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Physical and Functional Characterization of the *Bacillus subtilis spoIIM* Gene

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The *spoIIM* locus of *Bacillus subtilis* is the most recently discovered of six genetic loci in which mutations can prevent the synthesis of a normal asymmetric septum or prevent migration of the septal structure to engulf the forespore compartment of the sporangium. Ultrastructure studies of a *spoIIM* mutant confirmed a block prior to the completion of engulfment. Introduction of a *spoIIM* mutation into a panel of strains containing *lacZ* fusions belonging to different regulatory classes allowed us to determine that the *spoIIM* gene product is required for the efficient expression of genes transcribed by σ^G-associated RNA polymerase but is not required for the expression of σ^F-controlled genes, including *spoIIIG*, which encodes σ^G. The results of complementation studies, gene disruption analysis, and DNA sequencing revealed that the *spoIIM* locus contains a single sporulation-essential gene encoding a polypeptide with a predicted molecular mass of 24,850 Da. The predicted *spoIIM* gene product is highly hydrophobic and very basic, and it does not exhibit significant homology to sequence files in several major data bases.

During endospore formation in *Bacillus* and *Clostridium* species, two cell types are formed as the result of asymmetric septation followed by engulfment of the forespore compartment (29, 39). Septum formation, the removal of peptidoglycan from between the septum membranes, directed membrane synthesis to extend the septal structure during engulfment, and unknown mechanical forces needed to accomplish septum membrane migration are evidently complex and highly regulated processes which might be expected to require the concerted action of many gene products. Nevertheless, after decades of genetic analysis, only six genetic loci in which mutations block the formation or migration of a normal sporulation septum (stage II loci) are known: *spoIIA*, *spoIIB*, *spoIID*, *spoIIE*, *spoIIG*, and *spoIIM* (39, 45). The failure to identify more such loci could reflect the fact that some might encode functions essential for viability, e.g., functions also required for septation or membrane biosynthesis during growth, such as *ftsA* or *ftsZ* (4). Others might encode redundant functions.

As with mutations that cause distinct developmental blocks at other morphological stages of sporulation in *Bacillus subtilis*, stage II mutations predominantly seem to identify genes whose products play key regulatory roles (29). The *spoIIA* locus contains three cistrons, IIAA, IIAB, and IIAC, all of which are known to be crucial for establishing compartment-specific gene expression during the early stages of sporulation (7, 32). The IIAC cistron encodes σ^F (12, 66), apparently the first forespore-specific transcription factor to become active. Activation of σ^F in turn requires the functions of the IIAA and IIAB gene products (47). The *spoIIG* locus contains two cistrons, IIGA and IIGB (20, 54). The IIGB cistron encodes σ^E, apparently the first mother cell-specific transcription factor to become active. Activation of σ^E requires not only the function of IIGA, but also the function of σ^F (presumably the product of some gene transcribed by σ^F-associated RNA polymerase) (19, 28). The *spoIID* locus is monocistronic and encodes a 37-kDa protein of unknown function (15, 26). It has a very hydrophobic amino terminus, which could serve as a membrane anchor. This feature has led to speculation that it might be engulfase, a hypothetical protein which promotes septum migration (52). The *spoIIE* locus is also monocistronic, and it encodes a 92-kDa protein of unknown function (3). Like SpoIID, but even more extreme in this respect, the SpoIIE protein has a highly hydrophobic amino terminus, which would seem likely to be inserted into a membrane (3). Mutations in *spoIIE* cause the formation of a thick, asymmetric septum and prevent the activation of both σ^F and σ^E (16).

The present article concerns *spoIIM*, the most recently identified stage II locus. This locus was defined in previous studies by a single insertion of Tn*917*, which was not extensively characterized physically or phenotypically (45). We have examined the ultrastructure of a *spoIIM* mutant, and here we document the effects of a *spoIIM* mutation on several well-characterized sporulation-specific *lacZ* fusions. We also have cloned the locus, defined its functional boundaries through integrative gene disruption experiments, and determined its DNA sequence. We demonstrate that *spoIIM* mutants exhibit a bona fide stage II morphological phenotype and, like *spoIID* mutants, block forespore-specific gene expression without affecting the activity of σ^F directly.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains used in this work are listed in Table 1; plasmids and bacteriophages are listed in Table 2.

Culture media and genetic techniques. LB medium, prepared as described previously (61), was used for routine culture of both *B. subtilis* and *Escherichia coli*. DS medium (DSM), prepared as described previously (61), was used as the sporulation medium for *B. subtilis*. Transformation of *B. subtilis* strains was carried out as described by Anagnostopoulos and Spizizen (1, 14). Transformation of *E. coli* strains

