Sporulation protein SpoIVFB from Bacillus subtilis enhances processing of the sigma factor precursor Pro-sigma K in the absence of other sporulation gene products.

S Lu, S Cutting and L Kroos
Sporulation Protein SpoIVFB from *Bacillus subtilis* Enhances Processing of the Sigma Factor Precursor Pro-\(\sigma^K\) in the Absence of Other Sporulation Gene Products

SIJIE LU,† SIMON CUTTING,2 AND LEE KROOS1*

Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824,1 and Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 191042

Received 12 September 1994 / Accepted 2 December 1994

Processing of inactive pro-\(\sigma^K\) to active \(\sigma^K\) in the mother cell compartment of sporulating *Bacillus subtilis* is governed by a signal transduction pathway emanating from the forespore and involving SpoIVFB in the mother cell. Coexpression of spoIVFB and sigK (encoding pro-\(\sigma^K\)) genes in growing *B. subtilis* or *Escherichia coli* enhanced pro-\(\sigma^K\) processing in the absence of other sporulation-specific gene products. The simplest explanation of these results is that SpoIVFB is a protease that processes pro-\(\sigma^K\).

Sporulation of the gram-positive bacterium *Bacillus subtilis* involves the formation of an asymmetrically positioned septum that partitions the cell into a larger mother cell compartment and a smaller forespore. Both compartments receive a copy of the genome, but they express different sets of genes whose products drive a series of morphological changes that culminate with lysis of the mother cell to release a dormant spore (reviewed in reference 7). Gene expression is controlled, in part, by a cascade of sigma factors, and activation of sigma factors in the cascade depends on communication between the mother cell and the forespore (17). One communication pathway involves a signal from the forespore that governs the production of active \(\sigma^K\) in the mother cell (1, 2, 19). Synthesis of \(\sigma^K\) involves a chromosomal DNA rearrangement to generate the composite sigK gene (26) and transcription of this gene first by \(\sigma^B\) RNA polymerase and then by \(\sigma^K\) RNA polymerase, in the mother cell (5, 11, 14, 16). However, the primary translation product of sigK is an inactive precursor, pro-\(\sigma^K\) (14, 19). Activation of pro-\(\sigma^K\) involves removal of 20 amino acids from its N terminus to produce \(\sigma^K\), and this proteolytic processing reaction is coupled to events that occur in the forespore (1, 2, 19). Active \(\sigma^K\) directs transcription of genes encoding proteins involved in spore cortex and coat synthesis (1, 14, 31–33).

Genes whose products may respond to the forespore signal and govern pro-\(\sigma^K\) processing in the mother cell were identified by mutations that relieve the dependence of pro-\(\sigma^K\) processing on forespore events (2). These bof (bypass of forespore) mutations mapped to two loci, bofA and spoIVF. Genetic studies suggest that BofA negatively regulates pro-\(\sigma^K\) processing until a signal from the forespore is received (23). SpoIVF appears to negatively regulate processing until the forespore signal is received, but SpoIVFA also appears to regulate processing positively by stabilizing SpoIVFB, the product of the downstream gene in the same operon (4). SpoIVFB plays an important role in pro-\(\sigma^K\) processing. A missense mutation in spoIVFB blocks detectable processing (4, 19), and bofA mutations fail to relieve this block (2). Moreover, deletion of the pro-amino acid coding sequence from the sigK gene bypasses the requirement for spoIVFB in \(\sigma^K\)-dependent mother cell gene expression (2), and overproduction of pro-\(\sigma^K\) rescues sporulation of a spoIVF null mutant, apparently by allowing a small amount of \(\sigma^K\) to accumulate (20). SpoIVFB has been proposed to be either a protease that processes pro-\(\sigma^K\) or a regulator of the processing event (2, 4, 19). To test whether SpoIVFB could mediate pro-\(\sigma^K\) processing in the absence of other sporulation gene products, we engineered growing cells to coexpress the SpoIVFB and sigK genes.

