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Negative Regulator of σ^G-Controlled Gene Expression in Stationary-Phase *Bacillus subtilis*

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In some media, *Bacillus subtilis* can maintain a prolonged stationary growth phase; however, in other media, nutrient depletion triggers a complex differentiation that culminates in production of a dormant endospore. This differentiation requires the expression of many genes. We found that during the stationary phase in media in which the cells do not form endospores and do not normally express these sporulation-essential genes, a recessive mutation in *spoIIAB* caused increased transcription of a set of genes essential for sporulation. Evidently, the wild-type product of *spoIIAB* acts during the stationary phase to prevent expression of additional sporulation-specific genes.

During endospore formation by *Bacillus subtilis*, asymmetric cell division partitions the sporangium into two unequal daughter cells. The smaller cell (forespore) becomes engulfed by the larger cell (mother cell), where it develops into the mature endospore. Maturation of the endospore requires that different sets of genes be expressed in the forespore and mother cell (for reviews, see references 8 and 18 and other chapters in that book). This compartment-specific gene expression is regulated in part at the level of transcription. For example, the structural genes for the proteins that coat the outside of the endospore are transcribed exclusively in the mother cell (16), while the genes for glucose dehydrogenase (*gdh*) (14) and the small acid-soluble proteins (*ssp*) are transcribed exclusively in the forespore (9).

Compartment-specific transcription of at least some of these genes appears to result from compartment-specific synthesis of two RNA polymerase sigma factors. σ^K is produced exclusively in the mother cell (7), and σ^G is produced exclusively in the forespore (3, 20). They appear to direct transcription of several genes in their respective compartments. However, it is not known how synthesis of either sigma factor is limited to its specific compartment.

To identify factors that may regulate forespore-specific transcription, we isolated and characterized a mutant that exhibits increased transcription of *gdh*. Remarkably, this increased transcription occurs during the stationary phase in media that do not support the formation of endospores. Evidently, this mutation identifies a gene product that acts to antagonize expression of forespore-specific genes during the stationary phase when the cells do not form endospores.

MATERIALS AND METHODS

Bacterial strains. The *B. subtilis* strains used are listed in Table 1.

Nitrosoguanidine mutagenesis. *B. subtilis* JH642 containing plasmid pGL50.PM1 (14) was grown in LB medium containing chloramphenicol (5 μg/ml) to an optical density at 550 nm of 0.5. Cells were then pelleted and suspended in 2 ml of citrate buffer (10), and nitrosoguanidine was added to a final concentration of 96 μg/ml. After incubation for 30 min at 37°C, the cells were washed once with citrate buffer. Dilutions were plated on LB agar containing 5 μg of chloramphenicol per ml and 100 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml. The nitrosoguanidine treatment resulted in a survival rate of 0.05%.

Genetic mapping. To map the mutations that affected *gdh-lacZ* expression, the kit strains of Dedonder et al. (1) were transformed with chromosomal DNA from *B. subtilis* EU88925 to erythromycin resistance to introduce a *gdh-lacZ* fusion into each of the kit strains. Each of these strains was used in transduction experiments to map the mutation that affected *gdh* expression. PBS1 lysates and transductions were done as described previously (15). Transformants of competent cells with integrational plasmids were selected by growth on LB agar (tryptone [10 g/liter], sodium chloride [5 g/liter], yeast extract [5 g/liter] [pH 7.2], agar [15 g/liter]) containing chloramphenicol at 5 μg/ml. Congressions were done by transformation of strain EU2000 with 100 ng of chromosomal DNA from a *B. subtilis* prototroph and 4 μg of plasmid DNA. D-Alanine prototrophs, Dal^+ transformants, were selected by growth on LB agar.

Cloning of the mutant *spoIIAB* allele. Total chromosomal DNA from strain EU6001 was cut with *Bgl*II, and fragments in the 2- to 3-kilobase (kb) range were isolated and ligated to pJH101 (5, 14). Recombinant plasmids containing the 2.4-kb *Bgl*II fragment from the *B. subtilis* chromosome were identified by colony hybridization by using a 0.95-kb *Hin*dIII-*Eco*RI fragment from pPP51 (13) labeled with ^{32}P by random primers as described previously (5).

