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Novel *spoIIE* Mutation That Causes Uncompartmentalized σ^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 σ^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 σ^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 σ^F-dependent *spoIIQ* promoter was increased more than 10-fold and was no longer confined to the forespore. The excessive σ^F activity persisted even when asymmetric division was prevented. Disruption of *spoIIGB* 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 σ^E. Deletion of the gene encoding σ^F (*spoIIAC)* restored asymmetric division; however, a mutation that dramatically reduced the number of promoters responsive to σ^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 σ^F-dependent genes expressed in this background or to unregulated interaction of σ^F with some other factor. Our results indicate that regulation of SpoIIE plays a critical role in coupling asymmetric division to σ^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 σ^F in the forespore and σ^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 σ^F. SpoIIE dephosphorylates, and thus activates, the anti-anti-sigma factor SpoIIAA. Activated SpoIIAA can release inhibition of σ^F by the anti-sigma factor SpoIIAB, 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 σ^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 σ^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 σ^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 σ^F. Although it is not clear why σ^F impairs asymmetric division in this mutant, we determined that the effect does not depend on the mother cell-specific transcription factor σ^E. We have shown that a mutant version of σ^F that is unable to activate transcription of most known σ^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 σ^F-dependent genes expressed in this background or by unregulated interaction of σ^F with some other factor, possibly RNA polymerase or SpoIIAB. We observed that the *spoIIEV697A* mutant activates σ^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 σ^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 σ^F 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 σ^F 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 Schaffer'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 (*trpC2 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 pDH3. *E. coli* strain XL-mutS (Stratagene) was used for site-directed mutagenesis of pDH2 to produce pDH4.

The *spoIIE* gene was cloned as a 3.3-kb fragment by PCR with *Pfu* polymerase (Stratagene) with the primers AGCGAAGATCGCTTGTCA and ACCGTA ATCCCCTGCTCT and ligated into the *Hin*cII site of pBluescript SK−, destroying the *Hin*cII site and generating pDH1. The *spoIIE* gene encoded by pDH1 was able to restore sporulation to a strain in which *spoIIE* had been insertionally disrupted, indicating that no deleterious mutations were generated in the PCR amplification process (data not shown). To construct pDH2, pDH1 was linearized at the *Hin*cII site, located 418 bp upstream of the *spoIIE* start codon, and ligated with a 1.0-kb fragment derived from pIC177 Cm^r::Er^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 pIC156 (40) was ligated with the *Hin*cII-digested pDH1 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* XL-1 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 TTTTTGAAGG*C*TGGATC GACG (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 *spoIIQ-gfp* transcriptional fusion, the *spoIIQ* promoter region was cloned as a 0.5-kb fragment generated by PCR with *Taq* polymerase (Promega) with the primers GATGATGAATTCAATGAAGGCCATAAGTGA and GATGATGGATCCCACCACAGCAAGATTCGT. This PCR fragment was cloned into pGem T-Easy (Promega) by TA cloning. The resulting plasmid was digested with *Sal*I and ligated with a 0.8-kb *Sal*I fragment from pGreenTIR, which encodes the *mut1* allele of the *gfp* gene associated with an enhanced ribosome-binding site (28). PCR was used to confirm that the *gfp* gene and the *spoIIQ* promoter were in the same orientation. This plasmid was then digested with *Sca*I, cutting at a unique site in the *bla* (ampicillin resistance) gene, and ligated with a 1.2-kb *Sma*I fragment from pBEST501 containing the *neo* (neomycin resistance) gene (16). The resulting plasmid, pDH6, was designed to integrate at the *spoIIQ* locus by single crossover (Campbell-like integration) and generate a *spoIIQ-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 *Cla*I and *Xho*I and ligated with a 1.1-kb *Cla*I-*Xho*I 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 PY79 genetic background, was transformed with total DNA from SL8625, which has pDH3 integrated into the chromosome. The resulting strain, SL10242, has the *erm* 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.

