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Deactivation of the sporulation transcription factor Spo0A by the Spo0E protein phosphatase

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ABSTRACT **The *spo0E* locus of *Bacillus subtilis* codes for a negative regulator of sporulation that, when overproduced, represses sporulation and, if deleted, results in inappropriate timing of sporulation. The product of this locus, Spo0E, was purified and found to be a protein phosphatase, which specifically dephosphorylated the sporulation transcription factor Spo0A~*P*, converting it to an inactive form. Spo0E was not significantly active as a phosphatase on other components of the phosphorelay signal-transduction pathway producing Spo0A~*P*. A mutant Spo0E protein that results in sporulation deficiency was purified and found to be hyperactive as a phosphatase. The Spo0E phosphatase may provide an additional control point for environmental, metabolic, or cell-cycle regulation of phosphate flow in the phosphorelay. These results reinforce the concept that the phosphorelay is subject to a host of positive and negative signals for sporulation that are recognized and interpreted as a signal integration circuit that has the role of regulating the cellular level of active phosphorylated Spo0A sporulation transcription factor.**

The initiation of sporulation in *Bacillus subtilis* is under control of the Spo0A transcription factor; this protein is a member of the response regulator class of two-component systems (1) and is inactive unless phosphorylated (2). Environmental conditions conducive to sporulation promote the phosphorylation of Spo0A through a complex signal-transduction pathway, the phosphorelay (3) (Fig. 1). Signal recognition by either of two kinases, KinA or KinB, is thought to activate autophosphorylation of the kinases, resulting in phosphorylation of the Spo0F protein. Spo0F~*P* is a substrate for the Spo0B phosphotransferase, which, in turn, phosphorylates Spo0A. Spo0A~*P* acts both as a repressor of certain vegetative genes and as an activator of genes required for the initiation of sporulation. Because it appears that the level of Spo0A~*P* in the cell is the factor determining the decision to either grow or sporulate (4), it is important to judiciously control this level to reflect the environmental and metabolic potential monitored by the cell. Regulation of the phosphorelay pathway may be accomplished by either transcriptional control of the cellular concentration of the key components of the phosphorelay, Spo0F and Spo0A, or by control of the phosphate flux through the pathway to Spo0A (5–7). Although transcriptional controls have been well-documented, the activators of the kinases providing phosphate input into the pathway remain a mystery.

The *spo0E* gene is believed to code for a negative regulator of sporulation because overproduction of its gene product inhibits sporulation and deletion of the gene results in increased pressure to sporulate (8). A *spo0E* deletion mutation has no effect on the transcriptional control of the *kinA*, *spo0A*, *spo0F*, or *spo0B* genes that code for the phosphorelay components, suggesting that the target for Spo0E negative regulation is not transcription but rather the flow of phosphate in the phosphorelay. In this report, we present experiments showing that purified Spo0E is a phosphatase specific for Spo0A~*P*, and we suggest that Spo0E is a critical control point in the phosphorelay.

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MATERIALS AND METHODS

Bacterial Strains and Media. The λ DE3 lysogen of the *Escherichia coli* strain BL21 (F^-*ompT*$r_B^-m_B^-$) was purchased from Novagen along with plasmid pLysS. LB medium was prepared with 10 g of bactotryptone, 5 g of yeast extract from Difco, and 10 g of NaCl from Sigma per liter of culture. M9 salts were prepared with 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, and 1.0 g of NH_4Cl per liter of culture; the medium was supplemented with 100 μM $CaCl_2$/1 mM $MgSO_4$/0.2% glucose/0.2% Casamino acids.

Construction of Spo0E and Spo0E11 Expression Vectors. The wild-type *spo0E* locus was amplified from the chromosome via PCR with flanking oligonucleotides determined from the original sequence (9) and cloned into the *Nde* I site of pT7-7 to produce pMW67 (pT7-7 is a pUC19 derivative containing a T7 promoter and *E. coli* ribosome-binding site followed by a multicloning site derived from pT7-1) (10). The mutant spo0E11 expression vector was constructed by subcloning the 81-base *Stu* I–*Nsi* I fragment from plasmid pJM7139 (9), which included the mutant stop codon responsible for the truncation of the mutant protein, into these same sites on the wild-type expression vector pMW67 to produce pJK103. Both of these constructions code for complete proteins, containing no extra amino acids fused to the N terminus.

