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# Developmental Commitment in a Bacterium

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## Summary

**We investigated developmental commitment during sporulation in *Bacillus subtilis*. Sporulation is initiated by nutrient limitation and involves division of the developing cell into two progeny, the forespore and the mother cell, with different fates. Differentiation becomes irreversible following division when neither the forespore nor the mother cell can resume growth when provided with nutrients. We show that commitment is governed by the transcription factors $\sigma^F$ and $\sigma^E$, which are activated in the forespore and the mother cell, respectively. We further show that commitment involves *spoIIQ*, which is under the control of $\sigma^F$, and *spoIIP*, which is under the control of both $\sigma^F$ and $\sigma^E$. In the presence of nutrients, the forespore can exhibit rodlike, longitudinal growth when SpoIIQ and SpoIIP are absent, whereas the mother cell can do so when SpoIIP alone is absent. Thus, developmental commitment of this single-celled organism, like that of the cells of complex, multicellular organisms, ensures that differentiation is maintained despite changes in the extracellular milieu.**

## Introduction

A fundamental challenge in developmental biology is understanding the mechanisms that cause differentiation to become irreversible (Gilbert, 2000). As Hans Spemann (1918) observed almost a century ago, transplantation of prospective epidermal cells from an early newt gastrula to another region of the embryo changes their fate in accordance with their new location. In contrast, transplanted prospective epidermal cells from a later-stage gastrula retain their original fate (Spemann, 1918). Thus, as development progresses, the cells become committed to becoming epidermal cells despite the change in their environment. Similarly, more recent experiments in other systems, such as zebrafish, show that when single cells taken from an embryo at early stage of gastrulation are transplanted to a different location, their fate changes, but when cells at a later developmental stage are transplanted, they remain committed to their original fate (Ho and Kimmel, 1993). Whereas regulatory proteins that are involved in commitment are known in several systems (Tam et al., 2003), the identity of the target genes that directly act to prevent the reversal of cell fate have largely remained elusive.

An attractive developmental system in which to attempt to identify genes that mediate commitment is the process of sporulation in the bacterium *Bacillus subtilis*. In response to conditions of nutrient limitation, cells of *B. subtilis* cease growing and instead enter a developmental pathway that culminates in the formation of a spore. A hallmark of sporulation is the formation of an asymmetrically positioned (polar) septum that divides the developing cell into dissimilarly sized progeny called the forespore (the smaller cell) and the mother cell, which follow different pathways of differentiation (left hand of Figure 1A) (Errington, 2003; Piggot and Losick, 2002). The forespore is eventually engulfed by the mother cell, where it proceeds to develop into the spore. The mother cell facilitates the conversion of the forespore into a spore but ultimately undergoes lysis, releasing the spore, when development is complete. Cells that have started to sporulate but have not yet formed the polar septum are capable of resuming vegetative growth when provided with nutrients, that is, they are not committed. On the other hand, developing cells that have passed the point of asymmetric division (postdivisional sporangia) are obliged to complete spore formation even when transferred to nutrient-rich medium (Parker et al., 1996).

Shortly after the formation of the polar septum, the transcription factor $\sigma^F$ is activated in the forespore (Margolis et al., 1991), where it switches on the expression of about 16 genes (Piggot and Losick, 2002; S. Wang, P. Eichenberger, T. Sato, and R.L., unpublished data). Among the genes turned on by $\sigma^F$ is that (*spoIIR*) for an intercellular signaling protein (Londono-Vallejo and Stragier, 1995) that causes the activation in the mother cell of the transcription factor $\sigma^E$ (Figure 1A). The $\sigma^E$ factor, in turn, switches on an unusually large regulon consisting of at least 262 genes (Eichenberger et al., 2003; Eichenberger et al., 2004). Here we report that $\sigma^F$ and $\sigma^E$ are separately responsible for rendering differentiation in the forespore and the mother cell irreversible. We show that $\sigma^F$ can commit the forespore to its fate by switching on genes, including *spoIIP* and *spoIIQ*, that block growth and division in the forespore. We further show that *spoIIP* is under the control of both $\sigma^F$ and $\sigma^E$, allowing it to be expressed in both the forespore and the mother cell, and that the action of *spoIIP* commits the mother cell to its fate.

## Results

### Commitment in the Forespore Is Governed by $\sigma^F$

Bacteria lacking either $\sigma^F$ or $\sigma^E$ are unable to complete differentiation and instead undergo a second round of asymmetric division in which an additional polar septum is formed at the opposite pole (right hand of Figure 1A). This second division creates an aberrant three-chamber sporangium with two forespore-like compart-

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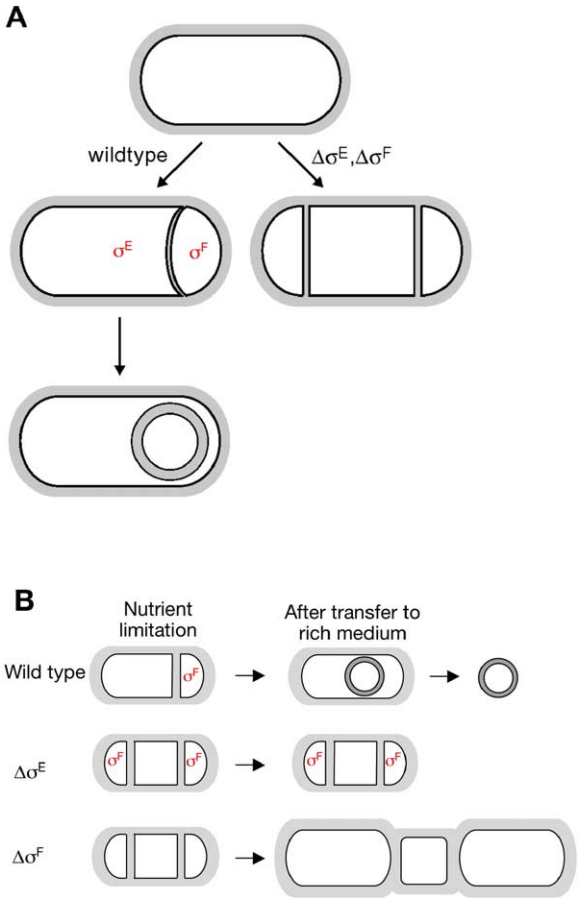


Figure 1. Cell Fate and Polar Division

(A) During sporulation of the wild-type (left), a single septum forms near one pole of the sporangium, creating a forespore (to the right of the septum) and a mother cell (to the left). In subsequent development, the forespore is engulfed by the mother cell and pinched off to create a cell within a cell. During sporulation of cells lacking $\sigma^F$ or $\sigma^E$ (due to a deletion of the *sigF* or *sigE* genes, respectively), a second polar septum is formed, creating a disporic sporangium that is blocked from further development (right).

