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Role of Sigma H in Expression of the Fumarase Gene (*citG*) in Vegetative Cells of *Bacillus subtilis* 168

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The fumarase gene (*citG*) of *Bacillus subtilis* is transcribed from two promoter regions, *citGp1* and *citGp2* (P1 and P2); the P2 promoter is used by the $E\sigma^H$ form of RNA polymerase. In order to study the role of P1 and P2 in *citG* expression, the promoter region and various deletion derivatives that effectively separate P1 and P2 were fused to the *Escherichia coli* β-galactosidase gene (*lacZ*) and introduced into the chromosome in single copy at the *amyE* locus. P1 functioned to provide a relatively low and stable basal level of fumarase activity throughout growth. In contrast, P2 activity was found to vary over at least a 50-fold range and was responsible for regulating fumarase activity during growth and sporulation in a rich medium and in response to changes in carbon source. To further investigate the role of sigma H in fumarase regulation, *citGp2-lacZ* fusions were introduced into a strain in which the expression of the chromosomal *spo0H* gene was under the control of the isopropylthiogalactopyranoside-inducible *spac* promoter. Induction of p_{spac} did not lead to P2 induction, suggesting that *citG* expression is not regulated at the level of *spo0H* transcription.

The tricarboxylic acid (TCA) cycle in *Bacillus subtilis* plays a dual role in metabolism, providing both energy (when glycolysis is unable to fulfill the cell's energy requirements) and biosynthetic intermediates. The cycle can be regarded as two pathways: the tricarboxylic pathway (citrate to 2-ketoglutarate) and the dicarboxylic pathway (succinyl coenzyme A to oxaloacetate). These pathways are subject to independent but overlapping regulation (15, 27). Levels of fumarase, a component of the dicarboxylic acid pathway, increase during vegetative growth in a rich medium to peak between 1 and 2 h after the end of exponential growth (9). Levels are highest in a lactate minimal medium (when both anabolic and energy-generating functions of the cycle are of prime importance) and are depressed by rapidly metabolizable carbon sources such as glucose and casamino acids (15).

The fumarase gene (*citG*) has been cloned and sequenced (22, 24, 25), and it has been shown to be transcribed from two promoter regions, *citGp1* (P1) and *citGp2* (P2); *citGp1* appears to be a complex of two overlapping promoters, P1a and P1b (9). *citGp1* and *citGp2* are separated by a short open reading frame (*orfA*), which has the potential to encode a small (61-residue) polypeptide. Transcription from *citGp2* is dependent on a functional *spo0H* gene, with the consequence that *spo0H* is required for efficient fumarase expression (9). *spo0H* encodes an alternative sigma factor (sigma H), which together with the RNA polymerase core enzyme directs transcription from *citGp2* (32). Thus, the analysis of the transcriptional control of fumarase in *B. subtilis* is complicated by the presence of multiple promoters and the involvement of differentially regulated multiple holoenzyme forms of RNA polymerase.

To tackle this problem, fusions of separated *citGp1* and *citGp2* promoters to the *Escherichia coli lacZ* gene were constructed, so that analysis of their individual contributions to fumarase expression under different growth conditions and in different mutant backgrounds could be made. The significance of *orfA* in fumarase expression was also investigated, and the role of changes in *spo0H* transcription in regulating transcription from *citGp2* is examined.

MATERIALS AND METHODS

Bacterial strains and media. Table 1 lists the *B. subtilis* strains used in this study. Two rich media were used for growth of *B. subtilis* strains: nutrient sporulation medium (NSMP) (11) and nutrient broth (NB; 1% peptone [Oxoid], 0.5% lab lemco [Oxoid], 0.1% yeast extract [Difco], and 0.9% NaCl). The minimal salts medium was that of Anagnostopoulos and Spizizen (3) supplemented with any required amino acids and the carbon sources referred to in the text. Routine subculture of strains was done on Oxoid nutrient agar, containing antibiotics as appropriate. *E. coli* DH1 (13) was used as the host for plasmid constructions except when unmethylated plasmid DNA was required. In this case, GM242 was used (*dam-3 recA1 sin-2 thr-1 leuB6 proA2 his-4 metB1 lacY1 galK1 ara-14 tsx-33 phi-1 deoB6 supE44 rpsL260*). *E. coli* strains were grown on L broth or agar (23) containing appropriate antibiotics. Colonies of both *E. coli* and *B. subtilis* were tested for β-galactosidase expression on plates containing 100 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal) per ml. Amylase production in *B. subtilis* was tested by growing colonies overnight on a nutrient broth plate containing 1% (wt/vol) starch and then staining with a solution containing 0.5% (wt/vol) iodine and 1.0% (wt/vol) potassium iodide.

