A sporulation membrane protein tethers the pro-σ^K processing enzyme to its inhibitor and dictates its subcellular localization

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The developmental transcription factor σ^K is derived from the inactive precursor protein pro-σ^K by regulated proteolysis during the process of sporulation in the bacterium *Bacillus subtilis*. The putative pro-σ^K processing enzyme SpoIVFB is a member of a family of membrane-embedded metalloproteases and is held inactive by two other integral membrane proteins, SpoIVFA and BofA. Herein we show that the processing enzyme and its two regulators exist in a multimeric complex that localizes to the membrane surrounding the developing spore (the forespore). We further show that one of the regulators, SpoIVFA, plays a central role in both the formation of this complex and its subcellular localization. Evidence is presented in support of a model in which SpoIVFA acts as a platform for bringing BofA and SpoIVFB together, whereby BofA inhibits pro-σ^K processing until a signal has been received from the forespore.

[*Key Words*: Regulated proteolysis; subcellular localization; sporulation; sigma factor; transcription]

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A common theme in intercellular signaling is the assembly of the components of signal transduction pathways into multimeric complexes that localize to particular sites within the cell. Thus, for example, the components of the *Drosophila* phototransduction cascade assemble into a multimeric complex, a transducisome, that localizes to the microvillar membranes of the rabdomeres in photoreceptor neurons (Tsunoda et al. 1997). Similarly, the STE2 receptor for α-factor in the mating transduction pathway of budding yeast localizes to the pheromone-induced mating projection (Jackson et al. 1991) and interacts with its heterotrimeric G-protein in a preactivation complex (Dosil et al. 2000). Here we are concerned with a signal transduction pathway that coordinates gene expression between the two cellular compartments of the developing sporangium during the process of sporulation in the bacterium *Bacillus subtilis*. We show that three components of the pathway assemble into a membrane-bound complex that localizes to a particular membrane surface within the developing cell and that one of the proteins is responsible both for the assembly of the complex and its subcellular localization.

During the process of sporulation in *B. subtilis*, an asymmetric division partitions the developing cell (or sporangium) into two daughter cells of unequal size: a large cell known as the mother cell and a small cell called the forespore (Stragier and Losick 1996). The two cells initially lie side-by-side, but later in development, the mother cell wholly engulfs the forespore to create a cell-within-a-cell (Fig. 1A). The forespore and the mother cell follow different programs of gene expression. Yet, the two programs are linked to each other by intercellular signal transduction pathways that ensure that gene expression in one compartment is kept in register with gene expression in the other compartment (Losick and Stragier 1992; Rudner and Losick 2001). These signaling pathways have been characterized by genetic and cytological approaches, but little information is available on the nature of the interactions among the protein components of the pathways. The present work focuses on a signaling pathway that operates after the completion of the process of engulfment and results in the activation of the mother-cell-specific transcription factor σ^K (Fig. 1A).

The σ^K factor is synthesized in the mother cell prior to the completion of engulfment as an inactive proprotein pro-σ^K, which contains an N-terminal extension of 20 amino acids (Kroos et al. 1989; Cutting et al. 1990). A signaling protein (SpoIVB) that is produced within the forespore under the control of the forespore-specific transcription factor σ^G is believed to be secreted into the intermembrane space between the forespore and mother-cell membranes, where it triggers the processing of pro-σ^K in the mother cell (Fig. 1A; Cutting et al. 1991a;

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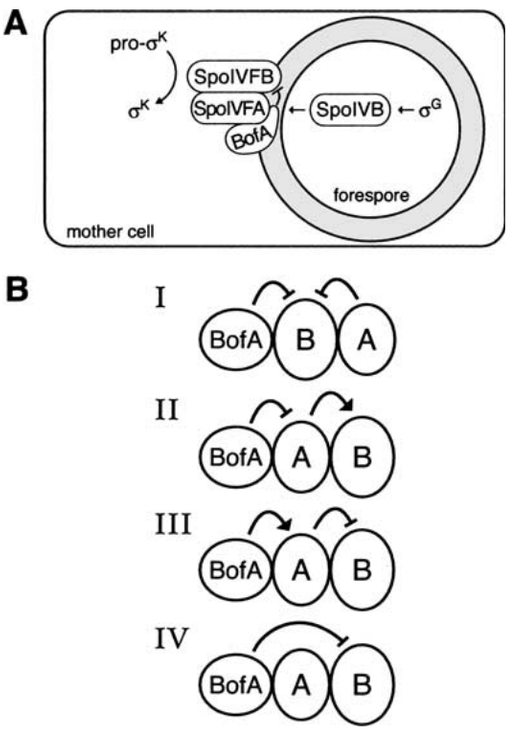


Figure 1. Models for the regulation of the SpoIVFB processing enzyme by BofA and SpoIVFA. (*A*) Proteolytic processing of pro-σ^K in the mother cell is catalyzed by the membrane-embedded metalloprotease SpoIVFB, which exists in a complex with its two regulators SpoIVFA and BofA. SpoIVFA acts as a platform bringing BofA and SpoIVFB together, whereby BofA inhibits SpoIVFB processing activity. The forespore signaling molecule SpoIVB activates processing by reversing the inhibition imposed on SpoIVFB by BofA. SpoIVB is made in the forespore under the control of σ^G. The space between the inner and outer forespore membranes that surround the forespore is in gray. (*B*) Four models for the regulation of SpoIVFB by SpoIVFA and BofA. (Segment *I*) SpoIVFA (*A*) and BofA separately contact and inhibit SpoIVFB (*B*). (Segment *II*) SpoIVFA is responsible for activating the SpoIVFB protease and BofA inhibits SpoIVFA. (Segment *III*) SpoIVFA is the inhibitor of SpoIVFB but is itself susceptible to proteolytic degradation. BofA regulates SpoIVFA by protecting it from degradation. (Segment *IV*) BofA is an inhibitor of SpoIVFB and is tethered to the protease by SpoIVFA (corresponding to the model in panel *A*).

Wakeley et al. 2000). The conversion of pro-σ^K to mature σ^K requires the polytopic membrane protein SpoIVFB (not to be confused with the aforementioned signaling protein SpoIVB), which is likely to be the processing enzyme (Cutting et al. 1991b; Lu et al. 1995; Resnekov and Losick 1998; Rudner et al. 1999; Green and Cutting 2000; Yu and Kroos 2000). SpoIVFB, henceforth referred to as the SpoIVFB processing enzyme, is a founding member of a family of putative membrane-embedded metalloproteases whose catalytic centers reside adjacent to or within the membrane (Rudner et al. 1999; Yu and Kroos 2000). The other founding member of this family is the Site-2 Protease (S2P; Rawson et al. 1997; Zelenski et al. 1999; Brown et al. 2000), which is required for the proteolytic activation of the sterol responsive element binding protein (SREBP), a transcription factor required for the activation of genes involved in cholesterol metabolism and uptake in mammalian cells (Brown and Goldstein 1997).

SpoIVFB is regulated by two other integral membrane proteins produced in the mother cell: SpoIVFA and BofA (Fig. 1A; Cutting et al. 1990, 1991b; Ricca et al. 1992). In cells mutant for SpoIVFA or BofA, SpoIVFB is capable of processing pro-σ^K in the absence of the forespore signaling protein. Thus, SpoIVFB is active for processing in its default state, and SpoIVFA and BofA are responsible for holding the processing enzyme inactive until a signal is received from the forespore. Consistent with the idea that the pro-σ^K signal transduction pathway ensures that gene expression in the two compartments remains in register, σ^K becomes active ~30 min prematurely in the BofA and SpoIVFA mutants, resulting in impaired sporulation efficiency (Cutting et al. 1990).

Cytological evidence indicates that SpoIVFA and SpoIVFB localize to the mother-cell membrane that surrounds the forespore (also known as the outer forespore membrane; Resnekov et al. 1996), where they are appropriately positioned to receive the SpoIVB signal from the forespore (Fig. 1A). Nonetheless, little is known about the basis for this pattern of subcellular localization, whether the proteins directly interact with each other, and how the SpoIVFB processing enzyme is kept inactive by BofA and SpoIVFA. Several models (Fig. 1B) have been considered for inhibition of the SpoIVFB processing enzyme by BofA and SpoIVFA, but these have not been subjected to detailed analysis. In the present study we have taken physiological, cytological, and biochemical approaches to investigate the interaction of the SpoIVFB processing enzyme with SpoIVFA and BofA. We show that all three proteins are in a multimeric complex that resides in the mother-cell membrane surrounding the engulfed forespore. We further show that SpoIVFA plays a central role in assembling the complex and anchoring it to the outer forespore membrane. These results in combination with our reexamination of previously proposed models led us to a new model (Fig. 1A and B, IV) for the regulation of SpoIVFB, in which SpoIVFA acts as a platform for bringing BofA and SpoIVFB together, whereby BofA inhibits the SpoIVFB processing enzyme until a signal has been received from the forespore.