Coexpression of spoIVFB and sigK in growing *B. subtilis*. The spoIVFB gene or the entire spoIVF operon was fused to an isopropyl-\(\beta\)-tiothiogalactopyranoside (IPTG)-inducible promoter, P\(_{\text{spac}}\), and integrated into the *B. subtilis* chromosome by selecting for transformants (6) on Luria-Bertani agar (21) containing chloramphenicol (5 \(\mu\)g/ml). In either case, both the integrative plasmid and the *B. subtilis* chromosome carried the bofB8 mutation (2). This mutation prematurely truncates the spoIVFA open reading frame, relieving the negative effect of SpoIVFA on SpoIVFB and eliminating the need for a signal from the forespore in order to process pro-\(\sigma^K\) (4). We reasoned that the presence of the bofB8 mutation might facilitate observing SpoIVFB activity in growing cells, since the forespore would be absent. These strains and their parent strain SC745 (bofB8 spoIIGA1) (2) were then transformed (6) with a multicopy P\(_{\text{spac}}\)-sigK plasmid (pSL1) that allows production of pro-\(\sigma^K\) during growth (19). Transformants were selected on Luria-Bertani agar containing kanamycin sulfate (5 \(\mu\)g/ml), resulting in the first three strains listed in Table 1. To monitor production of active \(\sigma^K\), the strains were then lysogenized with SPB phage bearing gerE-lacZ, a \(\sigma^B\)-dependent gene fusion (3). Cells were grown in 2× YT medium (21) at 37°C to the mid-exponential phase and then divided into two equal parts, one of which was induced with IPTG (1 mM). The IPTG-induced BSL57 derivative designed to coexpress spoIVFB and sigK showed twofold more gerE-directed \(\beta\)-galactosidase activity than the IPTG-induced BSL52 derivative capable of expressing only sigK (Fig. 1A). Neither strain expressed \(\beta\)-galactosidase.
when IPTG was omitted (Fig. 1A, open squares, and data not shown). Surprisingly, the IPTG-induced BSL58 derivative designed to coexpress the entire bofB8 mutant spoIVF operon and sigK exhibited a level of gerE-lacZ expression similar to that of the BSL52 derivative capable of expressing only sigK (data not shown). However, a BSL58w derivative (Table 1) [BK338 (spoIIGΔ1)] (15) was the parent strain designed to coexpress the wild-type spoIVF operon and sigK showed a pattern of gerE-lacZ expression comparable to that of the BSL57 derivative designed to coexpress spoIVF and sigK (data not shown). Thus, in contrast to our expectation from the behavior of the bofB8 mutation in sporulating cells (2), the bofB8 mutation appears to hinder accumulation of SpoIVF activity when the entire spoIVF operon is expressed from the Pspac promoter in growing cells.

To determine whether the twofold enhancement of gerE-lacZ expression was due to SpoIVF, an in-frame deletion of spoIVF lacking amino acids 149 to 276 (nearly half of the coding sequence) was fused to Pspac and integrated into the B. subtilis chromosome. Depending on the site of the homologous recombination, two outcomes were possible. One has the full-length spoIVF fused to Pspac (BSL59 [Table 1]), which is similar to the situation in the BSL57 derivatives. The other has the in-frame deletion of spoIVF fused to Pspac (BSL60 [Table 1]). Figure 1A shows that a BSL59 derivative had twofold-more gerE-lacZ expression than a BSL60 derivative. These results indicate that functional SpoIVF is required for the observed enhancement of σ^K-dependent gene expression during growth of B. subtilis.

To determine whether the SpoIVF-dependent enhancement of gerE-lacZ expression was due to increased accumulation of σ^K, we performed Western blot (immunoblot) analyses with anti-pro-σ^K and σ^K antibodies, which detect both pro-σ^K and σ^K (19). As shown in Fig. 1B, a polypeptide comigrating with σ^K was first detected 2 h after IPTG addition to the strain designed to coexpress spoIVF and sigK (BSL57) and increased thereafter. Very little of such a polypeptide was made in the strain capable of expressing only sigK (BSL52), even at 3.5 h after IPTG addition (Fig. 1B, lane 9). Polypeptides smaller than σ^K were detected for both strains, perhaps accounting for a portion of the gerE-lacZ expression (20). However, only the polypeptide that comigrated with σ^K was more abundant in the strain designed to coexpress spoIVF and sigK than in the strain capable of expressing only sigK, and this result was reproduced in two additional experiments (data not shown). We conclude that SpoIVF appears to enhance conversion of pro-σ^K to σ^K in the absence of other sporulation-specific gene products.

