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Regulation of *spo0H*, an Early Sporulation Gene in Bacilli

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The construction of *lacZ* fusions in frame with the *spo0H* gene of *Bacillus licheniformis* enabled us to study the expression of this gene under various growth conditions and in various genetic backgrounds. *spo0H* was expressed during vegetative growth, but the levels increased during early stationary phase and then decreased several hours later. Expression of the gene was not repressed by glucose, but was induced by decoyinine, an inhibitor of guanine nucleotide biosynthesis, which can induce sporulation. Of those tested, the only *spo0* gene required for the expression of *spo0H* was *spo0A*, and this requirement was eliminated by the *abrB* mutation, a partial suppressor of *spo0A* function. *spo0H-lacZ* expression was much higher in a strain with a deletion in the *spo0H* gene.

Organisms frequently encounter environments which are not optimal for their survival, and mechanisms must exist to allow cells to adjust to these adverse conditions. In recent years there have been great advances in understanding the global responses of microorganisms to environmental stresses such as unfavorable temperatures, pH, and anaerobiosis. The heat shock response, for example, includes the function of an alternate sigma factor, σ^{32}, the product of the *htpR* gene (15, 23), which is required for the elevated synthesis of other proteins involved with the basic biosynthetic machinery of the cell (30). Sporulation in the bacilli, elicited by an environment poor in nutrients, is a global response in which several new mRNAs (31) and proteins appear (25, 49). One of these proteins is the *spoIIG*-encoded σ^{29} (42), which appears in cells of *Bacillus subtilis* in the stationary phase of growth (43), and transcribes late *spo* genes (34).

One of the most fundamental problems in sporulation is to determine which genes are responsible for sensing the environmental signal(s) that conveys information to the cell about the concentrations of nutrients in the environment. The *spo0* genes, which block sporulation at the earliest morphological stage, i.e., the formation of the asymmetric septum, are candidates for such sensors. If they are sensors of nutrient deprivation or transducers of such information, they might be expected to be regulated by variations in growth conditions. They may also exhibit interdependence for expression, and analysis of these interrelationships may reveal the presence of regulational hierarchies. Much interest has centered on studies of these genetic determinants, and although several have been cloned and sequenced (3, 6, 9, 10, 41, 48), their function remains unknown. They are required for the expression of various late *spo* genes (27, 32, 36), development of competence (19), synthesis of certain transcripts which may be sporulation related (14), and production of certain late growth enzymes, such as extracellular proteases (8). As an example, most *spo0* genes are required for the full expression of *spoVG* (31, 51), a gene whose transcription is turned on early in sporulation, but whose product is not required until stage V (35). Recently, Yamashita et al. (47), using *spo0-lacZ* fusions, have shown that full expression of *spo0A* and *spo0F* requires the presence of gene products from *spo0* genes *B*, *D*, *E*, *F*, and *H*.

This report describes the expression of the cloned *spo0H* gene of *Bacillus licheniformis* (6) under various growth conditions and in various genetic backgrounds, utilizing a *spo0H-lacZ* fusion integrated into the chromosome of *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in these experiments are listed in Tables 1 and 2. Plasmid pIS9B contains the 1.2-kilobase *Bgl*II DNA fragment with the *spo0H* gene of *B. licheniformis* (6) cloned into pBD97 (deletion M-1 of pBD89) (17). pIS9A is a similar construct but with the cloned fragment in the opposite orientation with respect to the pBD97 vector. pSK10Δ6 is a derivative of pMC1403 which contains the *lacZ* gene of *Escherichia coli* with no transcriptional or translational signals and lacking the first eight codons (4). This plasmid was constructed and kindly provided to us by Jan Pero and has been used to make *lacZ* translational fusions with *B. subtilis* genes (51).

Plasmid DNA manipulations. Isolation, endonuclease restriction, ligation of plasmid DNA, and plasmid transformation were done as described by Gryczan et al. (16).

Plasmid constructions. (i) *spo0H-lacZ* fusion. Plasmids pIS9B and pSK10Δ6 were linearized at their unique *Eco*RI sites (Fig. 1). The ends were made blunt by the fill-in reaction of the Klenow fragment of DNA polymerase and ligated, and the mixture was used to transform a competent strain of *B. subtilis*. This resulted in the isolation of cointegrant pIS52, which confers resistance to ampicillin in *E. coli* and resistance to chloramphenicol in *B. subtilis*. It causes colonies to appear blue on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in both *E. coli* and *B. subtilis*. To create cointegrants in the other two possible reading frames, the plasmids were linearized with *Eco*RI and ligated directly, or pIS9B was linearized with *Eco*RI and the ends were made blunt as described above and ligated to pSK10Δ6 linearized with *Xma*I, the ends of which had also been made blunt. The only cloning procedure which resulted in a significant number of chloramphenicol-resistant

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TABLE 1. Bacterial strains

Strain	Relevant genotype	Source or reference
IS233	*trpC2 pheA1 spo0HΔHind*	46
IS234	*trpC2 pheA1 spo0HΔHind recE4*	46
IS250	*trpC2 thrA*(pE194)[a]	21 (strain "h")
IS253	*trpC2 thrA*(pIS52A)[b]	This work
IS352	*trpC2 thrA*(pIS52)	This work
IS372	*trpC2 pheA1 spo0HΔHind*(pE194)	This work
IS374	*trpC2 pheA1 spo0HΔHind*(pIS97)	This work
IS375	*trpC2 pheA1 spo0HΔHind*(pIS9A)	This work
IS376	*trpC2 pheA1 spo0HΔHind*(pBD97)	This work
IS390	*trpC2 thrA*(pIS123)	This work
IS391	*trpC2 thrA*(pIS125)	This work
IS392[c]	*trpC2 met*(pIS123)	This work
IS393[c]	*trpC2 spo0BΔPst*[d](pIS123)	This work
IS394[c]	*trpC2 spo0HΔHind*(pIS123)	This work
IS395[c]	*trpC2 spo0F221*(pIS123)	This work
IS396[c]	*trpC2 spo0G14*(pIS123)	This work
IS397[c]	*trpC2 spo0J87*(pIS123)	This work
IS399	*trpC2 met*	R. Yasbin (YB886)
IS400	*trpC2 met*(pIS125)	This work
IS401	*trpC2 pheA1*	J. Hoch (JH642)
IS402	*trpC2 pheA1 spo0AΔ204 abrB*	P. Zuber (ZB369)
IS403	*trpC2 pheA1 abrB*	P. Zuber (ZB449)
IS404	*trpC2 pheA1 spo0AΔ204*	J. Hoch (JH703)
IS406	*trpC2 pheA1*(pIS123)	This work
IS407	*trpC2 pheA1 spo0AΔ204 abrB*(pIS123)	This work
IS408	*trpC2 pheA1 abrB*(pIS123)	This work
IS409	*trpC2 pheA1 spo0AΔ204*(pIS123)	This work

[a] Parentheses indicate a plasmid integrated into the chromosome.

