SpoVM, a Small Protein Essential to Development in Bacillus subtilis, Interacts with the ATP-Dependent Protease FtsH

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Received 2 April 1997/Accepted 16 June 1997

The spoVM gene encodes a 26-amino-acid polypeptide that is essential for spore formation in Bacillus subtilis. A transposon insertion within the spoVM open reading frame has been shown to encode a chimeric protein which is biologically inactive and produces a phenotype identical to that of a deletion and insertion mutation. A genetic approach was used to identify possible interacting proteins, and the membrane-bound FtsH protease was identified. Mutations in ftsH suppressed the sporulation defect of certain spoVM mutants but not others. However, production of the mother cell sigma factors, σE and σK, was abnormal in the suppressed strains, and mutations in either spoVM or ftsH alone impaired sigma factor production and sporulation gene expression. Using FtsH purified from Escherichia coli, we demonstrated that in vitro (i) SpoVM inhibits FtsH protease activity and (ii) SpoVM is a substrate for the FtsH protease. We propose that during sporulation, SpoVM serves as a competitive inhibitor of FtsH activity. This interaction appears to be important for completion of the prespore engulfment step of sporulation, based on the phenotype of certain spoVM ftsH double mutants.

During development in Bacillus subtilis, an endospore is formed within the differentiating cell. This alternate cell type is initially formed as a protoplast, termed the forespore, and is separated from the mother cell by two phospholipid bilayers known as the inner and outer forespore membranes. As the forespore matures, it is encased in a thick layer of peptidoglycan (the cortex) and finally a tough proteinaceous outer coat. Driving development is a coordinated program of gene expression controlled by five sporulation-specific sigma factors. Four of these transcription factors act only in the compartmentalized cells (7). The two transcription factors to act exclusively in the mother cell chamber, σE and σK, are initially synthesized as inactive precursors, pro-σE and pro-σK. Activation is brought about by proteolytic cleavage of short N-terminal sequences; the pathways controlling activation of these sigma factors have been extensively studied, and most of the regulatory proteins have been identified (16).

One sporulation gene known to be involved in σK-controlled gene expression is spoVM (15, 28). This gene is intriguing because it encodes a small polypeptide of less than 3 kDa with an open reading frame (ORF) of just 26 codons (15). spoVM expression occurs in the mother cell chamber and is controlled by RNA polymerase associated with σK. A spoVM mutant consisting of a transposon insertion within the spoVM ORF arrests sporulation at stage IV-V and allows the formation of the forespore but impairs synthesis and assembly of the spore cortex. In addition, in spoVM::Tn917DU324 cells, there is a noticeable impairment of σK-directed gene expression. Most apparent is the severe reduction in expression of the cotA gene and a moderate reduction in gerE expression. However, expression of cotD, another member of the σK regulon, is unaffected by the spoVM mutation. Although expression of the σK regulon is not blocked in the spoVM mutant, SpoVM must be involved directly or indirectly in controlling σK-directed gene expression.

In this study, we used a combination of genetic and biochemical approaches to examine SpoVM function and have identified a potential interacting polyepitide, the FtsH protease.

MATERIALS AND METHODS

Bacterial strains. All strains used in this study were congenic derivatives of the prototrophic wild-type strain PY79 (33).

General methods. General B. subtilis methods were as described by Harwood and Cutting (10). Sporulation experiments used the resuspension method (21). Gene cloning techniques were as described by Sambrook et al. (27). For gene expression studies, 1.0-ml samples of sporulating cultures were harvested and assayed for β-galactosidase activity as described by Miller (20) with the exception that isopropyl (20 μg/ml) was used for cell permeabilization.