(B) During sporulation of the wild-type (upper row), transfer to rich medium after a polar septum has formed does not prevent sporulation from continuing to completion. Sporulation of cells lacking $\sigma^E$ ($\Delta\sigma^E$; middle row) yields disporic sporangia that are blocked in subsequent development and are unable to resume growth when transferred to rich medium. Finally, sporulation of cells lacking $\sigma^F$ ($\Delta\sigma^F$; bottom row) yields disporic sporangia that are able to reinitiate longitudinal growth when transferred to rich medium.

ments at the poles, which each receive a chromosome (leaving the middle compartment devoid of DNA) (Setlow et al., 1991). We found that when such "disporic" sporangia of a $\sigma^F$ mutant were transferred by dilution into rich medium, the polar compartments were able to reinitiate longitudinal growth (Figure 1B and Figure 2A, upper panels; Table 1, rows 2 and 4) followed by medial division. By contrast, disporic sporangia of a $\sigma^E$ mutant failed to grow or divide when transferred to rich medium (Figure 1B and Figure 2A, lower panels; Table 1, rows 1 and 3). To confirm that the vegetative-like outgrowth observed in the $\sigma^F$ mutants derived from fore-

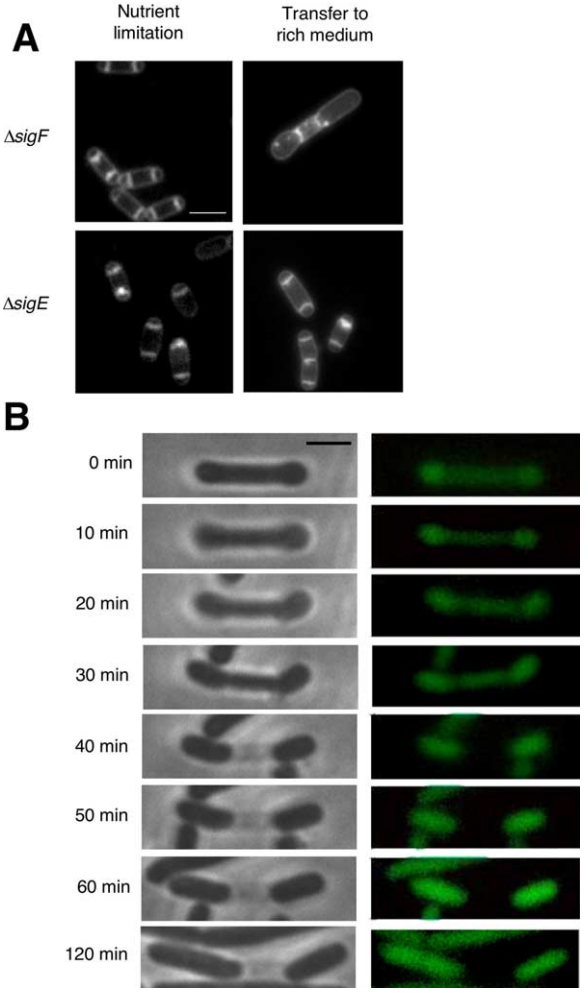


Figure 2. Disporic Sporangia Lacking $\sigma^F$ Are Able to Reinitiate Growth in the Presence of Excess Nutrients

(A) Shown are sporangia that have resumed rodlike growth from a strain (RL1265) lacking $\sigma^F$ (Δ*sigF*) or a strain (RL1061) lacking $\sigma^E$ (Δ*sigE*). Samples were collected for fluorescence microscopy after 3 hr in minimal (sporulation) medium or after an additional 2 hr following dilution into rich (growth) medium and treated with the vital membrane stain TMA-DPH. Scale bar = 3 μm.

(B) Shown are time-lapse phase (left) and fluorescent (right) images of a sporangium from a strain (MF2279) lacking $\sigma^F$ and carrying a fusion of *gfp* to the promoter $P_{abrB}$. The *abrB* promoter is active during growth and repressed during sporulation; thus, lower GFP fluorescence is indicative of a cell in sporulation, and higher GFP fluorescence is indicative of a cell that has resumed vegetative growth. Cells were grown for 3 hr in liquid minimal sporulation medium and then diluted 1:10 into rich medium and grown at 37°C for 45 min. Cells were concentrated by centrifugation, placed on an agarose pad (1% agarose in rich medium) maintained at 37°C, and visualized at regular intervals by time-lapse phase contrast and epifluorescence microscopy. As can be seen (left images), both polar forespores grew out into elongated cells, whereas the central mother-cell compartment, which in disporic sporangia lacks a chromosome, underwent lysis. At the time of dilution into rich medium, the disporic cell contained a comparatively fainter GFP fluorescence indicative of entry into sporulation (right images). At subsequent time points, the fluorescence grew brighter, indicative of a return to vegetative growth. Scale bar = 2 μm.