Construction of isogenic strains. Strain QB935, which contained pGL50 (14), was transformed to lysine prototropy (Lys^+) with chromosomal DNA prepared from strain PR100. Among the Lys^+ transformants, approximately 50% became blue on X-Gal plates, indicating that they had obtained the mutation in *spoIIAB*. A white Lys^+ transductant and a blue Lys^+ transductant were cured of pGL50 by growth without chloramphenicol. These two strains, designated EU6010 and EU6011, were used as an isogenic pair for further strain constructions. To introduce the *gdh-lacZ* fusion into the chromosome of each strain, this isogenic pair was transformed with chromosomal DNA from EU88925. Erm^r transformants were selected, creating EU6040 and EU6041. To introduce a *spo0H::cat* allele into the isogenic pair, each was transformed with chromosomal DNA from strain BH1-1, obtained from J. Healy, to create EU6020 and EU6021. For EU6050 and EU6051, the *gdh-lacZ* fusion was introduced before transformation with DNA from BH1-1 to introduce

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TABLE 1. *B. subtilis* strains

Strain	Genotype or relevant characteristics	Source or reference
JH642	*pheA1 trpC2*	
BH-1	*pheA1 trpC2 spo0H::cat*	R. Losick
IS233	*pheA1 trpC2 ΔspoOH* HindIII	17
QB928	*aroI926 purB33 dal-1 trpC2*	14
QB935	*aroD120 lys-1 trpC2*	14
PR100	*pheA1 trpC2 spoIIAB*	This study
EU88925	*pheA1 trpC2 gdh::lacZ ermC*	12
EU2000	*aroI926 purB33 dal-1 trpC2 gdh::lacZ ermC*	This study
EU6000	*aroD120 lys-1 trpC2 gdh::lacZ ermC*	This study
EU6001	*aroD120 trpC2 spoIIAB1 gdh::lacZ ermC*	This study
EU6010	*aroD120 trpC2*	This study
EU6011	*aroD120 trpC2 spoIIAB1*	This study
EU6020	*aroD120 trpC2 spo0H::cat*	This study
EU6021	*aroD120 trpC2 spoIIAB1 spo0H::cat*	This study
EU6030	*aroD120 trpC2 spoIIIG::cat*	This study
EU6031	*aroD120 trpC2 spoIIAB1 spoIIIG::cat*	This study
EU6040	*aroD120 trpC2 gdh::lacZ ermC*	This study
EU6041	*aroD120 trpC2 spoIIAB1 gdh::lacZ ermC*	This study
EU6050	*aroD120 trpC2 spo0H::cat gdh::lacZ ermC*	This study
EU6051	*aroD120 trpC2 spo0H::cat spoIIAB1 gdh::lacZ ermC*	This study
EU6071	*aroD120 trpC2 spo0A::cat spoIIAB1 gdh::lacZ ermC*	This study
EU6081	*aroD120 trpC2 spoIIAB1 gdh::lacZ ermC* pPP51	This study
EU7000	EU6010 lysogen SPβ *spoIIIG-lacZ*	This study
EU7001	EU6011 lysogen SPβ *spoIIIG-lacZ*	This study
EU7010	EU6020 lysogen SPβ *spoIIIG-lacZ*	This study
EU7011	EU6021 lysogen SPβ *spoIIIG-lacZ*	This study
EU7020	EU6030 lysogen SPβ *spoIIIG-lacZ*	This study
EU7021	EU6031 lysogen SPβ *spoIIIG-lacZ*	This study
EU8000	JH642 *spoIIAA-lacZ*	This study
EU8001	IS233 *spoIIAA-lacZ*	This study
EUR8900	*aroD trpC2 gdh::lacZ ermC*	This study
EUR8901	*aroD trpC2 gdh::lacZ ermC spoIIABΔ1*	This study
EUR8902	*aroD trpC2 gdh::lacZ ermC spoIIAB1*	This study
EUR8903	EUR8901 *spoIIA*::pPP51	This study
EUR8904	EUR8901(pHDAB)	This study

spo0H::cat. To construct a mutant *spoIIIG* allele, a 334-base-pair (bp) *Pst*I fragment internal to the *spoIIIG* structural gene was cloned into the *Pst*I site of pJH101 to create pJH101.IIIG. This plasmid was transformed into EU6010 and EU6011. Southern blot analysis was used to verify that the integrational plasmid disrupted the *spoIIIG* region of the chromosome. These strains were designated EU6030 and EU6031.

Construction of a specialized transducing bacteriophage carrying a *spoIIIG-lacZ* fusion. Plasmid pTK4 (5) was cut with *Hin*dIII and *Bam*HI to release a 448-bp fragment that contained the *spoIIIG* promoter. The ends of this fragment were made blunt by incubation with DNA polymerase (Klenow fragment) in the presence of all four deoxynucleoside triphosphates. This fragment was ligated to pZΔ326-GV34 (obtained from P. Youngman), which had been cut with *Sma*I. A recombinant plasmid containing the *spoIIIG* promoter region in the correct orientation was isolated. The plasmid was cut with *Hin*dIII and *Xba*I to remove a 2-kb region between the *spoIIIG* promoter and the β-galactosidase gene present in the plasmid. The plasmid was reclosed after the *Hin*dIII and *Xba*I sites had been blunt ended with Klenow treatment and was designated pDD3G. This plasmid was linearized with *Bal*I and used to transform *B. subtilis* ZB307A (23), which contains a heat-inducible SPβ prophage. Transformants were selected by growth on LB agar supplemented with 5 μg of chloramphenicol per ml. A transducing lysate was prepared from one transformant by heat induction as described in reference 5 and used for further experiments.

