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Sin, a Stage-Specific Repressor of Cellular Differentiation

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Sin is a *Bacillus subtilis* DNA-binding protein which is essential for competence, motility, and autolysin production but also, if expressed on a multicopy plasmid, is inhibitory to sporulation and alkaline protease synthesis. We have now examined the physiological role of Sin in sporulation and found that this protein specifically represses three stage II sporulation genes (*spoIIA*, *spoIIE*, and *spoIIG*) but not the earlier-acting stage 0 sporulation genes. *sin* loss-of-function mutations cause higher expression of stage II genes and result in a higher frequency of sporulation, in general. Sin binds to the upstream promoter region of *spoIIA* in vitro and may thus gate entry into sporulation by directly repressing the transcription of stage II genes. In vivo levels of Sin increase rather than decrease at the time of stage II gene induction, suggesting that posttranslational modification may play a role in downregulation of negative Sin function.

Bacillus subtilis escapes adverse environmental conditions, such as lack of nutrients, by differentiating into a dormant cell known as the endospore. This differentiation process can be viewed as a cellular decision to stop simple vegetative growth and to begin a series of physiological and morphological changes which result in a new cell type. Two major requirements must be satisfied for a successful completion of any procaryotic differentiation process: (i) the correct processing of multiple environmental signals and (ii) the successful coordination of subsequent morphological changes. Many sporulation genes which control the latter have been identified, and the network through which they communicate is just starting to reveal a sophisticated set of mechanisms which coordinate their temporal and spatial regulation (reviewed in references 17 and 34). In the case of the first requirement, which deals with whether the cell should enter a dormant way of life, it was shown recently that a *Bacillus* cell communicates environmental signals (low nutrients in the environment) to the sporulation genetic machinery by means of a multicomponent phosphorelay system (5). Although it is not clear as yet how the phosphorelay cascade is set into motion, i.e., how and through which component(s) the intercellular sensor KinA (also known as SpoIIJ) (2, 21) recognizes environmental change, the genetic evidence suggests that phosphorylation of the last phosphorelay protein, Spo0A, is a sporulation-triggering signal (20). The earliest known response to phosphorylation of Spo0A is downregulation of the negative regulator AbrB and the resultant increase in expression of late growth-regulated genes (35), including *spo0H* (42), which codes for transcription factor σ^H (9, 45). This in turn causes increased expression of *kinA*, *spo0F*, *spo0A*, and *spoVG*, which are transcribed by $E\sigma^H$ (6, 26). We have recently suggested that this double-negative control may serve as one of the first checkpoints for whether a stress-subjected cell will enter sporulation (33). The second major function of Spo0A phosphate is to increase the expression of the stage II sporulation genes, *spoIIG* (30), *spoIIE* (44), and *spoIIA* (38).

The requirement of Spo0A for initiation of stage II raises the question as to whether Spo0A phosphate is the only temporal regulator of stage II genes or whether additional factors play a role in expression of these genes. The experience from other developmental systems, e.g., *Drosophila* (36) and *Caenorhabditis elegans* (29) systems, as well as recent experiments in the sporulation field (27, 31) suggests that negative control could be an important factor in temporal regulation of the sporulation process. Besides AbrB, other factors, like Spo0E (23), Hpr (22), and Pai (15), have been shown to negatively control spore development. For example, Spo0E inhibits sporulation when overexpressed in the cell (23). Elevated levels of Hpr and Pai also inhibit sporulation, and loss-of-function (Lf) mutations in *hpr* and *pai* make sporulation insensitive to glucose inhibition (15, 22). Finally, we have shown that Sin inhibits sporulation and exoprotease production if overexpressed in the cell (*sin* gain-of-function [Gf] phenotype) (11).

Sin is a 14-kDa dual-function DNA-binding protein which has a negative function in sporulation and in *aprE* expression (*aprE* encodes the major alkaline protease) but also has a positive role in development of competence and motility [demonstrated in a *sin*(Lf) mutant] (11, 12). We have now examined the regulatory role of Sin in sporulation. In this report we provide evidence that the Sin protein negatively regulates sporulation by modulating the expression of three stage II-specific sporulation genes: *spoIIA*, *spoIIE*, and *spoIIG*. We also show that Sin binds to the *spoIIA* upstream promoter region in vitro. Thus, Sin may directly inhibit the transcription of its target genes. Finally, we examine the in vivo concentration of Sin during vegetative growth and sporulation.

MATERIALS AND METHODS

Strains. The bacterial strains used in this study are listed in Table 1. IS432 contains a large deletion (*Bal*I-*Nru*I) replaced by a chloramphenicol resistance (Cat^r) determinant insertion in the *sin* gene (12). IS720 (kindly provided by A. Sloma) carries the same deletion in the *sin* gene as IS432 except that a phleomycin resistance (Phl^r) determinant is inserted into the *Bal*I-*Nru*I region. IS620 and IS703 were constructed by transforming the *Hin*dIII-cut pIS158 plasmid (see below) into IS611 and IS686, respectively, selecting transformants for erythromycin resistant (Erm^r) colonies and scoring them for Cat^s. IS614, IS621, and IS705 were constructed by transforming IS432 chromosomal DNA into IS567, IS620,

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant genotype	Source or reference
Strains		
IS432	*Δsin::cat leuA8 metB5 hisA1*	12
IS567	*spoIIE*::Tn*917-lacZ erm*	PY463 (14)
IS611	*spoIIA*::(*spoIIA-lacZ*) *cat trpC2 metC3 rif-2*	MB24 (45)
IS614	*spoIIE*::Tn*917-lacZ erm Δsin::cat*	This work
IS620	*spoIIA*::(*spoIIA-lacZ*) *erm trpC2 metC3 rif-2*	This work
IS621	*spoIIA*::(*spoIIA-lacZ*) *erm Δsin::cat trpC2 metC3 rif-2*	This work
IS657	*spoIIG*::(*spoIIG-lacZ*) *cat trpC2 pheA1*	EU8901 (C. Moran)
IS673	*kinA*::Tn*917 erm leuA8 metB5 hisA1*	41
IS686	*spoIID*::(*spoIID-lacZ*) *cat*	SR10 (A. L. Sonenshein)
IS703	*spoIID*::(*spoIID-lacZ*) *erm*	This work
IS705	*spoIID*::(*spoIID-lacZ*) *erm Δsin::cat*	This work
IS715	*spoIIG*::(*spoIIG-lacZ*) *cat Δsin::phl trpC2 pheA1*	This work
IS720	*Δsin::phl leuA8 metB5 hisA1*	Alan Sloma
IS727	*kinA*::(*kinA-lacZ*) *cat leuA8 metB5 hisA1*	This work
IS780	*kinA*::Tn*917 erm Δsin::phl leuA8 metB5 hisA1*	This work
Plasmids[a]		
pE194	*erm*	Lab stock
pBD9	*erm kan*	D. Dubnau
pBD347	*cat*	41
pUB110	*kan phl*	Lab stock
pIS21	*erm sin* in pE194	11
pIS74	*cat sin* in pBD347	11
pIS158	*erm*	This work
pIS169	*erm sin* in pBD9	12
pIS184	*cat amp*	This work
pIS219	*cap amp spoIIA* promoter (−1300 to −30) in pIS184	This work
pIS229	*phl sin* in pUB110	This work
pIS247	*amp spoIIA* promoter (−168 to −30) in pUC19	This work

