Disappearance of the σ^E\ 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

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Received 17 October 1996/Accepted 20 February 1997

We used immunofluorescence microscopy to investigate mechanisms governing the establishment of cell-specific gene transcription during sporulation in the bacterium Bacillus subtilis. The transcription factors σ^E and σ^F are synthesized shortly after the start of sporulation but do not become active in directing gene transcription until after polar division, when the activity of σ^F is confined to the mother cell and the activity of σ^E is restricted to the forespore. We show that shortly after septation, σ^E and its proprotein precursor pro-σ^E appear to be absent from the forespore and that a null mutation in spoIIE, a gene known to be required for the translocation of a chromosome into the forespore, allows σ^E and/or pro-σ^E to persist and σ^E to become active in the forespore. These findings suggest that the loss of σ^E/pro-σ^E from the forespore contributes to the compartmentalization of σ^E-directed gene transcription. We also investigated the distribution of SpoIIE, a regulatory phosphatase required for the activation of σ^E which exhibits a bipolar pattern of localization shortly after the start of sporulation. Normally, SpoIIE rapidly disappears from the sporangium, first from the mother-cell pole and then from the forespore pole. Here we show that a null mutation in spoIIE causes the SpoIIE phosphatase to persist at both poles. The persistence of the SpoIIE phosphatase at the mother-cell pole could explain the lack of compartmentalization of σ^E activity observed in a spoIIE null mutant. We conclude that the establishment of cell-specific gene transcription involves the loss of σ^E/pro-σ^E from the forespore and the loss of the SpoIIE phosphatase from the mother-cell pole and that both processes are dependent upon the SpoIIE protein.

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A central issue in developmental biology is how the progeny of a single cell division assume dissimilar fates. An attractive experimental system in which this problem can be addressed is sporulation in Bacillus subtilis, in which the formation of an asymmetrically positioned septum partitions the developing cell or sporangium into a small, forespore compartment and a large, mother-cell chamber (27). The dissimilar fates of the forespore and the mother cell are determined by the RNA polymerase sigma factors σ^E and σ^F (recently reviewed in reference 35). Both transcription factors are synthesized in the predivisional sporangium, but σ^F and σ^E do not become active until after polar division, when they differentially switch on gene expression in the forespore and the mother cell, respectively.

The activity of σ^E is governed by a pathway involving the proteins SpoIAB, SpoIIB, and SpoIE (1, 2, 5–8, 10, 19, 24; reviewed in reference 35). SpoIAB is an inhibitor of σ^E, and SpoIIB is an inhibitor of SpoIAB. SpoIAB is also a protein kinase that inactivates SpoIIB by phosphorylating it on a serine residue. Thus, SpoIAB and SpoIIB are mutually antagonistic. Finally, SpoIE is a serine phosphatase that activates SpoIIB by dephosphorylation. It is not known how the SpoIAB-SpoIIB-SpoIE pathway restricts the activation of σ^E to one cell, but the subcellular localization of SpoIIE, which is an integral membrane protein, may provide a clue. In the predivisional sporangium, SpoIIE is localized to sites of potential polar septation near both ends of the predivisional sporangium, but shortly after polar division, when σ^E becomes active, the membrane-bound phosphatase is found exclusively at the sporulation septum (3, 4). This septal localization could contribute to cell-specific activation of σ^E by bringing about a relatively higher ratio of phosphatase to kinase in the forespore (6).

Like σ^E, σ^F is present in an inactive state in the predivisional sporangium but in this case as an inactive proprotein called pro-σ^F (16; reviewed in reference 35). Activation of pro-σ^F requires the proteolytic removal of an N-terminal extension of 27 amino acids, which is mediated by the integral membrane protein SpoIIGA (14, 25, 26, 34). Evidence indicates that SpoIIGA is a receptor-protease that catalyzes the conversion of pro-σ^F to mature σ^F in response to a signal protein, SpoIIR, that is produced in the forespore under the control of σ^F (12, 15, 21). It is believed that SpoIIR is secreted from the forespore and interacts with the external domain of SpoIIGA in the mother-cell membrane of the septum, triggering the cleavage of pro-σ^F. Thus, activation of σ^F is governed by an intercellular signal transduction pathway that ties the processing of pro-σ^F to the activation of σ^F, thereby delaying the onset of σ^F-directed gene transcription until after the formation of polar septum. But what is the nature of the mechanism(s) that restricts activation of σ^F to the mother cell?

Because pro-σ^E and SpoIIGA are synthesized prior to septation, both proteins could be expected to be present in the
forespore as well as in the mother cell after division. If so, then some mechanism must exist for preventing SpoIIR from triggering proteolytic processing of pro-αE in the forespore. Two hypotheses as to the nature of this mechanism are (i) that SpoIIR, which is secreted from the forespore, acts directionally, triggering processing only in the mother cell, and (ii) that some other protein produced in the forespore under the control of αE prevents pro-αE processing or otherwise inhibits αE activity in the small sporangial chamber (22). However, a recent experiment by Zhang et al. (42) indicates that the compartmentalization of αE activity is independent of αE activity. In their experiments, the normal dependence of spoIIR expression on αE activity was bypassed by fusing the spoIIR gene to a promoter active before septation. Thus, the authors were able to investigate whether either compartmentalized synthesis of SpoIIR or αE-dependent expression of any other gene was necessary for compartmentalization of αE activity. In about half of the sporangia, αE apparently became active prior to polar septation, indicating that αE activation occurred prematurely if SpoIIR was synthesized prior to septation. However, the remaining half of the engineered sporangia had normally compartmentalized αE activity, a finding that indicated the existence of a δ-protein-independent mechanism for the compartmentalization of αE activity (42).

