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Structural Alterations in the *Bacillus subtilis* Spo0A Regulatory Protein Which Suppress Mutations at Several *spo0* Loci†

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Secondary site mutations that restore sporulation to sporulation-defective *spo0F* or *spo0B* deletion mutants were found to reside in the *spo0A* gene. Sequence analysis of 23 such *sof* mutants showed that the *sof* mutations fell into six classes of missense codon changes, primarily in the conserved amino-terminal domain of the response regulator Spo0A protein. Changes were observed in codons 12, 14, 60, 92, and 121. The residues affected were predominantly located in the potential turn regions at one end of the amino-terminal conserved domain on the same topological face as the active site aspartate residues. The ability of *sof* mutations to suppress deficiencies in the transmitter kinases, KinA and KinB, of two-component regulatory systems was tested. All of the *sof* mutations suppressed the sporulation deficiency of *kinA* mutants but only two classes among five tested suppressed *kinB* mutations. *sof* mutants segregated Spo$^-$ colonies at high frequency. Five of these Spo$^-$ mutants were found to result from mutations in the *spo0A* locus that reversed the effect of the *sof* mutation. One of these was sequenced and found to have the original *sof* mutation and a new mutation, *sos*, at codon 105. The accumulation of *sos* mutations in *sof* strains suggested that the *sof* mutations have a subtle, yet deleterious, effect on the growth of the cell. The results suggested that the *sof* mutations increase the avidity for or reactivity with transmitter kinases in an allele-specific manner, although in some cases it is possible that the *sof* mutations obviate the need for phosphorylation to activate the Spo0A protein. An alternative hypothesis is presented in which the *sof* mutations play the role of bypass mutations for kinases.

Sporulation is the result of complex interactions of developmental processes which lead to the formation of dormant, heat-resistant spores. In *Bacillus subtilis*, the products of the following eight genes appear to control the onset of sporulation: *spo0A*, *spo0B*, *spo0E*, *spo0F*, *spo0H*, *spo0J*, *spo0K*, and *spo0L* (8, 20; and Hoch, unpublished data). Mutations in each of these genes block sporulation at the earliest stage, stage 0, and cause a wide variety of pleiotropic phenotypes (4). Since mutations in the *spo0A* gene are the most pleiotropic among the *spo0* genes, it is possible that the *spo0A* gene product plays a major role in transcriptional activation of the differentiation process. The Spo0A and Spo0F proteins have homology to response regulators of two-component regulatory systems which presumably respond to environmental or metabolic stimuli (6, 16, 29). Unlike most two-component regulatory systems, the *spo0A* and *spo0F* genes are not linked to a gene with homology to the signal transmitter kinases, which activate the response regulators by phosphorylation (7, 15). Thus, the identity of the kinase(s) responsible for phosphorylation of Spo0F and Spo0A is open to speculation. Recently, a kinase gene (also known as *kinA*, *spoIIJ*), which has a sporulation defective phenotype when mutated, was cloned and sequenced (2, 18). The purified kinase from this gene is a highly effective phosphate donor to the Spo0F protein and can phosphorylate the Spo0A protein, albeit at a lower catalytic rate (18). Based on enzymatic and genetic evidence, it was postulated that Spo0A is phosphorylated by more than one kinase in an environment-dependent manner (18).

Sporulation-proficient second-site suppressors (*sof*) of null mutations in the *spo0B* or *spo0F* gene have been isolated and mapped to the region of the *spo0A* gene (11, 23, 24). A missense point mutation near the amino terminus of the *spo0A* gene has been shown to be responsible for the restoration of sporulation by one *sof* suppressor (9). These subtle alterations in the structure of the Spo0A protein suppress the requirement for the products of the *spo0B*, *spo0E*, *spo0F*, and *spo0K* loci in sporulation (11, 24). Thus, it has been postulated that the products of *spo0* genes serve to activate the Spo0A protein (9), presumably by an environmentally sensitive signal transduction system. The exact mechanism of activation is unclear, although the ultimate step in Spo0A activation is thought to be a phosphorylation reaction (18), and none of the *spo0* suppressed genes encodes a protein with homology to the kinases of two-component systems (15, 16).

In this communication we report characterization of 23 *sof* mutations in the *spo0A* gene. These mutations suppress the *spo0* mutants to various degrees. In some cases the *sof* mutants suppress mutations in the genes for kinases that are known to be required for sporulation.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and transformation. *B. subtilis* strains used in this study are shown in Table 1. *B. subtilis* competent cells were prepared and transformed by the method of Anagnostopoulos and Spizizen (1). Samples of 0.1 μg of chromosomal DNA and approximately 1 to 2 μg of plasmid DNA were used in transformation. Selection for Cm^r or Km^r was on Schaeffer agar plates (22) supplemented

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

Strain	Genotype or description
W168	Prototroph
JH642	*trpC2 phe-1*
JH646MS	*trpC2 phe-1 spo0A12 abrB15*
JH647	*trpC2 phe-1 spo0E11*
JH648	*trpC2 phe-1 spo0B136*
JH649	*trpC2 phe-1 spo0F221*
JH651	*trpC2 phe-1 spo0H81*
JH12578	*trpC2 Δspo0F*
UOTO550	*trpC2 metB51 leuA8 nonB1 Δspo0F sof-1*
JH12704	*trpC2 phe-1 ermG*
JH12638	*trpC2 phe-1 kinA*::Tn*917ΩHU19*
JH12662	*kinA*::pJM8115 (in W168)
JH13170	*kinB*::pJH4906 (in W168)
JH13145	*kinA*::pJM8115 *kinB*::pJH4906
JH13051	*trpC2 phe-1 spo0B*::*cat*

