The interaction between coat morphogenetic proteins SafA and SpoVID

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Abstract

Morphogenetic proteins such as SpoVID and SafA govern assembly of the Bacillus subtilis endospore coat by guiding the various protein structural components to the surface of the developing spore. Previously, a screen for peptides able to interact with SpoVID led to the identification of a PYYH motif present in the C-terminal half of the SafA protein, and to the subsequent demonstration that SpoVID and SafA directly interact. spoVID and safA spores show deficiencies in coat assembly and are lysozyme-susceptible. Both proteins, orthologs of which are found in all Bacillus species, have LysM domains for peptidoglycan binding and localize to the cortex-coat interface. Here, we show that the interaction between SafA and SpoVID involves the PYYH motif (region B) but also a 13 amino acid region (or region A) just downstream of the N-terminal LysM domain of SafA. We show that deletion of region B does not block the interaction of SafA with SpoVID nor does it bring about spore susceptibility to lysozyme. Nevertheless, it appears to reduce the interaction and affects the complex. In contrast, lesions in region A impaired the interaction of SafA with SpoVID in vitro, and while not affecting the accumulation of SafA in vivo, interfered with the localization of SafA around the developing spore, causing aberrant assembly of the coat and lysozyme sensitivity. A peptide corresponding to region A interacts with SpoVID, suggesting that residues within this region directly contact SpoVID. Since region A is highly conserved among SafA orthologs, this motif may be an important determinant of coat assembly in the group of Bacillus sporeformers.
Introduction

A large number of proteins produced in the mother cell of sporulating cells of *B. subtilis* are targeted to the surface of the developing spore to form a structure known as the spore coat (11, 16, 18). Assembly of the coat confers protection against lytic enzymes and small noxious molecules, and allows efficient interaction of the spore with compounds able to trigger germination (11, 16, 18). Because of the resistance it imparts to the action of the lytic enzymes, the coat also protects phagocytosed spores from digestion by predatory microorganisms (22). Structurally, the coat consists of an amorphous undercoat that contacts the underlying cortex peptidoglycan (PG), a lamellar inner layer, and a thick electron-dense outer layer (11, 16, 18). The genes encoding the various coat components are expressed at different times during the deployment of a mother cell cascade of gene expression, and this temporal control is an important factor contributing to the ordered assembly of the coat (16). However, assembly of the coat relies to a large extent on the action of a class of so-called morphogenetic proteins that guide the assembly of the structural components (11, 16, 18, 29). One, called SpoIVA, localizes early to the mother cell side of the asymmetric septum, and is responsible for recruiting another morphogenetic protein, SpoVID, to the developing spore (11, 12, 33). A third morphogenetic protein, CotE, localizes in a SpoIVA-dependent manner close to the developing spore, around a region of unknown composition called the matrix (11, 12). The localization of SpoIVA, SpoVID, and CotE take place prior to the complete engulfment of the prespore by the mother cell when σE
and the ancillary transcription factors SpoIIID and GerR govern gene expression in the mother cell (13, 41). Following engulfment completion, and the activation of the late mother cell-specific regulators σK and later of GerE, the coat layers start gaining their final appearance (11, 13, 16, 18, 41). The prior localization of CotE at the edge of the matrix region is thought to be required to nucleate assembly of the outer coat, whereas the matrix region develops into the inner coat (11, 12). CotE appears to have a modular design, specifying information for its targeting to the surface of the developing spore, for oligomerization, and also for interaction with other coat proteins (3, 28).

Presumably, CotE controls outer coat assembly by means of complex network of direct or indirect interactions leading to the recruitment of many proteins to this coat layer (21, 28).

SpoVID does not influence the localization of CotE at an early stage, but following engulfment completion in cells mutant for spoVID, CotE and the rest of the coat detaches from the surface of the prespore and forms swirls of partially structured material dispersed throughout the mother cell cytoplasm (4, 12). Left with an exposed cortex, spoVID spores are sensitive to lysozyme (12). To study the function of SpoVID in the assembly process, Ozin et al. (32) used a phage display screening to identify peptides able to interact with SpoVID. They found a peptide motif, PYYH, occurring in the C-terminal half of another coat protein, SafA (387-residues long), the absence of which causes deficient formation of the coat and spore susceptibility to lysozyme (32, 40). At least three forms of SafA accumulate in sporulating cells: a full-length form (FL, 45 kDa), a C-terminal form derived from internal translation of the safA mRNA at
methionine 164 (C$_{30}$, 30 kDa) and a N-terminal form (N$_{21}$, 21 kDa) (31). Assays in vitro and in vivo showed that SafA and SpoVID directly interact, that SafA interacts with itself, and that each of the C and N terminal forms are capable of interacting with SafA-FL, with themselves, and at least N$_{21}$ with SpoVID (33). Both SpoVID (at its C-terminus) and SafA (at its N-terminus) contain a LysM domain, which has a general PG-binding function (2, 5, 20, 27, 35, 39), and both proteins localize to the cortex/coat interface (12, 32). Moreover, the localization of SafA depends on SpoVID (33). These results have led to a model according to which the interaction between SafA and SpoVID recruits the former to the coat region in close proximity to the cortex layer, and that the two proteins function in this region by promoting attachment of the nascent coat to the underlying cortex layer (33). However, in spite of the fact that it contains the PYYH motif, the C$_{30}$ form is not recruited to the prespore in the absence of SafA-FL, suggesting that a second region of interaction could exist in the N-terminal half of SafA (33). This second region of interaction between SafA and SpoVID was attributed to the LysM domain of both proteins, which is presumed to be involved in multimerization (2, 5, 20, 27, 35).

Here, we have analyzed the interaction between SafA and SpoVID. We present biochemical, cytological and genetic evidence that strongly suggests that the interaction of SafA with SpoVID via a region just downstream of the LysM domain is responsible for the targeting of SafA to the surface of the developing spore, and for the proper assembly and function of the coat structure.
Material and methods

Strains and general techniques. All the B. subtilis strains used in this study are congenic derivatives of the wild type strain MB24 (trpC2 metC3) (Table 1). The Escherichia coli strain DH5α (Bethesda Research Laboratories, BRL) was used for molecular cloning and CC118(DE3)(pLysS) for the overproduction of native or GST fusion proteins (Table 1). Luria-Bertani medium (LB) was used for maintenance and growth of E. coli and B. subtilis strains, with appropriate antibiotic selection when needed. Sporulation was induced by growth and nutrient exhaustion in Difco Sporulation medium (DSM) (30). Genetic manipulations of B. subtilis, and spore resistance or germination properties were assessed as previously described (9).