DNA manipulations. Methods for endonuclease digestion and DNA ligation were as described by Maniatis et al. (21), except that blunt-ended ligations were performed in low-melting-point agarose in the presence of hexamine cobalt chloride as described by Murray (26). Chromosomal DNA was isolated from *B. subtilis* by using CsCl gradients. Plasmid DNA was isolated from *E. coli* by the rapid boiling method of Holmes and Quigley (17). DNA sequencing was conducted by the method of Sanger et al. (29). Transformation of *E. coli* was carried out by the method of Viesturs Simanis as described by Hanahan (14), except that the RbCl in the transformation buffers was replaced with KCl. *B. subtilis* was made competent and transformed as described previously (3), and congression was carried out with a

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or markers	Reference or source
B. subtilis		
JH642	*trpC2 phe-1*	16
168t⁻	*trpC2*	R. Losick (18)
JH703	*trpC2 phe-1 ΔspoOA204*	J. Hoch
BH19	*trpC2 phe-1 spo0H*::Φ(P*spac-spo0H cat lacI*)	J. Healy
IS233	*trpC2 phe-1 spo0HΔHind*	33
CB100	*trpC2 sigD*::*cat*	Chamberlin
ML6	*trpC2 sigB*(*Hin*dIII-*Eco*RV)::*cat*	18
AM843	*trpC2 phe-1 spo0HΔHind amyE*::Φ(*citG-lacZ cat*)	This study
AM845	*trpC2 amyE*::Φ(*citG-lacZ cat*)	This study
AM846	*trpC2 phe-1 spo0HΔHind amyE*::Φ(Δ*citG94-lacZ cat*)	This study
AM848	*trpC2 amyE*::Φ(Δ*citG94-lacZ cat*)	This study
AM851	*trpC2 amyE*::Φ(Δ*citG96-lacZ cat*)	This study
AM852	*trpC2 phe-1 amyE*::Φ(*citG-lacZ cat*)	This study
AM854	*trpC2 phe-1 amyE*::Φ(Δ*citG94-lacZ cat*)	This study
AM870	*trpC2 amyE*::Φ(Δ*citG101-lacZ cat*)	This study
AM878	*trpC2 amyE*::Φ(Δ*citG88-lacZ cat*)	This study
AM909	*trpC2 phe-1 amyE*::Φ(Δ*citG101-lacZ cat*)	This study
AM910	*trpC2 phe-1 spo0HΔHind amyE*::Φ(Δ*citG101-lacZ cat*)	This study
AM911	*trpC2 phe-1 amyE*::Φ(Δ*citG88-lacZ cat*)	This study
AM912	*trpC phe-1 spo0HΔHind amyE*::Φ(Δ*citG88-lacZ cat*)	This study
AM936	*phe-1 spo0H*::Φ(P*spac-spo0H cat lacI*) *amyE*::Φ(Δ*citG88-lacZ cat*)	This study
AM952	*trpC2 phe-1 spo0HΔHind sigB*(*Hin*dIII-*Eco*RV)::*cat*	This study
AM953	*trpC2 phe-1 spo0HΔHind sigD*::*cat*	This study
AM966	*phe-1 spo0H*::Φ(P*spac-spo0H cat lacI*) *amyE*::Φ(Δ*citG94-lacZ*) *cat*	This study
Plasmids		
pAAM8	Ap^r Tc^r Cm^r	37
pAAM83	Ap^r	This study
pAAM88	Ap^r Δ*citG88* promoter derivative	This study
pAAM90	Ap^r	This study
pAAM91	Ap^r Cm^r *citG* promoter (intact)	This study
pAAM94	Ap^r Cm^r Δ*citG94* promoter derivative	This study
pAAM96	Ap^r Δ*citG96* promoter derivative	This study
pAAM97	Ap^r Cm^r	This study
pAAM101	Ap^r Cm^r Δ*citG101* promoter derivative	This study
pAAM129	Ap^r Cm^r *citG-lacZ*	This study
pAAM131	Ap^r Cm^r Δ*citG94-lacZ*	This study
pAAM133	Ap^r Cm^r Δ*citG96-lacZ*	This study
pAAM135	Ap^r Cm^r Δ*citG101-lacZ*	This study
pAAM137	Ap^r Cm^r Δ*citG88-lacZ*	This study
pAAM138	Ap^r Cm^r Δ*citG88-lacZ*	This study
pAAM144	Tc^r	This study
pAAM145	Tc^r Cm^r	This study
pVAP1	Ap^r Cm^r	This study
pDH32	Ap^r Cm^r	30
pSGMU31	Ap^r Cm^r	8
pMLB1010	Ap^r Cm^r	5
pJH101	Ap^r Tc^r Cm^r	10

saturating concentration (10 μg/ml) of donor DNA. Electrophoresis of DNA was done as described by Maniatis et al. (21). Preparative electrophoresis was done at 4°C with low-melting-point agarose (0.8%; Bethesda Research Laboratories). Phenol extraction of DNA from the agarose was done as recommended by the suppliers. DNA-DNA hybridizations were done as described by Southern (31), with the Boehringer Mannheim nonradioactive DNA labeling and detection kit (catalog no. 1093 657). Restriction enzymes, T4 DNA ligase, and the Klenow fragment of DNA polymerase I were purchased from commercial sources and used as directed by the manufacturers.

Enzyme assays. For assay of β-galactosidase, samples (100 μl to 1.6 ml) of *B. subtilis* cells were harvested by centrifugation for 1 min in an MSE microfuge and stored at −20°C. The frozen cells were suspended in 1 ml of Z buffer (23) and incubated at 37°C for 5 min with 100 μg of lysozyme per ml. After equilibration to 28°C for 5 min, samples were assayed for β-galactosidase activity by the method of Miller (23). Total cellular protein was measured by the method of Bradford (6). β-Galactosidase activity was expressed as nanomoles of orthonitrophenol produced per minute per milligram of protein. Preparation of extracts and fumarase assays were carried out as described previously (9). Fumarase specific activity was expressed as nanomoles of fumarate produced per minute per milligram of protein.