Coexpressing spoIVF and sigK from a multicopy plasmid is toxic to B. subtilis. We estimate that the level of σ^K produced in growing B. subtilis cells of strain BSL57 at 3.5 h after IPTG addition was about 10-fold less than the level of σ^K produced in sporulating cells at 6 h into development (Fig. 1B). One possible explanation for the low σ^K level in growing cells is that the synthesis and/or stability of SpoIVF does not allow it to accumulate sufficiently. To determine whether increasing the

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Plasmid description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSL52</td>
<td>bofB8 spoIIGGΔ1 (pSL1)</td>
<td>pSL1 contains Pspac-sigK in a multicopy vector (19)</td>
</tr>
<tr>
<td>BSL57</td>
<td>bofB8 spoIIGGΔ1 chr::pSL13 (pSL1)</td>
<td>pSL13 contains Pspac-spoIVF in an integrative vector (18)</td>
</tr>
<tr>
<td>BSL58</td>
<td>bofB8 spoIIGGΔ1 chr::pSL14 (pSL1)</td>
<td>pSL14 contains Pspac-spoIVF in an integrative vector (18)</td>
</tr>
<tr>
<td>BSL58w</td>
<td>spoIIGGΔ1 chr::pSL31 (pSL1)</td>
<td>pSL31 is similar to pSL14, except it does not contain the bofB8 mutation in the spoIVF operon (18)</td>
</tr>
<tr>
<td>BSL59</td>
<td>bofB8 spoIIGGΔ1 chr::pSL25 (pSL1)</td>
<td>pSL25 is similar to pSL13, except the spoIVF gene has an in-frame deletion; upon integration, recombination between sequences upstream of the deletion fused Pspac to a full-length spoIVF gene (18)</td>
</tr>
<tr>
<td>BSL60</td>
<td>bofB8 spoIIGGΔ1 chr::pSL25 (pSL1)</td>
<td>Upon integration of pSL25, recombination between sequences downstream of the deletion fused Pspac to an in-frame-deleted spoIVF gene (18)</td>
</tr>
</tbody>
</table>

**Table 1. B. subtilis strains constructed**

![Graph A](image1.png)  
**Graph A.** Coexpressing spoIVF and sigK in growing B. subtilis cells enhances σ^K-dependent gene expression and accumulation of σ^K. (A) gerE-directed β-galactosidase activity was measured as described previously (20) at the indicated times after IPTG addition to growing B. subtilis strains designed to coexpress spoIVF and sigK (○, BSL57 derivative; ▲, BSL52 derivative), express sigK alone (●, BSL52 derivative), or express sigK and an in-frame deletion of spoIVF (▲, BSL60 derivative). The level of gerE-lacZ expression without IPTG addition is also shown for the BSL57 derivative (△) and was comparable for all other strains (data not shown). Datum points are the averages of three determinations, and error bars indicate one standard deviation of the data. (B) Western blot analysis of whole-cell extracts (10 μg of protein) from growing B. subtilis BSL57 designed to coexpress spoIVF and sigK (lanes 1 to 7) and B. subtilis BSL52 that expresses only sigK (lane 9). Samples were collected at the indicated times after IPTG addition, and whole-cell extracts were prepared as described previously (19). Western blot analysis with anti-pro-σ^K and anti-σ^K antibodies was performed as described previously (19), except the primary antibody was detected with horseradish peroxidase-conjugated secondary antibody (Bio-Rad) and an enhanced chemiluminescence detection system (Amersham). A whole-cell extract (5 μg of protein) of sporulating (T6) wild-type PY79 (30) cells served as a control to indicate the positions of pro-σ^K and σ^K (lanes 8 and 10).
copy number of spoIVFB would further enhance \( \sigma^K \) production in growing *B. subtilis* cells, the spoIVFB gene was fused to \( p_{spac} \) in the multicopy vector pDG148 (24), resulting in pSL16 (18), and then \( sigK \) was cloned downstream of spoIVFB, resulting in pSL17 (18). Since no known transcriptional terminator exists between spoIVFB and \( sigK \) in pSL17, the \( p_{spac} \) promoter is expected to transcribe both genes. In pSL17, \( sigK \) is also fused to \( p_{L20} \), a ribosomal protein promoter located immediately downstream of spoIVFB (4). When we attempted to introduce pSL17 into *B. subtilis*, the transformants grew poorly on Luria-Bertani agar containing kanamycin sulfate (5 \( \mu \)g/ml) and could not maintain stably even in the absence of IPTG. In contrast, pSL16 (capable of expressing only spoIVFB) and pSL27 (a control plasmid similar to pSL17 but capable of expressing only \( sigK \) from \( p_{spac} \) and \( p_{L20} \)) (18) were maintained stably, and neither of these plasmids appeared to hinder growth. Thus, coexpression of spoIVFB and \( sigK \) from the multicopy pSL17 appears to be toxic to *B. subtilis*. Oke and Losick (22) have suggested that \( \sigma^K \) production during growth may be lethal because of the expression of lysis genes normally expressed late in sporulation.

