JOURNAL OF BACTERIOLOGY, Feb. 1995, p. 1082–1085 Vol. 177, No. 4
0021-9193/95/$04.00+0

NOTES

Sporulation Protein SpoIVFB from *Bacillus subtilis* Enhances Processing of the Sigma Factor Precursor Pro-σ^K in the Absence of Other Sporulation Gene Products

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Received 12 September 1994 / Accepted 2 December 1994

Processing of inactive pro-σ^K to active σ^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-σ^K) genes in growing *B. subtilis* or *Escherichia coli* enhanced pro-σ^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-σ^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 σ^K in the mother cell (1, 2, 19). Synthesis of σ^K involves a chromosomal DNA rearrangement to generate the composite *sigK* gene (26) and transcription of this gene first by σ^E RNA polymerase and then by σ^K RNA polymerase, in the mother cell (5, 11, 14, 16). However, the primary translation product of *sigK* is an inactive precursor, pro-σ^K (14, 19). Activation of pro-σ^K involves removal of 20 amino acids from its N terminus to produce σ^K, and this proteolytic processing reaction is coupled to events that occur in the forespore (1, 2, 19). Active σ^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-σ^K processing in the mother cell were identified by mutations that relieve the dependence of pro-σ^K processing on forespore events (2). These *bof* (bypass of forespore) mutations mapped to two loci, *bofA* and *spoIVFA*. Genetic studies suggest that BofA negatively regulates pro-σ^K processing until a signal from the forespore is received (23). SpoIVFA 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-σ^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 σ^K-dependent mother cell gene expression (2), and overproduction of pro-σ^K rescues sporulation of a *spoIVF* null mutant, apparently by allowing a small amount of σ^K to accumulate (20). SpoIVFB has been proposed to be either a protease that processes pro-σ^K or a regulator of the processing event (2, 4, 19). To test whether SpoIVFB could mediate pro-σ^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-β-D-thiogalactopyranoside (IPTG)-inducible promoter, P_{spac}, and integrated into the *B. subtilis* chromosome by selecting for transformants (6) on Luria-Bertani agar (21) containing chloramphenicol (5 μg/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-σ^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 spoIIIGΔ1*) (2) were then transformed (6) with a multicopy P_{spac}-*sigK* plasmid (pSL1) that allows production of pro-σ^K during growth (19). Transformants were selected on Luria-Bertani agar containing kanamycin sulfate (5 μg/ml), resulting in the first three strains listed in Table 1. To monitor production of active σ^K, the strains were then lysogenized with SPβ phage bearing *gerE-lacZ*, a σ^K-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 β-galactosidase activity than the IPTG-induced BSL52 derivative capable of expressing only *sigK* (Fig. 1A). Neither strain expressed β-galactosidase

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TABLE 1. *B. subtilis* strains constructed

Strain	Genotype	Plasmid description
BSL52	*bofB8 spoIIIGΔ1* (pSL1)	pSL1 contains P_{spac}-*sigK* in a multicopy vector (19)
BSL57	*bofB8 spoIIIGΔ1 chr*::pSL13 (pSL1)	pSL13 contains P_{spac}-*spoIVFB* in an integrative vector (18)
BSL58	*bofB8 spoIIIGΔ1 chr*::pSL14 (pSL1)	pSL14 contains P_{spac}-*spoIVF* in an integrative vector (18)
BSL58w	*spoIIIGΔ1 chr*::pSL31 (pSL1)	pSL31 is similar to pSL14, except it does not contain the *bofB8* mutation in the *spoIVF* operon (18)
BSL59	*bofB8 spoIIIGΔ1 chr*::pSL25 (pSL1)	pSL25 is similar to pSL13, except the *spoIVFB* gene has an in-frame deletion; upon integration, recombination between sequences upstream of the deletion fused P_{spac} to a full-length *spoIVFB* gene (18)
BSL60	*bofB8 spoIIIGΔ1 chr*::pSL25 (pSL1)	Upon integration of pSL25, recombination between sequences downstream of the deletion fused P_{spac} to an in-frame-deleted *spoIVFB* gene (18)

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 (*spoIIIGΔ1*) (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 *spoIVFB* 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 SpoIVFB activity when the entire *spoIVF* operon is expressed from the P_{spac} promoter in growing cells.