[b] pIS52A is similar to pIS52, but the *spo0H* gene is in the opposite orientation relative to the *lacZ* gene. The plasmid does not confer a Lac$^+$ phenotype on either *B. subtilis* or *E. coli*.

[c] An isogenic set of *trpC2 spo0* strains was prepared by Mark Albano and D. Dubnau by transforming YB886 with DNA prepared from the strains carrying the appropriate *spo0* mutation. IS*392-397* were then created by introducing integrated pIS123 by transformation into the appropriate *spo0* strain.

[d] The *Pst* deletion in the *spo0B* gene was produced in this laboratory (unpublished results).

transformants which were blue on X-gal plates was the one with the *Eco*RI restriction of both plasmids followed by the fill-in reaction. A similar construct, pIS123, was made with the *spo0H-lacZ* fusion in the orientation of pIS9A by cutting both pIS52 and pIS9A with *Hin*dIII, electroeluting the larger fragments of each from an agarose gel, ligating, and transforming to *E. coli* MC1061.

(ii) Disruption of the −35 region of the promoter of *spo0H*. pIS9A was partially digested with *Hin*cII and ligated in the presence of *Sal*I linkers, and the mixture was used to transform a competent strain of *B. subtilis*. Transformants were analyzed for plasmids containing a *Sal*I linker in the correct site. The resulting plasmid, pIS97, contains a *Sal*I site in place of the *Hin*cII site in the −35 region of the promoter (Fig. 2), as well as a small deletion which extends upstream past the *Bgl*II cloning site, 35 base pairs (bp) into the vector.

(iii) *spo0H-lac* fusion containing a disrupted −35 promoter region. pIS52 and pIS97 were each restricted with *Hin*dIII, and the large fragments of each were electroeluted from an agarose gel, ligated, and transformed into *E. coli*. The resultant cointegrant, pIS101, contains a *Sal*I linker inserted into the *Hin*cII site at the −35 promoter region of the *spo0H* gene and has the same *spo0H-lacZ* fusion as pIS52.

(iv) Repairing the deletion in pIS101. A *Sal*I site was inserted into the *Bgl*II site of pIS52 by restriction with *Bgl*II, followed by making the ends blunt with Klenow DNA polymerase and ligation in the presence of *Sal*I linkers. The DNA was used to transform *E. coli*, and an appropriate colony was obtained carrying the desired plasmid, pIS104. Insertion of the *Sal*I linker had no effect on *spo0H* expression. pIS101 and pIS104 were each restricted with *Sal*I and *Sph*I. The large fragment of pIS101 and the small fragment from pIS104 were electroeluted from an agarose gel, ligated, and transformed to *E. coli*. The resultant plasmid, pIS125, contains the disrupted −35 promoter sequence and a deletion from the original *Hin*cII site to the *Bgl*II cloning site of the *spo0H* gene, but the small deletion originally present in the vector sequence of pIS97 has been replaced by the normal sequence. The presence and subsequent repair of the small deletion in all plasmids were determined by analysis of small restriction fragments on sequencing gels.

In vivo S1 transcription mapping. RNA prepared from cultures of *B. licheniformis* IS352, IS374, and IS375, grown in nutrient sporulation medium (NSM) (37) and harvested at stage T_0, was purified as described previously (24). A radioactive DNA probe containing the 5′ promoter-proximal region of the *B. licheniformis spo0H* gene and labeled at its distal end was prepared as follows. pIS9B (Fig. 1 and 2) was restricted with *Hin*dIII, followed by removal of 5′-terminal phosphates by treatment with calf alkaline phosphatase, and 5′-end-labeled with polynucleotide kinase and [γ-^{32}P]ATP. The labeled fragments were further digested with *Hpa*I and separated on a 5% acrylamide gel, and the 770-bp *Hin*dIII-*Hpa*I fragment containing the 5′ portion of the *spo0H* gene and part of the vector was electroeluted. Approximately 200 μg of RNA from each strain was added to samples of the labeled DNA fragment (15,500 cpm) and dried in vacuo. The pellets were suspended in hybridization (6) buffer, heat denatured at 83°C for 15 min, and allowed to hybridize for 3 h at 52°C. They were then treated with S1 nuclease as described previously (6) and analyzed on a 5% acrylamide gel containing 7 M urea. pBR322 *Hin*fI restriction fragments 5′-end labeled with [γ-^{32}P]ATP and polynucleotide kinase were used as size standards.

β-Galactosidase determinations. The assays and units of activity for β-galactosidase are as described by Miller (28).

Media. Strains were grown in NSM in the presence or absence of 0.5% glucose. When specified, they were grown in S6C medium (13) containing 1% glucose and the required amino acids. Decoyinine, dissolved in 1 M KOH, was added at 1 mg/ml, and guanine was added at 50 μg/ml, as indicated.

TABLE 2. Plasmids

Plasmid	Relevant phenotype in:		Source or reference
	B. subtilis	*E. coli*	
pE194	Em^r		17
pBD97	Cm^r		17 (deletion MI of pBD89)
pSK10Δ6		Amp^r	51
pIS9A	Cm^r *spo0H*$^+$		6
pIS9B	Cm^r *spo0H*$^+$		6
pIS52	Cm^r Lac$^+$	Amp^r Lac$^+$	This work
pIS52A[a]	Cm^r	Amp^r	This work
pIS97	Cm^r *spo0H*$^\pm$		This work
pIS101	Cm^r Lac$^\pm$	Amp^r Lac$^\pm$	This work
pIS104	Cm^r Lac$^+$	Amp^r Lac$^+$	This work
pIS123	Cm^r Lac$^+$	Amp^r Lac$^+$	This work
pIS125	Cm^r Lac$^\pm$	Amp^r Lac$^\pm$	This work

[a] pIS52A is similar to pIS52, but the *spo0H* gene is in the opposite orientation relative to the *lacZ* gene. The plasmid does not confer a Lac$^+$ phenotype on either *B. subtilis* or *E. coli*.

