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Developmental Gene Expression in *Bacillus subtilis crsA47* Mutants Reveals Glucose-Activated Control of the Gene for the Minor Sigma Factor σ^H

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The presence of excess glucose in growth media prevents normal sporulation of *Bacillus subtilis*. The *crsA47* mutation, located in the gene for the vegetative phase sigma factor (σ^A) results in a glucose-resistant sporulation phenotype. As part of a study of the mechanisms whereby the mutation in σ^A overcomes glucose repression of sporulation, we examined the expression of genes involved in sporulation initiation in the *crsA47* background. The *crsA47* mutation had a significant impact on a variety of genes. Changes to stage II gene expression could be linked to alterations in the expression of the *sinI* and *sinR* genes. In addition, there was a dramatic increase in the expression of genes dependent on the minor sigma factor σ^H. This latter change was paralleled by the pattern of *spo0H* gene transcription in cells with the *crsA47* mutation. In vitro analysis of RNA polymerase containing σ^{A47} indicated that it did not have unusually high affinity for the *spo0H* gene promoter. The in vivo pattern of *spo0H* expression is not predicted by the known regulatory constraints on *spo0H* and suggests novel regulation mechanisms that are revealed in the *crsA47* background.

The activation of genes that are induced late in the growth cycle of *Bacillus subtilis* involves a network of interacting regulatory pathways. These pathways control the cellular response to conditions that include nutritional stress and high cell density (reviewed in references 13, 19, 24, 36, and 47). Under the appropriate environmental stimuli, *B. subtilis* will differentiate to form dormant endospores. The key factor in initiating sporulation is the accumulation and phosphorylation of the response regulator and transcription factor Spo0A (reviewed in references 13, 19, and 24). Phosphorylation of Spo0A takes place through a multicomponent pathway (the phosphorelay) (3) that appears to integrate multiple signals that act positively or negatively to regulate sporulation (24). Once a sufficient level of phosphorylated Spo0A (Spo0A~P) is reached, a complex series of feedback loops will drive differentiation forward (19, 24, 36).

One critical component of sporulation is the minor sigma factor, σ^H, encoded by a gene originally found as a stage zero sporulation mutant, *spo0H* (12, 20, 46). σ^H is required for the transcription of a variety of sporulation genes, including *spo0A, spo0F*, and *kinA* (20, 41). The activity of σ^H, which increases as cells enter stationary phase, is under complex, still-undefined regulatory controls (19, 45, 46). Transcription of the *spo0H* gene is repressed by the transition state regulator AbrB (11, 48, 50), and so the induction of *spo0H* seen at the transition between log growth and sporulation is influenced by Spo0A~P repression of the *abrB* gene (4, 11). Transcriptional and translational regulation of *spo0H* by the presence of nutrients has also been reported (8, 14).

A variety of experiments have shown that the increase in σ^H activity as measured by transcription of σ^H-dependent genes does not match the accumulation of the σ^H protein, implying the existence of posttranslational controls (8, 16, 17, 21, 23, 45, 50). Furthermore, evidence has been presented that external pH (8) and the activity of the tricarboxylic acid cycle (26) affect σ^H activity and that σ^H protein levels are affected by a Lon-type protease during the onset of sporulation and during stress responses (30). Recently, ClpX has been implicated in σ^H-dependent transcription activity. In vivo and in vitro experiments have suggested that ClpX may interact directly with RNA polymerase containing σ^H and stimulate transcription (31, 37). The level of σ^H-dependent transcription increases for 1 to 2 h after sporulation initiation and then declines (4, 11, 46). The decline of activity has been linked to another Clp protein, ClpP, and the decline is associated with the loss of σ^H protein (38).

Excess glucose in the growth medium represses sporulation, and some of the effects on sporulation have been linked to specific repression of σ^H activity (1, 7, 8, 16, 17, 50, 52). The *crsA47* mutation renders sporulation resistant to repression by glucose (49). The molecular basis for this phenotype is unknown, but the *crsA47* mutation is located within the gene for the σ^A subunit of RNA polymerase (*sigA* or *rpoD*) (27).

In this report, we describe experiments that suggest that the *crsA47* mutation in σ^A affects the expression of σ^H-dependent genes through its effect on the expression of *spo0H*. The effects include overexpression of the *spo0H* gene during the onset of sporulation and unusual extended expression past the time of normal shutoff during differentiation. As the pattern cannot be explained solely by increased promoter affinity, these effects suggest novel regulation of the *spo0H* gene.

