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Subcellular Localization of a Small Sporulation Protein in *Bacillus subtilis*

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SpoVM is an unusually small (26-residue-long) protein that is produced in the mother cell chamber of the sporangium during the process of sporulation in *Bacillus subtilis*. We investigated the subcellular localization of SpoVM, which is believed to be an amphipathic α-helix, by using a fusion of the sporulation protein to the green fluorescence protein (GFP). We found that SpoVM-GFP is recruited to the polar septum shortly after the sporangium undergoes asymmetric division and that the fusion protein localizes to the mother cell membrane that surrounds the forespore during the subsequent process of engulfment. We identified a patch of three residues near the N terminus of the proposed α-helix that is needed both for proper subcellular localization and for SpoVM function. We also identified a patch of residues on the opposite face of the helix and residues near both ends of the protein that are needed for SpoVM function but not for subcellular localization. Subcellular localization of SpoVM-GFP was found to require an unknown gene(s) under the control of the mother cell transcription factor σ^E. We propose that the N-terminal patch binds to an unknown anchoring protein that is produced under the control of σ^E and that other residues important in SpoVM function to recruit an unknown sporulation protein(s) to the mother cell membrane that surrounds the forespore. Our results provide evidence that SpoVM function depends on proper subcellular localization.

Subcellular localization is emerging as an important theme in understanding protein function in bacteria (11, 22). Often, elucidation of the function of a protein depends on knowledge of its location within the cell. In this study, we investigated the relationship of function to location in the context of the sporulation protein SpoVM of *Bacillus subtilis*. SpoVM is remarkable for its small size and the essential role it plays in the process of spore formation (9, 21). SpoVM, which is 26 residues in length, is believed to be an amphipathic α-helix whose charged surface mediates its interaction with the membrane (15). We wondered whether SpoVM localizes to a particular membrane surface in the sporulating cell and, if so, whether this localization is important for its function in spore formation. In this study, we addressed these questions by using a fusion of the sporulation protein to the green fluorescent protein (GFP).

Sporulation involves the formation of an asymmetrically positioned (polar) septum that divides the developing cell (the sporangium) into a small compartment called the forespore and a large compartment called the mother cell (2, 13, 14). The compartments exhibit dissimilar programs of gene transcription that are set in motion by the transcription factors σ^F in the forespore and σ^E in the mother cell. The forespore and mother cell initially lie adjacent to each other in the sporangium, but later in development, the forespore is taken up by, and wholly incorporated into, the mother cell in a phagocytic-like process called engulfment. During engulfment, the membrane on the mother cell face of the polar septum migrates around the membrane surrounding the forespore, eventually pinching the forespore off as a free protoplast within the mother cell cytoplasm. In subsequent morphogenesis, a thick layer of peptidoglycan is produced in the space between the forespore membrane and the mother cell membrane that surrounds the forespore (also known as the outer forespore membrane) and a multilayer protein shell known as the coat is deposited around the developing spore (the forespore) from within the mother cell. Eventually, the fully ripened spore is released from the sporangium by lysis of the mother cell.

SpoVM is produced in the mother cell compartment of the sporangium under the control of mother cell-specific transcription factor σ^E (9). SpoVM mutants are severely blocked in spore morphogenesis, being unable to produce a normal cortex and having only a thin, loosely attached coat (9). Here we report that SpoVM-GFP localizes to the mother cell membrane that surrounds the forespore. Evidently, this localization is important for function because alanine substitution mutants in which SpoVM-GFP is not restricted to the outer forespore membrane are severely impaired in spore formation. These mutations define a patch of three residues located near the N terminus of the proposed α-helix, which we propose is responsible for binding to an anchoring protein located in the outer forespore membrane. The identity of the anchoring protein is unknown, but experiments presented herein indicate that it is the product of a gene(s) that, like *spoVM*, is under the control of σ^E. We also report the identification of other residues that are important for SpoVM function but not for subcellular localization. We propose that these residues are responsible for recruiting an unknown sporulation protein to the outer surface of the engulfed forespore.

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MATERIALS AND METHODS

Strains and plasmids. The strains used in this study are described in Table 1. The parent strain for all experiments was *B. subtilis* strain PY79 (26). The cloning

TABLE 1. Strains used in this study

Strain	Relevant genotype	Source
PY79	Wild type	Laboratory collection
CVO1000	*spoVM-gfp*	This work
CVO1195	*amyE*::*spoVM-gfp*	This work
RL1075	Δ*spoIIGB*::*erm*	Kenney and Moran (7)
CVO1040	Δ*spoIIGB*::*erm amyE*::P_{spoVG}-*spoVM-gfp*	This work
CVO1110	Δ*spoIID*::*cat* Δ*spoIIP*::*kan*::*tet spoIIM*::Tn*917* ΩHU287::*erm*	Eichenberger et al. (3)
CVO1116	Δ*spoIID*::*cat* Δ*spoIIP*::*kan*::*tet spoIIM*::Tn*917* ΩHU287::*erm amyE*::P_{spoVG}-*spoVM-gfp kan*	This work
CVO1165	Δ*spoIID*::*cat* Δ*spoIIP*::*kan*::*tet spoIIM*::Tn*917* ΩHU287::*erm amyE*::P_{spoIIQ}-*spoVM-gfp kan*	This work
CVO1218	*amyE*::$P_{hyperspank}$-*spoVM-gfp spc*	This work
MLK947	*amyE*::P_{spoIIE}-*spoIIR*	Zhang et al. (27)
RL1275	Δ*spoIIAC*::*erm*	Laboratory collection
CVO1251	Δ*spoIIAC*::*erm amyE*::P_{spoIIE}-*spoIIR*	This work
CVO1252	Δ*spoIIAC*::*erm amyE*::P_{spoIIE}-*spoIIR spoVM-gfp spc*	This work

of all plasmids was performed in *E. coli* DH5α. Plasmids for single-recombination integration were isolated from *E. coli* TG1, which allows the isolation of concatenated plasmids, which provide a higher transformation frequency for single-recombination integration.

