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

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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, σ^E and σ^K, 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, σ^E and σ^K, are initially synthesized as inactive precursors, pro-σ^E and pro-σ^K. 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 σ^K-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 σ^E. 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*::Tn*917*Ω*HU324* cells, there is a noticeable impairment of σ^K-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 σ^K regulon, is unaffected by the *spoVM* mutation. Although expression of the σ^K regulon is not blocked in the *spoVM* mutant, SpoVM must be involved directly or indirectly in controlling σ^K-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 polypeptide, 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 cells were harvested and assayed for β-galactosidase activity as described by Miller (20) with the exception that lysozyme (20 μg/ml) was used for cell permeabilization.

Isolation of suppressor mutations. Strain SC1247 (*spoVM*::Tn*917*Ω*HU324*) 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 Tn*917*-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*::Tn*917*Ω*HU324*) by DNA-mediated transformation. From each cross, 100 Met^+ transformants were picked and checked for coinheritance, by congression, of the spo^+ suppressor allele. The congenic strains isolated were SC1509 (*spoVM*::Tn*917*Ω*HU324 vmb4*), SC1512 (*spoVM*::Tn*917*Ω*HU324 vmb3*), SC1513 (*spoVM*::Tn*917*Ω*HU324 vmb1*), SC1514 (*spoVM*::Tn*917*Ω*HU324 vmb9*), SC1516 (*spoVM*::Tn*917*Ω*HU324 vmb6*), SC1510 (*spoVM*::Tn*917*Ω*HU324 vmb17*), SC1511 (*spoVM*::Tn*917*Ω*HU324 vmb5*), and SC1515 (*spoVM*::Tn*917*Ω*HU324 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 introduced *spoVM*::Tn*917*Ω*HU324* into each of the nine kit strains. PBS1-generalized transducing lysates prepared from each mutant strain were used to infect the nine mapping-kit strains followed by selection for an appropriate Aux^+ phenotype.

(i) Extragenic mutations. Lysates prepared from five mutants (SC1509, SC1512, SC1513, SC1514, and SC1516) exhibited linkage of 26% to *purA26* and ca. 90% to *cysA14*, placing the mutations at about 4° on the chromosome. To further map these mutations, we used a combination of two- and three-factor transduction and transformational crosses 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 *ftsH* locus. To confirm this, we made use of the published sequence (23) of *ftsH* to amplify by PCR a 2.2-kb DNA fragment containing the entire *ftsH* gene from each of the five suppressor mutants. These DNAs were then introduced by DNA-mediated transformation into cells of strain SC1247 (*spoVM*::Tn*917*Ω*HU324*). Transformants were plated on sporulation agar plates in glass petri dishes and following 18 h of incubation exposed to chloroform to kill *spoVM*::Tn*917*Ω*HU324* cells. With each mutant, Spo^+ chloroform-resistant 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 *ftsH*. Using this procedure, we could more precisely position the suppressor alleles. A series of oligonucleotide primers was used to amplify (from the mutant genomes) overlapping segments of the *ftsH* gene by PCR. The approximate locations of the suppressor alleles were then determined by introduction of the PCR product into SC1247 cells followed by Spo^+ selection as described above.

(ii) Intragenic mutations. The remaining three mutations (strains SC1510, SC1511, and SC1515) showed high transductional linkage 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*::Tn*917lac*). This strain contains Tn*917lac* with the *lacZ* gene and a chloramphenicol marker, *cat*, inserted at the same position as Tn*917* in SC1247 (15). Therefore, SC1281 exhibits a SpoVM phenotype. Following selection for chloramphenicol resistance (Cm^r), all transformants exhibited a SpoVM phenotype, which could be explained only if the mutants contained an intragenic suppressor allele which had been removed following introduction of *spoVM*::Tn*917lac* by marker replacement. In addition, chromosomal DNA prepared from these three mutants when introduced into wild-type (WT) competent cells with selection for the *spoVM*::Tn*917* insertion always produced Spo^+ transformants, indicating that the mutant alleles were inseparable from the transposon insertion.

Sequence determinations. (i) *spoVM*::Tn*917*Ω*HU324*. To sequence *spoVM* DNA adjacent to the site of the Tn*917* insertion, we used two oligonucleotide primers, PTn (5′-CTCAAGCTTGTACCACTAATAACTCAC [restriction endonuclease sites are underlined]) and OL54 (5′-CGGGATCCGAACTCATTC ATTCACGAGGTAC), to amplify, by PCR, from strain SC1247 (*spoVM*::Tn*917*Ω*HU324*) a 330-bp fragment extending from the 5′ end of the *spoVM* gene to a point approximately 120 bp downstream of the 5′ end of Tn*917*Ω*HU324*. The PCR product was cloned (*Hin*dIII-*Bam*HI) into plasmid pSGMU2 (8), and the insert was sequenced on both strands by using primers PTn and OL54.

(ii) Intragenic suppressors. The procedure outlined above to sequence the *spoVM*-Tn*917* junction was used to sequence the intragenic suppressor alleles. A PCR product amplified from a mutant chromosome was cloned and sequenced. At least two independently isolated clones were sequenced for each mutation.

