NOTES

Regulation of σ^D Expression and Activity by *spo0*, *abrB*, and *sin* Gene Products in *Bacillus subtilis*

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Expression of σ^D protein and of the *hag* gene, which is transcribed by the σ^D holoenzyme, is not dependent on *spo0*, *abrB*, or *sin* gene products in *Bacillus subtilis*. Preliminary results, however, suggest that a signal mediated by the *spo0K* locus may be responsible for the inhibition of σ^D activity during the stationary phase.

The σ^D factor of *Bacillus subtilis* is responsible for the transcription of a family of genes required for flagellar assembly, motility, chemotaxis, and cell separation (8, 12, 16, 17, 21, 22). Expression of the structural gene for σ^D , *sigD*, as well as the genes transcribed by the σ^D holoenzyme, increases during logarithmic growth in sporulation medium and peaks shortly after entry into the stationary phase as the cell becomes limited for nutrients (4, 20, 21, 30, 37). These genes are not, however, expressed late in the stationary phase, when the cell has committed to the process of sporulation (4, 20, 21, 30, 37). The increase in both σ^D protein levels (22) and activity (4, 20, 21, 30, 35) throughout logarithmic growth and the fact that both peak shortly after entering the stationary phase indicate that the σ^D regulon is temporally regulated in much the same way as processes required for the initiation of sporulation and other stationary-phase phenomena (14, 31).

Several regulatory proteins play important roles in this transition from vegetative growth to sporulation. Among these, the *spo0* and *abrB* gene products have been best characterized (5, 10, 14, 26, 33), and the *sin* gene product has been implicated in the regulation of flagellin expression (2, 29). We therefore decided to determine the level and activity of σ^D protein found in mutant strains bearing lesions in these loci.

The *spo0* and *abrB* gene products are not required for σ^D expression or activity. Mutant strains bearing lesions in the *spo0A*, *spo0B*, *spo0E*, *spo0F*, *spo0K* (*spo0A*–*K*), and *abrB* loci (Table 1) were grown in 2XSG sporulation medium (34), and samples were withdrawn at the end of logarithmic growth (T_0). Total protein extracts were prepared from these samples, and the levels of σ^D and flagellin protein were determined by immunoblot analysis with enhanced chemiluminescence reagents for visualization of the reactive protein as previously described (18). Quantitative aspects of the immunoblot analysis are discussed in reference 18.

An anti- σ^D immunoblot of these samples (Fig. 1) demonstrated that the level of σ^D protein in these strains was very similar to that in the wild-type parent strain, CB25. Additionally, an anti-flagellin immunoblot (Fig. 2) indicated that with two exceptions, flagellin levels in the mutant strains were 70 to

100% of that in the parent strain. Densitometric analysis revealed that in the *spo0A abrB* strain, CB32, flagellin levels were twice that in the parent, whereas in the *spo0K* mutant strain, CB63, they were 10-fold higher. We do not believe that the twofold difference is significant, since the steady-state level of flagellin protein doubles in the 30 min following logarithmic growth (20) and may therefore be the result of sampling variation rather than strain-specific differences in flagellin expression. The 10-fold increase found in the *spo0K* strain, however, may be significant and is interesting in light of recent studies.

The *spo0K* locus is an operon of genes required for the transport of oligopeptides across the cell membrane (25, 28). It has been postulated that the transport system encoded by *spo0K* plays a role in sensing extracellular peptide factors required for efficient sporulation and the sensing of similar factors necessary for genetic competence, since *spo0K* mutants do not sporulate efficiently and are compromised for genetic competence (28). The fact that the flagellin level in a *spo0K* mutant, a strain unable to transport oligopeptides, is 10-fold higher than that in the parent strain (Fig. 2), although the level of σ^D protein is normal, indicates that σ^D activity may be negatively regulated by the accumulation of oligopeptide within the cell. We have determined that, following the rapid increase in both σ^D protein level and activity in the first 30 min of the stationary phase, σ^D activity is inhibited (20). This inhibition may be nutritionally mediated; in a separate study we have demonstrated that the addition of protein hydrolysate to cells growing vegetatively leads to a decrease in flagellin expression (20). It is unclear, however, which component of the hydrolysate is directly responsible for the inhibition and whether the hydrolysate contains peptide factors similar to those transported by the *spo0K* gene products. In addition, it remains to be demonstrated whether the effects of the *spo0K* mutation are actually exerted on flagellin transcription itself.