β-Galactosidase assays. β-Galactosidase assays were performed essentially as described previously (14), with lysozyme 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 μl of unfixed 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

TABLE 1. *B. subtilis* strains and plasmids used

Plasmid or strain	Relevant characteristics[a]	Origin or reference
Plasmids		
pDH1	pBluescript SK(−) *spoIIE*	This study
pDH2	*erm* @ *Hin*cII site of pDH1 upstream of *spoIIE* ORF	This study
pDH3	*spc* @ *Hin*cII site of pDH1 upstream of *spoIIE* ORF	This study
pDH4	*spoIIEV697A* in pDH2 background isolated from SL8978	This study
pDH5	*spoIIEV697A* inserted by site-directed mutagenesis in pDH3	This study
pDH6	*spoIIQ-gfp neo*	This study
pDH7	*spoIIEV697A neo*	This study
pEIA99	*amyE*::*spoIIQ-lacZ*	Edward Amaya
pVK59	Integrative vector with *neo*	Vasant Chary
pVK141	*thrC*::*spoIIQ-lacZ*	Vasant Chary
PVK228	*spoIIAC*::*neo*	Vasant Chary
Strains		
BR151	*trpC2 metB10 lys-3*	Laboratory stock
SL1102	*trpC2 metB10 spoIIAC561*	Laboratory stock
SL8410	*trpC2 metB10 lys-3 spoIIGB*::*erm*	This study
SL8603	*trpC2 metB10 lys-3 erm spoIIE amyE*::*spoIIQ-lacZ*	This study
SL8625	*trpC2 metB10 lys-3 erm spoIIE*::pSG1902 *amyE*::*spoIIQ-lacZ*	This study
SL8675	*trpC2 metB10 lys-3 spc spoIIE*::pSG1902 *amyE*::*spoIIQ-lacZ*	This study
SL8978	*trpC2 metB10 lys-3 erm spoIIEV697A*::pSG1902 *amyE*::*spoIIQ-lacZ*	This study
SL9055	*trpC2 metB10 spoIIAΔ4*	Laboratory stock
SL10002	*trpC2 metB10 lys-3 thrC*::*spoIIQ-lacZ*	This study
SL10008	*trpC2 metB10 lys-3 erm spoIIEV697A thrC*::*spoIIQ-lacZ*	This study
SL10174	*trpC2 metB10 lys-3 amyE*::*spoIIQ-lacZ*	This study
SL10221	*trpC2 metB10 lys-3 spoIIQ*::pDH6	This study
SL10222	*trpC2 metB10 lys-3 erm spoIIEV697A spoIIQ*::pDH6	This study
SL10312	*trpC2 metB10 lys-3 erm spoIIE div-355 amyE*::*spoIIQ-lacZ*	This study
SL10339	*trpC2 metB10 lys-3 erm spoIIEV697A amyE*::*spoIIQ-lacZ*	This study
SL10423	*trpC2 metB10 lys-3 erm spoIIEV697A div-355 amyE*::*spoIIQ-lacZ*	This study
SL10766	*trpC2 metB10 lys-3 erm spoIIEV697A*	This study
SL10767	*trpC2 metB10 lys-3 erm spoIIE48*	This study
SL10905	*trpC2 metB10 spc spoIIEV697A spoIIAΔ4*	This study
SL10912	*trpC2 metB10 lys-3 spc spoIIEV697A*	This study
SL11132	*trpC2 metB10 lys-3 spc spoIIEV697A spoIIGB*::*erm*	This study
SL11142	*trpC2 metB10 spc spoIIEV697A spoIIAC561*	This study
SL11173	*trpC2 metB10 lys-3 spoIIE*::pDH7	This study
SL11201	*trpC2 metB10 lys-3 erm spoIIE48*::pDH7	This study
SL11274	*trpC2 metB10 lys-3 spoIIAC*::*neo*	This study
SL11275	*trpC2 metB10 lys-3 spc spoIIEV697A spoIIAC*::*neo*	This study

[a] ORF, open reading frame.

