Prespore-specific gene expression in *Bacillus subtilis* is driven by sequestration of SpoIIE phosphatase to the prespore side of the asymmetric septum

Ling Juan Wu, Andrea Feucht, and Jeffery Errington¹

Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, UK

The *spoIIE* gene is essential for the compartment-specific activation of transcription factor σ^F during sporulation in *Bacillus subtilis*. SpoIIE is a membrane protein that is targeted to the potential sites of asymmetric septation near each pole of the sporulating cell. The cytoplasmic carboxy-terminal domain of SpoIIE contains a serine phosphatase that triggers the release of σ^F in the prespore compartment after septation. To understand how septum-located SpoIIE is activated selectively in the prespore, we examined the distribution of a SpoIIE-GFP fusion protein. We show that the polar bands of SpoIIE protein actually form sequentially and that the most prominent band develops at the pole where the prespore forms. We also show that the protein is sequestered to the prespore side of the asymmetric septum. Sequestration of SpoIIE into the prespore compartment provides a mechanism that could explain the cell specificity of σ^F activation.

[Key Words: *Bacillus subtilis*; sporulation; transcriptional regulation; SpoIIE protein; σ^F ; differentiation]

Received December 8, 1997; revised version accepted February 16, 1998.

The onset of sporulation of *Bacillus subtilis* is marked by the formation of the asymmetric septum, which divides the sporulating cell into a small prespore and a large mother cell. Soon after formation of the asymmetric septum, new transcriptional activities directed by σ factors σ^F and σ^E appear (for review, see Errington 1993; Stragier and Losick 1996). σ^F becomes active specifically in the prespore and initiates the prespore program of developmental gene expression. It also directs a signal across the septum, which leads to activation of σ^E in the mother cell. Mutations in several genes affect the morphology of sporulation in ways that can be used to probe the regulation of σ^F and σ^E . Thus, mutations in the *spoIIG* operon (encoding σ^E and its regulator) lead to sequential formation of two prespore-like cells, at opposite poles of the parent cell, in both of which σ^F becomes activated (Piggot and Coote 1976; Lewis et al. 1994). Mutations in the *spoIIIE* gene interfere with segregation of the prespore chromosome (Wu and Errington 1994). In the absence of SpoIIIE protein, only a segment comprising about one-third of the chromosome, centered approximately on *oriC*, enters the prespore compartment, leaving the remaining two-thirds of the chromosome in

the mother cell. Therefore, mutations in *spoIIIE* affect σ^F -directed gene expression in a manner dependent on chromosome position (Wu and Errington 1994, 1998). Also, null mutations in *spoIIIE* result in aberrant release of σ^F activity in the mother cell, for reasons that are not yet understood (Wu and Errington 1994).

σ^F is made before asymmetric septation, together with two coordinately expressed regulatory proteins, SpoIIAA and SpoIIAB (for review, see Errington 1996; Stragier and Losick 1996). At first, σ^F is held in an inactive complex by the anti- σ factor SpoIIAB (Duncan and Losick 1993; Min et al. 1993; Alper et al. 1994). The anti-anti- σ factor SpoIIAA is also maintained in an inactive state by the kinase activity of SpoIIAB, which phosphorylates it on a specific serine residue (Min et al. 1993; Diederich et al. 1994; Najafi et al. 1995; Magnin et al. 1996). After asymmetric septation, σ^F remains inactive in the mother cell, as in the pre-septation cell, but in the prespore it is released from SpoIIAB (Margolis et al. 1991; Lewis et al. 1996). This release is triggered by SpoIIE, a membrane-bound phosphatase, which dephosphorylates SpoIIAA-P and allows it to bind SpoIIAB, thereby releasing σ^F (Duncan et al. 1995; Arigoni et al. 1996; Feucht et al. 1996; Magnin et al. 1997). Thus, SpoIIE plays a critical role in the regulation of σ^F .

SpoIIE protein is synthesized soon after the initiation of sporulation, before asymmetric septation (Guzman et

¹Corresponding author.
E-MAIL erring@molbiol.ox.ac.uk; FAX 44-1865-275556.

al. 1988). Arigoni et al. (1995) reported that the protein is localized initially in a bipolar pattern, with bands (probably corresponding to circumferential rings of protein) located close to but not at both poles of the predivisional cell. Asymmetric septation then takes place at the site of one of the bands. Subsequently, the prespore-distal band disappears and the prespore-proximal SpoIIE protein, which presumably triggers activation of σ^F in the prespore, is retained. Eventually the prespore-proximal SpoIIE band also disappears. A crucial question that remains, however, concerns how the septum-located SpoIIE phosphatase selectively dephosphorylates SpoIIAA-P in the prespore but not in the mother cell. The asymmetric septum consists of two membranes flanking a thin layer of wall material (Illing and Errington 1991). As the septum forms, the SpoIIE protein, which is anchored to the membrane through its amino-terminal hydrophobic domain (Barak et al. 1996), could become equally distributed on both faces of the septum. Localized activation of σ^F could then occur as a result of the relatively higher concentration of the phosphatase in the small prespore compared with the much larger mother cell compartment (Duncan et al. 1995). Alternatively, there could be selective inactivation or degradation of the SpoIIE protein on the mother cell face of the septum. A third possibility is that the SpoIIE protein is sequestered specifically onto the prespore side of the asymmetric septum as it forms (Feucht et al. 1996). Biochemical fractionation experiments (Barak et al. 1996) have shown that most of the SpoIIE protein eventually associates with the prespore, but these experiments can only address the localization of the protein in completely engulfed prespores, long after the protein acts on septation and σ^F activation.

To better understand the mechanism by which the compartmentalization of σ^F activity and, therefore, cellular asymmetry is established, we have re-examined the distribution of the SpoIIE protein tagged with green fluorescent protein (GFP). Previous studies have established that GFP fusions provide a convenient and accurate means of determining the subcellular localization of proteins in bacteria (see, e.g., Glaser et al. 1997), although it is important to establish that the fusion protein functions normally. With a fully functional SpoIIE-GFP fusion we show first that the two polar SpoIIE bands (rings) form sequentially, suggesting that the limited availability of a component of the machinery required for septation could be responsible for the formation of only one asymmetric septum. We also show directly that the SpoIIE phosphatase is sequestered to the prespore side of the septum when it forms. This sequestration could explain the localized activation of σ^F in the prespore compartment.