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

Strain	Genotype or description[a]	Source or construction
JH642	*trpC2 phe-1*	J. Hoch
GLU-47	*crsA47 strA*	*Bacillus* Genetic Stock Center
JH12862	*trpC2 phe-l amyE*::(*spo0F-lacZ* Cm^r)	M. Perego
JH12866	*trpC2 phe-l amyE*::(*rapB-lacZ* Cm^r)	M. Perego
JH12961	*trpC2 phe-l amyE*::(*rapA-lacZ* Cm^r)	M. Perego
JH16124	*trpC2 phe-l amyE*::(*spoIIA-lacZ* Cm^r)	M. Perego
JH12664	*trpC2 phe-l kinA*::(1.7-kb *kinA-lacZ* Cm^r)	M. Perego
JH12638	*trpC2 phe-l kinA* W168::pJM8115 Cm^r	M. Perego
JH16304	*trpC2 phe-l amyE*::(*spoIIG-lacZ* Km^r)	M. Perego
IS688	*leuA8 metB5 hisA1 spoVG*::(*spoVG-lacZ* Cm^r)	I. Smith
IS875	*leuA8 metB5 hisA1* Δ*sinR*:: Cm^r	I. Smith
IS423	*leuA8 metB5 hisA1 sinI*::(pIS135 Cm^r)	I. Smith
IS424	*leuA8 metB5 hisA1 sinR*::(pIS142 Cm^r)	I. Smith
GBS10	*crsA47 trpC2 phe-l*	GLU-47DNA→JH642
GBS101	*crsA47 trpC2 phe-1 amyE*::(*spoIIG-lacZ* Km^r)	JH16304DNA→GBS10
GBS103	*crsA47 trpC2 phe-1 amyE*::(*rapB-lacZ* Km^r)	JH12866DNA→GBS10
GBS104	*crsA47 trpC2 phe-1 amyE*::(*rapA-lacZ* Km^r)	JH12961DNA→GBS10
GBS105	*crsA47 trpC2 phe-1 amyE*::(*spo0F-lacZ* Cm^r)	JH12862DNA→GBS10
GBS106	*crsA47 trpC2 phe-1 amyE*::(*spoIIA-lacZ* Cm^r)	JH16124DNA→GBS10
GBS107	*crsA47 trpC2 phe-1 kinA*::(1.7-kb *kinA-lacZ* Cm^r)	JH12664DNA→GBS10
GBS108	*crsA47 trpC2 phe-1 kinA*::(pJM8115 Cm^r)	JH12638DNA→GBS10
GBS109	*crsA47 trpC2 phe-1 spoVG*::(*spoVG-lacZ* Cm^r)	IS688 DNA→GBS10
GBS110	*trpC2 phe-1 spoVG*::(*spoVG-lacZ* Cm^r)	IS688 DNA→JH642
GBS111	*crsA47 trpC2 phe-1* Δ*sinR*::Cm^r	IS875 DNA→GBS10
GBS112	*trpC2 phe-1* Δ*sinR*::Cm^r	IS875 DNA→JH642
GBS113	*crsA47 trpC2 phe-1 sinI*::(pIS135 Cm^r)	IS423 DNA→GBS10
GBS114	*trpC2 phe-1 sinI*::(pIS135 Cm^r)	IS423 DNA→JH642
GBS115	*crsA47 trpC2 phe-1 sinR*::(pIS142 Cm^r)	IS424 DNA→GBS10
GBS116	*trpC2 phe-1 sinR*::(pIS142 Cm^r)	IS424 DNA→JH642
GBS150	*crsA47 trpC2 phe-1 amyE*::(*spo0H-lacZ* Cm^r)	pGS0H→GBS10
GBS151	*trpC2 phe-1 amyE*::(*spo0H-lacZ* Cm^r)	pGS0H→JH642

[a] Km, kanamycin; Cm, chloramphenicol.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains used in this study are shown in Table 1. The promoter-*lacZ* fusions used were created using a *B. subtilis amyE* integrative vector, with the exception of the *kinA-lacZ* fusions that were inserted in the *kinA* gene. Promoter-*lacZ* fusions provided in the JH642 background were transferred into GBS10 (containing the *crsA47* mutation in the *rpoD* gene but otherwise isogenic to JH642) by transformation with chromosomal DNA, and selection for both the antibiotic resistance and the *amyE* mutant phenotype was conferred with the acquisition of the construct. The plasmid pGS0H was created by the ligation of the *Eco*RI/*Bam*HI-digested Vent polymerase (New England Biolabs, Inc.) PCR product generated from chromosomal DNA and the primer pair 5′-AAGGATCCTGTTTCTGGCGAGTAG-3′ and 5′-ACGAATTCGGCA CGGACGTTAGAA-3′ that targets the *spo0H* gene into the *Eco*RI/*Bam*HI sites of the *B. subtilis* integrative vector pDH32 (44). The pDH32-based clone was linearized with *Pst*I prior to transformation into *B. subtilis*. Chloramphenicol-resistant transformants generated were confirmed to be amylase negative by using 1% starch agar plates prior to β-galactosidase analysis.

Bacterial transformation and growth conditions. *B. subtilis* transformations were performed by the method of Hoch (24) with 1 to 2 μg of plasmid DNA or 20 to 100 ng of chromosomal DNA. Transformants were selected on Schaeffer sporulation agar plates supplemented with 5 μg of chloramphenicol or kanamycin/ml. *Bacillus* cultures used to determine sporulation frequency were grown in Schaeffer spore media (SSM), pH 7.5, supplemented with tryptophan and phenylalanine (each 10 μg/ml), chloramphenicol or kanamycin (5 μg/ml), and, when appropriate, SSM with 1% glucose (SSMG). Cells were grown for 22 to 24 h, serially diluted in SSM, and plated before and after treatment with 0.1 volume of chloroform to obtain a viable cell count and a spore count.

β-Galactosidase assay. *B. subtilis* strains used for analysis of promoter-*lacZ* activity were inoculated into SSM, pH 7.5, containing 5 μg of the appropriate antibiotic/ml and supplemented with tryptophan and phenylalanine (each, 10 μg/ml) and, when appropriate, 0.2% glucose. Aliquots (0.5 ml) were removed hourly, the cells were collected by centrifugation, and cell pellets were frozen at −70°C until analyzed. β-Galactosidase assays and designation of time zero (T_0) in sporulation were done as previously described (15). Enzyme specific activity was expressed in Miller units (35). Assays of a minimum of three independent cultures were performed, and one representative pattern for each strain is shown. Each point in the assays is an average of duplicate samples that differed by no more than 5% from the mean.

In vitro transcription assay. The RNA polymerase preparations were isolated as described by Dobinson and Spiegelman (10) from logarithmic-stage cultures of JH642 and GBS10, except that the heparin-Sepharose column set was eliminated. Transcription assays used fractions from the glycerol gradient. Two DNA templates were used. The plasmid pUCA2trpA (6) that contains the bacteriophage ϕ29A2 promoter was treated with *Pvu*II, and the 600-bp fragment containing the promoter was isolated by electrophoresis through agarose and extracted from the agarose with a GeneClean kit from Qiagen. The *spo0H* promoter was isolated by amplifying a DNA fragment using 20 pmol (each) of two specific primers described above, 800 ng of chromosomal DNA from JH642, and an amplification protocol as follows: preincubation of the template and primers at 95°C for 5 min, and after the addition of 2.5 U of *Taq* polymerase (Promega), 30 cycles of 95°C for 1 min, 61°C for 1 min (with descending annealing temperatures of 0.3°C per cycle), and 72°C for 1 min. The product was purified by electrophoresis through 1% agarose and extracted from the gel as described above for the A2 promoter. The extracted product was digested with *Hin*dIII to provide a fixed endpoint for the transcription assays and then precipitated. The concentrations of promoter fragments were determined by measuring the absorbance at 260 nM.

