have been reported elsewhere (42, 67, 75).

Identification of the 332-codon ORF as the *spoIIB* gene. To localize the *spoIIB* locus, we subcloned fragments from the *B. subtilis* insert in pPM57 and tested their abilities to rescue the *spoIIB131* mutation. As summarized in Fig. 1, we found that a plasmid insert (present in pPM70) extending from a downstream *Eco*RI site through the *Hin*dIII site in the middle of the 332-residue ORF was capable of correcting the *spoIIB131* mutation (although the resulting *spoIIB*$^+$ transformants were only partially restored in their capacities to sporulate; see below). By contrast, plasmid DNA (pPM75; Fig. 1) extending from the same downstream *Eco*RI site through a *Sna*BI site in the middle of the 332-residue ORF failed to correct the *spoIIB131* mutant defect.

This analysis indicated that *spoIIB131* is close to or within a 370-bp interval between the *Hin*dIII and *Sna*BI sites. Nucleotide sequence analysis of the corresponding *Hin*dIII-to-*Sna*BI fragment cloned from the *spoIIB131* mutant revealed a single difference in nucleotide sequence, a G-to-A transition at nucleotide 2907 (Fig. 3), which we infer to be the *spoIIB131* mutation. The discovery that *spoIIB131* is a transition mutation is consistent with the fact that this allele was generated by the use of nitrosoguanidine (7). *spoIIB131* is a missense mutation that would cause a Gly-to-Asp substitution at residue 122, which is in the nonpolar region of the predicted product of the 332-codon ORF (Fig. 4).

Our sequence analysis indicated that both an *Eco*RI and a *Bam*HI restriction site should be internal to this 332-codon ORF and that plasmids carrying this *Eco*RI-to-*Bam*HI fragment should disrupt *spoIIB* upon integration. As expected, a plasmid bearing such a 740-bp fragment generates a *spoIIB* mutant phenotype (see below) upon integration into the chromosome of wild-type strains; the same plasmid is unable to rescue the defect of a *spoIIB131* mutant (47).

Finally, we constructed a deletion in the 332-codon ORF by replacing the *Sna*BI-to-*Bam*HI interval of this ORF with an erythromycin resistance gene (*erm*; see Materials and Methods). The phenotype of the resulting strain (PM735; Table 1) was indistinguishable from those of *spoIIB131* mutants (see below). Thus, the effect of the *spoIIB131* mutation is no less severe than that of a deletion mutation.

The severity of the *spoIIB* mutant phenotype depends on genetic background. As indicated above, correction of the *spoIIB131* mutation by transformation with pPM70 or other plasmids containing the 332-codon ORF did not restore a fully Spo$^+$ phenotype to the *spoIIB131* mutant strain 131.5 (47). Strain 131.5 presumably contains additional mutations that cause a partial defect in sporulation. Therefore, to characterize *spoIIB* further, we used *spoIIB*Δ::*erm* (Table 2) or an integrational disruption of the gene (47) to introduce null mutations of *spoIIB* into the sporulation-proficient strains JH642, 168, CU267, and PY79. Surprisingly, the severity of the effect of the *spoIIB* mutation was found to be dependent on the strain into which the mutation was introduced. Thus, *spoIIB*Δ::*erm* caused a 10- to 150-fold reduc-

TABLE 2. Spore production in *spoIIB* and *spoVG* mutants

Genotype[a]	No. of heat-resistant spores/ml[b]			
	PY79		JH642	
	37°C	25°C	37°C	25°C
+	8×10^8	3×10^8	6×10^8	3×10^8
spoVG::Tn*917*ΩHU265	2×10^8	3×10^8	1×10^8	3×10^8
*spoIIB*Δ::*erm*	5×10^8	3×10^6–6×10^7	4×10^6–6×10^7	6×10^4–2×10^5
*spoIIB*Δ::*erm spoVG*::Tn*917*ΩHU265	4×10^4	8×10^4–1×10^6	ND	ND

[a] Mutant strains were congenic derivatives of their respective wild-type genetic backgrounds: *spo*$^+$ (PY79 or JH642), *spoVG* (PM670 or PM799), *spoIIB* (PM735 or PM797), and *spoIIB spoVG* (PM740). +, the wild-type (*spo*$^+$) strain.