Isolation of suppressor mutations. Strain SC1247 (spoVM::Tn917DU324) was grown in LB medium until mid-log phase (optical density at 600 nm of 1.0). Cells were plated at a 10-fold dilution on sporulation agar (DS medium together with the antibiotics erythromycin and lincomycin to maintain selection for the Tn917-tagged spoVM mutation) contained in glass petri plates and exposed to 2 mJ of UV irradiation, using a Bio-Rad GS Gene Linker UV chamber. This procedure reduced the viable count of the culture by approximately 90%. Plates were incubated, in the dark, for approximately 18 h, after which they were inverted and exposed to chloroform vapor for 3 h at room temperature. Following a further 48 h of incubation, 27 chloroform-resistant colonies that were phenotypically Spo+ (i.e., produced phase-bright spores) were identified. Eight mutants were characterized further. Chromosomal DNA was prepared from each mutant and introduced into competent cells of strain SC1505 (metB5 spoVM::Tn917DU324) by DNA-mediated transformation. From each cross, 100 Met+ transformants were picked and checked for coinheritance, by conformation, of the spo+ suppressor allele. The congenic strains isolated were SC1509 (spoVM::Tn917DU324 vmb17), SC1511 (spoVM::Tn917DU324 vmb3), and SC1515 (spoVM::Tn917DU324 vmb27).

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Genetic mapping of suppressor alleles. To determine the chromosomal locations of the eight suppressor alleles, we used the B. subtilis mapping-kit strains to provide an approximate chromosomal position (4). The kit strains each contain different auxotrophic markers which together encompass the entire genome. We first transformed plasmid pEL13 into five different strains containing the kit strains (in order of increasing size: pEL12, pEL13, pEL13, pEL13, and pEL13) and the nine kit strains were used to transform each of the nine mapping-kit strains selected for each appropriate Aux’ phenotype as described above.

(i) Extragenic mutations. Lysates prepared from five mutants (SC1509, SC1512, SC1513, SC1514, and SC1516) exhibited linkage of 26% to araA26 and 90% to couA42, placing the mutations at about 4’ on the chromosome. To further map these mutations, we used a combination of two- and three-factor crosses, and interstitial crossing to position the mutations relative to other markers in this region of the chromosome. The results of these genetic crosses suggested that the mutations could be allelic to the fisH locus. To confirm this, we used the published sequence (23) of fisH to amplify by PCR a 2.7 kb DNA fragment containing the entire fisH gene from each of the five suppressor mutants. These DNAs were then introduced by DNA-mediated transformation into cells of strain SC1247 (spoVM::Tn917HU324). Transformants were plated on sporulation agar plates in glass petri dishes and following 18 h of incubation exposed to chloramphenicol to kill to 917HU324 cells. With each mutant, Spo’ chlororesistant survivors arose following exposure to solvent, demonstrating that the suppressor allele had been introduced into the SC1247 chromosome by marker replacement and was allelic to fisH. Using this procedure, we could precisely position the fisH locus. Oligonucleotide primers was used to amplify (from the mutant genomes) overlapping segments of the fisH gene by PCR. The approximate locations of the suppressor alleles were then determined by introduction of the PCR product into SC1247 cells followed by selection for complementation to WT. The results presented in Table 1 are consistent with the fisH allele being allelic to the fisH allele in the wild-type strain.

(ii) Intragenic mutations. The remaining three mutations (strains SC1510, SC1511, and SC1515) showed high transformation to pyrD1, which corresponded to the linkage of spoVM to pyrD1 (28). To determine whether these mutations were intragenic, we transformed each mutant with chromosomal DNA prepared from SC1281 (spoVM::Tn917HU17). This strain contains Tn917HU with the lacZ gene and a chloramphenicol marker, cat, inserted at the same position as Tn917 in SC1247 (15). Therefore, SC1281 exhibits a SpoVM phenotype. Following selection for chloramphenicol resistance, the transformants were subjected to PCR and hybridization with a SpoVM phenotype, which could be explained only if the mutants contained an intragenic suppressor allele which had been removed following selection for Cm’. Approximately 60% of colonies were spo’ due to removal of the suppressor allele. Second, DNA was isolated from one Spo’ transformant (strain SC1993) and sequenced to determine the spoVM::Tn917HU324 from this cross and introduced into the WT, spo’ strain 9579, with selection for Cm’. We confirmed that the spo’ mutants were WT colonies and mutant colonies that were more lytic and contained a very low percentage of phase-bright spores (60%). The lytic colonies were presumed to contain the spoVM suppressor allele. This was confirmed by using DNA prepared from these colonies to backcross into a spoVM::Tn917HU124 mutant which produced a spoVM phenotype in 60% of the transformants, demonstrating suppression.