Results

SpoIVFA, SpoIVFB, and BofA depend on each other for their accumulation

The purpose of this investigation was twofold. One purpose was to determine, as inferred from previous genetic analysis (Cutting et al. 1990; Resnekov et al. 1996; Resnekov and Losick 1998; Resnekov 1999; Green and Cutting 2000; Yu and Kroos 2000), whether SpoIVFA, SpoIVFB, and BofA exist as a multimeric complex in the membrane. The other purpose was to elucidate how such a complex, if it exists, regulates the SpoIVFB processing

enzyme. Frequently, the stability of proteins in multimeric complexes is influenced by the absence of other proteins normally present in the complex (Brown et al. 1994). As an initial test of whether SpoIVFA, SpoIVFB, and BofA reside in a complex, we analyzed the accumulation of each protein in the presence and absence of its putative partners. The levels of SpoIVFA, SpoIVFB, and BofA were monitored in mutant backgrounds by immunoblot analysis of whole-cell lysates from cells undergoing sporulation. SpoIVFA and SpoIVFB were visualized with antibodies raised against the proteins (Resnekov et al. 1996), whereas BofA (for which antibodies do not exist) was visualized using anti-GFP antibodies and a functional GFP–BofA fusion in place of the wild-type protein (see Materials and Methods; Supplemental Fig. 1, available online at www.genesdev.org). To avoid misinterpretation caused by the polar effects of deletions in *spoIVFA* or *spoIVFB* (the genes encoding SpoIVFA and SpoIVFB exist in an operon; Cutting et al. 1991b), we deleted the *spoIVF* operon and placed copies of *spoIVFA* and *spoIVFB* at separate nonessential loci. Both genes were placed under the control of the *spoIVF* operon promoter with an optimized ribosome binding site (see Material and Methods). Consistent with the idea that these proteins exist in a complex, the levels of SpoIVFA, SpoIVFB, and GFP–BofA were influenced by the absence of one or both their partners. The level of SpoIVFA was only mildly affected by the absence of SpoIVFB (Fig. 2), as was observed previously (Resnekov et al. 1996). The level of SpoIVFB, however, was greatly influenced by the absence of SpoIVFA. Meanwhile, the absence of BofA reduced the level of both SpoIVFA and SpoIVFB, an observation consistent with that of previous experiments in which these proteins were synthesized during growth (Resnekov 1999). Finally, the level of GFP–BofA was not affected by the absence of SpoIVFB but was greatly reduced by the absence of SpoIVFA. As a control, equivalent immunoblots were probed with anti-σ^K antibodies (Resnekov et al. 1996) to confirm that similar amounts of protein were loaded in each lane (Fig. 2). The simplest interpretation of these results is that SpoIVFA, SpoIVFB, and BofA protect each other from proteolytic degradation and, in extension of previous genetic observations (Cutting et al. 1990; Resnekov et al. 1996; Resnekov and Losick 1998; Resnekov 1999; Green and Cutting 2000; Yu and Kroos 2000), suggests that they exist in a complex.

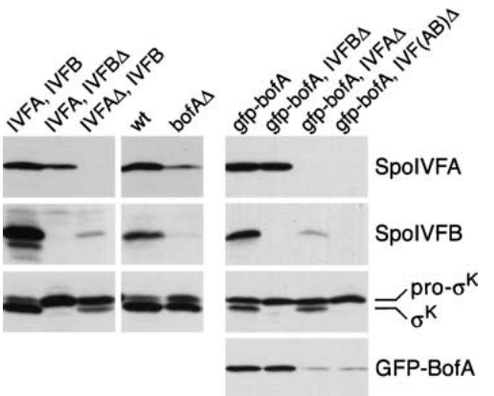


Figure 2. SpoIVFA, SpoIVFB, and BofA depend on each other for accumulation. Immunoblots of whole-cell extracts from cells undergoing sporulation. Lysates were prepared from sporulating cells at hour 3.5 and analyzed using polyclonal antibodies that recognize SpoIVFA, SpoIVFB, GFP, and σ^K (the anti-σ^K antibodies recognize both pro-σ^K and σ^K). The first two sets of panels were from the same immunoblot with intervening lanes deleted. The strains (BDR883, BDR891, BDR786) used for the first three lanes contained a deletion of *spoIVF* at its normal locus and either a copy of *spoIVFA* at the *amyE* locus or a copy of *spoIVFB* at the *zej-82* locus (Vandeyar and Zahler 1986), or both as indicated. The strains (BDR954, BDR961, BDR975, BDR963) used for the last four lanes contained a deletion of *bofA* at its normal locus and a copy of *gfp–bofA* at the *amyE* locus. The anti-σ^K immunoblot served as a control for loading and revealed the pro-σ^K processing efficiency. As has been observed previously (Rudner et al. 1999), low levels of SpoIVFB are sufficient to process pro-σ^K to a significant extent (third and fifth lane from left and second lane from right).

SpoIVFA, SpoIVFB, and BofA colocalize to the mother-cell membrane that surrounds the forespore

If SpoIVFA, SpoIVFB, and BofA form a multimeric complex, then all three proteins should show the same pattern of subcellular localization. Previous cytological analysis using a fusion of the original (slow-folding) form of GFP to SpoIVFB and immunofluorescence microscopy using anti-SpoIVFA antibodies revealed that both proteins do localize to the outer forespore membrane (Resnekov et al. 1996). Both the weak GFP signal and the inherent problems associated with visualizing membrane proteins using immunofluorescence (which requires permeabilization of the cytoplasmic membrane) prompted us to revisit this analysis with fusions of SpoIVFA, SpoIVFB, and BofA (see Materials and Methods) to a bright, fast-folding form of GFP (Cormack et al. 1996), which was not available at the time of the original analysis. All three of the newly constructed fusion proteins were functional and could fully (in the cases of GFP–SpoIVFA and SpoIVFB–GFP) or partially (in the case of GFP–BofA) substitute for the corresponding wild-type protein in the pro-σ^K signal transduction pathway (see Materials and Methods; Fig. 6, below; see Supplemental Table 1 and Supplemental Figs. 1 and 2 at www.genesdev.org).

SpoIVFA–GFP, SpoIVFB–GFP, and GFP–BofA exhibited bright signals and localized in a distinct ring that was coincident with the membranes that surround the forespore (Fig. 3A). Strictly speaking, we do not know whether localization was to the outer or to the inner membrane that surrounds the forespore because it is not possible to separate these two membranes. We presume, however, that localization was to the outer membrane because all three proteins are produced in the mother cell, and for present purposes we assume that this is the case. In any event, these cytological results confirm and extend earlier analysis (Resnekov et al. 1996) and are consistent with the idea that SpoIVFB, SpoIVFA, and

