Forespore Signaling Is Necessary for Pro-σ^K Processing during Bacillus subtilis Sporulation Despite the Loss of SpoIVFA upon Translational Arrest

Lee Kroos,* Yuen-Tsu Nicco Yu,† Denise Mills, and Shelagh Ferguson-Miller

Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, Michigan 48824

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The σ^K checkpoint coordinates gene expression in the mother cell with signaling from the forespore during Bacillus subtilis sporulation. The signaling pathway involves SpoIVB, a serine peptidase produced in the forespore, which is believed to cross the innermost membrane surrounding the forespore and activate a complex of proteins, including BoA, SpoIVFA, and SpoIVFB, located in the outermost membrane surrounding the forespore. Activation of the complex allows proteolytic processing of pro-σ^K, and the resulting σ^K RNA polymerase transcribes genes in the mother cell. To investigate activation of the pro-σ^K processing complex, the level of SpoIVFA in extracts of sporulating cells was examined by Western blot analysis. The SpoIVFA level decreased when pro-σ^K processing began during sporulation. In extracts of a spoIVB mutant defective in forespore signaling, the SpoIVFA level failed to decrease normally and no processing of pro-σ^K was observed. Although these results are consistent with a model in which SpoIVFA inhibits processing until the SpoIVB-mediated signal is received from the forespore, we discovered that loss of SpoIVFA was insufficient to allow processing under certain conditions, including static incubation of the culture and continued shaking after the addition of inhibitors of oxidative phosphorylation or translation. Under these conditions, loss of SpoIVFA was independent of spoIVB. The inability to process pro-σ^K under these conditions was not due to loss of SpoIVFB, the putative processing enzyme, or to a requirement for ongoing synthesis of pro-σ^K. Rather, it was found that the requirements for shaking of the culture, for oxidative phosphorylation, and for translation could be bypassed by mutations that uncouple processing from dependence on forespore signaling. This suggests that ongoing translation is normally required for efficient pro-σ^K processing because synthesis of the SpoIVB signal protein is needed to activate the processing complex. When translation is blocked, synthesis of SpoIVB ceases, and the processing complex remains inactive despite the loss of SpoIVFA. Taken together, the results suggest that SpoIVB signaling activates the processing complex by performing another function in addition to causing loss of SpoIVFA or by causing loss of SpoIVFA in a different way than when translation is blocked. The results also demonstrate that the processing machinery can function in the absence of translation or an electrochemical gradient across membranes.

Communication between cells is an essential feature of many biological processes. During sporulation of the gram-positive bacterium Bacillus subtilis, two cell types are produced that communicate extensively in order to coordinate the developmental process (19, 20, 35). The two cell types are produced within a sporangium by asymmetric division, resulting in a larger mother cell and a smaller forespore. The membranes of the division septum migrate around the forespore and pinch it off as a protoplast within the mother cell (Fig. 1A). Upon completion of this step, a new sigma factor, σ^K, becomes active in the forespore. σ^K RNA polymerase transcribes the spoIVB gene, whose product somehow signals to the mother cell that it is time to produce active σ^K by proteolytic processing of inactive pro-σ^K (3, 4, 24). This signaling pathway has been called the σ^K checkpoint (Fig. 1B) because it delays mother-cell gene expression under the control of σ^K RNA polymerase for about 1 h (4). A breakdown in communication between the forespore and the mother cell can lead to premature production of σ^K, which causes a sporulation defect (4).

Much is known about the components involved in the σ^K checkpoint, yet important questions remain. SpoIVB is a serine peptidase that is believed to cross the innermost membrane surrounding the forespore (14, 38). In the space between the two membranes surrounding the forespore, SpoIVB is thought to self-cleave, generating several species with slightly different N-terminal truncations (38). One or more of these species signals pro-σ^K processing, but the mechanism of signaling is unknown. For example, it is unclear whether further serine peptidase activity beyond the self-cleavage is required for signaling. SpoIVB has a PDZ domain likely to mediate protein-protein interactions (13, 27). These could include interactions between SpoIVB and other proteins which are important for signaling, but this is uncertain because the PDZ domain also plays a role in the autoproteolysis that activates the SpoIVB signaling function (13).

