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Serine proteases from two cell types target different components of a complex that governs regulated intramembrane proteolysis of pro- σ^K during *Bacillus subtilis* development

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Summary

Upon starvation *Bacillus subtilis* undergoes a developmental process involving creation of two cell types, the mother cell and forespore. A signal in the form of a serine protease, SpoIVB, is secreted from the forespore and leads to regulated intramembrane proteolysis (RIP) of pro- σ^K , releasing active σ^K into the mother cell. RIP of pro- σ^K is carried out by a membrane-embedded metalloprotease, SpoIVFB, which is inactive when bound by BofA and SpoIVFA. We have investigated the mechanism by which this complex is activated. By expressing components of the signalling pathway in *Escherichia coli*, we reconstructed complete inhibition of pro- σ^K RIP by BofA and SpoIVFA, and found that SpoIVB serine protease activity could partially restore RIP, apparently by targeting SpoIVFA. Pulse-chase experiments demonstrated that SpoIVFA synthesized early during *B. subtilis* sporulation is lost in a SpoIVB-dependent fashion, coincident with the onset of pro- σ^K RIP, supporting the idea that SpoIVB targets SpoIVFA to trigger RIP of pro- σ^K . Loss of BofA depended not only on SpoIVB, but also on CtpB, a serine protease secreted from the mother cell. CtpB appeared to cleave BofA near its C-terminus upon coexpression in *E. coli*, and purified CtpB degraded BofA. We propose that RIP of pro- σ^K involves a three-step proteolytic cascade in which SpoIVB first cleaves SpoIVFA, CtpB then cleaves BofA and finally SpoIVFB cleaves pro- σ^K .

Introduction

Communication between cells is crucial during the development of multicellular organisms, for interactions between hosts and pathogens or symbionts, and when microbial biofilms form. The endospore formation process of *Bacillus subtilis* provides an attractive experimental system to study signalling between two cell types. This starvation-induced developmental process (reviewed in Errington, 2003) involves formation of an asymmetrically located cell division septum, creating a larger mother cell and a smaller forespore, each with a copy of the genome (Fig. 1A). The two cell types differentiate further by activating different σ subunits of RNA polymerase, which direct transcription of different sets of genes (reviewed in Kroos and Yu, 2000; Piggot and Losick, 2002). Activation of σ^F in the forespore sends a signal that leads to activation of σ^E in the mother cell. Differential gene expression in the two cell types brings about engulfment of the fore-spore as the mother cell membrane migrates around the forespore, pinching it off as a protoplast within the mother cell (Fig. 1B). Hence, the forespore cytosol is separated from that of the mother cell by two membranes. Completion of engulfment activates σ^G in the forespore, initiating a signal transduction pathway that governs activation of σ^K in the mother cell (Fig. 1C). This signalling pathway, which has been called the σ^K checkpoint, is critical for efficient completion of endospore formation (Cutting *et al.*, 1990). Components

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of the σ^K checkpoint have been identified, but questions remain about the mechanism of signalling.

The σ^K checkpoint signal transduction pathway begins in the forespore when σ^G RNA polymerase transcribes the *spoIVB* gene, which encodes a serine protease with a PDZ domain (Cutting *et al.*, 1991a; Wakeley *et al.*, 2000). SpoIVB is believed to be secreted across the inner fore-spore membrane into the space between the two membranes surrounding the forespore (Wakeley *et al.*, 2000). There, its PDZ domain has been proposed to target the protease to a complex of proteins located in the outer forespore membrane (Dong and Cutting, 2004). The complex includes SpoIVFA, SpoIVFB and BofA (Rudner and Losick, 2002). All three proteins are expressed in the mother cell under the control of σ^E RNA polymerase (Cutting *et al.*, 1991b; Ricca *et al.*, 1992). SpoIVFA is critical for assembly of the complex and its localization to the outer forespore membrane, which may involve initial insertion in the mother cell membrane, diffusion to the septum and capture in the outer forespore membrane during the process of engulfment (Rudner and Losick, 2002; Rudner *et al.*, 2002; Doan *et al.*, 2005). SpoIVFB appears to be a metalloprotease that catalyses regulated intramembrane proteolysis (RIP) of pro- σ^K (Rudner *et al.*, 1999; Yu and Kroos, 2000), removing 20 amino acids from its N-terminal end (Kroos *et al.*, 1989; Zhou and Kroos, 2004) and releasing active σ^K from the outer forespore membrane into the mother cell (Zhang *et al.*, 1998). RIP is an important and widely conserved mechanism that controls signalling pathways in both prokaryotes and eukaryotes (reviewed in Brown *et al.*, 2000; Wolfe and Kopan, 2004). It involves cleavage of a protein within a membrane or near the membrane surface. Hence, proteases that perform RIP are referred to as intramembrane-cleaving proteases (I-CLiPs). SpoIVFB is a founding member of the site-2 protease (S2P) family of I-CLiPs, named after human S2P, which cleaves transcription factors that regulate sterol and fatty acid biosynthesis, and the endoplasmic reticulum stress response (reviewed in Brown *et al.*, 2000). BofA inhibits SpoIVFB protease activity, possibly by providing a metal ligand and blocking the active site (Zhou and Kroos, 2004) until the SpoIVB signal from the forespore relieves the inhibition.

One question that remains about the σ^K checkpoint is how SpoIVB relieves BofA-mediated inhibition of SpoIVFB. Recently, SpoIVB's PDZ domain has been shown to interact with a C-terminal segment of BofA *in vitro* (Dong and Cutting, 2004). Also, SpoIVB's serine protease activity can cleave SpoIVFA *in vitro* (Dong and Cutting, 2003). These findings suggest that SpoIVB might interact with the C-terminal segment of BofA in the space between the membranes surrounding the forespore (Fig. 1C), positioning SpoIVB to cleave SpoIVFA. After cleavage of SpoIVFA, the inhibitory effect of BofA on SpoIVFB would need to be relieved. We hypothesized that CtpB might be involved in this step. CtpB is a PDZ domain-containing serine protease expressed under σ^E control in the mother cell (Pan *et al.*, 2003). Because CtpB has a predicted N-terminal secretion signal, it is likely translocated across the mother cell membrane and present in the space between the membranes surrounding the forespore after engulfment, positioning it to cleave the C-terminal end of BofA (Fig. 1C).

To further investigate the signalling pathway that governs pro- σ^K RIP, one of the approaches we have employed is to coexpress components of the pathway in different combinations in *Escherichia coli* (Fig. 1D). Previously, we showed that BofA markedly, but not completely, inhibits RIP of pro- σ^K by SpoIVFB in *E. coli* (Zhou and Kroos, 2004). Here, we show that adding SpoIVFA to the system completely inhibits RIP, and RIP is partially restored when SpoIVB is also added. Additional results provide evidence that SpoIVB targets SpoIVFA to relieve inhibition of SpoIVFB in *E. coli*, supporting a model proposed by Dong and Cutting (2003) based on the ability of SpoIVB to cleave SpoIVFA *in vitro*. Furthermore, we use pulse-chase analysis of sporulating *B. subtilis* to demonstrate that SpoIVFA is lost in a SpoIVB-dependent fashion coincident with the onset of pro- σ^K RIP. We also use pulse-chase analysis of sporulating *B. subtilis*, and coexpression of proteins in *E. coli*, to obtain evidence that CtpB

plays a role in BofA degradation *in vivo*, and we demonstrate that purified CtpB degrades BofA *in vitro*. Our results support a new model for the σ^K checkpoint involving a cascade of three proteolytic events in which SpoIVB from the fore-spore first cleaves SpoIVFA in the space between the membranes surrounding the forespore (Fig. 1C), this exposes the C-terminal end of BofA to cleavage by CtpB from the mother cell, and finally SpoIVFB becomes active to perform RIP of pro- σ^K .