In the present communication, we report that αE and pro-αE are apparently absent from the forespore compartment of wild-type sporangia shortly after polar septation. This suggests that one mechanism by which αE activity is compartmentalized is the destruction or exclusion of pro-αE from the forespore. We also report that αE/proc-αE not only persists but that αE is also active in directing gene transcription in the forespore of sporangia bearing a null mutation in the spoIIE gene. This is a surprising finding in that SpoIIIIE is required for the transport of chromosomal DNA from the mother cell into the forespore; it has been demonstrated that a complete chromosome is transported into the forespore after the polar septum is formed and that in spoIIIIE mutant sporangia the forespore receives only about 30% of the origin-proximal region of the chromosome (38, 40).

Null mutations of spoIIIIE were previously reported to allow the release of δ-activity in the mother cell (38), an observation reminiscent of our present discovery that SpoIIE is required for the proper compartmentalization of αE activity. This led us to reexamine the localization of the SpoIIE phosphatase in spoIIIIE mutant sporangia, since elevated levels of SpoIIE phosphatase are known to cause premature activation of δ-activity (2, 10). We show that a spoIIIIE null mutation delays the normal loss of SpoIIE phosphatase from the mother-cell (forespore-distant) pole of the sporangium, a finding that could explain the misactivation of αE in the large sporangial chamber. Thus, the spoIIIIE null mutation apparently disrupts the compartmentalization of αE activity by inhibiting the loss of a key regulatory protein (SpoIIE) for αE from the mother-cell pole.

In summary, our findings suggest that the compartmentalization of gene expression during sporulation is facilitated, in part, by the loss of the SpoIIE phosphatase from the mother-cell pole, which precludes αE from becoming active in the large compartment of the sporangium, and by the loss of δ-pro-αE from the forespore, which precludes αE from becoming active in the small chamber of the sporangium.

### MATERIALS AND METHODS

**Antibodies and reagents.** Mouse monoclonal antibodies directed against β-galactosidase were obtained from Promega and used at a 1:1,500 dilution. Rabbit polyclonal antibodies against β-galactosidase were obtained from 5-3’ Inc. and used at a 1:1,500 dilution. Antibodies specific for αE (rabbit) were prepared by L. Duncan and used at a 1:200,000 dilution. Monoclonal antibodies that bind to both pro-αE and αE were a gift of W. Haldeman and were used at a 1:20 dilution. The affinity-purified SpoIIE-specific antibodies used in immunofluorescence experiments were a gift of F. Arigoni and P. Stragier and were used at 1:7 dilution. The SpoIIE antibodies used in Western blot (immunoblot) experiments were prepared by C. Webb and used at a 1:10,000 dilution. The secondary antibodies (from Jackson Immunolabs) were affinity-purified donkey anti-rabbit or anti-mouse antibodies conjugated either to fluorescein isothiocyanate (FITC) or to indocarbocyanine (Cy3). FITC-conjugated secondary antibodies were used at a 1:100 dilution, while Cy3-conjugated antibodies were used at a 1:200 dilution. 4’,6-Diamidino-2-phenylindole (DAPI) was obtained from Sigma and used at a final concentration of 0.2 μg/ml.

**Bacterial strains and growth conditions.** The bacterial strains used in these experiments are derivatives of PY79 and are described in Table 1. The strains containing the amyE::spoIID-lacZ::PspoIIE::spoIIR construct (KJP331, KJP335, and KJP337) were constructed by integrating pMLK30 (amyE::spoIIE::spoIIR) (42), a gift of P. Piggot, by a single recombination event into the amyE::spoIID-lacZ locus. pMLK30 was transformed into B2184 (amyE::spoIID-lacZ), and both neomycin resistance (conferring by pMLK30) and chloramphenicol resistance (conferring by amyE::spoIID-lacZ) were simultaneously selected. Transformsants were then screened for linkage between the two drug resistance markers by DNA transformation and for β-galactosidase activity. Chromosomal DNA from one such strain was transformed into strains containing spoIAC::kan, with or without spoIIE::spc and spoIIIIE::spc. Four independent isolates of each strain were tested in immunofluorescence experiments; all four behaved identically.

The spoIIE::spc mutation used in this study is an insertion-deletion mutation in which a spectinomycin resistance (spc) cassette was used to replace the DNA sequence between nucleotides 258 and 2001 from within the spoIIE coding sequence. It was constructed by P. Levin as follows. Fragments from within the spoIIE coding sequence extending from nucleotides 84 to 258 and from nucleotides 2001 to 2309 were cloned on either side of a spectinomycin resistance marker. The resulting plasmid was linearized and used to transform strain PY79, selecting for spectinomycin resistance. Southern blot analysis confirmed that the transformants had replaced the chromosomal copy of spoIIE with that from the plasmid. We presume that spoIIE::spc is a null mutation, but it could potentially produce the N-terminal 86 amino acids of SpoIIIIE (from a total of 787). The spoIIE::spc mutation behaved indistinguishably from the spoIIIIE::spc::kan mutation of Wu and Errington (38) with respect to DNA translacation, compartmentalization of αE activity, and the persistence of SpoIIIIE (data not shown).

For the results presented, sporulation was performed at 37°C and induced by the resuspension method (33) in medium supplemented with tryptophan and phenylalanine. Similar results were obtained in control experiments in DS medium (31).

**Immunofluorescence methods.** Immunofluorescence experiments were performed essentially as described previously (3, 11, 29). Cells were fixed in growth medium for 15 min at room temperature and for 30 min on ice, with final concentrations of 40 mM NaPO₃ (pH 7.5), 2.7% paraformaldehyde, and 0.0042 or 0.0065% glutaraldehyde. Samples were incubated overnight at 4°C with the primary antibodies and for several hours at room temperature with the secondary antibodies (with 50 ng of FITC-conjugated wheat germ agglutinin [FITC-WGA] as appropriate). Images were recorded on Ektachrome 400 slide film, and the images were scanned and processed with Adobe Photoshop.