To fuse *spoVM* and *gfp*, we amplified *spoVM* and part of its promoter by PCR with primers VM-Prom 5′ (GAGAATTCTATCAAGCAGAAAAAGATCAA) and VM3′ (CGCTCGAGATCTTTTCTAAATGAGCCCAG) from the genomic DNA of PY79. The resulting fragment was digested with *Eco*RI and *Xho*I (sites are underlined in the primer sequences) and cloned into pKL147 (8), producing pCVO110. pCVO110 was introduced by single-recombination integration into the chromosome of PY79 to produce strain CVO1000.

To place *spoVM-gfp* at the *amyE* locus, an *Eco*RI-*Hin*dIII fragment from pCVO110 was cloned into the integration vector pDG364 (6). The resulting plasmid, pCVO180, was introduced into PY79 by double-recombinant integration to produce strain CVO1195. To place *spoVM* at the *amyE* locus, *spoVM* was amplified by PCR from genomic DNA of PY79 with primers VM-Prom 5′ and VM3-5 (GGACGGATCCTTAATCTTTTCTAAATGAGCCCAG). The resulting fragment was digested with *Eco*RI and *Bam*HI and cloned into integration vector pDG364, producing pCVO206. This was introduced into strain EL200 (Δ*spoVM*) (1) to produce CVO1422.

Transcriptional fusion of P_{spoVG} and *spoVM* was accomplished by amplifying P_{spoVG} with primers VG5′Prom (GGACGAATTCGAGATCTTGTGTTGAAG TCGCGAG) and VG3′Prom (GGTCGTCGACTAAAAGCATTAGTGTATC AATTCC), and that of P_{spoVG} and *spoVM-gfp* was accomplished by amplifying P_{spoVG} with primers VM5′RBS (GGTCGTCGACGTTATAGGAGGGGACAA AAATG) and odr78 (GCCGGATCCTTATTTGTATAGTTCATCCATGCC) by PCR and digesting the resulting fragments with *Eco*RI, *Sal*I, and *Bam*HI. The fragments were cloned by triple ligation into the integration vector pER82 (19) to produce pCVO130. This vector was introduced into PY79 by double-recombination integration to yield CVO1035. RL1061 (Δ*spoIIGB*::*erm*) (7) was transformed with genomic DNA from CVO1035 to produce CVO1040. Genomic DNA from CVO1035 was also introduced into CVO1110 (Δ*spoIID*::*cat* Δ*spoIIP*::*kan*::*tet spoIIM*::Tn*917* ΩHU287::*erm*), a derivative of PE53 (3), to produce CVO1116.

To produce CVO1218, *spoVM-gfp* was amplified with spoVM5RBS (GGGC AAGCTTTTATAGGAGGGGACAAAAATG) and GFP3-3 (GGGCTGCATG CTTATTTGTATAGTTCATCCATGCC) by PCR and cloned into pDR111 to form a transcriptional fusion with the $P_{hyperspank}$ promoter (a kind gift of D. Rudner, Harvard University). $P_{hyperspank}$ is a modified version of $P_{hyperspac}$ (17) in which a second *lac* operator was placed 70.5 bp upstream (12). $P_{hyperspank}$ retains promoter strength similar to that of $P_{hyperspac}$ under inducing conditions but has significantly lower basal expression in the absence of an inducer. The resulting plasmid (pCVO196) was introduced into PY79 to form strain CVO1218.

To make strain CVO1165, pQP24 was made by amplifying the promoter region of *spoIIQ* by PCR with QPO133 (ATTGGAATTCGGCTGGCCTTCCA TAAAATGT) and QPO134 (CAATCGGATCCAGCAACATTCTGAACACT TTTCTG). The PCR product was digested with *Eco*RI and *Bam*HI and cloned into integration vector pER82. *spoVM-gfp* was amplified by PCR with spoVMRBS3 (GCGGGATCCTAAGGAGGGGACAAAAATGAAAATTTTA CACC) and odr78 and cloned into the *Bam*HI site of pQP24 to produce pCVO169. PY79 was transformed with pCVO169 to produce CVO1160. Genomic DNA from CVO1160 was used to transform CVO1110 (Δ*spoIID*::*cat* Δ*spoIIP*::*kan*::*tet spoIIM*::Tn*917* ΩHU287::*erm*) to produce CVO1165.

To construct strain CVO1251, RL1275 (*spoIIAC*::*erm*; in a PY79 background) was transformed with genomic DNA from strain MLK947 (*amyE*::P*spoIIE-spoII*) (27). This strain was transformed with genomic DNA from CVO1000 (*spoVM-gfp*) to produce strain CVO1252.