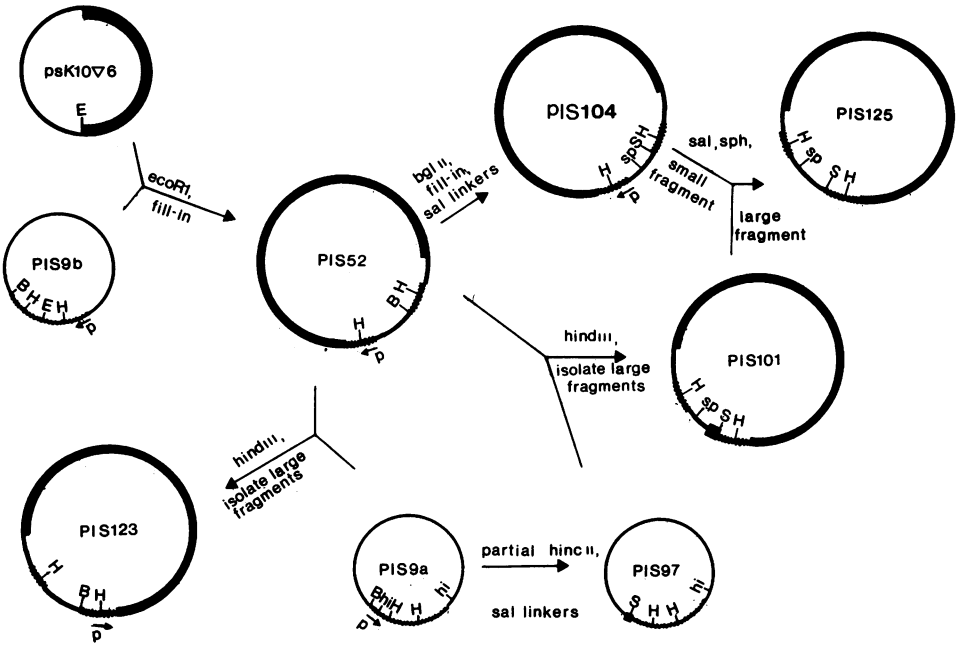

FIG. 1. Construction of *spo0H-lacZ* fusions with the wild-type *spo0H* gene and the *spo0H* gene containing a disruption of the −35 region of the promoter. The thick solid line represents the *lac* gene, and the wavy line represents the *spo0H* gene. The portion shown by a thin line represents the vector sequences, originating from either pBD97 or psK10Δ6. The *spo0H* promoter (p) and its direction of transcription (arrow) are indicated. The black square indicates a deletion in the promoter region of the *spo0H* gene which extends into the upstream vector sequences. Abbreviations: E, *Eco*RI; B, *Bgl*II; H, *Hin*dIII; S, *Sal*I; hi, *Hinc*II; sp, *Sph*I.

RESULTS

Construction of translational *lacZ* fusions. We found, by sequencing across the fusion junction, that a *spo0H-lac* fusion plasmid described previously (39), which was constructed by using *Eco*RI restriction of both plasmids followed by ligation, did not have the *spo0H* in phase with the *lacZ* gene. This result indicated that the amino acid sequence derived from our published sequence of the *B. licheniformis* gene (33) was in error. The correct sequence of the *spo0H* gene (E. Dubnau, G. Nair, and I. Smith, manuscript in preparation) predicts an open reading frame with a different reading phase than that reported previously. New cointegrants were constructed (see Materials and Methods) with plasmid pIS9B, which carries the *spo0H* gene of *B. licheniformis* (6), and pSK10Δ6, which carries the *lacZ* gene of *E. coli* with no transcriptional or translational signals and is missing the first eight codons for β-galactosidase (51). Although fusions to *lacZ* were attempted for each of the three potential *spo0H* reading frames, only the one made by *Eco*RI restriction of both plasmids followed by the fill-in reaction of the Klenow fragment of DNA polymerase resulted in a significant number of chloramphenicol-resistant transformants which were blue on plates containing X-gal

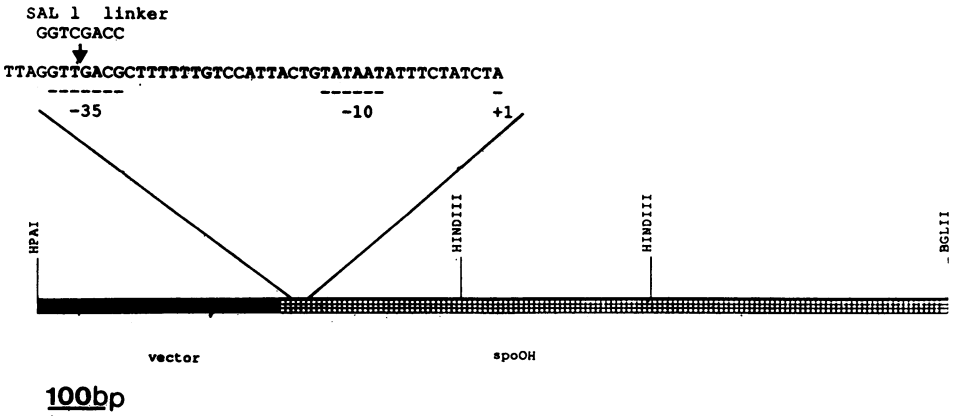

FIG. 2. Scheme for producing a promoter-proximal DNA probe for S1 RNA transcriptional mapping and for disruption of the promoter by introduction of a *Sal*I site into the promoter region of the *spo0H* gene. A portion of pIS9B is shown, with the cloned insert containing the *spo0H* gene indicated by cross-hatching and the vector portion indicated by a solid black line. The sequence of the promoter region (33) is shown as an expanded portion of the plasmid. The 770-bp *Hpa*I-*Hin*dIII fragment containing the promoter region was 5′-end labeled at the *Hin*dIII site and used as a probe to determine the transcription initiation site in various constructs. The −35 portion, GTTGACG, contains within it the *Hinc*II restriction site, GTTGAC, and a *Sal*I linker was inserted into this site to disrupt the promoter.

(3%, compared with less than 0.02% for the two other phases). This result was consistent with our new sequence.

pIS52 (Fig. 1), one of the fusion plasmids formed by *Eco*RI digestion followed by the Klenow fill-in reaction, having the *spo0H* gene in frame with the *lacZ* gene, was also used to create another construct, pIS123, in which the *spo0H-lacZ* portion had the opposite orientation with reference to the pBD97 moiety (Fig. 1). As expected, both cointegrants had the same β-galactosidase activity (data not shown).