(iii) Intragenic suppressors. The procedure outlined above to sequence the spoVM::Tn917 junction was used to sequence the intragenic suppressor alleles. A PCR reaction was prepared from a mutant chromosomal DNA adjacent to the site of Tn917 insertion, we used two oligonucleotide primers, Pts (5’-CTCAACGGTTGACTACTAAATACCTC [restriction/encoding sites are underlined] and OL54 (5’-CCGGATCCGAACTCATTCATCATTGCAAGTTAGTCATCGAAG), to amplify, by PCR (spoVM::Tn917HU324) to a point approximately 120 bp downstream of the 5’ end of the spoVM gene to a point approximately 120 bp downstream of the 5’ end of Tn917HU324. The PCR product was cloned (HindIII-BamHI) into plasmid pSGMU2 (8), and the insert was sequenced on both strands by using primers PTS and OL54.

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(v) In vitro proteolytic reactions with FtsH. Peptide synthesis. Peptides were synthesized by using standard solid-phase chemical synthesis techniques on an Applied Biosystems gas-phase synthesizer using Fmoc chemistry. The synthesized peptides were purified by reverse-phase HPLC using a C18 column and lyophilized for use in in vitro assays.

In vitro proteolytic reactions with FtsH. Reaction mixtures were carried out essentially as described previously (30). A complete reaction mixture for inhibition by SpoVM consisted of 50 mM Tris-acetate (pH 8.0), 5 mM magnesium acetate, 12.5 μM zinc acetate, 80 mM NaCl, 1.4 mM β-mercaptoethanol, 5 mM ATP, 0.2 mg of bovine serum albumin per ml, 185 μg of purified E. coli eL12.5-His per ml, and 44 μg of purified E. coli FtsH per ml. WT and PL9 synthetic polypeptides were added to the reaction mixtures at a concentration of 50 μM (peptide/ε222-Chi/FtsH molar ratio of 27:9:1). Reactions were performed at 42°C for 40 min. After SDS-PAGE and silver staining, the blots were scanned, and the bands were quantified using a densitometer (GLOC). The integrated densities were normalized to equal amounts of FtsH (using the standard curve for FtsH in Fig. 2).
SpoVM protein and mutant forms. Both the SpoVM WT polypeptide and the 37-aa chimeric encoded by spoVM::Tn917HU324 are shown. The 13 residues in boldface are those encoded by the transposon. Mutational changes are shown by arrows. spoVMPL9 was created by site-specific mutagenesis. The three intragenic suppressors restored WT activity to the SpoVM-Tn917 protein, as described in Results.

performed at 42°C for the indicated time, and samples were analyzed by Tricine-PAGE (TEFCO Technical Frontier Co., Tokyo, Japan) on a 16% polyacrylamide gel followed by silver staining.

**Immunological detection of σ^3^ and σ^2^ in whole-cell lysates.** Samples (1 ml) of sporulating cells were collected, and cell pellets were frozen at −70°C. Cell lysates were prepared by resuspending the cell pellet in lysis buffer (10 mM Tris-Cl [pH 8.4], 1 mM EDTA, 10 mM MgCl_2, 0.1 mM phenylmethylsulfonyl fluoride) containing lysisome (0.5 μg/ml), with incubation at 37°C for 10 min. SDS was added to a final concentration of 1%, and samples were boiled for 2 min. Protein concentrations were determined, and equal amounts of total protein (5 μg) were fractionated by SDS-PAGE (12.5% polyacrylamide gel).

**Size-fractionated proteins were transferred to a polyvinylidene difluoride membrane (Millipore), and immunoblotting was performed with the Amersham ECL Western blotting reagents.** For detection, anti-σ^3^ monoclonal antibody (obtained from W. Haldenwang [32]) was used at a dilution of 1:500, and a polyclonal antisera against pro-σ^3^ (17) was used at a 1:5,000 dilution.

**Electron microscopy.** Suspensions of bacteria (from cultures at approximately the 20th hour after the onset of sporulation) were fixed in 3% glutaraldehyde plus 4% paraformaldehyde in 0.1 M piperazine-N,N′-bis(2-ethanesulfonic acid) (Pipes) buffer (pH 7.2) according to method 1 of Page et al. (25). Silver sections of Spurr resin-embedded material were stained with uranyl acetate followed by Reynolds lead stain and viewed on a Zeiss EM 109 transmission electron microscope.

