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Engulfment-regulated proteolysis of SpoIIQ: evidence that dual checkpoints control σ^K activity

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Summary

During *Bacillus subtilis* sporulation, the engulfment checkpoint is thought to directly regulate late fore-spore transcription but to indirectly regulate late mother cell transcription, via the σ^G-produced pro-tease SpoIVB. We here demonstrate that SpoIIQ is subject to σ^G-independent, but engulfment-dependent, proteolysis that depends on SpoIVB. Thus, SpoIVB produced before engulfment supports some SpoIVB-dependent events, suggesting that its activity or access to substrates must be regulated by engulfment. Furthermore, a mutation (*bofA*) that allows σ^K to be active without σ^G does not allow σ^K activity in engulfment mutants, although the pro-σ^K processing enzyme (SpoIVFB) is localized to the septum in engulfment mutants, suggesting that engulfment comprises a second checkpoint for σ^K Finally, we find that SpoIIQ and another protein required for σ^G activity (SpoIIIAH), which directly interact and assemble helical structures around the forespore, recruit the σ^K-processing enzyme SpoIVFB to the forespore and these structures. We suggest that these foci serve a synapse-like role, allowing engulfment to simultaneously control both σ^G and σ^K, and integrating multiple checkpoints and signalling pathways.

Introduction

Transcription in the two cells required to make a *Bacillus subtilis* spore is governed both by intracellular signal transduction cascades, which ensure the coordinated development of the two cells, and by morphological checkpoints, which ensure that landmark morphological events are complete before the onset of transcription that could interfere with these events (reviewed by Rudner and Losick, 2001; Errington, 2003). The first morphological checkpoint of sporulation is polar septation (Fig. 1A), which generates the smaller forespore (the future spore), and the larger mother cell, which lyses to release the mature spore. The second is engulfment, a phagocytosis-like process during which the mother cell membrane migrates around the smaller forespore (Fig. 1B), until the engulfing membrane meets (Fig. 1C) and fuses to release the forespore into the mother cell cytoplasm (Fig. 1D). Engulfment is essential for spore assembly, which occurs within the mother cell cytoplasm. Uncoupling transcription from either checkpoint reduces the number of viable spores produced, and can directly block completion of these key morphological events (Cutting *et al*., 1990; Coppolecchia *et al*., 1991; Eichenberger *et al*., 2001; Fujita and Losick, 2002). For example, immediately after polar septation, a second polar division event commences in the mother cell that if unchecked will give rise to a defective sporangium with two forespores and an anucleate mother cell (Setlow *et al*., 1991; Lewis *et al*., 1994; Piggot *et al*., 1994). The early mother cell transcription factor σ^E therefore directs the synthesis of proteins that inhibit this division event (Pogliano

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et al., 1999; Eichenberger *et al*., 2001); premature synthesis of these proteins inhibits polar septation (Eichenberger *et al*., 2001). Thus, coupling σ^E activity to the morphological checkpoint of septation is likely essential for asymmetric division and the generation of daughter cells with distinct developmental cell fates.

The engulfment-regulated activation of the mother cell transcription factor σ^K occurs by regulated intramembrane proteolysis (RIP), resulting in the removal of a membrane associated N-terminal leader sequence from pro-σ^K (Zhou and Kroos, 2004). The pro-σ^K protease, SpoIVFB is a zinc metallopeptidase related to bacterial and eukaryotic proteases involved in RIP (Rudner *et al*., 1999; Yu and Kroos, 2000), including those involved in bacterial mating (Antiporta and Dunny, 2002), extracellular stress (Ades, 2004; Alba and Gross, 2004), eukaryotic development (Ebinu and Yankner, 2002), sterol biogenesis (Brown and Goldstein, 1997) and the unfolded protein response (Liu and Kaufman, 2003). In these pathways, signal transduction culminates in the cleavage of a protein within a membrane bilayer, often with an initiating cleavage event outside the membrane. These pathways can release signalling peptides or proteins that govern transcription, and in bacteria, the substrate is often an anti-sigma factor whose elimination allows transcription (Brown *et al*., 2000; Gottesman, 2003). For example, the extracytoplasmic stress response of *Escherichia coli* responds to elevated levels of unfolded outer membrane proteins, which bind to the PDZ domain of a secreted serine protease (Walsh *et al*., 2003). This protease then cleaves the extracellular domain of an anti-sigma factor (Walsh *et al*., 2003), allowing an intramembrane protease to inactivate the anti-sigma factor and release the active sigma factor (Ades *et al*., 1999). In the case of *B. subtilis* σ^K, the hydrophobic leader sequence of pro-σ^K acts as a covalently attached anti-sigma factor (Zhang *et al*., 1998), and there is no evidence for an initiating extracellular cleavage event.

One mechanism by which the activation of pro-σ^K is coupled to engulfment is by its dependence on the prior activation of the late forespore transcription factor σ^G, whose activity depends on engulfment (Cutting *et al*., 1990). The σ^G factor produces a secreted serine protease, SpoIVB, which activates the pro-σ^K processing machinery (Cutting *et al*., 1991a), comprised of three mother cell membrane proteins SpoIVFA, SpoIVFB and BofA (Resnekov *et al*., 1996; Rudner and Losick, 2002). SpoIVFA mediates the septal localization of SpoIVFB and BofA (Rudner and Losick, 2002) while SpoIVFB processes pro-σ^K (Rudner *et al*., 1999; Yu and Kroos, 2000; Zhou and Kroos, 2004). BofA is an inhibitor of SpoIVFB protease activity (Resnekov and Losick, 1998; Zhou and Kroos, 2004), whose elimination uncouples σ^K activity from forespore transcription and from SpoIVB (Cutting *et al*., 1990; Ricca *et al*., 1992). One model for the onset of pro-σ^K processing is that engulfment allows activation of σ^G, and the consequent production of the SpoIVB protease, which cleaves SpoIVFA *in vitro*, dissociating BofA from SpoIVFB and allowing processing (Dong and Cutting, 2003). However, SpoIVB is also produced before engulfment, because its expression is also mediated by the early transcription factor σ^F (Gomez and Cutting, 1996), suggesting that its activity or that of the SpoIVFB intramembrane protease might be regulated by engulfment. Indeed, given the detrimental effect of premature σ^K activation on sporulation (Cutting *et al*., 1990), one might expect its activity to be directly coupled both to the completion of engulfment and to σ^G activity.

A promising candidate for a protein that senses the completion of engulfment is the forespore-expressed membrane protein SpoIIQ, which is conserved in all endospore-forming bacteria (Stragier, 2002) and required for σ^G synthesis and activation (Londono-Vallejo *et al*., 1997; Sun *et al*., 2000). SpoIIQ assembles helical arcs surrounding the forespore, is degraded after the membrane fusion event that is the final step of engulfment (Rubio and Pogliano, 2004), and directly interacts with the mother cell protein SpoIIIAH, which is also involved in σ^G activation (Blaylock *et al*., 2004; Doan *et al*., 2005). We here demonstrate that SpoIIQ is subject to an engulfment-dependent but σ^G-independent proteolytic event involving at least two cleavage sites, one on each side of its transmembrane domain. Extracellular proteolysis requires

the SpoIVB serine protease, suggesting that the activity of this protease or its access to substrates is regulated by engulfment. We also provide preliminary evidence that σ^K activation is separately regulated by both the completion of engulfment and by σ^G activity. Finally, we find that SpoIIQ and its mother cell ligand SpoIIIAH direct the localization of the pro-σ^K processing enzyme, SpoIVFB, to the forespore and mediate its assembly into helical arcs and foci. This finding is in keeping with those of Doan *et al*. (2005), who noted that localization of the SpoIVFB interacting protein SpoIVFA depended on SpoIIQ and SpoIIIAH. We propose that the coassembly of proteins required for activation of post-engulfment transcription factors in both the forespore and the mother cell allows engulfment to simultaneously govern transcription in both cells.

Results

SpoIIQ is degraded after engulfment

SpoIIQ is a forespore-expressed protein with a single membrane spanning segment and a C-terminal extracellular domain (Londono-Vallejo *et al*., 1997). We previously noted that GFP-SpoIIQ assembles a complex structure of helical arcs and foci surrounding the forespore (Fig. 2A) and is degraded after engulfment to release soluble GFP, resulting in cytoplasmic fluorescence (Figs 2B and 3B; Rubio and Pogliano, 2004). Immunoblot analysis with antibodies to the C-terminus of SpoIIQ showed that native SpoIIQ was also degraded to release a product corresponding in size to the extracellular C-terminal domain (Fig. 3A). Fractionation analysis demonstrated that while full-length GFP-SpoIIQ and SpoIIQ-His_6 was in the insoluble membrane fraction, both the N-terminal (GFP-containing) and C-terminal (His-tagged) products released from the proteins were in the soluble fractions (Fig. 3C). The size and solubility of the degradation products suggest that GFP-SpoIIQ is subject to two proteolytic events, one on each side of the transmembrane domain (Fig. 3D), releasing the extracellular domain from native SpoIIQ and cytoplasmic GFP from the N-terminus of GFP-SpoIIQ.

