Compartmentalization of Gene Expression during Sporulation of
Bacillus subtilis Is Compromised in Mutants Blocked at
Stage III of Sporulation

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Mutations in the spoIII A and spoIII J loci disrupt the compartmentalization of gene expression during
sporulation of Bacillus subtilis. The breakdown in compartmentalization is not the cause of their being blocked in
spore formation. Rather, it appears to be a consequence of the engulfed prespore’s being unstable.

Cell differentiation is one of the fundamental processes in biology. Central to it is the compartmentalization of gene
expression, so that different genes are expressed in different cell
types. Spore formation by Bacillus subtilis has become a para-
digm for the study of cell differentiation in prokaryotes. During
sporulation, B. subtilis divides asymmetrically, resulting in two
unequal-size cells, the smaller prespore and the larger mother
cell. Following sporulation division, conventionally designated
unequal-size cells, the smaller prespore and the larger mother
cell. Following sporulation division, conventionally designated
stage II, gene expression becomes compartmentalized. Distinct
RNA polymerase sigma factors become active, $\sigma^F$ in the pre-
spore and $\sigma^E$ in the mother cell (12). The mechanisms for
compartmentalizing the activity of these sigma factors are not
fully understood. Following division, the prespore is engulfed
by the mother cell. The engulfed prespore (also called the
forespore) develops into the mature spore. The mother cell is
necessary for this process but ultimately lyse. Completion of
engulfment (stage III) is associated with activation of other
sigma factors, $\sigma^G$ in the prespore and $\sigma^K$ in the mother cell.
Mutants that are blocked at stage III do not activate $\sigma^G$ or $\sigma^K$
(12).

A genetic test to analyze the compartmentalization of gene
expression during sporulation was recently developed (7). The
test is based on the sacB/SacY transcriptional antitermination
system (2). The SacY protein regulates transcription of sacB
(encoding levansucrase) by antitermination. In the absence of
SacY, transcription of sacB terminates in the region 5' to the
sacB structural gene. When SacY is present, it binds to the
RAT site in the nascent 5' region, prevents termination, and so
allows transcription of the sacB structural gene (2). Thus, SacY
needs to be present during transcription of sacB in order to
obtain levansucrase or, in our case, $\beta$-galactosidase from a
sacB'-lacZ translational fusion. The SacY protein is ordinarily
subject to complex regulation, and we used a truncated deriv-
ative, SacY(1-55), that is free from this regulation (8). By
placing sacB'-lacZ and sacY(1-55) under the control of pro-
motors directed by the same or different sigma factors, we were
able to confirm that there was essentially complete compartment-
entalization of $\sigma^F$ and $\sigma^E$ activity during sporulation (7).

We have used mutagenesis with a Tn10 derivative (9) to
generate mutants with the compartmentalization of the activ-
ities of $\sigma^F$ and $\sigma^E$ disrupted and have obtained mutations in the
spoIII A and spoIII J loci. Here we explore the reason why
such mutants might have compartmentalization disrupted. We
distinguished two alternatives that would point to radically
different roles for these spoIII loci. The first is that the muta-
tions disrupt compartmentalization, and as a consequence
sporulation cannot proceed beyond stage III. The second is
that the mutations cause a block at stage III, and as a conse-
quence the engulfed prespores are prone to lyse, causing loss of
compartmentalization. In the first mechanism, disruption of
compartmentalization should be apparent as soon as $\sigma^F$ and
$\sigma^E$ become active. In the second mechanism, the activities of
$\sigma^F$ and $\sigma^E$ should be compartmentalized when they first ap-
pear, and compartmentalization should be lost only following
engulfment. We found the latter to be the case, as the loss of
compartmentalization follows the completion of engulfment.

Isolation of mutants disrupted in compartmentalization of
gene expression. A pool of B. subtilis mutants with mini-Tn10
insertions (9) was screened for compartmentalization of $\sigma^F$
and $\sigma^E$ activities. The mutants were obtained in strain SL7643,
which is a derivative of S501 cured of SPB (7); S501 (8) has
deletions of sacY, sacB, licT, and sacT to avoid possible inter-
ference with the antitermination system being used. Chromo-
somal DNA of the pooled mutants was purified and used to
transform the tester strain SL9106, also a derivative of SA501,
selecting for Tn10-associated chloramphenicol resistance.
Strain SL9106 contains $\text{P}_{cis}^{\text{galT}}, \text{SacB'}-\text{lacZ}/\text{SacY}(1-55)$
(the $\sigma^F/\sigma^E$ configuration) (7). Among approximately 30,000
Cm resistant transformants, 32 displayed $\beta$-galactosidase activity.
Of these, one activated the cryptic endogenous lacA gene, and one
mapped in spoIII E, where insertions have previously been
shown to disrupt compartmentalization (7, 17). The remaining
Tn10 mutations were tested by backcrossing into strain SL9106
and by transfer to strain SL9105 (which has the opposite con-
figuration, $\sigma^E/\sigma^F$). Nine mutations that caused substantial $\beta$-ga-
lactosidase expression in both strains were then transferred into
strains with $\sigma^E$- and $\sigma^F$-directed gfp fusions (SL9109 spoIID-gfp...
and SL10280 spoIIQ-gfp, respectively (kindly provided by David Hilbert and Vasant Chary). Eight of the mutations caused a substantial loss of compartmentalization, as visualized with bacteria grown on Schaeffer’s sporulation agar (11). This result indicated that the sacB/SacY screen was an effective predictor of loss of compartmentalization.