Mutagenesis. We used the QuikChange site-directed mutagenesis kit (Stratagene) to produce the site-directed *spoVM* and *spoVM-gfp* mutations. Primers were designed to change the targeted codon to a GCT codon flanked by 15 to 18 bases of complementarity on either side. Mutagenesis was carried out in accordance with the instructions of the manufacturer. To introduce mutations in *spoVM*, we used pCVO206 as the template. For mutations in *spoVM-gfp*, we used pCVO180 as the template. The presence of the mutations was confirmed by sequencing with the ABI Prism DNA sequencing kit (PE Applied Biosystems). Plasmids encoding mutagenized *spoVM* were introduced into the *amyE* locus of EL200 (Δ*spoVM*) by double-recombinant integration, producing strains CVO1398 to -1421. Plasmids encoding mutagenized *spoVM-gfp* fusions were introduced into the *amyE* locus of PY79 by double-recombinant integration, producing strains CVO1370 to -1397.

Sporulation conditions. For microscopy, sporulation was induced by resuspension in Sterlini-Mandelstam medium at 37°C (24). Cells were initially grown overnight at 25°C in growth medium (4) and resuspended in Sterlini-Mandelstam medium when the culture reached an optical density at 600 nm (OD_{600}) of 0.5 to 0.7. At the indicated times, a small aliquot was removed and resuspended in 1/10 volume of phosphate-buffered saline containing 1.5 μg of FM4-64 (Molecular Probes) per ml. Three microliters was placed on a microscope slide and covered with a coverslide that had been treated with poly-L-lysine (Sigma).

Heat resistance assays. Sporulation efficiency was determined by a heat resistance assay performed as described by Harwood and Cutting (4). Briefly, 5 ml of Difco sporulation medium was inoculated with the strain to be tested and incubated at 37°C for 24 to 30 h. Cells were serially 10-fold diluted in T base supplemented with 1 mM $MgSO_4$, and 100 μl was plated on Difco sporulation medium (DSM) agar. Cells were challenged at 80°C for 20 min, and again 100 μl was plated on DSM agar. Sporulation efficiency was determined by the ratio of heat-resistant CFU recovered from the mutant and the wild-type strain (PY79).

Induction of SpoVM-GFP synthesis during vegetative growth. To induce the synthesis of SpoVM-GFP during vegetative growth, strain CVO1218 was grown at 37°C in growth medium to an OD_{600} of 0.4. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. Cells were incubated at 37°C for 1 h and subsequently processed for microscopy as described previously, in the paragraph on sporulation conditions.

Microscopy. Fluorescence microscopy was performed as described by Eichenberger et al. (3) with an Olympus BX60 fluorescence microscope. Typical acquisition times were 400 to 1,000 ms for GFP and 1,000 ms for FM4-64. Images were captured and cropped with METAMORPH software. Some additional adjustments were made in Adobe Photoshop.

RESULTS

SpoVM localizes to the outer forespore membrane. To investigate the subcellular localization of SpoVM, we joined the *spoVM* gene at its 3′ terminus to the coding sequence for the GFP. The resulting gene fusion was introduced into the chro-

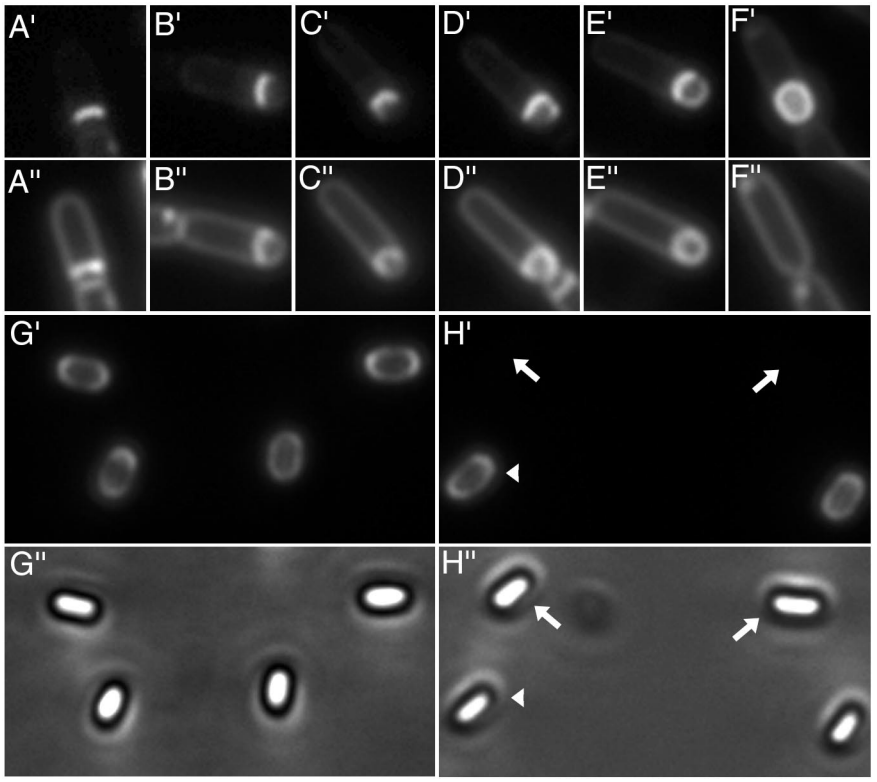

FIG. 1. Localization of SpoVM-GFP. (A′ to F″) Cells of strain CVO1000 (*spoVM-gfp*) were viewed 2.5 h after the start of sporulation. (A′ to F′) Fluorescence from SpoVM-GFP. (A″ to F″) Fluorescence from FM4-64 staining of the same cells. (G′ and G″) Fluorescence of CVO1000 spores. (G′) Fluorescence from SpoVM-GFP. (G″) The same field viewed by phase-contrast microscopy. (H′ and H″) Fluorescence from a mixture of PY79 (wild type) and CVO1000 spores. (H′) Fluorescence of the spores. (H″) The corresponding phase-contrast image. Arrows indicate nonfluorescing spores, and the arrowhead indicates a fluorescent spore.

