Novel spoIIE Mutation That Causes Uncompartmentalized $\sigma^F$ Activation in Bacillus subtilis

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During sporulation, Bacillus subtilis undergoes an asymmetric division that results in two cells with different fates, the larger mother cell and the smaller forespore. The protein phosphatase SpoIIE, which is required for activation of the forespore-specific transcription factor $\sigma^F$, is also required for optimal efficiency and timing of asymmetric division. We performed a genetic screen for spoIIE mutants that were impaired in sporulation but not $\sigma^F$ activity and isolated a strain with the mutation spoIIEV697A. The mutant exhibited a 10- to 40-fold reduction in sporulation and a sixfold reduction in asymmetric division compared to the parent. Transcription of the $\sigma^F$-dependent spoIIE promoter was increased more than 10-fold and was no longer confined to the forespore. The excessive $\sigma^F$ activity persisted even when asymmetric division was prevented. Disruption of spoIIEV697A did not restore asymmetric division to the spoIIEV697A mutant, indicating that the deficiency is not a consequence of predivisional activation of the mother cell-specific transcription factor $\sigma^F$. Deletion of the gene encoding $\sigma^F$ (spoIIAC) restored asymmetric division; however, a mutation that dramatically reduced the number of promoters responsive to $\sigma^F$, spoIIAC561 (spoIIACV233 M), failed to do so. This result suggests that the block is due to expression of one of the small subset of $\sigma^F$-dependent genes expressed in this background or to unregulated interaction of $\sigma^F$ with some other factor. Our results indicate that regulation of SpoIIE plays a critical role in coupling asymmetric division to $\sigma^F$ activation in order to ensure proper spatial and temporal expression of forespore-specific genes.

During sporulation, Bacillus subtilis undergoes a dramatic shift in the site of division from a medial site utilized during vegetative growth to a polar one. The result of this asymmetric division is two cells of unequal volumes with different fates, the larger mother cell and the smaller forespore (also known as the prespore). Immediately following asymmetric division, different programs of gene expression are initiated in the two cells by the cell-specific activation of the transcription factors $\sigma^F$ in the forespore and $\sigma^E$ in the mother cell (34). SpoIIE is a membrane-bound PP2C-like protein phosphatase (1, 38) that is essential for activation of the forespore-specific transcription factor $\sigma^F$. SpoIIE dephosphorylates, and thus activates, the anti-anti-sigma factor SpoIIAA. Activated SpoIIAA can release inhibition of $\sigma^F$ by the anti-sigma factor SpoIAB, resulting in transcription of forespore-specific genes (2, 9).

There is substantial evidence that SpoIIE has an additional role in asymmetric division that is independent of its phosphatase activity. Division in B. subtilis during growth and sporulation is preceded by the formation of a ring-like structure of the essential bacterial tubulin homologue FtsZ (43). FtsZ rings normally form at the midcell site during vegetative growth; however, during sporulation they are repositioned from this site to sites near both poles (22). It has recently been discovered that FtsZ ring switching occurs by the formation of a dynamic spiral-like intermediate. The spiral-like structures are a consequence of enhanced transcription of ftsZ as well as expression of spoIIE (6). B. subtilis strains bearing mutations in the phosphatase domain of SpoIIE that abolish $\sigma^F$ activation still efficiently form asymmetric septa, whereas spoIIE null mutants do not (5). Deletion of spoIIE results in both a reduction and a delay in polar Z-ring formation (19).

SpoIIE colocalizes to the asymmetric division site with FtsZ (23) and has been shown biochemically to interact with this protein (26). Deletion of either the N-terminal transmembrane domain or the extreme C terminus (beyond the phosphatase domain) results in soluble SpoIIE protein that efficiently activates $\sigma^F$ but poorly supports asymmetric division (3, 12). From this evidence, we speculated that SpoIIE plays a role in asymmetric division and performed a genetic screen to isolate spoIIE mutants that were deficient in this putative function but not in their ability to activate $\sigma^F$.

Below we describe the isolation and characterization of such a mutant having the mutation spoIIEV697A. However, the block in division is not caused by a specific loss of function of SpoIIE but rather is mediated by $\sigma^F$. Although it is not clear why $\sigma^F$ impairs asymmetric division in this mutant, we determined that the effect does not depend on the mother cell-specific transcription factor $\sigma^E$. We have shown that a mutant version of $\sigma^F$ that is unable to activate transcription of most known $\sigma^F$-dependent genes fails to restore asymmetric division in a spoIIEV697A mutant. This indicates that the block in division is caused either by expression of one of the small subset of $\sigma^F$-dependent genes expressed in this background or by unregulated interaction of $\sigma^F$ with some other factor, possibly RNA polymerase or SpoIAB. We observed that the spoIIEV697A mutant activates $\sigma^F$ even when asymmetric division is prevented, thereby uncoupling the two events. In addition, spoIIEV697A in cis restores sporulation to a spoIIE48 mutant that normally cannot activate $\sigma^F$ in response to asymmetric division.