with 5 μg of chloramphenicol per ml or 2 μg of kanamycin per ml, respectively. Selection for Erm^r was done as previously described (19). Strain JH12578 was constructed by transforming strain JH642 with 7.5 μg of chromosomal DNA from strain UOTO550 (11). Selection was for phenylalanine on minimal medium agar plates supplemented with glucose and tryptophan; integration of the unlinked marker *Δspo0F* by congression was checked on Schaeffer sporulation agar plates. Strain JH12704 was constructed by transformation of strain JH642 with pJB2000 linearized with *Acc*I. Selection was for Erm^r. The correct integration of the Erm^r marker via double-crossover recombination was checked with Southern blot analysis (data not shown). The linkage between *spo0A* and *ermG* was determined by two-factor cross recombination analysis. The recipient strain JH646MS (*spo0A* Erm^s) was transformed with 0.1 μg of chromosomal DNA from strain JH12704. Transformants were selected for Erm^r and then scored for Spo$^+$ on Schaeffer sporulation agar plates or selected for Spo$^+$ and then scored for Erm^r on Schaeffer plates containing 1 μg of erythromycin per ml. For the Spo$^+$ selection, competent cells were incubated in transformation medium in the presence of donor DNA for 1.5 h at 37°C. Then 3 ml of Schaeffer sporulation broth was added, and the culture was incubated overnight. Cells were then treated with $CHCl_3$ and plated on Schaeffer agar plates. The results of the linkage mapping are shown in Fig. 1. Construction of strain JH13051 was described previously by Trach and Hoch (30).

Efficiency of sporulation of *B. subtilis* strains carrying the *sof* mutations was measured by growing the cultures in 5 ml of Schaeffer medium at 37°C for 24 h. Serial dilutions were plated before and after treatment with $CHCl_3$, and the number of surviving cells was compared with the total number of viable cells.

Escherichia coli DH5α competent cells (Bethesda Research Laboratories, Inc.) were used for plasmids construction and propagation. *E. coli* cultures were grown in LB medium supplemented with 100 μg of ampicillin per ml or 20 μg of kanamycin per ml.

Mutagenesis. Suppressor mutations of *Δspo0F* and *Δspo0B* strains were isolated by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis. Cultures of strains JH12578 and JH13051 were grown to the midlog phase in Schaeffer medium. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine was added to 0.1 μg/ml (final concentration), and the cultures were subdivided in small samples. Cells were grown for 18 h at 37°C, treated with $CHCl_3$ for 30 min and then plated on Schaeffer agar plates. Strains designated SOF103, SOF108, etc., are genotypically *trpC2 Δspo0F sof-103*, *trpC2 Δspo0F sof-108*, etc., respectively.

DNA manipulations. *B. subtilis* chromosomal DNA was prepared by the method of Marmur (13) with some modifications. Plasmid DNA was purified by the alkaline method of Birnboim and Doly (3). Rapid plasmid DNA preparation was by the method of Holmes and Quigley (10). Plasmids used in this study were as follows: pJH1408 (9), which contained a truncated copy of the *spo0A* gene; pJB2000, which is a derivative of pBR328 carrying a 2.75-kilobase *Eco*RI fragment containing the carboxyl end of the *spo0A* gene and its downstream region; pJB2031, pJB2032, and pJB2033, which are derivatives of pJM113 (pUC19 carrying the Km^r gene from *Streptococcus faecalis* [31] in the *Nde*I site [Perego and Hoch, unpublished data]) containing, respectively, the *Cla*I-*Acc*I, *Cla*I-*Eco*RI, and *Cla*I-*Bgl*II fragments from the *spo0A* chromosomal region (Fig. 1).

Cloning of the *sof* mutations was carried out by transforming *sof* strains with plasmid pJH1408, selecting for Cm^r. Chromosomal DNA from one Spo$^+$ Cm^r colony was prepared; 1 μg was digested to completion with *Eco*RI, phenol extracted, ethanol precipitated, and suspended in 100 μl of 1× ligation buffer (DNA final concentration, 10 μg/ml), and T4 ligase (Bethesda Research Laboratories) was added. Religated plasmids were recovered by transformation into *E. coli* DH5α competent cells, selecting for ampicillin resistance.

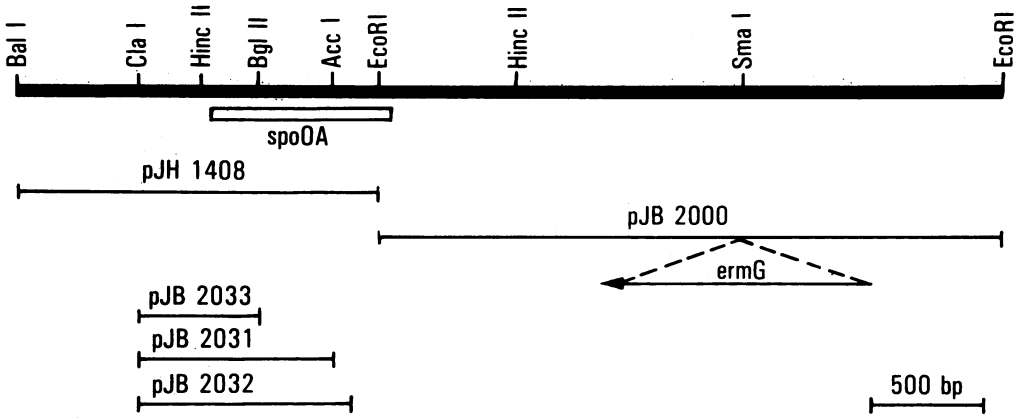

FIG. 1. Restriction map of the *spo0A* locus. The extents of the DNA inserts in the indicated plasmids are shown by lines.

Sequence analysis. Sequence analysis of the *sof* mutations was performed by the supercoiled sequencing method of Chen and Seeburg (5). Chain elongation reactions were conducted with Sequenase (U.S. Biochemical Corp.) or T7 Polymerase (Pharmacia Fine Chemicals). The following oligonucleotides were used as primers: 5'-GATATGCCAC TAATATTGG-3', 5'-CCCGATGTGCTCGTATT-3', and 5'-TGGGACGCCGATTTCAT-3'.

