Function of the SpoVAEa and SpoVAF Proteins of *Bacillus subtilis* Spores

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The *Bacillus subtilis spoVAEa* and *spoVAF* genes are expressed in developing spores as members of the *spoVA* operon, which encodes proteins essential for the uptake and release of dipicolinic acid (DPA) during spore formation and germination. SpoVAF is likely an integral inner spore membrane protein and exhibits sequence identity to A subunits of the spore's nutrient germinant receptors (GRs), while SpoVAEa is a soluble protein with no obvious signals to allow its passage across a membrane. However, like SpoVAD, SpoVAEa is present on the outer surface of the spore's inner membrane, as SpoVAEa was accessible to an external biotinylation agent in spores and SpoVAEa disappeared in parallel with SpoVAD during proteinase K treatment of germinated spores. SpoVAEa and SpoVAD were also distributed similarly in fractions of disrupted dormant spores. Unlike *spoVAD*, *spoVAEa* is absent from the genomes of some spore-forming members of the *Bacillales* and *Clostridiales* orders, although SpoVAEa's amino acid sequence is conserved in species containing *spoVAEa*. *B. subtilis* strains lacking SpoVAF or SpoVAEa and SpoVAF sporulated normally, and the spores had normal DPA levels. Spores lacking SpoVAF or SpoVAEa and SpoVAF also germinated normally with non-GR-dependent germinants but more slowly than wild-type spores with GR-dependent germinants, and this germination defect was complemented by ectopic expression of the missing proteins.

The *spoVA* operon of *Bacillus subtilis* is expressed only in the developing spore during sporulation and encodes seven proteins, with the genes in the order *spoVAA*, *-B*, *-C*, *-D*, *-Eb*, *-Ea*, and *-F*. At least five of these SpoVA proteins, SpoVAA, -B, -C, -D, and -Eb, are necessary for normal *B. subtilis* spore formation (1, 2). These proteins are essential for the uptake of dipicolinic acid (DPA) into the dormant spore (3–7); in turn, DPA is essential for spore stability, as DPA-less *B. subtilis* spores germinate spontaneously after their release from the sporangium and during spore purification (8). At least some SpoVA proteins are also essential for DPA release in spore germination, and one SpoVA protein, SpoVAD, binds DPA specifically, with this binding essential for DPA uptake in sporulation (6). SpoVAD is a soluble protein and has no obvious signal peptide or membrane-spanning or anchor sequences. However, assessment of SpoVAD's location and accessibility in spores indicates that this protein is on the outer surface of the spore's inner membrane (IM) (4, 9, 10).

In contrast to SpoVAD, SpoVAA, -B, -C, -Eb, and -F appear to be integral membrane proteins on the basis of predictions from primary sequences and in some cases the localization of proteins expressed in growing bacteria (3, 11, 12). Although SpoVAF exhibits significant sequence identity to the A subunits of the spore's nutrient germinant receptors (GRs) (2), SpoVAA, -B, -C, -Eb, and -Ea exhibit no significant identity to known proteins. However, SpoVAEa is also predicted to be a soluble protein. Consequently, in this work, we have examined the location and accessibility of SpoVAEa in *B. subtilis* spores compared to those of SpoVAD; the number of SpoVAEa molecules in spores; the role of this protein, as well as SpoVAF, in DPA uptake in sporulation and DPA release during spore germination; and the presence of a *spoVAEa* gene in other spore-forming members of the orders *Bacillales* and *Clostridiales*.

MATERIALS AND METHODS

Preparation of purified SpoVAEa and generation and purification of antiserum. The *spoVAEa* gene was amplified by PCR with genomic DNA from *B. subtilis* strain PS832, a prototrophic 168 strain derivative, as the template. The 5′ primer introduced a NotI site, and the 3′ primer introduced a KpnI site. Deletion of a predicted destabilizing loop corresponding to residues 124 to 135 was accomplished by overlap PCR (6), giving Δ*spoVAEa*. The resulting PCR products, Δ*spoVAEa* and the intact *spoVAEa* gene, were cloned into a modified pET15b vector containing a His$_6$ tag and a tobacco etch virus (TEV) protease cleavage site (13). The ΔSpoVAEa protein (residues 2 [Δ124-135] to 203) and intact SpoVAEa were expressed in *Escherichia coli* BL21 Star(DE3) (Invitrogen, Grand Island, NY) initially by growth at 37°C in Luria broth (LB) (14) plus ampicillin (100 μg/ml) and then by induction with 1 mM isopropyl-β-D-thiogalactopyranoside at an optical density at 600 nm (OD$_{600}$) of 0.8 and subsequent growth at 21°C for 16 h. Both SpoVAEa proteins were soluble and were purified by Ni^{2+}-nitrilotriacetic acid affinity chromatography under native conditions, followed by TEV protease cleavage of the His$_6$ tag and cation-exchange chromatography (SD200; GE Healthcare, Piscataway, NJ) (6, 13).

The purified ΔSpoVAEa protein was dialyzed against phosphate-buffered saline (PBS; 50 mM sodium phosphate, 150 mM NaCl, pH 7.2),

TABLE 1 Genotypes, spore phenotypes, and sources of strains used in this study

B. subtilis strain	Genotype; spore phenotype[a]	Source or reference
PS533	Wild type/pUB110 Km[r]	15
PS832	Wild-type 168 *trp*[+]	Laboratory stock
PS3406	*spoVA sleB*; no SpoVA proteins, stable DPA-less spores	16
PS3411	P*sspB-spoVA*; elevated SpoVA protein levels	4
PS4348	*spoVAEa*; Cm[r], lacks SpoVAEa and likely SpoVAF[a]	This work
PS4351	*spoVAF*; Cm[r], lacks SpoVAF	This work
PS4361	*spoVAF*; Tc[r], lacks SpoVAF	This work
PS4362	*spoVAEa*; Tc[r], lacks SpoVAEa and likely SpoVAF[a]	This work
PS4363	*spoVAF amyE*::P*sspB-spoVAEa*; has SpoVAEa but not SpoVAF	This work
PS4364	*spoVAEa amyE*::P*sspB-spoVAEa*; has SpoVAEa but not SpoVAF	This work
PS4374	*spoVAEa amyE*::P*spoVA-spoVAEa*; has SpoVAEa but not SpoVAF	This work
PS4381	*spoVAF amyE*::P*sspB-spoVAF*; has SpoVAF[a] and SpoVAEa	This work
PS4383	*spoVAEa amyE*::P*sspB-spoVAF*; has SpoVAF[a] but not SpoVAEa	This work
PS4390	*spoVAF amyE*::P*spoVA-spoVAF*; has SpoVAF[a]	This work
PS4391	*spoVAEa amyE*::P*sspB-spoVAEa-F*; has SpoVAF[a] and SpoVAEa	This work
PS4392	*spoVAF amyE*::P*sspB-spoVAEa-F*; has SpoVAF[a] and SpoVAEa	This work

[a] The presence of SpoVAF is based only on the finding that the *spoVAF* gene is present in a construct that should yield SpoVAF synthesis; there is no direct proof that SpoVAF is indeed synthesized.

adjusted to a concentration of 1 mg/ml in PBS, and supplied for poly-clonal antibody production in rabbits (Pocono Rabbit Farm and Laboratory, Canadensis, PA). The antibody was detected in a bleed 2 months after its initial injection and was affinity purified with Pierce AminoLink Plus Immobilization kit (Pierce, Rockford, IL) in accordance with the manufacturer's instructions. Briefly, 1 mg of purified SpoVAEa protein was added to an AminoLink Plus Resin column (~2 ml) in 0.1 M sodium citrate–0.05 M sodium carbonate coupling buffer (pH 10) and mixed by rocking at room temperature for 4 h, resulting in the formation of semi-stable Schiff base bonds. The column was washed with 4 ml of PBS, and reduction with sodium cyanoborohydride overnight at 4°C, followed by quenching with 1 M Tris-HCl (pH 7.4), was performed, resulting in stable secondary amine bonds. The antigen column was washed with 10 ml of 1 M NaCl and equilibrated in PBS, and then 2 ml of antiserum was added. After mixing by rocking for 1 h at room temperature, the column was washed with 8 ml of PBS. Bound antibody was eluted with 2 ml of 0.2 M glycine-HCl (pH 2.5) into a tube containing 100 μl of 1 M Tris-HCl (pH 8.9) to neutralize the glycine buffer. The purified antibody was finally dialyzed at 4°C against PBS and used for Western blot analysis.

B. subtilis strains used and spore preparation and germination. The *B. subtilis* strains used in this work are listed in Table 1; they are isogenic derivatives of strain PS832, a laboratory 168 strain, including (i) PS533 (15), which contains plasmid pUB110, providing resistance to kanamycin (10 μg/ml); (ii) PS3411 (4) (termed ↑*spoVA*), in which the *spoVA* operon is under the control of the strong forespore-specific promoter of the *sspB* gene (P*sspB*) (P*sspB-spoVA* is turned on at the same time in sporulation as the normal *spoVA* operon but gives ~5-fold higher levels of at least Spo-VAD); and (iii) PS3406 (16), which lacks most of the *spoVA* operon and the gene encoding the spore cortex lytic enzyme SleB. PS3406 spores lack

DPA, but because of their lack of SleB, these spores are stable and can be isolated.