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Taken together, our results indicate that regulation of SpoIIE plays a crucial role in coupling asymmetric division to σF activation and that this regulation is disrupted by the spoIIEV697A mutation. Uncoupling the two events leads to a severe reduction in asymmetric division, compartmentalization of σF activity, and spore formation, reinforcing the concept that tight coordination between morphology and gene regulation is crucial for the developmental process.

MATERIALS AND METHODS

Media. B. subtilis was grown in modified Schaeffer’s sporulation medium (MSSM) or on Schaeffer’s sporulation agar (SSA) or Luria-Bertani (LB) agar (33, 37). When required, the medium contained 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) at 40 μg/ml, chloramphenicol at 5 μg/ml, erythromycin at 1.5 μg/ml, neomycin at 3.5 μg/ml, and spectinomycin at 100 μg/ml. Escherichia coli was grown on LB agar containing ampicillin at 100 μg/ml.

Strains and plasmids. B. subtilis 168 strain BR151 (tpc2 metB10 lys-3) was used as the parent strain. Other B. subtilis strains and plasmids used are listed in Table 1. Escherichia coli strain DH5α (Gibco-BRL) was used to maintain plasmids. E. coli strain XL1-Red (Stratagene) was used for random mutagenesis of pH3. E. coli strain XL-mutS (Stratagene) was used for site-directed mutagenesis of pDH2 to produce pDH4.

To generate a plasmid encoding the C-terminal translational fusion, the spoIIQ gene was cloned as a 3.3-kb fragment by PCR with Pfu polymerase (Stratagene) with the primers AGCGGAAGATCGCTTGTCA and ACCGTA TGGATC CII site and generating pDH1. The resulting plasmid was named pDH5.

To construct pDH2, pDH1 was ligated with a 1.2-kb SmaI fragment that the GATGATGGATCCCACCACAGCAAGATTCGT. This PCR fragment was amplified with the primers GATGATGAATTCAATGAAGGCCATAAGTGA and TAq polymerase (Promega) with the primers GATGATGGATCCCACCACAGCAAGATTCGT. This PCR fragment was cloned into pGem T-Easy (Promega) by TA cloning. The resulting plasmid was digested with Sall and ligated with a 0.8-kb Sall fragment from pGreenTIR, which encodes the mstI allele of the gfp gene associated with an enhanced ribosome-binding site (28). PCR was used to confirm that the gfp gene and the spoIIE promoter were in the same orientation. This plasmid was then digested with Sall, cutting at a unique site in the blu (ampicillin resistance) gene, and ligated with a 1.2-kb Smal fragment from pBEST501 containing the neo (neomycin resistance) gene (16). The resulting plasmid, pDH6, was designed to integrate at the spoIIE locus by single crossover (Campbell-like integration) and generate a spoIIE-gfp transcriptional fusion.

To generate a vector that would introduce the spoIIEV697A mutation by Campbell-like integration at the 3’ end of the gene, we used pVK59 (kindly provided by V. Chary, Temple University, Philadelphia, Pa.), an integrative plasmid that has the neo gene. This plasmid was digested with ClaI and XhoI and ligated with a 1.1-kb ClaI-XhoI fragment of pDH4 encoding the C-terminal 292 residues of SpoIIEV697A. The resulting plasmid was named pDH7.

The div-355 mutant strain (kindly provided by R. Losick, Harvard University, Cambridge, Mass.) (21), which has the pyr79 genetic background, was transformed with total DNA from SL8625, which has pDH3 integrated into the chromosome. The resulting strain, SL10242, has the neo gene in the chromosomal region between divIC and spoIIE, which are separated by less than 1 kb (21). Total DNA was prepared from one of these clones and transformed into SL10174, selecting for erythromycin resistance and screening for cotransformation of the div-355 phenotype (Spo− at 37°C, filamentation at 45°C), generating SL10312. This strain has div-355 in the BR151 genetic background.

To construct pDH5, pDH6 was sequentially digested with Hinfi and HindIII (data not shown). To construct pDH2, pDH1 was linearized at the Hinbi site, located 418 bp upstream of the spoIIE start codon, and ligated with a 1.0-kb fragment derived from pFC177 Cm′:E′r (40) containing an erythromycin resistance (erm) gene. In a separate ligation, a 1.2-kb fragment encoding a spectinomycin resistance gene (spc) isolated from pC156 (40) was ligated with the Hinfl-digested pDH2 to generate pDH3. pDH4 is the name given to a derivative of pDH2 in which the spoIIEV697A mutation was introduced by passage through mutagenic E. coli XL1-Red competent cells (Stratagene) (see Results). pDH5 is a derivative of pDH3 in which the spoIIEV697A mutation was introduced into pDH2 by site-directed mutagenesis with the Gene Editor kit (Promega) with the mutagenic primer TTTTTGAGTTCTGGATGACG (base change in italic). pSG1902 (a gift from J. Errington, Oxford University, Oxford, United Kingdom) (44) was used to generate a spoIIE-green fluorescent protein gene (gfp) C-terminal translational fusion.