(iii) Extragenic suppressors. *ftsH* alleles were sequenced by using the *fmol* DNA sequencing system (Promega), using a PCR product as a sequencing template. Oligonucleotide primers used for mapping the *ftsH* mutations were used to amplify a PCR product from the mutant chromosome. These oligonucleotides were then used as sequencing primers and, together with additional primers, used to sequence the entire PCR product on both strands.

Construction of Δ*spoVM*::*spc* and *spoVMPL9*. (i) Δ*spoVM*::*spc*. A 700-bp *Eco*RI-*Hin*dIII fragment containing the entire *spoVM* ORF was cloned from pSC603 (15) into plasmid pTZ19R (Pharmacia) to produce pEL11. pEL11 was amplified by PCR using oligonucleotides OL56 (5′-AAACTGCAG**CAT**TTT TGTCCCCTCCTA) and OL57 (5′-GGCGGATCC**GATTAA**TGCCCAGGGG TT). The opposed orientation of these primers was such that the PCR would amplify both strands of the plasmid together with the additional restriction endonuclease sites provided by the PCR primers. OL56 contains the start codon sequence (boldface), and OL57 contains the terminal codon of the *spoVM* ORF and the translational stop signal (boldface). Next, the PCR product was digested with *Pst*I and *Bam*HI (provided by the oligonucleotide sequences) and ligated to a 1.1-kb spectinomycin-resistant (Sp^r) cassette (from pJL74 [14]). Following selection in *Escherichia coli*, we identified one plasmid, pEL12, that contained the Sp^r cassette flanked by the translational start and stop signals provided by *spoVM*, which was confirmed by DNA sequencing. pEL12 was linearized and introduced into *B. subtilis* by DNA-mediated transformation, with selection for Sp^r. All transformants were phenotypically arrested at stage IV-V (with a typical SpoVM phenotype) and would have arisen from a double-crossover recombination between the linearized plasmid and *spoVM* sequences on the chromosome. One isolate, EL200 (Δ*spoVM*::*spc*) was used in this work.

(ii) *spoVMPL9*. A chloramphenicol cassette was cloned into the *Xho*I site approximately 110 bp downstream of the *spoVM* ORF contained in plasmid pEL11 (see above) to create pEL13. pEL13 was introduced into *E. coli* BW313, and single-stranded DNA was prepared. The Kunkel method as described by Sambrook et al. (27) was used to generate a site-specific mutation creating a change in the ninth residue of the *spoVM* ORF from a proline to a leucine codon. The mismatch oligonucleotide M3b (5′-TCCTAAAAACTTCAGCAATTTA ATGGT) was used to introduce this mutation. We isolated one plasmid, pEL14, that contained the *spoVMPL9* mutation (confirmed by DNA sequencing). To introduce this mutation into *B. subtilis*, pEL14 was linearized and transformed into WT cells of *B. subtilis* (strain PY79), with selection for Cm^r (>95% linked to *spoVM*). Almost all transformants were Spo^- (showing a typical SpoVM phenotype) and would have arisen from cotransformation of the *spoVMPL9* allele with Cm^r by a double crossover recombination; one strain, EL315, was used for subsequent studies. To confirm that the *spo* mutation was in *spoVM*, we introduced chromosomal DNA from strain EL275 (*amyE*::*spoVM*[Erm^r]; see below) into EL315 and observed that transformants were Spo^+ due to complementation in *trans.*

Strain constructions. (i) SC2037 (*vmb4 chr*::*cat-1926*). To generate a strain containing only the *vmb4* suppressor mutation, we used a two-step procedure. First, a silent chloramphenicol marker (*chr*::*cat-1926*) approximately 60% linked by transformation to *ftsH* was introduced into strain SC1509 (*vmb4 spoVM*::Tn*917*Ω*HU324*), followed by selection for Cm^r. Approximately 60% of colonies were Spo^- due to removal of the suppressor allele. Second, DNA was isolated from one Spo^+ transformant (strain SC1993 *vmb4 chr*::*cat-1926 spoVM*::Tn*917*Ω*HU324*) from this cross and introduced into the WT, spo^+ strain PY79, with selection for Cm^r. From this cross, we could distinguish two colony types, WT colonies (40%) 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 *vmb4* suppressor allele. This was confirmed by using DNA prepared from these colonies to backcross into a *spoVM*::Tn*917*Ω*HU324* mutant which produced a Spo^+ phenotype in 60% of the transformants, demonstrating suppression.

(ii) SC2027(*vmb4 chr*::*cat-1926* Δ*spoVM*::*spc*). Chromosomal DNA from SC1993 (*vmb4 chr*::*cat-1926 spoVM*::Tn*917*Ω*HU324*; see above) was introduced into strain EL200 (Δ*spoVM*::*spc*), with selection for Cm^r. Two Spo^- colony types which were blocked at stage IV-V (40%) or stage II (60%) of development arose. By backcrossing (into strain SC1247 *spoVM*::Tn*917*Ω*HU324*), we confirmed that the stage IV-V-blocked cells contained only Δ*spoVM*::*spc* whereas the stage II-blocked colonies contained Δ*spoVM*::*spc* and *vmb4*; one strain (SC2027) with the genotype *vmb4 chr*::*cat-1926* Δ*spoVM*::*spc* was isolated.