SpoIIQ degradation commenced after the final step of engulfment, membrane fusion (Rubio and Pogliano, 2004), suggesting it was either directly regulated by engulfment or that it required the post-engulfment transcription factors σ^G or σ^K We therefore compared the localization and degradation of GFP-SpoIIQ in engulfment mutants and in a strain with a mutation in the gene encoding σ^G, *spoIIIG*, which also abolishes activity of the downstream transcription factor σ^K. GFP-SpoIIQ localized normally in the *spoIIIG* mutant, with cytoplasmic GFP fluorescence observed after membrane fusion (Fig. 2F, arrow), suggesting that neither σ^G nor σ^K directed gene expression is required for SpoIIQ proteolysis. Immunoblot analysis confirmed that in the *spoIIIG* mutant, both native SpoIIQ and GFP-SpoIIQ were degraded as in wild type (Fig. 3A and B). In the *spoIIP* mutant, engulfment is blocked at the first step, septal thinning, and the growing forespore pushes into the mother cell, forming a bulge (Frandsen and Stragier, 1995; Abanes-De Mello *et al*., 2002). In this strain, GFP-SpoIIQ stayed at the septum, sometimes localizing to the bulge, with no obvious release of soluble GFP (Fig. 2E, arrow; J). Immunoblot analysis showed that the *spoIIP* mutation abolished degradation of both SpoIIQ (Fig. 3A) and GFP-SpoIIQ (Fig. 3B). Thus, engulfment is a morphological checkpoint for SpoIIQ proteolysis.

Altered SpoIIQ proteolysis in *spoIIIA* and *spoIIIJ* mutants

A key event that also depends on engulfment is activation of σ^G, which requires SpoIIQ (Sun *et al*., 2000), SpoIIIJ (an Oxa1p/YidC homologue) (Errington *et al*., 1992; Yen *et al*., 2001; Serrano *et al*., 2003) and the eight mother cell proteins encoded by the *spoIIIA* operon (Kellner *et al*., 1996), one of which (SpoIIIAH) directly interacts with SpoIIQ (Blaylock *et al*., 2004). We were interested in determining if these proteins were involved in SpoIIQ proteoysis and therefore assessed GFP-SpoIIQ localization and degradation in a *spoIIIJ* mutant and a mutant

in which the last two genes in the *spoIIIA* operon were deleted, *spoIIIAG* and *spoIIIAH* (hereafter designated *spoIIIAG-H*). In both cases GFP-SpoIIQ localized to the septum, tracked the engulfing membrane and assembled foci early in sporulation, but remained membrane-bound even late in sporulation, with no GFP fluorescence in the forespore cytoplasm (Fig. 2C and D, arrows; I). Immunoblot analysis demonstrated an altered GFP-SpoIIQ degradation pattern in the *spoIIIA* and *spoIIIJ* mutants (Fig. 3B), with the accumulation of a product (Fig. 3B, asterisk) less abundant in wild type. Surprisingly, although fluorescence microscopy showed little cytoplasmic GFP fluorescence in *spoIIIA* and *spoIIIJ* mutants (Fig. 2C and D, arrows), a significant amount of soluble GFP-SpoIIQ degradation product was detected (Fig. 3B). This discrepancy might be due to cleavage of the insoluble product during sample preparation.

The absence of SpoIIIJ or SpoIIIAGH caused a more notable difference in the degradation pattern of native SpoIIQ. In the *spoIIIAG-H* mutant, the soluble extracellular domain was almost undetectable, although the level of full-length SpoIIQ decreased during sporulation (Fig. 3A). A somewhat weaker, but similar effect was seen in the *spoIIIJ* null mutant, where a reduced amount of the soluble extracellular domain was visualized (Fig. 3A). It is therefore possible that SpoIIIJ and SpoIIIAGH are required for both proteolysis events, and that the GFP-SpoIIQ fusion protein alters the proteolytic pathway (although providing a useful tag for cytoplasmic proteolysis). Alternatively, it is possible that the interaction between SpoIIQ and SpoIIIAH (Blaylock *et al*., 2004) stabilizes the SpoIIQ extracellular domain, so that in the absence of SpoIIIAH, this domain is subject to further degradation and undetectable.

In summary, after engulfment SpoIIQ is subject to at least two proteolysis events, one outside the forespore membrane that releases the extracellular domain (extracellular proteolysis) and a second inside the forespore membrane that releases GFP into the cytoplasm (intracellular proteolysis). Two proteins required for σ^G activation are required for either proteolysis or for the accumulation of proteolytic products, SpoIIIJ and SpoIIIAGH, suggesting that SpoIIQ proteolysis might play a role in activating σ^G after engulfment.

SpoIVB serine protease is required for extracellular proteolysis of SpoIIQ

While examining the lab collection of *spoIIIA* and *spoIIIJ* mutant strains, we found that one '*spoIIIA*' Tn917 insertion mutation completely inhibited GFP-SpoIIQ degradation in immunoblots, unlike the other *spoIIIA* mutations. The insertion site was identified using random primed polymerase chain reaction (PCR) and DNA sequencing, and we found that the transposon was actually within the *spoIVB* coding region. Indeed, a *spoIVB* deletion mutation had a phenotype identical to that of the s*poIVB::Tn917* mutation, strongly inhibiting extracellular proteolysis of native SpoIIQ (Fig. 3A) and intracellular proteolysis of GFP-SpoIIQ (Fig. 3B), with no decrease in the amount of full length protein. Fluorescence microscopy demonstrated that GFP-SpoIIQ localized normally in the *spoIVB* mutant, but no cytoplasmic GFP fluorescence was observed after membrane fusion (Fig. 2G, arrow, K). Thus, SpoIIQ degradation depends, directly or indirectly, on the forespore protein SpoIVB, a serine protease required for σ^K activation in the mother cell. The extracellular location of SpoIVB suggests that it is required for the extracellular proteolysis of SpoIIQ; the striking effect that the *spoIVB* null mutation also has on the intracellular proteolysis event could indicate that either SpoIVB is involved in both proteolysis events, or that intracellular proteolysis normally occurs only after extracellular proteolysis.

SpoIVB is a secreted signalling molecule that triggers σ^K activation by interacting with the σ^K processing complex in the mother cell membrane (Cutting *et al*., 1991a; Wakeley *et al*., 2000). SpoIVB cleaves SpoIVFA (Dong and Cutting, 2003), which has an extracellular domain with sequence similarity to that of SpoIIQ; it is possible that cleavage of SpoIIQ represents the second essential function of SpoIVB identified by genetic studies (Oke *et al*., 1997). SpoIVFA

is a component of the σ^K processing machinery and is required to localize the pro-σ^K protease SpoIVFB to the septum (Rudner and Losick, 2002). It is generally reported that σ^K activation is indirectly coupled to engulfment by its dependence on the production of SpoIVB by σ^G, whose transcriptional activity is regulated by engulfment by an unknown mechanism (Fig. 1E). However, SpoIVB is initially expressed by the early forespore transcription factor σ^F, although it is produced in higher levels after the engulfment-dependent activation of σ^G (Gomez and Cutting, 1996). Our results demonstrate that the initial, σ^F-dependent production of SpoIVB in the *spoIIIG* mutant is sufficient to mediate degradation of SpoIIQ at rates identical to wild type, which together with our observation that engulfment defective mutants block SpoIIQ degradation, suggests that SpoIVB activity might be regulated by engulfment.

A σ^G -independent role for SpoIIQ, SpoIIIA and engulfment proteins in σ^K activation

SpoIVB-dependent proteolysis of SpoIIQ occurred in the absence of σ^G activity, so we were interested in testing the possibility that engulfment and σ^G activity comprised two independent checkpoints for σ^K activation, and that SpoIIQ might be required both for σ^G and σ^K activity. To assess the possibility that SpoIIQ has a separate role in σ^K activation, we used a *bofA* mutant, in which σ^K is activated in the absence of σ^G activity and SpoIVB-mediated signalling (Cutting *et al*., 1990, 1991b; Ricca *et al*., 1992). If activating σ^G is the only role that SpoIIQ plays in σ^K activation, then the *bofA* mutation should completely rescue σ^K activity in the *spoIIQ* mutant. Surprisingly, in the *spoIIQ bofA* double mutant, no σ^K activity was detected (Fig. 4B, filled circle), suggesting that SpoIIQ has independent roles in the activation of σ^G and σ^K.