To generate a spoIIE-gfp transcriptional fusion, the spoIIE promoter region was cloned as a 0.5-kb fragment generated by PCR with Taq polymerase (Promega) with the primers GATGATGGATCCCACCACAGCAAGATTCGT and GATGATGGATCCCACCACAGCAAGATTCGT. This PCR fragment was cloned into pGem T-Easy (Promega) by TA cloning. The resulting plasmid was digested with Sall and ligated with a 0.8-kb Sall fragment from pGreenTIR, which encodes the mstI allele of the gfp gene associated with an enhanced ribosome-binding site (28). PCR was used to confirm that the gfp gene and the spoIIE promoter were in the same orientation. This plasmid was then digested with Sall, cutting at a unique site in the blu (ampicillin resistance) gene, and ligated with a 1.2-kb Sall fragment from pBEST501 containing the neo (neomycin resistance) gene (16). The resulting plasmid, pDH6, was designed to integrate at the spoIIE locus by single crossover (Campbell-like integration) and generate a spoIIE-gfp transcriptional fusion.

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Galactosidase assays. β-Galactosidase assays were performed essentially as described previously (14), with β-galactosidase used to permeabilize the cells. Specific activity is expressed as nanomoles of O-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolyzed per minute per milligram of bacterial dry weight.

Other methods. Cultures used for visualization of green fluorescent protein (GFP) were grown in MSSM at 33.5°C. Culture samples of 1 ml of un fused cells were transferred to slides and examined by fluorescence microscopy essentially as described previously (46).

Cultures used for visualization of asymmetric septa were grown in MSSM at 33.5°C. After half of the culture had divided, the cultures were fixed with formaldehyde. Cells were stained with DAPI or the propidium iodide and examined by fluorescence microscopy.
37°C. Culture samples of 10 μl were mixed with an equal volume of the vital membrane stain FM4-64 (Molecular Probes) (previously diluted 100-fold in water) and incubated at 37°C without shaking for 10 min. One-microliter samples were transferred to slides and visualized essentially as described previously (35).

Sporulation was assayed 20 h after the end of exponential growth by diluting cultures and determining the heat-resistant count (80°C, 20 min) and the viable count in the diluted cultures. *B. subtilis* transformation, sporulation by exhaustion in MSSM, and all other methods were essentially as described previously (14, 31, 32, 46).

RESULTS

Isolation of a novel spoIIE mutation that reduces sporulation efficiency. In order to isolate spoIIE mutants that were defective in asymmetric division, we performed a genetic screen, pDH2, encoding the full-length spoIIE gene on an integrative plasmid with the erm gene cloned upstream of the start codon of spoIIE, was randomly mutagenized by passage through *E. coli* XL1-Red mutagenic competent cells (Stratagene). The resulting transformants (greater than 10,000) were collected into pools of approximately 250 clones each for plasmid isolation. The plasmid pools were used to transform *B. subtilis* SL8625, selecting for erythromycin resistance. Strain SL8625 contains the spoIIE gene at the same site upstream of spoIIE as erm in pDH2. Therefore, the resulting transformants could be screened for single-crossover (spectinomycin resistant) or double-crossover (spectinomycin sensitive) integration at the spoIIE locus. The presence of either the erm or spoIIE gene at this site in the chromosome had no effect on growth or sporulation (data not shown). This strain also had a σF-dependent spoIIQ-lacZ transcriptional fusion (24) to avoid isolation of alleles that are defective in the phosphatase activity required for σF activation, a class that has already been described (5, 38). Lastly, the strain contained a C-terminal spoIIE-gfp translational fusion that would allow differentiation of nonsense (no GFP signal) versus missense (GFP signal) mutants (44).

Initial experiments indicated that potential mutants were unstable when selection for transformants with SL8625 was done on a medium (SSA) that supported sporulation. Subsequently, SL8625 transformants were selected on LB agar, which does not support sporulation. Two thousand such transformants were patched individually onto SSA plates containing X-Gal to screen for the desired phenotype, revealing one mutant that appeared to sporulate poorly but strongly activated σF. This mutant was the product of a single-crossover recombination event at the spoIIE locus. In order to generate a strain containing a double-crossover integration of the mutant allele presumed to be responsible for the phenotype, total DNA from bacteria from the same clone maintained on LB agar (designated SL8978) was used to transform *E. coli* DH5α, selecting for ampicillin resistance. The resulting *E. coli* transformants contained a plasmid designated pDH4, a mutant version of pDH3 that was capable of introducing the spoIIE allele responsible for the reduced sporulation, hyper-σF activity phenotype into *B. subtilis* (data not shown). Subsequent double-crossover integration of the mutant allele encoded by this plasmid resulted in a stable *B. subtilis* strain that exhibited the same phenotype (SL10008).