Integration of *spo0H-lac* fusions into the *B. subtilis* chromosome. Since cloned genes are often not regulated normally when present on multicopy plasmids (51) or in multiple copies on the chromosome (32), a study of the regulation of *spo0H* by using *lacZ* fusions requires their integration into the chromosome of *B. subtilis* in single copy. The *B. licheniformis spo0H* gene does not have sufficient homology with its *B. subtilis* counterpart to integrate into the *B. subtilis* chromosome (46), but we were able to integrate our plasmid constructs by using their homology with the pE194 replication region. The *lacZ* fusion plasmids described above contain pBD97, a vector with the replication region of pE194 (17). We were able to integrate the constructs into the *B. subtilis* chromosome by taking advantage of the fact that plasmid pE194 itself can integrate (21). Using competent cells of IS250, which contains pE194 integrated between *cysB* and *hisA* (21), we transformed in the relevant plasmids, selecting for chloramphenicol resistance, and passaged the transformants several times at 51°C to select against the free plasmid, whose replication in *B. subtilis* is temperature sensitive (38). After several such passages, the clones became sensitive to erythromycin and retained resistance to chloramphenicol, even when selection for that antibiotic was removed. Possibly the plasmids integrated into the pE194 site by homologous recombination with the replication region by a Campbell-like mechanism, followed by another "reverse Campbell" recombinational event at a different site, resulting in a substitution of the integrated pE194 with the selected construct. Alternatively, the plasmid may have originally integrated by a linear replacement. In any case, Southern hybridization experiments showed that no rearrangements occurred during the integration of the plasmids at the pE194 site, and the resulting structure was that of a linear replacement (data not shown). These experiments also showed that strains containing the integrated pE194, pIS123, and pIS125 (containing a deletion in the *spo0H* promoter region; see below) had the same chromosomal junction fragments. This indicated they all had the same chromosomal location. Transductional crosses with antibiotic resistance as an unselected marker showed that the integrated *lacZ* fusions had the same linkage to *cysB* (66%) and *hisA* (7%) as the originally integrated pE194 (21). No evidence for amplication of integrated plasmid sequences was observed, as has been reported for integrated plasmids when the strains carrying them are grown under conditions of increasing antibiotic concentration (32).

Disruption of the *spo0H* promoter. To ascertain whether the *spo0H-lacZ* fusions were using the *spo0H* promoter and not some upstream vector sequences, we constructed a *spo0H-lacZ* fusion containing a damaged *spo0H* promoter. The −35 region of the *spo0H* promoter contains a *Hin*cII restriction site, GTTGAC (Fig. 2) (33). We disrupted this sequence by ligating *Sal*I linkers into a partial *Hin*cII digest of pIS9A (there is an additional *Hin*cII site in the pBD97 moiety) and transforming into a competent strain of *B. subtilis*. Plasmid DNA from the chloramphenicol-resistant transformants was analyzed by restriction for insertion of the *Sal*I site into the proper *Hin*cII site. One of the plasmids with the correct insertion, pIS97, was used for further studies (Fig. 1).

Sporulation in strains with a disrupted *spo0H* promoter. The plasmids pIS97 (containing a disruption and deletion in the *spo0H* promoter), pIS9A (containing the entire *spo0H* gene), and pBD97 (the vector for both constructs) were assayed for their ability to cause sporulation in a *spo0H* mutant strain. First, the above plasmids were integrated into the chromosome of IS372, a strain with the *spo0HΔHind* (46), carrying pE194 integrated as in IS250 (described above). The strain was transformed with the various plasmids, and the Cm^r transformants were passaged at 51°C, resulting in strains IS374, IS375, and IS376, which we presume from analysis of analogous constructs (see above) now contain the respective plasmids integrated in the chromosome in single copy. These strains are chloramphenicol resistant and sensitive to erythromycin, as the incoming plasmid has replaced the host pE194, as described above. The frequencies of sporulation, measured by heat-resistant viable counts, in cultures grown overnight in NSM were 0.01% for IS374, 55% for IS375, and not detectable for IS376. The ability of the free plasmids to cause sporulation was then assayed in strain IS233, which carries the *spo0HΔHind* mutation, but no integrated pE194. Thus, plasmid transformants of IS233 cannot integrate into the chromosome but remain in multicopy form. The sporulation frequencies were, respectively, 33% for cells carrying pIS97, 74% for those with pIS9A, and not detectable for pBD97-containing cells. Similar results were obtained when pIS97 was used to transform IS234, a *recE spo0HΔHind* strain. We concluded that the damaged promoter was incapable of supporting sufficient transcription of the *spo0H* gene (see below) to allow sporulation unless present on a multicopy plasmid. This multicopy-plasmid effect could be due to gene dosage or to an alteration in the physical state of the promoter in the free plasmid.

We subsequently discovered that pIS97 had a small deletion which extended from the *Sal*I linker in the −35 region past the *Bgl*II cloning site 35 bp into the vector. A *lacZ* fusion cointegrant was made by using this plasmid, resulting in plasmid pIS101 (Fig. 1), and the part of the small deletion which extended into the vector was repaired as described in Materials and Methods. This cointegrant, pIS125, is shown in Fig. 1.

Initiation of transcription. To determine whether transcription of the *spo0H* gene initiated at the correct site in the various plasmid constructs, we performed S1 mapping experiments with in vivo RNA transcripts which protected restriction fragments containing the promoter-proximal portion of the *spo0H* gene. RNA was prepared from *B. licheniformis*, as well as from a set of *B. subtilis* strains which contained plasmid pIS52, pIS97, and pIS9A integrated into the chromosome (IS352, IS374, and IS375, respectively). A radioactive probe, 5′-end labeled at the proximal *Hin*dIII restriction site within the *spo0H* gene (Fig. 2), was used to hybridize with the RNA, and S1 nuclease-resistant hybrids were analyzed on acrylamide gels. The protected hybrids formed with the various RNAs are shown in Fig. 3.