[a] All plasmids replicate in *B. subtilis*, except for pIS247, which replicates only in *E. coli*. pIS148 and pIS219 replicate in both *B. subtilis* and *E. coli*.

and IS703, respectively, and selecting for Cat^r and the rough *Δsin* colony phenotype (11). IS715 was constructed by transforming IS720 DNA into IS657 and selecting for Phl^r and Cat^r. IS780 was constructed by transforming IS720 chromosomal DNA into IS673 (41) and selecting for Phl^r and Erm^r. IS781 was constructed by transforming IS720 chromosomal DNA into IS668 (41) and selecting for Phl^r and Cat^r. IS727, which contains a *kinA-lacZ* translational fusion integrated by Campbell insertion at the *kinA* locus, was made by transforming IS75 with pIS246 (see below), selecting for Cat^r. Transformation of chromosomal markers was performed as described by Anagnostopoulos and Spizizen (1).

Plasmids. Plasmids used in this study are listed in Table 1. Isolation of plasmid DNA, restriction, ligation, and plasmid transformation were done as described by Gryczan et al. (13). Plasmid pPP155 (kindly provided by P. Piggot) carries a 1,800-bp *Hin*dII-*Pvu*I fragment which spans the region from −1300 to +465 of the *spoIIA* operon (43). pIS219 was constructed by subcloning an *Eco*RI fragment from pPP155, containing the *spoIIA* promoter region from positions −1300 to −30, into pIS184 at the *Eco*RI site. pIS184 is a chimera between pUC19 and pBD347 (41) joined at their unique *Hin*dIII sites. pIS249 was constructed by subcloning the *Sca*I-*Eco*RI restriction fragment from pIS219, encompassing the *spoIIA* promoter region from −169 to −30, into the *Eco*RI and *Sma*I sites of pUC19. pIS229 was constructed by subcloning the *Mbo*I restriction fragment from pIS74 (11), containing the *sin* gene under the control of the *sin* P3 promoter (10), into the *Bal*I restriction site of pUB110 (13). pIS246 was made by cutting pDG580 (kindly supplied by P. Stragier and carrying the *kinA* structural gene and upstream regulatory sequence) with *Sac*I, making the end blunt with T4 DNA polymerase, and then cutting with *Dra*I. The resulting fragment was gel purified and ligated into pIS112 (18), giving an in-frame translational fusion of the *kinA* gene with the *lacZ* gene of *Escherichia coli*. Plasmid pIS158 was constructed by subcloning the *Taq*I restriction fragment containing the *erm* determinant from pE194 into the *Xmn*I restriction site of pC194, which is approximately in the middle of the *cat* gene. pIS158, cut at its unique *Hin*dIII restriction site, can be used to replace the *cat* determinant in the *B. subtilis* chromosome with *erm*.

Media and β-galactosidase determinations. Cells containing integrated *lacZ* fusions were grown in Schaeffer's nutrient sporulation medium (NSM) as described previously (8), and samples were removed at intervals to assay β-galactosidase activity. To test the effects of decoyinine on sporulation, cells were grown in S6C medium and sporulation was induced by decoyinine as previously described (3). The assays were performed with toluenized cells as described previously (8). Activity is expressed as units (1 U = 1 nmol of *o*-nitrophenyl hydrolyzed per min) per milligram of protein. Protein determinations were made with the Bio-Rad Laboratories protein assay.

Gel retardation assay. The Sin protein used in this study was purified to homogeneity as described previously (12). The *spoIIA* upstream promoter region DNA used in these studies was obtained from either pIS219 (see Fig. 4B) or from pIS249 (see Fig. 4C). Plasmids were 5′ end labeled and binding reactions were performed as described previously (12) except that poly(dI-dC) instead of salmon sperm DNA was added to the binding reaction.

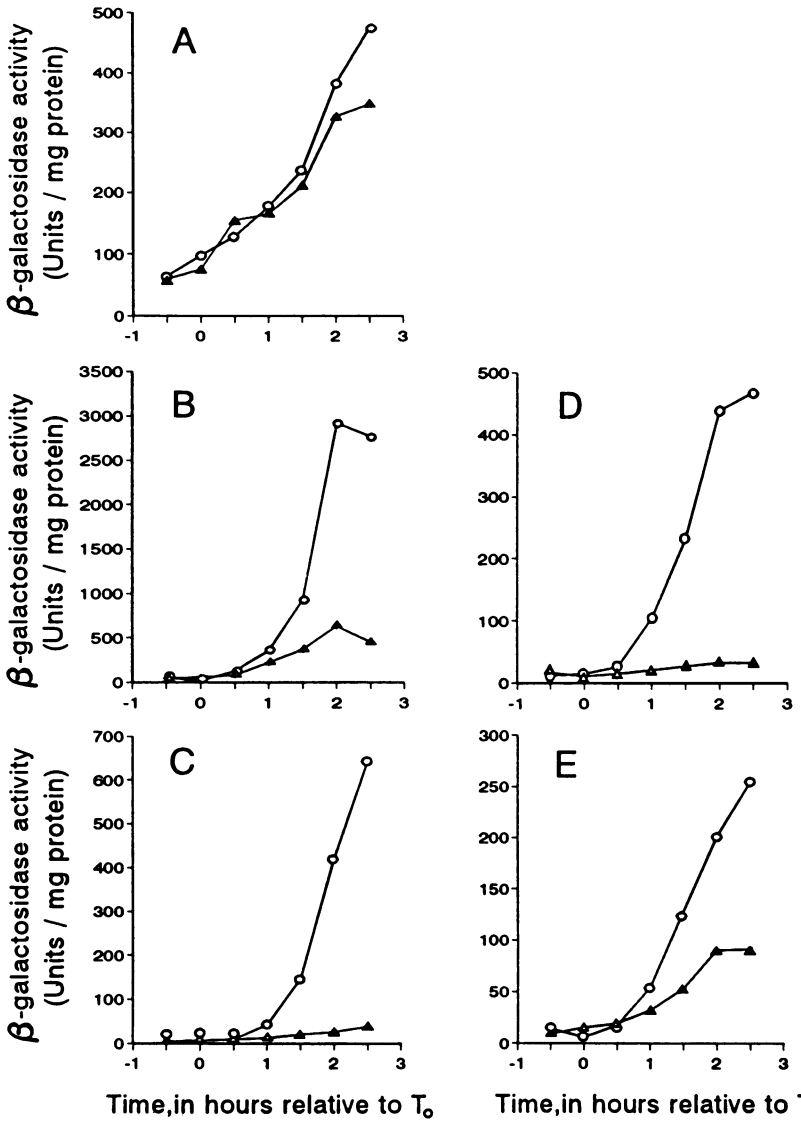

FIG. 1. Effect of Sin overexpression on sporulation genes. Various *spo-lacZ* fusion strains carrying either a plasmid containing the *sin* gene (▲) or the vector alone (○) were grown in NSM, and β-galactosidase activity was determined in cell aliquots withdrawn at the indicated time intervals. (A) IS727 (*kinA-lacZ*) bearing either pIS21 (*sin*$^+$) or pE194 (vector); (B) IS611 (*spoIIA-lacZ*) carrying either pIS169 (*sin*$^+$) or pBD9 (vector); (C) IS567 (*spoIIE-lacZ*) with pIS74 (*sin*$^+$) or pBD347 (vector); (D) IS657 (*spoIIG-lacZ*) with pIS229 (*sin*$^+$) or pUB110 (vector); (E) IS703 (*spoIID-lacZ*) bearing either pIS74 (*sin*$^+$) or pBD37 (*vector*).