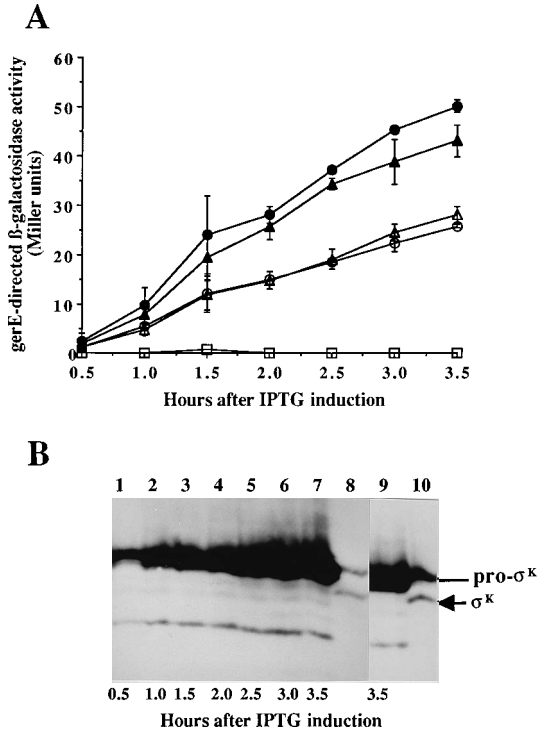

FIG. 1. Coexpressing *spoIVFB* and *sigK* in growing *B. subtilis* cells enhances σ^K-dependent gene expression and accumulation of σ^K. (A) *gerE*-directed β-galactosidase activity was measured as described previously (20) at the indicated times after IPTG addition to growing *B. subtilis* strains designed to coexpress *spoIVFB* and *sigK* (●, BSL57 derivative; ▲, BSL59 derivative), express *sigK* alone (○, BSL52 derivative), or express *sigK* and an in-frame deletion of *spoIVFB* (△, 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* BSL 57 designed to coexpress *spoIVFB* 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 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 (T_6) wild-type PY79 (30) cells served as a control to indicate the positions of pro-σ^K and σ^K (lanes 8 and 10).

To determine whether the twofold enhancement of *gerE-lacZ* expression was due to SpoIVFB, an in-frame deletion of *spoIVFB* lacking amino acids 149 to 276 (nearly half of the coding sequence) was fused to P_{spac} and integrated into the *B. subtilis* chromosome. Depending on the site of the homologous recombination, two outcomes were possible. One has the full-length *spoIVFB* fused to P_{spac} (BSL59 [Table 1]), which is similar to the situation in the BSL57 derivatives. The other has the in-frame deletion of *spoIVFB* fused to P_{spac} (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 SpoIVFB is required for the observed enhancement of σ^K-dependent gene expression during growth of *B. subtilis*.

To determine whether the SpoIVFB-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 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 *spoIVFB* 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 *spoIVFB* 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 SpoIVFB appears to enhance conversion of pro-σ^K to σ^K in the absence of other sporulation-specific gene products.

Coexpressing *spoIVFB* 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 SpoIVFB does not allow it to accumulate sufficiently. To determine whether increasing the