Oligonucleotides were provided by the Genentech Organic Synthesis Group or by the Scripps Clinic and Research Foundation Core Laboratory.

RESULTS

Isolation and genetic location of *sof* and *sob* suppressor mutations. We constructed a strain carrying the *spo0F* deletion mutation of Kawamura and Saito (11) to isolate extragenic rather than intragenic *spo0F* suppressors. Strain JH642 (*trpC2 phe-1*) was transformed with chromosomal DNA from strain UOTO550. Transformants were selected for an unlinked marker (Phe^+) and scored for the transfer of the Δ*spo0F* marker by congression. This strain, JH12578, was treated with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, grown in Schaeffer sporulation medium for 18 h at 37°C, exposed to $CHCl_3$ for 30 min, and plated on Schaeffer agar plates. Eighteen independent Spo^+ survivors were purified.

Genetic analyses on the putative *sof* suppressor mutants were first carried out by transforming each mutant strain with plasmid pJH1408, which contains a C-terminal truncated copy of the wild-type *spo0A* gene (Fig. 1). After integration of this plasmid by Campbell-type recombination, both Spo^+ and Spo^- colonies would be expected if the *sof* mutation resided in the chromosomal *spo0A* gene on the plasmid, since this type of recombination event would give rise to only one intact copy of the *spo0A* gene, which may or may not contain the *sof* allele. For the 18 suppressor strains tested, 50 to 90% of the Cm^r transformants obtained were Spo^-, indicating that all the *sof* suppressor mutations were localized in the *Bal*I-*Eco*RI chromosomal region carried by pJH1408. Since Spo^- colonies were obtained, these results also confirmed that these mutants still contained the original *spo0F* deletion mutation and therefore that they were authentic *spo0F* suppressors.

To isolate extragenic suppressors of an *spo0B* mutation (*sob*), we constructed a Δ*spo0B*-Cm insertion mutant strain, JH13051. Wild-type strain JH642 was transformed with linearized plasmid pJH4670 (30), selecting for Cm^r. The correct integration of pJH4670 via a double-crossover event resulting in the replacement of the wild-type *spo0B* gene with the chloramphenicol resistance gene inserted in a deleted copy of *spo0B* was checked by Southern blot analysis (data not shown).

JH13051 was subjected to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis, and sporulation-proficient colonies were selected; 131 independent Cm^r Spo^+ colonies were obtained, and all 95 of those tested were localized in the *spo0A* gene by transformation linkage mapping. In this case, suppressor strains (*sob*) were transformed with chromosomal DNA from strain JH12704, which contains an *erm* gene integrated downstream of the *spo0A* gene at the *Sma*I site. The construction of this strain is described below. Approximately 65 to 90% of the Erm^r transformants obtained were Spo^-, as expected for the introduction of a wild-type copy of the *spo0A* gene, which restored the Spo0 phenotype due to the *spo0B*::*cat* mutation. The remaining 36 suppressor strains showed transformability impairment, which prevented their mapping and analysis. A more precise localization of the *sob* mutations was obtained by transforming 33 *sob*-bearing strains with plasmids pJB2031, pJB2032, and pJB2033 (Fig. 1). After integration by Campbell-type recombination, these three plasmids gave rise to Spo^+ Km^r and Spo^- Km^r colonies, indicating that the *sob* mutations were localized in the *Cla*I-*Bgl*II fragment carried by pJB2033. This fragment contained the amino-terminal end of the *spo0A* gene.

TABLE 2. Codon and amino acid changes in suppressor mutations

Class	Suppressor allele(s)	Mutated codon	Codon change	Amino acid substitution
1	*sof-102*, *sof-103*, *sof-105*, *sof-113*, *sof-120*	12	AAT→AAA	Asn→Lys
2	*sof-107*, *sof-111*, *sof-115*, *sof-123*, *sof-124*, *sob-64*	14	GAG→GTG	Glu→Val
3	*sof-115*	14	GAG→GCG	Glu→Ala
4	*sof-118*, *sob-27*, *sob-87*, *sob-114*, *sob-137*, *sob-146*, *sob-160*, *sob-163*	60	CCG→TCG	Pro→Ser
5	*sof-114*	92	GAT→TAT	Asp→Tyr
6	*sof-108*	121	CAG→CGG	Gln→Arg

Cloning and sequencing of *sof* and *sob* mutations. To characterize the molecular origin of the suppressor mutations, we cloned and sequenced the mutant *spo0A* gene carrying the *sof* or *sob* mutation. Transformation of *sof* mutants with plasmid pJH1408 and selection for Cm^r gave rise to Spo^- and Spo^+ colonies. Since pJH1408 contains a truncated *spo0A* gene, only the Spo^+ transformants will have the *sof* mutation in the complete copy of the *spo0A* gene, which allows cloning of the mutant gene by rescue of the plasmid. To rescue the integrated plasmid carrying the *sof* mutation, this chromosomal DNA from one Spo^+ Cm^r colony was digested to completion with *Eco*RI and ligated to reform circular plasmids. Religated plasmids carrying the *sof* mutations (pSOF$_X$) were recovered by transformation into *E. coli* competent cells, selecting for ampicillin resistance. The plasmids obtained were checked for the presence of the correct insert by restriction enzyme digestions and then subjected to sequence analysis.

Cloning of *sob* mutations was carried out by the same method used for the cloning of *sof* mutations with a sporulation-proficient colony obtained after integration of plasmid pJB2032 in *sob* mutants. Plasmids from 14 *sof* and 9 *sob* mutants were recovered, and studies of their sequence revealed that all suppressor phenotypes could be attributed to missense mutations in the amino-terminal half of the *spo0A* gene (Table 2). The mutations were grouped into seven classes according to base substitutions and corresponding amino acid changes (Table 2).