### TABLE 1. Strain list

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH48</td>
<td>amyE::spoIID-lacZ spoIAC1 thrC::PspoIIR</td>
</tr>
<tr>
<td>AH110</td>
<td>spoIVF-lacZ spoIIIIE::spc</td>
</tr>
<tr>
<td>BZ184</td>
<td>amyE::spoIID-lacZ</td>
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<td>KJP86</td>
<td>amyE::spoIIIE::spoIID-lacZ spoIIGD1</td>
</tr>
<tr>
<td>KJP123</td>
<td>amyE::spoIIIE::spoIID-lacZ spoIIGD1 spoIIIIE::spc</td>
</tr>
<tr>
<td>KJP113</td>
<td>amyE::spoID-lacZ spoIIIIE::spc</td>
</tr>
<tr>
<td>KJP237</td>
<td>amyE::spoIIIE::spoIID-lacZ</td>
</tr>
<tr>
<td>KJP239</td>
<td>amyE::spoIIIE::spoIID-lacZ spoIIIIE::spc</td>
</tr>
<tr>
<td>KJP241</td>
<td>amyE::spoIIIE::spoIID-lacZ</td>
</tr>
<tr>
<td>KJP277</td>
<td>amyE::spoIIIE::spoIID-lacZ spoIIGD1 spoIIGB::erm</td>
</tr>
<tr>
<td>KJP286</td>
<td>amyE::spoID-lacZ spoIAC1 thrC::PspoIIR spoIIIIE::spc</td>
</tr>
<tr>
<td>KJP288</td>
<td>amyE::spoID-lacZ spoIIIIE::spc</td>
</tr>
<tr>
<td>KJP331</td>
<td>amyE::spoID-lacZ::pMLK230 (PspoIIE::spoIIR) spoIAC::kan</td>
</tr>
<tr>
<td>KJP335</td>
<td>amyE::spoID-lacZ::pMLK230 (PspoIIE::spoIIR) spoIIIIE::spc spoIAC::kan</td>
</tr>
<tr>
<td>KJP337</td>
<td>amyE::spoID-lacZ::pMLK230 (PspoIIE::spoIIR) spoIIIIE::spc spoIAC::kan</td>
</tr>
</tbody>
</table>

* All strains are derivatives of PY79 (41).
Staining of the cell wall with FITC-WGA. FITC-WGA (Molecular Probes) was used to stain the cell wall during immunofluorescence studies; a final concentration of 50 ng of FITC-WGA/ml was included with the secondary antibodies. FITC-WGA reliably revealed both vegetative septa and the outline of the cells, although the intensity of the staining was dependent upon the extent of digestion used to allow antibody permeabilization. Sporulation septa were only occasionally resolved from the cell poles, presumably because the short distance between the septum and the cell pole is close to the maximum resolution of the light microscope. Despite this difficulty, the lectin staining was superior to indirect staining for revealing the relative location of the cell pole and, for example, αE activity, especially in the spoIIE mutants, which have less DNA in the forespore than the wild type.

Immunostaining proteins in the forespore. During our initial immunofluorescence studies, we noted that many antibodies directed against proteins made in the forespore did not correlate with the presence of a fully translocated forespore nucleus (32). We now use just enough glutaraldehyde for fixation to keep the cells from lysing during our immunofluorescence protocol. First, we use just enough glutaraldehyde for fixation to keep the cells from lysing, as we previously noted that αE immunostaining in the forespore was unclear, in the experiments presented here, αE immunostaining was readily detected in most forespores, whereas αF appeared to be absent. This may have been achieved by modifications to our immunofluorescence protocol. First, we now use just enough glutaraldehyde for fixation to keep the cells from lysing during the staining procedure; a final concentration of 1 M Tris (pH 8.0), and resuspended in 60 μl of Tris-EDTA (33 mM Tris [pH 8.0], 40% sucrose, 1 mM EDTA) to which phenylmethylsulfonyl fluoride had just been added to a final concentration of 300 μg/ml. Lysozyme was added to a final concentration at 16,000 units/ml. Samples were incubated at 37°C for 5 min, washed once with 0.75 ml of 1 M Tris (pH 8.0), and resuspended in 60 μl of Tris-sucrose-EDTA (33 mM Tris [pH 8.0], 40% sucrose, 1 mM EDTA) to which phenylmethylsulfonyl fluoride had just been added to a final concentration of 300 μg/ml. Lysozyme was added to a final concentration at 16,000 units/ml. Samples were incubated at 37°C for 5 min, washed once with 0.75 ml of 1 M Tris (pH 8.0), and resuspended in 60 μl of Tris-sucrose-EDTA (33 mM Tris [pH 8.0], 40% sucrose, 1 mM EDTA) to which phenylmethylsulfonyl fluoride had just been added to a final concentration of 300 μg/ml. Lysozyme was added to a final concentration at 16,000 units/ml. Samples were incubated at 37°C for 5 min, washed once with 0.75 ml of 1 M Tris (pH 8.0), and resuspended in 60 μl of Tris-sucrose-EDTA (33 mM Tris [pH 8.0], 40% sucrose, 1 mM EDTA) to which phenylmethylsulfonyl fluoride had just been added to a final concentration of 300 μg/ml.

RESULTS

Loss of αF/pro-αE from the forespore. To investigate the mechanisms of compartmentalization of αF and αE activities, we examined the distribution of αF and αE proteins by immunofluorescence microscopy. We were able to simultaneously detect αF and αE by using αF-specific rabbit polyclonal antibodies and αE-directed mouse monoclonal antibodies. The monoclonal antibodies and antibodies to the forespore and the mother cell (Fig. 1A to P, short arrows; Table 2), and a few sporangia appeared to have more αE/pro-αE protein in the forespore than in the mother cell. Furthermore, αE was active in directing gene transcription in the forespores of many of the spoIIIE::spc mutant sporangia (Fig. 1M and O, long arrows; Table 2).
FIG. 1. Immunolocalization of $\sigma^F$, $\sigma^E$/$\sigma^H$, and $\sigma^E$ activity in wild-type and spoIIIE mutant sporangia. The sporangia were harvested 2 h after the start of sporulation and processed for immunofluorescence microscopy. Arrows point to the forespore compartment and are oriented perpendicularly to the long axis of the sporangia. The activity of $\sigma^E$ was monitored by immunostaining $\beta$-galactosidase produced from a spoIID-lacZ fusion that had been inserted at the amyE locus; the DNA was stained with DAPI (blue) (D, H, L, P, Q, R, V, and W). (A to D) Immunolocalization of $\sigma^F$ and $\sigma^E$/$\sigma^H$ in sporangia from a spoIIIG mutant (strain KJP86). Two sporangia displaying $\sigma^E$/$\sigma^H$ immunostaining (red) in the mother cell (B) and $\sigma^F$ immunostaining (green) in both the mother cell and the forespore (C) are
sporulation, 38% of the mutant sporangia scored as having α^E/pro-ac^E protein in both sporangial compartments exhibited α^E-directed β-galactosidase synthesis in both the forespore and the mother cell. Diagrams summarizing these results are presented in Fig. 2A.