We next assayed σ^G and σ^K activity in engulfment mutants (*spoIID*, *spoIIM* and *spoIIP*) and in *spoIIIA* mutants. Similar to the *spoIIQ* mutant, no σ^G activity (Fig. 4A, inverted triangles and diamonds) consequently, no σ^K activity was detected in these mutants (Fig. 4C and D, inverted triangles and diamonds) and the *bofA* mutation failed to rescue σ^K activity (Fig. 4C and D, filled inverted triangles, diamonds). Thus, at 37°C, the *bofA* mutation fails to rescue engulfment or *spoIIQ* or *spoIIIA* mutants, although the engulfment and *spoIIIA* mutants both produce pro-σ^K (Lu *et al*., 1990), and *spoIIQ* mutants have normal σE-directed gene expression (Sun *et al*., 2000). We next collected samples from cultures at 30°C, a more permissive temperature for many mutants, and performed β-galactosidase assays. Consistent with the results from 37°C experiments, *spoIIIG* and *spoIVB* mutants were bypassed by the *bofA* mutant, producing high levels of σ^K activity, while the engulfment mutants were not bypassed (Fig. 4E, filled triangle and square; Fig. 4F, filled inverted triangle). However, *spoIIQ bofA* and *spoIIIA bofA* double mutants showed small but reproducible increases in σ^K activity to ~5% of wild type levels (Fig. 4F, filled circle and filled diamond), perhaps explaining why previous publications observed that *bofA* could suppress the requirement for *spoIIIA* (Cutting *et al*., 1990). Because the *bofA* mutation completely bypasses the σ^G checkpoint for σ^K activation at both 37°C and 30°C, we conclude that SpoIIQ and SpoIIIA are required for both σ^G and σ^K activation and we speculate that engulfment might serve as a separate checkpoint for σ^K activation.

SpoIVFB localization requires SpoIIQ, SpoIIIA and engulfment

A simple mechanism by which both SpoIIQ and SpoIIIA could be involved in σ^K activation is that the SpoIIQ-SpoIIIAH zipper (Blaylock *et al*., 2004) might recruit the σ^K processing complex (SpoIVFA, SpoIVFB and BofA) to the sporulation septum. We tested this hypothesis by investigating the subcellular distribution of SpoIVFB-GFP in wild type and in *spoIIQ* and *spoIIIA* mutants. Early in engulfment, SpoIVFB-GFP localized to the septum, with discrete foci at the septum and very faint fluorescence in the mother cell cytoplasmic membrane (Fig. 5A, arrow), tracking the engulfing membrane (Fig. 5B, arrowhead) and assembling foci around the forespore before and after fusion (Fig. 5B, double arrowhead and arrow respectively; Fig. 5J). After three-dimensional reconstuction, SpoIVFB-GFP appeared as helical arcs

surrounding the forespore (Fig. 5I). The localization and structure formed by SpoIVFB-GFP is very similar to that of GFP-SpoIIQ and SpoIIIAH-flag, except GFP-SpoIIQ is degraded while the others persist as foci around the forespore (Blaylock *et al*., 2004; Rubio and Pogliano, 2004).

Similar effects were noted in both the *spoIIQ* and *spoIIIAG-H* mutants. In *spoIIQ* mutant sporangia early in engulfment, SpoIVFB-GFP showed a somewhat patchy distribution throughout the mother cell membrane (Fig. 5C, arrow) with a twofold enrichment at the leading edge of the engulfing membrane, which contains two layers of the mother cell membrane (Fig. 1). However, after membrane fusion, SpoIVFB-GFP fluorescence was more intense in the outer forespore membrane than in the cytoplasmic membrane (Fig. 5D, arrow). A similar result was obtained in the *spoIIIAG-H* mutant, with little septal localization of SpoIVFB-GFP during engulfment (Fig. 5E, arrow), but significant localization to the outer forespore membrane after membrane fusion (Fig. 5F, arrow). To quantitatively view the cytological data, we exported the deconvolved but unadjusted pixel data (photons/pixel) of select cells from SoftWoRx to Excel and visualized the pixel intensities as three-dimensional graphs (Fig. 5N). In wild type before and after fusion, little SpoIVFB-GFP fluorescence was visible in the mother cell cytoplasmic membrane, and peaks corresponding to foci were seen at the forespore. However, in *spoIIQ* before membrane fusion, SpoIVFB-GFP was uniformly distributed in the cytoplasmic and septal membrane domains. After fusion, the GFP intensity in the mother cell cytoplasmic membrane remained the same, but that in the outer forespore membrane increased about sevenfold (Fig. 5N), suggesting a continued accumulation of SpoIVFB-GFP in the outer forespore membrane after fusion. The requirement for SpoIIQ and SpoIIIAGH for localization of the pro-σ^K processing enzyme SpoIVFB to the forespore during engulfment could explain why they are required for σ^K activation.

Engulfment mutants showed only a slight increase in non-localized SpoIVFB in sporangia with flat septa (Fig. 5G, arrowhead), and in sporangia with a bulge, SpoIVFB clearly localized to the bulge, which is peptidoglycan-free (Fig. 5G, arrow). The *spoIID spoIIP* double mutant blocked bulge formation (Pogliano *et al*., 1999) and had a somewhat more severe defect in SpoIVFB-GFP localization (Fig. 5H, arrow). Thus, SpoIVFB appears to localize to the septum of engulfment mutants better than does SpoIIIAH, whose localization is strongly inhibited by the absence of septal thinning (Blaylock *et al*., 2004). This suggests the existence of an alternate mechanism for SpoIVFB localization, as is also supported by the SpoIIQ and SpoIIIAG-H independent recruitment of SpoIVFB to the outer forespore membrane after membrane fusion.

While this manuscript was in preparation, Doan *et al*. (2005) made similar observations regarding the dependence of the SpoIVFB interacting protein SpoIVFA to the septum, which they found to be dependent on SpoIIQ, SpoIIIAH and engulfment proteins. However, while we found that SpoIVFB (Fig. 5C) and SpoIIIAH (Blaylock *et al*., 2004) are completely dependent on SpoIIQ for septal localization during engulfment (Fig. 5N, iii), Doan *et al*. (2005) found substantial localization of SpoIVFA in the absence of either SpoIIQ or SpoIIIAH. These differences might be due to either the localization of different proteins (SpoIVFA vs. SpoIVFB) or to subtle differences in the ways the various fusion proteins were constructed.

GFP-SpoIIQ has a tracking defect in *bofA* and *spoIVFA* mutants

GFP-SpoIIQ tracks the engulfing mother cell membrane during engulfment, moving around the forespore together with the engulfing mother cell membrane. In the absence of mother cell gene expression, GFP-SpoIIQ moves around the forespore ahead of the engulfing membrane (Rubio and Pogliano, 2004), suggesting that an interaction between SpoIIQ and a mother cell membrane protein tethers SpoIIQ to the septum. We were interested in determining if the σ^K processing machinery (SpoIVFA, SpoIVFB or BofA) could be the SpoIIQ tether or involved in SpoIIQ degradation. We therefore investigated the localization of GFP-SpoIIQ in *bofA*,

spoIVFA, *spoIVFAB* and *spoIVFB* mutants. In each of these mutants, GFP-SpoIIQ initially localized to the septum, with discrete foci similar to wild type, so neither BofA, SpoIVFA nor SpoIVFB are required for SpoIIQ tethering. However, in *bofA*, *spoIVFA* and *spoIVFAB* mutants, GFP-SpoIIQ remained at the septum, failing to track the engulfing mother cell membrane around the forespore (Fig. 6B, C and E, arrows; Fig. 6F). Surprisingly, the *bofA*, *spoIVFA* and *spoIVFAB* sporangia failed to complete engulfment, remaining unfused (Fig. 6B, C and E, arrows). This unanticipated engulfment defect was observed in *bofA*, *spoIVFA* and *spoIVFAB* mutant strains expressing GFP-SpoIIQ (data not shown), but not in strains lacking GFP-SpoIIQ, suggesting a synergistic effect between GFP-SpoIIQ and the absence of either SpoIVFA or BofA. In these mutants, soluble GFP was observed even in sporangia that had not completed engulfment (Fig. 6E, arrowhead), demonstrating that the absence of these proteins partially uncouples SpoIIQ proteolysis from engulfment. In the mutant missing only *spoIVFB*, GFP-SpoIIQ localized normally and was degraded to release soluble GFP after engulfment (Fig. 6D, arrow and arrowhead). In summary, BofA and SpoIVFA but not SpoIVFB are required to allow GFP-SpoIIQ to track the engulfing mother cell membrane, but they are not required for SpoIIQ tethering or proteolysis.