The results of these immunoblot analyses demonstrate that expression of σ^D and flagellin protein is not dependent on *Spo0A*–*K* or *AbrB* proteins. Therefore, although both the initiation of sporulation and the increase in σ^D protein level and activity are induced following nutrient limitation, different but parallel regulatory factors are responsible for this regulation. The *spo0K* gene product, however, may be responsible for generating a common signal to which several stationary-phase processes respond, since a null mutation in the *spo0K* locus

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

Strain	Genotype ^a	Source or derivation ^b (Reference)
CB25	<i>trpC2 phe-1</i>	J. Hoch; JH642 ^c
CB30	<i>trpC2 phe-1 abrB::Tn917</i>	J. Hoch; JH12-575 ^c
CB32	<i>trpC2 phe-1 spo0A12 abrB::cat</i>	J. Hoch; JH12-588 ^c
CB35	<i>leu met his Sin⁺ Cm^r</i>	I. Smith; IS75(pIS119) ^c (2)
CB36	<i>leu met his sin::cat</i>	I. Smith; IS432 ^c (3)
CB56	<i>trpC2 phe-1 spo0A12</i>	J. Hoch; JH646 ^c (11)
CB57	<i>trpC2 phe-1 spo0A677</i>	J. Hoch; JH703 ^c (11)
CB58	<i>trpC2 phe-1 spo0B136</i>	J. Hoch; JH648 ^c (11)
CB59	<i>trpC2 phe-1 spo0E11</i>	J. Hoch; JH647 ^c (11)
CB60	<i>trpC2 phe-1 spo0F227</i>	J. Hoch; JH649 ^c (11)
CB61	<i>trpC2 phe-1 spo0H81</i>	J. Hoch; JH651 ^c (11)
CB62 ^d	<i>trpC2 phe-1 spo0J87</i>	BGSC ^e ; IS26 ^c (11)
CB63	<i>trpC2 phe-1 spo0K::pJM6174</i>	J. Hoch; JH12768 ^c (23)
CB64	<i>trpC2 phe-1 Δspo0A::cat</i>	A. Grossman; AG475 ^c (6)
CB143	<i>trpC2 phe-1 Δspo0A::cat</i>	Tfm(CB25:CB64, Cm ^r) (this study)
CB144	<i>trpC2 phe-1 abrB::Tn917</i>	Tfm(CB25:CB30, Ery ^r) (this study)
CB145	<i>trpC2 phe-1 Δspo0A::cat abrB::Tn917</i>	Tfm(CB143:CB30, Cm ^r Ery ^r) (this study)

^a Where a number appears after a *spo0* designation, it indicates the allele used, which in this study are all point mutations within the respective locus. A :: symbol indicates an insertion of either a plasmid, a chloramphenicol resistance cassette (*cat*) individually or as part of a plasmid, or a transposon (Tn917). The Δ symbol indicates a deletion of the respective locus.

^b Tfm(), transformation of (recipient strain:with chromosomal DNA of strain listed, selecting for given resistance).

^c Name given to strain previously.

^d We have been told that this strain has been identified as a *spoIII* mutant (1a).

^e *Bacillus* Genetics Stock Center.

results in an increase in flagellin expression (see above) but also in inefficient sporulation (25, 28) and a defect in the development of genetic competence (28). In fact, it has been proposed that the farthest upstream component of signal transduction between the external environment and the initiation of sporulation is the *spo0K* system (5).