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TABLE 1. Bacterial strains

Strain	Relevant genotype, characteristic(s), and/or derivation	Source or reference
Escherichia coli		
HB101	F$^-$ *mcrB mrr hsdS20*(r_B^- m_B^+) *recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20*(Sm^r) *supE44* λ^-	6
DH5αF′	F′ϕ80d*lacZ* ΔM15 Δ(*lacZYA-argF*) *endA1 recA1 supE44 thi-1 relA1 hsdR17*(r^- m^+) *gyrA* λ^-	GIBCO BRL
MM294	*endA thiA hsdR17 supE44*	2
Mri93	F$^-$ Δ*lacU169 araD139 thiA rpsL relA* Δ*rbs7 pcnB80 zad*::Tn*10*	27
PY1182	*endA thiA hsdR17 supE44 pcnB80 zad*::Tn*10*; transduction of MM294 with P1 lysate Mri93 selecting for Tc^r	This work
Bacillus subtilis		
PY79	Prototrophic	63
PY403	*spoIIM*::Tn*917*ΩHU287	45
PY742	*trpC2 thr-5 spoIVC*::Tn*917lac*	P. Youngman
PY744	*trpC2 thr-5 cotA*::Tn*917lac*	P. Youngman
BG158	*metC2*	B. Green
ZB307	Lysogen SPβc2Δ2::Tn*917*::pSK10Δ6	67
MO1248	*trpC2 pheA1*; transcriptional *spoIVF-lacZ* at *amyE*	P. Stragier
MO464	*trpC2 pheA1*; transcriptional *spoIIIG-lacZ* at *amyE*	21
MO1096	*trpC2 pheA1*; transcriptional *spoIIIA-lacZ* at *amyE*	P. Stragier
BK595	Prototrophic; translational *spoIIID-lacZ* at *spoIIID*	23
PS287	*trpC2*; translational *sspB-lacZ* at *amyE*	33
PS301	*trpC2*; translational *sspA-lacZ* at *amyE*	33
PS435	*trpC2*; translational *sspE-lacZ* at *amyE*	33
RS79	*spoIIIG*Δ1; P_{spac}-*spoIIAC*	47
EU88924	*pheA1 trpC2*; lysogen SPβc2Δ2::transcriptional *gdh-lacZ* fusion	40
KE5	Lysogen SPβc2Δ2::Tn*917*::pSK10Δ6 transcriptional *spoIID-lacZ* (pKSV1); transformation of ZB307 with pKSV1 selecting for Cm^r	This work
KE11	SPβ::pKSV1 (transcriptional *spoIID-lacZ*); transduction of PY79 with SPβ::pKSV1 from KE5 selecting for Cm^r	This work
KE38	*spoIIM*::Tn*917*ΩHU287 *spoIVC*::Tn*917lac*; transformation of PY742 with DNA from PY403 selecting for MLS^r	This work
KE40	*spoIIM*::Tn*917*ΩHU287 *cotA*::Tn*917lac*; transformation of PY744 with DNA from PY403 selecting for MLS^r	This work
KE48	*spoIIM*::Tn*917*ΩHU287 SPβ::pKSV1 (transcriptional *spoIID-lacZ*); transduction of PY403 with SPβ::pKSV1 from KE5 selecting for Cm^r	This work
KE51	*spoIIM*::Tn*917*ΩHU287 SPβ::transcriptional *gdh-lacZ*; transduction of PY403 with SPβ::*gdh-lacZ* from EU88924 selecting for Cm^r	This work
KE61	*spoIIM*::Tn*917*ΩHU287::pTV20; transformation of PY403 with pTV20 selecting for Cm^r	This work
KE62	*spoIIM*::Tn*917*ΩHU287::pTV21Δ2; transformation of PY403 with pTV21Δ2 selecting for Cm^r	This work
KE72	SPβ::transcriptional *gdh-lacZ*; transduction of PY79 with SPβ::*gdh-lacZ* from EU88924 selecting for Cm^r	This work
KE77	Translational *sspB-lacZ* at *sspB*; transformation of PY79 with DNA from PS287 selecting for Cm^r	49
KE79	Translational *sspA-lacZ* at *sspA*; transformation of PY79 with DNA from PS301 selecting for Cm^r	This work
KE81	Translational *sspE-lacZ* at *sspE*; transformation of PY79 with DNA from PS435 selecting for Cm^r	This work
KE82	*spoIIM*::Tn*917*ΩHU287; translational *sspA-lacZ* at *sspA*; transformation of PY403 with DNA from PS301 selecting for Cm^r	This work
KE83	*spoIIM*::Tn*917*ΩHU287; translational *sspB-lacZ* at *sspB*; transformation of PY403 with DNA from PS287 selecting for Cm^r	This work
KE84	*spoIIM*::Tn*917*ΩHU287; translational *sspE-lacZ* at *sspE*; transformation of PY403 with DNA from PS435 selecting for Cm^r	This work
KE90	*chr*::pKE11; transformation of PY79 with pKE11 selecting for Cm^r	This work
KE125	*spoIIM*::Tn*917*ΩHU287; translational *spoIIID-lacZ* at *spoIIID*; transformation of PY403 with DNA from BK595 selecting for Cm^r	This work
KE208	*spoIIIG*Δ*1*; transformation of BG158 with DNA from RS79 selecting for Met$^+$, screening for Spo$^-$	This work
KE209	*spoIIIG*Δ*1*; transcriptional *spoIIIG-lacZ* at *amyE*; transformation of KE208 with DNA from MO464 selecting for Cm^r	This work
KE210	*spoIIIG*Δ*1*; translational *gpr-lacZ* at *gpr*; transformation of KE208 with pMS16 selecting for Cm^r	This work
KE211	*spoIIIG*Δ*1 spoIIM*::Tn*917*ΩHU287; transcriptional *spoIIIG-lacZ* at *amyE*; transformation of KE209 with DNA from PY403 selecting for MLS^r	This work
KE212	*spoIIIG*Δ*1 spoIIM*::Tn*917*ΩHU287; translational *gpr-lacZ* at *gpr*; transformation of KE210 with DNA from PY403 selecting for MLS^r	This work
KE215	Transcriptional *spoIIIA-lacZ* at *amyE*; transformation of PY79 with DNA from MO1096 selecting for Cm^r	This work
KE217	Transcriptional *spoIVF-lacZ* at *amyE*; transformation of PY79 with DNA from MO248 selecting for Cm^r	This work
KE218	*spoIIM*::Tn*917*ΩHU287; transcriptional *spoIIIA-lacZ* at *amyE*; transformation of PY403 with DNA from MO1096 selecting for Cm^r	This work
KE220	*spoIIM*::Tn*917*ΩHU287; transcriptional *spoIVF-lacZ* at *amyE*; transformation of PY403 with DNA from MO248 selecting for Cm^r	This work

TABLE 2. Phages and plasmids

Phage or plasmid	Relevant characteristics or derivation	Source or reference
M13 phages		
M13mp19*cat*	M13mp19 carrying *cat* gene cloned into *Ava*II site of phage	14
M.5	M13mp19*cat* containing a 1,073-bp *Sph*I-*Hin*dIII fragment carrying entire *spoIIM* open reading frame minus the last 5 codons	This work
M.5Δ1	M.5 derivative containing deletion 111 bp inward from *Sph*I site	This work
M.5Δ3	M.5 derivative containing deletion 135 bp inward from *Sph*I site	This work
M.5Δ3.1	M.5 derivative containing deletion 242 bp inward from *Sph*I site	This work
M.5Δ3.2	M.5 derivative containing deletion 423 bp inward from *Sph*I site	This work
M.5Δ4	M.5 derivative containing deletion 701 bp inward from *Sph*I site	This work
M.6	M13mp19*cat* containing a 1,073-bp *Sph*I-*Hin*dIII fragment in opposite orientation to M.5	This work
M.6Δ1	M.6 derivative containing deletion 132 bp inward from *Hin*dIII site	This work
Plasmids		
pGV34	Integrational vector containing a promoterless version of the *E. coli lacZ* coding sequence for generating transcriptional fusions and *cat* gene for selection in *B. subtilis*	65
pSR5	Integrational vector containing 900-bp fragment carrying the *spoIID* promoter	42
pBG6	Integrational vector containing a polylinker cloning site, the ColE1 and M13 origins of replication, and a *cat* gene for selection in *B. subtilis*	65
pTV20	Contains left and right arms of Tn*917* interrupted by a ColE1-derived cloning vector carrying a *cat* gene selectable in *B. subtilis*	64
pTV21Δ2	Contains left and right arms of Tn*917* interrupted by a ColE1-derived cloning vector (opposite orientation to pTV20) carrying a *cat* gene selectable in *B. subtilis*	64
pMS16	Contains translational *gpr-lacZ* fusion and *cat* gene for selection in *B. subtilis*	57
pKSV1	900-bp *Hin*dIII fragment from pSR5 containing *spoIID* promoter inserted into *Hin*dIII site of pGV34	This work
pKE1	Plasmid rescued from KE62 after *Hin*dIII digestion; contains 2.0-kb DNA adjacent to Tn*917* (Fig. 4)	This work
pKE5	Plasmid rescued from KE61 after *Sph*I digestion; contains 4.3-kb DNA adjacent to Tn*917* (Fig. 4)	This work
pKE11	3.5-kb *Bgl*II fragment from pKE5 inserted into *Bam*HI site of pBG6 (Fig. 4)	This work
pKE18	Plasmid rescued from KE90 after *Sph*I digestion; contains entire *spoIIM* locus (Fig. 4)	This work