Sporangia from a strain carrying a spoIIIIE missense mutation (spoIIIIE36), which confers a DNA translocation defect similar to that of the null mutation (38), also frequently had α^E/pro-ac^E protein in the forespore as well as the mother cell. Among such sporangia, many exhibited compartmentalized α^E activity (Fig. 1I to L, arrows; Table 2). A low but significant population of missense mutant sporangia that had α^E activity in the forespore as well as the mother cell was observed (Table 2). Thus, the missense mutation exerted a milder effect than the null mutation in breaking the compartmentalization of α^E activity. Nonetheless, in toto, our results are consistent with the idea that the deletion of α^E/pro-ac^E from the forespore contributes to the compartmentalization of α^E activity and that the elimination of α^E/pro-ac^E from the forespore depends, directly or indirectly, on SpoIIIIE. At the same time, the existence of sporangia with α^E/pro-ac^E in both compartments but exhibiting α^E activity only in the mother cell could indicate the existence of an additional mechanism impeding α^E-directed gene expression in the forespore (e.g., a mechanism operating at the level of pro-ac^E processing in the forespore).

A spoIIIIE null mutation abolishes compartmentalization of α^E activity when SpoIR is synthesized before septation. Zhang et al. (42) reported that among cells engineered to synthesize SpoIR prior to septation and independently of α^E, sporangia are frequently observed that reach the stage of polar septation and exhibit compartmentalized α^E activity. Their experiments indicated the existence of a σ^E-independent mechanism to confine α^E activity to the mother cell (42). We therefore wondered whether the compartmentalization of α^E-directed gene expression observed in sporangia in which SpoIR synthesis had been uncoupled from α^E could be attributed to the SpoIIIIE-dependent loss of α^E from the forespore.

To carry out these experiments, we constructed a strain (AH48) harboring spoIIIIE fused to the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible spc promoter (Pspac) (21), lacZ fused to a gene under σ^E control (amyE::spolIId-lacZ), and a mutation in the gene (spolIAC) coding for σ^E. In addition, we constructed a derivative (KJP286) of AH48 that contained the spoIIIIE::spc null mutation. As the forespores of spoIIIIE mutant sporangia are often difficult to visualize due to the decreased DNA content, we simultaneously immunolocalized β-galactosidase and stained the cell wall with FITC-WGA, a lectin that binds to N-acetylglucosamine. While the lectin did not reliably detect the sporulation septum, it did allow the boundaries of the sporangium to be more clearly visualized.

Approximately 31% of spoIIIIE::spc sporangia exhibited compartmentalized α^E activity after induction of Pspac-spoIR (Fig. 1Q to U, short arrows; Table 3), whereas the remaining 69% appeared to have activated σ^E prior to septation (Fig. 1Q to U, long arrows). The α^E/pro-ac^E protein was absent from the forespores of the sporangia that exhibited compartmentalized α^E activity, demonstrating that the loss of α^E/pro-ac^E protein was not dependent upon α^E activity (data not shown). In sporangia from the spoIIIIE::spc null mutant (Fig. 1V to Z), both α^E activity (Fig. 1W to Y) and α^E/pro-ac^E protein (data not shown) were present throughout the sporangium. The forespore nucleoid was especially difficult to visualize in the spoIIIIE::spc mutant sporangia after synthesis of SpoIR before septation.

However, in those few mutant sporangia in which a clear forespore nucleoid was visualized, α^E was both present and active in the forespore (data not shown). In a second set of experiments, similar results were obtained with the strains of Zhang et al. (42), in which spoIIIIE was fused to a sporulation promoter (PspolIE) that is induced just prior to polar septation (data not shown).

We draw two conclusions from these results. First, in confirmation of the findings of Zhang et al. (42), the lack of α^E activity in the forespore can be attributed to a mechanism that does not depend on α^E-directed gene expression. Second, the compartmentalization of α^E activity that is observed when SpoIR is produced prior to septation is due, at least in part, to the loss of α^E/pro-ac^E from the forespore in a manner that appears to be dependent upon SpoIIIIE.

**spoIIIIE mutations cause the SpoIE phosphatase to persist.**

The observation that spoIIIIE null mutant sporangia have α^E activity in the forespore as well as the mother cell was reminiscent of an earlier, complementary observation that a spoIIIIE null mutant has α^E activity in the mother cell as well as the forespore (38). This led us to reexamine the effect of spoIIIIE mutations on the localization of the SpoIE phosphatase that is responsible for the activation of α^E (2, 6, 10). The SpoIE phosphatase initially localizes to the sites of potential polar septation near both ends of the predivisional sporangium and, after division, disappears first from the mother-cell pole and then from the forespore pole (3). It was previously observed that the bipolar localization of SpoIIIE is more evident in spoIIIIE null mutant sporangia than in wild-type sporangia. These observations suggested that the increased levels or persistence of the SpoIIIE phosphatase in the mother cell of a spoIIIIE null mutant may be responsible for the lack of compartmentalization of α^E-directed gene expression in the mutant sporangia. As a test of this hypothesis, we asked whether SpoIIIE persists at the mother-cell pole in the spoIIIIE missense mutant spoIIIIE36, in which misactivation of α^E in the mother cell does not occur.
To do this, we immunolocalized the SpoIIE phosphatase (Fig. 3, green stain) and β-galactosidase synthesized from the αE-dependent spoIE36POGLIANO ET AL. J. BACTERIOL.

