Forespore-specific transcription of a gene in the signal transduction pathway that governs Pro-σ^K processing in Bacillus subtilis

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We present studies on the regulation of a developmental gene (spolVB) whose product is required at a late stage of morphogenesis during the process of sporulation in Bacillus subtilis. Earlier work implicated the spolVB gene product in a signal-transduction pathway that governs the conversion of pro-σ^K to the mature and active form of the mother cell σ factor, σ^K, in response to a signal generated within the forespore chamber of the sporangium. We now show that (1) spoIVB is induced at the engulfment stage of sporulation, (2) this transcription is restricted to the forespore, and (3) spolVB is under the direct control of the forespore σ factor σ^K. The discovery that spolVB is a forespore-expressed gene suggests that the spoIVB gene product, or a developmental event under its control, triggers the processing of pro-σ^K and thereby mediates the coupling of σ^K-directed gene expression in the mother cell to σ^K-directed gene expression in the forespore. We also show that spoIVB transcription is partially dependent on the action of the mother cell regulatory gene spolIID, a finding that suggests that the transcription of certain forespore-expressed genes is influenced by events in the mother cell.

[Key Words: Bacillus subtilis; sporulation; σ factor; signal transduction]

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The earliest morphological manifestation of the process of sporulation in the Gram-positive bacterium Bacillus subtilis is the formation of an asymmetrically positioned transverse septum, which partitions the developing cell into separate mother cell and forespore compartments. The two compartments, each of which receives a chromosome from the last round of vegetative DNA replication, follow separate pathways of cellular differentiation. Thus, the forespore metamorphoses into the dormant cell type of the endospore, while the mother cell, which participates in the formation of the spore, lyases when maturation of the spore is complete.

Gene expression in the two compartments is regulated differentially, with certain genes being expressed in the forespore and others in the mother cell (for review, see Stragier and Losick 1990). This differential gene expression is, in turn, controlled by the action of at least three compartment-specific transcriptional regulatory proteins. Forespore gene expression is controlled by the regulatory gene spoIIIG, whose product is the RNA polymerase σ factor σ^K, which is present in the forespore, where it directs the transcription of forespore-expressed genes [Masuda et al. 1988; Karmazyn-Campelli et al. 1989; Sun et al. 1989]. Gene expression in the mother cell, on the other hand, is controlled in part by the action of two mother-cell regulatory genes, spoIIID and sigK. spoIIID encodes a small, DNA-binding protein that (1) governs the creation of sigK, a composite of two truncated genes, by a DNA rearrangement in the mother cell chromosome, and (2) directs the subsequent transcription of the composite gene (Kroos et al. 1989; Kunkel et al. 1989, 1990; Stragier et al. 1989; Sato et al. 1990; Stevens and Errington 1990). The product of sigK is the mother-cell σ factor σ^K, which directs the transcription of genes involved in the formation of the cortex and coat compartments of the spore [Kroos et al. 1989].

Although separately controlled by different transcriptional regulatory proteins, the mother cell and forespore lines of gene expression are not entirely independent. Thus, mutations in the forespore regulatory gene spoIIIG, as well as mutations in two other loci (spoIIIA and spoIIIE) whose products are needed for σ^K-directed gene expression, prevent σ^K-directed gene expression in the mother cell [Sandman et al. 1988; Cutting et al. 1989, 1990; Zheng and Losick 1990]. Recent work has shown that this intercompartmental coupling is mediated at the level of the conversion of an inactive pro-protein form.
[pro-σ^K] to the mature and active σ factor [Kroos et al. 1989; Cutting et al. 1990; Lu et al. 1990]. Genetic experiments identify the products of spoIVF [a two-cis-ron operon, S. Cutting, S. Roels, and R. Losick, unpubl.] as the pro-σ^K processing enzyme or as a regulator of the processing enzyme [Cutting et al. 1990]. It is believed that a signal emanating from within the developing forespore stimulates the activity of the spoIVF gene products in the mother cell, thereby promoting the conversion of pro-σ^K to its active form [Cutting et al. 1990]. This work also led to the identification of a fourth gene [spoIVB] of unknown function whose product evidently lies in the signal transduction pathway that leads from gene expression in the forespore to SpoIVF-mediated processing of pro-σ^K in the mother cell.

Here we report studies on the regulation of the spoIVB gene, whose product is a 46-kD polypeptide and whose transcription is known to commence at an intermediate stage of sporulation [Van Hoy and Hoch 1990]. Using a previously constructed transcriptional fusion of spoIVB to the lacZ gene of Escherichia coli [Van Hoy and Hoch 1990], we show that spoIVB is induced at approximately the engulfment stage of sporulation and that this transcription, which is shown herein to be under the direct control of the forespore σ factor σ^C, is confined to the forespore compartment of the developing sporangium. On the basis of these results and those published previously [Cutting et al. 1990], we infer that SpoIVB lies between σ^C and SpoIVF in the signal transduction pathway that controls pro-σ^K processing in the mother cell. Unexpectedly, spoIVB transcription and, to a lesser extent, transcription of certain other forespore-expressed genes, is found to depend partially on the product of the mother cell regulatory gene spoIID. This finding suggests that σ^C-directed gene expression in the forespore is influenced by events occurring within the mother cell.

