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Isolation and Characterization of *kinC*, a Gene That Encodes a Sensor Kinase Homologous to the Sporulation Sensor Kinases KinA and KinB in *Bacillus subtilis*

JOHN R. LEDEAUX AND ALAN D. GROSSMAN*

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

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Phosphorylation of the transcription factor encoded by *spo0A* is required for the initiation of sporulation in *Bacillus subtilis*. Production and accumulation of Spo0A~P is controlled by histidine protein kinases and the *spo0* gene products. To identify additional genes that might be involved in the initiation of sporulation and production of Spo0A~P, we isolated genes which when present on a multicopy plasmid could suppress the sporulation defect of a *spo0K* mutant. *kinC* was one gene isolated in this way. A multicopy plasmid containing *kinC* completely or partially suppressed the sporulation defect caused by mutations in *spo0K*, *kinA*, *spo0F*, and *spo0B*, indicating that at least when overexpressed, KinC is capable of stimulating phosphorylation of Spo0A independently of the normal phosphorylation pathway. The predicted product of *kinC* is 428 amino acids long and is most similar to KinA and KinB, the histidine protein kinases involved in the initiation of sporulation. In otherwise wild-type strains, *kinC* null mutations caused little or no defect in sporulation under the conditions tested. However, in the absence of a functional phosphorelay (*spo0F* or *spo0B*), KinC appears to be the kinase responsible for phosphorylation of the *sof-1* and *rvtA11* forms of Spo0A.

Cells of the gram-positive soil bacterium *Bacillus subtilis* differentiate to form heat-resistant metabolically dormant spores under appropriate conditions. The initiation of sporulation depends upon activation of the Spo0A transcription factor by phosphorylation (20), and a threshold concentration of Spo0A~P appears to be required (11). Spo0A~P is involved in the transcriptional regulation of many sporulation genes. It directly activates transcription of *spoIIA*, *spoIIE*, and *spoIIG*, which are necessary for sporulation (4, 6, 48, 49, 57, 62). Also, Spo0A~P represses transcription of *abrB* (56), which encodes a repressor of several genes involved in sporulation (44, 55, 59, 64).

Spo0A receives phosphate, albeit indirectly, from KinA (2, 38) and KinB (58), histidine protein kinases that belong to a conserved family of proteins known as sensor kinases. Sensor kinases are generally involved in signal transduction; they autophosphorylate and donate phosphate to particular proteins in response to changes in the environment, modifying the activity of those proteins. The proteins that receive phosphate from sensor kinases belong to a conserved family of proteins known as response regulators, which are often involved in transcriptional regulation. Together, a sensor protein and its cognate response regulator form a two-component system. There are many examples of two-component systems involved in signal transduction in bacteria (1, 37), and similar systems in plants (10) and yeasts (36) have recently been identified.

Although Spo0A belongs to the response regulator family of proteins, it does not normally obtain phosphate directly from a histidine protein kinase. Rather, Spo0A receives phosphate through a multicomponent phosphorelay (9). KinA (and other kinases) first donates phosphate to response regulator Spo0F. The phosphate is then transferred from Spo0F to Spo0B and finally to Spo0A. A major function of the phosphorelay seems to be to integrate multiple developmental signals that regulate the initiation of sporulation (9, 22, 24–26).

We have identified and characterized a third kinase gene, *kinC*, that is involved in the phosphorylation of Spo0A. This gene was identified as a multicopy suppressor of a *spo0K* mutant. The *spo0K* operon encodes an oligopeptide permease that is required for efficient initiation of sporulation (39, 43, 45) and the development of genetic competence (45, 46) and appears to affect activation of Spo0A (45). We describe the cloning and characterization of *kinC*, whose predicted product is most similar to the sensor kinases KinA and KinB.

Certain altered function mutations in *spo0A*, e.g., *sof-1* (*sur0F1*), *sur0B20*, and *rvtA11* (21, 29, 51–53), can bypass the need for *spo0F* and *spo0B* in sporulation. Presumably, in the absence of *spo0F* or *spo0B*, these altered forms of Spo0A obtain phosphate directly from some other source, probably one or more histidine protein kinases (26, 53). We show that KinC is one such kinase.

kinC was identified independently by Y. Kobayashi and his colleagues in a screen for mutations that abolished the suppressing activity of *sur0B20* (31).

MATERIALS AND METHODS

Media. Routine growth and maintenance of *Escherichia coli* and *B. subtilis* was done in LB medium (34). The nutrient sporulation medium used was 2×SG medium (32) or DS medium (50). Media in plates were solidified with 15 g of agar (Difco Laboratories) per liter. Sporulation proficiency was visualized on DS or 2×SG plates. Antibiotics were used at the following concentrations: ampicillin at 100 μg/ml, chloramphenicol (Cm) at 5 μg/ml, spectinomycin (Spec) at 100 μg/ml, neomycin at 5 μg/ml, and erythromycin and lincomycin together (MLS) at 0.5 and 12.5 μg/ml, respectively, to select for the *erm* gene. Cells were made competent in S7 minimal medium as described previously (33, 45).

Strains and plasmids. Standard *E. coli* strains were used for cloning and maintaining plasmids, as previously described (23, 45). The *B. subtilis* strains used are listed in Table 1, and all are derived from strain 168. The JH642 (42) or PB2 (7, 45) strains were used as the wild type, as indicated. The plasmids used are listed in Table 2, and some are illustrated in Fig. 1 and 2.

The Δ*spo0E*::*spc* allele contains the *spc* cassette from pUS19 (5) inserted into a deletion of *spo0E* from 13 bp upstream of the *spo0E* start codon to the G in the

* Corresponding author. Mailing address: Department of Biology, Building 68-530, Massachusetts Institute of Technology, Cambridge, MA 02139. Phone: (617) 253-1515. Fax: (617) 253-8699. Electronic mail address: adg@mit.edu.

TABLE 1. *B. subtilis* strains used in this study

Strain	Genotype	Comment(s) and/or reference
JH642	*trpC2 pheA1*	42
PB2	*trpC2*	7
AG676	JH642 *spo0BΔPst pheA*$^+$	*spo0BΔ* allele cotransformed with *pheA*$^+$ (59)
JRL237	JH642 pHP13	pHP13 from BGSC[a] (8, 18)
JRL357	JH642 Δ*spo0K357*::*neo*	
JRL358	JH642 Δ*spo0K358*::*erm* (Δ*spo0K*::*erm*)	
JRL407	PB2 *spo0K*::pJL58	
JRL408	PB2 Pspac-*spo0K*	
JRL417	PB2 Δ*spo0K*::*erm*	
JRL459	PB2 Pspac-*spo0K spoIIA*$^+$::(*spoIIA-lacZ neo*) pJL52	*spoIIA-lacZ* (24, 60)
JRL530	JH642 *spo0A9V* pLK2	*spo0A9V* (43)
JRL532	JH642 *spo0A9V* pHP13	
JRL550	JH642 *spo0J93* pLK2	*spo0J93* (43)
JRL552	JH642 *spo0J93* pHP13	
JRL555	JH642 Δ*spo0K*::*erm* pLK2	
JRL558	JH642 Δ*spo0K*::*erm* pHP13	
JRL595	JH642 pLK2	
JRL645	PB2 Δ*kinC645*::*spc* (Δ*kinC*::*spc*)	
JRL660	JH642 Δ*kinC*::*spc*	
JRL753	JH642 Δ*kinC*::*spc spo0A*$^+$*-cat*	
JRL763	JH642 *spo0BΔPst pheA*$^+$ *sof-1–cat*	*sof-1* (21, 29) ~90% linked to *cat* by transformation (16)
JRL764	JH642 *spo0BΔPst pheA*$^+$ *rvtA11-cat*	*rvtA11* (51)
JRL766	JH642 *spo0BΔPst pheA*$^+$ *spo0A*$^+$*-cat*	
JRL767	JH642 *spo0BΔPst pheA*$^+$ *sof-1–cat* Δ*kinC*::*spc*	
JRL768	JH642 *spo0BΔPst pheA*$^+$ *rvtA11-cat* Δ*kinC*::*spc*	
JRL770	JH642 *spo0BΔPst pheA*$^+$ *rvtA11-cat kinA*::Tn*917*	*kinA*::Tn*917* (2, 47)
JRL783	JH642 *spo0FΔS spo0BΔPst pheA*$^+$ *rvtA11-cat*	*spo0FΔS* (29)
JRL790	JH642 Δ*kinC*::*spc sof-1–cat*	
JRL791	JH642 Δ*kinC*::*spc rvtA11-cat*	
JRL792	JH642 *spo0FΔS spo0BΔPst pheA*$^+$ *rvtA11-cat* Δ*kinC*::*spc*	
JRL794	JH642 *spo0FΔS spo0BΔPst pheA*$^+$ *rvtA11-cat kinA*::Tn*917*	
JRL796	JH642 *spo0FΔS spo0BΔPst pheA*$^+$ pLK2	
JRL797	JH642 *spo0FΔS spo0BΔPst pheA*$^+$ pHP13	
JRL812	JH642 *thrC*::(*kinC-lacZ erm*)	
JRL923	JH642 *spo0FΔS* pHP13	
JRL925	JH642 *spo0FΔS* pLK2	
JRL951	PB2 Δ*spo0K*::*erm* pLK2	
JRL992	JH642 *spo0E11* pLK2	*spo0E11* (35, 40, 41)
JRL993	JH642 *spo0E11* pHP13	
JRL995	JH642 *spo0BΔPst pheA*$^+$ pLK2	
JRL996	JH642 *spo0BΔPst pheA*$^+$ pHP13	
JRL1010	JH642 *kinA*::Tn*917* pHP13	
JRL1011	JH642 *kinA*::Tn*917* pLK2	
JRL1124	JH642 Δ*spo0E*::*spc spo0FΔS spo0BΔPst pheA*$^+$ pHP13	
JRL1125	JH642 Δ*spo0E*::*spc spo0FΔS spo0BΔPst pheA*$^+$ pLK2	
KI644	JH642 *spo0E11 kinA*::Tn*917*	
KI1521	JH642 *spo0F221 rvtA11-cat*::*spc*	*spo0F221* (43)