spoIIIE spoIIAC

AH48

spoIIACΔ1

30.5

69.5

39

spoIIIE spoIIAC

KJP286

spoIIACΔ1

1.2

98.8

33

spoIIIE spoIIAC

KJP131

spoIIIE36

11

55

34

TABLE 2. spoIIIE mutants have αE/pro-αE protein and αE activity in the forespore and the mother cell

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Spoarangia with αE/pro-αE protein and αE activity in MC only (%)</th>
<th>Sporangia with αE/pro-αE protein in whole sporangium (%)</th>
<th>αE activity in MC only</th>
<th>αE activity in whole sporangium</th>
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<tbody>
<tr>
<td>BZ184</td>
<td>Wild type</td>
<td>91</td>
<td>9</td>
<td>90</td>
<td>0</td>
</tr>
<tr>
<td>KJP288</td>
<td>spoIIIE36</td>
<td>18</td>
<td>80</td>
<td>2</td>
<td>34</td>
</tr>
<tr>
<td>KJP131</td>
<td>spoIIIE:spc</td>
<td>11</td>
<td>55</td>
<td>34</td>
<td>2</td>
</tr>
</tbody>
</table>

* Sporangia were collected 2 h after the initiation of sporulation. A total of 509 wild-type, 345 spoIIIE36, and 327 spoIIIE:spc sporangia were scored. αE activity was monitored by immunofluorescence microscopy localizing β-galactosidase produced from the amyE:spoIIID-lacZ fusion. αE/pro-αE protein was monitored by immunolocalization. MC, mother cell.

1 In most of these sporangia, a clear forespore nucleoid could be detected, suggesting that the sporulation septum had formed.

2 When three additional experiments were scored for the compartmentalization of αE activity and the results were averaged with those of this experiment, 0.1% of wild-type sporangia, 9% of the spoIIIE36 mutant sporangia, and 44% of the spoIIIE:spc sporangia had noncompartmentalized αE activity.

3 A total of 1,264 wild-type sporangia and 1,030 spoIIIE36POGLIANO ET AL. J. BACTERIOL.

spoIIIE spoIIAC

AH48

spoIIACΔ1

30.5

69.5

39

spoIIIE spoIIAC

KJP286

spoIIACΔ1

1.2

98.8

33

spoIIIE spoIIAC

KJP131

spoIIIE36

11

55

34

TABLE 3. SpoIIIE is required for compartmentalization of αE activity in PspoIIIE

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>% Sporangia with αE activity present in</th>
<th>% Staining</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Mother cell</td>
<td>Whole sporangium</td>
</tr>
<tr>
<td>AH48</td>
<td>spoIIACΔ1</td>
<td>30.5</td>
<td>69.5</td>
</tr>
<tr>
<td>KJP286</td>
<td>spoIIACΔ1</td>
<td>1.2</td>
<td>98.8</td>
</tr>
<tr>
<td></td>
<td>spoIIIE:spc</td>
<td></td>
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</tr>
</tbody>
</table>

* Sporangia were collected 3 h after the initiation of sporulation. SpoIIR expression was induced either at the time of resuspension or one doubling prior to resuspension, with identical results.

* Percent of immunostaining sporangia in which β-galactosidase was confined to the mother cell.

* Percent of immunostaining sporangia in which β-galactosidase was present either in both the mother cell and forespore in the predivisional sporangium. Although these two classes were readily discerned in the wild type, the reduced amount of DNA in the forespores of spoIIIE mutant sporangia made such a distinction difficult.

* A total of 1,264 wild-type sporangia and 1,030 spoIIIE:spc mutant sporangia were scored from two independent experiments.

FIG. 2. Summary of the distribution of αE/pro-αE and the SpoIIE phosphatase in wild-type and mutant sporangia. (A) The results of immunofluorescence staining of wild-type and spoIIIE null mutant sporangia with αE-directed antibodies. The αE/pro-αE protein could be detected in the predivisional sporangia of both the mutant and the wild type. Among postdivisional sporangia, αE/pro-αE was absent or deficient in the forespore of the wild type but was present in both compartments of the mutant. (B) The distribution of the SpoIIE phosphatase is indicated by heavy and light rings, and the pattern of αE activity (αE-directed β-galactosidase synthesis) is indicated by light and dark shading. In predivisional sporangia, SpoIIE initially exhibited a strong bipolar pattern of distribution, as represented by the single cell on the left. Shortly after polar septation (middle column) all of the forespores (at the right ends of the sporangia) had low levels of αE activity (indicated by the light shading). Sporangia that had progressed further (right column) had higher levels of αE activity (indicated by heavier shading). Each of the spoIIE::erm mutant sporangia (bottom two rows), which lacked αE activity, formed a second forespore compartment and thus had both the original forespore (at the right end of the sporangium), which had high levels of αE activity, and a more recently formed forespore with lower levels of αE activity (at the left end). In wild-type sporangia, as soon as αE activity could be detected, the SpoIIE phosphatase was absent from the mother-cell pole; subsequently, SpoIIE disappeared from the forespore pole as well. In spoIIIE36 mutant sporangia, SpoIIE was rapidly lost from the mother-cell pole but persisted at the forespore pole. In spoIIIE::erm sporangia, the bipolar staining pattern persisted until after αE activity could be detected, although ultimately it was lost from the mother-cell pole, remaining only at the forespore pole. In spoIIE::erm mutants (which lack αE activity), the SpoIIE phosphatase disappeared normally from the forespore pole but persisted at the mother-cell pole until the formation of the second forespore. Mutants lacking αE activity behaved identically to those that lack αE activity, suggesting both that αE is not required for the loss of SpoIIE from the forespore pole and that αE is required for its loss from the mother-cell pole. In doubly mutant spoIIIE::erm spoIIE::erm sporangia, SpoIIE phosphatase persisted at both sites.

mutation leads to the persistence of the SpoIIE phosphatase in the mother cell.