(iii) SC2186 (*vmb4 chr*::*neo-1926 spoVMPL9 chr*::*cat*). We converted the *chr*::*cat-1926* marker in strain SC2037 (*vmb4 chr*::*cat-1926*; see above) to a neomycin-resistant marker by using plasmid pCm::Neo as described by Steinmetz and Richter (29). Chromosomal DNA from EL315 (*spoVMPL9 chr*::*cat*) was introduced into this strain, with selection for Cm^r. The *chr*::*cat* marker in EL315 is greater than 90% linked to *spoVMPL9*. Almost all transformants were Spo^- (stage IV-V) and had the genotype *vmb4 chr*::*neo-1926 spoVMPL9 chr*::*cat*. The genotype was confirmed by transforming competent cells of the Spo^- transformants with SC1247 (*spoVM*::Tn*917*Ω*HU324*) chromosomal DNA with selection for erythromycin resistance. Almost all transformants were Cm^s and Spo^+. One strain, SC2186, with the correct genotype (*vmb4 chr*::*neo-1926 spoVMPL9 chr*::*cat*) was isolated by this procedure.

(iv) EL275 (*amyE*::*spoVM*[Erm^r]). The chloramphenicol marker in strain PL80 (*amyE*::*spoVM*[Cm^r] [15]) was converted to erythromycin resistance by using plasmid pCm::Erm as described previously (29).

Peptide synthesis. Peptides were synthesized by using standard solid-phase synthetic methods and purified by reverse-phase high-pressure liquid chromatography, and their molecular weights were confirmed by laser desorption mass spectrometry. Buffer (40 mM potassium phosphate [pH 7.4]) was added to lyophilized SpoVM peptides in a polyethylene Eppendorf tube and vortexed to disperse undissolved solids into a turbid suspension. After overnight equilibration at room temperature, the suspension was centrifuged at 14,000 rpm for 5 min to pellet undissolved solid. The peptide concentration in the supernatant solution was measured by using the tyrosine absorbance at 275 nm, assuming a molar extinction coefficient of 1,400 A cm^{-1}.

In vitro proteolytic reactions with FtsH. Reactions 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* σ^{32}-C-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 μg/ml (peptide/σ^{32}-C-His/FtsH molar ratio of 27:9:1). Reactions were performed at 42°C for 40 min, and samples were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on a 12% polyacrylamide gel followed by staining with Coomassie blue. A complete reaction mixture for SpoVM degradation 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, 12 μg of WT peptide per ml, and 40 μg of purified *E. coli* FtsH per ml. Reactions were

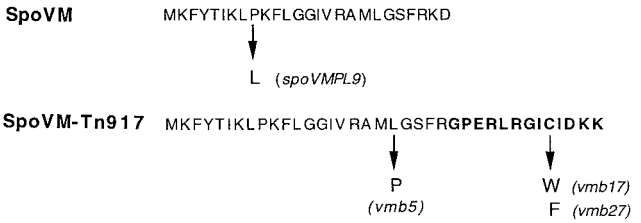

FIG. 1. SpoVM protein and mutant forms. Both the SpoVM WT polypeptide and the 37-aa chimera encoded by *spoVM*::Tn*917*Ω*HU324* are shown. The 13 residues in boldface are those encoded by the transposon. Mutational changes are shown by arrows. *spoVMPL9* was created by site-specific mutagenesis. The 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^E and σ^K 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 lysozyme (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^E monoclonal antibody (obtained from W. Haldenwang [32]) was used at a dilution of 1:500, and a polyclonal antiserum against pro-σ^K (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*::Tn*917* encodes a chimeric protein.** The SpoVM phenotype has been defined by one insertional mutation (*spoVM*::Tn*917*Ω*HU324*) consisting of a Tn*917* 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 insertional mutation by sequencing the junction of the Tn*917* insertion within the *spoVM* ORF. We found that insertion of Tn*917* 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 Tn*917* (the translational stop codon was provided by Tn*917*). Thus, following translation of *spoVM*-Tn*917* 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, Δ*spoVM*::*spc*, where the entire *spoVM* ORF had been replaced with an Sp^r cassette. Sporulating cells containing this allele (EL200) exhibited a morphological phenotype indistinguishable from that of a congenic strain containing *spoVM*::Tn*917*Ω*HU324*, i.e., production of immature, phase-grey spores which were completely sensitive to heat and solvents (Table 1). Figure 2 shows electron micrographs of *spoVM*::Tn*917*Ω*HU324* (Fig. 2C) and Δ*spoVM*::*spc* (Fig. 2D) spores. In both cases, an intact spore which is devoid of the electron-transparent cortex has formed. However, both inner and outer (electron-dense) coat layers encased the spore, as has been reported previously (15). We also created a mutant *spoVM* gene, in which codon 9, specifying proline, was changed to result in the substitution of leucine. *spoVMPL9* cells (strain EL315) exhibited a null phenotype indistinguishable from that of *spoVM*::Tn*917*Ω*HU324* and Δ*spoVM*::*spc* strains (Table 1). Interestingly, although completely sensitive to treatments with heat and chloroform, spores of all three mutants exhibited partial resistance to lysozyme.

