Temporal and spatial control of the mother-cell regulatory gene *spoIID* of *Bacillus subtilis*

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Gene expression during endospore formation in *Bacillus subtilis* is compartmentalized between the mother-cell and forespore chambers of the sporangium, which follow separate pathways of cellular differentiation. The earliest acting regulatory gene so far identified in the mother-cell line of gene expression is *spoIID*, whose product is required for the transcription of the composite gene (*sigK*) encoding the mother-cell RNA polymerase σ -factor σ^K and for the chromosomal rearrangement that gives rise to the composite gene. Here we report the nucleotide sequence of *spoIID* and studies on the temporal, spatial, and genetic control of its expression during sporulation. We show that the deduced *spoIID* gene product, a 93-residue-long polypeptide, is a previously identified transcription factor that is known to activate the promoter for the *sigK* gene in vitro. Expression of *spoIID* is largely confined to the mother-cell chamber of the sporangium and is turned on at, or shortly before, the time (hour 3 of sporulation) that the mother-cell chromosome is rearranged and transcription of the *sigK* gene commences. This gene expression depends strongly on the sporulation σ -factor σ^E and partially on the *spoIID* gene product, itself. We conclude that the timing and compartmentalization of the rearrangement and transcription of the *sigK* gene and, hence, of subsequent gene activation in the mother cell, are, in part, direct consequences of the temporal and spatial control of *spoIID* gene expression.

[Key Words: *Bacillus subtilis*; sporulation; transcription factor]

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Endospores of the gram-positive bacterium *Bacillus subtilis* are a dormant cell type that is produced by a process of cellular differentiation (Piggot and Coote 1976; Losick and Youngman 1984; Losick et al. 1986). The transformation of a vegetative cell into an endospore takes place within a sporangium that is composed of two compartments known as the forespore and the mother cell. The compartments each receive a chromosome from the last round of vegetative DNA replication but then follow divergent pathways of cellular differentiation. The forespore ultimately becomes the mature endospore and is the site of production of a family of small, basic proteins known as SASP (Francesconi et al. 1988; Setlow, 1988). The mother cell, on the other hand, is the site of production of the tough protein coat that encases the endospore (Aronson and Fitz-James 1976; Jenkinson et al. 1981; Donovan et al. 1987). After maturation of the endospore, the mother-cell compartment is discarded by lysis. Gene expression in the two compartments is regulated differentially (De Lencastre and Piggot 1979; Errington and

Mandelstam 1986b; Turner et al. 1986), with certain genes, such as the SASP-encoding *sspA*–*sspE* genes, being expressed from the forespore chromosome (Mason et al. 1988), and other genes, such as the spore coat protein genes *cotA*–*cotD*, being expressed from the mother-cell chromosome (Sandman et al. 1988; Cutting et al. 1989; L. Zheng and R. Losick, unpubl.). This differential gene regulation is governed, in part, by compartment-specific σ factors known as σ^G and σ^K . σ^G is present in the forespore, where it directs transcription of *sspA*–*sspE* and other forespore-expressed genes (Karmazyn-Campelli et al. 1989; Sun et al. 1989). σ^K , on the other hand, is present in the mother cell, where it governs the expression of *cotA*, *cotD*, and *gerE*, a regulatory gene whose product induces, in turn, the expression of *cotB* and *cotC* genes (Kunkel et al. 1988; Kroos et al. 1989; L. Zheng and R. Losick, unpubl.).

Here we consider mechanisms governing the mother-cell line of gene expression. It was known from previous work that the structural gene *sigK* for the mother-cell σ -factor σ^K is created by a DNA rearrangement in the mother-cell chromosome that brings together the coding sequences of two truncated genes, *spoIVCB* and *spoIIIC* (Stragier et al. 1989). The chromosomal rearrangement is

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due to a site-specific recombinational event, which is, in turn, dependent on the product of the sporulation regulatory gene *spoIIID* (Stragier et al. 1989). The precise role of the *spoIIID* gene product in the chromosomal rearrangement is not understood. It could turn on the expression of the recombinase gene and/or participate directly in the recombination process (see Discussion). In either case, the *spoIIID* gene product also directly governs expression of the rearranged gene by turning on transcription from the promoter for *sigK* (Kunkel et al. 1988; Kroos et al. 1989). On the other hand, the *spoIIID* gene product is not required for the transcription of genes whose expression is confined to the forespore (Errington and Mandelstam 1986b; De Lencastre and Piggot 1979; Mason et al. 1988; Panzer et al. 1989) or genes whose expression is switched on prior to the stage at which the sporangium is partitioned into forespore and mother-cell compartments (e.g., Errington and Mandelstam 1986a). Hence, *spoIIID* is a compartment-specific regulatory gene, representing, as far as is known, the earliest acting regulatory gene in the mother-cell line of gene expression (Turner et al. 1986).

To investigate the role of *spoIIID* in compartment-specific gene expression, we undertook the cloning of *spoIIID* to investigate the nature of its product and to study temporal, spatial, and genetic aspects of its regulation.