β-Galactosidase assays. β-Galactosidase assays for cells grown in liquid media were as described previously (14). To assay β-galactosidase in colonies grown on agar medium, plates containing approximately 100 colonies each were harvested with a glass spreader after addition of 3 ml of ice-cold LB. The optical density at 600 nm of the cell suspension was determined, and the cells were assayed for β-galactosidase as described previously (14).

RNA isolation and primer extensions analysis. RNA was prepared from cells grown on LB plates at a density of approximately 100 colonies per plate. After growth for 20 h at 37°C, 2 ml of ice-cold LB was added to each of 10 plates. The total cell suspension was harvested by centrifugation; the cell pellet was lysed, and RNA was isolated as described previously (14). The primer extension procedure was performed with the following oligonucleotides; 5'GCTGTA CGGCCCGCCGCC3' for *gdh*, 5'GCCAAATTGACCTTG ACC3' for *sspE*, 5'GTGATCCAGCGTGATGGAT3' for *spoVG*, and 5'GCTGCCTAAACAGCTTTC3' for *spoIIIG*. Conditions for primer annealing were as described previously (14), except for the *gdh* primer, in which the annealing reactions were incubated at 75°C for 2 min. Primer extensions were performed with avian myeloblastosis virus reverse transcriptase (1 U per reaction). Extension reactions were performed at 52°C. The products were subjected to electrophoresis and visualized by autoradiography as described previously.

Construction of a deletion in *spoIIAB*. The 1.17-kb *Eco*RI-*Sal*I DNA fragment from pPP51 (22) containing sequences from the *spoIIA* operon was cloned between the *Eco*RI and *Sal*I sites of M13mp18 to produce M13AB. Oligonucleotide-directed mutagenesis was used to delete 45 bp as described previously (4). This deletion removed a unique *Bgl*II site within *spoIIAB*. The oligonucleotide used for this mutagenesis had the sequence 5'AGAGCGCTCAAGCTCAATGCC TAAGCCTTC3'. The nucleotide sequence of the *spoIIAB* region of the resulting phage, M13Δ1, was determined to verify that the expected deletion had been obtained. The deletion was introduced into the chromosome of *B. subtilis* EU2000 by congression to Dal$^+$ and Lac$^+$ as described in the genetic mapping section. Chromosomal DNA was isolated from several Lac$^+$ transformants and used to transform EU6000 to Lys$^+$. Since *spoIIAB* is cotransformed with a frequency of 50% with *lys-1*, we expected and found that 50% of the Lys$^+$ transformants became Lac$^+$ (i.e., blue on X-Gal). Chromosomal DNAs from several Lac$^+$ and Lac$^-$ transformants were examined by Southern blot analysis. Only the Lac$^+$ transformants lacked the *Bgl*II site in *spoIIAB*.

Construction of *spoIIAB* expression plasmids. pJ89 is an expression plasmid containing pUCf1 (Pharmacia) joined to pUB110 (C. H. Jones and C. P. Moran, Jr., unpublished data). The *lac* promoter in this plasmid was changed to a consensus σ^A promoter by oligonucleotide-directed mutagenesis. To express the wild-type allele of *spoIIAB* in this

plasmid, it was necessary to clone both *spoIIAA* and *spoIIAB* into pJ89. Constructions that contain only *spoIIAB* appeared to be unstable. The 1.17-kb *Eco*RI-*Pst*I DNA fragment from M13AB was cloned between the *Pst*I and *Hin*dIII sites of pJ89 to produce pHDAB. The *spoIIAA-spoIIAB* region was oriented so that its transcription was directed from the modified *lac* promoter. This orientation was verified by sequence determination.

RESULTS

A mutation in *spoIIAB* affects *gdh* expression. We had previously shown that *gdh* is transcribed exclusively in the forespore compartment (14). To identify gene products that regulate this transcription, we isolated mutants with second-site mutations that increase *gdh* expression. Our strategy was to use a *B. subtilis* strain carrying plasmid pGL50.PM1, which contained *lacZ* fused to a derivative of the *gdh* promoter with a deleterious mutation (a transversion at position −10). Colonies of this strain are white when grown on DS (17) or LB agar supplemented with the β-galactosidase indicator X-Gal. After mutagenesis with nitrosoguanidine, 58 of 20,000 colonies turned blue. Bacteria from each blue colony were cured of the plasmid, and strains that lost the blue phenotype were retransformed with pGL50.PM1 or pGL50, which contains the wild-type *gdh* promoter. Each mutant strain accumulated more β-galactosidase than the otherwise isogenic parent, regardless of which plasmid it contained. One strain, designated PR100, that appeared to produce a high level of β-galactosidase from pGL50 was chosen for further study.

