Initiation of Sporulation in B. subtilis Is Controlled by a Multicomponent Phosphorelay

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Summary

Stage 0 sporulation (spo0) mutants of Bacillus subtilis are defective in the signal transduction system initiating sporulation. Two of the products of these genes, Spo0A and Spo0F, are related to response regulator components of two-component regulatory systems used to control environmental responses in bacteria. The Spo0F response regulator was found to be the primary substrate for phosphorylation by the sporulation-specific protein kinase, KinA. Phosphorylated Spo0F was the phosphodonor for a phosphotransferase, Spo0B, which transferred the phosphate group to the second response regulator, the transcription regulatory protein Spo0A. This phosphorelay provides a mechanism for signal gathering from several protein kinases using Spo0F as a secondary messenger. These divergent signals are integrated through Spo0B phosphotransferase to activate the Spo0A transcription factor. This system provides for many levels of control to prevent capricious induction of sporulation.

Introduction

The nature of the regulatory switch that determines whether a microbial cell continues in vegetative growth or enters a developmental cycle remains a fundamental problem of developmental biology. Sporulating bacteria such as Bacillus subtilis are thought to be capable of initiating development only during a small window in the cell cycle (Mandelstam and Higgs, 1974). This responsive interval exists from the end of the prior cell division until just after a new round of DNA synthesis has commenced. Presumably the cell monitors the environment and its own metabolic state during this interval and, based upon these inputs, decides whether to continue cell division or initiate the developmental program of sporulation. The nature of these metabolic and environmental signals and their intercellular form have yet to be elucidated.

An approach to unraveling this mystery was provided by mutants that are permanently coupled to cell division and unable to initiate the development cycle: the stage 0 (spo0) sporulation mutants (Hoch, 1976). These mutants map in several loci including spo0A, spo0B, spo0E, spo0F, and spo0H. The key regulatory protein in this group seems to be Spo0A. The Spo0A protein is a transcription regulator that can serve either as a repressor or an activator of transcription, depending on the target gene (Strauch et al., 1990; Bird, Burbulys, Hoch, and Spiegelman, unpublished data).

Spo0A is a negative regulator of the abrB gene, which codes for the AbrB repressor or transition state regulator (Perego et al., 1988; Zuber and Losick, 1987). The AbrB protein is not a classical repressor but is a rather unique DNA-binding protein showing cooperative binding kinetics with its promoter targets (Strauch et al., 1989). AbrB binds to and prevents expression from promoters of genes that are activated at the onset of stationary phase, including some genes essential for sporulation, presumably by recognizing the secondary structure of the promoter since it has no apparent consensus binding sequence. AbrB has been implicated in the control of at least ten operons that are normally expressed at the end of exponential growth. AbrB is a repressor of some genes (e.g., spo0E), but for most of the genes it serves as a backup or "preventer" that does not allow expression of the genes in vegetative conditions, even if the normal controls wish to do so. Since the level of AbrB protein in the cell is negatively regulated by the Spo0A protein, the regulatory state of the Spo0A protein is amplified through AbrB. During vegetative growth AbrB levels are high and its client genes are repressed, while at the end of vegetative growth Spo0A represses AbrB synthesis, releasing these genes from the repressive effects of AbrB. Thus a major factor in gene expression of many genes activated during the onset of stationary phase and sporulation is release of repression by falling AbrB levels, which results from Spo0A repression of AbrB transcription.

This controlling role for Spo0A was first revealed by studies of a mutant Spo0A protein (Sol-I) that suppressed the requirement for the spo0B, spo0F, and spo0E gene products in sporulation. The suppression pattern was consistent with the hypothesis that these latter genes served to modify Spo0A from an inactive to an active form, although at the time the nature of the modification was unknown (Hoch et al., 1985). Sequencing studies subsequently revealed that the amino-terminal region of the Spo0A protein was homologous to several other regulatory proteins, e.g., OmpR (Ferrari et al., 1985b). These proteins are now known to be response regulator components of so-called two-component regulatory systems (Ronson et al., 1987). The other component of such systems is a protein kinase that activates the response regulator by phosphorylation upon stimulation by an environmental signal (Ninfa and Magasanik, 1986). The majority of response regulator proteins are transcription activators that stimulate transcription of a specific set of genes unique to each response regulator. Two-component regulatory systems of this type have been found for a large variety of environmentally responsive genetic systems in bacteria (reviewed in Stock et al., 1989b, 1990).