GST-SafA fusions. Primer safA204D and primers safA-STOP51R, safA-STOP64R, safA-STOP78R, safA-STOP91R or safA-STOP99R (the sequences of all primers are available upon request) were used to PCR amplify the coding regions of safA between residues 1-50, 1-63, 1-77, 1-90 and 1-98, respectively. The PCR fragments were digested with BamHI and XhoI and cloned between the same sites of pGex4T-3 (Pharmacia) to form pTC138 (GST-SafAE50), pTC159 (GST-SafAS63), pTC160 (GST-SafAK77), pTC161 (GST-SafAK90), and pTC139 (GST-SafAP98), respectively. The region encompassing codons 1-162 of safA was amplified using primers safA204D and safA693R, digested with BamHI and cloned between the BamHI and a filled-in XhoI sites of pGex4T-3 (Pharmacia) to yield pTC141 (GST-SafAS162). pTC165 (GST-SafAΔG51-S63) was obtained after digestion with HincII and auto-ligation of a PCR obtained from pTC139 (see above) with primers safA50-64D.
and safA50-64R. pTC196 (GST-SafAΔG_{51-E_{57}}) and pTC197 (GST-SafAΔP_{58-S_{63}}) were constructed as following. First, the safA coding regions between residues 1-50 and 1-57 were PCR amplified with primer sets safA204D/safA51-57R and safA204D/safA58-63R, respectively. Second, the regions corresponding to residues 58-98 and 64-98 of safA were PCR amplified with primer sets safA51-57D/safA-STOP99R and safA58-63D/safA-STOP99R, respectively. The fragments (codons 1-50 and 58-98, or 1-57 and 64-98), were mixed and subjected to PCR using primers safA204D and safA-STOP99R, to obtain safA fragments comprising the region coding for the first 98 residues but excluding residues 51 to 57, or 58 to 63, respectively. The fragments were digested with BamHI and XhoI and introduced between identical sites of pGex4T-3 (Pharmacia) to create pTC196 and pTC197. All PCR products were sequenced. Plasmids pTC138, pTC159, pTC160, pTC161, pTC139, pTC141, pTC165, pTC196 and pTC197 were used to transform E. coli CC118(DE3)(pLysS) to obtain strains AH2934, AH2978, AH2979, AH2980, AH2935, AH2937, AH2985, AH4086 and AH4087, respectively (Table 1).

**GST-SpoVID fusions.** Strain AH2692 (Table 1) was obtained by transformation of E. coli CC118(DE3) (pLysS) cells with pOZ169 (GST-SpoVID_{FL}) in which the entire spoVID coding sequence was translationally fused to the gst gene (33). We used pOZ169 and primers pairs spoVIDSTOP1498D/spoVID-STOP1498R, spoVID-STOP1198D/spoVID-STOP1198R, spoVIDSTOP907D/spoVID-STOP907R, spoVID-STOP607D/spoVID-STOP607R, to substitute codons 500, 400, 303 and 203 in spoVID by nonsense codons using the QuickChange site-directed mutagenesis system (Stratagene) to obtain plasmid pTC52 (GST-SpoVID_{499}), pTC51 (GST-SpoVID_{399}), pTC50 (GST-SpoVID_{302}), and pTC49.
(GST-SpoVID\textsubscript{202}), respectively. Plasmids pTC52, pTC51, pTC50 and pTC49 were used to transform \textit{E. coli} CC118(DE3)(pLysS), yielding strains AH2691, AH2690, AH2689 and AH2688, respectively (Table 1). Fusion of GST to residues 201 to 575 and 201 to 399 of SpoVID was done as follows. We PCR amplified DNA fragments of spoVID (codons 201 to 575 and 201 to 399) using primers spoVID+800D and spoVID1935R, and the pOZ169 and pTC51 templates, respectively. The products were digested with \textit{BamHI} and \textit{XhoI} and cloned between the same sites of pOZ169, creating pTC61 (GST-SpoVID\textsubscript{201-575}) and pTC62 (GST-SpoVID\textsubscript{201-399}), which were then introduced into CC118(DE3)(pLysS), yielding AH2718 and AH2719, respectively (Table 1). In order to obtain strain AH2687, producing native GST (Table 1), we excised the spoVID coding region from pOZ169 (33) by digestion with \textit{BamHI} and \textit{XhoI}, and filled-in the ends before auto-ligation. The resulting plasmid, pTC55 was used to transform CC118(DE3)(pLysS) creating AH2687 (Table 1).

\textbf{Mutations in regions A and B of SafA.} A PCR fragment comprising the entire safA coding region and 595-bp upstream of its start codon, generated with primers safA-595D and safA+1176R, was introduced in pCR2.1TOPO (Invitrogen), to create pTC75. Primers safA791D and safA834R were used to delete codons 203 to 206 (the PYYH motif) of safA in pTC75 using the QuickChange method, yielding pTC78. A chloramphenicol resistance (Cm\textsuperscript{R}) cassette was released from pMS38 (43) with \textit{XbaI} and \textit{XhoI} and inserted between the same sites of pTC78, generating pTC90. A PCR fragment encompassing codons 1-162 of safA, including 595 bp upstream of the safA start codon was amplified with primers safA-595D and safA693R, inserted into pCR2.1TOPO (Invitrogen) to
obtain pTC93 (SafAN\textsubscript{1-162}). pTC93 was then digested with \textit{XbaI} and \textit{XhoI}, and a Cm\textsuperscript{R} cassette released from pMS38 (43) with the same enzymes was inserted, to form pTC95.

To fuse the \textit{safA} promoter directly to codons 164 to 387, codons 1 to 163 were eliminated by amplification of pTC88 (obtained by cloning of the Cm\textsuperscript{R} cassette from pMS38 between the \textit{XbaI} and \textit{XhoI} sites of pTC75) with primers \textit{safA203R} and \textit{safA677D}, digestion of the resulting product with \textit{HincII} and re-ligation to form pTC97 (SafA-C\textsubscript{164-387}). To delete residues 51 to 63 of SafA, a product generated from pTC88 with primers \textit{safA50-64D} and \textit{safA50-64R}, was cut with \textit{SacII} and re-ligated, to yield pTC170 (SafA\textsubscript{ΔG51-S63}). pTC198 (SafA\textsubscript{ΔG51-E57}) and pTC199 (SafA\textsubscript{ΔP58-S63}) were constructed by cloning 143- and 146-bp fragments released from pTC196 and pTC197 (see above) with \textit{BbvII} and \textit{EspI}, respectively, between the same sites of pTC88 (see above). Alanine substitutions in SafA (R\textsubscript{55A}, K\textsubscript{56A}, K\textsubscript{59A} and K\textsubscript{62A}) were obtained by the QuickChange method (Stratagene) using pTC75 and primers \textit{safAAlamut50-64D} and \textit{safAAlamut50-64R}. The result was pTC172 (SafA-4xAla), from which pTC173 was made by introducing a Cm\textsuperscript{R} cassette (see above) between its \textit{XbaI} and \textit{XhoI} sites. All PCR products were sequenced. pTC90, pTC95, pTC97, pTC170, pTC190, pTC191 and pTC173 were transformed into \textit{B. subtilis} AOB68 to form AH2781, AH2787, AH2788, AH4006, AH4090, AH4091 and AH4007, respectively (Table 1).