To determine the map location of this mutation (called *spoIIAB1* [see below]), two-factor transductional crosses were performed by using phage PBS1. PR100 was infected with PBS1, and a lysate was recovered as described in Materials and Methods. When this lysate was used to transduce EU6000 to lysine prototrophy (Lys^+), 190 colonies of a total of 217 Lys^+ transductants became blue on plates containing X-Gal, indicating that the mutation causing the increase in *gdh-lacZ* expression was approximately 90% linked to the *lys-1* marker. Transformations of EU6000 to Lys^+ with DNA from PR100 resulted in 229 blue colonies of a total of 488 Lys^+ transformants, demonstrating 47% linkage of *lys-1* and the mutation that caused overexpression of the *gdh-lacZ* fusion.

These mapping data were consistent with the notion that the mutation is in or near the *spoIIA* locus. To test this possibility, we performed fine-structure mapping by using three integrational plasmids, each of which carried DNA from the *spoIIA* region. Each of these integrational plasmids (pPP31 [13], pPP51 [22], and pPP72 [from P. J. Piggot]) contained the promoter region for *spoIIA* but carried a different amount of DNA extending into the *spoIIA* operon (Fig. 1). Strain EU6001, which carried the *spoIIAB1* mutation and a *gdh-lacZ* fusion and had the blue-colony phenotype, was used as the recipient for the transformations. If the mutation in the chromosome was present within the homologous region carried on the integrational plasmid, then a fraction of the transformants should correct the mutation and have a white-colony phenotype. Transformation of EU6001 with plasmid pPP72 resulted in 0 white colonies and 200 blue colonies. This indicated that the mutation was not within the fragment cloned in pPP72 or that the mutation was at the extreme 3′ end and was unable to be corrected by recombination (Fig. 1). Transformation with plasmid pPP31, which carries additional DNA extending into the *spoIIAB* gene, resulted in 64 white colonies and 178 blue colonies. This suggested that the mutation was within the additional DNA present in pPP31. Transformation with plasmid pPP51 resulted in 200 white colonies and 0 blue colonies. Since pPP51 contained the entire *spoIIAB* sequence, this is consistent with the model in which pPP51 complements, in *trans*, a defect in the *spoIIAB* gene. Moreover, these data suggest that the mutation is within the *spoIIAB* gene in the *Dra*I-to-*Bgl*II fragment present in pPP31 but not in pPP72 (Fig. 1).

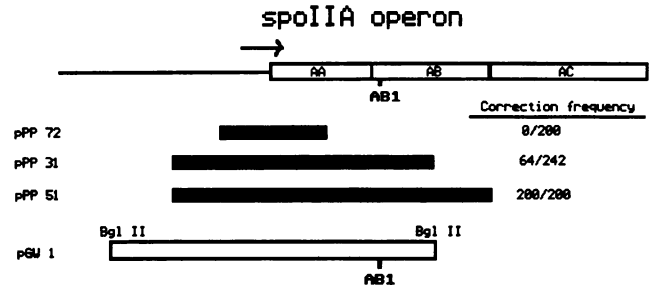

FIG. 1. Fine-structure mapping of the *spoIIAB1* allele. At the top are the three open reading frames in the *spoIIA* operon, AA, AB, and AC. Regions of DNA from the *spoIIA* region that are cloned into integrational plasmids are represented by solid bars. The correction frequency represents the number of white colonies divided by the total number of chloramphenicol-resistant transformants obtained when each of the plasmids was used to transform EU6001. Plasmid pGW1 contains a 2.4-kb *Bgl*II fragment with the *spoIIAB1* allele cloned into integrational plasmid pJH101. The location of the *spoIIAB1* mutation is indicated.

The restriction map of this region has been characterized in detail (13). The *Dra*I-*Bgl*II fragment that contained the mutation is within a 2.4-kb *Bgl*II fragment of the chromosome. This 2.4-kb fragment was cloned from the chromosome of mutant strain EU6001 into integrational plasmid pJH101, creating plasmid pGW1, as described in Materials and Methods. To verify that the cloned region contained the mutation, pGW1 was transformed into EU6000, which contained the *gdh-lacZ* fusion in the chromosome. This transformation resulted in 30 of 154 colonies with the blue-colony phenotype, demonstrating that the mutation was present within this cloned fragment. pGW1 also was used to transform EU6001, which contains the mutant *spoIIAB1* allele. As expected, all of the transformants exhibited the mutant phenotype.