The target of SpoIVB signaling is a complex of three proteins, BoA, SpoIVFA, and SpoIVFB, that localize to the outermost membrane surrounding the forespore (5, 9, 16, 29, 31, 33, 37). These proteins are normally produced under the transcriptional control of σ^K RNA polymerase (5, 16, 31), which directs early gene expression in the mother cell. However,
several insights have emerged from studies in which *B. subtilis* was engineered to produce different combinations of these proteins, as well as pro-σK*, during growth. First, SpoIVFB alone is sufficient for pro-σK processing, suggesting that it is the protease that processes pro-σK (23, 30). Subsequent mutational analyses have supported the idea that SpoIVFB is a member of a large family of membrane metalloproteases (32, 40). Second, BoaA and SpoIVFA together can inhibit processing by SpoIVFB (30). Third, BoaA stabilizes SpoIVFA (28). These observations led to the model that SpoIVFA is an inhibitor of SpoIVFB protease activity during sporulation and that the role of BoaA is to stabilize SpoIVFA (28).

We hypothesized that SpoIVB signaling from the forespore might target SpoIVFA, causing its level to decrease and relieving inhibition of SpoIVFB so that pro-σK processing would occur. We show that a decrease in the level of SpoIVFA in cell extracts coincides with the onset of pro-σK processing during sporulation and that in extracts of spoIVB mutant cells the SpoIVFA level fails to decrease normally. However, we also found that a decrease in the level of SpoIVFA was insufficient to allow pro-σK processing when shaking of the culture was discontinued or when shaking was continued after addition of inhibitors of oxidative phosphorylation or translation. Under these conditions, loss of SpoIVFA did not depend on SpoIVFB-mediated forespore signaling. Although these conditions blocked further synthesis of pro-σK*, this does not explain why processing was inhibited, because we confirmed a previous report that processing occurs posttranslationally (41). Moreover, we show that the dependence of pro-σK processing on shaking, oxidative phosphorylation, and translation can be relieved by mutations that bypass the need for forespore signaling.

From these results, we infer that synthesis of the SpoIVB signal protein is needed to activate the pro-σK* processing machinery by a mechanism that involves more than just the loss of SpoIVFA observed when translation is blocked. Our results also show that processing of pro-σK* can occur without ongoing translation and in the absence of a metabolic potential when the need for forespore signaling is bypassed.

**FIG. 1.** σK checkpoint. (A) Diagram of a sporangium in which the forespore (FS) has been pinched off as a protoplast within the mother cell (MC). (B) Expanded view of the two membranes separating the forespore and mother cell, depicting BoaA, SpoIVFA, and SpoIVFB in the outermost membrane surrounding the forespore. The three proteins form a complex in which SpoIVFB is inactive prior to signaling from the forespore (4, 5, 16, 30, 31, 33). σK RNA polymerase transcribes spoIVB in the forespore (3), and SpoIVB is secreted into the space between the two membranes (38), where it activates the processing complex, leading to the production of σK* in the mother cell.
We conclude that the SpoIVB-mediated signal from the forespore is required for the SpoIVFA decrease that normally accompanies the onset of pro-αK processing, but neither SpoIVFB protease activity nor αK is needed for the decrease in the level of extractable SpoIVFA.

When the blot shown in Fig. 3A was stripped of antibodies and reprobed with anti-pro-αK antibodies, only pro-αK was observed (Fig. 3D), consistent with previous results showing that SpoIVB is necessary for processing of pro-αK to αK (24). These results are consistent with the model that SpoIVB causes the level of SpoIVFA to decrease, releasing SpoIVFB from inhibition so that it can cleave pro-αK.

Effect of shaking the culture. A goal of our laboratory has been to observe pro-αK processing in extracts of sporulating cells. Although some apparent processing can be observed, it does not appear to depend on SpoIVFB (L. Kroos, unpublished data), the protein believed to be responsible for processing in vivo (5, 23, 30, 32, 40). As a control to investigate why extracts of sporulating cells fail to process pro-αK to αK during static incubation, we tested the effect of static incubation on intact cells. Normally, the culture is shaken at 400 rpm to promote good aeration and efficient sporulation (11). We discovered that if shaking was discontinued after T0, the level of SpoIVFA in cell extracts still decreased (Fig. 4A), but pro-αK processing was not observed (Fig. 4B).