[b] Heat resistance was determined as described in Materials and Methods. Strains were sporulated for about 24 h at 37°C or for about 50 h at 25°C. Values shown are the averages or range of values observed in at least two independent determinations. ND, not determined.

tion in sporulation efficiency when introduced into JH642 (Table 2), 168, or CU267 (47) but reduced sporulation by less than 2-fold when introduced into PY79 (Table 2). Thus, not only does the original *spoIIB* mutant strain contain more than one *spo* mutation, but certain wild-type, sporulation-proficient strains contain one or more allelic differences from PY79 that enhance the severity of the *spoIIB131* mutant phenotype. The nature of this allelic difference is considered below.

***spoIIB* mutants are cold sensitive for sporulation.** The severity of the *spoIIB* mutant phenotype depends not only on genetic background but also on temperature. Thus, when introduced into PY79, a strain in which *spoIIB*Δ::*erm* caused only a slight decrease in sporulation at 37°C, the *spoIIB*Δ::*erm* mutation reduced spore production by 10- to 100-fold when sporulation was carried out at 25°C (Table 2). Furthermore, when introduced into JH642, a strain in which *spoIIB*Δ::*erm* caused a significant reduction in spore production at 37°C, *spoIIB*Δ::*erm* caused a further reduction of 10- to 100-fold in spore formation when sporulation was carried out at 25°C (Table 2).

***spoIIB* mutant cells produce defective spores.** Thus, *spoIIB* mutant cells are oligosporogenous, allowing the production of spores to a greater or lesser extent, depending on genetic background and temperature. We have further observed that the spores that *spoIIB* mutant cells produce are aberrant in two respects. First, prolonged incubation (for several days) in sporulation medium results in spore lysis, as judged by losses in turbidity and in the number of heat-resistant spores (47). Second, spores purified from a *spoIIB*Δ::*erm* strain are slower to germinate than are *spoIIB*$^+$ spores (47). These observations suggest that *spoIIB* mutant spores are both unstable and defective in their ability to germinate.

Transcription of *spoIIB* is under sporulation control. The observation that *comC* is monocistronic (50) indicated that *spoIIB* is transcribed independently of the competence gene that precedes it. To study the regulation of the 332-codon ORF directly, we constructed a transcriptional fusion of *E. coli lacZ* to *spoIIB* (see Materials and Methods). The fusion was not measurably expressed during vegetative growth but was induced after the end of exponential growth in sporulation medium (Fig. 5A), although the level of expression (7 Miller units [compared with the endogenous β-galactosidase activity of 2 Miller units]) was lower than that observed for most other sporulation genes. Transcription of the *spoIIB-lacZ* fusion was strongly inhibited in *spo0A* or *spo0H* (*sigH*) mutant cells (Fig. 5B and C). In contrast, *spoIIB* expression was not reduced (and was in fact increased [Fig. 5D]) in a strain carrying a mutation in a gene (*spoIIE*) known to be required for gene expression after the septation stage of sporulation (43). Thus, its time of induction and pattern of dependence on other genes are consistent with the idea that *spoIIB* is under the control of regulatory events occurring at an early stage of sporulation.