TABLE 1. Sporulation phenotypes of *spoVM* and intergenic suppressor alleles[a]

Strain	Allele	Sporulation (% of CFU/ml of untreated culture)			Sporulation phenotype[b]
		Chloroform	Heat	Lysozyme	
PY79	*spo*$^+$	100	100	100	WT
SC1247	*spoVM*::Tn*917*Ω*HU324*	<0.0001	<0.0001	1.1	IV-V
EL200	Δ*spoVM*::*spc*	<0.0001	<0.0001	3.8	IV-V
EL315	*spoVMPL9*	<0.0001	<0.0001	0.18	IV-V
SC1509	*spoVM*::Tn*917 vmb4*	23	32	25	WT
SC2027	Δ*spoVM*::*spc vmb4*	<0.0001	<0.0001	0.16	II-III
SC2186	*spoVMPL9 vmb4*	<0.0001	<0.0001	0.17	II-III
SC2037	*vmb4*	71	60	47	WT

[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 spores; IV-V, stage IV-V block in morphogenesis; II-III, stage II-III block in morphogenesis.

Identification of an interacting polypeptide. We used a mutagenic selection to identify mutations that suppressed *spoVM*::Tn*917*Ω*HU324* and allowed production of chloroform-resistant spores. 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 Tn*917* sequences) to either phenylalanine (*vmb27*) or tryptophan (*vmb17*), while *vmb5* changed Leu20 to a proline (Fig. 1). Five suppressors (*vmb1*, *vmb3*, *vmb4*, *vmb6*, and *vmb9*) were extragenic and allelic to the *ftsH* locus, which encodes a 70-kDa member of the AAA (ATPases associated with diverse cellular activities) family of ATPases (2, 13). We sequenced one allele within *ftsH* (*vmb4*) and found it to change a glutamic acid to lysine seven residues upstream from the HEXGH motif found in the procaryotic members of the AAA family (Fig. 3). This motif is the putative Zn^{2+}-binding domain for metalloprotease activity possessed by the procaryotic FtsH proteins (2, 13).

Although all suppressor mutants were identified as restoring a Spo^+ phenotype to *spoVM*::Tn*917*Ω*HU324* cells, the intragenic and extragenic alleles behaved differently. During sporulation, cells carrying the intragenic suppressors produced spores at an efficiency indistinguishable from that of a congenic Spo^+ strain (data not shown). In contrast, cells carrying the extragenic alleles were approximately fourfold less efficient than WT cells in forming intact, phase-bright, heat-resistant spores (Table 1 shows data for the *spoVM*::Tn*917*Ω*HU324 vmb4* mutant SC1509).

Since *spoVM*::Tn*917*Ω*HU324* cells produced a null phenotype, we asked whether the extragenic alleles would suppress the Δ*spoVM*::*spc* mutation. After constructing strains carrying Δ*spoVM*::*spc* and each of the five extragenic mutations, we examined their sporulation phenotype. We found that in each case, no phase-bright, heat-resistant spores were produced (Table 1 shows data for a Δ*spoVM*::*spc vmb4* strain, SC2027). Similar results were also obtained with the *spoVMPL9* mutant (Table 1 shows data for SC2186 *spoVMPL9 vmb4*), suggesting