was carried out as described by Lederberg and Cohen (24). Propagation of M13 constructions as phage in *E. coli* and isolation of virion or replicative form I (RFI) DNAs was carried out as described by Messing et al. (34, 60). Techniques for the induction and transduction of SPβ derivatives were as described by Rosenthal et al. (44) and Zuber and Losick (67). Selections for antibiotic resistance employed the following concentrations: chloramphenicol, 5 μg/ml; macrolides-lincosamides-streptogramin B (MLS), 1 μg of erythromycin per ml and 25 μg of lincomycin per ml; ampicillin, 50 μg/ml; and tetracycline, 12 μg/ml.

In vitro manipulations of DNA. Chromosomal DNA was isolated from *B. subtilis* by a method described previously (14). Isolation of plasmid DNA from *E. coli* was carried out by the alkaline lysis procedure of Birnboim and Doly (5). Restriction enzymes, DNA ligase, calf intestinal phosphatase, T4 DNA polymerase, and the Klenow fragment of DNA polymerase were purchased from Bethesda Research Laboratories, Inc., Boehringer Mannheim Biochemicals, or New England Biolabs, Inc., and used as instructed by the suppliers. Deletions into DNA fragments cloned into M13mp19*cat* derivatives were obtained by the method of Dale et al. (11) with reagents purchased from International Biotechnologies, Inc. Sequencing of DNA fragments cloned into M13mp19*cat* derivatives was performed by the method of Sanger et al. (46) with reagents purchased from United States Biochemicals Corp. Oligonucleotide primers used for sequencing were purchased from either Genosys Biotechnologies, Inc., or Bethesda Research Laboratories, Inc.

Gene disruption and complementation experiments. Gene disruption experiments with *B. subtilis* were performed by transforming *B. subtilis* strains with integrational plasmids carrying various DNA segments from the *spoIIM* region of the chromosome with selection for Cm^r as described previously (14). The sporulation phenotypes of the transformants were determined by examination of the drug-resistant colonies after growth on DSM agar plates for 2 days at 37°C. Complementation analysis was performed by the introduction of SPβ derivatives containing deletions in the *spoIIM* locus into PY403 by specialized transduction with selection for Cm^r and MLS^r. Complementation of the Spo^- phenotype was judged by both visual examination of colonies and microscopic examination of bacteria after growth on a DSM agar plate for 2 days at 37°C. Spore titers were determined 15 h after the cessation of exponential growth in DSM broth at 37°C with vigorous shaking. Dilutions of culture samples were plated for viable count determination before and after being heated for 15 min at 85°C to select for spores. The spore titer represents the ratio of heat-resistant count to the overall viable count, and it was the average of two independent experiments.

Cloning and sequencing of *spoIIM*. To clone the *spoIIM* locus, the Tn*917*-derived cloning vectors pTV20 and pTV21Δ2 (63) were integrated into the chromosome of PY403 (*spoIIM*::Tn*917*ΩHU287) by recombination with the homologous sequences in Tn*917*. Chromosomal DNA was prepared from the resulting transformants and digested with *Sph*I or *Hin*dIII. The digestions were diluted to a concentration of 5 μg/ml, ligated, and used to transform *E. coli* HB101 to Ap^r. pKE1, containing 2.0 kb of *B. subtilis* chromosomal DNA adjacent to the Tn*917* insertion, was obtained from *Hin*dIII-digested chromosomal DNA of KE62 (*spoIIM*::Tn*917*::pTV21Δ2). pKE5, containing 4.3 kb of *B. subtilis* chromosomal DNA, was obtained from *Sph*I-digested chromosomal DNA of KE61 (*spoIIM*::Tn*917*::pTV20). As indicated in Fig. 4, various DNA fragments from pKE1 and pKE5 were subcloned into the *B. subtilis* integrational vectors pBG6 (65) and pBG14 (65) for gene disruption analysis to determine the functional boundaries of the *spoIIM* locus. To recover an intact copy of the entire *spoIIM*

locus, a 3.5-kb *Bgl*II DNA fragment from pKE5 containing sequences from the end of Tn*917* and the adjacent chromosomal DNA was cloned into pBG14, resulting in pKE11. pKE11 was integrated into the chromosome of PY79 by a single reciprocal (Campbell-like) recombination event with selection for Cm^r. Chromosomal DNA from the resulting Cm^r transformants was isolated and digested with *Sph*I. The digestion was diluted to 5 μg/ml, ligated, and transformed into *E. coli* PY1182 (a *pcnB* mutant) with selection for Ap^r. pKE18 was digested with *Hin*dIII and the 1,073-bp DNA fragment expected to carry the entire *spoIIM* locus was ligated to the *Hin*dIII backbones of pTK-lac (22) and M13mp19*cat* (14). The resulting M13mp19*cat* constructs, M.5 and M.6, contained the 1,073-bp DNA fragment in opposite orientations, such that deletions made by the method of Dale et al. (11) would extend inward from the *Sph*I site in M.5 and from the *Hin*dIII site in M.6. Deletion derivatives of M.5 and M.6 were sequenced such that both strands of the entire 1,073-bp DNA fragment were sequenced at least once. *Hin*dIII-*Eco*RI fragments containing the deletions were then subcloned into pTK-lac for complementation analysis by ligation to the *Eco*RI-*Hin*dIII backbone of pTK-lac. The remaining sequence of *spoIIM* was obtained by ligating a 2.0-kb *Pvu*II fragment from pKE18 that spans the 3′ end of *spoIIM* into the *Sma*I backbone of M13mp19*cat* in both orientations. Sequence was obtained from the *Pvu*II site within the *spoIIM* gene outward for an additional 95 bp beyond the *Hin*dIII site. The sequence was then confirmed on the opposite strand with a primer (5′GGCATGGAAGTTTTGCGGCA3′) to a sequence inward from an arbitrary point downstream of the *spoIIM* coding sequence. IFind programs of the Intelligenetics Suite were used to search sequence data bases for similarities between *spoIIM* and previously characterized genes or proteins; the most recent searches employed GenBank Release 72, SwissProt Release 23, EMBL Release 32, and PIR Release 33.

β-Galactosidase assays. *B. subtilis* strains were cultured and assayed for β-galactosidase activity as described previously (61, 62). At various intervals during growth and sporulation, 0.5-ml samples were removed and immediately frozen in either liquid nitrogen or a dry ice-ethanol bath. β-Galactosidase activity in these samples was measured fluorometrically. For each assay, an isogenic wild-type strain lacking a transcriptional *lacZ* fusion was assayed in parallel to determine background β-galactosidase activity. This background was subtracted from each assay sample to generate the datum points in Fig. 2 and 3. These datum points represent the averages of at least two independent experiments.