From these results, it appeared that the mutation was within a 320-bp *Dra*I-*Bgl*II fragment present at the 3′ end of the cloned *spoIIA* DNA in pPP31. To verify this, both the mutant and wild-type *Dra*I-*Bgl*II fragments were cloned into pUC18 to produce pUC18.IIAB and pUC18.WT, respectively. These plasmids were used in a congression experiment by transformation of a D-alanine auxotroph, strain EU2000, which contained the *gdh-lacZ* fusion, with chromosomal DNA from a D-alanine prototroph. None of the 1,284 Dal^+ transformants became blue when pUC18.WT was used. However, transformation with pUC18.IIAB resulted in 12 blue colonies of a total of 1,481 D-alanine prototrophs. From these results, we concluded that the mutation responsible for increased accumulation of β-galactosidase from the *gdh-lacZ* fusion was present within this 320-bp *Dra*I-*Bgl*II fragment.

The nucleotide sequence of both strands of the mutant insert indicated that a single base pair was different from that in the wild-type allele: a C-to-T transition at position 767

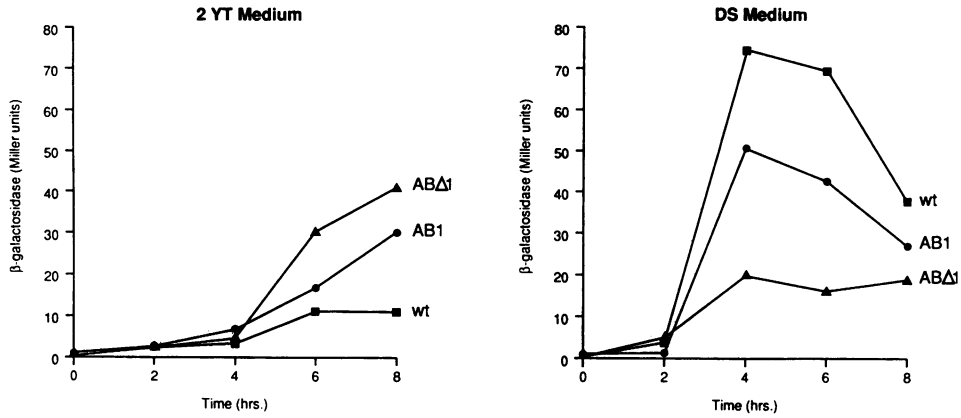

FIG. 2. Effects of *spoIIAB* on *gdh-lacZ* expression. Shown is the accumulation of β-galactosidase from three strains grown in sporulation (DS) medium or 2YT medium. Cells were harvested at intervals after the end of exponential growth (indicated as time zero). The strains were isogenic, except for the alleles of *spoIIAB* (EUR8900 [■], EUR8901 [▲], and EUR8902 [●]). wt, Wild type.

(according to the scheme of P. Fort and P. J. Piggot [2]). This mutation is expected to result in a substitution of valine for alanine at the amino acid 11 of the *spoIIAB* protein. This allele of *spoIIAB* was designated *spoIIAB1*.

***spoIIAB1* causes *gdh-lacZ* expression in stationary-phase cells that do not form endospores.** The *spoIIAB1* mutation was originally identified by an apparent increase in the accumulation of β-galactosidase from a *gdh-lacZ* fusion in colonies growing on LB agar. To quantitate this increase in *gdh-lacZ* expression, isogenic strains were constructed, each containing *gdh-lacZ* in the chromosome. Each of the strains was grown in DS (sporulation) liquid media (17), LB liquid medium, and 2YT liquid medium (10). The increased expression of *gdh-lacZ* caused by *spoIIAB1* was observed only in 2YT liquid medium (Fig. 2) and apparently in cells growing on LB agar in the original screen. To quantitate the effect of *spoIIAB1* on cells grown on solid media, we initially assayed individual colonies growing on LB plates for β-galactosidase. The amount of β-galactosidase was greater in the *spoIIAB1* mutant, strain EU6041, and there was no significant variation in the amount that each colony produced. The β-galactosidase activities (and standard deviations) of strains EU6040 and EU6041, respectively, were 0.9 (0.3) and 41.1 (6.2) Miller U after 24 h and 0.8 (0.3) and 36.1 (6.5) Miller U after 30 h (averages of 10 colonies grown on LB plates).

Subsequent β-galactosidase assays were done by pooling colonies from LB plates as described in Materials and Methods. In these experiments, the strain with the *spoIIAB1* mutation (EU6041) accumulated 30-fold more β-galactosidase than did EU6040, the isogenic wild-type strain (Table 2). This increase in β-galactosidase accumulation was shown to depend on the presence of the *gdh-lacZ* fusion, since strain EU6011, which contains no fusion, showed very little β-galactosidase activity. Evidently, the *spoIIAB1* allele caused increased expression of *gdh-lacZ* only in media in which the cells did not form endospores (both the wild type and the *spoIIAB1* mutant produced less than 10^2 heat-resistant spores per ml after 24 h in 2YT). The *spoIIAB1* allele did not cause an increase in *gdh-lacZ* expression in DS sporulation medium (Fig. 2) (in which 2×10^8 spores per ml were formed by the wild-type strain and 2×10^7 spores per ml were formed by the *spoIIAB1* mutant) or in resuspension medium (19) (data not shown).