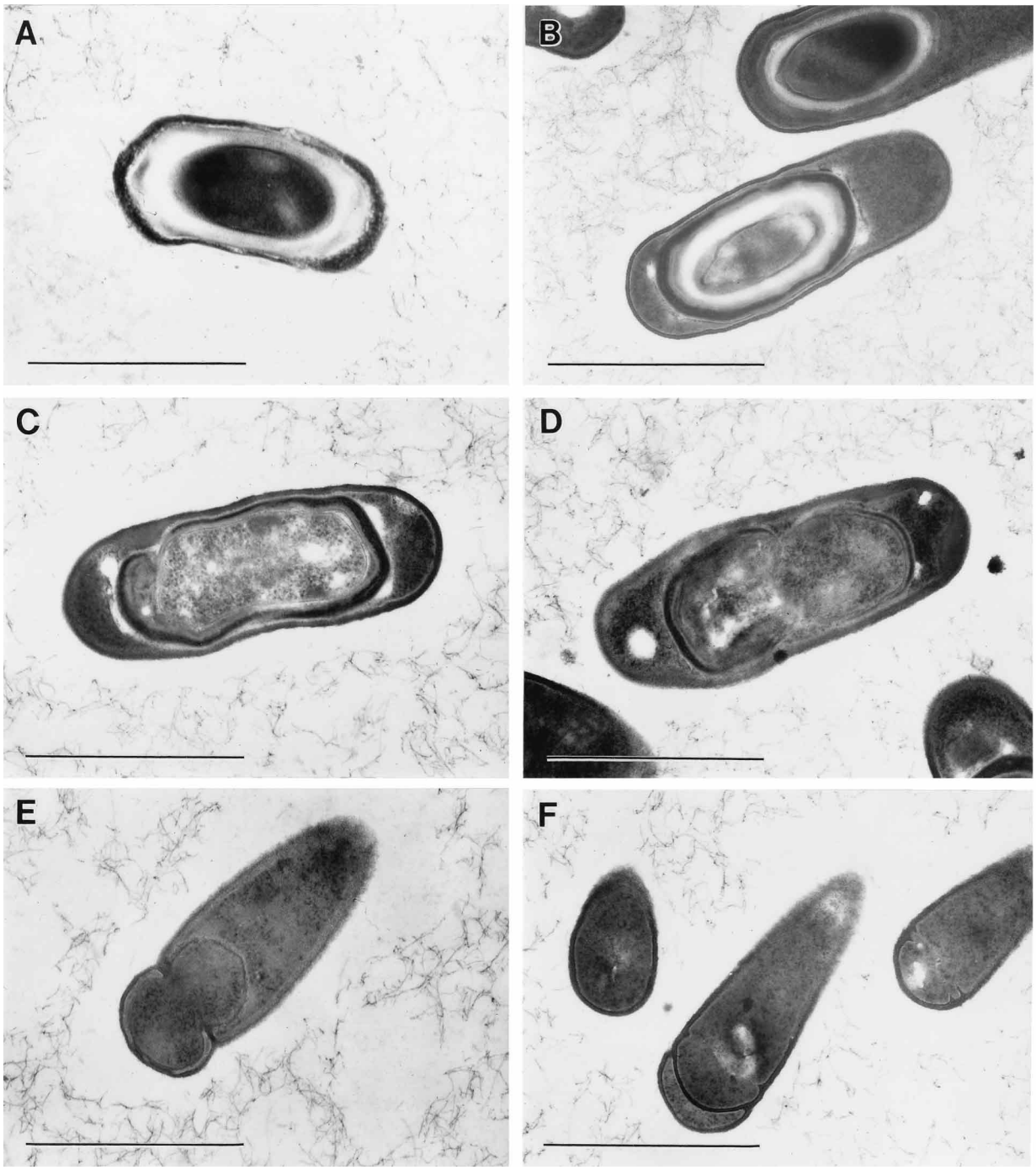

FIG. 2. Electron micrographs of mutant sporangia. (A) PY79 *spo*$^+$; (B) SC2037 *vmb4*; (C) SC1247 *spoVM*::Tn*917*Ω*HU324*; (D) EL200 Δ*spoVM*::*spc*; (E and F) SC2027 Δ*spoVM*::*spc vmb4*. The bar indicates 1 μm.

that the extragenic suppressors are allele specific. Unexpectedly, we also found that Δ*spoVM*::*spc* or *spoVMPL9* strains carrying a *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 Δ*spoVM*::*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 *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 Δ*spoVM*::*spc*, *spoVMPL9*, or *spoVM*$^+$ strains carrying the *ftsH*

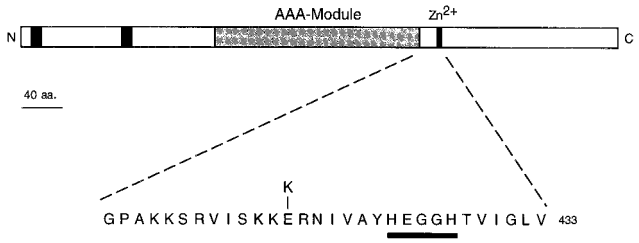

FIG. 3. Extragenic suppressor allele in FtsH. Shown is a schematic of the 70-kDa FtsH protein. The AAA module or ATP-binding region is shown (shaded box). The two N-terminal transmembrane domains which anchor FtsH to the phospholipid membrane are indicated by thick black bars. The expanded region shows the Zn^{2+}-binding motif (HEGGH) of the *B. subtilis* FtsH metalloprotease (accession no. D26185) and the glutamic acid-to-lysine substitution in the *vmb4* mutation.

suppressor alleles was impaired, although growth of *spoVM*::Tn*917ΩHU324* 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*::Tn*917ΩHU324* mutant impaired σ^K-directed gene expression (15). We examined expression of three σ^K-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*::Tn*917ΩHU324* or Δ*spoVM*::*spc* were impaired in σ^K-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*::Tn*917ΩHU324* mutant (13) and extends our observations reported above that the *spoVM*::Tn*917ΩHU324* and Δ*spoVM*::*spc* mutations produce indistinguishable sporulation defects. In *spoVM*::Tn*917ΩHU324 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*::Tn*917ΩHU324* mutant (heat sensitivity) was substantially suppressed by the *vmb4* allele (Table 1), σ^K-dependent gene expression remained defective in the double mutant. In Δ*spoVM*::*spc vmb4* cells, σ^K-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 σ^K-directed gene expression (3, 9). Lastly, in cells containing only