[a] BGSC, *Bacillus* Genetic Stock Center.

TAG stop codon and was constructed by K. Ireton. The *rvtA11* (51) and *sof-1* (29, 53) alleles of *spo0A* that were used are ~90% linked by transformation to *cat* or to *spc* (16, 22, 24), with the *spc* gene inserted at the *Nco*I site of the *cat* gene by using pJL62 (Table 2).

We made a null mutation in *spo0K* by deleting the first four genes of the *spo0K* operon, from *spo0KA* codon 18 (at the *Esp*I site) to *spo0KD* codon 127 (at the *Bgl*II site) (45), and inserting an *erm* cassette. The plasmid that contains this mutation is pJL50 (Table 2) and has the *cat* gene in the plasmid backbone. This mutation was introduced into the chromosome by transforming wild-type cells with linearized pJL50 and selecting for MLS^r. One of the MLS^r Cm^s transformants, resulting from a double crossover, was chosen as the Δ*spo0K*::*erm* mutant. A similar deletion-insertion mutation was made by inserting a *neo* cassette (Δ*spo0K*::*neo*) instead of *erm*.

A null mutation in *kinC* was made by deleting the region from 25 bp upstream of the putative *kinC* start codon (the *Pst*I site in Fig. 2) to codon 211 (the *Cla*I site in Fig. 2) and inserting the *spc* cassette from pJL74. This mutation, contained on pLK25, was recombined into the chromosome by double crossover by transforming the linearized plasmid into PB2 and selecting for Spec^r and screening for Cm^s.

We constructed a *kinC-lacZ* transcriptional fusion that contains the sequence from ~1.5 kb upstream of the putative *kinC* start codon to codon 211 and introduced it into the *thrC* locus of the chromosome, using *lacZ* fusion vector pDG793 (provided by P. Stragier). The plasmid containing this fusion is pLK114 (Table 2 and Fig. 2). pLK114 was linearized and transformed into wild-type cells selecting for MLS^r. Double-crossover events that resulted in the introduction of the *kinC-lacZ* fusion at *thrC* caused a Thr$^-$ phenotype.

Making the multicopy plasmid library. Two different multicopy plasmid libraries were made in the *B. subtilis-E. coli* shuttle vector pHP13 (8, 18). pHP13 has two drug markers suitable for selection in *B. subtilis*: *erm* (which confers MLS^r) and *cat* (which confers Cm^r). In *B. subtilis*, pHP13 has a copy number of approximately five or six per cell (8, 18). Chromosomal DNA from JH642 was partially digested with *Sau*3A and electrophoresed on an agarose gel. DNA was isolated from the size ranges of approximately 2 to 4.5 kb (library A) and approximately 4.5 to 9 kb (library B), on the basis of size markers run in parallel. Size-fractionated DNA was ligated into pHP13 that had been linearized with *Bam*HI and treated with phosphatase before transformation into *E. coli* selecting for Cm^r. The transformants were pooled and plasmid DNA was prepared for transformation into *B. subtilis.*

TABLE 2. Plasmids used in this study

Plasmid	Description (source or reference)[a]
Vectors	
pBluescriptII KS+	Ap; used for subcloning and sequencing (Stratagene)
pAG58	Ap Cm (28)
pJH101	Ap Tc Cm; integrative vector (15)
pGEM3Zf(+)::*cat-1* (pGEM*cat*)	Ap Cm; integrative vector (63)
pUC18::*erm*	Ap MLS; source of *erm* cassette (30)
pBEST501	Ap Neo; source of *neo* cassette (27)
pUS19	Ap Spec; integrative vector, source of *spc* cassette (5)
pJL62	Ap Spec; 1.1-kb *Bgl*I (blunted)-*Nde*I (blunted) *spc* cassette from pUS19 cloned into *Nco*I (blunted) of *cat* gene in pJH101; used to convert Cm^r $Spec^s$ strains to Cm^s $Spec^r$
pJL73	Ap Spec; 1.1-kb *Bgl*I (blunted)-*Nde*I (blunted) *spc* cassette from pUS19 cloned into *Sma*I site of pBluescript SK+
pJL74	Ap Spec; same as pJL73 except *spc* cassette is cloned in opposite orientation
pHP13	MLS Cm; *B. subtilis-E. coli* shuttle vector (8, 18)
pJL52	MLS; 0.5-kb *Bam*HI (blunted)-*Nco*I (blunted) deletion of pHP13; helper plasmid used in conjunction with pHP13 for homology assistance
pDG793	Ap MLS; vector used to construct *lacZ* transcriptional fusions and recombine into the chromosome at *thrC* (gift from P. Stragier)
Other plasmids	
pDR9	Ap Cm; clone of *spo0KB-E* in pJH101 (45)
pDR18	Ap Cm; clone of *spo0K* promoter region from *Pvu*II site upstream of promoter to *Eco*RI site in *spo0KA* in pJH101 (45)
pDR21	Ap; clone of *spo0K* promoter region (as in pDR18) in pBluescriptII SK+ (45)
pJL10	Ap Cm; 3.6-kb *Eco*RI fragment from pDR9 cloned into *Eco*RI of pDR18, putting all of *spo0KA-D* on a single integrational plasmid
pJL45	Ap Cm; 311-bp *Eco*RI (blunted)-*Sal*I fragment, containing Pspac, from pAG58 cloned into *Eco*NI (blunted)-*Sal*I of pJH101
pJL47	Ap Cm; Pspac replacement vector (Fig. 1); 1.3-kb *Bam*HI-*Sph*I fragment containing *lacI* from pAG58 cloned into pJL45 so transcription of *lacI* is opposite that from Pspac
pJL49	Ap Cm Neo; 1.3-kb *neo* cassette from pBEST501 cloned into *Esp*I-*Bgl*II (sites lost) of pJL10; used to make Δ*spo0K*::*neo*
pJL50	Ap Cm MLS; 2.3-kb *erm* cassette from pUC18::*erm* cloned into *Esp*I-*Bgl*II (sites lost) of pJL10; used to make Δ*spo0K*::*erm*
pJL51	Ap Cm; 0.9-kb *Xmn*I-*Cla*I (blunted) fragment from pDR21 5 bp downstream from *spo0K* promoter cloned into *Sal*I (blunted) site of pJL47
pJL58	Ap Cm; 0.6-kb *Bam*HI (blunted)-*Ssp*I fragment from pDR21 containing a 368-bp fragment upstream (23 bp) of the *spo0K* promoter cloned into *Cla*I (blunted)-*Bam*HI (blunted) of pJL51; used to make Pspac-*spo0K*
pLK2	MLS Cm; original *kinC* clone, ~3.5-kb *Sau*3A partial digest of JH642 chromosomal DNA cloned into *Bam*HI of pHP13
pLK21	MLS Cm; 2.1-kb *Pst*I fragment from pLK2 cloned into *Pst*I site of pHP13
pLK22	MLS Cm; 1.4-kb *Cla*I (blunted)-*Hin*dIII (blunted) deletion of pLK2
pLK23	MLS Cm; 1.4-kb *Pst*I deletion of pLK2
pLK24	Ap Cm; 3.5-kb *Eco*RI (blunted) fragment of pLK2 cloned into *Pst*I (blunted) of pGEM*cat*
pLK25	Ap Cm Spec; 1.1-kb *Pst*I-*Bam*HI (blunted) *spc* cassette of pJL74 cloned into *Pst*I-*Cla*I (blunted) of pLK24; used to make Δ*kinC*::*spc*
pLK56	Ap; 3.5-kb *Eco*RI fragment from pLK2 cloned into *Eco*RI of pBluescriptII KS+
pLK102	Ap; 2.2-kb *Cla*I deletion of pLK56
pLK104	Ap; 0.4-kb *Eco*RV-*Sma*I deletion of pLK102
pLK114	Ap MLS; *kinC-lacZ*; 2.3-kb *Eco*RI-*Cla*I (blunted) fragment of pLK2 cloned into *Eco*RI-*Bam*HI (blunted) of pDG793; used to make *thrC*::(*kinC-lacZ*)
pLK120	Ap Cm; 0.7-kb *Eco*RV fragment from pLK2 into *Sma*I of pGEM*cat*; contains *kinC* promoter
pLK121	Ap Cm; 0.2-kb *Nde*I (blunted)-*Eco*RI (blunted) deletion of pLK120
pLK124	Ap Cm; 0.4-kb *Bam*HI-*Pst*I fragment of pLK120 cloned into pGEM*cat*
pLK125	MLS Cm; 0.6-kb *Apa*I (blunted)-*Sal*I (blunted) deletion of pLK2
pLK126	Ap Cm; 102-bp *Apa*I (blunted)-*Bam*HI fragment of pLK104 cloned into *Sal*I (blunted)-*Bam*HI of pGEM*cat*; used to disrupt *orf277*

