FtsA Mutants of *Bacillus subtilis* Impaired in Sporulation

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Received 23 January 2002/Accepted 22 April 2002

Spore formation in *Bacillus subtilis* involves a switch in the site of cell division from the midcell to a polar position. Both medial division and polar division are mediated in part by the actin-like, cytokinetic protein FtsA. We report the isolation of an FtsA mutant (FtsA^{D265G}) that is defective in sporulation but is apparently unimpaired in vegetative growth. Sporulating cells of the mutant reach the stage of asymmetric division but are partially blocked in the subsequent morphological process of engulfment. As judged by fluorescence microscopy and electron microscopy, the FtsA^{D265G} mutant produces normal-looking medial septa but immature (abnormally thin) polar septa. The mutant was unimpaired in transcription under the control of Spo0A, the master regulator for entry into sporulation, but was defective in transcription under the control of σ^F , a regulatory protein whose activation is known to depend on polar division. An amino acid substitution at a residue (Y264) adjacent to D265 also caused a defect in sporulation. D265 and Y264 are conserved among endospore-forming bacteria, raising the possibility that these residues are involved in a sporulation-specific protein interaction that facilitates maturation of the sporulation septum and the activation of σ^F .

Most bacteria divide by binary fission, forming a septum at the midpoint of the long axis of the cell to produce identical-sized progeny. The gram-positive soil bacterium *Bacillus subtilis* exhibits an additional mode of division when it enters the pathway to sporulate. Under such conditions, it undergoes a process of asymmetric division in which a septum is formed at a polar rather than a medial position. The polar septum divides the cell asymmetrically into a forespore (the smaller cell) and a mother cell. The forespore and the mother cell each receive a chromosome but exhibit dissimilar programs of gene expression (reviewed in reference 37). Gene expression in the forespore is governed by the transcription factor σ^F , whose activation depends on the formation of the polar septum, whereas gene expression in the mother cell depends on the transcription factor σ^E . Following asymmetric division, the forespore is engulfed by the mother cell in a phagocyte-like process that results in the forespore becoming pinched off as a free protoplast within the mother cell.

The switch from medial division during growth to asymmetric division during sporulation involves a change in the subcellular localization of the cytokinetic protein FtsZ. This tubulin-like GTPase assembles into a ring-like structure, called the Z-ring, at the future site of septation (7, 49). During vegetative growth, the Z-ring assembles at the middle of the cell, fixing the position of the division site. The Z-ring is disassembled during cytokinesis and is largely absent from the completed septum. During sporulation, the Z-ring switches to a bipolar pattern of localization, forming a ring near each pole of the cell (29). Recent evidence indicates that the switch from medial to

bipolar Z-rings occurs via a spiral-like intermediate, which may serve to redeploy molecules of FtsZ from the midcell position to the poles (6). Both polar Z-rings have the potential to undergo cytokinesis, but only one ring is normally converted into a septum. A sporulation-specific pathway involving the proteins SpoIID, SpoIIM, and SpoIIP helps to ensure that a septum is formed at only one end of the developing cell (13, 37, 38). Mutants in which the pathway is disabled produce “disporic” sporangia with septa at both poles (36). The switch from medial to polar division is governed by the sporulation regulatory proteins Spo0A and σ^H , but little is known about the mechanism of the switch.

The Z-ring is a scaffold for the recruitment of additional proteins that help mediate septum formation. The same set of proteins appears to be involved in both medial and polar division. This set includes the actin-like protein FtsA as well as the membrane proteins FtsL, DivIB, DivIC, and PBP2B (3, 4, 5, 10, 22, 28, 50). FtsA, which interacts directly with FtsZ (48), localizes to the site of polar division during sporulation but is usually seen in a unipolar pattern, localizing preferentially to only one end of the cell, as opposed to the bipolar pattern observed with FtsZ (14).

The medial septum and the sporulation septum differ, however, not only in position but also in structure and composition. For example, the peptidoglycan layer is thinner in the polar septum than in the medial septum and is largely, if not entirely, dissolved prior to engulfment. Also, several proteins localize to the polar septum that are not present in cells undergoing binary fission. These include SpoIIE, SpoIIGA, SpoIVA, SpoIVFA, and SpoIVFB (37).