Although it seemed virtually certain that activation of the Spo0A protein occurred by phosphorylation in a manner similar to other response regulators, sequencing the
genes known to be responsible for SpoOA activation did not reveal a gene product with homology to transmitter kinases. The deduced products of the spoOB (Ferrari et al., 1985a; Bouvier et al., 1984) and spoOE genes (Perego and Hoch, 1987) did not show significant homology to any known proteins. The spoOF gene product was found to be a small protein entirely homologous to response regulators (Trach et al., 1985). The first transmitter kinase with effects on sporulation was discovered serendipitously as the product of the spoOII gene (Antoniewski et al., 1990). This protein, KinA, was shown to be a kinase capable of phosphorylating both SpoOF and Spo0A in vitro, although it was much less active on Spo0A (Perego et al., 1989). Thus, the mechanism of Spo0A phosphorylation was still unknown.

In this report we present evidence using purified components that Spo0A is phosphorylated by a phosphotransferase reaction catalyzed by the Spo0B protein. The phosphodonor in this reaction is phosphorylated SpoOF, which results from the activity of transmitter kinases. This sequential transfer of phosphate between four proteins is termed a multicomponent phosphorelay.

Results

In an earlier study it was found that the kinase, KinA, encoded by the spoOII gene was capable of phosphorylating both the SpoOF and Spo0A proteins (Perego et al., 1989). It was uncertain whether one or both of these proteins represented the true substrate for the kinase in vivo. Studies of the phosphorylation reaction between KinA and SpoOF and Spo0A were undertaken after purification of each of the components. In these experiments reaction mixtures with [γ-32P]ATP, kinase, and SpoOF or Spo0A proteins were separated on SDS–polyacrylamide gels, and the accumulation of label in the components was determined by autoradiography. An extensive analysis of the kinetics of this reaction (Burbulys, Hoch, and Grimshaw, unpublished data) is summarized in Table 1. SpoOF shows almost 60-fold lower $K_m$ with KinA than Spo0A, and a 60-fold higher $V_{max}$. The relative catalytic efficiency of KinA in vitro is 3450-fold higher with SpoOF than with Spo0A at equal concentrations. Thus the preferred or even exclusive substrate for KinA in the cell should be SpoOF.

The identity of the transmitter kinase responsible for phosphorylating Spo0A remained unknown. We considered two possibilities: Spo0A was phosphorylated by another, as yet undiscovered, kinase, or Spo0A needed to be modified by some effector molecule to become a suitable substrate for KinA. To test the latter possibility, we expressed and purified the product of the spo0B gene, which is involved in sporulation initiation, and assayed its effect on the phosphorylation of Spo0A by KinA. Figure 1 shows an autoradiograph of an SDS–polyacrylamide gel of the reaction products in this experiment. All the reactions contained KinA and [γ-32P]ATP. The kinase was highly active on SpoOF, while the phosphorylation of Spo0A or Spo0B was below detection. Mixing Spo0A with Spo0F or Spo0A with Spo0B did not result in phosphorylation of Spo0A or Spo0B. However, when Spo0F and Spo0B were incubated together, both Spo0F and Spo0B were labeled. If Spo0A was added to the mixture of Spo0F and Spo0B, it was also labeled and at the expense of label in Spo0F and Spo0B. A labeled band at the position of a dimer of Spo0B was also observed (Figure 1). On an HPLC sizing column Spo0B runs as a dimer. HPLC-purified Spo0B preparations used in these studies contain two minor bands at the position of dimer when analyzed on an SDS–polyacrylamide gel (Figure 2); we believe these bands to be partially denatured dimer that is active but does not dissociate in SDS. The results indicated that Spo0A could become highly phosphorylated by [γ-32P]ATP in the presence of KinA, Spo0B, and SpoOF.