[a] Ap, Tc, Cm, Neo, Spec, MLS refer to resistance to the particular drugs. All sizes in kilobases are approximate.

Approximately 80 to 90% of the plasmids in library A had inserts, and the chance that any particular fragment of the average 3.2-kb size is present in the library is ~87%, assuming a random distribution of *Sau*3A sites, no bias in selection in *E. coli*, and an average insert size of ~3.2 kb (probably an overestimate). For library B, ~60 to 70% of the plasmids had inserts and assuming an average 5-kb size the chance that a particular fragment of average size is represented is ~50%.

Construction of a conditional *spo0K* mutant (Pspac-*spo0K*). We constructed a conditional mutation of *spo0K* by replacing the normal *spo0K* promoter with the LacI-repressible, isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter Pspac (19, 28, 61), using the vector pJL47 (Fig. 1A). pJL47 can be used to replace the promoter of a gene with the *lacI* cassette and Pspac in a two-step process without leaving behind any drug resistance marker.

pJL58 contains DNA from immediately upstream and downstream of the *spo0K* promoter cloned into pJL47 (Fig. 1B and Table 2) and was used to replace the normal *spo0K* promoter with Pspac in two steps. Transformation of pJL58

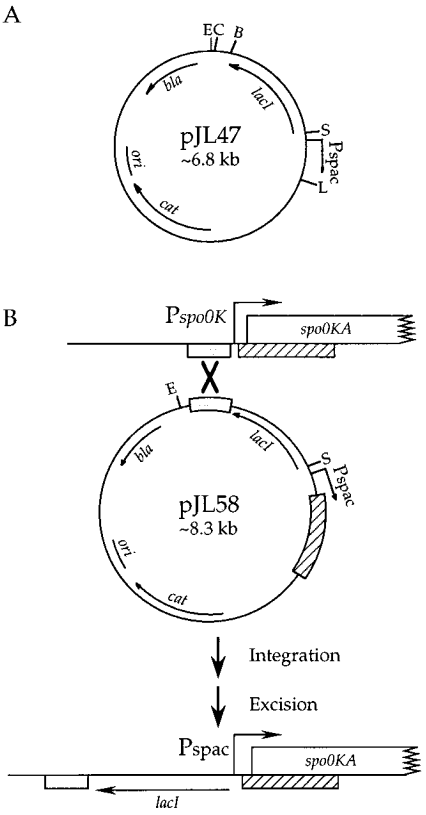

FIG. 1. Making a LacI-repressible, IPTG-inducible allele of *spo0K*. (A) pJL47 contains *lacI* under control of the constitutive P_{pen} promoter and the LacI-repressible, IPTG-inducible promoter Pspac (19, 61). (B) pJL58 contains sequences from both upstream (stippled box) and downstream (striped box) of the *spo0K* promoter cloned upstream and downstream, respectively, of the *lacI*-Pspac cassette of pJL47. When transformed into wild-type cells selecting for Cm^r (integration step), pJL58 can recombine either with the upstream *spo0K* sequence (shown), which would result in a phenotypically Spo^+ transformant, or with the downstream *spo0K* sequence (not shown), which would result in a transformant which was Spo^+ only in the presence of IPTG. Cells that had undergone a second recombination event that left behind the *lacI*-Pspac cassette in place of the *spo0K* promoter (excision step) were isolated as described in Materials and Methods. These cells were Cm^s and Spo^- in the absence of IPTG and Spo^+ in the presence of IPTG. Restriction site abbreviations: E, *Eco*RI; C, *Cla*I; B, *Bam*HI; S, *Sph*I; L, *Sal*I.

into wild-type cells yielded two types of transformants. One class was Spo^+ and most likely resulted from recombination with sequences upstream of the promoter. The other class was Spo^- in the absence of IPTG and Spo^+ in the presence of IPTG. This class most likely resulted from recombination with sequences downstream of the promoter. Three Spo^+ transformants were chosen and used to screen for a second recombination event that would leave behind the *lacI*-Pspac cassette in place of the *spo0K* promoter (Fig. 1B). The Spo^+ transformants were grown to stationary phase in LB medium in the absence of selection for the integrated plasmid, that is, in the absence of Cm. We screened ~13,000 colonies on sporulation plates (lacking IPTG and Cm) and identified two Spo^- colonies that were also Cm^s. The sporulation phenotypes of both of these colonies were completely dependent on IPTG; they were Spo^+ in the presence of 1 mM IPTG, and Spo^- in the absence of IPTG (data not shown). The sporulation and competence phenotypes in the absence of IPTG were indistinguishable from those of a *spo0K* mutant. One isolate, JRL408 (Pspac-*spo0K*), was chosen for further experiments. Strains containing the Pspac-*spo0K* fusion as the only copy of *spo0K* could be made competent under permissive conditions (in the presence of IPTG), and transformants could be screened under nonpermissive conditions (in the absence of IPTG).

Plasmid marker rescue transformation. To facilitate transformation of either of the libraries into *B. subtilis*, we constructed a helper plasmid that could be used in combination with pHP13 (8). The helper plasmid, pJL52, was made by deleting pHP13 from *Bam*HI to *Nco*I. This deletes part of *cat*, and pJL52 does not confer Cm^r but still confers MLS^r.

Conditions for the suppressor screen. Since *spo0K* mutations cause a more severe sporulation defect in the PB2 strain background than in the JH642 background (45), it seemed that a screen for multicopy suppressors of *spo0K* might be more sensitive in the PB2 background. Accordingly, we constructed a PB2 derivative that contained the Pspac-*spo0K* fusion, the helper plasmid pJL52, and a *spoIIA-lacZ* transcriptional fusion (24). This strain, JRL459, was grown in the presence of MLS to maintain pJL52. JRL459 was made competent in S7 minimal medium (plus tryptophan at 40 μg/ml) in 1 mM IPTG to allow expression of *spo0K*. Competent cells were transformed with the pHP13-based multicopy plasmid libraries, and Cm^r transformants were selected on 2×SG plates containing Cm or containing Cm and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 120 μg/ml). Transformants that had a more pronounced Spo^+ morphology or that were darker blue on X-Gal (indicating increased expression of *spoIIA-lacZ*) than the parent strain were chosen for further analysis.

Sporulation assays. Cells were grown in DS or 2×SG medium at 37°C unless otherwise indicated, and spores were assayed approximately 20 h after the end of exponential growth. The number of viable cells per milliliter of culture was determined as the total number of CFU on LB plates. The number of spores per milliliter of culture was determined as the number of CFU after heat treatment (80°C for 20 min). Sporulation frequency is the ratio of spores per milliliter to viable cells per milliliter.

DNA sequencing. The Sequenase V 2.0 kit (U.S. Biochemical Corp.) was used to sequence double-stranded plasmid DNA. The sequence of one strand or the other was determined from different subclones of pLK2 inserted into pBluescriptII KS by using either the universal or reverse primer. To determine the sequence of the opposite strand, primers complementary to the sequence determined with the pBluescript subclones were made, and these primers were used to sequence pLK2.

