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Regulation of *Bacillus subtilis* σ^H (Spo0H) and AbrB in Response to Changes in External pH

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The RNA polymerase sigma subunit, σ^H, of *Bacillus subtilis* is required for the transcription of genes that are induced in late-growth cultures at high cell density, including genes that function in sporulation. The expression of σ^H-controlled genes is repressed when nutrient broth sporulation medium (Difco sporulation medium [DSM]) is supplemented with high concentrations of glucose and glutamine (DSM-GG), preferred carbon and nitrogen sources of *B. subtilis*. Under these conditions, the pH of the DSM-GG medium decreases to ≈5. Raising the pH by the addition of morpholinepropanesulfonic acid (MOPS) or Tris-HCl (pH 7.5) results in a dramatic increase in the expression of *lacZ* fusions to σ^H-dependent promoters. Correspondingly, the level of σ^H protein was higher in cells of late-growth DSM-GG cultures treated with a pH stabilizer. When σ^H-dependent gene expression was examined in cells bearing a mutation in *abrB*, encoding the transition state regulator that negatively controls genes transcribed by the σ^H form of RNA polymerase, derepression was observed as well as an increase in medium pH. Reducing the pH with acetic acid resulted in repression, suggesting that AbrB was not functioning directly in pH-dependent repression but was required to maintain the low medium pH in DSM-GG. AbrB protein levels were high in late-growth, DSM-GG cultures but significantly lower when the pH was raised by Tris-HCl addition. An active tricarboxylic acid (TCA) cycle was required to obtain maximum derepression of σ^H-dependent transcription, and transcription of the TCA cycle enzyme gene *citB* was repressed in DSM-GG but derepressed when the pH was artificially raised. The negative effect of low pH on σ^H-dependent *lacZ* expression was also observed in unbuffered minimal medium and appeared to be exerted posttranslationally with respect to *spo0H* expression. However, the addition of amino acids to the medium caused pH-independent repression of both σ^H-dependent transcription and *spo0H-lacZ* expression. These results suggest that *spo0H* transcription or translation is repressed by a mechanism responding to the availability of amino acids whereas *spo0H* is posttranslationally regulated in response to external pH.

When the soil bacterium *Bacillus subtilis* encounters a growth-restricting environment, it chooses among a variety of strategems for coping with stressful situations. An environment depleted of nutrients will induce processes, such as the production of extracellular degradative enzymes and antibiotics, that will permit cells to acquire nutrition from complex organic material and to compete for limited resources. *B. subtilis* cells will also undergo sporulation or competence development in response to signals generated as a result of high cell density and nutrient limitation (22). These conditions lead to dramatic changes in the pattern of gene expression, which are due, in part, to the appearance of alternative RNA polymerase sigma subunits (25, 47). Thus, the minor sigma factor, σ^H, (encoded by *spo0H* [9, 14]), produced in late exponential growth, is required for the transcription of genes that function in diverse stress-induced processes (12, 45, 46). The product of the *sigB* gene, σ^B (31), functions in the transcription of genes activated in response to physical and chemical stress as well as reductions in intracellular ATP concentrations (1, 5–7, 53, 54, 56). The products of *spoIIGB* and *spoIIAC*, σ^E and σ^F, respectively, are activated to establish the characteristic patterns of mother cell and forespore-specific gene expression at the onset of sporulation (2, 10, 15, 16, 30, 35, 36, 40, 48). Much has been learned in recent years about how the activity of some sigma subunits is regulated, but control is often exerted at many levels of expression, from transcription initiation of the sigma-encoding gene to the turnover of the sigma protein. How this complex regulation is affected remains the subject of intense investigation.

The control of σ^H also appears to be exerted at multiple stages of expression. Transcription initiation of *spo0H* is under the negative control of the transition state regulator AbrB (50, 55), whose synthesis is repressed by the key regulator of late growth processes, Spo0A (52). Phosphorylated Spo0A, the end product of the *spo0*-dependent phosphorelay which is activated by signals that promote sporulation (8, 29), is a transcriptional regulator that can activate sporulation gene expression and repress *abrB* (52), whose product negatively controls genes induced by nutritional stress (38, 41, 43, 50, 51, 58). Spo0H accumulates in late-growth cultures, but its activity and stability also appear to be regulated (3, 21, 26, 28). In nutrient sporulation medium (Difco sporulation medium [DSM]), σ^H-dependent gene expression is induced as cultures enter the stationary phase but remains low in cultures supplemented with high concentrations of glucose and glutamine (DSM-GG) (3, 20, 21, 45), preferred carbon and nitrogen sources of *B. subtilis*. Although mutations in *pts* (phosphoenolpyruvate:sugar phosphotransferase) causing reduced glucose utilization resulted in elevated Spo0H protein levels (21), one report showed evidence that there is no difference in the level of σ^H in cells grown in DSM or DSM-GG (3). The repression resulting from nutritional supplementation was regarded as a form of catabolite control dependent on the synergistic effect of carbon and nitrogen sources (4, 11, 17, 20, 44, 45) and exerted at the level of σ^H activity (3, 20).

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

Strain	Genotype	Source or reference
JH642	*trpC2 pheA1*	J. Hoch
LAB569	*trpC2 pheA1* SPβ*c*2Δ2::Tn*917*::*spoVG-lacZ* Cm^r MLS^r	
LAB2571	*trpC2 pheA1 rpoDP4-lacZ* MLS^r	This study
LAB2572	*trpC2 pheA1 sigB::cat spoVG-lacZ*::pZL207 Cm^r Spc^r	
LAB2523	*trpC2 pheA1 abrB*::pJM5154 *spoVG-lacZ*::pZL207 Cm^r Spc^r	This study
OR172	*trpC2 pheA1 spo0H*::pIS173 Cm^r	O. Resnekov
OR180	*trpC2 pheA1 spo0H*::pIS162 Cm^r	O. Resnekov
ZB212S	*trpC2 pheA1*::pZL207 *spoVG-lacZ* Spc^r	23
LAB691	*trpC2 pheA1* SP*c*β2Δ2::Tn*917*::*rpsD-lacZ* Cm^r	59
LAB2575	*trpC2 pheA1 amyE::citBp23-lacZ* Cm^r	This study
LAB2576	*trpC2 pheA1 abrB*::pJM5154 *amyE::citBp23-lacZ* Cm^r Spc^r	This study
LAB2577	*trpC2 pheA1* Δ*citA* Δ*citZ* SPβ*c*2Δ2::Tn*917*::*spoVG-lacZ* Cm^r Neo^r	This study
LAB2578	*trpC2 pheA1* Δ*citA* Δ*citZ* SPβ*c*2Δ2::Tn*917*::*spoVG42-lacZ* Cm^r Neo^r	This study
LAB2579	*trpC2 pheA1 amyE::citBp23*::*lacZ*::pJL62 Spc^r	This study
LAB2580	*trpC2 pheA1 citZ-lacZ* Cm^r	This study
ZB456	*trpC2 pheA1* SPβ*c*2Δ2::Tn*917*::*spoVG42-lacZ* Cm^r MLS^r	57
JH12586	Δ*abrB* Cm^r	41
KH287	*rpoDP4-lacZ*	27
SMY::pAF23	*amyE*::*citBp23-lacZ*	18
SJB49	*citZ-lacZ* (Cm^r) *trpC2 pheA1*	33
SJB67	Δ*citA* Δ*citZ*	32

In this report, we describe experiments which were conducted to examine the expression of several σ^H-dependent *lacZ* gene fusions in wild-type and mutant cells grown in DSM-GG. Evidence is presented showing that the low-growth medium pH of DSM-GG cultures is primarily responsible for the reduced σ^H-dependent gene expression. Furthermore, the maintenance of low pH in late-growth cultures is dependent on AbrB. Evidence is also presented that high σ^H-dependent gene expression requires an active tricarboxylic acid (TCA) cycle and that the level of σ^H protein is significantly increased when the pH of DSM-GG culture is raised by the addition of pH stabilizer.