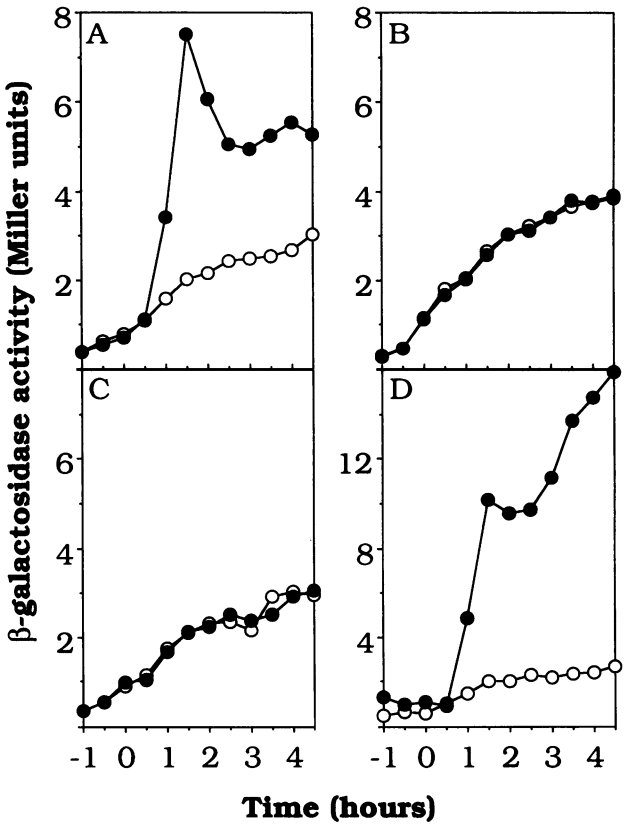

FIG. 5. *spoIIB*-directed β-galactosidase expression during sporulation. *spo*$^+$ cells (A) and *spo0A* (B), *spo0H* (C), and *spoIIE* (D) mutant cells were grown in DS medium. Samples were collected at the indicated times relative to the end of exponential growth (T_0) and assayed for β-galactosidase activity. The filled circles indicate the results from *spoIIB-lacZ*-bearing strains (PM794, PM853, PM809, and PM857 in panels A to D, respectively), and the open circles indicate the results from congenic strains lacking the fusion (PY79, PM854, PM855, and PY180 in panels A to D, respectively). The strains used in this experiment probably harbor more than one copy of the fusion. In other experiments (47), the peak expression observed for a *spoIIB-lacZ* fusion present in single copy was 2 Miller units above the level of endogenous activity.

Because of the low level at which *spoIIB* is expressed, it was not practical to determine the 5′ terminus of its transcript. Nevertheless, the promoter for *spoIIB* was expected to lie between the *comC* and *spoIIB* ORFs (Fig. 3), a region that contains the putative transcriptional terminator for *comC* (50). Examination of this 120-bp interval revealed no obvious match to any known (25) *B. subtilis* promoter consensus sequence (including that for promoters controlled by σ^H, the product of the *spo0H* gene), although sequences between positions 2470 and 2500 show weak similarity to the consensus sequence for σ^A promoters (68). However, as pointed out to us by P. Stragier (68), immediately downstream of the *comC* ORF (between positions 2411 and 2442) are three regions that resemble the consensus sequence binding site for the sporulation regulatory protein Spo0A [TG(A/T)CGAA] (71), a perfect "Spo0A box" flanked by two near-consensus (6 of 7 and 5 of 7 bp) binding sites. Since *spo0A* expression is under the control of σ^H (53, 76), the dependence of *spoIIB* transcription on *spo0H* could be an indirect consequence of the dependence of Spo0A synthesis on σ^H.

A *spoIIB spoVG* double mutant is strongly blocked at septation. The finding that mutations in *spoIIB* cause only a mild defect in sporulation is reminiscent of the observation that mutations in *spoVG* (62) and 18 other early-expressed genes that depend directly or indirectly on σ^H for their transcription (*csh* loci) (34) cause only a mild or undetectable impairment of sporulation. We suspected that certain members of this subset of early-expressed sporulation genes might play redundant roles in development, such that the sporulation defect of a strain mutant at any single locus would be concealed. Therefore, we constructed strains mutant in both *spoIIB* and each of these other genes. Strikingly, the combination of *spoIIB* and *spoVG* mutations generated a strong defect in sporulation. Either a *spoIIB* or a *spoVG* mutation alone caused a reduction by 4-fold or less in sporulation efficiency (at 37°C), but the double mutant was reduced by more than 10^4-fold in spore production (Table 2). This effect was unique to the combination of *spoIIB* and *spoVG*; none of the *spoIIB csh*::Tn*917-lacZ* double mutants (47) or the *spoVG csh*::Tn*917-lacZ* double mutants (26) was significantly impaired for sporulation, compared with the corresponding single mutants. Therefore, the *spoIIB* and *spoVG* genes are partially redundant for an important event in sporulation.

To identify the stage of blockage caused by the combination of the two mutations, we introduced fusions of *E. coli lacZ* to known developmentally regulated genes into a *spoIIB spoVG* double-mutant strain. As shown in Table 3, the double mutant was not impaired in the transcription of the σ^E- and σ^F-controlled genes *spoIID* (57) and *spoIIIG* (72), respectively, but was unable to support the transcription of the σ^G- and σ^K-controlled genes *sspB* (48) and *cotA* (61), respectively. Thus, *spoIIB spoVG* double mutants are able to direct the transcription of σ^E-controlled genes but are blocked in the activation of σ^G-directed gene expression, which is in turn required for the activation of σ^K (10, 45). Since the activation of σ^E is thought to require septation and that of σ^G engulfment (for a review, see reference 43), we infer that *spoIIB spoVG* double mutants are blocked prior to engulfment but after septum formation.

