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Transcription of *spoIVB* Is the Only Role of σ^G That Is Essential for Pro-σ^K Processing during Spore Formation in *Bacillus subtilis*

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Activation of pro-σ^K processing in the mother cell at late stages of sporulation in *Bacillus subtilis* requires the presence of active σ^G in the forespore. Placing the *spoIVB* gene under the control of σ^F, the early forespore transcription factor, allows σ^K to become active in the absence of σ^G. Therefore, transcription of *spoIVB* is the only role of σ^G that is essential for the signaling pathway between σ^G and σ^K.

Bacillus subtilis depends upon an ordered program of gene expression to drive the physiological and morphological changes that result in the production of a bacterial endospore (6). The most profound change to occur is the formation of two compartments in the sporulating cell, the forespore and the mother cell. The forespore, contained within the mother cell, is destined to become the mature endospore that is released at the end of development. The program of differential gene expression that drives this process is controlled temporally and spatially by the successive appearance of new sigma factors (11). In the forespore, two sigma factors, σ^F followed by σ^G, are responsible for directing transcription of distinct sets of genes. Similarly, σ^E and then σ^K direct their own programs of gene expression within the mother cell.

Although the forespore and mother cell follow different developmental fates, cell-cell signaling is used to coordinate gene expression between the two compartments (11). For instance, the mother cell transcription factor σ^K is synthesized first as an inactive proprotein (pro-σ^K [9, 18]). Proteolytic removal of 20 amino acids from the N terminus is essential for activation of σ^K and is coupled to events occurring within the forespore (3, 12). Specifically, expression in the forespore of *spoIVB*, a σ^G-dependent gene, triggers proteolysis of pro-σ^K (2). Processing occurs in the mother cell and requires the combined action of three additional proteins, SpoIVFA, SpoIVFB, and BofA, which are thought to form a heteroligomeric complex in the outer membrane of the forespore (5, 16).

The product of the *spoIVB* gene is a prime candidate for the direct activator of pro-σ^K processing for several reasons. First, *spoIVB* is transcribed by σ^G exclusively in the forespore (2). Second, a null mutation in *spoIVB* abolishes processing of pro-σ^K and results in a morphological phenotype similar to that of mutations in other genes directly involved in the synthesis of pro-σ^K (2, 12). Last, certain changes of function mutations (*bofB*) in the *spoIVFA* gene that allow constitutive processing of pro-σ^K bypass the requirement of the *spoIVB* gene product for activation of σ^K (3, 5).

Since it has not yet been possible to reconstitute the σ^K-activating pathway in artificial conditions, it is conceivable that other genes controlled by sigma G (*csg* genes) are involved in activating pro-σ^K processing. These genes might be essential and act epistatically to, or after, *spoIVB* in the signal transduction pathway that leads to pro-σ^K processing, or they might be nonessential and act as modulators of the signaling event. Conversely, if transcription of *spoIVB*, controlled by σ^G, is the only event required to activate processing of pro-σ^K, then expression of *spoIVB* in the forespore in the absence of σ^G should activate σ^K-directed gene expression. Here, we have used this genetic strategy to demonstrate that expression of *spoIVB*, controlled by σ^G, is the only event essential for the induction of pro-σ^K processing.

σ^G-Independent expression of *spoIVB* in the forespore. To allow forespore-specific expression of *spoIVB* in the absence of σ^G, we placed the *spoIVB* gene under the control of a promoter, *sspE**, efficiently recognized by σ^F (as well as by σ^G [see Fig. 1]). As shown in Fig. 1, the hybrid *spoIVB* gene started to be transcribed approximately 1.5 h earlier than wild-type *spoIVB*. When controlled by both σ^F and σ^G, *sspE***-spoIVB* expression was increased about fivefold compared with wild-type *spoIVB*, whereas it reached 40% of the wild-type level in a strain mutated for *spoIIIG*, the gene encoding σ^G. It can be concluded that the hybrid *spoIVB* gene allows premature and efficient synthesis of SpoIVB in the forespore compartment, even in the absence of σ^G.

