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Expression of *kinA* and Accumulation of σ^H at the Onset of Sporulation in *Bacillus subtilis*

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Induction of the *Bacillus subtilis kinA* gene, which codes for a major kinase of the phosphorelay pathway, required the *spo0H* gene, coding for the σ^H protein, but not the genes *spo0A*, *spo0B*, and *spo0F* at the onset of sporulation. Also, the levels of σ^H in *spo0A*, *spo0B*, and *spo0F* mutants were increased at the onset of sporulation, though induction of *spo0H* transcription in all of these mutants was appreciably inhibited. In addition, *kinA* expression was almost completely eliminated in a medium supplemented with excess glucose and glutamine, even though the usual stationary-phase-associated increase in σ^H was observed under these conditions.

The initiation of sporulation in *Bacillus subtilis* is induced under conditions of nutritional stress (22, 23) and high cell density (9), depending on at least eight *spo0* genes (11, 18). Among them, the *spo0A* gene plays a key role in the initiation of sporulation. Spo0A belongs to a family of response regulator proteins of prokaryotic two-component signal-transduction systems (7, 13, 24). It has been shown recently that Spo0A is converted to an active, phosphorylated form, Spo0A~P, at an early stage of spore development, via the so-called phosphorelay pathway, consisting of a sensor kinase, KinA, and Spo0B and Spo0F proteins as intermediate substrates (3). The phosphorylated Spo0A protein binds to DNA by recognizing the 7-bp sequence TGNCGAA (0A box) (27) and acts as a transcriptional repressor of a transition state regulator gene, *abrB* (17, 27), and as a transcriptional activator of sporulation genes, including *spo0F*, *spoIIA*, *spoIIG*, and *spoIIE* (21, 28, 29, 34).

The *spo0A* gene has two promoters which are differentially controlled: the relatively weak vegetative promoter (Pv, σ^A type) is shut off and the strong sporulation-specific promoter (Ps, σ^H type) is turned on during the initiation of sporulation (5). The Ps-directed transcription, which is induced at $T_{0.5}$ to $T_{1.0}$ after the end of exponential growth, depends absolutely on *spo0A*, *spo0B*, and *spo0F* gene products as well as the *spo0H* product and a sporulation-associated minor sigma factor, σ^H (4–6, 19, 32, 33), and depends partially on the *kinA* gene product (unpublished results). On the other hand, the *kinA* gene, coding for a protein kinase (1, 16) which is a major kinase of the phosphorelay pathway (3, 11, 12, 14, 30), is fully expressed with a σ^H-dependent promoter after T_0 (1, 15, 19). However, very little is known about the regulation of *kinA* gene expression, except that the promoter for *kinA* has a 0A box just downstream of the transcriptional start site and that there is no sign of AbrB binding (11).

Induction of *kinA* expression is independent of phosphorelay. To investigate further the control mechanism of induction of *kinA* expression at the initiation of sporulation, the effects on *kinA* expression of various mutations affecting the initiation events of sporulation were examined by using an integrated *kinA-lacZ* fusion (Fig. 1A). The *B. subtilis* strains used in this study are listed in Table 1. All of the strains are derivatives of UOT-1285. It is evident from Fig. 1A that *kinA-lacZ* expression in wild-type cells began to increase at or near T_0, the time at which cells reach stationary phase, while no induction of *kinA-lacZ* expression was seen in cells in which *spo0H*, the gene coding for σ^H, was deleted. These results are consistent with previous observations (1, 15, 19).

The products of the *spo0A*, *spo0B*, and *spo0F* genes are components of a phosphorelay pathway leading to the active form of Spo0A protein, Spo0A~P (3, 11). Therefore, we examined whether these *spo0* mutations affect induction of *kinA*. Expression of *kinA* was induced in these *spo0* mutants, albeit to lower levels than that in the wild-type strain (Fig. 1A). These results clearly indicate that the induction of *kinA* expression observed at the onset of sporulation is dependent on σ^H but does not absolutely require any component of the phosphorelay pathway and also strongly suggest that active σ^H can accumulate in the absence of Spo0A, Spo0B, and Spo0F functions.