We subsequently observed that integration of pSG1902, a plasmid encoding the C-terminal 184 residues of SpoIIE (44), was capable of restoring sporulation to SL10008 (data not shown). Sequencing of this region of pDH4 revealed a G-to-C transition at nucleic acid position 2090 of the coding strand of the spoIIE open reading frame (4), indicating a change of valine to alanine at residue 697. In order to verify that this mutation was causing the observed phenotype, it was generated in vitro with site-directed mutagenesis of pDH2 (see Materials and Methods) to produce pDH5. Introduction of the mutant spoIIE allele from this plasmid into *B. subtilis* resulted in a phenotype identical to that of SL10008 (data not shown).

The effect of spoIEV697A on sporulation was determined by heat survival (see Materials and Methods). The following data are the average of three independent experiments plus and minus the standard deviation. BR151, the spoIIE mutant, produced $3.1 \times 10^7 \pm 1.1 \times 10^6$ spores per ml. In contrast, SL10766, containing the spoIEV697A mutation, only produced $1.5 \times 10^7 \pm 0.5 \times 10^6$ spores per ml. Therefore, the spoIEV697A mutation caused a 10- to 40-fold reduction in sporulation.

**spoIEV697A severely impairs asymmetric division.** Our rationale for screening for spoIIE mutants that were impaired in sporulation but continued to activate σF was that these mutants would be deficient in some other putative function of SpoIIE, potentially its role in asymmetric division. To analyze asymmetric division, BR151 and the isogenic mutant SL10912 were induced to sporulate in MSSM, and samples were stained with the vital membrane stain FM4-64 and scored for the presence of asymmetric septa by fluorescence microscopy (35). The proportion of the BR151 population undergoing asymmetric division rose to 40% by $T_{a,5}$ and eventually reached a peak of greater than 60% by $T_6$ ($T_0$ indicates the end of exponential growth and the initiation of sporulation) (Fig. 1). In contrast, for strain SL10912 bearing the spoIEV697A mutation, the proportion with asymmetric septa rarely exceeded 10% (Fig. 1). In all of the samples analyzed after $T_a$, the mutant displayed at least a sixfold reduction in frequency of asymmetric division compared to BR151, indicating a severe defect in the morphological progression of sporulation in the spoIEV697A mutant (Fig. 1).

**spoIEV697A causes excessive, uncompartmentalized σF activity.** Colonies of the spoIEV697A mutant containing the σF-dependent spoIIQ-lacZ transcriptional fusion gave an in-
Mutation (SL10008) was induced to sporulate in MSSM, and results suggest that in addition to impairing asymmetric division, spoIIE also causes hyperactivation of σF (data not shown). These values are the sum of three independent experiments in which cultures were grown in MSSM at 33.5°C and samples were taken at T6. At least 100 cells were scored in each experiment.

The number of cells expressing GFP in the forespore was determined as a percentage of the total number of cells expressing GFP.

The presence of C-terminal fusions of gfp to either wild-type spoIIE or spoIIEV697A. The presence of the C-terminal fusion did not affect sporulation of the parent or the mutant. Western blots of the lysates with anti-GFP antibodies revealed little difference in SpoIE-GFP protein concentrations between the parent and the mutant (data not shown).

The activity of σF is normally confined to the forespore after asymmetric division occurs, and mutations that block asymmetric division prevent σF activation (12, 19, 21). This coupling of asymmetric division to σF activation establishes a developmental checkpoint that ensures proper spatial and temporal transcription of forespore-specific genes (34). However, in the spoIIEV697A mutant, we separately observed low levels of asymmetric division (Fig. 1) yet very high levels of σF activity (Fig. 2). This led us to speculate that the checkpoint had been disrupted and that in some cells in the spoIIEV697A population, σF activity would be un compartmentalized. In order to address this, we generated strains that had the spoIIE promoter transcriptionally fused to gfp, the gene encoding GFP, with pDH6 (see Materials and Methods). A spoIIE-gfp transcriptional fusion had previously been shown to be expressed exclusively in the forespore (24).

Strains SL10221 (spo+) and SL10222 (spoIIEV697A) were induced to sporulate in MSSM, and samples were examined by fluorescence microscopy and scored as either having no signal, signal confined to the forespore, or signal throughout the cell. In the parent, 96% of the fluorescent cells had signal present only in the forespore at T6 (Table 2). In contrast, only 2.2% of the mutant cells exhibiting signal at this time showed forespore-specific expression (Table 2). The rest of the fluorescent cells had a pattern of whole-cell fluorescence (Table 2). Therefore, spoIIEV697A largely abolished compartmentalization of σF activity. Similar results were observed with a spoIII-gfp transcriptional fusion located at an ectopic locus (data not shown). The fact that greater than 45% of the spoIIEV697A mutant cells exhibited whole-cell spoIII-gfp activity (Table 3) when less than 10% of these cells had undergone asymmetric division at a similar time in a previous assay (Fig. 1) strongly suggested that spoIIEV697A enables B. subtilis to activate σF in the absence of asymmetric division. We proceeded to test this possibility in the following experiment.