\textbf{GFP fusions.} pTC204 (SafA\textsubscript{wt-GFP}) and pTC205 (SafA\textsubscript{ΔG51-S63-GFP}) were constructed as following. First, the \textit{safA} 3\textsuperscript{'} coding region (684-bp) was PCR amplified with primers \textit{safA677D} and \textit{safA-gfpR}. Second, a 714-bp fragment comprising the coding region of the
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The gfp gene, was PCR amplified using pEA18 (a gift from Alan Grossman) and primers gfp-30D and gfpmut2/749R. The resulting fragments were mixed and subjected to PCR using primers safA677D and gfpmut2/749R. The resulting 1398-bp ‘safA-gfp fragment was cleaved with SmaI and EcoRI, and the 1110-bp fragment gel purified. DNA fragments corresponding to 5’ regions of safAwt or safAΔG51-S63 were obtained by digestion of pTC75 and pTC170 (see above), respectively, with SmaI and NotI. The safAwt or safAΔG51-S63 fragments were cloned together with the ‘safA-gfp fragment between the NotI and EcoRI sites of pBEST501 (19) to yield pTC204 and pTC205, respectively. All PCR products were verified by sequencing. Strains AH4102 and AH4103 resulted from the integration of pTC204 or pTC205, respectively, at the safA locus of AOB68 via a single reciprocal (Campbell-type) cross over (Table 1). The spoVID null mutant AH1910 was transformed with chromosomal DNA of strains AH4102 and AH4103 to yield AH4107 and AH4108, respectively (Table 1).

Pull-down assays. The in vitro pull down assays were performed as described previously (33), except that 60 µl of a 50% slurry of glutathione sepharose beads (Amersham Biosciences) were used, and the interaction mixtures were resuspended in a final volume of 30 µl of loading buffer, before analysis on 12.5% polyacrylamide gels containing sodium dodecyl sulfate (SDS-PAGE) and immunoblot.

Purification of spores and analysis of the spore coat. Spores were harvested by centrifugation of DSM cultures 24 hours after the onset of sporulation, washed, and purified on step gradients of 20% to 50% of Gastrografin (Schering) (8, 42). Coat
proteins were extracted from about 2 OD$_{580}$ units of purified spores and resolved on 15% SDS-PAGE gels (14, 15). The gels were stained with Coomassie brilliant blue R-250 or transferred to nitrocellulose for immunoblot analysis as described below.

**Western blot analysis.** Pull down samples or coat protein extracts were resolved by SDS-PAGE (12.5 and 15% gels, respectively) and immunoblot analysis conducted as previously described (8). Anti-SafA (32), anti-CotE (our unpublished work), and anti-CotG (43) antibodies were used at dilutions of 1:15000, 1:1000, and 1:7500 respectively, whereas a horseradish peroxidase-conjugated secondary antibody (Sigma) was used at a dilution of 1:6000 dilution. The immunoblots were developed with chemiluminescence reagents (Amersham Biosciences).

**Microscopy.** Samples (0.6 ml) of DSM cultures were collected about 2.5 and 4 hours after the initiation of sporulation, resuspended in 0.2 ml of PBS supplemented with 2 µl of a 1 mg ml$^{-1}$ solution of 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) for visualization of DNA. Microscopy was carried out as described before (8, 36). DAPI staining allowed the identification of the prespore region and the ability to distinguish between cells that had just completed asymmetric division (with highly condensed prespore chromosomes) and those that had completed the engulfment process (diffuse DAPI signal in the prespore) (8, 36).

**SPR experiments.** Surface plasmon resonance experiments were conducted on a BIACORE 2000 instrument with a CM5 sensor chip (Biacore International AB). For all measurements and immobilization procedures, the temperature was set to 25 ºC.
running buffer was HEPES-buffer saline (HBS, 150 mM NaCl, 10 mM Hepes pH 7.2, 3 mM EDTA, and 0.005% surfactant P20). Two flow cells (Fc) of the CM5 chip were prepared for the immobilization of GST (Fc3) and GST-SpoVID\textsubscript{202} (Fc4) using the standard amine coupling method provided by the manufacturer. Ligand immobilization was done at a flow rate of 10 µl/min, using a buffer containing 10 mM sodium acetate pH 3.9. The immobilization levels were established between 0.1-0.23 pmol/mm\textsuperscript{2} (about 3000-13500 resonance units, RUs). Two peptides were used as analytes: SafA-region\textsubscript{A} (GVPVRKEPKAGKS), and SafA-Ala (GVPVAEPAAGAS) (GenScript Corporation, Scotch Plains, USA). Binding assays were performed in triplicate in HBS buffer using 0.8 mM of each analyte with a flow rate of 10 µl/min. The flow cells were regenerated with 10 mM Glycine (pH 2.0) after each cycle. Injection times were 3 min for binding and 30s for regeneration.

Protein identification by peptide mass fingerprinting. Proteins were excised from Coomassie brilliant blue R-250 stained gels and sent to the Microchemical Facility at Emory University for MALDI-TOF (matrix-assisted laser desorption/ionisation time-of-flight) analysis.

Results

The PYYH motif is involved in formation of the SafA-SpoVID complex. The PYYH motif, found in a phage display screen for peptides able to interact with SpoVID is present in the C-terminal half of the SafA protein (residues 203 to 206) (Fig. 1A; 32). To investigate whether the PYYH motif is involved in the interaction of SafA with SpoVID,
we constructed a mutant expressing a form of SafA lacking residues 203 through 206, or
SafAΔPYYH (Fig. 1A). Then, we tested whether a GST-SpoVID purified protein was
able to pull down SafAΔPYYH. GST-SpoVID and GST alone were overproduced in E.
coli, bound to glutathione-sepharose beads and incubated with extracts from wild type
B. subtilis or the safAΔPYYH mutant prepared at hour 4 of sporulation. We also
incubated the extracts with beads alone as a control. After extensive washing, retained
proteins were resolved by SDS-PAGE, blotted and probed with an anti-SafA antibody
(see Material and methods). The three main forms of SafA that accumulate in
sporulating cells, full-length (FL, 45 kDa), C-terminal (C30, 30 kDa) and N-terminal (N21,
21 kDa), were pulled-down by the glutathione-sepharose beads to which GST-SpoVID
had been bound, but not by beads containing GST or by the beads alone (Fig. 1B).