MATERIALS AND METHODS

Bacterial strains. The *B. subtilis* strains used in this study are listed in Table 1. All strains are derivatives of JH642. LAB2523 was constructed by transforming ZB212S (59) with JH12586 DNA with selection for chloramphenicol resistance (Cm^r); The *rpoD*P4-*lacZ* fusion was obtained from A. Grossman (27). LAB2571 was constructed by transforming JH642 with KH287 DNA and selecting for MLS^r. Strain AG1332 (31a) bearing a *lacZ* fusion with the Ps (*spo0H*-dependent) promoter of the *spo0A* gene was obtained from A. Grossman. The fusion contains sequences from −134 to +1 of the *spo0A*Ps promoter in the *lacZ* fusion vector pKS2 (37), which allows integration of *lacZ* fusions into the *amyE* locus of the *B. subtilis* genome. LAB2575 was constructed by transforming JH642 with SMY::pAF23 (19) DNA and selecting for Cm^r. LAB2580 was constructed by transforming competent cells of JH642 with chromosomal DNA from strain SJB49 (33), which bears a *citZ-lacZ* transcriptional fusion at the *citZ* gene (*citZ* is not disrupted). LAB2577 and LAB2578 were constructed by transduction of SJB67 (*citZ citA*) (32) with SPβ*c*2Δ2::Tn*917*::*spoVG-lacZ* and SPβ*c*2Δ2::Tn*917*::*spoVG42-lacZ* (59), respectively. LAB2575 was transformed with *Eco*RI-linearized pJL62 and selection for Spc^r and screening for Cm^s to make LAB2579. LAB2579 was then transformed with JH12586 (41) DNA to construct LAB2576.

Strain OR172 is a JH642 derivative containing plasmid pIS173, which had recombined by Campbell recombination at the *spo0H* locus. pIS173 (55) contains a *spo0H::lacZ* translational fusion where the *lacZ* coding sequence is fused to codon 198 of *spo0H*. OR180 is also a JH642 derivative that contains plasmid pIS162 (55), also integrated at the *spo0H* locus. pIS162 bears a *spo0H::lacZ* fusion at codon 80 of *spo0H.*

Transformation and transduction. Competent *B. subtilis* cells were prepared and transformed by the method previously described (13). Transduction with specialized SPβ was done according to Zuber and Losick (58).

Culture medium. DSM was routinely used for cultivating *B. subtilis*, and Luria-Bertani medium (39) was used for growing cultures of *Escherichia coli*. Liquid 2× YT medium (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter) was used to grow cultures for plasmid and chromosomal DNA preparation. To make DSM-GG, sterile 50% glucose and 2.5% glutamine were added to final concentrations of 1.9 and 0.1%, respectively. To make solid medium, 1.2% agar was added. Minimal medium contained 20 mM K_2HPO_4 with the same concentrations of glucose, glutamine, and salts as DSM-GG. We added 0.2% Casamino Acids as indicated and adjusted to the minimal medium to a pH of 6.9. UBSS (unbuffered salt solution) contains the same ingredients as TSS (Tris-salts solution) (19) (0.36 g of K_2HPO_4 per liter, 10 ml of 1.2% $MgSO_4$ per liter, 10 ml of 0.4% $FeCl_3$-citrate per liter, 40 ml of 50% glucose per liter, 80 ml of 0.2% glutamine per liter), but Tris-HCl was omitted. UBSSCA is unbuffered salt solution containing 0.2% Casamino Acids.

Culture growth and β-galactosidase assay. All inocula except for LAB2577 and LAB2578 were grown on solid DSM at 30°C overnight with the appropriate antibiotics. For LAB2577 and LAB2578, frozen stocks were first streaked onto Luria-Bertani plates containing chloramphenicol and neomycin and incubated at 37°C overnight. Then a single colony was picked, streaked onto the same medium, and incubated at 30°C overnight. The cells were harvested by washing the plate surface with 1.0 ml of DSM, and broth media were inoculated to an initial absorbance at 595 nm (A_{595}) of about 0.17. Cultures were grown in baffled sidearm flasks (MRA, Clearwater, Fla.) at 37°C in a shaking water bath at approximately 360 rpm. When the culture A_{595} reached about 0.46, collection of 1.0-ml samples was started and continued at 30-min intervals for assays of β-galactosidase activity (42, 59). The samples were centrifuged for 5 min in a tabletop microcentrifuge, and supernatants were collected for pH measurement. The cell pellets were stored at −80°C until they were analyzed for β-galactosidase activity. In the experiments in Fig. 2, 3, and 5 (with the exception of Fig. 2C), parallel DSM-GG cultures were grown to an A_{595} of approximately 2.0 and then Tris-HCl (pH 7.5) or morpholinepropanesulfonic acid (MOPS) (pH 7.5) was added to one of the cultures to a final concentration of 25 mM. In other experiments, when the DSM-GG cultures reached an A_{595} of approximately 1.9 to 2.0, they were split into equal volumes and 1 M Tris HCl (pH 7.5) or 1 M MOPS (pH 7.5) was added to one of the two cultures to a final concentration of 25 mM to make DSM-GGTris and DSM-GGMOPS, respectively. Alternatively, 20% acetic acid (pH 4.65) was added to a final concentration of 8 mM to make DSM-GG-acetate.

Unbuffered minimal medium (described above) was supplemented with the same concentrations of glucose, glutamine, and salts as DSM-GG. Tryptophan and phenylalanine were also added to 0.01% (19). Casamino Acids (0.2%) was added as indicated, and the media were adjusted to pH 6.9. Inocula were prepared by picking a single colony from a freshly streaked DSM plate, streaking it onto TSS minimal medium, and incubating it at 37°C for 16 to 24 h. A single colony was then picked from this plate, streaked again onto TSS minimal medium, and incubated at 37°C for 16 to 18 h to produce a lawn of growth. The cells were harvested by washing the plate surface with 1.0 ml of phosphate buffer (pH 7.2). Minimal broth medium was then inoculated with this cell suspension to an initial A_{595} of ca. 0.05. Sample collection began when the cultures reached an A_{595} of ca. 0.3 and was continued at hourly intervals as indicated in the figures. MOPS or Tris buffer (25 mM final concentration) was added to the cultures as indicated by arrows.

Protein preparation, gel electrophoresis, and Western immunoblot analysis. Cultures were grown in DSM-GG or DSM-GGTris as described above. Samples were removed, centrifuged at 4°C, washed once in phosphate-buffered saline, centrifuged again, and stored at −80°C. The cell pellets were resuspended in 20 mM Tris-HCl (pH 7.5)–5 mM EDTA–1 mM dithiothreitol and placed at −80°C for at least 2 h. The samples were then thawed and added to an equal volume of glass beads, and the cells were disrupted with a mini-Beadbeater (Bio-Spec Products, Bartlesville, Okla.). The Beadbeater was set at maximum speed for six or seven 1-min pulses performed on each sample with at least a 1-min incubation on ice between pulses. Phenylmethylsulfonyl fluoride (1 mM) was added to the samples immediately before lysis. Cell disruption was determined by phase-contrast microscopy. The glass beads were allowed to settle, and the whole-cell lysate was removed, transferred to a separate tube, and stored at −20°C.