Activation of σ^K-directed gene expression in the absence of σ^G. σ^K-directed gene expression was measured in strains lysogenized with a modified SPβ bacteriophage containing a transcriptional fusion of *lacZ* to the σ^K-dependent *gerE* gene (4). As shown in Fig. 2, *gerE-lacZ* expression was induced between the third and fourth hours of sporulation in a wild-type strain and was essentially abolished in a *spoIIIGΔ1* and in a *spoIVB*Δ::*spc* mutant. In an otherwise wild-type background, the presence of the *sspE***-spoIVB* gene led to premature expression of *gerE-lacZ* which started 30 min earlier than in a strain containing only the normal *spoIVB* gene. In a *spoIVB*Δ::*spc* mutant, *gerE-lacZ* expression was fully restored by the presence of *sspE***-spoIVB*, with a similar premature accumulation of β-galactosidase. Most importantly, expression of *spoIVB* under σ^F control restored essentially wild-type levels of *gerE-lacZ* (and *cotA-lacZ* [7]) expression in a *spoIIIGΔ1* mutant, β-galactosidase synthesis starting about 30 min later than in a wild-type strain. Therefore, the requirement for σ^G in activation of σ^K-directed gene expression was bypassed when *spoIVB* was expressed from a σ^F-dependent promoter. Similar results were obtained in a *spoIIIA* mutant in which σ^G is synthesized but remains inactive (7).

Timing requirements for activation of σ^K-directed gene expression. Such a bypass of σ^G for σ^K-directed gene expression is reminiscent of the phenotypes of certain *bofB* mutations in the *spoIVFA* gene (3). As shown in Fig. 3, the *bofB8* mutation

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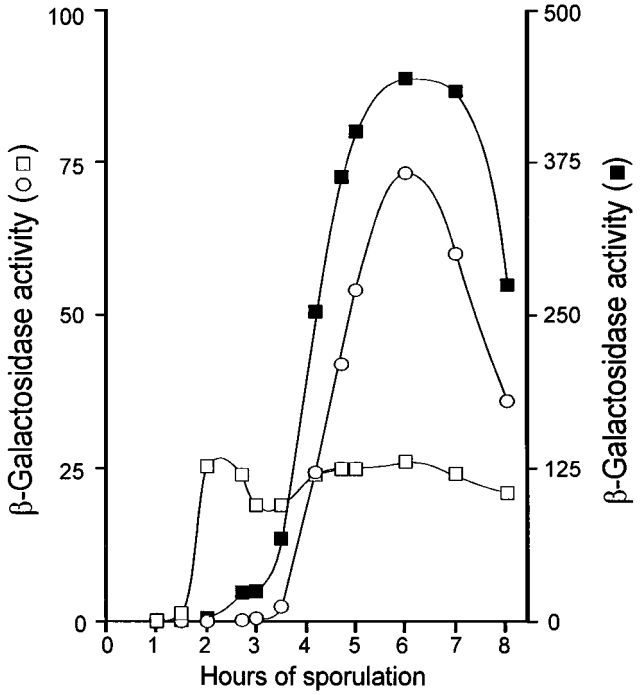

FIG. 1. The σ^F-controlled *sspE** promoter directs premature expression of *spoIVB*. The *spoIVB* coding sequence (from the *Nde*I site located immediately downstream of the proximal *spoIVB* promoter to the *Ssp*I site located 155 bp downstream of the *spoIVB* stop codon [20]) was cloned downstream of the *sspE*-2G promoter (recovered from plasmid pPS1280 [19] as a *Hin*cII-*Bam*HI fragment and hereafter named *sspE**). The recombinant *spoIVB* gene was introduced at the *amyE* locus, and its expression was compared with that of wild-type *spoIVB* by inserting through a Campbell-type recombination event a transcriptional *lacZ* fusion into one of the two *spoIVB* coding sequences. The fusion was constructed by cloning upstream of *lacZ*, in the pTK*lac* vector (14), a PCR-amplified fragment internal to the *spoIVB* reading frame. Depending on the locus in which recombination took place, the fusion disrupted either the wild-type or the recombinant *spoIVB* gene, which could easily be discriminated because of the different germination phenotypes generated (see text). β-Galactosidase synthesis was measured during sporulation (induced by the resuspension method [17]), at the indicated times, in strains containing a *lacZ* transcriptional fusion inserted by a single recombination event either into the *spoIVB* gene at its normal chromosomal position (empty circle) or into the *sspE*-spoIVB* gene at the *amyE* locus (filled square [*spoIIIG*$^+$] or empty square [*spoIIIG*Δ1]). Samples (0.4 ml) were taken at appropriate times and centrifuged, and the cell pellets were stored at −70°C. Frozen cells were thawed and permeabilized with lysozyme (200 μg/ml), and β-galactosidase was assayed as described previously (13), with a BioTek EL112 microplate reader. The background of β-galactosidase activity in wild-type cells without any fusion was subtracted. Note the two different scales.