Results

Cloning the *spoIIID* gene

The *spoIIID* gene was cloned in *B. subtilis* by selection for complementation of mutation *spoIIID83*, using a newly devised 'prophage transformation' system based on a derivative of the *B. subtilis* temperate phage SP β (Poth and Youngman 1988). One such recombinant phage that was capable of complementing *spoIIID83* contained a *B. subtilis* chromosomal insert of ~6 kb. To subclone *spoIIID* sequences from phage DNA, we took advantage of the presence of a derivative of the *E. coli* plasmid vector pBR322 in the SP β phage at a location adjacent to the site of the *B. subtilis* chromosomal DNA insert (Poth and Youngman 1988). As described in Methods, competent cells of *E. coli* were transformed with phage DNA that had been digested with *EcoRI* and then recircularized. One such transformant contained a plasmid (p β E-68) with a *B. subtilis* chromosomal insert of 5.7 kb (Fig. 1). This plasmid corrected the sporulation block of *spoIIID83* mutant cells when introduced by transformation.

To localize *spoIIID* on the cloned fragment, we tested the capacity of subcloned segments of the 5.7-kb insert to correct the *spoIIID83* mutation by transformation (Fig. 1). Subclones were generated by use of the indicated restriction sites and by use of the indicated deletion end points that had been generated by the single-stranded DNA deletion method of Dale et al. (1985; see Methods). The experiments of Figure 1 show that the wild-type allele of *spoIIID* lies within the ~300-bp interval between the *DraI* site and the deletion end point $\Delta 3$.

Localization of the *spoIIID* open reading frame

To localize *spoIIID* further, we determined the nucleotide sequence of DNA across the region found to exhibit *spoIIID*-transforming activity. This analysis revealed an open reading frame (ORF) of 93 codons that overlapped extensively with the *DraI*- $\Delta 3$ interval that contains the wild-type allele of *spoIIID83* (Fig. 2). The following lines of evidence strongly suggest that the ORF is the *spoIIID* gene:

1. Experiments (see below) in which the *lacZ* gene of *E. coli* was fused in-frame to the first codon of the ORF showed that the putative *spoIIID* ORF is used in vivo and directs β -galactosidase synthesis in cells undergoing sporulation.
2. No other ORF of >43 codons could be identified that overlapped with the region of the *spoIIID83* mutation.
3. Replacement of the ORF in the chromosome with an in vitro-constructed deletion mutation (*spoIIID* Δ erm) gave rise to mutant cells whose phenotype was indistinguishable from that of *spoIIID83* mutant cells. The deletion mutation was constructed by replacing the interval between the *RsaI* site at codon 15 and the *RsaI* site at codon 37 with an erythromycin-resistance cassette of ~1 kb. The wild-type chromosomal ORF was substituted with the Δ erm-bearing ORF by transformation of competent cells with linearized plasmid containing the deletion-mutated DNA, as described in Methods. Like *spoIIID83* mutant cells, cells containing the *spoIIID*- Δ erm mutation were blocked at stage III of sporulation and were unable to support *spoIVCB*-directed β -galactosidase synthesis (Fig. 3).
4. A 500-bp fragment that extended from an *XmnI* site located ~230 bp upstream of the first codon of the ORF to an *XmnI* site located 30 bp downstream of the end of the ORF was capable of complementing *spoIIID83*. The 500-bp *XmnI*-*XmnI* fragment was introduced into the *B. subtilis* chromosome by insertion into the amylase (*amy*) locus (Shimotsu and Henner 1986) by use of the *amy* insertion plasmid pDG268 (Karmazyn-Campelli et al. 1989; see Methods). Strains containing the *XmnI*-*XmnI* fragment at *amy* and the *spoIIID83* or the *spoIIID* Δ erm mutations at the *spoIIID* locus were found to sporulate normally.

Assuming, then, that the 93-codon ORF is the *spoIIID* gene, a further issue is whether the *spoIIID83* and *spoIIID* Δ erm mutations block sporulation by preventing the synthesis of the wild-type *spoIIID* gene product or by exerting a polar effect on the expression of an as yet unidentified downstream *spo* gene. A polar effect is, however, incompatible with the observation of complementation by the 500-bp *XmnI*-*XmnI* fragment, given that the *XmnI*-*XmnI* fragment only contains 30 bp of DNA downstream of the *spoIIID* ORF. As an independent test of polarity, a plasmid lacking a *B. subtilis* replicon (and, hence, incapable of autonomous replication in *B. subtilis*), but containing a *B. subtilis* DNA insert that extended from an *ApaI* site located at codon 1 of *spoIIID* to the *XmnI* site located 30 bp downstream of the 3' end of the ORF, was integrated into the *B. subtilis*

Figure 1. Physical map of the *spoIIID* region of the chromosome. The heavy black line represents a partial restriction site map of the 5.7-kb *B. subtilis* DNA insert in plasmid pBE-68. The left-hand boundary of the insert is the *Bam*HI/*Sau*3A junction generated by cloning chromosomal DNA fragments generated by partial digestion with *Sau*3A into the *Bam*HI site of the SP β prophage vector. The *Eco*RI site is the right-hand border of the insert and is ~1.7 kb downstream of the *Sst*I site. The thin lines represent subcloned segments of the *spoIIID* region generated by using the indicated restriction sites and the indicated deletion end points (see Methods). The transformation activity of each subclone was determined by transforming 0.3 ml of competent cells of strain BK395 (*spoIIID83*) harvested at stationary phase with 1–10 μ g of plasmid DNA. The transformed cells were added to 3.0 ml of DS medium and incubated at 37°C on a roller drum. The transforming activity of each clone is indicated at the right and was determined by measuring the number of heat-resistant cells present per milliliter after overnight incubation. The boundaries within which the *spoIIID83* mutation lies are indicated by the double-headed arrow above the restriction map.