A *B. subtilis* strain with a deletion in the *spoVAEa* cistron was constructed as follows. A DNA fragment consisting of bp +2 to −246 relative to the *spoVAEa* translation start codon was PCR amplified from PS832 chromosomal DNA with primers that inserted BamHI and PstI restriction sites upstream and downstream, respectively, of the translation start site. After digestion with BamHI and PstI, the fragment was cloned between these sites in plasmid pKG57 (10), which is pBluescript II KS with a chloramphenicol resistance (Cm[r]; 5 μg/ml) cassette (*cam*), and the recombinant plasmid, pPVE1, was isolated in *E. coli* DH5α. A DNA fragment consisting of bp −272 to +122 relative to the translation stop codon in *spoVAEa* was PCR amplified with primers that inserted HindIII and ClaI sites upstream and downstream, respectively, of the translation stop codon. This fragment was digested with HindIII and ClaI and cloned between these sites in plasmid pPVE1, giving plasmid pPVE2 (*spoVAEa*:: *cam*), which has fragments from either end of *spoVAEa* flanking the *cam* cassette. Plasmid pPVE2 was linearized by digestion with ClaI and used to transform *B. subtilis* strain PS832 to Cm[r]. PCR of one Cm[r] colony confirmed the expected chromosomal structure, and this strain was termed PS4348 (*spoVAEa* mutant).

A *B. subtilis* strain with a deletion of the *spoVF* gene was constructed by a two-step strategy that generated a derivative of plasmid pKG57 (10) termed pPVE4 in which upstream and downstream *spoVAF* fragments flanked a *cam* gene. In the first step, a DNA fragment consisting of bp +91 to −662 relative to the *spoVAF* translation start site was PCR amplified, the fragment was cut with BamHI and PstI (sites added in the upstream and downstream PCR primers, respectively), and the resultant fragment was inserted between the BamHI and PstI sites of plasmid pKG57 to create plasmid pPVE3. A PCR consisting of 275 bp upstream and 103 bp downstream of the *spoVAF* translation stop site was then amplified from PS832 genomic DNA, the fragment was cut with HindIII and ClaI (sites added in upstream and downstream PCR primers, respectively), and the resultant fragment was inserted between the BamHI and ClaI sites in plasmid pPVE3, giving plasmid pPVE4 (Δ*spoVAF*::*cam*). The individual steps in this plasmid's construction were confirmed by PCR and restriction enzyme digestion. Plasmid pPVE4 was then used to transform *B. subtilis* to Cm[r], and proper integration of the *spoVAF*::*cam* fragment at the *spoVAF* chromosomal locus was confirmed by PCR, giving strain PS4351 (*spoVAF*).

B. subtilis strains carrying the *spoVAEa* or *spoVAF* genes at the *amyE* locus under the control of P*sspB* (4) were constructed as follows. The region from bp −490 to −1 relative to the translation start site of the *B. subtilis sspB* gene (this region has P*sspB*, as well as a strong ribosome-binding site [RBS]) was amplified from PS832 DNA with primers with an EcoRI site in the upstream primer and a region of overlap with the *spoVAEa* translation start site in the downstream primer. The upstream primer for *spoVAEa* amplification was complementary to the downstream primer for P*sspB*, and the downstream *spoVAEa* primer would amplify the complete *spoVAEa* coding region and a 3′ BamHI site. For overlap PCR, we amplified a product that has P*sspB* plus a good RBS just upstream of the *spoVAEa* coding region and between the EcoRI and BamHI sites, with the amplified P*sspB* promoter and *spoVAEa* fragments as the template. The overlap PCR product of the expected size was purified, digested with EcoRI and BamHI, and ligated to similarly cut plasmid pDG364 (17), and the recombinant plasmid (pPVE5) was isolated in *E. coli* DH5α. This plasmid was used to transform *B. subtilis* strain PS4362 (derived from strain PS4348 with the Cm[r] cassette changed to a Tc[r] cassette [18]) to a Cm[r] Tc[r] amylase-negative phenotype by a double-crossover event, giving strain PS4364, and the expected genomic structure in the *amyE* region of this strain was confirmed by PCR. The construction of a *B. subtilis* strain in which *spoVAF* expression was under P*sspB* control was similar to that for strain PS4264 described above, except that the overlap PCR product was flanked by BamHI sites. Plasmid pPVE7 with the *spoVAF* overlap PCR product was used to transform *B. subtilis* strain PS4361 (derived from

strain PS4351 with the Cm^r cassette changed to a Tc^r cassette [18]) to a Cm^r Tc^r amylase-negative phenotype by a double-crossover event, giving strain PS4381, and the expected genomic structure in the *amyE* region of this strain was confirmed by PCR. Plasmids pPVE5 and pPVE7 were used to transform strains PS4361 and PS4362, respectively, giving strains PS4363 and PS4383.

The *spoVAEa-spoVAF* genes were also amplified together; the upstream primer for *spoVAEa* amplification was complementary to the downstream primer for P*sspB* (plus the strong RBS), and the downstream *spoVAF* primer would amplify the complete *spoVAEa-spoVAF* coding region plus a 3′-BamHI site, and the overlap PCR product was flanked by BamHI sites. Plasmid pPVE8 with the P*sspB-spoVAEa-spoVAF* overlap PCR product was used to transform *B. subtilis* strains PS4362 and PS4361, respectively, giving strains PS4391 and PS4392.

B. subtilis strains carrying the *spoVAEa* or *spoVAF* gene at the *amyE* locus under the control of the promoter for the *spoVA* operon (P*spoVA*) were constructed by a strategy identical to that described above. The region from bp −183 to −1 relative to the translation start site of the *B. subtilis spoVAA* gene (this is the first gene of the *spoVA* operon and includes P*spoVA*) was amplified from PS832 DNA, and this region was overlapped with either the *spoVAEa* or the *spoVAF* gene. The overlap amplicon P*spoVA-spoVAEa* or P*spoVA-spoVAF* was cloned into plasmid pDG364 (17), and the recombinant plasmid (pPVE6 or pPVE9, respectively) was isolated in *E. coli* DH5α. This plasmid was transformed into strain PS4362 or PS4361, respectively, giving strain PS4374 or PS4390.

Spores of all *B. subtilis* strains were prepared at 37°C with 2× Schaeffer's glucose (SG) medium either on agar plates or in liquid medium, and spores were purified as described previously (8, 19). The spores used in this work were free (>98%) of growing or sporulating cells, germinated spores, and cell debris as shown by phase-contrast microscopy.

Nutrient germination of spores was preceded by heat activation (75°C, 30 min) in water, followed by cooling on ice. Germination was in 10 mM L-valine–25 mM K-HEPES buffer (pH 7.4) at 37°C, 10 mM L-asparagine–10 mM D-glucose–10 mM D-fructose–10 mM KCl (AGFK) in 25 mM K-HEPES buffer (pH 7.4) at 37°C, a 1:1 mixture of 60 mM CaCl$_2$ and 60 mM DPA (CaDPA) adjusted to pH 8 with Tris base at 23°C, or 1 mM dodecylamine–25 mM K-HEPES buffer (pH 7.4) at 45°C. In germinations with AGFK, dodecylamine, and L-valine, spores were at an initial OD$_{600}$ of 0.5, and in CaDPA germination, spores were at an OD$_{600}$ of 2. Germination was routinely assessed by the presence of 50 μM TbCl$_3$ from the beginning of germination and continuously measuring the release of DPA from spores by its fluorescence with Tb^{3+} as described previously (L-valine) (20) and in some experiments by (i) adding TbCl$_3$ to 50 μM to aliquots of germination incubations taken at various times and measuring Tb-DPA fluorescence (AGFK and dodecylamine) or (ii) phase-microscopic analysis of ~100 spores either directly or in aliquots of CaDPA-germinating cultures taken at various times and made 0.1 M in EDTA to halt germination.

High-pressure (HP) germination of spores was carried out differently from the germination methods described above and was done essentially as described previously (21), with 1-ml samples of spores at an OD$_{600}$ of 1 at either 37°C and 150 MPa or 50°C and 550 MPa. Levels of spore germination in samples treated at HP for various hold times were assessed by first centrifuging the treated samples, suspending the pelleted spores in ~20 μl water, and examining ~100 spores by phase-contrast microscopy to determine the percentages of spores that had become phase dark and thus had germinated. The spores of the two strains whose HP germination was to be compared were prepared, purified, and HP treated at the same time.