Primer extension analysis. JRL951 (Δ*spo0K*::*erm* pLK2) was grown in 2×SG medium, and 50-ml samples were taken at various times for preparation of RNA essentially as described previously (3, 23). The sequence of the primer LKP16 was 5′-TTCTTCAGAAAGCTGTTTATACTTCCATTC-3′, and its complement is one of the underlined sequences in Fig. 3. The primer was end labeled with ^{32}P, essentially as described previously (3), and purified with a NICK spin column by the protocol supplied (Pharmacia). The protocol for hybridization extension

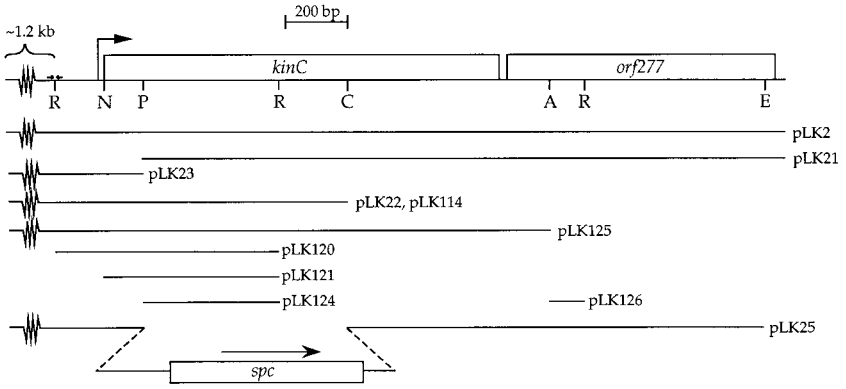

FIG. 2. Map of the *kinC* region and plasmids. Restriction site abbreviations: R, *Eco*RV; N, *Nde*I; P, *Pst*I; C, *Cla*I; A, *Apa*I; E, *Eco*RI.

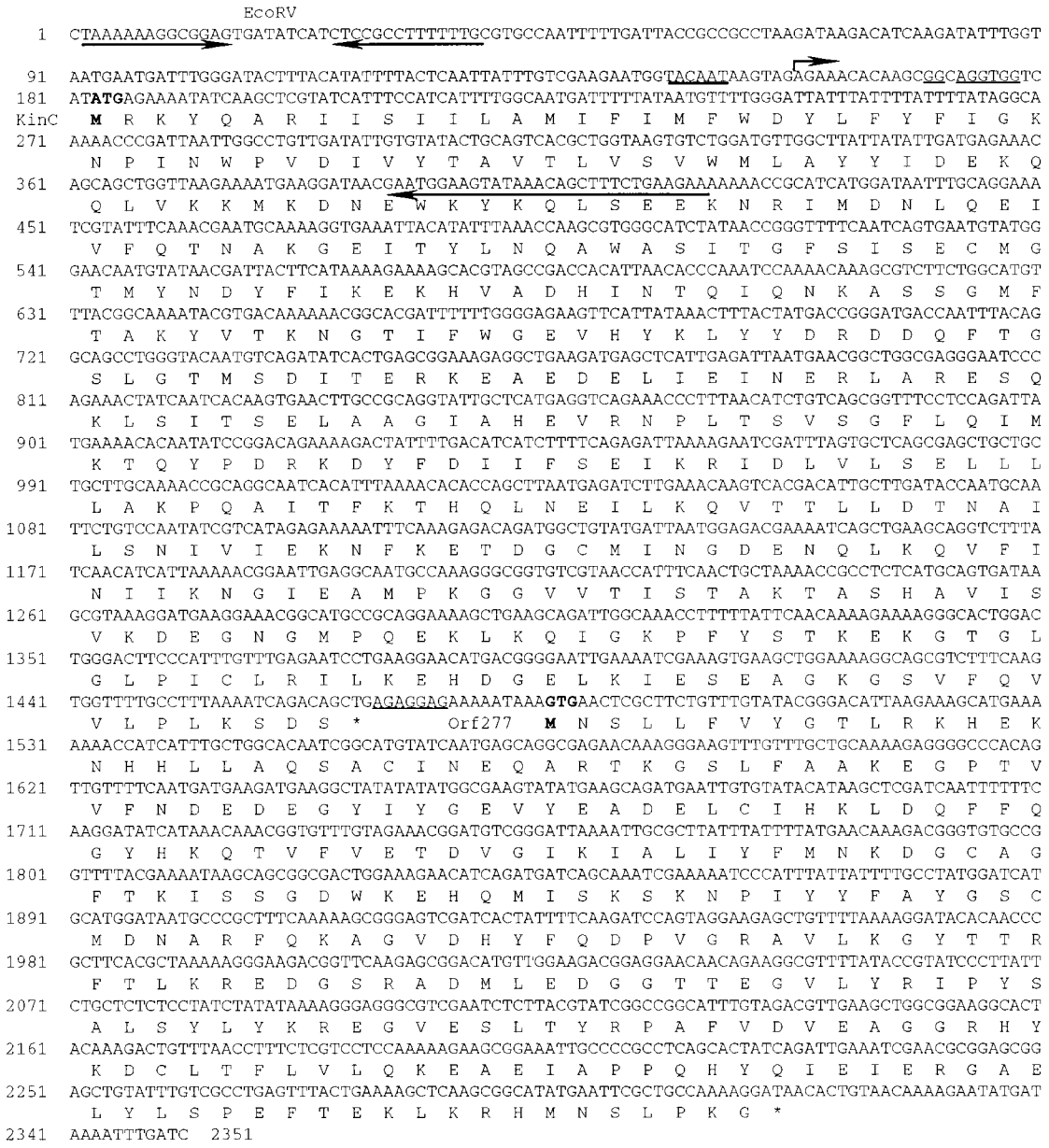

FIG. 3. Nucleotide and amino acid sequences of *kinC*. The DNA sequence was determined from both strands as described in Materials and Methods. An inverted repeat surrounding the *Eco*RV site upstream of *kinC* is indicated by a pair of arrows. A putative −10 region for a sigma-A promoter is shown with a thick underline. The transcriptional start site is indicated with an arrow at nucleotide 158. Putative ribosome binding sites for KinC and Orf277 are underlined, and putative translational start sites for KinC and Orf277 are in boldface type. A sequence complementary to primer LKP16, which was used for primer extension analysis, is underlined with a single arrow.

reactions was essentially as described previously (3, 23) except that we used 200 μg of RNA in each sample instead of 50 μg.

Transduction. We grew a PBS1-transducing lysate on JRL660 (Δ*kinC*::*spc trpC2 pheA1*) and used it to transduce some of the mapping kit strains (13) essentially as described earlier (12). In the three-factor cross between Δ*kinC*::*spc*, *spo0E11*, and *kinA*::Tn*917*, transductants were tested for MLS^r and the colony morphology was analyzed to distinguish between the different classes of recombinants. It was relatively easy to distinguish *spo0E11* from *kinA*::Tn*917* from the *spo0E11 kinA*::Tn*917* double mutant.

β-Galactosidase assays. For determination of β-galactosidase specific activity, cells were grown in 2×SG medium and samples were taken at appropriate times. Prior to the enzyme assay, cells were removed by centrifugation and resuspended in Spizizen salts (54). β-Galactosidase specific activity is expressed as (ΔA_{420} per minute per milliliter of culture per unit of optical density at 600 nm) × 1,000 (34).

Nucleotide sequence accession number. The *kinC* sequence shown in Fig. 3 has been assigned the data bank accession number L34803.

RESULTS

Isolation of genes which, when present on a multicopy plasmid, suppress *spo0K* mutants. *spo0K* null mutants are defective in competence development and have a decreased frequency of transformation. We constructed a conditional *spo0K* mutation so that a multicopy plasmid library could be introduced into the mutant under permissive conditions and be screened for the desired clones under nonpermissive conditions. The

conditional allele replaced the normal *spo0K* promoter with the LacI-repressible, IPTG-inducible promoter, Pspac, such that the Pspac-*spo0K* fusion was present as a single copy in the chromosome and was the only copy of *spo0K* (see Materials and Methods; Fig. 1B). Cells containing the Pspac-*spo0K* fusion (JRL408) were Spo$^-$ in the absence of IPTG and Spo$^+$ in the presence of IPTG. The sporulation frequency in the absence of IPTG was similar to that of a *spo0K* null mutant, while the frequency in the presence of IPTG was similar to that of wild-type cells. In addition, the Pspac-*spo0K* mutant was partly defective in transformation (Com$^-$) in the absence of IPTG and Com$^+$ in the presence of IPTG.