Purification of Spo0E and Spo0E11 Proteins. pMW67 and pJK103 were each transformed into the *E. coli* strain BL21 (DE3) which contained the pLysS plasmid. The pLysS plasmid encodes the T7 lysozyme gene, which inhibits T7 polymerase at low concentrations, whereas the DE3 λ lysogen contains the gene for T7 polymerase behind a *lacUV5* promoter (10). Four liters of cells were grown at 37°C in either M9 medium or LB medium, each containing ampicillin at 100 μg/ml and chloramphenicol at 30 μg/ml. When cultures reached an OD_{600} value of ≈0.5, induction was initiated with 1 mM isopropyl β-D-thiogalactopyranoside, and cells were allowed to continue growing for ≈2 hr. Cells were harvested by centrifugation, and cell pellets were frozen at −20°C overnight. Pellets were resuspended in ≈30 ml of buffer A (10 mM potassium phosphate, pH 6.8/10 mM KCl/1 mM dithiothreitol/1 mM fresh phenylmethylsulfonyl fluoride and briefly sonicated. Cell extracts were prepared by centrifugation at 27,000 × *g* for 30 min, and the pellets were discarded. ^{35}S-labeled "tracer" protein (10–30 × 10^6 cpm, prepared as outlined below) were added to the extract to enable location of Spo0E-containing fractions. All subsequent steps were monitored by scintillation counting of the fractions obtained. Whole-cell extract, 1040 mg of protein, was loaded on a 50-ml

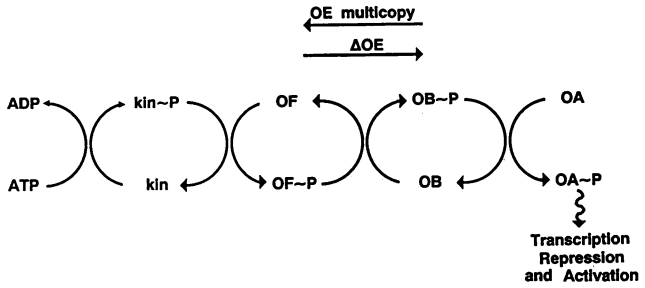

FIG. 1. The phosphorelay signal-transduction pathway. Two kinases, KinA and KinB, provide signal input into the phosphorelay. The apparent effects of Spo0E (OE) overproduction or deletion are indicated by the arrows. 0F, Spo0F; 0B, Spo0B; 0A, Spo0A.

hydroxyapatite column (2.6 × 10 cm column of Bio-Gel-HTP; Bio-Rad) equilibrated in 10 vol of buffer B (buffer A minus phenylmethylsulfonyl fluoride plus 1 mM ethylenediaminetetraacetic acid). The column was washed with 10 vol of buffer B overnight. Proteins were eluted in buffer C (10 mM potassium phosphate/1 M KCl/1 mM dithiothreitol/1 mM EDTA), and labeled fractions were pooled, dialyzed, and concentrated to ≈20 ml. The desalted sample (80 mg of protein) was then loaded on a 20-ml cation-exchange column (1.3 × 10 cm column of S-Sepharose; Pharmacia) equilibrated in 10 vol of buffer B, and labeled protein was collected in the flow-through fraction. Fractions were pooled, dialyzed, concentrated, and subsequently loaded (5 mg of protein) on a fast protein liquid chromatography Superdex-75 16/60 column (Pharmacia) equilibrated in buffer D (10 mM potassium phosphate, pH 7/100 mM KCl/1 mM dithiothreitol/1 mM EDTA), and labeled fractions were pooled and concentrated. Approximately 450 μg of 90% pure protein was obtained. Purity of the protein at various steps is illustrated in the SDS/tricine/18% polyacrylamide gel (11) shown in Fig. 2.