Two possible mechanisms for these results were entertained: the labeled phosphate was transferred from Spo0F~P to Spo0A via Spo0B~P in a phosphotransferase reaction, or, since [γ-32P]ATP was present in all reactions, one of the components of the reaction stimulated KinA to effectively phosphorylate Spo0A. To determine if KinA played a role in the phosphorylation of Spo0B and Spo0A, the phosphorylated Spo0F protein was isolated away from KinA and [γ-32P]ATP by DEAE chromatography. Purified Spo0F~P was mixed with the other compo-

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (µM)</th>
<th>Relative Catalytic Efficiency ($V_{max}/K_m$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spo0A</td>
<td>23</td>
<td>1</td>
</tr>
<tr>
<td>Spo0F</td>
<td>0.4</td>
<td>3450</td>
</tr>
</tbody>
</table>

Table 1. Kinetic Constants for Phosphorylation of Spo0A and Spo0F by KinA

Figure 1. Phosphotransfer Reactions between the Spo0 Proteins

Reactions contained 1 µM KinA and 10 µM each of the proteins listed above each lane in the autoradiograph. Reaction conditions were as described in Experimental Procedures. Abbreviations: OA, Spo0A; OB, Spo0B; OF, Spo0F.
Sporulation Initiation Phosphorelay

Figure 2. Purified Components of the Phosphorelay Reaction
Coomassie blue-stained gel of the purified protein components. Each lane contains approximately 5-10 μg of purified protein.

Figure 3. Transfer of Phosphate from Spo0F−P to Spo0B
The leftmost lane contains approximately 0.4 nmol of purified Spo0F−P. The middle lane contains purified Spo0F−P and an equal molar amount of unphosphorylated Spo0B. The rightmost lane contains purified Spo0F−P and 0.04 nmol of KinA. The reactions were allowed to proceed for 5 min at room temperature.

Figure 4. Transfer of Phosphate from Spo0B to Spo0A and Spo0F
All lanes contain approximately 0.4 nmol of purified Spo0B−P and equal molar amounts of the unphosphorylated proteins indicated. The reactions were incubated at room temperature for 5 min.

Components of the reaction to determine if transfer of the labeled phosphate to Spo0B would occur in the absence of KinA (Figure 3). Spo0F−P was the donor for phosphotransfer to Spo0B with these two components alone (Figure 3). Addition of KinA with or without ATP had no effect on this reaction.

Purified Spo0B−P was obtained by DEAE chromatography of a reaction with KinA and Spo0F. The phosphate of Spo0B−P was transferred from Spo0B−P to Spo0A with no other added components (Figure 4). Spo0B−P also transferred labeled phosphate back to Spo0F when these two proteins were incubated alone. If Spo0B−P, Spo0F, and Spo0A were incubated together, the label was found almost exclusively in Spo0A−P. Addition of KinA and ATP had no effect on these reactions.

The conclusion from these studies was that Spo0B is a protein phosphotransferase that catalyses the transfer of phosphate from Spo0F−P to Spo0A. The question arose as to whether the phosphate bond to Spo0A generated by the Spo0B phosphotransferase was similar to that expected for the product of transmitter kinase action. The CheA transmitter kinase is known to form an acyl-phosphate on aspartic acid residue 56 of CheY (Sanders et al., 1989), which is one of three Asp residues forming an aspartate pocket in the molecule (Stock et al., 1989a). We have determined that KinA generates an acyl-phosphate with the equivalent Asp residue (Asp-54) of Spo0F (Burbuly, Hoch, and Whiteley, unpublished data). Site-directed mutations of the aspartic acid pocket (Asp-10, Asp-11, and Asp-56) of Spo0A were generated by conversion of the aspartic acid residues to asparagine, individually and in combination. The mutant Spo0A proteins were purified and used as substrates for phosphorylation by KinA (Figure 5).