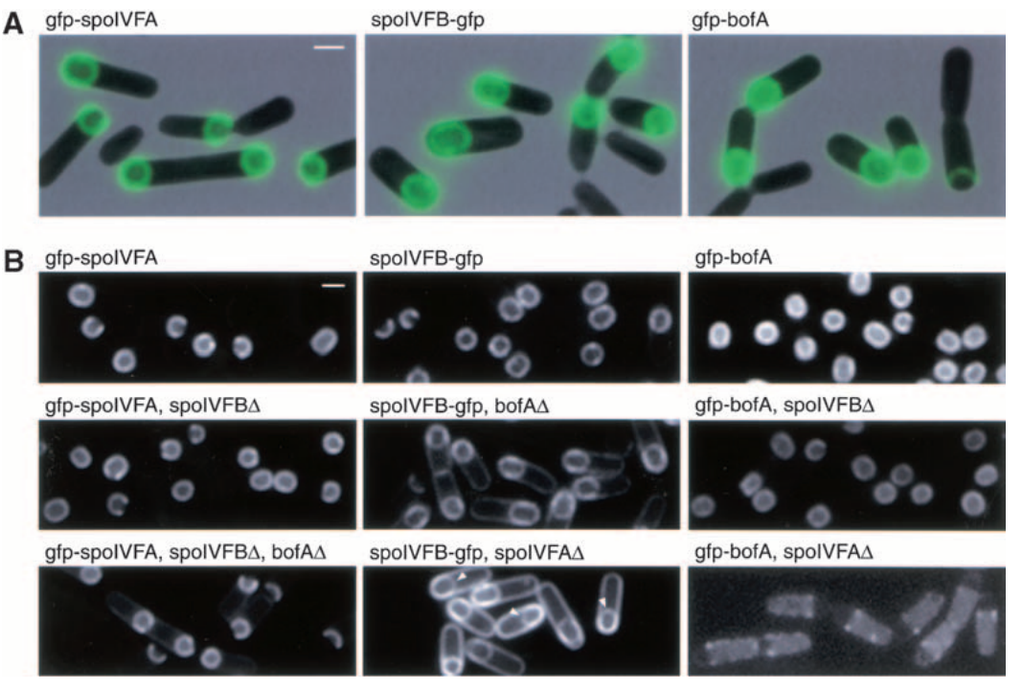


Figure 3. SpoIVFA, SpoIVFB, and BofA reside in the mother-cell membrane that surrounds the forespore; SpoIVFA is critical for this subcellular localization. (*A*) Cells were sporulated by resuspension and analyzed at hour 3 by fluorescence microscopy. The GFP-fluorescence images were false-colored green and overlaid on phase contrast images. The strains (BDR528, BDR347, BDR511) analyzed are indicated. (*B*) Cells were analyzed as in *A* but without false-coloring and phase-contrast overlays. The strain (BDR493) labeled *spoIVFB–gfp, spoIVFA*Δ contained a deletion of the *spoIVF* locus and the *spoIVFB–gfp* gene at the *amyE* locus. Similarly, the strain (BDR975) labeled *gfp–bofA, spoIVFA*Δ contained a deletion of the *spoIVF* locus and the *spoIVFB* gene at the *zej-82* locus (Vandeyar and Zahler 1986). The strains (BDR536, BDR557, BDR445, BDR961) analyzed are indicated. The region of the outer forespore membrane that does not overlap the cytoplasmic membrane is indicated by a caret. Bars, 1 μm.

BofA reside in a complex in the mother-cell membrane that surrounds the forespore, where they would be appropriately positioned to receive, and respond to, a signal from the forespore (SpoIVB).

SpoIVFA is required for the proper localization of SpoIVFB and BofA

Equipped with these bright and functional GFP fusions, we next asked whether SpoIVFA, SpoIVFB, and BofA required each other for proper localization. Consistent with previous cytological analysis, GFP–SpoIVFA was capable of proper localization to the outer forespore membrane in the absence of SpoIVFB (Fig. 3B; Resnekov et al. 1996). Interestingly, it retained proper localization in the absence of SpoIVFB and was only slightly impaired in its localization in the absence of both BofA and SpoIVFB (Fig. 3B). In contrast, SpoIVFB–GFP required SpoIVFA for proper localization. In the absence of SpoIVFA, SpoIVFB–GFP localized uniformly in the cytoplasmic and outer forespore membranes (Fig. 3B). In places where the cytoplasmic and outer forespore membranes were very close to each other, the SpoIVFB–GFP signal was brighter, giving the appearance of (partial) localization to the outer forespore membrane. However, the signal in regions (see the carets in Fig. 3B) of the outer forespore membrane that were not juxtaposed against the cytoplasmic membrane was indistinguishable in intensity from the signal in the rest of the cytoplasmic membrane. SpoIVFB–GFP also required BofA for proper localization. In the absence of BofA, SpoIVFB–GFP was partially mislocalized (Fig. 3B). Finally, GFP–BofA localized in the absence of SpoIVFB but, like SpoIVFB, required SpoIVFA. In the absence of SpoIVFA, the GFP–BofA signal was present in all the mother-cell membranes and in the mother-cell cytoplasm. The punctate staining pattern of GFP–BofA in the SpoIVFA mutant suggested partial aggregation of the fusion protein. Taken together, these results indicate that SpoIVFB and BofA require SpoIVFA for proper localization to the outer forespore membrane and provide further evidence that these proteins exist in a complex. These results also indicate that SpoIVFA is anchored to the outer forespore membrane independently of its partners.

A heteromeric membrane complex containing SpoIVFA, SpoIVFB, and BofA

Next, we attempted to obtain biochemical evidence that SpoIVFA, SpoIVFB, and BofA are bound to each other in a complex in the membrane. All three proteins are predicted to have transmembrane segments (Cutting et al.

1991b; Ricca et al. 1992), and topological analysis using fusions to PhoA and LacZ (Manoil and Beckwith 1986) indicates that in *Escherichia coli* the proteins contain one, six, and two transmembrane segments, respectively (Fig. 4A; Varcamonti et al. 1997; Green and Cutting 2000). Previous fractionation experiments in *B. subtilis* indicate that SpoIVFB and SpoIVFA behave as integral membrane proteins (Resnekov et al. 1996). We carried out fractionation experiments to characterize GFP–BofA and to identify conditions in which these proteins could be efficiently solubilized from the membrane. Soluble and insoluble proteins from whole-cell lysates of sporulating cells were separated by ultracentrifugation and then subjected to immunoblot analysis.

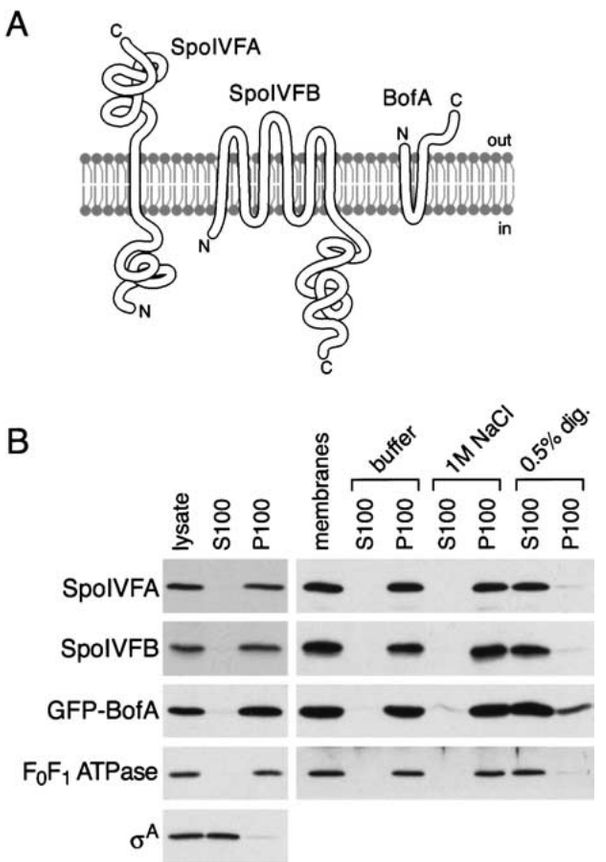


Figure 4. SpoIVFA, SpoIVFB, and BofA are integral membrane proteins. (*A*) Schematic diagram of the membrane topology of SpoIVFA, SpoIVFB, and BofA based on fusions to PhoA and LacZ assayed in *E. coli* (Varcamonti et al. 1997; Green and Cutting 2000). The *E. coli* cytoplasm (in) and periplasm (out) are indicated. The N and C termini of the proteins are shown. (*B*) Biochemical fractionation of SpoIVFA, SpoIVFB, GFP–BofA, and of a cytoplasmic (σ^A) and integral membrane (subunit a of the F_0F_1 ATP synthase) proteins as controls. The lysate was prepared from sporulating cells (strain BDR954) collected at hour 3.5. Soluble (S100) and insoluble (P100) protein fractions were separated by centrifugation at 100,000*g* and subjected to immunoblot analysis using polyclonal antibodies that recognize SpoIVFA, SpoIVFB, GFP, σ^A, and F_0F_1 ATP synthase. Crude membranes (membranes) derived from fractionation of the whole-cell lysate on two sucrose step-gradients (see Materials and Methods) were incubated with buffer, 1 M NaCl, or 0.5% nonionic detergent digitonin, and then fractionated and analyzed as described above. The soluble fraction after incubation with 0.5% digitonin was the source of the load in the coimmunoprecipitation experiments in Figure 5.

Consistent with the topological analysis and previous fractionation experiments, SpoIVFA, SpoIVFB, and GFP–BofA fractionated with the membrane-protein-containing insoluble fraction (Fig. 4B). As controls, the a subunit of the F_0F_1 ATP synthase, a well-characterized integral membrane protein (Santana et al. 1994; Long et al. 1998), was found in the insoluble fraction, and the RNA polymerase sigma factor σ^A, a cytoplasmic protein, was found in the soluble fraction (Fig. 4B).

As a further test of whether the regulators of pro-σ^K processing were integral membrane proteins, membranes from cells that contained SpoIVFA, SpoIVFB, and GFP–BofA were purified over two sucrose step-gradients (see Material and Methods) and then treated with buffer, buffer containing 1 M NaCl, or buffer containing 0.5% of the nonionic detergent digitonin. Soluble and insoluble material was separated by ultracentrifugation and then subjected to immunoblot analysis. Consistent with the idea that SpoIVFA, SpoIVFB, and GFP–BofA are integral membrane proteins and in confirmation and extension of previous fractionation experiments (Resnekov et al. 1996), all three proteins were found in the membrane fraction and were solubilized by digitonin but not by 1 M NaCl (Fig. 4B).