Preparation of ^{35}S-Labeled "Tracer" Proteins. BL21 (DE3)/pLysS/pMW67 or pJK103 was grown in LB medium with ampicillin at 100 μg/ml and chloramphenicol at 30 μg/ml as above; when the density reached an OD_{600} of ≈0.5, 10 ml of culture were removed, and Spo0E or Spo0E11 proteins, respectively, were specifically labeled as follows. Cells were centrifuged, and pellets were washed with 1× M9 salts. Washed cells were resuspended in medium containing M9 salts plus 0.2% glucose plus each of 18 amino acids (excluding methionine and cysteine) at 100 μg/ml. Cells were incubated with shaking for 30 min to allow exhaustion of endogenous methionine and cysteine, and then 1 mM isopropyl β-D-thiogalactopyranoside was added to induce T7 polymerase production. After a 30-min induction rifampicin at 200 μg/ml was added to inhibit the host RNA polymerase, and cells were incubated for another 30 min. At this time 100 μCi of ^{35}S-labeled L-methionine and L-cysteine (Tran^{35}S label; ICN; 1 Ci = 37 GBq) were added for a 5-min labeling, and cells were harvested by centrifugation. Cell pellets were frozen overnight at −20°C.

N-Terminal Sequencing. N-Terminal sequencing of the purified wild-type protein was done by Kerry Guinn in the Protein Sequencing Facility at this Institute, and the amino acid sequence Gly-Gly-Ser-Ser-Glu-Gln-Glu was obtained, corresponding to the first 7 amino acids of Spo0E. The initial methionine amino acid was not detected.

Phosphorelay Conditions. Phosphorelay assays were done in 50 mM [*N*-(2-hydroxyethyl)piperazine-*N*'-(3-propanesulfonic acid)] (EPPS), pH 8.5/20 mM $MgCl_2$/125 μM EDTA/5% glycerol/0.1% gelatin, pH 7.0/200 μM ATP in a 40-μl reaction volume at room temperature. Approximately 30 μCi of γ-labeled ATP (>7000 Ci/mmol; ICN) were added per reaction. Unless otherwise indicated, standard protein concentrations and incubation times are as follows. KinA (1.5 μM) was incubated with ATP for 15 min, and then 9.9 μM Spo0F/0.4 μM Spo0B/9.4 μM Spo0A was added for a 45-min incubation. After Spo0E addition (see figure legends for amounts) and an additional 15-min incubation, reactions were quenched by addition of 10 μl of concentrated loading buffer (250 mM Tris, pH 6.8/2 mM dithiothreitol/10% SDS/0.5% bromophenol blue/50% glycerol) and run on SDS/glycine/15% polyacrylamide gels (12) at 20 mA. In kinetic experiments, a 300-μl master reaction mix was prepared by using the standard reaction conditions indicated above. At time = 0, 1 μM Spo0E or Spo0E11 was added, and 40-μl samples were removed at the indicated times. Samples were immediately quenched by adding them to 10 μl of concentrated loading buffer and run on a SDS/glycine/15% polyacrylamide gel as above.

Unit Definition. One unit of activity is defined as that amount of Spo0E required to dephosphorylate 1 pmol of Spo0A~*P* in the standard reaction conditions in 1 min (assuming 100% of the Spo0A is phosphorylated at the beginning of the assay).

Preparation and Purification of ^{32}P-Labeled Spo0A~*P*. Spo0A was phosphorylated in a 2-ml reaction volume containing 3 μM KinA, 34 μM Spo0A, 50 mM EPPS (pH 8.5), 0.1 mM EDTA, and 5% (vol/vol) glycerol. The reaction was initiated by addition of 60 μCi [γ-^{32}P]ATP and enough unlabeled ATP to give a final ATP concentration of 1 mM. The mixture was incubated at 37°C for 4 hr and diluted with an equal volume of reaction buffer. The sample was loaded onto a Pharmacia fast protein liquid chromatography Mono Q HR 5/5 column equilibrated in 20 mM Tris·HCl, pH 8.3/10 mM KCl/1 mM EDTA/1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride.

The column was washed with 10 ml of loading buffer followed by a linear gradient from 10 mM KCl to 1 M KCl. Each fraction was analyzed by SDS/15% PAGE and by autoradiography. Under these conditions, KinA, Spo0A, and Spo0A~*P* were separated from one another. The Spo0A~*P*-containing fractions were pooled, concentrated to 1 ml, and immediately loaded onto a 120-ml Sephacryl S-200 column equilibrated in 25 mM Tris·HCl, pH 7.5/150 mM KCl. Fractions were analyzed by SDS/15% PAGE and autoradiography. Spo0A~*P*-containing fractions were pooled, concentrated by means of Centricon-3 ultrafiltration, and stored at −20°C until use.