To characterize this mutant phenotype further, we examined cells at the fourth hour of development by electron microscopy. A high proportion of wild-type cells at the fourth hour of sporulation were found to have advanced beyond the stage (stage III) of engulfment (16). As expected from the results presented above, *spoIIB spoVG* double-mutant sporangia were found to be blocked at the stage (stage II) of sporulation septum formation (Fig. 6D to F). This is consistent with the block ascribed to the original *spoIIB* mutant Z3 (7), which we now suspect contained a synergistic allele of the *spoVG* gene (see below).

TABLE 3. Gene expression in *spoIIB spoVG* double mutants

Fusion[a]	Synthesis of β-galactosidase (% wild type)[b]
spoIIE-lacZ	92
spoIIA-lacZ	79
spoIIIG-lacZ	220
spoIID-lacZ	92
sspB-lacZ	7
cotA-lacZ	<1

[a] Each indicated reporter fusion was introduced into a *spoIIB131 spoVG*::Tn*917*ΩHU265 double-mutant strain as described in Materials and Methods.

[b] Synthesis of β-galactosidase was determined as described in Materials and Methods. Values indicate the levels of expression observed in the double mutant expressed as percentages of the expression seen in the corresponding wild-type (*spoIIB*$^+$ *spoVG*$^+$) strain harboring the same fusion. The levels of β-galactosidase expression (in Miller units) observed in the wild-type strains were 75 (*spoIIE-lacZ* at T_3), 930 (*spoIIA-lacZ* at T_3), 7 (*spoIIIG-lacZ* at T_3), 330 (*spoIID-lacZ* at T_3), 2,000 (*sspB-lacZ* at T_5), and 90 (*cotA-lacZ* at T_7). Values are the averages of two independent determinations.

Illing and Errington (33) distinguish three substages of sporulation septum formation. These are stage II_i, in which the septum is straight and contains a thin layer of peptidoglycan; stage II_{ii}, in which hydrolysis of the peptidoglycan from the center of the septum correlates with the bulging of the septum into the mother cell; and stage II_{iii}, in which the septum is largely or entirely devoid of peptidoglycan and has begun to engulf the forespore. Our own observations confirm these stages, but in addition we find that about 5% of stage II cells exhibit a sporulation septum with a thick layer of peptidoglycan similar to that of the median septum of vegetative cells (16). Mutations in the sporulation gene *spoIIE* (33) (Fig. 6A) cause the accumulation of sporangia with similarly thick sporulation septa, a finding that raises the possibility that sporangia with thick septa are a normal intermediate in sporulation septum formation. Alternatively, and perhaps more likely, the sporulation septum may be initially produced with a thin layer of peptidoglycan, and the thick sporulation septum may be an aberrant structure (33).

The septa of *spoIIB spoVG* mutant sporangia were straight or sometimes curved and seemed to contain a narrow gap (presumably due to a thin layer of peptidoglycan) between the mother cell and forespore membranes (Fig. 6D to F). In contrast to the septa of stage II_{ii} sporangia, the septa of *spoIIB spoVG* mutant sporangia did not display a central, peptidoglycan-free region (that is, lacking any observable gap between the two membranes) that bulged into the mother cell. The morphological block of *spoIIB spoVG* mutant sporangia is therefore most appropriately assigned to stage II_i. From the results of Illing and Errington (33) and from our own analysis (Fig. 6B) (16), the morphological block in *spoIIA* and *spoIIG* mutant cells is similarly assigned to stage II_i. In contrast, *spoIIE* mutant sporangia exhibit a thick sporulation septum (Fig. 6A) (33), which, as suggested above, may be a normal precursor to the thin septum of cells at stage II_i or, alternatively, an aberrant structure caused by *spoIIE* mutations. Finally, *spoIID* mutant cells (in which the two membranes are in tight juxtaposition and are possibly