Having identified conditions in which SpoIVFA, SpoIVFB, and BofA could be solubilized from membrane preparations, we next performed coimmunoprecipitation experiments to determine whether these proteins exist in a complex. Detergent-solubilized membrane preparations (Fig. 4B) from strains containing GFP–SpoIVFA, GFP–BofA, or SpoIVFB–GFP were immunoprecipitated with anti-GFP antibodies, and the immunoprecipitates were subjected to immunoblot analysis (see Materials and Methods; Fig. 5; data not shown). As can be observed in Figure 5A, the anti-GFP antibodies efficiently precipitated GFP–SpoIVFA and SpoIVFB from extracts derived from a strain containing GFP–SpoIVFA (as well as a wild-type copy of SpoIVFA). A small but detectable amount of wild-type SpoIVFA was also observed. Importantly, an unrelated integral membrane protein (the a subunit of the F_0F_1 ATP synthase) was not present in the immunoprecipitate (Fig. 5A). As a control, a parallel immunoprecipitation from an extract derived from a wild-type strain lacking the GFP–SpoIVFA fusion did not precipitate SpoIVFB (Fig. 5A), indicating that the coimmunoprecipitation was specific. In similar experiments we observed that both SpoIVFA and SpoIVFB coimmunoprecipitated with GFP–BofA from extracts derived from a strain containing GFP–BofA (Fig. 5B). Moreover, SpoIVFA was efficiently coimmunoprecipitated with SpoIVFB–GFP from extracts derived from a strain containing SpoIVFB–GFP (data not shown). These results provide direct evidence that SpoIVFA, SpoIVFB, and BofA reside in a complex in the membrane.

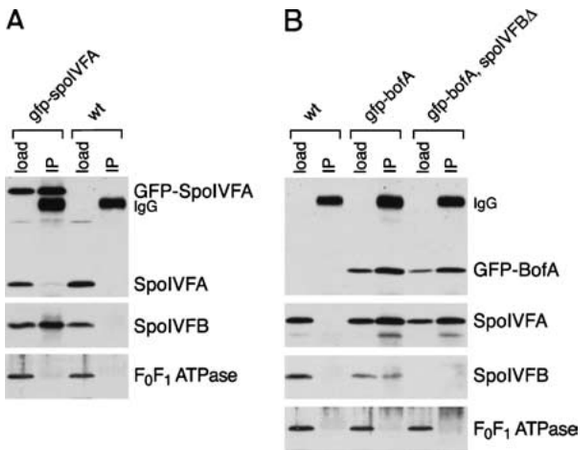


Figure 5. SpoIVFA, SpoIVFB, and BofA exist in a multimeric complex. (*A*) Immunoblots from coimmunoprecipitation assays. The strains (BDR528, PY79) analyzed are indicated. GFP–SpoIVFA was immunoprecipated from a detergent-solubilized membrane fraction (load; see Fig. 4B) from sporulating cells at hour 3.5 using affinity-purified anti-GFP antibody resin. Immunoprecipitates (IP) were subjected to immunoblot analysis using antibodies that recognize SpoIVFA, SpoIVFB, and the F_0F_1 ATP synthase. The strains analyzed are indicated. Anti-GFP antibody heavy chain (IgG) that leached off the resin (recognized by the secondary antibody) is indicated. (*B*) GFP–BofA was immunoprecipated as described above from strains (BDR954, BDR961) harboring a deletion of *bofA* at its normal locus and a copy of *gfp–bofA* at the *amyE* locus. Immunoprecipitates were subjected to immunoblot analysis as described in *A* except that anti-GFP antibodies were also used. The lower band in the SpoIVFA immunoblot is a proteolytic fragment of the full-length protein.

We infer that the stoichiometry of the SpoIVFB processing enzyme in the complex is one because we were unable to detect untagged SpoIVFB when anti-GFP antibodies were used to precipitate the complex from cells containing both SpoIVFB–GFP and wild-type SpoIVFB (data not shown). It is likely that the stoichiometry of SpoIVFA in the complex is also one, but we cannot be fully confident of this conclusion because a small amount of wild-type SpoIVFA was precipitated when anti-GFP antibodies were used to precipitate the complex from cells containing GFP–SpoIVFA and wild-type SpoIVFA (Fig. 5A).

We also used coimmunoprecipitation to investigate the requirements for complex formation. Importantly, coimmunoprecipitation of GFP–BofA and SpoIVFA was observed in the absence of SpoIVFB (Fig. 5B). This result is consistent with the idea that BofA directly or indirectly interacts with SpoIVFA in a manner that does not depend on SpoIVFB. It is also fully consistent with the results of cytological analysis, which showed that proper localization of GFP–BofA depended on SpoIVFA but not SpoIVFB (Fig. 3B), and with the results of physiological analysis, which showed that the accumulation of BofA depended upon SpoIVFA but not on SpoIVFB (Fig. 2). Technical limitations prevented us from determining whether SpoIVFB and SpoIVFA remain complexed in the absence of BofA as was suggested by the cytological and protein accumulation studies. In the absence of BofA, the levels of SpoIVFA and SpoIVFB (but not GFP–SpoIVFA or SpoIVFB–GFP) dropped below the limit of detection in our assay. We conclude that SpoIVFA, SpoIVFB, and BofA are components of a heteromeric, membrane-bound complex and that SpoIVFB is not required for retaining SpoIVFA and BofA in the complex.

Regulation of the SpoIVFB processing enzyme by SpoIVFA and BofA

Having definitively established that SpoIVFB, SpoIVFA, and BofA exist as a heteromeric complex in the membrane, we turned our attention to the regulation of pro-σ^K processing. Several models have been proposed for how SpoIVFB is regulated by BofA and SpoIVFA (Fig. 1B). In the first model, BofA and SpoIVFA separately contact and inhibit SpoIVFB (Fig. 1B, I). Three lines of evidence suggest that BofA and SpoIVFA interact with each other in the absence of SpoIVFB. First, SpoIVFA and BofA depend on each other for accumulation (Fig. 2). Moreover, the requirement for SpoIVFA for accumulation of BofA does not depend on SpoIVFB. Second, GFP–BofA depends on SpoIVFA for proper localization but does not depend on SpoIVFB (Fig. 3B). Third and most importantly, GFP–BofA and SpoIVFA can be coimmunoprecipitated in the absence of SpoIVFB (Fig. 5B). Thus, it is likely that BofA and SpoIVFA interact with each other, which would be inconsistent with a model in which BofA and SpoIVFA separately contact and inhibit SpoIVFB (Fig. 1B, I; Cutting et al. 1990; Resnekov et al. 1996; Resnekov and Losick 1998). We cannot rule out the possibility that BofA and SpoIVFA within the multimeric complex independently inhibit SpoIVFB and that inhibition by both proteins is necessary to prevent pro-σ^K processing.

The results of Figure 2, including the immunoblot analysis with anti-σ^K antibodies, are also inconsistent with a simple, linear model in which SpoIVFA is an activator of the SpoIVFB protease and BofA is an inhibitor of SpoIVFA (Fig. 1B, II). The results show that SpoIVFB was able to process pro-σ^K to a substantial extent in the absence of SpoIVFA (Fig. 2, lane 3). Therefore, SpoIVFA is not an essential activator of the protease, although it cannot be excluded that it has a stimulatory effect.

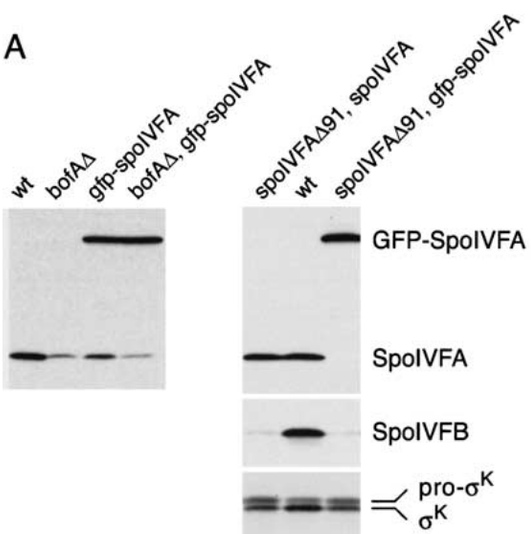
A third model holds that SpoIVFA is an unstable inhibitor of the SpoIVFB processing enzyme and that the role of BofA is to protect SpoIVFA from proteolytic degradation (Fig. 1B, III; Resnekov 1999). In this model, activation of the SpoIVFB processing enzyme is achieved by disruption of the interaction between BofA and SpoIVFA, resulting in the degradation of SpoIVFA and relief of the inhibition imposed on SpoIVFB. This model is based on experiments performed on *B. subtilis* cells that had been engineered to produce pro-σ^K, SpoIVFB, SpoIVFA, and BofA during growth (Resnekov and Losick 1998; Resnekov 1999). The model predicts that a SpoIVFA mutant that is resistant to degradation in the absence of BofA should prevent pro-σ^K processing. In our

characterization of a GFP–SpoIVFA fusion (see Materials and Methods), we discovered that the accumulation of this hybrid protein was unaffected by the absence of BofA. As can be observed in Figure 6A, the level of wild-type SpoIVFA during sporulation was greatly reduced by the absence of BofA, whereas the level of GFP–SpoIVFA was unaltered. Importantly, this GFP–SpoIVFA fusion protein is fully functional (Supplemental Fig. 2; Supplemental Table 1). If the instability of SpoIVFA were critical to the activation of SpoIVFB, then GFP–SpoIVFA should prevent pro-σ^K processing and reduce sporulation efficiency. Contrary to the predictions of this model, this stable form of SpoIVFA had no measurable effect on sporulation efficiency (Fig. 6B) or pro-σ^K processing (data not shown).