**RESULTS**

**spoVM::Tn917 encodes a chimeric protein.** The SpoVM phenotype has been defined by one insertion mutation (spoVM::Tn917H324) consisting of a Tn917 insertion following the 24th codon of the spoVM ORF (15). This mutation produces a strong block in spore formation leading to an asporogenous phenotype. We further characterized this insertion mutation by sequencing the junction of the Tn917 insertion within the spoVM ORF. We found that insertion of Tn917 had actually created a chimeric gene where 24 codons of the spoVM ORF had fortuitously fused, in frame, to 13 codons provided by the 5′ end of Tn917 (the translational stop codon was provided by Tn917). Thus, following translation of spoVM-Tn917 mRNA, a 37-amino-acid (aa) chimeric protein could be synthesized (Fig. 1). To establish a bona fide null SpoVM phenotype, we created a deletion-and-insertion mutation, ΔspoVM::spc, where the entire spoVM ORF had been replaced with an Sp^+^ cassette. Sporulating cells containing this allele (EL200) exhibited a morphological phenotype indistinguishable from that of spoVM::Tn917HU324 and ΔspoVM::spc strains (Table 1). Interestingly, although completely sensitive to treatments with heat and chloroform, SpoVM::Tn917HU324 and ΔspoVM::spc strains (Table 1). Interestingly, although completely sensitive to treatments with heat and chloroform, spoospores of all three mutants exhibited partial resistance to lysozyme.

**Identification of an interacting polypeptide.** We used a mutagenic selection to identify mutations that suppressed spoVM::Tn917HU324 and allowed production of chloroform-resistant spoospores. Since the transposon mutant encoded a chimeric protein containing over 90% of the full-length protein, we reasoned that our selection procedure had the potential to identify interacting gene products. Of 27 suppressor mutations isolated, 8 were characterized further. Surprisingly, three suppressors were intragenic. Two modified Cys33 (encoded by Tn917 sequences) to either phenylalanine (vmb27) or tryptophan (vmb17), while vmbs4 had changed to result in the substitution of leucine. spoVMPL9 cells (strain EL315) exhibited a null phenotype indistinguishable from that of spoVM::Tn917HU324 and ΔspoVM::spc strains (Table 1). Interestingly, although completely sensitive to treatments with heat and chloroform, spoospores of all three mutants exhibited partial resistance to lysozyme.

**TABLE 1. Sporulation phenotypes of spoVM and intragenic suppressor alleles**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Allele</th>
<th>Sporulation (% of CFU/ml of untreated culture)</th>
<th>Sporulation phenotype</th>
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<tr>
<td></td>
<td></td>
<td>Chloro-</td>
<td>Heat</td>
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<td></td>
<td></td>
<td>form</td>
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<tr>
<td>PY79</td>
<td>spo^+^</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>SC1247</td>
<td>spoVM::Tn917HU324</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>EL200</td>
<td>ΔspoVM::spc</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>EL315</td>
<td>spoVM::L9</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
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<td>spoVM::Tn917vmb4</td>
<td>23</td>
<td>32</td>
</tr>
<tr>
<td>SC2027</td>
<td>ΔspoVM::spc vmb4</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SC2186</td>
<td>spoVMPL9 vmb4</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SC2037</td>
<td>vmb4</td>
<td>71</td>
<td>60</td>
</tr>
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</table>

^a Sporulation was induced by the resuspension method. Nine hours following the initiation of spore formation, samples were removed and treated with chloroform, heat, or lysozyme as described previously (21). Following treatment, survival was determined. Averages of at least three experiments are given.

^b WT, phase-bright heat-resistant spoospores; IV-V, stage IV-V block in morphogenesis; II-III, stage II-III block in morphogenesis.
that the extragenic suppressors are allele specific. Unexpectedly, we also found that \( \Delta \text{spoVM}:\text{spc} \) or \( \text{spoVMPL9} \) strains carrying a \( \text{vmb} \) suppressor allele were arrested at a point prior to stage IV-V of sporulation. We were unable to discern any phase-grey bodies indicative of a block at stage IV-V in sporulating cells. Microscopic examination showed that many cells proceeded to the point of septum formation and produced abnormal pygmy cells indicative of abortive sporulation (Fig. 2E and F show \( \Delta \text{spoVM}:\text{spc} \)). This feature is attributable to late stage II of development and suggests that spore septation and engulfment are defective (7, 26).