One possible explanation of these results is that the putative SpoIVFB protease is not made or is unstable under these conditions. However, this was not the case. The *B. subtilis*...
strain OR758 used in our experiments has the gfp gene, encoding the GFP of *Aequorea victoria* fused to the 3′ end of *spoIVFB* (29). The *spoIVFB*-GFP fusion protein is fully active for pro-σK processing (Fig. 2B and data not shown) and can be detected with antibodies against GFP. Figure 4C shows that *SpoIVFB*-GFP was present at T₃₂₅, shortly after shaking was stopped, and its level remained constant until at least T₄₅₅. Similar results were observed when shaking was continued after T₃ (data not shown). Taken together, these results demonstrate that in the absence of shaking, the decrease in *SpoIVFA* is not sufficient to allow processing of pro-σK by *SpoIVFB-GFP*.

**Effect of inhibitors of oxidative phosphorylation.** Why is shaking of the culture needed for pro-σK processing? We hypothesized that good aeration of the culture permits energy generation via oxidative phosphorylation and that energy is needed to allow processing even when the *SpoIVFA* level is much diminished. To test this hypothesis, we added CCCP (5 mM), an H⁺ ionophore that uncouples oxidative phosphorylation, at T₂ and then continued shaking the culture at 400 rpm. The effects on the *SpoIVFA* level (Fig. 5A), on pro-σK processing (Fig. 5B), and on the *SpoIVFB-GFP* level (Fig. 5C) were similar to those obtained when shaking was discontinued after T₃ (Fig. 4). When CCCP was added at T₃₂₅ after processing had begun, no further processing was observed (Fig. 5D), in contrast to the further increase in the σK level seen in a parallel untreated culture (Fig. 5E). Similar results were obtained with an inhibitor of oxidative phosphorylation, sodium azide (0.2%); an ionophore that dissipates the pH gradient, nigericin (1 μM); and an ionophore that dissipates the membrane potential, valinomycin (1 μM) in the presence of KCl (0.1 M) (data not shown). We conclude that oxidative phosphorylation is necessary for pro-σK processing to occur despite the virtual absence of SpoIVFA from cell extracts within 1 h after blockage of oxidative phosphorylation and the continued presence of the putative processing protease *SpoIVFB-GFP*.

**Effect of protein synthesis inhibitors.** Why is oxidative phosphorylation needed for pro-σK processing? A clue came from comparing the pro-σK level in extracts of the *spoIVB* mutant (Fig. 3D) with that in extracts of wild-type OR758 (*spoIVFB-gfp*) cells that had been treated with inhibitors of oxidative phosphorylation (Fig. 5B and data not shown). Pro-σK continued to accumulate after T₃ in the *spoIVB* mutant but not in OR758 cells treated with inhibitor. This suggested that the inhibitors of oxidative phosphorylation block the energy-intensive process of protein synthesis. To test whether protein synthesis is required for pro-σK processing, the translation inhibitor Cm (200 μg/ml) was added at T₃ or T₄₅₅ and shaking was continued. In the parallel untreated culture (Fig. 6A, no addition), a small amount of σK was observed at T₃₂₅, considerably more was seen at T₄₅₅, and by T₆ or σK was more abundant than pro-σK. Addition of Cm at T₃ appeared to block processing (Fig. 6A, Cm at 3 h), but the interpretation was complicated by a decrease in the level of pro-σK, which was more rapid than when inhibitors of oxidative phosphorylation were added (Fig. 5B and data not shown) (see below).