The in vitro transcription assay followed published protocols (6). In brief, reaction tubes containing 16 μl of reaction mixture with template, transcription buffer (6), ATP, and [α-^{32}P]GTP (3 μCi/reaction mixture) were warmed to 37°C. The polymerase (2 μl of an appropriate dilution) was added. Two minutes later, 2 μl of a mixture of heparin (100 μg/ml, final concentration), UTP, and CTP was added to inactivate noninitiated RNA polymerase molecules and allow those that had initiated to elongate either to the terminator (in the case of the ϕ29A2 promoter) or to the end of the DNA fragment (in the case of the *spo0H* promoter). After 5 min, a stop buffer containing 7 M urea was added, and the reaction products were separated from free nucleotides on 5% polyacrylamide gels containing 7 M urea and 0.5× Tris-borate-EDTA (TBE). The gels were

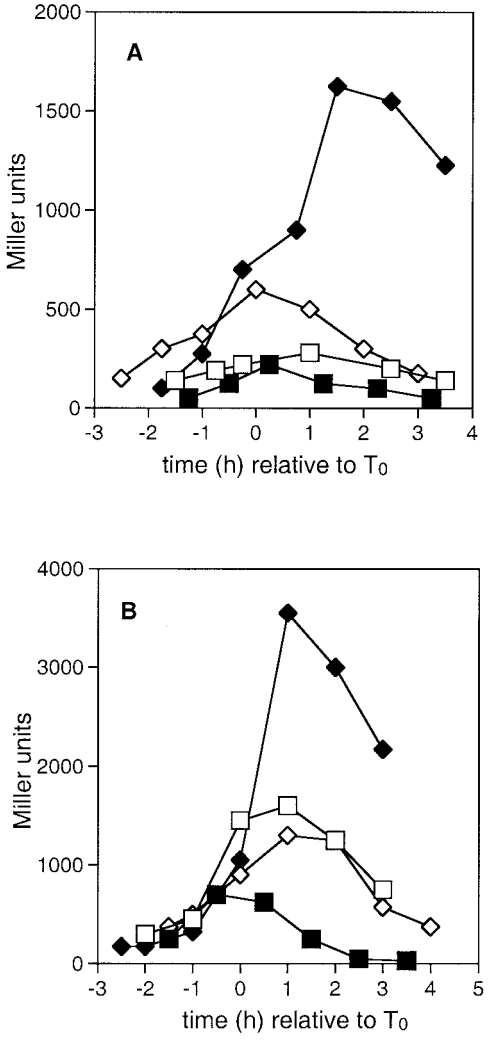

FIG. 1. The effect of the *crsA47* mutation on expression of *kinA* and *spoVG*. β-Galactosidase activities in strains carrying *kinA-lacZ* (A) or *spo0VG-lacZ* (B) fusions were measured as described in Materials and Methods. T_0 represents the onset of sporulation. Strains contained the wild-type σ^A gene (squares) or the *crsA47* mutation (diamonds) and were grown in either SSM (open symbols) or SSMG (filled symbols). (A) JH12664 (*kinA-lacZ*) and GBS107 (*crsA47 kinA-lacZ*); (B) GBS110 (*spoVG-lacZ*) and GBS109 (*crsA47 spoVG-lacZ*).

exposed to a Molecular Dynamics PhosphorImager screen, and the data were collected and analyzed with the ImageQuant 1.0 software on the instrument.

RESULTS

The *crsA47* mutation results in overexpression of σ^H-dependent *spo0* genes in glucose-containing media. To investigate the mechanism by which the *crsA47* mutation resulted in catabolite-resistant sporulation, we examined the expression of a variety of genes important in the sporulation initiation pathway in the presence and absence of glucose. Among these genes, the ones with σ^H-dependent promoters showed very unusual profiles of activity. Figure 1 shows the expression of the *kinA* and *spoVG* promoter fusions in wild-type (JH642) and *crsA47* (GBS10) backgrounds. The *kinA* promoter has not been previously shown to be subject to regulation other than that imposed by σ^H activity (1, 41). The *spoVG* gene is preceded by a σ^H promoter that is repressed during logarithmic growth by the transition state regulator AbrB (43, 48).

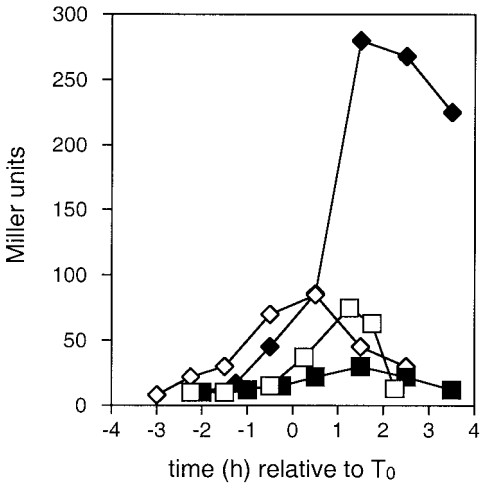

FIG. 2. The effect of the *crsA47* mutation on expression of *spo0F*. β-Galactosidase activities in strains carrying *spo0F-lacZ* fusions were measured as described in Materials and Methods. T_0 represents the onset of sporulation. Strains contained the wild-type σ^A gene (JH12862; squares) or the *crsA47* mutation (GBS105; diamonds) and were grown in either SSM (open symbols) or SSMG (filled symbols).