Samples of the whole-cell lysates were diluted in sodium dodecyl sulfate (SDS) sample buffer, boiled for 2 min, and applied to an SDS–15% (for AbrB) or SDS–12% (for Spo0H) polyacrylamide gel. The proteins were electrophoretically transferred to nitrocellulose filters with a Mini Trans-Blot transfer cell (Bio-Rad, Richmond, Calif.). Nonspecific binding of immunoglobulins to blots was blocked by incubation in Tris-buffered saline (TBS)–0.05% Tween 20–1% bovine serum albumin for 2 to 3 h at room temperature. Anti-AbrB was a gift from M. A. Marahiel and was absorbed against an acetone powder suspension of LAB2523 (Δ*abrB*). Anti-σ^H was a gift from R. Losick and was absorbed against a nitrocellulose filter impregnated with a whole-cell lysate of ZB249 (Δ*spo0H*) for 24 h at 4°C. The filters were then incubated with preabsorbed antibodies for 1 h at room temperature, washed thoroughly with TBS, incubated with secondary goat anti-rabbit alkaline phosphatase conjugate, and finally reacted with chomogenic

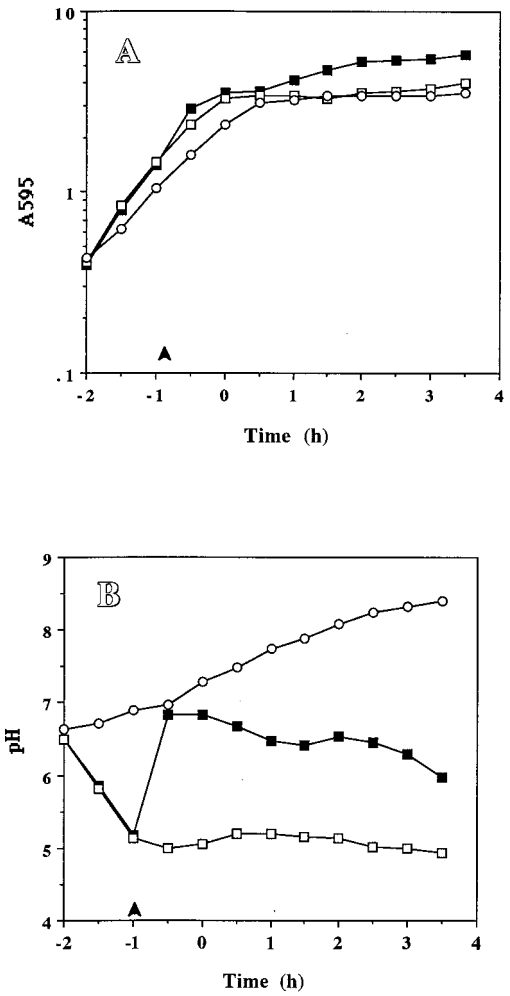


FIG. 1. *B. subtilis* JH642 growth in liquid DSM, DSM-GG, and DSM-GG-Tris. Tris-HCl (pH 7.5) at 1 M was added at an A_{595} of ca. 2.4 to a final concentration of 25 mM (the time of addition is denoted by the arrowhead). The time scale refers to hours before and after $T = 0$, the point marking the transition from the exponential to the stationary phase of growth. (A) Growth measured at A_{595}. (B) Culture pH. ○, DSM; □, DSM-GG; ■, DSM-GGTris.

alkaline phosphatase substrate as recommended by the manufacturer (Gibco/BRL).

RESULTS

Late-growth DSM and DSM-GG cultures show a marked difference in pH. *B. subtilis* JH642 cells, when propagated in DSM, will undergo sporulation when the culture enters the stationary phase of growth. However, when DSM is supplemented with high concentrations of glucose and glutamine (1.0 to 2.0% and 0.1 to 0.2%, respectively), sporulation is delayed; very few spores are detected after overnight growth. This is due primarily to low cell viability observed after overnight incubation (data not shown). A striking difference between parallel stationary-phase DSM and DSM-GG cultures is in the pH. In DSM, amino acids are a prominent source of carbon for energy production. The catabolism of amino acids does not give rise to acidic products characteristic of glucose metabolism (47). On the other hand, cultures of JH642 cells in DSM-GG show a marked decrease in pH, to below 5.0, when they enter stationary phase (Fig. 1B). The decrease in pH is the result of increased production of acidic glycolytic products such as acetate (24, 47). The pH of DSM-GG cultures can be artificially raised by the addition of Tris or MOPS at pH 7.0 to 7.5 to 25 mM at or immediately before T_0 (the time at which exponential growth ends). This causes the pH to remain between 6.0 and 7.0 (Fig. 1B) for several hours. It also causes the cell density (Fig. 1A) and total protein content of the culture (data not shown) to increase by approximately one-third.

Elevating the pH of DSM-GG cultures results in heightened σ^H-dependent gene expression. In previous reports, reduced expression of a *spoVG-lacZ* gene fusion (expression of which is dependent on σ^H) was observed in cells grown in DSM-GG compared to the expression in cells grown in DSM (20, 21). The target of this Ggr (glucose-glutamine-dependent repression) control was exerted at the region of *spoVG* required for the interaction of the σ^H RNA polymerase holoenzyme (20). One possible source of signals that affect σ^H activity and/or synthesis could by the low external pH of DSM-GG cultures. To test this possibility, cells of ZB212S (*spoVG-lacZ*, Spc^r) were grown in DSM and in two parallel DSM-GG cultures. When the DSM-GG cultures reached late-log growth, 1 M Tris (pH 7.0) (Fig. 2A) or 1 M MOPS (pH 7.0) (data not shown) was added to one of the flasks to a final concentration of 25 mM. Approximately 45 min after the addition of the pH stabilizer, *spoVG-lacZ* expression was observed to increase to levels that exceeded those in the sporulating, DSM culture (Fig. 2). The expression of *spoVG-lacZ* in DSM-GGTris was four- to fivefold higher than that observed in the untreated culture. The same induction of *spoVG-lacZ* expression was observed when a single culture of ZB212S in DSM-GG was grown to late log phase and then split into two cultures, one of which was treated with Tris-HCl (pH 7.5).

The expression of other fusions which are transcribed by σ^H RNA polymerase was examined to determine if the effect of pH on gene transcription might be a characteristic of σ^H-dependent transcription. Thus, the expression of *lacZ* fusions to the *rpoD* promoter P4 (Fig. 2B), and the *spo0A*Ps promoter (Fig. 2C), both shown to be used by the σ^H-form of RNA polymerase, was also examined in cells grown in DSM-GG and in DSM-GGTris. The expression of a σ^A-dependent fusion, *rpsD-lacZ* (23), which is not dependent on σ^H, was also examined (Fig. 2D). In cells grown in DSM-GG, the *rpoDP4-lacZ* fusion showed a threefold increase in expression when Tris was added late in exponential growth (Fig. 2B). A five- to sixfold-higher expression was observed in Tris-HCl-treated cells bearing *spo0A*Ps-*lacZ* grown in DSM-GG (Fig. 2C). The expression of *rpsD-lacZ* was examined in cells grown in DSM-GG and DSM-GGTris and in DSM. *rpsD-lacZ* expression was reduced in stationary-phase DSM cultures, but high-level expression was observed in cultures in DSM-GG (Fig. 2D). The addition of Tris to raise the pH did not result in an increase in expression, as was observed for the σ^H-dependent fusions, but caused a gradual decrease to about 60% of the level observed in the untreated DSM-GG culture.