A longer exposure of the blot shown in Fig. 6A revealed that a small amount of σK was present 15 min after Cm addition, but thereafter the amount of σK relative to the amount of pro-σK did not rise (Fig. 6B, Cm at 3 h) as it did in the untreated culture (Fig. 6A, no addition). Clearly, Cm strongly inhibited pro-σK processing. Likewise, Cm appeared to block pro-σK processing shortly after its addition at T₄₅₅ (Fig. 6A, Cm at 3.5 h), because the ratio of σK to pro-σK increased just slightly within 15 min after addition of the translation inhibitor, but thereafter the ratio did not increase (Fig. 6B, Cm at 3.5, shows a longer exposure) as it did in the untreated culture (Fig. 6A, no addition). We conclude that ongoing translation is required for efficient pro-σK processing.

Figure 6C shows that the level of *SpoIVFA* declined rapidly...
after addition of the protein synthesis inhibitor. In contrast, the SpoIVFB-GFP level remained fairly constant (Fig. 6D). These results demonstrate that the inhibition of pro-\(\sigma^K\) processing observed after Cm addition was not due to persistence of SpoIVFA or to loss of the proposed processing enzyme SpoIVFB-GFP. The entire experiment was also performed with another translation inhibitor, kanamycin (200 \(\mu\)g/ml), and similar results were observed (data not shown).

We suggested above that inhibitors of oxidative phosphorylation block the energy-intensive process of protein synthesis. However, the levels of pro-\(\sigma^K\) and \(\sigma^K\) declined much more rapidly after addition of translation inhibitors (Fig. 6A and data not shown) than after addition of oxidative phosphorylation inhibitors (Fig. 5B and 5D and data not shown). The inhibitors of oxidative phosphorylation either did not block translation completely or increased the stability of pro-\(\sigma^K\) and \(\sigma^K\).

To distinguish between these possibilities, we added both the oxidative phosphorylation inhibitor CCCP (5 \(\mu\)M) and the translation inhibitor Cm (200 \(\mu\)g/ml) at \(T_3\) or \(T_{3.5}\) and continued shaking the cultures. In this experiment, a small amount of \(\sigma^K\) had already accumulated at \(T_3\) in the parallel untreated culture (Fig. 7, no addition). The combination of inhibitors not only blocked pro-\(\sigma^K\) processing, as expected from the preceding experiments, but allowed pro-\(\sigma^K\) and \(\sigma^K\) to persist longer (Fig. 7, CCCP/Cm at 3 h or at 3.5 h) than when Cm alone was added (Fig. 6A, Cm at 3 h or at 3.5 h). Similar results were obtained when other inhibitors of oxidative phosphorylation, including sodium azide (0.2%) and nigericin (1 \(\mu\)M), were added in combination with Cm (data not shown). These results indicate that inhibition of oxidative phosphorylation increases the stability of pro-\(\sigma^K\) and \(\sigma^K\) in sporulating cells. We infer that degradation of pro-\(\sigma^K\) and \(\sigma^K\) may involve one or more ATP-dependent proteases.

**Effect of inhibitors on SpoIVFA level in a spoIVB mutant.** As shown in Fig. 8, the loss of SpoIVFA observed when translation or oxidative phosphorylation was inhibited did not require SpoIVB. Treatment of a spoIVB mutant with Cm, CCCP, azide, or nigericin at \(T_3\) resulted in decreases in SpoIVFA with similar kinetics as for strain OR758 (Fig. 5A and 6C and data not shown). These results demonstrate that sporulating cells possess a powerful SpoIVB-independent mechanism(s) that can cause loss of SpoIVFA when translation or oxidative phosphorylation is inhibited. Yet pro-\(\sigma^K\) processing was not observed (Fig. 5 and 6 and data not shown).

**Pulse-chase analysis of pro-\(\sigma^K\) processing.** We considered the possibility that ongoing translation is necessary for efficient pro-\(\sigma^K\) processing because processing occurs cotranslationally. According to this model, full-length pro-\(\sigma^K\) is not processed but is degraded, and processing occurs while nascent pro-\(\sigma^K\) is being synthesized. A prediction of this model is that \([^{35}S]\)methionine-labeled pro-\(\sigma^K\) should not be processed to \(\sigma^K\) during a chase period with unlabeled methionine. A previous pulse-chase experiment suggested that labeled pro-\(\sigma^K\) can be chased to \(\sigma^K\) (41), arguing against cotranslational processing, but in light of the results presented here, we were concerned about the adequacy of the chase ([i.e., the possibility of continued incorporation of label during the chase period]) in the previous experiment.