**Coexpressing spoIVFB and sigK in E. coli produces \( \sigma^K \) activity.** \( \sigma^K \)-dependent lysis genes presumably would not be present in *E. coli* cells, so we transformed strain JM103 (29) with pSL17, selecting for resistance to ampicillin (35 \( \mu \)g/ml). We found that pSL17 was maintained stably and did not hinder cell growth. As controls, pSL16 and both pSL27 and pSL1 were also introduced into JM103 cells. To determine whether \( \sigma^K \) was made, the strains were grown in 2\( \times \) YT medium (21) at 37\( ^\circ \)C to the mid-exponential phase, and then IPTG (1 mM) was added. Whole-cell extracts were prepared from cells collected 2 h after the IPTG addition by resuspending 5 \( \times \) 10\(^9\) cells per ml of sample buffer (0.125 M Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 5% 2-mercaptoethanol, 10% glycerol, 0.1% bromophenol blue) and boiling for 5 min. Western blot analyses with anti-\( \sigma^K \) antibodies were performed as described previously (19). Figure 2A shows that a polypeptide comigrating with \( \sigma^K \) was observed in the extract of cells containing pSL17 (lane 3). Very little such polypeptide was detected in extracts of cells containing pSL27 or pSL1 (Fig. 2A, lanes 4 and 2, respectively), and as expected, neither pro-\( \sigma^K \) nor \( \sigma^\alpha \) was detected in extracts of pSL16-containing cells (Fig. 2A, lane 5). Results similar to those shown in Fig. 2A were observed when the entire experiment was repeated (data not shown). These results suggest that SpoIVFB enhances accumulation of a polypeptide that comigrates with \( \sigma^K \) when \( sigK \) is expressed in *E. coli* cells.

To determine whether the polypeptide comigrating with \( \sigma^K \) possessed \( \sigma^K \) activity, proteins in whole-cell extracts were separated by SDS-polyacrylamide gel electrophoresis (10 to 20% polyacrylamide gradient), and polypeptides comigrating with pro-\( \sigma^\alpha \) and \( \sigma^K \) from each cell extract were recovered from the gel and renatured by the method of Hager and Burgess (8) and then added to *B. subtilis* core RNA polymerase prepared as described previously (14) to test their abilities to stimulate transcription from \( \sigma^K \)-dependent promoters in vitro (Fig. 2B). As a control, \( \sigma^K \) RNA polymerase partially purified from sporulating *B. subtilis* as described previously (14) was shown to transcribe cotD (14) and gerE (32) strongly (Fig. 2B, lanes 1 and 7). Strikingly, the polypeptide(s) comigrating with \( \sigma^K \) in the extract of cells containing pSL17 (designed to coexpress spoIVFB and \( sigK \)) stimulated transcription of cotD and gerE strongly (Fig. 2B, lanes 4 and 10). In contrast, the polypeptide(s) comigrating with \( \sigma^K \) in extracts of cells containing pSL27 or pSL1 (expressing only \( sigK \)) stimulated cotD and gerE transcription weakly (Fig. 2B, lanes 3, 5, 9, and 11), while the polypeptide(s) from cells containing pSL16 (expressing only spoIVFB) did not stimulate transcription (Fig 2B, lanes 6 and 12). Also, the polypeptide(s) comigrating with pro-\( \sigma^K \) did not stimulate transcription of either promoter (Fig. 2B, unmarked lanes), consistent with the idea that pro-\( \sigma^K \) is inactive as a sigma factor (14, 19). The results shown in Fig. 2B were reproducible in an independent repetition of the entire experiment (data not shown). These results suggest that SpoIVFB can enhance accumulation of active \( \sigma^K \) from pro-\( \sigma^K \) in *E. coli* cells.