Electron microscopy. Cultures of PY79 and PY403 were grown in 2× DSM at 37°C with vigorous aeration. Growth of the cultures was monitored by measuring turbidity, and 4.5-ml samples were removed for fixation at 2.5 and 5 h following the cessation of the exponential phase. Culture samples were fixed by the addition of 0.25 ml of 20% (vol/vol) paraformaldehyde–25% (vol/vol) glutaraldehyde. After incubation for 1 h at room temperature and overnight at 4°C, fixed cells were pelleted by centrifugation (5 min, 800 × *g*), washed in 0.1 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)]–0.05 M $CaCl_2$ (pH 7.2), centrifuged again (5 min, 800 × *g*), and resuspended in 5 ml of the same buffer with 1.0% OsO_4. After incubation for 5.5 h on ice, samples were pelleted again by centrifugation and encapsulated in 2% agar. Dehydration was carried out by successive washings at room temperature with increasing concentrations of acetone (once at 60%, 10 min; once at 80%, 10 min; twice at 90%, 10 min; and twice at 100%, 20 min), followed by overnight incubation in a 1:1 mixture of Spur's resin and acetone under a nitrogen atmosphere. This was followed by resuspension in full-strength resin and polymerization for 18 h at 60°C. Sections were cut with a diamond knife on a Sorvall ultramicrotome, stained with uranyl acetate and lead acetate, and viewed and photographed with a Philips 420 electron microscope.

TABLE 3. Morphological phenotype of *spoIIM*::Tn*917*ΩHU287

Strain	Description (morphological stage)[a]	No. of sections[b]
PY79	No septum	23
	Divisional septum	4
	Asymmetric septum (II_i)	18
	Engulfing septum (II_{ii} or II_{iii})	6
	Engulfed forespore	14
PY403	No septum	18
	Divisional septum	6
	Asymmetric septum (II_i)	8
	Engulfing septum (II_{ii} or II_{iii})	15
	Engulfed forespore	0

[a] Morphological stages were as defined by Illing and Errington (16).
[b] Full longitudinal sections only.

Nucleotide sequence accession number. The DNA sequence shown in Fig. 6 was deposited in the GenBank sequence data base under accession number L06664.

RESULTS

***spoIIM*::Tn*917*ΩHU287 results in a block at stage II of sporulation.** The original classification of *spoIIM*::Tn*917*ΩHU287 as a stage II mutation was based exclusively on biochemical criteria and on morphological features that could be detected by phase-contrast microscopy (45). The results of this analysis were consistent with a block at stage II, but a block at stage III could not be ruled out. To determine the morphological phenotype definitively, cultures of wild-type bacteria (PY79) and a *spoIIM* mutant (PY403) were grown to stationary phase in 2× DSM, and samples were fixed for electron microscopy 2.5 h after the cessation of exponential growth. Full longitudinal sections were scored for the presence of septal structures or engulfed forespores (Table 3). Adopting the nomenclature used by Illing and Errington (16) to describe different substages of the septation and engulfment processes, a distinction was made between cells containing asymmetric septa (stage II_i) and cells in which engulfment has initiated (stage II_{ii} or II_{iii}). In random fields of sections prepared from wild-type cultures, many cells containing fully engulfed forespores were found, and all substages of septum migration were observed (Table 3; Fig. 1A). No fully engulfed forespores were found in the mutant either in random fields recorded photographically or by direct screening of hundreds of cell sections at the microscope, although septal structures characteristic of stage II_i or II_{ii} were plentiful (Table 3; Fig. 1B). No disporic structures were seen. Thus, the *spoIIM* mutation caused a stage II block similar to that caused by *spoIID* and less pleiotropic *spoIIAC* mutations (16).

To investigate the possibility that complete engulfment was delayed but not absolutely prevented in a *spoIIM* mutant, sporulating cultures were also sampled for electron microscopy at 5 h after the end of log-phase growth (T_5). In