Elevation in culture pH raises the level of σ^H protein. The effect of elevated pH on σ^H-dependent transcription could be exerted at the level of *spo0H* transcription or translation or at the level of σ^H concentration or activity. To begin to test these possibilities, the expression of two *spo0H-lacZ* gene fusions in DSM-GG and in DSM-GGTris was examined. One fusion was constructed by joining the amino-terminal-coding end of *spo0H* to *lacZ*, and the other was constructed by joining the carboxy end of *spo0H* in frame to *lacZ*. Both fusions showed an approximately 30% increase in expression in DSM-GG-grown cells ca. 1.5 to 2 h after Tris addition compared to the untreated cells (Fig. 3). To determine if the pH significantly influenced the level of σ^H protein, immunoblot analysis was

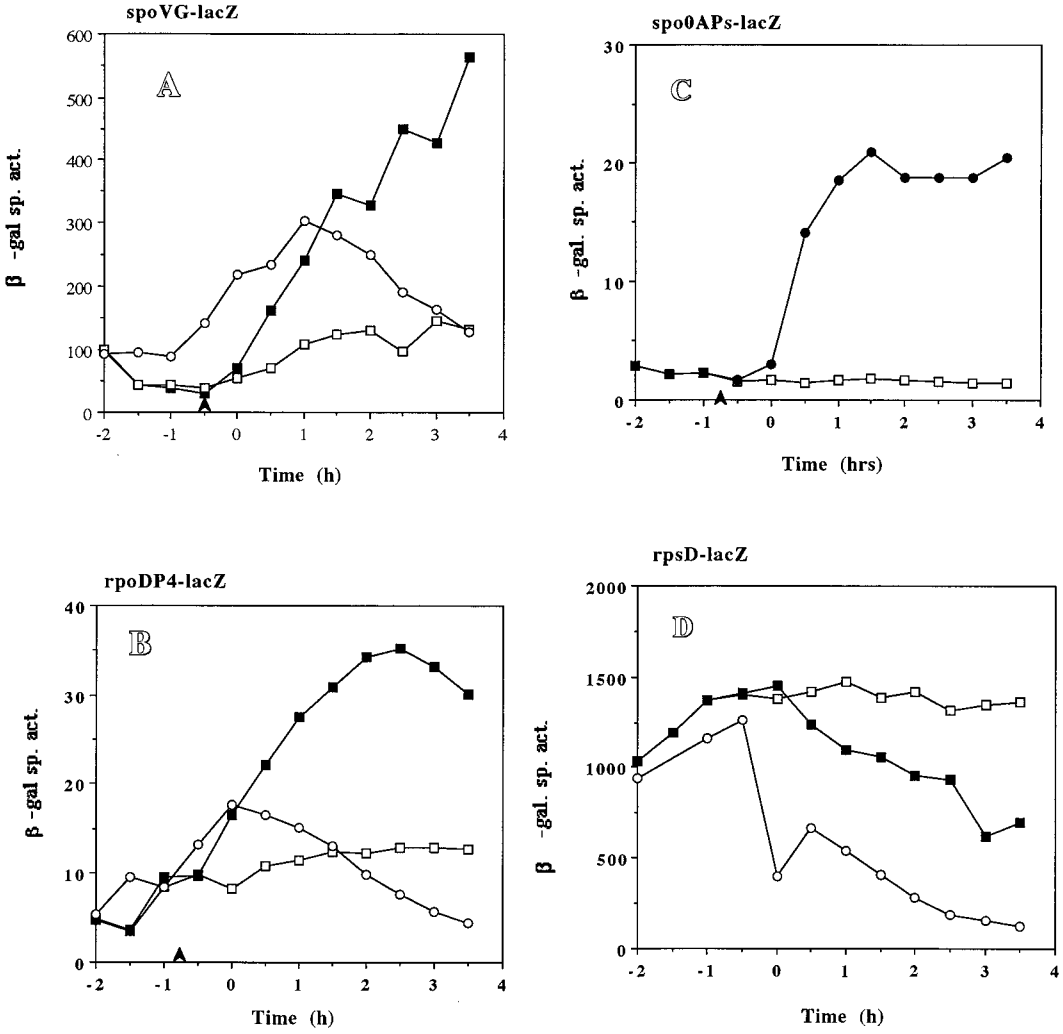


FIG. 2. Expression of σ^H- and σ^A-dependent *lacZ* fusions in JH642 cells grown in DSM, DSM-GG, and DSM-GGTris. (A) *spoVG-lacZ* in DSM (○), DSM-GG, (□), and DSM-GGTris (■). (B) *rpoDP4-lacZ* in DSM (○), DSM-GG (□), and DSM-GGTris (■). (C) *spo0A*Ps-*lacZ* in DSM-GG (□) and DSM-GGTris (●). (D) *rpsD-lacZ* in DSM (○), DSM-GG (□), and DSM-GGTris (■). β-Galactosidase specific activity was measured in samples collected at 0.5-h intervals from cultures grown in the media indicated. The time scale refers to hours before and after $T = 0$, the point marking the transition from the exponential to the stationary phase of the growth curve. The time of Tris-HCl or MOPS addition is denoted by the arrowhead ($T = -1$ in panel D).

performed with anti-σ^H antibody. A significant increase in the σ^H protein level could be detected by Western blot analysis (Fig. 4A), which contradicts a previous report (3). This increase was detected 1 h after Tris addition, at least 1 h before maximum *spo0H-lacZ* expression was observed. This suggests that σ^H protein levels are elevated when pH is raised, but this is only partly due to an increase in *spo0H* transcription and translation.

An *abrB* deletion mutation causes pH- and σ^H-dependent expression to increase. The expression of *spo0H* and some genes that are dependent on σ^H for their transcription is negatively controlled by *abrB* (50, 55), whose product is a repressor of many genes that are induced in cells of late-growth cultures (49). The possibility that pH-dependent control operates through *abrB* was examined. A culture of LAB2523 (*spoVG-lacZ* Δ*abrB*) cells was grown in DSM-GG until late log phase and then divided; half was treated with Tris HCl, and the other was untreated. The expression of *spoVG-lacZ* in the Tris-treated culture was high as cells entered the stationary phase, but an increase in fusion expression was also observed in the untreated culture (Fig. 5). An examination of DSM-GG culture pH over time revealed that this, too, increased from a low of 5.1 to a high of 6.6 as the culture proceeded into the stationary phase (Fig. 5B). Correspondingly, the cell density rose to a level similar to that observed in DSM-GGTris cultures (data not shown). The addition of acetic acid to reduce the pH of the *abrB* cell culture to a level observed in cultures of wild-type *B. subtilis* cells resulted in a reduction in *spoVG-lacZ* activity in the *abrB* mutant background (Fig. 5A). This suggests that AbrB does not participate in pH-dependent control but causes repression of σ^H-dependent gene expression in cells grown in DSM-GG by maintaining the pH at a low level during the stationary phase.

