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

Barbara Kunkel,1 Lee Kroos,1,3 Harold Poth,2,4 Philip Youngman,2 and Richard Losick1,5
1Department of Cellular and Developmental Biology, The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138 USA; 2Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104 USA

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 spoIIID, whose product is required for the transcription of the composite gene [sigK] encoding the mother-cell RNA polymerase σ-factor σK and for the chromosomal rearrangement that gives rise to the composite gene. Here we report the nucleotide sequence of spoIIID and studies on the temporal, spatial, and genetic control of its expression during sporulation. We show that the deduced spoIIID 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 spoIIID 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 σE and partially on the spoIIID 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 spoIIID gene expression.

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

Received June 5, 1989; revised version accepted August 10, 1989.

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 σE and σK. σE 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]; σK, on the other hand, is present in the mother cell, where it governs the expression of cotA, cotD, and getE, 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 σK 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
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 (Erntting 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., Erntting 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 spoIIIDAerm, using a newly devised 'prophage transformation' system based on a derivative of the B. subtilis temperate phage SP8 [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 SP8 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 [pBE-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 Δ3.

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–Δ3 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 [spoIIIDΔerm] 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 Rsal site at codon 15 and the Rsal site at codon 37 with an erythromycin-resistance cassette of ~1 kb. The wild-type chromosomal ORF was substituted with the Δerm-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-Δerm 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 spoIIIDΔerm 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 spoIIIDΔerm 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 ApaLI 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
spolIID encodes a transcription factor

Kroos et al. [1989] previously identified a sporulation transcription factor in the size range of 8–14 kDa [arbitrarily referred to as the 14-kDa protein] that alters the specificity of RNA polymerase containing the mother-cell σ-factor σK. 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 kDa) of spolIID. Therefore, we assign spolIID 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 sequence-specific DNA-binding protein, the spolIID 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 spolIID–lacZ

We studied the regulation of spolIID by using an in-frame fusion of spolIID to the lacZ gene of E. coli. The fusion was constructed by joining lacZ in-frame to the first codon of the spolIID ORF at the ApaLI site (see Methods). The spolIID–lacZ fusion was introduced into the B. subtilis chromosome at the spolIID locus by single-reciprocal recombination using a plasmid (pBK45) containing the fusion and adjacent B. subtilis chromosomal DNA extending to a HindIII 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 5' end of the transcription unit is located within the 700 bp upstream of the beginning of the ORF.

spolIID-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 Dral and ApaLI restriction sites and the A3 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.

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 SpoI^+ 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 (spoIAC1 and spoIGB55) 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 α-factor α^F (Stragier et al. 1984,1988; Labell et al. 1987; Trempy 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).
fraction. Our results are consistent with the findings of an earlier genetic study indicating that spoIID expression is a forespore-expressed gene; Mason galactosidase [sspB (Kunkel et al. 1988; Cutting et al. 1989; Panzer et al. 1988)] 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-specific 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). spoIIID, 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 σK (Kunkel et al. 1988; Kroos et al. 1989; Stragier et al. 1989). σK, 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* | Synthesis of β-galactosidase* |
|--------------------|------------------------------|
| spo+               | 100                          |
| spoIIAC1           | <5                           |
| spoIID298          | 100                          |
| spoIII::Tn917HU298 | 53                           |
| spoIIGB55          | <5                           |
| spoIIA53           | 175                          |
| spoIII::Tn917HU25  | 150                          |
| spoIIIC94          | 180                          |
| spoIID83           | 28                           |
| spoIIDerm          | 34                           |
| spoIIIE36          | 54                           |
| spoIIIGΔ1          | 180                          |
| spoIVCA133         | 140                          |
| spoIVCB23          | 170                          |

* Strains bearing the mutations spoIIAC1 [1.5], spoIID98 [292.4], and spoIIGB55 [55.3] were isogenic to SG38. Strains bearing spoIID::Tn917HU298 [KS298], spoIIA53 [SC615], spoIII::Tn917HU25 [KS25], spoIIIC94 [BK410], spoIID83 [BK395], spoIIDerm [BK541], spoIIIE36 [SC622], spoIIIGΔ1 [BK338], spoIVCA133 [BK558], and spoIVCB23 [BK556] were isogenic to PY79. An integrated copy of the spoIIID-lacZ fusion plasmid pBK45 was introduced into each strain as described in Methods.

