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Subcellular localization of a sporulation membrane protein is achieved through a network of interactions along and across the septum

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Summary

During the process of spore formation in *Bacillus subtilis* many membrane proteins localize to the sporulation septum where they play key roles in morphogenesis and cell-cell signalling. However, the mechanism by which these proteins are anchored at this site is not understood. In this report we have defined the localization requirements for the mother-cell membrane protein SpoIVFA, which anchors a signalling complex in the septal membrane on the mother cell side. We have identified five proteins (SpoIID, SpoIIP, SpoIIM, BofA and SpoIIIAH) synthesized in the mother cell under the control of σ^E and one protein (SpoIIQ) synthesized in the forespore under the control of σ^F that are all required for the proper localization of SpoIVFA. Surprisingly, these proteins appear to have complementary and overlapping anchoring roles suggesting that SpoIVFA is localized in the septal membrane through a web of protein interactions. Furthermore, we demonstrate a direct biochemical interaction between the extracellular domains of two of the proteins required to anchor SpoIVFA: the forespore protein SpoIIQ and the mother-cell protein SpoIIIAH. This result supports the idea that the web of interactions that anchors SpoIVFA is itself held in the septal membrane through a zipper-like interaction across the sporulation septum. Importantly, our results suggest that a second mechanism independent of forespore proteins participates in anchoring SpoIVFA. Finally, we show that the dynamic localization of SpoIIQ in the forespore is impaired in the absence of SpoIVFA but not SpoIIIAH. Thus, a complex web of interactions among mother cell and forespore proteins is responsible for static and dynamic protein localization in both compartments of the sporangium. We envision that this proposed network is involved in anchoring other sporulation proteins in the septum and that protein networks with overlapping anchoring capacity is a feature of protein localization in all bacteria.

Introduction

In spite of their small size and apparent simplicity, bacteria exhibit remarkably complex spatial organization, in which proteins localize to particular sites within the cell. Well-characterized examples include the cell division protein FtsZ, which assembles into a cytokinetic ring at mid cell in *Escherichia coli* (Lutkenhaus and Addinall, 1997); the chemotaxis receptor McpA, which localizes to the flagellated cell pole in *Caulobacter crescentus* (Alley *et al.*, 1992); the membrane phosphatase SpoIIE, which localizes to the asymmetrically positioned septum during sporulation in *Bacillus subtilis* (Arigoni *et al.*, 1995); and the actin polymerization protein IcsA which localizes to the older of the two cell poles of *Shigella flexneri* (Steinhauer *et al.*, 1999). Although the number of proteins that display specific patterns of localization continues to grow, the cellular landmarks that anchor them at particular sites remain unknown. We refer to these cellular landmarks as the 'ultimate anchors'.

An attractive system in which to define the ultimate anchors responsible for localizing proteins at particular sites in bacteria is the process of sporulation in *B. subtilis* (reviewed in Stragier and Losick, 1996; Piggot and Losick, 2002). Sporulation involves the formation of an asymmetrically positioned septum that divides the developing cell (or sporangium) into a small compartment called the forespore and a large compartment called the mother cell. These two cells follow completely different programs of gene expression that are set in motion by the transcription factors σ^F in the forespore and σ^E in the mother cell. Initially, the forespore and mother cell lie side-by-side but later in development the forespore is taken up by the mother cell in a phagocytic-like process called engulfment. During engulfment, the membrane on the mother cell face of the septum migrates around the forespore,

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pinching it off as a free protoplast within the mother cell. As a result of this process, the forespore is surrounded by two membranes: its own (called the inner forespore membrane or IFM) and a second membrane derived from the mother cell (called the outer forespore membrane or OFM). At this late stage, the mother cell packages the forespore in a protective protein coat while the forespore prepares for dormancy. Once the spore is fully mature it is released by lysis of the mother cell.

Many proteins synthesized in the mother cell under the control of σ^E have been found to localize to the OFM during the process of engulfment (Abanes-De Mello *et al*., 2002; Rudner and Losick, 2002; van Ooij and Losick, 2003; van Ooij *et al*., 2004). In addition, SpoIIQ, a forespore protein synthesized under the control of σ^F, has recently been shown to localize to the engulfing septal membrane on the forespore side (Rubio and Pogliano, 2004). We envision two possible models by which mother cell and forespore proteins are anchored in the engulfing septal membranes. In the first model, the polar septum serves as the major organizing centre. In this model, mother cell and forespore proteins are anchored through direct interaction with components of the septum (like the septal peptidoglycan) or the cell division machinery. Thus, the ultimate anchor would be the cellular landmark that specifies polar division. The protein that initiates asymmetric septation is the cell division protein FtsZ, which is redeployed from the mid cell through a spiral intermediate (Levin and Losick, 1996; Ben-Yehuda and Losick, 2002). However, it is not yet known what specifies the polar position for Z-ring assembly. In the second model, forespore and mother cell proteins are anchored in the IFM and OFM through interactions across the shared surface. In this model, the extracellular domains of integral membrane proteins on either side of the double-membrane septum interact in the intermembrane space. This zipper-like mechanism has recently been proposed for the localization of the forespore membrane protein SpoIIQ (Rubio and Pogliano, 2004). The localization of SpoIIQ to the forespore septum was found to require σ^E gene expression in the mother cell suggesting that mother cell proteins are responsible for anchoring it at this site. To determine how mother cell proteins localize to the OFM and whether they employ a similar zipper-like mechanism, we have investigated the mother-cell protein SpoIVFA.

SpoIVFA is an integral membrane protein that resides in a membrane complex with two other membrane proteins SpoIVFB and BofA (Rudner and Losick, 2002). These proteins are involved in a signal transduction pathway that links gene expression in the mother cell to gene expression in the forespore (Rudner and Losick, 2001 and references therein). All three membrane proteins are synthesized in the mother cell under the control of σ^E and localize with exquisite specificity to the mother cell septum during engulfment. SpoIVFA plays a central role in assembling and anchoring the signalling complex in the OFM. In its absence, both SpoIVFB and BofA are completely mislocalized (Rudner and Losick, 2002). Moreover, the localization of SpoIVFA to the OFM does not depend on SpoIVFB and is only slightly impaired in the absence of BofA. The mechanism by which the anchoring protein SpoIVFA is itself held in the OFM is not known. SpoIVFA is an ideal protein in which to dissect how mother cell proteins are specifically localized and to identify the ultimate anchors in the bacterial cell.

In this report, we have analysed the requirement for the localization of a functional GFP-SpoIVFA fusion protein to the OFM. We have identified five proteins (SpoIID, SpoIIP, SpoIIM, BofA and SpoIIIAH) synthesized in the mother cell under the control of σ^E and one protein (SpoIIQ) synthesized in the forespore under the control of σ^F that are all required to localize SpoIVFA. Surprisingly, these proteins appear to have overlapping anchoring capacity. Our results are most compatible with a model in which SpoIVFA is anchored in the OFM by a complex web of interactions. Furthermore, our data provides support for the idea that this web is anchored in the OFM by zipper-like interactions across the septal membrane as well as a second mechanism independent of these interactions.

Results

Subcellular localization of GFP-SpoIVFA requires σ^E

Previous studies using both immunofluorescence microscopy and GFP fusions in live cells have shown that SpoIVFA specifically localizes to the mother-cell membrane that engulfs the forespore (Fig. 1A and B) (Resnekov *et al*., 1996; Rudner and Losick, 2002). In addition, it has been shown that SpoIVFA is responsible for anchoring both SpoIVFB and BofA at this site, generating a three-partner heteromeric signalling complex (Rudner and Losick, 2002). However, nothing is known about how SpoIVFA itself is anchored in the engulfing septal membrane. Because SpoIVFA localizes to the septal membrane during sporulation, we wondered whether the anchor for SpoIVFA could be a component of the septum or the general cell-division machinery. To test this possibility, we investigated whether GFP-SpoIVFA was capable of localizing to the septum during vegetative growth.