σ^H accumulates in *spo0* mutants. Two mechanisms contribute to increased σ^H activity at the onset of the sporulation phase. Transcription of the *spo0H* gene is negatively regulated by AbrB during exponential growth and increases at the late exponential stage of growth, at which time the *abrB* gene is repressed by the increased concentration of Spo0A~P in the cell (11, 25–27, 29, 31). In addition, σ^H protein is stabilized by a posttranscriptional mechanism at the onset of sporulation (10). It seemed likely, therefore, that sufficient σ^H can accumulate after T_0 to activate *kinA* transcription whether or not the phosphorelay is active. We examined the expression of a *spo0H-lacZ* translational fusion and the levels of σ^H protein in various *spo0* mutant strains during exponential growth and at the onset of sporulation (Fig. 1B and 2A, respectively). As shown in Fig. 1B, the induction of

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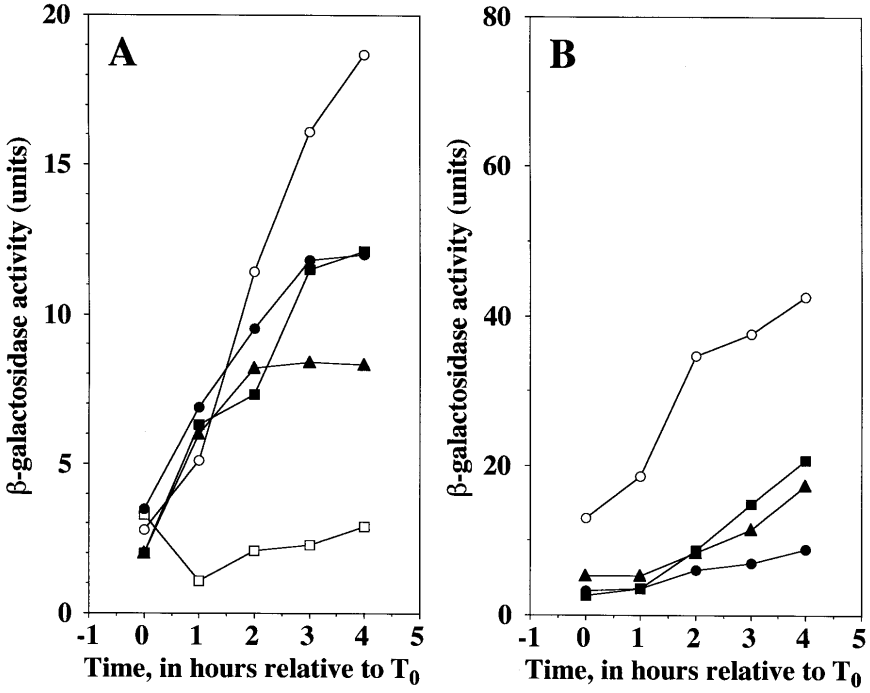

FIG. 1. Expression of β-galactosidase in *spo*$^+$ or *spo0* mutant strains. Shown are β-galactosidase activities for *spo*$^+$ and *spo0* mutants carrying *kinA-lacZ* translational fusions (kindly provided by I. Smith, The Public Health Research Institute, New York, N.Y. [15]) (A) and *spo0H-lacZ* translational fusions (B) integrated by Campbell insertion at the *kinA* and *spo0H* loci, respectively. The *spo0H-lacZ* translational fusion gene was constructed with the 413-bp *Sma*I fragment of *spo0H* containing the promoter region, the sequence downstream of which was exchanged with a *Sma*I site by PCR-directed mutagenesis, and a promoterless *lacZ* gene derived from a *Pst*I fragment of pMC1871 (purchased from Pharmacia). The abscissa shows incubation time (expressed in hours) preceding or following the end of exponential growth, which is designated as T_0. The cells were grown at 37°C in 2× SG containing 0.1% glucose sporulation medium as previously described (2) with shaking. Samples of the culture (0.5 ml before T_0 or 0.2 ml at various stages of the subsequent growth) were withdrawn for the assay of β-galactosidase activity as described previously (2). Symbols: ○, *spo*$^+$ (UOT-1285); ●, *spo0AΔHB*; ■, *spo0FΔS*; ▲, *spo0B136*; □, *spo0HΔHB*.

spo0H expression at the period of sporulation initiation in *spo0A*, *spo0B*, and *spo0F* mutants was inhibited. These results were similar to those reported previously by Weir et al. (31).