Three observations were common to the activity of the *kinA* and *spoVG* promoters. First, in a wild-type σ^A background promoter activity was depressed by the presence of glucose. Second, in a *crsA47* background the expression was elevated by the presence of glucose compared to that seen in the wild type in the absence of glucose, with transcription levels persisting long after the point of maximum activity in the wild type. Third, promoter activity in a *crsA47* background in the absence of glucose was only marginally affected compared to the effect in the wild type. The onset of promoter activity was not affected in the same way for the two promoters. *kinA* promoter activity (Fig. 1A) in GBS107 grown in the absence of glucose began earlier than in JH12664, whereas the timing of *spoVG* transcription activity (Fig. 1B) appeared similar in both GBS110 and GBS109. The logarithmic-phase repression of *spoVG* by AbrB did not differ between wild-type and *crsA47* mutants. The suggestion that AbrB regulation was not altered in the GBS109 was supported by the expression of an *abrB-lacZ* fusion which showed similar patterns in GBS10 and JH642 (data not shown).

The expression patterns of *spo0F* promoter-*lacZ* fusions are shown in Fig. 2. The *spo0F* gene is preceded by dual σ^A σ^H promoters, with the σ^H-dependent promoter requiring Spo0A~P as a transcription activator (28, 41, 51). In JH12862, grown in SSM, expression from the *spo0F* promoter-*lacZ* fusion increased during late-exponential-phase growth, peaked at roughly T_1, and decreased thereafter. The addition of glucose to the media resulted in a decrease in overall expression. In GBS105, the expression from *spo0F* began earlier and peaked at higher levels than were seen in JH12862, both with and without added glucose. As was seen with both the *kinA* and *spoVG* promoters (Fig. 1), transcription of *spo0F* in GBS105 in

cells grown with added glucose appeared to be stimulated after T_0 compared to levels seen in cells grown without glucose.

The *crsA47* mutation results in the expression of *spoII* genes despite the presence of glucose. The late- and post-exponential phase expression of σ^H-dependent *spo0* genes appeared to be elevated in the cells containing the *crsA47* mutation (Fig. 1 and 2), suggesting that σ^H was active in these cells despite the presence of glucose. The activation of σ^H during the transition state has been shown to be critical for the initiation of sporulation and is required for the maximal expression of genes encoding phosphorelay proteins and accumulation of a sufficient level of Spo0A~P to activate the transcription of stage II *spo* genes. As a test for whether or not Spo0A~P was fully activated, we examined the expression of the stage II genes *spoIIA* and *spoIIG* (Fig. 3).

In strain JH16124 cells grown in SSM (Fig. 3A), the expression from the σ^H-dependent *spoIIA* promoter-*lacZ* fusion began roughly at T_0 and peaked at T_2, dropping thereafter. In the presence of glucose, *spoIIA-lacZ* expression was depressed in stationary phase. In GBS106 grown in SSM (Fig. 3A), transcription activity from the *spoIIA* promoter began to increase at the same time as that observed in JH16124 and peaked at levels not substantially different from that in JH16124. In the presence of glucose, the expression of *spoIIA-lacZ* in GBS106 began immediately after the onset of stationary phase and rose to a level more than twice that seen in JH16124 grown in the absence of glucose.

Figure 3B depicts the expression from the σ^A-dependent *spoIIG* promoter-*lacZ* fusion in the wild type (JH16304) and cells containing the *crsA47* mutation (GBS101). As seen in Fig. 3A for the *spoIIA-lacZ* fusion, promoter activity from the *spoIIG* promoter was repressed by glucose in the wild type but not repressed in GBS101. Since effective expression of these two stage II operons directly requires high Spo0A~P levels, these cells must contain high levels of Spo0A~P. The *spo0A* gene is expressed from two promoters, one σ^A dependent and one σ^H dependent. It has been shown elsewhere that expression for the σ^H-dependent promoter is enhanced in *crsA47* mutants (9).

The presence of the *crsA47* mutation results in an alteration in the pattern of transcription from *sinI* and *sinR* promoters in the presence of glucose. SinR inhibits the expression of several *spo* genes, including *spo0A* (34), *spoIIG*, and *spoIIA* (5, 32, 33). The *sinR* gene is constitutively expressed from a σ^A-dependent promoter throughout exponential and post-exponential growth of *B. subtilis* (18). SinR inhibition of transcription is negatively regulated by Spo0A~P levels, which stimulate increased transcription of the σ^H-dependent *sinI* gene (the gene directly upstream of *sinR*) (18). SinI sequesters SinR via protein-protein interaction preventing SinR-mediated repression of promoter activity (2, 29). Gaur et al. showed that the presence of excess glucose in the media inhibits the transcription of the *sinI* gene (18). Presumably, inadequate transcription of *sinI* results in a SinI/SinR protein ratio insufficient to fully sequester SinR and relieve repression of sporulation genes (2, 29).

The data in Fig. 3 showed that σ^H- and σ^A-dependent stage II promoters were deregulated in cells containing the *crsA47* mutation grown in glucose. Because SinR is central to regulation of both the *spoIIG* and *spoIIA* operons, we examined the regulation of the *sinR* and *sinI* promoters. The data shown in