Plasmid constructions. All plasmids used in this study are listed in Table 1.

(i) Deletions of the promoter region. Four different deletions were introduced into the *citG* promoter region (Fig. 1 and 2). Δ*citG96* is a deletion of both P1 and P2 promoters, Δ*citG88* is a deletion of P1, leaving P2 and about 40 base pairs (bp) upstream of the −35 region intact (hereafter referred to as P2′), Δ*citG94* is another deletion of P1 to leave only 10 bp upstream of the P2 −35 region (hereafter referred to as P2″), and Δ*citG101* is a deletion of P2, leaving P1 intact. These deletions were produced in pAAM83, which was created by first deleting the *Sal*I fragment containing pC194 material from pAAM8 (which contains the 5.1-, 0.9-, and 0.8-kilobase [kb] *Eco*RI fragments from lambda BS*citG*II inserted into the unique *Eco*RI site of pHV33 [28, 37]). The Δ*citG88* derivative was generated by removal of the 862-bp *Tha*I-*Eco*RV fragment from pAAM83 to create plasmid pAAM88. The Δ*citG96* and Δ*citG94* derivatives were both created by first digesting pAAM83 with *Cla*I and *Nco*I to generate a large and a small (678-bp) fragment. (i) The small *Cla*I-*Nco*I fragment was digested with *Taq*I, and the resulting fragments were religated with the large fragment to create plasmid pAAM90 (which bears the Δ*citG94* derivative). The 1.6-kb *Eco*RI-*Sal*I fragment of pAAM90 containing Δ*citG94* was then subcloned into *Eco*RI-*Sal*I-cut pJH101 to generate pAAM94. (ii) The small *Cla*I-*Nco*I fragment was digested with *Sau*3A, and the resulting fragments were religated with the large fragment to create plasmid pAAM96 (which bears the Δ*citG96* derivative). The deletion of two *Sau*3A fragments creates a *Bgl*II site at the deletion junction. The Δ*citG101* derivative was created by first subcloning the 1.8-kb *Eco*RI-*Sal*I fragment from pAAM96 into *Eco*RI-*Sal*I-cut pJH101 to create pAAM97 and then inserting the 248-bp *Sau*3A fragment containing *citGp1* into the *Bgl*II site of pAAM97 at the junction of the pAAM96 deletion. In addition, the intact promoter complex was subcloned into *Eco*RI-*Sal*I-cut pJH101 as a 2.2-kb *Eco*RI-*Sal*I fragment from pAAM83 to create pAAM91. All the deletions were confirmed by DNA restriction analysis and, for Δ*citG101*, by DNA sequence analysis.

(ii) *citG-lacZ* fusions. Plasmid pDH32 is a single-copy integration vector in *B. subtilis* that facilitates the in vitro generation of transcriptional fusions to *lacZ* and their subsequent targeted integration into the *amyE* gene of *B. subtilis* by substitution of the wild-type *amy* locus (30). Digestion of

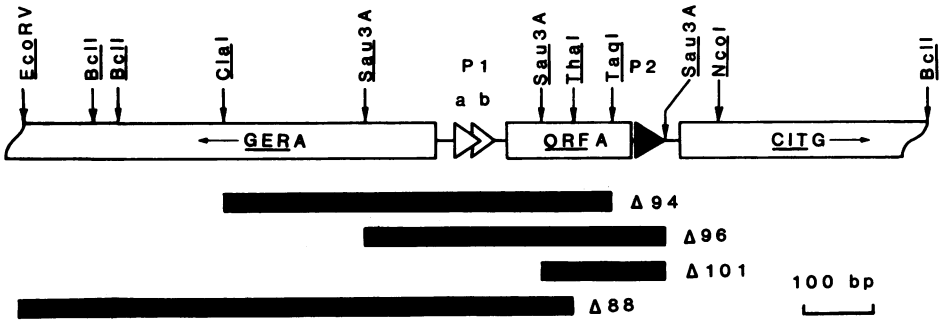

FIG. 1. *citG* promoter deletions. Schematic scale plan showing the deletions introduced into the *citG-gerA* intergenic region (solid bars). The *citG* promoters are represented by arrows; for clarity, the *gerA* promoter is not shown. The restriction sites relevant to the construction of the various deletions and fusion plasmids are also indicated.

plasmids pAAM91, -94, -96, and -101 (containing the *citG* promoter region and the various deletions described earlier) with *Bcl*I yielded a set of fragments each containing a different promoter configuration. These fragments were subcloned into the unique *Bam*HI site of pDH32 to generate a set of *citG-lacZ* transcriptional fusions; pAAM129 contained the intact promoter region from pAAM91, pAAM131 carried the Δ*citG94* derivative from pAAM94, pAAM133 carried the Δ*citG96* derivative from pAAM96, and pAAM135 carried the Δ*citG101* derivative from pAAM101. Because pAAM88 carries a deletion that spans the *gerA*-proximal *Bcl*I site, a different strategy had to be adopted. First, plasmid pVAP1 was constructed by ligating the 6.2-kb *Sma*I-*Bcl*I fragment from pSGMU31 with the 2.2-kb *Sma*I-*Bcl*I fragment from pMLB1010. The 1.4-kb *Nru*I fragment from pAAM88 was then subcloned into the unique *Sma*I site to create pAAM137, which was digested with *Bam*HI and *Bcl*I to yield a 0.94-kb fragment containing the Δ*citG88* promoter derivative. This was then cloned into the *Bam*HI site of pDH32 to create pAAM138. The ultimate fusion point of *citG* DNA to *lacZ* DNA in pAAM138 was therefore identical to that in the other constructs.