The fact that the same cytokinetic machinery governs the formation of two different kinds of septa raises the question of whether the involvement of FtsZ and FtsA is somewhat different in medial division and polar division. To investigate this question, we sought to isolate mutants of these proteins that are impaired in sporulation but not in growth. Here we report

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FIG. 1. Constructs with the *ftsAZ* operon inserted into the chromosome at the *amyE* locus. The strategy (see text) for mutagenizing the *ftsAZ* operon was based on the construction of donor (template) and recipient strains that each harbored a copy of the *ftsAZ* operon in opposite orientations at the *amyE* locus. The top panel shows the structure of the *ftsAZ* insert in *amyE* in the strain JKB101, which was the source of the template for amplification of the operon. The bottom panel shows the *ftsAZ* insert in *amyE* in the recipient strain JKB106. The arrows indicate the *ftsAZ* promoter region.

the isolation of a mutant of FtsA (FtsA^{D265G}) that exhibits little or no detectable defect in medial division but forms aberrant polar septa during sporulation and is defective in engulfment and in the activation of forespore transcription factor σ^F . D265 and an adjacent residue are conserved features of FtsA among endospore-forming bacteria and could define a patch on the surface of the protein that plays a dedicated role in development.

MATERIALS AND METHODS

General methods. Routine *B. subtilis* manipulations were performed as described (23). Cultures were grown in Luria broth medium (39), and sporulation was induced by nutrient exhaustion in Difco sporulation medium (40, 42) or by resuspension (34). Chromosomal DNA was prepared as described earlier (9). Competent cells were prepared and transformed by the two-step method (11) or the one-step method (27). Antibiotic concentrations used for selection on Luria broth agar were chloramphenicol at 5 μ g/ml, kanamycin at 10 μ g/ml, spectinomycin at 100 μ g/ml, and lincomycin at 25 μ g/ml plus erythromycin at 1 μ g/ml for macrolide-lincosamide-streptogramin B (MLS) resistance.

For nucleotide sequence analysis, DNA fragments containing the entire *ftsAZ* operon and promoter regions were amplified from mutant chromosomal DNAs. After gel purification, the fragments were sequenced using thermal cycle sequencing and the dideoxy method (41) with reagents from the BigDye Terminator Cycle Sequencing kit (PE/ABI). Prior to loading on an ABI Prism 310 Genetic analyzer, the sequencing reactions were purified using AutoSeq G-50 columns (Amersham Pharmacia) or Spin 50 columns (Biomax).

For β -galactosidase assays, samples (1 ml) from sporulating cultures were collected and used to determine β -galactosidase activity (23) using lysozyme to permeabilize the cells and *o*-nitrophenyl- β -D-galactopyranoside as the substrate.

Strain construction. Strain JKB101 ([RL1075] *ftsAZ::kan amyE::ftsAZ[spec]*) was constructed as a "donor" strain to provide a source of template DNA for PCR mutagenesis. First, a $\sim$ 3.6 kb DNA fragment containing the *ftsAZ* operon and promoter region was amplified from wild-type *B. subtilis* DNA (PY79[52]) with primers creating a *Bam*HI site at the 5' end and an *Eco*RI site at the 3' end (Fig. 1). The resulting DNA was digested with *Eco*RI and *Bam*HI and ligated into pLD30 (an *amyE* integration vector [17]) that had been digested with *Bam*HI and *Eco*RI, thereby inserting the operon into pLD30 in the same orientation as *amyE* and with the spectinomycin cassette downstream of the *ftsAZ* operon. Since the resulting construct could not be propagated through *Escherichia coli* without mutation (due to toxicity of the *B. subtilis* FtsZ and possibly FtsA produced from the plasmid), it was linearized with *Sca*I and transformed directly into *B. subtilis* strain RL1075 (*amyE::erm*), selecting for spectinomycin resistance and then screening for MLS sensitivity. One such spectinomycin-resistant, MLS-sensitive transformant is JKB171. Next, to delete the chromosomal copy of the *ftsAZ* operon from JKB171, pJTK101 was constructed to replace the *ftsAZ* operon with a kanamycin resistance cassette. First, the $\sim$ 3.6 kb *ftsAZ* fragment mentioned above was amplified by PCR and inserted into pCRII (Invitrogen), a TA cloning vector. Since complete integrity of the *ftsAZ* sequence