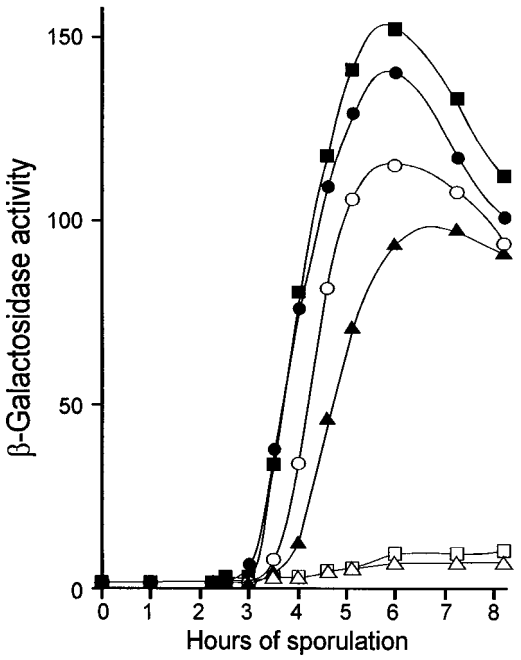

FIG. 2. Effect of premature synthesis of SpoIVB on *gerE-lacZ* expression. β-Galactosidase activity was measured during sporulation, at the indicated times, in PY79-derivative SPβ::*gerE-lacZ* lysogens, in the presence (filled symbols) or the absence (empty symbols) of the *sspE*-spoIVB* gene. Strains were either wild type (circles), *spoIVB*Δ::*spc* (squares), or *spoIIIG*Δ*1* (triangles). The background of β-galactosidase activity in wild-type cells without any fusion was subtracted.

also led to premature expression of a σ^K-dependent *lacZ* fusion, with kinetics very similar to those found with the *sspE*-spoIVB* recombinant gene in an otherwise wild-type strain. To determine the earliest possible time at which σ^K-directed gene expression could occur, we measured expression of a *gerE-lacZ* fusion in cells containing the *spoIVCB*Δ*19* mutation, which allows synthesis of an active σ^K protein lacking the pro-σ^K leader sequence (3). In the *spoIVCB*Δ*19* mutant, *gerE*-directed β-galactosidase synthesis started approximately 1 h earlier than in wild-type cells and 30 min earlier than in *bofB8* mutant cells. This difference in timing may reflect the time normally required for accumulation, insertion, and assembly of the SpoIVFA-SpoIVFB-BofA protein complex in the outer forespore membrane before pro-σ^K processing can commence. Alternatively, some critical point in the morphological development of the forespore may need to be reached before the SpoIVFA-SpoIVFB-BofA complex can activate pro-σ^K processing, as suggested by the fact that the *bofB* mutations do not bypass mutations in the *spoIID* gene which block the engulfment process (1). In any case, our results suggest that pro-σ^K processing cannot be advanced by more than 30 min, either by inactivation of the SpoIVFA inhibitor or by premature synthesis of SpoIVB.

The unique role of SpoIVB in activation of pro-σ^K processing. In a *spoIIIG*Δ*1* mutant containing the *sspE*-spoIVB* recombinant gene and where *spoIVB* was exclusively under σ^F control, *gerE-lacZ* expression was delayed by about 30 min compared with wild-type cells (Fig. 2). The simplest explanation for this delay is that the rate of accumulation of SpoIVB protein when expressed from the σ^F-dependent *sspE** promoter is lower than that from its own σ^G-dependent promoter. However, it cannot be excluded that there is at least one other gene, controlled by both σ^F and σ^G, that is required to activate processing of pro-σ^K. In a *spoIIIG* mutant containing the *sspE*-spoIVB* gene, the amount of this putative gene product would be dependent on σ^F activity alone and substantially reduced. Thus, a delay would be introduced in activating σ^K because of the time required for this protein to reach a critical concentration level before allowing SpoIVB-mediated activation of pro-σ^K processing. But even if there are such genes involved in the transduction pathway between the forespore and the mother cell at stage III, our results demonstrate that σ^G-controlled expression of *spoIVB* is the only event required for induction of σ^K activation. This is similar to the transduction pathway coupling gene expression in the forespore and the mother cell at stage II where the *csfX* gene (also known as *spoIIR* [8]) is the only σ^F-dependent gene required for activation of pro-σ^E processing (10).