The levels of σ^H protein in extracts of the cells harvested during exponential growth and at T_1 were estimated by Western blot (immunoblot) analysis (Fig. 2A). The accumulation of σ^H was found in the T_1-harvested cells of all strains except those of the *spo0H* null mutant. These results clearly indicate that σ^H protein can accumulate without phosphorelay function and support the idea that the activity of σ^H at the initiation of sporulation is regulated mainly at the posttranscriptional level rather than at the transcriptional level, as has been proposed previously (8, 10).

Figure 2A also shows that the altered σ^H protein produced in *spo0H17* mutant cells accumulates at the period of sporulation initiation. This result indicates that the *spo0H17* mutation, causing an Arg-to-Cys substitution in the C terminus of σ^H (unpublished data), does not affect the stabilization of σ^H protein but may affect its ability to interact with core RNA polymerase or to activate transcription from the σ^H-dependent promoters.

Effect of excess amounts of glucose and glutamine on induction of *kinA* expression at the onset of sporulation. It has been known since the studies of Schaeffer et al. that sporulation is subject to catabolite repression (22). We have previously shown that the induction of *spo0A* transcription from the Ps promoter is completely repressed in the presence of excess

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

Strain	Relevant genotype	Source or reference[a]
UOT-1276	*trpC2 lys1 pheA1 aprEΔ3 nprE18 nprR2*	Laboratory stock
UOT-1285	*trpC2 lys1 aprEΔ3 nprE18 nprR2*	32
UOT-1611	*trpC2 lys1 aprEΔ3 nprE18 nprR2 spo0AΔHB*	HUSA (12) $\xrightarrow{c}$ UOT-1276
UOT-1278	*trpC2 lys1 aprEΔ3 nprE18 nprR2 spo0B136*	UOT-1102 (33) $\xrightarrow{c}$ UOT-1276
UOT-1279	*trpC2 lys1 aprEΔ3 nprE18 nprR2 spo0FΔS*	UOT-0540 (33) $\xrightarrow{c}$ UOT-1276
UOT-1281	*trpC2 lys1 aprEΔ3 nprE18 nprR2 spo0H17*	UOT-1108 (33) $\xrightarrow{c}$ UOT-1276
UOT-1850[b]	*trpC2 lys1 aprEΔ3 nprE18 nprR2 spo0HΔHB*	This study

[a] c, congression.
[b] Strain UOT-1850, a *spo0H* null mutant, was carrying the *spo0H* gene that had been disrupted by substituting the *Hin*cII-*Bcl*I fragment of the *spo0H* gene with the *Pvu*II-*Bam*HI fragment of an erythromycin resistance gene cassette derived from pAE41 (kindly donated by Y. Kobayashi, Tokyo University of Agriculture and Technology, Tokyo, Japan).