Results

Construction and characterization of a *spoIIE-gfp* fusion strain

To examine the distribution of the SpoIIE protein, we constructed a plasmid carrying an in-frame *spoIIE-gfp*

fusion, which would allow easy introduction of the fusion onto the *B. subtilis* chromosome by Campbell-like recombination. A previously described SpoIIE-GFP strain (Arigoni et al. 1995) showed reduced sporulation efficiency, suggesting that the fusion protein is not fully functional. Cormack et al. (1996) have reported the derivation of mutant forms of GFP with improved folding properties and brightness. Therefore, we fused the SpoIIE protein to such a mutant GFP. Sporulating cells of the wild-type strain SG38 and the *spoIIE-gfp* derivative (strain 1305) showed similar frequencies of asymmetric septation at 80 min after initiation of sporulation (t_{80}) (46% and 45%, respectively) and spore formation at t_{420} (84% and 82%, respectively), suggesting that the fusion protein is fully functional. We also examined the fusion protein by Western blot analysis using both anti-SpoIIE and anti-GFP antibodies. Figure 1 shows that the time courses for production of SpoIIE or SpoIIE-GFP proteins were similar for both strains. In some experiments a faint fast migrating band that cross-reacted with anti-SpoIIE but not anti-GFP antibody could be detected from cultures of strain 1305. This band migrated at the same position as untagged SpoIIE protein and seemed to appear as early as t_{60} (broken arrow in Fig. 1). Because no free GFP protein was detected at this time (see below and Fig. 2A), this fast migrating band could be SpoIIE without the GFP tag, formed by a low level of translational arrest before the *gfp* coding sequence. Alternatively, it could be attributable to a low level of cleavage of the fusion protein, in which case the free GFP protein must either have been degraded or be present in quantities too low to be detected by Western blot analysis.

Figure 1. Accumulation and stability of SpoIIE and SpoIIE-GFP in sporulating cells and protoplasts. (A) Whole cell extracts of sporulating wild-type strain SG38 and the *spoIIE-gfp*-carrying strain 1305, taken at the times indicated, were analyzed by Western blot using anti-SpoIIE antibodies. (B) To demonstrate that protoplasting does not change the degradation pattern of SpoIIE-GFP, cells of strain 1305 were collected and protoplasted at t_{90} , then treated in various ways before being subjected to Western blot analysis using anti-SpoIIE antibodies (lanes 1–3) or monoclonal anti-GFP antibody (lane 4). The protoplasts were treated as follows: (lane 1) pelleted and frozen immediately; left for 30 min (lanes 2,4) at room temperature or (lane 3) on ice, then pelleted and frozen. The positions of SpoIIE and SpoIIE-GFP are indicated by solid arrows. The broken arrow indicates the position of a faster migrating band that cross-reacted with anti-SpoIIE, possibly representing near full-length SpoIIE without the GFP tag.

Figure 2. Time course of SpoIIE/SpoIIE-GFP degradation during sporulation. Whole cell extracts from sporulating cells were loaded on a 10% SDS-polyacrylamide gel and analyzed by Western blot using monoclonal anti-GFP antibody. Samples were prepared from the following strains: (A) 1305 (*spoIIE-gfp*); (B) 1211(*spoIIE-gfp spoIIGA::aph-A3*); (C) 1210 (*spoIIE-gfp spoIIE604*). Samples were taken and prepared as described in the legend to Fig. 1. The positions of full-length SpoIIE-GFP and the major SpoIIE-GFP degradation product are indicated by solid and broken arrows, respectively.

When the cultures of strain 1305 were probed with anti-GFP antibody, a small band of expected size for the GFP protein alone began to be detected from about t_{105} (Fig. 2A). Formation of this band was greatly reduced in *spoIIG* and *spoIIE* mutants (Fig. 2B,C). This proteolytic cleavage probably corresponds to developmentally regulated degradation of SpoIIE. Pogliano et al. (1997) showed that disappearance of the prespore-distal SpoIIE band was blocked by mutations in these genes. Although this developmental degradation of SpoIIE generates free, and thus untargeted, GFP, this should not affect interpretation of the results described below because it is not formed until well after σ^F and σ^E become active.

Kinetics of SpoIIE ring formation

Previous observations based on immunofluorescence microscopy suggested that SpoIIE bands form initially at both poles of the sporulating cell and that later they break down sequentially, first at the prespore-distal pole (Arigoni et al. 1995). When we looked at early sporulating cells containing the new *spoIIE-gfp* fusion, we were surprised to find that many cells had a single SpoIIE band (90% of the cells with localized SpoIIE at t_{70} , and 67% at t_{90}), or a bipolar pattern with one bright and one weaker band. Similar results were obtained with immunofluorescence microscopy (data not shown). At such an early stage of sporulation, it seemed unlikely that this was attributable to developmental degradation (i.e., loss of one band). Therefore, we examined the kinetics of the SpoIIE band formation in greater detail, using both live and fixed sporulating cells. Figure 3 shows examples of fields of cells of the wild type at different times of sporu-

lation and illustrating different patterns of SpoIIE-GFP localization. Cells with “1 band” were further divided into those exhibiting a weak straight band and those with a curving band. The curved bands were only observed at later time points, and we assume that they represent cells at a much later stage of development, either undergoing or having completed engulfment. The developmental stages of the cells were defined more precisely by staining the cells with DAPI to reveal their nucleoid morphology (Fig. 3B,D,F). It is clear from the images that in early samples (t_{70}), probably before or dur-

Figure 3. SpoIIE band formation and development. Strains carrying the *spoIIE-gfp* fusion were induced to sporulate, and live DAPI-stained cells were examined by fluorescence microscopy. (A,B) Wild-type (1305) cells at t_{70} ; (C,D) wild-type cells at t_{105} ; (E,F) *spoIIG spoIIE* double mutant (strain 1213) at t_{90} . (A,C,E) Localization of SpoIIE-GFP; (B,D,F) DAPI images showing the nucleoids of cells in the same fields as in A, C, and E, respectively. (0) Examples of cells without SpoIIE bands; (1) cells with one relatively weak and straight band, corresponding to cells that have not started to engulf; (1*) cells with one strong and curving fluorescent band, corresponding to cells undergoing or having completed engulfment; (2) cells with two SpoIIE bands. Arrows point to the asymmetric septa in cells that have not yet completed transfer of the prespore chromosome. Scale bar, 2.5 μ m.

ing asymmetric septation, often only one very weak SpoIIE band could be observed in each sporulating cell. In most of these cells (e.g., arrowed cells in Fig. 3A–D), the band was at the position of a constriction in the DNA, which corresponds to the site of a nascent asymmetric septum. In slightly later samples, cells with two bands could be observed, with one band brighter than the other (Fig. 3C,D). These cells generally showed a completely condensed prespore nucleoid, indicating that they had formed a septum and completed transfer of the prespore chromosome. Later still, cells with a visible prespore-distal band became less prevalent.