Immunological detection of Sin protein. Polyclonal anti-Sin antibodies were raised in guinea pigs by using the Sin protein purified to homogeneity (12) as an antigen. Crude extracts were prepared from *B. subtilis* strains grown in NSM at 37°C. Cells were harvested at intervals from a growing culture (200 ml of cells for each time point) and washed in harvest buffer (50 mM Tris HCl [pH 8.0], 10 mM EDTA, 10% glycerol, 1.0 M KCl, 1.7 mM phenylmethylsulfonyl fluoride). Cell pellets were suspended in buffer I (10 mM Tris HCl [pH 8.4], 1 mM EDTA, 1.7 mM phenylmethylsulfonyl fluoride, 10 mM $MgCl_2$, 0.3 mM dithiothreitol) and stored at −70°C until use. Cells were lysed by passing them twice through a French pressure cell at 1,250 lb/in^2. The protein concentration of each crude extract was determined by the Bio-Rad assay method. Crude extracts were suspended in denaturing tracking dye and boiled for 4 min. Samples (100 μg of total protein) were electrophoresed on a sodium dodecyl sulfate (SDS)–15 to 18% gradient polyacrylamide gel. Proteins, fractionated by SDS-polyacrylamide gel electrophoresis as described above, were transferred electrophoretically to nitrocellulose membranes (37). Immunodetection was performed by using IBI Enzygraphic Web film (Eastman Kodak) following the manufacturer's instructions and as described below. Nitrocellulose blots were blocked by BLOTTO (phosphate-buffered saline [PBS] containing 1.25% nonfat dry milk) at room temperature overnight. Diluted serum containing Sin antibodies (50 μl of serum and 4.5 ml of BLOTTO) was preincubated at room temperature for 2 h with 0.5 ml of cell extract prepared from IS690 (a strain carrying a total deletion of the *sin* gene). Blots were then incubated with pretreated serum at 37°C for 3 h. After a brief washing with PBS, the blots were incubated with a 1:7,000 dilution of biotinylated protein A (Amersham) at 37°C for 1 h. The blots were washed again and incubated

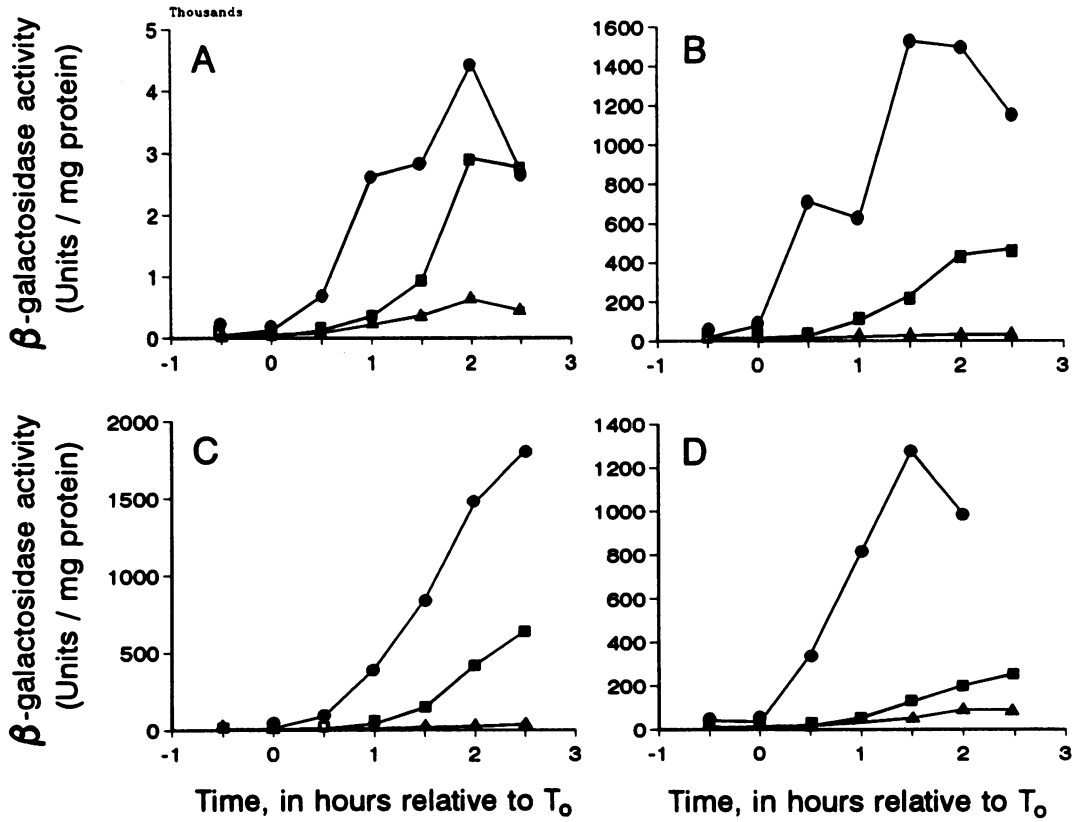

FIG. 2. Effect of Sin overexpression and Sin If mutations on sporulation genes. A set of isogenic *spo-lacZ* fusion strains with the wt *sin* gene (■), with the *sin* gene inactivated (●), or with the *sin* gene on a multicopy plasmid (▲) were grown in NSM, and at the indicated intervals samples were removed for β-galactosidase assays. (A) IS611 (*spoIIA-lacZ sin*$^+$), IS621 (*spoIIA-lacZ Δsin*), and IS611 with pIS169 (*sin*$^+$); (B) IS567 (*spoIIE-lacZ sin*$^+$), IS614 (*spoIIE-lacZ Δsin*), and IS567 with pIS74 (*sin*$^+$); (C) IS657 (*spoIIG-lacZ sin*$^+$), IS715 (*spoIIG-lacZ Δsin*), and IS657 with pIS229 (*sin*$^+$); (D) IS703 (*spoIID-lacZ sin*$^+$), IS705 (*spoIID-lacZ Δsin*), and IS703 with pIS74 (*sin*$^+$). Note the change of scale between Fig. 1 and 2.

with a 1:7,000 dilution of horseradish peroxidase-labeled streptavadin (Amersham) at 37°C for 1 h. Finally, the blots, treated with the protein A-biotin bridge and streptavadin-peroxidase secondary probe, were extensively washed in PBS, briefly dried with a paper towel, and exposed to IBI Enzygraphic Web film until the color developed (approximately 1 min). The blots were stored at −70°C to prevent fading of the obtained color reaction.