B. subtilis takes up transforming DNA as a linear single strand, making establishment of a multicopy circular plasmid rather inefficient. However, incoming DNA is able to recombine with homologous sequences very efficiently. To facilitate introduction and establishment of a multicopy plasmid library in *B. subtilis*, we constructed a helper plasmid, pJL52, that could be used in combination with the cloning vector pHP13 (see Materials and Methods). When a competent cell containing pJL52 takes up a plasmid monomer from a pHP13-based library and linearizes it, pJL52 provides the homology necessary for the plasmid to recombine and form a closed circular plasmid. One can select for these recombination events by using the Cm^r marker that is present on pHP13 but not on pJL52. This general process is known as plasmid marker rescue transformation or homology assistance (8, 17).

Two different multicopy libraries (see Materials and Methods) were used to screen for genes which when overexpressed could suppress the sporulation defect caused by mutations in *spo0K*. Strain JRL459 (Pspac-*spo0K* pJL52 *spoIIA-lacZ*) was grown and made competent in the presence of IPTG (phenotypically Spo0K$^+$), transformed with plasmid DNA from each library, and screened for sporulation phenotypes in the absence of IPTG (Spo0K$^-$). We screened for transformants that had a Spo$^+$ colony morphology on sporulation plates or that had increased expression of a *spoIIA-lacZ* fusion, as judged by their blue color on sporulation plates containing X-Gal.

We screened approximately 11,000 transformants (~4,300 from library A and ~6,700 from library B) and found 9 that contained plasmids that reproducibly suppressed the sporulation defect caused by the conditional *spo0K* mutation. To test whether these plasmids suppressed the sporulation defect caused by a true *spo0K* null mutation (Δ*spo0K*::*erm*), we isolated plasmid DNA from the candidates and introduced it into the wild type (PB2), a Pspac-*spo0K* mutant (JRL408), and a Δ*spo0K*::*erm* mutant (JRL417). All nine plasmids suppressed the sporulation defect caused by the Δ*spo0K*::*erm* mutation as well as that caused by the Pspac-*spo0K* mutation.

Restriction mapping of the nine plasmids showed that they fell into two classes, exemplified by the plasmids pLK2 and pLK11. The genes contained on these plasmids were called *mskA* and *mskB*, respectively, where *msk* stands for multicopy suppressor of *spo0K*. These two plasmids suppressed the sporulation defect but not the competence defect caused by null mutations in *spo0K*. Below, we describe the characterization of pLK2.

pLK2 contains a gene (*kinC*) encoding a histidine protein kinase homologous to KinA and KinB. Various fragments from the insert in pLK2 were subcloned into the multicopy vector (pHP13) to yield pLK21, pLK22, pLK23, and pLK125 (Fig. 2 and Table 2). These clones were used to identify the region responsible for suppression of the *spo0K* sporulation phenotype. The insert in pLK125 extends from the left end of pLK2 (as drawn in Fig. 2) to the *Apa*I site and completely suppressed the *spo0K* sporulation phenotype. pLK21 contains

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KinA  ...KSEKLSI AGQLAAGIAH EIRNPLTAIK GFLQLM.... KPTMEGNEHY  431
KinC  LARESQKLSI TSELAAGIAH EVRNPLTSVS GFLQIM.... KTQYPDRKDY  250
KinB  .LIHSEKMTI VSELAASVAH EVRNPLTVVR GFVQLLFNDE TLQNKSSADY  252

KinA  FDIVFSELSR IELILSELLM LAKPQQNAVK EYLNLKKLIG EVSALLETQA  481
KinC  FDIIFSEIKR IDLVLSELLL LAKPQAITFK TH.QLNEILK QVTTLLDTNA  299
KinB  KKLVLSELDR AQGIITNYLD MAKQQLYE.K EVFDLSALIK ETSSLMVSYA  301

KinA  NLNGIFIRTS Y.EKDSIYIN GDQNQLKQVF INLIKNAVES MPDG.GTVDI  529
KinC  ILSNIVIEKN FKETDGCMIN GDENQLKQVF INIIKNGIEA MPKG.GVVTI  348
KinB  NYKSVTVEAE ..TEPDLLIY GDATKLKQA. INLMKNSIEA VPHGKGMIHI  348

KinA  IITEDEHSVH VTVKDEGEGI PEKVLNRIGE PFLTTKEKGT GLGLMVTFNI  579
KinC  STAKTASHAV ISVKDEGNGM PQEKLKQIGK PFYSTKEKGT GLGLPICLRI  398
KinB  SAKRNGHTIM INITDNGVGM TDHQMQKLGE PYYSLKTNGT GLGLTVTFSI  398

KinA  IENHQGVIHV DSHPEKGTAF KISFPKK*    606
KinC  LKEHDGELKI ESEAGKGSVF QVVLPLKSDS* 428
KinB  IEHHHGTISF NSSFQKGTTV TIKLPADLPH* 428
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FIG. 4. Comparison of the C-terminal region of KinC to the C-terminal regions of KinA and KinB. The sequences are aligned according to the PILEUP program of the University of Wisconsin Genetics Computer Group (14). The first residues shown are amino acids 389, 204, and 205 for KinA, KinC, and KinB, respectively. In the regions shown, KinC is 51% identical to KinA and 36% identical to KinB. Residues that are highly conserved in the family of sensor kinases (1, 37) are indicated in slightly enlarged boldface type. In KinC, these residues include the conserved histidine that is thought to be the site of phosphorylation at position 223, the asparagine at position 331, and the two glycine-rich regions at positions 363 to 367 and 387 to 391.

the right end of the insert in pLK2 and ends at the *Pst*I site and partially suppressed the sporulation phenotype. pLK22 and pLK23 did not suppress the *spo0K* mutant. These results indicate that the suppressing activity is located upstream of the *Apa*I site.

We determined the DNA sequence from the leftmost *Eco*RV site shown in Fig. 2 to the right end of the pLK2 insert. Analysis of the sequence (Fig. 3) revealed two open reading frames. The predicted protein product of the first open reading frame, *kinC*, is 428 amino acids long and is homologous to histidine protein kinases. KinC is most similar to the two histidine protein kinases involved in sporulation, KinA (2, 38) and KinB (58). In the carboxy-terminal 213 amino acids of the proteins, KinC has 111 residues (51%) that are identical to those of KinA, and 81 (36%) that are identical to those of KinB (Fig. 4). The ability to suppress the *spo0K* sporulation defect was localized to the *kinC* open reading frame (as discussed above). pLK21, which partially suppresses the sporulation defect, is truncated in the 5′ end of the open reading frame and probably produces a protein fragment that is likely expressed from a plasmid promoter.

Just downstream of *kinC*, there is an open reading frame predicted to encode a protein of 277 amino acids, Orf277 (Fig. 3). The *orf277* gene product does not appear to be similar to any other protein in the database. In particular, it does not seem to be a member of the response regulator family of proteins (sensor proteins and their cognate response regulators often occur in the same operon [1]), nor is it similar to KapB, the product of the gene downstream of *kinB* (58).

Effects of overexpression of *kinC* in different *spo0* mutants. We tested the ability of multicopy *kinC* (pLK2) to suppress the sporulation defect caused by *spo0* mutations. In all cases, the

TABLE 3. Suppression of *spo0* mutants by multicopy *kinC*

Relevant genotype of strain harboring plasmid	Sporulation frequency[a]	
	pHP13 (vector)	pLK2 (multicopy *kinC*)
WT[b] (JH642)	0.41	0.81
Δ*spo0K*::*erm*	6.0×10^{-3}	0.63
kinA::Tn*917*	9.8×10^{-2}	0.28
spo0A9V	$<9.0 \times 10^{-8}$	$<8.2 \times 10^{-8}$
spo0J93	2.1×10^{-4}	0.21
spo0E11	2.5×10^{-3}	6.9×10^{-3}
*spo0F*Δ*S*	$<2.4 \times 10^{-7}$	9.8×10^{-3}
*spo0B*Δ*Pst*	$<4.1 \times 10^{-7}$	1.1×10^{-2}
*spo0F*Δ*S spo0B*Δ*Pst*	$<1.8 \times 10^{-7}$	2.5×10^{-2}
*spo0F*Δ*S spo0B*Δ*Pst* Δ*spo0E*::*spc*	8.5×10^{-7}	0.22

[a] Cells were grown in DS medium with Cm, and sporulation frequency was determined as described in Materials and Methods.
[b] WT, wild type.