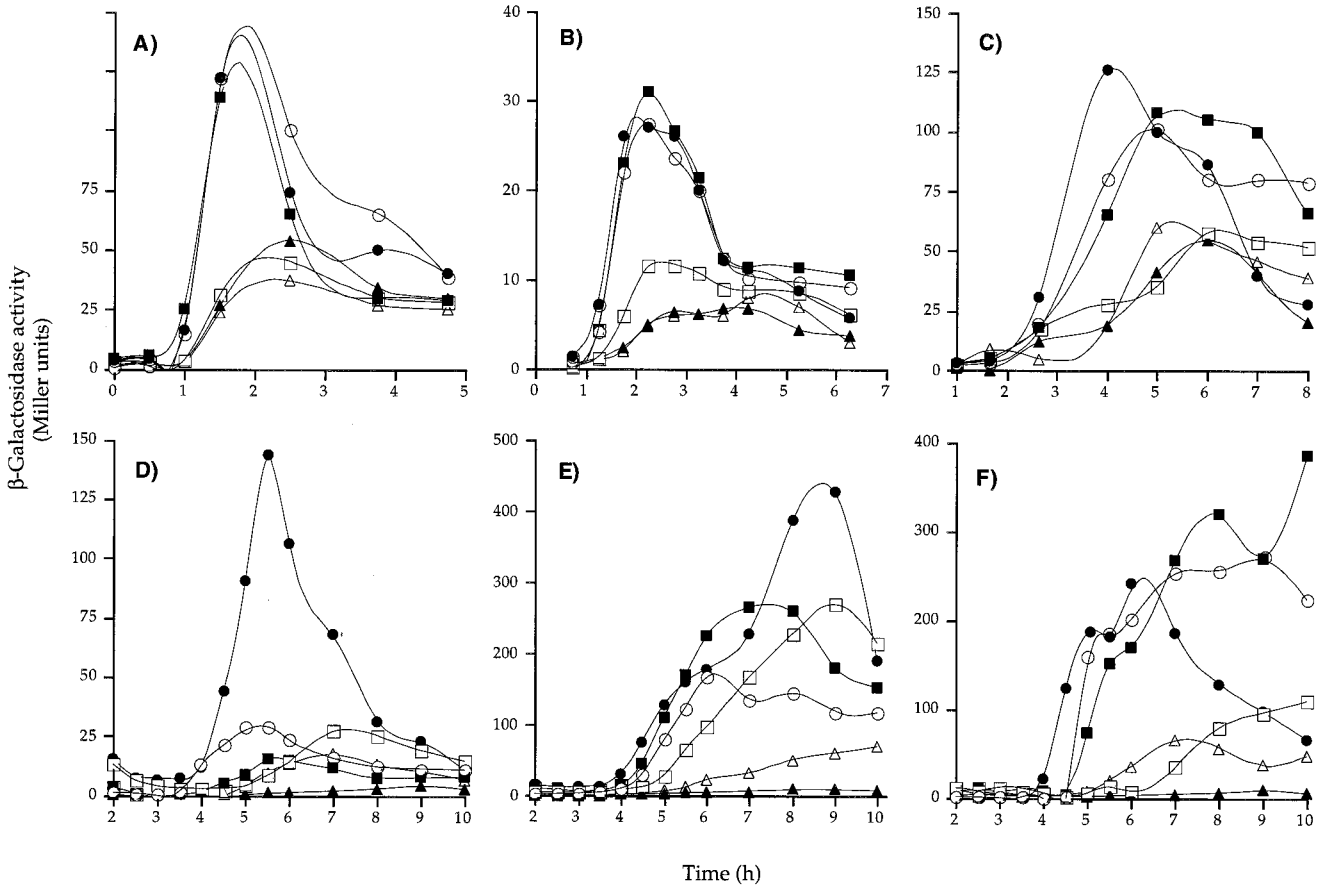

FIG. 4. Effect of *spoVM* and extragenic suppressors on developmental gene expression. β-Galactosidase activity was measured in strains containing different reporter genes: *spoIIG-lacZ* (A), *spoIVF-lacZ* (B), *spoIVB-lacZ* (C), *cotA-lacZ* (D), *gerE-lacZ* (E), and *cotD-lacZ* (F). Sporulation was induced by the resuspension method, and samples were measured for β-galactosidase activity at the indicated times after the initiation of sporulation (time zero). The strains were PY79 (*spo*$^+$; ●), SC1247 (*spoVM*::Tn*917ΩH324*; ○), EL200 (Δ*spoVM*::*spc*; ■), SC1509 (*spoVM*::Tn*917ΩHU324 vmb4*; □), SC2027 (Δ*spoVM*::*spc vmb4*; ▲), and SC2037 (*vmb4*; △). Background levels of enzyme activity present in cells containing no fusion have been subtracted.