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 *spc* 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 *spc* gene at this site in the chromosome had no effect on growth or sporulation (data not shown). This strain also had a σ^F-dependent *spoIIQ-lacZ* transcriptional fusion (24) to avoid isolation of alleles that are defective in the phosphatase activity required for σ^F 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 σ^F. 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-σ^F 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).

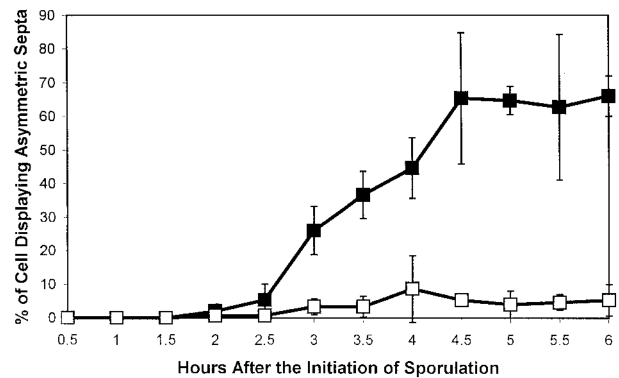

FIG. 1. Frequency of asymmetric division during sporulation in the parent strain BR151 (solid squares) and in the *spoIIEV697A* mutant SL10766 (open squares). Fifty cells were scored at each time. The results are the averages of three independent experiments plus and minus the standard deviation.

The effect of *spoIIEV697A* 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 *spo*$^+$ parent, produced $3.1 \times 10^8 \pm 1.1 \times 10^8$ spores per ml. In contrast, SL10766, containing the *spoIIEV697A* mutation, only produced $1.5 \times 10^7 \pm 0.5 \times 10^7$ spores per ml. Therefore, the *spoIIEV697A* mutation caused a 10- to 40-fold reduction in sporulation.

***spoIIEV697A* severely impairs asymmetric division.** Our rationale for screening for *spoIIE* mutants that were impaired in sporulation but continued to activate σ^F 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 $T_{4.5}$ 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 *spoIIEV697A* mutation, the proportion with asymmetric septa rarely exceeded 10% (Fig. 1). In all of the samples analyzed after T_4, 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 *spoIIEV697A* mutant (Fig. 1).

***spoIIEV697A* causes excessive, uncompartmentalized σ^F activity.** Colonies of the *spoIIEV697A* mutant containing the σ^F-dependent *spoIIQ-lacZ* transcriptional fusion gave an in-

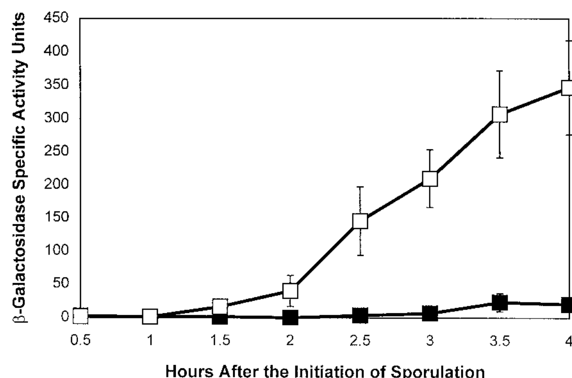

FIG. 2. Expression of *spoIIQ-lacZ* in the parent strain SL10002 (solid squares) and in the *spoIIEV697A* mutant SL10008 (open squares) in MSSM. Specific activity units are nanomoles of ONPG hydrolyzed per minute per milligram of bacterial dry weight. The results are the averages of three independent experiments plus and minus the standard deviation.

tensely blue color on SSA plates containing X-Gal, suggesting that σ^F activity was higher than in the parent (data not shown). Since it has been postulated that SpoIIE may play a role in the spatial and temporal regulation of σ^F (3, 11, 19), we wanted to characterize further the effect of *spoIIEV697A* on its activity. In order to quantitatively analyze σ^F activity in this mutant, the strain containing the *spoIIQ-lacZ* fusion and the *spoIIEV697A* mutation (SL10008) was induced to sporulate in MSSM, and samples were taken for analysis of β-galactosidase activity (see Materials and Methods). In the *spo*$^+$ parent, the *spoIIQ-lacZ* fusion became active at $T_{2.5}$, and β-galactosidase activity reached a peak specific activity of 30 nmol of ONPG hydrolyzed per min per mg at $T_{3.5}$ (Fig. 2). In contrast, the fusion in the *spoIIEV697A* mutant became active slightly earlier, at $T_{1.5}$, and β-galactosidase specific activity rose to over 275 nmol of ONPG hydrolyzed per min per mg by T_4, indicating at least a ninefold increase in expression of *spoIIQ-lacZ* (Fig. 2).