SpoIVFA is synthesized under the control of σ^E during sporulation and is not normally present in growing cells (Cutting *et al*., 1991). To study its localization during vegetative growth, we fused *gfp-spoIVFA* to the xylose-inducible promoter P_{xylA} (see *Experimental procedures*). When artificially produced during growth, GFP-SpoIVFA was present uniformly in all membranes (Fig. 1C). GFP-

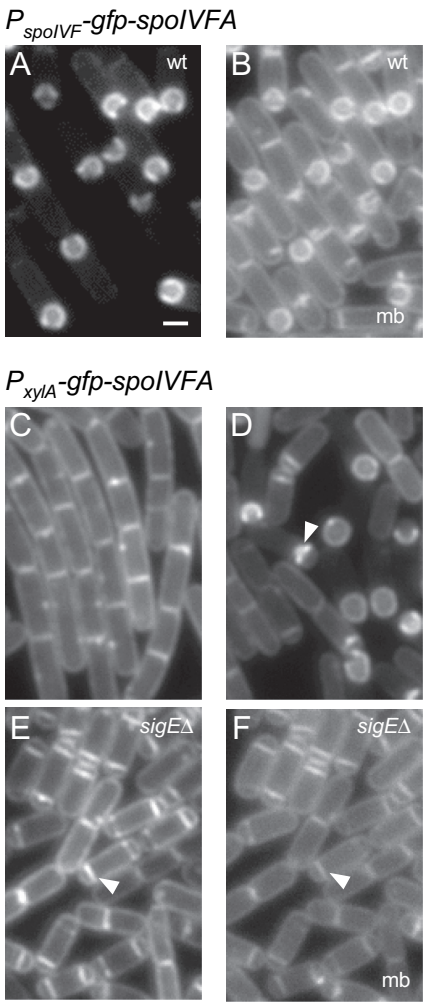

Fig. 1. Subcellular localization of GFP-SpoIVFA requires σ^E. Subcellular localization of GFP-SpoIVFA analysed in vegetative and sporulating cells (induced by resuspension) by fluorescence microscopy.
A. GFP-SpoIVFA in wild-type cells (strain BDR528) at hour 2 of sporulation.
B. The same field of cells as in (A) stained with the membrane dye TMA-DPH (mb).
C. GFP-SpoIVFA artificially synthesized in vegetatively growing cells (strain BDR776). The gene fusion was expressed during growth using the xylose-inducible promoter P_{xylA}.
D. The same cells as in (C) were induced to sporulate and GFP-SpoIVFA was analysed at hour 2 of sporulation.
E. The GFP-SpoIVFA was artificially synthesized at the initiation of sporulation (using the P_{xylA}-*gfp-spoIVFA* fusion) in a *sigE* mutant (strain BDR1101). GFP-SpoIVFA was analysed at hour 2 of sporulation.
F. The same field of cells as in (E) stained with the membrane dye TMA-DPH.
Polar septa are indicated by carets. Scale bar, 1 μm.

SpoIVFA appeared slightly enriched in the septal membranes and this is probably because the septum is composed of a double membrane. Consistent with this idea, increased GFP-SpoIVFA signal at the septum correlated with stronger membrane staining using the lypophylic membrane dye TMA-DPH (data not shown). Thus, the specific localization of GFP-SpoIVFA to the sporulation septum requires genes under sporulation control. Consistent with this idea, when the cells engineered to produce GFP-SpoIVFA during growth initiated sporulation, the fusion protein redistributed from the cytoplasmic membrane to the sporulation septum (Fig. 1D). Furthermore, the accumulation of GFP-SpoIVFA in the septal membrane was coincident with the onset of engulfment (see caret in Fig. 1D). From this we conclude that (i) as previously observed with SpoIVFB-GFP (Rudner *et al*., 2002), GFP-SpoIVFA is capable of diffusion from the cytoplasmic membrane and capture in the sporulation septum and (ii) the factors responsible for anchoring GFP-SpoIVFA in the septal membrane are produced at the time of engulfment.

Because the initiation of engulfment requires the mother cell transcription factor σ^E (Stragier and Losick, 1996), we wondered whether genes under σ^E control were required for anchoring GFP-SpoIVFA in the septal membrane. To explore this possibility, we examined GFP-SpoIVFA localization in the absence of the mother cell transcription factor. Because *spoIVFA* transcription is itself under the control of σ^E, we introduced the P_{xylA}-*gfp-spoIVFA* fusion into a *sigE* mutant and induced expression upon the initiation of sporulation. In the absence of σ^E, the process of engulfment is blocked and a second polar septum is frequently formed at the opposite cell pole (Fig. 1F) (Illing and Errington, 1991a). In support of the idea that genes under σ^E control are required for the specific localization of SpoIVFA, the fusion protein was present in all membranes after polar division (Fig. 1E). We note that GFP-SpoIVFA appeared slightly enriched in the septal membranes in the *sigE* mutant; however, we believe this is because GFP-SpoIVFA was produced prior to asymmetric division. Consequently, it would have been present in both the mother cell and forespore compartments and thus, on both sides of the septum. In support of this hypothesis, the stronger GFP-SpoIVFA signal at the polar septum correlated with the stronger membrane staining (see carets in Fig. 1E and F).

These results indicate that σ^E gene expression is required to localize GFP-SpoIVFA in the OFM. We note that as engulfment is blocked in a *sigE* mutant, it is possible that the process of engulfment is required to localize SpoIVFA. For example, the curvature of the engulfing membranes might provide a more favourable environment for the transmembrane segment of SpoIVFA (Bretscher and Munro, 1993). We do not favour this model because mutations in genes (*spoIIB, spoVG*) that are required for engulfment but are not under σ^E control (Margolis *et al*., 1993; Perez *et al*., 2000) have no discernible impact on the localization GFP-SpoIVFA (data not shown). Specifically, GFP-SpoIVFA was properly localized to both flat and bulged septa in the SpoIIB, SpoVG mutant (data not shown). We conclude that one or several proteins synthe-

sized under σ^E control are required to anchor GFP-SpoIVFA in the OFM.

Several σ^E-dependent genes are required for the localization of GFP-SpoIVFA

σ^E is the first mother-cell-specific transcription factor activated during the process of sporulation and it is responsible for the transcription of 157 loci (Eichenberger *et al*., 2003). In our effort to identify the gene(s) expressed under σ^E control that are required for anchoring SpoIVFA in the OFM, we systematically tested a library of σ^E-dependent gene knock-outs (kindly provided by P. Eichenberger and R. Losick) for effects on the localization of GFP-SpoIVFA. It has been shown previously that GFP-SpoIVFA is able to localize to the OFM without its partner proteins SpoIVFB and BofA (Rudner and Losick, 2002). However, the absence of BofA (but not SpoIVFB) leads to a slight but reproducible mislocalization of GFP-SpoIVFA to the cytoplasmic membrane (Fig. 2B). Thus, in order to cast the widest possible net in our search for mutants that impair the localization of GFP-SpoIVFA, we screened the σ^E regulon mutants in a strain lacking BofA. Individual null mutants were introduced into strain BDR706 (Table S1) and monitored for proper localization of GFP-SpoIVFA during sporulation. Most of the mutants had no detectable impact on the localization of the fusion protein (data not shown). However, we identified four mutants (*spoIID*, *spoIIM*, *spoIIP*, *spoIIIA*) that partially disrupted the septal localization of GFP-SpoIVFA (Fig. 2 and data not shown). SpoIID, SpoIIP and SpoIIM are all required for the process of engulfment and localize to the engulfing septal membrane (Smith *et al*., 1993; Frandsen and Stragier, 1995; Pogliano *et al*., 1999; Eichenberger *et al*., 2001). The *spoIIIA* locus is an eight-gene operon (*spoIIIAA-spoIIIAH*) required for the activation of the forespore transcription factor σ^G (Kellner *et al*., 1996). All eight genes are predicted to encode integral membrane proteins however,