* 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: Tn917HU25 (▲), 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 a-helix–β-turn–a-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 exclusive 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 spoIVC and spoIIIIC 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 nucleosomelike, synaptic complex [purified SpoIIID is known to bind to the spoIVC [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 colipage 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.

| 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 FY79 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 Tg–Tp 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-hydrolyzing activity 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 nmol/min per mg of protein) and forespore (≈1.5 nmol/min per mg of protein) fractions from cells lacking the gene fusion were subtracted. For comparison the ratio of mother-cell [270 nmol/min per mg of protein] to forespore [3200 nmol/min per mg of protein] specific activity for spoB-directed β-galactosidase synthesis analyzed in parallel was 0.08.
We infer that spoIIID is under the control of the sporulation a-factor σa [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 σa appears [Trempy et al. 1985a], and [2] because spoIIID expression is completely dependent on spoIIGB, which encodes the pro-σ precursor of σa [Stragier et al. 1987; Trempy et al. 1985a; Labell et al. 1987], and spoIIA, whose products are required for the processing of pro-σ 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 processing occurs. [The time of spoIIID induction may not be determined exclusively by the appearance of mature σa, 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 σa [Rong et al. 1986; Stragier et al. 1988].] It has been suggested that the conversion of pro-σ to mature σa 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 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 σa largely [if not entirely] explains the timing of spoIIID induction, transcription by σa-RNA polymerase presumably cannot be the basis for the compartmentalization of spoIIID expression. Subcellular fractionation experiments show that σa is present in both compartments of the sporangium (Carlson and Haldenwang 1989), and dependence studies show that σa 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 σa-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 a-factor gene spoIIG (sigG) and of the mother-cell a-factor gene sigK in enhancing compartmentalized synthesis of σa and σK, 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:: Tn917OHU25, Sandman et al. 1987], KS298 [spoIID:: Tn917OHU298, Sandman et al. 1987], SC615 [spoIIG; Cutten et al. 1989], SC622 [spoIIIE36, Cutten et al. 1989], BK338 [spoIIGA1; Kunkel et al. 1988], BK395 [spoIIID3; Kunkel et al. 1988], BK410 [spoIIIC94; Kunkel et al. 1988], BK556 [spoIVC82; this work], BK558 [spoVCA133; this work], and BK541 [spoIIGDerm; 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 [spoI/C23 trpC2], J. Errington (Oxford, University) and 1547 [spoI/C23 trpC2], Bacillus Genetic Stock Center, respectively, taking advantage of the linkage of spoI/C and spoI/VB (50% to aroD120. Strains 1.5 [spoIAC1 trpC2], 298.4 [spoI/C23 trpC2] and 55.3 [spoI/CG 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 spoIIID3 by use of the pro-phage transformation system of Poth and Youngman [1988]. A library of SPβ-transducing phages containing Sau3A-generated, partially digested fragments of B. subtilis DNA in the size range of 5–8 kb was used to infect spoIIID3 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 thermolysis of the Spo+ lysogens and used, once again, to infect spoIIID3 mutant cells. One such transducing phage (HP64) that was capable of complementing the spoIIID3 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 SPβ phage at a location adjacent to the BamHI site into which chromosomal DNA had been inserted and the presence of a unique EcoRI site in the insert distal to the pBR322 replicon. Phage DNA was cut with EcoRI 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...
Kunkel et al.

from the left end of the pBE-68 insert [i.e., the BamHI/Sau3A junction of insert and vector DNA] to the SstI 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 BamHI–SstI fragment that had been gel-purified from pBE-68 DNA into the polylinker of pBl30 (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 EcoRI, which cuts in the M13mp19 polylinker at a site adjacent to the deletion end point, and with the indicated restriction enzyme (BamHI, HindIII, or DraI).