RESULTS

Spo0E Specifically Decreases Spo0A~*P* Formation by the Phosphorelay System. Earlier studies with *spo0E* mutants showed no effect on the transcription of the genes for the components of the phosphorelay, suggesting that Spo0E may affect the flow of phosphate in the phosphorelay (8). The possibility that Spo0E interacted with one or more components of the phosphorelay was tested in a purified system *in vitro*. The Spo0E protein was produced using a T7 expression system in *E. coli*, and because the level of expression of Spo0E was low, it was purified by a radioactive tracer method (see *Materials and Methods*). The purity of the fractions obtained during this purification is illustrated in Fig. 2. Identity of the purified protein as Spo0E was confirmed via N-terminal sequence analysis of the protein from the Superdex-75 column; the first 7 amino acids conformed exactly to the amino acids predicted from the DNA sequence (9).

To ascertain whether Spo0E affected the reactions of the phosphorelay, the purified protein was added to a complete phosphorelay reaction. KinA was preincubated with [γ-^{32}P]-ATP for 15 min followed by the addition of the remaining components of the phosphorelay (Spo0F, Spo0B, Spo0A). After a 45-min incubation, increasing amounts of purified

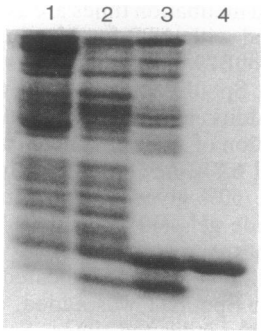

FIG. 2. Purification of wild-type Spo0E protein. Approximately 5 μg of protein was loaded in each lane. Lanes: 1, whole-cell extract; 2, 1 M KCl eluant of hydroxyapatite column; 3, S-Sepharose flow-through fraction; 4, Superdex-75 sizing column fractions.

Spo0E protein were added, and the reaction was allowed to proceed for 15 min, whereupon the reactions were stopped by adding loading buffer containing SDS (see *Materials and Methods*). The quenched reaction mix was subjected to electrophoresis in a SDS/polyacrylamide denaturing gel. The autoradiograph of this gel (Fig. 3) shows a dramatic decrease in the amount of labeled Spo0A~*P* correlating with the addition of the Spo0E protein. Little, if any, decrease in the amount of Spo0F~*P* was evident.

Spo0E Is a Specific Phosphatase of Spo0A. The overall effect of Spo0E addition to the prelabeled phosphorelay components was a decrease in the phosphorylation level of Spo0A. This effect could be either due to a specific dephosphorylation of Spo0A~*P* by Spo0E, by Spo0E stimulating another phosphorelay component to dephosphorylate Spo0A~*P*, or by specific inhibition or dephosphorylation of other components of the phosphorelay. To distinguish among these possibilities, purified radiolabeled Spo0A~*P* was incubated in the presence and absence of purified Spo0E protein. A decrease in the level of Spo0A~*P* was observed in the presence of Spo0E, showing that Spo0E alone can act as an effective phosphatase of Spo0A~*P* (Fig. 4). Because the dephosphorylation of Spo0A~*P* in the purified system did not appear to be as efficient as in the full phosphorelay, other components of the phosphorelay were added to the reaction to see whether the dephosphorylation rate could be enhanced. Spo0B did not affect the rate of dephosphorylation (Fig. 4, lane 3). Similarly, KinA and Spo0F had no effect in the purified system (data not shown).

The possible effects of Spo0E on the other components of the phosphorelay were tested in a build-up experiment as

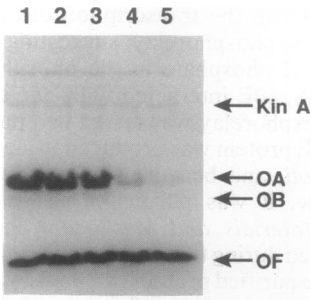

FIG. 3. Spo0E reduces Spo0A~*P* (0A) levels in the presence of the complete phosphorelay. All reactions contained the complete phosphorelay as defined in text. Protein concentrations are as in standard assay conditions. After a 15-min preloading of the KinA protein with the labeled ATP followed by a 45-min incubation with the remaining phosphorelay components, various amounts of Spo0E were added for a 15-min incubation. Lanes: 1, no Spo0E; 2, 206 nM Spo0E; 3, 412 nM Spo0E; 4, 825 nM Spo0E; and 5, 1.65 μM final concentration of Spo0E protein. 0B, Spo0B; 0F, Spo0F.