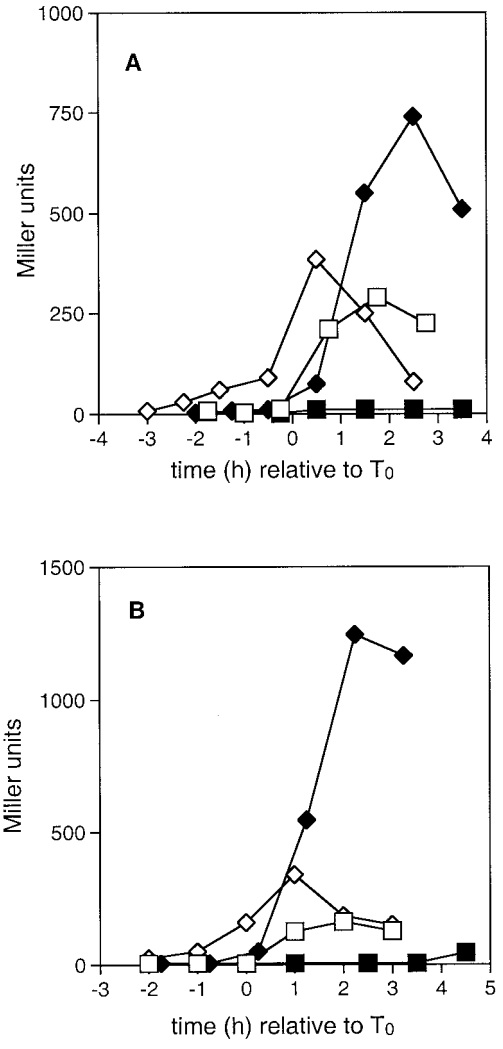

FIG. 3. The effect of the *crsA47* mutation on expression of *spoIIA* and *spoIIG*. β-Galactosidase activities in strains carrying *spoIIA-lacZ* (A) or *spoIIG-lacZ* (B) fusions were measured as described in Materials and Methods. T_0 represents the onset of sporulation. Strains contained the wild-type σ^A gene (squares) or the *crsA47* mutation (diamonds), and were grown in either SSM (open symbols) or SSMG (filled symbols). (A) JH16124 (*spoIIA-lacZ*) and GBS106 (*crsA47 spoIIA-lacZ*); (B) JH16304 (*spoIIG-lacZ*) and GBS101 (*crsA47 spoIIG-lacZ*).

Fig. 4 indicate that in cells containing wild-type σ^A (GBS114) grown in the absence of glucose, *sinI* transcription levels increased throughout late logarithmic growth to peak at T_0 (Fig. 4A). Transcription of *sinR* increased throughout late logarithmic growth and into stationary phase (GBS116) (Fig. 4B), presumably in part due to readthrough from the *sinI* promoter (18) as well as from the *sinR* promoter. The expression patterns in Fig. 4 are similar to those observed by others (18, 33). When glucose was added, *sinI* transcription remained relatively low during stationary phase in GBS114 (Fig. 4A), whereas *sinR* transcription in GBS116 (Fig. 4B) remained roughly the same as in the absence of glucose.

If transcription of the *sinI* and *sinR* genes reflects protein levels, then cells with wild-type σ^A grown in the absence of

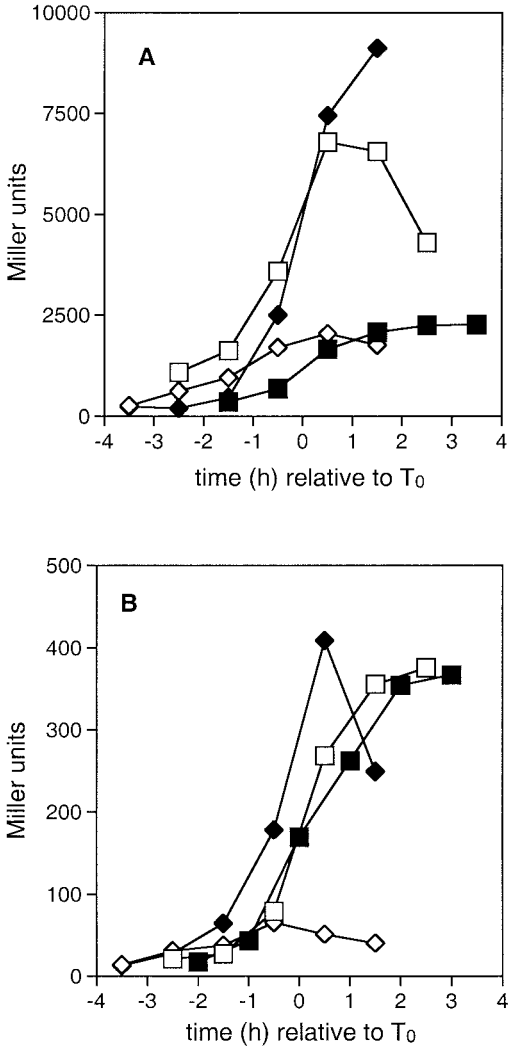

FIG. 4. The effect of the *crsA47* mutation on expression of *sinI* and *sinR*. β-Galactosidase activities in strains carrying *sinI-lacZ* (A) or *sinR-lacZ* (B) fusions were measured as described in Materials and Methods. T_0 represents the onset of sporulation. Strains contained the wild-type σ^A gene (squares) or the *crsA47* mutation (diamonds) and were grown in either SSM (open symbols) or SSMG (filled symbols). (A) GBS114 (*sinI-lacZ*) and GBS113 (*crsA47 sinI-lacZ*); (B) GBS116 (*sinR-lacZ*) and GBS115 (*crsA47 sinR-lacZ*).

glucose would contain a roughly 20-fold excess of SinI over SinR. Since these cells sporulate efficiently, this ratio should indicate the level of SinI needed to complex SinR between T_0 and $T_{1.5}$. In the presence of glucose, expression of the *sinI* promoter in cells with wild-type σ^A was reduced, with the implication that the ratio of SinI to SinR would not block SinR repression of sporulation.

sinI promoter activity in cells containing the *crsA47* mutation (GBS113) is also shown in Fig. 4A. In these cells, the activity from the *sinI* promoter in the absence of glucose rose slowly during logarithmic growth to peak at $T_{0.5}$ at levels 25 to 30% of that seen in GBS114. The *sinI-lacZ* activity was also altered in GBS113 grown in the presence of glucose (Fig. 4A), with the observed pattern of transcription similar to that seen in other σ^H-dependent promoters examined in the *crsA47* background.

TABLE 2. Sporulation efficiencies of *sinI-lacZ-*, *sinR-lacZ-*, and Δ*sinR*-containing strains

Strain	Sporulation efficiency[a]	
	SSM[b]	SSMG[c]
JH642	6.5×10^{-1}	1.0×10^{-5}
GBS114	7.1×10^{-1}	7.9×10^{-6}
GBS116	6.2×10^{-1}	9.3×10^{-6}
GBS112	7.4×10^{-1}	2.5×10^{-1}
GBS10	5.0×10^{-1}	1.0
GBS113	8.9×10^{-1}	9.8×10^{-1}
GBS115	9.1×10^{-1}	9.3×10^{-1}

[a] Sporulation efficiency was calculated as the number of chloroform-resistant cells per total cells.
[b] SSM, strain grown in Schaeffer's spore media, pH 7.5.
[c] SSMG, strain grown in Schaeffer's spore media (pH 7.5) plus 1.0% glucose.