chromosome by single-reciprocal (Campbell-like) recombination. If the *spoIIID* transcription unit is an operon that extends downstream of *spoIIID* and if the operon contains a downstream *spo* gene, then the *Apa*LI–*Xmn*I segment would be internal to the transcription unit, and integration of the plasmid would be expected to block expression of the *spo* gene and cause a Spo[−] phenotype. If, on the other hand, the *spoIIID* transcription unit does not contain a downstream *spo* gene, then Campbell integration would not cause a block in sporulation. Because cells containing an integrated copy of plasmid bearing the *Apa*LI–*Xmn*I fragment were fully sporulation proficient, we conclude that the *spoIIID* transcription unit does not contain a downstream *spo* gene and, hence, that the *spoIIID* gene product is required per se for spore formation.

spoIIID encodes a transcription factor

Kroos et al. (1989) previously identified a sporulation transcription factor in the size range of 8–14 kD (arbitrarily referred to as the 14-kD protein) that alters the specificity of RNA polymerase containing the mother-cell σ -factor σ^K . The first 34 amino acids of this factor were determined by sequential Edman degradation (underlined in Fig. 2). They conform exactly to the amino acid sequence of the predicted product (10.8 kD) of *spoIIID*. Therefore, we assign *spoIIID* as the structural gene for the transcription factor of Kroos et al. (1989). In agreement with the observation (R. Halberg and L. Kroos, unpubl.) that the transcription factor is a se-

quence-specific DNA-binding protein, the *spoIIID* gene product contains a region (residues 23–42) of striking similarity to the α -helix– β -turn– α -helix DNA-binding domains of many prokaryotic regulatory proteins (Pabo and Sauer 1984).

Expression of *spoIIID*–*lacZ*

We studied the regulation of *spoIIID* by using an in-frame fusion of *spoIIID* to the *lacZ* gene of *E. coli*. The fusion was constructed by joining *lacZ* in-frame to the first codon of the *spoIIID* ORF at the *Apa*LI site (see Methods). The *spoIIID*–*lacZ* fusion was introduced into the *B. subtilis* chromosome at the *spoIIID* locus by single-reciprocal recombination using a plasmid (pBK45) containing the fusion and adjacent *B. subtilis* chromosomal DNA extending to a *Hind*III site located ~700 bp upstream of the ORF. Competent cells of wild-type *B. subtilis* were transformed using pBK45 and selection for the plasmid-borne chloramphenicol-resistance gene. The resulting chloramphenicol-resistant transformants were Spo⁺, indicating that the *spoIIID* transcription unit had not been disrupted by the integration event and that the 5' end of the *spoIIID* transcription unit is located within the 700 bp upstream of the beginning of the ORF.

spoIIID-directed β -galactosidase synthesis was measured during sporulation and was found to commence 2–3 hr after the onset of sporulation, ~1 hr before the time of induction of a *lacZ* fusion to sporulation gene *spoIVCB* (Fig. 3) and ~30 min later than the time of in-

Figure 2. Nucleotide sequence of the nontranscribed strand of the *spoIIID* gene. The deduced amino acid sequence is shown beneath the nucleotide sequence. The putative ribosome binding site (GGAGG) is in boldface type. Numbers at right indicate positions in the amino acid sequence of the residues at the end of each line. The amino-terminal amino acid sequence of the 14-kD protein, as determined previously by sequential Edman degradation, is indicated by the thick line (residues 1–34). The *DraI* and *ApaLI* restriction sites and the $\Delta 3$ deletion end point correspond to those indicated in Fig. 1. Single-letter abbreviations for the amino acid residues: (A) Ala; (C) Cys; (D) Asp; (E) Glu; (F) Phe; (G) Gly; (H) His; (I) Ile; (K) Lys; (L) Leu; (M) Met; (N) Asn; (P) Pro; (Q) Gln; (R) Arg; (S) Ser; (T) Thr; (V) Val; (W) Trp; (Y) Tyr.

duction of a *lacZ* fusion to the sporulation gene *spoIID* (data not shown).

Use of the *spoIIID-lacZ* fusion to study the dependence of *spoIIID* gene expression on other *spo* genes

To study the dependence of *spoIIID* expression on the products of other *spo* genes, we introduced [by transformation of competent cells with chromosomal DNA from wild-type cells bearing an integrated copy of pBK45 (see Methods)] the *spoIIID-lacZ* fusion into 13 *spo* mutants blocked at stages II–IV of sporulation. The resulting fusion-bearing strains were induced to sporulate by resuspension in SM medium and assayed for *spoIIID*-directed β -galactosidase synthesis at various times after the onset of sporulation. Time-course experiments for each mutant strain were carried out in parallel with isogenic *Spo*⁺ cells bearing the *spoIIID-lacZ* fusion.

