JOURNAL OF BACTERIOLOGY, Nov. 2001, p. 6573–6578 Vol. 183, No. 22
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.22.6573–6578.2001
Copyright © 2001, American Society for Microbiology. All Rights Reserved.

In Vivo Effects of Sporulation Kinases on Mutant Spo0A Proteins in *Bacillus subtilis*

JOHN D. QUISEL, WILLIAM F. BURKHOLDER, AND ALAN D. GROSSMAN*

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Received 15 August 2000/Accepted 31 August 2001

The phosphorylated form of the response regulator Spo0A (Spo0A~P) is required for the initiation of sporulation in *Bacillus subtilis*. Phosphate is transferred to Spo0A from at least four histidine kinases (KinA, KinB, KinC, and KinD) by a phosphotransfer pathway composed of Spo0F and Spo0B. Several mutations in *spo0A* allow initiation of sporulation in the absence of *spo0F* and *spo0B*, but the mechanisms by which these mutations allow bypass of *spo0F* and *spo0B* are not fully understood. We measured the ability of KinA, KinB, and KinC to activate sporulation of five *spo0A* mutants in the absence of Spo0F and Spo0B. We also determined the effect of Spo0E, a Spo0A~P-specific phosphatase, on sporulation of strains containing the *spo0A* mutations. Our results indicate that several of the mutations relax the specificity of Spo0A, allowing Spo0A to obtain phosphate from a broader group of phosphodonors. In the course of these experiments, we observed medium-dependent effects on the sporulation of different mutants. This led us to identify a small molecule, acetoin, that can stimulate sporulation of some *spo0A* mutants.

In response to starvation and high cell density, *Bacillus subtilis* can initiate a developmental pathway leading to the formation of dormant endospores (3, 13, 46). The transcription factor Spo0A is a critical regulator of the shift from exponential growth to sporulation. Spo0A is activated by phosphorylation {reviewed in references 3 and 13), and sporulation begins with accumulation of a threshold amount of phosphorylated Spo0A (Spo0A~P) (5).

Spo0A is a response regulator, but unlike most members of this protein family, Spo0A does not obtain phosphate directly from a histidine kinase. Spo0A phosphorylation is controlled by a phosphotransfer pathway, known as the phosphorelay, composed of Spo0F, Spo0B, and at least four histidine kinases, KinA, KinB, KinC, and KinD (1, 2, 20, 21, 24, 27, 36, 47). The kinases donate phosphate to Spo0F, a response regulator with no output domain (2, 36). The phosphate from Spo0F~P is transferred to Spo0B and finally from Spo0B~P to Spo0A (2). *spo0B* and *spo0F* null mutants do not sporulate, indicating that phosphorelay-independent phosphorylation of Spo0A is normally insufficient to activate sporulation.

A range of intra- and extracellular signals control sporulation by affecting the flow of phosphate through the phosphorelay. KinA, KinB, KinC, and KinD all have different roles in Spo0A activation and probably respond to distinct stimuli (7, 28, 47). KinA is the most important kinase for sporulation in rich sporulation media (28, 47). KinB is the most important kinase when cells are grown in glucose minimal medium (28). KinC and KinD contribute very little to sporulation under any condition but appear to regulate Spo0A during vegetative growth (20, 21, 24, 27).

Spo0A~P levels are also regulated by phosphatases. Spo0E is a Spo0A~P-specific phosphatase (33). Several phosphatases indirectly decrease Spo0A~P levels by dephosphorylating Spo0F~P. At least three members of the Rap (response regulator aspartyl-phosphate phosphatase) family of proteins dephosphorylate Spo0F~P and are regulated by small peptides involved in cell-cell signaling (19, 26, 35, 37, 42).

Several *spo0A* mutations allow initiation of sporulation in the absence of *spo0F* and *spo0B*. These mutations have been isolated from many different screens and are named *sof* and *sur0F* (suppressors of *spo0F*), *rvt* (revertant), *coi* (control of initiation), *sur0B* (suppressor of *spo0B*), and *sad* (*spo0A* dominant) (18, 23, 34, 40, 41, 45). These mutations also bypass many of the regulatory signals that feed into the phosphorelay (15, 17, 18, 34, 40, 41, 45).

It is not fully understood how these *spo0A* mutations permit sporulation in the absence of *spo0F* and *spo0B*. Genetic analysis has shown that the *spo0A(N12K)* (*sof-1*) and *spo0A(Q90R)* (*rvtA11*) mutations alter Spo0A so that it depends on KinC to support sporulation without Spo0F or Spo0B (24, 27). This has generally been interpreted as evidence that altering residues on Spo0A can permit a better interaction with certain alternate sources of phosphate (4, 45), such as KinC (24, 27). Several lines of evidence suggest that even wild-type Spo0A can obtain a low level of phosphate directly from KinC in the absence of Spo0F (20, 27).

We performed a genetic screen to identify mutations that would allow Spo0A to obtain phosphate specifically from KinA, KinB, or KinC. Despite many differences between our screen and previous screens, all of the *spo0A* mutations that we isolated had been identified previously as suppressors of *spo0F* or *spo0B* null mutations. To assess the in vivo effects of the *spo0A* mutations on the interactions between Spo0A and various phosphodonors, we measured the ability of KinA, KinB, and KinC to activate sporulation of each *spo0A* mutant in the absence of *spo0F* and *spo0B*. Our results indicate that many of the mutations in *spo0A* relax specificity, allowing Spo0A to

* Corresponding author. Mailing address: Department of Biology, Building 68–530, Massachusetts Institute of Technology, Cambridge, MA 02139. Phone: (617) 253-1515. Fax: (617) 253-2643. E-mail: adg@mit.edu.