To confirm that the observation of single SpoIIE bands in early sporulating cells was not attributable to degradation in cells that initially had two bands, we examined cells of a *spoIIG spoIIIE* double mutant, in which developmental degradation is abolished (Pogliano et al. 1997; see above). As shown in Figure 3, cells with a single band at the site of nascent septation were readily detected in this mutant background. Therefore, we conclude that the polar bands of SpoIIE protein are generally formed sequentially, first appearing at the pole where the prespore septum forms.

Sequestration of SpoIIE phosphatase to the prespore side of the asymmetric septum

To examine the distribution of the SpoIIE phosphatase, we treated sporulating cells of strain 1305 with lysozyme. Treatment with lysozyme would digest away the thin peptidoglycan layer between the prespore and the mother cell membranes (before engulfment), as well as protoplasting the prespore and the mother cell. Therefore, upon protoplasting the distance between the prespore and the mother cell could be increased and it should be possible to distinguish between one-sided and two-sided distributions of the SpoIIE protein, as illustrated schematically in Figure 4A,B. Figure 4C–F shows the distribution of SpoIIE–GFP in protoplasted sporulating cells either just before (C,D) or just after (E,F) σ^F becomes active (t_{85} and t_{100} , respectively). Both samples were thus taken before developmental degradation of SpoIIE begins. The images show clearly that in both samples the SpoIIE–GFP signal was concentrated, often as a spot, in the prespore. The mother cell showed a weak signal throughout the cell. Weak, evenly distributed signals were also detected in mother cells of live (untreated) cultures from different times of sporulation, before and after developmental degradation of SpoIIE could be detected (Fig. 3A,C; data not shown), suggesting that this weak mother cell signal primarily came from cleaved (see above) or untargeted SpoIIE–GFP.

To check that the protoplasting procedure did not cause degradation of SpoIIE–GFP, we treated some sporulating wild-type cells at t_{90} to make protoplasts, then either pelleted and froze the protoplasts immediately, or left them on ice or at room temperature for 30 min before pelleting them. The samples were then analyzed by Western blot using both anti-SpoIIE and anti-GFP anti-

Figure 4. Localization of SpoIIE–GFP in protoplasted cells from sporulating cultures just before and after σ^F becomes active. (A,B) Schematic illustration of the consequences of treatment of (A) wild-type and (B) *spoIIG* mutant cells with lysozyme to make protoplasts. Removal of the cell wall material (shaded) allows the prespore (P), mother cell (M), or central (C) compartments to enlarge and round off and separate, allowing the fate of GFP initially located at the site of the thin division septum to be resolved. The remaining panels show images of typical cells. (C–F) Wild type (strain 1305); (G–J) *spoIIG spoIIIE* double mutant (strain 1213); (K,L) *spoIIG* mutant (strain 1211). (C,D,G,H) Cells at an early stage of sporulation (t_{85}), just before activation of σ^F . (E,F,I–L) Cells at slightly later stages of sporulation (t_{100}), just after σ^F becomes active. (C,E,G,I,K) Fluorescence images showing the distribution of SpoIIE–GFP; (D,F,H,J,L) phase-contrast images of same fields as C, E, G, I, and K, respectively. (N) Nonseptate cell. Arrows point to some of the SpoIIE spots in the mother cell protoplasts. Scale bar, 2.5 μ m.

bodies. As shown in Figure 1B, no degradation products could be detected even 30 min after the protoplasts were incubated at room temperature. This suggests that the fusion protein survived the protoplasting procedure and remained stable for a considerable period at room temperature. This excluded the possibility that the weak signal in the mother cell could be attributable to degradation of the fusion protein caused by the protoplasting procedure.

In the mother cell protoplast sometimes a small SpoIIE spot could also be detected at the pole opposite to the prespore (arrows in Fig. 4C), but no concentrated signals were seen on the face of the mother cell near to the

prespore. Presumably the prespore-distal SpoIIE spots were from the prespore-distal bands. The presence of these spots in some mother cell protoplasts suggested that the prespore-distal SpoIIE band did not always disassemble during protoplasting. However, to examine this further, as well as to exclude the possibility that SpoIIE did exist on the mother cell face but disassembled during protoplasting, we took advantage of a *spoIIG spoIIIE* double mutant. Western blot analysis showed that in both the *spoIIG* and the *spoIIIE* single mutants, developmental degradation of SpoIIE was greatly reduced or delayed (see Fig. 2). Pogliano et al. (1997) also reported that a *spoIIG spoIIIE* double mutant produces elevated levels of SpoIIE and has stable persistent SpoIIE bands at both poles. Therefore, if SpoIIE did exist on the mother cell face of the asymmetric septum and was disassembled upon protoplasting, the central compartment of the double mutant would be expected to show a stronger GFP signal, derived from two bands of SpoIIE rather than one. As shown in Figure 4G–J, this was not the case; the signal in the central compartment was actually much weaker than that observed in the mother cell of the wild-type cells. Furthermore, no SpoIIE spots could be observed in the central compartments. To check that the distribution pattern of the SpoIIE protein observed in the *spoIIG spoIIIE* double mutant was not attributable to some special effect of the *spoIIIE* mutation, we also examined the distribution of SpoIIE–GFP in a *spoIIG* single mutant (strain 1211) and obtained similar distribution patterns as in the double mutant (Fig. 4K,L). These results strongly suggest that there is no SpoIIE protein on the mother cell face of the completed asymmetric septum.