We noted one further difference between wild-type and spoIIIE mutant sporangia. In wild-type sporangia with intermediate to high levels of αE activity, the SpoIIE phosphatase was absent from the forespore pole as well as from the mother-cell pole (Fig. 3A to D, short arrows). However, in both spoIIIE mutants, the SpoIIE phosphatase persisted at the forespore pole, even when αE activity reached high levels (Fig. 3E to L). This indicates that both classes of spoIIIE mutants are defective in the loss of the SpoIIE phosphatase from the forespore pole. The similar effects of these two classes of mutations on
FIG. 3. Immunolocalization of the SpoIIE phosphatase and σF-directed β-galactosidase synthesis in spoIIE and spoIIG mutant sporangia. Sporangia were harvested 2 h after the start of sporulation and immunostained for the SpoIIE phosphatase (green) (A, C, E, G, I, K, M, O, Q, and S) and for σF-directed β-galactosidase synthesis from a sspE(2G)-lacZ fusion inserted at the amyE locus (red) (A, B, E, F, I, J, M, N, Q, and R). DAPI was used to stain the DNA (blue) (D, H, L, P, and T). The arrows point to the forespore and are oriented perpendicularly to the long axis of the sporangia. (A to D) In wild-type sporangia, once σF activity could be detected (red) (A and B), the SpoIIE phosphatase (green) (A and C) was almost entirely limited to the forespore pole (long arrows). In sporangia with higher levels of σF activity, the SpoIIE phosphatase was absent from the forespore as well as the mother-cell poles (short arrows). Predivisional sporangia, which lack σF activity, displayed bipolar SpoIIE staining (unfilled arrowheads). (E to H) spoIIIE36 mutant sporangia with both high and low levels of σF activity had SpoIIE phosphatase only at the forespore pole (arrows). (I to L) spoIIIE::spc mutant sporangia had bipolar SpoIIE phosphatase immunostaining in sporangia with low levels of σF activity (arrows) (the SpoIIE immunostaining at the mother-cell pole is indicated with arrowheads), whereas sporangia with high levels of σF activity had SpoIIE only at the forespore pole (data not shown). (M to P) In spoIIGB::erm sporangia, which lack σF activity, SpoIIE phosphatase persisted at the mother-cell pole (arrowheads), until the formation of a second septum at this site. (Q to T) In doubly mutant spoIIE::spc spoIIGB::erm sporangia, the SpoIIE phosphatase evenly stained both the forespore pole (arrows) and the mother-cell pole (arrowheads), even when both forespore compartments had σF activity. Bar (T), 5 μm. Magnifications are identical for all panels.

The panels in the far left column are double exposures, the yellow color resulting from the coincidence of red and green fluorescence.
the loss of SpoIIE phosphatase from the forespore pole suggests that this phenotype could be a consequence of their shared DNA translocation defect.

The rapid loss of SpoIIE phosphatase during sporulation of wild-type sporangia could also be detected by Western blot analysis, which revealed that the SpoIIE phosphatase reached maximal levels about 2 h after the start of sporulation and was undetectable 2.6 h after the start of sporulation (Fig. 4A). However, in spoIIE::spc mutant sporangia, the SpoIIE phosphatase persisted at elevated levels until at least 4 h after the start of sporulation (Fig. 4). This demonstrates that the SpoIIE phosphatase is normally only transiently present during sporulation and that it persists longer in the spoIIE mutant than in the wild type, in accordance with our immunofluorescence studies.

**Effect of σ^K on loss of SpoIIE phosphatase.** Finally, we consider the contribution of σ^K to the loss of the SpoIIE phosphatase. Mutants lacking σ^K produce aberrant, dispersive sporangia that form septa at both polar sites (27, 35). Earlier work showed that in the absence of σ^K, the SpoIIE phosphatase persists at the mother-cell pole but disappears normally from the forespore as well as in the mother cell when sporangium (3) is also harvested (see also Fig. 3M to P). We now report that the SpoIIE phosphatase, which is normally only transiently present during sporulation (Fig. 4). This demonstrates that the SpoIIE phosphatase reaches its maximal levels early in the life cycle of the chromosome and therefore would be among the last to be achieved if synthesis of pro-σ^K processing of the SpoIIR protein, whose synthesis depends on σ^K (15, 21). This has raised the possibility that another σ^K-transcribed gene may serve as a forespore-specific inhibitor of pro-σ^K processing or σ^K activity or that SpoIIR may act directionally, only allowing pro-σ^K processing in the mother cell (22). However, our present results, in confirmation and extension of those of Zhang et al. (42), indicate that when expression of spoIIR is engineered to occur prior to septation, many sporangia that exhibit compartmentalized σ^Kprotein and activity even in the absence of σ^K can be observed; the remaining sporangia that exhibit σ^K activity seem to lack a forespore, suggesting that in these sporangia, σ^K becomes active before formation of the sporulation septum. Two inferences can be drawn from these observations. First, the σ^K-dependent expression of spoIIR is a timing device that ensures that activation of σ^K occurs only after septation (42). Second, a separate, σ^K-independent mechanism(s) exists to eliminate pro-σ^K protein and to block σ^K-directed gene expression in the forespore.

**Activation of σ^K normally requires σ^F activity due to the dependence of pro-σ^K processing on the SpoIIR protein, whose synthesis depends on σ^K (15, 21). This has raised the possibility that another σ^K-transcribed gene may serve as a forespore-specific inhibitor of pro-σ^K processing or σ^K activity or that SpoIIR may act directionally, only allowing pro-σ^K processing in the mother cell (22). However, our present results, in confirmation and extension of those of Zhang et al. (42), indicate that when expression of spoIIR is engineered to occur prior to septation, many sporangia that exhibit compartmentalized σ^K protein and activity even in the absence of σ^K can be observed; the remaining sporangia that exhibit σ^K activity seem to lack a forespore, suggesting that in these sporangia, σ^K becomes active before formation of the sporulation septum. Two inferences can be drawn from these observations. First, the σ^K-dependent expression of spoIIR is a timing device that ensures that activation of σ^K occurs only after septation (42). Second, a separate, σ^K-independent mechanism(s) exists to eliminate pro-σ^K protein and to block σ^K-directed gene expression in the forespore.