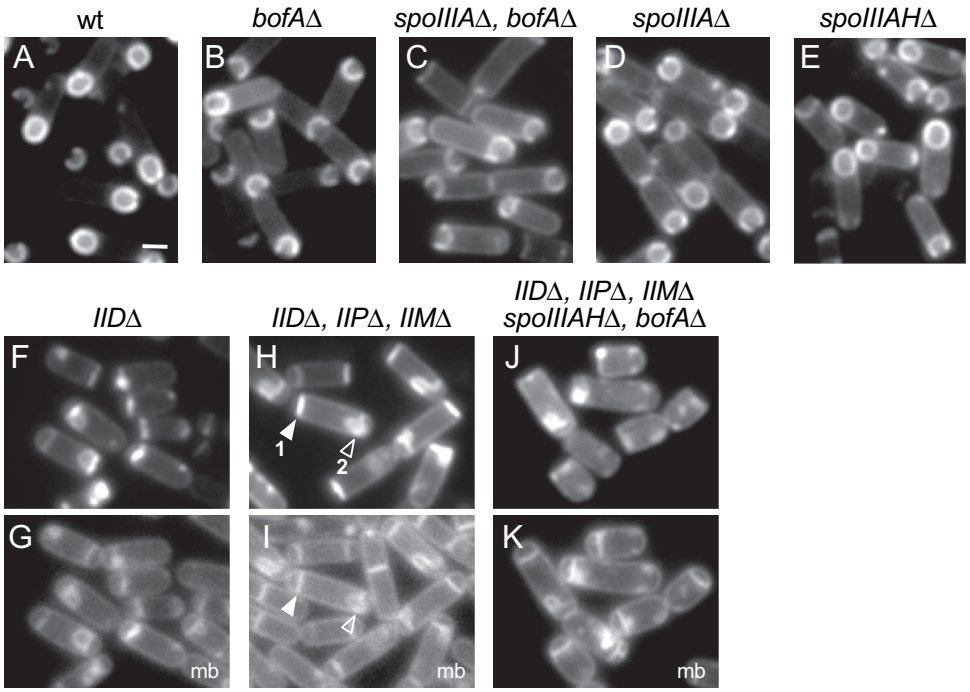

Fig. 2. Several σ^E-dependent genes are required for the localization of GFP-SpoIVFA. Sporulation was induced by resuspension in wild-type and mutant strains and GFP-SpoIVFA localization was monitored between hours 2 and 2.5 by fluorescence microscopy.
A. Wild-type (strain BDR528).
B. *bofAΔ* (strain BRD706).
C. *spoIIIAΔ*, *bofAΔ* (strain BDR779).
D. *spoIIIAΔ* (strain BDR718).
E. *spoIIIAHΔ* (strain BTD569).
F. *spoIIDΔ* (strain BTD75).
G. The same field as in (F) stained with the membrane dye TMA-DPH (mb).
H. *spoIIDΔ*, *spoIIPΔ*, *spoIIMΔ* (strain BTD3).
I. The same field as in (H) stained with the membrane dye TMA-DPH.
J. *spoIIIAHΔ*, *bofAΔ*, *spoIIDΔ*, *spoIIPΔ*, *spoIIMΔ* (strain BTD561).
K. The same field as in (J) stained with the membrane dye TMA-DPH.
Scale bar, 1 μm. Larger fields of cells comparing GFP-SpoIVFA localization in the absence of BofA and/or SpoIIIAH can be found in Fig. S1.

nothing is known about their function or their subcellular localization (Illing and Errington, 1991b).

The mislocalization of GFP-SpoIVFA was most pronounced in the absence of the *spoIIIA* locus. In our sensitized strain that lacked both BofA and SpoIIIA, we observed a decreased signal in the engulfing septal membrane accompanied by an increased signal in the cytoplasmic membranes (Fig. 2C and Fig. S1). To determine whether this mislocalization phenotype was resulting from the absence of both BofA and SpoIIIA or entirely a result of the absence of SpoIIIA, we examined GFP-SpoIVFA in a *spoIIIA* single mutant (Fig. 2D). In this background the fusion protein was impaired in its localization but not as severely as in the double mutant. These results suggest that BofA and SpoIIIA act in parallel rather than in a linear localization pathway and therefore, it is likely that they have independent or partially overlapping roles in anchoring GFP-SpoIVFA.

To determine which of the genes in the *spoIIIA* locus were required for the proper localization of GFP-SpoIVFA we analysed the fusion protein in strains harbouring in-frame deletions in each of the eight genes in the operon (kindly provided by P. Stragier). In the absence of any of the first seven genes in the *spoIIIA* locus (*spoIIIAA-AG*), GFP-SpoIVFA remained properly localized in the OFM (data not shown). However, the fusion protein was impaired in its localization in the absence of *spoIIIAH*. This mislocalization was similar to what we observed in the original *spoIIIA* mutant (Fig. 2E and Fig. S1). We therefore conclude that, among the eight proteins encoded by the *spoIIIA* operon, only SpoIIIAH is required for the proper localization of GFP-SpoIVFA.

The weak but reproducible mislocalization of GFP-SpoIVFA in the absence of SpoIID, SpoIIP or SpoIIM prompted us to test the triple mutant combination. The absence of any one of these three proteins partially impairs the engulfment process generating septa with bulges, presumably because dissolution of the septal peptidoglycan is incomplete (Fig. 2G) (Abanes-De Mello *et al*., 2002). In the triple mutant, the block to engulfment is much more pronounced and frequently a second septum forms at the opposite cell pole (see filled and unfilled carets in Fig. 2H and I) (Pogliano *et al*., 1999; Eichenberger *et al*., 2001). Consistent with a role for these engulfment proteins in the localization of SpoIVFA, the mislocalization of GFP-SpoIVFA was also more pronounced in the triple mutant (Fig. 2H). In addition to the partial localization of GFP-SpoIVFA to the first polar septum in the triple mutant, we observed a strong concentration of GFP-SpoIVFA at the second polar septum (see carets in Fig. 2H). We can distinguish between the first and second septum because σ^E becomes active in the mother cell after completion of the first septum but before the completion of the second. Subsequently, some GFP-SpoIVFA is trapped in the second forespore compartment (Fig. 2H). We believe that the second septum, which often appears blotchy and disordered by membrane staining, acts as a non-specific sink for integral membrane proteins. In support of this idea, an unrelated integral membrane protein (*E. coli* MalF fused to GFP) was similarly enriched at the second septum (but not the first) in the triple mutant (Fig. S2).

We have described five mother cell proteins synthesized under the control of σ^E (BofA, SpoIIIAH, SpoIIP, SpoIID, SpoIIM) that are all required for robust localization of GFP-SpoIVFA to the OFM. To assess whether these proteins act in a linear pathway or parallel pathways in anchoring the fusion protein, we examined GFP-SpoIVFA in the quintuple mutant. Predictably, the quintuple mutant sporangia were unhealthy looking with a disordered second polar septum (Fig. 2K); nonetheless, GFP-SpoIVFA was almost completely mislocalized in this background (Fig. 2J). This result is most consistent with the idea that these proteins act in parallel or have overlapping anchoring capacity.

SpoIIIAH temporally and spatially co-localizes with SpoIVFA

The *spoIIIAH* gene is predicted to encode an integral membrane protein with one transmembrane segment (Illing and Errington, 1991b), however, its subcellular localization is not known. The requirement for SpoIIIAH in the localization of GFP-SpoIVFA suggested that it could reside in a membrane complex with SpoIVFA in the OFM. If this is the case, then SpoIIIAH should also be present in the engulfing septal membrane. To test this, we fused CFP to the amino-terminus of SpoIIIAH. The gene encoding CFP was resynthesized with codons optimized for translation in *B. subtilis* (see *Experimental procedures*). The gene fusion was placed under the control of the native *spoIIIA* promoter and inserted into the nonessential *sacA* locus (Middleton and Hofmeister, 2004) in the *B. subtilis* chromosome. The CFP-SpoIIIAH fusion restored sporulation to near wild-type levels in cells lacking SpoIIIAH. Consistent with the idea that SpoIIIAH anchors SpoIVFA through a direct interaction, CFP-SpoIIIAH localized specifically to the engulfing septal membranes (Fig. 3). Furthermore, CFP-SpoIIIAH retained proper localization in the absence of *spoIVF* and in a *spoIIIA* mutant (Fig. S3). These results indicate that SpoIIIAH, like SpoIVFA, localizes to the OFM and does so independently of SpoIVFA and the proteins encoded by the *spoIIIA* operon.

