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Perturbations to engulfment trigger a degradative response that prevents cell–cell signalling during sporulation in *Bacillus subtilis*

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Summary

During sporulation in *Bacillus subtilis*, the mother cell membranes migrate around the forespore in a phagocytic-like process called engulfment. Developmental gene expression requires the successful completion of this key morphological event. Here we show that perturbations to engulfment block the accumulation of proteins secreted into the space between the mother cell and forespore membranes. Our data support a model in which engulfment defects cause the proteolytic clearance of these secreted proteins. Importantly, we show that this degradative response is reversible; once proper engulfment is restored, secreted proteins again accumulate. In particular, we have found that the forespore signalling protein SpoIVB fails to accumulate when engulfment is impaired and, as a result, late mother cell gene expression under the control of σ^K is blocked. If engulfment is restored, SpoIVB accumulates and cell–cell signalling resumes. Thus, this degradative pathway functions like a developmental checkpoint ensuring that mother cell gene expression does not commence unless morphogenesis proceeds normally.

Introduction

During the course of cellular differentiation, the activation of gene expression is linked to the successful completion of key morphological events (Rudner and Losick, 2001). The surveillance mechanisms that couple gene expression to morphogenesis are referred to as developmental checkpoints. In principal, these surveillance mechanisms can monitor the completion of a morphological event or defects that arise during the morphological process itself. In other words, the point being checked by the cell can be during the event or upon its completion. In either case, if morphogenesis is perturbed, these control mechanisms prevent activation of gene expression and thus halt the developmental programme. Here we are concerned with the morphological process of engulfment during sporulation in the bacterium *Bacillus subtilis*. We have identified a degradative response akin to a developmental checkpoint that couples late gene expression to this landmark morphological event.

In response to nutrient limitation, *B. subtilis* differentiates into a dormant stress-resistant spore (Stragier and Losick, 1996; Piggot and Losick, 2002). Upon entry into sporulation, the developing cell divides asymmetrically generating two cells of unequal size and dissimilar developmental fates: a small cell (the prospective spore) referred to as the forespore and a large cell called the mother cell. At first, these two cells lie side-by-side but shortly after polar division the mother cell membranes migrate around the forespore in a phagocytic-like process called engulfment. As a result of this morphological process, the forespore ends up as a free protoplast inside the mother cell surrounded by two membranes: its own, called the inner forespore membrane and a second membrane derived from the mother cell, called the outer forespore membrane. Together, these two membranes define an intermembrane space. After the completion of engulfment, a thick layer of peptidoglycan is assembled in the intermembrane space and the mother cell lays down a protective protein coat on the outside of the forespore. Once the spore has fully matured the mother cell lyses releasing it into the environment.

During this elaborate developmental process, the mother cell and forespore follow completely different programmes of gene expression but these programmes are linked through signal transduction pathways to ensure that gene expression in one cell is kept in register with gene expression in the other (Losick and Stragier, 1992; Stragier and Losick, 1996; Rudner and Losick, 2001). In particular, the forespore-specific transcription factor σ^F is required for the activation of the σ^E transcription factor in the mother cell. In turn, σ^E is required for the activation of σ^G in the forespore. Finally, σ^G is required for the activation of σ^K in the mother cell. Here we describe a degradative response that prevents the activation of developmental

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gene expression under the control of σ^K when the morphological process of engulfment is impaired. The discovery of this response arose from our analysis of the cell–cell signalling pathway that results in the activation of σ^K in the mother cell under the control of σ^G in the forespore.

σ^K is synthesized as an inactive membrane-associated precursor protein known as pro-σ^K and is activated by proteolysis and release from the membrane (Kroos *et al*., 1989; Zhang *et al*., 1998). The protease responsible for the activation of σ^K is the mother cell membrane protein SpoIVFB (referred to as B) (Lu *et al*., 1995; Rudner *et al*., 1999). B is held inactive by two other mother cell membrane proteins SpoIVFA (referred to as A) and BofA (Cutting *et al*., 1991a; Ricca *et al*., 1992). These three proteins reside in a complex that localizes to the mother cell membrane that surrounds the forespore (see Fig. 5A) (Resnekov *et al*., 1996; Rudner and Losick, 2002). The signalling protein SpoIVB (called IVB and not to be confused with the pro-σ^K processing enzyme B) is itself a protease (Hoa *et al*., 2002). It is made in the forespore under the control of σ^G (Cutting *et al*., 1991b; Gomez and Cutting, 1996) and is secreted into the intermembrane space where it triggers pro-σ^K processing by cleaving A (Dong and Cutting, 2003; Campo and Rudner, 2006). We have previously shown that the proper localization of the σ^K signalling complex requires a network of protein–protein interactions along and across the septal membrane (Doan *et al*., 2005). In particular, the mother cell proteins SpoIID (IID), SpoIIM (IIM), SpoIIP (IIP) and SpoIIIAH (IIIAH) and the forespore protein SpoIIQ (IIQ) are all required for the proper localization of this complex (Blaylock *et al*., 2004; Doan *et al*., 2005; Jiang *et al*., 2005). We wondered whether these proteins might play a direct regulatory role in σ^K activation beyond localizing the signalling complex. As all of these proteins act early in the sporulation pathway (IID, IIM, IIP and IIQ are all required for proper engulfment while IIIAH is required for σ^G activation) (Smith *et al*., 1993; Frandsen and Stragier, 1995; Kellner *et al*., 1996; Londono-Vallejo *et al*., 1997; Abanes-De Mello *et al*., 2002), a role in σ^K signalling (a later event) might have been missed.

Surprisingly, our analysis of these 'anchoring proteins' revealed that perturbations to engulfment trigger the degradation of proteins secreted into the intermembrane space. In particular, we have found that the signalling protein IVB fails to accumulate when engulfment is impaired, and as a result, σ^K is not activated. Importantly, this degradative response is reversible and, once proper engulfment is restored, secreted proteins again accumulate and σ^K is activated. Our results are most consistent with a model in which transient impairments to engulfment lead to a degradative response that halts cell–cell signalling and the progression of sporulation until engulfment is restored to normal.

Results

The IVB signalling protein fails to accumulate when engulfment is impaired

We have shown previously that the sporulation proteins IIQ, IID, IIM, IIP and IIIAH are required for the proper localization of the σ^K signalling complex to the mother cell membranes that surround the forespore (Doan *et al*., 2005). We wanted to explore whether these proteins play a more active role in regulating pro-σ^K processing beyond passively anchoring the signalling complex in the outer forespore membrane. Mutations in the genes encoding these proteins prevent the activation of the forespore transcription factor σ^G (Partridge and Errington, 1993; Smith *et al*., 1993; Frandsen and Stragier, 1995; Kellner *et al*., 1996; Londono-Vallejo *et al*., 1997). In the absence of σ^G, the forespore signalling protein IVB is not synthesized to high levels and pro-σ^K is not processed (Fig. 1A) (Gomez and Cutting, 1996). Thus, in order to determine whether mutants in these proteins play a direct role in σ^K activation, we needed to bypass the requirement for σ^G in this signalling pathway.