mosome at the *spoVM* locus by single-reciprocal recombination, creating strain CVO1000, and at the *amyE* locus by double recombination, creating strain CVO1195. Both strains were merodiploid, harboring both wild-type *spoVM* and the *spoVM-gfp* fusion. As described below, the SpoVM-GFP fusion protein exhibited a striking pattern of subcellular localization. This localization did not depend on the presence of a wild-type copy of the gene, but the capacity of the strains to sporulate did, indicating that the gene fusion was nonfunctional (data not shown). SpoVM is an unusually small protein, and efforts to raise antibodies against it have been unsuccessful (15). Therefore, we could not carry out immunofluorescence microscopy as an independent assessment of the pattern of SpoVM localization. Nonetheless, genetic analyses presented below reveal a close correlation between residues important for the localization of SpoVM-GFP and for the function of SpoVM itself.

Fluorescence microscopy of SpoVM-GFP was carried out with sporulating cells that had been stained with the vital membrane stain FM4-64. Figure 1 shows the results for strain CVO1000, with panels A′ to F′ corresponding to the fusion protein and panels A″ to F″ corresponding to the membrane dye (essentially identical results were obtained with strain CVO1195). In cells at the asymmetric division stage of sporulation, fluorescence from SpoVM-GFP colocalized with the polar septum and was seen as a straight line across the short axis of the cell (Fig. 1A′ and A″). During the process of engulfment, SpoVM-GFP remained associated with the septal membrane as it migrated around the forespore (Fig. 1B′ to E′ and B″ to E″). In many cases, fluorescence from SpoVM-GFP was weaker in the region of the engulfing membrane that was closest to the cytoplasmic membrane of the mother cell than elsewhere in the membrane. Conceivably, the juxtaposition of the engulfing membrane against the cytoplasmic membrane temporarily impeded the ability of SpoVM-GFP to track with the septal membrane as it migrated around the forespore. In any event, SpoVM-GFP eventually formed a uniform shell around the outer forespore membrane once engulfment was complete and the fusion protein remained associated with the outer forespore membrane during the subsequent stages of spore maturation. An example of a postengulfment sporangium in which SpoVM-GFP has formed a uniform shell around the forespore is shown in Fig. 1F′ and F″. Note the absence of FM4-64 staining of the forespore in this sporangium, which is diagnostic of completion of engulfment. FM4-64 is membrane impermeable, and hence the forespore becomes inaccessible to the dye once engulfment is complete and the forespore is topologically isolated from the cytoplasmic membrane (23).

Finally, fluorescence from SpoVM-GFP could be detected in mature spores that had been released from the sporangia by lysis of the mother cell (Fig. 1). The fusion protein was distributed in a nonhomogeneous manner, being present as caps at both ends of the oblong spores, a pattern similar to that

observed previously for the spore coat protein SpoIVA (16) and CotE (25). Because spores are known to be autofluorescent, it was important to verify that the fluorescence observed in Fig. 1G′ was principally due to the fusion protein and not due to autofluorescence. To do this, we compared the proportion of highly fluorescent spores among spores of SpoVM-GFP-producing strain CVO1000 to that of a 1:1 mixture of spores from wild-type parent strain PY79 and spores from strain CVO1000. Approximately half of the spores from the mixture exhibited a fluorescence signal (Fig. 1H′), whereas in a sample that contained only CVO1000 spores, all of the spores were fluorescent (Fig. 1G′).

Residues in the N-terminal region of SpoVM are required for its proper localization. We then turned to the task of identifying the region(s) of SpoVM-GFP responsible for its pattern of subcellular localization. Because of the unusually small size of SpoVM, it was convenient to assess the function of almost all of its 26 residues by a site-directed mutagenesis strategy in which we replaced every codon with an alanine codon (except for the initiation codon and codon 18, which is already an alanine codon). The localization of the resulting mutant proteins was assessed by the use of fusions to *gfp*, which were integrated into the chromosome at the *amyE* locus. Of the 24 substitution mutant forms created, three (F3A, I6A, and P9A) detectably altered the pattern of SpoVM localization (Fig. 2A). The most severe was P9A, which caused SpoVM-GFP to be substantially mislocalized. The F3A and I6A substitutions significantly increased the amount of fluorescence along the periphery of the mother cell compared to wild-type SpoVM-GFP. Nonetheless, fusion proteins with the F3A and I6A substitutions were enriched at the outer membrane of the forespore, albeit to a lesser degree than the wild-type protein.

Because all three substitution mutants were unimpaired in their membrane association (cytoplasmic or engulfment), we infer that the substitutions interfered with specific localization but not with the general tendency of SpoVM to associate with membranes. Immunoblotting experiments with anti-GFP antibodies confirmed that the mutant proteins were indistinguishable in size from the wild-type protein and had not undergone degradation (data not shown). Evidently, the three substitutions had neither altered the overall conformation of SpoVM in a way that blocked its membrane association capacity nor rendered the protein subject to proteolysis. Rather, it appears that F3A, I6A, and P9A disrupted a specific region of SpoVM that is responsible for targeting the protein to the outer forespore membrane.

Prajapati and colleagues have suggested that SpoVM forms an amphipathic α-helix along its entire length and that association with the membrane is mediated by positively charged residues on one face of the helix (15). In their model, F3, I6, and P9 lie near each other on the same face of the helix, forming a patch near the N-terminal end of the helix (Fig. 2B, top; although the proline residue at position 9 could interrupt the α-helix*)*. Interestingly, this is the same surface of the helix that is thought to interact with the membrane. Extending the analysis of Prajapati and colleagues (15), we propose that F3, I6, and P9 represent a patch on the surface of the SpoVM helix that interacts with an unknown anchoring protein in the outer forespore membrane.