spoIIEV697A uncouples σF activation from polar division. div-355 is a conditional mutation in the essential cell division gene divIC. This mutation is very useful because it allows vegetative division to occur at 37°C but blocks asymmetric division during sporulation at that temperature (21). Therefore, it would allow us to test our hypothesis that spoIIEV697A activates σF in the absence of polar division without the complication of the approximately 10% of cells that undergo asymmetric division in the population (Fig. 1). We constructed a double mutant strain with div-355 and spoIIEV697A as well as a spoIIE-lacZ transcriptional fusion (SL10423). In addition, we also generated isogenic strains with the mutations singly

![Graph](http://jb.asm.org/)

**FIG. 2.** Expression of spoIIE-lacZ in the parent strain SL10002 (solid squares) and in the spoIIEV697A mutant SL10008 (open squares) in MSSM. Specific activity units are nanomoles of ONPG hydrolyzed per minute per milligram of bacterial dry weight. The results are the averages of three independent experiments plus and minus the standard deviation.
(SL10312 with div-355 and SL10339 with spoIEV697A). These three strains, along with the spo+ parent also containing the spoIQ-lacZ fusion (SL8603), were induced to sporulate in MSSM, and samples were taken for analysis of β-galactosidase activity. This experiment was performed at 37°C, a temperature that we had previously determined was permissive for vegetative division but nonpermissive for asymmetric division for the strain with the div-355 mutation in the BR151 background (data not shown).

As in a previous experiment (Fig. 2), we observed that the spoIQ-lacZ fusion became active at T_{3.5} and specific β-galactosidase activity reached a peak of between 15 and 25 nmol of ONPG hydrolyzed per min per mg in the spo+ parent (Fig. 3A). This stands in stark contrast to the div-355 mutant, in which β-galactosidase activity was completely abolished (Fig. 3A). The spoIEV697A mutation in the wild-type divIC background displayed the same pattern as described in a previous experiment (Fig. 2), becoming active slightly earlier (at T_{2}) and with β-galactosidase activity reaching a far higher level (at least 300 nmol of ONPG hydrolyzed per min per mg at T_{3.5}) than with the spo+ parent (Fig. 3B). The div-355 spoIEV697A double mutant had a pattern of spoIQ-lacZ activity that was not significantly different from that of the spoIEV697A single mutant (Fig. 3B). From these results, we can conclude that the spoIEV697A mutation uncouples σ^F activation from asymmetric division, allowing σ^F to become active even when division is blocked.

**spoIEV697A is an intragenic suppressor of spoIE48.** Based on the previous experiments, we strongly suspected that spoIEV697A bypassed the developmental checkpoint coupling σ^F activation to asymmetric division (Fig. 3B). As an independent test to confirm this, we wanted to determine if this mutation could restore sporulation to a mutant that is normally incapable of passing this checkpoint. Such a mutant, carrying spoIE48 (spoIES361F), has been described as being capable of substantially dephosphorylating SpoIIA but unable to activate σ^F and as a consequence is severely impaired in spore formation (19).

To test this possibility, the spo+ parent, the spoIE48 mutant (SL10767), the spoIEV697A mutant (SL11173), and the spoIE48 spoIEV697A double mutant (SL11201) were induced to sporulate in MSSM, and their ability to form spores was analyzed by heat survival. It is important to note that SL11201 contains a single copy of the spoIE gene harboring both mutations. Whereas the spoIE48 mutant was severely impaired in sporulation (53 ± 40 spores per ml [Table 3]), the spoIE48 spoIEV697A double mutant was Spo+, producing almost as many spores as the spo+ parent (5.9 × 10^8 ± 1.0 × 10^8, compared to 7.4 × 10^8 ± 0.4 × 10^8 spores per ml [Table 3]). In turn, the double mutant produced nearly 10 times as many spores as the spoIEV697A single mutant (5.9 × 10^8 ± 1.0 × 10^8 compared to 4.5 × 10^7 ± 0.8 × 10^7 spores per ml [Table 3]). These results indicate that spoIEV697A can function as an intragenic suppressor of spoIE48. Since spoIE48 is normally incapable of activating σ^F in response to asymmetric division, this provides further evidence that spoIEV697A severely interferes with the checkpoint coupling the two events.