We also tested the effect of *spoIIEV697A* on expression of two other σ^F-dependent promoters, *spoIIR* (17) and *spoIIIG* (42). In both cases, the mutation caused at least a threefold to fourfold increase in peak activity (data not shown). These results suggest that in addition to impairing asymmetric division, *spoIIEV697A* causes hyperactivation of σ^F, a phenotype that is consistent with the role of SpoIIE as a phosphatase in triggering σ^F activation (2, 9).

It had been previously demonstrated that overexpression of SpoIIE can cause abnormally high levels of σ^F activation and severely impair sporulation (2), a phenotype reminiscent of that observed in the *spoIIEV697A* mutant. In order to determine if the level of SpoIIE protein was disturbed by this mutation, whole-cell lysates were isolated at various times during sporulation from strains containing C-terminal translational 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 SpoIIE-GFP protein concentrations between the parent and the mutant (data not shown).

The activity of σ^F is normally confined to the forespore after asymmetric division occurs, and mutations that block asymmetric division prevent σ^F activation (12, 19, 21). This coupling of asymmetric division to σ^F 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 σ^F activity (Fig. 2). This led us to speculate that the checkpoint had been disrupted and that in some cells in the *spoIIEV697A* population, σ^F activity would be uncompartmentalized. In order to address this, we generated strains that had the *spoIIQ* promoter transcriptionally fused to *gfp*, the gene encoding GFP, with pDH6 (see Materials and Methods). A *spoIIQ-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 T_6 (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 σ^F activity. Similar results were observed with a *spoIIIG-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 *spoIIQ-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 σ^F in the absence of asymmetric division. We proceeded to test this possibility in the following experiment.

***spoIIEV697A* uncouples σ^F 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 σ^F 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 *spoIIQ-lacZ* transcriptional fusion (SL10423). In addition, we also generated isogenic strains with the mutations singly

TABLE 2. Pattern of *spoIIQ-gfp* expression in the parent strain and *spoIIEV697A* mutant

Strain	No. of cells showing indicated fluorescence pattern[a]:			% Compartmentalization[b]
	Forespore	Whole cell	No signal	
SL10221 (parent)	102	4	219	96
SL10222 (mutant)	4	178	145	2.2

[a] 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 T_6. At least 100 cells were scored in each experiment.

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

TABLE 3. Sporulation frequency of parent strain and *spoIIE* mutants

Strain	Relevant genotype	Avg no. heat survivors, CFU (±SD)/ml	Heat survivors as % of parent value
BR151	spo^+	$7.4 (\pm 0.4) \times 10^8$	100
SL10767	*spoIIE48*	$5.3 (\pm 4.0) \times 10^1$	0.0000074 ± 0.0000057
SL11173	*spoIIEV697A*	$4.5 (\pm 0.8) \times 10^7$	6.2 ± 1.5
SL11201	*spoIIE48 spoIIEV697A*	$5.9 (\pm 1.0) \times 10^8$	71 ± 18

(SL10312 with *div-355* and SL10339 with *spoIIEV697A*). These three strains, along with the spo^+ parent also containing the *spoIIQ-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 *spoIIQ-lacZ* fusion became active at $T_{2.5}$ 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 *spoIIEV697A* 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 spoIIEV697A* double mutant had a pattern of *spoIIQ-lacZ* activity that was not significantly different from that of the *spoIIEV697A* single mutant (Fig. 3B). From these results, we can conclude that the *spoIIEV697A* mutation uncouples σ^F activation from asymmetric division, allowing σ^F to become active even when division is blocked.