The fact that σ^D activity is not dependent on the Spo0A-K and AbrB proteins was unexpected, since a previous study had

FIG. 1. σ^D protein in wild-type and *spo0 abrB* mutant strains. Cells were grown in sporulation medium, and samples were withdrawn at the end of logarithmic growth (T_0). Equal amounts of total protein extract (20 μ g) were loaded per lane.

FIG. 2. Flagellin protein in wild-type and *spo0 abrB* mutant strains. Cells were grown in sporulation medium and samples withdrawn at the end of logarithmic growth (T_0). Equal amounts of total protein extract (20 μ g) were loaded per lane.

demonstrated that two σ^D -dependent promoters were dependent on several *spo0* gene products for their utilization in vivo (4). One of these promoters has since been identified as the sole promoter for a *B. subtilis* motility locus (21). However, the *spo0A-K* strains used in this study are motile, as determined by microscopic observation. The discrepancy in the results could therefore be due to either (i) the acquisition of an *abrB* suppressor mutation in the *spo0* strains used in this study, since *abrB* mutations have been shown to reverse many of the pleiotropic effects of *spo0* mutations (14, 33), or (ii) the acquisition of second-site flagellar mutations in the strains used in the previous study. We therefore decided to study σ^D activity in *spo0A*, *abrB*, and *spo0A abrB* strains in a way that would allow us to include an internal control for *abrB* suppression.

Primer extension analysis confirms the lack of *hag* regulation by *spo0A* and *abrB*. To avoid any ambiguities in the interpretation of the results obtained by primer extension analysis, we decided to use *spo0A* and *abrB* mutations that would lead to the complete lack of Spo0A and/or AbrB proteins in the resultant strains. Strains CB143 to CB145 (Table 1) were constructed by transforming a wild-type strain, CB25, with chromosomal DNA prepared from a mutant strain bearing a *spo0A* insertion deletion (CB64) and/or with chromosomal DNA prepared from a strain bearing an *abrB* transposon insertion (CB3). The CB25 strain used was first screened for motility and then transformed by the method of Anagnostopoulos and Spizizen (1). Chromosomal DNA was prepared as previously described (19).

As an internal control, we chose to monitor the transcription of the *B. subtilis* neutral protease gene (*npr*). Expression of the neutral protease is repressed in a *spo0A* strain (10, 26), and this repression is relieved in a *spo0A abrB* double mutant (33). Furthermore, the *npr* gene has been cloned (36) and its transcription start site has been mapped (32), enabling the design of an oligonucleotide for measuring *npr* transcription by primer extension analysis. The *npr* primer used has the sequence 5'-CCTTCAGCAGCCTGAACACCTGG-3'.

We then used primer extension analysis to measure the transcription of the *npr* and flagellin (*hag*) genes in strains CB25, CB143, CB144, and CB145. These strains were grown in 2XSG sporulation medium (34), and samples were withdrawn

FIG. 3. *hag* and *npr* primer extension analysis of mRNA from wild-type and *spo0A abrB* mutant strains. Cells were grown in sporulation medium, and samples were withdrawn 1 h after the end of logarithmic growth (T_1). Lanes: 1 to 5, *npr* primer; 6 to 10, *hag* primer. Primer extension products from RNA samples (5 μ g per reaction) are as follows: lane 1, RNA transcribed in vitro by σ^A holoenzyme with pNPRsubR1 (36) as the template; lanes 2 and 6, mRNA from strain CB25; lanes 3 and 7, mRNA from strain CB143; lanes 4 and 8, mRNA from strain CB144; lanes 5 and 9, mRNA from strain CB145; lane 10, RNA transcribed in vitro by σ^D holoenzyme and the *hag* gene as the template. Start sites of transcription are indicated by arrows.

at T_1 since *npr* and *hag* mRNAs are both present in the cell at this time (20, 32). Total mRNA was isolated from the four strains, and equal amounts were subjected to *npr* and *hag* primer extension analysis as previously described (20) (Fig. 3). The specific activities of the two primers were approximately the same; however, the exposure time for the *npr* primer extension was 10 times that for *hag* primer extension. The start site of transcription of the *npr* gene was confirmed by the analysis of RNA transcribed in vitro by σ^A RNA polymerase with pNPRsubR1 (36) as the template and by the use of sequencing ladders.