Genetic evidence for sequestration of SpoIIE to the prespore face of the asymmetric septum

Previously we reported that *spoIIIE* null mutants have σ^F activity in the mother cell compartment (Wu and Errington 1994). Studies by Pogliano et al. (1997) showed that the prespore-distal SpoIIE band persists in a *spoIIIE* null mutant and thus that the activation of σ^F in the mother cell of this mutant could be attributable to persistence of the SpoIIE band in the mother cell. If the SpoIIE protein were distributed on both sides of the asymmetric septum, rather than being restricted to the prespore side, one would expect that σ^F would also become active in the central compartment of *spoIIG spoIIIE* double mutants, in which both SpoIIE bands persist. To test this we examined the expression of a σ^F -dependent *gpr-lacZ* fusion in the central compartment during sporulation of the double mutant. *spoIIIE* mutations block prespore chromosome translocation and the *gpr* locus lies in the part of the chromosome that is excluded from the prespore (Wu and Errington 1994). As shown in Figure 5, the reporter gene was expressed in both the *spoIIG* and *spoIIIE* single mutants, but not in the *spoIIG spoIIIE* double mutant. When the reporter gene was moved to the prespore-located *amyE* locus it became highly expressed in the double mutant, suggest-

Figure 5. Absence of σ^F activity from the central compartment of a *spoIIG spoIIIE* double mutant. Various strains carrying a σ^F -dependent *gpr-lacZ* fusion located at the *gpr* locus (::pPS1395) or at *amyE* (::pPS1326) were induced to sporulate and β -galactosidase was assayed. (●) Wild-type strain SG38::pPS1395; (▲) *spoIIG spoIIIE* double mutant with the fusion located at the *gpr* locus (919::pPS1395); (△) *spoIIG spoIIIE* double mutant with the fusion located at the *amyE* locus (919::pPS1326); (○) *spoIIG* single mutant 901::pPS1395; (□) *spoIIIE* single mutant 604.6::pSG1395.

ing that σ^F is active in the prespores in this mutant. This clearly demonstrates that in the double mutant σ^F activity is restricted to the prespores despite the presence of the *spoIIIE* mutation. Again these results would be consistent with SpoIIE being sequestered to the prespore face of the asymmetric septum.

Discussion

Previous work showed that the SpoIIE protein localizes in bands at the potential sites of asymmetric septation near each pole early in sporulation (Arigoni et al. 1995). Localization at these sites was likely to play an important role in SpoIIE function because the protein is required for both formation of the asymmetric septum and for activation of σ^F in the polar compartment once formed (Barak and Youngman 1996; Feucht et al. 1996). Now we have used a fully functional *spoIIIE-gfp* fusion to examine SpoIIE band formation, development, and degradation in more detail. The results obtained from observations of wild-type and mutant strains are summarized in schematic form in Figure 6A.

Sequential formation of SpoIIE bands

In previous work, mainly on the basis of immunofluorescence microscopy (Arigoni et al. 1995), it was reported that SpoIIE assembles initially in a bipolar pattern. We were surprised to find that when cells containing the SpoIIE–GFP fusion were examined early in sporulation, a monopolar pattern was predominant. Thus, at t_{70} when

Figure 6. Schematic summary of the localization of SpoIIE protein in wild-type and mutant cells (A) and possible models for sequestration of SpoIIE to the prespore compartment (B,C). (A) 1 represents a pre-septational cell soon after the initiation of sporulation; open arrowheads indicate the potential sites for asymmetric septation close to each pole. σ^F is present throughout the cytoplasm but in an inactive state (indicated by outline text). SpoIIE protein (○) then begins to be made (2) and is localized initially in a band at the polar septation site where the asymmetric septum will be formed, presumably by association with a component of the division apparatus. When the septum has formed (3) the SpoIIE protein is sequestered specifically into the membrane on the prespore side, and a second band of SpoIIE starts to appear at the prespore-distal potential division site. Sequestration of most of the cellular SpoIIE into the small prespore compartment results in activation of σ^F (boldface type). In wild-type cells SpoIIE in the mother cell is then degraded (4), probably as a result of activation of σ^E and synthesis of one or more proteases encoded by genes controlled by the new form of RNA polymerase. In *spoIIIE* mutants (5), the second SpoIIE ring persists in the mother cell, leading to aberrant activation of σ^F in that compartment. In *spoIIG* single or *spoIIG spoIIIE* double mutant (6), the absence of σ^E allows the second polar septum to form. The second SpoIIE ring is thus sequestered into its prespore, leaving the central compartment devoid of SpoIIE, and hence preventing σ^F from being activated in this compartment.

(B) SpoIIE protein localizes initially to the site of incipient septation. As the septal annulus closes, flux of membrane lipid from the large compartment (where the bulk of new lipid synthesis could conceivably take place) drives the membrane-bound SpoIIE protein into the prespore compartment. (C) The SpoIIE band forms just to the polar side of the division site, so that as the septum forms the protein is captured by the prespore.

only the first few cells have formed an asymmetric septum, and long before developmental degradation of SpoIIE could be detected (Fig. 2), most such cells had only one prominent band, at the position of the septum (Fig. 3). The polar constriction in the DNA of these cells shows that they had just formed a septum but had not yet translocated the whole of the prespore chromosome into the small compartment and thus had not yet activated σ^F (Partridge and Errington 1993; Lewis et al. 1994). In later samples, the majority of cells did have two SpoIIE bands, but such cells were generally at a later stage of development, with a completely segregated prespore chromosome (Fig. 3). Eventually, as reported previously (Arigoni et al. 1995), the SpoIIE bands disappeared, first from the prespore-distal pole. Presumably, this reflects the developmental degradation of the protein seen in the Western analysis from about t_{105} (Fig. 2). We conclude, particularly from the analysis of cells in the early samples, that SpoIIE band formation occurs sequentially, rather than in a simultaneous bipolar manner. Because targeting of SpoIIE to these bands requires interaction with one or more components of the division apparatus (Levin et al. 1997), the differential development of the SpoIIE bands probably reflects differential assembly of the division apparatus at the two poles. This would accord well with the previous finding that in disporic mutants, the second asymmetric septum is formed about 15 min later than the first one (Lewis et al. 1994). Taken together, these observations are consistent with the notion that the choice of pole for formation of the

prespore septum is attributable to the limited availability of one or more components of the division apparatus (Lewis et al. 1994). Moreover, the finding that in many cells, most of the SpoIIE protein is located at the pole where septation occurs, accords with its proposed role in modification of the form of the sporulation septum (Illing and Errington 1991).