Nucleotide sequencing

The spoIIID gene was sequenced across both strands by the dideoxy 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 Δ21 and Δ3 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 HindIII–PstI insert of pCK5 had been cloned [Fig. 1]. The following synthetic oligonucleotides were used as primers for sequencing the 5’ strand 5’-GATGAAATGAGGTGTCTG-3’ (centered at 375 bp upstream of the spoIIID ORF), 5’-CCTAAACAAAGAGAGTGT-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 spoIIIDΔerm was constructed in vitro by replacing the interval between the Rsal site at codon 15 and the Rsai site at codon 37 with an erythromycin-resistance cassette of ~1 kb. A 500-bp Rsal–Rsai fragment containing the 5’ region of spoIIID and extending to the Rsai site at codon 15 (Fig. 2) was cloned into the polylinker of pUC18 at the HindIII site in the orientation such that the spoIIID ORF read toward the EcoRI site in the polylinker. The resulting plasmid, pCK13, was linearized with KpnI and BamHI [which cut uniquely in the polylinker at sites adjacent to the Rsai site at codon 15], and a gel-purified 1-kb KpnI–BamHI fragment carrying the erm cassette isolated from pUC18erm [Kenney and Moran 1987] was inserted to create pBK43. A 360-bp Rsal–EcoRI fragment isolated from pBK39, extending from the Rsai site at codon 37 to the deletion end point Δ21 [Fig. 1], was gel-purified and inserted into pBK43 that had been linearized at the ClaI 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 EcoRI. 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 HindIII, which cuts outside of spoIIIDΔerm, and was used to transform competent cells of strain PY79 to erythromycin resistance (25 μg/ml lincomycin, 1 μg/ml erythromycin) to generate the spoIIIDΔerm, 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 XmnI site located ~230 bp upstream of the first codon of the spoIIID ORF to an XmnI 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 SmaI to generate plasmid pBK49. The 500-bp insert containing the spoIIID gene was then released from pBK49 by digestion with EcoRI and HindIII and inserted into pDC268 [Karmazyn-Campelli et al. 1989], a derivative of the amy-integration vector of Shimotsuma and Henner [1986] that had been linearized with both EcoRI and HindIII. The resulting plasmid, pBK51, was linearized with Xhol 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 [Shimotsuma and Henner 1986]. Chromosomal DNA from one chloramphenicol-resistant Amy+ transformant (BK607) was purified and used to introduce the wild-type spoIIID gene into spoIIIDΔerm 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 ApaI, 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 HindIII and the 730-bp HindIII–ApaI [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 Xhol, filled in with Klenow enzyme, and digested with HindIII. 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 μmole ONPG/min per A595 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 spoIIID–lacZ fusions.

Acknowledgments

We thank S. Cutting, L. Zheng, A. Grossman, A.L. Sonenshein, and P. Stragier for helpful discussions and advice on the manuscript. We also thank A.L. Sonenshein for help with the analysis of the spoIIID sequence and C. Kroeger for technical assistance. B.K. was a predoctoral fellow of the National Science Foundation, and L.K. was a postdoctoral fellow of the Helen Hay Whitney Foundation. This work was supported by National Institutes of Health grants GM-18568 to R.L. and GM-35495 to P.Y.

Note added in proof

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

References

Aronson, A.l. and P.C. Fitz-James. 1976. Structure and morphogenesis of the bacterial spore coat. Bacteriol. Rev. 40: 360–402.

Better, M., S. Wickner, J. Auerbach, and H. Echols. 1983. Role of the Xis protein of bacteriophage λ in a specific reactive complex at the attR prophage attachment site. Cell 32: 161–168.

Carlson, H.C. and W.G. Haldenwang. 1989. The σ^6 subunit of Bacillus subtilis RNA polymerase is present in both forespore and mother cell compartments. J. Bacteriol. 171: 2216–2218.

Cutting, S., S. Panzer, and R. Losick. 1989. Regulatory studies on the promoter for a gene governing synthesis and assembly of the spore coat in Bacillus subtilis. J. Mol. Biol. 207: 393–404.

Dale, R.M.K., B.A. McClure, and J.P. Houchins. 1985. A rapid single-stranded cloning strategy for producing a sequential series of overlapping clones for use in DNA sequencing: Application to sequencing the 16S rDNA. Plasmid 13: 31–40.

De Lencastre, H. and P.J. Piggot. 1979. Identification of different sites of expression for spo loci by transformation of Bacillus subtilis. J. Gen. Microbiol. 114: 377–389.

Donovan, W.P., L. Zheng, K. Sandman, and R. Losick. 1987. Genes encoding spore coat polypeptides from Bacillus subtilis. J. Mol. Biol. 196: 1–10.