Similar experiments showed that the full-length form of SafA deleted for the PYYH
motif (SafAΔPYYH), was also retained by GST-SpoVID, but not by GST or the beads
(Fig. 1B). In contrast, the C30 and N21 forms in the SafAΔPYYH strain were not retained
by the GST-SpoVID-beads (Fig. 1B). Yeast two-hybrid experiments have shown that N21
can directly interact with SpoVID, but the interaction seemed weaker than the
interaction of N21 with SafA-FL (32; see also below). N21 may not bind directly to
SpoVID in the pull down assay because it is out-competed by SafA-FL or by
SafAΔPYYH. This suggests that N21 is recruited by the SpoVID-SafA complex but not by
the SpoVID-SafAΔPYYH complex (Fig. 1B). This also suggests that a site able to interact
with SpoVID is likely to exist in the N-terminal region of SafA (see also below). In the
yeast two-hybrid analysis of Ozin et al. (32) no interaction was detected between C30 and
SpoVID and GST-C₃₀ was only able to pull down itself from extracts of sporulating cells (33). Thus, it appears that the C₃₀ form interacts with the SpoVID-SafA complex but not with the SpoVID-SafAΔPYYH complex (Fig. 1B). Together, the results suggest that the PYYH motif in SafA (hereinafter named region B) is not essential for the interaction of full-length SafA with SpoVID but appears to affect the SpoVID-SafA complex in a way that interferes with its capacity to recruit the N₂₁ and C₃₀ forms of SafA.

Residues 51-63 of SafA are essential for its interaction with SpoVID in vitro. The results in preceding section together with previous results showing that N₂₁ interacts with SpoVID in a yeast two-hybrid assay (32), strongly suggest that a second site in SafA (presumably in its N-terminal region) must exist that promotes its interaction with SpoVID. To test this, we performed pull-down assays using B. subtilis mutants producing only the SafA N₂₁ (residues 1-162) or C₃₀ (residues 164-387) forms (Fig. 1A). The results in figure 1C show that only SafA N₂₁ (first 162 residues) was retained by GST-SpoVID, indicating that this region contains residues sufficient for the interaction with SpoVID. The observation that when produced alone “wild type” C₃₀ (i.e., carrying region B) was not retained by GST-SpoVID (Fig. 1C), is consistent with results of previous studies in which C₃₀ and SpoVID did not interact (32, 33).

To determine whether a specific region within the first 162 residues of SafA was involved in the interaction with SpoVID, we overproduced several truncated forms of SafA as GST fusion proteins in E. coli. The forms of SafA produced contained residues 1-162 (herein designated S₁₆₂, the letter and number referring to the identity and position
of the last residue in the SafA fragment), 1-98 (P98), 1-90 (K90), 1-77 (K77), 1-63 (S63), or 1-
50 (E50) (Fig. 2A). All the truncated forms of SafA were able to pull down untagged
SpoVID overproduced in *E. coli*, except the form containing just the first 50 residues of
SafA (E50), which correspond to its LysM domain (Fig. 2B). The results indicate that the
region of 13 amino acids comprised between residues 51 to 63 in SafA
(GVPVRKEPKAGKS, hereinafter referred to as region A), just downstream of the LysM
domain, is sufficient for the interaction with SpoVID *in vitro*. In confirmation of this
assumption, deletion of residues 51 to 63 (∆G51-S63) from the P98 form prevented its
interaction with SpoVID (Fig. 2A and B). In an attempt to define region A further,
residues 51 to 57 (∆G51-E57, i.e., the N-terminal half of region A), or 58 to 63 (∆P58-S63, its
C-terminal half) were removed from the P98 form (Fig. 2A). Neither of the new versions
of P98 was able to pull-down SpoVID (Fig. 2B), indicating that residues on both halves of
region A are required for the interaction with SpoVID *in vitro*.

**Region A of SafA directly contacts SpoVID.** All three deletion forms of SafA (∆G51-S63,
∆G51-E57, or ∆P58-S63) accumulate to wild type levels in extracts from *B. subtilis* cells
harvested at hour 4 of sporulation (Fig. 3B), but failed to bind to immobilized GST-
SpoVID (not shown). Therefore, the complete or partial deletion of region A does not
alter the stability of SafA significantly. (Note that for reasons we do not presently
understand, the accumulation of the N21 and C30 forms varied among the three safA
deletion mutants and in comparison to the wild type, as shown in Fig. 3B; however, we
also note that only SafA-FL is required for the formation of lysozyme-resistant spores

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We also tested the effect of alanine substitutions within region A on the interaction with SpoVID. The results shown in Fig. 3B indicate that the substitution of residues R_{55}, K_{56}, K_{59} and K_{62} in region A by alanines (R_{55}A, K_{56}A, K_{59}A and K_{62}A) to produce the Saf4xAla form (Fig. 3A), did not interfere with the accumulation of SafA in extracts of sporulating cells. However, Saf4xAla was not pulled down by GST-SpoVID \textit{in vitro} (Fig. 3C), suggesting that residues R_{55}, K_{56}, K_{59} and K_{62} in SafA are directly involved in the interaction with SpoVID.

To independently test whether region A of SafA is directly involved in the interaction with SpoVID, we conducted surface plasmon experiments with immobilized GST or GST-SpoVID_{202} (a truncated form of SpoVID that binds SafA in pull down assays as well as the wild type protein; see below) and peptides corresponding to either region A (SafA-region A, GVPVRKEPKAGKS) or to region A bearing the four alanine substitutions (SafA-Ala, GVPVAAEPAAGAS). GST and GST-SpoVID_{202} was immobilized in flow cells 3 and 4 of a CM5 sensor chip, respectively (see Material and methods), and the SafA-region A or SafA-Ala peptides were tested for binding using a BIAcore system (see Material and methods). We obtained 27.7 ± 4.0 RU as the response for binding of SafA-region A to GST-SpoVID_{202} (in flow cell 4), but only −0.7 ± 0.4 RU for binding of the SafA-Ala peptide (note that the negative values are caused by differences in the refractive indices between the buffer with or without the ligand). No binding was detected for either the SafA-region A or SafA-Ala peptides (0.9 ± 0.4 RU and −1.7 ± 0.4 RU, respectively) to immobilized GST alone (in flow cell 3). These results
strengthen our inference that residues in region A of SafA are involved in the interaction with SpoVID.