Table 1. Quantitative Analysis of Commitment

| Strain | Genotype | Time in Rich Medium (hr) | Disporic | Vegetative | Outgrown |
|---|---|---|---|---|---|
| RL1061 | $\Delta\sigma^E$ | 0 | 92 | 36 | 0 |
| RL1265 | $\Delta\sigma^F$ | 0 | 130 | 28 | 0 |
| RL1061 | $\Delta\sigma^E$ | 1.5 | 40 | 39 | 0 |
| RL1265 | $\Delta\sigma^F$ | 1.5 | 2 | 46 | 24 |
| RL1061 | $\Delta\sigma^E$ | 0 | 61 | 22 | 0 |
| JDB967 | $\Delta\sigma^E$Δ*spoIIQ*Δ*spoIIP* | 0 | 53 | 21 | 0 |
| RL1061 | $\Delta\sigma^E$ | 2.5 | 30 | 102 | 0 |
| JDB967 | $\Delta\sigma^E$Δ*spoIIQ*Δ*spoIIP* | 2.5 | 16 | 198 | 33 |

Samples were collected for fluorescence microscopy after 3 hr in minimal sporulation medium and after an additional 1.5 hr or 2.5 hr following dilution into rich growth medium; they were then treated with the vital membrane stain TMA-DPH. Cells that contained two recognizable, complete, asymmetric septa were categorized as "disporic," cells with medial septa were categorized as "vegetative," and cells with at least one polar compartment that had become rodlike were categorized as "outgrown."

spores, we conducted time-lapse microscopy. When a disporic $\sigma^F$-mutant sporangium placed on an agarose pad composed of a rich medium was visualized at regular intervals over 2 hr, longitudinal outgrowth of the forespore compartments was observed (Figure 2B, left panels). These cells contained, in addition, a fusion of *gfp* to a promoter (*abrB*) that is active during vegetative growth and repressed during sporulation (Strauch et al., 1990). Consistent with our interpretation that the polar compartments were forespores that were resuming vegetative growth, fluorescence from GFP in the two polar compartments was comparatively faint at the start of the time-lapse sequence and became progressively brighter as the polar cells elongated and returned to vegetative growth (Figure 2B, right panels).

### *spoIIQ* and *spoIIP* Contribute to Blocking Growth and Division of the Forespore

Although $\sigma^E$-mutant sporangia are similar in appearance to $\sigma^F$-mutant sporangia, $\sigma^E$-mutant sporangia are able to activate $\sigma^F$ in both polar compartments (Figure 1B). This suggested that the difference between $\sigma^F$ and $\sigma^E$ mutants in their potential to resume growth was due to a gene(s) under the control of the forespore-specific transcription factor that prevented growth. To identify this gene(s), we constructed a series of strains that were mutant for $\sigma^E$ and that also carried a mutation in one of the previously known (Piggot and Losick, 2002) members of the $\sigma^F$ regulon (*spoIIQ*, *spoIIIG*, *dacF*, *csfA*, *csfB*, *spoIIR*, *sspA*, *sspB*, *sspE*, and *rsfA*) or in one of the genes that were recently assigned to the regulon through microarray analysis (*yffL*, *yisN*, *ywmF*, *ykwF*, *yqhH*, and *yphA* [S. Wang, P. Eichenberger, T. Sato, and R.L., unpublished data]). *spoIIQ* was a particularly attractive candidate because its product, a membrane protein with similarity to metallo-endopeptidases, plays an important role in remodeling of the cell wall of the forespore (Londono-Vallejo et al., 1997; Rubio and Pogliano, 2004). However, neither a *spoIIQ* mutation (Figure 3A, upper panels) nor a mutation in any of the other $\sigma^F$-controlled genes tested allowed any disporic sporangia to undergo longitudinal growth when placed in rich growth medium.

Another attractive candidate was *spoIIP*, which encodes a membrane protein that is involved in preventing a second round of asymmetric division in wild-type sporulating cells. Strains lacking *spoIIP* exhibit a significantly higher frequency of bipolar and partially bipolar septa, and premature expression of *spoIIP* along with two other genes, *spoIID* and *spoIIM*, inhibits formation of the initial sporulation septum (Eichenberger et al., 2001; Pogliano et al., 1999). Whereas *spoIIP* is normally considered to be a $\sigma^E$-controlled gene (Frandsen and Stragier, 1995), and its transcription in the mother cell contributes to preventing the disporic phenotype (Eichenberger et al., 2001; Pogliano et al., 1999), it lies immediately downstream (62 bp) of a gene under $\sigma^F$ control (*gpr*), and there are no obvious transcriptional terminators in the intergenic region between the two genes (Figure 4A). Also, prior genetic evidence (Frandsen and Stragier, 1995) as well as gene microarray analysis (S. Wang, P. Eichenberger, T. Sato, and R.L., unpublished data) suggested that *spoIIP* might exhibit a second mode of regulation in which it is transcribed under the control of the forespore-specific transcription factor by readthrough from *gpr*. Accordingly, we built a $\sigma^E$-mutant strain that also carried a *spoIIP* mutation, but the resulting sporangia were indistinguishable in their terminal differentiation phenotype from those of a strain that was mutant for $\sigma^E$ alone (Figure 3A, middle panels).

We hypothesized that commitment could be due to the combined activity of more than one gene under $\sigma^F$ control. We therefore constructed a series of $\sigma^E$-mutant strains that carried mutations in two $\sigma^F$-controlled genes. Whereas almost all of these strains failed to exhibit growth of their forespores when transferred into rich medium, in forespores of a $\sigma^E$-mutant strain that additionally carried mutations in both *spoIIP* and *spoIIQ*, swelling and/or pronounced rodlike, longitudinal elongation could be observed (Figure 3A, lower panels; Table 1, rows 6 and 8). In fact, some of the elongating forespores of the disporic sporangia eventually underwent medial division (Figure 3A, lower right panel), a hallmark of vegetative growth, and by 2.5 hr after transfer to rich medium, comparatively few disporic sporangia remained (Table 1, row 8). These findings suggest that the action of both genes contributes to preventing postdivisional sporangia from undergoing outgrowth. We note, however, that cells lacking *spoIIQ* and *spoIIP* were noticeably less efficient in exhibiting outgrowth than cells lacking $\sigma^F$ (Table 1, rows 4 and 8),