Transcription from the *sinI* promoter rose from $T_{-1.5}$ to peak after T_2 at levels five times higher than was seen in cells with wild-type σ^A.

Figure 4B shows *sinR-lacZ* activity in cells containing the *crsA47* mutation (GBS115). Without excess glucose, transcription of the *sinR* gene was reduced from that seen in cells containing wild-type σ^A (GBS116), peaking at roughly T_0 and decreasing thereafter. In the presence of glucose, transcription rose sharply from T_{-2} to peak at T_0 at levels similar to those achieved in GBS116 in the presence or absence of glucose.

Continuing the assumption that the activity of the *sinI* and *sinR* promoter fusions reflects protein levels, then even though SinR and SinI levels were reduced in cells containing the *crsA47* mutation (in media without excess glucose), the ratio would be similar to that seen in wild-type cells. Thus, SinR activity would be blocked by SinI during early stationary phase. When glucose was added, the large induction of the *sinI* promoter would further reduce SinR activity. Thus, unlike cells with wild-type σ^A where the addition of glucose decreased the ratio of expression of *sinI*/*sinR*, in the cells with the *crsA47* mutation the ratio of *sinI*/*sinR* would increase with added glucose. This alteration in *sin* operon transcription in the *crsA47* mutant could contribute to the ability of these cells to express the *spoIIA* and *spoIIG* operons in the presence of glucose.

The importance of SinR negative regulation in glucose repression of sporulation was examined by determining the sporulation efficiency of a Δ*sinR* mutant, as shown in Table 2. In the *crsA47* mutant (GBS10), the sporulation efficiencies of cells grown in the presence and absence of glucose were comparable, clearly indicating a glucose-resistant sporulation phenotype. In JH642, the addition of excess glucose to the medium resulted in a 10^4-fold decrease in sporulation efficiency. However, in GBS112 (*sigA*$^+$ Δ*sinR*) the sporulation efficiency in medium with excess glucose was only three-fold less than that observed in the absence of glucose. These results, combined with those shown in Fig. 2 through 4, suggest that a decrease in SinR repression whether by *sinR* gene deletion or by altering the ratios of *sinI* and *sinR* transcription increased expression of *spoII* genes and contributed to the glucose-resistant sporulation phenotype.

The *crsA47* mutation results in increased transcription from the *spo0H* gene in late- and post-exponential-phase growth. The results shown in Fig. 3 and 4 demonstrate increased ex-

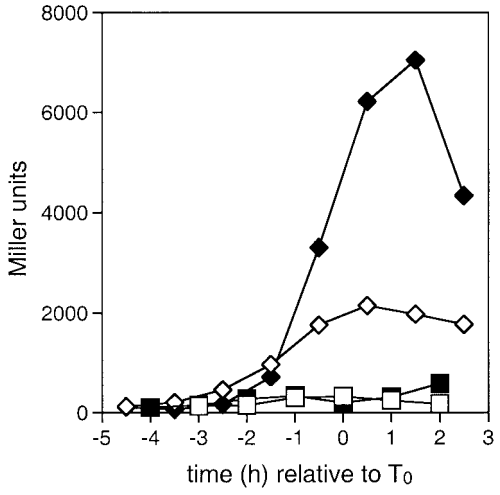

FIG. 5. Effect of the *crsA47* mutation on the expression of *spo0H*. β-Galactosidase activities in a strain carrying *spo0H-lacZ* fusions were measured as described in Materials and Methods. T_0 represents the onset of sporulation. Strains contained the wild-type σ^A gene (GBS151; squares) or the *crsA47* mutation (GBS150; diamonds) and were grown in either SSM (open symbols) or SSMG (filled symbols).

pression of both σ^A- and σ^H-dependent stage II genes in the *crsA47* background when the cells were grown in the presence of excess glucose. The implication from the analysis of the *sinI-sinR* operon was that the increase in stage II gene expression reflected the increase in σ^H activity, which allowed SinI to inactivate SinR. Thus, overexpression of the *spoIIG* operon (σ^A dependent) has the same fundamental mechanism as overexpression of the *spoIIA* operon (σ^H dependent); that is, increased activity of σ^H. However, the reason for the high levels of stationary-phase transcription from all of the σ^H-dependent promoters examined in the *crsA47* mutant in the presence of glucose was not clear. Previous studies have shown higher-than-normal σ^H-dependent promoter activity during the stationary phase either by using an increased copy number of *spo0H* or by placing *spo0H* under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible P_{SPAC} promoter (21). Thus, the increased σ^H activity could be due to overexpression of the *spo0H* gene in the *crsA47* mutant, and this was examined using a *spo0H-lacZ* fusion in the presence and absence of added glucose.

Figure 5 shows the expression patterns of the σ^A-dependent *spo0H-lacZ* fusion in strains containing wild-type *sigA* (GBS151) or the *crsA* mutation (GBS150). In GBS151 the activity of the *spo0H* promoter began to increase at T_{-2} to peak at roughly T_1, with the maximum activity not substantially different in the presence or absence of glucose. This pattern of transcription is similar to that seen elsewhere (1, 50). In GBS150 the timing of transcription from the *spo0H* promoter was the same as in GBS151, but peak activity was roughly eight times higher in the absence of glucose and 19 times higher in the presence of glucose than was seen in the GBS151.