We repeated the previous experiment, labeling cells with \([^{35}S]\)methionine for 5 min at \(T_3\) and then chasing with a 1,000-fold molar excess of unlabeled methionine (Fig. 9A, lanes 1 to 3). As reported previously (41), pro-\(\sigma^K\) was processed to \(\sigma^K\) during the chase period. Quantification of the signals revealed little change in the total amount of pro-\(\sigma^K\) plus \(\sigma^K\) (data not shown). In contrast, Western blot analysis of whole-cell extracts of samples collected in parallel showed a greater than twofold increase in the total amount of pro-\(\sigma^K\) plus \(\sigma^K\) by 60 min after the pulse (data not shown). Taken together, these results indicate that synthesis of unlabeled pro-\(\sigma^K\) continued during the chase period and suggest that synthesis of labeled pro-\(\sigma^K\) was prevented by the 1,000-fold molar excess of unlabeled methionine.

To be certain that continued synthesis of labeled pro-\(\sigma^K\) did not occur during the chase period, cells were collected by centrifugation immediately after the 5-min pulse-labeling and resuspended in fresh medium containing a 10,000-fold molar excess (relative to the concentration of labeled methionine used during the pulse) of unlabeled methionine. Similar results were observed (Fig. 9A, lanes 4 to 6). When spoIVB or spoIVFB mutant cells were pulse-labeled at \(T_3\) and chased with a 1,000-fold molar excess of unlabeled methionine, pro-\(\sigma^K\) persisted and was not processed to \(\sigma^K\) (Fig. 9A, lanes 7 to 12). We conclude that pro-\(\sigma^K\) is processed to \(\sigma^K\) posttranslationally.

We next addressed the possibility that ongoing translation is needed to synthesize the SpoIVB signaling protein in the forespore. This would explain our results if SpoIVB signaling is needed to activate pro-\(\sigma^K\) processing despite the loss of SpoIVFA upon translational arrest. The normal dependence of processing on SpoIVB can be bypassed by bof (which stands

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**FIG. 7.** Effect of a combination of inhibitors on pro-\(\sigma^K\) and \(\sigma^K\) levels. _B. subtilis_ strain OR758 was induced to sporulate, and the culture was split at \(T_3\) into portions to which nothing was added or CCCP (5 \(\mu\)M) and Cm (200 \(\mu\)g/ml) were added immediately (CCCP/Cm at 3 h) or after 30 min (CCCP/Cm at 3.5 h). Shaking was continuous except when the culture was split and inhibitors were added. Samples were collected at the indicated times (hours) after sporulation was induced, and whole-cell extracts were subjected to Western blot analysis with antibodies against pro-\(\sigma^K\).

**FIG. 8.** Effect of inhibitors on the level of SpoIVFA in a spoIVB mutant. _B. subtilis_ strain LK1 (spoIVBΔ::spc) was induced to sporulate, and the culture was split at \(T_3\) into portions to which nothing was added or CCCP (5 \(\mu\)M), sodium azide (0.2%), nigericin (1 \(\mu\)M), or Cm (200 \(\mu\)g/ml) was added. Shaking was continuous except when the culture was split and inhibitors were added. Samples were collected at the indicated times (hours) after sporulation was induced, and whole-cell extracts were subjected to Western blot analysis with antibodies against SpoIVFA.
pro-/H9268
quantification of the signals showed that the ratio of pro-σK to σK increased from 0.35 (lane 1) to 0.83 (lane 2) during the first 30 min of the chase period in wild-type cells. The ratio of σK to pro-σK after the pulse at T2 was higher in the bypass mutant (lane 4) than in wild-type cells (lane 1) because processing was not delayed by dependence on forespore signaling. During the chase, the ratio of σK to pro-σK in the bypass mutant increased more than twofold in the presence of Cm (lane 6) as well as in its absence (lane 5). A similar result was observed for a bofA mutant (data not shown). These results suggest that the need for ongoing translation to observe efficient processing in wild-type cells reflects the need to synthesize SpoIVB.