Results

Timing and compartmentalization of spoIVB-lacZ expression

The time course experiment of Figure 1 shows that spoIVB-lacZ expression was induced slightly later than a lacZ fusion to the forespore-expressed gene sspB and significantly later than lacZ fusions to the forespore regulatory gene spoIIIG and the mother cell regulatory gene spoIID. These results and other findings presented below indicate that spoIVB transcription commences during engulfment, the stage [III] at which the forespore becomes pinched off as a free protoplast within the mother cell.

Since spoIVB is induced after the sporangium has been partitioned into two compartments, we wondered whether its transcription was compartmentalized. We therefore compared the accessibility of fusion-encoded β-galactosidase to ONPG [o-nitro-phenyl-β-D-galacto-side] substrate in cells treated with toluene to that observed in cells treated with lysozyme [Mason et al. 1988]. β-Galactosidase sequestered in the forespore is partially "masked" from the action of toluene but not from lysozyme because the forespore is somewhat resistant to the permeabilizing effect of the organic solvent, but not to the digestive effect of the cell wall-degrading enzyme. In contrast, because toluene readily permeabilizes the mother cell, fusion-encoded β-galactosidase produced in the mother cell is fully accessible to ONPG in cells that have been treated with either toluene or lysozyme. The time course experiment of Figure 2B shows that treatment of cells bearing a lacZ fusion to the forespore-expressed gene sspB with lysozyme revealed considerably more β-galactosidase activity (open squares) than did treatment of the cells with toluene (solid squares). As a further indication of sequestration, treatment of the cells with SDS, which inactivates enzyme in the mother cell, followed [after washing to remove the detergent] by lysozyme treatment to release the contents of the forespore, showed that after hour 6 of sporulation, at least some sspB-lacZ fusion-encoded β-galactosidase had been rendered inaccessible to the action of the detergent (open circles). In contrast, lysozyme and toluene treatment released similar levels of fusion-encoded β-galactosidase from cells harboring a lacZ fusion to the mother cell-expressed gene gerE (Fig. 2C). Furthermore, essentially all of the gerE–lacZ-produced enzyme was inactivated by treatment of the cells with SDS. As the patterns [Fig. 2A] of accessibility of β-galactosidase produced under the direction of the spoIVB–lacZ fusion closely mimicked those observed with the sspB–lacZ fusion, we infer that spoIVB is a forespore-expressed gene.

Use of immuno-electron microscopy to localize spoIVB-directed β-galactosidase synthesis

As an independent test of compartmentalization, we used immuno-electron microscopy to visualize the

![Figure 1. A comparison of the time course of expression of spoIVB–lacZ to that of other spo–lacZ fusions. Isogenic strains (Spo^+) containing different lacZ fusions were induced to sporulate by resuspension in SM medium, and samples were taken at the indicated times after the onset of sporulation (T0). Cells were permeabilized by lysozyme treatment, and β-galactosidase-specific activity was determined. Levels of β-galactosidase activity are shown as a percentage of the maximum levels obtained for each fusion. The fusions were spoIIIG–lacZ (○; PM30), spoIID–lacZ (■; BK55S), spoIVB–lacZ (□; SC1080), and sspB–lacZ (●; SC780).](genesdev.cshlp.org)
location of spolVB-directed β-galactosidase synthesis [Francesconi et al. 1988]. Because the level of expression of spolVB–lacZ (~100 Miller units) is too low to be detected by immuno-electron microscopy, we increased the copy number of the gene fusion by subjecting cells (strain SC1080) containing a chromosomally integrated copy of the spolVB–lacZ-bearing plasmid pJB2026 to successive rounds of growth in the presence of increasing concentrations of chloramphenicol to select for amplification of the integrated plasmid. pJB2026 contains the cat chloramphenicol-resistance gene.) Tandem chromosomal arrays selected in this manner are known to be relatively stable and are maintained for several generations of growth in the absence of drug selection [Janniere et al. 1985]. At a level of resistance to 200 μg/ml of drug, a strain was obtained that was capable of accumulating β-galactosidase to a sp. act. of 900 Miller units when grown and sporulated in the absence of chloramphenicol. Despite the increase in copy number, the temporal pattern of spolVB–lacZ expression was similar to that of the parental strain SC1080 [data not shown].