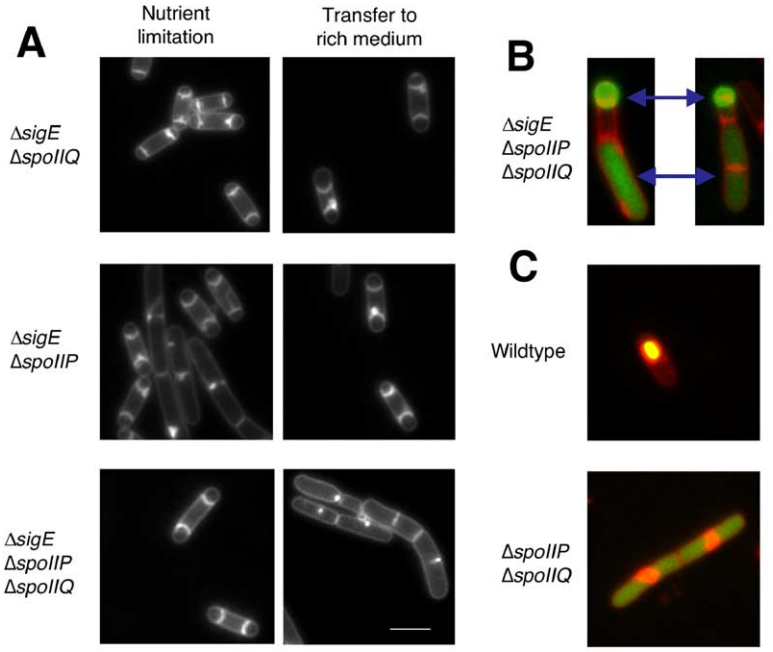


Figure 3. The *spoIIP* and *spoIIQ* Genes Contribute to Commitment in the Forespore

(A) Shown are sporangia that have resumed longitudinal growth from strains lacking $\sigma^E$ and mutant for *spoIIQ* (Δ*spoIIQ*; JDB919), *spoIIP* (Δ*spoIIP*; JDB963), or doubly mutant for both *spoIIP* and *spoIIQ* (Δ*spoIIP* Δ*spoIIQ*; JDB967). Scale bar = 3μm.

(B) Shown are two sporangia after 2.5 hr in rich medium from a strain (JDB972) lacking $\sigma^E$, containing mutations of *spoIIP* and *spoIIQ*, and carrying a fusion of *gfp* to a promoter under $\sigma^F$ control ($P_{spoIIQ}$-*gfp*). One forespore compartment of each of the disporic sporangia (bottom arrow) resumed longitudinal growth and contains GFP fluorescence that had been diluted during growth, whereas the other forespores (top arrow) had not undergone growth and therefore contain brighter GFP signals that have not been diluted. Note that the middle compartments lack GFP fluorescence, as would be expected for mother cells in which $\sigma^F$ is not active. Scale bar = 3 μM. Of cells exhibiting green fluorescence observed in this experiment, 9 (33%) were forespores of disporic sporangia that had not grown, 16 (60%) were outgrowing forespores, and 2 (7%) were cases in which fluorescence was seen in the mother cell, which evidently arose from leakage from the forespore.

(C) Shown are a sporangium from a wild-type strain (RL2382) that had continued through engulfment in rich medium (top) and a sporangium from a strain (JDB1047) carrying mutations in *spoIIP* and *spoIIQ* (Δ*spoIIP* Δ*spoIIQ*) that had resumed vegetative growth after 2 hr in rich medium (bottom). Both strains carried $P_{spoIIQ}$-*gfp*. Of 152 wild-type cells examined, 122 were rodlike and lacked significant green fluorescence indicative of the progeny of vegetative cells that had not committed to sporulation at the time of transfer to rich medium and were able to resume (or continue) vegetative growth rapidly and undergo multiple rounds of division during the period of growth in rich medium. Thirty cells in the control population were undergoing or had undergone engulfment, as indicated by the green fluorescence signal in their forespore. Of 168 Δ*spoIIP* Δ*spoIIQ* cells examined, 80 were rodlike and contained green fluorescence above background, and 88 were rodlike and lacked green fluorescence. The latter is indicative of cells that had initiated sporulation but returned to vegetative growth after transfer to rich medium.

suggesting that one or more additional genes under $\sigma^F$ control contribute to commitment.

### Cytoplasmically Inherited Green Fluorescent Protein as a Lineage Reporter

To confirm that these elongating, rod-shaped cells arose from cells that had entered sporulation, we introduced a reporter containing the gene (*gfp*) for the green fluorescent protein (GFP) under control of $\sigma^F$, the forespore-specific transcription factor. Two examples of rod-shaped cells in which the presence of green fluorescence indicates that the cells originated from forespores can be seen in Figure 3B (bottom arrow). In each of these disporic sporangia, a forespore compartment (bottom arrow) resumed longitudinal growth (and in one case had undergone binary fission [right panel]). This growth diluted the GFP fluorescence so that it was weaker than that seen in the other forespores (top arrow) that had not grown out and whose GFP therefore had not been diluted. The middle compartments lacked GFP fluorescence, as would be expected for mother cells in which $\sigma^F$ is not active. Although leakage of GFP from the forespore to mother cell has been observed in some sporulation mutants, this phenomenon typically only occurs following extended periods (>8 hr) in sporulation medium (Li et al., 2004), and while we could observe some sporangia with GFP fluorescence in the mother cell, such sporangia were rare. Thus, *spoIIQ spoIIP*-mutant cells exhibiting green fluorescence were most likely to have derived from a forespore cell that had entered sporulation and activated $\sigma^F$ but had not proceeded further into sporulation. In other words, in this strain, GFP can be considered to be a cytoplasmically inherited lineage reporter that certifies that a growing cell arose from a cell that had $\sigma^F$ activity.

We examined whether mutations in *spoIIP* and *spoIIQ* would prevent commitment in sporangia that were otherwise wild-type, that is, sporangia that were not lacking $\sigma^E$. When wild-type cells harboring a construct in which *gfp* was under the control of $\sigma^F$ were transferred into rich medium at 2.25 hr after the start of sporulation, postdivisional sporangia in which $\sigma^F$ was activated were observed to continue through further stages of sporulation, including engulfment of the forespore by the mother cell (note the fluorescence of the engulfed forespore in the upper panel of Figure 3C). In contrast, sporangia of a *spoIIQ spoIIP* double mutant (once again carrying the *gfp* construct) that were treated in the same way could revert to growth (note the fluorescence of the dividing cells in the lower panel of Figure 3C). Thus, otherwise wild-type cells lacking *spoIIP* and *spoIIQ* that have initiated sporulation and have activated $\sigma^F$ are not necessarily committed to sporulation and appear to be able to resume rodlike elongation when exposed to excess nutrients.