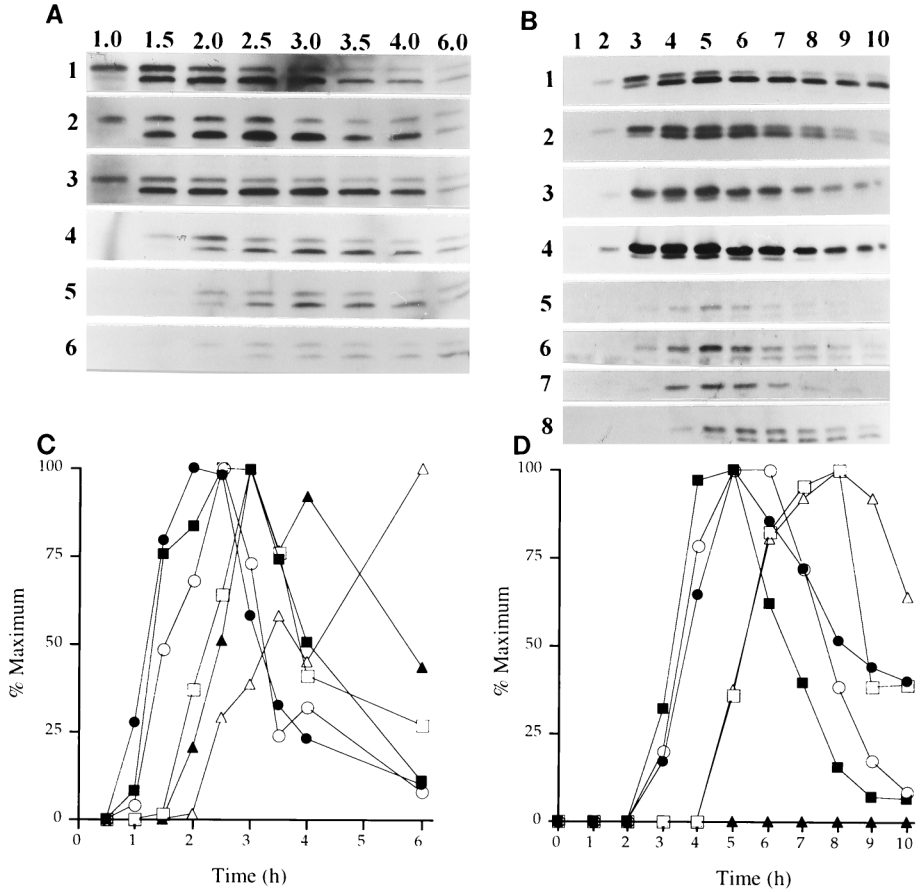

FIG. 5. σ^E and σ^K production during sporulation. (A and B) Cells containing the indicated alleles were collected at 30-min (A) or 60-min (B) intervals after the onset of sporulation, as indicated by the numbers at the top. Whole-cell extracts were prepared and analyzed by using either monoclonal anti-σ^E antibody (A) or polyclonal anti-pro-σ^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-σ and the lower band is the mature σ. (A) 1, *spo*$^+$ (30-min exposure); 2, *spoVM*::Tn*917ΩHU324* (30 min); 3, Δ*spoVM*::*spc* (30 min); 4, *spoVM*::Tn*917ΩHU324 vmb4* (30 min); 5, Δ*spoVM*::*spc vmb4* (30 min); 6, *vmb4* (30 min). (B) 1, *spo*$^+$ (30-s exposure); 2, *spoVM*::Tn*917ΩHU324* (30 s); 3, Δ*spoVM*::*spc* (30 s); 4, Δ*spoVM*::*spc* (60 s); 5, *spoVM*::Tn*917ΩHU324 vmb4* (10 min); 6, *spoVM*::Tn*917ΩHU324 vmb4* (45 min); 7, Δ*spoVM*::*spc vmb4* (10 min); 8, *vmb4* (10 min). (C and D) σ^E (C) or σ^K (D) 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*::Tn*917ΩHU324*; ■, Δ*spoVM*::*spc*; □, *spoVM*::Tn*917ΩHU324 vmb4*; ▲, Δ*spoVM*::*spc vmb4*; △, *vmb4*. In panel C, the maximum intensities relative to the maximum intensity of the *spo*$^+$ strain were as follows: *spoVM*::Tn*917ΩHU324*, 1.5; Δ*spoVM*::*spc*, 1.0; *spoVM*::Tn*917ΩHU324 vmb4*, 0.48; Δ*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*::Tn*917ΩHU324*, 0.78; Δ*spoVM*::*spc*, 0.25; *spoVM*::Tn*917ΩHU324 vmb4*, 0.0011; Δ*spoVM*::*spc vmb4*, 0.0; and *vmb4*, 0.0097.

the *vmb4* mutation, σ^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*::Tn*917ΩHU324 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* (σ^A dependent) (Fig. 4A), *spoIVF* (σ^E dependent) (Fig. 4B), and *spoIVB* (σ^G dependent) (Fig. 4C). Expression of these genes was unaffected by the *spoVM*::Tn*917Ω HU324* and Δ*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 σ^K-dependent gene expression. In results not shown, we have found that the other *ftsH* 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, σ^E and σ^K, in these cells. Production of both of these sigma factors involves proteolytic processing of an inactive precursor called a pro-σ. We used antibodies against pro-σ^E and pro-σ^K to examine production of mature σ^E and σ^K by Western blot analysis of extracts prepared from sporulating cells.