Sequestration of SpoIIE protein to the prespore-face of the asymmetric septum

Both direct microscopic observations and indirect genetic experiments suggested that after formation of the asymmetric septum, the SpoIIE protein in the band marking the site of impending septation is sequestered into the prespore compartment. Microscopic examination of whole living cells did not provide sufficient resolution to be able to distinguish between localization on one or both sides of the septum. However, treatment with lysozyme, leading to formation of protoplasts, resulted in expansion of the space between the septal membranes of the prespore and mother cell, and revealed a strong enrichment of the SpoIIE-GFP in the prespore compartment. In many of the treated cells, especially those in which protoplasting was only partial (e.g., Fig. 4C), the SpoIIE-GFP signal remained highly concentrated, as in the bands of untreated cells, and the concentrated spots were clearly associated with the prespore, rather than with the mother cell. Control experiments showed that the background fluorescence associated

with the mother cell was probably not attributable to degradation of the fusion protein (at least in the early samples). Probably, it represents untargeted protein, as a diffuse background signal was generally visible in the living untreated cells (Fig. 3).

The conclusion that SpoIIE becomes sequestered into the prespore was reinforced by examination of *spoIIG* mutant cells. These mutant studies were useful for two reasons. First, *spoIIG* mutants form a second prespore-like cell at the opposite pole of the cell (see above), therefore, the SpoIIE protein associated with the prespore-distal band should either remain partly associated with the central compartment, or be sequestered into the second prespore leaving only a low background signal in the central compartment. Second, as shown previously by Pogliano et al. (1997), the prespore-distal SpoIIE band persists in *spoIIG* mutants (and both bands in *spoIIG spoIIIE* double mutants), therefore, SpoIIE localization after septation should be more readily evident in these mutants than in the wild type. Microscopic examination of such mutants showed strong signals in the prespore protoplasts but not in the central compartment, strongly supporting our contention that the SpoIIE-GFP from the polar bands is sequestered to the membrane of the prespore compartment after septation (see Fig. 6A).

The disporic phenotype of *spoIIG* mutants also provided an indirect genetic test for sequestration of SpoIIE protein to the prespore side of the septum. If sequestration results in removal of most of the SpoIIE phosphatase activity from the central compartment, σ^F should not become active there. Although the central compartment of these mutants is normally devoid of DNA and thus would not normally contain a σ^F -dependent reporter gene, it was possible to test activity in this compartment by use of a *spoIIIE* mutation, which results in most of the chromosome failing to be segregated into the prespore compartment (Wu and Errington 1994; Wu et al. 1995). As shown in Figure 5, in a *spoIIG spoIIIE* double mutant, strong σ^F activation was detected with a reporter gene segregated into the prespore compartment but not with the same reporter positioned in the central compartment. Although this result strongly supports the sequestration of SpoIIE protein out of the central compartment and into the polar ones, interpretation of the data is made slightly complicated by the 15- or 20-min delay between formation of the first and second polar septa in *spoIIG* mutants (Lewis et al. 1994). The absence of detectable σ^F activity in the large (later central) compartment of the double mutant suggests that σ^F does not become active in the interval before the second septum forms. The notion that aberrant σ^F activation in the large compartment of *spoIIIE* null mutants is delayed would be consistent with the *lacZ* fusion data; for example, the open squares versus open triangles in Figure 5 (also L.J. Wu, P.J. Lewis, and J. Errington, unpubl.). Unfortunately, we do not yet understand the molecular basis for mother cell activation of σ^F in *spoIIIE* null mutants and this aspect of the mutant phenotype may be a misleading, indirect consequence of the unbalanced genetic and physiological state of the cells. Nevertheless, in results to

be presented elsewhere, we have found that dephosphorylation of SpoIIAA (attributable to SpoIIE activity) occurs predominantly in the prespore compartment of disporic *spoIIG* mutant cells, even in the absence of the complicating *spoIIIE* mutation (P.J. Lewis and J. Errington, unpubl.). Therefore, we conclude, on the basis of both direct and indirect evidence, that formation of the asymmetric septum results in sequestration of SpoIIE protein onto the prespore face of the asymmetric septum.

How might sequestration of SpoIIE to the prespore face of the septum be achieved? One possibility would be for the SpoIIE protein to be transferred through the septum as it forms (Fig. 6B). As we suggested previously (Feucht et al. 1996), the asymmetric positioning of the septum probably results in a net influx of membrane lipid through the septal annulus as it constricts. Transport of the SpoIIE protein could be driven by this flux. Alternatively, it is possible that the SpoIIE ring is slightly offset from the division apparatus, toward the pole, so that when the septum forms, the topology dictates that SpoIIE becomes trapped in the prespore (Fig. 6C). Our present methods do not provide sufficient resolution to distinguish between the relative positions of SpoIIE and other proteins that contribute to formation of the septum, such as FtsZ, before septation.

Whatever the mechanism of sequestration, our finding that the strongest SpoIIE band tends to lie at the pole where prespore septation takes place, means that most of the cellular SpoIIE is trapped in the small prespore compartment after septation. The resultant high concentration of SpoIIE in the prespore conceivably could provide the trigger for σ^F activation by overwhelming the opposing kinase reaction that maintains the inhibitory state (see introductory section). It could also provide a simple explanation for the dependence of σ^F activation on formation of the septum (Levin and Losick 1994).