The LysM motifs in both SpoVID and SafA are not involved in their interaction. The observation that SafA-N21 interacted with SpoVID in a yeast two-hybrid assay led Ozin and co-workers to propose that the two proteins could interact via their LysM domains (32). However, the results presented above indicate that SafA does not interact with SpoVID via its LysM domain (fusion E50 in Fig. 2). To examine whether the LysM domain present at the C-terminus of SpoVID was involved in the interaction with SafA, we conducted pull-down assays using GST fusions to full-length SpoVID (FL) or to SpoVID deleted for its last 76 residues (T499), which encompasses the LysM motif (Fig. 4A). The results shown in Fig. 4B indicate that both versions of SpoVID were able to pull-down all main forms of SafA present in B. subtilis extracts, that is, SafA-FL, N21, and C30. These experiments suggest that the LysM domain SpoVID is not involved in the interaction with SafA. To determine whether SafA binds to a specific section of the 575 residues-long SpoVID protein, additional pull-down assays were carried-out using fusions of GST to truncated forms of SpoVID corresponding to residues 1 to 202 (R202), 1 to 302 (A302), and 1 to 399 (N399) (Fig. 4A). We found that all the truncated forms of SpoVID were able to pull-down the main forms of SafA present in B. subtilis extracts (Fig. 4B). Hence, the first 202 residues of SpoVID are sufficient for the interaction with SafA. To determine whether this region is necessary for the interaction, we performed similar pull down experiments using fusions of GST to truncated forms of SpoVID
lacking either its first 200 residues (L$_{201}$-A$_{375}$) or comprising only residues 201 to 399 (L$_{201}$-N$_{399}$) (Fig. 4A). Neither of these fusion proteins retained SafA (Fig. 4B). We infer from these results that LysM domain of SpoVID is not involved in the interaction with SafA. We further infer that the N-terminal 202 residues of SpoVID are necessary for the interaction with SafA (see also below).

Region A but not region B of SafA is required for proper coat assembly. Spores of a safA null mutant have abnormal coats, from which several coat components are missing, and are susceptible to lysozyme (32, 40). Because the SafA$\Delta$G$_{51}$-S$_{63}$, SafA$\Delta$G$_{51}$-E$_{57}$, SafA$\Delta$P$_{58}$-S$_{63}$ and SafA4xAla mutant forms accumulated to normal levels in B. subtilis yet failed to interact with SpoVID, we wanted to assess the impact of mutations in region A in the assembly of the spore coat. Spores of the various region A mutants, along with wild type and safA null mutant spores, were purified and the collection of coat polypeptides extracted and analyzed by SDS-PAGE (Fig. 5A). safA null mutant spores lack the 36 kDa CotG protein as judged by Coomassie-staining of gels of coat protein extracts (40; Fig. 5A). A 36 kDa band, and to a lesser extent a band of about 32 kDa, seen in wild type coat extracts appeared reduced from the extracts of the various safA mutants (Fig. 5A). These represent 36- and 32 kDa forms of CotG (43), as verified by immunoblot analysis (not shown). In agreement with the reduction in the representation of CotG, the 66-kDa form of CotB (as verified by immunoblot analysis; data not shown), whose formation depends on CotG (43), was also reduced in all safA mutants (Fig. 5A). Two other bands that appeared reduced in all the safA mutants are
labeled a and b in figure 5A. Mass spectrometry analysis indicated that these bands

 contained CotJC and YhcN (band a; 38) and CotC sequences (band b; 10). In contrast,

 band c appeared more abundant in the extracts from SafA∆G51-S63 spores, relative to the
 wild type (Fig. 5A), whereas band d was more abundant in the extracts from SafA∆G51-
 E57 spores (Fig. 5A). We found band c to contain GerQ, and band d to contain SodA,
 previously found in association with the spore coat and involved in the oligomerization
 of CotG (17, 25). The differences between the various SafA deletion mutants may reflect
 subtle differences in the capacity of residues in the N- and C-terminal halves of region
 A to promote the interaction of SafA with SpoVID (see also below). In contrast, deletion
 of region B did not affect in a discernible way the profile of Coomassie-stained coat
 polypeptides (data not shown). Together, the results show alterations of the coat
 structure caused by lesions in region A of SafA that are largely coincident with those
 caused by a safA null allele. We infer that the coat structural deficiencies seen in region
 A mutants are caused by the impaired interaction of SafA with SpoVID.

 Region A in SafA is required for the formation of a functional coat. Spores of a safA
 null mutant are susceptible to lysozyme (32, 40). To examine whether the mutations in
 region A and the associated perturbations at the level of the coat polypeptide
 composition, were sufficient to affect this functional property of the coat relies, spores
 of the various region A mutants were assayed for lysozyme resistance. The results in
 Table 2 show that spores produced by mutants lacking residues 51-63 (region A), 51-57
 or 58-63 of SafA have a degree of susceptibility to lysozyme treatment in the range of
that observed for spores of a safA null mutant (about $10^7$ lysozyme-resistant spores/ml of culture, as compared to $10^8$ for wild type spores). None of the mutations in region A affected spore heat resistance (Table 2), a property that relies on proper cortex formation, and hence the effects herein described are specifically related to the assembly of the spore coat. We have also found that spores of the SafAΔPYYH mutant are fully resistant to lysozyme (Table 2), an observation that is in agreement with absence of any discernible alteration in the profile of Coomassie-stained coat polypeptides (see above). This reinforces the suggestion that the PYYH motif is not a critical determinant for the SafA-SpoVID interaction (see above). Surprisingly however, spores of the SafA4xAla mutant were also found to be resistant to lysozyme (Table 2). Presumably, the alanine substitutions within region A still permit some degree of interaction of SafA with SpoVID in vivo, sufficient to support spore lysozyme resistance. In support of this interpretation, we found that when examined for their germination in response to a mixture of asparagine, glucose, fructose and KCl (AGFK), a property that is dependent on safA (40), spores of the SafA4xAla mutant showed an intermediate phenotype between the wild type and the safA null mutant (Fig. 5B). In contrast, the results in figure 5B show that spores of the SafAΔG51-E57 and SafAΔG51-S63 mutants were as impaired in AGFK-induced germination as were spores of the safA null mutant. Spores of the SafAΔP58-E63 mutant however, showed a response to AGFK that closely paralleled that of the SafA4xAla mutant (Fig. 5B), suggesting that residues in the N-terminal half of region A have a greater impact on the SafA-SpoVID interaction, at least as assessed by this spore property. Overall, the finding that mutations in region A led to
a germination phenotype that largely mimics that of a safA null mutant strengthens the view that the function of SafA critically depends on its interaction with SpoVID.