(i) Class 1. Transversion from T to A at codon 12, which changed an asparagine residue into a lysine residue. This mutation was observed in five suppressors. We designated the *sof-103* mutation as the representative of this class. This mutation was the same as the previously described *spo0F* suppressor mutations *sof-1* (11, 32) and *sur0F1* (24). We observed a difference in colony morphology, also mentioned by Shoji et al. (24), between the class 1 *sof* mutants and the *sof-1* mutant described by Kawamura and Saito (11), which may be due to differences in genetic backgrounds.

(ii) Class 2. Transversion from A to T at codon 14, resulting in a glutamate-to-valine substitution. This mutation

TABLE 3. Suppression of the sporulation-deficient phenotype of strain JH12578 (Δ*spo0F*) by *sof* mutations

Strain	Suppressor class	Total cells/ml	Spores/ml	%[a]
JH12578::pJH1408		2.0×10^8	0	0
JH12578::pSOF103	1	4.0×10^8	2.4×10^7	6
JH12578::pSOF111	2	1.6×10^8	8.0×10^7	6
JH12578::pSOF115	3	1.1×10^8	5.4×10^7	49
JH12578::pSOF118	4	3.5×10^8	1.3×10^6	0.37
JH12578::pSOF114	5	2.6×10^8	8.8×10^5	0.33
JH12578::pSOF108	6	5.9×10^8	2.8×10^3	0.0004

[a] Percentage is expressed as 100 × the ratio between spores per milliliter and total cells per milliliter.

occurred in six suppressors. The *sof-111* mutation was designated as the representative of class 2. This mutation is identical to the *sur0B20* mutation of Shoji et al. (24).

(iii) Class 3. Transversion from A to C at codon 14, changing glutamate into alanine. This mutation was found in one strain, *sof-115*.

(iv) Class 4. Transition from C to T at codon 60, which substituted a proline with a serine. This mutation was very common among the *sob* mutations analyzed. *sof-118* was used as the representative of this class.

(v) Class 5. Transversion from G to T at codon 92, which resulted in an aspartate to tyrosine change. *sof-114* was the only mutation for this class.

(vi) Class 6. Transition from A to G at codon 121, which changed a glutamine residue into an arginine residue. *sof-108* was the only mutation found for this class.

(vii) Class 7. Transversion from A to T at codon 145, changing a glutamate to a valine residue. *sob-2* was the only representative of this class.

Efficiency of suppression of the ΔSpo0F phenotype by the *sof* mutations. To quantitate the level of suppression of the ΔSpo0F phenotype by the various classes of *sof* mutations, we transformed strain JH12578 with one pSOF plasmid for each class of mutation (pSOF103, pSOF108, pSOF111, pSOF114, pSOF115, pSOF118), selecting for Cm^r. The class 7 suppressor was not tested in this and subsequent experiments. Since pSOF plasmids carry an interrupted copy of the *spo0A* gene with the *sof* mutations, after integration by a single crossover, Spo$^-$ and Spo$^+$ colonies were obtained. One JH12578 Spo$^+$ transformant for each pSOF plasmid was grown, and the efficiency of sporulation was determined (Table 3). Sporulation frequencies ranging from 0.0004 to 49% were obtained, depending on the *sof* mutation. No spores were obtained from the control strain JH12578 carrying an integrated copy of plasmid pJH1408.

Construction of isogenic *sof* strains. To insure against a possible effect of the truncated *spo0A* gene product on the regulation of sporulation and interpretation of the phenotypes of *sof* mutations, we constructed isogenic strains with a *sof* mutation within a single-copy *spo0A* gene. A strain was designed with a selectable marker linked to the *spo0A* gene, which could be used in genetic manipulations. To this end we constructed a plasmid (pJB2000) carrying the *ermG* gene from *Bacillus sphaericus* (14) in the *Sma*I site located 1.5 kilobases downstream of the stop codon of the *spo0A* gene. Linearized plasmid pJB2000 (Fig. 1) was transformed into strain JH642, selecting for Erm^r. An Erm^r Cm^s transformant resulting from a double-crossover event as determined by Southern blot analysis (data not shown) placed the *ermG* marker on the *B. subtilis* chromosome and became strain JH12704.

The linkage between the *spo0A* and *ermG* markers was tested by transformation in strain JH646MS with chromosomal DNA from strain JH12704. Analysis of two-factor transformation crosses with selection for Erm^r or Spo$^+$ gave an average of 20% recombination. JH12704 chromosomal DNA was used to transform strains SOF103, SOF108, SOF111, SOF114, SOF115, SOF118, and UOTO550, selecting for Erm^r. Approximately 15% of the Erm^r transformants remained sporulation proficient, as expected from the linkage analysis. A Spo$^+$ (i.e., Sof$^+$) transformant was used as a donor in a backcross to the parent strain, JH12578, to eliminate unlinked mutations introduced during the mutagenesis procedure.

Chromosomal DNA from one Erm^r Spo$^+$ transformant for each of the seven *sof* mutants was prepared and used to transform into strains JH12578 and JH642. The Erm^r transformants from strain JH12578 were scored for the ability to sporulate on Schaeffer agar plates as the result of the cotransfer of the *sof* mutation with the *ermG* gene. The isogenic JH12578 *sof* strains exhibited the *sof*-associated phenomenon of Spo$^-$ segregation, which is described below. Cotransfer of the *sof* mutations with the erythromycin marker into strain JH642 was detected by the occurrence of Spo$^-$ segregants, since both donor and recipient were Spo$^+$. The sporulation proficiency associated with the *sof* mutations was cotransferred with the Erm^r marker at a frequency of 85%. One representative Erm^r Spo$^+$ colony for each *sof* mutation (JH12578 *sof-103*, JH12578 *sof-108*, JH12578 *sof-111*, JH12578 *sof-114*, JH12578 *sof-115*, JH12578 *sof-118*, JH12578 *sof-1*) was analyzed for the level of suppression of the Δ*spo0F* phenotype by carrying out a sporulation frequency assay as previously described. The results were comparable to those previously obtained with strain JH12578 derivatives harboring the integrated pSOFχ plasmids (Table 3).