Results

Complete inhibition of pro- σ^K RIP by BofA and SpoIVFA in *E. coli*

Both BofA and SpoIVFA are necessary for complete inhibition of pro- σ^K RIP in sporulating *B. subtilis*, prior to fore-spore signalling that relieves the inhibition (Cutting *et al.*, 1990; Cutting *et al.*, 1991a,b; Ricca *et al.*, 1992). To determine whether coexpression of BofA and SpoIVFA could bring about complete inhibition of pro- σ^K RIP in *E. coli*, we built on a system established previously (Zhou and Kroos, 2004). In this system, *B. subtilis* proteins are produced in *E. coli* under the control of T7 RNA polymerase (Fig. 1D). Coexpression of the tagged I-CLiP, H10SpoIVFB-GFP, with a C-terminally truncated substrate, pro- $\sigma^K(1-126)$ H6, which makes it easier to separate the unprocessed and processed forms by SDS-PAGE, resulted in abundant RIP (Fig. 2, lane 1), as observed previously (Zhou and Kroos, 2004). Also, addition of SpoIVFA to the system had no effect (Fig. 2, lane 2), while GFP Δ 27BofA, a functional fusion of green fluorescent protein (GFP) to amino acid 28 of BofA (Rudner and Losick, 2002), markedly, but not completely, inhibited RIP of pro- $\sigma^K(1-126)$ H6 (Fig. 2, lane 3) (Zhou and Kroos, 2004). When both SpoIVFA and GFP Δ 27BofA were simultaneously added to the system, we observed complete inhibition of RIP (Fig. 2, lane 4). Under these conditions, both SpoIVFA and GFP Δ 27BofA were detected in complexes with H10SpoIVFB-GFP (data not shown). Complexes were isolated by cobalt affinity chromatography (to bind the H10-tag on H10SpoIVFB-GFP) after detergent (1% digitonin) solubilization of membranes. Using this technique, we showed previously that GFP Δ 27BofA forms a complex with H10SpoIVFB-GFP in the absence of SpoIVFA, but SpoIVFA fails to form a stable complex with H10SpoIVFB-GFP in the absence of GFP Δ 27BofA, when proteins are coexpressed in *E. coli* (Zhou and Kroos, 2004). Our new results suggest that SpoIVFA, GFP Δ 27BofA and H10SpoIVFB-GFP can form a complex in *E. coli*, consistent with evidence that the three proteins form a complex in sporulating *B. subtilis* (Rudner and Losick, 2002). The enhanced inhibition of pro- $\sigma^K(1-126)$ H6 RIP observed when SpoIVFA and GFP Δ 27BofA were coexpressed in *E. coli* (Fig. 2, lane 4), relative to inhibition by GFP Δ 27BofA alone (Fig. 2, lane 3), may reflect a larger number of inhibitory complexes being formed, greater stability of trimeric complexes than binary complexes of GFP Δ 27BofA and H10SpoIVFB-GFP, and/or a direct effect of SpoIVFA on SpoIVFB I-CLiP activity when it is in a trimeric complex.

SpoIVB partially restores RIP of pro- σ^K in *E. coli* and destabilizes SpoIVFA

SpoIVB has an N-terminal signal sequence capable of promoting translocation of alkaline phosphatase across the *E. coli* inner membrane (Wakeley *et al.*, 2000). Therefore, we expected SpoIVB to be secreted into the periplasm upon expression in *E. coli*, placing it in a position to activate SpoIVFA/BofA/SpoIVFB complexes (Fig. 1D). We tagged SpoIVB at its C-terminus with hexahistidine to permit detection with anti-His antibodies. When SpoIVB-H6 was coexpressed with SpoIVFA, GFP Δ 27BofA, H10SpoIVFB-GFP and pro- $\sigma^K(1-126)$ H6 in *E. coli*, RIP was partially restored and the level of SpoIVFA was decreased (Fig. 2, lane 5). Coexpression of SpoIVB-H6 with just pro- $\sigma^K(1-126)$ H6 did not result in RIP (data not shown). SpoIVB-H6 migrated as two or three distinct species in different experiments, consistent with the observation of multiple SpoIVB species in sporulating *B. subtilis* and evidence that SpoIVB synthesized *in vitro* self-cleaves at several distinct sites near its N-terminus (Wakeley *et al.*, 2000). SpoIVB-H6 S378A, with a substitution of alanine for the putative catalytic serine

residue of the protease (Wakeley *et al.*, 2000), migrated as a single species of higher molecular weight than the multiple species observed for wild-type SpoIVB-H6, suggesting that the mutant enzyme fails to self-cleave (Fig. 2, lane 6). The mutant enzyme failed to restore RIP of pro- $\sigma^K(1-126)H6$, and SpoIVFA accumulated normally. These results suggest that SpoIVB serine protease activity can target SpoIVFA and partially relieve the complete inhibition of SpoIVFB I-CLiP activity brought about by the combined action of SpoIVFA and BofA in *E. coli*.

To further investigate the target of SpoIVB, we tested whether it could relieve the partial inhibition of RIP mediated by GFP $\Delta 27$ BofA alone (Fig. 2, lane 3). In the absence of SpoIVFA, SpoIVB-H6 had no effect on RIP and no effect on the level of GFP $\Delta 27$ BofA (Fig. 2, lanes 7 and 8). In contrast, SpoIVB-H6 had a dramatic effect on SpoIVFA accumulation in *E. coli* when GFP $\Delta 27$ BofA was not coexpressed (Fig. 2, lane 9). This effect was not observed with the SpoIVB-H6 S378A mutant enzyme (Fig. 2, lane 10). Hence, SpoIVB-H6 serine protease activity appears to target SpoIVFA, not GFP $\Delta 27$ BofA, in *E. coli*. This is consistent with *in vitro* transcription-translation experiments that suggest SpoIVB can cleave SpoIVFA but not BofA (Dong and Cutting, 2003). Based on their *in vitro* data, Dong and Cutting proposed that SpoIVB cleavage of SpoIVFA in complexes would relieve BofA-mediated inhibition of SpoIVFB. In support of this model, SpoIVB-H6 appears to target SpoIVFA in *E. coli* and reverse the extra inhibition of pro- σ^K RIP brought about by coexpressing SpoIVFA with GFP $\Delta 27$ BofA, restoring RIP to a level similar to that observed when GFP $\Delta 27$ BofA alone is expressed (Fig. 2, lanes 3–5). However, RIP is not restored to the level seen in the absence of GFP $\Delta 27$ BofA (Fig. 2, lane 2), perhaps because some SpoIVFA persists (Fig. 2, lane 5) and/or another mechanism is involved in relieving BofA-mediated inhibition of SpoIVFB (see below).

Loss of SpoIVFA synthesized early during sporulation coincides with onset of pro- σ^K RIP

If SpoIVB cleavage of SpoIVFA leads to activation of SpoIVFB and RIP of pro- σ^K , one might expect that the steady-state level of SpoIVFA measured by Western blot analysis would decrease when pro- σ^K RIP begins during *B. subtilis* sporulation. This expectation appeared to be met in experiments reported previously (Kroos *et al.*, 2002). Also as expected, the level of SpoIVFA did not decrease at the normal time and RIP of pro- σ^K did not commence in a *spoIVB* mutant (Kroos *et al.*, 2002; Dong and Cutting, 2003). However, we found that in some experiments RIP of pro- σ^K appeared to precede loss of SpoIVFA (see below). Because the synchrony of sporulation can vary depending on experimental conditions, we reasoned that the lack of correlation between loss of SpoIVFA and the onset of pro- σ^K RIP in some experiments might be attributed to SpoIVFA synthesis in cells that are delayed in the sporulation process relative to other cells in the population.