Forespore gene expression plays an important role in the localization of SpoIVFA and SpoIIIAH

Our results clearly demonstrate that σ^E-dependent gene

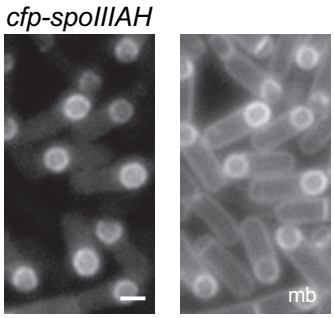

Fig. 3. SpoIIIAH localizes to the outer forespore membrane. Cells harbouring *cfp-spoIIIAH* (strain BTD23) were monitored by fluorescence microscopy at hour 2.5 of sporulation. The membranes (mb) from the same field were visualized using the fluorescent dye TMA-DPH. Scale bar, 1 μm.

expression in the mother cell is required for the proper localization of GFP-SpoIVFA. But does forespore gene expression under σ^F control also play a role? σ^E activity in the mother cell is triggered by the signalling protein SpoIIR synthesized in the forespore under the control of σ^F (Karow *et al*., 1995; Londono-Vallejo and Stragier, 1995)**.** Therefore, in cells lacking σ^F, the mother cell transcription factor σ^E fails to become active. Because GFP-SpoIVFA requires σ^E-dependent gene expression for proper localization, it was not possible to assess the role of σ^F in a simple *sigF* mutant background. To circumvent this problem, we took advantage of a strain in which SpoIIR is synthesized independently of σ^F. In this *sigF* mutant strain, the gene encoding the signalling protein is fused to an early sporulation promoter (P_{spoIIE}) resulting in its expression prior to asymmetric division (Zhang *et al*., 1996). Because of the inappropriate spatial and temporal synthesis of SpoIIR, the cells of this strain exhibit pleiotropic phenotypes during sporulation. Many of the cells fail to undergo polar division presumably because of premature activation of σ^E while others make a polar septum but never activate σ^E. A small proportion of the cells (5–10%) activates σ^E in the mother cell compartment (after asymmetric division) and initiates the process of engulfment (for example, see carets in Fig. 4). The requirement for σ^F in the localization of GFP-SpoIVFA can be addressed in these normal-looking sporangia. This strain has been used previously to investigate the requirement for forespore gene expression in the localization of the sporulation protein SpoVM (van Ooij and Losick, 2003). In the normal-looking sporangia, GFP-SpoIVFA was incompletely anchored in the OFM and was present in the cytoplasmic membranes of the mother cell (Fig. 4). However, the degree of localization at the engulfing septal membrane varied considerably. In some cells, there was almost no enrichment of the fusion protein at the engulfing septal membrane, while in others, there was significant localization. We do not understand why the localization of GFP-SpoIVFA is variable, however, because in all normal-looking sporangia we observed some mislocalization of GFP-SpoIVFA to the cytoplasmic membranes, we tentatively conclude that σ^F plays a role in anchoring SpoIVFA in the OFM.

Because GFP-SpoIVFA requires SpoIIIAH for proper localization to the OFM, we wondered whether σ^F also plays a role in anchoring SpoIIIAH. To investigate this possibility, we analysed the localization CFP-SpoIIIAH in the *sigF* mutant background described above. In the normal-looking sporangia, we observed some specific localization of the fusion protein, however, a significant amount of the protein was mislocalized (Fig. 4). Unlike GFP-

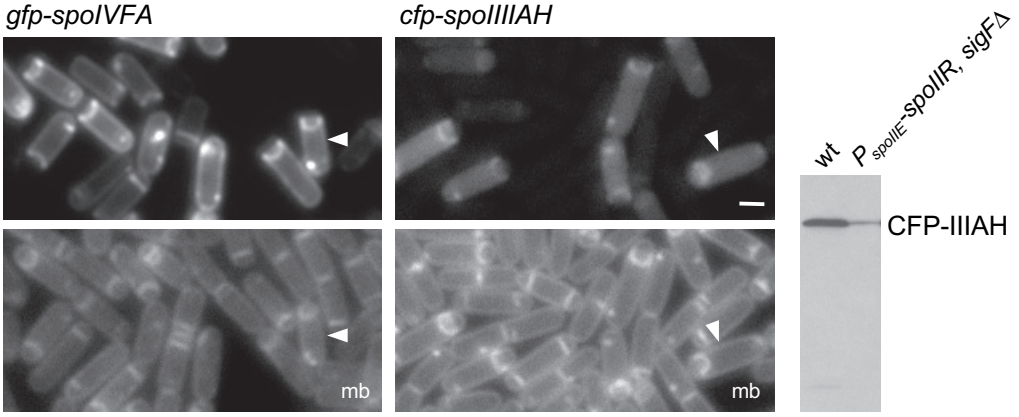

Fig. 4. Forespore gene expression plays an important role in the localization of SpoIVFA and SpoIIIAH. The localization of GFP-SpoIVFA (strain BKM162) and CFP-SpoIIIAH (strain BKM164) was analysed at hour 2.5 of sporulation in a *sigF* mutant strain engineered to express *spoIIR* under the control of an early sporulation promoter (P_{spoIIE}). The membranes (mb) from the same fields were visualized using the fluorescent dye TMA-DPH. Immunoblot of whole-cell lysates from a wild-type strain harbouring *cfp-spoIIIAH* (strain BTD23) and strain BKM164 are shown. CFP-SpoIIIAH was detected using anti-GFP polyclonal antibodies. The carets indicate examples of normal-looking sporangia. Scale bar, 1 μm.

SpoIVFA, the mislocalized CFP-SpoIIIAH appeared as a fluorescent haze in the mother-cell cytoplasm. To rule out the possibility that this cytoplasmic signal was a result of proteolysis and release of soluble CFP, we analysed CFP-SpoIIIAH by immunoblot. Consistent with the idea that the cytoplasmic signal represents full-length CFP-SpoIIIAH, we did not observe significant proteolysis of the fusion protein (Fig. 4). We note that the CFP-SpoIIIAH protein level is lower in the *sigF* mutant compared with the wild type control and this is probably because of the reduced number of sporangia that successfully activate σ^E. We do not know why CFP-SpoIIIAH appears to be present in the mother cell cytoplasm rather than in the cytoplasmic membrane. However, we do not think that this is a result of the strain background because we observed a similar localization pattern in a *spoIIQ* mutant (see below).

In summary, these results suggest that both GFP-SpoIVFA and CFP-SpoIIIAH require forespore gene expression for proper localization to the engulfing septal membrane. However, because we observed some septal localization of both SpoIVFA and SpoIIIAH in most sporangia, our data also suggest that forespore gene expression is not solely responsible for anchoring these proteins.

The forespore-specific protein SpoIIQ is required for proper localization of both SpoIVFA and SpoIIIAH

The experiments described above are consistent with the idea that a gene or genes under the control of σ^F are required for the localization of GFP-SpoIVFA and CFP-SpoIIIAH. There are only three genes under σ^F control known to be required for efficient sporulation. Among these is *spoIIQ*. The SpoIIQ protein contains a single amino-terminal transmembrane segment and a carboxyl-terminal extracellular domain that is similar to domains involved in peptidoglycan remodelling (Londono-Vallejo *et al*., 1997). Interestingly, SpoIVFA also contains this putative peptidoglycan remodelling domain (Rudner and Losick, 2002). Based on this similarity, we decided to investigate whether SpoIIQ is required for anchoring SpoIVFA and SpoIIIAH in the OFM. During our analysis, Rubio and Pogliano (2004) reported that SpoIIQ localizes to the IFM during the process of engulfment. Thus, SpoIIQ is well-positioned to participate in anchoring SpoIVFA and/or SpoIIIAH.

To determine whether SpoIIQ was required for the localization of GFP-SpoIVFA and CFP-SpoIIIAH, we analysed their localization in a *spoIIQ* mutant. Both fusions were significantly mislocalized in the absence of this forespore protein (Fig. 5). Interestingly, the patterns of mislocalization were similar to what we observed in the *sigF* mutant background described above. The mislocalized GFP-SpoIVFA was in the cytoplasmic membranes of the mother cell while the mislocalized CFP-SpoIIIAH was found in the mother cell cytoplasm. To eliminate the possibility that this cytosolic haze resulted from degradation of CFP-SpoIIIAH, we analysed the fusion protein by immunoblot (Fig. 5). We observed a small amount of proteolysis of CFP-SpoIIIAH in the *spoIIQ* mutant. However, at later time points we have seen similar proteolysis in wild-type cells and do not believe this amount of degradation is sufficient to explain the cytoplasmic signal. Finally, we note that, in the absence of SpoIIQ, there is still some enrichment of both GFP-SpoIVFA and CFP-SpoIIIAH in the engulfing septal membrane as we observed in the *sigF* mutant background (Fig. 4). We conclude that SpoIIQ plays a major role in anchoring both proteins in the OFM. We note that Rubio and Pogliano (2004) recently described a similar requirement for SpoIIQ in the localization of SpoIIIAH.