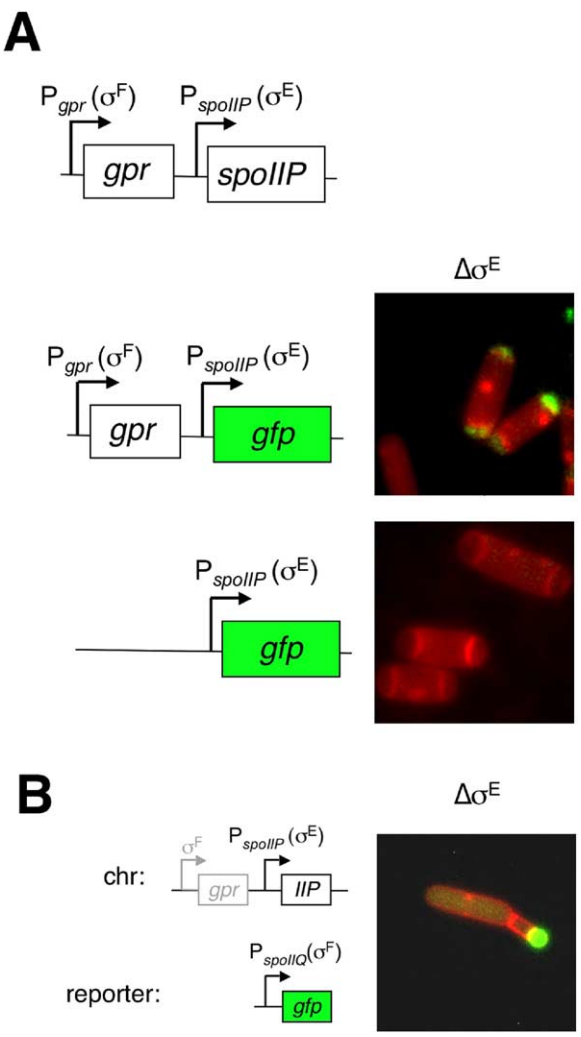


Figure 4. Readthrough from the *gpr* Promoter Drives Transcription of *spoIIP* in the Forespore

(A and B) Sporangia were processed for fluorescence microscopy after 3 hr in sporulation medium as in Figure 2.

(A) The *spoIIP* gene is transcribed from a $\sigma^E$-controlled promoter ($P_{spoIIP}$) located immediately upstream of *spoIIP* and a $\sigma^F$-controlled promoter ($P_{gpr}$) located just upstream of *gpr*. Shown are sporangia from strains lacking $\sigma^E$ and carrying *gfp* fused to the 1462 bp of DNA upstream of *spoIIP* including the $\sigma^F$-dependent $P_{gpr}$, the *gpr* gene, and the $\sigma^E$-dependent $P_{spoIIP}$ (JDB1080; upper) or carrying a fusion of *gfp* to the 183 bp of DNA upstream of *spoIIP* including the $\sigma^E$-dependent $P_{spoIIP}$ (JDB1082; lower).

(B) Sporangium from a strain (JDB1025) lacking $\sigma^E$, *spoIIQ*, and *gpr* and containing a fusion of *gfp* to a promoter under $\sigma^F$ control ($P_{spoIIQ}$-*gfp*).

## Expression of *spoIIP* in the Forespore

The idea that *spoIIP* is involved in the developmental commitment of the forespore requires that *spoIIP* be in fact expressed in the forespore under the control of $\sigma^F$. We confirmed previous work (Frandsen and Stragier, 1995) suggesting that some transcription of *spoIIP* arises by readthrough from the upstream, $\sigma^F$-controlled *gpr* gene (data not shown) and extended this analysis further by fusing the chromosomal region upstream of *spoIIP*, including the entire *gpr* gene and its promoter, to a promoterless copy of *gfp*. We then introduced the *gfp*-containing construct into the chromosome (at a nonessential locus) of a $\sigma^E$ mutant. The fusion was expressed in the forespore as would be expected if expression of the fusion were solely under control of $\sigma^F$ (Figure 4A, middle panel). Consistent with this interpretation, when the construct was introduced into cells that were mutant for $\sigma^F$ (and therefore also lacked $\sigma^E$ activity since activation of $\sigma^E$ is dependent on $\sigma^F$), little or no production of GFP was detected (data not shown). When *gfp* was fused to the intergenic region between *gpr* and *spoIIP* (and hence lacked the $\sigma^F$-dependent *gpr* promoter), once again little or no production of GFP was detected in the absence of $\sigma^E$ (Figure 4A, lower panel). In toto, these results confirm that *spoIIP* is subject to two modes of expression: $\sigma^E$-directed transcription from a promoter located immediately upstream of the gene and $\sigma^F$-directed readthrough transcription from the promoter of the adjacent upstream gene.

If this readthrough transcription is critical for the contribution of *spoIIP* to commitment, then a deletion mutation that removes *gpr* and its promoter but leaves *spoIIP* intact should, when tested in combination with a *spoIIQ* mutation, result in a defect in commitment. *gpr* is dispensable for sporulation (Sussman and Setlow, 1991), and, indeed, a deletion that spans both *gpr* and its promoter had no measurable effect on sporulation, which indicates that development does not depend on expression of *spoIIP* in the forespore. However, when the *gpr* deletion was introduced into a strain that was mutant for *spoIIQ* and the resulting double-mutant strain allowed to sporulate, sporangia that had reached the stage of asymmetric division were capable of exhibiting rodlike elongation when introduced into rich medium (Figure 4B).