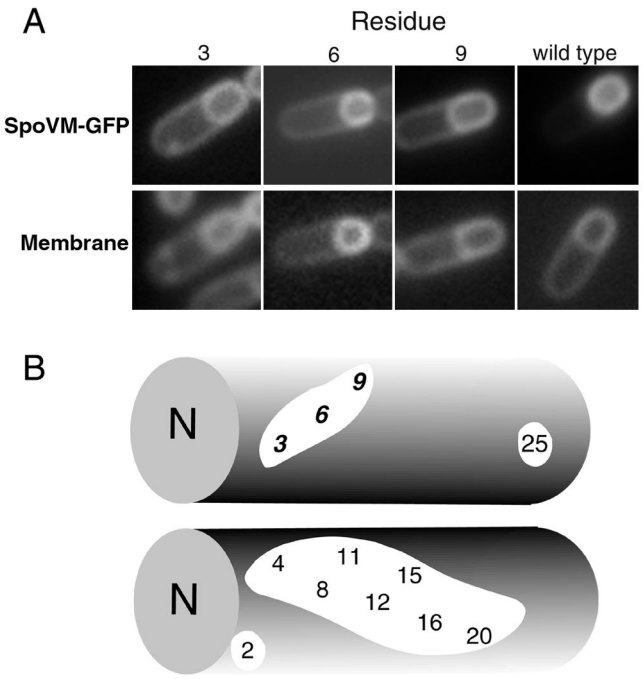

FIG. 2. Mislocalization of SpoVM-GFP mutant proteins. (A) Fluorescence from the F3A, I6A, and P9A SpoVM-GFP substitution mutant proteins and from wild-type SpoVM-GFP. Images were collected 3 h after the induction of sporulation. The top panels show the fluorescence from SpoVM-GFP, and the bottom panels show the membrane (FM4-64) staining of the same cells. Note the higher level of fluorescence from SpoVM-GFP at the cytoplasmic membrane of the mutant proteins compared to that of the wild type. (B) Model of SpoVM. SpoVM is represented as a cylinder on the basis of the model of Prajapati et al. (15). The two panels are rotated 180° along the long axis relative to each other, with the face thought to be exposed to the membrane at the top and the face thought to be exposed to the cytoplasm at the bottom. Residues involved in localization are in boldface, whereas residues involved in function but not localization are not.

Localization of SpoVM is required for its function. Next, we investigated the effect of the alanine substitution mutations on SpoVM function as assessed by the ability of cells harboring the mutant gene to sporulate. For this purpose, the mutant genes, in this case not fused to *gfp*, were introduced into the chromosome at the *amyE* locus in a strain with *spoVM* deleted. The resulting mutants were tested for the ability to sporulate as judged by the production of heat-resistant CFU. As shown in Table 2, the F3A, I6A, and P9A alanine substitution mutants were markedly impaired in sporulation. Therefore, it appears that proper localization is a requirement for SpoVM function. Conversely, the demonstration that F3A, I6A, and P9A both impair the function of SpoVM and cause mislocalization of SpoVM-GFP is consistent with the view that the subcellular localization properties of the fusion protein are physiologically significant. Underscoring their importance, I6 and P9 are identically conserved in SpoVM orthologs from other endospore-forming species, including the distantly related *Clostridium* species (Fig. 3).

Interestingly, several alanine substitutions that had no measurable effect on the localization of SpoVM-GFP nonetheless impaired sporulation (Table 2). Leaving aside substitutions

TABLE 2. Effects of alanine substitutions on sporulation efficiency and localization of SpoVM-GFP[a]

Position	Effect on sporulation	SpoVM-GFP localization
2	Severe	+
3	Severe	±
4	Severe	+
5	None	+
6	Severe	±
7	None	+
8	Severe	+
9	Severe	−
10	Mild	+
11	Intermediate	+
12	Severe	+
13	None	+
14	None	+
15	Severe	+
16	Intermediate	+
17	None	+
19	Mild	+
20	Intermediate	+
21	None	+
22	None	+
23	Mild	+
24	Mild	+
25	Intermediate	+
26	Mild	+

[a] The sporulation efficiency of *spoVM* alanine substitution mutants was measured as described in Materials and Methods. The mutant strains contained a single copy of *spoVM* that was not fused to *gfp*. The efficiency of the wild type was set at 1.0. Severe, sporulation efficiency of $<10^{-5}$ relative to the wild type; intermediate, efficiency of 10^{-2} to 10^{-5}; mild, efficiency of 0.5 to 10^{-2}; none, efficiency of >0.5. Subcellular localization was determined by fluorescence microscopy of strains producing fusions of SpoVM substitution mutants with GFP. Localization was scored on the basis of the extent to which fluorescence was restricted to the polar septum. +, pattern similar to that of the wild type; ±, localization to the cytoplasmic membrane detected; −, fusion protein more or less uniformly distributed along all membranes.

that had only a mild effect on sporulation, these localization-competent but functionally impaired mutants fell into three groups: one (L2A) that was located near the N terminus, one (K25A) that was near the C terminus, and a group of seven substitutions that defined a patch on the proposed cytosol-exposed face of the α-helix. Reinforcing the view that they are important in the function of SpoVM, many of these residues are conserved in SpoVM orthologs from other endospore-forming species (Fig. 3). We postulate that the proposed cytosol-exposed face of the helix represents a surface that is responsible for interaction with another protein(s) that plays an important role in sporulation.