Discussion

We here demonstrate that the forespore membrane protein SpoIIQ is subject to a proteolysis event regulated by the phagocytosis-like process of engulfment. Complete SpoIIQ proteolysis requires SpoIVB, a protease secreted from the forespore, SpoIIIAH, a mother cell membrane protein with which SpoIIQ directly interacts (Blaylock *et al*., 2004), and SpoIIIJ, a homologue of the YidC translocase subunit. SpoIIQ proteolysis involves at least two proteolysis events, one on each side its transmembrane domain. Our results suggest that extracellular proteolysis requires the SpoIVB serine protease, which is also essential for the engulfment-dependent activation of the mother cell transcription factor σ^K Although it remains possible that SpoIVB activates a second extracellular protease that cleaves SpoIIQ, we favour the hypothesis that it directly cleaves SpoIIQ, which is in the same protein family as the only other SpoIVB substrate described to date, SpoIVFA (Dong and Cutting, 2003).

While the role of SpoIIQ proteolysis remains unclear, our studies impact our understanding of the regulated RIP event that activates the mother cell transcription factor σ^K by removing the inhibitory leader sequence from pro-σ^K (Rudner *et al*., 1999; Yu and Kroos, 2000). Proteolytic activation of pro-σ^K by the SpoIVFB protease depends on SpoIVB, which is produced in high levels after engulfment by the late forespore transcription factor σ^G(Cutting *et al*., 1991a). Thus, σ^K activity is considered to be indirectly coupled to engulfment by its dependence on σ^G for production of the SpoIVB signal transduction protein (Fig. 1E). However, SpoIVB is also produced before engulfment by σ^F (Gomez and Cutting, 1996) so the simple view that activation of σ^G produces, for the first time, this key signal transduction protein, is unlikely to be correct. Indeed two of our results lead us to speculate that σ^K activation might be directly regulated by engulfment. First, SpoIVB-dependent proteolysis of SpoIIQ does not depend on σ^G, demonstrating that the SpoIVB protein produced by σ^F before engulfment is sufficient to support certain SpoIVB-dependent events (Fig. 7A). Further, SpoIIQ degradation commences after engulfment and is blocked by engulfment defective mutants, suggesting that SpoIVB activity or access to its substrates might be regulated by engulfment. Second, the *bofA* mutation, which completely bypasses the requirement for σ^G activity and for SpoIVB to activate σ^K, does not bypass the requirement for engulfment to activate σ^K Thus, while engulfment-defective mutants (*spoIID*, *spoIIM* and *spoIIP*) lack σ^G activity, this is unlikely to be the only reason they lack σ^K activity. We therefore speculate that the ability of the SpoIVFB intramembrane protease to activate σ^K is separately coupled to both the completion of engulfment and σ^G activity (Fig. 7C). The efficiency of SpoIVFB-mediated pro-σ^K processing in *E. coli* cells expressing SpoIVFB and pro-σ^K (Zhou and Kroos, 2004) suggests that the

engulfment checkpoint might be governed by an inhibitor of σ^K processing, rather than an activator.

Our results further emphasize the importance that the SpoIIQ membrane protein plays directing mother cell membrane proteins to the sporulation septum. We previously demonstrated that the extracellular domain of SpoIIQ directly interacts with that of the mother cell protein SpoIIIAH within the septal space, thereby tethering SpoIIIAH to the sporulation septum (Blaylock *et al*., 2004). Doan *et al*. (2005) also made similar findings regarding the interaction of SpoIIQ with SpoIIIAH and observed that the absence of these proteins reduced, but did not abolish, septal localization of SpoIVFA (which forms a complex with SpoIVFB). We here demonstrate that SpoIIQ/SpoIIIAH are essential for the localization of the pro-σ^K protease, SpoIVFB to the sporulation septum during engulfment, as in the absence of this complex SpoIVFB-GFP is randomly distributed within the mother cell membrane. However, after membrane fusion, SpoIVFB-GFP shows improved localization in the absence of SpoIIIAH or SpoIIQ, suggesting that after fusion, a SpoIIQ and SpoIIIAH independent mechanism recruits SpoIVFB to the outer forespore membrane. This proposal would require SpoIVFB to be directly inserted into this location, as membrane fusion generates a mother cell with two separate membrane systems, the mother cell cytoplasmic membrane and the outer forespore membrane. Thus, SpoIIQ recruits the SpoIIIAH membrane protein to the sporulation septum by virtue of a direct interaction between their extracellular domains, and SpoIIIAH in turn recruits SpoIVFA and SpoIVFB, perhaps via another protein since thus far, no interaction has been demonstrated between SpoIIIAH and either SpoIVFB (our data not shown) or SpoIVFA (Doan *et al*., 2005).

The SpoIIQ-SpoIIIAH-SpoIVFB localization pathway involves key proteins required for activation of engulfment-dependent transcription factors in both the forespore and the mother cell (Fig. 7B). We propose that the coassembly of these proteins into a single protein complex allows the coordinated regulation of both σ^G and σ^K activity by engulfment. Remarkably, each of these signal transduction proteins assemble foci in what appears to be a roughly icosahedral arrangement around the forespore (Blaylock *et al*., 2004;Rubio and Pogliano, 2004;Fig. 2A, arrowhead, Fig. 5B, double arrowhead), suggesting that these forespore and mother cell membrane proteins interact only at discrete contact sites (Fig. 7B). The restriction of membrane-localized signal transduction proteins to discrete points of contact between two cells also occurs during the assembly of neurological and immunological synapses, which are thought to allow prolonged signalling between the cells and the integration of multiple signalling pathways and checkpoints (Davis and Dustin, 2004;Jacobelli *et al*., 2004). It is tempting to speculate that the SpoIIQ/SpoIIIAH/SpoIVFB complex plays a similar role during *B. subtilis* development.

Experimental procedures

Bacterial strains

Bacillus subtilis strains (Table 1) are PY79 derivatives (Youngman *et al*., 1984), constructed by transformation (Dubnau and Davidoff-Abelson, 1971). *EZ::TN(kan)* insertions were isolated as described (Blaylock *et al*., 2004). Sporulation was induced by resuspension at 30° C or 37°C with t_n representing the hours after the onset of sporulation (Sterlini and Mandelstam, 1969). Plasmids were constructed by PCR (using primers listed below) in *E. coli* DH5α selecting ampicillin resistance (100 μg ml^{-1}) and sequenced by the Shared Source UCSD Cancer Center (funded in part by NCI Cancer Center Support Grant #2 P30 CA23100-18). Sporulation efficiency was determined as described (Rubio and Pogliano, 2004).

Microscopy and image analysis

Live cells were stained with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (0.2 μg ml^{-1}; Molecular Probes) and Mitotracker Red (0.1 μg ml^{-1}; Molecular Probes) or FM 4–64 (5 μg ml^{-1}; Molecular Probes) (Pogliano *et al*., 1999; Sharp and Pogliano, 1999). Images were collected with a Spectris optical sectioning microscope with 15 iterations of the Delta Vision constrained-iterative deconvolution program (Applied Precision, Issaquah, WA). The three-dimensional model of SpoIVFB was constructed using 12 sections (z = 0.15 μm) and the volume builder function of SoftWoRx (v. 3.3).

SpoIIQ antibody production, membrane fractionation and Western blot analysis

Polyclonal rabbit anti-SpoIIQ antibodies were raised against purified C-terminal SpoIIQ (amino acids 63–283), expressed from a clone constructed by PCR using primers 5′-GGAAACTCCG**CATATG**GACAACAAC and 5′-TTTTCT**CTCGAG**AGACTGTTCAGT (NdeI and XhoI in bold) cloned into pET28b (Novagen; Madison, WI). This plasmid (pAR92) was electroporated into BL21 (λDE3) by selecting on kanamycin (50 μg ml^{-1}). A growing culture was induced with 1 mM IPTG for 24 h at 25°C. Subsequent purification of His_6-$SpoIIQ_{63–283}$-His_6 was performed by nickel affinity chromatography (HIS-Select Nickel Affinity Gel, Sigma; St. Louis, MO) yielding ~2.7 mg ml^{-1} purified protein which was sent to Antibodies (Davis, CA).