As a final test of the role of readthrough transcription in commitment, we examined a strain in which *spoIIP* (but not *gpr*) was moved to another site on the chromosome. At this ectopic location, *spoIIP* is fully functional in sporulation (Abanes-De Mello et al., 2002), but when a *spoIIQ* mutation was introduced, postdivisional sporangia from the resulting strain were able to reinitiate growth in the presence of excess nutrients despite the presence of a functional copy of *spoIIP* (data not shown). We conclude that transcription of *spoIIP* from its own promoter suffices for sporulation but that readthrough transcription from the *gpr* promoter is necessary for the role of *spoIIP* in commitment.

## *spoIIP* Blocks Growth and Division in the Mother Cell

We then examined the requirements for commitment in the mother cell. To identify cells that had initiated the mother-cell program of gene expression, we introduced a reporter containing *gfp* under the control of $\sigma^E$, the mother-cell-specific transcription factor. When sporulating cells that had reached the stage at which this reporter had been activated were transferred to rich medium, they proceeded through the later stages of sporulation, such as engulfment (Figure 5, upper panels), and no sporangia that resumed vegetative growth were seen. By contrast, when postdivisional sporangia

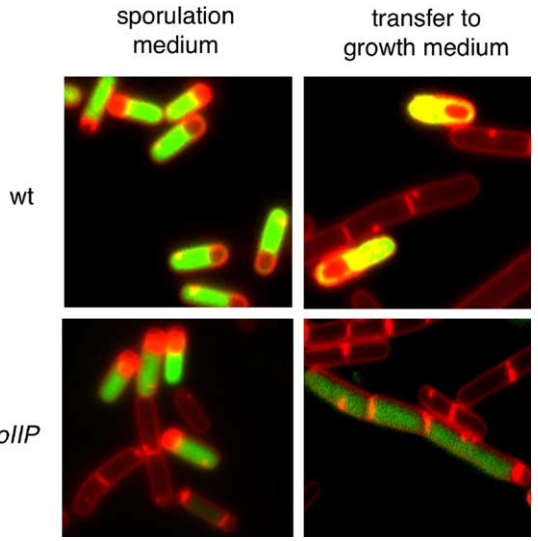


Figure 5. Role of *spoIIP* in Mother-Cell Commitment

Shown are sporangia from an otherwise wild-type strain (JDB869) and a strain (JDB973) mutant for *spoIIP* (Δ*spoIIP*), each carrying *gfp* fused to a promoter under the control of $\sigma^E$ ($P_{spoIID}$-*gfp*). Sporangia were processed for fluorescence microscopy as in Figure 2. For quantitative analysis of the *spoIIP* mutant strain, 221 cells were examined microscopically at hour 2 of sporulation. Of these rodlike cells, 54 (or 25%) exhibited a GFP signal above background.

of a *spoIIP*-mutant strain (once again carrying the *gfp* reporter under $\sigma^E$ control) were exposed to excess nutrients, vegetative-like cells with medial septa that exhibited green fluorescence (and hence were of mother-cell origin) were readily observed (Figure 5, lower panels). Whereas the absence of both *spoIIP* and *spoIIQ* was necessary to prevent forespore elongation, the absence of *spoIIP* alone was sufficient to disrupt commitment in the mother cell. Indeed, the absence of *spoIIP* was more effective in abrogating commitment in the mother cell than was the corresponding effect on the forespore of the absence of both *spoIIP* and *spoIIQ*.

### Cell-Specific Expression of *spoIIP* Orthologs from *B. anthracis*

Interestingly, the related spore-forming bacterium *Bacillus anthracis* has two genes, BA2068 and BA3102, that are orthologous to *B. subtilis spoIIP*, and both are turned on during sporulation (Liu et al., 2004). There is extensive synteny between the *B. subtilis* and *B. anthracis* genomes (Read et al., 2003), but neither ortholog is located near *gpr* in *B. anthracis* (Figures 6Aa and 6Ab) even though the chromosomal regions around *gpr* are otherwise highly similar in both organisms (Figures 6Ac and 6Ad). We note, however, that the upstream regions of BA2068 and BA3102 contain sequences that match the consensus for $\sigma^E$ (Eichenberger et al., 2003) and $\sigma^F$ (S. Wang, P. Eichenberger, T. Sato, and R.L., unpublished data) controlled promoters, respectively. To investigate the regulation of BA2068 and BA3102 directly, we fused their putative promoter regions to *gfp* and introduced those constructs into a nonessential chromosomal locus of *B. subtilis*. In sporulating cells carrying a fusion of *gfp* to DNA corresponding to the region upstream of BA3102, a GFP signal was observed in the forespore in some cells (Figure 6B, right panel). In contrast, fusion of *gfp* to DNA corresponding to the region upstream of BA2068 resulted in mother-cell-specific production of GFP (Figure 6B, left panel). Thus, *B. anthracis* apparently has evolved a strategy different from that of *B. subtilis* to ensure that *spoIIP* expression occurs in both compartments: it uses two copies of the gene, one under $\sigma^F$ and one under $\sigma^E$ control. It remains to be seen, however, whether commitment in *B. anthracis* is governed by a mechanism similar to that described here for *B. subtilis*. In another related spore-forming bacterium, *Clostridium difficile*, the ortholog of the $\sigma^F$-controlled *spoIIQ* gene is located immediately adjacent to a gene (*spoIID*) that is known to be under $\sigma^E$ control, and *spoIIP* is even closer to *gpr* (15 bp) than it is in *B. subtilis* (62 bp). Thus, in *C. difficile* both *spoIIQ* and *spoIIP* are likely to be expressed in both compartments of the sporangium (Stragier, 2002).

## Discussion

Viewed in light of our results, $\sigma^F$ is emerging as the master regulator for the establishment of cell fate during sporulation. The $\sigma^F$ factor directs the expression of genes that drive differentiation of the forespore as well as the expression of the signaling gene that triggers the activation of $\sigma^E$ in the mother cell and hence unleashes the mother-cell program of differentiation (Errington, 2003; Piggot and Losick, 2002). Now we see that two of the genes switched on by $\sigma^F$, *spoIIP* and *spoIIQ*, have a previously unrecognized role in causing the forespore program of differentiation to become irreversible (Figure 7). One of these genes, *spoIIP*, is also expressed in the mother cell, where it plays a parallel role in preventing the reversal of differentiation (Figure 7). Thus, *spoIIP* plays a critical role in commitment in both compartments, but its function in commitment of the forespore is masked by redundancy with *spoIIQ*. This requirement for an additional factor(s) in the forespore may reflect the weak level of expression of *spoIIP* in the forespore. In addition, the less-efficient outgrowth observed in a *spoIIP spoIIQ*-mutant strain as compared to a $\sigma^F$-mutant strain indicates that an additional, yet-to-be-identified, forespore-expressed gene(s) contributes to preventing outgrowth.