RESULTS

Sin is a stage II-specific repressor of sporulation. We have previously shown that elevated levels of the Sin protein inhibit sporulation (11). To investigate the means by which Sin acts, we searched for sporulation genes whose expression would be lowered by overexpression of Sin. The idea was to screen for Sin target genes by introducing either a multicopy plasmid encoding the *sin* gene or a control plasmid into available *spo-lacZ* fusion strains and then to monitor specific β-galactosidase expression in these cells during growth in sporulation medium.

We have previously reported that the *sin*(Gf) phenotype (caused by overexpression of Sin) inhibits alkaline phosphatase expression (11). Alkaline phosphatase is a marker for successful completion of sporulation stage II (defined by completion of an asymmetric septum) (4). To test more specifically the effect of Sin overexpression on this stage of sporulation, we examined *spoIID* expression. The *spoIID* gene is expressed at the same time as alkaline phosphatase, after asymmetric septation, and it is indispensable for sporulation (28). As expected, *spoIID-lacZ* expression was greatly inhibited in the strain carrying multiple copies of the *sin* gene, compared with that in a strain bearing a control plasmid (Fig. 1E). This observation supported our idea that Sin inhibits sporulation prior to or during asymmetric septation.

Genes examined next for their response to high levels of Sin were *spo0H*, *spo0F*, *kinA*, *spoVG*, *spoIIA*, *spoIIG*, and *spoIIE*. The first four genes show maximum expression at or about T_0, the earliest sporulation stage, and the last three are transcriptionally activated at the end of stage 0 (about 1 h later), immediately prior to the formation of the asymmetric septum. None of the early sporulation genes (*spo0H*, *spo0F*, *kinA*, and *spoVG*) were repressed by overexpression of Sin (Fig. 1A, only *kinA* shown). However, expression of *spoIIA*, *spoIIE*, and *spoIIG-lacZ* was severely inhibited in strains carrying a multicopy *sin* plasmid (Fig. 1B, C, and D). Our results demonstrated that *sin*(Gf) mutations specifically downregulate stage II sporulation genes.

The multicopy *sin* effect on *spoII* genes seemed like a reasonable explanation for its sporulation inhibition phenotype but did not necessarily indicate the physiological role of Sin in sporulation when present at normal levels. To explore the normal role of Sin in this developmental process, we

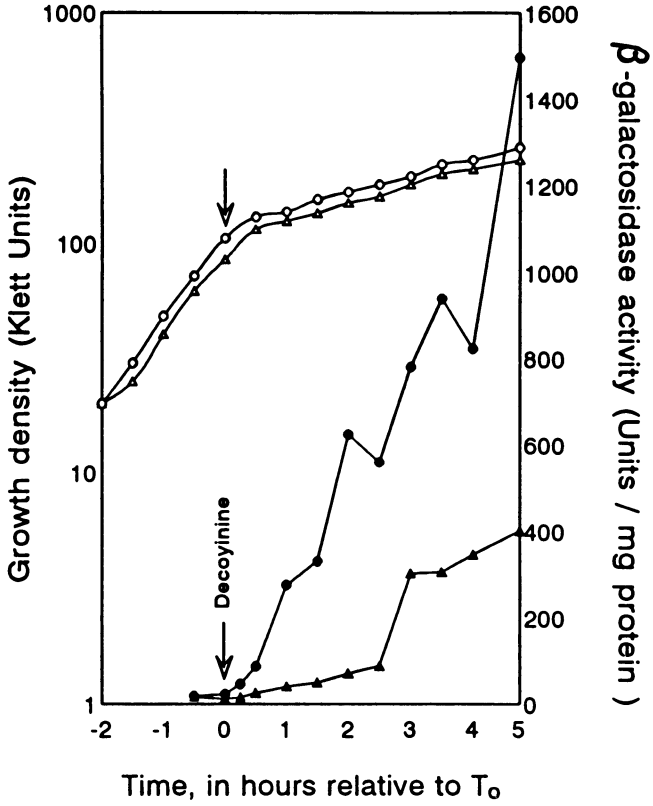

FIG. 3. Effect of *sin* null mutations on timing of *spoIIG-lacZ* induction. Strains were grown in S6C medium, and sporulation was initiated by the addition of decoyinine (0.5 mg/ml). The β-galactosidase specific activity was determined at indicated time intervals in IS657 (*spoIIG-lacZ*) (▲) and in IS715 (*spoIIG-lacZ* Δ*sin*) (●). The growth of IS657 (△) and IS715 (○) is also shown.

introduced a *sin*(Lf) mutation (a *sin* null lesion) into strains carrying integrated *spoIIE*, *spoIIA*, or *spoIIG-lacZ* fusion genes. All three genes were expressed in longer amounts in a *sin* null background than in a wild-type (wt) strain. In addition, we observed that lack of Sin in *B. subtilis* results in expression of stage II genes 1 h earlier (Fig. 2A, B, and C). Expression of alkaline phosphatase and *spoIID-lacZ*, which we were able to monitor in the same culture, responded to the *sin*(Lf) lesion in a way similar to that of the above three genes (Fig. 2D, only data for *spoIID* shown).

The above-described experiments were performed in nutrient broth sporulation medium, in which cells entered the stationary phase and sporulation after exhausting nutrients in the medium. Although this is a standard way to induce sporulation, it was possible that cells were reaching T_0 earlier in the *sin*(Lf) strains. Thus, there was uncertainty in defining the sporulation start point and the beginning of stage II transcription. To examine further the Sin effect upon the timing of expression of its target gene(s), we tested the effect of the *sin*(Lf) mutation on expression of *spoIIG-lacZ* fusions in cultures which were synchronized for their entry into sporulation by the rapid lowering of GTP levels. Decoyinine added to an exponentially growing *B. subtilis* culture causes synchronous entry into the stationary phase by immediately inhibiting GMP synthetase activity and lowering GTP levels (19). Figure 3 shows that *spoIIG*-directed β-galactosidase activity became detectable about an hour earlier in a strain

TABLE 2. Effect of *sin*(Lf) mutations on sporulation[a]

Strain	Cells/ml	Spores/ml	% Spores
IS75 (wt)	1.6×10^8	6.7×10^7	41.8
IS720 (Δ*sin*)	1.0×10^8	0.9×10^8	90.0
IS673 (*kinA*::Tn*917*)	6.7×10^8	1.1×10^7	1.6
IS780 (*kinA*::Tn*917* Δ*sin*)	1.2×10^8	1.0×10^8	83.3

[a] Cells were grown in NSM at 37°C, and after 24 h, dilutions of the cell cultures were made and aliquots were plated on tryptose blood agar base agar. This gave total viable cell counts (cells per milliliter). The diluted samples were then heated at 85°C for 30 min, and aliquots were plated. This gave heat-resistant spores. The percentage of heat-resistant spores was then calculated.

bearing a *sin*(Lf) lesion than in the wt *sin* strain when decoyinine initiated sporulation. Our results are in agreement with the idea that Sin has a physiological role in sporulation, acting as a controller which prevents premature expression of stage II sporulation genes and thus delays entry into sporulation.