It was conceivable that the GFP–SpoIVFA fusion protein was not fully resistant to proteolysis and that a small but undetectable amount of GFP–SpoIVFA was destroyed during sporulation (as a result, according to the model, of disruption of the interaction of GFP–SpoIVFA with BofA in response to the forespore signaling protein). Such a small reduction in GFP–SpoIVFA levels would have allowed a small quantity of SpoIVFB to escape inhibition. This is a valid concern because it has previously been reported that the amount of SpoIVFB necessary for pro-σ^K processing and efficient sporulation is well below the wild-type level of the processing enzyme (Rudner et al. 1999; Yu and Kroos 2000). To address this possibility, we used a strain in which the amount of the SpoIVFB processing enzyme had been reduced to a low level. The *spoIVFAΔ91* mutation is an in-frame deletion of *spoIVFA* that has a polar effect on *spoIVFB* (Supplemental Fig. 3; Cutting et al. 1990; Resnekov et al. 1996). In a *spoIVFAΔ91* mutant with a wild-type copy of *spoIVFA* present in *trans*, the level of SpoIVFB was ~5- to 10-fold lower than that in the wild type (Fig. 6A; Supplemental Fig. 3). As a result, the efficiency of pro-σ^K processing was slightly reduced (Fig. 6A). If SpoIVFA is an inhibitor of SpoIVFB and if the processing enzyme is activated by the proteolytic degradation of SpoIVFA, then we would expect to observe at least some reduction in the level of pro-σ^K processing when GFP–SpoIVFA is present in a strain with only very low levels of SpoIVFB. In fact, we observed nearly identical levels of processing activity in the presence of GFP–SpoIVFA or wild-type SpoIVFA (Fig. 6A). Thus, even under conditions in which the level of processing enzyme had been greatly reduced, GFP–SpoIVFA showed no measurable capacity to inhibit pro-σ^K processing. We conclude that even if GFP–SpoIVFA undergoes a small amount of sporulation-specific degradation, it seems unlikely that a decrease in GFP–SpoIVFA levels is responsible for triggering SpoIVFB-dependent processing. These results therefore indicate that the accumulation of SpoIVFA, although influenced by the presence of BofA (see Figs. 2 and 6A), does not play an active role in regulating the SpoIVFB processing enzyme.

Our analysis of the pro-σ^K processing complex is most consistent with a new model in which SpoIVFA is neither a direct activator nor an inhibitor of SpoIVFB. Instead, BofA is responsible for inhibiting the processing enzyme, and SpoIVFA serves as a platform for bringing SpoIVFB and BofA into close proximity so that BofA blocks SpoIVFB-mediated processing of σ^K (Fig. 1A and B, IV). An important prediction of this model is that in the absence of SpoIVFA, the BofA inhibitor would not be tethered near the SpoIVFB protease and pro-σ^K processing would be constitutive. It was previously reported that an in-frame deletion in *spoIVFA* (*spoIVFAΔ91*) severely impaired pro-σ^K processing (Cutting et al. 1991b; Green and Cutting 2000), which would be inconsistent with our model. However, it was not appreciated at the time that *spoIVFAΔ91* has a polar effect on the downstream gene *spoIVFB*, as we have now shown to be the case (Fig. 6A; Supplemental Fig. 3). When *spoIVFB* was, instead, placed under the control of the *spoIVF* promoter at a nonessential locus in an SpoIVFA mutant, approximately wild-type levels of σ^K activity were observed in the absence of a forespore signal (Supplemental Fig. 4).

A



B

strain	sporulation efficiency
wild-type	100%
spoIVFΔ::cat	0.0002%
spoIVFΔ::cat, zej-82::spoIVFB	11%
spoIVFΔ::cat, zej-82::spoIVFB, amyE::spoIVFA	78%
spoIVFΔ::cat, zej-82::spoIVFB, amyE::gfp-spoIVFA	67%

Figure 6. GFP–SpoIVFA is stable in the absence of BofA but does not inhibit pro-σ^K processing. (*A*) Immunoblots of whole-cell extracts from cells undergoing sporulation. Lysates were prepared from sporulating cells at hour 3.5 and analyzed using polyclonal antibodies that recognize SpoIVFA, SpoIVFB, and σ^K. The strains analyzed (PY79, RL774, BDR528, BDR706, BDR426, BDR704) are indicated. The *gfp–spoIVFA* and *spoIVFA* genes were placed at the *amyE* locus. The *spoIVFAΔ91* allele contains an in-frame deletion of *spoIVFA* and results in reduced levels of SpoIVFB protein (Supplemental Fig. 3). (*B*) Indicated strains (PY79, RL976, BDR786, BDR883, BDR955) were sporulated by nutrient exhaustion, and sporulation efficiency (as compared with wild type) was determined.

This constitutive, "bypass-of-forespore" phenotype supports our model that the SpoIVFB protease is active in the absence of SpoIVFA and that the role of SpoIVFA is to bring BofA into close proximity to the processing enzyme.

Discussion

We have shown that the pro-σ^K processing enzyme SpoIVFB exists in a complex with its partner proteins BofA and SpoIVFA. The evidence indicates that the complex is likely to contain one molecule of SpoIVFB and one molecule of SpoIVFA. We have also shown that the complex resides in the mother-cell membrane that surrounds the forespore and hence that it is appropriately positioned to receive, and respond to, the signaling protein (SpoIVB) that is produced in the forespore. SpoIVFA plays a central role in both maintaining the complex and anchoring it to the outer forespore membrane. In the absence of SpoIVFA, both BofA and SpoIVFB are mislocalized. Conversely, SpoIVFA localizes properly in the absence of both BofA and SpoIVFB, indicating that SpoIVFA serves as the primary anchor for the complex and that it is localized by a mechanism that is independent of its partners.

A model for regulation of SpoIVFB processing activity

The SpoIVFA–SpoIVFB–BofA complex is responsible for keeping the pro-σ^K processing enzyme inactive until the signal is received from the forespore (Cutting et al. 1990). What is the mechanism by which SpoIVFB is held inactive by SpoIVFA and BofA? Our results support arguments against several previously proposed models and suggest a new model for the regulation of the pro-σ^K processing enzyme. Because SpoIVFA and BofA interact with each other, a model in which BofA and SpoIVFA separately contact and inhibit SpoIVFB (Fig. 1B, I) is likely to be incorrect. The observation that SpoIVFB is capable of processing pro-σ^K in the absence of SpoIVFA is also incompatible with a simple linear model, in which BofA inhibits SpoIVFA and SpoIVFA activates SpoIVFB (Fig. 1B, II), be correct. Finally, a model in which SpoIVFA is an inhibitor of SpoIVFB and BofA serves to protect SpoIVFA from proteolysis (Fig. 1B, III) was ruled out using a stabilized version of SpoIVFA (GFP–SpoIVFA), which was fully functional but did not detectably inhibit pro-σ^K processing.

This third model merits further discussion because two previous observations suggested that SpoIVFA does inhibit SpoIVFB. The existence of point mutants of SpoIVFA (the result of alleles called *bofB* mutations, for bypass of forespore) that bypass the requirement for the forespore signal (and hence result in constitutive pro-σ^K processing) was interpreted to indicate that SpoIVFA inhibits the SpoIVFB processing enzyme and that the *bofB* mutant form of SpoIVFA was defective in this inhibitory effect (Cutting et al. 1990). An alternative interpretation consistent with our present finding that SpoIVFA is responsible for localizing both SpoIVFB and BofA to the outer forespore membrane is that *bofB* mutants of SpoIVFA are defective in proper localization of BofA. Consistent with this interpretation, GFP–BofA showed only partial localization in *bofB5* and *bofB8* mutants of SpoIVFA at hour 2.5 of sporulation and became completely mislocalized at later times (data not shown). Thus, the constitutive processing activity of the bypass mutants could be caused by impaired interaction of SpoIVFA with BofA rather than by a failure of SpoIVFA to directly inhibit the SpoIVFB processing enzyme.