TABLE 4. *kinC* requirements of strains for suppression of *spo0B*Δ*Pst* by *sof-1* or *rvtA11*

Strain	Relevant genotype	Sporulation frequency[a]
JH642	WT[b]	0.59
JRL753	Δ*kinC*::*spc*	0.92
JRL791	Δ*kinC*::*spc rvtA11*	0.51
JRL790	Δ*kinC*::*spc sof-1*	0.70
JRL766	*spo0B*Δ*Pst*	$<4.1 \times 10^{-8}$
JRL763	*spo0B*Δ*Pst sof-1*	3.6×10^{-4}
JRL767	*spo0B*Δ*Pst sof-1* Δ*kinC*	1.4×10^{-7}
JRL764	*spo0B*Δ*Pst rvtA11*	2.1×10^{-3}
JRL768	*spo0B*Δ*Pst rvtA11* Δ*kinC*	$<2 \times 10^{-8}$
JRL783	*spo0F*Δ*S spo0B*Δ*Pst rvtA11*	0.73
JRL792	*spo0F*Δ*S spo0B*Δ*Pst rvtA11* Δ*kinC*	$<1 \times 10^{-7}$
JRL770	*spo0B*Δ*Pst rvtA11 kinA*	1.2×10^{-5}
JRL794	*spo0F*Δ*S spo0B*Δ*Pst rvtA11 kinA*	0.34

[a] Cells were grown in 2×SG medium, and sporulation frequency was determined as described in Materials and Methods.
[b] WT, wild type.

sporulation frequency was measured in otherwise isogenic strains containing the specific sporulation mutation and either pLK2 or the cloning vector, pHP13. pLK2 significantly suppressed the sporulation defect caused by *spo0K*, *spo0J*, *spo0F*, and *spo0B* mutations (Table 3). In addition, it also suppressed the sporulation defect of a *kinA* mutant (Table 3). Multicopy *kinC* did not suppress the sporulation defect of the *spo0A9V* mutant or that of the *spo0E11* mutant. Because multicopy *kinC* is able to significantly bypass the need for *spo0F* and *spo0B* in sporulation, it appears that KinC, at least when overexpressed, is able to act directly on Spo0A.

The effect of pLK2 on the *spo0B* mutant makes it possible to determine the likely target of Spo0E in vivo. Recent in vitro experiments demonstrate that Spo0E is a phosphatase that removes phosphate from Spo0A~P (35). *spo0E11* is a nonsense mutation that produces an N-terminal fragment of Spo0E and inhibits sporulation (41), most likely because of increased phosphatase activity (35).

If the in vivo target of Spo0E is one of the components of the phosphorelay (Spo0F or Spo0B), then *spo0E* mutations should have little or no effect in the absence of a functioning phosphorelay. On the other hand, if the in vivo target of Spo0E is Spo0A, as suggested by the in vitro results (35), then *spo0E* mutations are likely to have effects in the absence of the phosphorelay, if they can be measured. pLK2 partially suppressed the sporulation defect of a *spo0F spo0B* double mutant (Table 3), and this suppression was enhanced by a null mutation in *spo0E* (Table 3; *spo0F spo0B* Δ*spo0E*::*spc*). Since the *spo0E* mutation causes a phenotype in the absence of Spo0F and Spo0B, Spo0E is probably inhibiting Spo0A directly, consistent with the in vitro results.

***kinC* null mutations have no significant effect on sporulation.** To characterize the phenotypes caused by loss of *kinC*, we constructed a strain (JRL660) containing a deletion-insertion mutation in *kinC*, Δ*kinC*::*spc* (Fig. 2; see Materials and Methods). The Δ*kinC*::*spc* mutation had little or no effect on the sporulation frequency, compared with that of the wild type (Table 4). Sporulation conditions tested included 2×SG medium (Table 4), DS medium, minimal exhaustion medium, and minimal medium with decoyinine (data not shown). In addition, the Δ*kinC*::*spc* mutation did not alter the sporulation defect caused by mutations in *kinA*, *spo0K*, or *spo0J* (data not shown). The Δ*kinC*::*spc* mutation also had little or no effect on competence development or expression of *comG-lacZ* compared with that of otherwise isogenic *kinC*$^+$ cells (data not shown).

KinC is required for *sof-1* and *rvtA11* to suppress the sporulation defect of *spo0B* mutants. The *sof-1* and *rvtA11* alleles of *spo0A* bypass or partially bypass the sporulation defect caused by mutations in *spo0F* and *spo0B*, possibly because these altered forms of Spo0A will accept phosphate from kinases independently of Spo0F and Spo0B. To determine whether KinC is one such kinase, we introduced the Δ*kinC*::*spc* mutation into a *spo0B* mutant that had either the *sof-1* or *rvtA11* allele of Spo0A. The *kinC* mutation completely abolished the suppressing effect of either allele (Table 4). In addition, *kinC* was required for *sof-1* and *rvtA11* to suppress *spo0F* mutants (data not shown). *kinC*, and not *orf277* or any other gene downstream of *kinC*, was responsible for this phenotype. Disruption of *orf277* by integrating pLK126 (Fig. 2) by a single crossover had no effect on the ability of *rvtA11* to suppress *spo0F221* (data not shown). These results indicate that KinC is the major kinase that donates phosphate to the *sof-1* and *rvtA11* forms of Spo0A in the absence of Spo0F or Spo0B.

Additional results suggest that KinC can also act on Spo0F and, in some conditions, might prefer Spo0F to Spo0A. The incomplete suppression of the *spo0B* mutant by *sof-1* or *rvtA11* was due to the presence of *spo0F*$^+$. *rvtA11* allowed *spo0F spo0B* double mutants to sporulate at a much higher frequency than the otherwise isogenic *spo0B* single mutant, and this suppression was completely dependent on *kinC* (Table 4). Null mutations in *kinA* significantly reduced the sporulation frequency of the *spo0B rvtA11* strain but had no significant effect on sporulation of the otherwise isogenic *spo0F spo0B rvtA11* strain (Table 4). Together, these results indicate that KinC probably interacts with Spo0F and that the presence of Spo0F inhibits the ability of KinC to donate phosphate to Spo0A^{rvtA11}. Furthermore, the absence of KinA probably increases the amount of unphosphorylated Spo0F, increasing the KinC diverted from Spo0A^{rvtA11} and decreasing the ability of the cells to sporulate. These results suggested that KinC normally can interact with Spo0F and probably contributes to the initiation of sporulation.

Genetic mapping of *kinC*. We used generalized transduction with PBS1 to determine the chromosomal map location of *kinC*. A PBS1 lysate was made from JRL660 (Δ*kinC*::*spc*) and used to transduce the mapping kit strains (13) to Spec^r. The initial results indicated that *kinC* was ~15 to 20% cotransduced with *pyrD*$^+$. A series of two-factor crosses indicated that *kinC* was ~50% linked to *spo0E* and ~50% linked to *kinA*. To map *kinC* with greater resolution, we did a three-factor cross

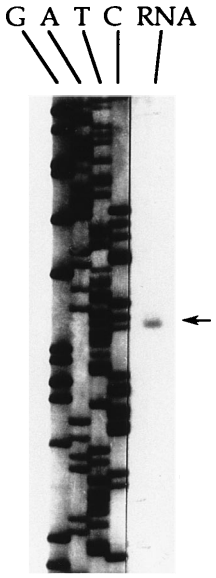

FIG. 5. Primer extension analysis of *kinC* mRNA. RNA was obtained as described in Materials and Methods, and extensions were performed with primer LKP16 (Fig. 3). The sequencing ladder (lanes G, A, T, and C) was constructed from pLK2 by using primer LKP16. The KinC band is indicated by an arrow.

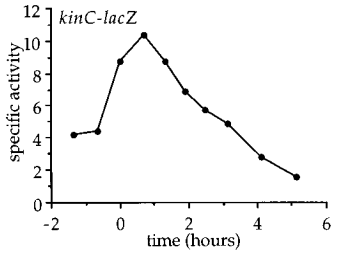

FIG. 6. Expression of *kinC-lacZ*. Strain JRL812 [*thrC*::(*kinC-lacZ erm*)] was grown in 2×SG medium, and samples were taken at the indicated times for determination of β-galactosidase specific activity. Similar results were obtained with cells grown in DS medium, except that specific activity levels were reduced (data not shown).

with Δ*kinC*::*spc*, *spo0E11*, and *kinA*::Tn*917*. Strain KI644 (*kinC*$^+$ *spo0E11 kinA*::Tn*917*) was transduced to Spec^r with a PBS1 lysate grown on JRL660 (Δ*kinC*::*spc spo0E*$^+$ *kinA*$^+$). *spo0E*$^+$ was cotransduced with Δ*kinC*::*spc* in 107 of 199 transductants, and *kinA*$^+$ was cotransduced with Δ*kinC*::*spc* in 147 of 199 transductants. Every *spo0E*$^+$ transductant was also *kinA*$^+$ (MLS^s), indicating that the gene order is *spo0E-kinA-kinC*.