**Reduction in asymmetric division in the spoIEV697A mutant is dependent upon the spoIIA operon but independent of σ^F activity.** We had separately observed un compartmentalized σ^F activity (Table 2) and a severe reduction in asymmetric division (Fig. 2) in the spoIEV697A mutant. We wanted to investigate the possibility that these two phenotypes were re-

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FIG. 3. Expression of spoIQ-lacZ in strains containing mutations in spoIE and/or divIC. (A) Expression in the spoIE+ divIC+ parent strain SL10174 (solid squares) and the spoIEV697A div-355 mutant SL10312 (solid triangles). (B) Expression in the spoIEV697A divIC+ mutant SL10339 (open squares) and in the spoIEV697A div-355 mutant SL10423 (open triangles). Specific activity units are nanomoles of ONPG hydrolyzed per minute per milligram of bacterial dry weight. The results are the averages of three independent experiments plus and minus the standard deviation.
lated. It had been determined previously that activation of the mother cell-specific transcription factor $\sigma^F$ is required to block a second asymmetric division from occurring in the mother cell (15). $\sigma^F$ is synthesized as an inactive precursor, pro-$\sigma^F$, which is proteolytically cleaved into the active form by the inferred aspartyl protease SpoIIGa (20, 39). This activation is coupled to asymmetric division because expression of spoIIR directed by $\sigma^F$ is required for proteolysis to occur (17, 25). However, uncoupling spoIIR expression from asymmetric division causes uncompartmentalized activation of $\sigma^F$ in some cells (41, 46). A different study has demonstrated that predivisional expression of three $\sigma^F$-dependent genes, spoIID, spoIIM, and spoIIP, impairs asymmetric division (10).

Taken together, these results suggest that one possible cause of the defect in asymmetric division observed in the spoIIEV697A mutant would be predivisional transcription of spoIIR leading to premature activation of $\sigma^F$. In order to test this possibility, we constructed a spoIIGB::erm (13) spoIIEV697A double mutant (SL11132). This strain was induced to sporulate in MSSM along with the isogenic single mutant carrying spoIIGB::erm (SL8410). Samples were stained with FM4-64 and scored for the presence of asymmetric septa. Whereas 55% of the spoIIGB::erm single-mutant cells exhibited asymmetric septa by $T_5$, they were present in less than 12% of the spoIIGB::erm spoIIEV697A double-mutant cells at all times analyzed (Fig. 4A). Comparison of the frequency of asymmetric division in the spoIIEV697A mutant with that in the spoIIGB::erm spoIIEV697A double mutant revealed no significant differences at any time analyzed (data not shown). We conclude that predivisional expression of $\sigma^F$-dependent genes inhibitory to asymmetric division is not responsible for the deficiency in asymmetric division observed in the spoIIEV697A mutant. Consistent with this conclusion, we have observed that transcription from the $\sigma^E$-dependent cotEP1 (8) and spoIIID (36) promoters is drastically reduced in the spoIIEV697A mutant, making it unlikely that any aspect of the spoIIEV697A phenotype is due to inappropriate $\sigma^F$ activity (data not shown).

From previous studies, we had speculated that SpoIIE had a role in promoting asymmetric division (5, 18, 23, 26). The mutation that we isolated (spoIIEV697A), caused deficiency in asymmetric division but also excessive, uncompartmentalized $\sigma^F$ activity (Fig. 1 and 2, Table 3). We wanted to determine whether the asymmetric division phenotype was directly attributable to the loss of a specific division function encoded by spoIIE or if it was a consequence of the hyper-$\sigma^E$ activity. In order to do this, we used a spoIIA\Delta4 mutant (33), carrying a deletion of the spoIIA operon encoding the structural gene for $\sigma^F$, spoIAC, as well as the spoIIA and spoIAB genes, encoding regulators of $\sigma^E$ (34). The spoIIEV697A spoIIA\Delta4 double mutant (SL10905) and the single spoIIA\Delta4 mutant
SL9055) were induced to sporulate in MSSM, and samples were stained with FM4-64 and scored for the presence of asymmetric septa.

Compared to the spoIIAΔ4 single mutant, the spoIEV697A spoIIAΔ4 double mutant exhibited no significant reduction in frequency of asymmetric division and actually demonstrated a modest increase at several times after T5 (Fig. 4B). Whereas rarely more than 10% of the cells in the spoIEV697A single-mutant population exhibited asymmetric septa at the times analyzed (Fig. 1), the spoIEV697A spoIIAΔ4 double-mutant cells underwent asymmetric division at a much higher frequency, reaching 50% by T4, and greater than 65% by T9 (Fig. 4B). There was at least a fivefold increase in the frequency of asymmetric division in the spoIEV697A spoIIAΔ4 double mutant compared to the spoIEV697A single mutant at all times analyzed after T5 (Fig. 1 and 4B). These results indicate that the deficiency in asymmetric division observed in the spoIEV697A mutant is dependent upon some product of the spoIIA operon.