A reduction in AbrB protein levels is observed when the pH is elevated. It was possible that the addition of a pH stabilizer to a culture that had accumulated acidic glycolytic products not only initially elevated the pH but also affected cellular processes that could further influence growth medium pH. These other effects could contribute to high pH levels during prolonged incubation in DSM-GG after Tris addition. Increased

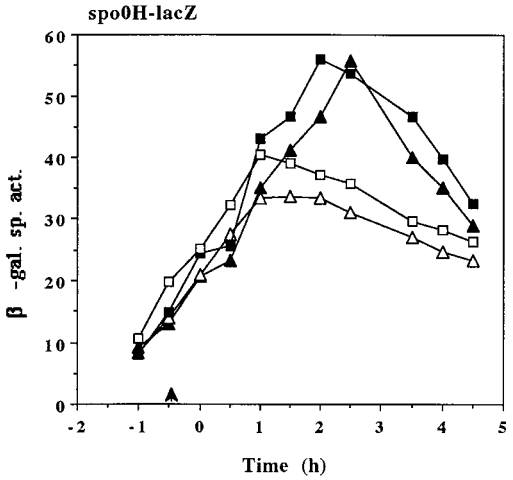


FIG. 3. Expression of *spo0H-lacZ* gene fusions in JH642 cells grown in DSM-GG and DSM-GGTris. Cells bearing a translational fusion of the 5′ end of the *spo0H* coding sequence (from strain OR172) or the 3′ end (from strain OR180) were assayed for β-galactosidase specific activity. Refer to the legend to Fig. 2 for an explanation of the sample collection and time scale. The time of Tris-HCl addition is denoted by the arrowhead. 5′ *spo0H-lacZ*, DSM-GG (□) and DSM-GGTris (■); 3′ *spo0H-lacZ*, DSM-GG (△) and DSM-GGTris (▲).

σ^H levels would result in higher *spo0A* expression and thus in higher concentrations of Spo0A. Since Spo0A is a repressor of *abrB*, lower *abrB* expression would be expected, which would favor an increase in pH. In fact, a decrease in *abrB* expression was observed when the pH is raised. The level of AbrB protein was examined in DSM-GG and DSM-GGTris cultures by immunoblot analysis with anti-AbrB antiserum (provided by M. A. Marahiel). Figure 4B shows that AbrB protein levels remained high in DSM-GG cultures but substantially decreased in DSM-GGTris cultures. The decrease in AbrB protein levels began 2 to 3 h after Tris addition, well after the increase in σ^H protein was observed (Fig. 4A). Therefore the increase in σ^H protein concentration is not likely due to reduced AbrB levels, which would lead to *spo0H* derepression.

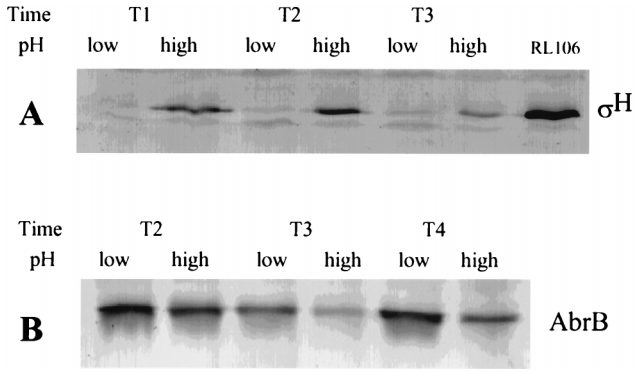


FIG. 4. Western immunoblot filters of AbrB and σ^H from whole-cell extracts of JH642 cells grown in DSM-GG and DSM-GGTris. Time points T1, T2, etc., refer to hours after Tris-HCl addition. (A) σ^H (16 μg of protein per lane). Lanes labeled "low" contain samples from a DSM-GG culture harvested at T1, T2, and T3. Lanes labeled "high" contain samples from a DSM-GGTris culture harvested at the same time points. The right-hand lane contains *B. subtilis* RL106, which carries *spo0H* on the plasmid pJOH25 and overproduces σ^H. (B) AbrB (15 μg of protein per lane). Lanes labeled "low" contain samples from a DSM-GG culture harvested at T2, T3, and T4, respectively. Lanes labeled "high" contain samples from a DSM-GGTris culture harvested at the same time points.

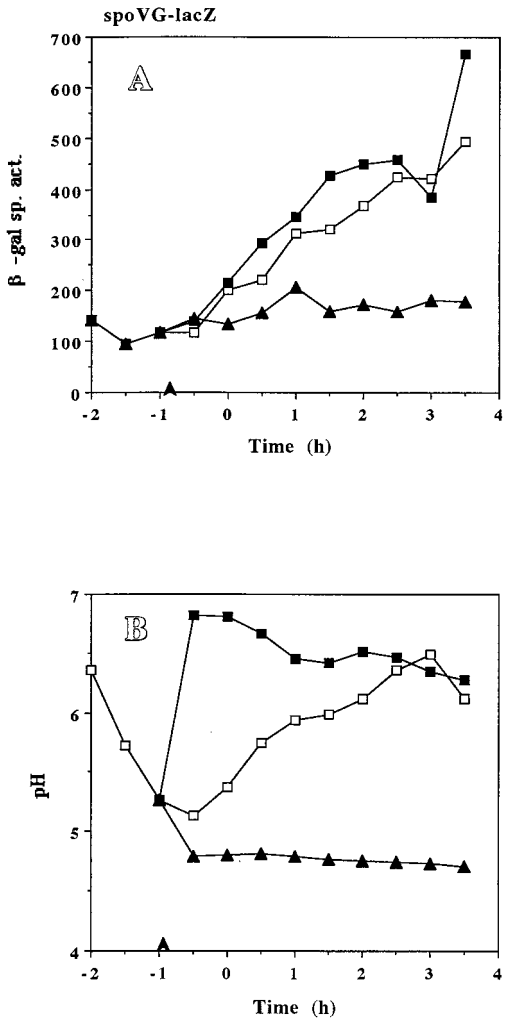


FIG. 5. *spoVG-lacZ* activity in LAB2523 (Δ*abrB*). Cultures were grown in DSM-GG and DSM-GGTris as in Fig. 1. One culture was grown in DSM-GG to an A_{595} of 2.4, and then 20% acetic acid (pH 4.65) was added to a final concentration of 8 mM. Refer to the legend to Fig. 2 for an explanation of the time scate and sample collection. (A) β-Galactosidase specific activity; (B) culture pH. □, DSM-GG; ■, DSM-GGTris; ▲, DSM-GG plus acetate.

The maintenance of high pH upon Tris-HCl addition might be due to reduced expression of *abrB*.

The genes encoding TCA cycle enzymes are required for pH-dependent induction of *spoVG-lacZ*. The mechanism of the pH increase in an *abrB* mutant could be the utilization of acetate facilitated by the enzyme acetyl coenzyme A synthetase (acetyl-CoA synthetase), which catalyzes the formation of acetyl-CoA from acetate. The acetyl-CoA thus generated could then enter the TCA cycle. Citrate would be formed from oxalacetate and acetyl-CoA in a reaction catalyzed by citrate synthase, and this would cause the induction of *citB*, the gene encoding the second enzyme of the TCA cycle, aconitase (18). To determine if the TCA cycle contributes to the pH-dependent control of σ^H activity, the expression of a σ^H-dependent gene fusion, *spoVG42-lacZ*, was examined in a TCA cycle mutant. The *spoVG42* mutation rendered transcription of *spoVG* independent of AbrB control. Therefore, any changes observed in the expression of *spoVG42-lacZ* is likely to be the result of altered *spo0H* expression or σ^H levels and/or activity.