At an early stage in sporulation, IVB is synthesized at a low level under the control of the early acting forespore transcription factor σ^F (Fig. 1A) (Gomez and Cutting, 1996). The high levels of IVB that are needed to trigger pro-σ^K processing are only achieved at later times under the control of σ^G (Fig. 1A). The groups of Stragier and Cutting have previously reported that the requirement for σ^G can be bypassed in this signalling pathway if IVB is synthesized at high levels under σ^F control (Gomez *et al*., 1995). Accordingly, we fused the gene encoding IVB to a strong σ^F-dependent promoter (P_{IIQ}) and inserted it at a nonessential locus in a σ^G mutant strain. This promoter fusion resulted in high levels of IVB in the absence of σ^G (Fig. 1A). IVB undergoes autoproteolysis after translocation into the intermembrane space and these autoproteolytic products are hypothesized to be the active species (Wakeley *et al*., 2000). Synthesis of the signalling protease under the control of σ^F resulted in a similar (although not identical) autoproteolytic profile (Fig. 1A). Importantly, these proteolytic products efficiently triggered pro-σ^K processing in the absence of σ^G (Fig. 1A). We observed a small delay in the conversion of pro-σ^K to its mature form in the bypass strain compared with wild type (Fig. 1A). However, a similar delay in σ^K activity was reported in the original bypass experiments (Gomez *et al*., 1995).

We used the σ^G bypass strain to investigate whether mutants in the proteins required to anchor the σ^K signalling complex impact pro-σ^K processing. Conversion of pro-σ^K to its mature form was monitored in a sporulation time-course by immunoblot. Surprisingly, in the absence of IIQ, IID, IIM or IIP, pro-σ^K processing was completely

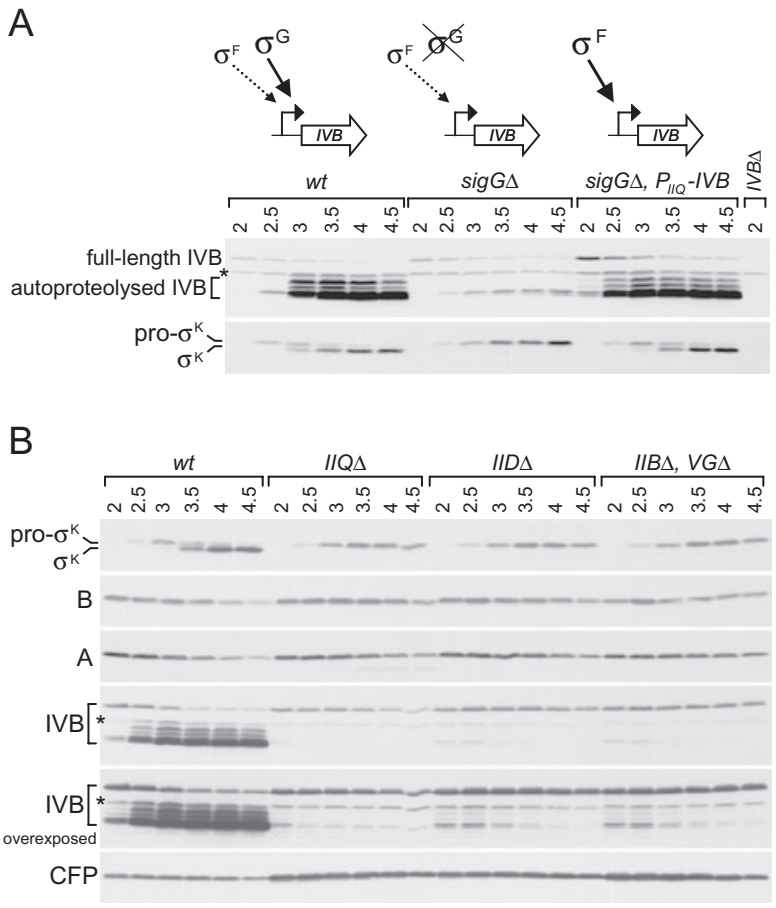

Fig. 1. Autoproteolysed IVB fails to accumulate when engulfment is impaired. Immunoblot analysis of whole cell lysates from sporulating cells. Cells were induced to sporulate by resuspension and samples were collected at indicated times (in hours).
A. IVB levels and pro-σ^K processing in wild-type (PY79), a *sigG* null (BDR104) and the *sigG*Δ bypass strain in which the *IVB* gene was fused to the σ^F-responsive promoter P_{spoIIQ} (BTD409).
B. Comparison of pro-σ^K processing and the levels of A, B, IVB and CFP in the *sigG*Δ bypass strain (BTD1017) and three mutants in which engulfment is impaired: *IIQ*Δ (BTD1053), *IID*Δ (BTD1057) and *IIB*Δ, *VG*Δ (BTD2019). The genes encoding IVB and CFP were fused to the identical σ^F-responsive promoter P_{spoIIQ}. A non-specific band recognized by the anti-IVB antibody is indicated by an asterisk.

blocked (Fig. 1B and data not shown). These results cannot be entirely explained by the mislocalization of the σ^K signalling complex because the absence of IID, IIM or IIP only mildly affects its localization (Doan *et al*., 2005). We therefore wondered whether the block to pro-σ^K processing in the absence of IIQ, IID, IIM or IIP could have resulted from instability of the processing enzyme B and/or the regulatory protein A. To investigate this possibility, we monitored the levels of these proteins by immunoblot in the same sporulation time-course. In all mutant backgrounds tested, the levels of A and B were similar to wild type (Fig. 1B and data not shown). Next, we investigated whether accumulation of the IVB signalling protein might be affected in these mutants. Remarkably, in the absence of any of the four 'anchoring proteins', full-length IVB was made but the autoproteolytic cleavage products failed to accumulate (Fig. 1B and data not shown). This result was surprising because we anticipated that the proteins that anchor the signalling complex would act directly on the components of this complex. Instead, our data indicate that the absence of IIQ, IID, IIM or IIP impact the accumulation of the secreted signalling protein IVB.