Analysis of the germination of multiple individual spores by differential interference contrast (DIC) microscopy was done as described previously (22, 23). Briefly, heat-activated spores (1 μl; ~10^8 spores/ml of water) were spread on the surface of a microscope coverslip that was then dried in a vacuum desiccator for 5 to 10 min. Coverslips were then mounted on and sealed to a microscope sample holder kept at 37°C. The DIC microscope was set such that the polarizer and analyzer were crossed and thus the DIC bias phase was zero. After the addition of preheated (37°C) germinant-buffer solution (10 mM L-valine in 25 mM K-HEPES buffer, pH 7.4) to spores on the coverslips, a digital charge-coupled device camera (16 bit, 1,600 by 1,200 pixels) was used to record the DIC images at a rate of 1 frame/15 s for 60 to 120 min. The images obtained were analyzed with a computation program in Matlab to locate each spore's position and to calculate the average pixel intensity of an area of 20 by 20 pixels that covered the whole individual spore in the DIC image. The DIC image intensity of each individual spore was plotted as a function of the incubation time (with a resolution of 15 s), and the initial intensity at T_0 (the first DIC image recorded after the addition of the germinant) was normalized to 1 and the intensity at the end of measurements was normalized to 0. These data allowed the determination of kinetic parameters of the germination of individual spores defined previously (22, 23), including the following: (i) T_{lag}, the time between germinant addition and initiation of fast CaDPA release; (ii) $T_{release}$, the time of completion of fast CaDPA release; (iii) $\Delta T_{release}$, equal to $T_{release} - T_{lag}$; (iv) T_{lys}, the time when a spore's DIC image intensity stops decreasing and when spore cortex hydrolysis and core swelling are complete; (v) ΔT_{lys}, equal to T_{lys} minus $T_{release}$; (vi) I_{lag}, the relative DIC image intensity at T_{lag}; (vii) $I_{release}$, the relative DIC image intensity at $T_{release}$.

Analysis of DPA accumulation during the sporulation of various strains was carried out in liquid 2× SG medium at 37°C. Cultures were inoculated to an OD$_{600}$ of 0.1 from an overnight culture (the inoculation time was defined as time zero), and at various times, 1-ml aliquots were taken, centrifuged in a microcentrifuge, washed twice with 1 ml of water, suspended in 1 ml of water, and boiled for 30 min to extract DPA. The samples were centrifuged after cooling on ice for >15 min, and the supernatant fluid was saved. The DPA in these supernatant fluids was assayed by its fluorescence with Tb^{3+} as described previously (20).

For analysis of the biotinylation or proteinase K sensitivity of germinated spores, heat-activated spores were germinated with L-valine as described above but without TbCl$_3$ and at an OD$_{600}$ of 2. After ~60 min of incubation when germination was >90% complete as determined by phase-contrast microscopy, the spores were harvested by centrifugation and suspended in a small volume, and the remaining dormant spores were removed by centrifugation through 50% HistoDenz as described previously (9).

Analytical procedures. Dormant spores were decoated to remove some protein from the spore's outer coat, as well as the outer membrane; the cortex layer surrounding the spore's IM and the core was disrupted with lysozyme, followed by mild sonication with glass beads; the lysate obtained was centrifuged at low speed to isolate the integument (I) fraction; and the supernatant fluid was centrifuged at high speed to give the IM (M) fraction and a soluble (S) fraction, all as described previously (24–26). In some cases, the sonicated, lysozyme-ruptured spores (defined as the lysate) were made 1% in SDS and 55 mM in dithiothreitol, and the mixture was stored frozen. Germinated spores were extracted similarly but without prior decoating.

SpoVAD and SpoVAEa, as well as GR subunits and the GerD protein, were detected in the lysate or various fractions of disrupted spores described above by Western blotting with specific antiserum to either SpoVAEa prepared as described above or GerD, SpoVAD, or GR subunits (4, 25, 26). The levels of these proteins in spores were determined by Western blot analyses of spore fractions or lysates, and comparisons of the intensities of protein bands with those of known amounts of the purified protein antigens with ImageJ were all done as described previously (4, 25, 26). The concentrations of the antigens were determined by measuring the OD$_{280}$ of these proteins and their predicted molar extinction coefficients at this wavelength. In a number of experiments, different amounts of protein from some spore lysates were run on the same Western blot to facilitate the quantitation of specific antigen levels in lysates from spores of different strains.

Assessment of the accessibility of SpoVAD and SpoVAEa in chemically
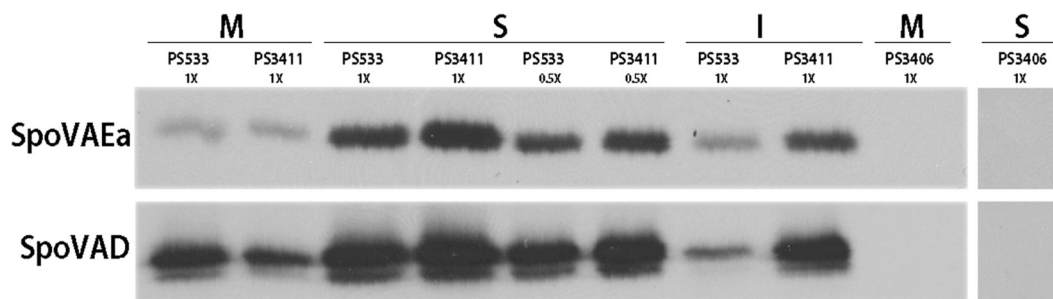
FIG 1 Detection of SpoVAEa and SpoVAD in spores and their distribution in different spore fractions. Spores of *B. subtilis* strains PS533 (wild type), PS3411 (↑ *spoVA* mutant), and PS3406 (*sleB spoVA* mutant) were purified and decoated, removing some spore coat protein, as well as the spore outer membrane. The spores were then disrupted and fractionated as described in Materials and Methods, giving the spore IM fraction (M), the soluble fraction (S) consisting of soluble proteins from the spore core, and the integument fraction (I) consisting of insoluble coat protein, partially degraded spore cortex, and IM that is not sheared off the I fraction (9, 25–27). Aliquots of the various fractions from equal numbers of spores (~10^8) were then subjected to Western blot analysis with antiserum against SpoVAEa and SpoVAD. All samples were run on the same Western blot, except the S fraction from PS3406 spores. The purified SpoVAD protein migrated identically to the SpoVAD antigen detected in spores, while the SpoVAEa antigen detected in spores ran more slowly than the His-tagged full-length SpoVAEa protein used as a marker (data not shown).

decoated dormant spores and intact germinated spores (isolated as described above) to the biotinylation agent sulfo-*N*-hydroxysuccinimide-SS-biotin (EZ-Link Sulfo-NHS-SS-Biotin reagent; Pierce) was assayed in both M fractions and lysates as described previously (9). Treatment of intact germinated spores with proteinase K (Sigma Chemical Co., St. Louis, MO), stopping of proteinase K digestion by the addition of the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF), and subsequent Western blot analysis of SpoVAD and SpoVAEa in M fractions or lysates were all done as described previously (9).

RESULTS

Characteristics and distribution of SpoVAEa in spore formers. The *spoVA* operon of *B. subtilis* was originally annotated as containing six genes, *spoVAA-spoVAF*. However, there was a sequencing error in the *spoVAE* gene in earlier work, and *B. subtilis spoVAE* actually comprises two genes now annotated as *spoVAEa* and *spoVAEb*. The gene order in the *spoVAE* region is *spoVAD-spoVAEb-spoVAEa-spoVAF*. While *spoVAEa* is present in many members of the order *Bacillales*, it is less prevalent in those of the order *Clostridiales*, although the amino acid sequence of SpoVAEa is well conserved in those species that have a *spoVAEa* gene, with 42% of all residues conserved ≥86% in the 36 species examined (see Fig. S1A and Table S1 in the supplemental material; data not shown). However, the SpoVAEa sequence shows no obvious similarity to other proteins in available databases. Notably, a *spoVAF* gene is routinely associated with a *spoVAEa* gene only in *Bacillus* species and is found only infrequently with a *spoVAEa* gene in genera such as *Alicyclobacillus*, *Amphibacillus*, *Brevibacillus*, *Geobacillus*, *Lysinibacillus*, and others, as well as members of the order *Clostridiales* (7; data not shown). In contrast, the gene upstream of *spoVAEa*, *spoVAEb*, is found in the genomes of all of the spore-forming members of the orders *Bacillales* and *Clostridiales* (7), and the sequence of the SpoVAEb protein is also well conserved, with ~35% of all residues conserved ≥86% in the 26 species examined (see Fig. S1B and Table S2 in the supplemental material).

Like many of the *spoVA*-encoded proteins, SpoVAEb is predicted to be relatively hydrophobic and is further predicted to have four membrane-spanning domains by multiple prediction programs (data not shown). In contrast, the SpoVAEa protein is predicted to be relatively hydrophilic and its sequence contains no obvious predicted membrane-spanning domains (data not shown). In the latter regard, SpoVAEa is similar to the SpoVAD protein, which also lacks any obvious membrane-spanning or attachment signals, even though SpoVAD has been shown to associate with the spore's IM (4). SpoVAD is also a soluble globular protein and has recently been shown to be on the outer surface of the spore's IM (6, 9). Given that SpoVAEa is a soluble protein, it was thus of interest to determine if this protein is also associated with the spore's IM and, if so, whether the protein is on the outer or the inner surface of this membrane.