To focus on SpoIVFA synthesized early during the period of σ^E RNA polymerase activity in the population, we pulse-labelled proteins at 1.75 h post starvation by adding [^{35}S]methionine for 5 min. This was followed by incubation in the presence of excess unlabelled methionine and collection of samples for immunoprecipitation of SpoIVFA at different times during the chase period. Samples were also collected to monitor pro- σ^K RIP and the steady-state level of SpoIVFA by Western blot analysis. During the chase with excess unlabelled methionine, the amount of labelled SpoIVFA rose initially, perhaps because of increasing SpoIVFA synthesis in the population and incomplete dilution of labelled methionine (Fig. 3). Even so, in wild-type cells, SpoIVFA decreased markedly between 75 and 105 min after labelling, coincident with the onset of pro- σ^K RIP, and while the steady-state level of SpoIVFA increased. By 135 min post labelling, SpoIVFA decreased further, RIP of pro- σ^K was substantial, and the steady-state level of SpoIVFA was unchanged. In a *spoIVB* mutant, SpoIVFA decreased little even 135 min after labelling and RIP of pro- σ^K was not observed. We conclude that SpoIVFA synthesized early during the period of σ^E RNA polymerase activity is cleaved in a SpoIVB-

dependent fashion at a time close to the onset of pro- σ^K RIP, consistent with the model that SpoIVB cleavage of SpoIVFA triggers RIP of pro- σ^K .

Loss of GFP Δ 27BofA also correlates with onset of pro- σ^K RIP

How does cleavage of SpoIVFA relieve BofA-mediated inhibition of SpoIVFB? One possibility is that loss of SpoIVFA from complexes with BofA and SpoIVFB, makes BofA susceptible to proteolysis. To test whether GFP Δ 27BofA is lost in a SpoIVB-dependent fashion, *B. subtilis* expressing GFP Δ 27BofA in a wild-type or *spoIVB* mutant background were treated with [³⁵S]methionine to pulse-label proteins at hour 2 of sporulation and analysed as described above, except using antibodies against GFP to immunoprecipitate GFP Δ 27BofA or detect it by Western blot analysis. For comparison, labelled SpoIVFA was also immunoprecipitated. The level of labelled SpoIVFA decreased markedly between 1 and 2 h after labelling (Fig. 4), in agreement with experiments like that shown in Fig. 3. Labelled GFP Δ 27BofA showed a similar pattern of decrease as SpoIVFA in wild type, but neither GFP Δ 27BofA nor SpoIVFA decreased much even 3 h after labelling in the *spoIVB* mutant (Fig. 4). RIP of pro- σ^K commenced as the levels of labelled SpoIVFA and GFP Δ 27BofA decreased in wild type. As observed for the steady-state level of SpoIVFA (Fig. 3), the steady-state level of GFP Δ 27BofA (determined by Western blot analysis) did not decrease when σ^K first appeared (Fig. 4), perhaps because of asynchronous sporulation. We conclude that like SpoIVFA, GFP Δ 27BofA made early during the period of σ^E RNA polymerase activity is degraded in a SpoIVB-dependent fashion at a time close to the onset of pro- σ^K RIP. This is consistent with the idea that BofA in complexes with SpoIVFA and SpoIVFB becomes susceptible to proteolysis after SpoIVB cleaves SpoIVFA. Loss of BofA would relieve inhibition of the SpoIVFB I-CLiP, allowing RIP of pro- σ^K .

Loss of GFP Δ 27BofA, but not loss of SpoIVFA, is delayed in a *ctpB* mutant that is delayed for RIP of pro- σ^K

The SpoIVB serine protease did not appear to target GFP Δ 27BofA in *E. coli* (Fig. 2, lanes 7 and 8) or *in vitro* (Dong and Cutting, 2003). How, then, might SpoIVB cleavage of SpoIVFA render BofA susceptible to proteolysis? CtpB, like SpoIVB, is a PDZ-domain-containing serine protease that has been shown to influence RIP of pro- σ^K (Pan *et al.*, 2003). A *ctpB* mutant exhibits delayed pro- σ^K RIP. The target of CtpB's protease activity is unknown, but it is likely that CtpB acts in the space between the membranes surrounding the forespore as it has a predicted N-terminal signal sequence for secretion (Pan *et al.*, 2003). In Fig. 1C, CtpB is depicted being translocated across the outer forespore membrane after engulfment. Alternatively or in addition, prior to engulfment, CtpB is likely translocated across the mother-cell membrane, may diffuse into the asymmetric septum, and be captured between the membranes surrounding the forespore during engulfment. We hypothesized that CtpB in the space between the membranes surrounding the forespore targets the C-terminal region of BofA.

To test whether loss of GFP Δ 27BofA and/or SpoIVFA is delayed in a *ctpB* mutant, we performed pulse-chase, immunoprecipitation experiments as described above. In the *ctpB* mutant, labelled SpoIVFA decreased normally, but loss of labelled GFP Δ 27BofA was delayed; its level changed little between 1 and 2 h after labelling (Fig. 4). The onset of pro- σ^K RIP was also delayed in the *ctpB* mutant by about 1 h, being first observed at hour 5 of sporulation, rather than at hour 4 as in wild type. The correlation between delayed loss of labelled GFP Δ 27BofA and delayed onset of pro- σ^K RIP in the *ctpB* mutant is consistent with the idea that CtpB targets BofA, relieving its inhibitory effect on SpoIVFB and allowing RIP of pro- σ^K . Loss of SpoIVFA at the normal time was insufficient to trigger pro- σ^K RIP at the normal time in the *ctpB* mutant. This finding, taken together with the observation that neither SpoIVFA nor GFP Δ 27BofA decreased normally in the *spoIVB* mutant, strongly suggests that SpoIVB

must cleave SpoIVFA before CtpB can cleave BofA, which in turn allows SpoIVFB to cleave pro- σ^K .

CtpB causes BofA cleavage in *E. coli* and degrades H6GFP Δ 27BofA *in vitro*

To test the idea that CtpB cleaves BofA, we expressed tagged versions of the two proteins in *E. coli*. CtpB was tagged with hexahistidine at its C-terminus (CtpB-H6). As a control, CtpB-H6 S309A, with a substitution that inactivates the serine protease (Pan *et al.*, 2003), was also tested. The mutant form accumulated much better than wild-type CtpB-H6 (Fig. 5A). This does not appear to be attributed to poor expression of the wild-type enzyme, as an abundant 20 kDa polypeptide was detected with antibodies against the C-terminal H6 tag (data not shown). Rather, CtpB appears to undergo autoproteolysis, as observed for SpoIVB (Wakeley *et al.*, 2000) (Fig. 2). Coexpression of GFP Δ 27BofA with wild-type CtpB-H6, but not CtpB-H6 S309A, resulted in a decrease in the level of full-length GFP Δ 27BofA and the appearance of at least one and possibly two lower molecular weight species (Fig. 5A, lane 2). To ask whether the lower molecular weight species is attributed to cleavage of BofA or GFP, we tagged GFP Δ 27BofA at its N-terminus with hexahistidine and coexpressed H6GFP Δ 27BofA with CtpB-H6 in *E. coli*. Western blot analysis with anti-His antibodies yielded a similar result as shown in Fig. 5A (lane 2) (data not shown), suggesting that H6GFP Δ 27BofA is cleaved near its C-terminus. The lower band could be purified by affinity chromatography with cobalt resin (which binds H6) and then detected by anti-GFP antibodies (data not shown), demonstrating that it was not a breakdown fragment of CtpB-H6. These results suggest that BofA is cleaved near its C-terminus in a CtpB-dependent fashion when the two proteins are coexpressed in *E. coli*. Because at least the C-terminal 29 amino acids of BofA appear to be present in the periplasm when BofA-alkaline phosphatase fusion proteins are expressed in *E. coli* (Varcamonti *et al.*, 1997), CtpB would presumably have to be secreted into the periplasm in order to directly cleave BofA near its C-terminus (Fig. 1D). As noted previously (Pan *et al.*, 2003), CtpB has an N-terminal amino acid sequence that could serve as a signal sequence for secretion. In contrast, CtpB-H6 had no effect on SpoIVFA when the two proteins were coexpressed in *E. coli* (Fig. 5B, lanes 1 and 2). As SpoIVFA, like BofA, has a C-terminal periplasmic domain when expressed in *E. coli* (Green and Cutting, 2000), we conclude that CtpB specifically causes BofA to be cleaved.