We constructed strains containing only the \( \text{ftsH} \) suppressor alleles. These strains were able to form phase-bright, heat-resistant spores at a fairly high efficiency, although less so than WT cells (Table 1 and Fig. 2B). Examination of sporulating cultures showed that spore formation was markedly (ca. 2 h) slower than for the WT strain. We also found that growth of \( \Delta \text{spoVM}:\text{spc} \), \( \text{spoVMPL9} \), or \( \text{spoVM}^+ \) strains carrying the \( \text{ftsH} \)
suppressor alleles was impaired, although growth of spoVM::Tn917VHU324 cells was not. In liquid culture, this resulted in lower maximum cell densities, while on solid media, colonies were extremely lytic. This characteristic has been observed in cells containing a partially inactivated ftsH gene (5, 6, 19).

Sporulation-specific gene expression and sigma factor production. In previous work, it was shown that the spoVM::Tn917VHU324 mutant impaired σK-directed gene expression (15). We examined expression of three σK-dependent genes (cotA, gerE, and cotD) in strains containing mutations in spoVM and/or the ftsH extragenic suppressor alleles (Fig. 4 shows data for the vmb4 allele). As determined by the level of β-galactosidase produced from lacZ fusions, strains containing spoVM::Tn917VHU324 or ΔspoVM::spc were impaired in σK-directed gene expression, with cotA expression being severely reduced, gerE expression being partially reduced, and cotD expression being essentially unaffected. This finding is consistent with data reported previously for the spoVM::Tn917VHU324 mutant (13) and extends our observations reported above that the spoVM::Tn917VHU324 and ΔspoVM::spc mutations produce indistinguishable sporulation defects. In spoVM::Tn917VHU324 vmb4 cells, expression of cotA, gerE, and cotD was delayed by about 1 to 2 h, and relative to spo+ cells, expression of cotA and cotD was severely reduced and expression of gerE was moderately reduced. Thus, although the sporulation defect of the spoVM::Tn917VHU324 mutant (heat sensitivity) was substantially suppressed by the vmb4 allele (Table 1), σK-dependent gene expression remained defective in the double mutant. In ΔspoVM::spc vmb4 cells, σK-directed gene expression was completely abolished, consistent with the earlier morphological block (stage II-III) observed for this mutant and its failure to form an intact forespore chamber, a prerequisite for σK-directed gene expression (3, 9). Lastly, in cells containing only...
FIG. 5. $\sigma^E$ and $\sigma^K$ production during sporulation. (A and B) Cells containing the indicated alleles were collected at 30-min (A) or 60-min (B) intervals after the onset of sporulation, as indicated by the numbers at the top. Whole-cell extracts were prepared and analyzed by using either monoclonal anti-$\sigma^E$ antibody (A) or polyclonal anti-pro-$\sigma^K$ antiserum (B) as described in Materials and Methods. Western blots are numbered at the left. In some cases, more than one exposure of the same blot is shown to highlight quantitative differences. In each Western blot, the upper band is the pro-$\sigma$ and the lower band is the mature $\sigma$. (A) 1, spo$^+$ (30-min exposure); 2, spoVM::spc :: D (30 min); 3, spoVM::spc :: D (30 min); 4, spoVM::Tn917::HU324 vmb4 (30 min); 5, $\Delta$spoVM::spc vmb4 (30 min); 6, vmb4 (30 min). (B) 1, spo$^+$ (30-s exposure); 2, spoVM::Tn917::HU324 (30 s); 3, spoVM::spc :: D (30 s); 4, spoVM::spc :: D (60 s); 5, spoVM::Tn917::HU324 vmb4 (10 min); 6, spoVM::Tn917::HU324 vmb4 (45 min); 7, $\Delta$spoVM::spc vmb4 (10 min); 8, vmb4 (10 min). (C and D) $\sigma^E$ or $\sigma^K$ from each blot shown in panel A or B was quantified by using a Visage Digital Imager and plotted as a percentage of the maximum level reached during sporulation. Symbols: ●, spo$^+$; ○, spoVM::Tn917::HU324; ■, $\Delta$spoVM::spc; ▲, spoVM::spc vmb4; △, vmb4. In panel C, the maximum intensities relative to the maximum intensity of the spo$^+$ strain were as follows: spoVM::Tn917::HU324, 1.5; $\Delta$spoVM::spc, 1.0; spoVM::Tn917::HU324 vmb4, 0.48; $\Delta$spoVM::spc vmb4, 0.35; and vmb4, 0.14. In panel D, the maximum intensities relative to that of the spo$^+$ strain were as follows: spoVM::Tn917::HU324, 0.78; $\Delta$spoVM::spc, 0.25; spoVM::Tn917::HU324 vmb4, 0.0011; $\Delta$spoVM::spc vmb4, 0.0; and vmb4, 0.0097.