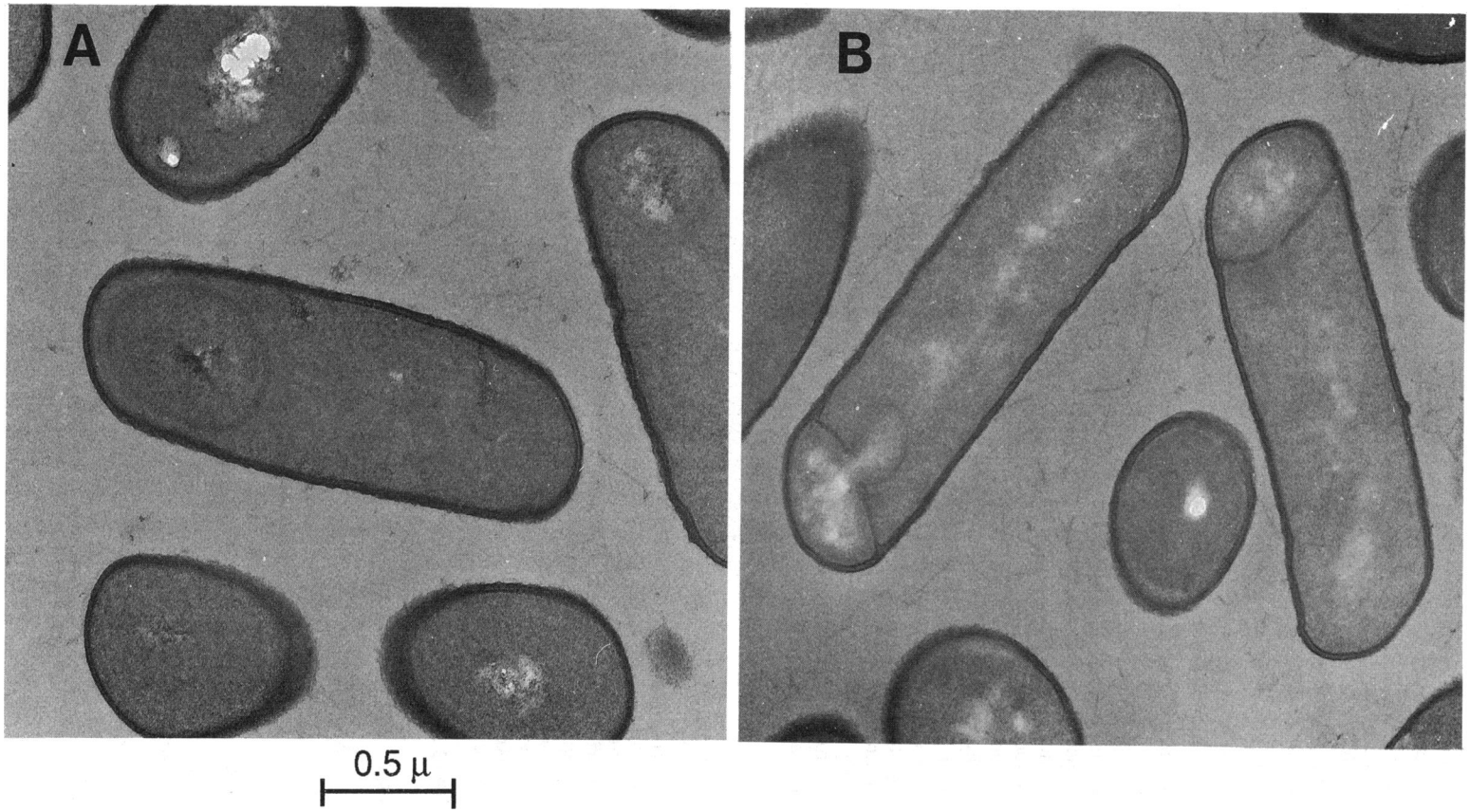

FIG. 1. Ultrastructural features of a *spoIIM* mutant. Isogenic wild-type (PY79) (A) or *spoIIM* mutant (PY403) (B) bacteria were harvested for fixation 2.5 h after the initiation of sporulation in DS medium (see Materials and Methods). (A) Typical appearance of a longitudinal section scored as containing a completely engulfed forespore (Table 3); (B) two typical examples of longitudinal sections scored as containing asymmetric septa (Table 3). Magnification, ×37,000.

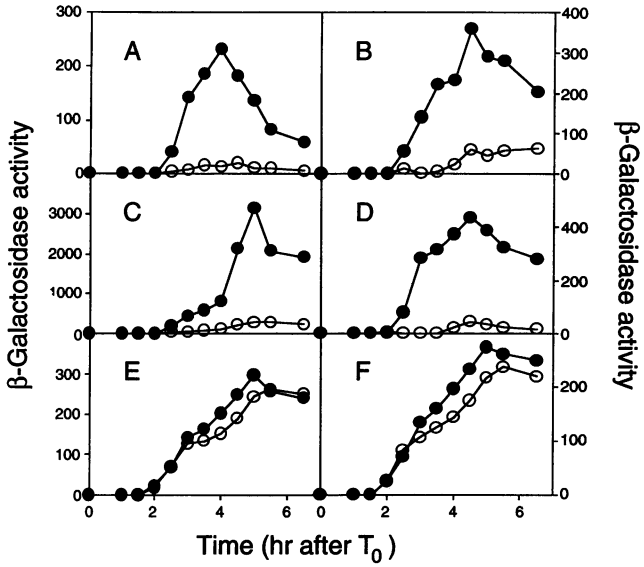

FIG. 2. Effects of *spoIIM* mutation on forespore-specific gene expression. Cultures of strains carrying a wild-type copy of the *spoIIM* locus (●) or isogenic strains carrying the *spoIIM*::Tn*917*ΩHU287 mutation (○) and containing various *lacZ* fusions were grown in DS medium and sampled at the indicated times after the end of exponential growth (T_0) for the determination of β-galactosidase activity as described by Youngman (61). The following *lacZ* fusions were assayed (strain designations are for wild-type *spoIIM* locus and *spoIIM* mutant strains, respectively): *sspA* in KE79 and KE82 (A), *sspB* in KE77 and KE83 (B), *sspE* in KE81 and KE84 (C), *gdh* in KE72 and KE51 (D), *spoIIIG* in KE209 and KE211 (E), and *gpr* in KE210 and KE212 (F).

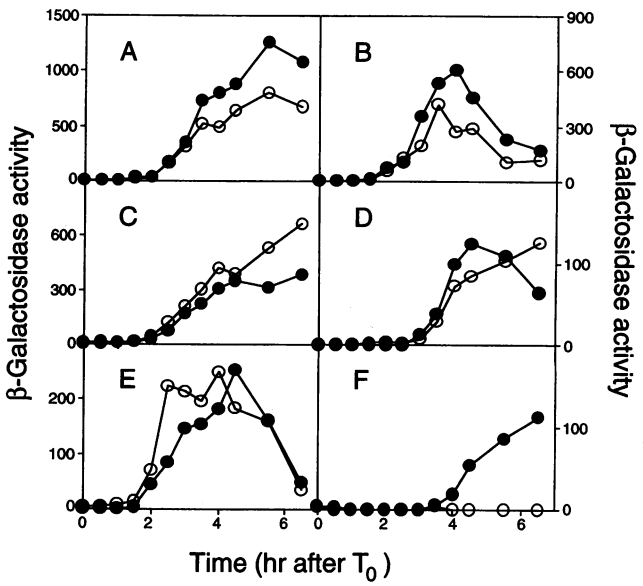

FIG. 3. Effect of a *spoIIM* mutation on mother cell-specific gene expression. Cultures of wild-type bacteria (●) or isogenic *spoIIM* mutants (○) containing various *lacZ* fusions were grown in DS medium and sampled at the indicated times after the end of exponential growth (T_0) for the determination of β-galactosidase activity as described by Youngman (61). The following *lacZ* fusions were assayed (strain designations are for wild-type and *spoIIM* mutant strains, respectively): *spoIID* in KE11 and KE48 (A), *spoIIIA* in KE215 and KE218 (B), *spoIIID* in KE90 and KE125 (C), *spoIVCB* in PY742 and KE38 (D), *spoIVF* in KE217 and KE220 (E), and *cotA* in PY744 and KE40 (F).

samples from wild-type cultures, many cells were in later stages of spore formation (stage IV or later). However, no septal structures or engulfed forespores were found in samples from the *spoIIM* mutant (48). We conclude from this that either the septal structures themselves or the cells blocked in septum migration are prone to lysis and disintegration.