Results of this analysis showed that Asp→Asn mutations of residues Asp-10 and Asp-56 rendered the Spo0A proteins inactive as substrates for KinA. The same mutation of Asp-11, on the other hand, diminished the activity of the protein in phosphorylation, but significant activity remained. Using these same proteins as substrates for the Spo0B phosphotransferase gave identical results (Figure 6). The Asp-11 mutant Spo0A was still active with Spo0B, although less so, and Asp-10 and Asp-56 mutant
proteins were inactive. Thus it can be concluded, tenta-
tively, that the product of Spo0B phosphotransferase on
Spo0A is an acyl-phosphate on the same aspartate resi-
due of Spo0A as would be found with a transmitter kinase.
This conclusion is consistent with the acid/base stability
properties of Spo0A~P. While not conclusive evidence, it
is possible to determine which type of residue in a protein
is phosphorylated by its stability to acid or base treatment.

It has been shown that acyl-phosphates such as phos-
phoaspartate are very labile at either pH extreme as well
as increasingly labile with increasing temperature (Kosh-
land, 1951). Phosphoramidates such as phosphohistidine,
phospholysine, and phosphoarginine are extremely acid
labile and relatively base stable (Fujitaki and Smith, 1984;
Hultquist, 1968), and O-linked phosphates such as phos-
phoserine and phosphothreonine are very base labile and
exceedingly acid stable (Martensen, 1984). The phos-
phate of Spo0A~P is labile to both acidic and basic condi-
tions, as are acyl-phosphates (Figure 7). This acyl-phos-
phate linkage is much more stable in Spo0A than in CheY,
which most likely reflects amino acid differences around
the aspartic acid pocket of the molecules. Spo0B~P was
found to be extremely sensitive to acidic conditions but
stable in basic conditions, which strongly suggests the
presence of an N-linked phosphate, probably His~P.

Discussion

The core of the signal transduction system for the initiation
of sporulation is a series of phosphotransfer reactions that
we have termed a phosphorelay (Figure 8). The phos-
phorelay is a significant variant of the two-component
stimulus–response mechanism used to control environ-
mental responses in bacteria (see reviews by Stock et al.,
Sporulation Initiation Phosphorelay

In their simpler forms such systems use a signal-transducing transmitter protein kinase to phosphorylate a response regulator; the latter protein in many systems is a transcriptional activator. In the phosphorelay, on the other hand, the phosphate group from one response regulator is transferred to a second response regulator by the Spo0B protein phosphotransferase. The Spo0B protein phosphotransferase has no homology to any other protein phosphotransferase. All the protein components of the phosphotransferase resemble transmitter kinases in the ultimate location and nature of the phosphate group transferred to the target response regulator. The phosphate of Spo0A~P has the properties of an acyl-phosphate in its acid and base stability, and mutations in Spo0A affect its ability to be phosphorylated by KinA or Spo0B. Thus the phosphotransferase reaction of Spo0B closely resembles the phosphotransfer reaction of transmitter kinases.

The phosphorelay described here is reminiscent of the chain of phosphorylatable proteins that comprise the phosphoenolpyruvate:sugar phosphotransferase (PTS) system (Saier, 1989). In the PTS system a phosphoryl group derived from phosphoenolpyruvate is transferred between four proteins, with the enzyme III component acting as a protein phosphotransferase. All the protein components of the chain are phosphorylated on the imidazole ring of histidine, giving rise to high energy phosphoramidate bonds. A histidine phosphoramidate bond has been implicated in the autophosphorylation of two-component transmitter kinases (Hess et al., 1988). This phosphoryl group is transferred to the response regulator, where it is bonded to an aspartyl residue as a high energy mixed anhydride (Sander et al., 1989). The Spo0B protein phosphotransferase transfers the phosphoryl group from the aspartate of one response regulator to the aspartate of another response regulator through the formation of a phosphoramidate intermediate. Whether this is a phosphohistidine or another N-linked phosphoamino acid remains to be elucidated. However, the phosphoryl group of Spo0B~P was freely transferable back to Spo0F, suggesting that the energy of the phosphoramidate bond of Spo0B was equivalent to that of the acyl-phosphate of Spo0F.