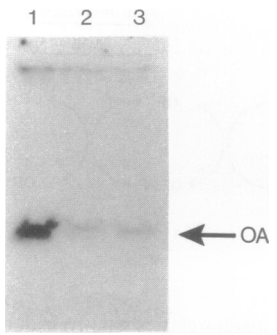

FIG. 4. Spo0E alone dephosphorylates Spo0A~*P*. All reactions contained purified ^{32}P-labeled Spo0A~*P* incubated in the standard reaction buffer for 30 min. Lanes: 1, labeled Spo0A~*P* (0A) alone; 2, labeled Spo0A~*P* plus 0.75 μM Spo0E; 3, labeled Spo0A~*P* plus 0.75 μM Spo0E plus 0.4 μM Spo0B.

follows. Pairs of reactions were run containing only the first component, the first two, the first three, or all four components of the phosphorelay and allowed to equilibrate with [γ-^{32}P]ATP for a total of 1 hr under the standard reaction conditions. Purified Spo0E protein was added to one of each pair for a 15-min incubation before electrophoresis. The results of these experiments showed that Spo0E had no phosphatase activity toward KinA when it was the sole phosphorelay component in the reaction (Fig. 5, lane 1 vs. lane 2). Similarly, addition of Spo0E protein did not result in dephosphorylation of Spo0F (Fig. 5, lane 3 vs. lane 4). When Spo0B was added to the reaction, no specific dephosphorylation of this protein was noted either (Fig. 5, lane 5 vs. lane 6). Finally, when Spo0A was added, the phosphatase activity of Spo0E was observed (Fig. 5, lane 7 vs. lane 8). Thus, the overall reduction in Spo0A~*P* levels seen upon Spo0E addition to the complete phosphorelay is apparently due to Spo0E-mediated direct dephosphorylation of the response regulator Spo0A~*P*.

Mutant Spo0E Responsible for Spo$^-$ Phenotype Has an Enhanced Phosphatase Activity. The original Spo$^-$ mutations of the *spo0E* locus were shown to be nonsense mutations giving rise to C-terminal truncations of the wild-type protein (9). These gene products were deduced to be constitutive or hyperactive repressors of sporulation. In this study the protein encoded by the gene containing one of these truncated alleles, *spo0E11*, which lacks 14 amino acids from the C terminus of the wild-type 85-amino acid protein, was cloned, expressed, and purified. This purified truncated protein migrated somewhat faster on a denaturing gel than the wild type, as would be expected from the shorter coding region. The rates of reaction for the mutant protein and the wild-type protein were compared in a kinetic experiment. First, the phosphorelay proteins were incubated with [γ-^{32}P]ATP for a total of 1 hr under the standard reaction

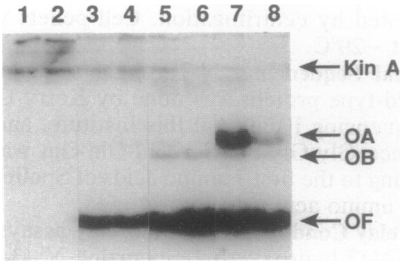

FIG. 5. Spo0E is not a phosphatase for other phosphorelay proteins. Lanes: 1–8, 1 μM KinA; 3–8, 1 μM KinA plus 10 μM Spo0F (0F); 5–8, 1 μM KinA plus 0.4 μM Spo0B (0B); 7–8, 1 μM KinA plus 10 μM Spo0A (0A). Spo0E protein was present at 0.75 μM in the reactions assayed in lanes 2, 4, 6, and 8.