TABLE 1. *B. subtilis* strains used

Strain	Genotype
BB1012	*spo0F*Δ*S spo0B*Δ*Pst kinA*::Tn*917*(*mls*) Δ*kinB*::*phl* Δ*kinC*::*kan spo0A(Q90R)-spc* Δ*spo0E*::*tet* Δ*pta*::*cat*
JH642	Wild type (*trpC2 pheA1*)
JQ33	*spo0F*Δ*S spo0B*Δ*Pst* Δ*kinC*::*kan kinA*::Tn*917*(*neo*) *kinB*::*pJQ8* (*Pspac-kinB-cat*)
JQ38	*spo0F*Δ*S spo0B*Δ*Pst* Δ*kinC*::*kan kinA*::Tn*917*(*neo*) *kinB*::*pJQ8* (*Pspac-kinB-cat*) *spo0A*$^+$*-spc*
JQ51	*spo0F*Δ*S spo0B*Δ*Pst kinA*::Tn*917*(*neo*) Δ*kinB*::*phl kinC*::*pJQ7* (*Pspac-kinC-cat*)
JQ53	*spo0F*Δ*S spo0B*Δ*Pst* Δ*kinC*::*kan* Δ*kinB*::*phl kinA*::*(Pspac-kinA-cat) spo0A*$^+$*-spc*
JQ61	*spo0F*Δ*S spo0B*Δ*Pst* Δ*kinC*::*kan* Δ*kinB*::*phl kinA*::*(Pspac-kinA-cat)*
JQ62	*spo0F*Δ*S spo0B*Δ*Pst kinA*::Tn*917*(*neo*) Δ*kinB*::*phl kinC*::*pJQ7 (Pspac-kinC-cat) spo0A*$^+$*-spc*
JQ63	*spo0F*Δ*S spo0B*Δ*Pst kinA*::Tn*917*(*neo*) Δ*kinB*::*phl kinC*::*pJQ7* (*Pspac-kinC-cat*) *spo0A*$^+$*-spc* Δ*spo0E*::*tet*
JQ64	*spo0F*Δ*S spo0B*Δ*Pst* Δ*kinC*::*kan kinA*::Tn*917*(*neo*) *kinB*::*pJQ8* (*Pspac-kinB-cat*) *spo0A*$^+$*-spc* Δ*spo0E*::*tet*
JQ65	*spo0F*Δ*S spo0B*Δ*Pst* Δ*kinC*::*kan* Δ*kinB*::*phl kinA*::*(Pspac-kinA-cat) spo0A*$^+$*-spc*Δ*spo0E*::*tet*
JQ82	*spo0F*Δ*S spo0B*Δ*Pst kinA*::Tn*917*(*neo*) Δ*kinB*::*phl kinC*::*pJQ7* (*Pspac-kinC-cat*) *spo0A(Q90R)-spc*
JQ95	*spo0F*Δ*S spo0B*Δ*Pst kinA*::Tn*917*(*neo*) Δ*kinB*::*phl kinC*::*pJQ7* (*Pspac-kinC-cat*) *spo0A*(*Q90R*)*-spc* Δ*spo0E*::*tet*
JQ97	*spo0F*Δ*S spo0B*Δ*Pst kinA*::Tn*917*(*mls*) Δ*kinB*::*phl* Δ*kinC*::*kan*
JQ134	*spo0F*Δ*S spo0B*Δ*Pst kinA*::Tn*917*(*mls*) Δ*kinB*::*phl* Δ*kinC*::*kan spo0A*$^+$*-spc* Δ*spo0E*::*tet*
JQ137	*spo0F*Δ*S spo0B*Δ*Pst kinA*::Tn*917*(*mls*) Δ*kinB*::*phl* Δ*kinC*::*kan spo0A(Q90R)-spc* Δ*spo0E*::*tet*
JQ214	*spo0F*Δ*S spo0B*Δ*Pst kinA*::Tn*917*(*mls*) Δ*kinB*::*phl* Δ*kinC*::*kan spo0A(N12K)-spc*
JQ219	*spo0F*Δ*S spo0B*Δ*Pst* Δ*kinC*::*kan* Δ*kinB*::*phl kinA*::*(Pspac-kinA-cat) spo0A(N12K)-spc*
JQ220	*spo0F*Δ*S spo0B*Δ*Pst* Δ*kinC*::*kan kinA*::Tn*917*(*neo*) *kinB*::*pJQ8* (*Pspac-kinB-cat*) *spo0A(N12K)-spc*
JQ221	*spo0F*Δ*S spo0B*Δ*Pst kinA*::Tn*917*(*neo*) Δ*kinB*::*phl kinC*::*pJQ7* (*Pspac-kinC-cat*) *spo0A(N12K)-spc*
JQ225	*spo0F*Δ*S spo0B*Δ*Pst kinA*::Tn*917*(*mls*) Δ*kinB*::*phl* Δ*kinC*::*kan spo0A(N12K)-spc* Δ*spo0E*::*tet*
JQ227	*spo0F*Δ*S spo0B*Δ*Pst* Δ*kinC*::*kan* Δ*kinB*::*phl kinA*::*(Pspac-kinA-cat) spo0A(D92Y)-spc*
JQ235	*spo0F*Δ*S spo0B*Δ*Pst* Δ*kinC*::*kan* Δ*kinB*::*phl kinA*::*(Pspac-kinA-cat) spo0A(E14A)-spc* Δ*spo0E*::*tet*
JQ236	*spo0F*Δ*S spo0B*Δ*Pst* Δ*kinC*::*kan* Δ*kinB*::*phl kinA*::*(Pspac-kinA-cat) spo0A(P60S)-spc*
JQ263	*spo0F*Δ*S spo0B*Δ*Pst kinA*::Tn*917*(*neo*) Δ*kinB*::*phl kinC*::*pJQ7* (*Pspac-kinC-cat*) *spo0A(N12K)-spc* Δ*spo0E*::*tet*
JQ363	*spo0F*Δ*S spo0B*Δ*Pst kinA*::Tn*917*(*mls*) Δ*kinB*::*phl* Δ*kinC*::*kan spo0A(E14A)-spc* Δ*spo0E*::*tet*
JQ367	*spo0F*Δ*S spo0B*Δ*Pst kinA*::Tn*917*(*mls*) Δ*kinB*::*phl* Δ*kinC*::*kan spo0A(E14A)-spc*
JQ370	*spo0F*Δ*S spo0B*Δ*Pst kinA*::Tn*917*(*neo*) Δ*kinB*::*phl kinC*::*pJQ7* (*Pspac-kinC-cat*) *spo0A(E14A)-spc* Δ*spo0E*::*tet*
JQ372	*spo0F*Δ*S spo0B*Δ*Pst* Δ*kinC*::*kan kinA*::Tn*917*(*neo*) *kinB*::*pJQ8 (Pspac-kinB-cat) spo0A(E14A)-spc* Δ*spo0E*::*tet*
JQ387	*spo0F*Δ*S spo0B*Δ*Pst kinA*::Tn*917*(*mls*) Δ*kinB*::*phl* Δ*kinC*::*kan spo0A(D92Y)-spc*
JQ388	*spo0F*Δ*S spo0B*Δ*Pst kinA*::Tn*917*(*mls*) Δ*kinB*::*phl* Δ*kinC*::*kan spo0A(D92Y)-spc* Δ*spo0E*::*tet*
JQ395	*spo0F*Δ*S spo0B*Δ*Pst kinA*::Tn*917*(*mls*) Δ*kinB*::*phl* Δ*kinC*::*kan spo0A(P60S)-spc*
JQ396	*spo0F*Δ*S spo0B*Δ*Pst kinA*::Tn*917*(*mls*) Δ*kinB*::*phl* Δ*kinC*::*kan spo0A(P60S)-spc* Δ*spo0E*::*tet*
JQ397	*spo0F*Δ*S spo0B*Δ*Pst kinA*::Tn*917*(*neo*) Δ*kinB*::*phl kinC*::*pJQ7 (Pspac-kinC-cat) spo0A(E14A)-spc*
JQ532	*spo0F*Δ*S spo0B*Δ*Pst kinA*::Tn*917*(*mls*) Δ*kinB*::*phl* Δ*kinC*::*kan spo0A(Q90R)-spc* Δ*spo0E*::*tet acoA*::*pLGSA700 (cat)*
JQ536	*spo0F*Δ*S spo0B*Δ*Pst* Δ*kinC*::*kan* Δ*kinB*::*phl kinA*::*(Pspac-kinA-cat) spo0A(Q90R)-spc*
JQ537	*spo0F*Δ*S spo0B*Δ*Pst* Δ*kinC*::*kan kinA*::Tn*917*(*neo*) *kinB*::*pJQ8 (Pspac-kinB-cat) spo0A(Q90R)-spc*
JQ538	*spo0F*Δ*S spo0B*Δ*Pst* Δ*kinC*::*kan kinA*::Tn*917*(*neo*) *kinB*::*pJQ8 (Pspac-kinB-cat) spo0A(P60S)-spc*
JQ539	*spo0F*Δ*S spo0B*Δ*Pst kinA*::Tn*917*(*neo*) Δ*kinB*::*phl kinC*::*pJQ7 (Pspac-kinC-cat) spo0A(P60S)-spc*
JQ540	*spo0F*Δ*S spo0B*Δ*Pst* Δ*kinC*::*kan kinA*::Tn*917*(*neo*) *kinB*::*pJQ8 (Pspac-kinB-cat) spo0A(D92Y)-spc*
JQ541	*spo0F*Δ*S spo0B*Δ*Pst kinA*::Tn*917*(*neo*) Δ*kinB*::*phl kinC*::*pJQ7 (Pspac-kinC-cat) spo0A(D92Y)-spc*