Thin sections of cells of the amplified, drug-resistant strain that had been harvested at hour 8 of sporulation were prepared. The sections were treated with anti-β-galactosidase antibodies, and antibodies bound on the surface of the sections were then visualized by applying gold-conjugated secondary antibodies. The level of decoration varied from cell to cell, but among cells exhibiting a significant level of decoration, gold granules were far more prevalent in the forespore than in the mother cell. Several examples of decorated sporangia are shown in Figure 3. As a control, little decoration was observed when cells lacking spolVB–lacZ were subjected to immuno-electron microscopy [A. Driks, data not shown]. Also, when cells containing lacZ fusions to genes [cotA and gerE] under the control of the mother cell transcription factor αK were subjected to immuno-electron microscopy at hour 8 of sporulation, decoration by gold granules was principally restricted to the mother cell [A. Driks, data not shown].

**Dependence of spolVB expression on the products of other spo genes**

spolVB–lacZ was introduced by transformation into a collection of isogenic strains, each containing a different sporulation mutation. The resulting fusion-bearing strains were induced to sporulate by suspension in SM [Sterlini–Mandelstam] medium. Samples were taken at various times throughout sporulation and assayed after lysozyme extraction for spolVB–lacZ-directed β-galactosidase synthesis. Time course experiments were done in parallel with an otherwise isogenic Spo+ strain containing the spolVB–lacZ fusion [strain SC1080]. The pattern of spolVB expression in all mutants is summarized in Table 1, and some representative time course experiments are shown in Figure 4. In addition to spolVB, we determined the dependence patterns of the forespore-expressed genes sspA, sspB, and sspE (Table 1; Fig. 4).

Our results first show that the dependence pattern of spolVB expression was similar to that of the other forespore-expressed genes. Second, three levels of dependence were observed for the mutations examined: those that caused a strong block in spolVB and ssp expression [e.g., spolIG35, spolIHA35, spolIIE36, spolIIGA1], those that had no effect on expression [e.g., spolIIIC94, spolIVCB23], and those that caused a partial impairment of expression (i.e., spolIIDD83 and spolIIDΔ :: erm).

These results are generally consistent with previous reports on the regulation of ssp genes [Mason et al. 1988]. Furthermore, the observed block in spolVB [and ssp] expression caused by spoIIA, spoIIG, and spoIIIE mutations is consistent with the view that expression of this sporulation gene is confined to the forespore. Both the spoIIIG and spoIIIE gene products are known to be needed for efficient forespore-specific gene expression [Mason et al. 1988; Foulger and Errington 1989; Karmazyn-Campelli et al. 1989]. A recent report indicates that the spoIIA gene products are not important regulators of forespore gene expression [Illiing and Errington 1990], but our results show that spoIIA mutations substantially impaired the transcription of the known forespore-expressed ssp genes while also preventing spolVB transcription.

The partial impairment of spolVB and ssp expression caused by mutations in the mother cell regulatory gene spoIID merits further comment. First, the extent of the
impairment of spoIVB expression varied from experiment to experiment, ranging from a strong block (3%) to a partial block (31%). We do not understand the basis for this variability. Second, spoIVB expression was impaired more severely by spoIIIID mutations than was ssp expression. Nevertheless, spoIIIID mutations did cause a noteworthy (two- to threefold) inhibition of sspA, sspB, and sspE expression, the extent of inhibition being somewhat greater than that observed in previous dependence studies of sspA and sspE expression (Mason et al. 1988).

Use of mosaic sporangia to verify that the requirement for the spoIIIID gene product is restricted to the mother cell

The partial dependence of spoIVB and sspA, sspB, and sspE expression on the spoIIIID gene product was surprising in light of earlier genetic experiments, which indicated that expression of spoIIIID is only required in the mother cell (De Lencastre and Piggot 1979). As a further test of whether the requirement for spoIIIID expression is confined to the mother cell, we used the recently devised, integrational plasmid excision method of Illing et al. (1990) to create "mosaic sporangia" bearing a wild-type chromosome in one compartment and a chromosome with a disrupted spoIIIID gene in the other compartment. An integrational plasmid (pBK56) containing an internal fragment (200 bp) of spoIIIID was used to transform wild-type cells (Spo+) of strain PY79 to CmR (pBK56 contains the cat gene). The resulting transformants were Spo− due to integration of the plasmid into the chromosome at the spoIIIID locus by a Campbell-like recombination. Two independent transformants were then grown and sporulated. In each case, the number of
Table 1. Effect of spo mutations on spoIVB, sspA, sspB, and sspE-directed β-galactosidase synthesis

| Relevant mutation* | spoIVB | sspA | sspB | sspE |
|--------------------|--------|------|------|------|
| spoIIC94           | 103    | 105  | 95   | 102  |
| spoIID83           | 6-31   | 30-38|
| spoIIA35           | 9      | 9    | 5    | 5    |
| spoIIA53           | 6      | 5    | 3    | 4    |
| spoIIC49           | 8      | 11   | 6    | 8    |
| spoIIA65           | 5      | 2    |      |      |
| spoIIC94           | 103    | 105  | 95   | 102  |
| spoIID83           | 6-31   | 30-38|
| spoIIA35           | 9      | 9    | 5    | 5    |
| spoIIA53           | 6      | 5    | 3    | 4    |
| spoIIC49           | 8      | 11   | 6    | 8    |
| spoIIA65           | 5      | 2    |      |      |
| spoIVA67           | 98     |      |      |      |
| spoIVA178          | 128    |      |      |      |
| spoIVB :: erm      | 41     |      |      |      |
| spoIVA1933         | 123    |      |      |      |
| spoIVC823          | 111    | 113  | 83   |      |
| spoIVF152          | 108    |      |      |      |

*Strains carrying the indicated allele were transformed with chromosomal DNA containing either a spoIVB-lacZ, sspA-lacZ, sspB-lacZ, or sspE-lacZ fusion.