The high level of transcription of the *spo0H* gene seen in GBS150 grown in the presence of glucose could lead to excess σ^H protein and thus explain the unusual patterns of σ^H-dependent transcription seen in cells containing the *crsA47* mutation. These data raise the question whether the extended transcription from the σ^A-dependent promoter of the *spo0H* gene reflected a general phenomenon or whether it was specific to the *spo0H* promoter. The only known regulator of the *spo0H* promoter is AbrB. We would not expect AbrB levels to play a role in this regulation of *spo0H* beyond T_1 as the gene is repressed earlier, and this repression was not changed in cells containing the *crsA47* mutation (9). However, it was possible that the *crsA47* mutation altered the reduction of σ^A activity that is normally seen in sporulation (46).

As part of the characterization of the *crsA47* mutation, we examined several other σ^A-dependent promoters. The *rapA*-encoded phosphatase removes phosphate from the phosphorelay by dephosphorylating phosphorylated Spo0F (reviewed in reference 40). Transcription from the *rapA* gene promoter also showed a *crsA47*- and excess-glucose-specific increase in transcription after T_0 (Fig. 6A). In other work, the same effect for the σ^A-dependent *spo0APv* promoter has been seen (9). In contrast, expression of a second σ^A-dependent gene encoding a phosphatase, *rapB*, did not show increased transcription in strains containing the *crsA47* mutation and grown in excess glucose (Fig. 6B). Thus, the effect of the *crsA47* mutation was promoter specific as well as being specific to growth conditions.

In vitro analysis of transcription from the *spo0H* promoter by RNA polymerase containing σ^{A47}. One possible explanation for the increase in σ^H activity seen in strains containing the *crsA* mutation is that the mutation in σ^A increases the affinity of the polymerase for the *spo0H* gene promoter. To directly measure this affinity, we purified RNA polymerase from wild-type and GBS10 strains and tested them in vitro in single-round transcription assays (as described in Materials and Methods) (Fig. 7). As a means of measuring the specific activity of the RNA polymerase preparations, we compared the activity of the two preparations on a promoter from bacteriophage ϕ29 that has been cloned into the plasmid pUCA2trpA (Fig. 7A). Aliquots of the polymerase preparations were mixed with DNA and initiating nucleotides (ATP plus GTP) and challenged with a mixture of heparin and CTP plus UTP. The heparin inactivates noninitiated RNA polymerase molecules while the CTP plus UTP allow enzymes that have initiated to elongate. Over a range of DNA concentrations of 0.5 to 8 nM, the wild-type RNA polymerase was three- to five-fold more active than the preparation from GBS10. We then repeated this experiment using a DNA fragment containing the *spo0H* promoter. This DNA fragment was produced by amplification of the promoter region. Transcription from this promoter produced a single transcript (data not shown). The activity of both polymerase preparations was significantly lower on the *spo0H* promoter than on the phage promoter. However, as with the ϕ29A2 promoter, the polymerase containing σ^{A47} was three- to five-fold less active on this template than was the wild-type polymerase. By this assay, the change in σ^A caused by the *crsA* mutation did not increase the affinity of the polymerase for the *spo0H* promoter.

DISCUSSION

We began the study of the *crsA47* mutation to uncover the mechanism by which it makes sporulation resistant to the presence of excess glucose in the growth medium. During the

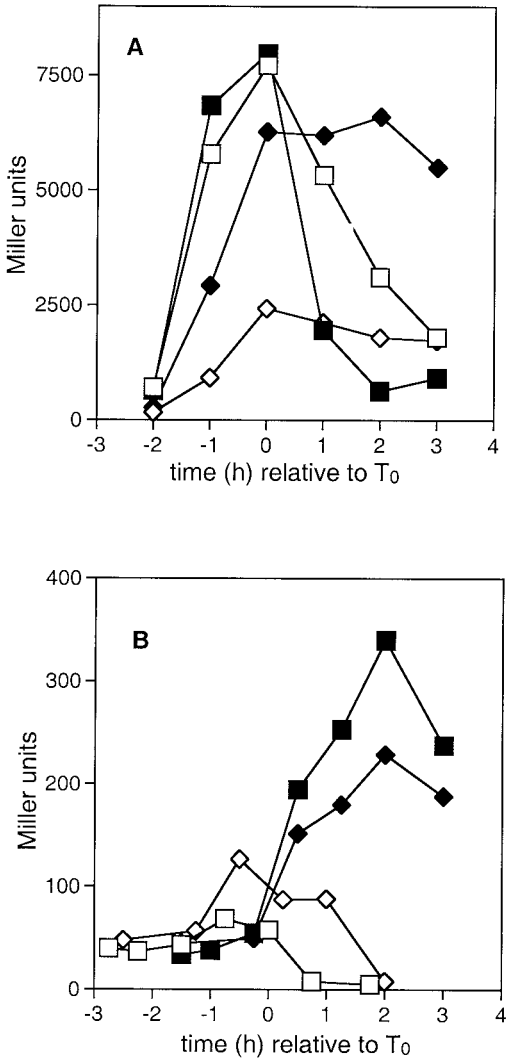

FIG. 6. Effect of the *crsA47* mutation on the expression of *rapA* and *rapB*. β-Galactosidase activities in strains carrying *rapA-lacZ* (A) or *rapB-lacZ* (B) fusions were measured as described in Materials and Methods. T_0 represents the onset of sporulation. Strains contained the wild-type σ^A gene (squares) or the *crsA47* mutation (diamonds) and were grown in either SSM (open symbols) or SSMG (filled symbols). (A) JH12961 (*rapA-lacZ*) and GBS104 (*crsA47 rapB-lacZ*); (B) JH12866 (*rapB-lacZ*) and GBS103 (*crsA47 rapB-lacZ*).