Deletion of spoIAC but not a point mutation that largely abolishes promoter recognition by σF restores asymmetric division in a spoIEV697A mutant. We thought it was most likely that the asymmetric division phenotype in the spoIEV697A mutant was being mediated by σF because the major role for the other products of the spoIIA operon, SpoIIAa and SpoIAB, is to regulate its activity (34). In order to test this possibility, we utilized a spoIAC::neo deletion, which does not interfere with either the spoIIAa or the spoIAB open reading frame. We constructed a spoIAC::neo single mutant (SL11274) and a spoIEV697A spoIAC::neo double mutant (SL11275) and induced them to sporulate in MSSM. Samples were stained with FM4-64 and scored for the presence of asymmetric septa. The frequency of asymmetric septation in both strains was very similar, reaching a peak of about 40% (Fig. 4C). This was a dramatic increase over that of the spoIEV697A single mutant, in which the frequency rarely exceeded 10% of the population (Fig. 1). At all times analyzed after T5, the frequency of asymmetric septation in the spoIEV697A spoIAC::neo double mutant was at least threefold higher than that in the spoIEV697A single mutant (Fig. 1 and 4C). This indicates that the block in division is indeed mediated by σF.

We next wished to address the question of why hyperactivation of σF might inhibit asymmetric division. The most likely explanation seemed that its activity as a sigma factor would lead to hyperexpression of some gene whose product inhibits division. We refined the analysis further by testing the effect of the spoIAC561 mutation (15). This mutation causes a V233M change in the 4.2 promoter recognition region of σF (45). As a consequence, most σF-directed promoters are not recognized, although at least one σF-controlled gene, spoIR, becomes hyperexpressed (17).

We used a spoIAC561 mutant (SL1102) as well as a spoIEV697A spoIAC561 double mutant (SL11142) and induced these strains to sporulate in MSSM. Samples were stained with FM4-64 and scored for the presence of asymmetric septa. Whereas the spoIAC561 mutant formed asymmetric septa at a high frequency, reaching nearly 50% by T5, the frequency in the spoIEV697A spoIAC561 double mutant population rarely exceeded 10% (Fig. 4D). Compared to the spoIAC561 mutant, the spoIEV697A spoIAC561 double mutant exhibited a reduction of between three- and eightfold in asymmetric division between T5 and T9. The frequency of asymmetric division in the spoIEV697A single mutant and the spoIEV697A spoIAC561 double mutant was not significantly different at any time analyzed (Fig. 1 and 4D). This indicates that the block in division observed in the spoIEV697A mutant results either from expression of one of the σF-dependent genes whose promoter is still recognized by σF or from interaction of σF with some other factor.

DISCUSSION

The exact mechanism by which B. subtilis switches from a medial division site to a polar one during sporulation remains unclear. There is genetic evidence that both a burst of ftsZ transcription and the expression of SpoIE are required for this switch to occur (6, 18). However, the mechanism by which SpoIE facilitates asymmetric division remains unknown. Here we report the isolation and characterization of a mutation, spoIEV697A, that causes a severe impairment in the formation of polar septa during sporulation. However, it appears that this impairment is indirect, mediated via σF in the spoIEV697A mutant. This is consistent with previous observations that deletion of the gene encoding the anti-sigma factor SpoIAB caused unregulated σF activity and a concomitant block in asymmetric division (7). Although our findings do not provide additional evidence for a direct role of SpoIE in promoting asymmetric division, they suggest that one function of SpoIE may be to delay σF activation until after polar division is complete, thereby tightly coupling these two events to ensure the proper spatial and temporal expression of forespore-specific genes.

SpoIE has a C-terminal PP2C-like phosphatase domain that has a well-characterized role in activation of the forespore-specific transcription factor σF (1, 2, 9, 38). SpoIE dephosphorylates, and thus activates the anti-anti-sigma factor SpoIIAa, which can then relieve inhibition of σF by the anti-sigma factor SpoIAB (2, 9). In turn, SpoIAB is a serine kinase that can phosphorylate, and thus inactivate, SpoIIAa (29). Although the biochemistry underlying these interactions is reasonably well understood, it is not yet clear why σF becomes active only after asymmetric division and only in the forespore (34). It has been proposed that SpoIE itself is regulated by a number of different mechanisms in order to explain the temporal and spatial regulation of σF activity.

Two broad models have emerged. In the first, SpoIE is inactive in its default state and is activated by interaction with cell division proteins (19). In the second, SpoIE is active in the default state and is negatively regulated to prevent inappropriate σF activation (3). The first, activator, model was developed from three lines of research (19). It was demonstrated that the early cell division protein FtsZ was required for SpoIE to efficiently dephosphorylate SpoIIAa. Next, it was demonstrated that the div−355 mutation of divIC allows efficient dephosphorylation of SpoIIAa by SpoIE but not activation of σF. Lastly, the spoIE48 (spoIE3631F) mutant was found to have the same phenotype with regards to σF activation as the div−355 mutant (i.e., dephosphorylation of SpoIIAa but no σF activation) (19). These results suggested a regulatory step in which SpoIE prevents dephosphorylated SpoIIAa from at-
tackling the SpoIIB-A complex and activating $\sigma^F$ until asymmetric division is complete (19).

