Control of Initiation of Sporulation by Replication Initiation Genes in Bacillus subtilis

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Initiation of spore formation in Bacillus subtilis appears to depend on initiation of DNA replication. This regulation was first identified using a temperature-sensitive mutation in dnaB. We found that mutations in the replication initiation genes dnaA and dnaD also inhibit sporulation, indicating that inhibition of sporulation is triggered by general defects in the function of replication initiation proteins.

Under conditions of starvation and high cell density Bacillus subtilis can enter a developmental pathway that produces environmentally resistant endospores. Spore formation is characterized by an asymmetric division that results in two distinct cell types, the mother cell (larger cell) and forespore (smaller cell), each of which requires a chromosome and each of which has a distinct pattern of gene expression (29). Formation of the asymmetric septum requires phosphorylation and activation of the transcription factor Spo0A, a member of the response regulator protein family (17). Phosphate for Spo0A comes from the histidine protein kinases KinA, KinB, and KinC, which autophosphorylate. Unlike typical two-component systems, the kinases do not donate phosphate directly to Spo0A. Rather, phosphate is donated to the response regulator Spo0F, then from Spo0F to the phosphotransferase Spo0B, and, finally, from Spo0B to Spo0A (2, 7). The multiple steps of the phosphorelay allow for integration of multiple signals that modulate sporulation. In addition to environmental conditions, physiological conditions are known to modulate initiation of sporulation. These include the status of the tricarboxylic acid cycle (11) and chromosome integrity, which consists of chromosome organization (10, 24), DNA damage (8), and initiation of DNA replication (9). It is presumed that chromosome integrity is monitored to ensure that cells do not initiate sporulation unless both the mother cell and forespore receive a complete genome.

In B. subtilis, three genes are known to be required for initiation, but not elongation, of DNA replication: dnaA, dnaB, and dnaD (12, 20, 21). DnaA is the highly conserved DNA replication initiator in bacteria. It recognizes and binds specific sequences in the origin of DNA replication (14, 30). On the basis of a comparison with available sequences in genome databases, dnaB and dnaD homologues are found in other low-G+C-content gram-positive bacteria, Staphylococcus aureus, Enterococcus faecalis, Streptococcus pyogenes, and Streptococcus pneumoniae. In B. subtilis, DnaB and DnaD are implicated in primosome assembly (1).

Initiation of sporulation appears to depend on initiation of DNA replication. B. subtilis must initiate a new round of DNA replication under starvation conditions in order to initiate sporulation (3, 19). In these experiments, dnaB134 temperature-sensitive mutants were shifted to the restrictive temperature, which blocked new initiation while allowing ongoing rounds of replication to finish. Results from experiments where cells were synchronized, using dnaB134, prior to induction of sporulation suggest that there might be a limited window in the DNA replication cycle during which initiation of sporulation can occur (4, 18).

Inhibiting initiation of DNA replication appears to generate a signal that impinges on the phosphorelay and inhibits initiation of sporulation (9). When a dnaB19 mutant is induced to sporulate at the restrictive temperature, Spo0A-dependent gene expression is inhibited. This inhibition is observed immediately upon the shift to the nonpermissive temperature, even as elongation continues, indicating that initiation of replication or the functions of the initiation proteins are key to this regulation (9). A mutant allele of spo0A, rvtA11, bypasses this regulatory coupling in a kic-dependent manner. It appears that the mutant Spo0A<sup>rvtA11</sup> protein can receive phosphate directly from KinC, eliminating the requirement for the phosphorelay (13, 15, 27). Thus, information regarding the status of DNA replication initiation impinges upon the phosphorelay. It is not known whether Spo0F, Spo0B, or the kinases KinA and KinB are targets of this regulation. The phosphatases known to modulate accumulation of Spo0A<sup>~P</sup> (RapA, RapB, and Spo0E) did not appear to be targets of this regulation (data not shown).

Inhibiting the elongation phase of DNA replication also causes a defect in initiation of sporulation by inhibiting the phosphorelay (8). This sporulation defect is largely recA dependent, in contrast to the recA-independent defect caused by inhibition of initiation of DNA replication (8, 9).

dnaA mutants and sporulation. We have now found that sporulation is inhibited in dnaA and dnaD mutants. This indicates that the inhibition of sporulation by inhibition of initiation of DNA replication is not specific to dnaB mutants. At the permissive temperature dnaAI mutants have a sporulation defect (S. Moriya, personal communication).

When the dnaAI mutant was grown in DS medium (nutrient broth [26]) at 30°C (permissive for growth), its sporulation frequency was reduced approximately 2,000-fold relative to that of the wild type (Table 1). We found a similar defect in the richer sporulation medium 2×SG (twice the nutrient broth of DS medium plus 0.1% glucose [16]) at 30°C. Interestingly, the sporulation defect of the dnaAI mutant was worse at 30°C than at 37°C. In DS medium at 37°C there was an ~100-fold decrease in the level of sporulation. We do not know the nature of this temperature effect.