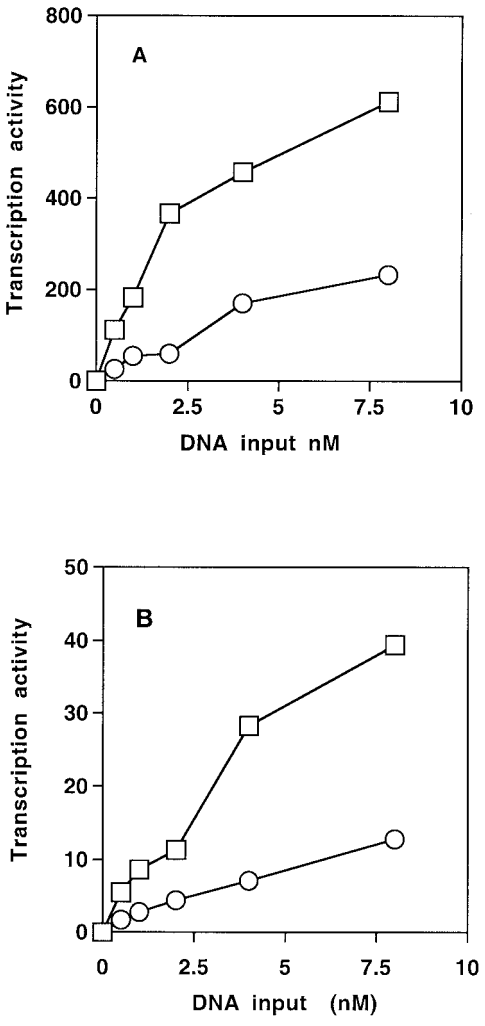

FIG. 7. In vitro transcription activity of RNA polymerase isolated from JH642 and GBS10. In vitro transcription reactions were carried out using a control promoter (pUCA2trpA) (A) or a DNA fragment containing the *spo0H* gene promoter (B). Reaction mixtures contained a constant amount of RNA polymerase from either JH642 (wild type; squares) or GBS10 (*crsA*; circles) and increasing amounts of template. Transcription products were separated by electrophoresis through polyacrylamide gels containing 7 M urea. The level of product produced was determined using a Molecular Dynamics PhosphorImager and ImageQuant 1.0 software and is reported in arbitrary units.

course of these studies, we observed the consistent overexpression of σ^H-dependent genes in strains carrying *crsA47* that we have described in this paper. The overexpression occurred after T_0 and only in strains grown in the presence of excess glucose. The overexpression peaked 2 to 3 h later than the normal peak of σ^H-dependent expression and then declined. This combination of features suggests a novel feature of σ^H transcription regulation that is revealed in the *crsA47* genetic background.

The regulation of σ^H activity appears to be complex. There is a low level of transcription of the *spo0H* gene during vegetative growth, and at least a few σ^H-dependent genes are transcribed during this time (19, 42). In wild-type cells, transcription dependent on σ^H is induced during the transition stage, and it normally peaks within 1 to 2 h after the onset of sporulation and decays after that time (46). Comparison of the levels of σ^H protein with σ^H-dependent transcription in vegetative growth and in early sporulation indicates the presence of post-transcriptional regulation of σ^H (1, 8, 16, 17, 21, 23, 42, 50). Later in sporulation, the decrease in σ^H activity is associated with loss of σ^H protein due to the activity of ClpC protease (38). Another Clp family protein, ClpX, influences the induction of activity of σ^H during early sporulation, although not through changes in the level of σ^H protein. It has been reported that ClpX stimulates σ^H-dependent transcription by direct interaction with the polymerase containing σ^H (31, 37). It is known that σ^H activity is regulated at transcription, since *spo0H* gene transcription is repressed by AbrB (11, 43). It has

been recently shown that σ^H-dependent expression of a limited set of genes is enhanced by amino acid starvation and that this enhancement requires the *relA* gene product (14). While this finding is yet another illustration of the complexity of controls over σ^H activity, it is probably not related to our findings, which appeared to be specific to the presence of glucose.

It seems likely to us that the overexpression of σ^H-dependent genes in GBS10 and its derivatives grown in the presence of glucose cannot be explained by loss of repression by AbrB, since AbrB repression was normal in GBS10 (9), and σ^H-dependent transcription continues to increase after the time when AbrB is repressed (9). Given the similarity of the expression patterns of the *spo0H* gene (Fig. 5) and the σ^H-dependent genes (Fig. 1 and 3), we suggest that overexpression of *spo0H* in cells containing the *crsA47* mutation is a sufficient explanation for the unusually high transcription of σ^H-dependent genes.

In a direct in vitro test of the activity of RNA polymerase isolated from GBS10 and wild-type cells (Fig. 7), there was no evidence that the *crsA47* mutation increased the activity at the *spo0H* promoter. Furthermore, several lines of in vivo evidence support the finding that the *crsA47* mutation does not simply increase the affinity of the polymerase for the *spoH* promoter. First, GBS10 does not grow unusually slowly, as might be expected if the *crsA47* mutation altered the polymerase specificity. Second, the overexpression of the *spo0H* promoter in GBS10 happened only in stationary phase and only in the presence of excess glucose in the growth medium, suggesting a specific regulatory mechanism. Third, two other σ^A-dependent promoters studied (for the *abrB* and *rapB* genes) did not show unusual expression patterns in GBS10 (9). We note in passing that the regulation of AbrB, which is known to control expression of a number of genes (48), must be particularly important to the overall physiology of cells with a *crsA47* mutation, because it was found that a *crsA47 abrB* double mutant grew so poorly even in rich media that regulation in the strain could not be studied (9).

Our results indicate that there must be a regulator of the *spo0H* promoter (and possibly other similar promoters) whose activity is changed in GBS10 grown in excess glucose. We cannot provide any indication whether the regulator is an activator of the *spo0H* promoter that is hyperactive or a repressor whose activity is reduced. The latter seems more likely, since the extended high expression can be viewed as a lack of shutoff of *spo0H* transcription. The existence of such a regulator implies that other controls of σ^H are yet to be discovered.

A consequence of σ^H overexpression in the presence of glucose was the change in the SinI-to-SinR ratio, with the predicted result that SinR repression of stage 0 and stage II sporulation genes would be reduced. There is little question that reduction of SinR in the cell would increase the glucose resistance of sporulation (32). This is likely to be a major contributor to the glucose-resistant sporulation in GBS10 and illustrates the redundant pathways that regulate entry into sporulation.

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