Effect of a *spoIIM* mutation on gene expression in the forespore and mother cell compartments. As a first step toward understanding the function of the *spoIIM* gene product during sporulation, we examined the effect of *spoIIM*::Tn*917*ΩHU287 on the expression of several developmentally regulated genes. It was known from previous work that *spoIIM* mutants lacked glucose dehydrogenase activity (45). This sporulation-specific enzyme is encoded by *gdh*, a forespore-specific gene transcribed by σ^G-associated RNA polymerase (36, 37). To determine whether *spoIIM* mutations blocked the expression of *gdh* and other σ^G-dependent genes at the level of transcription, *spoIIM*::Tn*917*ΩHU287 was introduced into strains containing transcriptional *lacZ* fusions to *gdh*, *sspA*, *sspB*, and *sspE*. In all of these strains, expression of the *lacZ* fusions was severely inhibited (Fig. 2). To determine whether mutations in *spoIIM* prevent the synthesis of σ^G itself, the *spoIIM*::Tn*917*ΩHU287 mutation was introduced into a strain, KE211, containing a *lacZ* fusion to *spoIIIG*, the gene encoding σ^G (21, 56). The *spoIIIG* gene is transcribed predominantly by σ^F-associated RNA polymerase (31, 32, 38, 47, 55), but its expression may be autoregulated to some extent (21). In KE211, the *spoIIIG-lacZ* fusion is integrated at the *amyE* locus and a deletion of *spoIIIG* eliminates any contribution of σ^G to its own expression; under these circumstances, expression of the fusion presumably depends exclusively on σ^F. As shown by the results in Fig. 2E, the *spoIIM*::Tn*917*ΩHU287 mutation had no significant effect on the *spoIIIG-lacZ* fusion in KE211. To test the effect of *spoIIM*::Tn*917*ΩHU287 on another σ^F-dependent promoter, we examined a *gpr-lacZ* fusion (57). Again, only slight inhibition was observed (Fig. 2F). We conclude from these results that mutations in *spoIIM* severely inhibit the utilization of σ^G-dependent promoters but do not significantly affect σ^F-dependent promoters, including the σ^F-dependent promoter primarily responsible for σ^G synthesis.

To investigate the possible effect of a *spoIIM* mutation on gene expression in the mother cell compartment, we examined *lacZ* fusions to the σ^E-dependent promoters for *spoIID*, *spoIIIA*, *spoIIID*, *spoIVCB*, and *spoIVF*. In no case did we observe significant inhibition or enhancement of expression (Fig. 3A to E). However, expression of a σ^K-dependent mother cell gene, *cotA*, was strongly inhibited (Fig. 3F), presumably reflecting the lack of pro-σ^K processing expected in the absence of σ^G activity (9, 10, 30).

Genetic organization of the *spoIIM* locus. To recover cloned DNA spanning the *spoIIM* locus, vectors pTV20 and pTV21Δ2 were integrated by recombination at the site of the *spoIIM*::Tn*917*ΩHU287 insertion as described by Youngman et al. (64). DNA adjacent to the site of the insertion was rescued by transformation of an *E. coli* strain with ligated restriction digests of chromosomal DNA from pTV20 or pTV21Δ2 integrants. Information from restriction analysis of rescued DNA is summarized in Fig. 4. The results of Southern hybridizations indicated that the *spoIIM*::

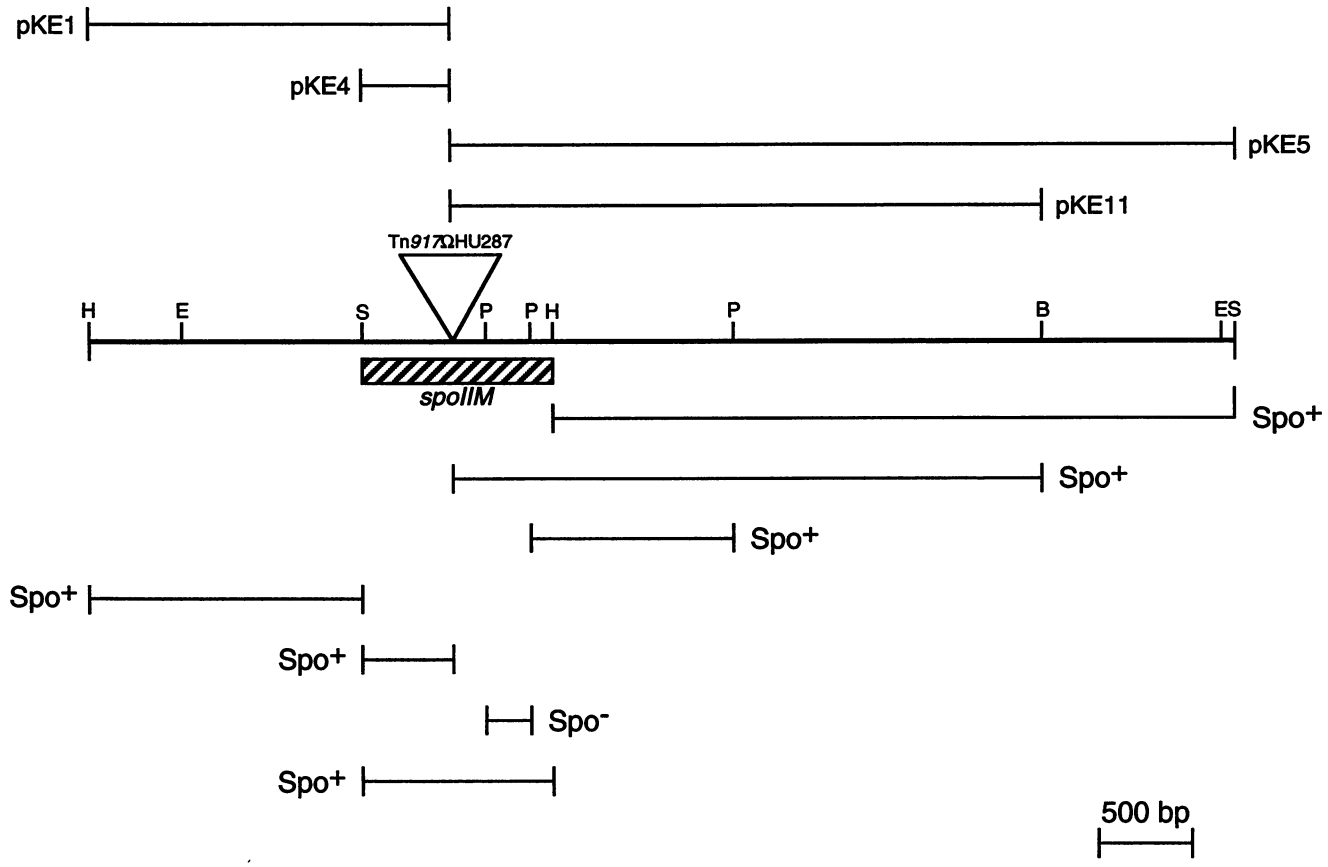

FIG. 4. Physical map of the chromosomal region encoding *spoIIM* gene function. Shown above the restriction map of the *spoIIM* region are chromosomal DNA fragments (with the corresponding plasmid indicated) rescued from strains containing the Tn*917*-derived vector pTV20 (KE61) or pTV21Δ2 (KE62) integrated into *spoIIM*::Tn*917*ΩHU287 (64). Plasmid pKE11, a *B. subtilis* integrational vector containing a 3.5-kb *Bgl*II DNA fragment from pKE5 encompassing the end of Tn*917* and the adjacent chromosomal sequences, was used to rescue a segment of the chromosome containing the entire *spoIIM* locus (see Materials and Methods). Indicated below the restriction map are DNA fragments subcloned into *B. subtilis* integrational vectors for gene disruption experiments to define the functional boundaries of the *spoIIM* locus. The sporulation phenotype resulting from the integration of each vector into the chromosome of a wild-type strain (PY79) is indicated next to each DNA fragment. The results of the gene disruption analysis localized the *spoIIM* gene to a 1,073-bp *Sph*I-*Hin*dIII fragment (hatched box). The approximate location of the Tn*917* insertion was determined from restriction analysis. The relevant restriction sites are shown. H, *Hin*dIII; E, *Eco*RI; S, *Sph*I; P, *Pvu*II; B, *Bgl*II.