Can a bypass mutation also alleviate the need for oxidative phosphorylation and shaking of the culture in order to observe processing? Figure 9C shows that CCCP addition (lane 3) and static incubation of the culture (lane 4) inhibited pro-σK processing to a similar extent as Cm addition (Fig. 9B, lane 3) during the first 30 min of the chase period in wild-type cells but had little effect on processing in the bypass mutant (Fig. 9C, lanes 5 to 8). Taken together, the results of our pulse-chase analysis indicate that processing of pro-σK to σK occurs post-translationally and its dependence on shaking, oxidative phosphorylation, and translation can be relieved by a mutation that bypasses the need for forespore signaling.

**DISCUSSION**

Our results provide several new insights into the signaling pathway that leads to activation of pro-σK processing. First, the SpoIVB-mediated signal from the forespore causes a decrease in the level of extractable SpoIVF in the culture, which coincides with the onset of pro-σK processing during sporulation. Second, loss of SpoIVF was induced by static incubation of the culture or inhibition of oxidative phosphorylation or translation, but this does not result in processing of pro-σK, even though the putative processing site, SpoIVFB-GFP, is present. Third, this inhibition of processing can be overcome by mutations that bypass the dependence of processing on forespore signaling. We conclude that forespore signaling is necessary to activate the pro-σK processing complex despite loss of SpoIVF under conditions that inhibit translation. We discuss two possible explanations for this novel finding below.

One possibility is that although SpoIVB-mediated signaling normally causes loss of SpoIVF from cell extracts, it also performs another function necessary to activate the pro-σK processing complex. What might that function be? Rudner and Losick (33) showed recently that SpoIVF interacts with both BoF and SpoIVF, possibly enabling BoF to inhibit SpoIVF complex formation, whereas static incubation and inhibition of translation do not result in processing of pro-σK, even though the putative processing site, SpoIVFB-GFP, is present. We conclude that forespore signaling is necessary to activate the pro-σK processing complex despite loss of SpoIVF under conditions that inhibit translation. We discuss two possible explanations for this novel finding below.

For bypass of forespore (4, 5, 16, 31). We repeated the pulse-chase experiment, including Cm to block translation during the chase period, and compared the wild-type strain with a bofB8 spoIIIG double mutant in which the spoIIIG mutation blocks production of SpoIVB for signaling (3) and the bofB8 mutation in spoIVF nevertheless permits processing (4).

Figure 9B shows that Cm inhibited processing of pro-σK to σK during the first 30 min of the chase period in wild-type cells. Quantification of the signals showed that the ratio of σK to pro-σK increased from 0.35 (lane 1) to 0.83 (lane 2) during the 30-min chase in the absence of Cm, but in the presence of Cm it increased only to 0.56 (lane 3). In contrast, Cm did not inhibit processing in the bypass mutant (Fig. 9B, lanes 4 to 6). The ratio of σK to pro-σK after the pulse at T2 was higher in the bypass mutant (lane 4) than in wild-type cells (lane 1) because processing was not delayed by dependence on forespore signaling. During the chase, the ratio of σK to pro-σK in the bypass mutant increased more than twofold in the presence of Cm (lane 6) as well as in its absence (lane 5). A similar result was observed for a bofA mutant (data not shown). These results suggest that the need for ongoing translation to observe efficient processing in wild-type cells reflects the need to synthesize SpoIVB.

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hibitors (Fig. 6A and data not shown) than after addition of oxidative phosphorylation inhibitors (Fig. 5B and 5D and data not shown), and we showed that the effect of the oxidative phosphorylation inhibitors was dominant, allowing pro-\(\sigma^K\) and \(\sigma^K\) to persist longer when a combination of the two types of inhibitors was added (Fig. 7) than when translation inhibitor alone was added (Fig. 6A).