Our demonstration of the importance of residues P9 and L25 is consistent with previous reports that a proline-to-leucine substitution at residue 9 and deletion of the codons for residues 24 to 26 render *B. subtilis* asporogenous (1, 15).

Localization of SpoVM is sporulation specific. SpoVM is normally produced only during sporulation. We wondered whether it would localize in a specific manner in vegetative cells. We investigated this by placing the *spoVM-gfp* fusion under control of the IPTG-inducible $P_{hyperspank}$ promoter. When synthesis of SpoVM-GFP was induced for 1 h during the mid-exponential phase of growth (OD_{600} = 0.4), we observed that SpoVM-GFP was uniformly distributed along the cell membrane (Fig. 4A′ and A″). The pattern of SpoVM-GFP staining was indistinguishable from that of FM4-64 staining, without any detectable enrichment of the fusion protein at the division septum. This is in contrast to the situation in sporulating cells in which SpoVM-GFP selectively localized to the polar septum and later to the engulfment membrane (which is derived from the polar septum). The absence of specific localization of SpoVM-GFP during vegetative growth supports the idea that a sporulation-specific gene product is required for the proper localization of SpoVM.

SpoVM-GFP is unlocalized in the absence of σ^E. Because SpoVM is produced under the control of σ^E, we wondered whether its localization is dependent upon another gene in the σ^E regulon. To investigate this possibility, we engineered cells to produce SpoVM-GFP in a σ^E-independent manner by placing *spoVM-gfp* under the control of P_{spoVG}, a sporulation promoter that is activated at the onset of sporulation (28). Thus, when SpoVM-GFP was produced under the control of P_{spoVG}, synthesis of the fusion protein commenced earlier than normal. In sporangia that had not yet undergone polar division, the fusion protein was found to be unlocalized, being distributed uniformly along the cytoplasmic membrane (data not shown). Later, however, after asymmetric division had taken place, localization of the fusion protein was found to be restricted to the polar septum. Thus, by the time the polar septum had formed, the pattern of localization of SpoVM-GFP produced under the control of P_{spoVG} was indistinguishable from that observed for SpoVM-GFP synthesized under the direction of its own promoter. A simple interpretation of the results is that SpoVM-GFP had undergone redistribution from the cytoplasmic membrane to the septal membrane once the polar septum had formed.

When, however, SpoVM-GFP was produced under the control of P_{spoVG} in a strain lacking the gene for σ^E (*spoIIGB*), the fusion protein was found to localize in a nonspecific manner even after polar division, being uniformly distributed along the cytoplasmic membrane and the septal membrane (Fig. 5, left).

	1	**2**	**3**	**4**	5	**6**	7	**8**	**9**	10	**11**	**12**	13	14	**15**	**16**	17	18	19	**20**	21	22	23	24	**25**	26
B. subtilis	M	K	F	Y	T	I	K	L	P	K	F	L	G	G	I	V	R	A	M	L	G	S	F	R	K	D
B. halodurans	M	K	F	Y	T	I	K	L	P	K	F	L	G	G	V	V	R	A	V	L	N	S	F	K	K	-
B. anthracis	M	R	F	Y	T	I	K	L	P	K	F	L	G	X	I	V	R	A	M	L	N	T	F	K	K	D
C. perfringens	M	R	I	M	T	I	K	L	P	K	F	L	A	K	I	V	R	M	F	K	G	N	K	K	S	D
C. acetobutylicum	M	K	I	V	A	I	K	L	P	K	F	L	S	N	I	I	K	F	F	F	R	-	-	K	K	S
C. botulinum	M	K	I	V	A	I	K	L	P	K	F	L	S	N	I	I	K	F	F	F	R	-	-	K	K	S

FIG. 3. Amino acid sequences of *Bacillus* and *Clostridium* SpoVM orthologs. The numbering at the top refers to positions in the *B. subtilis* SpoVM amino acid sequence, and the numbers in boldface are positions at which substitutions caused a strong defect in sporulation. The alignment was performed with ClustalW (available at http://www.ebi.ac.uk/clustalw/). The X in the *B. anthracis* sequence is a position of sequence uncertainty.