To test whether CtpB could proteolyse BofA *in vitro*, we purified wild-type CtpB-H6 and the S309A mutant enzyme from *E. coli*. Although it was difficult to purify much of the full-length, wild-type enzyme, presumably because of autoproteolysis, it caused a marked decrease in the level of H6GFP Δ 27BofA upon co-incubation, whereas a much larger amount of the S309A mutant enzyme had no effect (Fig. 5A, lanes 4–6). Wild-type CtpB-H6 had no effect on the level of H6SpoIVFA upon co-incubation (Fig. 5B, lanes 3–5). These results support the idea that CtpB serine protease activity can specifically target BofA.

Discussion

Our results support a new model for events governing RIP of pro- σ^K (Fig. 6). In agreement with a model proposed previously based on *in vitro* data (Dong and Cutting, 2003), our findings in *E. coli* suggest that SpoIVB serine protease activity targets SpoIVFA in complexes that otherwise inhibit pro- σ^K RIP. Careful examination revealed a good correlation between loss of SpoIVFA and onset of pro- σ^K RIP during *B. subtilis* sporulation (Fig. 3), consistent with the idea that SpoIVB from the forespore initiates events leading to RIP of pro- σ^K by cleaving SpoIVFA (Fig. 6A). We also found that loss of GFP Δ 27BofA depends on SpoIVB (Fig. 4), yet SpoIVB failed to cleave BofA in *E. coli* (Fig. 2) or *in vitro* (Dong and Cutting, 2003). Our evidence suggests that CtpB cleaves BofA in the second step of a proteolytic cascade (Fig.

6B). Loss of BofA would free SpoIVFB from inhibition, allowing RIP of pro- σ^K (Fig. 6C). Below, we discuss further the evidence for this three-step cascade model and its implications.

Reconstruction of the σ^K checkpoint in *E. coli*

Expressing components of the σ^K checkpoint in different combinations in *E. coli* has proven to be very effective for elucidating the function of proteins in this signal transduction pathway (Zhou and Kroos, 2004), especially as reconstitution of pro- σ^K RIP *in vitro* has not yet been successful. Expressing SpoIVB-H6 in *E. coli* along with other components of the σ^K checkpoint provided evidence that SpoIVB-H6 targets SpoIVFA to partially relieve the complete inhibition of H10SpoIVFB-GFP brought about by the combined action of SpoIVFA and GFP Δ 27BofA (Fig. 2). The model in Fig. 6 may explain why pro- σ^K RIP was only partially restored. As CtpB was not coexpressed, GFP Δ 27BofA remains, and can substantially inhibit RIP on its own. In addition, GFP Δ 27BofA appeared to partially protect SpoIVFA from SpoIVB-dependent proteolysis in *E. coli* (Fig. 2). Perhaps in sporulating *B. subtilis*, interactions between SpoIVFA and other proteins (Doan *et al.*, 2005) render it more susceptible to cleavage by SpoIVB.

When we attempted to coexpress CtpB-H6 along with other components of the σ^K checkpoint in *E. coli*, we obtained a surprising result. σ^K was abundantly produced in a SpoIVFB-independent fashion (data not shown). This depended on CtpB serine protease activity, as it was not observed for CtpB-H6 S309A (data not shown). If CtpB-H6 is secreted across the *E. coli* inner membrane into the periplasm, it is unlikely that CtpB-H6 directly cleaves pro- σ^K . Perhaps periplasmic CtpB-H6 somehow leads to activation of RseP (formerly YaeL), an *E. coli* homologue of SpoIVFB (Alba *et al.*, 2002; Kanehara *et al.*, 2002; Akiyama *et al.*, 2004). In any case, we were unable to investigate the role of CtpB in overcoming the complete inhibition of pro- σ^K RIP mediated by the combined action of GFP Δ 27BofA and SpoIVFA in *E. coli*, or the partial inhibition of pro- σ^K RIP mediated by GFP Δ 27BofA alone.

Evidence that CtpB cleaves BofA in the second step of a proteolytic cascade

Loss of labelled SpoIVFA occurred at the normal time in the *ctpB* mutant, yet RIP of pro- σ^K was delayed (Fig. 4). This implies that the first step of the cascade, SpoIVB cleavage of SpoIVFA leading to its loss, is intact in the *ctpB* mutant, but is insufficient to allow RIP of pro- σ^K . Loss of labelled GFP Δ 27BofA was delayed in the *ctpB* mutant, and coincided with onset of pro- σ^K (Fig. 4). This suggests a second step in the cascade, CtpB cleavage of BofA leading to its loss. Support for this idea was obtained by coexpressing CtpB-H6 and GFP Δ 27BofA in *E. coli* (Fig. 5A). Also, by coexpressing N-terminally tagged H6GFP Δ 27BofA with CtpB-H6, we obtained evidence that BofA is cleaved near its C-terminus in a CtpB-dependent fashion (data not shown). Interestingly, CtpB shares sequence similarity with members of the carboxyl-terminal processing protease family (Pan *et al.*, 2003). One member of the family, Tsp of *E. coli*, has been shown to use its PDZ domain to recognize non-polar C-terminal residues of its substrates (Keiler and Sauer, 1996; Beebe *et al.*, 2000). As BofA has non-polar C-terminal residues (Ricca *et al.*, 1992), it is tempting to speculate that CtpB uses its PDZ domain to recognize these amino acids. Deletion of as little as three amino acids from its C-terminal end inactivates BofA (Varcamonti *et al.*, 1997), so these residues are also important for BofA-mediated inhibition of pro- σ^K RIP. Except for its C-terminal tail, the rest of GFP Δ 27BofA is expected to be inaccessible to CtpB when the proteins are coexpressed in *E. coli*, based on membrane topological analysis of BofA fusion proteins in *E. coli* (Varcamonti *et al.*, 1997) and assuming that CtpB's predicted N-terminal signal sequence (Pan *et al.*, 2003) mediates its translocation across the inner membrane to the periplasm (Fig. 1D). This might explain the limited digestion of GFP Δ 27BofA in *E. coli* coexpressing CtpB-H6 (Fig. 5A). In contrast, purified CtpB-H6 caused a loss of full-length H6GFP Δ 27BofA (Fig. 5A) and partial degradation products were not detected under the conditions we tested (data not shown),

perhaps because H6GFP Δ 27BofA had been purified after detergent solubilization from membranes. On the other hand, SpoIVFA was not recognized by CtpB in *E. coli* or *in vitro* (Fig. 5B). It is noteworthy that SpoIVFA has a charged residue at its C-terminus (Cutting *et al.*, 1991b), which might prevent its recognition by CtpB. Taken together, our *in vivo* experiments in sporulating *B. subtilis* and in *E. coli*, and our *in vitro* experiments with purified CtpB-H6 strongly support the idea that CtpB cleaves BofA after SpoIVB has cleaved SpoIVFA.

Loss of BofA would allow the third step in the cascade, RIP of pro- σ^K by SpoIVFB. Loss of GFP Δ 27BofA and RIP of pro- σ^K are not absolutely dependent on CtpB, but both are significantly delayed in a *ctpB* null mutant (Fig. 4), resulting in a twofold reduction in sporulation efficiency (Pan *et al.*, 2003). This implies that there is a CtpB-independent mechanism(s) capable of ridding SpoIVFB of its inhibitor, BofA, albeit less efficiently. It is unlikely that CtpA, a paralogue of CtpB, is completely responsible for BofA proteolysis in the absence of CtpB, because a *ctpA ctpB* double mutant is only slightly more defective in sporulation than a *ctpB* mutant (Pan *et al.*, 2003).

CtpB-H6 was extremely unstable when synthesized in *E. coli* (Fig. 5). It is attractive to speculate that CtpB is also unstable in sporulating *B. subtilis*, because it provides a rationale for the second step of the cascade; namely, that the second step monitors ongoing protein synthesis capability of the mother cell. CtpB, like SpoIVFA, BofA and SpoIVFB, is expressed in the mother cell under the control of σ^E RNA polymerase (Cutting *et al.*, 1991b; Ricca *et al.*, 1992; Pan *et al.*, 2003). If CtpB is less stable than the other mother cell-produced components of the σ^K checkpoint, it could provide a check on mother-cell protein synthesis capacity, prior to activation of σ^K and the ensuing final phase of protein synthesis in the mother cell. We propose that the cascade, by sensing SpoIVB from the forespore via cleavage of SpoIVFA, and sensing CtpB from the mother cell via BofA cleavage, ensures the proper timing and rate of pro- σ^K RIP, which is important for efficient sporulation (Cutting *et al.*, 1990; Pan *et al.*, 2003).