Why does the cell bother to use a phosphorelay? The answer must lie in the seriousness of the commitment to shut down cell division and initiate sporulation. Compared with a simple environmental response like chemotaxis, the initiation of sporulation is a nontrivial event involving the ultimate activation of hundreds of genes and the expenditure of a great deal of energy. Furthermore, complex morphological structures are initiated within the cell, and the capricious reversal of the process may not be in the cell's best interest. The rationale for the phosphorelay mechanism is presented in Figure 9. This model has two major features: Spo0F acts as a secondary messenger gathering phosphate groups from several kinases and therefore serving as an accumulation point for a variety of stress inputs; and Spo0B serves not only to couple this input to the transcription regulator Spo0A but also as a further control point for regulatory action.

The major signal transduction event for sporulation in the laboratory occurs by the autophosphorylation of KinA, which is activated by unknown signals. kinA mutants are defective delayed for sporulation and sporulate more slowly than wild-type cells (Perego et al., 1989). The residual sporulation in kinA mutants is due to the action of KinB, which is a membrane-bound kinase that has general homology to transmitter kinases but is unrelated to any other known kinases (Trach and Hoch, unpublished data) and to some minor extent to the ComP kinase (Weinrauch et al., 1990). These kinases feed their signals to Spo0F, which assumes the role of a secondary messenger (Figure 9). KinA is an effective phosphatase of Spo0F~P when stimulated with high ATP levels, and this activity may form a control point for the phosphorelay. It is unknown whether other protein or effector molecules can stimulate this phosphatase activity of KinA. In addition to this phosphatase activity, the level of flux through the phosphorelay will be dependent on the stimulation of KinA and KinB by the level of the postulated unknown signals and by the activity of the Spo0E protein, which negatively regulates this pathway in an unknown manner (Perego and Hoch, unpublished data).
In our model the primary role of SpoOF^−P is to serve as a signal gatherer and substrate for the Spo0B phosphotransferase. We have no evidence that SpoOF or SpoOF^−P has any other function. If it has another role, it is dispensable for sporulation since sof mutations (mismatch mutations of SpoOA that bypass the phosphorelay by making the SpoOA protein a kinase target) can completely suppress an spoOF deletion (Spiegelman et al., 1990).

The Spo0B phosphotransferase is postulated to be a critical control point of the phosphorelay. Spo0B may be a phosphatase for Spo0A^−P, and this activity may be subject to external control not unlike that observed for P_II activation of the phosphatase activity of NR_II in the nitrogen regulation signal transduction pathway (Niño and Magasanik, 1986; Keener and Kustu, 1988). The Spo0B operon consists of two genes, spoOB and obg, the latter of which codes for a GTP-binding protein (Trach and Hoch, 1989). Obg is a G protein that is essential for growth and superficially resembles the Era protein of Escherichia coli (Ahnn et al., 1986; March et al., 1988) and the ras genes of eukaryotes. Like these other proteins, Obg is a GTPase (Fogder and Hoch, unpublished data). It is possible that Obg, like ras, stimulates growth in its GTP-bound form and simultaneously prevents sporulation by stimulating the phosphatase activity of Spo0B. It is known that lowering GTP levels by antibiotic (decoynine) inhibition of GTP synthase leads to the induction of sporulation (Zain-ul-Abedin et al., 1983). Perhaps the lowered GTP levels deactivate Obg. Alternatively, Obg may be a vector for communicating cell cycle signals to the sporulation initiation mechanism. Whether Obg itself or some other protein or small molecule controls the activity of the Spo0B phosphotransferase, we postulate that this enzyme must be an important control point in sporulation initiation.