Dubnau, D. and R. Davidoff-Abelson. 1971. Fate of transforming DNA following uptake by competent Bacillus subtilis. I. Formation and properties of the donor-recipient complex. J. Mol. Biol. 56: 209–221.

Errington, J. 1986. A general method for fusion of the Escherichia coli lacZ gene to chromosomal genes in Bacillus subtilis. J. Gen. Microbiol. 132: 2953–2966.

Errington, J. and J. Mandelstam. 1986a. Use of a lacZ gene fusion to determine the dependence pattern of sporulation operon spoⅢA in spo mutants of Bacillus subtilis. J. Gen. Microbiol. 132: 2976–2976.

Errington, J. and J. Mandelstam. 1986b. Use of a lacZ gene fusion to determine the dependence pattern and the spore compartment expression of sporulation operon spoⅣA in spo mutants of Bacillus subtilis. J. Gen. Microbiol. 132: 2977–2985.

Farquhar, R. and M.D. Yudkin. 1988. Phenotypic and genetic characterization of mutations in the spoⅢC locus of Bacillus subtilis. J. Gen. Microbiol. 134: 9–17.

Fort, P. and J. Errington. 1985. Nucleotide sequence and complementation analysis of a polycistronic sporulation operon, spoⅣA, in Bacillus subtilis. J. Gen. Microbiol. 131: 1091–1105.

Francesconi, S.C., T.J. MacAlister, B. Setlow, and P. Setlow. 1988. Immunoelectron microscopic localization of small, acid-soluble spore proteins in sporulating cells of Bacillus subtilis. J. Bacteriol. 170: 5963–5967.

Friedman, D. 1988. Integration host factor: A protein for all reasons. Cell 55: 545–554.

Fujita, M. and Y. Kobayashi. 1985. Cloning of sporulation gene spoⅢC in Bacillus subtilis. Mol. Gen. Genet. 199: 471–475.

Jenkinson, H. F., W. D. Saver, and J. Mandelstam. 1981. Synthesis and order of assembly of spore coat proteins in Bacillus subtilis. J. Gen. Microbiol. 123: 1–16.

Johnson, R.C., M.F. Bruist, and M.L. Simon. 1986. Host protein requirements for in vitro site-specific DNA inversion. Cell 46: 531–539.

Karmazyn-Campelli, C., C. Bonamy, B. Savelli, and P. Stragier. 1989. Tandem genes encoding σ-factors for consecutive steps of development in Bacillus subtilis. Genes Dev. 3: 150–157.

Kenney, T.J. and C.P. Moran. 1987. Organization and regulation of an operon that encodes a sporulation-essential sigma factor in Bacillus subtilis. J. Bacteriol. 169: 3329–3339.

Kroos, L., B. Kunkel, and R. Losick. 1989. Switch protein alters specificity of RNA polymerase containing a compartment specific sigma factor. Science 243: 526–529.

Kunkel, B., K. Sandman, S. Panzer, P. Youngman, and R. Losick. 1988. The promoter for a sporulation gene in the spoⅢC locus of Bacillus subtilis and its use in studies of temporal and spatial control of gene expression. J. Bacteriol. 170: 3513–3522.

Labell, T.L., J.E. Trempy, and W.G. Haldenwang. 1987. Sporulation specific σ factor σ^39 of Bacillus subtilis is synthesized from a precursor protein, P^39. Proc. Natl. Acad. Sci. 84: 1784–1788.

Losick, R. and L. Kroos. 1989. Dependence pathways for the expression of genes involved in endospore formation in Bacillus subtilis. In Regulation of procaryotic development. [ed. I. Smith, R. Slepecky, and P. Setlow], pp. 223–241. American Society for Microbiology, Washington, D.C.

Losick, R. and P. Youngman. 1984. Endospore formation in Bacillus. In Microbial development. [ed. R. Losick, and L. Shapiro], pp.63–88. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Losick, R., P. Youngman, and P.J. Piggot. 1986. Genetics of endospore formation in Bacillus subtilis. Annu. Rev. Genet. 20: 625–669.

Mason, J.M., R.H. Hackett, and P. Setlow. 1988. Studies on the regulation of expression of genes coding for small, acid-sol-
Kunkel et al.

uble proteins of Bacillus subtilis spores using lacZ gene fusions. J. Bacteriol. 170: 239–244.