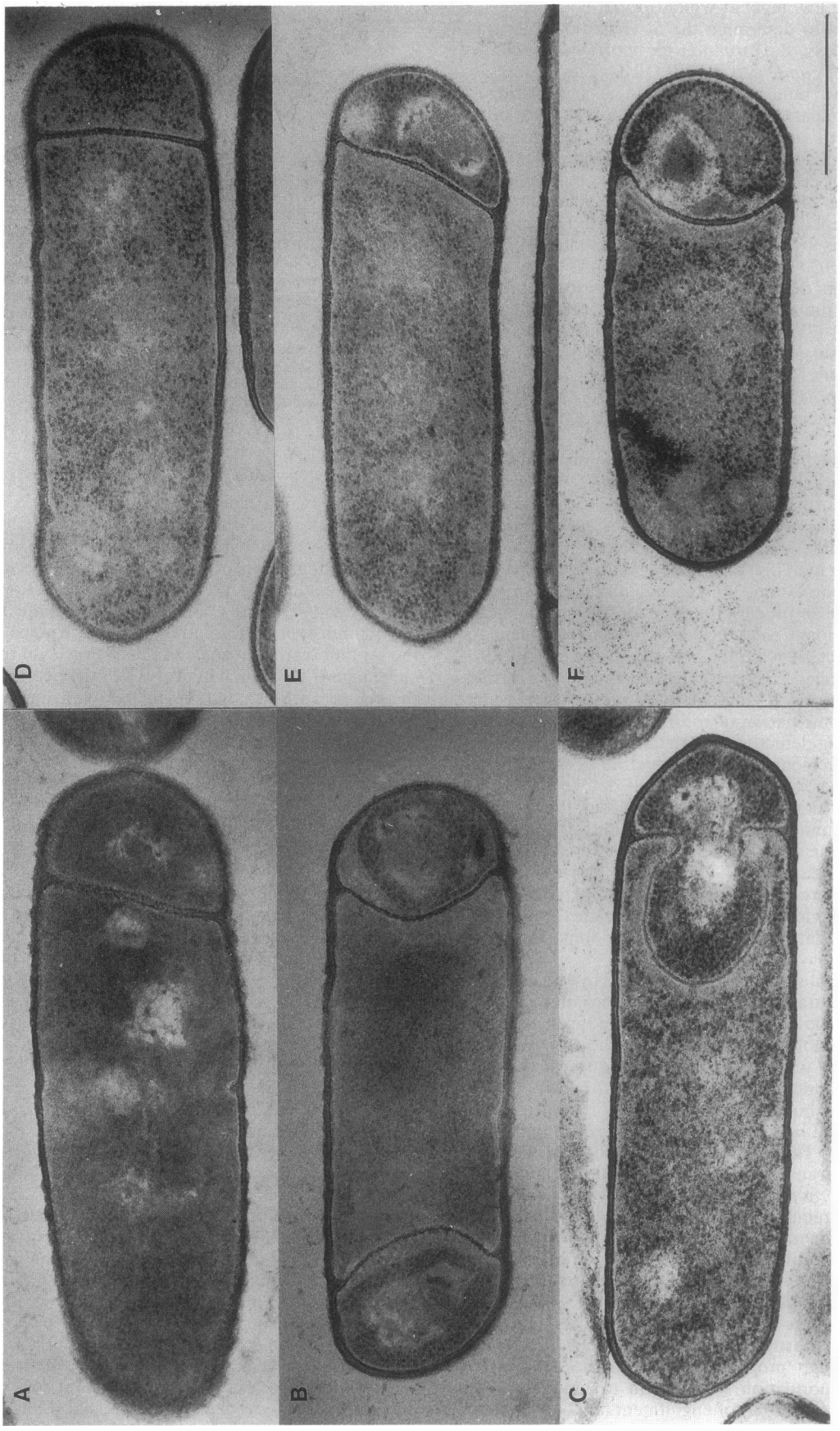

FIG. 6. Electron microscopy of mutant sporangia. Sporangia from *spoIIE* (SC1161 [A]), *spoIIG* (PM806 [B]), *spoIID* (SC1160 [C]), and *spoIIB spoVG* (PM740 [D to F]) mutant cells were grown at 37°C until the 4th hour of sporulation and then fixed and sectioned for examination by transmission electron microscopy (9). Scale bar, 500 nm.

fused into a single membrane [31]) are blocked at stage II_{ii} (Fig. 6C) (33).