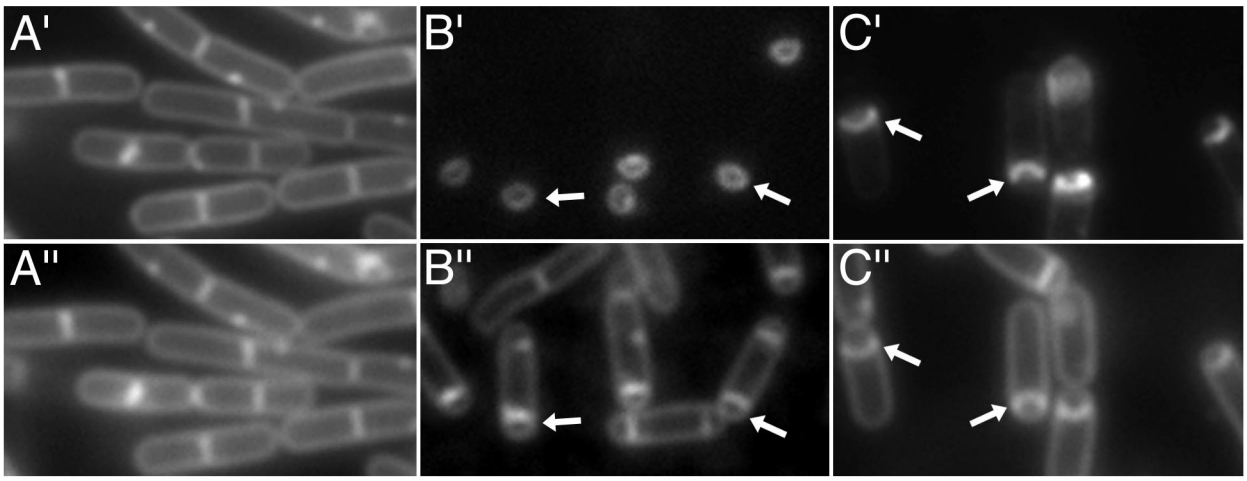

FIG. 4. Localization of ectopically produced SpoVM-GFP. (A′ and A″) Localization of SpoVM-GFP produced during vegetative growth. Expression of SpoVM-GFP was induced in strain CVO1218 (*amyE*:: $P_{hyperspank}$-*spoVM-gfp*) by addition of IPTG and incubation for 1 h. (A′) Localization of SpoVM-GFP. Compare staining of SpoVM-GFP with the FM4-64 membrane staining (A″). (B′ and B″) Localization of SpoVM-GFP when synthesized in the forespore. Strain CVO1165 (*amyE*:: P_{spoIIQ}-*spoVM-gfp*) was induced to sporulate and viewed 2 h later. (B′) Fluorescence from SpoVM-GFP. (B″) Fluorescence from FM4-64 staining. Arrows in panels B′ and B″ point to corresponding cells. (C′ and C″) Localization of SpoVM-GFP in the absence of σ^F activity. Strain CVO1252 (*amyE*:: P*spoIIE-spoIIR* Δ*spoIIAC*::*erm spoVM-gfp*) was induced to sporulate and viewed after 3 h. (C′) Fluorescence from SpoVM-GFP. (C″) FM4-64 staining of the same cells. Arrows point to the same cells in both panels.

SpoVM-GFP appears to be somewhat enriched at the septum in the σ^E mutant, but this is explained by the fact that the fusion protein was produced prior to asymmetric division. Hence, it would have been present in both sporangial compartments and thus on both the mother cell and forespore faces of the septum. The conclusion that SpoVM-GFP was unlocalized in the absence of σ^E is reinforced by the similarity of the pattern of fluorescence from the fusion protein to that of the membrane stain FM4-64 (Fig. 5).

A complication in the interpretation of the experiment of Fig. 5 is that sporangia from a σ^E mutant differ from the wild type in their inability to undergo engulfment (5). So that the effect of the absence of σ^E could be examined in a manner that avoided this complication, we introduced the P_{spoVG}-*spoVM-gfp* fusion into a *spoIID spoIIM spoIIP* triple mutant strain. Cells with mutations in these σ^E-controlled genes are blocked at the earliest stage of engulfment, producing straight polar septa that do not arch around the forespore, a phenotype similar to that of a σ^E mutant (3). The results show that SpoVM-GFP readily localized to the polar septum in sporangia of the triple mutant (Fig. 5, right). Thus, proper subcellular localization depends on an unknown gene(s) under the control of σ^E but not on the process of engulfment.

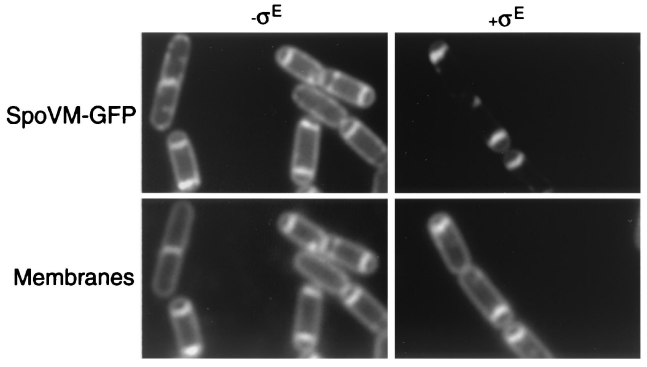

FIG. 5. Mislocalization of SpoVM-GFP in the absence of σ^E. Strains CVO1040 (*spoIIGB*::*erm* P_{spoVG}-*spoVM-gfp*), on the left, and CVO1116 (Δ*spoIID*::*cat* Δ*spoIIP*::*kan*::*tet spoIIM*::Tn*917* ΩHU287::*erm* P_{spoVG}-*spoVM-gfp*), on the right, were induced to sporulate and viewed 3 h later. The top panels show the localization of SpoVM-GFP; the bottom panels show FM4-64 staining of the same cells. Note the similarity of the SpoVM-GFP staining (top) and the FM4-64 staining in the absence of σ^E for strain CVO1040 (left panels), indicating that SpoVM-GFP was not restricted to the septum. As a control, the panels on the right show that SpoVM-GFP localization was tightly restricted to the septum in σ^E-producing cells that were blocked from progressing beyond polar septation.

SpoVM-GFP is unable to localize in the forespore. Because SpoVM-GFP localization is dependent on σ^E, we anticipated that the fusion protein would be unable to localize in the forespore because σ^E is a mother cell-specific transcription factor. To investigate this possibility, we constructed a strain (CVO1265) that transcribes *spoVM-gfp* from a promoter (P_{spoIIQ}) that is under the control of the forespore-specific transcription factor σ^F (10). To simplify the analysis, we carried out the experiment in the presence of mutations in *spoIID*, *spoIIM*, and *spoIIP* that block engulfment at an early stage (3). The results show that SpoVM-GFP uniformly decorated the membrane surrounding the forespore without any significant enrichment at the septum (Fig. 4B′ and B″).