was unimportant for this construct, routine *E. coli* cloning techniques were used. Next, a $\sim$ 3.0-kb *Acc*I fragment, containing most of the *ftsAZ* operon, was removed and replaced with a $\sim$ 1.4-kb fragment from pDG792 (20) containing a kanamycin resistance cassette. Finally, the resulting construct, pJTK101, was linearized with *Bsa*AI and transformed into the above *B. subtilis* strain JKB171, selecting for kanamycin resistance. The resulting strain, JKB101, was indistinguishable from the wild type in growth rate and sporulation efficiency, indicating that the *ftsAZ* operon inserted at the *amyE* locus was able to complement the wild-type operon that had been deleted.

Strain JKB106 ([PY79] *ftsAZ::kan amyE::ftsAZ[cat]* SP β ::*gerE-lacZ[cat, erm]*) was constructed as a "recipient" strain (for a nonbiased screen) into which the mutagenized DNA from JKB101 would be transformed. The *ftsAZ* operon was amplified with an *Eco*RI site at the 5' end and a *Bam*HI site at the 3' end. Next, it was ligated into the *amyE* integration vector pDG364 (24), which had been digested with *Bam*HI and *Eco*RI, thereby inserting the operon into pDG364 in the orientation opposite that of *amyE* and with the chloramphenicol resistance cassette upstream of the *ftsAZ* operon. The resulting construct was linearized and transformed directly into *B. subtilis* RL1075 as above, again bypassing *E. coli*. To move the construct into a PY79 background, chromosomal DNA from the resulting strain was transformed into PY79, selecting for chloramphenicol resistance. Next, chromosomal DNA from JKB101 was transformed into the above strain, selecting for kanamycin resistance, to remove the chromosomal copy of the *ftsAZ* operon. Finally, JKB106 was created by transducing the above strain with phage prepared from RL13 (SP β ::*gerE-lacZ[cat, erm]*), selecting for MLS resistance.

PCR mutagenesis. Localized mutagenesis of the *ftsAZ* operon was achieved by amplifying chromosomal DNA from strain JKB101 with TaqPlus Long Polymerase (Stratagene). To amplify the entire *ftsAZ* operon, PCR primers were located in the *amyE* upstream and *amyE* downstream regions. The resulting mutagenized DNA was transformed into JKB106, selecting for spectinomycin resistance. Since the orientation of the *ftsAZ* operon (with respect to *amyE*) differed in the donor and recipient strains, homologous recombination resulting in marker replacement could occur only at the *amyE* upstream and downstream regions, thereby forcing uptake of the entire *ftsAZ* operon (Fig. 1). Colonies were screened for sporulation defects on DSM plates containing 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) at a concentration of 60 μ g/ml. Colonies that failed to fully activate the *gerE-lacZ* reporter were light blue or white and were selected as candidates.

About 30,000 transformants were screened, of which 78 exhibited a defect in *gerE-lacZ* activation. Of these, 17 transformants were identified that exhibited little or no defect in vegetative growth and for which impaired activation of *gerE-lacZ* could be shown to be the result of a mutation that exhibited linkage to *ftsAZ* in DNA-mediated transformation experiments. Some of the mutants contained multiple mutations, and three of the mutants contained mutations in *ftsZ*. The FtsA^{D265G} mutant was chosen for further study because of its pronounced phenotype and because it contains a substitution of a conserved residue.

Microscopy and image acquisition. For epifluorescence microscopy, an Olympus BX 60 microscope (as described in reference 21) equipped with a MicroMax (Princeton Instruments, Trenton, N.J.) cooled charge-coupled device camera driven by the Meta-Morph software package (version 4.0; Universal Imaging,

Media, Pa.) was used. The fluorescein isothiocyanate and FM1-43 images were captured using a U-MWIB excitation cube unit (Olympus) with a band-pass excitation filter (460 to 490 nm) and a long-pass barrier filter (≥ 515 nm). Electron microscopy was carried out as described previously (32).