Localization of the promoter and primer extension analysis. Preliminary analysis indicated that the *kinC* promoter was between the *Eco*RV and *Nde*I sites upstream of the *kinC* open reading frame (Fig. 2). This was determined by integrating various plasmids into a *spo0F rvtA11* strain (KI1521). If the insert in the plasmid was internal to the *kinC* transcription unit, then integrating the plasmid would disrupt *kinC* and the strain would become Spo$^-$. If the plasmid extended past the 5′ end of the transcription unit (or past the 3′ end of *kinC*), then integrating the plasmid would not disrupt *kinC* and the cells would remain Spo$^+$. Integration of pLK121 and pLK124 resulted in a Spo$^-$ phenotype, while integration of pLK120 maintained the Spo$^+$ phenotype. These results indicated that the promoter region of *kinC* was probably between the upstream endpoint of pLK120 (*Eco*RV) and the upstream endpoint of pLK121 (*Nde*I).

We performed primer extension analysis, using primer LKP16 (see Materials and Methods; Fig. 3), to localize the 5′ end of the *kinC* mRNA. RNA was prepared from strains grown in 2×SG medium. Despite repeated attempts, we could not detect *kinC*-specific RNA from wild-type cells. However, a *kinC* transcript was easily detected from a strain (JRL951) with multicopy *kinC* (Fig. 5). The 5′ end of the mRNA is ~25 nucleotides upstream of the *kinC* start codon (Fig. 3 and 5). Just upstream of the putative start site is a sequence that matches the consensus for the −10 region of sigma-A promoters in five of six positions (Fig. 3). However, sequences located in the −35 region do not show a striking resemblance to the −35 consensus sequence for sigma-A promoters, perhaps explaining the evidently low level of expression of *kinC*. Just upstream of the putative −10 region is a perfect 0A box, TGNCGAA, the consensus binding site for Spo0A (56). On this basis, one might expect Spo0A or Spo0A~P to act as a repressor of *kinC*. However, a *spo0A* null mutation had relatively little effect on *kinC-lacZ* expression (as discussed below). This could indicate that Spo0A does not control expression of *kinC* or that the effect of the *spo0A* mutation was masked by other regulatory factors.

***kinC* is expressed as cells enter stationary phase.** To determine how *kinC* is normally expressed, we made a *kinC-lacZ* fusion and introduced it into the chromosome by double-crossover recombination at the *thrC* locus by using pLK114 (Fig. 2; see Materials and Methods). The *kinC-lacZ* fusion contains all of the sequences upstream of the *kinC* open reading frame that are present in pLK2. The level of expression of *kinC-lacZ* was low during exponential growth in 2×SG medium and increased as the cells approached stationary phase (Fig. 6). The low level of β-galactosidase specific activity made it difficult to reliably determine quantitative effects of various regulatory mutations on *kinC-lacZ* expression. However, there seemed to be no significant effect of mutations in *spo0A*, *spo0B*, *spo0F*, *spo0H*, *spo0K*, *kinA*, *abrB*, *comP*, *comA*, and *sin* on expression of the *kinC-lacZ* fusion (data not shown). Of course, we cannot rule out the possibility that there is an effect under other conditions or in other strain backgrounds.

DISCUSSION

We have isolated and characterized *B. subtilis kinC*, a gene which when present on a multicopy plasmid suppresses the sporulation defect caused by a null mutation in *spo0K*. Identification of genes on the basis of phenotypes caused by increased expression is a relatively general approach that is easier than and can sometimes be used in place of isolating gain-of-function mutations. In addition, genes that might be difficult to identify on the basis of phenotypes caused by loss-of-function mutations are sometimes easier to identify on the basis of phenotypes caused by overexpression.

The *kinC* gene product is homologous to histidine protein kinases and is most similar to KinA and KinB, the histidine protein kinases involved in the initiation of sporulation (2, 38, 58). On the basis of this homology and the phenotypes caused by overexpression and deletion of *kinC*, we infer that the *kinC* gene product is a histidine protein kinase. *kinC* in multicopy partly bypasses the need for *spo0F* and *spo0B* in sporulation, suggesting that KinC, at least when overexpressed, is able to donate phosphate directly to Spo0A. Mutations in *spo0A* (*sof-1* and *rvtA11*) that bypass the need for the phosphorelay require *kinC* in order to support sporulation. Thus, KinC appears to be the primary kinase responsible for phosphorylation of the *sof-1*

and *rvtA11* forms of Spo0A and for sporulation in these strains in the absence of the phosphorelay.

We suspect that KinC is also able to donate phosphate to Spo0F. Spo0F is a response regulator protein and presumably obtains phosphate in vivo from KinA and KinB. We found that the presence of *spo0F*$^+$ inhibited the ability of a *spo0B rvtA11* mutant to sporulate (Table 4). A simple interpretation of this finding is that Spo0F can compete with Spo0A^{rvtA} for access to KinC~P. This would suggest that KinC might normally play a role in donating phosphate to Spo0F in wild-type cells.

In addition to the three kinases and Spo0F and Spo0B, other proteins also control accumulation of Spo0A~P in vivo, either by regulating activity of the phosphorelay or by acting on Spo0A directly. *spo0E* encodes a phosphatase that is an inhibitor of the initiation of sporulation (35, 41). In vitro, the target of the Spo0E phosphatase is Spo0A~P, suggesting that in vivo Spo0A~P might also be the primary target (35). We used multicopy *kinC* to show that Spo0E can function independently of the phosphorelay (Table 3), indicating that the in vivo target is probably Spo0A~P, consistent with the in vitro results.

One function of the phosphorelay is to integrate the multiple developmental signals that regulate the initiation of sporulation (9, 22, 24–26). One of the developmental signals controlling the activity of the phosphorelay is related to the state of DNA replication. The activity of the phosphorelay is inhibited when DNA replication is inhibited. We were able to use multicopy *kinC* and a *kinC* mutant (previously called *mskA*) to show that the target of the DNA replication control is not Spo0A but probably Spo0F or Spo0B (24).

A role for KinC in sporulation? Null mutations in *kinC* do not cause any obvious defect in sporulation under standard laboratory conditions, suggesting that KinC might not normally be involved in sporulation. However, as discussed above, we suspect that KinC normally contributes some phosphate to Spo0F and is partly redundant with KinA and KinB. While KinA and KinB appear to be responsible for most of the Spo0A~P that accumulates during the initiation of sporulation, there is some Spo0A~P in even a *kinA kinB* double mutant (58) and we suspect that this comes from KinC. In addition, there are many ways for cells to deplete nutrients and enter the sporulation pathway. It seems possible that the relative contributions of the individual kinases might vary depending on the specific condition that induces sporulation.