The final product of this phosphorelay system is Spo0A^−P. This phosphorylated form of Spo0A is a transcriptional activator and repressor. Repression of the synthesis of the AbrB regulator occurs by Spo0A^−P binding to tandem copies of the "OA box," which is the 7 bp sequence TGNCGAA (Strauch et al., 1990). Nonphosphorylated Spo0A is effective in binding and preventing transcription leading to the induction of sporulation (Zain-ul-Abedin et al., 1983). Perhaps the lowered GTP levels deactivate Obg. Alternatively, Obg may be a vector for communicating cell cycle signals to the sporulation initiation mechanism. Whether Obg itself or some other protein or small molecule controls the activity of the Spo0B phosphotransferase, we postulate that this enzyme must be an important control point in sporulation initiation.

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**Experimental Procedures**

**Assay Conditions**

The phosphorylation assays were performed in a 40 μl reaction volume containing 100 mM Tris–HCl (pH 8.0), 0.1 mM EDTA, 1.5 mM MgCl_2, 1 mM CaCl_2, 100 mM KCl, 0.5 mM dithiothreitol, 20 μM [γ-^32P]ATP (6000 Ci/mmol; ICN Biomedicals, Inc.) and enough unlabeled ATP (neutralized with an equal molar amount of MgCl_2) to give a final ATP concentration of 100 μM. The reaction mixture was incubated at room temperature for 60 min and was stopped by the addition of 0.2 vol of 5x protein loading dye (250 mM Tris–HCl [pH 6.8], 10% glycerol, 0.02% bromophenol blue, 1% SDS, and 150 mM β-mercaptoethanol) and placed immediately on dry ice until just prior to loading onto a 15% SDS-polyacrylamide gel. Electrophoresis was carried out at a constant voltage of 200 V for approximately 3 hr. The lower portion of the gel was removed to reduce background radiation due to unincorporated [γ-^32P]ATP. The gel was dried and exposed for approximately 15 min to Kodak X-Omat RP film at room temperature with one intensifying screen.

**SpoOF^−P/Spo0B^−P Purification**

To demonstrate the path of phosphotransfer from KinA to Spo0A, it was necessary to purify the phosphorylated intermediates from the reaction mix. The phosphorylated form of SpoOF was purified as follows. A 10x standard assay containing only KinA and SpoOF was set up as described above. After incubation at room temperature for 60 min, the reaction was diluted with 5 vol of 0.1 M Tris–HCl (pH 8.0), 0.1 mM EDTA, 1 mM MgCl_2, 10 mM KCl, 7 mM β-mercaptoethanol, and 20 μM [γ-^32P]ATP containing no KCl and then applied to a 1 ml DEAE-Triacryl M column (IBF–Biotechnics) previously equilibrated with buffer A. The flowthrough was reapplied three times to ensure maximal protein binding to the column. The column was washed with three column volumes of buffer A, and 0.5 ml fractions were collected. It was then washed with three column volumes of buffer A containing 50 mM KCl, and 0.5 ml fractions were again collected. The SpoOF^−P eluted from the column at this point, while the KinA^−P remained bound. The counts in each fraction were determined, and the SpoOF^−P-containing fractions were pooled. This separation was later confirmed via SDS–PAGE followed by autoradiography. The purification of Spo0B^−P was done in a similar manner, with the Spo0B^−P eluting from the DEAE-Triacryl M column at 400 mM KCl. KinA^−P eluted at 500 mM KCl.

**SpoOA Phosphorylation Assays In the Presence of Purified SpoOF^−P or Spo0B^−P**

Purified SpoOF^−P or Spo0B^−P was mixed with equal molar amounts of SpoOF, Spo0B, and/or Spo0A and/or with 1/10th molar amounts of KinA as described in the legends to Figures 3 and 4. The reaction mixture was incubated for 60 min at 30°C. The reaction was stopped by the addition of 5 volumes of 12% trichloroacetic acid, followed by the addition of 100 μl of 5x SDS–PAGE sample buffer. The mixture was boiled for 5 min, and then 20 μl aliquots were run on an 8% SDS–PAGE gel. The gel was dried, exposed to X-Omat film, and developed.