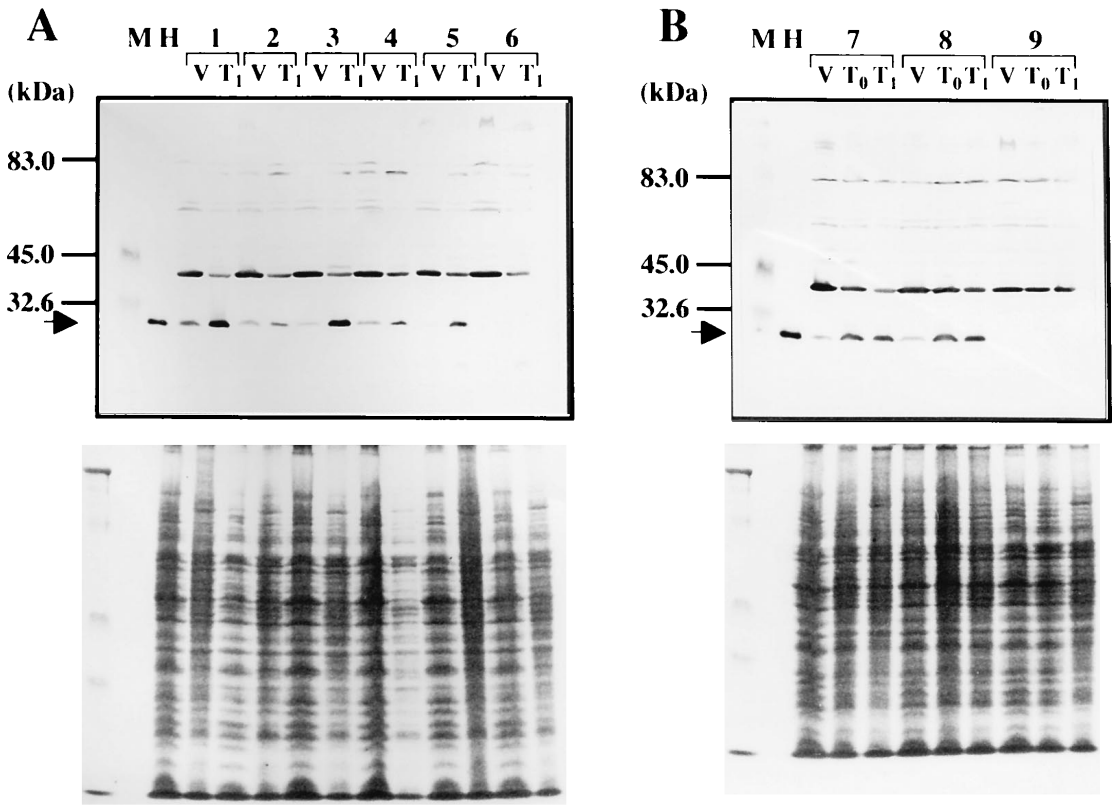

FIG. 2. Western blot analysis of σ^H protein in *spo*$^+$ or *spo0* mutant strains (A) and in *spo*$^+$ (UOT-1285) cells growing in 2× SG medium supplemented with 0.1% glucose or 1% glucose and 0.1% glutamine (B). The cells which were collected at the times indicated as T_x (where x is the number of hours after the end of exponential growth) and V (vegetative growth phase, about 1 h before the end of exponential growth) were incubated at 37°C for 12 min in lysis buffer (50 mM Tris-HCl, pH 7.0, 1 mM EDTA, 5% glycerol, 0.1 mM dithiothreitol, 0.1 M NaCl, 2 mg of lysozyme per ml, 10 mM $MgCl_2$, 0.2 mg of DNase I per ml, and 0.5 mg of RNase A per ml). The cell lysate was mixed with an equal volume of 2× sodium dodecyl sulfate (SDS) loading buffer containing 100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol. Aliquots of cell extracts containing 50 μg of total protein, whose concentrations were determined by a Bio-Rad protein assay, were boiled for 10 min and electrophoresed through SDS-12% (wt/vol) polyacrylamide minigels. Then, the proteins were blotted with nitrocellulose filter and immunodetection procedures were performed as described elsewhere (20), with primary anti-σ^H antibodies and a secondary goat anti-rabbit alkaline phosphatase conjugate antibody (BioMakor). A polypeptide consisting of the C-terminal third of σ^H and an N-terminal extension of six tandem histidine residues was purified by using the QIA expression system (Qiagen) and used to immunize a rabbit (20). Crude serum prepared from the rabbit was used for the Western blot analysis shown. The lower panels show Coomassie brilliant blue-stained total protein samples corresponding to the respective lanes of the upper panels. Arrow indicicates position of ς^H. Lanes: M, molecular mass markers (kaleidoscope-prestained standards [Bio-Rad]); H, *Escherichia coli*-overexpressed, purified σ^H (kindly donated by M. Fujita, National Institute of Genetics, Mishima, Japan); 1, *spo*$^+$ (UOT-1285); 2, *spo0AΔHB*; 3, *spo0B136*; 4, *spo0FΔS*; 5, *spo0H17*; 6, *spo0HΔHB*; 7, *spo*$^+$ with 0.1% glucose; 8, *spo*$^+$ with 1% glucose and 0.1% glutamine; 9, *spo0HΔHB* with 0.1% glucose. See the legend to Fig. 1 for other information.

glucose (5). The *spo0A* Ps induction was thought to be one of the earliest sporulation events sensitive to excess glucose. However, the induction of *kinA* transcription occurred approximately 1 h earlier than that of *spo0A* Ps transcription (Fig. 1A) (32). Therefore, we examined whether the *kinA* induction was under the control of catabolite repression. Interestingly, *kinA* expression was almost completely repressed in the presence of 1% glucose and 0.1% glutamine but was not affected by the addition of 0.1% glucose and 0.1% glutamine (Fig. 3A). When only the carbon source was added in an excess amount (1% glucose), the repression of *kinA* induction was incomplete.