The end-labeled probe was about 770 bp and the size of the S1 nuclease-resistant fragments was about 300 bp with RNA from *B. licheniformis* (lane 1), IS352 (lane 2), or IS375 (lane 4). No protected DNA was observed with RNA from IS374, which contains the disrupted *spo0H* promoter (lane 3). We conclude from this that the *spo0H* gene initiates transcription from the same sequence, within the limits of detection

on a low-resolution gel, whether the gene is on the *B. licheniformis* chromosome in its usual location, cloned in the pBD97 vector, or as part of a *spo0H-lacZ* fusion and integrated into the *B. subtilis* chromosome. This sequence is the normal *spo0H* promoter, as the size of the protected hybrid (approx. 300 bp) (Fig. 3) was consistent with our earlier high-resolution mapping of the *spo0H* promoter (33) and because disruption of the −35 sequence abolished *spo0H* transcription.

***spo0H*-directed β-galactosidase activity.** *B. subtilis* strains containing *spo0H-lacZ* fusions integrated in the chromosome were grown in NSM. Samples were removed at intervals and assayed for β-galactosidase activity. The level of activity rose, going up about threefold as the cells entered stationary phase, and then decreased to its original level (Fig. 4). This pattern remained the same when glucose was added to the medium, except that the activity was higher. We found that *B. subtilis* had a low level of endogenous β-galactosidase activity in NSM which increased at the end of exponential growth and reached a level of about 1 to 3 Miller units. This activity, which was repressed by glucose to barely detectable levels, has been noted by other workers (P. Zuber, personal communication), and it must be taken into consideration when measuring low-activity *lacZ* fusions. The strain containing the promoter disrupted in the −35 sequence had no activity above that found in IS253, a *B. subtilis* strain carrying the integrated pIS52A, which is similar to pIS52 but with the pIS9B moiety in the opposite orientation with relation to the pSK10Δ6 part. Thus, the *spo0H* promoter is incorrectly placed for transcription of the *lacZ* gene. This strain produced the same low endogenous levels of β-galactosidase observed in *B. subtilis* strains with no *lacZ* fusions (see Fig. 8).

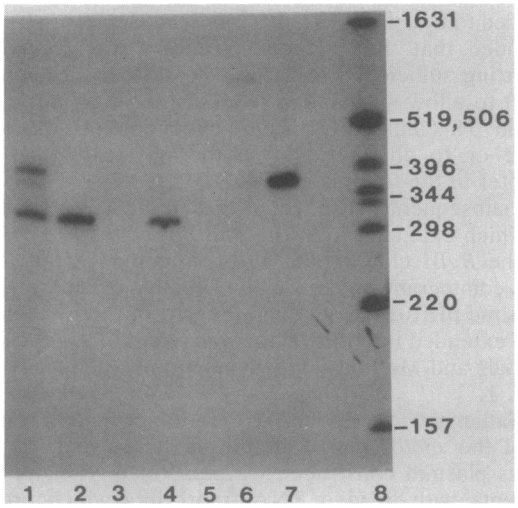

FIG. 3. S1 nuclease mapping of in vivo *spo0H* transcripts. RNA isolated from various strains was used to protect a 770-bp *Hin*dIII-*Hpa*I fragment (Fig. 2) containing the promoter-proximal segment of the *spo0H* gene, 5′ end-labeled with [γ-^{32}P]ATP at the *Hin*dIII site. Lanes: 1, RNA from *B. licheniformis*; 2, RNA from IS352, which contains pIS52 integrated into the *B. subtilis* chromosome; 3, RNA from IS374, which contains pIS97 integrated into the *B. subtilis* chromosome; 4, RNA from IS375, which contains pIS9A integrated into the *B. subtilis* chromosome; 5, no RNA; 6, 770-bp DNA probe not treated with S1 nuclease and no RNA; 7, 350-bp *Hin*dIII-*Hin*dIII fragment from the *B. licheniformis spo0H* clone labeled at both ends with [γ-^{32}P]ATP, no RNA, and not treated with S1 nuclease; 8, pBR322 *Hinf* fragments end labeled with [γ-^{32}P]ATP as molecular weight standards. RNA was added at 200 μg per lane. The 770-bp probe was slightly contaminated with the 350-bp *Hin*dIII fragment (lanes 6 and 7), and a protected band corresponding to this is visible in lanes 1 and 4. An extra band (which ran more slowly than the 350-bp *Hin*dIII fragment) was observed (lane 1) when RNA from *B. licheniformis* was used, but its presence was variable (33).

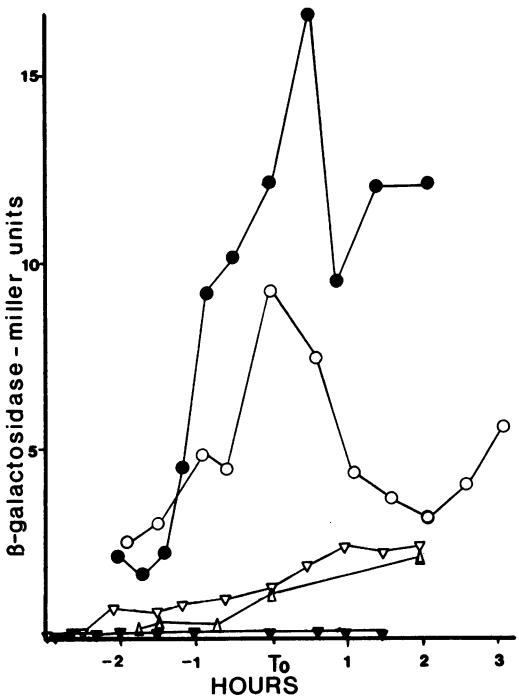

FIG. 4. *spo0H-lacZ* activity during growth. β-Galactosidase activity (Miller units) was measured during growth of various strains in NSM with (solid symbols) and without (open symbols) 0.5% glucose. Symbols: ●,○, IS390 (containing the integrated *spo0H-lacZ* fusion, pIS123); △, strain IS391, containing the integrated *spo0H-lacZ* fusion, pIS125, which has a disrupted promoter; ▼,▽, IS253, an isogenic strain with integrated pIS52A (see Table 1). For clarity, growth curves, measured turbidimetrically, are omitted. T_0 connotes the beginning of stationary phase, and the other times refer to this point.