A characteristic of the stage II_i *spoIIA* and *spoIIG* mutants is the frequent formation of septa at both poles of the sporangium (Fig. 6B) (52). Such mutants are said to be disporic. The *spoIIB spoVG* mutant cells, by contrast, are not disporic. They occasionally produce a second polar septum (16), but we believe that the presence of the second septum is not a true disporic phenotype but is instead the result of minicell formation. In other work, O. Resnekov (54) has found that the *spoVG* mutation (by itself) causes the formation of minicells during vegetative growth. Consistent with our interpretation, *spoIIB spoVG* mutant cells having a forespore at one end sometimes display a second polar cell, which we interpret as undergoing cytokinesis because it has begun to invaginate and separate from the sporangium (16).

The 332-codon ORF complements a *spoIIB* mutation. The strong combinatorial effect of *spoIIB* and *spoVG* mutations provided an easily detectable phenotype for *spoIIB* mutants. That is, in the presence of a *spoVG* mutation, the additional presence of a *spoIIB* mutation causes a strong defect in sporulation. We employed this observation to demonstrate that the 332-residue ORF alone is sufficient to complement *spoIIB* mutations. A 1.5-kb *Pvu*II fragment that contains the complete *spoIIB* ORF but only part of the flanking genes (Fig. 1 and 3) was placed at a second site (*amyE*) elsewhere in the *B. subtilis* chromosome (see Materials and Methods). *amyE*::*spoIIB*$^+$ chromosomal DNA from the resulting strain complemented the severe sporulation defect of a *spoIIB*Δ::*erm spoVG*::Tn*917*ΩHU265 double mutant, restoring the sporulation efficiency to that seen in a *spoVG* mutant alone (47). Thus, the phenotype of a *spoIIB spoVG* double mutant is the result of the absence of the 332-residue ORF gene product and not the result of polarity of the *erm* insertion upon the expression of a downstream gene. In addition, this experiment demonstrates that this 1.5-kb fragment contains all of the information necessary for *spoIIB* expression and function.

Heterogeneity at or near the *spoVG* gene of wild-type strains. As noted above, the sporulation defect of a *spoIIB* mutant varies with genetic background. The observed synergy between mutations in *spoIIB* and *spoVG* suggested that the strain variability of the *spoIIB* single-mutant phenotype may be due to allelic differences among the *spoVG* genes of various laboratory strains. Such an inference predicts that the *spoVG* locus from a PY79 strain should partially correct the sporulation defect of a JH642 strain bearing the *spoIIB*Δ::*erm* mutation (PM797). To test this hypothesis, chromosomal DNA from a PY79 strain carrying a *cat* gene integrated at a locus (*ctc* [30]) known to be approximately 50% linked to *spoVG* was used to transform competent cells of PM797 to Cm^r (47). About half of the Cm^r transformants were no longer impaired for sporulation at 37°C (47), as if a mutation at or near *spoVG* had been corrected. Thus, JH642 (and presumably 168 and CU267, by extension) apparently contains a partial loss-of-function allele of the *spoVG* gene compared with that of PY79. Therefore, we infer that *spoIIB131* was originally detected and assigned a stage II mutant phenotype only because the strain 168 genetic background used by Coote (7) contained an allele of *spoVG* that acted in synergy with *spoIIB131* to cause a block in sporulation.

DISCUSSION

Twenty contiguous genes in the *phe* region of the chromosome are oriented in the same direction as that of DNA replication. The cloning and characterization of DNA in the vicinity of *spoIIB* extend to 16 kb the contiguous stretch of DNA in the *phe* region of the chromosome that has been subjected to nucleotide sequence analysis (11, 23, 42, 67, 74, 75). This chromosomal region contains 20 ORFs, of which 17 correspond to genes whose functions have been inferred from genetic analysis or from the similarity of their predicted products to other proteins of known function. The order of these genes is as follows: *valS folC comC spoIIB orfA orfB mreB mreC mreD minC minD spoIVFA spoIVFB L20 orfX L24 spo0B obg pheB pheA*. Strikingly, all 20 genes are transcribed in the same direction, with an orientation that corresponds to the direction of DNA replication in the *phe* region of the chromosome. As noted previously, a high proportion of *B. subtilis* (and *E. coli*) genes (for which this information is available) are oriented in the chromosome such that the direction of their transcription corresponds to the direction of movement of the DNA replication fork (6, 79).