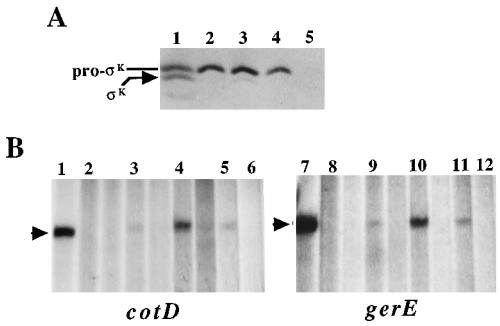

FIG. 2. Coexpressing *spoIVFB* and *sigK* in *E. coli* generates σ^K activity. (A) Levels of pro-σ^K and σ^K in *E. coli* strains containing different plasmids. Proteins in whole-cell extracts (1 μl of a 1:10 dilution in sample buffer) of strains containing pSL1 (lane 2), pSL17 (lane 3), pSL27 (lane 4), or pSL16 (lane 5) were subjected to Western blot analysis with anti-pro-σ^K antibodies. A whole-cell extract (10 μg of protein) of sporulating (T_4) wild-type PY79 (30) *B. subtilis* cells served as a control to indicate the positions of pro-σ^K and σ^K (lane 1). (B) σ^K activity of polypeptides generated in *E. coli*. Polypeptides that comigrated with pro-σ^K and σ^K were recovered from the *E. coli* whole-cell extracts described for panel A and reconstituted with *B. subtilis* core RNA polymerase (1 pmol) and then added to in vitro transcription reaction mixtures containing linearized plasmid DNA (0.5 pmol) as described previously (31). Lanes 1 to 6 *cotD* transcription from pLRK100 (14) digested with *Hin*dIII (225-base transcript); lanes 7 to 12, *gerE* transcription from pSC146 (32) digested with *Hin*dIII (204-base transcript). Run-off transcripts (denoted by arrowheads) of the expected sizes (as judged with end-labeled pBR322/*Msp*I fragments as markers) were produced with partially purified *B. subtilis* σ^K RNA polymerase (lanes 1 and 7). Other reaction mixtures contained core RNA polymerase alone (lanes 2 and 8) or core RNA polymerase plus a polypeptide(s) that comigrated with σ^K from *E. coli* strains bearing pSL1 (lanes 3 and 9), pSL17 (lanes 4 and 10), pSL27 (lanes 5 and 11), or pSL16 (lanes 6 and 12). Unmarked lanes are reaction mixtures containing core RNA polymerase plus a polypeptide(s) that comigrated with pro-σ^K (in each case from the same *E. coli* strain as in the next lane to the right).

copy number of *spoIVFB* would further enhance σ^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 μg/ml) and could not be maintained 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 σ^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 σ^K activity. σ^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 μg/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 σ^K was made, the strains were grown in 2× YT medium (21) at 37°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 5×10^9 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-pro-σ^K antibodies were performed as described previously (19). Figure 2A shows that a polypeptide comigrating with σ^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-σ^K nor σ^K 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 σ^K when *sigK* is expressed in *E. coli* cells.

To determine whether the polypeptide comigrating with σ^K possessed σ^K activity, proteins in whole-cell extracts were separated by SDS-polyacrylamide gel electrophoresis (10 to 20% polyacrylamide gradient), and polypeptides comigrating with pro-σ^K and σ^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 σ^K-dependent promoters in vitro (Fig. 2B). As a control, σ^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 σ^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 σ^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 2 B, lanes 6 and 12). Also, the polypeptide(s) comigrating with pro-σ^K did not stimulate transcription of either promoter (Fig. 2B, unmarked lanes), consistent with the idea that pro-σ^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 σ^K from pro-σ^K in *E. coli* cells.

How might SpoIVFB enhance σ^K accumulation in growing *B. subtilis* or *E. coli*? One possibility is that SpoIVFB stabilizes σ^K produced by fortuitous proteolysis. However, we think this explanation is unlikely because σ^K accumulates in the absence of SpoIVFB when σ^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-σ^K to σ^K. Genetic studies suggest that SpoIVFB is intimately involved in pro-σ^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-σ^K or a regulator of the processing reaction (2, 4, 19).

The idea that SpoIVFB is the protease that processes pro-σ^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 α-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-σ^K, why is the σ^K 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-σ^K 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). Stragier et al. (24) proposed an analogous hypothesis to explain the low level of expression of a σ^E-dependent gene observed when *sigE* (encoding pro-σ^E, the precursor of the early-acting mother cell-specific sigma factor, σ^E) (5, 25, 27) and *spoIIGA* (encoding the pro-σ^E 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 σ^K 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 σ^K in both *B. subtilis* and *E. coli* cells expressing only *sigK*. Consistent with this observation, a low level of σ^K activity was recovered from *E. coli* cells expressing only *sigK* (Fig. 2B), and expression from a *gerE-lacZ* fusion was considerable in *B. subtilis* cells expressing only *sigK* (Fig. 1A). Perhaps SpoIVFB enhances pro-σ^K processing by interacting with pro-σ^K to make the processing site more accessible to protease activity. Alternatively, SpoIVFB may stimulate a weak autoprocessing activity inherent to pro-σ^K.

We thank R. Losick, A. Grossman, and G. Zeikus for providing bacterial strains. We thank R. Halberg for providing σ^K RNA polymerase.

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

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