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 *kinC*-dependent manner. It appears that the mutant Spo0A*rvtA11* 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–P (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 *dnaA* mutants have a sporulation defect (S. Moriya, personal communication).

When the *dnaA* 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 2xSG (twice the nutrient broth of DS medium plus 0.1% glucose [16]) at 30°C. Interestingly, the sporulation defect of the *dnaA1* 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 *dnaA1* sporulation defect was partially bypassed by the *rvtA11* mutation in *spo0A*, and this bypass was dependent on *kinC* (Table 1). Whereas the *dnaA1 rvtA11* strain sporulated...
~75-fold better than the dnaA1 strain, the dnaA1 rvtA11 kinC triple mutant sporulated as poorly as the dnaA1 single mutant (Table 1). Since this rvtA11 bypass was kinC dependent, it is unlikely that rvtA11 relieves a direct inhibition of Spo0A. Most likely, the primary target of inhibition is either Spo0F, Spo0B, or both of the primary kinases KinA and KinB. We also observed the partial bypass by rvtA11 and dependence on kinC in the richer 2×5G medium (data not shown).

The partial bypass of the sporulation defect in the dnaA1 mutant by rvtA11 may indicate that there is a target of inhibition other than the phosphorylation pathway. However, we speculate that the bypass is incomplete because the regulation inhibits only one of the phosphorelay proteins, either Spo0F or Spo0B or both KinA and KinB. rvtA11 only partly bypasses spo0B and spo0F single mutants but completely bypasses the sporulation defect of a spo0F spo0B double mutant in the rich 2×5G medium (15). In the spo0F single mutant, Spo0B can probably synthesize phosphatase away from Spo0A VTA1. In the spo0B mutant, Spo0F can compete with Spo0A VTA1 for access to KinC (15). A similar result probably occurs if either Spo0B or Spo0F (or KinA and KinB) is inhibited rather than missing.

**dnaD mutants and sporulation.** Initiation of sporulation was also inhibited when initiation of 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 nonpermissive temperature, whereas the dnaD23 rvtA11 kinC triple mutant had a sporulation defect similar to that of the dnaD23 single mutant (Table 2). 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 con founded by a decrease in sporulation efficiency in our wild-type strain at 42°C and above.

Similar to what occurred with sporulation at the nonpermis-

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**TABLE 1. Sporulation of dnaA1 mutants**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotypea</th>
<th>% Sporulationb</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 (tpc2 pheA1) (22).

**TABLE 2. Sporulation of dnaD mutants**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotypea</th>
<th>% Sporulationb at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>32°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 are derivatives of the wild-type strain JH642 (tpc2 pheA1) (22). In addition, all strains have a silent spectinomycin cassette insertion that is ~90% linked by transformation to spo0A (5, 15), a silent Tn917 insertion (chr::Tn917HU163 [mls] [25]) linked to dnaA1, and a spo0A-lacZ (cat) fusion integrated by single crossover at the spo0A locus (6). The following mutations were used: dnaA1 (21), rvtA11 (27), and kinC (15). The ΔkinC:Cam is a deletion and insertion that replaces codons 45 to 285 of kinC (428 codons total) with a kanamycin resistance cassette.

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FIG. 1. Expression of the Spo0A-dependent spo0A-lacZ fusion. Strains were grown in DS medium at 32°C and transferred to 42°C approximately 0.5 h before the onset of sporulation. Samples were collected at the indicated times for β-galactosidase assays. Time zero indicates sporulation onset, taken as the time at which the culture left exponential growth. Squares, wild type; circles, dnaD23 strain; diamonds, dnaD23 rvtA11 strain; triangles, dnaD23 rvtA11 kinC strain. β-Galactosidase-specific activity is expressed as the change in ΔA$_{600}$ per minute per milliliter of culture per unit of optical density at 600 nm times 1,000.
The initiation of DNA replication to sporulation should provide us and elucidating the mechanism by which initiation of DNA replication. The onset of development in should allow cells to initiate sporulation in the absence of developmental checkpoint. If so, then deleting the checkpoint genes abolibly monitor initiation of DNA replication to avoid the disarrays of gene expression in the compartments. Cells presum-mably the inhibition of initiation of DNA replication is leaky at 42°C. It is possible that the decrease in viability in the dnaB rva11 mutants upon sporulation is due to a more severe block in rep-iniation initiation caused by the dnaB mutations. Alternatively, DnaB might have a function in addition to its role in initiation of replication.

The mechanism that couples initiation of DNA replication to initiation of sporulation appears to sense general defects in the functions of the proteins required for initiation of DNA replication. The most severe defect was found in response to perturbing the function of either DnaA or DnaB. It is possible that both of these are required for a common step during initiation of DNA replication, perhaps in a single complex. It is unclear why the effect is so mild in the dnaD23 mutant; possibly the inhibition of initiation of DNA replication is leaky at 42°C. Previous work has implicated both DnaB and DnaD in primosome assembly (1). The cell may monitor the function of each protein separately. However, it seems more likely that the cell monitors either the function of a complex or the effect of each protein on the activity of a single target protein. This information may then be transduced by a signaling pathway and impinge on the phosphorylase. It will be of interest to identify the genes required to inhibit sporulation in response to defects in dnaA, dnaB, and dnaD.

During development, both the mother cell and the forespore require a complete genome and there are very different patterns of gene expression in the compartments. Cells presumably monitor initiation of DNA replication to avoid the disassembly of the replication apparatus under conditions where both cells do not receive a complete chromosome. The coupling between initiation of DNA replication and sporulation may be a developmental checkpoint. If so, then deleting the checkpoint genes should allow cells to initiate sporulation in the absence of initiation of DNA replication. The onset of development in many organisms appears to be coupled to key cell cycle events, and elucidating the mechanism by which B. subtilis couples initiation of DNA replication to sporulation should provide us with some general insights.

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REFERENCES