Strain constructions. *B. subtilis* strains bearing single-copy transcriptional fusions of *citG* and the various promoter derivatives were generated by transforming competent cells with the relevant linearized (*Pst*I-digested) fusion plasmids described earlier, selecting for transformants on NA plates supplemented with chloramphenicol to 5 μg/ml. The Cm^r transformants were then picked onto starch plates to check the Amy phenotype; in all cases, Cm^r transformants were also Amy^-. The location and ultimate structure of the integration event were confirmed in each case by Southern analysis (data not shown). Once established, the insertions were stable in the absence of chloramphenicol and were moved to different genetic backgrounds by transformation of competent cells, selecting for Cm^r.

RESULTS AND DISCUSSION

Fumarase activity is regulated at the transcriptional level. To study further the role of P1 and P2 in *citG* expression,

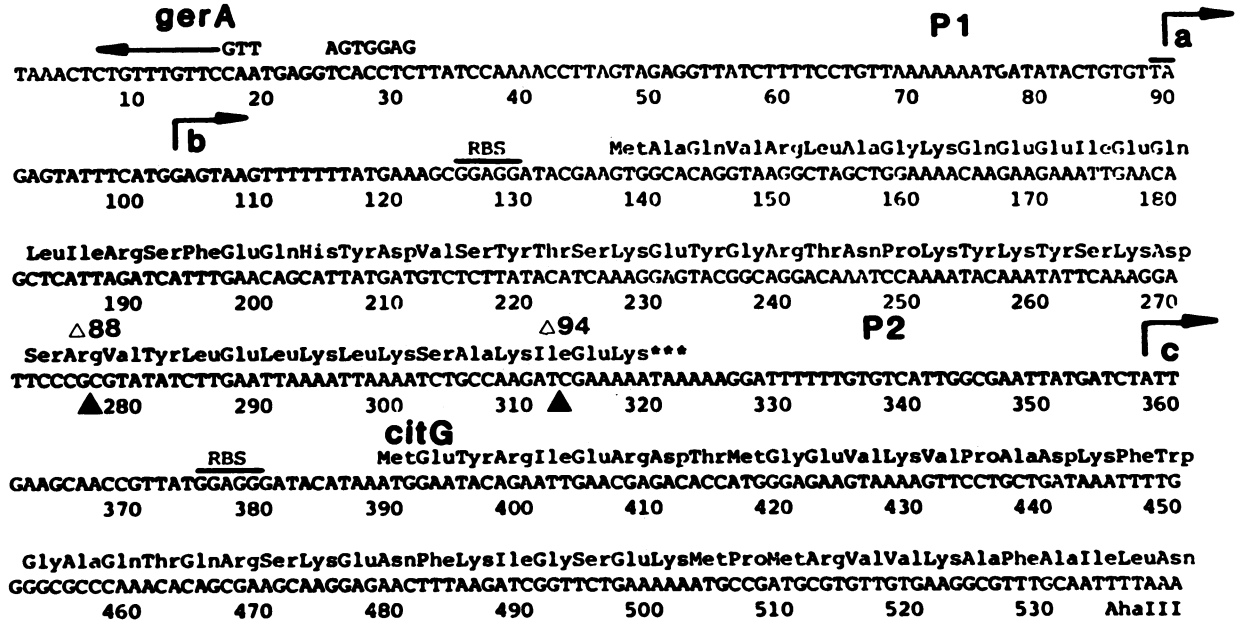

FIG. 2. Nucleotide sequence of the *citG-gerA* intergenic region, showing the transcriptional startpoints (a, b, c) and the implied promoter regions P1 and P2. The *citGp2*-proximal deletion endpoints are also indicated as solid arrowheads (the Δ*citG88* derivative is referred to as *citGp2'*, and the Δ*citG94* derivative is referred to as *citGp2''* in the text). Potential ribosome-binding sites (RBS) and the translational starts of *citG* and *gerA* are also indicated.

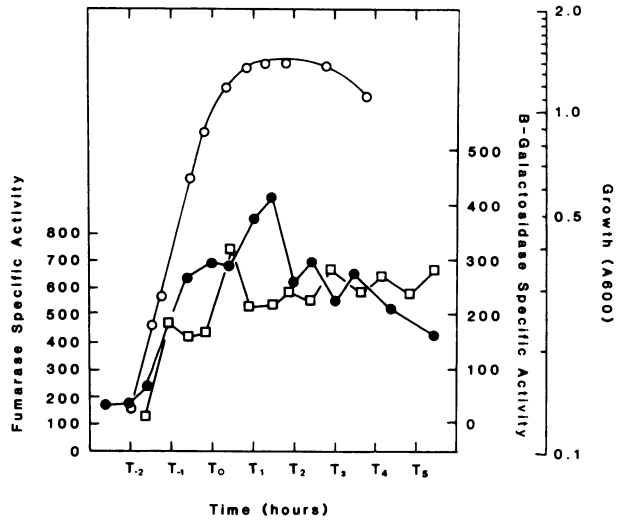

FIG. 3. Activity profiles of fumarase and β-galactosidase in *B. subtilis* AM845 grown at 37°C in NSMP medium. Symbols: ○, growth; □, fumarase specific activity; ●, β-galactosidase specific activity. T_0, End of exponential phase.