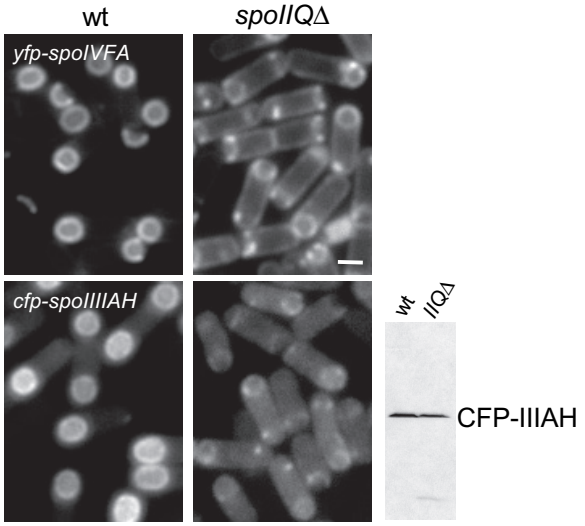

Fig. 5. The forespore-specific protein SpoIIQ is required for proper localization of both SpoIVFA and SpoIIIAH. YFP-SpoIVFA localization was analysed in a wild-type strain (BKM119) and a *spoIIQΔ* mutant (strain BTD147) at hour 2.5 of sporulation. CFP-SpoIIIAH localization was analysed at hour 2.5 of sporulation in the same backgrounds (strains BTD23 and BTD149 respectively). Immunoblots of whole-cell lysates from the same time point used for microscopy are shown. CFP-SpoIIIAH was detected using anti-GFP polyclonal antibodies. Scale bar, 1 μm.

In summary, it has been previously shown that the septal localization of SpoIIQ requires mother cell gene expression under the control of σ^E (Rubio and Pogliano, 2004) and here, we provide evidence that the forespore protein SpoIIQ is required for the localization of mother cell proteins under σ^E control.

The localization of GFP-SpoIVFA requires SpoIIQ in the forespore and SpoIIIA in the mother cell

We have presented evidence that SpoIIQ is required for the proper localization of both CFP-SpoIIIAH and GFP-

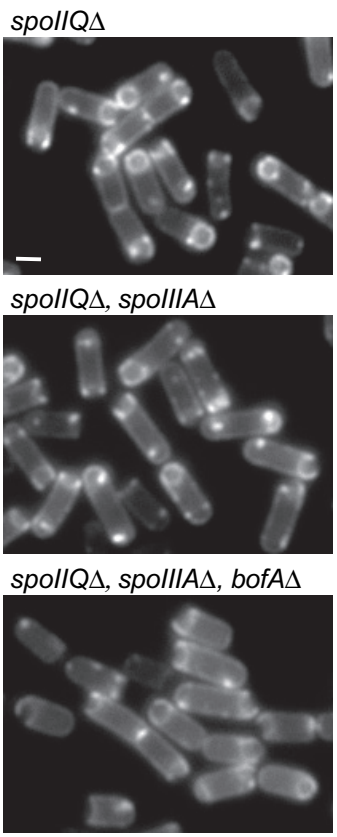

Fig. 6. The subcellular localization of SpoIVFA requires SpoIIQ in the forespore and SpoIIIA in the mother cell. The localization of YFP-SpoIVFA was monitored by fluorescence microscopy at hour 2.5 of sporulation in *spoIIQΔ* (strain BTD505), *spoIIQΔ*, *spoIIIAΔ* (strain BTD507), and *spoIIQΔ*, *spoIIIAΔ*, *bofAΔ* (strain BTD511) mutants. Scale bar, 1 μm.

SpoIVFA and we have also shown that SpoIIIAH plays a role in localizing GFP-SpoIVFA. We therefore wondered whether SpoIIQ acts through SpoIIIAH in anchoring SpoIVFA. We note that because the mislocalization of GFP-SpoIVFA was more pronounced in the absence of SpoIIQ (Fig. 5) than in the absence of SpoIIIAH (Fig. 2), it is clear that SpoIIQ must also play some role independent of SpoIIIAH. To investigate whether the requirement for SpoIIQ in the localization of SpoIVFA is mediated, in part, through the SpoIIIAH protein, we examined the localization of GFP-SpoIVFA in the absence of both mother cell and forespore proteins. Surprisingly, in the *spoIIIA, spoIIQ* double mutant the fusion protein was slightly more mislocalized than in the *spoIIQ* single mutant (Fig. 6 and Fig. S4). Specifically, the GFP-SpoIVFA signal in the septal membrane compared with the signal in the cytoplasmic membrane was lower in the double mutant. This result suggests that SpoIIIAH plays a role in anchoring SpoIVFA independent of SpoIIQ. Moreover, it suggests that the residual localization of CFP-SpoIIIAH in the absence of SpoIIQ (Fig. 5) is required to anchor SpoIVFA and is therefore functionally significant. Finally, we tested whether the absence of BofA would even further mislocalize GFP-SpoIVFA. Consistent with the idea that all three proteins are required to localize SpoIVFA, the fusion protein was even more mislocalized in the *spoIIQ*, *spoIIIA*, *bofA* triple mutant (Fig. 6). These results suggest that the anchoring of SpoIVFA in the OFM is mediated by a network of interactions across the septum and adjacent to it.

SpoIIIAH and SpoIIQ interact in vitro

Our results indicate that SpoIIQ, which localizes in the IFM, is required to anchor SpoIVFA and SpoIIIAH in the OFM. This raised the intriguing possibility that these proteins might interact directly in the space between the inner and outer forespore membranes and thus become localized by the zipper-like mechanism proposed by Rubio and Pogliano (2004). To investigate this possibility, we tested whether the extracellular domain of SpoIIQ could bind directly to the extracellular domains of SpoIVFA and SpoIIIAH. All three proteins are predicted to have one transmembrane segment and, in the case of SpoIVFA and SpoIIQ, the carboxyl-terminal domains have been shown to reside outside the cell (Londono-Vallejo *et al*., 1997; Green and Cutting, 2000). The transmembrane segment of SpoIIIAH is close to the amino-terminus of the protein but its orientation in the membrane is not known. We reasoned that if there was a direct interaction between SpoIIIAH and SpoIIQ, it would likely involve the larger carboxyl-terminal domain. To test for interaction, we used a GST-pull-down assay. We overproduced the carboxyl-terminal domains of SpoIIIAH and SpoIVFA as fusions to Glutathione-S Transferase (GST-SpoIIIAH and GST-SpoIVFA) and immobilized the fusion proteins on glutathione resin. The extracellular domain of SpoIIQ was overproduced as an untagged protein in *E. coli* and a crude lysate was incubated with the immobilized GST fusions (Fig. 7A, Load). Thus, the *E. coli* proteins in the crude lysate served as competitors for interaction with the immobilized proteins. After extensive washing, the GST-fusion proteins were released from the resin by SDS or by cleavage between the carboxyl-terminal domains and GST using the PreScission Protease. Released proteins were then analysed by SDS-PAGE and coomassie staining. The GST-SpoIIIAH fusion efficiently bound the extracellular domain of SpoIIQ and both domains were found in the eluate (Fig. 7A, PP). This interaction, which appears almost stoichiometric, was resistant to 2 M NaCl. By contrast, we could detect no interaction between the extracellular domains of SpoIVFA and SpoIIQ (Fig. 7B, PP) nor could we detect an interaction between SpoIIQ and GST alone (data not shown). These results indicate that the extracellular domain of SpoIIQ can bind the carboxyl-

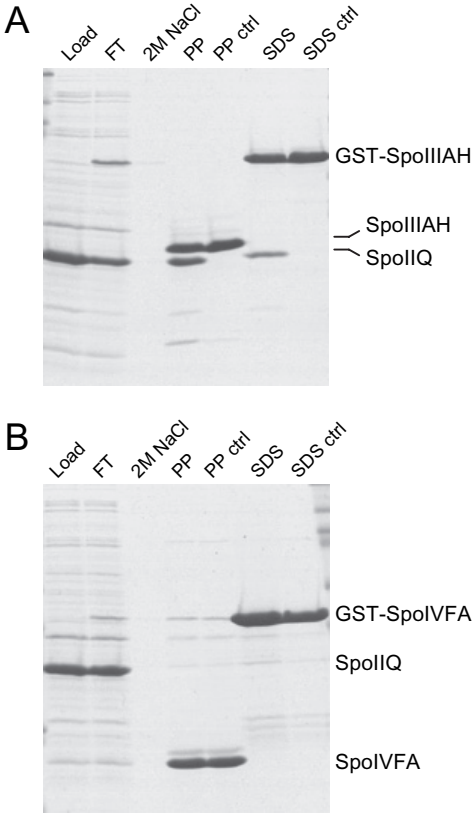

Fig. 7. SpoIIQ and SpoIIIAH interact *in vitro*. Coomassie stained SDS-polyacrylamide gels of immobilized-protein interaction assays.
A. GST-SpoIIIAH and SpoIIQ.
B. GST-SpoIVFA and SpoIIQ.
An *E. coli* lysate containing the extracellular domain of SpoIIQ (Load) was mixed with glutathione resin containing Glutathione-S Transferase (GST) fusions to the carboxyl-terminal domains of SpoIIIAH or SpoIVFA (GST-SpoIIIAH and GST-SpoIVFA). Load, Flow Through (FT), elution with 2 M NaCl (2 M NaCl), PreScission Protease (PP) and SDS (SDS) are shown. Control elutions with PreScission Protease (PP ctrl) and SDS (SDS ctrl) of the fusion proteins without the SpoIIQ lysate are included.

terminal domain of SpoIIIAH and suggests that this domain is indeed located in the space between the two membranes. Furthermore, these findings strongly support the idea that SpoIIQ anchors SpoIIIAH through a direct zipper-like interaction in the intermembrane space.