Cultures of KP845 and KP980 were induced to sporulate by resuspension, 4 ml harvested at $t_{2.5}$ (KP980) and 25 ml at t_4 (KP845) and fractionated (Blaylock *et al*., 2004). Samples were prepared (Pogliano *et al*., 1997), heated for 10 min at 50°C, loaded on a 12.5% SDS-polyacrylamide gel, transferred to PVDF (Perez *et al*., 2000) and probed with 0.4 μg ml^{-1} mouse monoclonal anti-GFP antibodies (Roche) or 1:10 000 dilution of rabbit polyclonal anti-SpoIIQ antibodies, followed by 1:1500 HRP-labelled anti-mouse or anti-rabbit antibodies and visualized with enhanced chemiluminescence (Amersham).

β-Galactosidase assay

Cultures were sporulated at 37°C or 30°C (with the 30°C cultures approximately 2 h slower), and β-galactosidase assays performed (Miller, 1972; Pogliano *et al*., 1997).

Plasmid construction

The *spoIIQ-his*$_6$ gene was constructed by cloning a EcoRI-HindIII-digested PCR product amplified from PY79 chromosome using primers 5′-ATGTCAT**GAATTC**ACGTTTTTGGCACTCCTCTC and 5′-CGTACGT**AAGCTT**ATGTAACTACATAGACGGTA (bold, EcoRI and HindIII sites, respectively) into pDG1662 (Guerout-Fleury *et al*., 1996) to yield pCH505 (*amyE::*P_{spoIIQ}*spoIIQΩcat*). The hexa-histidine tag was introduced at the *spoIIQ* termination codon by site-directed mutagenesis (Sawano and Miyawaki, 2000) using the primer (5′-CAGAAGACACTGAACAGTCT**CATCACCATCACCATCAC**TAATGAAGAAAACGTCTATC-3′; bold, hexa-histidine sequence).

SpoIVFB-GFP was constructed using pPL51 (Levin *et al*., 1999), containing *gfp* downstream and in frame with EcoRI and XhoI restriction sites with a chloramphenicol resistance gene for selection in *B. subtilis*. The 3′ ~500 bp of *spoIVFB* was amplified using 5′-CCC**AAGCTT**CTGCCGATCTGGCCGCTG and 5′-CCG**CTCGAG**GTAGGGCAGAAGCAGTTC (HindIII and XhoI sites in bold), digested with HindIII and filled in using T4 polymerase. pPL51 was digested with EcoRI and filled in with T4 polymerase, and the PCR product and plasmid digested with XhoI and ligated to yield pXJ58, which was integrated at *spoIVFB*, selecting Cm^R.

The *spoIVFB::pAR98(kan)* insertion mutant was constructed using a derivative of pMOD2 (Epicenter; Madison, WI), pXJ52, containing a kanamycin resistance gene (*aphIII*) amplified from pUK19 and cloned into the BamHI and XbaI restriction sites of pMOD2 (Trieu-Cout and Courvalin, 1983). The 5′ end of *spoIVFB* was amplified using 5′-ACC**CTGCAG**TCAGATTAAACCCGCCGT and 5′-AAG**GTCGAC**CCATTTATTCAAATGAAA, digested with PstI and SalI (bold) and ligated to pXJ52 to yield pAR98. The plasmid contained the 3′ end of *spoIVFA* and the first four codons of *spoIVFB* and was integrated at *spoIVFA* selecting Kan^R, producing a phenotype identical to that of *spoIVFB152*.

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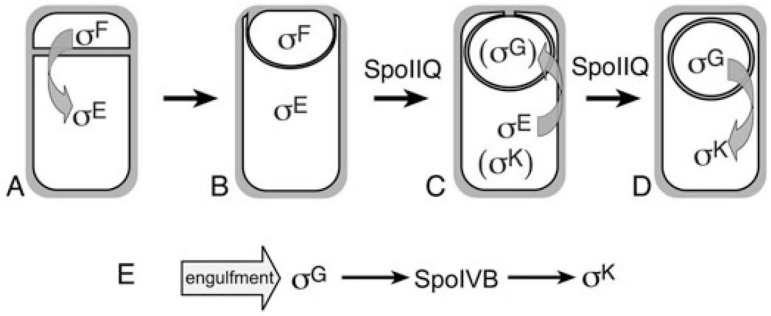

Fig. 1.

Engulfment pathway of *B. subtilis* and cell-specific gene expression.

A. Asymmetric division allows activation of σ^F in the smaller forespore, followed by activation of s^E in the larger mother cell.

B. During engulfment, the mother cell membrane migrates around the forespore, meets (C) and fuses (D) to release the forespore into the mother cell cytoplasm. The forespore protein SpoIIQ is required to synthesize the second forespore transcription factor (σ^G) and for σ^G activation, which also requires the completion of engulfment and the mother cell proteins encoded by the *spoIIIA* operon (arrow in C). The σ^G factor then produces the secreted signal transduction protein SpoIVB (arrow in D), allowing σ^K activation.

E. Summary of checkpoints controlling late gene expression: engulfment directly controls σ^G activity, which produces SpoIVB, allowing σ^K activity.

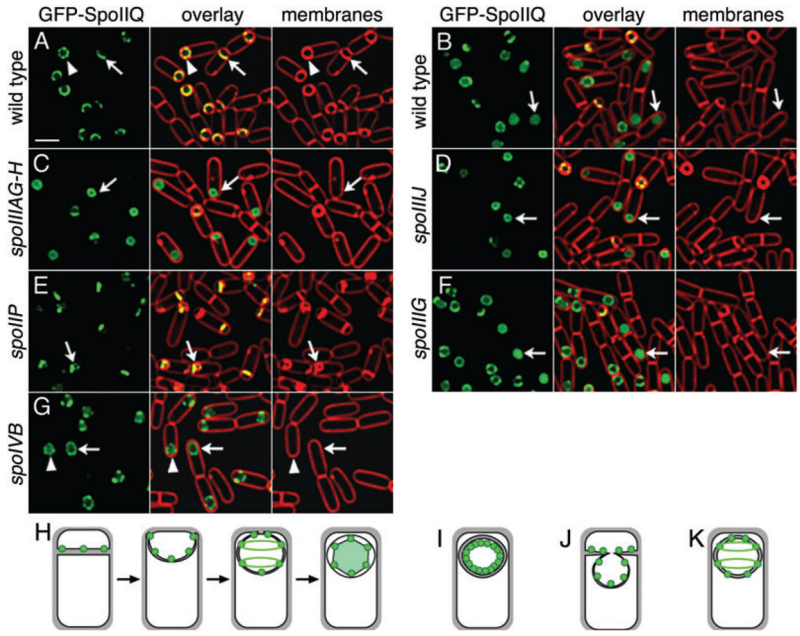

Fig. 2.

Localization and proteolysis of GFP-SpoIIQ. Live cells expressing either GFP-SpoIIQ (A–G) or MalF-GFP (H) (green), with FM 4–64 stained membranes (red).

A. Wild type (KP845) at t_2. Arrow shows GFP-SpoIIQ early in engulfment. Arrowhead, the punctate structure assembled prior to membrane fusion. Bar = 2 μm.

B. GFP-SpoIIQ (KP845) at t_3. After membrane fusion (arrow), FM 4–64 is excluded from the forespore and soluble GFP is observed (C) *spoIIIAG-H* (KP872) at t_3. GFP-SpoIIQ remains membrane associated after fusion (arrow).

D. *spoIIIJ* (KP873)at t_3. GFP-SpoIIQ remains membrane associated after fusion (arrow).

E. In *spoIIP::tet* (KP848) at t_3, engulfment is blocked, and the forespore bulges into the mother cell, forming bulges to which GFP-SpoIIQ localizes (arrow).