The expression of *spoVG42-lacZ* was examined in a strain

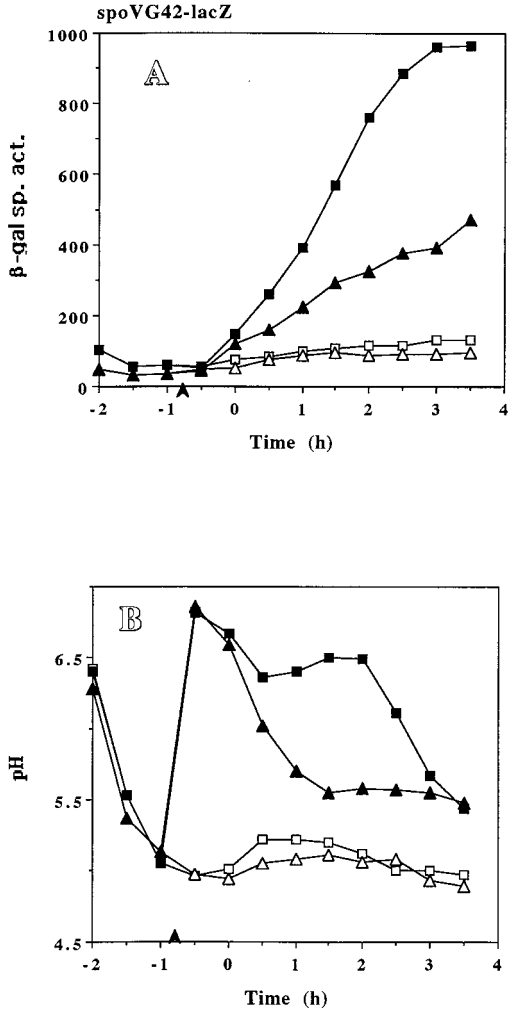


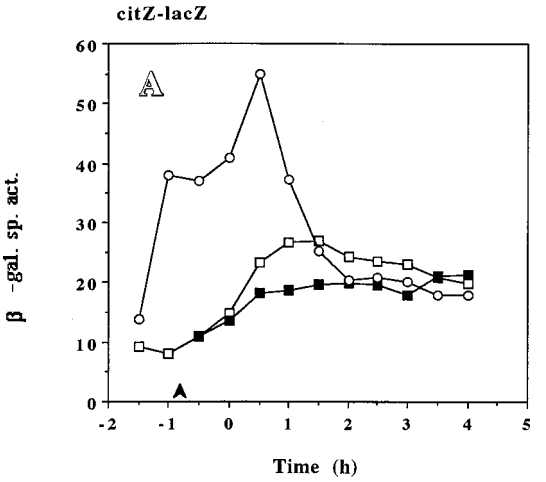
FIG. 6. Effect of a TCA cycle mutant on the expression of *spoVG42-lacZ* (a mutant *spoVG* promoter that is not subject to AbrB repression). ZB456 (Spβ*c2*Δ*2*::Tn*917*::*spoVG42-lacZ*) and LAB2578 (Δ*citZ* Δ*citA* SPβ*c2*Δ*2*::Tn*917*::*spoVG42-lacZ*) were grown in DSM-GG or DSM-GGTris. Refer to the legend to Fig. 2 for an explanation of sample collection and time scale. The time of Tris-HCl addition is denoted by the arrowhead. (A) β-Galactosidase specific activity. (B) pH of cultures. ZB456, DSM-GG (□) and DSM-GGTris (■); LAB2578, DSM-GG (△) and DSM-GGTris (▲).

(LAB2568) bearing mutations in *citA* and *citZ* (32), encoding the two forms of citrate synthase made in vegetatively growing cells. Cells of strain LAB2568 (*citA citZ*) and ZB456 (*cit*$^+$) were grown in DSM and DSM-GG cultures. At the end of exponential growth, the DSM-GG culture was divided as described above, and one of the cultures was treated with Tris-HCl (pH 7.5). Figure 6 shows the effect of the *citA* and *citZ* mutations on *spoVG42* expression (Fig. 6A) and culture pH (Fig. 6B). The culture pH in DSM-GG is low, as is the case for the wild-type culture, but the addition of Tris resulted in only a transient increase in pH. The culture pH began to decrease about 1 h after Tris addition and continued decreasing until it nearly equaled that observed in the untreated DSM-GG culture. The expression of *spoVG42-lacZ* was higher in DSM-GGTris than in DSM-GG in both the wild-type and the *cit* double-mutant backgrounds, but the extent of the increase was smaller in the mutant cells. There was a six- to ninefold increase in *spoVG42-lacZ* expression in the wild-type cells after Tris addition, in contrast to the *cit* mutant cells, which exhibited a three- to fourfold increase. These data suggests that there is a requirement for TCA cycle activity to maintain an elevated pH following Tris addition, resulting in heightened σ^H activity. This could be brought about by the utilization of acetate carbon through the TCA cycle or perhaps some other TCA cycle-dependent activity mobilized to cope with acid stress.

Elevated pH causes an increase in *citB-lacZ* expression in DSM-GG. Because the TCA cycle is necessary for the optimal expression of *spoVG42-lacZ* in DSM-GGTris, the possibility that TCA cycle enzyme gene expression is also induced by elevated pH was explored. The expression of the genes *citZ*, encoding the major citrate synthetase of *B. subtilis*, and *citB*, encoding aconitase, was examined by using *lacZ* fusion constructs (obtained from A. L. Sonenshein [see Materials and Methods]). Cells of strain LAB2575 (*amyE::citBp23-lacZ*) and strain LAB2580 (*citZ-lacZ*) were propagated in two cultures of DSM-GG and one culture of DSM. Prior to T_0, one of the DSM-GG cultures was divided in half, and Tris-HCl (pH 7.5) was added to one half. Examination of *citZ*- and *citB*-directed β-galactosidase activity revealed that both fusions were expressed in the later stages of the exponential phase in DSM cultures but were repressed during growth in DSM-GG (see Fig. 8). Addition of Tris caused little change in *citZ-lacZ* activity, which was observed to increase slightly in both DSM-GG and DSM-GGTris cultures upon entry into the stationary phase (Fig. 7A). Very little *citB*-directed β-galactosidase activity was detected in the stationary-phase DSM-GG culture. However, the DSM-GGTris culture showed an 8- to 10-fold increase in β-galactosidase activity (Fig. 7B). These data suggest that raising the pH relieves glucose-glutamine-dependent repression of *citB* expression.

It was observed that raising the pH reduced the expression of *abrB-lacZ* (data not shown) and resulted in lower levels of AbrB protein. If *citB* is negatively controlled by *abrB*, it is possible that this repression was relieved by raising the pH and thereby reducing *abrB* expression. We examined the possibility that an *abrB* mutation relieves the apparent repression of *citB* in untreated DSM-GG cultures (low pH). Cells of strains LAB2575 (*citBp23-lacZ*) and LAB2576 (*citBp-lacZ* Δ*abrB*) were grown in duplicate DSM-GG cultures and a DSM culture, and then one of the DSM-GG cultures was treated with Tris-HCl (pH 7.5) as described above. Examination of *citB*-directed β-galactosidase activity revealed that the pH-dependent reduction in *citB-lacZ* expression was partially relieved in an *abrB* mutant, as evidenced by the fourfold increase in activity of the *abrB* mutant compared to the *abrB*$^+$ parent (Fig. 7C). Treatment with Tris did not further increase the *citB-lacZ* expression in the *abrB* mutant (data not shown). This suggests that raising the pH relieves *abrB*-dependent repression of *citB* transcription.