the vmb4 mutation, $\sigma^K$-directed gene expression was delayed (by about 1 to 2 h) and substantially reduced. This pattern is most similar to that observed for the spoVM::Tn917::HU324 vmb4 double mutant, and like the double mutant, the vmb4 single mutant forms heat-resistant spores, though not as efficiently as WT cells (Table 1).

We also examined expression of some other developmental genes, spoIIG ($\sigma^+$ dependent) (Fig. 4A), spoIVF ($\sigma^E$ dependent) (Fig. 4B), and spoIVB ($\sigma^G$ dependent) (Fig. 4C). Expression of these genes was unaffected by the spoVM::Tn917::HU324 and $\Delta$spoVM::spc mutations; however, expression was similarly delayed and reduced in all strains carrying the vmb4 allele. Hence, the vmb4 allele impairs early sporulation-specific gene expression, as well as later $\sigma^K$-dependent gene expression. In results not shown, we have found that the other fnbH suppressors behaved similarly.

Since sporulation-specific gene expression was impaired in cells containing the vmb4 alleles, we examined production of the mother cell sigma factors, $\sigma^E$ and $\sigma^K$, in these cells. Production of both of these sigma factors involves proteolytic processing of an inactive precursor called a pro-$.\sigma$. We used antibodies against pro-$\sigma^E$ and pro-$\sigma^K$ to examine production of mature $\sigma^E$ and $\sigma^K$ by Western blot analysis of extracts prepared from sporulating cells.

Strains containing spoVM::Tn917::HU324 or $\Delta$spoVM::spc produced $\sigma^E$ normally (Fig. 5A and C), as expected since spoIVF-lacZ expression was unimpaired (Fig. 4B). The timing of $\sigma^K$ appearance was normal, but the maximum level was reduced (Fig. 5B and D). The moderate reduction in $\sigma^K$ production most likely accounts for the differential effects on expression of $\sigma^K$-dependent genes (cotA, gerE, and cotD in Fig. 4D, E, and F), as has been reported previously for other strains with diminished $\sigma^K$ production (18, 24).

In contrast, all strains carrying the vmb4 allele exhibited a delay in the timing of $\sigma^K$ appearance and a reduction in the amount of $\sigma^K$ produced (Fig. 5A and C). The delay in the appearance of $\sigma^K$ correlates with the delayed expression of the $\sigma^K$-dependent spoIVF operon in these mutants (Fig. 4B). In addition, the maximum level of spoIVF expression was also reduced in all strains bearing the vmb4 mutation (Fig. 4B), as
we asked what effect SpoVM would have on restoration of σK production (Fig. 4D to F) or form heat-resistant spores (Table 1). Only 1% of the level in WT cells [Fig. 5B]) and delayed (Fig. 5D), as chimera appears to be biologically inactive, since both WT and SpoVM-Tn917 mutant cells by the vmb4 allele does not result from restoration of σK production. Finally, σK was not even detected in ΔspoVM::spc vmb4 mutant cells (Fig. 5B), consistent with the inability of this mutant to express σK-dependent genes (Fig. 4D to F) or form heat-resistant spores (Table 1).

SpoVM inhibits FtsH-mediated degradation of E. coli σ32.