We went to this considerable effort to construct single-copy *sof* mutants in *spo0A* to determine whether the C-terminal truncated Spo0A protein generated by the Campbell insertion of integrative plasmid was affecting our results. It is clear that this was not the case.

Suppression of *spo0* mutations by *sof-111*. One representative *sof* mutation (*sof-111*) in codon 14 was used to test the ability of *sof* mutations to suppress the sporulation-deficient phenotype of stage 0 mutants. Chromosomal DNA from strain JH12578 *sof-111* was used to transform strains JH647 (*spo0E*), JH648 (*spo0B*), JH649 (*spo0F*), and JH651 (*spo0H*), selecting for Erm^r. Cotransfer of the *sof-111* mutation with the Erm^r marker was detected on Schaeffer sporulation medium: approximately 80 to 85% of the transformants obtained lost the typical Spo0 phenotype (transparency), became more or less opaque depending on the level of suppression by *sof-111* on each *spo0* mutant, and began to segregate *sos* suppressor strains (see below). One such colony for each strain was used in a liquid quantitation analysis of the efficiency of suppression of the Spo0 phenotype by the *sof-111* mutation (Table 4). The absolute number of spores observed can vary considerably from one experiment to the next. Much of this variability is caused by the accumulation of *sos* mutations, which are phenotypically Spo$^-$ and are described below.

sof-111 can partially restore the ability to sporulate in *spo0E*, *spo0B*, and *spo0F* mutant strains, but it cannot overcome the requirement for the *spo0H* gene product, the sigma factor *sigH*. It can also suppress an Spo0K mutant (data not shown). This is similar to the results found with the *sur0B20* allele (24). Similar results were obtained when the

TABLE 4. Suppression of sporulation-deficient phenotypes in isogenic stage 0 mutants by *sof-111*

Strain	Total cells/ml	Spores/ml	%[a]
JH642	2.0×10^8	1.6×10^8	80
JH647	5.8×10^8	9.0×10^4	0.02
JH647 *sof-111*	1.3×10^9	1.1×10^7	0.85
JH648	7.0×10^8	0	0
JH648 *sof-111*	6.7×10^8	8.7×10^7	13
JH649	1.0×10^9	0	0
JH649 *sof-111*	1.4×10^8	8.7×10^7	62
JH651	4.8×10^8	0	0
JH651 *sof-111*	5.8×10^8	0	0

[a] Percentage is expressed as 100 × the ratio between spores per milliliter and total cells per milliliter.

remaining five classes of *sof* mutations were tested for their ability to suppress the stage 0 sporulation mutants (data not shown).

Characterization of *sos* mutations. Strains carrying a copy of the *spo0A* gene containing a *sof* mutation segregated sporulation-deficient colonies at a high rate. These segregants were called *sos* mutants for suppressor of *sof*. Their occurrence was associated with the appearance of small, translucent papillae on the surface of the colonies (Fig. 2). These could be isolated by simply streaking out the segregating colony on sporulation agar plates to obtain single colonies: sporulation-deficient segregants appeared among the sporulation-proficient colonies.

Several possibilities exist for the occurrence of such segregants, including *sof* revertants and intragenic or extragenic suppressors of *sof* mutations. We investigated the nature of several of the *sos* mutations by mapping them, followed by cloning and sequencing one of them. One *sos* segregant was isolated for each of the seven *sof* alleles in the JH642 background. These strains were called JH642 *sofX sosX*, where *X* indicates the *sof* and *sos* allele. Strains JH642 *sofX sosX* were first transformed with the corresponding parental *sof* allele by using the pSOFχ plasmids to check the possibility that *sos* mutations were the result of an intragenic suppression event. If this were the case, Spo^+ and Spo^- Cm^r colonies would be obtained after integration of the plasmid via Campbell-type recombination. In fact these were the results obtained for six (JH642 *sof-1 sos-1*, JH642 *sof-103 sos-103*, JH642 *sof-108 sos-108*, JH642 *sof-111 sos-111*, JH642 *sof-114 sos-114*, JH642 *sof-118 sos-118*) of the seven *sof sos* mutants tested, indicating that the *sos* mutations occurred within the *spo0A* locus. Due to the poor efficiency of transformation of strain JH642 *sof-115 sos-115*, insufficient Cm^r transformants were obtained to reach a mapping conclusion.

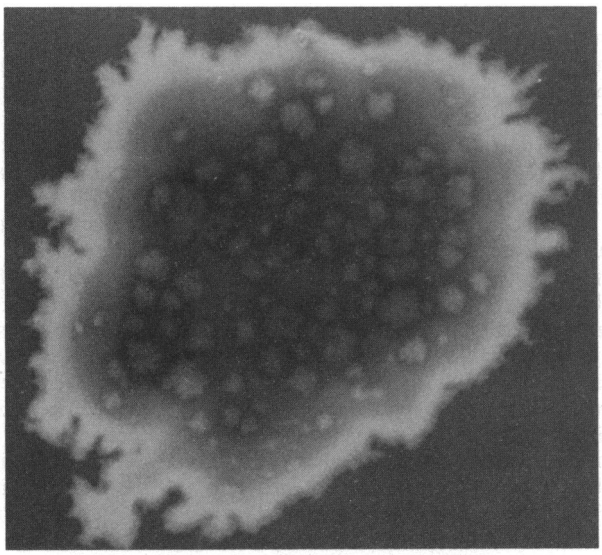

FIG. 2. Photograph of a *sof* mutant colony. The papillae segregating from the colony are *sos* mutants.