***spoIIEV697A* is an intragenic suppressor of *spoIIE48*.** Based on the previous experiments, we strongly suspected that *spoIIEV697A* 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 *spoIIE48* (*spoIIES361F*), has been described as being capable of substantially dephosphorylating SpoIIAA 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 *spoIIE48* mutant (SL10767), the *spoIIEV697A* mutant (SL11173), and the *spoIIE48 spoIIEV697A* 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 *spoIIE* gene harboring both mutations. Whereas the *spoIIE48* mutant was severely impaired in sporulation (53 ± 40 spores per ml [Table 3]), the *spoIIE48 spoIIEV697A* double mutant was Spo^+, producing almost as many spores as the spo^+ parent ($5.9 \times 10^8 \pm 1.0 \times 10^8$, compared to $7.4 \times 10^8 \pm 0.4 \times 10^8$ spores per ml [Table 3]). In turn, the double mutant produced nearly 10 times as many spores as the *spoIIEV697A* single mutant ($5.9 \times 10^8 \pm 1.0 \times 10^8$ compared to $4.5 \times 10^7 \pm 0.8 \times 10^7$ spores per ml [Table 3]). These results indicate that *spoIIEV697A* can function as an intragenic suppressor of *spoIIE48*. Since *spoIIE48* is normally incapable of activating σ^F in response to asymmetric division, this provides further evidence that *spoIIEV697A* severely interferes with the checkpoint coupling the two events.

Reduction in asymmetric division in the *spoIIEV697A* mutant is dependent upon the *spoIIA* operon but independent of σ^E activity. We had separately observed uncompartmentalized σ^F activity (Table 2) and a severe reduction in asymmetric division (Fig. 2) in the *spoIIEV697A* mutant. We wanted to investigate the possibility that these two phenotypes were re-

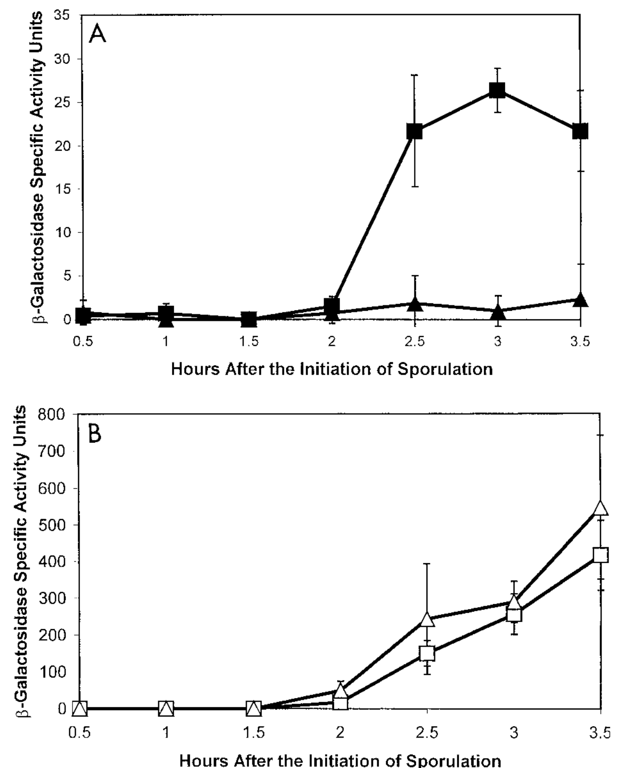

FIG. 3. Expression of *spoIIQ-lacZ* in strains containing mutations in *spoIIE* and/or *divIC*. (A) Expression in the $spoIIE^+$ $divIC^+$ parent strain SL10174 (solid squares) and the *spoIIEV697A div-355* mutant SL10312 (solid triangles). (B) Expression in the *spoIIEV697A* $divIC^+$ mutant SL10339 (open squares) and in the *spoIIEV697A 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.