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REFERENCES

1. **Albright, L. M., E. Huala, and F. M. Ausubel.** 1989. Prokaryotic signal transduction mediated by sensor and regulator protein pairs. Annu. Rev. Genet. **23:**311–336.
2. **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.
3. **Ausubel, F., R. Brent, R. Kingston, D. Moore, J. Seidman, J. Smith, and K. Struhl (ed.).** 1990. Current protocols in molecular biology. John Wiley & Sons, New York.
4. **Baldus, J. M., B. D. Green, P. Youngman, and C. P. Moran, Jr.** 1994. Phosphorylation of *Bacillus subtilis* transcription factor Spo0A stimulates transcription from the *spoIIG* promoter by enhancing binding to weak 0A boxes. J. Bacteriol. **176:**296–306.
5. **Benson, A. K., and W. G. Haldenwang.** 1993. Regulation of σ^B levels and activity in *Bacillus subtilis*. J. Bacteriol. **175:**2347–2356.
6. **Bird, T. H., J. K. Grimsley, J. A. Hoch, and G. B. Spiegelman.** 1993. Phosphorylation of Spo0A activates its stimulation of *in vitro* transcription from the *Bacillus subtilis spoIIG* operon. Mol. Microbiol. **9:**741–749.
7. **Boylan, S. A., K. T. Chun, B. A. Edson, and C. W. Price.** 1988. Early-blocked sporulation mutations alter expression of enzymes under carbon control in *Bacillus subtilis*. Mol. Gen. Genet. **212:**271–280.
8. **Bron, S.** 1990. Plasmids, p. 75–174. *In* C. R. Hardwood and S. M. Cutting (ed.), Molecular biological methods for *Bacillus*. John Wiley and Sons, Chichester, England.
9. **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.
10. **Chang, C., S. F. Kwok, A. B. Bleecker, and E. M. Meyerowitz.** 1993. *Arabidopsis* ethylene-response gene *ETR1*: similarity of product to two-component regulators. Science **262:**539–544.
11. **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.
12. **Cutting, S. M., and P. B. Vander Horn.** 1990. Genetic analysis, p. 27–74. *In* C. R. Hardwood and S. M. Cutting (ed.), Molecular biological methods for *Bacillus*. John Wiley and Sons, Chichester, England.
13. **Dedonder, R. A., J. A. Lepesant, J. Lepesant-Kejzlarova, A. Billault, M. Steinmetz, and F. Kunst.** 1977. Construction of a kit of reference strains for rapid genetic mapping in *Bacillus subtilis* 168. Appl. Environ. Microbiol. **33:**989–993.
14. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
15. **Ferrari, F. A., A. Nguyen, D. Lang, and J. A. Hoch.** 1983. Construction and properties of an integrable plasmid for *Bacillus subtilis*. J. Bacteriol. **154:** 1513–1515.
16. **Grossman, A. D., T. Lewis, N. Levin, and R. De Vivo.** 1992. Suppressors of a *spo0A* missense mutation and their effects on sporulation in *Bacillus subtilis*. Biochimie **74:**679–688.
17. **Gryczan, T., S. Contente, and D. Dubnau.** 1980. Molecular cloning of heterologous chromosomal DNA by recombination between a plasmid vector and a homologous resident plasmid in *Bacillus subtilis*. Mol. Gen. Genet. **177:**459–467.
18. **Haima, P., S. Bron, and G. Venema.** 1987. The effect of restriction on shotgun cloning and plasmid stability in *Bacillus subtilis* Marburg. Mol. Gen. Genet. **209:**335–342.
19. **Henner, D. J.** 1990. Inducible expression of regulatory genes in *Bacillus subtilis*. Methods Enzymol. **185:**223–228.
20. **Hoch, J. A.** 1993. Regulation of the phosphorelay and the initiation of sporulation in *Bacillus subtilis*. Annu. Rev. Microbiol. **47:**441–465.
21. **Hoch, J. A., K. Trach, F. Kawamura, and H. Saito.** 1985. Identification of the transcriptional suppressor *sof-1* as an alteration in the Spo0A protein. J. Bacteriol. **161:**552–555.
22. **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.
23. **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.
24. **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.
25. **Ireton, K., N. W. Gunther IV, and A. D. Grossman.** 1994. *spo0J* is required for normal chromosome segregation as well as the initiation of sporulation in *Bacillus subtilis*. J. Bacteriol. **176:**5320–5329.
26. **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.
27. **Itaya, M., K. Kondo, and T. Tanaka.** 1989. A neomycin resistance cassette selectable in a single-copy state in *B. subtilis*. Nucleic Acids Res. **17:**4410.
28. **Jaacks, K. J., J. Healy, R. Losick, and A. D. Grossman.** 1989. Identification and characterization of genes controlled by the sporulation regulatory gene *spo0H* in *Bacillus subtilis*. J. Bacteriol. **171:**4121–4129.
29. **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.
30. **Kenney, T. J., and C. P. Moran, Jr.** 1987. Organization and regulation of an operon that encodes a sporulation-essential sigma factor in *Bacillus subtilis*. J. Bacteriol. **169:**3329–3339.
31. **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.

32. **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.
33. **Magnuson, R., J. Solomon, and A. D. Grossman.** 1994. Biochemical and genetic characterization of a competence pheromone from *B. subtilis*. Cell **77:**207–216.
34. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
35. **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.
36. **Ota, I. M., and A. Varshavsky.** 1993. A yeast protein similar to bacterial two-component regulators. Science **262:**566–569.
37. **Parkinson, J. S., and E. C. Kofoid.** 1992. Communication modules in bacterial signaling proteins. Annu. Rev. Genet. **26:**71–112.
38. **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.
39. **Perego, M., C. F. Higgins, S. R. Pearce, M. P. Gallagher, and J. A. Hoch.** 1991. The oligopeptide transport system of *Bacillus subtilis* plays a role in the initiation of sporulation. Mol. Microbiol. **5:**173–185.
40. **Perego, M., and J. A. Hoch.** 1987. Isolation and sequence of the *spo0E* gene: its role in initiation of sporulation in *Bacillus subtilis*. Mol. Microbiol. **1:**125–132.
41. **Perego, M., and J. A. Hoch.** 1991. Negative regulation of *Bacillus subtilis* sporulation by the *spo0E* gene product. J. Bacteriol. **173:**2514–2520.
42. **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 *spo0A* sporulation gene in *Bacillus subtilis*. Mol. Microbiol. **2:**689–699.
43. **Piggot, P. J., and J. G. Coote.** 1976. Genetic aspects of bacterial endospore formation. Bacteriol. Rev. **40:**908–962.
44. **Robertson, J. B., M. Gocht, M. A. Marahiel, and P. Zuber.** 1989. AbrB, a regulator of gene expression in *Bacillus*, interacts with the transcription initiation regions of a sporulation gene and an antibiotic biosynthesis gene. Proc. Natl. Acad. Sci. USA **86:**8457–8461.
45. **Rudner, D. Z., J. R. LeDeaux, K. Ireton, and A. D. Grossman.** 1991. The *spo0K* locus of *Bacillus subtilis* is homologous to the oligopeptide permease locus and is required for sporulation and competence. J. Bacteriol. **173:**1388–1398.
46. **Sadaie, Y., and T. Kada.** 1983. Formation of competent *Bacillus subtilis* cells. J. Bacteriol. **153:**813–821.
47. **Sandman, K., R. Losick, and P. Youngman.** 1987. Genetic analysis of *Bacillus subtilis spo* mutations generated by Tn*917*-mediated insertional mutagenesis. Genetics **117:**603–617.
48. **Satola, S., P. A. Kirchman, and C. P. Moran, Jr.** 1991. Spo0A binds to a promoter used by sigma-A RNA polymerase during sporulation in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **88:**4533–4537.
49. **Satola, S. W., J. M. Baldus, and C. P. Moran, Jr.** 1992. Binding of Spo0A stimulates *spoIIG* promoter activity in *Bacillus subtilis*. J. Bacteriol. **174:**1448–1453.
50. **Schaeffer, P., J. Millet, and J. Aubert.** 1965. Catabolite repression of bacterial sporulation. Proc. Natl. Acad. Sci. USA **54:**704–711.
51. **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.
52. **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.
53. **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.
54. **Spizizen, J.** 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. Proc. Natl. Acad. Sci. USA **44:**1072–1078.
55. **Strauch, M. A., G. B. Spiegelman, M. Perego, W. C. Johnson, D. Burbulys, and J. A. Hoch.** 1989. The transition state transcription regulator *abrB* of *Bacillus subtilis* is a DNA binding protein. EMBO J. **8:**1615–1621.
56. **Strauch, M. A., V. Webb, G. Spiegelman, and J. A. Hoch.** 1990. The Spo0A protein of *Bacillus subtilis* is a repressor of the *abrB* gene. Proc. Natl. Acad. Sci. USA **87:**1801–1805.
57. **Trach, K., D. Burbulys, M. Strauch, J.-J. Wu, N. Dhillon, R. Jonas, C. Hanstein, C. Kallio, M. Perego, T. Bird, G. Spiegelman, C. Fogher, and J. A. Hoch.** 1991. Control of the initiation of sporulation in *Bacillus subtilis* by a phosphorelay. Res. Microbiol. **142:**815–823.
58. **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.
59. **Weir, J., M. Predich, E. Dubnau, G. Nair, and I. Smith.** 1991. Regulation of *spo0H*, a gene coding for the *Bacillus subtilis* sH factor. J. Bacteriol. **173:**521–529.
60. **Wu, J.-J., M. G. Howard, and P. J. Piggot.** 1989. Regulation of transcription of the *Bacillus subtilis spoIIA* locus. J. Bacteriol. **171:**692–698.
61. **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.
62. **York, K., T. J. Kenney, S. Satola, C. P. Moran, Jr., H. Poth, and P. Youngman.** 1992. Spo0A controls the σ^A-dependent activation of *Bacillus subtilis* sporulation-specific transcription unit *spoIIE*. J. Bacteriol. **174:**2648–2658.
63. **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. A. Slepecky, and P. Setlow (ed.), Regulation of procaryotic development. American Society for Microbiology, Washington, D.C.
64. **Zuber, P., and R. Losick.** 1987. Role of AbrB in Spo0A- and Spo0B-dependent utilization of a sporulation promoter in *Bacillus subtilis*. J. Bacteriol. **169:**2223–2230.