Materials and methods

Bacterial strains and plasmids

The bacterial strains and the plasmids used in this study are listed in Table 1. Plasmid pSG1151 (P.J. Lewis, unpubl.) is a derivative of pSG1137 (Lewis and Errington 1996) with an additional mutation (F64L) in the *gfp* gene (Cormack et al. 1996). To make a new *spoIIE-gfp* fusion a 871-bp fragment was amplified by PCR from chromosomal DNA of CW314 (Arigoni et al. 1995), in which the 3' end of *spoIIE* was joined in-frame to *gfp* through a polylinker sequence. The PCR product was digested with *HindIII* and *MscI* and the resulting 770 bp fragment was subcloned into *HindIII*-*MscI*-digested pSG1151. The oligonucleotides used for the PCR were 5'-CAGCTACAGCATGATGGAGC-3' and 5'-AGTGACAAGTGTGGCCATGG-3'. Plasmid pSG1901 was constructed by cloning the 1571-bp *spoIIE* fragment amplified from SG38 chromosomal DNA using PCR and digested with *Bam*HI into pQE-30 (Qiagen), thereby fusing codon 325 of *spoIIE* in-frame with the polyhistidine-encoding sequence of pQE-30. The oligonucleotides used for PCR were 5'-ACGCGGATCCACGAGGAAAGTGGCGAG-3' and 5'-GCGGATCCCATATATTTCCCATCTTCGCCAGAAG-3', which would create a *Bam*HI site at each end of the PCR product.

Table 1. Bacterial strains and plasmids

Strain/plasmid		Relevant genotype ^a	Construction, source, or reference
<i>B. subtilis</i>			
SG38	<i>trpC2 amyE</i>		Errington and Mandelstam (1986)
CW314	<i>spoIIE::pCW28 (spoIIE-gfp aph-A3)</i>		Arigoni et al. (1995)
1305	<i>trpC2 amyE spoIIE::pSG1902 (spoIIE-gfpF64L S65T cat)</i>		pSG1902 transformed into SG38, with selection for chloramphenicol resistance
604.6	<i>trpC2 spoIIE604</i>		M. Deadman (unpub.)
901	<i>trpC2 Δ (spoIIGA::aph-A3)901</i>		Wu and Errington (1994)
919	<i>trpC2 spoIIE604 Δ (spoIIGA::aph-A3)901</i>		Wu and Errington (1998)
1210	<i>trpC2 spoIIE604 spoIIE::pSG1902 (spoIIE-gfpF64L S65T cat)</i>		pSG1902 transformed into 604.6, with selection for chloramphenicol resistance
1211	<i>trpC2 Δ (spoIIGA::aph-A3)901 spoIIE::pSG1902 (spoIIE-gfpF64L S65T cat)</i>		pSG1902 transformed into 901, with selection for chloramphenicol resistance
1213	<i>trpC2 spoIIE604 Δ (spoIIGA::aph-A3)901 spoIIE::pSG1902 (spoIIE-gfpF64L S65T cat)</i>		pSG1902 transformed into 919, with selection for chloramphenicol resistance
<i>E. coli</i>			
DH5α	<i>F⁻ endA1 hsdR17 supE44 λ⁻ thi-1 recA1 gyrA96 relA1 Δ(lacZYA-argF)U169(φ80dIac) Δ(lacZ)M15</i>		GIBCO BRL
MC1061	<i>F⁻ hsdR mcrB araD139 Δ(araABC-leu)7679 galU galK Δ(lac)X74 rpsL thi</i>		Meissner et al. (1987)
NM554 (pREP4)	<i>F⁻ hsdR mcrB araD139 Δ(araABC-leu)7679 galU galK Δ(lac)X74 rpsL thi recA13 (pREP4) lacI⁺</i>		pREP4 (Qiagen) transformed into NM554 of Raleigh et al. (1988)
Plasmids			
pSG1151	<i>bla cat gfpF64L S65T</i>		P.J. Lewis (unpub.)
pSG1901	<i>bla spoIIE (973-2484)</i>		this paper
pSG1902	<i>bla cat spoIIE (1932-2481)-gfpF64L S65T</i>		this paper
pPS1395	<i>grp-lacZ</i> in pJF751		P. Setlow (University of Connecticut Health Center, Farmington)
pPS1326	<i>grp-lacZ</i> in ptrpBG1		Sun and Setlow (1991)

^aNumbers in parentheses after *spoIIE* refer to the first and last nucleotides of the insert from the *spoIIE* coding sequence.

Plasmids pSG1901 and pSG1902 were constructed using *Escherichia coli* strains DH5α and MC1061, respectively. Both plasmids were sequenced to ensure that the cloned *spoIIE* fragments did not contain any mutations.

Growth media

Nutrient agar (Oxoid) was used as a solid medium for growing *B. subtilis*. Chloramphenicol (5 μg/ml) or kanamycin sulfate (5 μg/ml) were added as required. Media used for growing *E. coli* were 2× TY (Sambrook et al. 1989) and nutrient agar (Oxoid) supplemented with ampicillin (100 μg/ml) and kanamycin sulfate (25 μg/ml) as necessary.

General methods

B. subtilis cells were made competent for transformation with DNA by the method of Anagnostopoulos and Spizizen (1961), as modified by Jenkinson (1983). *B. subtilis* chromosomal DNA was prepared by a scaled-down method based on the one described by Errington (1984). DNA manipulations and *E. coli* transformations were carried out using standard methods (Sambrook et al. 1989).

Induction of sporulation

B. subtilis cells grown in hydrolyzed casein growth media at 37°C were induced to sporulate by the resuspension method of Sterlini and Mandelstam (1969), as specified by Nicholson and Setlow (1990) and Partridge and Errington (1993). Times (minutes) after resuspension of cells in the starvation medium were denoted t_0 , t_{60} , t_{75} , and so forth. β-Galactosidase activity was measured by the method of Errington and Mandelstam (1986). One unit of β-galactosidase catalyzes the production of 1 nmole of 4-methylumbelliferone per minute under the standard reaction conditions.

Measurement of septum formation and sporulation frequency

The frequency of asymmetric septation of strain 1305 and the wild-type strain SG38 were determined at t_{80} , as described by Feucht et al. (1996). The sporulation frequency was determined by counting spores and total cells with a phase contrast microscope at t_{420} .