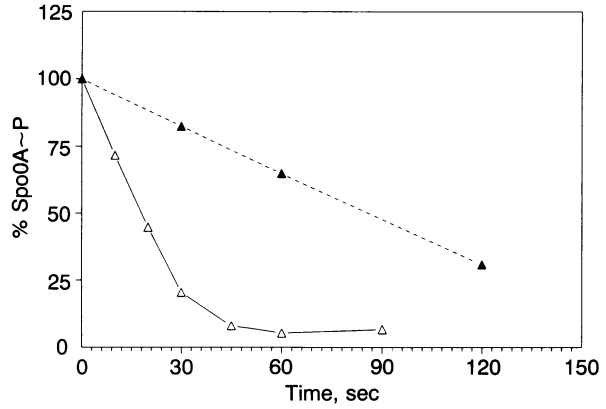

FIG. 6. Mutant Spo0E11 protein is hyperactive compared with Spo0E. Percent Spo0A~*P* per μM Spo0E or Spo0E11 remaining after addition of Spo0E or Spo0E11 protein to complete phosphorelay is as defined in text. Spo0E (1 μM) or Spo0E11 (1 μM) was added at time = 0. ▲, Spo0E; △, Spo0E11.

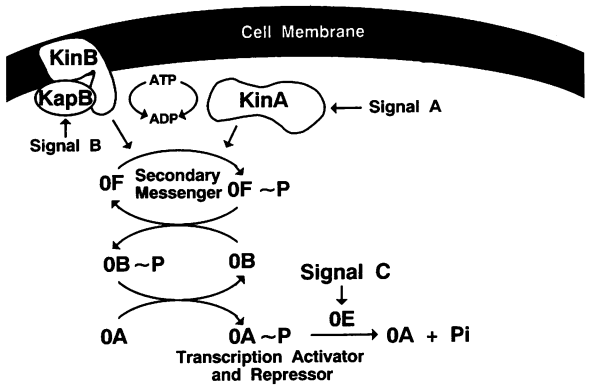

FIG. 7. Signal input into the phosphorelay. The core phosphorelay reactions are subject to activation or inhibition signals A, B, and C at the indicated steps. 0F, Spo0F; 0B, Spo0B; 0A, Spo0A; 0E, Spo0E.

conditions. Then Spo0E or Spo0E11 proteins were added, and rapid time points were taken. These samples were run on SDS/glycine/polyacrylamide gels, and the level of residual Spo0A~*P* was quantitated by densitometry of the resultant autoradiograph. Spo0E11 addition induced a rapid decrease in Spo0A~*P* levels (Fig. 6), reflecting a dramatic increase in the rate of dephosphorylation. Quantitation of these activities revealed that Spo0E11 is ≈5-fold more active as a phosphatase than the wild-type Spo0E, as indicated by the relative specific activities of the proteins, 2256 units/nmol and 452 units/nmol, respectively.

DISCUSSION

The studies reported here indicate that the Spo0E protein is a phosphoprotein phosphatase that cleaves the phosphate from the phosphoaspartate of the Spo0A transcription regulator, converting it from an active to an inactive form. The enzyme appears to have high specificity for Spo0A~*P* and little, if any, activity toward Spo0F~*P* or other components of the phosphorelay. Discovery of this enzymatic activity resolves the enigmatic negative regulator role of Spo0E in sporulation and provides a rational basis for explanation of the genetics of the *spo0E* locus.

Overproduction of the Spo0E protein was known to severely inhibit sporulation, whereas deletion of this locus caused premature sporulation and the accumulation of mutations in the phosphorelay (8). Because of the phosphatase activity of Spo0E, it should be expected that overproduction of this enzymatic activity would likely result in greatly decreased Spo0A~*P* levels and, therefore, lower probability of sporulation. Conversely, deletion of a regulatory phosphatase might be expected to result in abnormally elevated levels of Spo0A~*P* under conditions that might not be optimal for sporulation. The observation that a *spo0E* deletion has a hypersporulation phenotype eliminates the trivial explanation for the effects of phosphatase overproduction that it nonspecifically dephosphorylates response regulators and points to an important role for Spo0E in the overall control and timing of the phosphate flux in the phosphorelay. In addition, the inappropriate sporulation observed with this phenotype implies that Spo0E is involved in sensing some inhibitory signal for sporulation (Fig. 7).