Although the precise biochemical nature of the growth and division block mediated by the *spoIIP* and *spoIIQ* gene products is not known, both are membrane proteins that play a direct role in the modification of the peptidoglycan that surrounds the forespore during engulfment (Abanes-De Mello et al., 2002; Rubio and Pogliano, 2004), and the *spoIIP* gene product is part of the mechanism that prevents formation of a second septum in the mother cell (Eichenberger et al., 2001; Pogliano et al., 1999). In fact, premature expression during sporulation of *spoIIP*, along with *spoIIM* and *spoIID*, inhibits formation of the initial polar septum and causes distension of the cell wall (Eichenberger et al., 2001; Pogliano et al., 1999). Conceivably, the effect of SpoIIP and SpoIIQ on growth results from an ability to directly inhibit enzymes involved in peptidoglycan syn-

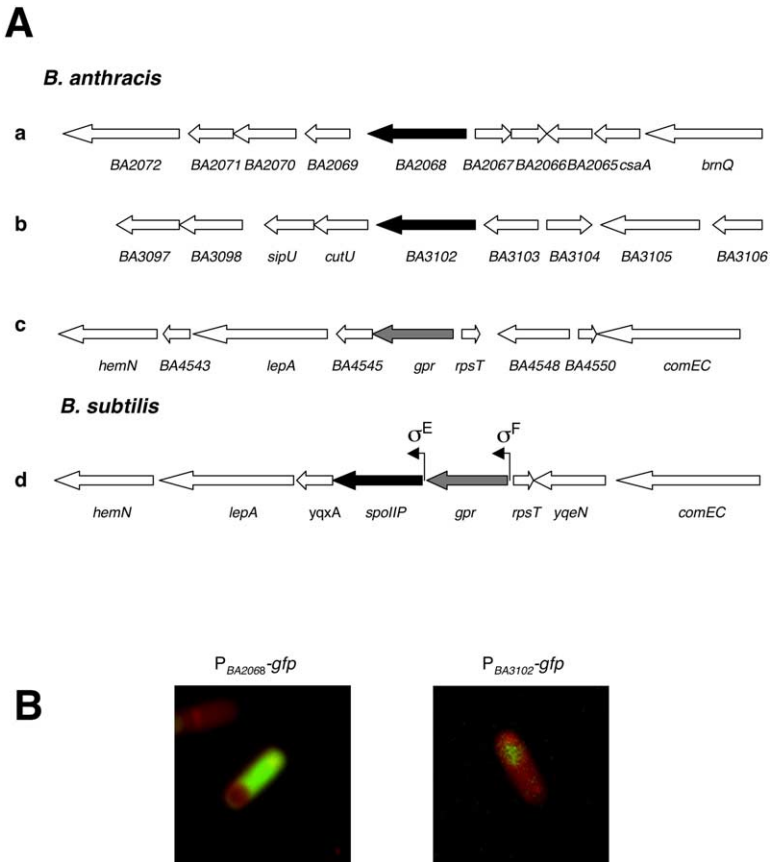


Figure 6. The Presence of Two *spoIIP* Genes in *B. anthracis*

(A) Organization of the chromosome in the vicinity of the *B. anthracis spoIIP* orthologs BA2068 (a) and BA3102 (b), near the *B. anthracis* ortholog of *gpr* (c), and near *B. subtilis gpr* (d).

(B) Shown are sporangia from *B. subtilis* strains carrying *gfp* fused to the promoters of the *B. anthracis spoIIP* orthologs BA3102 (JDB1104) and BA2068 (JDB1105) after 3 hr in sporulation medium. Sporangia were processed for fluorescence microscopy as in Figure 2.

thesis, as has been described for the E lysis protein of phage $\phi$X174 (Bernhardt et al., 2001).

Our findings take on added significance in view of the recent discovery that cells that have entered sporulation (predivisional sporangia) secrete factors that induce sibling cells that have not yet initiated sporulation to lyse (Gonzalez-Pastor et al., 2003). Nutrients released by lysis delay predivisional sporangia from proceeding further into sporulation. However, once the sporangia have reached the stage of asymmetric division and have activated $\sigma^F$ and $\sigma^E$, they employ expression of *spoIIP* and *spoIIQ* as a means to ensure that they are committed to completing spore formation even in the presence of nutrients released by lysing sibling cells. Thus, developmental commitment of a single-celled organism, like that of the cells of complex, multicellular organisms, ensures that differentiation is maintained despite changes in the extracellular milieu.

## Experimental Procedures

### Strains

*B. subtilis* strains were derivatives of the wild-type strain PY79 and are listed in Table S1. JDB919 was constructed by transforming RL1061 with genomic DNA from RL2022 (*spoIIQΔ::spc*, laboratory stock). JDB963 was constructed by transforming RL1061 with genomic DNA from RL2373 (*spoIIPΔ::tet*, laboratory stock). JDB967 was constructed by transforming JDB919 with genomic DNA from RL2373. JDB972 was constructed by transforming JDB967 with genomic DNA from a strain derived from RL2382 where the spec$^R$ was switched to a cm$^R$ gene through the use of pCm::Sp (Steinmetz and Richter, 1994). JDB973 was constructed by transforming JDB869 with genomic DNA from RL2373. JDB1025 was constructed in several steps. First, long-flanking-homology PCR (Wach, 1996) was used to replace codons 1–324 of *gpr* as well as 248 bp upstream of the start codon with a spec$^R$ gene. Genomic DNA from this strain was then used to transform JDB919. Finally, this strain was transformed with genomic DNA from a strain carrying $P_{spoIIQ}$-*gfp* where spec$^R$ was switched to tet$^R$ through the use of plasmid pCm::Tc (Steinmetz and Richter, 1994). JDB1047 was constructed by transforming RL2022 with genomic DNA from RL2373. JDB1080 was constructed by transforming RL1061 with pCB47. JDB1082 was constructed by transforming RL1061 with pCB48. JDB1104 was constructed by transforming PY79 with pCB52. JDB1105 was constructed by transforming PY79 with pCB54. MF2279 was constructed by transformation of RL1265 with chromosomal DNA from MF1179 ($P_{abrB}$-*gfp spc*) that was generated by introducing plasmid pMF175 into *amyE* by double recombination.