Targeting of SafA to the surface of the developing spore depends on its interaction with SpoVID. Lastly, we examined the impact of eliminating region A on the subcellular localization of SafA in developing cells. SafA is targeted to the surface of the developing spore early in the process of coat assembly, just after formation of the asymmetric septum that partitions the sporulating cell into the mother cell and prespore compartments (33). Targeting of SafA occurs in two stages, as defined by immunofluorescence microscopy: first, SafA localizes as a spot at the mother cell/prespore border in a SpoVID-independent manner, and later SafA migrates around the developing spore in a SpoVID-dependent manner (33). Both wild type SafA and SafA∆G51-S63 were fused to GFP and the localization of both fusion proteins studied by fluorescence microscopy (Fig. 6A). First, we established that spores of a strain expressing SafA-GFP were resistant to lysozyme indicating that the fusion protein is largely functional (data not shown). Then, we studied the pattern of localization of both fusion proteins in the wild type and in a spoVID congenic mutant 2.5 and 4 h after the onset of sporulation. In wild type cells, SafA-GFP was first targeted to the mother cell/prespore border as a spot (Fig. 6A, panel a) and later started migrating around the prespore to form a cap covering the spore end distal to the cell pole (Fig. 6A, panel e) in a spoVID-dependent mode (Fig. 6A, panel f). In the wild type, the “spot” pattern was associated with cells that had just undergone asymmetric division but had not initiated
engulfment, whereas the “cap” pattern was found for cells that had initiated
engulfment (the morphological stages were scored by DAPI staining, as described in the
Material and methods section). The quantification of these results is shown in figure 6B.

Note that the “spot” pattern predominated in samples at hour 2.5 of sporulation and
was not affected by mutation of spoVID, whereas accumulation of the “cap” pattern,
which predominated at hour 4, required expression of spoVID (Fig. 6B). These results
agree with those of Ozin and co-workers (33), except that in their experiments SafA was
found as caps covering both spore ends. However, the signal at the pole proximal spore
end was always weaker than the signal at the spore end away from the cell pole (33).

We emphasize that our SafA-GFP fusion is functional, and we presume that the
fluorescence at the pole proximal spore end is not detected under our conditions. In any
event, a strikingly different pattern was found for the SafAΔG51-S63-GFP fusion protein.
SafAΔG51-S63-GFP was still targeted at an early time (hour 2.5) to the asymmetric
septum where it accumulated as a spot (Fig. 6A, panels c and d). However, for this
protein the incidence of the “spot” pattern persisted at hour 4, at the expense of the
“cap” pattern whose representation was severely curtailed regardless of the presence of
a functional spoVID locus (Fig. 6A, panels g and h, and Fig. 6B). We conclude that
deletion of region A in SafA strongly interferes with the SpoVID-dependent stage of
SafA localization, and we infer that the interaction of SafA with SpoVID via region A is
required for the correct localization of SafA to the developing coat.
Discussion

Previous work has suggested that the PYYH motif (region B) in the C-terminal half of SafA, found in a phage display screen for peptides able to interact with SpoVID, could mediate the interaction of SafA with SpoVID in vivo (32, 33; Fig. 7A). However, a second region of interaction was postulated to exist in the N-terminal half of SafA (33). We now show that such a region indeed exists in the N-terminal half of SafA (Fig. 7A).

SafA-N$_{21}$ (but not SafA-C$_{30}$) could be pulled out by immobilized GST-SpoVID, and deletion mapping indicated that a region of 13 amino acids encompassing residues 51 to 63 of SafA, or region A, is sufficient for the interaction with SpoVID in vitro (Fig. 2). Moreover, region A is required for the interaction with SpoVID and for the proper subcellular localization and function of SafA in vivo (Figs. 5 and 6). Importantly, variants of SafA with the complete or partial deletion of region A accumulated in B. subtilis, but failed to promote proper assembly of the coat, lysozyme resistance and normal spore germination, mimicking the phenotypes of a safA null mutant (32, 40). That region A of SafA is directly involved in the interaction with SpoVID, is supported by two lines of evidence. First, a form of SafA with alanine substitutions in region A accumulated in vivo, but could not be pulled down by GST-SpoVID. Second, a peptide consisting of the wild type region A sequence, but not a related peptide bearing the same four alanine substitutions (above), was able to bind to immobilized GST-SpoVID.

Region B is also involved in SpoVID-SafA complex formation (33; Fig. 7A), as its deletion did not allow immobilized SpoVID to pull down SafA-N$_{21}$ or SafA-C$_{30}$ (Fig. 7A).
This suggests that N$_{21}$ and C$_{30}$ are not bound directly by SpoVID, but rather are recruited by the complex formed by SafA and SpoVID. N$_{21}$ binds to SpoVID when made alone but may be out-competed by SafA-FL or by SafAΔPYYH, in agreement with the yeast two-hybrid analysis of Ozin and co-authors which suggested a N$_{21}$-SpoVID interaction weaker than that of N$_{21}$ with SafA-FL (33). C$_{30}$ in turn, was not pulled-down by GST-SpoVID when made alone (Fig. 1C), also in agreement with an earlier study which found that GST-C$_{30}$ could not bind to SpoVID in the absence of SafA-FL, and that no interaction was detected between C$_{30}$ and SpoVID in a yeast two-hybrid assay (33).

In any case, while deletion of region A causes spore susceptibility to lysozyme, deletion of region B does not (Table 2; see also below). Taken together, the results suggest that region A is essential for the interaction of SafA with SpoVID, and that region B (while not essential), favors the interaction and/or allows the SafA-SpoVID complex to attain the correct topology for binding of SafA-N$_{21}$ and SafA-C$_{30}$ (Fig. 7B).

The consequences of lesions in region A were reminiscent of the effects of a safA null allele. First, spores of the various mutants had aberrant coats, from which CotG (both the 32- and 36-kDa forms), CotB, CotC, and possibly CotJC (10, 15, 37, 38) were reduced or missing (Fig. 5A). Second, as those of a safA null mutant (40), spores of all region A mutants showed impaired germination in response to AGFK (Fig. 5B). Lastly, with the exception of the 4xAla mutant, the partial or complete deletion of region A results in spores that were, like those of the safA null mutant (32, 40), susceptible to lysozyme (Table 2). We take these results as indicating that the interaction of SafA (via region A) with SpoVID is responsible for its morphogenetic function in coat assembly.
The differences in the composition of the coat extracts as well as in the germination response among the various mutants, and the lysozyme resistance of the 4xAla mutant suggest that the various mutations affect the interaction in vivo to various degrees, influencing the assembly of other downstream components.