obtain phosphate from a broader range of phosphodonors. In the course of these experiments we observed medium-dependent effects on the sporulation of different mutants. This led us to identify a small molecule, acetoin, that can stimulate sporulation of strains carrying certain *spo0A* alleles.

MATERIALS AND METHODS

Media. Routine growth and maintenance of *B. subtilis* and *Escherichia coli* was done in Luria-Bertani medium (32). Cells were grown and allowed to sporulate in two different nutrient sporulation media: DS medium (a nutrient broth) (39) and 2×SG (29) (2×SG contains twice the nutrient broth of DS medium plus 0.1% glucose). Acetoin (Fluka, Buchs, Switzerland) was melted in an 80°C water bath, cooled to room temperature, and mixed with water to make a 500 mM stock. The stock was made fresh for each use and was diluted into DS medium to a final concentration of 20 mM prior to inoculation. Acetoin did not affect the exponential growth of cells but did permit more residual growth during the transition to stationary phase. IPTG (isopropyl-β-D-galactopyranoside) was used at a concentration of 1 mM.

Strains. The *B. subtilis* strains used (Table 1) are derivatives of JH642 (38) and contain the *trpC2* and *phe-1* mutations. Strains with *spo0B*Δ*Pst* are *phe*$^+$. Transformations and strain constructions were performed by standard methods (12). *Pspac-kinA* (16), *kinA*::Tn*917*(*mls*) and *kinA*::Tn*917*(*neo*) (essentially the *neo* cassette replacing *mls*) (16), *spo0B*Δ*Pst* (48), *spo0F*Δ*S* (23), and *acoA*::*pLGSA700* (14) have been previously described. *spo0A-spc* was constructed similarly to the previously described *spo0A-cat* (10), and the *spc* cassette is approximately 90% linked to *spo0A* by transformation.

pJQ4, carrying the Δ*kinB*::*phl* deletion-insertion, was constructed as follows. pNY113 (28), carrying the *kinB-kapB* operon, was digested with *Sac*II and *Cla*I to remove the kinase domain of *kinB*. The *phl* cassette was cut from pUC18-*ble*-1 (50) with *Hin*dIII and *Eco*RI. Both the digested pNY113 and the *phl* cassette were blunted and ligated to give pJQ4.

kinC deletion-insertions were constructed using the pLK24 (27) backbone. pLK24, containing *kinC*, was digested with *Pst*I and *Bgl*II to remove the kinase domain of *kinC* and ligated with either a *kan* cassette to make pJQ2 (Δ*kinC*::*kan*) or a *phl* cassette to make pJQ3 (Δ*kinC*::*phl*). The *kan* cassette was obtained by *Pst*I-*Bgl*II digestion of pDG792 (11) and the *phl* cassette was obtained by *Hin*dIII-*Eco*RI digestion of pUC18-*ble*-1.

pJQ7 (*Pspac-kinC*) and pJQ8 (*Pspac-kinB*) were constructed by amplifying a portion of the genes (+3 to +357 for *kinC*; −1 to +306 for *kinB* [+1 indicating the transcription start site]) and cloning into pDH88 (49), to place the gene fragments downstream of the LacI-repressible IPTG-inducible promoter *Pspac*. These plasmids were inserted by single crossover into the chromosome, disrupting the native locus and placing the intact kinase gene under the control of *Pspac*.

Sporulation assays. Cells were grown in DS or 2×SG medium at 37°C. Spores were assayed approximately 20 to 25 h after the end of exponential growth. Viable cells and spores were counted by plating before and after a heat treatment (80°C for 20 min) as previously described (27). Percent sporulation is calculated as 100 times the number of spores per milliliter divided by the number of viable cells per milliliter. Data presented are from representative experiments. Similar results were obtained in at least two and usually three or more independent experiments.

Mutagenesis. Six different strains, JQ33, JQ51, JQ61, JQ63, JQ64, and JQ65 (Table 1; see also Results), were mutagenized. Each contained loss-of-function

TABLE 2. *spo0A* mutations used

Allele names	Amino acid change	Reference(s)
spo0AN12K; sof1; sof103; sur0F1	Asparagine 12 to lysine	4, 23, 41, 45
spo0AE14A; sof3; sof115	Glutamate 14 to alanine	4, 45
spo0AP60S; sof4; sof118; coi-1	Proline 60 to serine	4, 34, 45
spo0AQ90R; rvtA11	Glutamine 90 to arginine	40
spo0AD92Y; sof5; sof114	Aspartate 92 to tyrosine	4, 45

mutations in *spo0F*, *spo0B*, and two of the three kinase genes (*kinA*, *kinB*, and *kinC*). The third kinase gene was fused to the LacI-repressible IPTG-inducible promoter *Pspac*. Three strains were *spo0E*$^+$ and three were *spo0E*$^-$. For mutagenesis, strains were grown to mid-exponential phase in Luria-Bertani medium, pelleted by brief centrifugation, and resuspended in 10 mM $(NH_4)_2SO_4$–5 mM K_2PO_4–50 mM MOPS (morpholinepropanesulfonic acid)-KOH, pH 7. Ethyl methanesulfonate (Sigma) was added (1%), and cells were incubated for 30 min at 37°C. Mutagenized cells were collected by centrifugation, washed in DS medium, inoculated into DS medium with 1 mM IPTG, and grown until 20 h after the end of exponential growth, at which point cells capable of sporulating should have done so. Cells were killed by heating at 80°C for 20 min, and the surviving spores were plated and allowed to grow on plates containing DS medium plus 1 mM IPTG. The unmutagenized parent strains sporulate extremely poorly. Mutations were back-crossed to determine linkage to *spo0A* (*spc* linked) or *Pspac* (*cat* linked).