Detection, location, and quantitation of SpoVAEa in spores. In order to detect SpoVAEa in spore extracts, the *B. subtilis* ΔspoVAEa gene was cloned and the ΔSpoVAEa protein, which lacks amino acids 124 to 135 of the SpoVAEa protein (see Materials and Methods), was overexpressed in *E. coli*. The protein was soluble and was purified and used to immunize rabbits to generate a polyclonal antiserum against SpoVAEa. The antiserum was then purified and used in a Western blot analysis of various fractions of dormant wild-type and ↑ *spoVA* and *spoVA sleB* mutant spores (Fig. 1). A band corresponding to the expected size of SpoVAEa was not detected in the material removed from wild-type spores by the decoating procedure used prior to spore lysis and subsequent fractionation (data not shown), as also found previously for SpoVAD (4). However, a band of ~22 kDa, the expected size of SpoVAEa, was detected in wild-type spore fractions, but this band was absent from *spoVA* mutant (strain PS3406) spore fractions. The intensity of the putative SpoVAEa band was also significantly higher in the soluble (S) and integument (I) fractions of spores of strain PS3411, in which the *spoVA* operon is overexpressed ~5-fold from the strong forespore-specific P*sspB* promoter (4). SpoVAD was also absent from *spoVA sleB* mutant spores, and its level was elevated in ↑ *spoVA* mutant spores, as expected (4). Thus, the band detected by the anti-SpoVAEa serum was almost certainly SpoVAEa.

While we expected that most SpoVAEa protein would be associated with the spore's outer layer, the coat, cortex, outer membrane, or IM on the basis of previous localization experiments with intact spores (10), most was actually found in the S fraction, which previous work has shown is derived largely from the spore core (9, 25, 26), with slightly less in the insoluble I fraction that pellets upon low-speed centrifugation of decoated spore lysates (Fig. 1). The I fraction contains the large amount of coat protein not removed from spores by decoating and fragments of spore
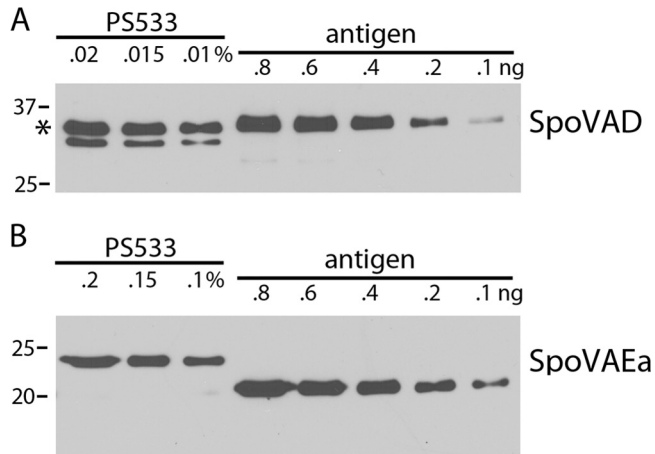
FIG 2 (A, B) Levels of SpoVAD (A) and SpoVAEa (B) in wild-type spores. Various amounts of the total lysate from 8.2×10^8 spores of strain PS533 (wild type) were subjected to Western blot analysis as described in Materials and Methods alongside various amounts of purified SpoVAD or SpoVAEa protein. (A) The intensities of the SpoVAD band in various amounts of the lysate (upper band at 36 kDa [asterisk]) were compared to the intensities of known amounts of purified SpoVAD. (B) The intensities of the SpoVAEa band at 23 kDa in various amounts of lysate were compared to the intensities of various amounts of purified ΔSpoVAEa that ran at ~20 kDa. The molecular mass of the purified ΔSpoVAEa protein was lower than that of the SpoVAEa protein in the lysate, probably because the purified ΔSpoVAEa protein had an internal deletion, as described in Materials and Methods. Analysis of the various band intensities by ImageJ gave values of 5,800 molecules of SpoVAD/spore and 740 molecules of SpoVAEa/spore. The numbers of SpoVAD and SpoVAEa molecules per spore determined in three independent Western blot analyses were all within ±20% of these values. The values to the left of both panels are molecular sizes in kilodaltons.

cortex peptidoglycan, as well as much of the spore's IM that is not sheared off the spore cortex during fractionation (25–27). Similar results were obtained when the same Western blot was probed for SpoVAD (Fig. 1). These results were not unexpected on the basis of recent work that also found much SpoVAD in the spore's soluble fraction and further that much spore IM and integral IM proteins partition with the I fraction (27). Presumably, the hydrophilic SpoVAD and SpoVAEa proteins are only loosely bound to the IM in intact spores and readily dissociate in spore lysates. Analysis of the levels of SpoVAD and SpoVAEa in the various spore fractions gave a value of ~6,000 molecules of SpoVAD per spore, similar to the value determined recently (27), while the level of SpoVAEa was lower, at ~750 molecules per spore (Fig. 2).

Topology of SpoVAEa in spores. Previous work has shown that SpoVAD is largely, if not completely, on the outer surface of the intact decoated spore's IM, as in germinated spores this protein is accessible to a biotinylation agent, as well as to proteinase K, while a soluble spore core protein is not (9). Analysis of the labeling of the SpoVAEa and SpoVAD proteins by this same biotinylation agent found that the biotinylation of both proteins in dormant and germinated wild-type spores was quite similar, as ~40% of both proteins was biotinylated in dormant spores, with ~65% of both proteins biotinylated in germinated spores (Fig. 3A and B). Similar results were obtained when proteins obtained in the M fraction (Fig. 3A and B) or the spore lysate were examined (data not shown). SpoVAE and SpoVAD were also both sensitive to proteinase K in intact germinated spores when proteins in either the M fraction (Fig. 3C) or the spore lysate were examined (data
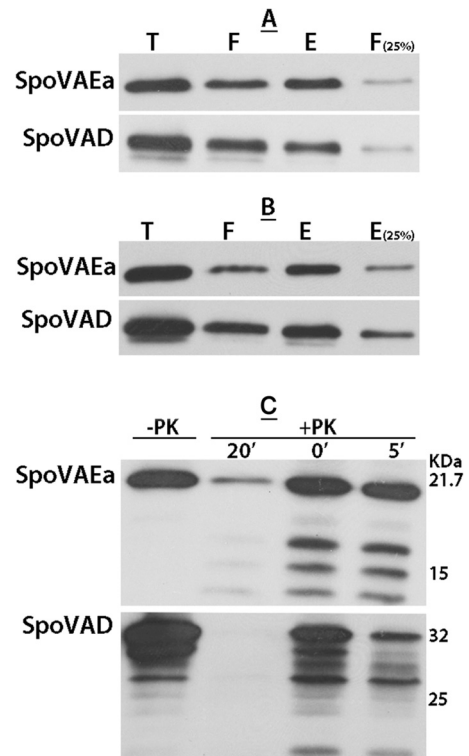

FIG 3 (A to C) Levels of biotinylation of SpoVAEa and SpoVAD in dormant (A) and germinated (B) spores and proteinase K susceptibility of SpoVAEa and SpoVAD in germinated spores (C). Decoated dormant (A) or intact germinated (B) spores of *B. subtilis* strain PS533 (wild type) were biotinylated, the biotinylation reagent was quenched, spores were lysed, IM fractions were isolated, and proteins were solubilized, giving the total (T) fraction. These proteins were adsorbed to NeutrAvidin beads (Pierce); proteins that did not absorb were termed the flowthrough (F) fraction, and proteins eluted with dithiothreitol were termed the eluted (E) fraction. Aliquots of these three fractions from the same amount of spores, as well as 25% of the amounts of the F fraction (A) and the E fraction (B) were subjected to Western blot analysis with either anti-SpoVAEa or anti-SpoVAD serum. ImageJ analysis of the intensities of the bands showed that the levels of biotinylation of SpoVAEa in dormant and germinated spores were 40 and 65%, respectively, while the levels of SpoVAD biotinylation in dormant and germinated spores were 36 and 65%, respectively. (C) Germinated *B. subtilis* PS533 (wild-type) spores were treated with proteinase K (25 μg/ml) for various times, and proteinase K was inactivated as described in Materials and Methods immediately after addition or after 5 or 20 min of digestion at 25°C. IMs were then isolated, and equal aliquots of the IM fraction were subjected to Western blot analysis with antiserum against SpoVAEa or SpoVAD. Note that some SpoVAD and SpoVAEa was digested with proteinase K even if PMSF was added immediately after the protease was added, presumably because proteinase K inactivation was not instantaneous.

not shown). Importantly, the conditions used for the biotinylation of dormant and germinated spores and the proteinase K treatment of germinated spores have been shown not to biotinylate or digest a soluble spore core protein, in particular, one that is very proteinase K sensitive in spore extracts (9; data not shown). These results together indicate that, like SpoVAD, SpoVAEa is also on the outer surface of the spore's IM.