Antibodies and Western blots

To raise antibodies against SpoIIE the histidine-tagged, carboxy-

terminal fragment of SpoIIE was overexpressed using plasmid pSG1901 in *E. coli* NM554 (pREP4) and the resulting insoluble fusion protein was purified as described in the Qiagen protocol. A rabbit polyclonal antiserum was raised by standard procedures (Harlow and Lane 1988). For the purification of the anti-SpoIIE antibodies the truncated SpoIIE protein was further purified by a Prep Cell (model 491, Bio-Rad) as described by the manufacturer. The antibodies were affinity purified as described by Reznikov et al. (1996) using 4.5 M MgCl_2 as eluant. The final purified antiserum was used at a dilution of 1:200 for Western blot analysis (Harlow and Lane 1988). Monoclonal anti-GFP antibody (Clontech) was used at a dilution of 1:1750 for Western blot analysis. The amount of protein samples loaded on SDS-PAGE for Western blot analysis was determined by a Bio-Rad protein assay.

Protoplasting and fluorescence microscopy

Samples of sporulating cultures were centrifuged to harvest the cells (at different times after induction of sporulation) and the cell pellet was resuspended quickly in about half volume of the original sample of $1 \times \text{SMM}$ [0.5 M sucrose, 20 mM maleic acid; 20 mM MgCl_2 (pH 6.5)] containing 4 mg/ml lysozyme, as described by Errington (1990). The mixture was mixed gently for 1 min, then $\sim 1.5 \mu\text{l}$ was loaded onto a polylysine-treated microscope slide. For live cell examination, a $1.5\text{-}\mu\text{l}$ drop of fresh culture was placed directly onto a polylysine-treated microscope slide. For fixed cells, the conditions described by Harry et al. (1995) were used except that glutaraldehyde was reduced to 0.005%. For either live or fixed cells or protoplasts, DAPI solution (1 $\mu\text{g}/\text{ml}$) was sometimes added directly onto the slides to visualize the nucleoids. Microscopic examination of GFP fluorescence and image grabbing was performed as described previously (Lewis and Errington 1996).

Acknowledgments

We thank Chris D. Webb and Peter Lewis for providing strain CW314 and plasmid pSG1151, respectively. This work was supported by the Biotechnology and Biological Sciences Research Council.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

- Alper, S., L. Duncan, and R. Losick. 1994. An adenosine nucleotide switch controlling the activity of a cell type-specific transcription factor in *B. subtilis*. *Cell* **77**: 195–205.
- Anagnostopoulos, C. and J. Spizizen. 1961. Requirements for transformation in *Bacillus subtilis*. *J. Bacteriol.* **81**: 741–746.
- Arigoni, F., K. Pogliano, C.D. Webb, P. Stragier, and R. Losick. 1995. Localization of protein implicated in establishment of cell type to sites of asymmetric division. *Science* **270**: 637–640.
- Arigoni, F., L. Duncan, S. Alper, R. Losick, and P. Stragier. 1996. SpoIIE governs the phosphorylation state of a protein regulating transcription factor σ^F during sporulation in *Bacillus subtilis*. *Proc. Natl. Acad. Sci.* **93**: 3238–3242.
- Barak, I. and P. Youngman. 1996. SpoIIE mutants of *Bacillus subtilis* comprise two distinct phenotypic classes consistent with a dual functional role for the SpoIIE protein. *J. Bacteriol.* **178**: 4984–4989.
- Barak, I., J. Behari, G. Olmedo, P. Guzman, D.P. Brown, E. Castro, D. Walker, J. Westpheling, and P. Youngman. 1996. Structure and function of the *Bacillus* SpoIIE protein and its localization to sites of sporulation septum assembly. *Mol. Microbiol.* **19**: 1047–1060.
- Cormack, B.P., R.H. Valdivia, and S. Falkow. 1996. FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173**: 33–38.
- Diederich, B., J.F. Wilkinson, T. Magnin, S.M.A. Najafi, J. Errington, and M.D. Yudkin. 1994. Role of interactions between SpoIIAA and SpoIIAB in regulating cell-specific transcription factor σ^F of *Bacillus subtilis*. *Genes & Dev.* **8**: 2653–2663.
- Duncan, L. and R. Losick. 1993. SpoIIAB is an anti- σ factor that binds to and inhibits transcription by regulatory protein σ^F from *Bacillus subtilis*. *Proc. Natl. Acad. Sci.* **90**: 2325–2329.
- Duncan, L., S. Alper, F. Arigoni, R. Losick, and P. Stragier. 1995. Activation of cell-specific transcription by a serine phosphatase at the site of asymmetric division. *Science* **270**: 641–644.
- Errington, J. 1984. Efficient *Bacillus subtilis* cloning system using bacteriophage vector $\phi 105\text{J9}$. *J. Gen. Microbiol.* **130**: 2615–2628.
- . 1990. Gene cloning techniques. In *Molecular biological methods for Bacillus* (ed. C.R. Harwood and S.M. Cutting), pp. 175–220. Wiley, Chichester, UK.
- . 1993. *Bacillus subtilis* sporulation: Regulation of gene expression and control of morphogenesis. *Microbiol. Rev.* **57**: 1–33.
- . 1996. Determination of cell fate in *Bacillus subtilis*. *Trends Genet.* **12**: 31–34.
- Errington, J. and J. Mandelstam. 1986. Use of a *lacZ* gene fusion to determine the dependence pattern of sporulation operon *spoIIA* in *spo* mutants of *Bacillus subtilis*. *J. Gen. Microbiol.* **132**: 2967–2976.
- Feucht, A., T. Magnin, M.D. Yudkin, and J. Errington. 1996. Bifunctional protein required for asymmetric cell division and cell-specific transcription in *Bacillus subtilis*. *Genes & Dev.* **10**: 794–803.
- Glaser, P., M.E. Sharpe, B. Raether, M. Perego, K. Ohlsen, and J. Errington. 1997. Dynamic, mitotic-like behavior of a bacterial protein required for accurate chromosome partitioning. *Genes & Dev.* **11**: 1160–1168.
- Guzman, P., J. Westpheling, and P. Youngman. 1988. Characterization of the promoter region of the *Bacillus subtilis* *spoIIE* operon. *J. Bacteriol.* **170**: 1598–1609.
- Harlow, E. and D.P. Lane. 1988. *Antibodies: A laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Harry, E.J., K. Pogliano, and R. Losick. 1995. Use of immunofluorescence to visualize cell-specific gene expression during sporulation in *Bacillus subtilis*. *J. Bacteriol.* **177**: 3386–3393.
- Illing, N. and J. Errington. 1991. Genetic regulation of morphogenesis in *Bacillus subtilis*: Roles of σ^E and σ^F in prespore engulfment. *J. Bacteriol.* **173**: 3159–3169.
- Jenkinson, H.F. 1983. Altered arrangement of proteins in the spore coat of a germination mutant of *Bacillus subtilis*. *J. Gen. Microbiol.* **129**: 1945–1958.
- Levin, P.A. and R. Losick. 1994. Characterization of a cell division gene from *Bacillus subtilis* that is required for vegetative and sporulation septum formation. *J. Bacteriol.* **176**: 1451–1459.
- Levin, P.A., R. Losick, P. Stragier, and F. Arigoni. 1997. Localization of the sporulation protein SpoIIE in *Bacillus subtilis* is dependent upon the cell division protein FtsZ. *Mol. Microbiol.* **25**: 839–846.