Effect of *sin*(Lf) mutations on sporulation. We have now examined the effect of *sin*(Lf) mutations on the sporulation frequency of *B. subtilis*. Table 2 shows that the strain lacking Sin (IS720) sporulates with higher efficiency than does the wt strain (IS75). This observation has been repeated in several independent experiments using either the same strains or strains with other genetic backgrounds (data not shown). The higher frequency of sporulation in *sin*(Lf) strains raised a question as to whether elimination of the repressor bypasses requirements for any sporulation genes which act before Sin in the sporulation network. We found that *sin*(Lf) mutations do not bypass mutations in *spo0A*, *spo0F*, and *spo0B* (unpublished observations). On the other hand, the same mutations in *sin* do bypass the requirement for *kinA* (17a) (Table 2, compare IS673 and IS780). This result indicates that removing Sin can compensate for the sporulation defect caused by the lack of KinA.

Sin binds to spoIIA. We have previously shown that Sin is a DNA-binding protein with a typical helix-turn-helix DNA-binding motif and that it binds to the *aprE* promoter region (12). It was therefore reasonable to see whether Sin binds to its *spo* target genes. We studied the binding of purified Sin to the *spoIIA* gene by gel shift assays. We initially screened for Sin-binding DNA sequences on a large *spoIIA* fragment cloned into plasmid pPP155. After finding that Sin binds to a site somewhere within positions −1300 to −30 of *spoIIA* (data not shown), we subcloned the −1300 to −30 DNA fragment into pIS184. The new plasmid, named pIS219, was used in subsequent studies. Figure 4B, lane 1, shows 5′-labeled fragments resolved on a polyacrylamide gel in the absence of Sin protein. If Sin (0.1 μg) was added to the binding reaction mixture containing labeled DNA, only fragment "b," which covers the region between −30 and −110, was retarded on the gel (Fig. 4B, lane 2).

Next we purified the labeled probe spanning the −169 to −30 region of the *spoIIA* promoter from pIS219 (see Materials and Methods) and examined the Sin affinity for this sequence in vitro by varying the amounts of Sin protein in a binding reaction while keeping the DNA concentration constant (0.1 μg of labeled DNA per ml). Figure 4C indicates that the amount of retarded DNA increases with higher concentrations of Sin protein in the binding reaction (from right to left), reaching its maximum at 0.1 μg of Sin added to the reaction. Since we were unable to get complete shifting of the *spoIIA* promoter fragment, it has not been possible, as

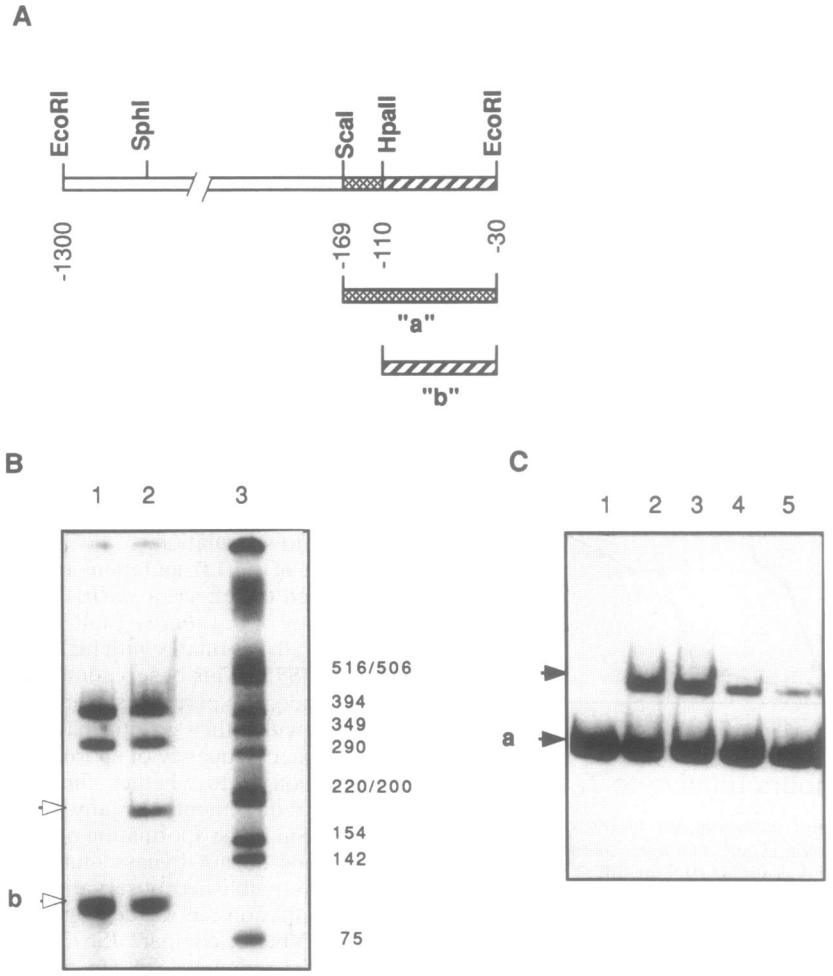

FIG. 4. Binding of Sin protein to the *spoIIA* promoter. (A) Restriction map of a 1.3-kb fragment in pIS219 that contains a part of the *spoIIA* promoter. DNA fragments "a" and "b" are indicated below the map. (B) The *Eco*RI restriction fragments from pIS219 were 5′ end labeled and then cut with *Sph*I and *Hpa*II. This resulted in four labeled fragments which arose from insert and vector sequences. The largest fragment (~400 bp) is the *Eco*RI-*Sph*I fragment from the insert. The second largest fragment (~300 bp) comes from the vector. The labeled "b" fragment is the *Eco*RI-*Hpa*II fragment as indicated in panel A. The other labeled fragment is only 13 bp and has run out of the gel. Restricted DNA was allowed to bind to Sin protein and then resolved on a 5% polyacrylamide gel. Lane 1, DNA alone; lane 2, DNA incubated with Sin protein (0.1 μg per reaction) (the bound and unbound DNA molecules are indicated by white arrows); lane 3, DNA size standards (indicated in base pairs). (C) Fragment "a" was 5′ labeled at the *Eco*RI restriction site and gel purified. A constant amount of labeled DNA (2 ng per reaction) was allowed to bind to various concentrations of Sin protein. Poly(dI-dC) DNA (50 μg) was also added to each reaction. Lane 1, no Sin; lane 2, 200 ng; lane 3, 100 ng; lane 4, 20 ng; lane 5, 2 ng of Sin protein.

yet, to get reproducible DNase footprints of the Sin binding region.

Sin concentration in vivo. Since our results indicated that the Sin protein was acting on certain stage II genes, it was important to determine relative levels of Sin during this time. Previous experiments have indicated that the *sin* gene is expressed at relatively constant levels during growth and sporulation (10). To extend these observations, since results with translational *lacZ* fusions may not indicate true protein levels, we have now examined the wt levels of Sin during the exponential and stationary phases of growth by immunoassay. These levels were measured in a *spo*$^+$ strain carrying the *spoIIG-lacZ* fusion, allowing us to simultaneously examine Sin concentration and transcriptional activity of one of its target genes. Figure 5A shows that Sin concentration increased at the beginning of the stationary phase (at T_0), i.e., 1 h before *spoIIG* is expressed (Fig. 5B), and remained rather constant or even increased slightly until the third hour of sporulation. This indicated that there was no decrease in Sin concentration at the time of induction of stage II genes. These results are consistent with our *sin-lacZ* fusion data (10) and suggest that Sin function may be regulated post-translationally.