Lack of essential function of SpoVAEa and SpoVAF in sporulation. The fact that SpoVAEa is not encoded in the genomes of many spore-forming members of the orders *Bacillales* and *Clostridiales* suggests that this protein may not be essential for DPA movement in *B. subtilis* spore formation and germination, as is
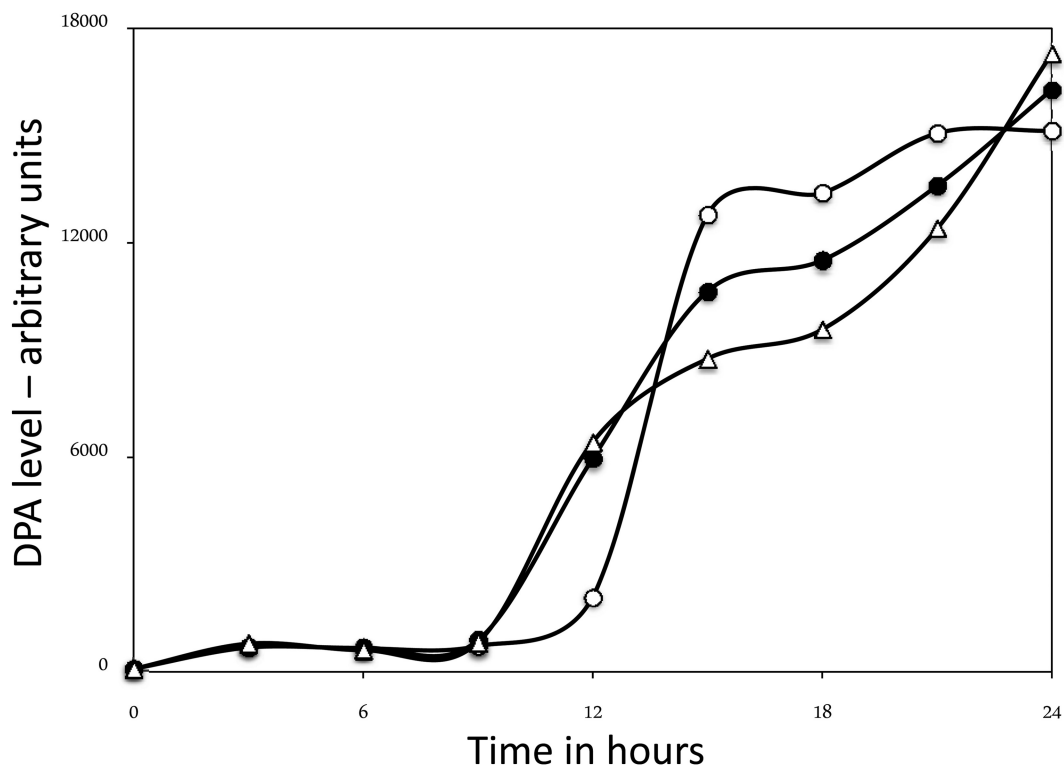
FIG 4 DPA accumulation during sporulation of wild-type and *spoVAEa* and *spoVAF* mutant spores. Spores of strains PS533 (wild type), PS4348 (*spoVAEa* mutant), and PS4351 (*spoVAF* mutant) were sporulated in liquid $2 \times$ SG medium and 37°C. At various times, samples were taken, washed, and extracted and DPA was analyzed in extracts as described in Materials and Methods. Time zero is the time when cultures were inoculated to an OD_{600} of ~0.1. Symbols: ○, wild type; ●, *spoVAEa* mutant; △, *spoVAF* mutant.

also reported to be the case for SpoVAF (2). To test SpoVAEa's function in spore formation in light of evidence that SpoVA proteins are important in DPA uptake by the developing spore, a *spoVAEa* deletion replacement mutation was integrated into the wild-type *B. subtilis* chromosome, giving strain PS4348. Analysis of the sporulation of this strain indicated that the strain sporulated normally and accumulated DPA levels in the resultant spores that were within ~5% of those in the parental wild-type spores and that the rate of DPA accumulation by developing forespores during sporulation of the *spoVAEa* strain was quite similar to that of the wild-type strain (Fig. 4; data not shown). Spores of the *spoVAEa* mutant strain lacked SpoVAEa, as expected, but had normal levels of SpoVAD (Fig. 5A). Levels of GerD and the GR subunits GerAA, GerAC, GerBC, and GerKA were also normal in *spoVAEa* mutant spores when spore lysates were examined by Western blot analysis with various specific antisera (data not shown). Since the mutation generating the *spoVAEa* strain would be expected to eliminate the expression of *spoVAF*, we also constructed a *spoVAF* strain. The latter strain had normal SpoVAD and SpoVAEa levels, as expected (Fig. 5B), and also accumulated DPA during sporulation relatively similarly to the wild-type strain and to essentially wild-type levels in spores (Fig. 4; data not shown).

Germination of *spoVAEa* and *spoVAF* mutant spore populations. While the sporulation, in particular the DPA accumulation, of strains lacking SpoVAEa and/or SpoVAF was normal, there is evidence that SpoVA proteins are also involved in DPA release during spore germination (5). Consequently, it was of interest to examine the germination of *spoVAEa* and *spoVAF* mutant spores.

Germination of *B. subtilis* spores can be triggered either by a nutrient germinant such as L-valine or the AGFK mixture via GRs located in the spore's IM or by compounds that do not act via GRs, including CaDPA and dodecylamine (1, 3). The *spoVAEa* and *spoVAF* mutant spores germinated essentially identically to wild-type spores with the non-GR-dependent germinants CaDPA and dodecylamine (Fig. 6A and B; data not shown). However, the germination of the *spoVAEa* and *spoVAF* mutant spores with the GR-dependent nutrient germinant L-valine was slower than that of wild-type spores (Fig. 6C), with the *spoVAEa* mutant spores exhibiting the slowest germination. The *spoVAEa* mutant spores also exhibited slower germination than wild-type spores with AGFK, although *spoVAF* mutant spores did not (Fig. 6D). These mutant spore germination phenotypes were complemented by the ectopic expression of *spoVEAa* and *spoVAF* (see below). The slower valine germination of the *spoVAEa* and *spoVAF* mutant spores was also seen when germination was measured solely by phase-contrast microscopy (data not shown). In the latter experiment, it took ~1 h for wild-type spore germination with L-valine to reach ~95% completion, while *spoVAEa* and *spoVAF* mutant spores took 3 to 4 h to reach ~95% completion of L-valine germination (data not shown). This overall germination behavior was seen with several independent preparations of *spoVAEa* and *spoVAF* mutant spores, and the dependence of the rate of *spoVAEa* mutant spore germination on the L-valine concentration was essentially identical to that of wild-type spores (data not shown).

The slower germination of *spoVAEa* mutant spores than wild-type spores with GR-dependent germinants was also seen when
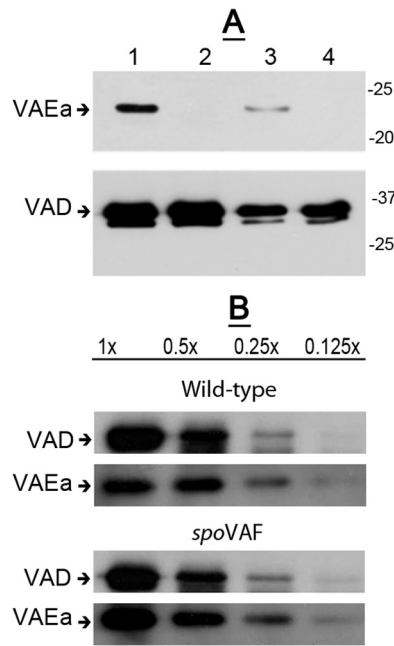
FIG 5 (A, B) Levels of SpoVAD and SpoVAEa in lysates of wild-type and *spoVAEa* and *spoVAF* mutant spores. Spores of *B. subtilis* strains PS533 (wild type) (A, B), PS4348 (*spoVAEa* mutant) (A), and PS4351 (*spoVAF* mutant) (B) were decoated, and lysates were prepared as described in Materials and Methods. Various amounts of protein in the lysates from the spores of the two strains (~5 × 10^7 spores for analysis of SpoVAEa or ~5 × 10^6 spores [each defined as 1×] for analysis of SpoVAD) were analyzed by Western blotting with antiserum against either SpoVAEa or SpoVAD as described in Materials and Methods. The lanes in panel A are as follows: 1, 1× protein from wild-type spores; 2, 1× protein from *spoVAEa* mutant spores; 3, 0.25× protein from wild-type spores; 4, 0.25× protein from *spoVAEa* mutant spores. The arrows labeled VAEa and VAD on the left of the panels denote the migration positions of SpoVAEa and SpoVAD, respectively, and the values to the right of panel A are the masses of molecular size marker proteins in kilodaltons.

germination was triggered by 150 MPa of HP, which also acts via GRs (1, 3), although the germination of the *spoVAF* mutant spores was only slightly slower than that of wild-type spores (Fig. 6E and G). However, with a HP of 550 MPa, which acts primarily on the SpoVA protein channel and largely in a GR-independent manner (1, 3), the germination of the spores of the two mutant strains was only slightly slower than that of wild-type spores (Fig. 6F and H).

Germination of individual *spoVAEa* mutant spores. It was clear from the results described above that SpoVAEa, SpoVAF, or both proteins are important in normal GR-dependent spore germination. However, these experiments measured the germination of spore populations and additional information can be gleaned about the germination process by examining the germination of individual spores (22, 23). In particular, a variety of kinetic germination parameters can be obtained by monitoring the germination of individual spores. Consequently, we compared the L-valine germination of large numbers of wild-type and *spoVAEa* mutant spores (Fig. 7A to C; Table 2). As expected from analyses of the L-valine germination of spore populations, the germination of individual *spoVAEa* mutant spores was also slower than that of wild-type spores (Fig. 7A) and a significant percentage of the *spoVAEa* mutant spores did not germinate in the observation period. Examination of the DIC image intensity curves of large numbers of individual germinating wild-type and *spoVAEa* mutant spores

further allowed the determination of the average values of the germination kinetic parameters T_{lag}, T_{release}, $\Delta T_{\text{release}}$, and ΔT_{lys}, which were defined in Materials and Methods (Fig. 7B and C; Table 2). Notably, the average T_{lag}, the time prior to initiation of rapid CaDPA release, of *spoVAEa* mutant spores was >2-fold longer than that of wild-type spores, and the difference is actually larger than 2-fold, as while 97% of wild-type spores germinated during the observation period, only 74% of the *spoVAEa* mutant spores did so and these spores thus have T_{lag} values of >90 min. The *spoVAEa* mutation also had effects on the average $\Delta T_{\text{release}}$ and ΔT_{lys} values, which are the times for fast CaDPA release and for cortex lysis plus core expansion, respectively (Table 2). However, these effects were relatively small, certainly smaller than the >2-fold effects on the average T_{lag}, and the effects on the $\Delta T_{\text{release}}$ and ΔT_{lys} values are probably not significant.