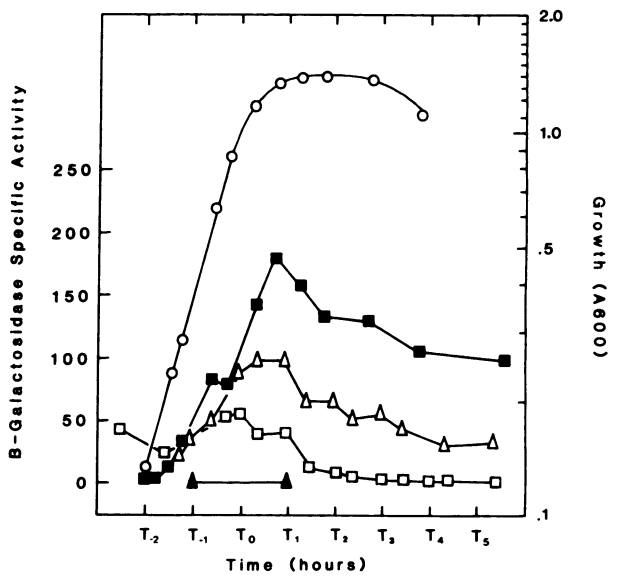

FIG. 4. Activity profiles of β-galactosidase in strains bearing fusions of deletion derivatives of the *citG* promoter region to *lacZ*. Cells were grown at 37°C in NSMP. Samples were removed periodically throughout growth and sporulation and assayed for optical density and β-galactosidase activity. β-Galactosidase specific activities are represented by the following symbols: ■, AM878 (*citGp2'-lacZ* fusion); △, AM848 (*citGp2"-lacZ* fusion); □, AM870 (*citGp1-lacZ* fusion); ▲, AM851 (*citGp1+p2* deletion fused to *lacZ*). A representative growth curve is also shown (○).

plasmids carrying the *citG* promoter region and various deletion derivatives were constructed as described in Materials and Methods (Fig. 1). The promoter derivatives were then fused to the *E. coli lacZ* gene in plasmid pDH32 and introduced into *B. subtilis* 168t$^-$ by transformation of competent cells (see Materials and Methods). These strains carry transcriptional fusions of *lacZ* to the promoter derivatives but retain an intact copy of the fumarase gene, so that the regulation of *citG* promoter-driven β-galactosidase activity could be studied in Cit$^+$ cells. To compare the levels of fumarase activity and *citG* promoter activity, strain AM845 was grown in NSMP, and samples were taken throughout growth and sporulation. These were then assayed for both β-galactosidase and fumarase activity. Figure 3 shows the kinetics of appearance of β-galactosidase and fumarase activity; both increased throughout vegetative growth in an essentially parallel manner, peaking during the first hour of sporulation and between T_1 and T_2, respectively. This suggests that fumarase activity is regulated primarily at the transcriptional level. The difference in timing of peak activity presumably reflects differences in the rates of turnover of the two enzymes.

P1 and P2 are differentially regulated. Since the deletions introduced into the *citG* promoter complex effectively separated P1 and P2, this allowed us to assess the contribution made in vivo by each promoter under different growth conditions. In order to test whether P1 and P2 are differentially regulated, the kinetics of appearance of β-galactosidase activity during growth in NSMP was determined in strains bearing fusions of separated P1 and P2 promoters to *lacZ* (Fig. 4). Levels of P1-directed β-galactosidase activity were relatively low, remaining stable throughout vegetative growth and decaying only after T_1. This suggests that P1 is responsible for maintaining a relatively uniform basal level of fumarase activity during vegetative growth. Since it has been reported that β-galactosidase is relatively unstable in early sporulating cells (J. Errington, personal communication), the post-T_1 fall in activity may result from increased protein turnover rather than a reduction in P1 activity.

In contrast, P2-directed β-galactosidase activity was subject to dramatic regulation in both AM878 (which bears a *citGp2'-lacZ* fusion) and AM848 (which bears a *citGp2"-lacZ* fusion); very little β-galactosidase activity could be detected at T_{-2}, but thereafter levels rose steeply to peak during the first hour of sporulation. While *citGp2'* and *citGp2"* derivatives showed qualitatively similar expression profiles, the levels of *citGp2"*-directed β-galactosidase activity were consistently lower (equivalent to about 60% of the P2' levels); the presence of an additional 37 bp in *citGp2'* increased expression levels. This suggests that the transcriptional regulation of *citG* is exerted via P2 and does not require extensive sequence upstream of the −35 region (the *citGp2"* derivative present in strain AM848 contains only 12 bp upstream of the *citGp2* −35 region). It may be that the DNA upstream of *citGp2* functions in *cis* to facilitate utilization by Eσ^H. Since the 34 bp immediately preceding the *citGp2* −35 region are relatively A+T rich (81% of the bases are A or T) and all but 12 of these base pairs are deleted in the *citGp2"* derivative (Fig. 2), this might be accomplished by an enhancement of melting at the promoter. In this model, the upstream DNA would function as an A+T box similar to that described for *spoVG* (4).