DISCUSSION

In this report we have shown that Sin is a repressor of sporulation, because *sin*(Gf) mutations inhibit spore development and *sin*(Lf) mutations cause higher frequency of sporulation. This regulatory role of Sin in endospore development is consistent with the observation that *sin* null mutations bypass the requirement for an essential sporulation gene, *kinA*. We have further shown that Sin negatively controls expression of stage II sporulation genes (*spoIIA*,

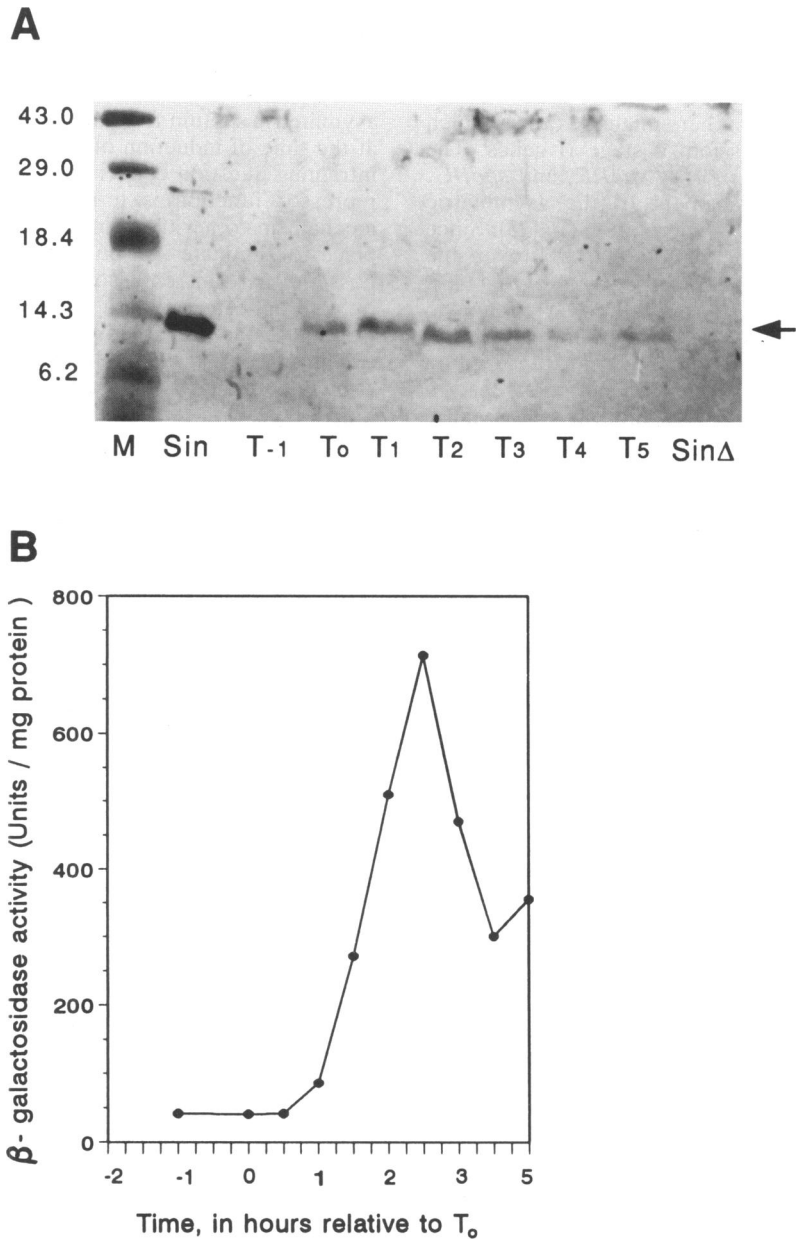

FIG. 5. Immunodetection of Sin during growth and sporulation. (A) Total cellular proteins were isolated from aliquots of IS657 grown in NSM. Samples (100 μg of protein) from different time points were electrophoresed on an SDS-polyacrylamide (15 to 18%) polyacrylamide gradient) gel. The proteins from the gel were transferred to nitrocellulose paper and probed with anti-Sin antibodies, as described in Materials and Methods. Lane M, molecular weight markers; lane Sin, approximately 50 ng of purified Sin protein, which served as a positive control; lanes T_{-1} through T_5, IS657 cell extracts at the indicated times; lane SinΔ, cell extract (T_2) from IS715 which carries a large deletion in the *sin* gene and which served as a negative control. (B) β-Galactosidase activity of *spoIIG-lacZ* (a Sin target gene) was measured in IS657. Samples for β-galactosidase assays were withdrawn at the indicated time intervals from the same culture used for monitoring the Sin levels during growth and sporulation (in panel A).

spoIIE, and *spoIIG*) but not the earlier-acting stage 0 genes, which suggests that Sin is a stage II-specific repressor of sporulation. This conclusion is in agreement with Sin being epistatic on KinA and with our Sin-DNA-binding studies which show that Sin binds to the *spoIIA* promoter region.

Why would a cell need a stage II repressor? Entry into the dormant life cycle may be controlled at several levels. The first level is at the earliest stage of sporulation, defined as T_0. This is the time at which cells cease logarithmic growth and enter the stationary phase. External and internal changes are sensed through the signal transduction system, an elaborate phosphorelay composed of several components, including KinA, Spo0F, Spo0B, and Spo0A (5). This multicomponent signal transduction system could allow multiple rechecking and thus correct channeling of incoming signals. Any proteins which negatively control either induction of genes encoding phosphorelay components or their phosphorylation may be needed to prevent an inappropriate commitment

to dormancy. Indeed, several negative regulators, AbrB (24, 46), Hpr (22), Pai (15), and Spo0E (23), could control the progression into sporulation at this time.

If the initial checkpoints are intimately connected to transduction of external signals, there might be others which act later by controlling expression of stage II genes. The stage II sporulation operons, *spoIIA*, *spoIIE*, and *spoIIG*, are important for correct placement of the asymmetric septum and for the later stages of sporulation (16, 25). Since it is known that stage II completion marks irreversible commitment to sporulation (19, 32), induction of *spoIIA* (43), *spoIIE* (14), and *spoIIG* (39) may be the last chance to prevent an inappropriate entry into the dormant way of life. We have shown that *sin*(Gf) mutations inhibit transcription of these three genes and also that *sin*(Lf) mutations lead to their higher expression. Results presented in Fig. 2 and 3 also suggest that *sin*(Lf) mutations cause earlier expression of three stage II genes and of the later-expressed *spoIID*. The effect on *spoIID* is particularly interesting because this gene is believed to be induced after asymmetric septation. *spoIID* is transcribed by σ^E and expressed predominantly in the mother cell (7). If Sin helps set the time at which stage II genes are induced, it should also affect the timing of later sporulation processes. The effect on *spoIID* is consistent with this idea, and we are now further examining whether *sin* null mutations cause earlier asymmetric septation and related events. As a working model, we propose that Sin plays the role of a stage II sporulation gatekeeper, which may act to delay transcriptional activation of stage II genes and thus the entry into sporulation until all other possibilities for survival are exhausted.