TABLE 5. Effect of *sof* alleles on sporulation in a *kinA* mutant

Strain[a] genotype	Total cells/ml	Spores/ml	% Spores
kinA sof-103	1×10^9	1×10^8	10
kinA	5×10^8	1×10^7	2
kinA sof-108	2×10^8	1×10^8	50
kinA	3.5×10^8	1×10^7	2.8
kinA sof-111	4×10^8	1×10^8	25
kinA	5×10^8	3×10^7	6
kinA sof-114	1.8×10^8	1×10^8	55
kinA	5.5×10^8	8×10^6	1.4
kinA sof-115	2.5×10^8	6×10^7	24
kinA	4.5×10^8	7.5×10^7	16
kinA sof-118	6.3×10^8	7.5×10^7	12
kinA	5.7×10^8	2.8×10^6	0.5
kinA sof-1	3.5×10^8	8×10^7	22.8
kinA	7.4×10^8	3.5×10^7	4.7
kinA[b]	7.8×10^8	6×10^6	0.7
kinA[b]	6.7×10^8	8×10^6	1.2

[a] The background for all strains is JH12638.
[b] This strain is JH12638 transformed with plasmid pJH1408, which does not contain a *sof* allele in the insert.

One *sos* mutation (*sos-118*) was cloned by rescuing the plasmid from an Spo^- colony obtained after transformation of strain JH642 *sof-118 sos-118* with plasmid pSOF118. The method used was the same as the one described above for the cloning of the *sof* mutations. Sequencing of the plasmid obtained, pSOS118, showed that the *sof-118* mutation was still present and that the *sos* mutation was the result of a secondary missense mutation that changed a phenylalanine into a serine residue at codon 105 (transition of T to C) of the *spo0A* gene.

Effect of *sof* mutations on sporulation in kinase mutants. One candidate for a kinase with activity on the Spo0A protein is the product of the *kinA* gene (18). Mutations in the *kinA* gene result in a 20- to 50-fold diminution in the frequency of sporulation. We tested whether *sof* mutations would increase the sporulation frequency of a *kinA* mutant. To this end strain JH12638, containing a Tn*917* insertion in the *kinA* locus, was transformed with the pSOF plasmids, giving rise to Spo^+ and Spo^- colonies that were Sof^+ and Sof^-, respectively. One of each type of transformant from each cross was grown in Schaeffer medium, and spores were counted after 24 h (Table 5). All the *sof* alleles tested gave increased frequencies of sporulation in the *kinA* strain, compared with the same strain with the non-*sof* plasmid insertion pJH1408. Some *sof* alleles, notably *sof-108* and *sof-114*, were especially efficient in increasing the sporulation frequency.

TABLE 6. Effect of *sof* alleles on the sporulation of kinase double mutants

Strain	Relevant genotype or description	Antibiotics	Total cells/ml	Spores/ml	% Spores
W168	Prototroph	−	1.8×10^8	1.9×10^8	100
		−	5.1×10^8	2.4×10^8	47
JH12662	*kinA*	+	4.3×10^8	1.1×10^7	2.5
		−	1.0×10^7	6.2×10^5	6.2
JH13170	*kinB*	+	7.2×10^8	1.3×10^8	18
		−	1.9×10^8	1.3×10^8	68
JH13145	*kinA kinB*	+	6.5×10^7	<10	0
		−	6.0×10^7	<10	0
JH13148	*kinA kinB sof-103*	+	4.6×10^8	3.5×10^2	0.00008
		−	8.0×10^7	1.1×10^3	0.0014
JH13151	*kinA kinB sof-111*	+	7.4×10^8	9.0×10^2	0.00012
		−	2.0×10^8	1.1×10^2	0.00006
JH13153	*kinA kinB sof-115*	+	6.0×10^8	4.5×10^2	0.00008
		−	1.7×10^8	8.0×10^1	0.00005
JH13154	*kinA kinB sof-118*	+	1.5×10^8	1.0×10^7	6.7
		−	6.1×10^8	1.4×10^7	2.2
JH13163	*kinA kinB sof-114*	+	3.8×10^8	5.0×10^7	13
		−	2.4×10^8	1.2×10^8	50

Through the use of probes designed to hybridize to conserved regions of transmitter kinase genes we have isolated a number of new kinase genes of this class (Trach, unpublished data). One of these genes codes for a protein, KinB, with high homology to the transmitter class of kinases but no identity to any particular transmitter kinase described for any organism. Inactivation of this gene by an insertional inactivation plasmid (pJH4906) in a strain carrying a *kinA* mutation reduces the sporulation efficiency of the double mutant strain to practically zero (Trach, unpublished data).

We examined the effect of the *sof* mutants in *kinA kinB* double mutants. Strains were constructed that contained a CAT gene insert in the *kinA* gene (18) and a kanamycin resistance marker in the *kinB* gene generated by an insertional inactivation Campbell recombination with an internal fragment of the *kinB* locus (Trach et al., unpublished data). The double-mutant strain was then used to construct strains carrying a representative of each of the five *sof* mutations. DNA from the Erm^r *sof* strains (JH12578 *sof-X*) described above served as donor DNA in transformation of strain JH13145 for Erm^r *sof*. The sporulation frequency of these strains was tested in Schaeffer medium (Table 6). In these experiments antibiotics were added to the medium in some experiments to insure the integrity of the strain with regard to the kanamycin resistance plasmid. In some cases this decreased the overall yield of spores. The *kinA kinB* double mutant strain did not make detectable spores under most conditions of growth. When *sof* mutations of class 1 (*sof-103*), 2 (*sof-111*), or 3 (*sof-115*) were inserted into the double-mutant strain, the level of spores increased to a detectable level, but usually fewer than 1,000 spores per ml are formed in a overnight culture of at least 10^8 cells. Thus the sporulation was only slightly enhanced by these three classes of *sof* alleles. On the contrary, the addition of either class 4 (*sof-118*) or class 5 (*sof-114*) *sof* alleles to the *kinA kinB* double-mutant strain dramatically raised the level of sporulation of this strain. Thus the effects of *sof* mutations on the sporulation of *kinA kinB* double mutants was allele specific, and at least two classes of alleles have been found in this regard.