***spoIIAB1* results in loss of *spoIIAB* function.** Since the effects of the *spoIIAB1* mutation were limited to stationary-phase cells in media in which spores are not formed, we tested whether β-galactosidase expression was subject to sporulation-specific controls. Isogenic strains carrying the *spoIIAB1* mutation with either a *spo0A*::*cat* or a *spo0H*::*cat* insertional mutation were constructed. Expression of β-galactosidase was abolished in EU6071, which contained the *spo0A* mutation and the *spoIIAB1* allele (Table 2), demonstrating that *gdh-lacZ* expression in the *spoIIAB1* background was dependent on *spo0A*. In contrast, high levels of β-galactosidase were produced by EU6051, which contained both the *spo0H* mutation and *spoIIAB1*, and by EU6050, which carried only the *spo0H* mutation (Table 2). This effect could be due to the absence of the *spoIIAB* product in the *spo0H* strains because expression of the *spoIIA* operon is dependent on *spo0H*.

To test this model, plasmid pPP81, containing a *spoIIAA-lacZ* fusion (12), was introduced into wild-type *B. subtilis* (EU8000) and an isogenic *spo0H* mutant (EU8001). In cells grown on solid medium, β-galactosidase expression from the *spoIIAA-lacZ* fusion was 13-fold lower in the *spo0H* mutant than in the wild-type strain (Table 2), consistent with the model in which the increase in *gdh* expression in *spoIIAB1* mutant strains results from loss of *spoIIAB* function. Furthermore, the *spoIIAB1* defect can be complemented in *trans* by wild-type *spoIIAB* allele. When the wild-type *spoIIAB* allele was introduced into a *spoIIAB1* mutant on integrative plasmid pPP51 (see beginning of previous sec-

TABLE 2. Effects of mutations on β-galactosidase activities in colonies grown on LB plates

Strain	Relevant genotype	β-galactosidase activity (Miller U)[a]
EU6010	Wild type	0.0
EU6011	*spoIIAB1*	0.0
EU6040	*gdh-lacZ*	1.0
EU6041	*spoIIAB1 gdh-lacZ*	31.0
EU6050	*spo0H*::*cat gdh-lacZ*	26.0
EU6051	*spo0H*::*cat spoIIAB1 gdh-lacZ*	41.0
EU6071	*spo0A*::*cat spoIIAB1 gdh-lacZ*	0.6
EU6081	*spoIIAB1 gdh-lacZ* pPP51	1.0
EU8000	*spoIIAA-lacZ*	8.0
EU8001	*spo0H spoIIAA-lacZ*	0.6

[a] Values were obtained after subtraction of the basal levels (0.5 Miller U) in EU6010 and EU6011.

tion) or on replicative plasmid pHDAB (data not shown), expression of *gdh-lacZ* was reduced to wild-type levels.

The *spoIIAB1* allele results in increased accumulation of *gdh* mRNA. There are several explanations for the increase in *gdh-lacZ* expression resulting from a *spoIIAB* defect. One possibility is that *spoIIAB* could encode a protease that degrades β-galactosidase. This is unlikely, since no difference in β-galactosidase accumulation was seen from a *spoVG-lacZ* fusion in either the wild type or the *spoIIAB1* mutant (data not shown). An alternative explanation is that the *spoIIAB1* mutation results in increased accumulation of *gdh* mRNA. To test this possibility, we analyzed the amount of *gdh* mRNA present in both the wild type and the *spoIIAB1* mutant strain by primer extension analysis. The amount of *gdh* message was greatly increased in the *spoIIAB1* mutant relative to the wild type (Fig. 3, lanes a and b). Accumulation of mRNA from another sporulation gene, *spoVG*, was found to be unaffected by the *spoIIAB1* allele. Expression of *spoIIAB* is also prevented by mutations in *spo0H*, which encodes the sigma factor that directs transcription of the *spoIIA* operon. We investigated whether a mutation in *spo0H* which prevented *spoIIAB* expression would result in accumulation of *gdh* mRNA. EU7010 (Fig. 3, lane c) and EU7011 (lane d), which contained *spo0H*::*cat* and the wild-type or mutant *spoIIAB* allele, respectively, also accumulated *gdh* mRNA.