Additional evidence that has been interpreted as indicating that SpoIVFA is a negative regulator of SpoIVFB comes from experiments in which the synthesis of SpoIVFA, SpoIVFB, and pro-σ^K was engineered to occur during vegetative growth (Resnekov 1999). Under these conditions, SpoIVFA accumulates to a low level and SpoIVFB-dependent processing of pro-σ^K processing can be readily detected (Resnekov and Losick 1998; Resnekov 1999; Supplemental Fig. 5). However, in the absence of the protease FtsH, SpoIVFA accumulates to a high level and pro-σ^K processing is impaired (Resnekov 1999; Supplemental Fig. 5). Evidently, SpoIVFA is degraded in an FtsH-dependent manner. The inverse correlation between the level of SpoIVFA and SpoIVFB-dependent processing was interpreted to indicate that SpoIVFA is responsible for inhibiting the SpoIVFB processing enzyme (Resnekov 1999). To determine whether impaired processing in the FtsH mutant was a direct consequence of stabilizing SpoIVFA, we engineered cells to synthesize SpoIVFB and pro-σ^K, but not SpoIVFA, during growth, and we did so in the presence and absence of FtsH. Importantly, SpoIVFB processing activity was significantly reduced when FtsH was absent, even though the cells lacked SpoIVFA (Supplemental Fig. 5). The reduced level of SpoIVFB processing activity in the absence of FtsH is sufficient to explain the low level of pro-σ^K processing observed in the previously reported experiments in which SpoIVFB and SpoIVFA were cosynthesized. Apparently, the absence of FtsH, which is known to have pleiotropic effects, adversely affects the SpoIVFB processing enzyme even when SpoIVFA is not present. Therefore, these experiments do not provide support for the idea that SpoIVFA is an inhibitor of SpoIVFB.

Based on our analysis of previous models and the data in the present study we propose that BofA is the true inhibitor of SpoIVFB and that SpoIVFA is neither an activator nor inhibitor of processing. Rather, SpoIVFA brings BofA into close proximity to the processing enzyme, allowing BofA to inhibit SpoIVFB (Fig. 1A and B, IV). In our model, SpoIVFA serves as a platform upon which BofA and SpoIVFB are able to interact with each other. Although favoring the idea that BofA directly inhibits SpoIVFB, we cannot rule out a more complicated allosteric model in which BofA does not touch SpoIVFB. Instead, it induces a conformational change in SpoIVFA, and it is this altered state of SpoIVFA that is responsible for inhibiting the SpoIVFB processing enzyme. In either view, BofA is responsible for blocking processing, whether directly by contact with the process-

Figure 7. SpoIVFA contains a region that is conserved in a family of peptidoglycan remodeling enzymes. Conserved amino acids are shown in black and gray boxes. A black box was assigned if 50% of the proteins analyzed contained the identical residue at that position. A gray box was assigned if 50% of the proteins had a similar residue at that position. Similar residues were I, L, V, and M; F and Y; D and E; N and Q; S and T; A and G; R and K. Amino acid positions are indicated. Gene names are shown on the *left*. The two conserved histidines implicated in Zn^{2+} coordination and peptidase activity are indicated with carets. Lysostaphin (GenBank accession no. P10547) from *Staphylococcus simulans* encodes lysostaphin precursor (glycyl-glycine endopeptidase); lytM (GenBank accession no. AAB62278) from *Staphylococcus aureus* encodes peptidoglycan hydrolase; zooA (GenBank accession no. AAC46072) from *Streptococcus zooepidemicus* encodes ZoocinA endopeptidase; tagE (GenBank accession no. VC0843) from *Vibrio cholerae* encodes TagE protein; nlpD (GenBank accession no. P33648) from *E. coli* encodes novel lipoprotein D; spoIIQ (B69713) from *B. subtilis* encodes the sporulation protein SpoIIQ.

ing enzyme or indirectly by transmitting a signal through SpoIVFA.

A cell-wall anchoring model for the localization of the pro-σ^K processing complex to the outer forespore membrane

We have presented evidence that SpoIVFA anchors the pro-σ^K processing complex to the mother-cell membrane that surrounds the forespore. What feature of SpoIVFA is responsible for this specific localization? Searches of amino-acid-sequence databases identified a region of SpoIVFA that is present in a family of proteins involved in peptidoglycan remodeling (Fig. 7). All the characterized members of this family are Zn^{2+} metalloproteases that cleave peptide bonds in interpeptide bridges in peptidoglycan (Sugai et al. 1997). It is unlikely, however, that SpoIVFA has endopeptidase activity because it lacks two conserved histidines that are thought to be required for coordinating Zn^{2+} (Gustin et al. 1996; see the carets in Fig. 7). Nonetheless, the conserved region present in SpoIVFA might be sufficient for interaction with peptidoglycan (Baba and Schneewind 1996). Importantly, the membrane topology of SpoIVFA analyzed in *E. coli* predicts that this conserved region resides in the space between the mother-cell and forespore membranes (Green and Cutting 2000). These observations raise the intriguing possibility that SpoIVFA is anchored to the outer forespore membrane through an interaction with a feature of the peptidoglycan that resides in the space between the mother-cell and forespore membranes. The conserved domain in SpoIVFA could interact with remnants of the septal peptidoglycan, which is degraded following polar division, or to newly synthesized peptidoglycan produced in the space between the forespore and mother-cell membranes during the process of engulfment (Illing and Errington 1991; Stragier and Losick 1996; Driks 1999; Perez et al. 2000).

In summary, the pro-σ^K processing enzyme SpoIVFB and its partner proteins SpoIVFA and BofA exist in a heteromeric complex that is localized in the mother-cell membrane surrounding the forespore. We propose that SpoIVFA serves as a platform for bringing BofA into close proximity to SpoIVFB, thereby enabling BofA to inhibit the pro-σ^K processing enzyme. SpoIVFA also dictates the subcellular localization of the multimeric complex, perhaps through an interaction with peptidoglycan in the space between the mother-cell and forespore membranes.

Materials and methods

General methods

All *B. subtilis* strains were derived from the prototrophic strain PY79 (Youngman et al. 1983) and are listed in Supplemental Table 2. The *E. coli* strains used were TG1 and DH5α. Antibiotic resistance genes present in preexisting chromosomal insertion mutations were switched using the plasmids of Steinmetz and Richter (1994). The oligonucleotide primers used for PCR are listed in Supplemental Table 3. To insert genes at the nonessential locus *zej-82* (Vandeyar and Zahler 1986), plasmids used for integrations at *amyE* marked with *kan* were transformed into the strain JDB309 [*zej-82*::Tn*917*::*pTV21Δ2*::*pD177.1 (spc, cat)*] selecting for Kan(R) and screening for Spc(S), Cm(R), and the ability to catabolize starch (Amy$^+$; Dworkin and Losick 2001). The *zej-82* locus has recently been found to be the *proJ* gene (P. Stragier, pers. comm.).

GFP fusion proteins

All three fusions were to a bright, fast-folding mutant of GFP (mutant 2; Cormack et al. 1996). The C-terminal SpoIVFB–GFP fusion was identical to an earlier version (Resnekov et al. 1996) except that the original GFP was replaced with the enhanced form. Like the earlier version, this fusion was functional for sporulation and was inhibited by BofA and SpoIVFA in the absence of the forespore signaling protein (Resnekov and Losick 1998; Supplemental Table 1). Based on topological analysis that indicated that the N-terminal end of SpoIVFA is located on the cytoplasmic side of the membrane (Green and Cutting 2000), we fused GFP to the N terminus of SpoIVFA. This GFP–SpoIVFA fusion protein was functional in that it inhibited pro-σ^K processing in the absence of a forespore signal and it complemented

a *spoIVFA* null for sporulation (Supplemental Fig. 2; Supplemental Table 1). Fusions of GFP to the N or C termini of BofA were nonfunctional and showed dominant-negative phenotypes (perhaps owing to attempted translocation of GFP across the membrane). Based on the observation that the first transmembrane segment of BofA was dispensable for activity (Ricca et al. 1992), we fused GFP to the twenty-seventh amino acid of BofA, deleting the first transmembrane segment of the protein. When produced in the mother cell under the control of a σ^E-regulated promoter (see below), the GFP–BofA fusion protein, like the original transmembrane segment deletion, was found to be functional in that it inhibited pro-σ^K processing in the absence of the forespore signaling protein SpoIVB and complemented a *bofA* null mutation for sporulation (Supplemental Fig. 1; Supplemental Table 1).

Plasmid construction

A complete description of the plasmids used can be found online in Supplemental Material at www.genesdev.org.