DISCUSSION

sof mutations that suppress the requirements for the *spo0B*, *spo0E*, *spo0F*, and *spo0K* gene products in the sporulation process have been found to be missense alterations in the Spo0A protein located in the conserved amino-terminal domain of this protein. The most commonly recovered mutations were found at codons 12 and 14 and at codon 60. The codon 12 and 14 mutations reduced the negative charge of the protein region adjacent to the conserved aspartic acid residues 10 and 11. The proline-to-serine substitution observed in codon 60 mutations occurs next to aspartic acid residue 56, which could be implicated in *spo0A* phosphorylation by analogy to CheY (21). *sof* mutations were also found at residues 92 and 121. These amino acids are part of the amino-terminal domain but they are not immediately adjacent to the putative phosphorylation site.

The residues altered by *sof* mutation at codons 12, 14, 60, and 92, although seemingly scattered within the amino-terminal domain, are in fact located in the turn regions connecting β sheets and α helixes when the tertiary structure of the domain is considered (Fig. 3) (25). Furthermore, the turn regions affected are all located on one topological face of the molecule: the top as envisioned by Stock et al. (25). Residues 10, 11, and 56, which make up the presumed aspartate pocket and phosphorylation site (21), are part of this region of the molecule. The proximity of the active site and the *sof* mutations suggests that this is the region that intimately contacts the activating kinase molecule; therefore, these suppressor mutations could influence the activity of the Spo0A protein with its kinase or change the specificity of interaction to allow alternative kinases to phosphorylate the protein.

To test this hypothesis, we determined the ability of the suppressor mutations to reverse the sporulation defect in kinase mutants. The *kinA* gene codes for a protein that phosphorylates Spo0F and Spo0A proteins in vitro (18). The *kinA* gene on a multicopy plasmid, which presumably raises the intracellular level of KinA, suppresses *spo0B*, *spo0E*, and *spo0F* mutations but not *spo0A* mutations (18), suggesting that KinA phosphorylates *spo0A* in vivo as well. The *sof* effect, however, is unlikely to be due to altered activity of mutant Spo0A proteins toward KinA, because introduction of all classes of *sof* alleles into a *kinA* mutant restored efficient sporulation. This suggests that *sof* mutations alter Spo0A so it is a substrate for another kinase in the cell or makes the mutant Spo0A protein independent of kinase action.

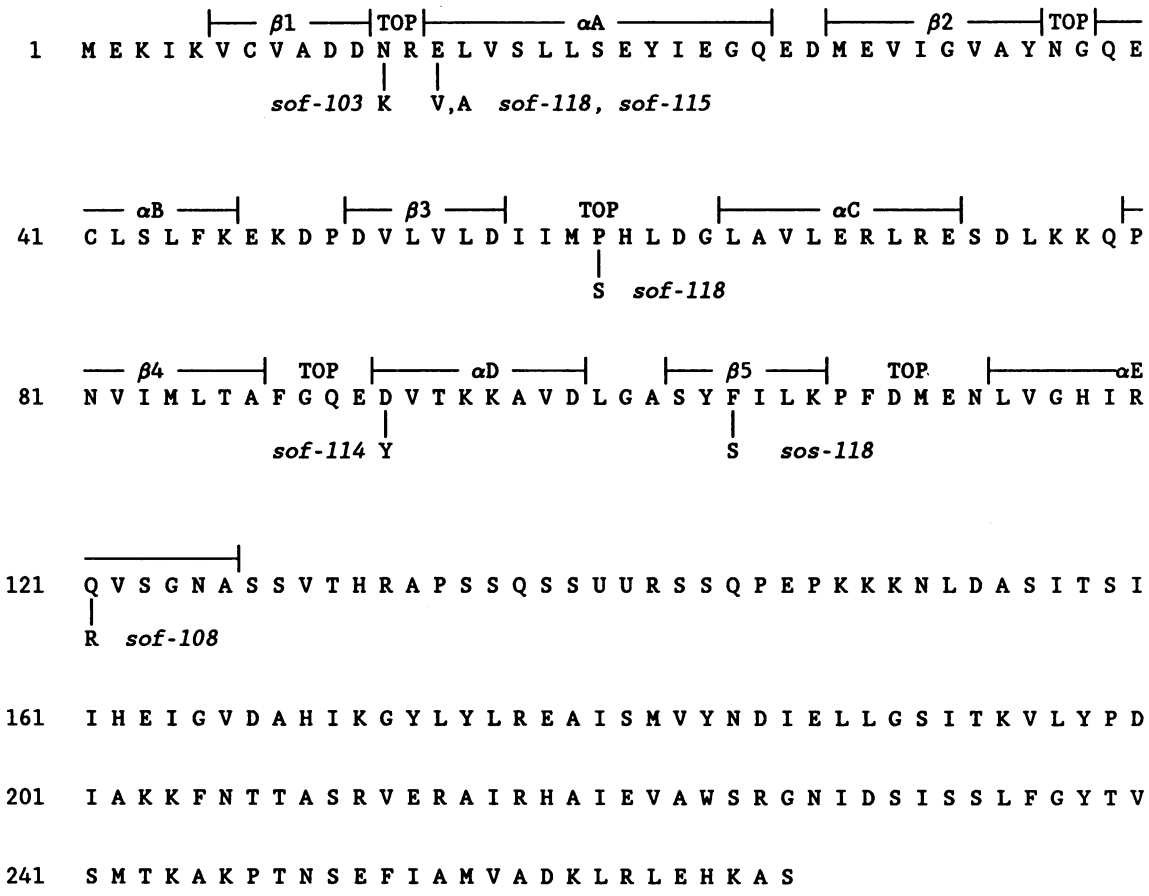

FIG. 3. Location of *sof* mutations in the *spo0A* gene. The amino acid alterations for each class of *sof* mutation are shown. The extent of the α-helical segments and β strands are derived from the structure of CheY (25) in the format of Stock et al. (26). The top designation indicates turn regions at the top end of the molecule as displayed by Stock et al. (25).