All four of these proteins are involved in the morphological process of engulfment (Smith *et al*., 1993; Frandsen and Stragier, 1995; Londono-Vallejo *et al*., 1997; Abanes-De Mello *et al*., 2002). We therefore hypothesized that the effect of these mutants on IVB accumulation was due to perturbations to engulfment and that the block in pro-σ^K processing was not due to the mislocalization of the signalling complex. To investigate this, we analysed IVB levels in an engulfment mutant (a *spoIIB*Δ, *spoVG*Δ double mutant) that does not affect the localization of the signalling complex (Margolis *et al*., 1993; Doan *et al*., 2005). IVB autoproteolytic products also failed to accumulate in this mutant and pro-σ^K processing was completely blocked (Fig. 1B). All together, these results are consistent with the idea that when the morphological process of engulfment is impaired, autoproteolysed IVB does not accumulate.

To investigate whether the dramatic reduction in IVB levels was due to a defect in transcription of *IVB*, we fused the gene encoding CFP to the identical P_{IIQ} promoter and monitored its accumulation in the absence of IIQ or IID. The level of CFP in these engulfment mutants was similar to or slightly higher than wild type (Fig. 1B). Thus, the failure to accumulate IVB when engulfment is impaired cannot be explained by reduced transcription from the P_{IIQ} promoter. Examination of a longer exposure of the anti-

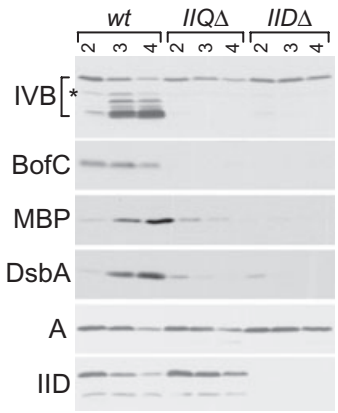

Fig. 2. All secreted proteins fail to accumulate when engulfment is perturbed. Immunoblot analysis of whole cell lysates from sporulating cells. Cells were induced to sporulate by resuspension and samples were collected at indicated times (in hours). The levels of four secreted proteins (IVB, BofC, MBP and DsbA) and two integral membrane proteins (A and IID) were monitored. The levels of these proteins were compared in the *sigG*Δ bypass strain (wt) and two mutants (*IIQ*Δ and *IID*Δ) in which engulfment is perturbed. A non-specific band recognized by the anti-IVB antibody is indicated by an asterisk.

IVB immunoblot (Fig. 1B) revealed small amounts of autoproteolysed IVB at early time points that disappeared as sporulation progressed. This result supports a model in which perturbations to engulfment leads to degradation of the autoproteolysed IVB products in the intermembrane space (see below).

All secreted proteins fail to accumulate when engulfment is impaired

We wondered whether engulfment defects specifically block the accumulation of the signalling protein IVB (and the subsequent activation of σ^K) or if impaired engulfment causes a more general defect in the accumulation of proteins in the intermembrane space. To address this, we monitored several other secreted proteins in the absence of IIQ or IID. The genes encoding these secreted proteins were placed under the control of the σ^F-dependent P_{IIQ} promoter and inserted into the *sigG*Δ bypass strain. First, we tested the accumulation of BofC, a secreted sporulation protein that is normally made in the forespore under the control of σ^F and σ^G (Gomez and Cutting, 1997). BofC was easily detectable in wild type sporulating cells but was undetectable when engulfment was impaired (Fig. 2). Next we analysed the accumulation of two secreted proteins completely unrelated to sporulation. For this, we used two well-characterized secreted proteins from *Escherichia coli*: the maltose binding protein (MBP) (Bassford, 1990; Boos and Shuman, 1998) and DsbA, a protein involved in disulphide bond formation in the periplasm (Bardwell *et al*., 1991). To ensure that these proteins would be efficiently secreted in *B. subtilis*, we fused a *B. subtilis* signal sequence (from AmyE) to the mature form of these proteins (see *Experimental procedures*). These fusion proteins were efficiently secreted because only a small amount of full-length protein containing its signal sequence (with an apparent molecular mass greater than the mature protein) was detected using anti-MBP or anti-DsbA antibodies (data not shown). In the wild-type background, the secreted forms of both MBP and DsbA accumulated during sporulation (Fig. 2). By contrast, in the absence of IIQ or IID, the levels of MBP and DsbA were much lower (Fig. 2). In both cases, a small amount of protein was detectable at early times but disappeared as sporulation continued. We analysed two additional secreted proteins (the ribose binding protein from *E. coli* and CtpB from *B. subtilis*) and both also failed to accumulate when engulfment was impaired (data not shown).

All together, these results indicate that perturbations to engulfment do not specifically affect the accumulation of the IVB signalling protein. In fact, defects to engulfment appear to elicit a more general response that blocks the accumulation of all proteins secreted into the intermembrane space. This response seems to be restricted to secreted proteins because integral membrane proteins with large extracellular domains that reside in the intermembrane space (IID, A and IIQ) were not affected when engulfment was impaired (Fig. 2 and data not shown).

The inability to accumulate secreted proteins is not due to leakage into the medium

We considered two possible explanations for the failure to accumulate secreted proteins in the intermembrane space when engulfment was impaired: (i) secreted proteins were specifically targeted for degradation or (ii) proteins that were secreted into the space between the forespore and mother cell membranes were able to leak out into the cultured medium. Indeed, it is not known whether this space is sealed off from the environment, even during normal engulfment. The second model predicts that proteins normally resident in the intermembrane space should be found in the cell supernatant in the engulfment mutants. To test this, we analysed IVB accumulation in the medium during sporulation. At hour 3 of sporulation cell supernatants were collected; concentrated by TCA precipitation; and analysed by immunoblot. Under all conditions tested IVB was undetectable in the medium (data not shown). This result suggests that secreted proteins do not leak out from the intermembrane space when engulfment is impaired. However, as we were unable to detect any IVB protein, it was possible that the IVB that leaked into the medium was degraded by extracellular proteases. To address this concern, we analysed *E. coli* MBP. Equivalent amounts of cell lysate and cell