The dynamic localization of SpoIIQ requires SpoIVFA

It has been shown previously that SpoIIQ exhibits a dynamic localization pattern during the process of engulfment (Rubio and Pogliano, 2004). Initially, GFP-SpoIIQ is found in the septal membrane on the forespore side. During the process of engulfment, the fusion protein tracks along the IFM. Later, it assembles into helical arcs around the forespore and finally, it is released into the cytoplasm probably through proteolysis. In addition, Rubio and Pogliano have shown that the localization of GFP-SpoIIQ to the IFM requires mother-cell gene expression under the control of σ^E. In light of our findings that SpoIVFA and SpoIIIAH require SpoIIQ to localize to the OFM and that the carboxyl-terminal domains of SpoIIQ and SpoIIIAH can interact *in vitro*, we wondered whether these mother-cell proteins are necessary for the localization of SpoIIQ to the IFM. Rubio and Pogliano (2004) reported that SpoIIIAH was not required for the specific localization of SpoIIQ and we have confirmed this result (data not shown). In addition, the absence of SpoIVFA had no discernible impact on the localization of CFP-SpoIIQ to the IFM (Fig. 8). However, the dynamic localization of the fusion protein was significantly affected in the absence of SpoIVFA (Fig. 8). Initially, CFP-SpoIIQ localized properly to the engulfing septal membrane in the *spoIVF* mutant (Fig. 8, left panels). However, as engulfment continued in the mutant, CFP-SpoIIQ did not efficiently track with the engulfing septal membrane and rarely assembled into the arc-like structures (Fig. 8, middle and right panels). Instead, CFP-SpoIIQ appeared stalled in its initial localization pattern. After the completion of engulfment, CFP-SpoIIQ was released normally as was observed in wild type (Fig. 8, right panels). These defects were also observed in the *spoIVF* mutant with *spoIVFB* supplied *in trans* (data not shown). Thus, we conclude that SpoIIQ is required for the localization of SpoIVFA in the OFM (Fig. 5) and, in turn, SpoIVFA is required for the dynamic localization of SpoIIQ in the IFM (Fig. 8).

Discussion

Here we report the identification of five mother cell proteins (BofA, SpoIIIAH, SpoIIP, SpoIID and SpoIIM) and one forespore protein (SpoIIQ) that are all required to localize SpoIVFA in the engulfing septal membrane. At this time, with the exception of BofA, we do not know whether these proteins are complexed with SpoIVFA or act indirectly to anchor it in the OFM. However and importantly, we have found that all of these proteins appear to act independently or have partially overlapping function in localizing SpoIVFA. Thus, this result suggests that a complex web of interactions is necessary for anchoring SpoIVFA in the OFM. Moreover, our data suggest that this network is composed of interactions between proteins embedded in the OFM as well as interactions across the sporulation septum (Fig. 9). In addition, we have characterized SpoIIIAH, one of the proteins in this anchoring network. We have found that, like SpoIVFA, SpoIIIAH localizes to the OFM and requires SpoIIQ (but not SpoIVFA) for this localization pattern. Furthermore, we have shown that the extracellular domains of SpoIIQ and SpoIIIAH interact *in vitro* suggesting that SpoIIQ anchors SpoIIIAH in the OFM through a direct interaction in the space between the mother cell and forespore mem-

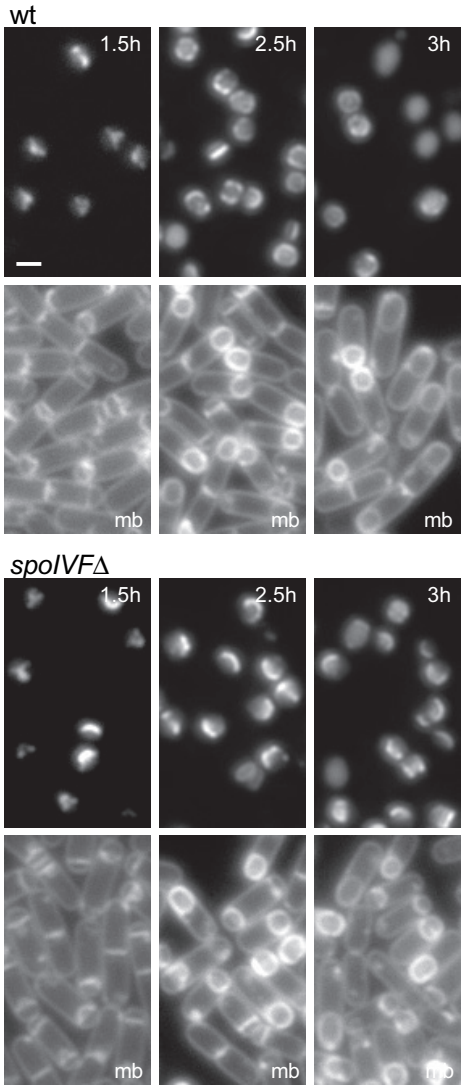

Fig. 8. SpoIVFA is required for the dynamic localization of the forespore protein SpoIIQ. CFP-SpoIIQ localization was monitored during sporulation (by resuspension) in wild-type (strain BTD231) and a *spoIVFΔ* mutant (strain BTD277). The membranes (mb) from the same fields were visualized using the fluorescent dye TMA-DPH. Time (h) after the initiation of sporulation is indicated. Initially, CFP-SpoIIQ localized properly to the inner forespore membrane in the *spoIVFΔ* mutant (left panels). However, CFP-SpoIIQ did not track with the engulfing septal membrane nor assemble into the arc-like structures in the absence of *spoIVF* (middle and right panels). After the completion of engulfment CFP-SpoIIQ was released normally in the *spoIVFΔ* mutant. Scale bar, 1 μm.

branes. Finally, we have shown that SpoIIQ can localize properly in the absence of either SpoIVFA or SpoIIIAH but requires SpoIVFA for its full range of dynamic localization. Thus, a complex web of non-reciprocal interactions among mother cell and forespore proteins is responsible for static and dynamic protein localization in both compartments of the sporangia.

One of the major conclusions of this work is that the mechanism by which proteins are anchored at particular subcellular sites in the bacterial cell is complicated! Our original models of linear localization pathways akin to the precursor-product relationship in the assembly of a phage particle (Berget and King, 1983; Hartwell and Weinert, 1989) were clearly too simple. The results presented here reinforce the idea that higher order organization of proteins in bacteria can be as sophisticated as the organization of proteins in eukaryotes.

How is SpoIVFA anchored in the OFM?