Genetic crosses between the eight strains with impaired compartmentalization indicated that the mutations mapped in two distinct loci. Flanking DNA from the inserts was recovered and sequenced, and the loci were identified as spoIII (seven mutations) and spoIIIAB (one mutation; Tn10 insertion 428 nucleotides downstream from the presumed start of translation). The ninth mutation, which resulted in β-galactosidase activity in SL9105 and SL9106 but had little effect on the compartmentalization of σ^E- and σ^K-directed gfp expression, mapped in ybdB (Tn10 insertion 457 nucleotides downstream from the presumed start of translation); it was not studied further. The ybdB locus, now designated skf, is associated with production of an antibiotic that kills nonsporulating bacteria in the same population (4). The spoIIIAB mutation and a spoIIIJ mutation with Tn10 inserted 472 nucleotides downstream from the presumed start of translation were used for further study. Mutations in the spoIII and spoIIIA loci block sporulation at stage III and prevent activation of σ^I and hence σ^K (3, 10, 12, 14, 15); the engulfed prespores of spoIIIA mutants have been reported to be unstable (10, 16).

**Compartmentalization of gene expression is gradually lost during sporulation of spoIIA and spoIIIJ mutants.** The following questions arise for spoIIA and spoIII: do the mutations directly affect the compartmentalization of σ^I and σ^K activity, or do they indirectly influence the process, because they block sporulation at the stage of engulfment, and the mutant prespore is unstable and breaks down, resulting in loss of compartmentalization? To distinguish between these possibilities, compartmentalization of gene expression during sporulation was monitored by observing expression of spoIIQ-gfp (directed by σ^I) and spoIID-gfp (directed by σ^K). We reasoned that if the primary effect of spoIIA and spoIIIJ mutations is on compartmentalization, then we should be able to observe impaired compartmentalization at the earliest times of green fluorescent protein (GFP) expression and that the extent of impairment should not increase with time during sporulation. In contrast, if loss of compartmentalization was a consequence of an engulfment or other defect, then GFP expression should initially be compartmentalized; bacteria displaying uncompartmentalized expression should appear only after they reach the impaired stage. It is thought that σ^I and σ^K continue to be active in the absence of σ^I and σ^K activity (7), so that either mechanism for the breakdown in compartmentalization would result in β-galactosidase activity with the sacB/SacY system. The sacB/SacY system was used to analyze populations and not single cells and so did not satisfactorily distinguish between the two possibilities; expression of gfp was found to be more suitable for analyzing single cells.

Strains with spoIIQ-gfp and spoIID-gfp transcriptional fusions were induced to sporulate in modified Schaeffer’s sporulation medium (11). They were examined by fluorescence microscopy at various times after the start of spore formation, defined as the end of exponential growth. Conditions for microscopy were essentially as previously described (5, 13, 18).

When GFP fluorescence was first detected, about 3 h after the end of exponential growth, it was almost completely compartmentalized in the spoIIA and spoIIIJ mutants as well as the spo^+ strain. This observation applies to both σ^I-directed (spoIIQ-gfp) and σ^K-directed (spoIID-gfp) fusions. Expression remained largely compartmentalized for several hours (Table 1). However, by 8 to 10 h after the start of sporulation, fluorescence became uncompartmentalized in an increasing proportion of the mutant bacteria (Table 1). The transition from compartmentalized to uncompartmentalized expression was observed consistently for the spoIIA and spoIIIJ mutants, although the extent of the breakdown in compartmentalized expression varied from experiment to experiment (data not shown).