The pH of a minimal medium affects σ^H-dependent gene expression, but addition of amino acids to minimal medium affects *spo0H* expression. DSM is a complex medium, and it is possible that reduced pH is affecting *spo0H* activity and expression quite indirectly by altering the composition or effective concentration of some component within the medium. An attempt was made, therefore, to examine the effect of pH on σ^H activity in a minimal medium. Because the minimal media that are often used for growth of *B. subtilis* are based on a pH stabilizer such as Tris or phosphate, an unbuffered version of the minimal medium TSS (called here UBSS) was used (see Materials and Methods) to create the conditions of low pH observed in late-growth cultures is DSM-GG. Casamino Acids were added to 0.2% in an attempt to approximate the medium



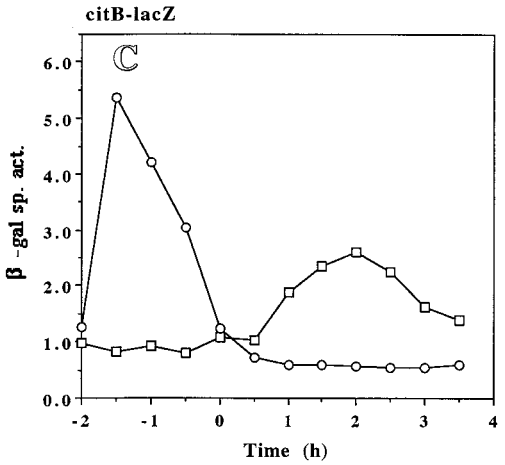
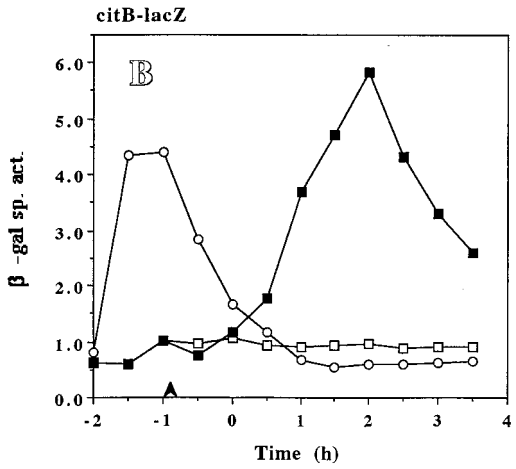


FIG. 7. Effect of pH on TCA cycle enzyme gene expression. JH642 cells containing *amyE*::*citBp23-lacZ* (LAB2579), *citZ-lacZ* (LAB2580), and *amyE*::*citBp23-lacZ abrB* were grown in DSM, DSM-GG, and DSM-GGTris.Refer to the legend to Fig. 2 for an explanation of sample collection and time scale. In panels A and B, the time of Tris-HCl addition is denoted by the arrowhead. (A) LAB2580, (B) LAB2579, DSM (○), DSMGG (□), and DSMGGTris (■). (C) LAB2576, DSM (○) and DSMGG (□).



conditions that exist in DSM-GG cultures. In these experiments, the expression of *spoVG-lacZ*, *spoVG42-lacZ*, and *spo0H-lacZ* (both *spo0H-lacZ* translational fusions) was examined in UBSS with and without 0.2% Casamino Acids (Fig. 8). The pH of the UBSS culture decreased from a starting value of 6.9 to 5.2 (Fig. 8B), but the UBSS culture containing Casamino Acids showed a further decrease to 4.3. The addition of MOPS to 25 mM before the beginning of the stationary phase resulted in an increase in the pH to 7.3 in UBSS and 6.6 in UBSS plus Casamino Acids (Fig. 8B). The expression of *spoVG-lacZ* and *spoVG42-lacZ* in cells grown in UBSS remained high in medium treated with MOPS (pH 7.5) before the start of the stationary phase, while the UBSS culture without MOPS showed a reduction in the expression of both fusions as the pH decreased (Fig. 8A). A similar experiment was performed with UBSS, this time adding MOPS (pH 7.5) to the UBSS culture of *spoVG-lacZ*-bearing cells. Again, the addition of MOPS resulted in a two- to threefold increase in *spoVG-lacZ* expression. However, the fusion was severely repressed when Casamino Acids were present in the medium, with expression levels below that observed in UBSS without added MOPS (Fig. 8C). The addition of MOPS to UBSS plus Casamino Acids had little effect on the level of *spoVG-lacZ* expression. This suggested that there are two mechanisms of *spoVG* regulation, a pH-dependent mechanism and an amino acid-dependent mechanism.

The effect on *spoVG* expression could be exerted at the level of *spo0H* expression or at the level of σ^H levels and activity. To begin to examine the level at which pH and Casamino Acids exert their effect, the expression of a *spo0H-lacZ* fusion (from OR180) was measured in UBSS- and UBSS-plus-Casamino-Acids-grown cells. The expression of the fusion showed no change when the culture was grown in UBSS and subsequently treated with Tris (Fig. 8D). However, substantially lower *spo0H-lacZ* activity was observed in cells grown in UBSS plus Casamino Acids, and the addition of Tris did not raise the level of expression (Fig. 8D). That the expression of *spo0H* appeared not to be influenced by culture pH while *spoVG-lacZ* expression showed marked pH dependence suggests that σ^H activity or stability is influenced by pH. The reduced *spoVG-lacZ* expression in UBSS plus Casamino Acids can be explained by the repression of *spo0H-lacZ*, which appears to be due to an amino acid-dependent mechanism.

DISCUSSION

The data presented above can be summarized as follows. (i) The transcription of *spo0H*-controlled genes is maintained at a low level due to the low pH of cultures grown in DSM-GG. Raising the pH by the addition of a pH stabilizer results in elevated transcription of *spo0H*-controlled genes. (ii) The levels of σ^H increase as the pH is raised, but this is only partly due to an increase in *spo0H* transcription and translation. (iii) *abrB* is required to maintain the pH at a low level in stationary-phase DSM-GG cultures. (iv) The AbrB concentration is reduced when the pH is adjusted to neutrality, but the reduction in protein level is observed after the increase in σ^H concentration. (v) Enzymes of the TCA cycle are required to maintain high pH levels after addition of Tris-HCl to the stationary-phase DSM-GG cultures. The expression of the *citB* gene is sensitive to changes in medium pH; low expression levels are observed at low pH, but this apparent repression is partially relieved by mutations in *abrB*. (vi) The expression of *spo0H* is