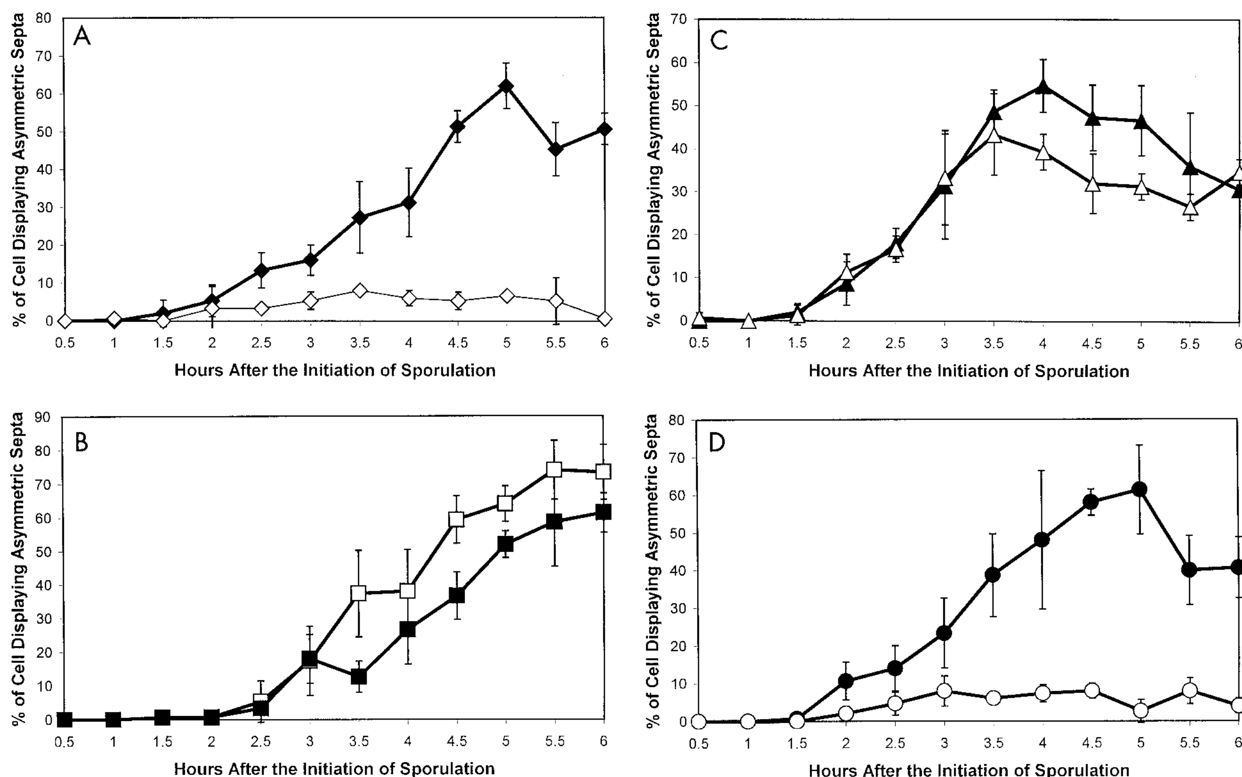

FIG. 4. Frequency of asymmetric division during sporulation in strains containing mutations in *spoIIE*, *spoIIGB*, and/or *spoIIA*. (A) Frequency of asymmetric division in the *spoIIGB*::*erm* mutant SL8410 (solid diamonds) and in the *spoIIEV697A spoIIGB*::*erm* mutant SL11132 (open diamonds). (B) Frequency of asymmetric division in the *spoIIA*Δ*4* mutant SL9055 (solid squares) and in the *spoIIEV697A spoIIA*Δ*4* mutant SL10905 (open squares). (C) Frequency of asymmetric division in the *spoIIAC*::*neo* mutant SL11274 (solid triangles) and the *spoIIEV697A spoIIAC*::*neo* mutant SL11275 (open triangles). (D) Frequency of asymmetric division in the *spoIIAC561* mutant SL1102 (solid circles) and in the *spoIIEV697A spoIIAC561* mutant SL11142 (open circles). Fifty cells were scored at each time. 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 σ^E is required to block a second asymmetric division from occurring in the mother cell (15). σ^E is synthesized as an inactive precursor, pro-σ^E, 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 σ^F is required for proteolysis to occur (17, 25). However, uncoupling *spoIIR* expression from asymmetric division causes uncompartmentalized activation of σ^E in some cells (41, 46). A different study has demonstrated that predivisional expression of three σ^E-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 σ^E. 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 σ^E-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 σ^E-dependent *cotEP1* (8) and *spoIID* (36) promoters is drastically reduced in the *spoIIEV697A* mutant, making it unlikely that any aspect of the *spoIIEV697A* phenotype is due to inappropriate σ^E 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 σ^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-σ^F activity. In order to do this, we used a *spoIIA*Δ*4* mutant (33), carrying a deletion of the *spoIIA* operon encoding the structural gene for σ^F, *spoIIAC*, as well as the *spoIIAA* and *spoIIAB* genes, encoding regulators of σ^F (34). The *spoIIEV697A spoIIA*Δ*4* double mutant (SL10905) and the single *spoIIA*Δ*4* 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 *spoIIEV697A spoIIAΔ4* double mutant exhibited no significant