The environmental or metabolic signals affecting Spo0E activity are obscure. However, a mutant Spo0E protein, Spo0E11, which suffered deletion at its C end, is a hyperactive phosphatase *in vitro*. The phenotype of the spo0E11 mutant is sporulation deficient, suggesting that it is hyperactive *in vivo* as well (9). Perhaps this mutant protein has lost a controlling site and is unable to respond to a signal that modulates Spo0E phosphatase activity. Because several mutants lacking portions of the C region of the protein have the same phenotype, this region of the molecule might be responsible for signal interpretation that may take the form of a discrete signal or may delineate a region interacting with another component of the phosphorelay.

There are many similarities between the enzymatic activities and roles of Spo0E in sporulation and of CheZ in the chemotaxis system of *E. coli*. Strains with mutations in *cheZ* continually tumble (13), and those that overproduce CheZ swim smoothly (14), indicating an increased or decreased level of CheY~*P*, respectively. Purified CheZ protein induces dephosphorylation of CheA kinase (15, 16) and acts as a specific phosphatase of CheY~*P* (17). In contrast to CheZ, which can dephosphorylate CheA~*P* (although poorly), Spo0E showed no phosphatase activity for KinA~*P* in any of our experiments. Although CheY~*P* is inherently very unstable due, presumably, to an autophosphatase activity (18), the activity of CheZ phosphatase is required to enhance the response time to signals generated by the chemotaxis signal-transduction pathway. Spo0A~*P* is very stable compared with CheY~*P* and may need to accumulate to levels required to repress or initiate transcription at the many promoters it regulates. Reversal of this process to take advantage of new opportunities for growth may require an active dephosphorylation mechanism responding to specific signals.

The initiation of sporulation results from the accumulation of a threshold level of Spo0A~*P*, which is produced only by the phosphorelay (3). Phosphate input into the phosphorelay originates from two kinases that are regulated by as-yet-undetermined factors. One kinase, KinA, is inhibited by cis-unsaturated fatty acids, and nothing else is known about functional regulation of either kinase (19). Both activators and inhibitors of these kinases may exist, and their accumulation to significant levels could result from several environmental and metabolic processes, each of which affects the decision to sporulate (i.e., the accumulation of Spo0A~*P*) in an incremental manner. Some signal input must also be transduced through Spo0E, which has a direct ability to influence Spo0A~*P* levels. Thus, the phosphorelay serves as the activated phosphate-producing core of a much larger signal-interpretation system, which we would like to think of as a signal integration circuit that is a more complex version of the original concept of Parkinson and Kofoid (20).

The major concept that the signal integration circuit is designed to convey is the cumulative influence of individual signals and events on the initiation of sporulation. This process is not controlled by a single event or signal, but rather

by the sum of a legion of signals that individually reflect the entire physiological and biochemical makeup of the cell. The kinases KinA and KinB act as the ultimate signal transducers, as they produce the phosphate group that activates Spo0A, but it is very likely that activation or deinhibition of these kinases is itself subordinate to a host of positive and negative signals interpreted by other complex signal-transduction pathways. Control of the activity of Spo0E phosphatase may also depend on signaling pathways different from those controlling the kinases. The end result of this complexity is that no individual signal or event is likely to cause sporulation in the absence of several other sporulation signals. Furthermore, it suggests that no individual environmental or metabolic signal is necessary or sufficient, but rather the cell integrates the available information from a variety of environmental influences.

Since the original identification of mutants in KinA as partially sporulation-defective *spoIIJ* mutants (21) that were simply slower to sporulate (22), it has been clear that other kinases and, therefore, other signal inputs could initiate sporulation. The sources of such signals are diverse and probably involve metabolic, environmental, and density-dependent signals (23) among others, that may activate two-component systems such as the *comAP* and *degSU* systems (24, 25) and kinases that are thought to directly phosphorylate Spo0F, such as KinA and KinB (26). Furthermore, it is known that the cell cycle (3), the state of DNA synthesis (27), and probably a host of other processes have effects that may influence the activity of other components of the phosphorelay but manifest as control of the Spo0A~*P* level. The identification of the Spo0E protein as a phosphatase of Spo0A~*P* and, therefore, an inactivator of sporulation-specific transcription gives another point of entry for control of the phosphorelay by these processes.

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