The analyses of multiple individual spores also revealed that because of their longer average T_{lag}, the *spoVAEa* mutant spores exhibited a period of a slow decrease in DIC image intensity prior to T_{lag} that was much longer than that of wild-type spores. As a consequence, the *spoVAEa* mutant spore I_{lag} value, the average relative DIC image intensity at T_{lag}, was much lower than that of wild-type spores, although the average DIC image intensities at T_{release}, the I_{release} values, were relatively similar for wild-type and *spoVAEa* mutant spores. However, the precise meaning of the slow decrease in DIC image intensity prior to T_{lag} for either wild-type or *spoVAEa* mutant spores is not clear (see Discussion).

Commitment of *spoVAEa* and *spoVAF* mutant spores to germination. The analysis of the germination of multiple individual wild-type and *spoVAEa* mutant spores noted above indicated that the defect in the mutant spores was their much longer average T_{lag} values. One event that is known to take place in T_{lag} is the process of commitment, whereby a spore is committed to continue through germination even if a nutrient germinant is removed (20). Therefore, we examined the kinetics of commitment in L-valine germination of wild-type and *spoVAEa* and *spoVAF* mutant spores (Fig. 8A and B). As found previously (20), commitment took place 5 to 15 min before CaDPA release with wild-type spores. However, the average times between commitment and germination were 2- to 3-fold longer for *spoVAEa* and *spoVAF* mutant spores, respectively. Given the much slower germination of these two mutant spore types, it thus appears that the defect in these mutant spores is primarily in the period prior to commitment, especially since the length of time between commitment and CaDPA release is larger when germination is slower (20).

Complementation of the germination defect of *spoVAEa* and *spoVAF* mutant spores. The results described above suggested that perhaps both SpoVAEa and SpoVAF are essential for normal DPA release during spore germination by nutrients. To further examine this possibility, we integrated copies of *spoVAEa*, *spoVAF*, or both genes under the control of either the *spoVA* promoter or the very strong forespore-specific P*sspB* promoter at the *amyE* locus of the *spoVAEa* and *spoVAF* mutant strains and examined the L-valine germination and SpoVAEa levels of spores of the resultant strains (Fig. 9; data not shown). The results showed that ectopic expression of *spoVAEa* plus *spoVAF* under P*sspB* control restored relatively normal germination and ~5-fold higher-than-normal SpoVAEa levels in *spoVAEa* or *spoVAF* mutant spores, while expression of either *spoVAEa* or *spoVAF* alone did not (data not shown). Surprisingly, ectopic expression of *spoVAEa* alone from P*sspB* gave levels of SpoVAEa in spores that were ~10-fold
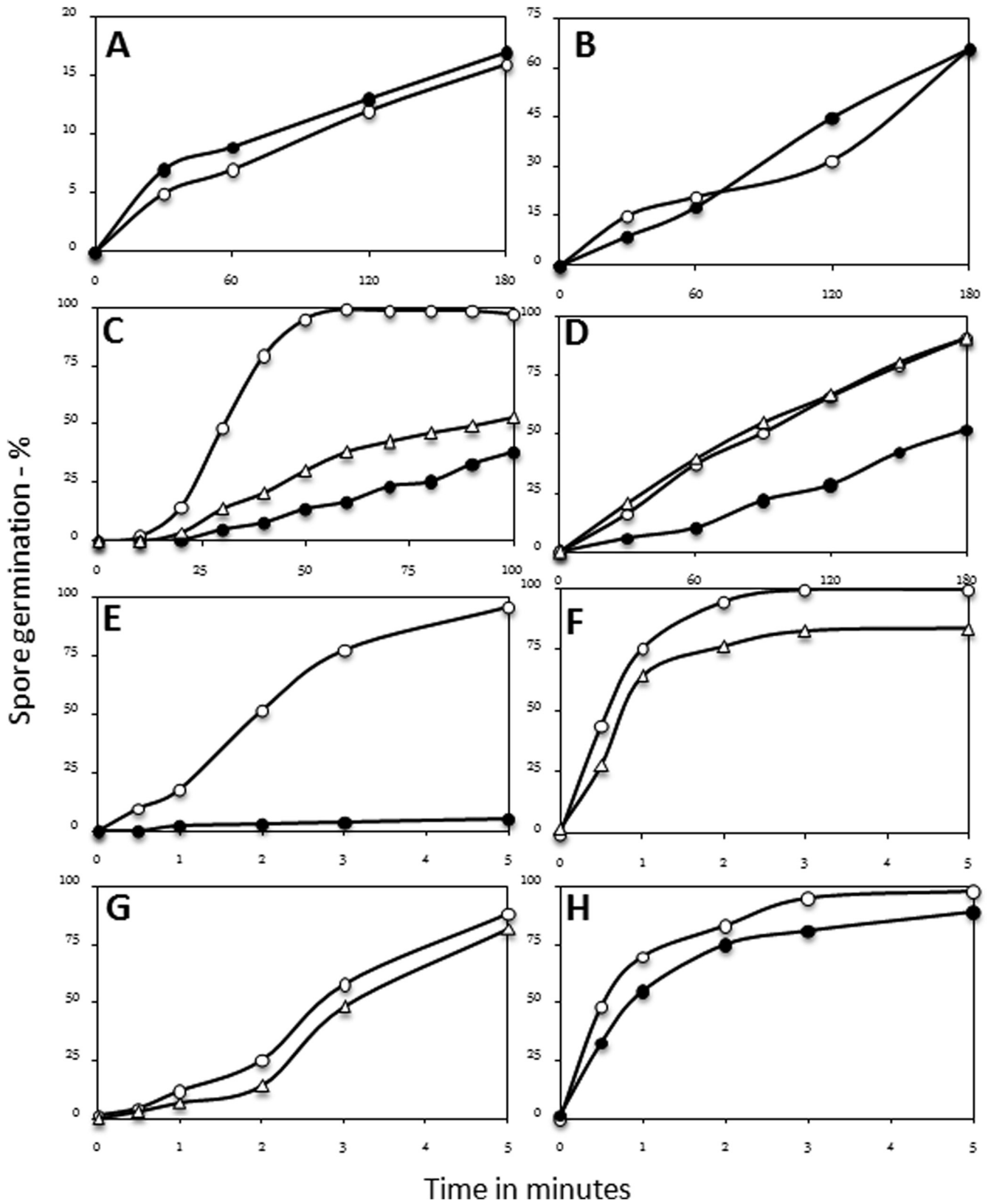
FIG 6 (A to H) Germination of wild-type and *spoVAEa* and *spoVAF* mutant spores with various agents. Wild-type (PS533) and *spoVAEa* (PS4348) and *spoVAF* (PS4351) mutant spores were germinated with CaDPA (A), dodecylamine (B), L-valine (C), AGFK (D), HP at 150 MPa (E, G), or HP at 550 MPa (F, H), and spore germination was assessed as described in Materials and Methods. Symbols: ○, wild-type spores; ●, *spoVAEa* mutant spores; △, *spoVAF* mutant spores.
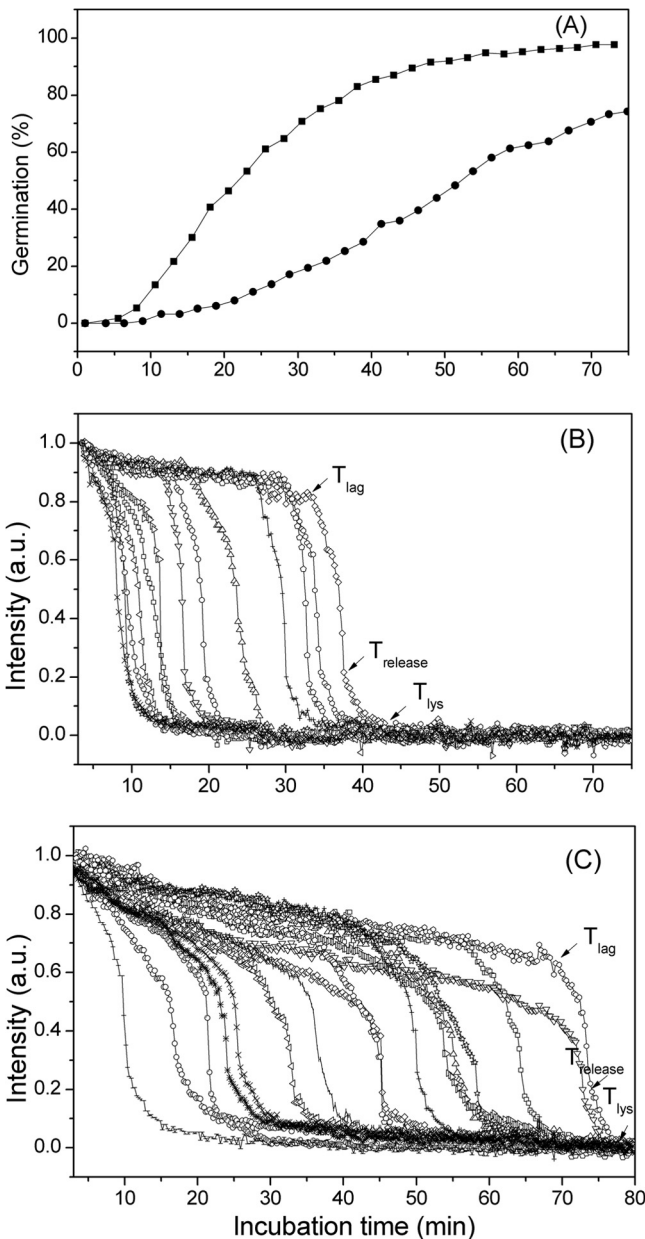
FIG 7 (A to C) Analysis of the ʟ-valine germination of individual wild-type and *spoVAEa* mutant *B. subtilis* spores. Heat-inactivated spores of strains PS533 (wild type) and PS4348 (*spoVAEa* mutant) were germinated with ʟ-valine, the germination of individual spores was monitored by DIC microscopy, and various germination parameters were determined as described in Materials and Methods and illustrated in panels B and C. (A) The percent germination of >300 individual wild-type (■) and *spoVAEa* mutant (●) spores was monitored for 75 to 80 min. (B, C) The DIC image intensities of 13 to 16 randomly chosen wild-type (B) or *spoVAEa* mutant (C) spores were monitored for 75 to 80 min. The T_{lag}, $T_{release}$, and T_{lys} values of one selected spore are shown in panels B and C. a.u., arbitrary units.