reduction in frequency of asymmetric division and actually demonstrated a modest increase at several times after T_3 (Fig. 4B). Whereas rarely more than 10% of the cells in the *spoIIEV697A* single-mutant population exhibited asymmetric septa at the times analyzed (Fig. 1), the *spoIIEV697A spoIIAΔ4* double-mutant cells underwent asymmetric division at a much higher frequency, reaching 50% by $T_{4.5}$ and greater than 65% by T_6 (Fig. 4B). There was at least a fivefold increase in the frequency of asymmetric division in the *spoIIEV697A spoIIAΔ4* double mutant compared to the *spoIIEV697A* single mutant at all times analyzed after T_4 (Fig. 1 and 4B). These results indicate that the deficiency in asymmetric division observed in the *spoIIEV697A* mutant is dependent upon some product of the *spoIIA* operon.

Deletion of *spoIIAC* but not a point mutation that largely abolishes promoter recognition by σ^F restores asymmetric division in a *spoIIEV697A* mutant. We thought it was most likely that the asymmetric division phenotype in the *spoIIEV697A* mutant was being mediated by σ^F because the major role for the other products of the *spoIIA* operon, SpoIIAA and SpoIIAB, is to regulate its activity (34). In order to test this possibility, we utilized a *spoIIAC*::*neo* deletion, which does not interfere with either the *spoIIAA* or the *spoIIAB* open reading frame. We constructed a *spoIIAC*::*neo* single mutant (SL11274) and a *spoIIEV697A spoIIAC*::*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 *spoIIEV697A* single mutant, in which the frequency rarely exceeded 10% of the population (Fig. 1). At all times analyzed after T_4, the frequency of asymmetric septation in the *spoIIEV697A spoIIAC*::*neo* double mutant was at least threefold higher than that in the *spoIIEV697A* single mutant (Fig. 1 and 4C). This indicates that the block in division is indeed mediated by σ^F.

We next wished to address the question of why hyperactivation of σ^F 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 *spoIIAC561* mutation (15). This mutation causes a V233M change in the 4.2 promoter recognition region of σ^F (45). As a consequence, most σ^F-directed promoters are not recognized, although at least one σ^F-controlled gene, *spoIIR*, becomes hyperexpressed (17).