Oxidative phosphorylation generates ATP (and, indirectly, GTP), needed for translation. Certain proteases also require ATP (8). Blocking ATP synthesis with inhibitors of oxidative phosphorylation is expected to block not only translation but also ATP-dependent mechanisms of proteolysis. The stabilizing effect of oxidative phosphorylation inhibitors on pro-\(\sigma^K\) and \(\sigma^K\) in the presence of a translation inhibitor (Fig. 7 and data not shown) suggests that degradation of pro-\(\sigma^K\) and \(\sigma^K\) may involve an ATP-dependent protease(s). FtsH is a membrane-bound ATP- and Zn\(^{2+}\)-dependent protease that might be involved in pro-\(\sigma^K\) and \(\sigma^K\) turnover. In \textit{E. coli}, FtsH degrades the heat shock transcription factor \(\sigma^D\) (12, 36). Also, the SpoV protein of \textit{B. subtilis} can inhibit FtsH-mediated degradation of \textit{E. coli} \(\sigma^D\) in vitro, and certain mutations in the \textit{B. subtilis} \textit{ftsH} gene can partially suppress the sporeulation defect of certain \textit{spoVM} mutants, leading to speculation that SpoVM might antagonize FtsH-dependent degradation of sigma factors during sporulation (2).

FtsH has also been implicated in the turnover of SpoIVFA in \textit{B. subtilis}. A null mutation in \textit{ftsH} enhances SpoIVFA accumulation in cells engineered to produce SpoIVFA during growth (28). However, our results showing that the SpoIVFA level declines as rapidly after treatment of sporulating cells with inhibitors of oxidative phosphorylation (Fig. 5A and data not shown) as after treatment with translation inhibitors (Fig. 6C and data not shown) suggest that degradation of SpoIVFA under these conditions can occur by an ATP-independent mechanism, so FtsH is not involved.

Although FtsH does not appear to be involved in the loss of SpoIVFA in sporulating cells treated with inhibitors of oxidative phosphorylation or translation, we cannot exclude the involvement of FtsH in untreated sporulating cells, because the mechanism of SpoIVFA loss caused by SpoIVB signaling is unknown and, as noted above, it could be different. A simple model would be that the serine peptidase activity of SpoIVB (14, 38) acts directly on SpoIVFA, leading to degradation of SpoIVFA. Perhaps a product of this specific degradation pathway is necessary to activate the SpoIVFB protease. Alternatively, SpoIVB might cause sequestration rather than degradation of SpoIVFA.

In addition to its signaling role in the \(\sigma^K\) checkpoint, SpoIVB has a second distinct function required for heat-resistant spore formation, which may involve a role in synthesis of the germ cell wall deposited between the membranes surrounding the forespore (26). We showed that the SpoIVFA decrease was not due to sequestration brought about by \(\sigma^K\)-dependent gene expression and morphological change (Fig. 3B), but it remains possible that SpoIVB causes sequestration of SpoIVFA (e.g., related to the postulated role of SpoIVB in germ cell wall synthesis). Interestingly, Rudner and Losick (33) noted a region of SpoIVFA with similarity to proteins involved in peptidoglycan remodeling and proposed that SpoIVFA interacts with peptidoglycan between the membranes surrounding the forespore. Perhaps SpoIVB directly or indirectly causes linkage of SpoIVFA to peptidoglycan, resulting in activation of the pro-\(\sigma^K\) processing complex and loss of SpoIVFA from cell extracts.

Further studies are needed to determine the mechanism of SpoIVFA loss caused by SpoIVB signaling and how it differs from that caused by static incubation or treatment with inhibitors of oxidative phosphorylation or translation. The significance of such studies hinges on whether the first explanation of our results, offered above, is correct, because in this model, SpoIVFA loss is a mere consequence of activation of the processing complex, not a cause of activation. Instead, BofA is the key to regulation of the complex. Rudner and Losick (33) favor this model because they showed that a functional GFP-SpoIVFA fusion protein, which accumulates in the absence of BofA better than native SpoIVFA, did not impair sporulation or pro-\(\sigma^K\) processing. They inferred that the instability of SpoIVFA is not critical to activation of the SpoIVFB protease. However, as Rudner and Losick (33) pointed out, it is conceivable that a small amount of the GFP-SpoIVFA fusion protein was destroyed (or linked to peptidoglycan) during sporulation in their experiments. This leaves open the possibility that loss of SpoIVFA plays a role in the regulation of pro-\(\sigma^K\) processing. Rudner and Losick (33) believed this was unlikely because GFP-SpoIVFA failed to inhibit pro-\(\sigma^K\) processing even in a strain that made 5- to 10-fold less SpoIVFB. However, this only strengthens the argument if a molecule of GFP-SpoIVFA not present in a complex initially can replace a molecule of GFP-SpoIVFA lost from a complex upon its activation.