Effect of excess amounts of glucose and glutamine on accumulation of σ^H. Frisby and Zuber have isolated a mutation (*ggr-31*) which results in the catabolite-resistant sporulation phenotype in the presence of excess glucose and glutamine (8). They have shown that this mutation, which probably resides within the *ptsG* gene, and a defined *ptsI* mutation both result in significant increases in the level of σ^H protein in early stages of sporulation when cells are cultured in sporulation medium containing an excess of glucose and glutamine. On the basis of these results together with those from previous studies, Frisby and Zuber (8) suggested that a glucose- and glutamine-dependent control mechanism is operating at the level of translation or σ^H protein stability, as has been proposed by Healy et al. (10).

Taking these suggestions into consideration together with the fact that expression of *kinA-lacZ* depends on σ^H activity, we tested the expression of the *spo0H-lacZ* fusion and the levels of σ^H protein in the cells supplemented with excess amounts of glucose and glutamine. As shown in Fig. 3B, induction of *spo0H* occurred regardless of the supplements. The level of σ^H protein also increased at the beginning of stationary phase under all conditions tested (Fig. 2B). These results suggest that the reduction in *kinA* expression seen in the presence of excess glucose and glutamine is due neither to the repression of *spo0H* transcription nor to a decrease in σ^H protein but suggest instead the existence of an additional factor that regulates the expression of the *kinA* gene in response to nutritional conditions.

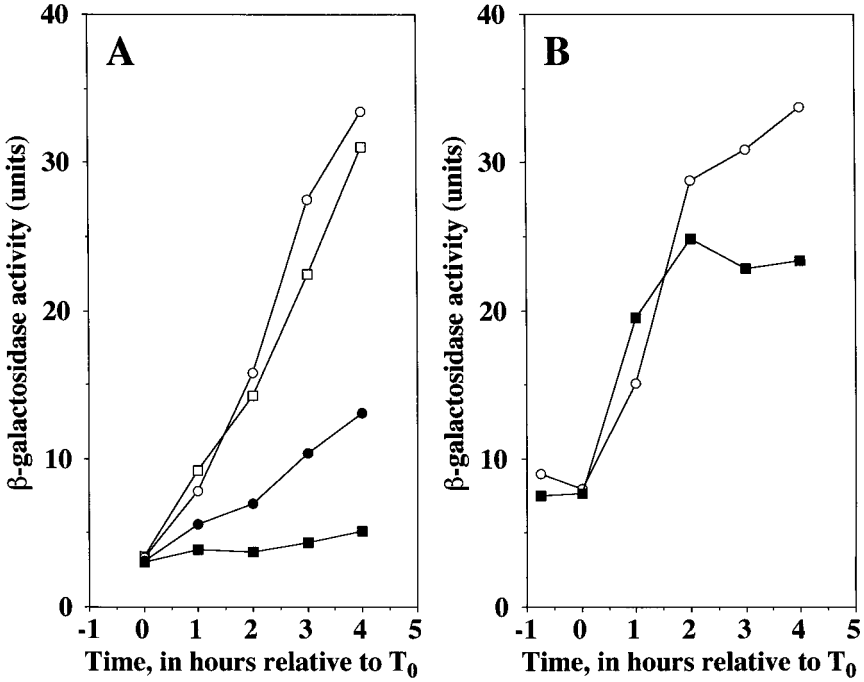

FIG. 3. Expression of *kinA-lacZ* (A)- and *spo0H-lacZ* (B)-directed β-galactosidase in *spo*$^+$ (UOT-1285) cells growing under various nutritional conditions. UOT-1285 cells were grown in 2× SG medium supplemented with 0.1% glucose (○), 0.1% glucose and 0.1% glutamine (□), 1% glucose (●), or 1% glucose and 0.1% glutamine (■). See the legend to Fig. 1 for other information.

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