Isoelectric focusing and Western blot analysis. Isoelectric focusing was performed as described earlier (18). Strains were sporulated by resuspension at 37°C, and 1-ml samples were harvested at the indicated times. Samples were incubated in lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM MgCl₂, 0.3 mg of phenylmethylsulfonyl fluoride/ml, 0.1 mg of DNase I/ml, and 0.5 mg of lysozyme/ml) at 37°C for 10 min. Isoelectric focusing sample buffer containing urea was added to samples on ice 15 min prior to loading approximately equal numbers of cells (as determined by optical density) onto an isoelectric focusing gel (2). Protein was electroblotted to Immobilon-P membranes (Millipore). Immunodetection was performed using polyclonal anti-SpoIIAA antibodies (12) followed by secondary antibodies conjugated to horseradish peroxidase (Promega).

RESULTS AND DISCUSSION

Isolation of FtsA and FtsZ mutants impaired in sporulation.

To determine whether FtsA and FtsZ, the genes for which are contained in the *ftsAZ* operon, play unique roles in sporulation, we screened for mutants of the cell division proteins that were defective in sporulation but were able to undergo vegetative growth with little or no impairment. Our strategy was to perform PCR mutagenesis on the *ftsAZ* operon and then screen for mutants that were impaired in the expression of *lacZ* fused to a promoter that is activated at a late stage of development. To do this, we inserted the *ftsAZ* operon and a downstream drug resistance gene (*spec*) into the chromosome at the *amyE* locus (Fig. 1; see Materials and Methods). Next, a 6.8-kb segment of DNA that included the operon, the drug resistance gene, and flanking *amyE* sequences was amplified by PCR under conditions that are known to introduce mutations. The amplified DNA was directly introduced by transformation into competent cells of a recipient strain that contained a wild-type copy of the *ftsAZ* operon and a flanking chloramphenicol resistance gene (*cat*) at the *amyE* locus (Fig. 1). The recipient strain also contained a deletion of the *ftsAZ* operon at its normal chromosomal location and a *lacZ* fusion to a gene (*gerE*) under the control of the sporulation transcription factor σ^K . We then selected for recombinants that were spectinomycin resistant, thereby exchanging the entire wild-type *ftsAZ* operon (see Materials and Methods) with mutagenized *ftsAZ*. Transformants were screened for those exhibiting normal growth but also exhibiting impaired expression of *gerE-lacZ* on sporulation medium containing X-Gal.

To verify that the mutant phenotype was due to a mutation in or near the *ftsAZ* operon, chromosomal DNA from each mutant was used to transform the recipient strain, once again selecting for spectinomycin resistance. Only mutants in which the sporulation-impairing mutation showed 100% linkage to the spectinomycin resistance gene were considered further.

Mutants impaired at the stage of polar septation. The predominant category of mutants exhibited a partial block in sporulation at the stage of polar septum formation. The growth rates of the mutants were indistinguishable from that of the wild type in liquid medium, a representative example of which is shown in Fig. 2A. Also, the cells appeared normal during growth as judged by staining with the vital membrane dye FM1-43, except that the mutant cells (as measured from division septum to division septum) appeared slightly longer than the wild type's. The mutants exhibited a conspicuous pheno-

FIG. 2. Reporter gene expression in wild-type and FtsA^{D265G} mutant strains. (A) Wild-type (circles; RL13) and FtsA^{D265G} mutant (squares; JKB124) strains harboring a *gerE-lacZ* fusion, a reporter for σ^K activity, were grown and sporulated in Difco sporulation medium at 37°C. At the indicated times, samples were taken for measurement of cell density (dashed lines; optical density at 600 nm) and β -galactosidase activity (solid lines). (B) Wild-type (circles; RL1740) and mutant (squares; JKB162) strains containing a *spoIIA-lacZ* fusion, a reporter for SpoIIA activity, were sporulated by suspension in Sterlini-Mandelstam medium at 37°C, and samples were taken for β -galactosidase assays at the indicated times after suspension. (C) Wild-type (circles; RL1131) and mutant (squares; JKB163) containing a *sspE(2G)-lacZ* fusion, a reporter for σ^F activity, were sporulated and assayed as in panel B.

type, however, during sporulation: many of the mutant cells reached the stage of polar septation, but few, if any, underwent engulfment by h 2.5 of sporulation (as shown for one such mutant in Fig. 3), a time at which many cells of the wild-type recipient strain exhibited engulfed forespores (Fig. 3). At later times a minority of the cells did successfully complete engulfment and proceed through spore formation. The mutants were oligosporogenous and produced heat-resistant spores about 10-fold less efficiently than did the wild type.