***Pspac*(hy) mutation.** Mutations that were not linked to *spo0A* were tightly linked to the chloramphenicol resistance gene linked to *Pspac-kinC*. The integrated plasmid (containing *Pspac-kinC*) and nearby portions of *kinC* were rescued from the mutant and its parent by digesting total DNA from the mutant strains (using *Sph*I), ligating, and transforming into *E. coli*, selecting for Amp^r. Plasmid isolates were sequenced, and the mutation was identified as a G-to-T change at the −1 position of *Pspac* relative to the transcription start site (mapped in reference 49). This mutation, called *Pspac*(hy), raised the level of expression 10- to 20-fold over that from *Pspac* without affecting the fold induction by IPTG. As previously shown, overexpression of *kinC* permits sporulation in the absence of the phosphorelay (27). *Pspac*(hy) is a useful promoter for overexpression of a variety of genes of interest. Plasmids have been constructed for gene expression from *Pspac*(hy) either as a single crossover (pJQ43) or as a double crossover at the *amyE* locus (pPL82). Another mutation (A to G at +1) in *Pspac* that increases expression in the absence of Lac repressor has been described (22).

RESULTS AND DISCUSSION

Phosphorelay bypass mutations are identical to previously isolated mutations. We performed a genetic screen to identify mutations that would allow Spo0A to accept phosphate from KinA, KinB, or KinC in the absence of the phosphotransfer proteins, Spo0F and Spo0B. We constructed strains lacking *spo0F*, *spo0B*, and two of the three kinases. The remaining kinase was expressed from the LacI-repressible IPTG-inducible promoter, *Pspac*. The *Pspac* kinase expression constructs were all functional, since each could complement a null mutation of the corresponding kinase (data not shown). The strains expressing one kinase from *Pspac* and lacking the other two kinases, and *spo0F* and *spo0B*, were unable to sporulate. These strains (JQ33, JQ51, and JQ61) were mutagenized with ethyl methanesulfonate and then grown in DS medium supplemented with IPTG. Spores were selected by a heat treatment that killed nonsporulated cells. This procedure strongly selected for mutations that permitted sporulation in the absence of *spo0F* and *spo0B*. To identify weaker alleles, the screen was also performed in strains (JQ63, JQ64, and JQ65) null for *spo0E*, the gene coding for a phosphatase that can dephosphorylate Spo0A~P. This did not change the types of mutations isolated.

All of the mutations characterized were linked to *spo0A*, except for one which was in *Pspac* (see Materials and Methods). The *spo0A* gene from mutant cells was amplified by PCR, cloned, and sequenced to identify the mutations. All of the isolates carried mutations that were identical to those previously isolated as *sof*, *coi*, or *rvt* mutations (Table 2). Although we independently obtained some mutations 10 or more times, other previously identified mutations were never isolated. This selectivity has been observed previously and may depend upon the mutagen used (45). Nonetheless, there is overlap in the alleles isolated from different screens. This overlap suggests that there are very few single mutations that allow Spo0A to stimulate sporulation in the absence of *spo0F* and/or *spo0B*. We examined the ability of the five different *spo0A* alleles to support sporulation in the presence of KinA, KinB, or KinC in vivo.

Cells with *spo0A* mutations have various sensitivities to the sporulation kinases. The abilities of *kinA*, *kinB*, and *kinC*, expressed from *Pspac*, to stimulate sporulation of the various *spo0A* mutants was assessed in a Δ*spo0F* Δ*spo0B* background (Table 3). Wild-type Spo0A was not sufficiently activated by any of the kinases to allow sporulation. *spo0A(N12K)* and *spo0A(Q90R)* could be activated only by KinC, as has been described before when the kinases were expressed from their native promoters (24, 27). Sporulation of the *spo0A(N12K)* and *spo0A(Q90R)* strains was lower than reported previously because we used DS medium instead of 2×SG as the sporulation medium. This medium effect is discussed below. *spo0A (E14A)* could also be activated by KinC (Table 3).

spo0A(P60S) and *spo0A(D92Y)* permitted a moderate level of sporulation in the absence of the three kinases and the phosphorelay (Table 3). There was no further stimulation of sporulation in the presence of any of the three kinases. These data indicate that the *spo0A(P60S)* and *spo0A(D92Y)* mutations do not improve the ability of Spo0A to accept phosphate directly from KinA, KinB, or KinC, consistent with in vitro results (4). The *spo0A(P60S)* and *spo0A(D92Y)* gene products might obtain phosphate from another source. Alternatively, in the absence of the phosphorelay, they probably stimulate sporulation independently of phosphorylation (see below).

Cells with *spo0A* mutations exhibit differing sensitivities to the phosphatase Spo0E. Spo0E negatively regulates sporulation by directly dephosphorylating Spo0A~P (33). We tested the sensitivity of the different *spo0A* mutations to Spo0E in vivo. If *spo0E* has an effect on sporulation, then we can infer

TABLE 3. Effects of *kinA, kinB*, and *kinC* on sporulation of *spo0A* mutants

spo0A allele[a]	% Sporulation[b]			
	Pspac-kinA Δ*kinBC*	*Pspac-kinB* Δ*kinAC*	*Pspac-kinC* Δ*kinAB*	Δ*kinABC*
spo0A$^+$	$<1.3 \times 10^{-6}$	$<2.9 \times 10^{-6}$	1.0×10^{-6}	$<1.9 \times 10^{-6}$
spo0A(N12K)	3.6×10^{-6}	$<5.0 \times 10^{-6}$	1.9×10^{-3}	$<5.0 \times 10^{-6}$
spo0A(E14A)	ND	ND	1.5×10^{-3}	9.4×10^{-5}
spo0A(P60S)	3.1	2.2	4.4	4.7
spo0A(Q90R)	1.6×10^{-6}	1.8×10^{-5}	1.4×10^{-2}	ND
spo0A(D92Y)	0.10	0.20	0.36	0.13

[a] All strains are Δ*spo0F* Δ*spo0B* in addition to the indicated *spo0A* and kinase alleles.