The levels of β-galactosidase activity from separated P1 and P2 promoters did not add up to the activity generated by the intact promoter region (Fig. 3). The lack of additivity of the separated P1 and P2 promoters might be a trivial consequence of the precise structure of the fusions, or it might be that additional sequence upstream further increases levels of transcription from P2. It is also possible that initiation of transcription at P1 potentiates that at P2.

***citGp2* can be active throughout vegetative growth and is regulated by carbon source.** In order to study further the roles of P1 and P2 in *citG* transcription, strains AM870 and AM878 were grown in a minimal salts medium supplemented with various carbon sources, and samples were taken during

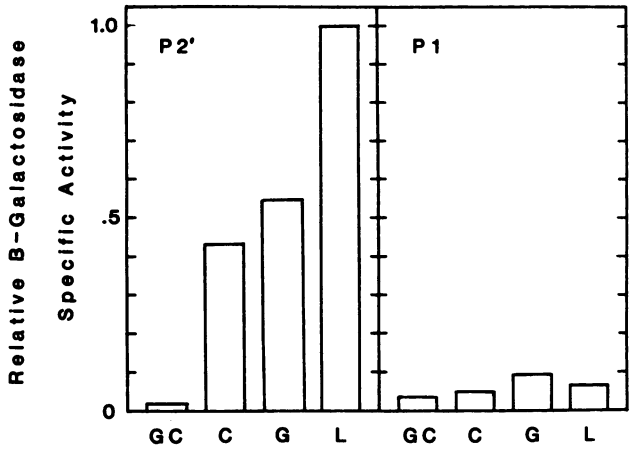

FIG. 5. Effect of supplements to a minimal medium on the specific activity of β-galactosidase in strains bearing fusions of *citGp1* and *citGp2* to *lacZ*. Relative specific activity represents the specific activity for a given medium divided by the specific activity measured in AM878 cells grown in a minimal lactate medium. In these cells, the β-galactosidase specific activity was 292 U. Symbols on the abscissa are: GC, glucose (0.5%) plus casamino acids (0.2%); G, glucose (0.5%); C, casamino acids (0.5%); L, lactic acid (0.5%). Panel P2', AM878 (*citGp2'-lacZ* fusion); panel P1, AM870 (*citGp1-lacZ* fusion).

early vegetative growth (A_{600}, 0.2). Figure 5 shows that the levels of P1-directed β-galactosidase activity remained relatively low irrespective of the carbon source, whereas P2-directed activity varied over at least a 50-fold range; levels were highest in a minimal lactate medium and lowest in a minimal glucose-casamino acids medium. This variation in P2-driven activity was too great to be accounted for by differences in growth rate in the various media tested and presumably reflects changes in *citGp2* activity.

Samples taken later in growth (data not shown) revealed that the levels of *citGp2*-directed β-galactosidase specific activity increased continuously throughout growth—levels were about twofold higher at the end of exponential growth in these media, except for that supplemented with both glucose and casamino acids; in this medium, levels increased in a manner similar to that seen in NSMP (Fig. 4). The P2-directed activity was dependent on an intact *spo0H* gene, as is discussed below and in the accompanying paper (32). The suppression of fumarase activity by readily metabolizable carbon sources is therefore exerted at the level of *citGp2* transcription. This sigma H-dependent promoter has an important role in maintaining fumarase levels throughout vegetative growth on lactate, glucose, and casamino acids.

The apparent differences in *citGp2* activity seen in the various media can be rationalized by considering the contribution made by each supplement to the cell's metabolism. Both the biosynthetic and energy-generating roles of the TCA cycle are required during growth on lactate (a nonfermentable substrate), so that TCA cycle enzyme activity must be relatively high. However, during growth on either glucose or casamino acids, the energy-generating and biosynthetic function, respectively, is spared, consistent with some reduction in TCA cycle activity. Both anabolic and catabolic functions become less important during growth on a mixture of glucose and casamino acids—energy can be derived via glycolysis, and biosynthetic demands can be met by the utilization of amino acids in the medium. This situation is therefore associated with a suppression of TCA cycle activity.