Decoyinine, an inhibitor of guanine nucleotide biosynthesis and an inducer of sporulation in glucose-containing media (13), was extremely effective in stimulating *spo0H*-directed β-galactosidase activity (Fig. 5). IS390, containing the integrated *spo0H-lacZ* fusion, was grown in the minimal medium used for decoyinine induction of sporulation (13), and samples were taken at intervals after addition of (i) decoyinine, (ii) decoyinine plus guanosine, (iii) guanosine, or (iv) no addition to determine whether the effect of decoyinine would be reversed by supplementation with guanosine, as has been shown for sporulation (13). The samples were assayed for β-galactosidase, and the specific activity remained low unless decoyinine was added, which caused a 10-fold increase in the specific activity. This increase was apparent within an hour after decoyinine addition. The cultures were incubated overnight and assayed for sporulation. In the absence of decoyinine there were 3.6×10^6 spores per ml out of 1.5×10^9 total cells (0.2%), and in the presence of decoyinine there were 1.3×10^7 spores per ml out of 2.7×10^8 total cells (5%).

We next examined the effect of various *spo0* mutations on expression of the *spo0H-lacZ* fusion. A set of otherwise isogenic strains (IS392, IS393, IS394, IS395, IS396, and IS397, Table 1) was constructed which contained the integrated pIS123 and mutations in *spo0A*, *spo0B*, *spo0F*,

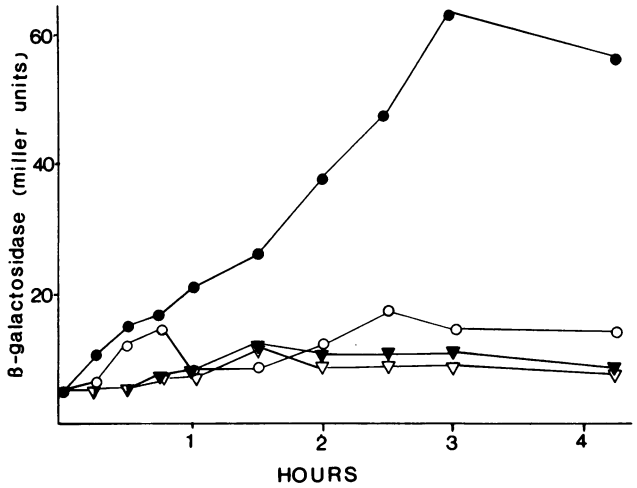

FIG. 5. Effect of decoyinine on *spo0H-lacZ* activity. β-Galactosidase activity was measured during growth of strain IS390 (containing the integrated *spo0H-lacZ* fusion, pIS123) in S6C (13) medium with the addition of decoyinine, 1 mg/ml (●), guanine, 50 μg/ml, plus decoyinine, 1 mg/ml (▼), guanine, 50 μg/ml (▽), or no addition (○).

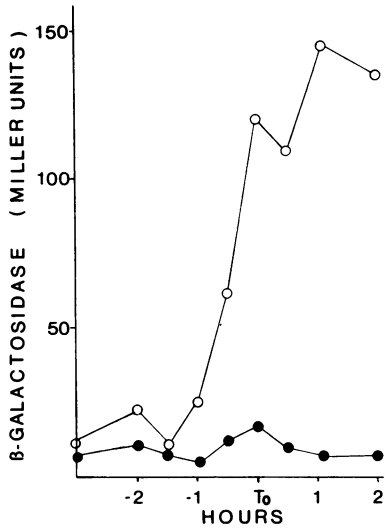

FIG. 7. Effect of a *spo0H* mutation on *spo0H-lacZ* activity. β-Galactosidase levels were measured during growth in NSM of IS392 (●) and IS394 (○), which carries the *spo0HΔHind* mutation.

spo0G, *spo0H*, and *spo0J*, respectively. These were created by transforming different *spo0* recipients with DNA isolated from IS390, which has pIS123 integrated, and selecting for chloramphenicol resistance. The strains were grown in NSM, and β-galactosidase was assayed at intervals during growth. The results for strains with mutations in *spo0J*, *spo0B*, and *spo0F* are shown in Fig. 6. All these strains showed patterns similar to that of the Spo$^+$ parent. However, when the same experiment was performed with a strain carrying the *spo0HΔHind* mutation, levels of β-galactosidase rose much higher (Fig. 7), reaching a maximum of about 150 Miller units, compared with 10 to 15 units in the Spo$^+$ strain. *spo0H*-driven β-galactosidase activity in this *spo0H* deletion strain remained at a high level, not declining after T_0 as with the wild type.

In contrast, we found very low *spo0H-lac* fusion activity in strain IS409, which has a deletion in the *spo0A* gene (Fig. 8). This activity was only slightly higher than in IS404, an isogenic strain with no *lac* fusion, and did not show the usual

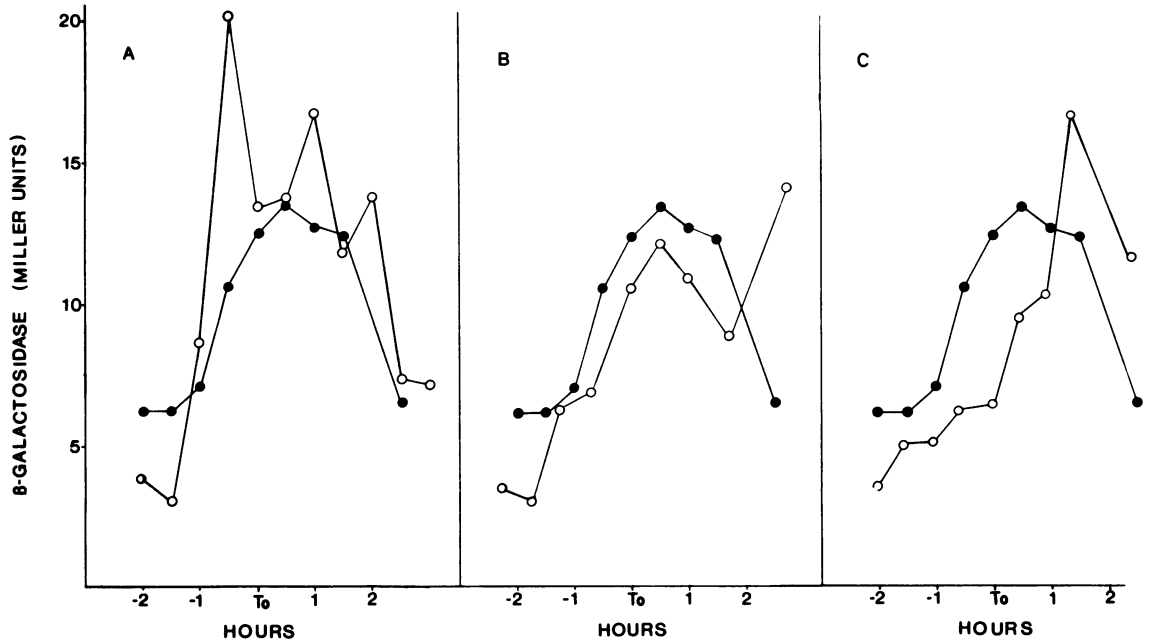

FIG. 6. Effect of *spo0* mutations on *spo0H-lacZ* activity. Various isogenic strains were grown in NSM, and at intervals samples were removed for β-galactosidase assays. (A) IS392, a *spo*$^+$ strain containing integrated pIS123. (●), and IS397, an otherwise isogenic strain containing the *spo0J87* mutation (○); (B) IS392 (●) and IS395, with the *spo0F221* mutation (○); (C) IS392 (●) and IS393, containing *spo0BΔPst* (○).