Components of the σ^K checkpoint in related bacteria and comparison with other cascades governing RIP

Homologues of the components of the σ^K checkpoint are found in spore-forming bacteria closely related to *B. subtilis*, including *B. licheniformis*, *B. anthracis* (causes anthrax), *B. cereus* (causes food poisoning), *B. thuringiensis*, *B. halodurans*, *B. clausii*, *Geobacillus kaustophilus* and *Oceanobacillus iheyensis* (data not shown), so the mechanism governing RIP of pro- σ^K is likely conserved in close relatives. In contrast, components of the σ^K checkpoint are missing or significantly different in spore-forming bacteria of the genus *Clostridium*. SpoIVFA does not appear to be present in the genome of *Clostridium perfringens*, *C. thermocellum*, *C. acetobutylicum*, or *C. tetani*. SigK homologues are present and appear to have pro sequences (data not shown), but these sequences lack a lysine residue that has been shown to be critical for RIP of *B. subtilis* pro- σ^K (Prince *et al.*, 2005). Likewise, potential BofA homologues can be found (with the exception of *C. tetani*), but lack a histidine residue essential for inhibition of pro- σ^K RIP in *B. subtilis* (Zhou and Kroos, 2004). Potential SpoIVFB and CtpB homologues exist, but these could just as well carry out other functions, because they are about as similar to SpoIVFB or CtpB, as to their functionally distinct paralogues, YluC and YydH (paralogues of SpoIVFB) or CtpA (paralogue of CtpB). It remains to be seen how different the mechanisms regulating σ^K activity are in these *Clostridia*. In *C. difficile*, SigK has no pro sequence, and the production of σ^K is regulated by excision of a prophage-like insertion element in the *sigK* gene (Haraldsen and Sonenshein, 2003).

Most known RIP events involve a proteolytic cascade in which a substrate is first cleaved in a cytosolic or lumenal/ extracellular domain before the I-CLiP can cleave the substrate in a transmembrane segment. For example, S2P, the human homologue of SpoIVFB, cleaves

SREBPs and ATF6 only after initial cleavage by site-1 protease, a serine protease (reviewed in Brown *et al.*, 2000). Similarly, S2P family member RseP (formerly YaeL) of *E. coli* catalyses intramembrane proteolysis of RseA only after serine protease DegS cleaves RseA's periplasmic domain (Alba *et al.*, 2002; Kanehara *et al.*, 2002). This theme extends beyond the S2P family. I-CLiPs in the presenilin and signal peptide peptidase families of aspartyl proteases also require prior cleavage of their substrates by another protease (reviewed in Wolfe and Kopan, 2004). In contrast, the Rhomboid family of serine protease I-CLiPs appear to function independently, cleaving their substrates without a prior cleavage. Rhomboid regulation seems to occur mainly at the level of translocation of its substrate from the endoplasmic reticulum to the Golgi (Lee *et al.*, 2001). SpoIVFB is unique among S2P family members in that there is no evidence to suggest that pro- σ^K is cleaved before RIP by SpoIVFB. Yet, our results provide evidence that regulation of SpoIVFB activity, like that of other S2P family members, is achieved by a proteolytic cascade involving serine proteases. However, in the case of SpoIVFB, our model proposes that the cascade involves two serine proteases from different cell types that target a localization/assembly factor (SpoIVFA) and an I-CLiP inhibitor (BofA), rather than targeting the I-CLiP's substrate (pro- σ^K). This elaborate mechanism governing SpoIVFB I-CLiP activity would allow the forespore and mother cell to communicate about the decision to activate pro- σ^K . Previously, RIP has been shown to initiate inter-cellular communication by generating a signal (reviewed in Brown *et al.*, 2000; Wolfe and Kopan, 2004). Our results provide new insight into how intercellular signalling can culminate in RIP of a transcription factor. It will be interesting to see whether intercellular signalling mechanisms involving proteolytic cascades govern RIP in organisms other than spore-forming bacteria closely related to *B. subtilis*.

Experimental procedures

General methods

The plasmids used in this study are described briefly in Table 1. A complete description of plasmid construction and polymerase chain reaction (PCR) primers is available in Tables S1 and S2. DNA sequencing of all cloned PCR products and all genes subjected to mutagenesis (QuikChange kit, Stratagene) confirmed the presence of the desired sequences. All *B. subtilis* strains were derived from the prototrophic strain PY79 and are listed in Table 2. ZR264, ZR266 and ZR368 were constructed by transformation of BK754, ZR264 and BDR954 with DNA from BDR88, BDR954 and QPB161, respectively, selecting with appropriate antibiotics. Sporulation was induced by resuspending growing cells in SM medium (Harwood and Cutting, 1990). The time of resuspension is considered the onset of sporulation.

Cotransformation of plasmids into *E. coli* and induction of *B. subtilis* proteins

Two plasmids bearing different antibiotic resistance genes and different *B. subtilis* genes fused to a T7 RNA polymerase promoter were cotransformed into *E. coli* BL21(DE3), and expression of T7 RNA polymerase and *B. subtilis* proteins was induced as described previously (Zhou and Kroos, 2004).

Western blot analysis

Equivalent amounts of *E. coli* cells from different cultures were collected and whole-cell extracts were prepared and subjected to Western blot analysis as described previously (Zhou and Kroos, 2004). For *B. subtilis*, samples (1 ml) were collected at the indicated times after the onset of sporulation, cells were collected by centrifugation (12 000 g), and the cell pellet was stored at -80°C . Whole-cell extracts were prepared and subjected to Western blot analysis as described previously (Zhou and Kroos, 2004). Antibodies against pentahistidine (penta-His) (Qiagen), GFP and SpoIVFA (Kroos *et al.*, 2002) and pro- σ^K (Lu *et al.*, 1990), were used at a 1:5000 dilution, except the antipenta-His antibodies were used at a 1:10 000 dilution.

Purification of proteins

To purify the membrane proteins H6SpoIVFA and H6GFP Δ 27BofA for use as substrates, *E. coli* from cultures (4 l) induced with isopropyl β -D-thiogalactopyranoside (IPTG) as described previously (Zhou and Kroos, 2004) were harvested by centrifugation and resuspended in 40 ml phosphate buffered saline (PBS) pH 7.2 (Invitrogen) containing 1 mM Pefabloc, 10 μ g ml⁻¹ DNase I and 10 μ g ml⁻¹ RNase A, then passed twice through a French pressure cell at 14 000 lb in⁻². The lysate was centrifuged at 4°C (12 000 g, 10 min). The supernatant was further centrifuged at 4°C for 1.5 h at 200 000 g in a Ti 42.1 rotor (Beckman). The pellet, representing a crude membrane fraction, was solubilized with 6 ml of 1% digitonin buffer (PBS pH 7.2, 150 mM NaCl, 10% glycerol, 1 mM Pefabloc) by rotating for 2 h at 4°C. The sample was centrifuged at 4°C for 1 h at 100 000 g in a Ti 42.1 rotor (Beckman). The supernatant was incubated with cobalt resin (Clontech) and purification of H6-tagged proteins was performed according to the manufacturer's instructions except elution was with 0.1% digitonin buffer (PBS pH 7.2, 200 mM imidazole, 150 mM NaCl). Purified proteins were dialysed in 0.05% digitonin buffer (PBS pH 7.2, 50 mM NaCl) at 4°C for 6 h before use as substrates for *in vitro* proteolysis.