The levels of *spoIIID*-directed β -galactosidase synthesis in each of the 13 strains are listed in Table 1. Examples of the time-course experiments from which the data in Table 1 are based are presented in Figure 4. The level of expression of *spoIIID-lacZ* in different mutants

ranged from almost complete absence of expression (*spoIIAC1* and *spoIIGB55*) to moderate overexpression (e.g., *spoIIIA53* and *spoIVCB23*). Several mutations, including *spoIIID83*, caused a partial impairment of *spoIIID-lacZ* expression. The two mutations that severely impaired *spoIIID* expression are known to prevent the appearance of the sporulation σ -factor σ^E (Stragier et al. 1984,1988; Labell et al. 1987; Trempey et al. 1985a,b).

Compartmentalization of *spoIIID* expression

Because the sporangium is partitioned into mother-cell and forespore chambers by the time that *spoIIID* expression commences, we investigated the extent to which *spoIIID* expression is compartmentalized. This was determined by measuring the accumulation of *spoIIID-lacZ*-specified β -galactosidase in the mother-cell and forespore compartments of sporulating wild-type cells bearing the *spoIIID-lacZ* fusion. Fusion-bearing cells were harvested at 8–9 hr after the onset of sporulation, and the contents of the mother-cell and forespore compartments fractionated as described previously (Kunkel et al. 1988; Cutting et al. 1989; Panzer et al. 1989). Table

Figure 3. *spoIID*-directed and *spoIVCB*-directed β -galactosidase synthesis. The specific activity of β -galactosidase [Miller units] was determined after resuspension in SM medium at the indicated times of *spo*⁺ cells (strain PY79) containing an integrated copy of the *spoIID*-*lacZ*-bearing plasmid pBK45 (■); *spo*⁺ cells (strain PY79) containing the SPB :: *spoIVCB*-*lacZ* prophage (Kunkel et al. 1988) (▲); and *spoIID* Δ erm mutant cells (strain BK541) containing the SPB :: *spoIVCB*-*lacZ* prophage (●).

2 presents the specific activities of β -galactosidase observed in the mother-cell and forespore fractions in three independent experiments. It can be seen that the specific activity of β -galactosidase in the mother-cell fraction was about 6- to 11-fold higher (depending on the experiment) than in the forespore fraction. Thus, we conclude that *spoIID* is expressed preferentially (or, more precisely, that *spoIID*-*lacZ*-encoded β -galactosidase accumulates preferentially) in the mother cell. The extent of this compartmentalization is similar to that observed previously for other genes, such as *gerE*, *spoIVCB*, and *cotA* (Kunkel et al. 1988; Cutting et al. 1989; Panzer et al. 1989), whose expression is considered to be specific to the mother cell. In contrast, *sspB*-*lacZ*-specified β -galactosidase (*sspB* is a forespore-expressed gene; Mason et al. 1988) was found in a parallel experiment (see Table 2 footnote) to accumulate to 12-fold higher specific activity in the forespore fraction than in the mother-cell fraction. Our results are consistent with the findings of an earlier genetic study indicating that *spoIID* expression is required in the mother-cell but not in the forespore chamber of the sporangium (De Lencastre and Piggot 1979).

Discussion

Gene expression in the mother-cell chamber of the sporangium is controlled, in part, by a hierarchical regulatory cascade (for review, see Losick and Kroos 1989). *spoIID*, the earliest acting regulatory gene so far identified in the cascade (Turner et al. 1986; Kunkel et al. 1988; L. Zheng and R. Losick, unpubl.), governs the construction (by a chromosomal rearrangement) and transcription of the structural gene *sigK* for the mother-cell σ -factor σ^K (Kunkel et al. 1988; Kroos et al. 1989; Stragier et al. 1989). σ^K , in turn, switches on the transcription of *gerE* (Cutting et al. 1989; L. Kroos, unpubl.), a regulatory gene whose product governs gene expression at a late stage of development in the mother cell (L. Zheng and R. Losick, unpubl.). Our finding that *spoIID*

is preferentially expressed in the mother cell explains, at least in part, why the rearrangement (Stragier et al. 1989; B. Kunkel, R. Losick, and P. Stragier, unpubl.) and transcription (Kunkel et al. 1988) of *sigK* and, hence, subsequent gene expression (Cutting et al. 1989) in the cascade are compartmentalized. Also, our demonstration that expression of *spoIID* commences at or shortly before the time at which the rearrangement occurs and at

Table 1. Effect of *spo* mutations on *spoIID*-directed β -galactosidase synthesis

Relevant mutation ^a	Synthesis of β -galactosidase ^b
<i>spo</i> ⁺	100
<i>spoIIAC1</i>	<5
<i>spoIID298</i>	100
<i>spoIID</i> :: Tn917 Ω HU298	53
<i>spoIIGB55</i>	<5
<i>spoIIIA53</i>	175
<i>spoIIIA</i> :: Tn917 Ω HU25	150
<i>spoIIIC94</i>	180
<i>spoIID83</i>	28
<i>spoIID</i> Δ erm	34
<i>spoIIIE36</i>	54
<i>spoIIIGΔ1</i>	180
<i>spoIVCA133</i>	140
<i>spoIVCB23</i>	170

^a Strains bearing the mutations *spoIIAC1* (1.5), *spoIID298* (298.4), and *spoIIGB55* (55.3) were isogenic to SC38. Strains bearing *spoIID* :: Tn917 Ω HU298 (KS298), *spoIIIA53* (SC615), *spoIIIA* :: Tn917 Ω HU25 (KS25), *spoIIIC94* (BK410), *spoIID83* (BK395), *spoIID* Δ erm (BK541), *spoIIIE36* (SC622), *spoIIIG Δ 1* (BK338), *spoIVCA133* (BK558), and *spoIVCB23* (BK556) were isogenic to PY79. An integrated copy of the *spoIID*-*lacZ* fusion plasmid pBK45 was introduced into each strain as described in Methods.