Tn*917*ΩHU287 insertion had not been accompanied by any significant deletion (data not shown). To define the functional boundaries of the *spoIIM* locus, DNA fragments from the region were subcloned into an integrational vector, either pBG6 (65) or pBG14 (62, 65), and used to transform a wild-type *B. subtilis* strain to Cm^r. Transformation was followed by examination for the Spo phenotype. The results of this analysis localized *spoIIM* gene function to a 1,073-bp *Sph*I-*Hin*dIII fragment (Fig. 4). To recover cloned DNA containing the intact *spoIIM* coding sequence, an integrational plasmid, pKE11, containing a fragment of chromosomal DNA adjacent to the *spoIIM*::Tn*917*ΩHU287 locus was inserted into the chromosome of a wild-type strain of *B. subtilis* (PY79) and then was rescued into *E. coli* after digestion with *Sph*I (see Materials and Methods). This was expected to retrieve a 3.5-kb chromosomal segment including the *Sph*I-*Hin*dIII fragment (pKE20, Fig. 4). Initial rescue attempts were unsuccessful, indicating that the 3.5-kb segment might contain a gene whose product is toxic to *E. coli*. We were subsequently successful, however, using an *E. coli* transformation recipient, PY1182, containing a mutation (*pcnB80*) reported to reduce significantly the copy number of ColE1-derived replicons (25, 27). Further analysis of the 3.5-kb fragment indicated that a gene adjacent to *spoIIM* and not *spoIIM* itself was responsible for the toxic effect (51).

To confirm that the *Sph*I-*Hin*dIII fragment contained an intact copy of the *spoIIM* gene, the fragment was subcloned into pTK-lac (22), a vector which permitted recombinational transfer of the fragment into a thermoinducible prophage derivative of SPβ (67). A recombinant phage containing the *Sph*I-*Hin*dIII fragment was obtained by heat induction and then used to lysogenize a strain (PY403) containing the *spoIIM*::Tn*917*ΩHU287 insertion. Colonies from the resulting lysogens exhibited a Spo^+ phenotype. To further delineate the functional boundaries of the *spoIIM* gene, SPβ derivatives containing deletions extending into the *Sph*I-*Hin*dIII fragment were tested for complementation of the Spo^- phenotype produced by *spoIIM*::Tn*917*ΩHU287. DNA fragments containing deletions extending into the fragment from both directions were generated by the method of Dale et al. (11), subcloned into pTK-lac, and transferred into SPβ by recombination. SPβ derivatives containing the

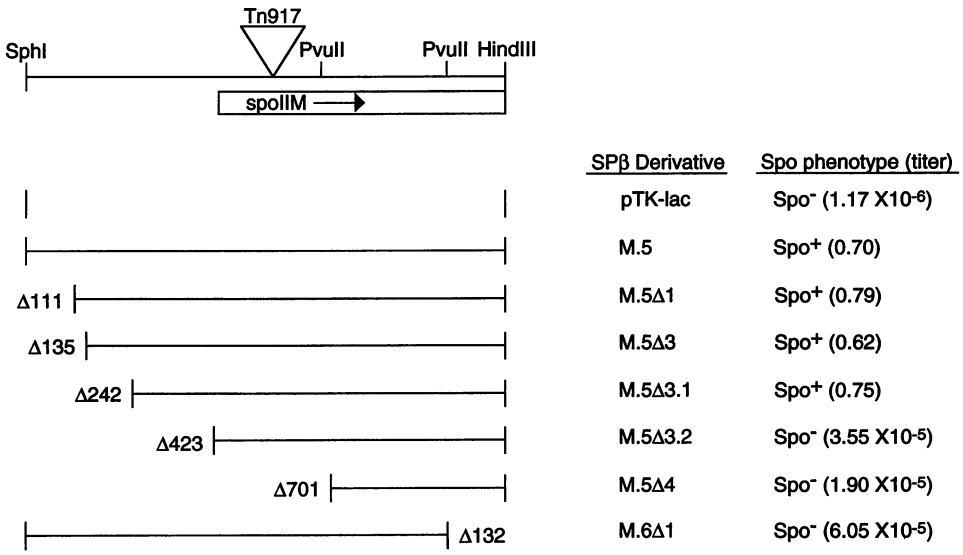

FIG. 5. Complementation analysis of the *spoIIM* locus. Deletions in the 1,073-bp *Sph*I-*Hin*dIII fragment thought to contain the *spoIIM* gene were obtained as described by Dale et al. (11) and then introduced into a prophage SPβ derivative by recombination (67). Some of the relevant deletions are represented by a capital delta and a number, the corresponding size of the deletion in base pairs. SPβ derivatives containing the deleted fragments were introduced into PY403 (*spoIIM*::Tn*917*ΩHU287) by specialized transduction and tested for complementation of its sporulation defect. The spore titer was determined as described in Materials and Methods, and it represents the average of two independent experiments. The open box represents the apparent limits of DNA encoding *spoIIM* function.

various deleted fragments were then used to lysogenize a strain (PY403) containing the *spoIIM*::Tn*917*ΩHU287 insertion. As indicated by the ability to complement the *spoIIM* mutation, deletions extending inward from the *Sph*I site as far as 242 bp left the *spoIIM* gene unimpaired (Fig. 5). A deletion of 423 bp inward from the *Sph*I site abolished complementation, however, as did a small deletion (132 bp) inward from the *Hin*dIII site.

DNA sequence of the *spoIIM* gene. DNA sequence analysis of the *Sph*I-*Hin*dIII fragment revealed an open reading frame starting 458 bp from the *Sph*I site and extending through the *Hin*dIII site, oriented such that its transcription would proceed through the *Hin*dIII site (Fig. 6). Because the extent of this open reading frame corresponds very closely to the functional boundaries of the *spoIIM* gene and because no other large open reading frame is disrupted by the *spoIIM*::Tn*917*ΩHU287 insertion, we conclude that this open reading frame corresponds to the *spoIIM* coding sequence. However, this indicates that the *Sph*I-*Hin*dIII fragment does not contain the entire *spoIIM* coding sequence, since no in-frame termination codon precedes the *Hin*dIII site. When we determined the DNA sequence beyond the *Hin*dIII site, it was apparent that the putative *spoIIM* open reading frame would extend an additional 5 amino acids before termination. In the pTK-lac construction used for complementation analysis, these 5 codons would be replaced by 4 codons derived from the vector (50). Evidently, this truncation-substitution does not significantly impair the activity of the *spoIIM* protein.