higher than when *spoVAEa* and *spoVAF* were expressed together from P*sspB* (data not shown). Ectopic expression of *spoVAEa* and *spoVAF* from P*spoVA* in the *spoVAEa* mutant strain gave no complementation of the spore germination defect, and Western blot analysis showed that the levels of SpoVAEa in these spores were <5% of the levels in wild-type spore (data not shown). Unfortu-

nately, we have no antiserum against SpoVAF, so the levels of this protein in spores of the strains complemented by *spoVAF* expression alone or with *spoVAEa* are not known.

DISCUSSION

There are a number of interesting features of the SpoVAEa protein. First, SpoVAEa is made in the forespore, where the *spoVA* operon is transcribed, and is present on the outer surface of the spore's IM yet has no obvious signal sequence or transmembrane segments that might direct this hydrophilic protein to the IM's outer surface. In this regard, SpoVAEa behaves similarly to the hydrophilic SpoVAD protein. Second, SpoVAEa distribution in spore fractions is similar to that of the SpoVAD protein, which has been shown to be an IM protein by several methods (4, 10). However, SpoVAEa and SpoVAD are bound only loosely to the IM, since in fractionated spore lysates most of these proteins are found in the supernatant fraction, although SpoVAD and SpoVAEa do not interact directly, as assessed in a pulldown assay (6). This lack of interaction in a pulldown assay has also been seen between SpoVAEa and the auxiliary germination protein GerD (Y.-Q. Li and B. Hao, unpublished data). In contrast to the hydrophilic SpoVAD and SpoVAEa proteins, the other SpoVA proteins are most likely to be integral IM proteins. While the arrangement of the SpoVA proteins in the IM is not known, it seems likely that (i) all of these proteins are in a complex in the IM, with SpoVAD and SpoVAEa on the outer surface; and (ii) this protein complex comprises a channel for CaDPA movement into and out of the developing and germinating spore.

Third, the level of SpoVAEa in spores is much lower than that of SpoVAD. While the reason for this is not clear, the *spoVAEa* mRNA may be translated more poorly than the *spoVAD* mRNA, as the RBS for the latter gene is a good match to the optimal RBS for translation of *B. subtilis* mRNAs, as is the RBS for *spoVAEb* (28) (Fig. 10). In contrast, the *spoVAEa* RBS is by no means a perfect match to the optimal RBS, and this is also the case for the *spoVAF* RBS (Fig. 10).

The other notable feature of SpoVAEa is that this protein is not essential for DPA uptake in *B. subtilis* sporulation, as is also the case for SpoVAF, while SpoVAA, -B, -C, -D, and -Eb are essential (2, 4, 6). Notably, not only do several temperature-sensitive *spoVAC* mutants exhibit alterations in DPA uptake during sporulation at the nonpermissive temperature, but spores of these mutants made at the permissive temperature also exhibit slow DPA release during germination (5, 29). *spoVAEa* and *spoVAF* mutant spores do not exhibit any major defect in DPA uptake in sporulation, while *spoVAEa* mutant spores and, to a slightly lesser extent, *spoVAF* mutant spores do exhibit germination defects, but only in GR-dependent germination. A possible function of these proteins is thus some role in linking GR activation to the opening of the SpoVA DPA channel, although SpoVAEa and SpoVAF are clearly not essential for the opening of this channel. This lack of an essential role for SpoVAEa and SpoVAF in spore formation and germination is consistent with the absence of the *spoVAEa* and *spoVAF* genes in many members of the orders *Bacillales* and *Clostridiales*.

Probably the major findings from this overall work are that SpoVAEa, SpoVAF, or both proteins (i) are not essential for normal DPA uptake in sporulation (ii) but are essential for normal DPA release in GR-dependent spore germination, although not in GR-independent germination. The latter finding is consistent with SpoVA proteins being components of a DPA channel in the

TABLE 2 Kinetic parameters of the L-valine germination of wild-type and *spoVAEa* mutant spores[a]

Spores	T_{lag} (min)	$T_{release}$ (min)	$\Delta T_{release}$ (min)	ΔT_{lys} (min)	I_{lag}	$I_{release}$
PS533 (wild type)	21 ± 14[b]	25 ± 14	3.8 ± 1.2	7 ± 4	0.81 ± 0.08	0.21 ± 0.07
PS4348 (*spoVAEa*)	45 ± 18[c]	49 ± 19	4.9 ± 1.8	10 ± 6	0.60 ± 0.09	0.16 ± 0.07

[a] The germination of individual spores with L-valine was analyzed as described in Materials and Methods, and kinetic parameter values were determined as described previously and as indicated in Fig. 7B and C.

[b] Spores were observed for 90 min, and the data are average values and standard deviations from analyses of ≥80 spores that germinated.

[c] This value is a significant underestimate of the T_{lag} value for the entire *spoVAEa* mutant spore population, since only 74% of the spores examined germinated during the 90-min observation period while 97% of the wild-type spores did so.

spore's IM, with a possible function of SpoVAEa being to coordinate communication between GRs and the gating of this DPA channel, as suggested above. The effect of SpoVAEa, SpoVAF, or both proteins appears to be to decrease the T_{lag} value for GR-dependent germination, while the CaDPA release process itself, as measured by $\Delta T_{release}$ values, was relatively unaffected by a *spoVAEa* mutation. The absence of SpoVAEa also lengthens the time between germinant addition and commitment. This was also seen previously when times for commitment and DPA release for spores of several mutants in the *spoVAC* gene were determined, but this may be because there are generally longer times between commitment and germination with lower rates of germination