The nature of the mutations in the mutants was determined by nucleotide sequence analysis. In each case, the entire *ftsAZ* operon, including the promoter region, was sequenced. Some

FIG. 3. Fluorescence microscopy of sporulating cells. (A) Wild-type (PY79) and (B) *FtsA*^{D265G} mutant (JKB124) strains were sporulated by suspension in Sterlini-Mandelstam medium at 37°C. At 2.5 h after the start of sporulation, samples were stained with the vital membrane dye FM1-43 and visualized by fluorescence microscopy (38). Arrows indicate polar septa or engulfed forespores. The scale bar, which represents 1 μ m, was approximated from other images.

mutants contained multiple mutations and were not considered further. Three of the mutants contained single nucleotide substitutions in *ftsA* that were expected to cause the following amino acid substitutions: D265G, S242P, and L434P.

To verify that the mutant phenotypes described above were indeed caused by the predicted amino acid changes, we used site-directed mutagenesis to recreate mutant genes for *FtsA*^{D265G}, *FtsA*^{S242P}, and *FtsA*^{L434P}. All three of the newly created mutants were indistinguishable in their phenotypes from the original mutants.

D265 and Y264 are conserved in FtsA among endospore-forming bacteria. A sequence alignment of FtsA proteins from a variety of bacteria revealed that the aspartic acid at residue 265 in *B. subtilis* FtsA is present at the equivalent position in FtsA from four other endospore-forming species of *Bacillus* and *Clostridium* as well in FtsA from two closely related but non-spore-forming species, *Listeria monocytogenes* and *Listeria innocua* (Fig. 4). Aspartic acid is not, however, present at the

corresponding position in FtsA from several other nonsporulating species of gram-positive bacteria and in gram-negative species. Additionally, we noticed that the residue immediately upstream of D265 in *B. subtilis* FtsA, Y264, is conserved in three of the endospore-forming species (Fig. 4). This raised the possibility that residues 264 and 265 define a patch on the surface of *B. subtilis* FtsA that plays a distinctive role in sporulation. Loss-of-side chain (alanine) substitutions were created at D265 and Y264, but neither the D265A nor the Y264A mutant exhibited a defect in sporulation. However, replacement of the negatively charged aspartic acid at residue 265 with a lysine residue or the replacement of the tyrosine at residue 264 with a phenylalanine resulted in mutants with sporulation defects similar to those of the original D265G mutant.

In keeping with the idea that D265 and Y264 represent a patch on the surface of *B. subtilis* FtsA, the equivalent residues (N269 and Y268, respectively) in FtsA from *Thermotoga maritima*, an unrelated thermophilic eubacterium, are located in a loop (between helix 7 and strand 12 in subdomain 2B) on an exposed face of the recently determined X-ray crystallographic structure of the protein (47) (Fig. 5).

Mutant *FtsA*^{D265G} is defective in the activation of σ^F . Given the conservation of *FtsA*^{D265} among endospore-forming bacteria and other closely related species, the *FtsA*^{D265G} mutant was chosen for further study. Figure 2A shows that σ^K -directed synthesis of β -galactosidase from *gerE-lacZ* was considerably reduced and delayed in the mutant compared to in the wild type, indicating a partial block at or before a late stage of sporulation. To further define the defect caused by *FtsA*^{D265G}, we introduced into the mutant *lacZ* fused to *spoIIE*, which is activated at the onset of sporulation under the control of Spo0A (51), the master regulator for entry into sporulation, and fused to *sspE(2G)*, which is activated after polar septation under the control of σ^F (44). The pattern of *spoIIE-lacZ* expression in the mutant was not significantly different from that of the wild type, indicating that entry into sporulation and Spo0A-directed gene expression were unimpaired (Fig. 2B). However, expression of the *sspE(2G)-lacZ* fusion was somewhat reduced and delayed compared to that of the wild type, indicating that the cells were defective in the activation of σ^F (Fig. 2C).