Synergistic interaction between mutations in *spoIIB* and *spoVG*. We have shown that the *spoIIB131* allele is a missense mutation in a 332-codon ORF. Complementation experiments and the use of an in vitro-constructed deletion mutation confirm that this ORF is *spoIIB* and that the phenotype of mutations in this gene is due to the absence of the *spoIIB* gene product rather than to a polar effect on the expression of downstream genes. Studies based on the use of a *spoIIB-lacZ* fusion show that the transcription of *spoIIB* is induced at and is under the control of regulatory events occurring at the start of sporulation.

The requirement for the *spoIIB* gene product in sporulation is conditional on genetic background and temperature. Thus, when introduced into the sporulation-proficient strain PY79, a *spoIIB* deletion mutation, *spoIIB*Δ::*erm*, caused no measurable impairment of the efficiency of sporulation at 37°C. However, when introduced into *spoVG* mutant cells, which are only slightly impaired in sporulation, *spoIIB*Δ::*erm* reduced spore formation at 37°C by more than 4 orders of magnitude, causing the cells to arrest development at the stage of asymmetric septation. Interestingly, when introduced into several other genetic backgrounds, the *spoIIB* deletion mutation caused a significant defect in spore formation. In at least one case (that of strain JH642), the difference from strain PY79 could be attributed to an allelic difference at or near *spoVG*. Thus, the variation in the severity of the *spoIIB* mutant phenotype in different strains may be due to allelic variation in the *spoVG* gene.

As noted above, the requirement for the *spoIIB* gene product in sporulation is also dependent on temperature. Thus, when introduced into PY79, a strain in which *spoIIB*Δ::*erm* caused little reduction in sporulation at 37°C, the deletion mutation reduced sporulation efficiency by 1 to 2 orders of magnitude at 25°C. Knowing that the requirement for *spoIIB* in sporulation is strongly influenced by the state of the *spoVG* gene, we speculate that the cold-sensitive phenotype of *spoIIB*Δ::*erm* is due to reduced synthesis or activity of the *spoVG* gene product at low temperatures.

spoVG was originally designated a stage V gene because a null mutation of the gene causes enhanced sporulation pigmentation and a mild impairment at a late stage of development (58, 62), even though it is under σ^H control and its transcription is induced at the start of sporulation (81).

Under the conditions of our present experiments, little effect of the *spoVG* mutation alone on spore formation was observed. Moreover, our results show that *spoVG* functions during or prior to the septation stage of sporulation. The stage II developmental block observed in the *spoIIB spoVG* double mutant is consistent with the fact that both *spoVG* and *spoIIB* are under σ^H control (directly or indirectly), and their products are therefore expected to be present prior to septation (70).

The synergy between *spoVG* and *spoIIB* mutations reveals that the primary function of *spoVG* is normally concealed by redundancy with the wild-type product of *spoIIB*. Redundancy of sporulation gene products has been observed previously for the *cot* loci, which encode the spore coat proteins (14, 80), and for the *ssp* loci, which encode the small acid-soluble DNA-binding proteins of the spore nucleoid (66). Our results, like those for the *cot* and *ssp* loci, imply the possible existence of additional *spo* genes that have not been detected by traditional genetic approaches because they are members of functionally redundant gene sets.

Two possibilities for the nature of the redundancy between *spoIIB* and *spoVG* are as follows. In the first, SpoIIB and SpoVG have similar (homologous) functions at stage II of sporulation and can largely substitute for each other in modifying the asymmetric septum. This seems unlikely, in that the proteins exhibit little similarity to each other either in amino acid sequence or size (36 kDa for SpoIIB versus 11 kDa for SpoVG [32]). Also, *spoIIB* is weakly expressed and *spoVG* is expressed strongly (81). The second possibility, which we favor, is that *spoIIB* and *spoVG* are members of redundant pathways governing a common critical event in septation but that the proteins play nonhomologous functions in the two pathways. This possibility predicts the existence of additional gene products with functions that are redundant to SpoIIB or SpoVG.