To purify CtpB-H6, *E. coli* from a culture (2 l) induced with IPTG as described previously (Zhou and Kroos, 2004) were harvested by centrifugation and resuspended in 30 ml PBS pH 7.2 containing 1 mM Pefabloc, 10 μ g ml⁻¹ DNase I and 10 μ g ml⁻¹ RNase A, then lysed as described above. The lysate was centrifuged at 4°C for 1.5 h at 200 000 g in a Ti 42.1 rotor (Beckman). The supernatant was incubated with cobalt resin (Clontech) and purification of H6-tagged proteins was performed according to the manufacturer's instructions. The purified protein was dialysed in PBS pH 7.2 containing 50 mM NaCl at 4°C for 6 h prior to use in assays for *in vitro* proteolysis.

Pulse-chase and immunoprecipitation

Pulse-labelling of cells was performed as described previously (Zhang and Kroos, 1997). The chase involved addition of a 2000-fold molar excess of unlabelled methionine. Immunoprecipitation was performed as described previously (Kroos *et al.*, 2002). Polyclonal anti-SpoIVFA antibodies (6 μ l) or anti-GFP antibodies (14 μ l) were used and these amounts were sufficient to quantitatively precipitate SpoIVFA or GFP Δ 27BofA, respectively, from 1 ml culture lysates in control experiments. Immunoprecipitates were separated on sodium dodecyl sulphate – 14% Prosieve polyacrylamide gels (FMC) with Tris-Tricine electrode buffer (0.1 M Tris, 0.1 M Tricine, 0.1% SDS). SpoIVFA or GFP Δ 27BofA bands were visualized by fluorography with En³Hance (NEN) as enhancing fluors and quantified with a Storm 820 PhosphorImager (Molecular Dynamics), with the background of each lane subtracted from the band intensity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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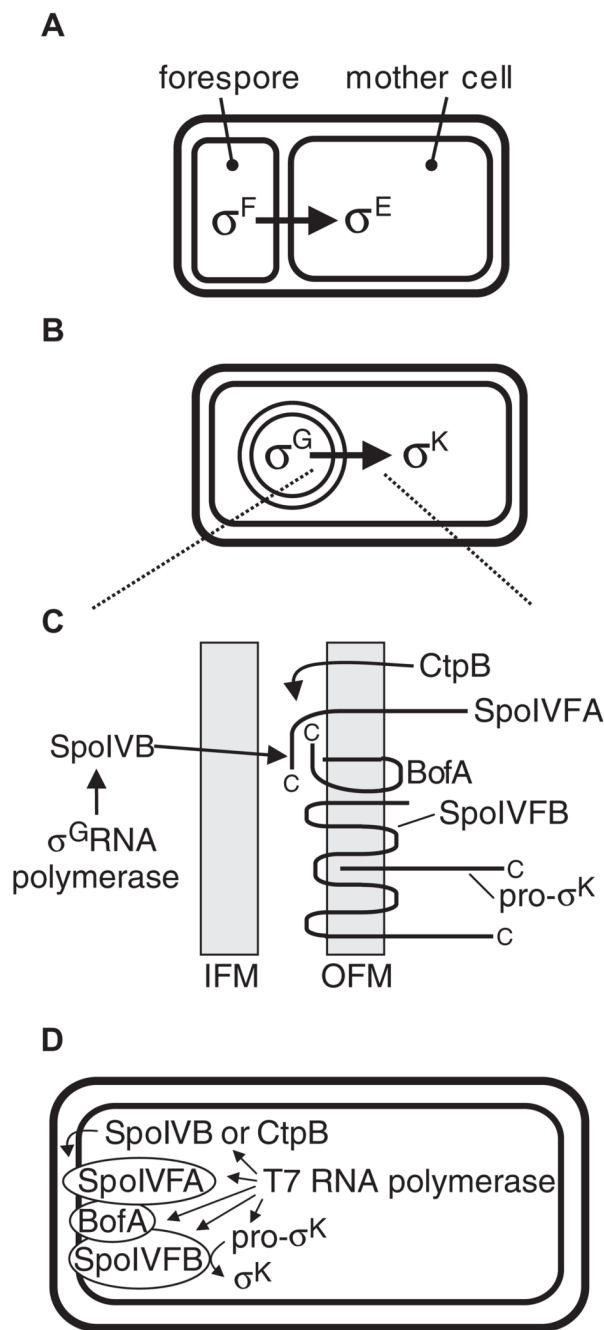


Fig. 1. Morphological changes and cell–cell signalling pathways during *B. subtilis* sporulation, and reconstruction of the σ^K checkpoint in *Escherichia coli*

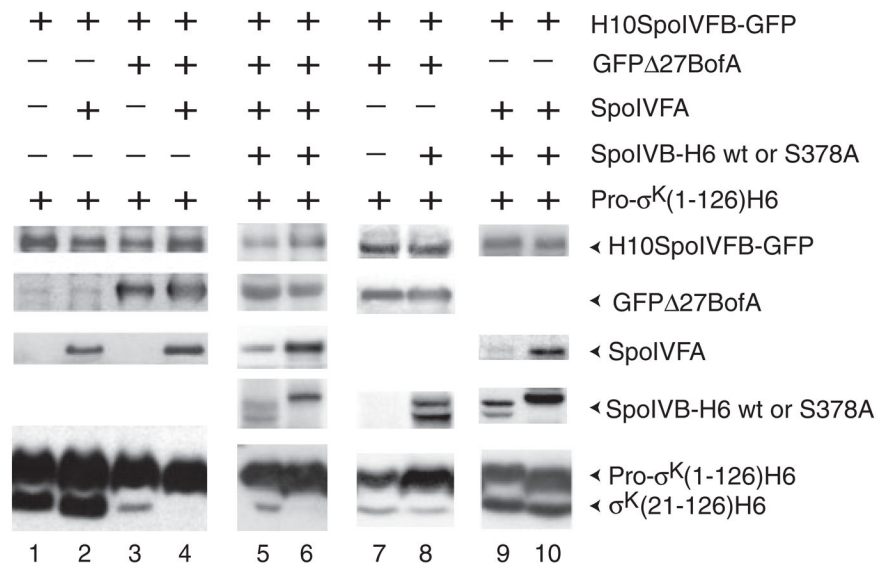
A. After the sporulation septum forms, σ^F RNA polymerase activity in the forespore generates a signal that leads to activation of σ^E in the mother cell.

B. Later during sporulation, the forespore is engulfed by the mother cell, σ^G becomes active in the forespore, and this signals activation of σ^K in the mother cell.

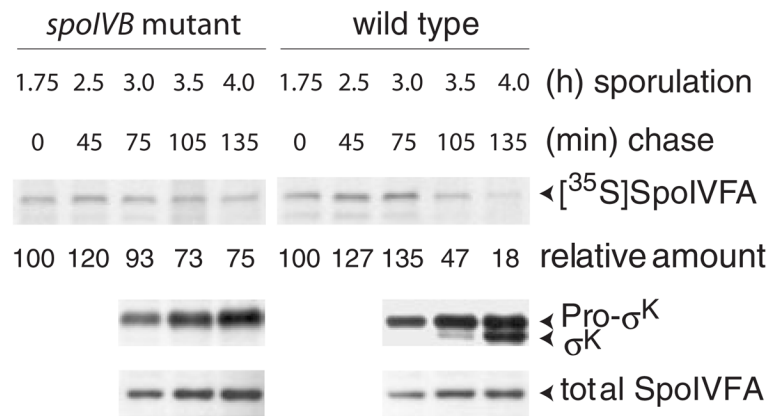
C. Expanded view of the σ^K checkpoint signalling pathway. See text for explanation. IFM, inner fore-spore membrane; OFM, outer fore-spore membrane. Proteins depicted in the OFM are labelled at their C-terminus and their topologies are based on analysis of *lacZ* and *phoA* fusions in *E. coli* (Varcamonti *et al.*, 1997; Green and Cutting, 2000), with the exception of

pro- σ^K , for which analysis of *gfp* fusions in *B. subtilis* suggests that its N-terminal prosequence somehow mediates membrane association (Zhang *et al.*, 1998; Prince *et al.*, 2005). A–C are adapted from the study by Prince *et al.* (2005).