β-expressed as a percentage of the level of β-galactosidase activity observed in wild-type cells at peak activity (~6-8 hr) after resuspension in SM medium. The values given are means of at least two independent experiments, with the exception of the spoIIC94 alleles in which case the range of values obtained are shown. Typical wild-type levels of β-galactosidase activity (in Miller units) for each lacZ fusion were spoIVB, 80-200, sspA/E, 400-500; and sspB, 500-5000.

CmR (Spo-) heat-resistant cells/ml was determined 24 hr after the start of sporulation. The results of these experiments showed that the excision of pBK56 occurred at a very low frequency (1 excision per 10³ to 10⁴ cells) during growth and sporulation and generally resulted in the production of normal [Spo+] heat-resistant cells, presumably due to recombination of the wild-type spoIID gene (Table 2). However, a small but significant number of the heat-resistant spores were found to be both CmR and Spo-. These presumably arose from mosaic sporangia bearing a wild-type copy of spoIID in the mother-cell chromosome. Their existence indicates that expression of spoIID is required only in the mother cell, a finding in agreement with the original experiments of De Lencastre and Piggot (1979). Thus, spoIID action is confined to the mother cell, and the partial dependence of forespore gene expression on the spoIID product is presumably an indirect effect, reflecting an influence of mother cell development on the level of gene transcription in the forespore.

spolVB is under the direct control of the forespore σ factor σ²

Despite the unexpected inhibitory effects of spoIIDD mutations on forespore gene expression, the dependence studies indicate that spolVB [like sspA, sspB, and sspE] is under the direct control of the forespore transcription factor σ². As an independent approach to testing whether spolVB transcription is under σ² control, we investigated whether σ² is capable of directing spolVB transcription in cells that have been engineered to produce the forespore transcription factor during vegetative growth. Accordingly, we introduced the autonomously replicating plasmid pDG298 into cells containing a spolIIGΔI mutation and the spolIVB-lacZ fusion by selection for kanamycin resistance (10 μg/ml). pDG298 carries lacI and a modified spoIIG gene that lacks its own promoter but is instead fused to the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible spo promoter (Sun et al. 1989), such that transcription of spoIIG can be induced by addition of IPTG. Since spoIIG is autoregulatory, the spolIIGΔI mutation was included in our strain construction to ensure that no transcription of the chromosomal spoIIG gene occurred. Figure 5 shows that addition of IPTG caused a rapid induction of spolIVB-directed β-galactosidase synthesis.

Consistent with the view that spolIVB is transcribed by σ²-RNA polymerase is the similarity of the −10 and −35 sequences of its P1 promoter [the principal promoter governing spolIVB transcription (Van Hoy and Hoch 1990)] to the corresponding consensus sequences for σ²−recognized promoters (Nicholson et al. 1989). Thus, the spolIVB −10 region [tATcCaA] matches the consensus −10 region [CATACTA] in four of seven po-
As a demonstration that the Cm R (Spo) cells did not arise from leakiness in the requirement for the medium with no antibiotic selection.

For comparison, the parent strain PY79 produced \(1 \times 10^3\) CFU/ml to determine the proportion of cells that still contained an integrated plasmid. Sporulation was induced in experiments 1 and 2 by resuspension in SM medium containing chloramphenicol (5 \(\mu\)g/ml) and in experiment 3 by exhaustion in DS medium with no antibiotic selection.

The indicated strains were induced to sporulate, and samples were taken 24 hr after the onset of sporulation (\(T_2n\)). Each sample was heat-treated (82°C, 15 min), and appropriate dilutions were plated on DS agar plates with and without chloramphenicol (5 \(\mu\)g/ml) to determine the proportion of cells that still contained an integrated plasmid. Sporulation was induced in experiments 1 and 2 by resuspension in SM medium containing chloramphenicol (5 \(\mu\)g/ml) and in experiment 3 by exhaustion in DS medium with no antibiotic selection.

For comparison, the parent strain PY79 produced 1 \(\times 10^4\) heat-resistant CFU/ml.

A demonstration that the Cm R (Spo) cells did not arise from leakiness in the requirement for the spoIID gene product, a strain bearing a nonreversible (nonexcisable) spoIID mutation (spoIID\(\Delta\)erm: Kunkel et al. 1989) produced <10 heat-resistant CFU/ml in parallel experiments.