The second, repressor, model is based on studies utilizing a spoIIE mutant, in which the region encoding the N-terminal transmembrane domains has been deleted, rendering the protein cytoplasmic. Surprisingly, the mutant protein supported relatively high levels of asymmetric division, compartmentalization of $\sigma^F$ activity, and sporulation (3). The authors concluded that there is a cytoplasmic inhibitor of SpoIIE that is capable of interacting with and regulating (albeit less efficiently) the cytoplasmic form of SpoIIE. Indeed, the in vitro phosphatase activity of SpoIIE, which activates SpoIIE, is 100 times stronger than the kinase activity of SpoIIB, which inactivates it; since the levels of the SpoIIE and SpoIIB proteins are very similar during the early part of sporulation, an additional factor may be involved in negatively regulating the phosphatase activity of SpoIIE (27).

The spoIIEV697A mutation does not help us to distinguish between the two models. Consistent with the first, the mutant protein no longer needs activation by a division component and activates $\sigma^F$ in the div-355 background. This mutant is the converse of another phosphatase-competent mutant, SpoIIE48, which cannot activate $\sigma^F$ even when division occurs. The SpoIIEV697A phenotype is reminiscent of a hybrid MalF-SpoIIE protein in which the SpoIIE transmembrane domains are replaced by the two MalF transmembrane domains. The hybrid protein strongly activated $\sigma^F$ and could do so in a div-355 background (19). However, consistent with the second model, the mutant protein could be thought of as an inhibitor-resistant form of SpoIIE. Furthermore, a combination of the two models is also possible, with SpoIIE being subject to both positive and negative regulation and SpoIIEV697A being insensitive to just one type of regulation. This might explain why spoIIEV697A causes a milder phenotype than an in-frame spoIIE deletion (which also causes hyper-$\sigma^F$ activity) (7). Consistent with the idea that the spoIIEV697A mutant is less sensitive to some regulator, we found that the spoIIEV697A allele is trans-dominant to wild-type spoIIE as well as to the loss-of-function alleles spoIIE48 and spoIIE64 (unpublished observations).

The effect of the spoIIEV697A mutation on sporulation division is mediated by $\sigma^F$. This is indicated by our observation that deletion of the spoIIA operon and of spoIAC, the structural gene for $\sigma^F$, largely overcame the effect of the spoIIEV697A mutation on sporulation division. We showed that the effect was not a secondary consequence of $\sigma^F$ activation. We also determined that if the effect was caused by expression of a $\sigma^F$-dependent gene, it must be one that continues to be expressed in a spoIAC561 mutant, in which expression of most known $\sigma^F$-dependent genes is abolished. The $\sigma^F$ protein interacts with core RNA polymerase and also with the SpoIAB protein. Interaction of $\sigma^F$ with either (or with some unknown protein), rather than its role as a sigma factor, could somehow affect septation. We saw no impairment of transcription of the key division gene ftsZ (unpublished observations) as a possible consequence of an increase in $\sigma^F$ sequencing core polymerase but cannot exclude an effect on transcription elsewhere. SpoIAB has a pivotal role in $\sigma^F$ activation (34), and it may be part of the link between activation and septation. The presence of $\sigma^F$ can affect SpoIAB stability (30).

Although speculative, the presence of the mutant $\sigma^F$ protein might affect septation through its interaction with SpoIAB.

Feucht et al. (11) recently described mutations in a different region of spoIIE, the hinge region mutations spoIIEG334R and spoIIEQ344P, which give rise to a phenotype similar to that of the spoIIEV697A mutant in that they dissociate $\sigma^F$ activation from septation, cause uncompartmentalized hyper-$\sigma^F$ activity, and impair spore formation. The purified mutant proteins had in vitro phosphatase activity similar to that of wild-type SpoIIE, and it was concluded that the mutant proteins were refractory to a regulatory step unrelated to their phosphatase activity (11). The hinge region is involved in oligomerization and in the interaction with FtsZ (26), but the mutants retained the ability to localize to a septum (11). It is possible that the hinge region, located close to the membrane, is subject to one type of regulation, whereas the phosphatase domain, which is thought to project into the cytoplasm (4), is subject to a distinct cytoplasmic regulator. Loss of response to either type of regulator may result in an effect similar to that seen here with the phosphatase domain V697A mutation and by Feucht et al. (11) with the hinge domain G334R and Q344P mutations. Consistent with the idea of interdependence of regulation of the domains, we observed that a strain with a single copy of spoIIE containing both the spoIIE48 mutation (hinge domain, no $\sigma^F$ activation) and the spoIIEV697A mutation was Spo . In addition, the spoIIEV697A mutation has been independently isolated as a spontaneous suppressor of a hinge spoIIE mutation associated with a phenotype identical to that of the spoIIE49 mutant (K. Carniol and R. Losick, personal communication).

Our data, and those of Feucht et al. (11) and Carniol and Losick, strongly support the general theme that regulation of SpoIIE plays a crucial role in both coupling $\sigma^F$ activation to asymmetric division and confining $\sigma^F$ activity to the forespore. These studies reinforce the concept that precise coupling of asymmetric division to $\sigma^F$ activation is critical for efficient spore formation.

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