Quantitative sporulation assay

Sporulation was induced by resuspension or exhaustion (in supplemented DS medium; Schaeffer et al. 1965) as described previously (Rudner et al. 1999). Sporulation efficiency was determined in 36-h cultures as the total number of heat-resistant (80°C for 20 min) colony forming units (CFUs) compared with the total number of CFUs before heat treatment.

Immunoblot analysis

Proteins were separated by SDS-PAGE on 12% polyacrylamide gels and electroblotted onto Immobilon-P membrane (Millipore) and blocked in 5% nonfat milk in phosphate-buffered saline (PBS)–0.5% Tween-20. The blocked membrane was probed with anti-σ^K (Resnekov et al. 1996), anti-SpoIVFA (Resnekov et al. 1996), anti-σ^A (Fujita and Sadaie 1998), anti-F_0F_1 ATP synthase polyclonal antiserum, or affinity-purified anti-SpoIVFB (Resnekov et al. 1996) or anti-GFP antibodies. Primary antibodies were diluted 1:10,000 (anti-σ^K, anti-SpoIVFA, anti-GFP, and anti-σ^A), 1:2000 (anti-F_0F_1 ATP synthase), and 1:1000 (anti-SpoIVFB) into 3% BSA or 5% nonfat milk in PBS–0.05% Tween-20. Primary antibody was detected using horseradish peroxidase-conjugated goat, anti-rabbit immunoglobulin G (Bio-Rad) with the Supersignal Substrate as described by the manufacturer (Pierce).

Whole-cell extract preparation

At indicated times after the initiation of sporulation (by resuspension), the OD_{600} was measured (for equivalent loading) and samples (1.0 mL) were collected by centrifugation. Cell pellets were stored at −80°C. Whole-cell extracts were prepared by resuspension of cell pellets in 50 µL of lysis buffer (20 mM Tris at pH 7.0, 10 mM EDTA, 1 mg/mL lysozyme, 10 µg/mL DNase I, 100 µg/mL RNase A, with protease inhibitors: 1 mM PMSF, 1 µg/mL leupeptin, 1 µg/mL pepstatin) and incubation at 37°C for 10 min followed by addition of 50 µL of sodium dodecyl sulfate (SDS) sample buffer (0.25 M Tris at pH 6.8, 6% SDS, 20% glycerol) containing 10% 2-mercaptoethanol. Samples were heated at 50°C for 20 min prior to loading.

Fractionation of membrane proteins

The 50-mL cultures were harvested at 3.5 h after the initiation of sporulation (by resuspension) and washed two times with 1× SMM (0.5 M sucrose, 20 mM $MgCl_2$, 20 mM maleic acid at pH 6.5; Harwood and Cutting 1990) at room temperature. Cells were resuspended in 1/10 volume of 1× SMM and protoplasted with lysozyme (0.5 mg/mL). Protoplasts were collected by centrifugation and flash frozen in liquid N_2. Thawed protoplasts were disrupted by osmotic lysis with 5 mL of hypotonic buffer (buffer H) (20 mM HEPES at pH 7.6, 25 mM NaCl, 1 mM DTT, with protease inhibitors: 1 mM PMSF, 1 µg/mL leupeptin, 1 µg/mL pepstatin). $MgCl_2$ and $CaCl_2$ were added to 1 mM, and lysates were treated with DNase I (10 µg/mL) and RNase A (20 µg/mL) for 1 h on ice. Soluble and insoluble proteins were separated by centrifugation at 100,000*g* at 4°C for 1 h.

Crude membrane preparation

Nuclease-treated extracts as described above were loaded on a 3-mL 0.8 M sucrose cushion and spun at 100,000*g* at 4°C for 1 h in an SW41Ti rotor (Beckman). The supernatant was carefully removed, and the pellet was dispersed in 1 mL of buffer H and loaded on a step-gradient containing 0.6 M, 1.2 M, 1.6 M sucrose and spun at 100,000*g* at 4°C for 1 h in an SW55Ti rotor (Beckman). The membrane fraction was collected from the interface between the 1.2 M and 1.6 M steps and diluted fivefold in buffer G (buffer H with 10% glycerol) and spun at 100,000*g* at 4°C for 1 h in an SW55Ti rotor. The pellet was dispersed in 500 µL of buffer G, distributed in 50-µL aliquots, and flash-frozen in liquid N_2.

Solubilization of membrane proteins

The 50-µL crude membranes were diluted 10-fold with buffer S (buffer H + 1.5 mg/mL *E. coli* phospholipids [Avanti], 20% glycerol) (Driessen and Wickner 1990), buffer S containing 1 M NaCl, or buffer S containing 0.5% digitonin. Diluted membranes were incubated on ice for 1 h with periodic mixing, and soluble and insoluble fractions were separated by centrifugation at 100,000*g* at 4°C for 1 h in a Tft80.4 rotor (Sorvall).

Coimmunoprecipitation from detergent-solubilized membrane fractions

The soluble fraction from the digitonin-treated membrane preparation was transferred to a microfuge tube and incubated with 30 µL of uncoupled affigel-Hz (Bio-Rad) at 4°C for 1 h. Then 15 µL of affinity-purified anti-GFP antibody coupled to affigel-Hz (as described by the manufacturer) was added to the precleared extract and rotated at 4°C for 2–4 h. The antibody resin was pelleted at 3000 rpm and washed four times with 1 mL of buffer S. Finally, 50 µL of SDS-sample buffer was added to the pellet and heated at 50°C for 20 min.

GFP purification and antibody production

The *gfp* gene, subcloned into pQE9 (QIAGEN) (a gift from A. Straight, Harvard Medical School, Boston, MA) was expressed in *E. coli* TG1. Cells were grown in LB at room temperature to an OD_{600} of 0.6, induced by the addition of IPTG to 1 mM, and harvested after 3 h. All subsequent manipulations were carried out at 4°C. Cells were harvested by centrifugation and resuspended in 1/20 volume of buffer I (20 mM Tris-HCl at pH 8.0, 100 mM NaCl, 5 mM 2-mercaptoethanol, 1 mM PMSF). A crude extract was prepared by freeze-thawing the cells followed by the addition of DNase I (10 µg/mL), RNase A (10 µg/mL), and lysozyme (1 mg/mL). A soluble fraction was prepared by a 100,000*g* spin and was loaded on an HR5/5 (Pharmacia) Ni^{2+}-NTA agarose (QIAGEN) column equilibrated with buffer II (20 mM Tris-

HCl at pH 8.0, 500 mM NaCl, 10% glycerol, 5 mM 2-mercaptoethanol). Bound protein was washed with buffer II containing 20 mM imidazole and eluted in buffer II containing 100 mM imidazole. Peak fractions were pooled and loaded on a Sephacryl 200 (Pharmacia) preparative sizing column equilibrated with buffer III (20 mM HEPES at pH 7.6, 100 mM NaCl, 10% glycerol, 5 mM 2-mercaptoethanol). Peak fractions were pooled and concentrated on an HR5/5 monoQ column (Pharmacia) equilibrated with buffer III. Bound protein was eluted with a linear NaCl gradient from 100 mM to 1 M. Peak fractions were used to generate anti-GFP, rabbit polyclonal antibodies (Covance).

Fluorescence microscopy

Fluorescence microscopy was performed with an Olympus BX60 microscope as described previously (King et al. 1999), except that a Xenon lamp (Sutter Instruments, Novato, CA) was used in place of a Mercury lamp. Fluorescent signals were viewed using the phase contrast objective UplanF1 100×. GFP was visualized using a U-WIBA filter cube (excitation filter 460–490 nm, barrier filter >590 nm). Images were captured using Metamorph software version 4.0 (Universal Imaging, Media, PA). Exposure times were typically 1–3 sec. Metamorph images were converted to 8-bit images and imported as TIFF files into Adobe Photoshop. Images were false-colored and overlaid in Photoshop.

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References

Baba, T. and Schneewind, O. 1996. Target cell specificity of a bacteriocin molecule: A C-terminal signal directs lysostaphin to the cell wall of *Staphylococcus aureus*. *EMBO J.* **15:** 4789–4797.

Brown, J.D., Hann, B.C., Medzihradszky, K.F., Niwa, M., Burlingame, A.L., and Walter, P. 1994. Subunits of the *Saccharomyces cerevisiae* signal recognition particle required for its functional expression. *EMBO J.* **13:** 4390–4400.

Brown, M.S. and Goldstein, J.L. 1997. The SREBP pathway: Regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* **89:** 331–340.

Brown, M.S., Ye, J., Rawson, R.B., and Goldstein, J.L. 2000. Regulated intramembrane proteolysis: A control mechanism conserved from bacteria to humans. *Cell* **100:** 391–398.

Cormack, B.P., Valdivia, R.H., and Falkow, S. 1996. FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173:** 33–38.