**Expression of Spo0A and Mutant Spo0A Proteins**

The expression of the Spo0A protein was placed under the control of the inducible hybrid tac promoter of pKK223-3 (Pharmacia, Inc.). The 1.4 kb Hpal–HincII fragment containing the complete Spo0A open reading frame (Ferrari et al., 1985b) was subcloned into the Smal site of the expression vector. The correct orientation of the insert was verified by restriction analysis, and the plasmid was designated pH4555. E. coli strains carrying pH4555 grew poorly and segregated phenotypically distinct colonies at a high rate, even in a lac^− background. The segregants were found to result from a host mutation(s). To strengthen the repression of P_{lac} under noninducing conditions, the lac^− gene, available in cassette form on plasmid pMBR1850 (Amer- sham Corporation), was cloned into the unique NruI site in a nonessen-
To determine the acid/base stability of the phosphorylated residues of Spo0A proteins, the 850 bp EcoRI fragment from pJH4530 that contains the majority of the Spo0A coding sequence was subcloned into the EcoRI site of the replicative form of M13mp18. Oligonucleotide-directed in vitro mutagenesis was performed based on the method of Kunkel (1985) using the Bio-Rad Muta-Gen M13 in vitro-mutagenesis kit. The conserved aspartate residues at positions 10 and 11 (codon GAT) were altered to asparagines (codon AAT) either separately or together using a mixture of oligonucleotides of the following sequences: 5'-CCAGCCTCTGATATTCTGATATTCTGACGACAAACACTTAA-3'. The third conserved aspartate, at position 56 (GAT), was also changed to an asparagine (AAT) utilizing the oligonucleotide 5'-TGGAGGCGATATATGATGATAGGGCGGACGACG-3' as a primer on both the wild-type and the position 10 and 11 Asp→Aan double-mutant templates. Following verification of the mutations by DNA sequence analysis, the mutant EcoRI fragments were isolated from the replicative-form DNA and reintroduced to the EcoRI-cut vector fragment of pJH4530. Proper orientation of the EcoRI fragment was verified by restriction analysis, and the constructs were again sequenced to verify the desired mutation(s).

Purification of the wild-type and mutant Spo0A proteins will be described in another publication (Burbulys et al., unpublished). In all, five mutant proteins were purified: Spo0A-10, Spo0A-11, Spo0A-56, Spo0A-10+11, and Spo0A-10+11+56. Each of these mutants was tested in two separate phosphorylation assays. One was identical to the standard assay previously described with the substitution of the mutant Spo0A protein for the wild-type Spo0A. The autoradiograph was exposed to Kodak X-Omat RP film for 15 min at room temperature with one intensifying screen. The other test was performed by the direct phosphorylation of Spo0A or Spo0A mutants by KinA. For the direct phosphorylation of Spo0A by KinA, 2 μM KinA was used versus 1 μM KinA. In both experiments, the reactions were incubated at 37°C for 120 min and the autoradiograph was exposed for 120 min at ~70°C with one intensifying screen.

**Acid/Base Stability**

To determine the acid/base stability of the phosphorylated residues of KinA, Spo0F, Spo0B, and Spo0A, four identical reactions were performed containing all the proteins as described for the general assay. Each of these reactions was subjected to SDS-PAGE on separate but identical 15% gels. All four gels were fixed by soaking in 40% isopropanol for 30 min at room temperature. To the first gel, which served as the control, nothing further was done. The second gel was treated by soaking in 0.2 M HCl at 55°C for 45 min. The third gel was treated with 1.0 M NaOH at 55°C for 45 min, and the final gel was treated with 5% trichloroacetic acid at 90°C for 15 min. After the acid/base treatments, the gels were again soaked in 40% isopropanol to reduce the gels to the size of the control. The gels were then dried under a vacuum at 80°C and exposed to Kodak X-Omat RP film for approximately 30 min at room temperature with one intensifying screen.

**Expression of Spo0B**

The generation of the plasmid to express the Spo0B protein and the method of purification of Spo0B will be the subject of a later publication.

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