The E. coli FtsH protein has been well characterized (11, 30). In these studies, FtsH has been shown to degrade the heat shock transcription factor σ32, and this protease activity is ATP and zinc dependent. FtsH, by degrading σ32, is thought to serve as a mechanism for regulating gene expression under stress conditions.

Our genetic experiments described above suggest that SpoVM may interact with FtsH. Using the purified E. coli FtsH protein, we asked what effect SpoVM would have on σ32 degradation in vitro. We found that WT synthetic SpoVM protein strongly inhibited degradation of σ32 by FtsH, whereas the PL9 mutant form of SpoVM had no effect under the conditions used (Fig. 6A).

SpoVM acts as a substrate for E. coli FtsH.

To understand the interaction between SpoVM and FtsH further, we examined the possibility that SpoVM may act as a competitive substrate for the FtsH protease. As shown in Fig. 6B, SpoVM was actually degraded by FtsH in the presence of ATP. We tried to detect the degradation products by using reverse-phase high-pressure liquid chromatography and found several peptides (data not shown). However, their amounts were very small, and we were unable to recover them for further analysis. These results, though, indicate that SpoVM is indeed a substrate for the FtsH protease, and so it is possible that SpoVM competitively inhibits FtsH-mediated proteolysis of other substrates.

DISCUSSION

We have discovered that the spoVM::Tn917HU324 mutant encodes a chimeric protein 37 aa in length. By constructing a deletion-and-insertion mutation, ΔspoVM::spc, we have also determined that in otherwise WT cells, the SpoVM-Tn917 chimera appears to be biologically inactive, since both spoVM mutants arrest sporulation at stage IV-V and exhibit similar defects in sigma factor production and expression of σK-dependent genes. Together with its developmental function, the extremely small size of SpoVM makes this polypeptide particularly intriguing.

An important finding of this work is the identification of extragenic suppressor alleles of spoVM::Tn917HU324 that are allelic to ftsH. ftsH encodes a member of the AAA protein family of ATPases (2, 13). The AAA proteins are involved in a multitude of varied activities, including protein secretion, protein assembly and proteolysis, peroxisome biogenesis, and cell cycle control. All members of this protein family have in common a domain of about 200 aa containing an ATP-binding site, termed the AAA module. FtsH contains two hydrophobic segments at the N terminus which anchor it to the phospholipid membrane, exposing the C-terminal AAA module to the cytoplasm (1, 22, 31). Near the C terminus is a domain which is similar to the active site motif of zinc metalloproteases. Recently, the E. coli FtsH protein has been shown to be directly involved in the proteolytic degradation of the heat shock transcription factor σ32 (11, 30). E. coli FtsH has also been shown to be involved in the degradation of uncomplexed SecY, an essential component of the protein translocation machinery of E. coli, which may partially explain its pleiotropic effects on protein assembly and secretion (12). In other work, it has been shown that B. subtilis FtsH is required for sporulation (5, 19). In an ftsH mutant encoding a C-terminally truncated FtsH protein, spore formation does not initiate under conditions of nutrient starvation. As in E. coli and Lactococcus lactis, we would expect B. subtilis FtsH to be associated with the cytoplasmic membrane in vegetative cells. Since the forespore protoplast is constructed by a process of membrane invagination, it is quite possible that FtsH is also be assembled into the forespore membranes.

The allele-specific nature of the suppressor mutations in ftsH supports the idea that FtsH and the SpoVM-Tn917 chimeric protein interact in vivo. All three spoVM mutations studied here (spoVM::Tn917HU324, ΔspoVM::spc, and spoVMPL9) produce similar stage IV-V blocks and severely reduce the formation of resistant spores (Table 1), yet the suppressor mutations in ftsH restore sporulation only to the spoVM::Tn917HU324 mutant. One possible explanation for the suppression is that FtsH, acting as a protease, fortuitously cleaves the C-terminal extension of SpoVM-Tn917.Alternatively, the SpoVM-Tn917 chimera may possess feasible biological activity, and the delay in early sporulation events caused by the suppressor mutations in ftsH may permit sufficient activity of the chimera to allow sporulation. Although we cannot rule out these possibilities, our data provide additional reasons to believe that FtsH and SpoVM interact functionally.