The spoIVB –35 region [TGC\(\Delta\)AcA] matches the consensus –35 region [TGAATA] in four of six positions.

**The spoIVB gene product is required for normal cortex and coat formation**

Having established that spoIVB is a forespore-expressed gene, we were interested in investigating the nature of the requirement for its product in morphogenesis. The structure of a spoIVB :: ermG mutant sporangium [Fig. 6B] is compared with that of wild-type [Fig. 6A] and spoIVCB23 mutant sporangia [Fig. 6C]. spoIVCB23 is a mutation in the gene for the amino-terminal portion of \(\sigma^K\). The electron micrographs show that the spoIVCB mutant, the spoIVB mutant was defective in cortex and coat formation. We also examined the block caused by the original spoIVB mutation of Coote (1972), who did not observe a defect in cortex formation in spoIVB165 cells. Our results showed that like the null mutant, spoIVB165 cells are defective in cortex as well as coat formation [data not shown].

**Discussion**

We have studied temporal, spatial, and genetic aspects of the regulation of the stage-IV sporulation gene spoIVB. Our principal conclusion is that transcription of spoIVB is induced in the forespore chamber of the sporangium. This conclusion rests on the following evidence: [1] Transcription of spoIVB is turned on at the engulfment stage of sporulation; [2] \(\beta\)-galactosidase produced by a spoIVB–lacZ fusion is sequestered in the forespore; and [3] spoIVB transcription depends on, and is under the direct control of, the forespore \(\sigma\) factor \(\sigma^{\omega}\). Thus, spoIVB is to be added to the growing list of genes and operons [e.g., spoIIIG, spoVA, sspA-E, gdh, gcrA, and 0.3 kb (Errington and Mandelstam 1986b, Mason et al. 1988; Rather and Moran 1988; Karmazyn-Campelli et al. 1989, Panzer et al. 1989; Feavers et al. 1990)], whose transcription are confined to the forespore. However, spoIVB is only the third example [the other two being spoIIIG (Karmazyn-Campelli et al. 1989) and spoVA (Errington and Mandelstam 1986b)] of a forespore-expressed gene whose product is required for spore formation [i.e., spoIVB mutations are Spo\(^{-}\)]

The discovery that spoIVB is a forespore-expressed gene provides an important insight into the signal transduction pathway that couples \(\sigma^K\)-directed gene expression in the mother cell to \(\sigma^{\omega}\)-directed gene expression in the forespore. Earlier work showed that the mother-cell \(\sigma\) factor \(\sigma^K\) is initially produced as an inactive pro-protein, pro-\(\sigma^K\), which is then converted to the mature and active transcription factor \(\sigma^{\omega}\).

![Figure 5](image-url)

**Figure 5.** IPTG-dependent induction of spoIVB–lacZ-directed \(\beta\)-galactosidase synthesis. spoIIIGA1 cells containing the spoIVB–lacZ fusion and the Pspac–IIIG plasmid pDG298 [strain RS208] were grown in 2XYT medium [with kanamycin 10 \(\mu\)g/ml]. Plasmid pDG298 carries the spoIIIG gene under the control of the spac promoter. At an OD\(_{600}\) of 0.3, the culture was split, and IPTG [to a final concentration of 1 mM] was added to one portion and growth was continued. Samples were removed and assayed for \(\beta\)-galactosidase at the indicated times after IPTG induction. Symbols: |O| Without the addition of IPTG, |\(|\) with the addition of IPTG.
Processing of pro-σ^K depends on the products of five stage III–IV loci, spoIIB, spoIIE, spoIIG, spoIVB, and spoIVF (Cutting et al. 1990; Lu et al. 1990). Based in part on the discovery of change-of-function mutations (bofB, Cutting et al. 1990) in spoIVF that bypass the requirement for the other four gene products in σ^K-directed gene expression, we inferred that spoIVF encodes the pro-σ^K protease or a regulator of the protease and that the spoIIB, spoIIE, and spoIIG gene products generate a signal within the forespore that is transduced to the mother cell where it stimulates the activity of the spoIVF gene products.