Prior to this study, a mutation of *ftsA* (originally referred to as *spoIIN279* and causing the substitution of an asparagine at Ser9) was known that impairs sporulation (24, 31). In contrast to *FtsA*^{D265G}, which is impaired in gene transcription at or after the stage of polar division, the *FtsA*^{S9N} mutant is defective in Spo0A-controlled transcription at the onset of sporulation. Additionally, the *FtsA*^{S9N} mutant is filamentous at high temperatures, indicating that it causes a defect in vegetative cell division in addition to the sporulation defect.

***FtsA*^{D265G} forms aberrant polar septa during sporulation.** Activation of σ^F is known to be dependent upon formation of the polar septum (28). Because σ^F activation was impaired in the *FtsA*^{D265G} mutant, we decided to take a closer look at septum formation. As indicated above, the *FtsA*^{D265G} mutant formed normal-looking polar septa as judged by FM1-43 staining (Fig. 3). Next, we performed immunolocalization using antibodies against the cell division protein FtsZ, which is known to assemble into a so-called Z-ring at the future site of cytokinesis (7, 49). In wild-type cells, FtsZ transiently localizes

		<i>B. subtilis</i> residues
		264 265
Spore formers	<i>B. stearothermophilus</i>	KHGHAF-YDYASEEEV
	<i>B. anthracis</i>	KYGHAF-YDTASEEEM
	<i>B. subtilis</i>	QLGHAY-YDEASEDEI
	<i>B. halodurans</i>	THGHAY-IDEASEEDR
	<i>C. acetobutylicum</i>	KRNYAN-IDASINNGE
Non-spore formers	<i>L. monocytogenes</i>	DHGYAF-YDDASPDDEV
	<i>L. innocua</i>	EHGYAF-YDDASPDDEV
	<i>S. pneumoniae</i>	NYGEAYPP--LASKET
	<i>E. faecalis</i>	NYGDAY-PERTSANEE
	<i>E. coli</i>	RHGCAL-GSIVGKDES
	<i>H. influenzae</i>	NYSAFYPARLHGDKK
	<i>P. aeruginosa</i>	RYACAL-AKLAGAGET
	<i>T. maritima</i>	THGNVAVY--NDLKEEE

FIG. 4. Alignment of the region containing D265 in FtsA of *B. subtilis* with the corresponding regions in FtsA from other bacterial species. The amino acid sequences are from references 8, 16, 19, 26, 33, 35, 43, 45, and 46 and from databases accessible through the Institute for Genomic Research (<http://www.tigr.org>). Tyrosine and aspartic acid residues corresponding to Y264 and D265 of *B. subtilis* FtsA in other endospore-forming and closely related (*Listeria*) species are in unshaded boxes. Widely conserved alanine and glutamate residues are highlighted in dark shading.

near both poles of the sporangium even though a septum is normally formed at only one pole (29). Immunofluorescence microscopy experiments readily revealed cells with a similar pattern of bipolar FtsZ localization in cells of the FtsA^{D265G} mutant; indeed cells with a bipolar pattern of FtsZ localization appeared to be even more abundant in the mutant than in the corresponding wild-type strain (data not shown).

To examine polar septa at high resolution, we performed electron microscopy on sporulating cells. Electron microscopy revealed that the polar septa in FtsA^{D265G} mutant cells were thinner and more irregularly formed than those of the wild type (some examples of which are shown in Fig. 6). Neverthe-

less, as suggested by FM1-43 staining, the polar septa did appear to extend entirely across the short axis of the cell (Fig. 6). In some cases, partial septa that had initiated but not completed formation were observed next to the thin, irregular septa. Occasionally, a polar septum was observed that had branched to form an extra, small compartment. Medial septa in vegetative cells of the FtsA^{D265G} mutant appeared normal (data not shown). We conclude that FtsA^{D265G} is impaired in the proper maturation of the sporulation septum and, as a consequence, is impaired in progressing to the engulfment stage of sporulation.

Localization and activity of the sporulation phosphatase

FIG. 5. Location of residues corresponding in position to Y264 and D265 of *B. subtilis* FtsA in the crystal structure of FtsA (Y268 and N269, respectively) from *T. maritima* (47). The backbone (left) and space-filling (right) models were generated using RasMol version 2.7.2.1 for Windows.