[b] Strains were grown and allowed to sporulate in DS medium with 1 mM IPTG to induce expression from *Pspac*. Wild-type (JH642) cultures produced 50 to 80% spores. ND, not determined.

TABLE 4. Effects of *spo0E* on sporulation of *spo0A* mutants

Strains[a] (*spo0E*$^+$/Δ*spo0E*)	*spo0A* allele	Other genotype	% Sporulation[b]	
			spo0E$^+$	Δ*spo0E*
JQ62/JQ63	*spo0A*$^+$	*Pspac-kinC*	1.0×10^{-6}	3.0×10^{-4}
JQ221/JQ263	*spo0A(N12K)*	*Pspac-kinC*	2.0×10^{-3}	44
JQ397/JQ370	*spo0A(E14A)*	*Pspac-kinC*	1.5×10^{-3c}	64
JQ367/JQ363	*spo0A(E14A)*	Δ*kinC*	9.4×10^{-5c}	0.78
JQ395/JQ396	*spo0A(P60S)*	Δ*kinC*	2.4	1.6
JQ82/JQ95	*spo0A(Q90R)*	*Pspac-kinC*	7.6×10^{-2}	46
JQ387/JQ388	*spo0A(D92Y)*	Δ*kinC*	0.33	0.24

[a] All strains are Δ*kinA* Δ*kinB* Δ*spo0F* Δ*spo0B*. Strains carrying *spo0A(P60S)* and *spo0A(D92Y)* were unaffected by *kinC*, and therefore the assay was done in a Δ*kinC* background. *Pspac-kinC* was induced with 1 mM IPTG added when the culture was inoculated.

[b] Strains were grown in DS medium. Wild-type (JH642) cultures produced 50 to 80% spores.

[c] Data are from the experiment shown in Table 3.

that mutant Spo0A is phosphorylated in vivo. If *spo0E* has no effect, then either the mutant Spo0A is not effectively phosphorylated in vivo or it is resistant to dephosphorylation by Spo0E.

We found that wild-type *spo0A* and the *spo0A* mutants (N12K, Q90R, and E14A) that were responsive to one or more sporulation kinases (Table 3) were also sensitive to Spo0E (Table 4). *Pspac-kinC* was used to drive Spo0A phosphorylation in the absence of *kinA*, *kinB*, *spo0F*, and *spo0B*. Wild-type *spo0A* did not allow significant sporulation in the presence of *spo0E*$^+$, but in the Δ*spo0E* background it allowed a low level of sporulation (Table 4). Even in the absence of *spo0E*, wild-type Spo0A could not obtain sufficient phosphate from KinA or KinB to stimulate sporulation (data not shown). These results indicate that wild-type Spo0A can obtain a low level of phosphate from KinC and that Spo0E normally acts to remove this phosphate.

The absence of *spo0E* caused increased sporulation of *spo0A (N12K)*, *spo0A(E14A)*, and *spo0A(Q90R)* strains (Table 4), indicating that these altered forms of the Spo0A protein need to be phosphorylated to have significant activity in vivo and that they are substrates for the Spo0E phosphatase in vivo. These in vivo findings are consistent with results indicating that the phosphorylated forms of Spo0A(N12K) and Spo0A(E14A) are dephosphorylated by Spo0E in vitro as efficiently as wild-type Spo0A (4). Even though these mutant proteins are substrates for Spo0E, the *spo0A(N12K)* and *spo0A(E14A)* alleles are known to suppress the sporulation defect caused by the hyperactive *spo0E* mutation, *spo0E11* (34, 41, 45). Taken together, these results indicate that these Spo0A mutants can receive phosphate from sources other than Spo0B~P, overwhelming the hyperactive phosphatase produced in a *spo0E11* strain.

spo0A(E14A) can moderately activate sporulation in the absence of KinA, KinB, or KinC, but this residual sporulation was virtually abolished in the presence of *spo0E*$^+$ (Table 4). Therefore, the residual sporulation was due to phosphorylation of Spo0A(E14A) by another, unidentified phosphodonor. Given that *spo0A(E14A)* exhibits relaxed specificity for phosphodonors in the absence of *spo0E*, we asked if sporulation of a *spo0A(E14A)* Δ*spo0E* strain could be stimulated by KinA or KinB and found that KinA significantly stimulated sporulation (Table 5).

In contrast to mutants that were affected by one or another of the kinases and *spo0E*, sporulation of the *spo0A(P60S)* and *spo0A(D92Y)* strains was not significantly affected by *spo0E* (Table 4). This indicates that either these mutant *spo0A* gene products are not significantly phosphorylated in vivo in the absence of the phosphorelay or that they are resistant to dephosphorylation by Spo0E. Previous work showed that Spo0A (D92Y) is efficiently dephosphorylated by Spo0E in vitro (4). Therefore, we favor the interpretation that Spo0A(D92Y) and probably Spo0A(P60S) function independently of phosphorylation. They could do this by stabilizing interactions between Spo0A and RNA polymerase, as has been observed in vitro (4). Though these two mutant proteins appear to function in the absence of phosphorylation, they clearly function better when phosphorylated. Phosphorylation in vitro stimulates their ability to activate transcription (4). In vivo, these mutant proteins allow higher levels of sporulation in the presence of the phosphorelay than in its absence, indicating that activity in vivo is also stimulated by phosphorylation (45). Together, the in vivo and in vitro results are most consistent with the interpretation that, in vivo, these mutant forms of Spo0A do not obtain phosphate from sources other than the phosphorelay.

These experiments divide the *spo0A* alleles into two groups. The first group of *spo0A* alleles, *spo0A(N12K)*, *spo0A(E14A)*, and *spo0A(Q90R)*, code for proteins that can support sporulation in the absence of *spo0F* and *spo0B* by accepting phosphate directly from KinC. As seen in the absence of *spo0E*, *spo0A(E14A)* can also weakly accept phosphate from KinA and at least one unidentified phosphodonor. Sporulation of these *spo0A* mutant strains is highly sensitive to the phosphatase Spo0E. Consistent with these mutants having a relaxed specificity for alternative phosphodonors, in vitro data indicate that Spo0A(N12K) and Spo0A(E14A) can be phosphorylated directly by KinA more efficiently than wild-type Spo0A (4, 20). The second group of *spo0A* alleles, *spo0A(P60S)* and *spo0A (D92Y),* appear to support a modest level of sporulation in the absence of the phosphorelay without being phosphorylated.