Investigation of the dependence of SpoVM-GFP localization on σ^F. Finally, we investigated the question of whether the localization of SpoVM-GFP in the mother cell depends on σ^F-dependent gene expression in the forespore. This is a difficult question to address because SpoVM-GFP localization, as we have seen, depends on σ^E and because the appearance of σ^E in the mother cell depends on σ^F via the synthesis of a signaling protein (SpoIIR) that is produced in the forespore

under the control of σ^F (14). To circumvent this complication, we took advantage of a previously described construct (*amyE*::P_{spoIIE}-*spoIIR* in strain MLK947) that allows SpoIIR to be produced in a σ^F-independent manner (27). We introduced the *amyE*::P_{spoIIE}-*spoIIR* construct into strain RL1275 (*spoIIAC*::*erm*), which is a σ^F mutant, to create CVO1251. Finally, we introduced *spoVM-gfp* into CVO1251 to create strain CVO1252. Because of the misregulation of σ^E, cells of CVO1252 frequently failed to undergo asymmetric division but about 12% of the cells did produce normal-looking sporangia (those with a single polar septum) in which a fluorescent signal from SpoVM-GFP could be detected. A large majority (82%, $n = 180$) of these sporangia exhibited a clear pattern of subcellular localization in which SpoVM-GFP was enriched at, or substantially restricted to, the polar septum (Fig. 4C). In about 5% of the sporangia, SpoVM-GFP was uniformly distributed along all membranes, and in the remainder, the fusion protein displayed a heterogeneous staining pattern. Because localization could be studied in only a minority of the population, we regard our findings as preliminary. Nonetheless, the simplest interpretation of the results is that localization of SpoVM-GFP is not dependent on the expression of a σ^F-controlled gene in the forespore (other than the indirect dependence of σ^E activation on σ^F-directed synthesis of SpoIIR).

DISCUSSION

Despite its remarkably small size, SpoVM plays a critical role in sporulation. A principal contribution of the present work is the demonstration that the function of this small protein is strongly dependent upon its ability to localize to the mother cell membrane that surrounds the forespore. We identified a patch of amino acids near the N terminus of the protein, which is proposed to have an α-helical conformation, that is needed both for tightly restricting SpoVM-GFP to the outer forespore membrane and for the normal function of SpoVM in sporulation. We also identified residues that are required for SpoVM function but are not needed for localization. The simplest interpretation of these results is that the N-terminal patch interacts with an anchoring protein located in the outer forespore membrane and that the other residues important for SpoVM function help to recruit an unidentified sporulation protein(s) needed for morphogenesis.

A candidate for a sporulation protein recruited to the outer forespore membrane by SpoVM is the morphogenetic protein SpoIVA. SpoIVA is needed for cortex formation and for proper assembly of the coat around the forespore. SpoIVA has been shown by immunofluorescence microscopy and by use of a GFP fusion (GFP-SpoIVA) to surround the forespore in a shell-like structure that is believed to serve as a basement layer for the coat and that somehow helps to trigger cortex formation in the space between the inner and outer forespore membranes (3, 15). Localization of the GFP-SpoIVA fusion is aberrant in an *spoVM* mutant (16), but recent work indicates that, in the absence of SpoVM, the fusion protein transiently localizes in a partial crescent around the forespore before misassembling in the mother cell cytoplasm (C.V.O., unpublished results). This could indicate that SpoIVA does not interact with SpoVM directly or that SpoVM is one of two or more proteins involved in the adherence of SpoIVA to the region around the forespore. In any event, the partial localization of SpoIVA in the absence of SpoVM could provide an explanation for the finding that an *spoVM* mutant has a less severe coat defect than does an *spoIVA* mutant (9).

We do not know the identity of the anchoring protein for SpoVM, but our evidence indicates that it is the product of a gene under the control of σ^E or a protein whose localization is σ^E dependent. A further issue is whether localization of SpoVM additionally depends on a gene(s) under the control of σ^F in the forespore. This is a difficult question to address because the appearance of σ^E in the mother cell depends on σ^F via synthesis of the SpoIIR signaling protein in the forespore (14). An experiment based on the use of a strain in which the appearance of σ^E was uncoupled from its normal dependence on σ^F favors the view, at least provisionally, that the localization of SpoVM-GFP does not depend on σ^F-directed gene expression in the forespore. If this is so, then the topological information for distinguishing the outer forespore membrane from the cytoplasmic membrane is generated from within the mother cell with no significant contribution from the forespore.

SpoVM joins the growing list of proteins produced in the mother cell that colocalize with the outer forespore membrane. Among these are the integral membrane proteins SpoIVFA, SpoIVFB, and BofA, which form a heterotrimeric complex in the outer forespore membrane that governs the appearance of the late-appearing mother cell transcription factor σ^K (18, 19). Localization of SpoIVFB, which has been studied in the greatest detail, commences at the stage of polar septation. Recent work favors the view that SpoIVFB reaches its target by a diffusion-and-capture mechanism in which the protein (alone or in a complex with SpoIVFA and BofA) is initially inserted into the cytoplasmic membrane of the mother cell and then reaches the polar septum by diffusion, where it is retained (captured) (20). Localization of SpoVM, as we have seen, also commences at the stage of polar division, and it will be interesting to see if its pattern of localization is similarly achieved by a diffusion-and-capture mechanism.

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