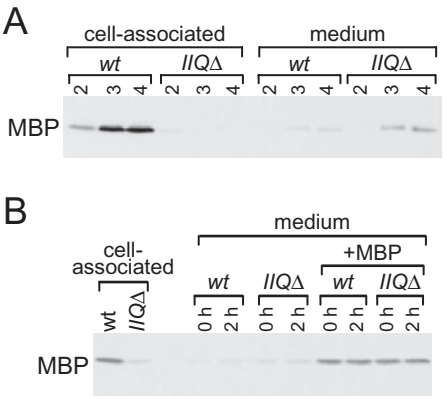

Fig. 3. Secreted proteins do not leak into the medium when engulfment is impaired. Immunoblot analysis of whole cell lysates from sporulating cells. Cells were induced to sporulate by resuspension and samples were collected at indicated times (in hours).
A. MBP does not leak into the medium. The levels of MBP present in the cell-associated and medium fractions were compared in the *sigG*Δ bypass strain (BTD1209) and a *IIQ*Δ mutant (BTD1221).
B. MBP is stable in the medium fraction. The amount of MBP present in the culture medium at hour 3 of sporulation was monitored before and after a 2 h incubation. Incubations were performed in the presence (+MBP) or absence of exogenously added MBP.

supernatant were separated by SDS-PAGE and MBP was monitored by immunoblot. A small amount of MBP was detected in the cell supernatant in wild type sporulating cultures (Fig. 3A) and this could be due to a small amount of leakage, cell lysis, or secretion directly out of the forespore compartment before the completion of engulfment. However and importantly, in an engulfment mutant, the level of MBP in the medium was only slightly higher than in wild type (Fig. 3A). These results support the idea that there is no significant leakage of proteins from the intermembrane space when engulfment is impaired. As a final control, we analysed the stability of MBP in the cell supernatant. If most but not all of MBP was degraded in the culture medium, this would compromise our interpretation of the above experiment. We analysed the stability of MBP in two ways. First, we monitored the amount of MBP present in the culture medium before and after 2 h of incubation. Second, we added exogenous MBP to the cell supernatant (an amount equivalent to the MBP present in the cell-associated wild-type fraction) and analysed it before and after a 2 h incubation. In both experiments the level of MBP remained unchanged after 2 h (Fig. 3B). Thus, we conclude that MBP is stable in the medium of *B. subtilis* sporulating cells. All together, these results demonstrate that the failure to accumulate secreted proteins when engulfment is impaired is not due to leakage. Instead, our data are most consistent with a model in which perturbations to engulfment cause the degradation of proteins secreted into the space between the mother cell and the forespore membranes. This analysis also argues against a model in which proteins are exclusively secreted out of the forespore and into the medium when engulfment is blocked.

Secreted proteins are efficiently degraded only after release from the membrane

Figure 1B shows that when engulfment is impaired autoproteolysed IVB was efficiently degraded but full-length (unprocessed) IVB appeared unaffected. Fractionation experiments indicate that full-length IVB is membrane associated while the autoproteolysed species are not (Fig. S1) (Hoa *et al*., 2002). Together, these observations support a model in which membrane-anchored IVB is not susceptible to degradation and that IVB is degraded only after it is released from the membrane. This idea is in line with what we have observed for integral membrane proteins, which are stable when engulfment is impaired (Fig. 2). To more rigorously investigate whether or not full-length IVB is degraded when engulfment is impaired we analysed a catalytically inactive IVB mutant (*IVB*^{S378A}) (Wakeley *et al*., 2000). This mutant is incapable of autoproteolysis and thus IVB is not released from the membrane. Under these conditions full-length IVB^{S378A} accumulates at early times during sporulation. As sporulation progresses, IVB^{S378A} is proteolysed due to the action of other proteases in the intermembrane space (Fig. 4A) (Wakeley *et al*., 2000). Consistent with the idea that membrane-anchored IVB is resistant to proteolysis, full-length IVB^{S378A} remained intact while the smaller proteolytic products failed to accumulate in the IIQ and IID mutants (Fig. 4A). These results support the idea that proteins are only degraded upon release from the membrane when engulfment is impaired.

However, it was formally possible that full-length IVB was inefficiently translocated into the intermembrane space in these engulfment mutants and thus accumulated in the forespore cytoplasm. To address this, we analysed the location of full-length IVB using a protease susceptibility assay. *B. subtilis* cells were harvested prior to the completion of engulfment and protoplasted such that translocated proteins that reside in the intermembrane space would be accessible to Trypsin. Virtually all of IVB^{S378A} was susceptible to Trypsin proteolysis whether or not engulfment was impaired (Fig. 4B). As controls, the integral membrane protein YFP-A was also susceptible to Trypsin while the cytoplasmic protein σ^F was fully protected (Fig. 4B). Moreover, all three proteins were efficiently degraded if the integrity of the protoplasts was compromised by the addition of the mild detergent Triton X-100 prior to the addition of Trypsin (Fig. 4B). These results demonstrate that full-length IVB^{S378A} is properly translocated into the intermembrane space. Moreover,