While *sin*(Lf) mutations cause higher expression of stage II genes, they do not abolish their temporal regulation. Sin is therefore not the only factor which contributes to the proper timing of their transcriptional activation. There must be additional stage II-specific negative or positive factors which are necessary for temporal regulation of stage II genes. One of them is Spo0A, whose levels increase at T_0 and which is necessary for *spoIIA*, *spoIIG*, and *spoIIE* expression (30, 38, 44). Since none of the stage 0 genes tested so far are affected by *sin*(Gf) or *sin*(Lf) mutations, it is probable that Sin does not regulate *spo0A* expression. However, this has not been tested directly, but placing *spo0A* under inducible pSPAC promoter control does not bypass the *sin*(Gf) phenotype (19a). A Spo0A requirement for activation of stage II gene transcription could explain why stage II genes remain temporally regulated in *sin* null strains. However, the existence of additional stage II regulators cannot be excluded. Redundancy of certain functions in developmental control mechanisms is not uncommon. *aprE* transcription, for example, is regulated by three repressors (Sin, Hpr, and AbrB) and also by several positive factors (40).

We have now provided evidence that Sin binds to the −110 to −30 region of the *spoIIA* gene in vitro. In addition, we have recently identified by exonuclease footprinting that Sin, which is an autogenous repressor of its own synthesis, protects a 47-bp promoter region in the *sin* operon (33). We had previously shown that Sin also protects a region of 48 bp in the region upstream of the *aprE* promoter (12). Although it is clear that Sin is a DNA-binding protein, little is known about the mechanism by which Sin represses transcription of its target genes. It is possible that Sin antagonizes the positive role of Spo0A in *spoIIA*, *spoIIE*, and *spoIIG* expression. Confirmation of this or other equally plausible mechanisms for Sin action must await the precise mapping of the Sin binding site on its stage II target genes.

Immunochemical experiments presented here show that Sin, being undetectable during vegetative growth, first appears in the cell around T_0. Rising slowly in concentration as development proceeds, it remains in the cell long after asymmetric septum formation. The presence of Sin in a cell at the time of induction of stage II genes leaves us with an intriguing question concerning the mechanism by which its repressor function is terminated. Our data suggest that mechanisms other than simple degradation of Sin must take place to eliminate its sporulation inhibition function. It is possible that Sin repressor function is passively overcome by the appearance of a positive factor such as Spo0A phosphate, as discussed above. On the other hand, the termination of negative Sin function may still be actively regulated either by chemical modification of Sin or another kind of posttranslational alteration. The observation that *sin*(Lf) mutations bypass the requirement for an essential sporulation gene, *kinA*, suggests that this protein is involved in downregulation of Sin. Recent data from our laboratory indicate that the open reading frame 1 protein of the *sin* operon (10) is involved in downregulation of Sin function and that *kinA* may act through open reading frame 1 (3a).

To conclude, the formation of the asymmetric septum marks an irreversible commitment to sporulation. The stage II genes, *spoIIA*, *spoIIE*, and *spoIIG*, are essential for this process and later stages of sporulation, and they are induced prior to this event. Sin, as a stage II-specific repressor which negatively controls stage II gene induction, may thus prevent premature commitment to the dormant way of life.

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ADDENDUM IN PROOF

Recent experiments have shown that Sin represses expression of *spo0A*, especially the rise in activity observed at T_0 (I. Mandic-Mulic and I. Smith, unpublished results).

REFERENCES

1. **Anagnostopoulos, C., and J. Spizizen.** 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81**:741–746.
2. **Antoniewski, C., B. Savelli, and P. Stragier.** 1990. The *spoIIJ* gene, which regulates early developmental steps in *Bacillus subtilis*, belongs to a class of environmentally responsive genes. J. Bacteriol. **172**:86–93.
3. **Bai, U., M. Lewandoski, E. Dubnau, and I. Smith.** 1990. Temporal regulation of the *Bacillus subtilis* early sporulation gene *spo0F*. J. Bacteriol. **172**:5432–5439.

3a. **Bai, U., I. Mandic-Mulec, and I. Smith.** Unpublished experiments.

4. **Bookstein, C., C. W. Edwards, N. V. Kapp, and F. M. Hulett.** 1990. The *Bacillus subtilis* 168 alkaline phosphatase III gene: impact of a *phoAIII* mutation on total alkaline phosphatase