We have shown that several mother cell proteins contribute to the localization of SpoIVFA and we hypothesize that these proteins are part of a web of interactions that have overlapping anchoring capacity (Fig. 9). Our data indicate that this web is anchored in the septal membrane, in part, through the forespore protein SpoIIQ. One of the links between the mother cell web and SpoIIQ is SpoIIIAH, which we have shown interacts directly with SpoIIQ *in vitro*. However, our data suggest there is at least one other link between the mother cell web and SpoIIQ. This is because the absence of SpoIIQ disrupts the localization of SpoIVFA to a greater extent than the absence of SpoIIIAH. Moreover, we have shown that SpoIVFA (but not SpoIIIAH) is required for the dynamic localization of

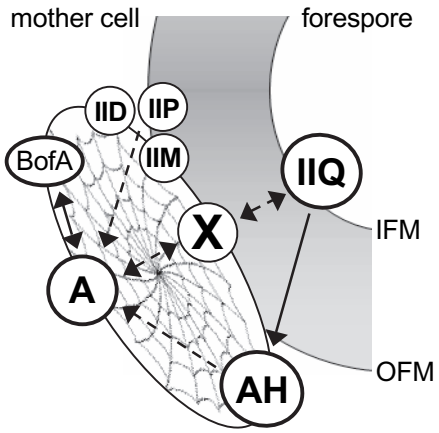

Fig. 9. A complex web of interactions anchors SpoIVFA in the OFM. The mother cell web (drawn schematically as a spider web) includes SpoIVFA (A), SpoIIP (IIP), SpoIID (IID), SpoIIM (IIM), SpoIIIAH (AH), BofA and a hypothetical protein X. Arrows indicate a requirement for protein localization (or dynamic localization). Single-headed arrows show non-reciprocal requirements and double-headed arrows indicate reciprocal requirements. Solid lines represent a direct interaction between two proteins. Dashed lines indicate it is not known whether the requirement for localization is direct or indirect. A proposed second link (X) between the mother cell web and the forespore protein SpoIIQ (IIQ) is shown. SpoIID, SpoIIP and SpoIIM are probably anchored in the sporulation septum through their interaction with the septal peptidoglycan. They are drawn in close proximity but could reside in a complex. SpoIVFA and BofA reside in a complex although direct interaction between these proteins has not been demonstrated.

SpoIIQ. We therefore hypothesize that a mother cell protein (protein X in Fig. 9) is the second link between the anchoring web and SpoIIQ. Finally, because SpoIVFA remains partially localized in the absence of forespore gene expression, a second mechanism independent of the zipper-like interactions must also participate in anchoring the mother cell web (see below). We envision that this proposed web is responsible for anchoring other mother cell proteins in the OFM and probably other forespore proteins in the IFM. In support of this idea, we have found that the mother cell protein SpoVM is partially mislocalized in the absence of SpoIIIAH and more significantly (but not completely) in the absence of SpoIIQ (data not shown). We further hypothesize that protein networks with overlapping anchoring capacity are employed in the localization of proteins in both vegetative *B. subtilis* as well as in other bacteria.

What are the ultimate anchors in the cell?

It has been proposed previously that interaction between extracellular domains that reside in the space between the double membrane septum could serve to anchor proteins in the septal membrane (Rubio and Pogliano, 2004). Here we provide cytological and biochemical evidence to support this zipper-like anchoring model. However, our data also suggest that the interaction across the septal membrane is not sufficient to fully explain the subcellular localization of SpoIIIAH or SpoIVFA. In particular, we have found that both proteins remain partially localized in the absence of SpoIIQ and retain some septal enrichment in the complete absence of forespore gene expression. A similar finding was made for another mother cell protein SpoVM that also localizes to the OFM (van Ooij and Losick, 2003). Thus, some other mechanism must participate in anchoring these proteins in the septal membrane. One possible candidate is the septum itself. We hypothesize that some σ^E-dependent protein(s) interacts with a component of the cell division machinery or the septal peptidoglycan and this protein (or set of proteins), in turn, participates in anchoring SpoIVFA and SpoIIIAH. Consistent with this idea, SpoIID, SpoIIP and SpoIIM are thought to localize to the septum through their interaction with the septal peptidoglycan (Abanes-De Mello *et al*., 2002). Thus, these proteins could be part of a septal peptidoglycan anchoring mechanism. In summary, our data provide strong evidence that zipper-like interactions across the septum serve as one of the ultimate anchors in the cell but also suggests that a second fundamental anchor participates in localizing proteins to the septal membrane.

A dynamic network

Rubio and Pogliano (2004) previously reported that the absence of any one of the major engulfment proteins (SpoIID, SpoIIP or SpoIIM) impairs the dynamic localization pattern of SpoIIQ. This defect is similar to what we have observed in the absence of SpoIVFA (Fig. 8). These observations further link SpoIVFA and the three engulfment proteins. Moreover, because engulfment proceeds normally in the absence of SpoIVFA, our results suggest that engulfment is necessary but not sufficient for the dynamic redistribution of SpoIIQ. How these mother cell proteins impact SpoIIQ localization and function and whether they act separately or together remain outstanding questions for the future.

In conclusion, we have identified the major proteins that impact the localization of the SpoIVFA/SpoIVFB/BofA signal transduction module. We envision that these proteins anchor the signalling complex in the OFM through a network of interactions across and adjacent to the sporulation septum. We are now poised to determine whether any of these anchoring proteins plays a direct regulatory role in the signal transduction pathway that employs this signalling complex.

Experimental procedures

General methods

All *B. subtilis* strains were derived from the prototrophic strain PY79 (Youngman *et al*., 1983) and are listed in Table S1. *E. coli* strains used were TG1, DH5α, NB42 and BL21. Introduction of gene fusions at ectopic integration sites in *B. subtilis* were confirmed by marker replacement or by the inability to catabolize starch. Sporulation was induced by resuspension at 37°C by the method of Sterlini-Mandelstam (Harwood and Cutting, 1990). Sporulation efficiency was determined in 36 h cultures as the total number of heat resistant (80°C for 20 min) colony-forming units (cfu) compared with the total number of cfu before heat treatment. To visualize GFP-SpoIVFA during vegetative growth, BDR776 was grown in growth medium (CH) in the presence of 20 mM xylose.

The trains (Table S1), plasmids (Table S2) and oligonucleotide primers (Table S3) used in this study are available in *Supplementary material*.

Plasmid construction

pKM23 [*pelB::*P_{spoIVF}*-gfp-spoIVFA* (*cat*)] was generated by inserting P_{spoIVF}*-gfp-spoIVFA* from pDR104 (Rudner and Losick, 2002) into pKM20 between *Eco*RI and *Bam*HI. pKM20 [*pelB::cat*] is an ectopic integration vector for double crossover insertions into the nonessential *pelB* locus (K. A. Marquis and D. Z. Rudner, unpubl.).

pKM30 [*lacA::*P_{spoIVF}*-yfp(JF)-spoIVFA (erm)*] was generated by inserting P_{spoIVF}*-yfp-spoIVFA* from pKM26 into pDR183 between *Eco*RI and *Bam*HI. pDR183 [*lacA::erm*] is an ectopic integration vector for double crossover insertions into the nonessential *lacA* locus based on pAX01 (Hartl *et al*., 2001) (D. Z. Rudner, unpubl.). pKM26 [*amyE::*P_{spoIVF}*-yfp(JF)-*

spoIVFA (spec)] was created in a three-way ligation with a *Hin*dIII-*Xho*I PCR fragment containing the Jelly Fish version of *yfp* (Lemon and Grossman, 2000) and an optimized ribosome binding site (RBS) (Vellanoweth and Rabinowitz, 1992) (oligonucleotide primers odr107 and odr108) and a *Xho*I-*Bam*HI fragment containing *spoIVFA* from pDR104 and pDR77 (Rudner and Losick, 2002) cut with *Hin*dIII and *Bam*HI.

pKM32 [*sacA::*P_{spoIVF}*-yfp-spoIVFA (phleo)*] was generated by inserting P_{spoIVF}*-yfp(JF)-spoIVFA* from pKM26 into pNC15 (N. Campo and D. Z. Rudner unpubl.) between *Eco*RI and *Bam*HI. pNC15 [*sacA::phleo*] is an ectopic integration vector for double crossover insertions into the nonessential *sacA* locus based on pRM58 (Middleton and Hofmeister, 2004).

pDR124 [*amyE*::P_{xylA}*-gfp-spoIVFA (cat*)] was generated in a three-way ligation with a *Xho*I-*Hin*dIII fragment containing *gfp* and an optimized RBS (Vellanoweth and Rabinowitz, 1992) from pDR95 (Rudner and Losick, 2002), a *BamH*I-*Xho*I fragment containing *spoIVFA* from pDR104 (Rudner and Losick, 2002) and pOR277 (Resnekov *et al*., 1996) cut with *Hin*dIII and *Bam*HI.

pDT9 [*sacA::*$P_{spoIIIA}$*-cfp(Bs)-spoIIIAH (phleo*)] was generated in a three-way ligation with a *Hin*dIII-*Xho*I PCR fragment containing *cfp* (with codons optimized for *B. subtilis*) and an optimized RBS (Vellanoweth and Rabinowitz, 1992) (oligonucleotide primers oTD4 and oTD5 and template DNA pKM8) and a *Xho*I-*Bam*HI PCR fragment containing *spoIIIAH (*oligonucleotide primers oTD10 and oTD11) and pDT7 cut with *Hin*dIII and *Bam*HI. pDT7 [(*sacA::*$P_{spoIIIA}$ *(phleo*)] was generated by cloning an *Eco*RI-*Bam*HI PCR fragment containing the *spoIIIA* promoter (oligonucleotide primers oTD1 and oTD2) into pNC15.