SafA localizes to the developing spore in two stages (33). First it localizes as a dot at the mother cell/prespore border in a SpoVID-independent manner. Second, under the guidance of SpoVID, it encircles the prespore. Our results indicate that while a functional fusion of SafA to GFP undergoes assembly following the two-stages pathway described for the native protein, a SafA-GFP fusion lacking region A did not initiate the SpoVID-dependent stage (Fig. 6). These results suggest that SafA interacts with SpoVID via region A after the initial targeting of both proteins to the nascent coat, and that this interaction allows SafA to follow SpoVID in its movement around the prespore as engulfment proceeds. We suggest that SpoVID directly interacts with a factor localized close to or at the prespore outer membrane, that itself is pulled around the prespore during engulfment. This factor could be morphogenetic protein SpoIVA, which localizes close to the prespore outer membrane, and upon which the localization of SpoVID is dependent (12, 33; Fig. 7B).

We do not yet know how is SafA initially targeted to the surface of the developing spore. It is possible that the initial (SpoVID-independent) targeting of SafA, which then permits the interaction with SpoVID, involves its LysM domain. For example, the tandem LysM domains of another coat protein component, YaaH, are sufficient to direct β-lactamase to the surface of the developing spore (23, 24). However,
we could not yet test for the involvement of the LysM domain in the initial targeting of SafA because deletion of this region results in an unstable protein that does not accumulate in sporulating cells (our unpublished results).

Formation of the SafA-SpoVID complex involves defined regions in SafA (regions A and B), and SpoVID (first 202 residues), suggesting that SafA and SpoVID have a modular design. Other morphogenetic proteins, for example SpoIVA and CotE, also appear to have a modular design, with regions specifically involved in their targeting, multimerization, and interactions with additional coat components (3, 7, 21, 28, 34). Both SafA and SpoVID localize to the cortex-coat interface where they promote attachment of the coat to the cortex (4, 12, 32, 40; this work), and both proteins and their complex are likely to interact with additional coat components (6, 10, 23-26, 33, 38; Fig. 7B).

SpoVID and SafA orthologs are present in all Bacillus species whose genomes have been sequenced to date, as well as in other related species (not shown). Importantly, the recent study of a SafA ortholog, called ExsA, revealed its role in promoting anchoring of both the coat and exosporium layers to the spore in B. cereus (1). Together with the observation that region A is highly conserved among SafA orthologs (Fig. 7A), this suggests that the overall role and perhaps some of the mechanistic details of the functioning of SafA in coat morphogenesis are conserved in the Bacillus group of sporeformers.
Acknowledgments

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References


Table 1. Bacterial strains used in this study.

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<sup>a</sup>Sp<sup>r</sup>, spectinomycin resistant; Cm<sup>r</sup>, chloramphenicol resistant; Nm<sup>r</sup>, neomycin resistant.
Table 2. Spore resistance properties.

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*Total (viable), heat- and lysozyme-resistant cell counts were determined as described in the Material and Methods section.*
Figure Legends

**Figure. 1. Interaction of the N-terminal of SafA with GST-SpoVID.** Panel A represents the 387 residues-long SafA protein (wt), and SafA variants deleted for residues 203 to 206 (ΔPYYH), for the N-terminal first 163 residues (C164-387, corresponding to the C30 form), or missing the last C-terminal 225 residues (N1-162, corresponding to the N21 form). The striped pattern represents the LysM domain (first 50 residues) of SafA. Other relevant residues are also indicated. SafA accumulates in sporulating cells as three main forms: full-length (FL; 45 kDa), a C-terminal form (C30; 30 kDa) and a N-terminal form (N21; 21 kDa). Panels B and C show the interaction of immobilized GST-SpoVID with wild type SafA and SafAΔPYYH (B), and with the C30 and N21 forms (C) present in extracts at hour 4 of sporulation. The extracts were incubated with GST-SpoVID (VID for simplicity), with GST bound to glutathione sepharose beads, or with the beads alone (Bd). Pulled-down proteins were resolved by SDS-PAGE, and immunoblotted with an anti-SafA antibody. The position of FL, C30 and N21 (black arrowheads), of cross-reactive species (asterisks) and of molecular mass markers (MW, in kDa), is indicated. The last three lanes in panel C show the immunoblot analysis of the extracts from strains producing N21 or C30, used in the pull down assays, and of a ΔsafA mutant, with the anti-SafA antibody.

**Figure 2. Deletion mapping of region A in SafA.** Panel A shows the various GST-SafA fusions used, with the white box representing GST (not to scale) and the striped pattern
(residues 1 to 50), the LysM motif. The plus (+) or minus (-) symbols below each

diagram indicate interacting or non-interacting fusion proteins, respectively. Panel B
shows the results of pull-down assays using truncated forms of GST-SafA and SpoVID.
A cell extract prepared from an *E. coli* strain overproducing native SpoVID was
incubated with the various purified GST-SafA truncated forms containing the following
residues, as indicated: 1 to 162 (S₁₆₂), 1 to 98 (P₉₈), 1 to 90 (K₉₀), 1 to 77 (K₇₇), 1 to 63 (S₆₃),
1 to 50 (E₅₀), 1 to 98 but excluding residues 51 to 63 (ΔG₅₁-S₆₃), 1 to 98 but excluding
residues 51 to 57 (ΔG₅₁-E₅₇) and residues 1 to 98 but excluding residues 51 to 63 (ΔP₅₈-
S₆₃). The same extracts were incubated with GST bound to glutathione sepharose beads
or with beads alone (Bd), as controls. Pulled down proteins were resolved by SDS-
PAGE, and immunoblotted with an anti-SpoVID antibody (see Material and methods).
The position of SpoVID is indicated by the black arrowhead.