***gdh* expression requires σ^G.** Transcription of *gdh* is probably directed by RNA polymerase containing σ^G, the product of the *spoIIIG* gene (3, 20). To test this, we constructed strains bearing a *spoIIIG*::*cat* insertion in both the wild type and the *spoIIAB1* mutant, i.e., strains EU7020 and EU7021. Primer extension analysis demonstrated that disruption of the *spoIIIG* coding sequence eliminated the accumulation of transcripts seen from the *gdh* promoter in the *spoIIAB1* mutant (Fig. 3, lane f). This demonstrated that increased accumulation of the *gdh* message was dependent on *spoIIIG*, suggesting that σ^G directs transcription of *gdh* on LB agar. The transcription of *gdh* observed in the *spoIIAB* mutant grown in 2YT liquid medium was also dependent on *spoIIIG* (data not shown).

To investigate whether loss of *spoIIAB* function specifically affected *gdh* or also other genes expressed in the forespore compartment, we examined the expression of another forespore-specific gene, *sspE*. Primer extension analysis showed increased accumulation of the *sspE* message in the *spoIIAB1* mutant (Fig. 3, lanes a and b), as well as in the *spo0H* and *spo0H spoIIAB1* double mutant (lanes c and d). As previously seen with *gdh*, *spoIIIG* was required for accumulation of *sspE* mRNA (lanes e and f).

***spoIIAB1* affects transcription of the σ^G structural gene.** Since both *gdh* and *sspE* showed similar patterns of message accumulation in each of the strains tested, it was possible that *spoIIAB* affected a regulatory protein common to both of these genes. Because *spoIIIG* encodes the sigma factor responsible for transcription of both *gdh* and *sspE* (3, 20) and *spoIIIG* disruption eliminated accumulation of mRNA from these genes, it seemed possible that *spoIIIG* was the target of *spoIIAB*. Primer extensions using an oligonucleotide complementary to *spoIIIG* showed that its message was present in much greater amounts in the *spoIIAB1* mutant (Fig. 3, lane b). Additionally, the *spoIIIG* message was increased in the *spo0H* and *spo0H spoIIAB1* double mutant (lanes c and d). Transcription of *spoIIIG* was eliminated by a *spoIIIG*::*cat* insertion (lanes e and f), as expected, since σ^G directs transcription of *spoIIIG* (3, 20). A mutation in *spoIIAB* appears to increase the amount of *spoIIIG* mRNA.