Implications for the mechanism of activation of σ^F and σ^E. The phenotype of the *spoIIB spoVG* double mutant is relevant to models of the ways in which the activities of σ^F and σ^E are controlled. According to current thinking, σ^F is a compartment-specific transcription factor that is present prior to septation but becomes active specifically in the forespore after the sporulation septum is formed (21, 43, 46, 47, 65). Like *spoIIE* mutant sporangia (33), *spoIIB spoVG* mutant cells are blocked at the septation stage of sporulation. However, in contrast to *spoIIE* mutant bacteria (46), *spoIIB spoVG* mutant cells are not blocked in σ^F-directed gene expression. Thus, our results and previous results with *spoIIG* mutants (33, 46) demonstrate that the activation of σ^F correlates with the stage (stage II_i) of sporulation at which the sporangium acquires a straight septum with a thin layer of cell wall material.

Like σ^F, σ^E is present in the predivisional cell, but it becomes active only after septation when its action is believed to be largely confined to the mother cell (15, 21, 43). The activity of σ^E is controlled at the level of the processing of its inactive precursor pro-σ^E (35, 40, 69), possibly in response to a signal from the forespore (43, 46). Because a *spoIIB spoVG* mutant is not blocked in σ^E-directed gene expression, we infer that the double mutant is not blocked in pro-σ^E processing. Thus, the hypothetical processing signal that emanates from the forespore would have to traverse a barrier that consists of both the forespore and the mother cell membranes as well as a small gap between the membranes that probably represents a thin layer of cell wall material. However, we cannot exclude the possible existence in the sporulation septum of the double mutant of localized patches (junctions) that are devoid of peptidoglycan and in which the two membranes are in tight juxtaposition. The intercompartmental signal transduction pathway that couples the processing of the precursor (pro-σ^K) of the late-acting mother cell transcription factor σ^K to the action of the late-acting forespore transcription factor σ^G similarly traverses a barrier that consists of the forespore and mother cell membranes (9, 10).

Successive stages in the dissolution of the cell wall layer from the sporulation septum. As noted above, the sporulation septum of *spoIIB spoVG* mutant cells exhibits a small gap (possibly consisting of a thin layer of peptidoglycan) between the two membranes. This phenotype is similar to that observed for *spoIIA* and *spoIIG* mutant cells (Fig. 6B) (33). Since *spoIIA* and *spoIIG* mutant cells are blocked in σ^E-directed gene expression, we infer that elimination of the gap between the membranes (possibly corresponding to the degradation of the residual layer of peptidoglycan) requires the concerted action of *spoIIB* or *spoVG* and a gene that has not yet been identified under the control of σ^E. Conceivably, SpoIIB is a peptidoglycan-degrading enzyme (or a modifier of such an enzyme) that is involved (together with the product of a σ^E-controlled gene) in removing the remaining layer of cell wall material from the septum. Consistent with such a hypothesis is the observation that the predicted product of *spoIIB* (but not that of *spoVG* [32]) contains a nonpolar segment, which could indicate that SpoIIB is an integral membrane protein. In addition, the carboxy terminus of SpoIIB shows weak but possibly significant similarity to a muramidase encoded by the *Bacillus licheniformis cwlM* gene (39, 47). Kuroda et al. (39) found that this region of the CwlM protein was essential for the substrate specificity but not for the enzymatic activity of this enzyme.

Taken together, our results and those of Illing and Errington (33) suggest that the transition from stage II (septation) to stage III (engulfment) may involve maturation of the sporulation septum in at least two steps. In the first step, the thin layer of cell wall material in the septa of sporangia at stage II_i is degraded, permitting the septal membranes to bulge into the mother cell (stage II_{ii}). This step requires either *spoIIB* or *spoVG* and an unidentified gene under the control of σ^E. In the second step, the septal membranes begin to migrate around (engulf) the prespore to generate the stage II_{iii} sporangium. This step may involve degradation of peptidoglycan in the forespore envelope at the leading edge of the migrating septum; the product of the *spoIID* gene, which is required for entry into stage II_{iii}, shows significant sequence similarity to a modifier of muramidase activity (38, 41a).

In summary, we have cloned and characterized *spoIIB* and have shown that its function in sporulation is largely redundant to that of another early-expressed sporulation gene, *spoVG*. Apparently, the effect of mutations in *spoIIB* or *spoVG* alone is largely concealed by the presence of a wild-type copy of the other gene. We infer from electron microscopy and dependence studies that the *spoIIB* and *spoVG* gene products are involved in maturation of the sporulation septum at a stage subsequent to events involved in the activation of the compartment-specific transcription factors σ^F and σ^E.

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