F. A mutant lacking σ^G (KP850; *spoIIIG::neo*) at t_3, showing cytoplasmic GFP after fusion (arrow).

G. *spoIVB::Tn917Ωmls* (KP949) at t_3. No cytoplasmic GFP is observed (arrow, arrowhead).

H. SpoIIQ (green circles) localization and degradation (green shading).

I. GFP-SpoIIQ in *spoIIIA* and *spoIIIJ* mutants.

J. Cartoon of SpoIIQ in a *spoIIP* mutant.

K. GFP-SpoIIQ remains punctate and membrane associated after fusion in the *spoIVB* mutant.

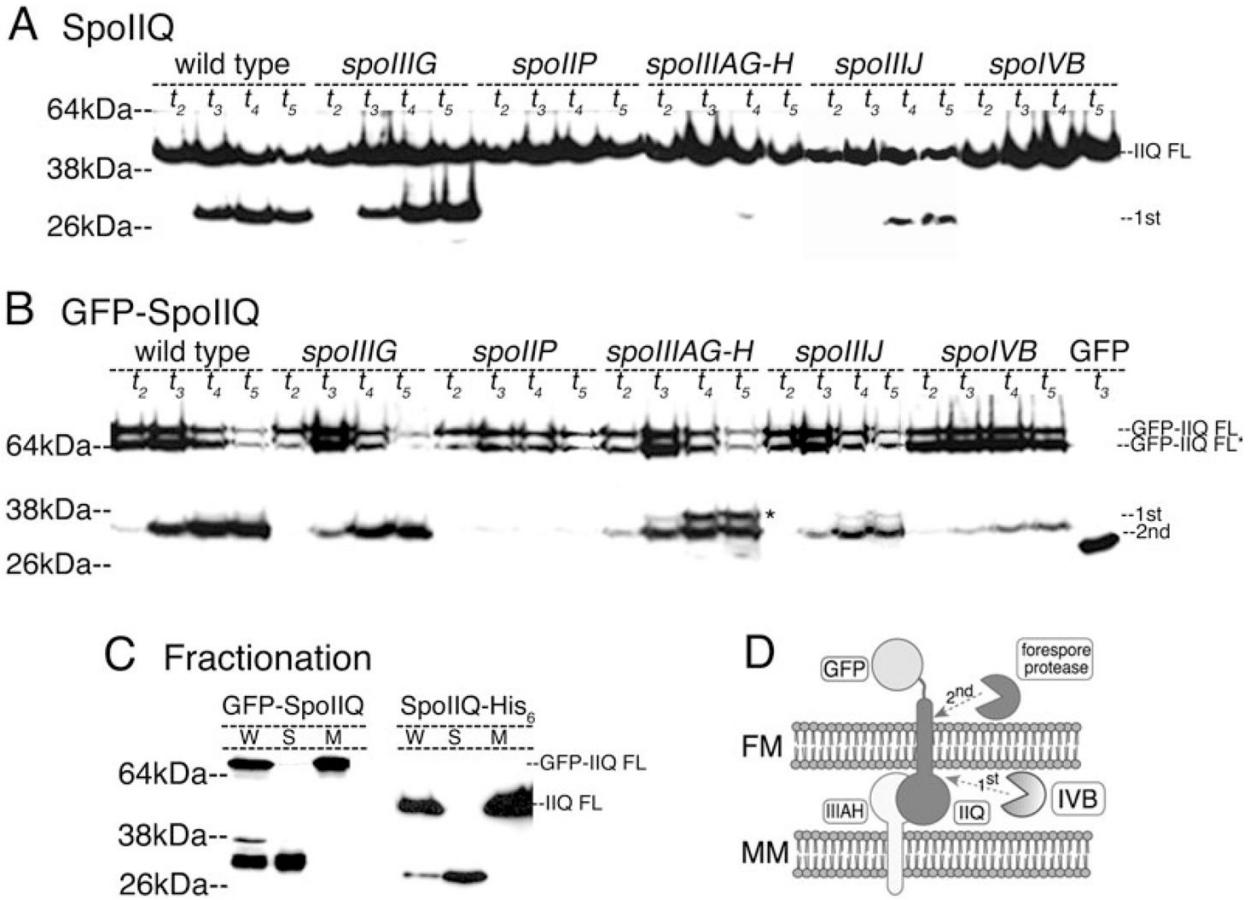

Fig. 3.

Regulated proteolysis of SpoIIQ.

A. Immunoblot and fractionation analysis of GFP-SpoIIQ and native SpoIIQ.

A. Native SpoIIQ in wild type (PY79), *spoIIIG* (KP85), *spoIIP* (KP719), *spoIIIAG-H* (KP896), *spoIIIJ* (KP901) and *spoIVB* (KP468). The ~30 kDa product does not accumulate in s*poIIP* or *spoIVB* and is reduced in *spoIIIAG-H* and *spoIIIJ* mutants.

B. GFP-SpoIIQ in wild type (KP845), *spoIIIG* (KP850), *spoIIP* (KP848), *spoIIIAG-AH* (KP872), *spoIIIJ* (KP873) and KP949 (*spoIVB*) and a strain expressing soluble GFP from the *spoIIQ* promoter (KP835; labelled GFP). Full length GFP-SpoIIQ often migrates as a doublet (~68 kDa, GFP-IIQ FL and GFP-IIQ FL*), although the use of additional protease inhibitors eliminates the smaller product, suggesting proteolysis during sample preparation. By t_3, a ~32 kDa N-terminal fragment of GFP-SpoIIQ is produced. *spoIIIAG-H* and *spoIIIJ* mutant strains produce the ~32 kDa product and a 38 kDa product (*).

C. Fractionation analysis of GFP-SpoIIQ at t_4 (KP845), and SpoIIQ-His_6 at $t_{2.5}$ (KP980). W = whole cells, S = soluble, M = insoluble membrane fraction. Full length GFP-SpoIIQ and SpoIIQ-His_6 are in the membrane fraction, while the N-terminal, GFP-containing product of GFP-SpoIIQ and the C-terminal, His_6-containing product of SpoIIQ-His_6 are soluble.

D. Model for SpoIIQ proteolysis. GFP-SpoIIQ in the forespore membrane (FM) interacts with SpoIIIAH in the mother cell membrane (MM) (Blaylock *et al*., 2004). Extracellular proteolysis depends on the secreted serine protease SpoIVB (light pac-man), allowing a second cleavage within the cytoplasm by an unknown protease (dark pac-man).

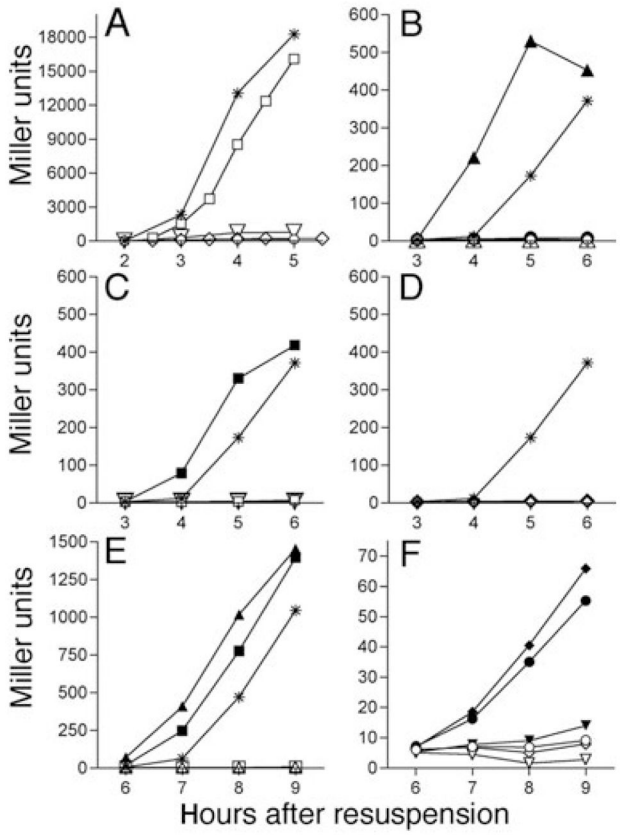

Fig. 4.

Effect of various spo mutations on σ^G and σ^K activity. Strains were induced to sporulate via resuspension with samples removed and β-galactosidase activity measured at hourly intervals.

A. Activity of σ^G-dependent *sspB-lacZ* at 37°C. Wild type (*; KP6); *spoIIQ* (○ KP950); *spoIVB* (□; KP957); *spoIIIAG-H* (◇; KP952); *spoIIP* (▽; KP951).

B–D. Activity of the σ^K-dependent *cotD–lacZ* fusion at 37°C.

B. Wild type (*; KP946), *spoIIIG* (△; KP947), *spoIIIG bofA* (▲; KP948), *spoIIQ* (○; KP953), *spoIIQ bofA* (●; KP955).

C. Wild type (*; KP946), *spoIIP* (▽; KP958), *spoIIP bofA* (▼; KP959), *spoIVB* (□; KP954); *spoIVB bofA* (■; KP956).

D. Wild type (*; KP946), *spoIIIAG-H* (◇; KP962); *spoIIIAG-H bofA* (◆; KP963), *spoIVB* (□; KP954); *spoIVB bofA* (■; KP956).