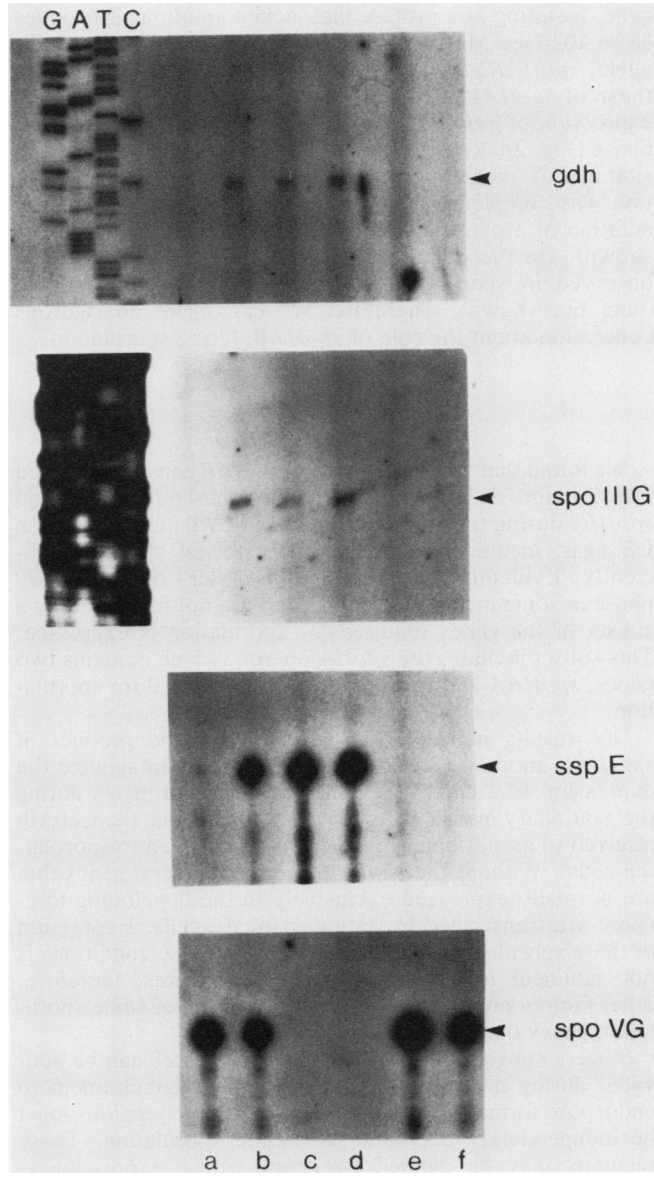

FIG. 3. Primer extension analysis. Oligonucleotide primers complementary to each of four sporulation-specific genes, *gdh*, *spoIIIG*, *sspE*, and *spoVG*, were hybridized to total RNA prepared from cells growing on solid media. Lanes: a, EU7000; b, EU7001; c, EU7010; d, EU7011; e, EU7020; f, EU7021. For *gdh* and *spoIIIG*, the same primers were used to generate a dideoxy sequencing ladder using plasmid DNA containing the cognate promoter. For *gdh* and *spoIIIG*, the starting point of transcription was identical to that seen when RNA prepared from cells grown to 4 h after the start of sporulation in liquid sporulation media was used in the primer extensions.

This could lead to greater amounts of σ^G in the cell and therefore increased transcription of *gdh* and *sspE*.

A deletion in *spoIIAB* has effects similar to those of *spoIIAB1* during the stationary phase. The *spoIIAB1* allele was found to be recessive to the wild-type allele and therefore probably results in loss of *spoIIAB* function. However, since this mutation causes only a single amino acid substitution, the mutant protein may retain partial activity. Therefore, we isolated a mutant (EUR8901) that contained an allele of *spoIIAB* in which 45 bp had been deleted from within the

gene, resulting in a protein that lacked amino acid residues 88 to 102 (see Materials and Methods). The effects of this allele, *spoIIABΔ1*, were similar to but more extreme than those of *spoIIAB1* (Fig. 2). For example, in 2YT medium, expression of *gdh-lacZ* was increased during the stationary phase (Fig. 2). As expected, the increased *gdh-lacZ* expression in 2YT medium caused by the *spoIIAB* deletion mutation was complemented in *trans* to the wild-type level by addition of a plasmid carrying *spoIIAB*, pHDAB (data not shown). On the other hand, the defect in *gdh* expression observed in sporulation medium was not complemented (data not shown). Therefore, we can make no rigorous conclusion about the role of *spoIIAB* during sporulation.

DISCUSSION

We found that two mutations in *spoIIAB* caused increased transcription of sporulation-specific genes *gdh*, *sspE*, and *spoIIIG* during the stationary phase in 2YT medium and on LB agar, media in which the cells do not sporulate efficiently. Evidently, when *B. subtilis* enters the stationary phase in some media in which spores are not formed, only a subset of the genes required for sporulation is expressed. This subset includes the *spoIIA* operon, which contains two genes, *spoIIAA* and *spoIIAC*, that are essential for sporulation.

Our results indicate that one role for the product of *spoIIAB*, another gene in this operon, is to antagonize the expression of a class of essential sporulation genes during the stationary phase in nonsporulation media (henceforth referred to as stationary-phase cells as opposed to sporulating cells). Without the *spoIIAB* product, several genes that are normally expressed exclusively in the developing forespore are transcribed in stationary-phase cells. Expression of these sporulation-specific genes under these conditions is not sufficient for development of endospores; therefore, other factors must act to prevent expression of some sporulation genes under these conditions.

Since a subset of sporulation-essential genes can be activated during the stationary phase without commitment to endospore formation, it appears that multiple regulons must be independently activated to initiate sporulation. These regulons may be defined by genes whose expression is directly dependent on σ^H (21) and those that are expressed after the end of exponential growth by a form of RNA polymerase containing σ^A (6). It is not known how cells sense the differences between rich media like 2YT and sporulation media such as DS medium.

Some aspects of the mechanism by which the *spoIIAB* product inhibits expression of the forespore-specific genes can be predicted, but several questions remain unanswered. *spoIIIG* encodes σ^G, which directs transcription from the *gdh*, *sspE*, and *spoIIIG* promoters (3, 11, 20). We found that inactivation of *spoIIIG* prevented increased transcription of these genes in the *spoIIAB* mutants. Therefore, increased expression of *spoIIIG* in the *spoIIAB* mutants probably leads to accumulation of σ^G and therefore expression of *gdh* and *sspE*. In stationary-phase cells, *spoIIAB* appears to antagonize the use of the *spoIIIG* promoter. This promoter is used in an autocatalytic manner by σ^G RNA polymerase (3). *spoIIAB* may antagonize this utilization. Because σ^G synthesis is autocatalytic (3), a mechanism for establishing σ^G synthesis is essential. The product of *spoIIAB* could antagonize this establishment synthesis. It is not known whether the *spoIIAB* product acts directly at the *spoIIIG* promoter or whether it modifies RNA polymerase, but in either case, a defective allele of *spoIIAB* would result in amplification of σ^G within the cell and a concomitant increase in *gdh* transcription.

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