A second kinase implicated in sporulation is the product of the *kinB* gene (Trach, unpublished data). This kinase has homology to the transmitter class of kinases in two-component systems, but its activity in vitro on either Spo0A or Spo0F proteins has not been determined. KinB-deficient mutants are, by themselves, not sporulation defective. However, *kinA kinB* double mutants are completely sporulation defective, indicating that KinB must play a role in the sporulation of *kinA* mutants. Introducing the *sof* alleles into the *kinA kinB* double kinase mutant strain showed that *sof* alleles at codons 12 and 14 were able to only weakly restore sporulation, whereas *sof* alleles at codons 60 and 92 reinstated efficient sporulation. These results can be interpreted to mean that suppressors of classes 1, 2, and 3 increase the avidity for or reactivity with kinase B, and that is why they are effective suppressors of *kinA* mutants but not of *kinA kinB* double mutants. Suppressors of classes 4 and 5 suppress *kinA* mutants or *kinA kinB* double mutants equally well, and therefore they must either increase the reactivity of the altered Spo0A protein for a new, unknown kinase or they modify the Spo0A protein such that it no longer requires a phosphorylation event to be active.

In an alternative model for the interaction of *sof* suppressors and the other *spo0* genes, KinA and KinB are envisioned as not acting directly on *spo0A*. The class 1, 2, and 3 *sof* mutations are postulated to obviate the need for KinA function by changing the interaction of *spo0A* with other proteins, presumably other Spo0 proteins. The form of *spo0A* created by a class 1, 2, or 3 *sof* mutation still would require KinB function on the pathway to final activation. Class 4 and 5 *sof* mutations would alter the Spo0A protein to a form that is able to bypass the functions of both KinA and KinB. This general model implies that the targets of KinA and KinB are gene products whose combined function is required to carry out *spo0A* activation. The model is partially supported by the observations that none of the suppressors of *spo0F* or *spo0B* mutations were in KinA or KinB, yet the *sof* and *sob* mutations suppress *kinA* and *kinA kinB* mutants. The relationship suggests that the kinases act through a complex of proteins that includes the *spo0F* and *spo0B* gene products at least. The end product of the pathway is a form of the Spo0A protein that drives the cell toward sporulation. Deletion of *spo0F* or *spo0B* prevents the action of the kinases, and only mutations in the end product (Spo0A) can overcome the *spo0F* or *spo0B* defect.

With either of the two general models, it is clear that the *sof* forms of *spo0A* are not equivalent to the constitutively activated form of the protein. In other regulator proteins, such as CheB and NtrC, the N-terminal domain acts as an inhibitor of C-terminal activity, so that N-terminal mutations may reduce or eliminate the inhibition (12). We would be unlikely to find an analogous mutant of *spo0A*, because such a mutant would be an obligate sporulation mutant and may not produce a colony. The *sof* mutants we have described must prepare the *spo0A* for the final modification step without committing the cell to sporulation.

In our original study of *sof* mutations we postulated that the Spo0A protein must exist in two forms, active and inactive, and that the other *spo0* gene products served to produce an activating effect (9). The involvement of the *kinA* and *kinB* genes in these early events indicates that the process involves phosphorylation, although the details are not yet clear. The *sof* mutants appear to be able to grow and regulate sporulation without any one of the other *spo0* genes except *spo0H*, raising the question of the role of the other *spo0* gene products. A key observation is that the *sof* mutants segregate *spo* mutants at a high frequency. All of the *spo* mutants tested, which we designated as *sos*, mapped within *spo0A*, and one that we sequenced retained the *sof* mutation and had a second missense mutation in codon 105. This mutation may restore the mutant Spo0A protein to a wild-type configuration, or it may destroy the ability of Spo0A to activate transcription. Backcrosses to the wild type would resolve this question.

The propensity to segregate Spo^- mutants in *sof* strains indicates that the *sof* mutations are deleterious to some normal cellular function, even though they do not dramatically alter colony size or growth rates. We have not tested whether they affect premature sporulation or whether they accumulate only in stationary-phase cells or during logarithmic growth. If the cellular function of *spo0A* during the initiation of sporulation is to inhibit normal growth, as well as to induce the synthesis of transition and stage 0 genes, then inappropriate activation of Spo0A would provide pressure to gather mutations in the gene. It follows then that the timing of the induction of Spo0A modification is likely to be an important property of the *spo0* gene products whose genes are suppressed by *sof* mutants.

Spo^+ *sof* strains also segregate Spo^+ mutants that do not segregate further. We have not examined any of these further in detail, but we predict that some of them would be transcription down mutations in the *spo0A* gene or mutations in Spo0A that compensate for the enhanced propensity for activation of the *sof* mutations while mutants remain Spo^+.

Cells reaching the end of logarithmic growth enter a state where genes are both induced and repressed. Although the ultimate signal for this process almost certainly involves the GTP pools in the cell (17), it is likely that there are several input circuits that modulate the environmental signals. We have shown that Spo0A regulates expression of genes during the transition between growth cells and commitment to sporulation through products such as AbrB (27, 28). The genes that are regulated are temporally associated with sporulation but in many cases are not essential for sporulation. Thus, Spo0A functions in at least two physiological states: transition and the sporulation pathway. The fact that these two states are sequential implies that there is likely to be an ordered set of events that affect the state and activity of the central regulatory protein, Spo0A.

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