Strains containing *spoVM*::Tn*917ΩHU324* or Δ*spoVM*::*spc* produced σ^E normally (Fig. 5A and C), as expected since *spoIVF-lacZ* expression was unimpaired (Fig. 4B). The timing of σ^K appearance was normal, but the maximum level was reduced (Fig. 5B and D). The moderate reduction in σ^K production most likely accounts for the differential effects on expression of σ^K-dependent genes (*cotA*, *gerE*, and *cotD* in Fig. 4D, E, and F), as has been reported previously for other strains with diminished σ^K production (18, 24).

In contrast, all strains carrying the *vmb4* allele exhibited a delay in the timing of σ^E appearance and a reduction in the amount of σ^E produced (Fig. 5A and C). The delay in the appearance of σ^E correlates with the delayed expression of the σ^E-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

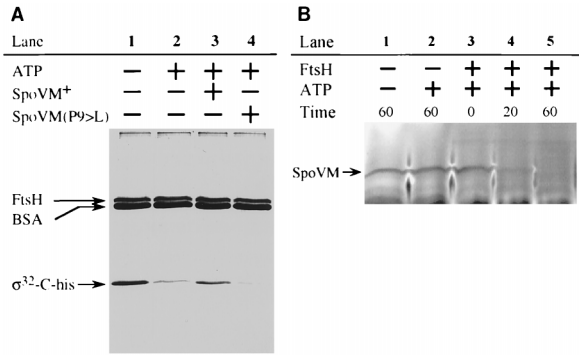

FIG. 6. SpoVM inhibits *E. coli* FtsH protease and acts as a substrate for FtsH. (A) Inhibitory effects of SpoVM WT and PL9 peptides. BSA, bovine serum albumin. (B) Degradation of SpoVM at 42°C.

was production of pro-σ^K (Fig. 5B), presumably due to reduced transcription of the σ^E-dependent *sigK* gene. In the *vmb4* single mutant and the *spoVM*::Tn*917ΩHU324 vmb4* double mutant, σ^K production was both severely reduced (less than 1% of the level in WT cells [Fig. 5B]) and delayed (Fig. 5D), as was expression of σ^K-dependent genes (Fig. 4D to F). Clearly, suppression of the sporulation defect of *spoVM*::Tn*917Ω HU324* mutant cells by the *vmb4* allele does not result from restoration of σ^K production. Finally, σ^K was not even detected in Δ*spoVM*::*spc vmb4* mutant cells (Fig. 5B), consistent with the inability of this mutant to express σ^K-dependent genes (Fig. 4D to F) or form heat-resistant spores (Table 1).

SpoVM inhibits FtsH-mediated degradation of *E. coli* σ^{32}. 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 σ^{32}, and this protease activity is ATP and zinc dependent. FtsH, by degrading σ^{32}, 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 σ^{32} degradation in vitro. We found that WT synthetic SpoVM protein strongly inhibited degradation of σ^{32} 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*::Tn*917ΩHU324* mutant encodes a chimeric protein 37 aa in length. By constructing a deletion-and-insertion mutation, Δ*spoVM*::*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 σ^K-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*::Tn*917ΩHU324* 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 σ^{32} (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*::Tn*917ΩHU324*, Δ*spoVM*::*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*::Tn*917ΩHU324* 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 feeble 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 Δ*spoVM*::*spc vmb4* and *spoVMPL9 vmb4* cells. This phenotype differs from that of *vmb4* (Spo$^+$) or Δ*spoVM*::*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 II-III function. As expected for a mutant blocked at stage II-III, expression of σ^K-dependent genes (Fig. 4D to F) was undetectable in the *spoVM*Δ::*spc vmb4* mutant. Neither the Δ*spoVM*::*spc* mutation nor the *vmb4* mutation alone completely blocked σ^K 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 σ^{32}. 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 λCIII protein acts as a competitive inhibitor of FtsH-mediated degradation of σ^{32}, and it is thought that λCIII may also act as a proteolytic substrate for FtsH (11, 30). SpoVM, by analogy to λCIII, 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 σ^E and σ^K 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 σ^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 σ^E production, but accumulation of σ^K was reduced by 22 to 75% (Fig. 5). Expression of two σ^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 σ^K, consistent with previous observations (18, 24).

It seems unlikely, however, that reduced σ^K production accounts for the stage IV-V sporulation block of the *spoVM* mutants. These mutants produce much more σ^K than *vmb4* mutants (Fig. 5) yet fail to sporulate (Table 1). On the other hand, the *vmb4* mutation restores sporulation when present in *spoVM*::Tn*917ΩHU324* mutant cells, but this suppression clearly does not result from restoration of normal σ^K 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*::Tn*917ΩHU324* 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 this interaction may involve SpoVM acting as an antagonist and/or substrate of FtsH protease. The interaction first becomes crucial during the prespore engulfment 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*::Tn*917ΩHU324* mutant cells to progress beyond stage IV-V of sporulation.

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