^b Expressed as a percentage of the level (~45 Miller units) of *spoIID*-directed β -galactosidase synthesis observed in the corresponding isogenic wild-type (*Spo*⁺) strain at peak activity (6–8 hr) after resuspension in SM medium. Background levels of β -galactosidase activity of the respective isogenic fusionless *Spo*⁺ strain (1–3 Miller units) were subtracted.

Figure 4. *spoIIID*-directed β -galactosidase synthesis in various *spo* mutants. The specific activity of β -galactosidase was determined at the indicated times after resuspension in SM medium of *spo*⁺ cells (■) or cells of mutant strains containing the following mutations: *spoIIGB55* (△), *spoIIID83* (●), *spoIIIA*: Tn917ΩHU25 (▲), and *spoIVCB23* (□). Each strain contained an integrated copy of the *spoIIID-lacZ* fusion-bearing plasmid pBK45 that had been introduced as described in Methods.

which the transcription of *sigK* (as monitored by the use of the *spoIVCB-lacZ* fusion; Fig. 3) is induced suggests that the activation of the regulatory cascade is directly attributable to the induction of *spoIIID*.

spoIIID encodes an activator of the *sigK* promoter

The *spoIIID* gene encodes a 93-residue-long polypeptide of 10.8 kD. The predicted amino acid sequence of this protein conforms exactly to the partial amino-terminal amino acid sequence of a previously identified transcription factor that binds to the promoter for *sigK* (R. Halberg and L. Kroos, unpubl.) and strongly stimulates its transcription (Kroos et al. 1989). Therefore, we assign *spoIIID* as the structural gene for this transcription factor and infer that the strong dependence of the induction of *sigK* transcription on the *spoIIID* gene product (Kunkel et al. 1988) is due to direct activation of the *sigK* promoter by SpoIIID protein. The presence of a region (residues 23–42 in Fig. 2) of striking similarity to the α -helix- β -turn- α -helix DNA-binding domain of many other prokaryotic transcriptional regulatory proteins (Pabo and Sauer 1984) is consistent with this view. Recently, *spoIIID* was cloned independently and sequenced by C. Stevens and J. Errington (pers. comm.), who confirm its assignment as the structural gene for the transcription factor of Kroos et al. (1989).

Role of the *spoIIID* gene product in the *sigK* chromosomal rearrangement

The discovery that the *spoIIID* gene product is a small, DNA-binding protein suggests two, nonmutually exclu-

sive explanations for its requirement in the mother-cell chromosomal rearrangement. One possibility is that SpoIIID governs the transcription of the site-specific recombinase gene whose product catalyzes the juxtaposition of *spoIVCB* and *spoIIIC* sequences to create the composite *sigK* gene. Several lines of investigation (B. Kunkel, R. Losick, and P. Stragier, unpubl.) strongly suggest that the recombinase gene is *spoIVCA*, a sporulation gene that is located immediately adjacent to and in convergent orientation with *spoIVCB* (Fujita and Kobayashi 1985; Farquhar and Yudkin 1988; Kunkel et al. 1988; Stragier et al. 1989). It will be of considerable interest to learn whether *spoIVCA* expression, like *spoIVCB* expression, depends on the *spoIIID* product. The other possibility is that SpoIIID is directly involved in the rearrangement process as part of a nucleosome-like, synaptic complex [purified SpoIIID is known to bind to the *spoIVCB* (i.e., *sigK*) promoter region in vitro; R. Halberg and L. Kroos, unpubl.] that facilitates the recombination event. This would be analogous to the involvement of small host proteins in *Salmonella* phase variation and in excisive and integrative recombination by coliphage λ (Better et al. 1983; Pollack and Nash 1983; Johnson et al. 1986; Thompson et al. 1987; Friedman 1988).

Temporal and spatial regulation of *spoIIID* expression

Finally, we consider the temporal and spatial regulation of *spoIIID* expression. Because *spoIIID* is at or near the top of the hierarchical regulatory cascade, the mechanisms that govern the timing and compartmentalization of its expression can be considered to be important determinants of the mother-cell line of gene expression.