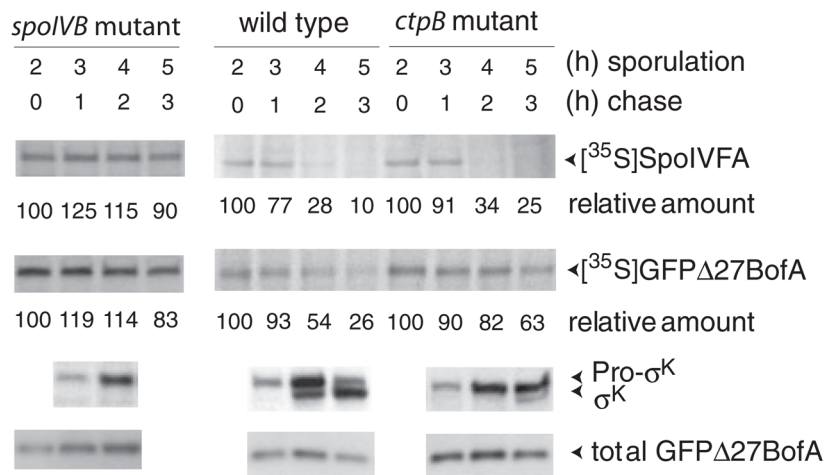
D. Components of the σ^K checkpoint can be expressed from plasmids in *E. coli* by creating fusions to a T7 RNA polymerase promoter. SpoIVFA, BofA and SpoIVFB appear to insert into the inner membrane with the topologies depicted in C (Varcamonti *et al.*, 1997; Green and Cutting, 2000) (i.e. with the *E. coli* cytosol equivalent to the *B. subtilis* mother cell cytosol and the *E. coli* periplasmic space equivalent to the space between the *B. subtilis* OFM and IFM). Pro- σ^K is predominantly membrane-associated (presumably with the inner membrane) when expressed in *E. coli* (Zhang *et al.*, 1998; Zhou and Kroos, 2004). A SpoIVB-PhoA fusion protein appears to be translocated across the inner membrane to the periplasmic space (Wakeley *et al.*, 2000) and the same localization is expected for CtpB, based on its predicted N-terminal secretion signal (Pan *et al.*, 2003).

**Fig. 2.**

The combination of BofA and SpoIVFA completely inhibits RIP of pro- σ^K in *E. coli*, and SpoIVB partially restores RIP and can destabilize SpoIVFA. Samples were collected 2 h after IPTG induction of the indicated proteins in *E. coli* bearing pZR12 and pZR13 (lane 1), pZR12 and pZR33 (lane 2), pZR12 and pZR67 (lane 3), pZR12 and pZR69 (lane 4), pZR57 and pZR69 (lane 5), pZR71 and pZR69 (lane 6), pZR2 and pZR63 (lane 7), pZR57 and pZR67 (lane 8), pZR33 and pZR57 (lane 9), or pZR33 and pZR71 (lane 10). Whole-cell extracts were subjected to Western blot analysis using antibodies against GFP to detect H10SpoIVFB-GFP and GFP Δ 27BofA, antibodies against SpoIVFA to detect SpoIVFA, and antibodies against penta-His to detect SpoIVB-H6, SpoIVB-H6 S378A, pro- $\sigma^K(1-126)$ H6 and $\sigma^K(21-126)$ H6.

**Fig. 3.**

Loss of SpoIVFA coincides with onset of pro- σ^K RIP during *B. subtilis* sporulation. Wild-type *B. subtilis* PY79 and *spoIVB* mutant LK1 were induced to sporulate by resuspending growing cells in SM medium, pulse-labelled for 5 min with [³⁵S]methionine at 1.75 h after sporulation induction, and chased with a 2000-fold molar excess of unlabelled methionine for the indicated time. Samples were immunoprecipitated with antibodies against SpoIVFA, subjected to SDS-PAGE, visualized by fluorography, and labelled SpoIVFA was quantified (numbers below fluorograms indicate the per cent relative to the amount at the beginning of the chase for each strain). Samples were also collected at the indicated times for preparation of whole-cell extracts that were subjected to Western blot analysis with antibodies against pro- σ^K and SpoIVFA.

**Fig. 4.**

Loss of GFPΔ27BofA correlates with onset of pro-σ^K RIP in wild-type *B. subtilis* and both are delayed in a *ctpB* mutant. *B. subtilis* BDR954, ZR266 and ZR368 express GFPΔ27BofA but not native BofA in otherwise wild-type, *spoIVB* mutant, and *ctpB* mutant backgrounds respectively. These strains were induced to sporulate by resuspending growing cells in SM medium, pulse-labelled for 5 min with [³⁵S]methionine at 2 h after sporulation induction, and chased with a 2000-fold molar excess of unlabelled methionine for the indicated time. Samples were immunoprecipitated with antibodies against SpoIVFA or GFP (to immunoprecipitate GFPΔ27BofA), subjected to SDS-PAGE, visualized by fluorography, and labelled proteins were quantified (numbers below fluorograms indicate the per cent relative to the amount at the beginning of the chase for each strain). Samples were also collected at the indicated times for preparation of whole-cell extracts that were subjected to Western blot analysis with antibodies against pro-σ^K and GFP (to detect total GFPΔ27BofA).

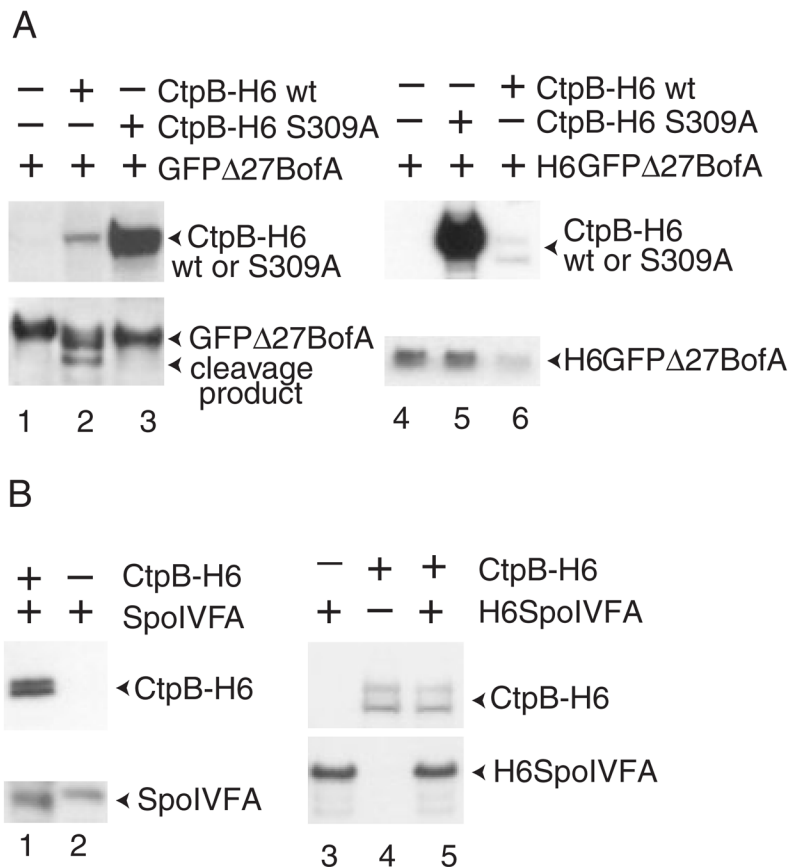


Fig. 5. Serine protease CtpB causes BofA cleavage in *E. coli* and purified CtpB degrades H6GFP Δ 27BofA *in vitro*

A. Effect of CtpB on GFP Δ 27BofA in *E. coli* and *in vitro*. Samples were collected 2 h after IPTG induction of the indicated proteins in *E. coli* bearing pZR62 alone (lane 1), pZR62 and pZR114 (lane 2), or pZR62 and pZR119 (lane 3). Whole-cell extracts were subjected to Western blot analysis using antibodies against penta-His to detect CtpB-H6 and antibodies against GFP to detect GFP Δ 27BofA. For *in vitro* analysis, purified H6GFP Δ 27BofA was incubated at 37°C for 2 h alone (lane 4) or in combination with purified CtpB-H6 S309A mutant enzyme (lane 5) or wild-type CtpB-H6 (lane 6). After incubation, Western blot analysis was performed using antibodies against penta-His.