In the sporulation system used, there is a substantial period, about 3 h, between the end of exponential growth and the formation of the spore septum, and by 5 h after the end of exponential growth, 86 to 100% of the GFP-expressing population were at stage II of sporulation. Cultures were examined in detail 7 h after the end of exponential growth, before there was significant breakdown in compartmentalization. Engulfment was complete in about 50% of the GFP-expressing bacteria of the spoIIAB and spoIIIJ strains and in a substantial portion of the spoIIAB strain (Table 2) (in other experiments, that portion also reached 50%). Similar results were obtained with spoIIIJ-gfp (σ^K-directed) and spoIID-gfp (σ^K-directed) fusions. Thus, completion of engulfment occurred well before substantial numbers of mutant bacteria displayed uncompartmentalized expression. It is inferred that the effect of the spoIIAB and spoIIIJ mutations on compartmentalization was most likely a consequence of the instability of the engulfed prespore that led to mixing of mother cell and prespore cytoplasm. This effect contrasts with the loss of compartmentalization caused by insertional inactivation of spoIIIJ, where the defect occurs at stage II and is most likely the consequence of a small pore in the septum permitting small proteins such as sigma factors and GFP to cross the septum (17; D. Hilbert, V. Chary, and P. Piggot, unpublished obser-

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>σ factor</th>
<th>Proportion (%) of GFP-expressing bacteria displaying uncompartmentalized expression at time (h) after the end of exponential growth</th>
</tr>
</thead>
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<tr>
<td>SL10280</td>
<td>+</td>
<td>σ^F</td>
<td>0 0 0 1 0 1</td>
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<tr>
<td>SL10271</td>
<td>spoIIAAB</td>
<td>σ^F</td>
<td>0 0 0 3 21 31</td>
</tr>
<tr>
<td>SL10253</td>
<td>spoIII</td>
<td>σ^F</td>
<td>0 0 0 0 5 23</td>
</tr>
<tr>
<td>SL11371</td>
<td>+</td>
<td>σ^E</td>
<td>0 0 0 0 0 0</td>
</tr>
<tr>
<td>SL10307</td>
<td>spoIIAAB</td>
<td>σ^E</td>
<td>0 0 0 0 24 62</td>
</tr>
<tr>
<td>SL10319</td>
<td>spoIIIJ</td>
<td>σ^E</td>
<td>1 0 2 5 14 50</td>
</tr>
</tbody>
</table>

^σ^I factor directing gfp transcription; σ^K activity was detected with a spoIIQ-gfp fusion, and σ^K activity was detected with a spoIID-gfp fusion.

^b At least 40% of bacteria were expressing GFP, except for SL10251 (5 h, 14%) and SL10280 (5 h, 38%). At least 200 GFP-expressing organisms were counted for each sample. Samples were stained with FM4-64 (Molecular Probes) to enhance visualization of sporulation septa (13).
TABLE 2. Stage of sporulation reached by bacteria displaying compartmentalized expression of αF- and αE-directed gfp fusions 7 h after the end of exponential growth

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>α factor</th>
<th>Proportion (%) of bacteria displaying compartmentalized GFP expression at the indicated stage</th>
</tr>
</thead>
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<tr>
<td>SL10280</td>
<td>+</td>
<td>αE</td>
<td>IIi, IIi-IIIi, IIIi</td>
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<tr>
<td>SL10271</td>
<td>spoIIIJ</td>
<td>αF</td>
<td>18, 17, 65</td>
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<td>spoIIIJ</td>
<td>αF</td>
<td>35, 40, 25</td>
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<tr>
<td>SL11371</td>
<td>+</td>
<td>αE</td>
<td>15, 18, 67</td>
</tr>
<tr>
<td>SL10307</td>
<td>spoIIIJ</td>
<td>αF</td>
<td>11, 38, 51</td>
</tr>
<tr>
<td>SL10319</td>
<td>spoIIIJ</td>
<td>αE</td>
<td>21, 23, 56</td>
</tr>
</tbody>
</table>

* At least 100 GFP-expressing organisms were counted for each sample; αF activity was detected with a spoIIIJ-gfp fusion, and αE activity was detected with a spoIIAB-gfp fusion.

The stages of sporulation are as follows: IIi, spore septum formed; IIi-IIIi, partial engulfment of the prespore by the mother cell; IIIi, complete engulfment (6, 10). In bacteria scored as having reached stage IIIi, the prespore had clearly detached from the mother cell (10), and the prespore was not detected by FM4-64 staining (13); the prespore was visualized by prespore- or mother cell-specific GFP expression.

Inactivation of the spoIIAB locus results in uncompartmentalized αF activity and blocks spore septum formation (1); the mutants display little αE activity and so would not be detected with the sacB/SacY system. The only mutations causing severe defects in compartmentalization that we detected in 30,000 Tn10 mutated screens, with a system requiring both αF and αE activity, were located in spoIIIJ (7), spoIIIJ, and spoIIIJ. The transposon insertion could have a polar effect on genes downstream in the spoIIIJ and spoIIIJ operons. We have not tested for this possibility. Disruption of the gene downstream of spoIIIJ does not affect spore formation (3, 12), so that spoIIIJ inactivation is the most likely cause of the effect described here. Nonpolar mutations in each of the eight genes in the spoIIIJ operon result in the same stage III sporulation phenotype (12, 15), suggesting that disruption of other genes in the operon would have the effect described here. Our results suggest that insertional inactivation of a very limited number of loci results in uncompartmentalized activities of both αF and αE.

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REFERENCES