The dnaAI sporulation defect was partially bypassed by the rvtA11 mutation in spo0A, and this bypass was dependent on kinC (Table 1). Whereas the dnaAI rvtA11 strain sporulated...
DNA replication was blocked after shifting of a dnaD23 mutant to the restrictive temperature, though the defect was less severe. At 42°C a dnaD23 mutant had a three- to fourfold decrease in the level of sporulation in DS medium (Table 2). This defect was completely bypassed by rvtA11 in a kinC-dependent manner. The dnaD23 rvtA11 double mutant was able to sporulate as well as, or better than, the wild type at the permissive temperature (32°C), sporulation of the dnaD23 mutant was similar to that of the wild-type strain (Table 2). Although 42°C is restrictive for growth of the dnaD23 mutant, inhibition of initiation of DNA replication may be leaky. This might explain why the sporulation defect in the dnaD23 mutant is less severe than that in the dnaB mutants. Attempts to observe whether the dnaD23 sporulation defect was more severe at higher temperatures were confounded by a decrease in sporulation efficiency in our wild-type strain at 42°C and above.

### TABLE 1. Sporulation of dnaA1 mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype*</th>
<th>% Sporulationa</th>
</tr>
</thead>
<tbody>
<tr>
<td>JW120</td>
<td>Wild type</td>
<td>76.6 (4.2)</td>
</tr>
<tr>
<td>JW116</td>
<td>dnaA1</td>
<td>0.04 (0.01)</td>
</tr>
<tr>
<td>JW114</td>
<td>dnaA1 rvtA11</td>
<td>2.97 (0.6)</td>
</tr>
<tr>
<td>IRN367</td>
<td>dnaA1 rvtA11 kinC</td>
<td>0.05 (0.02)</td>
</tr>
</tbody>
</table>

* All strains are derivatives of the wild-type strain JH642 (trpC2 pheA1) (22).

** a All strains were derivatives of the wild-type strain JH642 (trpC2 pheA1) (22).

### TABLE 2. Sporulation of dnaD mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype*</th>
<th>% Sporulationa at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>32°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>42°C</td>
</tr>
<tr>
<td>IRN378</td>
<td>Wild type</td>
<td>66.6 (8.5)</td>
</tr>
<tr>
<td>IRN377</td>
<td>dnaD23</td>
<td>57.5 (13.1)</td>
</tr>
<tr>
<td>IRN385</td>
<td>dnaD23 rvtA11</td>
<td>51.4 (7.7)</td>
</tr>
<tr>
<td>IRN390</td>
<td>dnaD23 rvtA11 kinC</td>
<td>31.8 (14.9)</td>
</tr>
</tbody>
</table>

* All strains were derivatives of the wild-type strain JH642 (trpC2 pheA1) (22).

** a All strains were derivatives of the wild-type strain JH642 (trpC2 pheA1) (22).

### Notes

The sporulation defect of a spo0F spo0B double mutant in the rich SG medium (15). In the spo0F single mutant, Spo0B can probably siphon phosphate away from Spo0A (29). Using a transcriptional fusion of spoI IA to lacZ (23), a decrease in Spo0A-dependent gene expression was observed in the dnaD23 mutant at the nonpermissive temperature (Fig. 1). This defect was rescued by rvtA11, and that rescue was dependent on kinC (Fig. 1). We observed a mild temperature-sensitive defect in the sporulation of the wild-type strain at 42°C (Table 2) that was not present in the rvtA11 dnaD23 double mutant. This may explain the apparent overexpression of the spoI IA-lacZ fusion in the double mutant at 42°C (Fig. 1).

### Replication and sporulation

Bypassing the sporulation initiation block with rvtA11 in the dnaD23 mutant at the permissive temperature (Table 1) or the dnaD23 mutant at the nonpermissive temperature (Table 2) restored production of mature endospores. In contrast, when the dnaD19 rvtA11 mutant is induced to sporulate at the restrictive temperature, Spo0A-dependent gene expression is restored to wild-type levels or above but cell viability decreases to 0.01 to 0.1% of that of the wild type. Sporulation efficiency, Spo0A-dependent gene expression was decreased in the dnaD23 mutant at the nonpermissive temperature. Phosphorylated Spo0A activates transcription of spoI IA (29). Using a transcriptional fusion of spoI IA to lacZ (23), a decrease in Spo0A-dependent gene expression was observed in the dnaD23 mutant at the nonpermissive temperature (Fig. 1). This defect was rescued by rvtA11, and that rescue was dependent on kinC (Fig. 1). We observed a mild temperature-sensitive defect in the sporulation of the wild-type strain at 42°C (Table 2) that was not present in the rvtA11 dnaD23 double mutant. This may explain the apparent overexpression of the spoI IA-lacZ fusion in the double mutant at 42°C (Fig. 1).

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and elucidating the mechanism by which many organism appears to be coupled to key cell cycle events, developmental checkpoint. If so, then deleting the checkpoint genes ably monitor initiation of DNA replication to avoid the disas-
require a complete genome and there are very different pat-
itiation of the growth of dnaB19 mutant; pos-
and its effect on sporulation, p. 233–237.

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