The primer extension results confirm those obtained previously by immunoblot analysis. There is *hag* mRNA in both the *spo0A* insertion-deletion strain and the *spo0A abrB* double mutant (Fig. 3, lanes 7 and 9). Additionally, the lack of *npr* mRNA in the *spo0A* strain (lane 3) indicates that expression of *hag* mRNA in this strain is not due to suppression by *abrB*. We therefore conclude that σ^D expression and activity are not dependent on the Spo0A or AbrB proteins and that the results obtained in the previous study (4) may have been due to a second-site flagellar mutation (*fla*) not linked to the *spo0* or *abrB* mutations being studied. Many laboratory strains of *Escherichia coli* have been found to spontaneously accumulate *fla* mutations, presumably as a result of the large amount of biosynthetic energy saved (15). This phenomenon may also occur in *B. subtilis*.

The *sin* gene product is not essential for flagellin expression.

Having determined that the *spo0A-K* and *abrB* gene products are not required for σ^D expression or activity, we decided to study the role of another candidate regulatory factor. Sin is a DNA-binding protein which has been shown to both repress the expression of certain genes (3) and activate other genes and physiological functions (2, 7). We were particularly interested in this locus since alleles of the *sin* locus, *flaD1* and *flaD2*, give rise to a nonflagellate phenotype (27, 29), while inactivation of the *sin* gene results in a pleiotropic phenotype that includes the lack of motility in the strain bearing this mutation (2).

FIG. 4. *hag* primer extension analysis of mRNA from *sin* mutant strains. Cells were grown in sporulation medium, and samples were withdrawn during vegetative growth (V), at the end of logarithmic growth (T_0), and 1 h after the end of logarithmic growth (T_1). Primer extension products from RNA samples (5 μ g per reaction) are as follows: lane 1, mRNA from strain CB25; lanes 2 to 4, mRNA from strain CB35; lanes 5 to 7, mRNA from strain CB36. The start site of transcription is indicated by the arrow.

Two mutant strains, one (CB35) overexpressing the Sin protein and the second (CB36) containing an insertional inactivation within the *sin* gene, were grown in 2XSG sporulation medium (34), and samples were withdrawn during vegetative growth (V), at the end of logarithmic growth (T_0), and 1 h after the end of logarithmic growth (T_1). Total mRNA was isolated from these strains, and equal amounts were subjected to *hag* primer extension analysis as described previously (20). As a positive control for flagellin expression, mRNA from a wild-type strain, CB25, was included in this analysis. In all cases flagellin mRNA was detected (Fig. 4), indicating that the *sin* gene product is not essential for expression of the *hag* gene. However, there is a reduction in *hag* mRNA levels in the *sin* disruption, suggesting that *sin* levels do affect flagellin synthesis.

The results of this analysis are similar to those obtained with the *degS* and *degU* genes. Missense mutations within these loci, *degS*(Hy) and *degU*(Hy) [originally designated as *sacU*(Hy) mutations] (9), give rise to a pleiotropic phenotype that includes a lack of flagella in the strains bearing these mutations (13). However, disruption of the *degS* or *degU* gene does not affect motility, indicating that their gene products are not directly responsible for motility functions (23). It has been postulated that the lack of flagella in the *degS*(Hy) and *degU*(Hy) mutants is a result of cross talk between the mutant regulators and cellular components that directly control expression of motility functions (24). Perhaps the lack of flagella in strains bearing the *flaD1* and *flaD2* mutations is due to a similar phenomenon since we have demonstrated that the Sin protein is not essential for flagellin expression. Interestingly, whereas the *flaD1* and *flaD2* mutant regulators lead to a lack of flagellin expression (27, 29), overexpression of wild-type Sin protein results in a severalfold increase (Fig. 4, lanes 2 to 4) relative to the null mutant (lanes 5 to 7), indicating that the postulated cross talk may result in a negative or positive interaction with the flagellar machinery.

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