E–F. Activity of the σ^K-dependent *cotD–lacZ* fusion at 30°C.

E. Wild type (*; KP946), *spoIIIG* (△; KP947), *spoIIIG bofA* (▲; KP948), *spoIVB* (□; KP954); *spoIVB bofA* (■; KP956).

F. *spoIIQ* (○; KP953), *spoIIQ bofA* (●; KP955), *spoIIP* (▽; KP958), *spoIIP bofA* (▼; KP959), *spoIIIAA-H* (◇; KP960); *spoIIIAA-H bofA* (◆; KP961). Identical results were obtained with other engulfment mutants (*spoIID* or *spoIIM*).

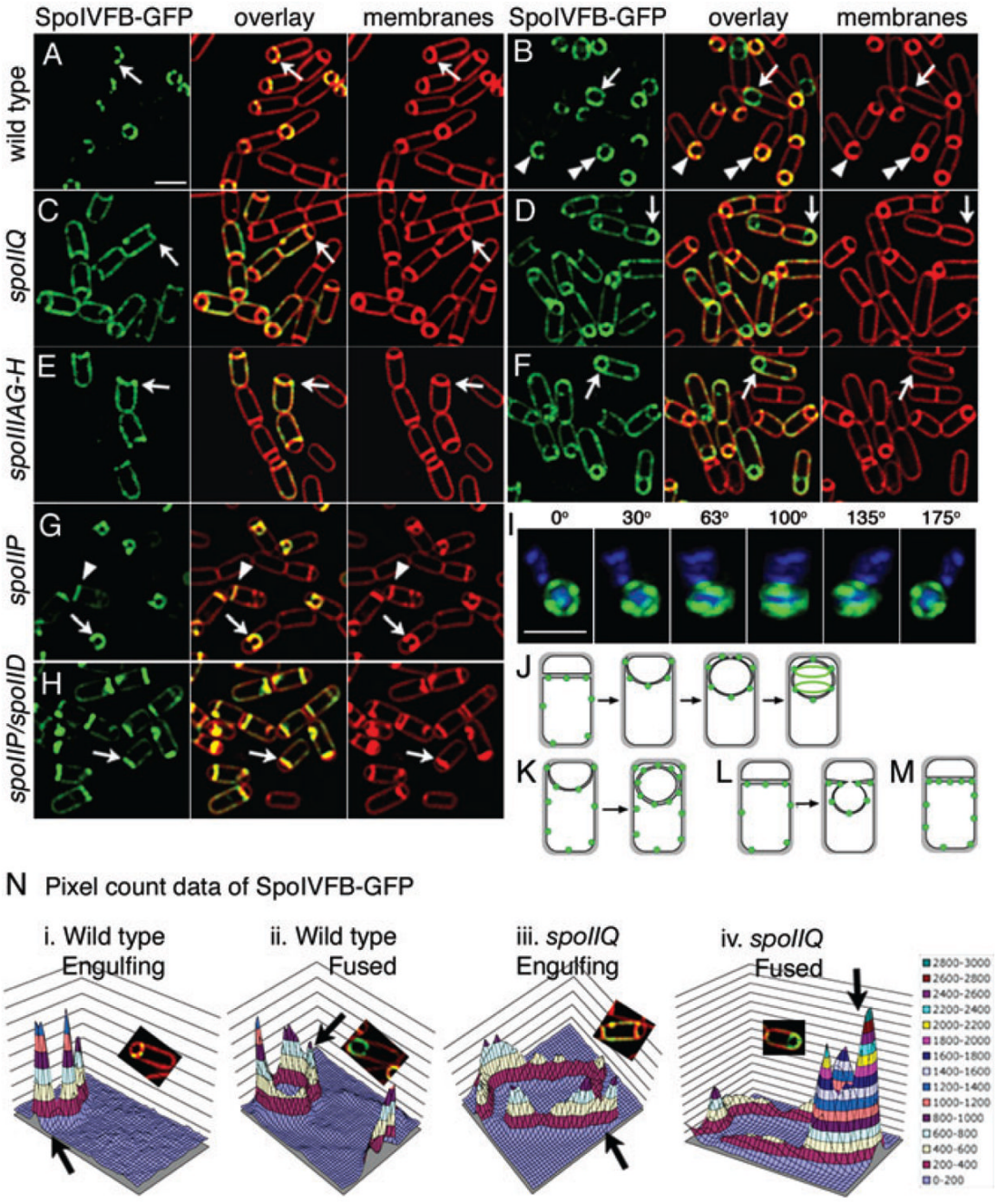

Fig. 5.

Localization of mother cell-expressed SpoIVFB-GFP (green). Live cells expressing SpoIVFB-GFP (green) at t_2 (A, C, E) or t_3 (B, D, F, G, H). FM 4–64 stained membranes are red.

A. Wild type (KP969) at t_2. Arrow shows punctate localization early in engulfment. Bar = 2 μm.

B. Wild type (KP969) at t_3. Sporangia before (arrowhead, double arrowhead) and after (arrow) membrane fusion.

C. *spoIIQ* (KP970) at t_2. SpoIVFB-GFP is randomly distributed in mother cell membrane (arrow).

D. *spoIIQ* (KP970) at t_3. SpoIVFB-GFP enriched at the forespore after fusion (arrow).

E. *spoIIIAG-H* (KP971) at t_2. SpoIVFB-GFP randomly distributed (arrow).
F. *spoIIIAG-H* (KP971) at t_3. SpoIVFB-GFP enriched at the forespore after fusion (arrow).
G. *spoIIP* (KP972) is engulfment defective. SpoIVFB localizes to flat septa (arrowhead) and bulges (arrow).
H. *spoIIP spoIID* double mutant (KP973).
I. Three dimensional reconstruction of SpoIVFB-GFP from 12 focal planes (z = 0.15 μm); rotated around the *Y*-axis. DAPI-stained DNA (blue). Bar = 2 μm.
J. SpoIVFB-GFP (green) localization during engulfment.
K. SpoIVFB-GFP (green) in the absence of either SpoIIQ or SpoIIIA.
L. SpoIVFB-GFP (green) in the absence of either SpoIIP, M or D or (M) both SpoIIP and SpoIID.
N. Quantification of SpoIVFB-GFP fluorescence intensity in wild type or *spoIIQ*. Deconvolved pixel count data from cells indicated by arrows in panels A (i), B (ii), C (iii) and D (iv) were imported into Microsoft Excel and three-dimensional graphs created. Scale on right.

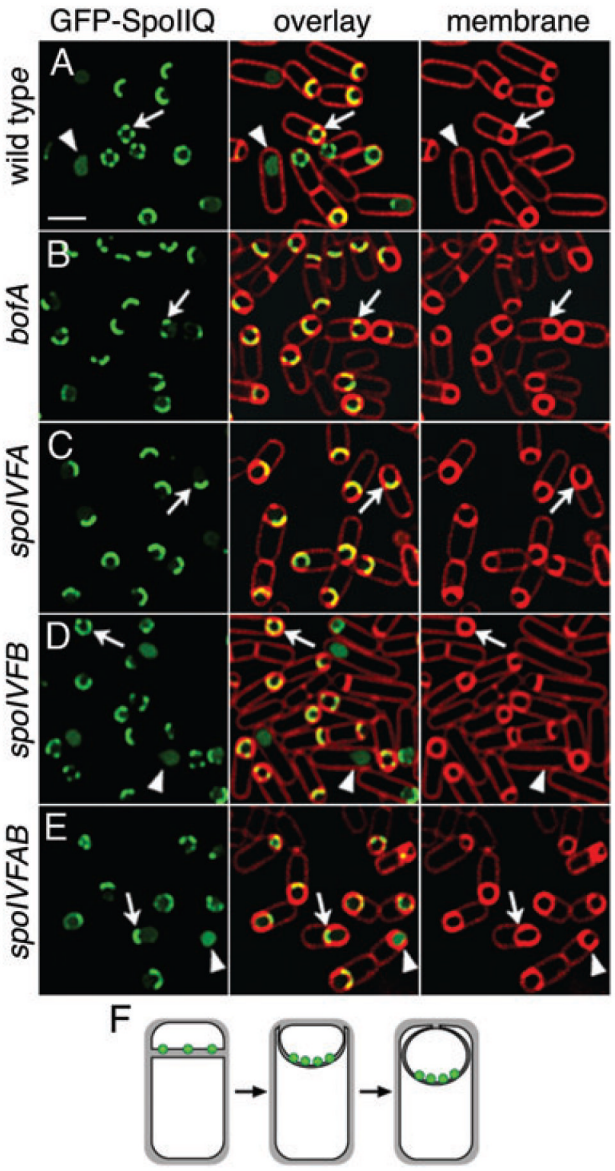

Fig. 6.