**How might σ^K/pro-σ^K be depleted from the forespore?** One attractive possibility is that the depletion of pro-σ^K protein from the forespore is mediated by a protease that is either more active or more concentrated in the forespore than in the mother cell. A similar hypothesis has been proposed for the degradation of the methyl-accepting chemotaxis receptor (McPA) of Caulobacter crescentus, which occurs specifically in just one of the two daughter cells of the Caulobacter cell cycle (13). However, the forespore-specific instability of pro-σ^K protein may not be necessary for the depletion of pro-σ^K from the forespore. Depletion of pro-σ^K protein could also be achieved if synthesis of pro-σ^K is specifically inhibited in the forespore. In this respect, it is interesting to note that the spoIIG operon, which encodes both pro-σ^K and SpoIIGA (the putative pro-σ^K protease), is located in the origin-distal region of the chromosome and therefore would be among the last
genes to enter the forespore during the 10 to 15 min required to translocate a chromosome into the forespore (9, 28). Thus, the spoIIG operon cannot be transcribed in the forespore until 10 to 15 min after septation. If, during this time, the preexisting pro-\(\sigma^F\) protein and its mRNA were degraded (perhaps in a SpoIIA-dependent manner) and some mechanism to prevent spoIIG transcription were implemented, depletion of \(\sigma^F\)/pro-\(\sigma^F\) from the forespore could be achieved.

**Depletion of the SpoIIE phosphatase from the mother-cell pole.** In contrast to \(\sigma^F\)/pro-\(\sigma^F\), \(\sigma^E\) appears to be present in the mother cell as well as the forespore, and thus the compartmentalization of \(\sigma^E\) activity does not seem to rely on the loss of \(\sigma^E\) from the mother cell. Instead, our results suggest that the loss of the SpoIIE phosphatase from the mother cell pole contributes to the compartmentalization of \(\sigma^E\) activity. The SpoIIE phosphatase is synthesized before the sporulation septum is formed and initially localizes to potential division sites near each pole of the sporangium (3). After septation, SpoIIE rapidly becomes sequestered to the sporulation septum, prior to disappearing entirely from the sporangium (3). Our present results indicate a close correlation between this disappearance of SpoIIE from the mother-cell pole and the compartmentalization of \(\sigma^E\) activity. In a spoIIIE null mutant, in which \(\sigma^E\) becomes active in the mother cell (38), the SpoIIE phosphatase is observed to persist at the mother-cell pole. In contrast, in a spoIII E missense mutant (spoIII E36), in which \(\sigma^E\) activity is strictly compartmentalized, SpoIIE disappears normally from the mother-cell pole. Thus, the rapid disappearance of SpoIIE from the mother-cell pole correlates with, and evidently contributes to, the compartmentalization of \(\sigma^E\) activity. An important unanswered question is whether the disappearance of SpoIIE occurs only at the mother-cell pole of the sporangium or from the mother-cell face of the septum as well. If SpoIIE is entirely eliminated from the mother cell, then the phosphatase would be present only in the forespore, thereby ensuring that \(\sigma^E\) is activated exclusively in one cell. Even if SpoIIE is eliminated only from the mother-cell pole of the sporangium, however, and hence persists on both faces of the septum, the septal localization of SpoIIE could nonetheless contribute to the cell-specific activation of \(\sigma^E\): because of the small size of the forespore, the ratio of the SpoIIE phosphatase to the SpoIIAB kinase will be relatively higher in the small chamber of the sporangium, thereby contributing to the selective activation of \(\sigma^E\) in the compartment (6).

The elimination of the SpoIIE phosphatase from the mother-cell pole requires the concerted action of two proteins, \(\sigma^E\) and SpoIII E. A null mutation in the spoIII E gene causes a delay in the loss of the SpoIIE phosphatase from the mother-cell pole and also strongly inhibits the loss of SpoIIE from the forespore pole. In contrast, a mutation in the gene (spoIIG B) coding for \(\sigma^B\) has no effect on the loss of SpoIIE from the septum but strongly inhibits loss of SpoIIE from the potential division site within the mother cell distal to the forespore (3). Mutants lacking \(\sigma^E\) activity ultimately die at this second site, forming a disporic sporangium with two chromosome-containing forespore compartments and a central compartment that lacks a chromosome (27, 32, 20). After the formation of the second forespore compartment in disporic mutants, the SpoIIE phosphatase is rapidly lost from the newly formed forespore, representing the original mother-cell pole (3). These results suggest that SpoIII E is involved in the loss of the SpoIIE phosphatase from both poles of the sporangium, being required for the loss of the SpoIIE phosphatase from the forespore pole of the sporangium and acting together with \(\sigma^F\) to mediate the loss of SpoIIE from the mother-cell pole. Consistent with the hypothesis that both proteins contribute to the loss of the SpoIIE phosphatase, a doubly mutant strain lacking both \(\sigma^E\) and SpoIII E showed stably bipolar SpoIIE immunostaining.

How might these proteins act to promote the timely disappearance of the SpoIIE phosphatase from the sporangium? One model is that they directly or indirectly increase the rate of proteolysis of the SpoIIE phosphatase in either the mother cell or the forespore. For example, since both SpoIII E and SpoIII E are known to be located in the sporulation septum (3, 39), it is conceivable that the proteins are in contact with each other in the septum, allowing SpoIII E to directly enhance the instability of SpoIIE. Alternatively, the SpoIIE phosphatase may be equally unstable at all stages of sporulation and in both mutants, and the two proteins (\(\sigma^E\) and SpoIII E) may prevent the continued synthesis of SpoIIE as sporulation proceeds. A third possibility is suggested by recent findings demonstrating a connection between the SpoIIE phosphatase and the FtsZ protein, an essential and evolutionarily conserved cell division protein (23, 37). Like SpoIIE, FtsZ initially localizes to the two potential sites of septation in the early sporangium but is rapidly lost from the second potential division site after septation (18). The loss of FtsZ from the second division site depends on \(\sigma^E\) activity, perhaps explaining why mutants lacking \(\sigma^E\) activity are disporic. Intriguingly, localization of SpoIIE to potential division sites requires FtsZ (17), suggesting that the \(\sigma^E\)-dependent loss of FtsZ from the second division site may be sufficient to allow the loss of the SpoIIE phosphatase from this site.