Table 2. Compartmentalization of *spoIIID* expression

Experiment	Mother-cell	Forespore	Mother-cell/forespore ratio
1	7.9	0.7	11.3
2	12.8	2.2	5.8
3	8.8	0.9	9.8

Cells of strain PY79 containing an integrated copy of the *spoIIID-lacZ* fusion plasmid pBK45 were sporulated in DS medium (experiments 1 and 3) or in SM medium (experiment 2). Five-milliliter samples were collected at T₈–T₉ and fractionated into mother-cell and forespore fractions as described by Panzer et al. (1989). The specific activity of β -galactosidase in each fraction was determined and is expressed as nanomoles of ONPG hydrolyzed/min per milligram of protein. Specific activities from each of 3 independent experiments are shown. The data are averages of 4, 2, and 4 sets of measurements for experiments 1, 2, and 3, respectively. The background specific activities of ONPG-hydrolyzing activity in the mother-cell (~2 nmole/min per mg of protein) and forespore (~1.5 nmole/min per mg of protein) fractions from cells lacking the gene fusion were subtracted. For comparison the ratio of mother-cell (270 nmole/min per mg of protein) to forespore (3200 nmole/min per mg of protein) specific activity for *sspB*-directed β -galactosidase synthesis analyzed in parallel was 0.08.

We infer that *spoIIID* is under the control of the sporulation σ -factor σ^E (although biochemical experiments to determine if this control is direct have not yet been carried out) (1) because *spoIIID* expression commences shortly after the time that σ^E appears (Trempey et al. 1985a), and (2) because *spoIIID* expression is completely dependent on *spoIIGB*, which encodes the pro- σ precursor of σ^E (Stragier et al. 1984; Trempey et al. 1985a; Labell et al. 1987), and *spoIIA*, whose products are required for the processing of pro- σ^E to its mature and active form (Stragier et al. 1988). Therefore, we suggest that the time at which *spoIIID* is induced is determined principally by the time at which pro- σ^E processing occurs. [The time of *spoIIID* induction may not be determined exclusively by the appearance of mature σ^E , however, because *spoIIID* induction occurs slightly later (see Results) than the time of induction of *spoIID*, a stage II gene whose transcription is known to be under the direct control of σ^E (Rong et al. 1986; Stragier et al. 1988).] It has been suggested that the conversion of pro- σ^E to mature σ^E is coupled to the formation of the sporulation septum at morphological stage II of development (Labell et al. 1987; Stragier et al. 1988). If this hypothesis is correct, the dependence of pro- σ^E processing on septum formation would ensure that *spoIIID* is not induced until after the sporangium has been partitioned into forespore and mother-cell compartments.

Although regulation by σ^E largely (if not entirely) explains the timing of *spoIIID* induction, transcription by σ^E -RNA polymerase presumably cannot be the basis for the compartmentalization of *spoIIID* expression. Subcellular fractionation experiments show that σ^E is present in both compartments of the sporangium (Carlson and Haldenwang 1989), and dependence studies show that σ^E is required both for mother-cell and forespore gene expression (Errington and Mandelstam 1986b; Turner et al. 1986; Kunkel et al. 1988; Mason et al. 1988; Sandman et al. 1988; Karmazyn-Campelli et al. 1989). Therefore, we infer the existence of an additional regulatory mechanism superimposed on the recognition of the *spoIIID* promoter by σ^E -RNA polymerase that limits *spoIIID* expression to the mother cell.

The nature of this compartmentalization mechanism is unknown, but our dependency studies suggest the possible existence of a positive feedback loop that could contribute to the differential expression of *spoIIID* in the mother cell. We interpret the observation that *spoIIID* expression is partially dependent on its own gene product, but not on the product of the downstream regulatory gene *sigK*, to indicate that expression of *spoIIID* is stimulated by its own gene product; that is, that *spoIIID* expression is subject to autoregulation. Autoregulation could be expected to set up a positive feedback loop in which an underlying mother-cell bias in *spoIIID* expression (of unknown mechanistic basis) would preferentially stimulate further expression of *spoIIID* in the mother cell. This is analogous to the postulated role of autoregulation of the forespore σ -factor gene *spoIIG* (*sigG*) and of the mother-cell σ -factor gene *sigK* in enhancing compartmentalized synthesis of σ^G

and σ^K , respectively (Karmazyn-Campelli 1989; Kroos et al. 1989). A possible additional feature of such a positive feedback loop that would reinforce the differential expression of *spoIIID* in the mother cell is suggested by the observation that the *spoIIID* gene product oligomerizes (Kroos et al. 1989; L. Kroos and R. Losick, unpubl.). If the active form of the *spoIIID* gene product in autoregulation is an oligomer, then positive regulation of *spoIIID* by SpoIIID protein would be highly sensitive to the *spoIIID* protein concentration and, hence, would occur much more efficiently in the mother cell than in the forespore, assuming an underlying mother-cell bias in *spoIIID* expression.

Methods

Bacterial strains

B. subtilis strains KS25 (*spoIIIA* :: Tn917 Ω HU25; Sandman et al. 1987), KS298 (*spoIID* :: Tn917 Ω HU298; Sandman et al. 1987), SC615 (*spoIIIA53*; Cutting et al. 1989), SC622 (*spoIIIE36*; Cutting et al. 1989), BK338 (*spoIIIGA1*; Kunkel et al. 1988), BK395 (*spoIIID83*; Kunkel et al. 1988), BK410 (*spoIIIC94*; Kunkel et al. 1988), BK556 (*spoIVCB23*; this work), BK558 (*spoIVCA133*; this work), and BK541 (*spoIIID Δ erm*; this work, see below) were isogenic with strain PY79. Strains BK556 and BK558 were constructed by PBS1-mediated transduction of BK407 (*aroD120*; Kunkel et al. 1988) to prototrophy using PBS1 lysates grown on strain 23.1 (*spoIVCB23 trpC2*; J. Errington, Oxford University) and 1S47 (*spoIVCA133 trpC2*; Bacillus Genetic Stock Center), respectively, taking advantage of the linkage of *spoIVCA* and *spoIVCB* (50%) to *aroD120*. Strains 1.5 (*spoIIAC1 trpC2*), 298.4 (*spoIID298 trpC2*) and 55.3 (*spoIIGB trpC2*) were isogenic with strain SG38 and were provided by J. Errington (Errington and Mandelstam 1986a).