Most compelling is the stage II-III block found in ΔspoVM::spc vmb4 and spoVMPL9 vmb4 cells. This phenotype differs from that of vmb4 (Spo32) or ΔspoVM::spc/spoVMPL9 (stage IV-V block) cells. If FtsH function was normally unrelated to SpoVM, then we would expect a stage IV-V block in these cells. Our interpretation is that FtsH and SpoVM are involved in some aspect of prespore engulfment and synthesis of the forespore. In the absence of SpoVM or in the presence of a defective SpoVM peptide, the modified FtsH protein cannot carry out its stage I-II function. As expected for a mutant blocked at stage II-IIII, expression of σK-dependent genes (Fig. 4D to F) was undetectable in the spoVM::spc vmb4 mutant. Neither the ΔspoVM::spc mutation nor the vmb4 mutation alone completely blocked σK production, which is consistent with the ability of these mutants to advance to a later stage of sporulation. The vmb4 allele, as well as the other suppressor
mutations in ftsH, delayed and reduced expression of all of the developmental genes that we tested, including spoIIG, which is expressed very early during sporulation (Fig. 4A). It seems likely that FtsH may provide critical functions at several different times during the sporulation process, just as it is thought to play several different roles during vegetative cell growth, based on the pleiotropic effects of mutations in bacterial ftsH genes (2).

Our in vitro experiments with the purified E. coli FtsH protein provide additional evidence that FtsH and SpoVM might interact in vivo. We have shown that WT SpoVM can inhibit the E. coli FtsH-mediated degradation of the heat shock transcription factor α K. This inhibition is highly specific since it is not observed with the SpoVM PL9 peptide. In addition, we have shown that SpoVM is actually degraded by the FtsH protease and thus can act as a substrate. In E. coli, the small α CIII protein acts as a competitive inhibitor of FtsH-mediated degradation of α K, and it is thought that α CIII may also act as a proteolytic substrate for FtsH (11, 30). SpoVM, by analogy to α CIII, could serve a similar role during development by antagonizing FtsH function.

It is attractive to speculate that FtsH may be involved in the degradation and/or processing of sigma factors during sporulation. The appearance of α K and α E was delayed in the vmb4 mutant, and production of both sigma factors was markedly impaired (Fig. 5). Even so, vmb4 cells formed resistant spores at a high frequency (Table 1). The ability of cells to sporulate despite a severely reduced α K level has been observed previously (18).

If SpoVM antagonizes a function of FtsH that affects sigma factor production, then mutations in spoVM might also affect sigma factor production. The spoVM mutations did not affect α K production, but accumulation of α E was reduced by 22 to 75% (Fig. 5). Expression of two α K-dependent genes was reduced (Fig. 4). In particular, expression of the cotA gene seems to be affected by a modest reduction in the level of α E, consistent with previous observations (18, 24).

It seems unlikely, however, that reduced α E production accounts for the stage IV-V sporulation block of the spoVM mutants. These mutants produce much more α E than vmb4 mutants (Fig. 5) yet fail to sporulate (Table 1). On the other hand, the vmb4 mutation restores sporulation when present in spoVM::Tn917HU324 mutant cells, but this suppression clearly does not result from restoration of normal α E production (Fig. 5). It remains to be determined why spoVM mutants are blocked at stage IV-V of sporulation and how the vmb4 mutation in ftsH allows the spoVM::Tn917HU324 mutant to overcome this block.

In conclusion, we have shown that the small polypeptide encoded by spoVM is essential for B. subtilis development, and we have genetically identified FtsH as a protein with which SpoVM is likely to interact. Our in vitro results suggest that this interaction may involve SpoVM acting as an antagonist and/or substrate of FtsH protease. The interaction first becomes crucial during the presporangium step of sporulation, based on the stage II-III block observed for spoVMΔ:spc vmb4 and spoVMPL9 vmb4 mutant cells, and also plays a role later, since the vmb4 mutation in ftsH allows spoVM::Tn917HU324 mutant cells to progress beyond stage IV-V of sporulation.

ACKNOWLEDGMENTS

This work was supported by the Medical Research Council (S.C.), grants from the Ministry of Education, Science, Sports and Culture of Japan to T.O., and NIH grant GM43585 to L.K.

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