Phosphorylation-dependent *spo0A* mutants are differentially stimulated by various carbon sources. In the course of these experiments, we observed that cells carrying *spo0A(N12K)*, *spo0A(Q90R)*, and *spo0A(E14A)* all sporulated substantially better in 2×SG medium than in DS medium (data not shown). The difference in sporulation was caused by the 0.1% glucose in 2×SG. A *spo0A(Q90R)* strain sporulated as well in DS medium plus 0.1% glucose as in 2×SG but did not sporulate well in DS medium or DS medium with twice the nutrient broth (data not shown). Similar results were seen with *spo0A(N12K)*

TABLE 5. Effects of *kinA, kinB*, and *kinC* on sporulation of the *spo0A(E14A)* Δ*spo0E* mutant

Strain[a]	Other genotype[b]	% Sporulation[c]
JQ363	Δ*kinABC*	0.33
JQ235	*Pspac-kinA* Δ*kinBC*	35
JQ372	*Pspac-kinB* Δ*kinAC*	0.09
JQ370	*Pspac-kinC* Δ*kinAB*	47

[a] All strains are *spo0A(E14A)* Δ*spo0F* Δ*spo0B* Δ*spo0E*.

[b] Expression of kinases from the *Pspac* promoter was induced with 1 mM IPTG added when the culture was inoculated.

[c] Strains were grown in DS medium. Wild-type (JH642) cultures produced 50 to 80% spores.

TABLE 6. Acetoin affects sporulation of *spo0A* mutants

Strain[a]	*spo0A* allele	% sporulation	
		DS medium	DS medium + 20 mM acetoin
JQ134	Wild type	3.3×10^{-6}	2.9×10^{-6}
JQ225	*spo0A(N12K)*	1.6×10^{-5}	0.5
JQ363	*spo0A(E14A)*	0.33	11
JQ396	*spo0A(P60S)*	1.3	0.25
JQ137	*spo0A(Q90R)*	2.4×10^{-5}	1.5
JQ388	*spo0A(D92Y)*	0.37	0.048

[a] All strains are Δ*kinA* Δ*kinB* Δ*kinC* Δ*spo0B* Δ*spo0F* Δ*spo0E* in addition to the indicated *spo0A* allele.

and *spo0A(E14A)*. We found that other carbon sources (fructose, glycerol, and ribose) that are metabolized to pyruvate through part of the glycolytic pathway also stimulated sporulation. Pyruvate itself had little effect on sporulation, perhaps because it is poorly taken up (44). Malate, which is metabolized through the citric acid cycle, did not affect sporulation. It is important to note that high levels of some sugars (>0.5%) inhibit sporulation. The activating effect described here occurs with low levels (0.1%) of sugars.

A metabolic by-product, acetoin, differentially activates Spo0A mutants. We reasoned that various carbon sources might affect sporulation by stimulating production of a metabolic by-product. *B. subtilis* growing on DS medium plus 0.1% glucose secretes primarily pyruvate, acetate, acetoin, isovalerate, and isobutyrate into the medium (44). Upon entering stationary phase, the citric acid cycle is activated and acetate and acetoin are rapidly consumed (43, 44).

Acetoin substantially affected sporulation of some *spo0A* mutants (Table 6). Acetate and pyruvate had little effect on sporulation, and isovalerate and isobutyrate had no effect (data not shown). When supplemented with exogenous acetoin, *B. subtilis* cultures exhibit increased residual growth after entry into stationary phase (in fact, the growth on acetoin might more properly be called biphasic) (14). Sporulation of *spo0A*$^+$, *spo0A(P60S)*, and *spo0A(D92Y)* strains was slightly inhibited by acetoin, perhaps due to increased growth. In contrast, sporulation of *spo0A(N12K)*, *spo0A(E14A)*, and *spo0A(Q90R)* mutants was stimulated by the addition of acetoin to the medium (Table 6). The fact that acetoin affected some *spo0A* mutants differently from others indicates that it might be contributing to activation of Spo0A. Any effect on downstream sporulation events or transcription and translation of *spo0A* should affect all the *spo0A* mutants similarly. Acetoin probably stimulates sporulation by increasing phosphorylation of Spo0A. Acetoin stimulated sporulation of all the *spo0A* mutants with relaxed specificity for KinC [*spo0A(N12K)*, *spo0A(E14A)*, and *spo0A(Q90R)*] and did not affect the mutants with normal specificity for the upstream kinases [*spo0A(P60S)* and *spo0A (D92Y)*]. Additionally, the acetoin effect on *spo0A(Q90R)* was decreased in the presence of the phosphatase, Spo0E (data not shown), further supporting the notion that phosphorylation of the mutant Spo0A proteins was affected.

Acetoin must be metabolized to affect sporulation. Acetoin metabolism was required for acetoin to stimulate sporulation. A set of proteins encoded by the *aco* operon metabolizes acetoin to acetyl coenzyme A (acetyl-CoA) and acetaldehyde (14). Acetoin did not stimulate sporulation of a strain containing the *acoA* and *spo0A(Q90R)* mutations (Table 7). Strains containing a disruption of *acoA*, the first gene in the *aco* operon, no longer showed growth stimulation by acetoin (data not shown). The *acoA* mutation is probably polar on the entire operon (14).

It is unclear how the metabolism of acetoin stimulates sporulation. One possibility is that cells metabolizing acetoin have increased levels of acetyl-phosphate, which is known to donate phosphate to some response regulators (8, 30, 31). Our data do not support this model, however. Both acetyl-CoA and acetaldehyde are readily converted into acetyl-phosphate in *E. coli* and probably in *B. subtilis* as well (6, 9). The conversion of acetyl-CoA to acetyl phosphate requires the product of the *pta* gene. Thus, deletion of *pta* might attenuate the acetoin effect if the effect depends on the conversion of acetoin to acetyl phosphate. However the effect of acetoin on the sporulation of JQ137 (Tables 6 and 7) is unaffected by deleting *pta* (strain BB1012) (data not shown).

Altered forms of Spo0A have decreased discrimination among phosphate donors. Our results indicate that the *spo0A (N12K)*, *spo0A(E14A)*, and *spo0A(Q90R)* mutations cause the production of altered forms of Spo0A that can activate sporulation in response to phosphorylation from a broader range of possible phosphodonors than wild-type Spo0A. All of the mutants and wild-type Spo0A can support strong sporulation when activated by Spo0B~P (the phosphorelay) (27, 34, 45). In addition, KinC allows weak sporulation of a *spo0A*$^+$ strain in the absence of the phosphatase Spo0E. *spo0A(N12K)*-, *spo0A (E14A)*-, and *spo0A(Q90R)*-containing strains sporulate well in the presence of KinC and are also stimulated by acetoin. These data suggest that Spo0A may have evolved to be generally stringent so as to prevent all but the most efficient phosphodonor, Spo0B~P, from stimulating sporulation. Mutations such as *spo0A(N12K)*, *spo0A(Q90R)*, and *spo0A(E14A)* may alter Spo0A so as to relax the general stringency of Spo0A activation. They may allow a more efficient interaction between Spo0A and various phosphodonors, or they may increase the stability of Spo0A~P. Increased stability would allow lower levels of phosphorylation from alternative phosphodonors to activate sporulation despite the poor efficiency of the interaction. These mechanisms are not mutually exclusive.