Cutting, S., Oke, V., Driks, A., Losick, R., Lu, S., and Kroos, L. 1990. A forespore checkpoint for mother cell gene expression during development in *B. subtilis*. *Cell* **62:** 239–250.

Cutting, S., Driks, A., Schmidt, R., Kunkel, B., and Losick, R. 1991a. Forespore-specific transcription of a gene in the signal transduction pathway that governs Pro-sigma K processing in *Bacillus subtilis*. *Genes & Dev.* **5:** 456–466.

Cutting, S., Roels, S., and Losick, R. 1991b. Sporulation operon spoIVF and the characterization of mutations that uncouple mother-cell from forespore gene expression in *Bacillus subtilis*. *J. Mol. Biol.* **221:** 1237–1256.

Dosil, M., Schandel, K.A., Gupta, E., Jenness, D.D., and Konopka, J.B. 2000. The C terminus of the *Saccharomyces cerevisiae* α-factor receptor contributes to the formation of preactivation complexes with its cognate G protein. *Mol. Cell. Biol.* **20:** 5321–5329.

Driessen, A.J. and Wickner, W. 1990. Solubilization and functional reconstitution of the protein-translocation enzymes of *Escherichia coli*. *Proc. Natl. Acad. Sci.* **87:** 3107–3111.

Driks, A. 1999. *Bacillus subtilis* spore coat. *Microbiol. Mol. Biol. Rev.* **63:** 1–20.

Dworkin, J. and Losick, R. 2001. Differential gene expression governed by chromosomal spatial asymmetry. *Cell* **107:** 339–346.

Fujita, M. and Sadaie, Y. 1998. Rapid isolation of RNA polymerase from sporulating cells of *Bacillus subtilis*. *Gene* **221:** 185–190.

Green, D.H. and Cutting, S.M. 2000. Membrane topology of the *Bacillus subtilis* pro-σ^K processing complex. *J. Bacteriol.* **182:** 278–285.

Gustin, J.K., Kessler, E., and Ohman, D.E. 1996. A substitution at His-120 in the LasA protease of *Pseudomonas aeruginosa* blocks enzymatic activity without affecting propeptide processing or extracellular secretion. *J. Bacteriol.* **178:** 6608–6617.

Harwood, C.R. and Cutting, S.M. 1990. *Molecular biological methods for* Bacillus. Wiley, New York, NY.

Illing, N. and Errington, J. 1991. Genetic regulation of morphogenesis in *Bacillus subtilis*: Roles of σ E and σ F in prespore engulfment. *J. Bacteriol.* **173:** 3159–3169.

Jackson, C.L., Konopka, J.B., and Hartwell, L.H. 1991. *S. cerevisiae* α pheromone receptors activate a novel signal transduction pathway for mating partner discrimination. *Cell* **67:** 389–402.

King, N., Dreesen, O., Stragier, P., Pogliano, K., and Losick, R. 1999. Septation, dephosphorylation, and the activation of σF during sporulation in *Bacillus subtilis*. *Genes & Dev.* **13:** 1156–1167.

Kroos, L., Kunkel, B., and Losick, R. 1989. Switch protein alters specificity of RNA polymerase containing a compartment-specific σ factor. *Science* **243:** 526–529.

Long, J.C., Wang, S., and Vik, S.B. 1998. Membrane topology of subunit a of the F_1F_0 ATP synthase as determined by labeling of unique cysteine residues. *J. Biol. Chem.* **273:** 16235–16240.

Losick, R. and Stragier, P. 1992. Crisscross regulation of cell-type-specific gene expression during development in *B. subtilis*. *Nature* **355:** 601–604.

Lu, S., Cutting, S., and Kroos, L. 1995. Sporulation protein SpoIVFB from *Bacillus subtilis* enhances processing of the σ factor precursor Pro-σ K in the absence of other sporulation gene products. *J. Bacteriol.* **177:** 1082–1085.

Manoil, C. and Beckwith, J. 1986. A genetic approach to analyzing membrane protein topology. *Science* **233:** 1403–1408.

Perez, A.R., Abanes-De Mello, A., and Pogliano, K. 2000. SpoIIB localizes to active sites of septal biogenesis and spatially regulates septal thinning during engulfment in *Bacillus subtilis*. *J. Bacteriol.* **182:** 1096–1108.

Rawson, R.B., Zelenski, N.G., Nijhawan, D., Ye, J., Sakai, J.,

Hasan, M.T., Chang, T.Y., Brown, M.S., and Goldstein, J.L. 1997. Complementation cloning of S2P, a gene encoding a putative metalloprotease required for intramembrane cleavage of SREBPs. *Mol. Cell* **1:** 47–57.

Resnekov, O. 1999. Role of the sporulation protein BofA in regulating activation of the *Bacillus subtilis* developmental transcription factor σK. *J. Bacteriol.* **181:** 5384–5388.

Resnekov, O. and Losick, R. 1998. Negative regulation of the proteolytic activation of a developmental transcription factor in *Bacillus subtilis*. *Proc. Natl. Acad. Sci.* **95:** 3162–3167.

Resnekov, O., Alper, S., and Losick, R. 1996. Subcellular localization of proteins governing the proteolytic activation of a developmental transcription factor in *Bacillus subtilis*. *Genes Cells* **1:** 529–542.

Ricca, E., Cutting, S., and Losick, R. 1992. Characterization of bofA, a gene involved in intercompartmental regulation of pro-σ K processing during sporulation in *Bacillus subtilis*. *J. Bacteriol.* **174:** 3177–3184.

Rudner, D.Z. and Losick, R. 2001. Morphological coupling in development. Lessons from prokaryotes. *Dev. Cell* **1:** 733–742.

Rudner, D.Z., Fawcett, P., and Losick, R. 1999. A family of membrane-embedded metalloproteases involved in regulated proteolysis of membrane-associated transcription factors. *Proc. Natl. Acad. Sci.* **96:** 14765–14770.

Santana, M., Ionescu, M.S., Vertes, A., Longin, R., Kunst, F., Danchin, A., and Glaser, P. 1994. *Bacillus subtilis* F_0F_1 ATPase: DNA sequence of the atp operon and characterization of atp mutants. *J. Bacteriol.* **176:** 6802–6811.

Schaeffer, P., Millet, J., and Aubert, J.P. 1965. Catabolite repression of bacterial sporulation. *Proc. Natl. Acad. Sci.* **54:** 704–711.

Steinmetz, M. and Richter, R. 1994. Plasmids designed to alter the antibiotic resistance expressed by insertion mutations in *Bacillus subtilis*, through in vivo recombination. *Gene* **142:** 79–83.

Stragier, P. and Losick, R. 1996. Molecular genetics of sporulation in *Bacillus subtilis*. *Annu. Rev. Genet.* **30:** 297–241.

Sugai, M., Fujiwara, T., Akiyama, T., Ohara, M., Komatsuzawa, H., Inoue, S., and Suginaka, H. 1997. Purification and molecular characterization of glycylglycine endopeptidase produced by *Staphylococcus capitis* EPK1. *J. Bacteriol.* **179:** 1193–1202.

Tsunoda, S., Sierralta, J., Sun, Y., Bodner, R., Suzuki, E., Becker, A., Socolich, M., and Zuker, C.S. 1997. A multivalent PDZ-domain protein assembles signalling complexes in a G-protein-coupled cascade. *Nature* **388:** 243–249.

Vandeyar, M.A. and Zahler, S.A. 1986. Chromosomal insertions of Tn917 in *Bacillus subtilis*. *J. Bacteriol.* **167:** 530–534.

Varcamonti, M., Marasco, R., De Felice, M., and Sacco, M. 1997. Membrane topology analysis of the *Bacillus subtilis* BofA protein involved in pro-σ^K processing. *Microbiology* **143:** 1053–1058.

Wakeley, P.R., Dorazi, R., Hoa, N.T., Bowyer, J.R., and Cutting, S.M. 2000. Proteolysis of SpolVB is a critical determinant in signalling of pro-σ^K processing in *Bacillus subtilis*. *Mol. Microbiol.* **36:** 1336–1348.

Youngman, P.J., Perkins, J.B., and Losick, R. 1983. Genetic transposition and insertional mutagenesis in *Bacillus subtilis* with *Streptococcus faecalis* transposon Tn917. *Proc. Natl. Acad. Sci.* **80:** 2305–2309.

Yu, Y.T. and Kroos, L. 2000. Evidence that SpoIVFB is a novel type of membrane metalloprotease governing intercompartmental communication during *Bacillus subtilis* sporulation. *J. Bacteriol.* **182:** 3305–3309.

Zelenski, N.G., Rawson, R.B., Brown, M.S., and Goldstein, J.L. 1999. Membrane topology of S2P, a protein required for intramembranous cleavage of sterol regulatory element-binding proteins. *J. Biol. Chem.* **274:** 21973–21980.