synthesis. J. Bacteriol. **172:**3730–3737.
5. **Burbulys, D., K. A. Trach, and J. A. Hoch.** 1991. Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay. Cell **64:**545–552.
6. **Carter, H. L., III, and C. P. Moran, Jr.** 1986. New RNA polymerase σ factor under *spo0* control in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **83:**9438–9442.
7. **Driks, A., and R. Losick.** 1991. Compartmentalized expression of a gene under the control of sporulation transcription factor σ^E in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **88:**9934–9938.
8. **Dubnau, E., K. Cabane, and I. Smith.** 1987. Regulation of *spo0H*, an early sporulation gene in bacilli. J. Bacteriol. **169:** 1182–1191.
9. **Dubnau, E., J. Weir, G. Nair, L. Carter III, C. Moran, Jr., and I. Smith.** 1988. *Bacillus* sporulation gene *spo0H* codes for σ^{30} (σ^H). J. Bacteriol. **170:**1054–1062.
10. **Gaur, N. K., K. Cabane, and I. Smith.** 1988. Structure and expression of the *Bacillus subtilis sin* operon. J. Bacteriol. **170:**1046–1053.
11. **Gaur, N. K., E. Dubnau, and I. Smith.** 1986. Characterization of a cloned *Bacillus subtilis* gene that inhibits sporulation in multiple copies. J. Bacteriol. **168:**860–869.
12. **Gaur, N. K., J. Oppenheim, and I. Smith.** 1991. The *Bacillus subtilis sin* gene, a regulator of alternate developmental processes, codes for a DNA-binding protein. J. Bacteriol. **173:**678–686.
13. **Gryczan, T. J., S. Contente, and D. Dubnau.** 1978. Characterization of *Staphylococcus aureus* plasmids introduced by transformation into *Bacillus subtilis*. J. Bacteriol. **134:**318–329.
14. **Guzman, P., J. Westpheling, and P. Youngman.** 1988. Characterization of the promoter region of the *Bacillus subtilis spoIIE* operon. J. Bacteriol. **170:**1598–1609.
15. **Honjo, M., A. Nakayama, K. Fukazawa, K. Kawamura, K. Ando, M. Hori, and Y. Furutani.** 1990. A novel *Bacillus subtilis* gene involved in negative control of sporulation and degradative-enzyme production. J. Bacteriol. **172:**1783–1790.
16. **Illing, N., and J. Errington.** 1991. Genetic regulation of morphogenesis in *Bacillus subtilis*: roles of σ^E and σ^F in prespore engulfment. J. Bacteriol. **173:**3159–3169.
17. **Kunkel, B.** 1991. Compartmentalized gene expression during sporulation in *Bacillus subtilis*. Trends Genet. **7:**167–172.
17a. **Leighton, T.** Personal communication.
18. **Lewandoski, M., and I. Smith.** 1988. Use of a versatile *lacZ* vector to analyze the upstream region of the *Bacillus subtilis spo0F* gene. Plasmid **20:**148–154.
19. **Lopez, J. M., A. Dromerick, and E. Freese.** 1981. Response of guanosine 5′-triphosphate concentration to nutritional changes and its significance for *Bacillus subtilis* sporulation. J. Bacteriol. **146:**605–613.
19a. **Mandic-Mulec, I., and I. Smith.** Unpublished data.
20. **Olmedo, G., E. G. Ninfa, J. Stock, and P. Youngman.** 1990. Novel mutations that alter the regulation of sporulation in *Bacillus subtilis*. Evidence that phosphorylation of regulatory protein Spo0A controls the initiation of sporulation. J. Mol. Biol. **215:**359–372.
21. **Perego, M., S. P. Cole, D. Burbulys, K. Trach, and J. A. Hoch.** 1989. Characterization of the gene for a protein kinase which phosphorylates the sporulation-regulatory proteins Spo0A and Spo0F of *Bacillus subtilis*. J. Bacteriol. **171:**6187–6196.
22. **Perego, M., and J. A. Hoch.** 1988. Sequence analysis and regulation of the *hpr* locus, a regulatory gene for protease production and sporulation in *Bacillus subtilis*. J. Bacteriol. **170:**2560–2567.
23. **Perego, M., and J. A. Hoch.** 1991. Negative regulation of *Bacillus subtilis* sporulation by the *spo0E* gene product. J. Bacteriol. **173:**2514–2520.
24. **Perego, M., G. B. Spiegelman, and J. A. Hoch.** 1988. Structure of the gene for the transition state regulator, *abrB*: regulator synthesis is controlled by the *spo0A* sporulation gene in *Bacillus subtilis*. Mol. Microbiol. **2:**689–699.
25. **Piggot, P. J., and J. G. Coote.** 1976. Genetic aspects of bacterial endospore formation. Bacteriol. Rev. **40:**908–962.
26. **Predich, M., G. Nair, and I. Smith.** 1992. *Bacillus subtilis* early sporulation genes *kinA*, *spo0F*, and *spo0A* are transcribed by the RNA polymerase containing σ^H. J. Bacteriol. **174:**2771–2778.
27. **Rather, P. N., R. Coppolecchia, H. DeGrazia, and C. P. Moran, Jr.** 1990. Negative regulator of σ^G-controlled gene expression in stationary-phase *Bacillus subtilis*. J. Bacteriol. **172:**709–715.
28. **Rong, S., M. Rosenkrantz, and A. L. Sonenshein.** 1986. Transcriptional control of the *spoIID* gene of *Bacillus subtilis*. J. Bacteriol. **165:**771–779.
29. **Ruvkun, G., and J. Giusto.** 1989. The *Caenorhabditis elegans* heterochronic gene *lin-14* encodes a nuclear protein that forms a temporal developmental switch. Nature (London) **338:**313–319.
30. **Satola, S., P. A. Kirchman, and C. P. Moran, Jr.** 1991. Spo0A binds to a promoter used by σ^A RNA polymerase during sporulation in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **88:**4533–4537.
31. **Schmidt, R., P. Margolis, L. Duncan, R. Coppolecchia, C. P. Moran, Jr., and R. Losick.** 1990. Control of developmental transcription factor σ^F by sporulation regulatory proteins SpoIIAA and SpoIIAB in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **87:**9221–9225.
32. **Smith, I.** 1991. Choice and commitment in sporulation initiation: the role of positive and negative control elements. Semin. Dev. Biol. **2:**13–20.
33. **Smith, I., I. Mandic-Mulec, and N. Gaur.** 1991. The role of negative control in sporulation. Res. Microbiol. **142:**831–839.
34. **Stragier, P., and R. Losick.** 1990. Cascades of sigma factors revisited. Mol. Microbiol. **4:**1801–1806.
35. **Strauch, M., V. Webb, G. Spiegelman, and J. A. Hoch.** 1990. The Spo0A protein of *Bacillus subtilis* is a repressor of the *abrB* gene. Proc. Natl. Acad. Sci. USA **87:**1801–1805.
36. **Struhl, G.** 1989. Differing strategies for organizing anterior and posterior body pattern in *Drosophila* embryos. Nature (London) **338:**741–744.
37. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
38. **Trach, K., D. Burbulys, M. Strauch, J. Wu, R. Jonas, C. Hanstein, P. Kallio, M. Perego, T. Bird, G. Spiegelman, C. Fogher, and J. A. Hoch.** 1991. Control of the initiation of sporulation in *Bacillus subtilis* by a phosphorelay. Res. Microbiol. **142:**815–823.
39. **Trempy, J. E., J. Morrison-Plummer, and W. G. Haldenwang.** 1985. Synthesis of σ^{29}, an RNA polymerase specificity determinant, is a developmentally regulated event in *Bacillus subtilis*. J. Bacteriol. **161:**340–346.
40. **Valle, F., and E. Ferrari.** 1989. Subtilisin: a redundantly temporally regulated gene?, p. 131–146. *In* I. Smith, R. A. Slepecky, and P. Setlow (ed.) Regulation of procaryotic development. American Society for Microbiology, Washington, D.C.
41. **Weinrauch, Y., R. Penchev, E. Dubnau, I. Smith, and D. Dubnau.** 1990. A *Bacillus subtilis* regulatory gene product for genetic competence and sporulation resembles sensor protein members of the bacterial two-component signal-transduction systems. Genes Dev. **4:**860–872.
42. **Weir, J., M. Predich, E. Dubnau, G. Nair, and I. Smith.** 1991. Regulation of *spo0H*, a gene coding for the *Bacillus subtilis* σ^H factor. J. Bacteriol. **173:**521–529.
43. **Wu, J., M. G. Howard, and P. J. Piggot.** 1989. Regulation of transcription of the *Bacillus subtilis spoIIA* locus. J. Bacteriol. **171:**692–698.
44. **York, K., T. J. Kenney, S. Satola, C. P. Moran, Jr., H. Poth, and P. Youngman.** 1992. Spo0A controls the σ^A-dependent activation of *Bacillus subtilis* sporulation-specific transcription unit *spoIIE*. J. Bacteriol. **174:**2648–2658.
45. **Zuber, P., J. Healy, H. L. Carter III, S. Cutting, C. P. Moran, Jr., and R. Losick.** 1989. Mutation changing the specificity of an RNA polymerase sigma factor. J. Mol. Biol. **206:**605–614.
46. **Zuber, P., and R. Losick.** 1987. Role of *abrB* in Spo0A- and Spo0B-dependent utilization of a sporulation promoter in *Bacillus subtilis*. J. Bacteriol. **169:**2223–2230.