B. Effect of CtpB on SpoIVFA in *E. coli* and *in vitro*. Samples were collected 2 h after IPTG induction of the indicated proteins in *E. coli* bearing pZR73 and pZR114 (lane 1) or pZR73 alone (lane 2). Whole-cell extracts were subjected to Western blot analysis using antibodies against penta-His to detect CtpB-H6 and antibodies against SpoIVFA to detect SpoIVFA. For *in vitro* analysis, the indicated purified proteins were incubated at 37°C for 2 h (lanes 3–5). After incubation, Western blot analysis was performed using antibodies against penta-His to detect CtpB-H6 and antibodies against SpoIVFA to detect H6SpoIVFA.

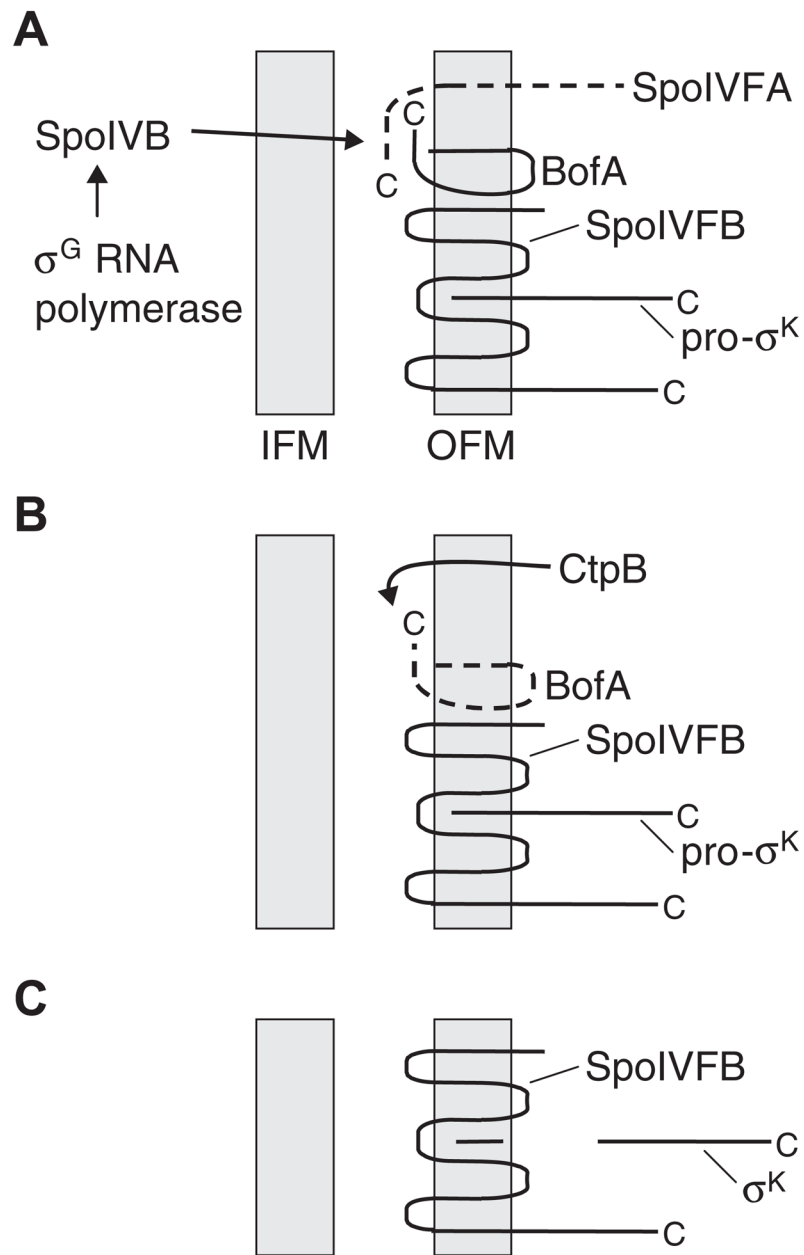


Fig. 6. Model for events governing RIP of pro- σ^K

A. SpoIVB from the forespore initiates a proteolytic cascade by cleaving SpoIVFA, leading to its loss from complexes with BofA and SpoIVFB.

B. In the second step, CtpB from the mother cell normally cleaves BofA near its C-terminus, leading to its loss from complexes with SpoIVFB. In a *ctpB* null mutant, another protease(s) causes loss of BofA, albeit less efficiently.

C. Free SpoIVFB performs intramembrane cleavage of pro- σ^K , releasing σ^K into the mother cell to direct transcription. See the Fig. 1 legend for additional explanation.

Table 1

Plasmids used in this study.

Plasmid	Description
pZR2	Ap ^R , T7-H10SpoIVFB-GFP ^a
pZR12	Km ^R , T7-pro-σ ^K (1-126)H6
pZR13	Ap ^R , T7-H10SpoIVFB-GFP
pZR33	Ap ^R , T7-H10SpoIVFB-GFP/SpoIVFA
pZR42	Ap ^R , T7-H10SpoIVFB-GFP/T7-BofA/SpoIVFA
pZR50	Km ^R , T7-pro-σ ^K (1-126)H6/T7-SpoIVB
pZR53	Ap ^R , T7-SpoIVB-H6
pZR57	Km ^R , T7-pro-σ ^K (1-126)H6/T7-SpoIVB-H6
pZR62	Ap ^R , T7-GFPΔ27BofA
pZR63	Km ^R , T7-pro-σ ^K (1-126)H6/T7-GFPΔ27BofA
pZR67	Ap ^R , T7-H10SpoIVFB-GFP/T7-GFPΔ27BofA
pZR69	Ap ^R , T7-H10SpoIVFB-GFP/T7-GFPΔ27BofA/SpoIVFA
pZR71	Km ^R , T7-pro-σ ^K (1-126)H6/T7-SpoIVB-H6 S378A
pZR73	Ap ^R , T7-SpoIVFA
pZR74	Km ^R , T7-H6SpoIVFA
pZR114	Km ^R , T7-CtpB-H6
pZR115	Km ^R , T7-H6GFPΔ27BofA
pZR119	Km ^R , T7-CtpB-H6 S309A

^aThe T7 RNA polymerase promoter (T7) plus the optimal *E. coli* translation initiation sequence and DNA coding for 10 histidine (H10) residues in pET-16b was fused to the *B. subtilis* gene *spoIVFB*, which had been fused to a gene coding for the green fluorescent protein (GFP). The descriptions of other plasmids are abbreviated in a similar fashion and details of their construction are in Tables S1 and S2 or have been described previously (Zhou and Kroos, 2004).

Ap^R, ampicillin-resistant; Km^R, kanamycin-resistant.

Table 2*Bacillus subtilis* strains used in this study.

Strain	Relevant characteristics	Reference or construction
PY79	Spo ⁺ prototroph	Youngman <i>et al.</i> (1984)
LK1	<i>spoIVBA::spc</i>	Kroos <i>et al.</i> (2002)
BK754	<i>spoIVB165</i>	Cutting <i>et al.</i> (1991a)
BDR88	<i>bofA::erm</i>	Rudner and Losick (2002)
BDR954	<i>bofA::erm, amyE::PspoIID-gfp(mut.2)-Δ27bofA (spc)</i>	Rudner and Losick (2002)
QPB161	<i>ctpB::tet</i>	Pan <i>et al.</i> (2003)
ZR264	<i>spoIVB165 bofA::erm</i>	This study
ZR266	<i>spoIVB165 bofA::erm, amyE::PspoIID-gfp(mut.2)-Δ27bofA (spc)</i>	This study
ZR368	<i>ctpB::tet, bofA::erm, amyE::PspoIID-gfp(mut.2)-Δ27bofA (spc)</i>	This study