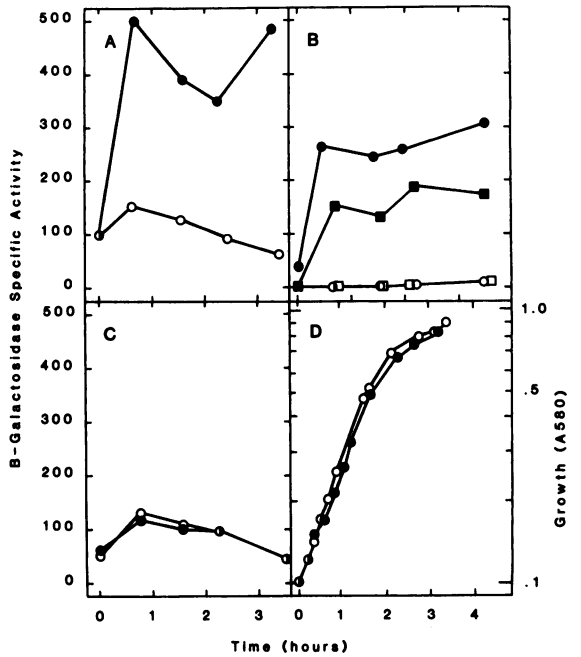

FIG. 6. Effect of *spo0H* on *citG* expression. (A–C) Activity profiles of β-galactosidase in *B. subtilis spo0H*$^+$ (solid symbols) and *spo0HΔHind* (open symbols) cells. Strains were grown in NB medium at 37°C, and samples were removed periodically for assay of turbidity and β-galactosidase activity. (A) AM852 (wild type; ●) and AM843 (*spo0H*; ○) (strains bearing a *citG'-lacZ* fusion, i.e., both P1 and P2 are present). (B) AM911 (wild type; ●) and AM912 (*spo0H*; ○) (strains bearing a *citGp2'-lacZ* fusion) and also AM854 (wild type; ■) and AM846 (*spo0H*; □) (strains bearing a *citGp2''-lacZ* fusion). (C) AM909 (wild type; ●) and AM910 (*spo0H*; ○) (strains bearing a *citGp1-lacZ* fusion). (D) Representative growth curves of *spo0H*$^+$ (solid symbols) and *spo0HΔHind* (open symbols) cells.

As *citG* expression is suppressed in the presence of glucose, it can be considered a catabolite-repressible gene. Little is known of the mechanism(s) mediating this response in *B. subtilis*, and as yet it is impossible to tell whether the repression of *citGp2* by readily metabolizable carbon sources is specific to this gene or mediated via a global regulatory system.

Effect of sigma factor and *spo0* mutations on *citG* expression. In order to explore further the role of alternative sigma factors in *citG* transcription, the expression of fumarase and *citG-lacZ* fusions was studied in strains bearing deletions of the genes *sigB*, encoding sigma B (sigma 37); *sigD*, encoding sigma D (sigma 28); and *spo0H*, encoding sigma H (sigma 30). Figure 6 shows the β-galactosidase activity profiles of strains bearing *citG-lacZ* fusions in both wild-type and *spo0H* genetic backgrounds. The level of P1-directed β-galactosidase activity was unaffected by a *spo0H* mutation, while P2-directed activity was completely abolished. Thus, P2 but not P1 is sigma H dependent.

The activity profile generated by the intact promoter region containing both P1 and P2 in a *spo0H* mutant was similar to that generated by one containing P1 alone. We conclude that residual *citG* expression in a *spo0H* mutant reflects P1 activity. This allowed us to test whether sigma B or sigma D is involved in transcription from P1 by simply measuring fumarase activity in *spo0H sigB* and *spo0H sigD* double mutants. Neither *sigB* nor *sigD* mutations had any effect on the *spo0H*-independent (P1-generated) fumarase

TABLE 2. Dependence pattern of *spo0H*-dependent promoters

Promoter (reference)	Expression[a]			
	spo0A	*spo0B*	*spo0F*	*spo0E*
citGp2 (9)	+	+	+	+
spo0F (20, 35)	−	−	−	−
spoVG (36)	−	−	±	±
sinp1 (12)	−	ND	ND	ND
spoIIA (34)	−	−	−	ND
com-39 (1)	−	ND	−	ND

[a] Symbols: +, the gene is expressed; −, the gene is not expressed; ±, the gene is expressed at a lower level; ND, not determined.

activity (data not shown), suggesting that these sigma factors have no role in *citG* expression. It therefore seems likely that the major vegetative sigma factor, sigma A, is involved in transcription from at least one of the *citGp1* promoters, as similarities in the −10 region of these promoters to the sigma A consensus has been reported previously (9).

Measurement of fumarase activity in strain JH703 revealed that fumarase expression during late exponential growth in nutrient broth (when 70 to 80% of fumarase activity arises from *citGp2* activity) is unaffected by a deletion in *spo0A* (data not shown); this is consistent with our failure to see an effect of the *spo0A12* mutation in minimal glucose medium, as reported previously (9). In contrast, levels of fumarase in a *spo0H* mutant under the same conditions were severely suppressed (data not shown). Thus, *spo0A* and *spo0H* do not have equivalent effects on fumarase expression. Other workers, however, have reported that *spo0H* expression is dependent to a large degree on *spo0A* function (7). Our work implies that there must be significant residual sigma H levels in *spo0A* strains. We have also shown previously (9) that fumarase expression is not dependent on the products of the *spo0B*, *spo0F*, and *spo0E* genes. In this respect, *citG* differs from other *spo0H*-dependent genes. Table 2 lists the *spo0H*-dependent promoters and genes that have been tested for activity in various *spo0* mutants. In the case of *spoVG*, *citGp2*, and *spoIIA*, the direct involvement of sigma H in transcription has been demonstrated by in vitro transcription experiments (32). In the other cases, inference of direct $E\sigma^H$ transcription is based on evidence of *spo0H* dependence of *lacZ* fusions and similarities of the mapped promoters with those of *spoVG* and *spoIIA*.