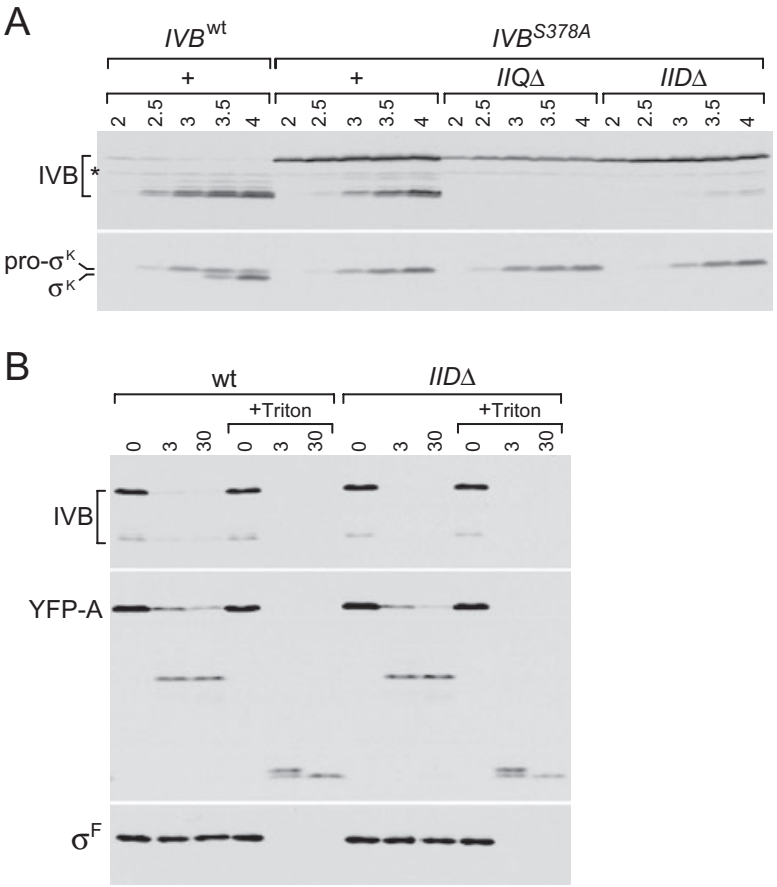

Fig. 4. Secreted proteins are efficiently degraded only after release from the membrane.
A. Immunoblot analysis of whole cell lysates from sporulating cells. Cells were induced to sporulate by resuspension and samples were collected at indicated times (in hours). The levels of full-length (upper band) and proteolysed IVB^{S378A} (a catalytically inactive mutant) were compared in the *sigG*Δ bypass strain (+) (BTD1099) and two engulfment mutants: *IIQ*Δ (BTD1245) and *IID*Δ (BTD1101). The levels of wild-type IVB in the *sigG*Δ bypass strain (*IVB*^{wt}) (BTD489) are shown for comparison. A non-specific band recognized by the anti-IVB antibody is indicated by an asterisk.
B. Full-length IVB^{S378A} is efficiently translocated across the forespore membrane when engulfment is impaired. Immunoblots from protease susceptibility assays. Cells were induced to sporulate by resuspension. At hour 2.2 of sporulation, prior to the completion of engulfment, the sporangia were protoplasted and incubated for indicated times (in minutes) with Trypsin or Trypsin and Triton X-100 (+ Triton). Full-length IVB^{S378A}, YFP-A (an integral membrane protein) and σ^F (a cytoplasmic protein) were compared in the *sigG*Δ bypass strain (wt) (BTD2081) and an engulfment mutant (*IID*Δ) (BTD2089). Both strains contained a *IIIAH* mutation to prevent protoplast engulfment (Broder and Pogliano, 2006) (see Fig. S2).

they suggest that the secretion apparatus is not affected by perturbations to engulfment. We conclude that when engulfment is impaired secreted proteins are only degraded after release from the membrane.

The degradation of IVB is reversible

We wondered whether the degradation of secreted proteins caused by perturbations to engulfment served a protective function for the sporulating cell or if it simply resulted from non-specific proteolysis in cells permanently arrested in the sporulation pathway. We reasoned that if this response is protective then it should be reversible. In other words, a transient impairment to engulfment should trigger the degradation of secreted proteins and these proteins should accumulate once engulfment is restored (Fig. 5A).

To test this hypothesis, we engineered a strain that transiently impairs engulfment by delaying the synthesis of the engulfment protein IID. The gene encoding IID is transcribed in the mother cell at hour 1.5 of sporulation (Clarke *et al*., 1986; Rong *et al*., 1986). To delay *IID* expression, we fused the *IID* gene to a mother cell promoter ($P_{spoIVCB}$) that is turned on at about hour 2.5 of sporulation (Kroos *et al*., 1989; Stragier *et al*., 1989). This promoter fusion resulted in a delay of at least 30 min in the synthesis of IID (Fig. 5B). Importantly, this delay generated a transient block to the process of engulfment. At hour 1.5 of sporulation when IID levels were very low, the polar septa in this strain formed a characteristic bulge that protruded into the mother cell cytoplasm. This engulfment defect was indistinguishable from a IID null strain (Fig. 5C). However, at late time points once IID had accumulated (Fig. 5B), the engulfing septal membranes appeared smooth and the cells completed engulfment in manner similar to wild type. By contrast, the engulfment defect persisted in the IID mutant (Fig. 5C).

Using this strain we monitored the level of IVB to investigate whether the degradation of this secreted signalling protein was reversible. At early time points when IID was present at low levels and engulfment was impaired, autoproteolysed IVB levels were significantly reduced compared with wild type (Fig. 5B). However, once IID was synthesized to high levels and engulfment was restored, IVB began to accumulate. Autoproteolysed IVB never reached wild-type levels in this background; however, the level achieved was sufficient to trigger pro-σ^K processing (Fig. 5B). Importantly, the P_{IIQ}