rise in specific activity at T_0. Similar results were obtained when a strain with the *spo0G14* mutation was used to measure *lacZ* fusion activity (data not shown). This is consistent with the recent finding that *spo0G* mutations actually lie in the *spo0A* gene (J. Hoch, personal communication). However, the requirement for the *spo0A* gene product could be suppressed in IS407, which contains both the *spo0A* deletion and the *abrB* mutation, which is a partial suppressor of *spo0A* mutations (44). In addition, the presence of the *abrB* mutation alone or in combination with the *spo0A* deletion caused an increase in the levels of β-galactosidase in samples collected during vegetative growth from 3 to 5 Miller units in Spo^+ strain IS406 to 9 to 15 units in strains IS407 and IS408, which contain the *abrB* mutation (Fig. 8).

We found that the decoyinine stimulation of *spo0H-lacZ* activity described above still occurred in strain IS409, which has the *spo0AΔ204* mutation, albeit the absolute activity is 5 to 10 times lower than in strains IS407 (*spo0AΔ204 abrB*), IS408 (*spo*$^+$ *abrB*), or IS406 (*spo*$^+$ *abr*$^+$). The stimulation increased the activity 5 to 10 times above the levels obtained in the absence of decoyinine in all the strains (data not shown).

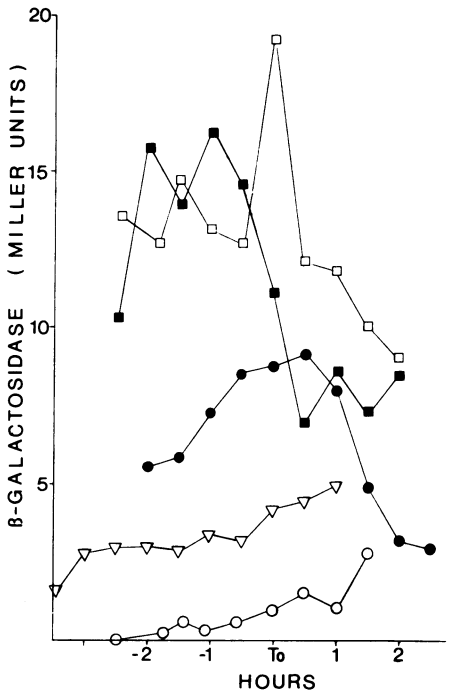

FIG. 8. Effect of *spo0A* and *abrB* mutations on *spo0H-lacZ* activity. Strains were grown in NSM, and β-galactosidase levels were measured at intervals. IS406, a Spo^+ strain containing the integrated pIS123 (●); IS407, isogenic to IS406 and carrying the mutations *spo0AΔ204* and *abrB* (□); IS408, isogenic to IS406 with the *abrB* mutation (■); IS409, isogenic to IS406 carrying the *spo0AΔ204* mutation (▽); and IS404, isogenic to IS406 but without pIS123 and containing the *spo0AΔ204* mutation (○).

DISCUSSION

Losick and Pero (26) have proposed that sporulation in *B. subtilis* is controlled at the transcriptional level by a sequential replacement of various sigma factors in the RNA polymerase holoenzyme. Thus, holoenzymes with differing promoter specificities will be functional at different times during the developmental cycle. Although several sigma factors have been described for *B. subtilis* (reviewed in reference 5), thus far only σ^{29}, coded for by the *spoIIG* gene (42), is known to be essential for sporulation. The sequence of the *spoIIAC* gene suggests that this gene may also code for a sigma factor (7, 11), but a holoenzyme containing the *spoIIAC* gene product has not been isolated.

Studies on *spoVG*, a gene which is expressed early in sporulation, led Losick and co-workers to propose that the *spo0H* gene product is a positive regulatory element required for the transcription of *spoVG* (50), which in vitro can be transcribed by an RNA polymerase holoenzyme containing σ^{37} (18). Expression of this gene is highly dependent on the presence of *spo0H*, and although it also requires most of the other *spo0* genes for maximum expression (52), there are conditions under which it requires only *spo0H*. For example, the requirement for *spo0A* can be suppressed by the *abrB* mutation (P. Zuber and R. Losick, personal communication), a mutation which does not suppress the sporulation-negative phenotype of *spo0A* mutants. In addition, the *sof-1* mutation, which suppresses the Spo^- phenotype of *spo0B*, *spo0E*, and *spo0F* mutations (22) and maps in the *spo0A* open reading frame (20), also allows the in vivo expression of *spoVG-lacZ* fusions in *spo0F* cells (C. Moran, personal communication). When the *spoVG-lacZ* fusion is present on a multicopy plasmid, the only *spo0* gene required for its expression is *spo0H* (P. Zuber and R. Losick, personal communication). There is evidence that the product of *spo0H* interacts directly with the promoter region of *spoVG*, based on the observation that deletion of the AT-rich region upstream of the *spoVG* promoter, which has very little residual activity, is still totally dependent on *spo0H* (50). In addition, a particular mutant allele of *spo0H* is capable of suppressing a promoter mutation of *spoVG* (P. Zuber and R. Losick, personal communication).