The putative *spoIIM* open reading frame would encode a protein of 215 amino acids with a predicted molecular mass of 24,850 Da. Centered 10 bp upstream of the proposed initiation codon is a sequence that would be expected to function efficiently as a ribosome-binding site in *B. subtilis* (35, 59). Centered 55 bp upstream from the proposed initiation codon is a sequence closely resembling the consensus sequence for promoters utilized by $E\sigma^E$ (13, 41, 58). A *lacZ* transcriptional fusion constructed with a portion of the *spoIIM* open reading frame exhibits a pattern of expression consistent with transcription by $E\sigma^E$ (50). A translational fusion constructed by joining the 39th codon of the putative *spoIIM* coding sequence to the 13th codon of *lacZ* exhibits a pattern of expression indistinguishable from that of the transcriptional fusion (51). The predicted product of the *spoIIM* gene would be a very hydrophobic and very basic protein (pI of 10.8) (Fig. 6). Its amino acid sequence does not show significant similarity to sequences represented in the GenBank, EMBL, SwissProt, and PIR data bases (see Materials and Methods).

DISCUSSION

It has become apparent from recent work in several laboratories that key morphological events in sporulation serve as signals or checkpoints to coordinate gene expression programs being executed in the two sporangium compartments (28). One such checkpoint may be the formation of the spore septum, which has previously been proposed to control the forespore-specific activation of σ^F (31, 32, 47). Expression of *spoIIIG*, which encodes σ^G, would be the direct consequence of σ^F activation. Expression of σ^G-dependent genes, in turn, serves as a signal to promote the posttranslational modification of pro-σ^K, which activates it to transcribe genes in the mother cell (8, 9). Phenotypes caused by mutations in several genes, including *spoIID* and *spoIIIA*, suggest another level of regulation mediated in a less well-defined manner. In addition to causing their morphological phenotypes, mutations in these genes block transcription of σ^G-dependent genes, apparently without significantly impairing expression of σ^G itself (8, 33). In the present work, we show that a mutation in *spoIIM* exhibits the same properties. Whether this level of regulation involves morphology-based signals is not known, but other investigators have suggested that σ^G activation could be

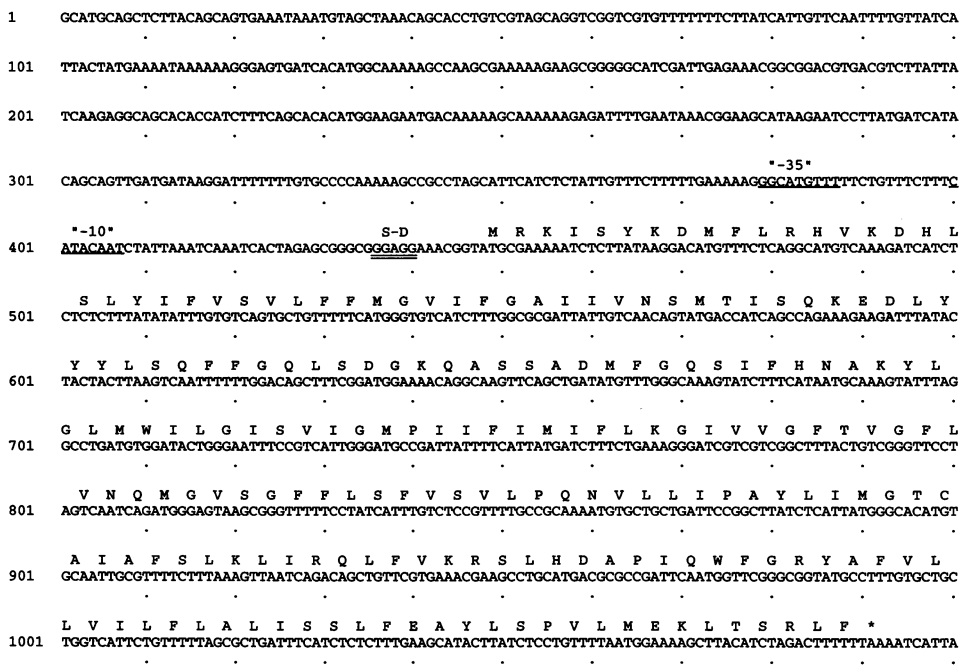

FIG. 6. Nucleotide sequence of the *spoIIM* gene and adjacent DNA. The beginning of the sequence is the upstream functional boundary of the *spoIIM* transcriptional unit. The deduced amino acid sequence of the *spoIIM* gene is shown above the nucleotide sequence. The proposed Shine-Dalgarno (S-D) sequence for *spoIIM* mRNA is underlined twice (35, 59). Sequences strongly resembling the recognition sequences for σ^E-associated RNA polymerase are underlined once (13, 18).

dependent upon completion and normal engulfment of the forespore compartment (28). It is interesting that nearly normal expression levels of a *spoIIIG-lacZ* transcriptional fusion were seen in a strain (KE211) containing a *spoIIIG* mutation; other investigators have previously reported an anomalous reduction in expression of *spoIIIG-lacZ* transcriptional fusions under similar circumstances (55). This discrepancy could be due to differences in the genetic backgrounds of the strains used; previously studied strains were derived from JH642 or 168 (55), while those in the present work were derived from PY79 (63).

To refine our understanding of the morphological defect caused by *spoIIM* mutations, we examined ultrastructural features of mutant bacteria fixed at two time points expected to capture events following asymmetric septation. Bacteria sampled at earlier times ($T_{2.5}$) exhibited classic stage II features. An exhaustive search failed to identify any full longitudinal sections containing engulfed forespores. However, bacteria sampled at later times (T_5) lacked septal structures altogether. We conclude from this that the asymmetric septa found in mutant bacteria must be grossly defective in some way that is not evident at a level of resolution afforded by electron microscopy. This defect must cause either extensive disintegration of the septum itself or lysis of septum-containing cells. We note that the predicted amino acid sequence of the *spoIIM* gene product is highly hydrophobic. Conceivably, the SpoIIM protein is a critical component of the septum membrane itself. Disintegration of septal structures or rapid lysis of the spore septum has not been previously described for *spoIID* mutants (39).

Few conclusions can be drawn from the *spoIIM* coding sequence, as its predicted translation product is not significantly similar to sequences in the GenBank, EMBL, SwissProt, or PIR data base. However, sequences immediately upstream from the *spoIIM* coding sequence establish the likelihood that the gene is transcribed by RNA polymerase associated with σ^E, as are *spoIID*, *spoIIIA*, and *spoIIID* (17, 23, 43, 53, 58). Thus, *spoIIM* apparently belongs to a family of genes expressed in the mother cell compartment whose products are required for full activity of σ^G-associated RNA polymerase in the forespore compartment. If a common mechanism is responsible for linkage of this presumptive mother cell regulon to the activation of σ^G, all that we can conclude at the present about its nature is that it must be independent of the pathway responsible for activation of σ^F in the forespore.

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