pDT36 [*sacA::*P_{spoIIQ}*-cfp(Bs)-spoIIQ (tet)*] was created in a three-way ligation with a *Hin*dIII-*Xho*I fragment containing *cfp* and an optimized RBS from pDT9, a *Xho*I-*Bam*HI PCR fragment containing *spoIIQ* (oligonucleotide primers oTD29 and oTD30) and pDT29 cut with *Hin*dIII and *Bam*HI. pDT29 was generated by inserting P_{spoIIQ}*-cfp* from pKM8 into pNC17 between EcoRI and BamHI. pNC17 (*sacA::tet*) is an ectopic integration vector for double crossover insertions into the nonessential *sacA* locus based on pRM58 (Middleton and Hofmeister, 2004) (N. Campo and D. Z. Rudner, unpubl.).

pDT34 [*amyE::*P_{spoIIQ}*-cfp(Bs)-spoIIQ (cat*)] was generated in the same way as pDT36 using pDT28 instead of pDT29. pDT28 was generated by inserting P_{spoIIQ}*-cfp* from pKM8 into pDG364 (Karmazyn-Campelli *et al*., 1992) between *Eco*RI and *Bam*HI.

pDR171 was created by inserting a *Bam*HI-*Xho*I PCR fragment encoding the extracellular domain of SpoIVFA (oligonucleotide primers oDR202 and oDR203) into pGEX-6P-2 (Amersham) between *Bam*HI and *Xho*I.

pDT60 was generated by inserting a *Bam*HI-*Xho*I PCR fragment encoding the carboxyl-terminal domain of SpoIIIAH (oligonucleotide primers oDR348 and oDR349) into pGEX-6P-2 (Amersham) between *Bam*HI and *Xho*I.

pDT58 was generated by inserting a *Nhe*I-*Xho*I PCR fragment encoding the extracellular domain of SpoIIQ (oligonucleotide primers oTD50 and oTD51) into pET24b (Invitrogen) between *Nhe*I and *Xho*I.

Fluorescence microscopy

Fluorescence microscopy was performed with an Olympus BX61 microscope as previously described (Rudner and Losick, 2002). Fluorescent signals were visualized with a phase contrast objective UplanF1100× and captured with a monochrome CoolSnapHQ digital camera (Photometrics) using Metamorph software version 6.1 (Universal Imaging). Exposure times were typically 500 ms (SpoIVFA and SpoIIQ) and 2000 ms (SpoIIIAH). The lipophylic membrane dye TMA-DPH (Molecular Probes) was used at a final concentration of 0.01 mM and exposure times were typically 200 ms. Images were analysed, adjusted and cropped using Metamorph software. Final figure preparation was performed in Powerpoint (Microsoft).

Immunoblot analysis

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. Whole cell extracts were prepared by resuspension of cell pellets in 50 μl of lysis buffer [20 mM Tris pH 7.0, 10 mM EDTA, 1 mg ml^{-1} lysozyme, 10 μg ml^{-1} DNase I, 100 μg ml^{-1} RNase A, with protease inhibitors: 1 mM PMSF, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin] and incubation at 37°C for 10 min followed by addition of 50 μl of sodium dodecyl sulphate (SDS) sample buffer [0.25 M Tris pH 6.8, 4% SDS, 20% glycerol, 10 mM EDTA] containing 10% 2-Mercaptoethanol. Samples were heated for 5 min at 80°C prior to loading. Proteins were separated by SDS-PAGE on 12.5% polyacrylamide gels, electroblotted onto Immobilon-P membrane (Millipore) and blocked in 5% non-fat milk in phosphate-buffered saline (PBS)-0.5% Tween-20. The blocked membrane was probed with affinity-purified anti-GFP antibodies (Rudner and Losick, 2002) diluted 1 : 10 000 into 3% BSA 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).

GST interaction assay

The untagged extracellular domain of SpoIIQ was overproduced in *E. coli* strain BL21(DE3) *plysS* and the extracellular domains of SpoIIIAH and SpoIVFA fused to GST were overproduced in *E. coli* strain NB42. Cells were grown in Luria–Bertani at 37°C to an OD_{600} of 0.4, induced by the addition of IPTG to 1 mM, and harvested after 2 h. All subsequent manipulations were carried out at 4°C. Cells were harvested by centrifugation and resuspended in 1/20th vol Buffer S (20 mM Tris-HCl pH 8.0, 25 mM NaCl, 1 mM EDTA, 5 mM 2-Mercaptoethanol) containing 1 mM PMSF. Crude extracts were prepared by freeze–thawing the cell suspension, followed by incubation with lysozyme and sonication. The soluble fractions were prepared by 100 000× *g* spin. GST-SpoIVFA and GST-SpoIIIAH were bound to Glutathione Sepharose (Amersham) and washed with 40 column vols of Buffer S. The soluble lysate containing the extracellular domain of SpoIIQ was incubated for 2 h with the resins con-

taining GST-SpoIVFA or GST-SpoIIIAH. The resins were then washed with 40 column vols of Buffer S. Bound proteins were eluted with Buffer S containing 2 M NaCl, the PreScission Protease (10 U) (Amersham) or SDS sample buffer. Eluted proteins were separated by SDS-PAGE and visualized with coomassie blue.

Codon-optimized CFP

The gene encoding eCFP (Clonetech) was resynthesized by DNA TwoPointO (http://www.dnatwopointo.com/) with codons optimized for *B. subtilis* (http://www.kazusa.or.jp/codon/) and cloned into pDrive (Qiagen) to generate plasmid pDR200. An optimized RBS and amino acid substitution A206K that prevents dimerization (Zacharias *et al*., 2002) were included in the synthesis (Fig. S5). The synthesized gene was inserted into pKM1 between *Hin*dIII and *Bam*HI to generate pKM8 [*amyE::*P_{spoIIQ}*-cfp(Bs) (spec)*]. pKM1 [*amyE::*P_{spoIIQ} *(spec)*] was generated by subcloning a PCR fragment containing the *spoIIQ* promoter (oligonucleotide primers oDR234 and oDR235) into pLD30 (Garsin *et al*., 1998) between EcoRI and *Hin*dIII. The fluorescence intensity of this codon-optimized version of *cfp* was compared to the *Aequorea victoria* (Jelly Fish) *cfp(JF)* (Lemon and Grossman, 2000) and *ecfp* (Clonetech) placed under the identical *spoIIQ* promoter and optimized RBS. The *B. subtilis* codon-optimized *cfp* was ~3× brighter than the Jelly Fish version and the ecfp version was barely detectable above background (Fig. S6). The *ecfp* gene has been codon-optimized for mammals and contains several codons that are underrepresented in *B. subtilis*. The sequence of *cfp*(*Bs*) can be found on our website: http://rudnerlab.med.harvard.edu/

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Supplementary material

The following material is available from
http://www.blackwellpublishing.com/product/journals/suppmat/mmi/mmi4501/mmi4501sm.htm

Fig. S1. GFP-SpoIVFA is more mislocalized in the absence of both BofA and SpoIIIAH. Sporulation was induced by resuspension in wild-type and mutant strains and GFP-SpoIVFA localization was monitored by fluorescence microscopy between hours 2 and 2.5.

Fig. S2. MalF-GFP accumulates at the second polar septum in the spoIID, spoIIP, spoIIM triple mutant.

Fig. S3. SpoIIIAH localization to the OFM does not require the proteins encoded by the *spoIII*A locus or SpoIVFA.

Fig. S4. The subcellular localization of GFP-SpoIVFA requires SpoIIQ in the forespore and SpoIIIA in the mother cell.

Fig. S5. Codon-optimized *cfp* [*cfp*(*Bs*)].

Fig. S6. Comparison of CFP fluorescence.

Table S1. Strains used in this study.

Table S2. Plasmids used in this study.

Table S3. Oligonucleotides used in this study.

Note added in proof

Blaylock *et al.* (2004) have recently reported that epitope-tagged versions of SpoIIIAH and SpoIIQ co-localize to foci in the membranes that surround the forespore by immunofluorescence microscopy. Under certain conditions we can also detect co-localization of CFP-SpoIIIAH and YFP-SpoIIQ to specific foci around the forespore in live cells. Images and details can be found on our website (http://rudnerlab.med.harvard.edu/resources.html).

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