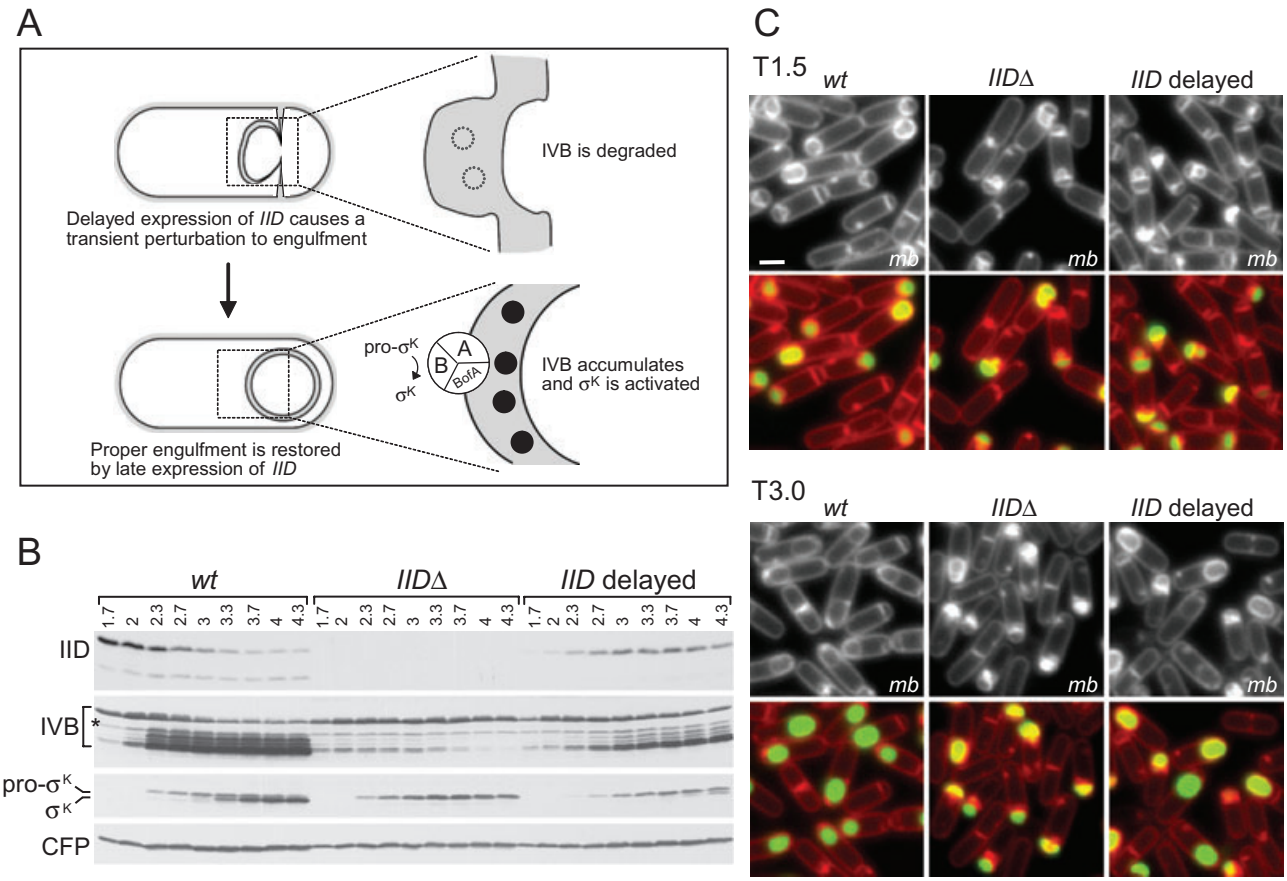

Fig. 5. The degradation of IVB is reversible.
A. Schematic diagram of a transient perturbation to engulfment and the effect on IVB and pro-σ^K processing. A delay in the expression of the *IID* gene generates a transient perturbation to engulfment. This perturbation triggers the degradation of IVB in the intermembrane space. Proper engulfment is restored by late expression of *IID*, resulting in the accumulation of IVB and pro-σ^K processing.
B. Immunoblot analysis of whole cell lysates from sporulating cells. The levels of IID, IVB and CFP and pro-σ^K processing were compared in the *sigG*Δ bypass strain (wt) (BTD1575), a *IID*Δ mutant (BTD1595) and a strain in which *IID* expression is delayed (*IID* delayed) (BTD1597). This strain contains two copies of the *IID* gene fused to the $P_{spoIVCB}$ promoter (see *Experimental procedures*). Cells were induced to sporulate by resuspension and samples were collected at indicated times (in hours). The genes encoding IVB and CFP were fused to the identical σ^F-responsive promoter P_{IIQ}. A non-specific band recognized by the anti-IVB antibody is indicated by an asterisk.
C. Transient and permanent blocks to engulfment. BTD1575 (wt), BTD1595 (*IID*Δ) and BTD1597 (*IID* delayed) were induced to sporulate by resuspension. At indicated times (in hours) the engulfing membranes (*mb*) and σ^F activity (CFP) were visualized by fluorescence microscopy. Scale bar, 1 μm.

promoter driving expression of *IVB* was not affected by this transient block to engulfment because the same promoter fused to *cfp* resulted in levels of CFP that were comparable to controls (Fig. 5B). Consistent with the idea that the degradation of secreted proteins is reversible and protective, this transient block to engulfment had no impact on sporulation efficiency (data not shown). All together, these results support a model in which perturbations to engulfment trigger a general degradative response; this response is reversible and is turned off when engulfment resumes to normal.

Discussion

Here we report that when engulfment is impaired proteins secreted into the space between the mother cell and forespore membranes do not accumulate. In particular, we have found that the signalling protein IVB fails to accumulate when engulfment is defective and, as a result, pro-σ^K processing is blocked. The failure to accumulate secreted proteins is not due to their leakage into the medium. Instead, our data support a model in which perturbations to engulfment lead to their proteolytic degradation. The protease responsible for this degradation appears to target proteins only after their release from the membrane because integral membrane proteins and membrane-tethered proteins are not affected. Finally, our data indicate that this degradative response is reversible. It is triggered by perturbations to engulfment and shut down when proper engulfment is restored. We propose

that this pathway serves a protective function by preventing cell–cell signalling and the activation of developmental gene expression while this critical morphological process is impaired. We envision that a surveillance mechanism exists that monitors the engulfment process itself. If it is perturbed, this degradative response is triggered, halting the activation of late gene expression. It is also possible that proteins secreted into the intermembrane space are constitutively degraded *during* the process of engulfment and the protease responsible is inactivated upon its completion. In this scenario, the completion of engulfment, rather than perturbations to it, would be monitored by the surveillance mechanism. We do not favour this model because in our bypass strain IVB appears to accumulate at a time prior to the completion of engulfment (Fig. 1A and data not shown).

We have hypothesized that this degradative response serves as protection against defects that arise during the normal programme of engulfment and in this way is akin to a developmental checkpoint. However, it is also possible that stimuli from or variations in the environment experienced by the sporulating cell could trigger this degradative response. For example, a sudden change in temperature, osmolarity or pH could impair the process of engulfment. Alternatively, metabolites secreted by other organisms within a biofilm in which *B. subtilis* is sporulating could impair this morphological process. It has been reported that extreme changes in osmolarity or antibiotics like penicillin block sporulation prior to the activation of the mother cell transcription factor σ^E (Jonas *et al*., 1990; Ruzal *et al*., 1998). However, we imagine that less severe perturbations might allow low levels of activated σ^E. This could result in a transient defect in engulfment. Under these conditions the degradative response described here would be analogous to a stress response. The activation of this stress response would ensure that cell–cell signalling (and progression through the sporulation process) does not occur until more favourable conditions allow for the successful completion of this key step in sporulation.

In an attempt to identify the protease responsible for degrading secreted proteins, we tested mutants in proteases [including WprA, HtrA, HtrB, HtrC, CtpA, CtpB, AprE and IVB itself (see Fig. 4)] known to act outside the cell that might be present in the intermembrane space. Strains lacking one (or several) of these proteases were analysed for their ability to maintain high levels of IVB when engulfment was perturbed. None significantly stabilized the signalling protein (data not shown). In addition, we performed a genetic screen for mutants that would accumulate IVB and thus trigger σ^K activation even when engulfment was impaired. However, none of the mutants that activated σ^K significantly stabilized IVB. Our analysis is consistent with the idea that several redundant proteases are activated when engulfment is impaired or that the protease we are looking for is essential for viability or sporulation. The identification of this protease (or proteases) remains the principal challenge for the future.

Perturbations to engulfment do not impair B processing activity

Here we have shown that when engulfment is impaired, σ^K activation is blocked due to the failure to accumulate the IVB signalling protein. It is noteworthy that Pogliano and coworkers have recently hypothesized that perturbations to engulfment *directly* inhibit the pro-σ^K processing enzyme B (Jiang *et al*., 2005). In their analysis, they used the same engulfment mutants but a different σ^G bypass strain. Importantly, in this bypass mutant, the levels of B are much lower than wild type and we have discovered are artificially sensitive to engulfment defects (Fig. S3A) (Rudner and Losick, 2002). In particular, we have found that if a more stable version of B is used in this bypass mutant background, pro-σ^K processing is completely unaffected even when engulfment is impaired (Fig. S3B). For a detailed description of these experiments see *Supplementary material*. These results indicate that perturbations to engulfment do not significantly impact B processing activity and further emphasize that engulfment defects prevent σ^K activation primarily at the level of expression (Gomez *et al*., 1995; Rudner and Losick, 2001) and accumulation of the signalling protein IVB.