FIG. 6. Electron microscopy of sporulating cells. Wild-type (top panel; PY79) and *FtsA*^{D265G} mutant (bottom panels; JKB124) cells collected 1.5 h after suspension in Sterlini-Mandelstam medium were visualized using transmission electron microscopy. Open arrowheads indicate incomplete or aberrant septa; filled arrowheads indicate septa that extend completely across the cell. Scale bar, 1 μ m.

SpoIIE in the *FtsA*^{D265G} mutant. Several sporulation-specific proteins are known to localize to the polar septum. One of these is SpoIIE, an integral membrane protein that is indirectly responsible for activating σ^F by dephosphorylating the phosphoprotein SpoIIAA (1, 30). To determine whether the defect in σ^F activation in *FtsA*^{D265G} could be explained by failure of SpoIIE to localize to the aberrant polar septa, we constructed a strain containing a SpoIIE-GFP fusion in the *FtsA*^{D265G} mutant background. However, fluorescence microscopy on sporulating cells showed that SpoIIE-GFP was able to localize to the polar septa in the *FtsA*^{D265G} mutant (data not shown), demonstrating that the σ^F activation defect in *FtsA*^{D265G} could not be attributed to mislocalization of the phosphatase. Next, we tested whether the SpoIIE phosphatase was active in the *FtsA*^{D265G} mutant by examining its ability to dephosphorylate its substrate SpoIIAA~P in vivo. Protein was extracted from samples of sporulating cultures and subjected to isoelectric focusing to separate phosphorylated and unphosphorylated forms of SpoIIAA. SpoIIAA and SpoIIAA~P were then visu-

alized by probing an immunoblot with anti-SpoIIAA antibodies (Fig. 7). SpoIIE activity can be measured by the accumulation of SpoIIAA throughout sporulation; no accumulation of SpoIIAA is observed in a strain containing a *spoIIE* deletion. In wild-type cells, accumulation of unphosphorylated SpoIIAA commenced about 1.5 h after the start of sporulation, in keeping with previous observations (2). No defect in the accumulation of unphosphorylated SpoIIAA was observed in cells producing *FtsA*^{D265G}, with the dephosphorylated protein appearing at about h 1.5 of sporulation and accumulating to levels as high as or higher than those observed in the wild-type.

Recent studies indicate that the appearance of unphosphorylated SpoIIAA, though necessary, is insufficient to trigger the activation of σ^F (15, 25). Indeed, certain cell division mutants allow unphosphorylated SpoIIAA to accumulate to high levels even though σ^F activation is prevented. Furthermore, certain mutants of the SpoIIE phosphatase itself allow high levels of dephosphorylation to take place while preventing σ^F -directed transcription. These findings indicate that septum formation

FIG. 7. Accumulation of unphosphorylated SpoIIAA during sporulation. Cells of a *spoIIE* null mutant (RL2220) and of the corresponding wild-type strain (PY79) and cells of an *FtsA*^{D265G} mutant (JKB124) and a corresponding wild-type strain (JKB106) were sporulated by suspension at 37°C. Samples were collected at the indicated times, and lysates were subjected to isoelectric focusing (see Materials and Methods). Antibodies directed against SpoIIAA were used to visualize SpoIIAA (AA) and SpoIIAA~P (AA-P). The leftmost lane contains 6 ng of purified SpoIIAA.

and SpoIIE play some additional role in σ^F activation beyond simply permitting the conversion of SpoIIAA~P to SpoIIAA. These studies and our present results with FtsA^{D265G} suggest that the dependence of σ^F activation on septum formation is a morphological checkpoint and that the σ^F activating machinery monitors and is sensitive to a relatively late stage in the process of septum formation.

ACKNOWLEDGMENTS

We thank O. Resnekov for strategic advice during the early stages of this project. We also thank P. Levin for anti-FtsZ antiserum and N. King, D. Garsin, and K. Carniol for anti-SpoIIAA antibody and purified SpoIIAA. We are grateful to P. Stragier for insightful discussions and to Sigal Ben-Yehuda for advice on the manuscript.

This work was supported by National Institutes of Health grants GM18568 to R.L. and GM53989 to A.D.

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