Inappropriate activation of Spo0A impairs cell growth and can be lethal (18, 45). *B. subtilis* contains genes for 34 putative response regulators and 36 putative histidine kinases (7, 25). Residues that help reject interactions with nonspecific kinases may be important in preventing inappropriate activation of Spo0A and other response regulators.

TABLE 7. Acetoin metabolism affects sporulation

Strain[a]	Relevant genotype	% sporulation in DS medium[b]		
		Alone	+ 0.1% glucose	+ 20 mM acetoin
JQ137	*spo0A(Q90R)*	4.9×10^{-5}	4.1×10^{-3}	3.2
JQ532	*spo0A(Q90R) acoA::pLGSA700*	7.5×10^{-5}	3.1×10^{-3}	6.5×10^{-5}

[a] Both strains are Δ*kinA* Δ*kinB* Δ*kinC* Δ*spo0F* Δ*spo0B* Δ*spo0E*.
[b] Strains were grown in the indicated medium. Spores were assayed approximately 20 h after the end of exponential growth.

ACKNOWLEDGMENTS

We thank I. Kurtser for excellent technical assistance, K. P. Lemon and E. Küster-Schöck for critical readings of the manuscript, and A. Steinbüchel and F. Oppermann for providing the *aco* mutation.

J.D.Q. was supported in part by a predoctoral fellowship from NSF, and W.F.B. was supported in part by a postdoctoral fellowship from the American Cancer Society. This work was also supported by Public Health Service grant GM41934 to A.D.G.