Figure 3. Accumulation of SafA variants in sporulating *B. subtilis* and analysis of
their interaction with GST-SpoVID. Panel A represents the 387aa-long SafA protein
(wt) and SafA variants deleted for residues 51 to 63 (ΔG₅₁-S₆₃), 51 to 57 (ΔG₅₁-E₅₇), 58 to
63 (ΔP₅₈-S₆₃), or with alanine substitutions of residues R₅₅, K₅₆, F₆₂ and K₅₉ (4xAla). The
LysM domain (residues 1 to 50) in SafA is indicated by the striped pattern. Panel B
shows the accumulation of SafA variants in cells extracts prepared from cultures of *B.
subtilis* at hour 4 of sporulation. The proteins were resolved by SDS-PAGE, and
immunoblotted with an anti-SafA antibody. The position of the FL, C₃₀, and N₂₁ forms
of SafA (black arrowheads), a cross-reactive species (asterisk), and of molecular mass
markers (MW, in kDa) is indicated. Panel C shows the interaction of wild type SafA (wt) or the SafA 4xAla variant present in extracts from sporulating *B. subtilis*, with GST-SpoVID (VID). Cells extracts prepared from wild type *B. subtilis* and from strains producing SafAwt or SafA4xAla were incubated with GST-SpoVID or GST immobilized on glutathione sepharose beads, or with the beads (Bd), and the pulled down proteins resolved by SDS-PAGE and immunoblotted with an anti-SafA antibody (see Material and methods). The position of full-length SafA (FL) is indicated by the black arrowhead.

**Figure 4. SafA interacts with a defined region of SpoVID.** Panel A shows a schematic representation of fusions of the 575 residues-long SpoVID protein (FL) as well as forms of SpoVID containing residues 1 to 499 (T<sub>499</sub>), 1 to 399 (N<sub>399</sub>), 1 to 302 (A<sub>302</sub>), 1 to 202 (R<sub>201</sub>), 201 to 575 (L<sub>201</sub>-A<sub>575</sub>) and 201 to 399 (L<sub>201</sub>-N<sub>399</sub>), to GST. The white box represents GST (not to scale) and the LysM motif (residues 525 to 575) corresponds to the striped pattern. The plus (+) or minus (-) symbols on the right side of each diagram indicate proteins interacting or non-interacting with SafA, respectively. Panel B shows the results of pull down assays performed using the various purified truncated forms of SpoVID fused to GST and extracts from wild type *B. subtilis AOB90* (Table 1) prepared at hour 4 of sporulation. The immunoblot was probed with an anti-SafA antibody. The same extracts were also incubated with immobilized GST or with glutathione beads alone (Bd). The black arrowheads indicate the position of the FL, C<sub>30</sub> and N<sub>21</sub> forms of SafA, and the asterisk indicates a degradation product of SafA. Molecular mass markers (MW, in kDa) are also indicated.
Figure 5. Spores of region A mutants have an altered coat and impaired germination.

Panel A shows a Coomassie-stained gel of SDS-PAGE-resolved coat protein extracts, prepared from purified spores of the wild type (lane 1) or the SafA∆G51-S63 (lane 2), SafA∆G51-E57 (lane 3), SafA∆P58-S63 (lane 4), SafA4xAla (lane 5) or ∆safA (lane 6) mutants. Open arrowheads point to bands (a to d) that were excised and subjected to mass spectrometry analysis (see Material and methods). The position of bands corresponding to the 66 kDa form of CotB, and to the 32 and 36 kDa forms of CotG is indicated by black arrowheads. Panel B shows the germination response to AGFK of purified spores of the wild type (closed diamonds), or the SafA4xAla (closed squares), SafA∆G51-S63 (open squares), SafA∆G51-E57 (closed triangles), SafA∆P58-S63 (crosses) or ∆safA mutants (open circles). Germination was monitored by following the decrease in absorbance at 580 nm over time (in min) and shown as the percentage of the OD580 at the beginning of the assay.

Figure 6. Region A is important for the SpoVID-dependent localization of SafA. In panel A, cells expressing a functional fusion of SafA to GFP (SafA-GFP) or SafA∆G51-S63-GFP in the wild type (panels a, c, e and g) or in a spoVID background (panels b, d, f and h) were observed by fluorescence microscopy 2.5 (panels a to d) and 4 hours (panels e to h) after the onset of sporulation in DSM. Overlay images of GFP fluorescence and phase contrast are shown in all panels. White arrowheads point to the region showing fluorescence in a selected cell, and the position of the prespore (as...
The interaction between SafA and SpoVID

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determined by DAPI chromosomal staining, not shown) is shown in the schematic representation of the same cell included in each panel. The scale bar represents 1 µm.

Panel B shows the quantification of the GFP decoration patterns. A minimum of 75 cells were scored for fluorescence patterns designed by spot, septum or cap, at hour 2.5 and 4, in cultures of both the wild type or spoVID mutant expressing either SafA-GFP or SafAΔG51-S63-GFP: AH4102 (SafA-GFP; black), AH4103 (SafAΔG51-S63 –GFP; striped bar), AH4107 (SafA-GFP spoVID; white bar), and AH4108 (SafAΔG51-S63 –GFP spoVID; grey bar).

Figure 7. The interaction of SafA with SpoVID and role of the complex in spore coat assembly. Panel A illustrates the structure of SafA and SpoVID, with the LysM motifs (striped pattern) shown in each protein, as well as regions A (residues 51-63) and B (PYTH motif) in SafA (white boxes). Also shown is an alignment of the sequence of region A in the following SafA orthologs: Bs, B. subtilis 168 (accession number NP_390662); Bl, B. licheniformis ATCC14580 (YP_080055); Bt, B. thuringiensis serovar konkukian str. 97-27 (YP-038478); Ba, B. anthracis “Ames ancestor” (YP_021306); Bc, B. cereus ATCC 10987 (NP_980805); Bcl, B. clausii KSM-K16 (YP_175046); Bh, B. halodurans C-125 (NP_242087); Oi, Oceanobacillus oeiensis HTE831 (NP_692961). Identical residues are highlighted and asterisks indicate the alanine substitutions in SafA4xAla. Panel B illustrates a model for the localization of SafA and SpoVID. Both SafA and SpoVID localize at the cortex/coat interface, delimited by the outer forespore membrane (OFM).

Targeting of SpoVID to this region requires SpoIVA and may involve a direct
interaction between the two proteins. SafA is initially targeted to the cortex/coat interface independently of SpoVID, a step that may involve its LysM domain. In a second stage, SafA interacts with SpoVID via regions A and B to form the SpoVID-SafA complex (shown within a box), which allows SafA to encircle move around the spore. SafA or the SpoVID-SafA complex controls the assembly of a sub-set of inner and outer coat proteins (i.e., CotE-controlled), and SpoVID is essential for maintaining the coat anchored to the cortex, in part because of its requirement for keeping CotE at the inner coat/outer coat interface. Solid arrows indicate a direct interaction, whereas broken arrows indicate direct or indirect interactions.
Figure 2 - Costa et al.
Figure 4 - Costa et al.
Figure 6 - Costa et al.