A new RNA polymerase (containing a sigma factor with an apparent molecular weight of approximately 30,000) has been isolated in *B. subtilis* which transcribes *spoVG* in vitro (3a). This holoenzyme presumably transcribes *spoVG* in vivo since deletions in the gene coding for σ^{37} (2) do not prevent expression of *spoVG* (R. Losick, personal communication; C. Price, personal communication) and, significantly, do not affect sporulation (2). The σ^{30}-containing RNA polymerase is not observed in strains with a deletion in the *spo0H* gene (H. L. Carter and C. Moran, personal communication), and preliminary experiments have indicated that antibodies raised against the *B. licheniformis spo0H* protein react specifically with σ^{30} (I. Smith et al., unpublished observations). New sequencing data indicate that the *spo0H* gene of *B. licheniformis* and *B. subtilis* codes for a sigma factor (Dubnau et al., manuscript in preparation). These results indicate a central role for the *spo0H* gene product in the initiation of the sporulation process as a sigma factor required for the transcription of early sporulation genes.

We have studied the regulation of the cloned *spo0H* gene of *B. licheniformis* by constructing translational *lacZ* gene fusions, integrating them into the chromosome in single copy, and measuring the β-galactosidase produced under various conditions. The cloned *spo0H* gene of *B. licheniformis* functions both in *trans* (6) and, when integrated into the chromosome in single copy, to promote sporulation in *B. subtilis* strains lacking a functional *spo0H* gene. It is transcribed from the same promoter sequence used in *B. licheniformis*. However, we should qualify our conclusion on the regulation of this gene, since sequences

upstream to the cloned gene may reveal additional controlling factors.

spo0H-lacZ fusion activity, while present during vegetative growth, rises at T_0 and then declines, which is similar to the pattern observed with *spoVG-lacZ* fusions (51). This suggests that while the *spo0H* gene product may function during vegetative growth, it is regulated so that more *spo0H* protein is available during the early stages of sporulation. The pattern is the same in the presence or absence of glucose, but β-galactosidase levels rise to higher levels in glucose-grown cultures. This contrasts with the glucose repressibility of *spoVG-lacZ* fusion activity (51) and indicates that the control of *spoVG* transcription is not determined solely by regulation of the *spo0H* product. This is already known, because in addition to the requirement for *spo0A* and *spo0H*, *spoVG* transcription requires *spo0B*, *spo0F*, *spo0E*, and *spo0K* for maximal expression (51). Certainly the fact that *spo0H-lacZ* activity is not repressed by glucose implies that the *spo0H* gene is not a target for catabolite sensitivity of the sporulation process. The somewhat elevated levels of *spo0H-lacZ* fusion activity in glucose-grown cultures may be due to lower levels of proteases in such cultures. The decline in *spo0H-lacZ* activity after T_0 could be caused by increased turnover due to protease production appearing during late growth, by downregulation of *spo0H* expression coupled with a high level of instability of the *spo0H* or *spo0H-lacZ* fusion protein, or by a combination of these factors. The regulation of *spo0H-lacZ* expression in a *spo0H* deletion strain (Fig. 6) is interesting in this regard. Much higher levels (10-fold) of *spo0H-lacZ* activity were found in the mutant than in the wild type, and these activities did not decline after T_0. Similar results were described previously (39) with a different *spo0H* mutation. These results could be explained by *spo0H*-dependent protease or nuclease activities, but they are also consistent with autoregulation of *spo0H* expression by its gene product. Although the σ^{70} of *E. coli* is apparently not autoregulated (1), it would not be too surprising if this type of control were involved in expression of the *spo0H* gene since overproduction of a minor sigma might be harmful to the cell. Experiments are now in progress which will enable us to decide on these alternative explanations for the decline in *spo0H-lacZ* fusion activity after T_0. In contrast to the pattern in NSM-grown cultures, the *spo0H-lacZ* fusion activity did not decline in the minimal salts-glucose medium used for decoyinine induction (Fig. 5), and at present this result is unexplained.

Our experiments with *spo0H-lacZ* fusions of the *B. licheniformis spo0H* gene indicate that its expression does not require *spo0B*, *F*, *H*, or *J* (*spo0E* and *spo0K* were not tested). However, its expression does depend on *spo0A*, since strains with lesions in this gene have very little activity above the background endogenous β-galactosidase levels found in *B. subtilis*. Previously we reported (39) that a strain with a *spo0A* mutation had slightly elevated levels of *spo0H-lacZ* fusion activity. The strain used in those experiments contained the *spo0A12* mutation, and although the small increase in activity was not reproducible, we found that unlike strains with the *spo0G* or *spo0AΔ204* mutations, this strain behaved like the Spo^+ strain, producing normal levels of *spo0H-lacZ* fusion activity. Albano and Dubnau (personal communication) have shown that although the *spo0A12* mutation caused a 100-fold decrease in competence compared with the wild type, *spo0G* and *spo0AΔ204* caused a decrease of 1,000-fold. Therefore, we feel that the variation in phenotype observed between strains containing various alleles of the *spo0A* gene may reflect differences in the amount of residual *spo0A* activity and that meaningful results on the interdependence of these genes require the use of null mutations.

The requirement for the *spo0A* gene can be bypassed if the *spo0A* mutant strains also carry an *abrB* mutation, a partial suppressor of *spo0A* mutations, which restores the production of antibiotic protease and ability to develop competence, but does not restore the ability to sporulate (45). On the basis of the observation that the dependence of *spoVG* transcription on the *spo0A* gene can be suppressed by mutations in the *abrB* gene (52), Losick has proposed that the *abrB* gene codes for a repressor of *spo0H* transcription and that one of the functions of the *spo0A* gene product is to inactivate this repressor (personal communication). The facts that the presence of a σ^{30}-containing RNA polymerase holoenzyme is dependent on *spo0A* and that this requirement can be suppressed by an *abrB* mutation (Carter and Moran, in press), as well as the results presented here, are consistent with this proposal.

The decoyinine induction of *spo0H-lac* expression is the first indication that guanine nucleotide starvation, which has been implicated as a nutritional signal for sporulation (12, 29), affects the expression of a specific gene required for the early stages of sporulation. We found that decoyinine stimulation is independent of the *spo0A* requirement, since the low residual *spo0H-lacZ* fusion activity present in the strain with the *spo0AΔ204* mutation could still be increased by incubation with decoyinine. Decoyinine also rapidly induced the expression of *spoVG*, which is totally dependent on the *spo0H* gene (P. Zuber and R. Losick, personal communication). It therefore seems probable that the stimulation of sporulation by guanine nucleotide starvation is mediated at some level through the *spo0H* gene. It is interesting to postulate the presence of a gene(s) whose function is to detect the guanine levels in the cell, a system similar to the G-binding proteins involved in yeast sporulation (40). Such a system may be involved in functions other than sporulation, but one of its activities could be to turn on the *spo0H* gene.

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