Role of *orfA* in *citG* expression. The short open reading frame immediately following *citGp1* (*orfA*) is preceded by a potential ribosome-binding site and so could encode a 61-residue polypeptide. Since there are precedents for the involvement of very small proteins in the regulation of gene expression in *B. subtilis* (e.g., SacQ [2]), we tested the possibility that *orfA* is translated in vivo by constructing a translational fusion of the *orfA* to *lacZ* and introducing it into the chromosome in single copy. We found that there was no significant expression of β-galactosidase above that attributable to the very low level endogenous β-galactosidase activity in *B. subtilis*; β-galactosidase activities in cells containing an *orfA-lacZ* fusion were similar to those in the parent strain lacking the fusion (data not shown). To test whether *orfA* encodes a protein involved in *citG* regulation, a chloramphenicol resistance gene (*cat*) was inserted into *orfA*. No effect on fumarase levels could be detected (data not shown). Thus, it seems that *orfA* is not translated significantly in vivo and is not involved in the regulation of fumarase expression in *trans*.

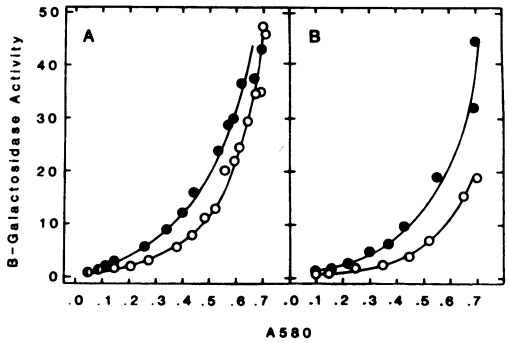

FIG. 7. Effect of P*spac*-directed *spo0H* transcription on *citGp2* activity. Differential plots of β-galactosidase activity per milliliter against culture A_{585} (determined by using the 585-nm filter of a Corning 252 colorimeter) of cells grown in a minimal glucose-casamino acids medium supplemented with 1 mM IPTG at 37°C. The solid symbols indicate cells in which *spo0H* is under the control of a fully induced *spac* promoter, and the open symbols indicate cells in which *spo0H* is under normal regulation. (A) AM936 (●) and AM911 (○) (strains bearing a *citGp2'-lacZ* fusion); (B) AM966 (●) and AM854 (○) (strains bearing a *citGp2"-lacZ* fusion).

***citGp2* is not regulated at the level of *spo0H* transcription.** A simple model for the regulation of *citGp2* is that its activity is determined by the level of *spo0H* transcription. To test this model, we introduced the *citGp2-lacZ* fusions into competent cells of strain BH19, in which the chromosomal copy of the *spo0H* gene has been placed under the control of the isopropylthiogalactopyranoside (IPTG)-inducible *spac* promoter. (BH19 was constructed in R. Losick's laboratory by J. Healy in order to test whether *spoVG* was controlled at the level of *spo0H* transcription. It has also been used to find other *spo0H*-controlled genes [19].) This was accomplished by congression with trp^+ DNA and saturating quantities of AM878 DNA and AM854 chromosomal DNA to generate strains AM936 and AM966, respectively. Congressants carrying the *citGp2-lacZ* fusions were identified by screening Trp^+ transformants in situ for Lac^+ on minimal plates containing both 1 mM IPTG and Xgal. All of the congressants were Amy^-, indicating that the fusions had been efficiently targeted to the *amyE* gene (see Materials and Methods). Simple transformation to Cm^r could not be used in this case because BH19 already contained a Cm^r marker at the *spo0H* locus. Strains AM936 and AM966 were stable in the absence of chloramphenicol and showed a Lac^+ Spo^+ phenotype only in the presence of IPTG. We could therefore study the effect of establishing P*spac*-directed transcription of *spo0H* on *citGp2* activity by including IPTG in the medium used to culture AM936 and AM966. We used a minimal glucose-casamino acids medium, in which *citGp2* activity is normally very low during early vegetative growth (Fig. 5) but increases thereafter in a manner similar to that seen in NSMP. The data were plotted as differential plots so that comparisons of the differential rates of synthesis of β-galactosidase could be made easily.

We found that adding saturating quantities of IPTG to induce P*spac*-directed transcription of *spo0H* caused a small stimulation of promoter activity (Fig. 7). However, the β-galactosidase induction curves remained essentially similar to those of the otherwise isogenic parent strains in which *spo0H* was expressed normally. This was the case even when only 10 bp upstream of the −35 region was retained in the fusion construct (Fig. 7B). These data suggest that the regulation of *citGp2* is not exerted at the level of *spo0H* transcription.

The expression of *spoVG* has also been found to be essentially unperturbed by induction of P*spac*-directed *spo0H* transcription (J. Healy, personal communication). Either sigma H activity is regulated posttranscriptionally (e.g., at the level of sigma H stability), or regulatory factors in addition to sigma H are involved in determining the pattern of both *citG* and *spoVG* expression.

The construction of an isolated *citGp2-lacZ* fusion has enabled us to demonstrate the dramatic regulation to which this $E\sigma^H$-transcribed promoter is subject during vegetative growth. The observed high fumarase levels in postexponential-phase, nutrient broth-grown cells has been taken to suggest that fumarase expression is growth phase (i.e., temporally/developmentally) regulated (27). However, the simplest explanation of these observations is that fumarase levels are fluctuating in response to changes in the concentration of available nutrients which necessarily occur during batch growth in a rich medium. There is no need to invoke temporal regulation or to imply any association with sporulation; the *spo0H* product fulfills the role of a vegetative sigma factor.

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