The present finding that the transcription of spoIVB depends on the products of spoIIB, spoIIE, and spoIIG and is under the control of σ^K indicates that SpoIVB acts after SpoIIB, SpoIIE, and SpoIIG [σ^K] in the pathway leading to the stimulation of the spoIVF gene products. Likewise, as noted above, the finding (Cutting et al. 1990) that the requirement for SpoIVB in σ^K-directed gene expression can be eliminated by bofB mutations in spoIVF indicates that the action of SpoIVB precedes that of the spoIVF gene products. Thus, in our model (Fig. 7), the chain of events leading to the processing of pro-σ^K is as follows: First, SpoIIB, SpoIIE, and SpoIIG turn on the transcription of spoIVB in the forespore. Next, the product of spoIVB, or a developmental event under its control, stimulates the activity of the spoIVF gene products, thereby promoting the processing of pro-σ^K. An important feature of our model is that SpoIVB or an event under its control is the signal that stimulates pro-σ^K processing and thereby links σ^K-directed gene expression in the forespore to σ^K-directed gene expression in the mother cell. In support of our model, spoIVB is the only σ^K-controlled gene known whose product is required for pro-σ^K processing. Also, in agreement with the view that an important aspect of SpoIVB function is to stimulate pro-σ^K processing, the phenotype of a spoIVB mutant (Fig. 6B; impaired in cortex and coat formation) is similar to that of mutants in mother cell genes, such as spoIVCB (Figs. 6C, spoIVC, spoIVCA, and spoIVF [Piggot and Coote 1976], that are directly involved in the production of σ^K. [However, stimulation of pro-σ^K processing is not the only function of SpoIVB, since the block in spore formation caused by spoIVB:ermG is not relieved by mutations that bypass the requirement for SpoIVB in σ^K-directed gene expression (Cutting et al. 1990, S. Cutting, unpubl.)].]

A remaining mystery is the question of exactly how SpoIVB stimulates the activity of the spoIVF gene products. No clue is provided by computer analysis of the 425-amino-acid-long predicted product of spoIVB, which does not resemble a known bacterial protein (Van Hoy and Hoch 1990). A simple hypothesis is that SpoIVB interacts directly with SpoIVF across the membranes separating the two compartments, with SpoIVB being localized in the forespore membrane and SpoIVF being integrated into the mother cell membrane that engulfs the forespore.
Although its precise role in the pro-σK signal transduction pathway is not known, spoIVB is to be added to the list of genes whose products are intimately involved in the production of σK. These genes are spoIVCB and spoIII C, the truncated coding elements for σK [Stragier et al. 1989]; spoIVCA, the recombinate gene that governs the construction of the composite σK-coding sequence [Kunkel et al. 1990, Sato et al. 1990]; spoIII D, the regulatory gene that governs both the rearrangement and the transcription of the rearranged gene [Kunkel et al. 1989, 1990]; and the two members (A and B) of the spoIVF operon, which govern the processing of pro-σK [Cutting et al. 1990, S. Cutting, S. Roels, and R. Losick, unpubl.]. Thus, the activation of cortex and coat genes in the mother cell may depend on the action of as many as seven gene products that participate in the production of the mother cell σ factor.

A further aspect of the present work that bears on the issue of interactions between the mother cell and the forespore is the unexpected finding that spoIVB transcription is significantly dependent on the product (SpoIII D) of the mother cell regulatory gene spoIII D. Indeed, the transcription of several forespore-expressed genes (e.g., sspA, sspB, and sspE) was found to be partially impaired in spoIII D mutant cells, although not as severely as spoIVB transcription. Earlier experiments of various kinds indicate that spoIII D is a mother cell-expressed gene and that its product is required exclusively in the mother cell [De Lancastre and Piggot 1979; Errington and Mandelstam 1986b; Kunkel et al. 1989, Stevens and Errington 1990]. The most important of these are the experiments of De Lancastre and Piggot (1979), which showed that mosaic sporangia in which the forespore chromosome harbors a spoIII D mutation produce heat-resistant (but genotypically spo−) spores, a finding that we have confirmed by use of the integrational plasmid, gene disruption method of Illing et al. [1990]. We therefore infer that the partial requirement of SpoIII D in forespore gene expression is an indirect effect, being the consequence of SpoIII D action in the mother cell. Since the forespore at engulfment and later stages is entirely contained within the mother cell, it is logical to suppose that normal forespore metabolism (and, hence, efficient forespore gene transcription) depends on the normal functioning of the mother cell. Thus, the block in mother cell development caused by spoIII D mutations could indirectly hamper macromolecular synthesis in the forespore, which may depend on the mother cell for energy and metabolites. In this view, spoIVB transcription is impaired more severely by spoIII D mutations than is transcription of ssp genes simply because spoIVB is induced slightly later than these other forespore-expressed genes. Alternatively, SpoIII D or the product of a gene under its control may be part of a specific signal transduction pathway that somehow influences the transcription of certain forespore-expressed genes.

SpoIII D is not the only example of a mother cell gene whose product is required for efficient forespore gene expression. Genetic mosaic experiments indicate that expression of the spoIII A operon is exclusively required in the mother cell [De Lancastre and Piggot 1979, Illing et al. 1990]. Yet, as observed here and reported previously [Mason et al. 1988], spoIII A mutations substantially inhibit sspA, sspB, and sspE transcription as well as transcription of spoIVB. Once again, the requirement for spoIII A gene products in efficient transcription of forespore gene expression may reflect the dependence of the forespore on the overall metabolism of the surrounding mother cell or on a specific regulatory mechanism that links σC-directed gene expression to events occurring within the mother cell [Illing and Errington 1990].