How might SpoIVFB enhance \( \sigma^K \) accumulation in growing *B. subtilis* or *E. coli*? One possibility is that SpoIVFB stabilizes \( \sigma^K \) produced by fortuitous proteolysis. However, we think this explanation is unlikely because \( \sigma^K \) accumulates in the absence of SpoIVFB when \( \sigma^K \) is produced from a truncated \( sigK \) gene in growing cells (9, 22) or in cells during the early stages of sporulation (2, 10). It seems more likely that SpoIVFB increases the processing of pro-\( \sigma^K \) to \( \sigma^K \). Genetic studies suggest that SpoIVFB is intimately involved in pro-\( \sigma^K \) processing and that this is its only role in sporulation (2, 4, 20). Thus, SpoIVFB has been proposed to be either the protease that processes pro-\( \sigma^K \) or a regulator of the processing reaction (2, 4, 19).

The idea that SpoIVFB is the protease that processes pro-\( \sigma^K \) provides a simple explanation for our results. Consistent with this idea, we note that amino acids 40 to 49 (VLI HELGHAA) of SpoIVFB match a motif found in zinc-dependent endopeptidases (13). The hydrophobicity and predicted \( \alpha \)-helical structure of this segment led to its identification as a potential transmembrane domain (4), but these features are also found in the corresponding segment of zinc-dependent endopeptidases (28). If SpoIVFB is the protease that processes
pro-σK, why is the σK level so low in growing cells coexpressing spoIVFB and sigK? It is possible that the synthesis and/or stability of SpoIVFB in growing cells does not allow it to accumulate sufficiently in order to process pro-σK efficiently. Alternatively, the conditions for optimal SpoIVFB activity may not be met during growth. Optimal activity may require that SpoIVFB inserts in the outer forespore membrane of sporulating cells and receives a signal from the forespore (2, 4). Straiger et al. (24) proposed an analogous hypothesis to explain the low level of expression of a σK-dependent gene observed when sigE (encoding pro-σK, the precursor of the early-acting mother cell-specific sigma factor, σK') (5, 25, 27) and spoIIIA (encoding the pro-σ2 processing enzyme or a regulator of processing) (12, 24) were coexpressed in growing B. subtilis cells.

Our results do not exclude the possibility that SpoIVFB functions as a regulator of the processing reaction rather than as the protease. The results we obtained by expressing sigK alone in growing B. subtilis or E. coli cells suggest that these cells possess a mechanism(s) for producing a very small amount of σK in the absence of SpoIVFB. Though not obvious in the Western blots shown in Fig. 1B and 2A, we consistently detected a very small amount of a polypeptide that comigrated with σK in both B. subtilis and E. coli cells expressing only sigK. Consistent with this observation, a low level of σK activity was recovered from E. coli cells expressing only sigK (Fig. 2B), and expression from a gerE-lacZ fusion was considered to be in B. subtilis cells expressing only sigK (Fig. 1A). Perhaps SpoIVFB enhances pro-σK processing by interacting with pro-σK to make the processing site more accessible to protease activity. Alternatively, SpoIVFB may stimulate a weak autoprocessing activity inherent to pro-σK.

We thank R. Losick, A. Grossman, and Z. Zeikus for providing bacterial strains. We thank R. Halberg for providing σK RNA polymerase.

This research was supported by grants GM43585 to L.K. and GM49206 to S.C. from the National Institutes of Health and by the Michigan Agricultural Experiment Station.

REFERENCES