Miller, J.H. 1972. In Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Pabo, C.O. and R.T. Sauer. 1984. Protein-DNA recognition. Annu. Rev. Biochem. 53: 293–321.

Panzer, S., R. Losick, D. Sun, and P. Setlow. 1989. Evidence for an additional temporal class of gene expression in the forespore compartment of sporulating Bacillus subtilis. J. Bacteriol. 171: 561–564.

Piggott, P.J. and J.G. Coote. 1976. Genetic aspects of bacterial endospore formation. Bacteriol. Reviews. 40: 908–962.

Pollack, T.J. and H.A. Nash. 1983. Knotting of DNA caused by a genetic rearrangement: Evidence for a nucleosome-like structure in site-specific recombination of bacteriophage lambda. J. Mol. Biol. 170: 1–18.

Poth, H. and P. Youngman. 1988. A new cloning system for Bacillus subtilis comprising elements of phage, plasmid and transposon vectors. Gene 73: 215–226.

Rong, S., M.S. Rosenkrantz, and A.L. Sonenshein. 1986. Transcriptional control of the Bacillus subtilis spoIID gene. J. Bacteriol. 165: 771–779.

Sandman, K., R. Losick, and P. Youngman. 1987. Genetic analysis of spo mutations generated by Tn917-mediated insertional mutagenesis. Genetics 117: 603-617.

Sandman, K., L. Kroos, S. Cutting, P. Youngman, and R. Losick. 1988. Identification of the promoter for a spore coat protein gene in Bacillus subtilis and studies on the regulation of its induction at a late stage of sporulation. J. Mol. Biol. 200: 461–473.

Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. 74: 5463–5467.

Setlow, P. 1988. Small, acid-soluble spore proteins of Bacillus species: Structure, synthesis, genetics, function, and degradation. Annu. Rev. Microbiol. 42: 319–338.

Shimotsu, H. and D.J. Henner. 1986. Construction of a single-copy integration vector and its use in analysis of regulation of the trp operon of Bacillus subtilis. Gene 43: 85–94.

Sterlini, J.M. and J. Mandelstam. 1969. Commitment to sporulation in Bacillus subtilis and its relationship to the development of actinomycin resistance. Biochem. J. 113: 29–37.

Stragier, P., C. Bonamy, and C. Karmazyn-Campelli. 1988. Processing of a sporulation sigma factor in Bacillus subtilis: How morphological structure could control gene expression. Cell 52: 697–704.

Stragier, P., J. Bouvier, C. Bonamy, and J. Szulmajster. 1984. A developmental gene product of Bacillus subtilis homologous to the sigma factor of Escherichia coli. Nature 312: 376–378.

Stragier, P., B. Kunkel, L. Kroos, and R. Losick. 1989. Chromosomal rearrangement generating a composite gene for a developmental transcription factor. Science 243: 507–512.

Sun, D., P. Stragier, and P. Setlow. 1989. Identification of a new σ-factor involved in compartmentalized gene expression during sporulation of Bacillus subtilis. Genes Dev. 3: 141–149.

Thompson, J.F., L. Moitoso de Vargas, S.E. Skinner, and A. Landy. 1987. Protein-protein interactions in a higher-order structure direct lambda site-specific recombination. J. Mol. Biol. 195: 481–493.

Trempy, J.E., J. Morrison-Plummer, and W.G. Haldenwang. 1985b. Bacillus subtilis sigma factor σ 29 is the product of the sporulation-specific gene spoIII G. Proc. Natl. Acad. Sci. 82: 4189–4192.

Turner, S.M., J. Errington, and J. Mandelstam. 1986. Use of a lacZ gene fusion to determine the dependence pattern of sporulation operon spoIII C in spo mutants of Bacillus subtilis: A branched pathway of expression of sporulation operons. J. Gen. Microbiol. 132: 2995–3003.
Temporal and spatial control of the mother-cell regulatory gene spoIID of Bacillus subtilis.

B Kunkel, L Kroos, H Poth, et al.

Genes Dev. 1989, 3: Access the most recent version at doi:10.1101/gad.3.11.1735

References

This article cites 45 articles, 18 of which can be accessed free at: http://genesdev.cshlp.org/content/3/11/1735.full.html#ref-list-1

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or click here.