In summary, our study of spoIVB expression and regulation has provided evidence for reciprocal pathways of interaction between the mother cell and the forespore chambers of the sporangium: On the one hand, the action of SpoIVB in the forespore promotes σK-directed gene expression in the mother cell; on the other hand, the action of SpoIII D in the mother cell influences the level of expression of certain σC-transcribed genes in the forespore. Thus, the mother cell and forespore lines of gene expression are not independent; rather, they are coordinated by at least two distinct intercellular interac-
tions that may be responsible for keeping the course of development in the sister cell types of the sporangium in register.

Materials and methods

Bacterial strains

Strains of B. subtilis are listed in Table 3. All strains used in this study were isogenic to the prototrophic wild-type (Spo+) strain PY79 and were constructed previously or by congression into strain PY78 [Sandman et al. 1988]; PY79 is the prototrophic derivative of PY78.

General methods

Competent cells were prepared and transformed by the method of Dubnau and Davidoff-Abelson [1971]. Selection for CmR was made on agar plates containing 5 μg/ml of chloramphenicol.

Sporulation was induced on solid medium using DS (Difco sporulation) agar and in liquid by either resuspension in SM medium, using the method of Sterlini and Mandelstam [1969].

Table 3. Bacillus subtilis strains

| Strain | Genotype | Source or reference |
|--------|----------|---------------------|
| PY79   | Prototrophic | lab stock (Youngman et al. 1984) |
| PY78   | glnA100   | lab stock           |
| SC1080a| spoIVB :: pJB2026 (spoIVB-lacZ) | this work           |
| SC262  | sspA :: sspA-lacZ trpC2       | P. Setlow [Mason et al. 1988] |
| SC263  | sspB :: sspB-lacZ trpC2       | P. Setlow [Mason et al. 1988] |
| SC264  | sspE :: sspE-lacZ trpC2       | P. Setlow [Mason et al. 1988] |
| SC780a | sspB :: glnE-lacZ             | from SC263 into PY79 |
| RS208a | spoIIGA1 spoIVB-lacZ (pDG298) | lab stock (Cutting et al. 1989) |
| BK595a | spoIID :: pBK45 (spoIID-lacZ)  | this work           |
| PM30a  | amyE :: spoIIC-lacZ            | lab stock (Kunkel et al. 1989) |
| 1.5    | spoIAC1 trpC2                  | P. Stragier [Karmazyn-Campelli et al. 1989] |
| SC1159a| spoIAC1                          | J. Errington [Errington and Mandelstam 1986a] |
| 1S33a  | spoID66 trpC2 rpoB2             | from 1.5 into PY78b |
| SC1158a, e| spoID66                             | BGSC               |
| 298.4c | spoIID298 trpC2                  | J. Errington [Errington and Mandelstam 1986a] |
| SC1160a, c| spoIID298                         | from 298.4 into PY78b |
| KS298a | spoIID :: Tn917IHU298            | lab stock [Sandman et al. 1987] |
| 48.7   | spoIIE48 trpC2                   | J. Errington [Errington and Mandelstam 1986a] |
| SC1161a| spoIIE48                          | from 48.7 into PY78b |
| 55.3   | spoIG55 trpC2                    | J. Errington [Errington and Mandelstam 1986a] |
| SC1163a| spoIG55                          | from 55.3 into PY78b |
| SC614a | spoIIA35                          | lab stock (Cutting et al. 1989) |
| SC615a | spoIIA53                          | lab stock (Cutting et al. 1990) |
| SC620a | spoIIA59                          | lab stock (Cutting et al. 1989) |
| SC616a | spoIIA65                          | lab stock (Cutting et al. 1989) |
| KS25a | spoIIA :: Tn917IHU25              | lab stock [Sandman et al. 1987] |
| BK410a | spoIIC94                          | lab stock (Kunkel et al. 1988) |
| BK395a | spoIID83                          | lab stock (Kunkel et al. 1988) |
| BK541a | spoIIDA :: erm                   | lab stock (Kunkel et al. 1989) |
| SC622a | spoIIE36                          | lab stock (Cutting et al. 1989) |
| SC500a | spoIIGA1                          | lab stock (Cutting et al. 1990) |
| 67    | spoIVA67 trpC2                     | J. Errington [Errington and Mandelstam 1986a] |
| SC1164a| spoIVA67                          | from 67 into PY78b |
| 1S46  | spoIVA178 pheA12                   | BGSC               |
| SC1165a| spoIVA178                         | from 1S46 into PY78b |
| SL765  | spoIVB165 trpC2                   | J. Hoch [Van Hoy and Hoch 1990] |
| BK754a | spoIVB165                         | from SL765 into PY78b |
| JH12719| spoIVB :: ermG trpC2 phe-1         | J. Hoch [Van Hoy and Hoch, 1990] |
| BK750a | spoIVB :: ermG                      | from JH12719 into PY79 |
| BK558a | spoIVCA133                        | lab stock [Kunkel et al. 1989] |
| BK556a | spoIVCB23                          | lab stock [Kunkel et al. 1989] |
| SC834a | spoIVFI52                          | lab stock (Cutting et al. 1990) |

aIsogenic with PY79.

bConstructed in the present work using the congression procedure of Sandman et al. (1988) using PY78, a gln- derivative of PY79.