Cloning of *spoIIID*

The *spoIIID* gene was cloned in *B. subtilis* by selection for complementation of mutation *spoIIID83* by use of the prophage transformation system of Poth and Youngman (1988). A library of SP β -transducing phages containing *Sau3A*-generated, partially digested fragments of *B. subtilis* DNA in the size range of 5–8 kb was used to infect *spoIIID83* mutant cells. Lysogens were selected by resistance to chloramphenicol. After growth under sporulation conditions, Spo⁺ lysogens were selected by resistance to chloroform. A lysate was prepared by thermoinduction of the Spo⁺ lysogens and used, once again, to infect *spoIIID83* mutant cells. One such transducing phage (HP64) that was capable of complementing the *spoIIID83* mutation contained an insert of ~6 kb. To rescue the *spoIIID* gene from the phage, we took advantage of the presence of a derivative of the *E. coli* plasmid vector pBR322 in the SP β phage at a location adjacent to the *Bam*HI site into which chromosomal DNA had been inserted and the presence of a unique *Eco*RI site in the insert distal to the pBR322 replicon. Phage DNA was cut with *Eco*RI and circularized by ligation at low DNA concentration and used to transform competent *E. coli* cells to ampicillin resistance.

Generation of nested deletions

A series of nested deletions of the *spoIIID* gene used for sequencing and subcloning was generated by the method of Dale et al. (1985). A fragment of *B. subtilis* DNA of ~4 kb, extending

from the left end of the pBE-68 insert (i.e., the *Bam*HI/*Sau*3A junction of insert and vector DNA) to the *Sst*I site indicated in Figure 1 was subcloned into phage M13mp19. Single-stranded DNA was isolated from the recombinant phage and used to create nested deletions whose end points are indicated in Figure 1.

Construction of plasmid subclones

Plasmid pBK32 was constructed by inserting the 4-kb *Bam*HI-*Sst*I fragment that had been gel-purified from pBE-68 DNA into the polylinker of pIB30 (International Biotechnologies). Plasmids pCK1, pCK3, pCK5, and pCK7 were constructed by gel-purifying the indicated restriction fragments from pBK32 and cloning them into the polylinker of pSGMU2 (Fort and Errington 1985). Deletion subclones pCK9, pBK34, pBK39, pBK41, and pBK42 were constructed by transferring the indicated fragments from phage M13mp19 bearing the indicated deletions into pUC18. The inserts were gel-purified from replicative form (double-stranded) phage DNA that had been digested with *Eco*RI, which cuts in the M13mp19 polylinker at a site adjacent to the deletion end point, and with the indicated restriction enzyme (*Bam*HI, *Hind*III, or *Dra*I).

Nucleotide sequencing

The *spoIIID* gene was sequenced across both strands by the deoxy chain termination method of Sanger et al. (1977). The 3' strand of the gene was sequenced by using the universal M13 sequencing primer and phage M13mp19 DNA bearing the $\Delta 21$ and $\Delta 3$ deletion-mutated DNAs (Figs. 1 and 2) as templates. The 5' strand of the gene was sequenced by using as templates single-stranded phage M13mp18 DNA into which the 1.5-kb *Hind*III-*Pst*I insert of pCK5 had been cloned (Fig. 1). The following synthetic oligonucleotides were used as primers for sequencing the 5' strand 5'-GATGAATGAGGTGTCTG-3' (centered at 375 bp upstream of the *spoIIID* ORF), 5'-CCTAA-CAAAGAGAGTGT-3' (centered at 150 bp upstream of the ORF), and 5'-CAGTACACAAGGATTTA-3' [centered at codon 39 within the ORF (Fig. 2)].

Construction of mutant cells bearing a *spoIIID* deletion mutation

The deletion mutation *spoIIID* Δ *erm* was constructed in vitro by replacing the interval between the *Rsa*I site at codon 15 and the *Rsa*I site at codon 37 with an erythromycin-resistance cassette of ~1 kb. A 500-bp *Rsa*I-*Rsa*I fragment containing the 5' region of *spoIIID* and extending to the *Rsa*I site at codon 15 (Fig. 2) was cloned into the polylinker of pUC18 at the *Hinc*II site in the orientation such that the *spoIIID* ORF read toward the *Eco*RI site in the polylinker. The resulting plasmid, pCK13, was linearized with *Kpn*I and *Bam*HI (which cut uniquely in the polylinker at sites adjacent to the *Rsa*I site at codon 15), and a gel-purified 1-kb *Kpn*I-*Bam*HI fragment carrying the *erm* cassette isolated from pUC18*erm* (Kenney and Moran 1987) was inserted to create pBK43. A 360-bp *Rsa*I-*Eco*RI fragment isolated from pBK39, extending from the *Rsa*I site at codon 37 to the deletion end point $\Delta 21$ (Fig. 1), was gel-purified and inserted into pBK43 that had been linearized at the *Cla*I site (located in the polylinker adjacent to the end of the *erm* cassette distal to the 5' end of the *spoIIID* ORF), treated with Klenow enzyme to generate blunt ends, and then digested with *Eco*RI. The resulting plasmid, pBK46, contained the 5' and 3' portions of the *spoIIID* ORF in the same orientation, as was verified by endonuclease restriction analysis. pBK46 was linearized with *Hind*III, which cuts outside of *spoIIID* Δ *erm*, and was used to transform