### Plasmids

Plasmid constructions were performed in *E. coli* DH5$\alpha$ using standard methods. pCB47 contains $P_{1462}$-*gfp*, which is the 1462 bp upstream of the *spoIIP* ribosome binding site (RBS) fused to *gfp*, and was created by amplifying genomic DNA from PY79 using primers ojd787 (5′-GGCGCTAGCGATTGCAGTACGCATAGCAG-3′) and ojd790 (5′-GTCGCATGCGCGCTTGTCTAGTAATTACTC-3′). This PCR-amplified DNA was digested with NheI and SphI and cloned into pCB45 that contains NheI and SphI sites upstream of *gfpmut2* with an optimized RBS in the *amyE* integration vector pLD30 (Garsin et al., 1998). pCB48 contains $P_{183}$-*gfp*, which is the 183 bp upstream of the *spoIIP* RBS fused to *gfp*, and was created by amplifying genomic DNA from PY79 using primers ojd788 (5′-GGCGCTAGCGGC CACAACTTAATGGTTAC-3′) and ojd790. This PCR-amplified DNA was digested with NheI and SphI and cloned into pCB45. pCB52 contains the 580 bp upstream of the *B. anthracis* BA3102 RBS

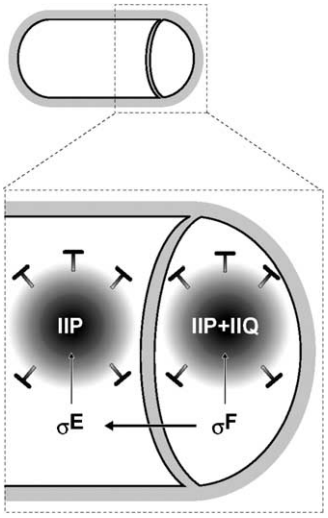


Figure 7. Role of *spoIIP* and *spoIIQ* in Establishment of Cell Fate during Sporulation

The $\sigma^F$ factor turns on the synthesis of the *spoIIP* (IIP) and *spoIIQ* (IIQ) gene products in the forespore as well as the synthesis of an intercellular signaling protein (horizontal arrow) that causes the activation of $\sigma^E$ in the mother cell. The $\sigma^E$ factor, in turn, turns on the synthesis of the *spoIIP* (IIP) gene product in the mother cell. The *spoIIP* and *spoIIQ* gene products likely block cell-wall growth and septation.

fused to *gfp* and was created by amplifying genomic DNA from *B. anthracis* strain Sterne (kind gift of P. Hanna, University of Michigan) using primers ojd775 (5′-GGCGAATTCCCTTGTCTTGATTGG AGTG-3′) and ojd776 (5′-GGCAAGCTTCTTCATTTACTATATTCTT ACG-3′). This PCR-amplified DNA was digested with EcoRI and HinDIII and cloned into pCB44 that contains *gfpmut2* with an optimized RBS cloned into the HinDIII and BamHI sites of the *amyE* integration vector pLD30 (Garsin et al., 1998). pCB54 contains the 565 bp upstream of the *B. anthracis* BA2068 RBS fused to *gfp* and was created by amplifying genomic DNA from *B. anthracis* strain Ames using primers ojd777 (5′-GGCGAATTCGAATGCTACC AACAATCAGC-3′) and ojd778 (5′-GGCAAGCTTCTCTCTATTTCA AGTTATGTAC-3′). This PCR-amplified DNA was digested with EcoRI and HindIII and cloned into pCB44. To construct pMF175 ($P_{abrB}$-*gfp spc*), a larger DNA fragment derived from pMF35 ($P_{spacC}$-*gfp spc*; Fujita and Losick, 2002) digested with EcoRI and HindIII was ligated to a smaller DNA fragment derived from pMF172 ($P_{abrB}$-*lacZ spc*; Fujita et al., 2005) digested with the same restriction enzymes.

### General Methods

All PCR reactions were performed with *pfu* DNA polymerase (Stratagene). Preparation and transformation of *B. subtilis* competent cells were as described (Harwood and Cutting, 1990). Sporulation and growth were carried out at 37°C. Cells were grown in hydrolyzed casein (CH) growth medium (Harwood and Cutting, 1990). Experimental cultures were inoculated with a portion of an overnight culture grown in CH at 25°C to an OD600 of 0.05, and when cultures reached an OD600 of ~0.6, they were resuspended in an equal volume of preheated resuspension medium (Sterlini and Mandelstam, 1969) and allowed to grow with aeration. To transfer sporulating cells to a rich medium, 1 ml of a sporulating culture was added to 9 ml of Luria Broth (LB) preheated to 37°C and then allowed to grow with aeration.

### Fluorescence Microscopy

Fluorescence microscopy was performed as described previously (Dworkin and Losick, 2002).

### Comparative Genomics

*B. subtilis* genome sequence was obtained from the SubtiList website (http://genolist.pasteur.fr/SubtiList/). *B. anthracis* strain Ames genome sequences were obtained from the NCBI website (http://www.ncbi.nlm.nih.gov/genomes/static/eub_g.html). To identify orthologs of *B. subtilis spoIIP* in related genomes, its sequence was blasted against the selected bacterial genome using the tblastn program on the NCBI website.

### Supplemental Data

Supplemental Data include one table and are available with this article online at http://www.cell.com/cgi/content/full/121/3/401/DC1/.

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