We used a *spoIIAC561* mutant (SL1102) as well as a *spoIIEV697A spoIIAC561* 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 *spoIIAC561* mutant formed asymmetric septa at a high frequency, reaching nearly 50% by T_5, the frequency in the *spoIIEV697A spoIIAC561* double mutant population rarely exceeded 10% (Fig. 4D). Compared to the *spoIIAC561* mutant, the *spoIIEV697A spoIIAC561* double mutant exhibited a reduction of between three- and eightfold in asymmetric division between T_3 and T_5. The frequency of asymmetric division in the *spoIIEV697A* single mutant and the *spoIIEV697A spoIIAC561* double mutant was not significantly different at any time analyzed (Fig. 1 and 4D). This indicates that the block in division observed in the *spoIIEV697A* mutant results either from expression of one of the σ^F-dependent genes whose promoter is still recognized by σ^{F561} or from interaction of σ^F 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 SpoIIE are required for this switch to occur (6, 18). However, the mechanism by which SpoIIE facilitates asymmetric division remains unknown. Here we report the isolation and characterization of a mutation, *spoIIEV697A*, that causes a severe impairment in the formation of polar septa during sporulation. However, it appears that this impairment is indirect, mediated via σ^F in the *spoIIEV697A* mutant. This is consistent with previous observations that deletion of the gene encoding the anti-sigma factor SpoIIAB caused unregulated σ^F activity and a concomitant block in asymmetric division (7). Although our findings do not provide additional evidence for a direct role of SpoIIE in promoting asymmetric division, they suggest that one function of SpoIIE may be to delay σ^F 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.

SpoIIE has a C-terminal PP2C-like phosphatase domain that has a well-characterized role in activation of the forespore-specific transcription factor σ^F (1, 2, 9, 38). SpoIIE dephosphorylates, and thus activates the anti-anti-sigma factor SpoIIAA, which can then relieve inhibition of σ^F by the anti-sigma factor SpoIIAB (2, 9). In turn, SpoIIAB 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 σ^F becomes active only after asymmetric division and only in the forespore (34). It has been proposed that SpoIIE itself is regulated by a number of different mechanisms in order to explain the temporal and spatial regulation of σ^F activity.

Two broad models have emerged. In the first, SpoIIE is inactive in its default state and is activated by interaction with cell division proteins (19). In the second, SpoIIE is active in the default state and is negatively regulated to prevent inappropriate σ^F 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 SpoIIE to efficiently dephosphorylate SpoIIAA. Next, it was demonstrated that the *div-355* mutation of *divIC* allows efficient dephosphorylation of SpoIIAA by SpoIIE but not activation of σ^F. Lastly, the *spoIIE48* (*spoIIES361F*) mutant was found to have the same phenotype with regards to σ^F activation as the *div-355* mutant (i.e., dephosphorylation of SpoIIAA but no σ^F activation) (19). These results suggested a regulatory step in which SpoIIE prevents dephosphorylated SpoIIAA from at-

tacking the SpoIIAB-σ^F complex and activating σ^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 σ^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 SpoIIAA, is 100 times stronger than the kinase activity of SpoIIAB, which inactivates it; since the levels of the SpoIIE and SpoIIAB 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 σ^F in the *div-355* background. This mutant is the converse of another phosphatase-competent mutant, SpoIIE48, which cannot activate σ^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 σ^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 *spoIIAB* deletion (which also causes hyper-σ^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 σ^F. This is indicated by our observation that deletion of the *spoIIA* operon and of *spoIIAC*, the structural gene for σ^F, largely overcame the effect of the *spoIIEV697A* mutation on sporulation division. We showed that the effect was not a secondary consequence of σ^E activation. We also determined that if the effect was caused by expression of a σ^F-dependent gene, it must be one that continues to be expressed in a *spoIIAC561* mutant, in which expression of most known σ^F-dependent genes is abolished. The σ^F protein interacts with core RNA polymerase and also with the SpoIIAB protein. Interaction of σ^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 σ^F sequestering core polymerase but cannot exclude an effect on transcription elsewhere. SpoIIAB has a pivotal role in σ^F activation (34), and it may be part of the link between activation and septation. The presence of σ^F can affect SpoIIAB stability (30). Although speculative, the presence of the mutant σ^F protein might affect septation through its interaction with SpoIIAB.

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 σ^F activation from septation, cause uncompartmentalized hyper-σ^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 σ^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 *spoIIE48* 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 σ^F activation to asymmetric division and confining σ^F activity to the forespore. These studies reinforce the concept that precise coupling of asymmetric division to σ^F activation is critical for efficient spore formation.

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