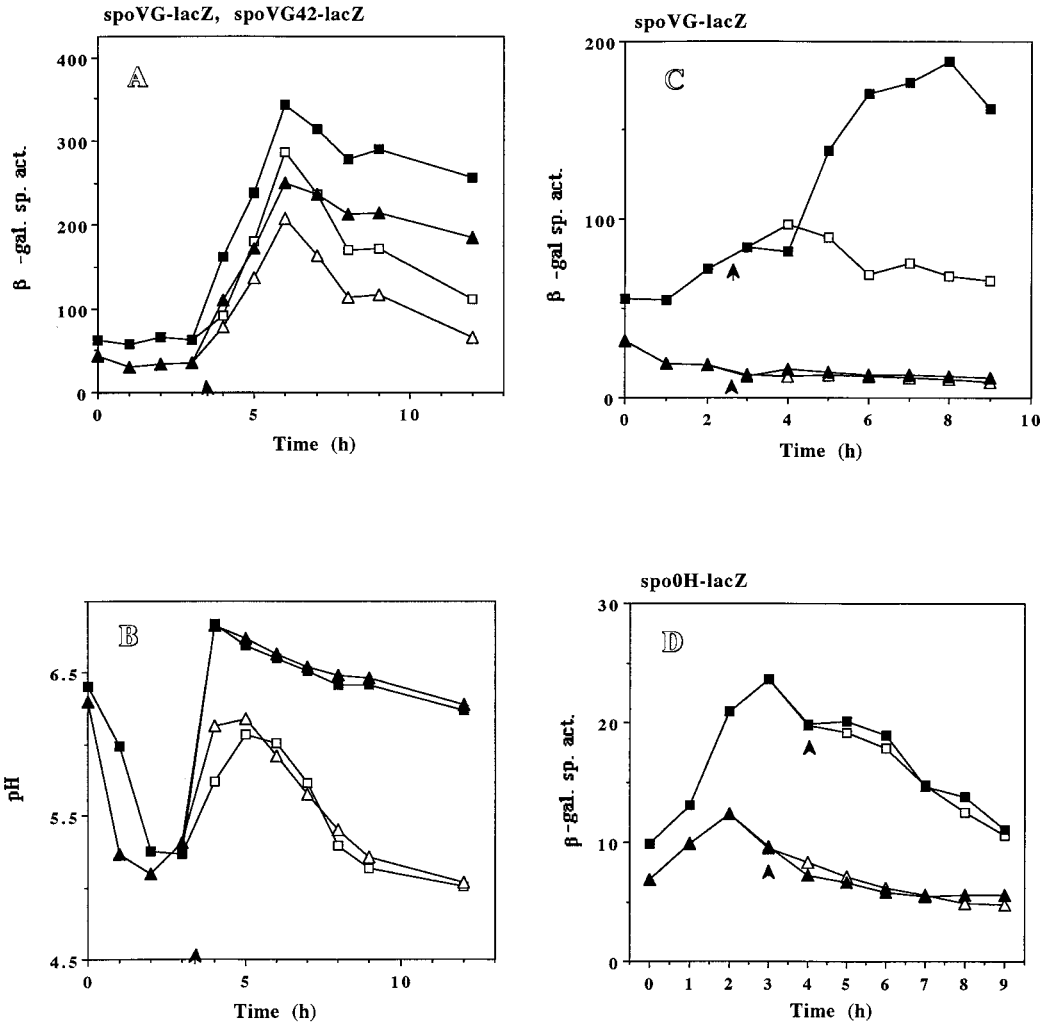


FIG. 8. Effect of pH and amino acids on expression of *spoVG-lacZ*, *spoVG42-lacZ*, and *spo0H-lacZ* in cells grown in UBSS. Samples were collected at hourly intervals, and the β-galactosidase specific activity was determined. (A) ZB212S *spoVG-lacZ*, UBSS (△), UBSS plus MOPS (▲); ZB456 *spoVG42-lacZ*, UBSS (□) and UBSS plus MOPS (■). (B) pH of culture. Symbols correspond to those in panel A. (C) ZB212S *spoVG-lacZ*, UBSS (□), UBSS plus MOPS (■), UBSS plus Casamino Acids, and (△), UBSS plus Casamino Acids plus MOPS (▲). (D) 3′ *spo0H-lacZ* UBSS (□), UBSS plus MOPS (■), UBSS plus Casamino Acids, and (△), UBSS plus Casamino Acids plus MOPS (▲).

only modestly affected by changes in the pH of DSM-GG cultures and little affected in unbuffered minimal medium. However, the addition of Casamino Acids to the minimal medium causes a severe reduction in *spo0H-lacZ* expression which is not relieved by the addition of Tris or MOPS. Therefore, the level of σ^H protein is sensitive to changes in culture pH and the expression of *spo0H* is reduced when amino acids are present in the growth medium. How the presence of amino acids causes the repression of *spo0H* is unknown, but *abrB* does not appear to be involved (data not shown).

It is known that the cultivation of *B. subtilis* cells in the presence of excess glucose results in acetate accumulation and reduced TCA cycle activity (47). Acetate production might be more pronounced if glutamine is also present, since this results in further reduced TCA cycle activity, possibly because of the reduced need for α-ketoglutarate, a necessary precursor of glutamine synthesis. In highly aerated cultures, the pH at T_0 is less than 5.0. It is possible that this results in a reduction in the cytoplasmic pH due to the diffusion of protonated acetate and subsequent ionization within the cell. Such changes might be modest since the cytoplasm of *B. subtilis* is reported to possess a higher buffering capacity that of other bacteria (34). Normally, the diffusion of acetate would be expected to induce TCA cycle activity, but this is not observed in DSM-GG, as shown by the low levels of *citB* expression.

If Tris or MOPS (pH 7.5) is added to DSM-GG, there is a sharp increase in culture pH followed by a gradual reduction to ca. 6.5. Raising the pH causes reduced *abrB* expression, which would lead to enhanced expression of *citB*, encoding the TCA cycle enzyme aconitase. This results in accelerated acetate utilization or in the mobilization of some other TCA cycle-dependent activity and maintenance of the high pH level. A culture of a *citZ citA* double mutant, defective in the production of citrate synthase and thus of citrate, the inducer of *citB*, does not exhibit a prolonged period of high pH following Tris-HCl addition, highlighting the need for an active TCA cycle to maintain the pH near neutrality after pH elevation.

The increase in pH as a result of the addition of pH stabilizer leads to an increase in σ^H levels within 1 h. This is probably due to enhanced protein stability rather than to in-

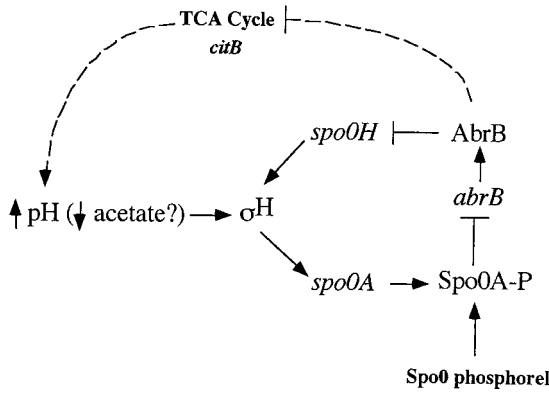


FIG. 9. Possible effects of pH on σ^H in *B. subtilis*. Changes in pH affect the level of σ^H protein [↑pH (↓ acetate?)→σ^H]. Active σ^H results in enhanced *spo0A* transcription and an increase in Spo0A-phosphate (σ^H→*spo0A*→Spo0A-P), leading to repression of *abrB* (Spo0A-P—| *abrB*). AbrB causes repression of *spo0H* (AbrB—| *spo0H*) and *citB* (AbrB----| TCA Cycle, *citB*) and reduced TCA cycle activity, favoring low external pH. Elevated TCA cycle activity tends to increase culture pH (TCA Cycle ---> pH).

creased *spo0H* transcription and translation. An examination of *spo0H-lacZ* expression shows that *spo0H* is expressed at a higher level in DSM-GGTris, but not before there is an observed elevation in σ^H protein. The increase in σ^H stability would, in turn, cause heightened transcription of *spo0A*, whose product would repress *abrB*, thus completing a regulatory loop (Fig. 9). Reduced AbrB protein levels, occurring well after the increase in σ^H levels, would result in higher *spo0H* transcription, possibly accounting for part of the observed increase in *spo0H-lacZ* expression.

It had been reported that glucose and glutamine repress genes that are induced when a culture approaches the stationary phase (11, 17, 20, 21, 45). It was thought that this was a form of catabolite-dependent control resulting from the synergistic effect of these preferred carbon and nitrogen sources. From the study reported herein, it now appears that the reduction of culture pH, perhaps by the accumulation of acetate, is primarily responsible for the repression of genes dependent on *spo0H* (σ^H) for their expression. This reduction in expression can be explained by the reduced concentration of σ^H. It is not known how a low pH causes the reduction in σ^H levels. The *sigB* regulon is activated in response to low pH (6, 54), but a *sigB* mutation has no effect on expression of σ^H-controlled genes at low or neutral pH.

For years, it was thought that sporulation and the expression of sporulation genes were under glucose catabolite repression. Whereas this might be true in exponentially growing cultures, it has not been shown for cultures that have entered the stationary phase and reached a high cell density. Nutritional signals play a role in the initiation of sporulation, as do signals derived from culture cell density and chromosomal DNA synthesis. Perhaps the external pH is yet another condition from which signals arise to become integrated through the signal transduction network governing prokaryotic development.

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