competent cells of strain PY79 to erythromycin resistance (25 μ g/ml lincomycin, 1 μ g/ml erythromycin) to generate the *spoIIID* Δ *erm*, deletion-mutated strain BK541. That the wild-type *spoIIID* gene in the chromosome had been substituted by marker replacement (double) recombination with mutant DNA in strain BK541 was verified by Southern hybridization analysis.

Insertion of *spoIIID* at the amy locus

A fragment of ~500 bp that extended from an *Xmn*I site located ~230 bp upstream of the first codon of the *spoIIID* ORF to an *Xmn*I site located 30 bp downstream of the end of the ORF was gel-purified from pBK39 (Fig. 1) and subcloned into pUC18 that had been linearized with *Sma*I to generate plasmid pBK49. The 500-bp insert containing the *spoIIID* gene was then released from pBK49 by digestion with *Eco*RI and *Hind*III and inserted into pDG268 (Karmazyn-Campelli et al. 1989), a derivative of the *amy*-integration vector of Shimotsu and Henner (1986) that had been linearized with both *Eco*RI and *Hind*III. The resulting plasmid, pBK51, was linearized with *Xho*I and used to transform competent wild-type cells of strain PY79 to chloramphenicol resistance. The resulting transformants were screened for those exhibiting an *Amy*⁻ phenotype (Shimotsu and Henner 1986). Chromosomal DNA from one chloramphenicol-resistant *Amy*⁻ transformant (BK607) was purified and used to introduce the wild-type *spoIIID* gene into *spoIIID*83 and *spoIIID* Δ *erm* mutant cells by transformation.

Construction of *spoIIID*-*lacZ* fusion

The *spoIIID*-*lacZ* fusion was created by joining the *lacZ* gene of *E. coli* in-frame to the first codon of the *spoIIID* ORF. pBK39 (see Fig. 1 and above) was linearized with *Apa*LI, partially filled in with Klenow enzyme in the presence of only dTTP and dGTP, and then mildly digested with mung bean nuclease (New England Biolabs) to generate blunt-ended fragments. Then the DNA was digested with *Hind*III and the 730-bp *Hind*III-*Apa*LI (blunt) fragment containing the 5' portion of the gene and extending into the ORF to include only the first codon of *spoIIID* was gel-purified and inserted into the *lacZ*-*cat* vector pSGMU37 (Errington 1986) that had been digested with *Xho*I, filled in with Klenow enzyme, and digested with *Hind*III. The resulting plasmid, pBK45, contained an in-frame fusion of *spoIIID*-*lacZ*, as was verified by DNA sequencing across the junction of *spoIIID* and *lacZ* sequences.

Introduction of *spoIIID*-*lacZ* gene fusion into the chromosome

The *spoIIID*-*lacZ* fusion was inserted into the chromosome by single-reciprocal (Campbell-like) recombination at the *spoIIID* locus by transformation of competent cells of the strain PY79 with plasmid pBK45 (see above) and selection for chloramphenicol resistance. To introduce the *spoIIID*-*lacZ* fusion into other wild-type and mutant strains, chromosomal DNA was purified from one chloramphenicol-resistant transformant (BK533) and used to transform competent cells of the indicated strains to chloramphenicol resistance.

Transformation of *B. subtilis*

Competent cells were prepared and transformed as described by Dubnau and Davidoff-Abelson (1971).

Growth and sporulation

Growth and sporulation in Difco sporulation (DS) medium were carried out as described by Sandman et al. (1988). Induc-

tion of sporulation induced by resuspension in SM medium was carried out by the method of Sterlini and Mandelstam (1969). Samples to be analyzed for β -galactosidase activity were collected at the indicated times after the end of exponential growth and stored frozen at -70°C .

Measurement of β -galactosidase activity

The specific activity of β -galactosidase was determined as described by Miller (1972) with the substrate *o*-nitrophenol- β -D-galactoside (ONPG). One unit of enzyme hydrolyzes 1 μ mole ONPG/min per A_{595} unit. The background activity of ONPG-hydrolyzing activity (~ 2 Miller units) in cells of the *Spo*⁺ parental strains PY79 and SG38 lacking the gene fusion was subtracted from the values obtained for cells containing the *spoIID-lacZ* fusions.

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Sequence data described in this paper have been submitted to the EMBL/GenBank Data Libraries under accession number X15520.

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Temporal and spatial control of the mother-cell regulatory gene *spoIIID* of *Bacillus subtilis*.

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