Premature expression of *spoIVB* leads to formation of defective spores. As has been reported previously, early processing of pro-σ^K impairs spore formation both by reducing the efficiency of sporulation and by producing spores that are germination defective (3). We examined the effect of premature

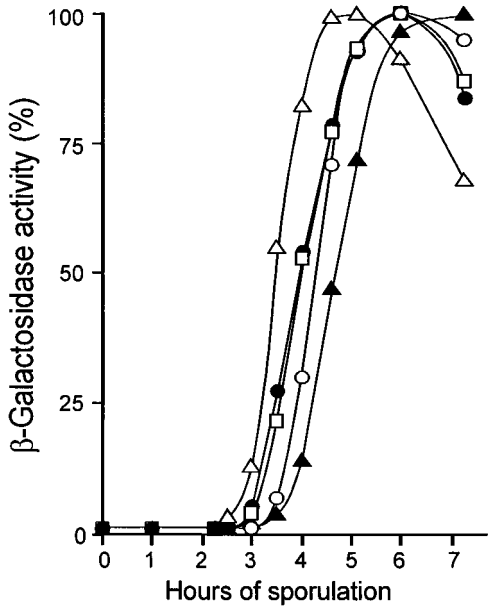

FIG. 3. Effect of various mutations on the timing of *gerE-lacZ* expression. β-Galactosidase activity was measured during sporulation, at the indicated times, in PY79-derivative SPβ::*gerE-lacZ* lysogens. Strains were either wild type (empty circle), *spoIVCBΔ19* (empty triangle), *bofB8* (empty square), *amyE*::[*sspE*-spoIVB*] (filled circle), or *spoIIIGΔ1 amyE*::[*sspE*-spoIVB*] (filled triangle). β-Galactosidase activity is shown as a percentage of the maximum level obtained for each fusion.

spoIVB expression on spore formation and spore germination (Table 1). Our results show that early synthesis of SpoIVB in an otherwise wild-type background reduced the efficiency of spore formation by almost 50-fold. In addition, those spores that were produced were found to germinate defectively.

This effect on spore formation and spore germination is very similar to that seen in cells containing the *spoIVCBΔ19* mutation and in cells containing the *bofB8* mutation (Table 1).

TABLE 1. Effect of premature *spoIVB* expression on spore formation and spore germination

Relevant genotype[a]	Spore formation[b]	Germination[c]
spo$^+$	100	Normal
sspE-spoIVB*	2	Defective
spoIVBΔ::spc	<0.01	ND
spoIVBΔ::spc amyE::[*sspE*-spoIVB*]	2.5	Defective
spoIIIGΔ1	<0.01	ND
spoIIIGΔ1 amyE::[*sspE*-spoIVB*]	<0.01	ND
spoIVCBΔ19	3	Defective
bofB8	10	Defective

[a] In the strains containing the alleles *sspE*-spoIVB*, *spoIVCBΔ19*, and *bofB8*, σ^K is activated at least 30 min earlier than in a wild-type strain.

[b] The percentage of heat-resistant spores was determined by removing 1-ml samples of sporulating cultures 9 h after resuspension in sporulation medium and comparing the CFU per milliliter before and after incubation at 65°C for 30 min. Values are expressed as the percentage of CFU obtained from the same cultures immediately prior to heat treatment. The results shown are the average from at least two independent experiments.

[c] Spore germination response was measured by preparing suspensions of highly purified spores by gradient centrifugation as described previously (15). Suspensions containing greater than 95% phase-bright spores were adjusted in volume to give an optical density at 600 nm of 3.0. A total of 25 μl of this concentrated spore suspension was spotted onto nitrocellulose filter discs, and the germination response (i.e., the rate of germination compared with that of wild type) of spores embedded in the filter was determined by the tetrazolium plate transfer test (15). ND, not determined.

Such a phenotype is indicative of deregulated activation of σ^K which leads to premature synthesis and assembly of the spore coats onto the forespore (3). Similarly, in cells containing the *spoIVBΔ::spc* mutation and *sspE*-spoIVB*, even though the defect caused by the *spoIVB* mutation was complemented (since heat-resistant spores were produced), early processing of pro-σ^K caused a 50-fold reduction in sporulation efficiency compared with the wild type and with the formation of germination-defective spores. These results strongly suggest that the purpose of coupling forespore and mother cell gene expression is to ensure that σ^K is activated at a precise time in the developmental cycle such that σ^K-directed gene expression is held in register with development of the forespore.

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