- Lewis, P.J. and J. Errington. 1996. Use of green fluorescent protein for detection of cell-specific gene expression and subcellular protein localization during sporulation in *Bacillus subtilis*. *Microbiology* **142**: 733–740.
- Lewis, P.J., S.R. Partridge, and J. Errington. 1994. σ factors, asymmetry, and the determination of cell fate in *Bacillus subtilis*. *Proc. Natl. Acad. Sci.* **91**: 3849–3853.
- Lewis, P.J., T. Magnin, and J. Errington. 1996. Compartmentalized distribution of the proteins controlling the prespore-specific transcription factor σ^F of *Bacillus subtilis*. *Genes Cells* **1**: 881–894.
- Magnin, T., M. Lord, J. Errington, and M.D. Yudkin. 1996. Establishing differential gene expression in sporulating *Bacillus subtilis*: Phosphorylation of SpoIIAA (anti-anti- σ^F) alters its conformation and prevents formation of a SpoIIAA/SpoIIAB/ADP complex. *Mol. Microbiol.* **19**: 901–907.
- Magnin, T., M. Lord, and M.D. Yudkin. 1997. Contribution of partner switching and SpoIIAA cycling to regulation of σ^F activity in sporulating *Bacillus subtilis*. *J. Bacteriol.* **179**: 3922–3927.
- Margolis, P., A. Driks, and R. Losick. 1991. Establishment of cell type by compartmentalized activation of a transcription factor. *Science* **254**: 562–565.
- Meissner, P.S., W.P. Sisk, and M.L. Berman. 1987. Bacteriophage λ cloning system for the construction of directional cDNA libraries. *Proc. Natl. Acad. Sci.* **84**: 4171–4175.
- Min, K.-T., C.M. Hilditch, B. Diederich, J. Errington, and M.D. Yudkin. 1993. σ^F , the first compartment-specific transcription factor of *B. subtilis*, is regulated by an anti- σ factor that is also a protein kinase. *Cell* **74**: 735–742.
- Najafi, S.M.A., A.C. Willis, and M.D. Yudkin. 1995. Site of phosphorylation of SpoIIAA, the anti-anti-sigma factor for sporulation-specific σ^F of *Bacillus subtilis*. *J. Bacteriol.* **177**: 2912–2913.
- Nicholson, W.L. and P. Setlow. 1990. Sporulation, germination and outgrowth. In *Molecular biological methods for Bacillus* (ed. C.R. Harwood and S.M. Cutting), pp. 391–450. Wiley, Chichester, UK.
- Partridge, S.R. and J. Errington. 1993. The importance of morphological events and intercellular interactions in the regulation of prespore-specific gene expression during sporulation in *Bacillus subtilis*. *Mol. Microbiol.* **8**: 945–955.
- Piggot, P.J. and J.G. Coote. 1976. Genetic aspects of bacterial endospore formation. *Bact. Rev.* **40**: 908–962.
- Pogliano, K., A.E. Hofmeister, and R. Losick. 1997. Disappearance of the σ^F transcription factor from the forespore and the SpoIIE phosphatase from the mother cell contributes to establishment of cell-specific gene expression during sporulation in *Bacillus subtilis*. *J. Bacteriol.* **179**: 3331–3341.
- Raleigh, E.A., N.E. Murray, H. Revel, R.M. Blumenthal, D. Westaway, A.D. Reith, P.W.J. Rigby, J. Elhai, and D. Hanahan. 1988. McrA and McrB restriction phenotypes of some *E. coli* strains and implications for gene cloning. *Nucleic Acids Res.* **16**: 1563–1575.
- Reznikov, O., S. Alper, and R. Losick. 1996. Subcellular localization of proteins governing the proteolytic activation of a developmental transcription factor in *Bacillus subtilis*. *Genes Cells* **1**: 529–542.
- Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- 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. and R. Losick. 1996. Molecular genetics of sporulation in *Bacillus subtilis*. *Annu. Rev. Genet.* **30**: 297–341.
- Sun, D. and P. Setlow. 1991. Cloning, nucleotide sequence, and expression of the *Bacillus subtilis* *ans* operon, which codes for L-asparaginase and L-aspartase. *J. Bacteriol.* **173**: 3831–3845.
- Wu, L.J. and J. Errington. 1994. *Bacillus subtilis* SpoIIIE protein required for DNA segregation during asymmetric cell division. *Science* **264**: 572–575.
- . 1998. Use of asymmetric cell division and *spoIIIE* mutants to probe chromosome orientation and organization in *Bacillus subtilis*. *Mol. Microbiol.* **27**: 777–786.
- Wu, L.J., P.J. Lewis, R. Allmansberger, P.M. Hauser, and J. Errington. 1995. A conjugation-like mechanism for prespore chromosome partitioning during sporulation in *Bacillus subtilis*. *Genes & Dev.* **9**: 1316–1326.

Prespore-specific gene expression in *Bacillus subtilis* driven by sequestration of SpoIE phosphatase to the prespore side of the asymmetric septum

Ling Juan Wu, Andrea Feucht and Jeffery Errington

Genes Dev. 1998, **12**:

References

This article cites 42 articles, 25 of which can be accessed free at:
<http://genesdev.cshlp.org/content/12/9/1371.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](#).