IVB activity is not coupled to the completion of engulfment

Although the low level of IVB protease made under σ^F control is not able to trigger pro-σ^K processing, it has been found recently that there is sufficient IVB to cleave IIQ, another IVB substrate (Jiang *et al*., 2005). Interestingly, this small amount of protease cleaves IIQ at a time when engulfment is complete even though IVB is synthesized early during the engulfment process. Based on this observation, Pogliano and coworkers proposed that IVB protease activity is coupled to the completion of engulfment (Jiang *et al*., 2005; Chiba *et al*., 2006). Consistent with this idea, these researchers found that IIQ is not cleaved when engulfment is impaired (Jiang *et al*., 2005; Chiba *et al*., 2006). Our findings that IVB is degraded when engulfment is impaired suggests that the failure to cleave IIQ might not be due to regulation of IVB activity but rather the absence of the IVB protease itself. In support of this, we have found that when IVB is expressed under the control of the P_{IIQ} promoter it is capable of cleaving IIQ in the absence of IID (a complete block to engulfment) (Fig. S4). IVB levels are quite

low in this bypass strain when engulfment is impaired and were not sufficient to trigger pro-σ^K processing (Fig. 1B); however, it appears there was enough IVB to cleave some IIQ. This result indicates that IVB activity is not linked to the completion of engulfment. We speculate that during the normal programme of engulfment IIQ is inaccessible to IVB and only becomes susceptible to cleavage when engulfment is complete. In support of this idea, Pogliano and coworkers have recently found that IIQ is relatively immobile in discrete foci during engulfment but becomes more mobile after engulfment is complete (Chiba *et al*., 2006).

A fail-safe mechanism ensures timely cell–cell signalling

In this work, we have shown that σ^K activation is coupled to proper engulfment through the degradation of IVB. We note that the accumulation of IVB (and the activation of σ^K) is separately linked to engulfment through σ^G-dependent expression of the *IVB* gene. Specifically, it has been shown that when engulfment is blocked, the activity of the forespore transcription factor σ^G is significantly impaired (Margolis *et al*., 1993; Partridge and Errington, 1993; Smith *et al*., 1993; Frandsen and Stragier, 1995; Londono-Vallejo *et al*., 1997). Thus, when engulfment is perturbed, the accumulation of IVB is blocked both at the level of its synthesis under the control of σ^G and its degradation in the pathway described here. This degradative response is therefore a fail-safe mechanism to ensure that late mother cell gene expression is kept completely off during perturbations to this crucial morphological process. Interestingly, it has recently been shown that in certain genetic backgrounds, σ^G can become activated in the forespore even when engulfment is impaired (Chary *et al*., 2006). Under these conditions, the degradation pathway described here would be critical to ensure that mother cell gene expression under σ^K control is not turned on.

We hypothesize that this degradative response is also important to remove other secreted proteins from the intermembrane space when engulfment is impaired. For example, secreted morphogenetic proteins involved in synthesis or maturation of the spore peptidoglycan would be degraded if engulfment is perturbed and thus prevent premature remodelling of the spore envelope. In addition, the activation of σ^G in the forespore requires the successful completion of engulfment and a signal from the mother cell under the control of σ^E (Rudner and Losick, 2001). If a secreted protein is required for either of these two pathways, our results suggest that it would also be targeted for degradation when engulfment is impaired. Thus, it is possible that the function of this degradative response is to block σ^G activation in the forespore in addition to σ^K activation in the mother cell.

Experimental procedures

General methods

All *B. subtilis* strains were derived from the prototrophic strain PY79 (Youngman *et al*., 1983). *E. coli* strain DH5α was used for cloning. Insertions of gene fusions at ectopic integration sites in *B. subtilis* were confirmed by marker replacement or by the inability to catabolize starch. Sporulation was induced by resuspension at 37°C by the method of Sterlini-Mandelstam (Harwood and Cutting, 1990). Tables of strains, plasmids and oligonucleotide primers used in this study are available as supplementary data at http://www.blackwell-synergy.com

Plasmid construction

The plasmids used in this study are listed in Table S2. A description of how they were created can be found in *Supplementary material*.

Immunoblot analysis

At indicated times after the initiation of sporulation by resuspension, the OD_{600} was measured for equivalent loading and samples (1.0 ml) were collected by centrifugation. Whole cell extracts were prepared by resuspension of cell pellets in 50 μl lysis buffer (20 mM Tris pH 7.0, 10 mM EDTA, 1 mg ml^{-1} lysozyme, 10 μg ml^{-1} DNase I, 100 μg ml^{-1} RNase A, with protease inhibitors: 1 mM PMSF, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin) and incubation at 37°C for 10 min followed by addition of 50 μl SDS sample buffer (0.25 M Tris pH 6.8, 4% SDS, 20% glycerol, 10 mM EDTA) containing 10% 2-mercaptoethanol. Samples were heated for 5 min at 80°C prior to loading. Proteins were separated by SDS-PAGE on 12.5% polyacrylamide gels, electrobloted onto Immobilon-P membranes (Millipore) and blocked in 5% non-fat milk in phosphate-buffered saline (PBS)–0.5% Tween-20. The blocked membranes were probed with affinity-purified anti-SpoIVB (IVB) (1:10 000) (Campo and Rudner, 2006), anti-σ^K (1:10 000) (Resnekov *et al*., 1996), anti-SpoIVFA (A) (1:10 000) (Resnekov *et al*., 1996), anti-SpoIVFB (B) (1:2000) (Resnekov *et al*., 1996), anti-GFP (1:10 000) (Rudner and Losick, 2002), anti-SpoIID (IID) (1:10 000), anti-MBP (1:10 000) (NEB), anti-BofC (1:2000) and anti-DsbA (1:10 000) (Bardwell *et al*., 1993) antibodies diluted into 3% BSA in 1× PBS-0.05% Tween-20. Primary antibodies were detected using horseradish peroxidase-conjugated goat, anti-rabbit immunoglobulin G (Bio-Rad) and the Western Lightning reagent kit as described by the manufacturer (PerkinElmer).

Assay for leakage

One millilitre of culture samples was collected during a time-course of sporulation (by resuspension). The cells were pelleted by centrifugation and the supernatant was transferred to fresh microfuge tube. Lysates from the cell pellet were made as described above. The proteins in the medium (supernatant) were concentrated by TCA precipitation. One

hundred and fifty microlitres of TCA was added to the supernatant and incubated for 15 min on ice and then pelleted by centrifugation at 10 000 *g* for 15 min at 4°C. The pellet was washed with ice-cold acetone and proteins were resuspended in 100 µl 2× sample buffer. Equivalent amounts of whole cell lysate and supernatant were analysed by immunoblot.