If BofA is the key to regulation of pro-\(\sigma^K\) processing, how does SpoIVB signaling overcome BofA-mediated inhibition of the SpoIVFB protease? It is possible that BofA is a direct target of SpoIVB protease activity (14, 38). Alternatively, a protein-protein interaction via SpoIVB’s PDZ domain (13, 27) might somehow relieve BofA inhibition of SpoIVFB processing activity.

In bof mutant cells, the SpoIVFB protease is not inhibited, so there would be no need for translation to produce SpoIVB, and our results show that processing occurs in the presence of Cm in bof mutants (Fig. 9B and data not shown). Moreover, the processing inhibition caused by static incubation and inhibitors of oxidative phosphorylation was relieved by a bypass mutation in a similar fashion (Fig. 9C). These results demonstrate that processing of pro-\(\sigma^K\) does not require ongoing translation or an electrochemical gradient across membranes if the need for forespore signaling is bypassed mutagenically. These insights are guiding our efforts to reconstitute pro-\(\sigma^K\) processing in vitro.

We favor the model that ongoing translation is normally required for efficient pro-\(\sigma^K\) processing because synthesis of the SpoIVB signal protein is needed to activate the processing complex. The bof mutant used in the experiments shown in Fig. 9B and C also contained a mutation in the spoIIIH gene that encodes \(\sigma^E\), which is necessary to direct sufficient expression of the spoIVB gene in order for SpoIVB to carry out its signaling function in the \(\sigma^K\) checkpoint (3, 7). Indeed, transcription of spoIVB is the only role of \(\sigma^E\) that is essential for pro-\(\sigma^K\) processing (6). This is one reason we propose that the need for ongoing translation in wild-type cells reflects the need to syn-
thesize SpoIVB. Another reason is that the active SpoIVB species may be unstable (13, 14, 38).

SpoIVB self-cleaves, generating several species with slightly different N-terminal truncations. One or more of these was proposed to be active in signaling pro-αK processing (38). However, these intermediate (44 to 46 kDa) forms of SpoIVB are subject to secondary proteolysis by one or more unidentified enzymes that may inactivate SpoIVB signaling function. Because secondary proteolysis began almost immediately after SpoIVB autoproteolysis during sporulation, it was proposed that active SpoIVB intermediates have a short half-life (38). If this model is correct, it might explain the rapid cessation of processing that we observed after adding inhibitors of oxidative phosphorylation or translation preventing any more metabolism, the response to unfolded proteins in the endo-

G protein receptor. The recent development of a pharmacological approach to the characterization of these receptors opens up new possibilities for the identification of novel modulators of these pathways.

Understanding the molecular mechanism governing pro-αK processing is important because it is a model for a type of processing that we observed after adding inhibitors of oxidative phosphorylation or translation on synthesis and persistence of SpoIVB (38). The heat shock regulator in Escherichia coli, which rapidly signals complete (or nearly complete) processing of all the pro-αK in that cell. According to this model, the inhibitors of oxidative phosphorylation or translation prevent any more cells in the population from attaining the threshold level of SpoIVB needed to initiate rapid signaling and processing.

Interestingly, the addition of Cm or rifampin (an inhibitor of transcription) has been shown to arrest pro-αK processing (17). Pro-αK processing requires the synthesis of SpoIR to activate the apparent protease SpoIIGA (15, 18, 22). Hence, the inhibition of pro-αK processing by Cm or rifampin may reflect inhibition of SpoIR synthesis.

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