The spo allele was complemented, in trans, by the spoIIG+ bacteriophage φ105J85 [Errington and Jones 1987].

4Bacillus Genetic Stock Center.

The spo allele was complemented, in trans, by the spoIIG+ bacteriophages, φ105J83 and/or φ105J112 [Errington and Jones 1987; J. Errington, pers. comm.].
or by growth and exhaustion in DS medium, as described by Sandman et al. (1988).

**Introduction of lacZ fusions into isogenic strains**

An integrational plasmid [pBB2026] containing the spoIVB–lacZ transcriptional fusion [Van Hoy and Hoch 1990] was used to transform wild-type [Spo⁺] cells of strain PY79, followed by selection for CmR [pBB2026] carries the cat gene]. One CmR transformant was isolated, purified, and used to prepare chromosomal spoIVB–lacZ DNA. This DNA was used to transform various mutant strains with selection for CmR. In addition, we transformed the wild-type strain [PY79] with this DNA and used the resulting derivative [strain SCI800] as an isogenic, Spo⁻ control in time course experiments. Likewise, strains SC262, SC263, and SC264 were used to prepare chromosomal DNA containing the spoA–lacZ, spoB–lacZ, and spoE–lacZ fusions, respectively. As with spoIVB–lacZ, these DNAs were used to transform both mutant strains and the isogenic wild-type strain PY79.

**Measurement of β-galactosidase activity**

Samples (1.0 ml) to be analyzed for β-galactosidase activity were collected at appropriate times and stored at −70°C until the time of assay. Cells were permeabilized either by treatment with toluene [Miller 1972], lysozyme [Mason et al. 1988], or with SDS followed by lysozyme treatment [Mason et al. 1988]. β-Galactosidase activity was assayed using the substrate ONPG as described by Miller (1972). One unit of enzyme hydrolyzes 1 μmole of ONPG/min per A$_{405}$ unit.

**Immuno-electron microscopy**

Strain SCI800 was subjected to increasing concentrations of chloramphenicol by growth on Luria broth agar plates containing progressively higher concentrations of chloramphenicol in increments of 10–25 μg/ml to a final concentration of 200 μg/ml of drug. After overnight growth, the resulting lawn of cells was scraped into 5 ml of DS medium without chloramphenicol and used to inoculate 50 ml of DS medium. After incubation for 16 hr at 37°C without the drug, the culture was used to inoculate a second culture of DS medium, which was allowed to enter sporulation by growth and nutrient exhaustion at 37°C.

Cells [15 ml] at hour 8 of sporulation were collected by centrifugation and suspended in 1.0 ml of phosphate-buffered Karnovsky’s fixative [Karnovsky 1965] and left at 4°C for 18 hr. The fixed cells were washed once in 0.5 M NH$_4$Cl, suspended in 2% agarose in water, and dehydrated by a progressive lowering of temperature as follows. Cells were sequentially maintained in 30% ethanol at 4°C for 30 min, 50% ethanol at −20°C for 2 hr, 70% ethanol at −35°C for 1 hr, and 100% ethanol for 1 hr, Lowicryl K4M/ethanol [1:1] at −35°C for 1 hr, and finally, twice in 100% Lowicryl K4M at −35°C for 1 hr. After adding fresh resin, polymerization was carried out with UV irradiation at −35°C in flat embedding molds covered with Saran Wrap over night. After polymerization, the blocks were sectioned (gold-silver sections) with a glass knife, and sections were placed on Formvar-coated nickel grids. The grids were placed on droplets of 1% glycine, 1% gelatin, for 30 min, and then onto a 1 : 25,000 dilution of rabbit anti-β-galactosidase antibodies [U.S. Biochemical] overnight in a hydrated chamber. Grids were washed five times by floating on droplets of 10 mM Tris-HCl [pH 8.0]. 0.1 mM EDTA, for 10 min, and treated with a 1 : 50 dilution of goat anti-rabbit antibodies conjugated to 5 nm gold particles [Bio-Rad] for 3 hr. After a second wash, the grids were stained as described previously [Francesconi et al. 1988].

**Electron microscopy**

Wild-type cells [strain PY79] and otherwise isogenic derivatives of PY79 bearing the mutations spoIVC[BK556] and spoIVB [ermG [BK750] were harvested 12 hr after suspension in SM medium. The cells were fixed and embedded as described previously [Rosenbluh et al. 1981], except that Spurr’s resin was substituted for Vestopal and phosphate buffer [pH 6.7] was used throughout. Staining was by treatment with 1% uranyl acetate for 5 min and Reynold’s lead [Hayat 1972] for 1 min.

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