Protease susceptibility assay

At 2.2 h after the onset of sporulation (by resuspension), 2 ml of samples was collected for protoplasting. Cells were washed with 1× SMM buffer (0.5 M sucrose, 20 mM $MgCl_2$, 20 mM maleic acid pH 6.5) (Harwood and Cutting, 1990) at room temperature and resuspended in 1 ml 1× SMM and protoplasted with lysozyme (0.5 mg ml^{-1}) for 10 min. Protoplasts were washed with 1× SMM and resuspended in 200 µl 1× SMM. Twenty microlitres of fresh protoplasts (200 µl equivalent of cell culture) was incubated with Trypsin (30 µg ml^{-1}) (Worthington) or Trypsin and 1% Triton X-100. Reactions were terminated by the addition of 20 µl of 2× SDS-sample buffer and analysed by immunoblot.

Purification of His_6-IID and His_6-BofC for antibody production

Both gene fusions were expressed in *E. coli* BL21 DE3 pLysS. Cells were grown in Luria–Bertani at 37°C to an OD_{600} of 0.8 and induced by addition of IPTG to 1 mM. After 2 h of induction, cells were harvested by centrifugation and resuspended in 1/100th volume buffer I (50 mM Tris-HCl pH 8, 300 mM NaCl, 5 mM 2-mercaptoethanol, 10 mM imidazole) and flash-frozen in N_2(l). A crude extract was prepared by freeze-thawing the cells followed by the addition of lysozyme (1 mg ml^{-1}) and sonication. A soluble fraction was made by 100 000 *g* spin and was loaded on a 1 ml Ni^{2+}-NTA agarose (Qiagen) column equilibrated with buffer I. Bound protein was washed with buffer I containing 20 mM imidazole and eluted in buffer I containing 120 mM imidazole. Peak fractions were used to generate anti-IID and anti-BofC, rabbit polyclonal antibodies (Covance). Crude serum was affinity purified as described previously (Campo and Rudner, 2006).

Fluorescence microscopy

Fluorescence microscopy was performed with an Olympus BX61 microscope as previously described (Rudner and Losick, 2002). Fluorescent signals were visualized with a phase contrast objective UplanF1 100× and captured with a monochrome CoolSnapHQ digital camera (Photometrics) using Metamorph software version 6.1 (Universal Imaging). Exposure times were typically 500 ms for CFP. The lipophylic membrane dye TMA-DPH (Molecular Probes) was used at a final concentration of 0.01 mM and exposure times were typically 200 ms. Images were analysed, adjusted and cropped using Metamorph software. Final figure preparation was performed in Adobe Illustrator.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Full-length IVB is membrane-associated.
Biochemical fractionation of IVB and A (an integral membrane control). Lysates were prepared from sporulating cells (PY79

and BDR94) collected at hour 3. Soluble (S100) and membrane fractions (P100) were separated by centrifugation at 100 000× g and subjected to immunoblot analysis. A nonspecific (soluble) protein recognized by the anti-IVB antibody is indicated by an asterisk.

Fig. S2. Protoplast engulfment protects membrane-anchored proteins that localize to the intermembrane space from protease digestion.

Immunoblots from protease susceptibility assays. Cells were induced to sporulate by resuspension. At hour 2.2 of sporulation, prior to the completion of engulfment, the sporangia were protoplasted and incubated for indicated times (in minutes) with Trypsin or Trypsin and Triton X-100 (+Triton). Full-length IVB^{S378A}, YFP-A (an integral membrane protein) and σ^F (a cytoplasmic protein) were analyzed in the *sigG*Δ bypass strain (wt) (BTD2073), in a *IIIAH*Δ mutant (BTD2081), and in a *IIIA*Δ, *IID*Δ double mutant (BTD2089). Broder and Pogliano have recently reported that zipper-like interactions between IIQ and IIIAH allow engulfment to proceed even upon protoplast treatment (Broder and Pogliano, 2006). Thus, in the *IIIAH*+ strain (BTD2073), membrane-anchored proteins that localize to the intermembrane space are protected from trypsin degradation.

Fig. S3. Perturbations to engulfment do not affect B-dependent pro-σ^K processing activity.

Immunoblot analysis of whole lysates from sporulating cells. Cells were induced to sporulate by resuspension and samples were collected at indicated times (in hours). (A) Perturbations to engulfment reduce B levels in a *bofA*Δ bypass background. B levels and pro-σ^K processing were compared in *bofA*Δ (BDR103), *bofA*Δ, *IIQ*Δ (BTD2021) and *bofA*Δ, *IID*Δ (BTD2023) mutants. Levels of B and pro-σ^K processing in a *bofA*+ strain (wt) (PY79) at hour 3.25 are shown for comparison. (B) A stable version of B (B-GFP) supports efficient pro-σ^K processing in the *bofA*Δ bypass strain even when engulfment is impaired. Pro-σ^K processing and B-GFP levels were compared in *bofA*Δ (BTD2025), *bofA*Δ, *IIQ*Δ (BTD2029) and *bofA*Δ, *IID*Δ (BTD2031) mutants. Levels of B-GFP and pro-σ^K processing in a *bofA*+ strain (BDR347) at hour 3.25 are shown for comparison. (C) B-GFP is a functional fusion. B-GFP, like wild-type B, is held inactive by A and BofA in the absence of the IVB signal. Pro-σ^K processing and the levels of B and B-GFP were compared in wild-type (PY79), *B-GFP* (BDR347), *IVB*Δ (BDR94) and *IVB*Δ, *B-GFP* (BTD2027) strains.

Fig. S4. IVB activity is not coupled to the completion of engulfment.

Immunoblot analysis of whole lysates from sporulating cells. Cells were induced to sporulate by resuspension and samples were collected at indicated times (in hours). IVB levels and IVB-dependent cleavage of IIQ were compared in the *sigG*Δ bypass strain in which the *IVB* gene was fused to the σ^F-responsive promoter P_{IIQ} (BTD409) and this same strain containing the engulfment mutants *IID*Δ (BTD1057) or *IIQ*Δ (BTD1053). The IIQ cleavage product is indicated by an arrow. A nonspecific band recognized by the anti-IVB antibody is indicated by an asterisk. The immunoblots were obtained from samples collected in the same experiment and analyzed on the same day but on separate gels. Exposure times were the same for all three strains.

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