REFERENCES

1. **Antoniewski, C., B. Savelli, and P. Stragier.** 1990. The *spoIIJ* gene, which regulates early developmental steps in *Bacillus subtilis*, belongs to a class of environmentally responsive genes. J. Bacteriol. **172:**86–93.
2. **Burbulys, D., K. A. Trach, and J. A. Hoch.** 1991. Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay. Cell **64:**545–552.
3. **Burkholder, W. F., and A. D. Grossman.** 2000. Regulation of the initiation of endospore formation in *Bacillus subtilis*, p. 151–166. *In* Y. V. Brun and L. J. Shimkets (ed.), Prokaryotic development. American Society for Microbiology, Washington, D.C.
4. **Cervin, M. A., and G. B. Spiegelman.** 1999. The Spo0A *sof* mutations reveal regions of the regulatory domain that interact with a sensor kinase and RNA polymerase. Mol. Microbiol. **31:**597–607.
5. **Chung, J. D., G. Stephanopoulos, K. Ireton, and A. D. Grossman.** 1994. Gene expression in single cells of *Bacillus subtilis*: evidence that a threshold mechanism controls the initiation of sporulation. J. Bacteriol. **176:**1977–1984.
6. **Clark, D. P., and J. E. Cronan.** 1996. Two-carbon compounds and fatty acids as carbon sources, p. 343–357. *In* F. C. Neidhardt et al. (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
7. **Fabret, C., V. A. Feher, and J. A. Hoch.** 1999. Two-component signal transduction in *Bacillus subtilis*: how one organism sees its world. J. Bacteriol. **181:**1975–1983.
8. **Feng, J., M. R. Atkinson, W. McCleary, J. B. Stock, B. L. Wanner, and A. J. Ninfa.** 1992. Role of phosphorylated metabolic intermediates in the regulation of glutamine synthetase synthesis in *Escherichia coli*. J. Bacteriol. **174:** 6061–6070.
9. **Fortnagel, P.** 1993. Glycolysis, p. 171–180. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. ASM Press, Washington, D.C.
10. **Grossman, A. D., T. Lewis, N. Levin, and R. DeVivo.** 1992. Suppressors of a *spo0A* missense mutation and their effects on sporulation in *Bacillus subtilis*. Biochimie **74:**679–688.
11. **Guerout-Fleury, A. M., K. Shazand, N. Frandsen, and P. Stragier.** 1995. Antibiotic-resistance cassettes for *Bacillus subtilis*. Gene **167:**335–336.
12. **Harwood, C. R., and S. M. Cutting.** 1990. Molecular biological methods for *Bacillus*. John Wiley & Sons, Chichester, England.
13. **Hoch, J. A.** 1995. Control of cellular development in sporulating bacteria by the phosphorelay two-component signal transduction system, p. 129–144. *In* J. A. Hoch and T. J. Silhavy (ed.), Two-component signal transduction. ASM Press, Washington, D.C.
14. **Huang, M., F. B. Oppermann-Sanio, and A. Steinbuchel.** 1999. Biochemical and molecular characterization of the *Bacillus subtilis* acetoin catabolic pathway. J. Bacteriol. **181:**3837–3841.
15. **Ireton, K., and A. D. Grossman.** 1992. Coupling between gene expression and DNA synthesis early during development in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **89:**8808–8812.
16. **Ireton, K., and A. D. Grossman.** 1992. Interactions among mutations that cause altered timing of gene expression during sporulation in *Bacillus subtilis*. J. Bacteriol. **174:**3185–3195.
17. **Ireton, K., and A. D. Grossman.** 1994. A developmental checkpoint couples the initiation of sporulation to DNA replication in *Bacillus subtilis*. EMBO J. **13:**1566–1573.
18. **Ireton, K., D. Z. Rudner, K. J. Siranosian, and A. D. Grossman.** 1993. Integration of multiple developmental signals in *Bacillus subtilis* through the Spo0A transcription factor. Genes Dev. **7:**283–294.
19. **Jiang, M., R. Grau, and M. Perego.** 2000. Differential processing of propeptide inhibitors of Rap phosphatases in *Bacillus subtilis*. J. Bacteriol. **182:**303–310.
20. **Jiang, M., W. Shao, M. Perego, and J. A. Hoch.** 2000. Multiple histidine kinases regulate entry into stationary phase and sporulation in *Bacillus subtilis*. Mol. Microbiol **38:**535–542.
21. **Jiang, M., Y. L. Tzeng, V. A. Feher, M. Perego, and J. A. Hoch.** 1999. Alanine mutants of the Spo0F response regulator modifying specificity for sensor kinases in sporulation initiation. Mol. Microbiol **33:**389–395.
22. **Jin, S., P. A. Levin, K. Matsuno, A. D. Grossman, and A. L. Sonenshein.** 1997. Deletion of the *Bacillus subtilis* isocitrate dehydrogenase gene causes a block at stage I of sporulation. J. Bacteriol. **179:**4725–4732.
23. **Kawamura, F., and H. Saito.** 1983. Isolation and mapping of a new suppressor mutation of an early sporulation gene *spo0F* mutation in *Bacillus subtilis*. Mol. Gen. Genet. **192:**330–334.
24. **Kobayashi, K., K. Shoji, T. Shimizu, K. Nakano, T. Sato, and Y. Kobayashi.** 1995. Analysis of a suppressor mutation *ssb* (*kinC*) of *sur0B20* (*spo0A*) mutation in *Bacillus subtilis* reveals that *kinC* encodes a histidine protein kinase. J. Bacteriol. **177:**176–182.
25. **Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, et al.** 1997. The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. Nature **390:**249–256.
26. **Lazazzera, B. A., J. M. Solomon, and A. D. Grossman.** 1997. An exported peptide functions intracellularly to contribute to cell density signaling in *B. subtilis*. Cell **89:**917–925.
27. **LeDeaux, J. R., and A. D. Grossman.** 1995. Isolation and characterization of *kinC*, a gene that encodes a sensor kinase homologous to the sporulation sensor kinases KinA and KinB in *Bacillus subtilis*. J. Bacteriol. **177:**166–175.
28. **LeDeaux, J. R., N. Yu, and A. D. Grossman.** 1995. Different roles for KinA, KinB, and KinC in the initiation of sporulation in *Bacillus subtilis*. J. Bacteriol. **177:**861–863.
29. **Leighton, T. J., and R. H. Doi.** 1971. The stability of messenger ribonucleic acid during sporulation in *Bacillus subtilis*. J. Biol. Chem. **252:**268–272.
30. **Lukat, G. S., W. R. McCleary, A. M. Stock, and J. B. Stock.** 1992. Phosphorylation of bacterial response regulator proteins by low molecular weight phospho-donors. Proc. Natl. Acad. Sci. USA **89:**718–722.
31. **McCleary, W. R., J. B. Stock, and A. J. Ninfa.** 1993. Is acetyl phosphate a global signal in *Escherichia coli*? J. Bacteriol. **175:**2793–2798.
32. **Miller, J.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Ohlsen, K. L., J. K. Grimsley, and J. A. Hoch.** 1994. Deactivation of the sporulation transcription factor Spo0A by the Spo0E protein phosphatase. Proc. Natl. Acad. Sci. USA **91:**1756–1760.
34. **Olmedo, G., E. G. Ninfa, J. Stock, and P. Youngman.** 1990. Novel mutations that alter the regulation of sporulation in *Bacillus subtilis*: evidence that phosphorylation of regulatory protein Spo0A controls the initiation of sporulation. J. Mol. Biol. **215:**359–372.
35. **Perego, M.** 1997. A peptide export-import control circuit modulating bacterial development regulates protein phosphatases of the phosphorelay. Proc. Natl. Acad. Sci. USA **94:**8612–8617.
36. **Perego, M., S. P. Cole, D. Burbulys, K. Trach, and J. A. Hoch.** 1989. Characterization of the gene for a protein kinase which phosphorylates the sporulation-regulatory proteins Spo0A and Spo0F of *Bacillus subtilis*. J. Bacteriol. **171:**6187–6196.
37. **Perego, M., C. Hanstein, K. M. Welsh, T. Djavakhishvili, P. Glasser, and J. A. Hoch.** 1994. Multiple protein-aspartate phosphatases provide a mechanism for the integration of diverse signals in the control of development in *B. subtilis*. Cell **79:**1047–1055.
38. **Perego, M., G. B. Spiegelman, and J. A. Hoch.** 1988. Structure of the gene for the transition state regulator, *abrB*: regulator synthesis is controlled by the *spo*0A sporulation gene in *Bacillus subtilis.* Mol. Microbiol. **2:**689–699.
39. **Schaeffer, P., J. Millet, and J. Aubert.** 1965. Catabolite repression of bacterial sporulation. Proc. Natl. Acad. Sci. USA **54:**704–711.
40. **Sharrock, R. A., S. Rubenstein, M. Chan, and T. Leighton.** 1984. Intergenic suppression of *spo0* phenotypes by the *Bacillus subtilis* mutation *rvtA*. Mol. Gen. Genet. **194:**260–264.
41. **Shoji, K., S. Hiratsuka, F. Kawamura, and Y. Kobayashi.** 1988. New suppressor mutation *sur0B* of *spo0B* and *spo0F* mutations in *Bacillus subtilis*. J. Gen. Microbiol. **134:**3249–3257.
42. **Solomon, J. M., B. A. Lazazzera, and A. D. Grossman.** 1996. Purification and characterization of an extracellular peptide factor that affects two different developmental pathways in *Bacillus subtilis*. Genes Dev. **10:**2014–2024.
43. **Sonenshein, A. L.** 1989. Metabolic regulation of sporulation and other stationary phase phenomena, p. 109–130. *In* I. Smith, R. Slepecky, and P. Setlow (ed.), Regulation of procaryotic development. American Society for Microbiology, Washington, D.C.
44. **Speck, E. L., and E. Freese.** 1973. Control of metabolite secretion in *Bacillus subtilis.* J. Gen. Microbiol **78:**261–275.
45. **Spiegelman, G., B. Van Hoy, M. Perego, J. Day, K. Trach, and J. A. Hoch.** 1990. Structural alterations in the *Bacillus subtilis* Spo0A regulatory protein which suppress mutations at several *spo0* loci. J. Bacteriol. **172:**5011–5019.
46. **Stragier, P., and R. Losick.** 1996. Molecular genetics of sporulation in *Bacillus subtilis*. Annu. Rev. Genet. **30:**297–341.
47. **Trach, K. A., and J. A. Hoch.** 1993. Multisensory activation of the phosphorelay initiating sporulation in *Bacillus subtilis*: identification and sequence of the protein kinase of the alternate pathway. Mol. Microbiol. **8:**69–79.
48. **Weir, J., M. Predich, E. Dubnau, G. Nair, and I. Smith.** 1991. Regulation of *spo0H*, a gene coding for the *Bacillus subtilis* σ^H factor. J. Bacteriol. **173:** 521–529.
49. **Yansura, D. G., and D. J. Henner.** 1984. Use of the *Escherichia coli lac* repressor and operator to control gene expression in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **81:**439–443.
50. **Youngman, P., H. Poth, B. Green, K. York, G. Olmedo, and K. Smith.** 1989. Methods for genetic manipulation, cloning, and functional analysis of sporulation genes in *Bacillus subtilis*, p. 65–87. *In* I. Smith, R. Slepecky, and P. Setlow (ed.), Regulation of procaryotic development. American Society for Microbiology, Washington, D.C.