(20, 29). The fact that ectopic expression of *spoVAEa-spoVAF* could complement deletions of *spoVAF* or *spoVAEa* is consistent with the proteins they encode together playing a role in nutrient germination, although this interpretation is not definitive since (i) we cannot analyze levels of SpoVAF in spores and (ii) expression of *spoVAF* or *spoVAEa* alone did not complement the L-valine germination defect of *spoVAEa* or *spoVAF* mutant spores. However, the larger effect on L-valine germination of the loss of both SpoVAEa and SpoVAF than that of the loss of SpoVAF alone, the minimal effect of the loss of SpoVAF alone on AGFK and HP germination at 150 MPa, and the very large effect of the loss of both SpoVAEa and SpoVAF on HP germination at 150 MPa all
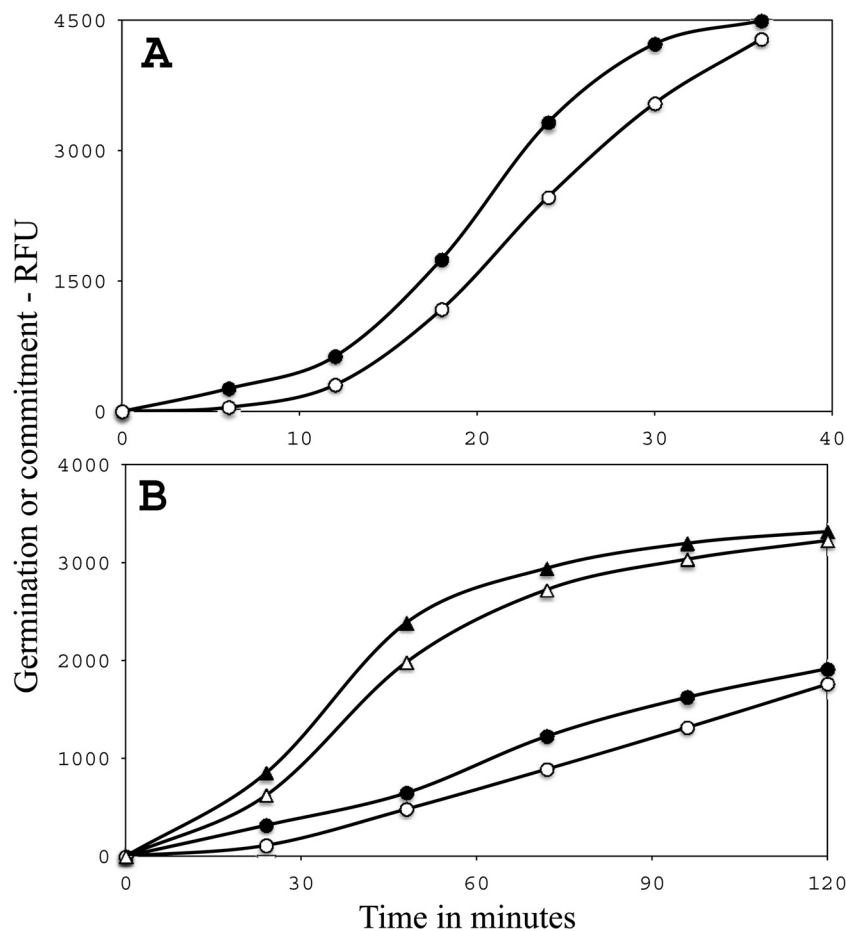

FIG 8 (A, B) Commitment to germination of spores of various strains. Spores of strain PS533 (wild type) (A) and strains PS4348 (*spoVAEa* mutant) (○, ●) and PS4351 (*spoVAF* mutant) (△, ▲) (B) were germinated with L-valine, and both commitment (●, ▲) and CaDPA release (○, △) were measured as described in Materials and Methods. Measurement of the times between commitment and CaDPA release for wild-type spores at 6, 12, 28, 24, and 30 min (A) and *spoVAEa* and *spoVAF* mutant spores at 24, 48, 72, and 96 min (B) gave average values of ~5, ~10, and ~14 min, respectively. RFU, relative fluorescence units.
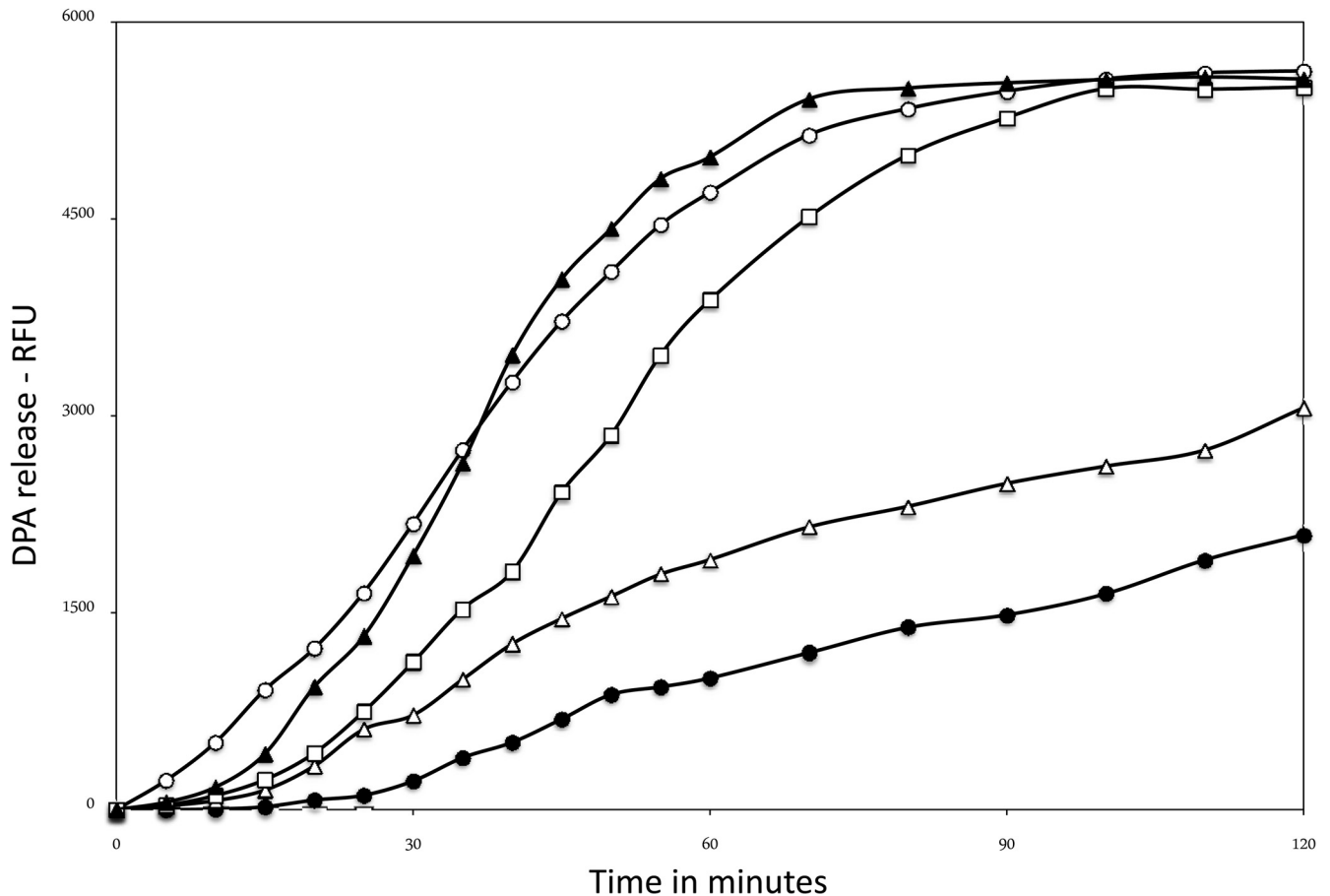
FIG 9 Nutrient germination of *spoVAEa* or *spoVAF* mutant spores with or without ectopic expression of *spoVAEa* and *spoVAF*. Heat-activated spores of various strains were germinated with L-valine, and DPA release was measured by determining Tb-DPA fluorescence as described in Materials and Methods. Results are presented in relative fluorescence units (RFU). Numbers of RFU of spores of different strains were corrected for the amounts of DPA in different spore preparations. Symbols: ○, PS832 (wild type); ●, PS4348 (*spoVAEa* mutant); △, PS4351 (*spoVAF* mutant); □, PS4391 (*spoVAEa* P*sspB-spoVAEa-spoVAF* mutant); ▲, PS4392 (*spoVAF* P*sspB-spoVAEa-spoVAF* mutant).

suggest that both of these SpoVA proteins are involved in some fashion in GR-dependent spore germination.

One of the intriguing results of this work is that the main effect of loss of SpoVAEa on spore germination was a much longer slow decrease in the spore's DIC image intensity prior to the end of T_{lag}. Previous work suggested that this slow DIC image intensity de-

spoVAC-spoVAD	*TA*GGAGGAGAAGAAA**ATG**
spoVAD-spoAEb	AGGAGGTGCATCT*TAAT***ATG**
spoVAEb-spoVAEa	AAACCGAAAGGA*TAAT*GCCGAC**ATG**
spoVAEa-spoVAF	AAAGGAGCGGGTATC**ATG**
Optimal	AAAGGAGGTG-(7)-**ATG**

FIG 10 Intergenic regions of the *spoVA* operon showing translational start regions for *spoVAD*, *spoVAEb*, and *spoVAEa*. Triplets in italics are the stop codons for the upstream gene, the translation initiation codon for the downstream gene is in bold, and underlined residues are matches to the optimal *B. subtilis* RBS (28), which is shown below the sequence along with the optimal spacing between the RBS and the translation initiation codon. Note that the translation stop codon for *spoVAEa* is within the coding sequence of *spoVAF*.

crease is largely due to slow CaDPA release, and therefore the absence of SpoVAEa greatly prolongs this slow CaDPA release compared to that in wild-type spores. Interestingly, this same extended slow CaDPA release was also observed in the nutrient germination of spores with one of several mutations in the *spoVAC* gene (29). The major questions that arise from these observations are then (i) how is this slow CaDPA release mediated by GR activation, (ii) how does the presence of SpoVAEa lead to a more rapid transition between slow CaDPA release and the rapid CaDPA release in $\Delta T_{release}$, and (iii) mechanistically, what is sensed in the slow CaDPA release period that ultimately triggers very rapid CaDPA release? It is also notable that, given the longer average T_{lag} in L-valine germination of *spoVAEa* mutant spores and that commitment of wild-type spores to germination occurs only a few minutes prior to fast CaDPA release (20), there is still much CaDPA release from *spoVAEa* mutant spores in particular, even before commitment takes place. Consequently, a major period when important events are taking place during spore germination is the period prior to rapid CaDPA release, and understanding what is taking place in spores during this period will be crucial to a more complete understanding of spore germination.

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