GFP-SpoIIQ tracking defect in spoIVF and bofA mutants. GFP-SpoIIQ (green) at t_3 with FM 4–64 stained membranes (red).

A. Wild type (KP845). Cells before fusion show punctate localization (arrow), while after fusion, soluble GFP (arrowhead).

B. *bofA* (KP964).

C. *spoIVFA* (KP966).

D. *spoIVFB* (KP976).

E. *spoIVFAB* (KP965). In the absence of BofA or SpoIVFA, GFP-SpoIIQ fails to track the engulfing mother cell membrane around the forespore, remaining at the septum (arrows in B, C, E or F). In the absence of only SpoIVFB, GFP-SpoIIQ localizes normally (arrow and arrowhead in D). Scale, 2 μm.

F. Localization of GFP-SpoIIQ in the absence of BofA or SpoIVFA.

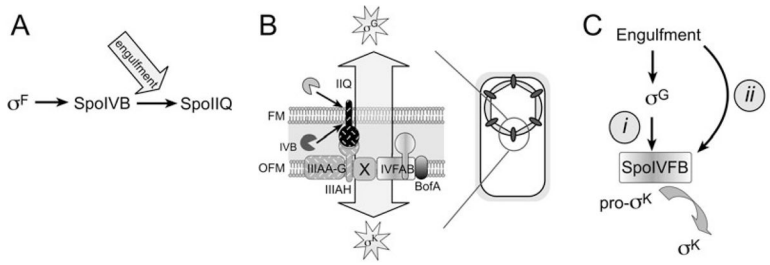

Fig. 7.

Summary and model of engulfment regulated events.

A. SpoIIQ degradation depends on the SpoIVB serine protease produced before engulfment by σ^F, but commences after engulfment, which must regulate either protease activity or access to its substrate.

B. The forespore protein SpoIIQ recruits the mother cell proteins SpoIIIAH and SpoIVFB to the outer forespore membrane (OFM) thereby assembling a protein complex containing proteins required for the engulfment-dependent activation of transcription in both cells (arrow). We propose that this synapse-like complex serves as a dedicated location for cell-cell communication.

C. We speculate that σ^K activity is governed by two checkpoints, the previously described σ^G checkpoint (i), in which the production of high levels of SpoIVB by σ^G relieves BofA-mediated inhibition of SpoIVFB and indirectly couples σ^K to engulfment, and a separate checkpoint (ii) that directly couples σ^K to engulfment, which could be comprised of a second inhibitory pathway or an activator.

Table 1

Strains used in this study.

Strain	Genotype	Reference
PY79	Wild type	Youngman *et al*. (1984)
KP6	*sspB-lacZΩcat*	Sun *et al*. (1991a)
KP85	*spoIIIGΔ1*	Sun *et al*. (1991b)
KP265	*spoIVFB152, Tn917ΩHU144*	Cutting *et al*. (1991b)
KP270	*spoIVFAΔ91*	Cutting *et al*. (1991b)
KP468	*ΔspoIVB::spc*	Oke *et al*. (1997)
KP575	*ΔspoIIQ::spc*	Sun *et al*. (2000)
KP719	*ΔspoIIP::tet*	Frandsen and Stragier (1995)
KP835	*amyE::*P_{spoIIQ}*-gfpΩcat*	Rubio and Pogliano (2004)
KP845	*ΔspoIIQ::spc amyE::*P_{spoIIQ}*gfp-spoIIQΩcat*	Rubio and Pogliano (2004)
KP848	*ΔspoIIQ::spc amyE::*P_{spoIIQ}*gfp-spoIIQΩcat ΔspoIIP::tet*	Rubio and Pogliano (2004)
KP850	*ΔspoIIQ::spc amyE::*P_{spoIIQ}*gfp-spoIIQΩcat ΔspoIIIG::neo*	Rubio and Pogliano (2004)
KP872	*ΔspoIIQ::spc amyE::*P_{spoIIQ}*gfp-spoIIQΩcat ΔspoIIIAG-H::kan*	Blaylock *et al*. (2004)
KP873	*ΔspoIIQ::spc amyE::*P_{spoIIQ}*gfp-spoIIQΩcat ΔspoIIIJ-jag::cat::tet*	Blaylock *et al*. (2004)
KP896	*ΔspoIIIAG-H::kan*	Blaylock *et al*. (2004)
KP901	*ΔspoIIIJ-jag::cat::tet*	Blaylock *et al*. (2004)
KP946	*thrC::cotD-lacZΩmls*	Sharp and Pogliano (1999)
KP947	*thrC::cotD-lacZΩmls spoIIIGΔ1*	This study
KP948	*thrC::cotD-lacZΩmls spoIIIGΔ1 ΔbofA::cat*	This study
KP949	*ΔspoIIQ::spc amyE::*P_{spoIIQ}*gfp-spoIIQΩcat spoIVB::Tn917Ωmls*	This study
KP950	*sspB-lacZΩcat ΔspoIIQ::spc*	This study
KP951	*sspB-lacZΩcat ΔspoIIP::tet*	This study
KP952	*sspB-lacZΩcat ΔspoIIIAG-H::kan*	This study
KP953	*thrC::cotD-lacZΩmls ΔspoIIQ::spc*	This study
KP954	*thrC::cotD-lacZΩmls ΔspoIVB::spc*	This study
KP955	*thrC::cotD-lacZΩmls ΔspoIIQ::spc ΔbofA::cat*	This study
KP956	*thrC::cotD-lacZΩmls ΔspoIVB::spc ΔbofA::cat*	This study
KP957	*sspB-lacZΩcat spoIVB::Tn917*	This study
KP958	*thrC::cotD-lacZΩmls ΔspoIIP::tet*	This study
KP959	*thrC::cotD-lacZΩmls ΔspoIIP::tet ΔbofA::cat*	This study
KP960	*thrC::cotD-lacZΩmls ΔspoIIIAA-H::kan*	This study
KP961	*thrC::cotD-lacZΩmls ΔspoIIIAA-H::kan ΔbofA::cat*	This study
KP962	*thrC::cotD-lacZΩmls ΔspoIIIAG-H::kan*	This study
KP963	*thrC::cotD-lacZΩmls ΔspoIIIAG-H::kan ΔbofA::cat*	This study
KP964	*ΔspoIIQ::spc amyE::*P_{spoIIQ}*gfp-spoIIQΩcat bofA::erm*	This study
KP965	*ΔspoIIQ::spc amyE::*P_{spoIIQ}*gfp-spoIIQΩcat ΔspoIVFAB::cat::tet*	This study
KP966	*ΔspoIIQ::spc amyE::*P_{spoIIQ}*gfp-spoIIQΩcat spoIVFAΔ91*	This study
KP967	*ΔbofA::cat*	Ricca *et al*. (1992)
KP969	*spoIVFB-gfpΩcat*	This study

Strain	Genotype	Reference
KP970	*spoIVFB-gfpΩcat ΔspoIIQ::spc*	This study
KP971	*spoIVFB-gfpΩcat ΔspoIIIAA-H::kan*	This study
KP972	*spoIVFB-gfpΩcat ΔspoIIP::tet*	This study
KP973	*spoIVFB-gfpΩcat ΔspoIIP::tet spoIID::Tn917ΩHU298*	This study
KP975	*spoIVFB::pAR98(kan)*	This study
KP976	*ΔspoIIQ::spc amyE::P_{spoIIQ}gfp-spoIIQΩcat spoIVFB::pAR98(kan)*	This study
KP978	*ΔspoIIQ::spc amyE::P_{spoIIQ}gfp-spoIIQΩcat spoIVFB::pAR98(kan) ΔbofA::cat::tet*	This study
KP979	*ΔspoIIP::tet amyE::P_{spoIID}gfp-spoIIPΩcat ΔspoIIQ::spc ΔbofA::erm*	This study
KP980	*ΔspoIIQ::spc, amyE::$P_{spoIIQ}$$his_6$-spoIIQΩcat*	This study
AR391	BL21 (λDE3)/pAR92	This study