**Role of SpoIII E in compartmentalizing the activities of \(\sigma^E\) and \(\sigma^F\).** The most surprising result we have presented is that the same spoIII E null mutation that allows \(\sigma^E\)/pro-\(\sigma^E\) protein to persist and become active in the forespore also allows the SpoIIE phosphatase to persist at the mother-cell pole and thus for \(\sigma^E\) to become active in the mother cell. How might a protein whose primary role is to promote the translocation of the chromosome into the forespore compartment cause defects in the compartmentalization of the activities of both \(\sigma^E\) and \(\sigma^F\)? One obvious possibility is that these compartmentalization defects are a secondary consequence of the DNA translocation defect, which could prevent some key gene from entering and being expressed in the forespore. However, spoIII E36 mutant sporangia have a DNA translocation defect evidently similar to that of spoIII E null mutant sporangia (38) yet have a less severe compartmentalization defect. Although it remains possible that the two mutations may have subtle differences in their DNA translocation defects, this suggests that some other function of SpoIII E may be differentially affected by these two classes of mutations.

Another possibility is that septa formed by spoIII E null mutant sporangia are leaky due to the presence of an incompletely translocated chromosome traversing the polar septum, allowing the diffusion of proteins between the two cells. Three observations argue against this possibility, however. First, when a lacZ gene fusion whose synthesis depends on \(\sigma^E\) (spoIVF-lacZ) was located in the origin-distal portion of the chromosome and was thereby trapped in the mother cell of spoIII E mutant sporangia, 94% of spoIII E::spc mutant sporangia had \(\beta\)-galactosidase confined to the mother cell, indicating that \(\beta\)-galactosidase produced in the mother cell does not leak into the forespore compartment (data not shown). Second, in spoIII E::spc sporangia, in which \(\sigma^E\) becomes active in the mother cell (38) (see below), \(\beta\)-galactosidase produced from a \(\sigma^E\)-dependent lacZ fusion (spoIIIG-lacZ) trapped in the mother cell (by virtue of its location on the origin-distal region of the chromosome) was present only in the mother cell (data not shown). Thus, even those spoIII E sporangia in which compartmentalization of \(\sigma^E\) activity is disrupted have a septum that prevents
diffusion of β-galactosidase from the mother cell to the forespore. Finally, some spoIIIE::spc mutant sporangia have normal compartmentalization of both σF and σE activities, suggesting that at least in these spoIIIE mutant sporangia, neither β-galactosidase, nor active σF or σE, nor any small molecule effectors of their activity leak either from the forespore to the mother cell or from the mother cell to the forespore. It therefore seems unlikely that diffusion of proteins or molecules through the septum explains the defective compartmentalization of σF or σE activities in spoIIIE mutants. We therefore favor the hypothesis, at least provisionally, that the two classes of spoIIIE mutations differentially affect some other unknown function of SpoIIIIE that contributes, directly or indirectly, to the loss of σF/pro-σE from the forespore and of the SpoIIIIE phosphatase from the mother-cell pole.

A model for the establishment of cell type. In summary, our present findings in extension of previous reports (for a review, see reference 35) point to the following updated model for the establishment of cell type (Fig. 5). The forespore transcription factor σF is present in the predivisional sporangium and in both compartments of the postdivisional sporangium. Only in the forespore does σF escape the inhibitory effect of the SpoIAB anti-sigma factor through a pathway involving the regulatory phosphatase SpoIIIE. We have only a partial understanding of how this happens, but our present results indicate that loss of the SpoIIIE phosphatase from the mother cell or at least from the mother-cell pole contributes to the forespore-specific activation of σF. Moreover, our present results show that disappearance of the SpoIIIE phosphatase from the mother-cell pole depends in some unknown fashion upon the action of SpoIIIIE. Meanwhile, the mother-cell transcription factor σE, which is present in the predivisional sporangium as the inactive proprotein, pro-σE, comes to be excluded from or destroyed in the small chamber of the postdivisional sporangium. Once again, and in an unknown fashion, the elimination of σF/pro-σE from the forespore depends on the action of SpoIIIIE. Finally, once σF has become active in the forespore and σF/pro-σE has been eliminated from the small sporangial chamber, an intercompartmental signal transduction pathway that is dependent upon σF-directed synthesis of the secreted signal protein SpoIIR triggers the conversion of pro-σE to mature and active σE in the mother cell. In this model, SpoIIIIE-dependent elimination of σE/pro-σE from the forespore contributes to cell-specific gene expression, whereas the σF-dependent signal transduction system serves as a timing device to ensure that pro-σE is not activated until after polar division.

An important feature of this model is that the SpoIIIIE protein contributes to the proper spatial regulation of both σF and σE activities. Just how a protein that is required for the transport of a chromosome into the forespore could be involved in cell-specific activation of σE and σF raises intriguing new questions about the nature of SpoIIIIE and the underlying basis for compartmentalized gene expression.

ACKNOWLEDGMENTS

We thank F. Arigoni, P. Stragier, W. Haldenwang, L. Duncan, and C. Webb for their invaluable gifts of antibodies, P. Levin for the spoIIIE null mutation, P. Piggot for the PspoIIE::spoIIR-bearing plasmid pMLK230, and P. Stragier, J. Errington, and P. Piggot for their many helpful comments and discussions.

This work was supported by NIH grant GM18568 to R.L. K.P. was a postdoctoral fellow of the Damon Runyon-Walter Winchell Cancer Foundation, and A.H. was an Alexander von Humboldt postdoctoral fellow.
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