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Transcriptional Regulation of *Bacillus subtilis* Glucose Starvation-Inducible Genes: Control of *gsiA* by the ComP-ComA Signal Transduction System

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The *Bacillus subtilis* glucose starvation-inducible transcription units, *gsiA* and *gsiB*, were characterized by DNA sequencing, transcriptional mapping, mutational analysis, and expression in response to changes in environmental conditions. The *gsiA* operon was shown to consist of two genes, *gsiAA* and *gsiAB*, predicted to encode 44.9- and 4.8-kDa polypeptides, respectively. The *gsiB* locus contains a single cistron which encodes a protein of unusual structure; most of its amino acids are arranged in five highly conserved, tandemly repeated units of 20 amino acids. The 5′ ends of *gsiA* and *gsiB* mRNAs were located by primer extension analysis; their locations suggest that both are transcribed by RNA polymerase containing sigma A. Expression of both *gsiA* and *gsiB* was induced by starvation for glucose or phosphate or by addition of decoyinine, but only *gsiA* was induced by exhaustion of nutrient broth or by amino acid starvation. Regulation of *gsiA* expression was shown to be dependent upon the two-component signal transduction system ComP-ComA, which also controls expression of genetic competence genes. Mutations in *mecA* bypassed the dependency of *gsiA* expression on ComA. Disruption of *gsiA* relieved glucose repression of sporulation but did not otherwise interfere with sporulation, development of competence, motility, or glucose starvation survival. We propose that *gsiA* and *gsiB* are members of an adaptive pathway of genes whose products are involved in responses to nutrient deprivation other than sporulation.

When available nutrients fall below the levels necessary to sustain rapid vegetative growth of *Bacillus subtilis*, several developmental programs are initiated. In one program, individual cells undergo a process of morphogenesis that culminates in the differentiation of rod-shaped vegetative cells into spherical, environmentally resistant, dormant cells known as spores. The regulation of this response depends on a number of gene products which appear to be part of a complex, intertwined signal transduction network that controls not only initiation of sporulation but also other programs, such as development of genetic competence, motility and chemotaxis, degradative enzyme synthesis, and antibiotic production. Some of the regulatory genes required for these various adaptive responses are members of the two-component family of bacterial signal transduction systems; others are known to be transcription factors (6, 7, 13). Only limited information exists as to the specific conditions that trigger each response or the order of events as cells enter the stationary phase. It is also not clear whether the responses are mutually exclusive, whether each type of response constitutes an endpoint, or whether at least some of them represent sequential steps in an ordered pathway. It is clear, however, that many gene products induced at the onset of sporulation perform functions that are dispensable for or even inimical to spore formation (46).

Bacterial two-component regulatory systems consist of a histidine protein kinase and a response regulator (47). The histidine kinases, in response to environmental or intracellular information, undergo autophosphorylation and subsequently transfer their phosphate groups to their cognate response regulators, in many cases modulating the activity of that protein as a transcriptional activator or repressor. At least four interconnected, two-component regulatory pathways are known to govern transcription of nutrient stress response genes in *B. subtilis*. KinA and Spo0A are the histidine kinase and regulator proteins thought to be responsible for activation of the sporulation pathway (2, 34); ComP and ComA function as the regulatory pair for the competence cascade (6, 7, 49, 51). Other two-component family members in *B. subtilis* are DegU and DegS, which control extracellular enzyme production, and PhoP and PhoR, which control alkaline phosphatase and phosphodiesterase synthesis (for a review, see reference 45).

Much recent interest in *B. subtilis* has been directed toward elucidation of the environmental cues that initiate development and the search for genes and gene products that mediate it. Several genes that are activated shortly after nutrient deprivation of growing cells have been identified (for reviews, see references 10 and 46). To examine regulation of gene expression during the onset of sporulation in more detail, we have been isolating genes on the basis of their differential expression under conditions of nutrient deprivation (21, 27). This approach involves synthesis of cDNA probes and enrichment for desired sequences by subtractive hybridization. We recently reported the application of this direct method to the identification of two *B. subtilis* genes rapidly induced in response to glucose deprivation (27). Glucose deprivation is one of the conditions that induce sporulation and several other adaptive responses.

In this report, we characterize the structure and regulation of glucose starvation-inducible genes *gsiA* and *gsiB*. Nutri-

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TABLE 1. Bacterial strains and plasmids used in this study

Organism or plasmid	Trait or relevant genotype (plasmid size [kb])	Derivation or reference
Bacillus subtilis strains		
SMY	Prototrophy	Laboratory stock
BD1626	*hisA1 leuA8 metB5 comA124*::pTV55Δ2*cat*	D. Dubnau; 49
BD1692	*aroD120 mecB31 comA124*::pTV55Δ2*cat comG12*::Tn*917lac*	D. Dubnau; 37
BD1697	*aroD120 mecA42 comA124*::pTV55Δ2*cat comG12*::Tn*917lac*	D. Dubnau; 37
BD1853	*hisA1 leuA8 metB5 comPΔK1*[a]	D. Dubnau; 14
MB25	Φ(*gsiA-lacZ*)*27 cat*	pJPM27→SMY[b]
MB28	*trpC2 pheA1*	J. Hoch (JH642); 21
MB39	Δ*amyE*::Φ(*gsiA-lacZ*)*42 ermC*	pJPM42→SMY
MB59	*gsiB*::pJPM22 *cat*	pJPM22→SMY
MB60	Φ(*gsiB-lacZ*)*70 cat*	pJPM70→SMY
MB61	Δ*gsiA69*::*cat*	pJPM69→SMY
MB72	*gsiA*::pJPM20 *cat*	pJPM20→SMY
MB82	Δ*gsiB60*::*neo*	pJPM60→SMY
MB83	Δ*gsiA69*::*cat* Δ*gsiB60*::*neo*	MB61→MB82
MB84	MB28 Δ*gsiA69*::*cat*	MB61→MB28
MB186	*trpC2* SPβ[s]	H. Taber
MB223	MB39 *comA124*::pTV55Δ2*cat*	BD1626→MB39
MB225	MB39 *comPΔK1*	BD1853→MB39
MB258	MB39 *mecA42 comA124*::pTV55Δ2*cat*	BD1697→MB39
MB259	MB39 *mecB31 comA124*::pTV55Δ2*cat*	BD1692→MB39
MB350	MB84 SPβ*c2del2*::Tn*917*::pSK10Δ6::pJPM117	SPβ*gsiA*[+][c] × MB84
ZB307A	SPβ*c2del2*::Tn*917*::pSK10Δ6	P. Zuber; 53
Escherichia coli strains		
DH5α	F[−] ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44* λ[−] *thi-1 gyrA relA1*	P. Miller
JM106	JM107 F[−]	Laboratory stock
JM107	Δ(*lac-proAB*) *thi gyrA96 endA1 hsdR17* (r_K^- m_K^-) *supE44* (F′ *traD36 proAB lacI*[q]ZΔM15) *mcrA mcrB*	Laboratory stock
Plasmids		
pAF1	*bla cat* (11.1)	A. Fouet; 12
pBEST501/502	*bla neo* (4.3)	M. Itaya; 17
pBS[−]	*bla* (3.2)	Stratagene, Inc.
pJPM1	*bla cat* (3.8)	This work
pJPM3	*bla cat lacZ* (8.0)	This work
pJPM8/9	*bla erm* (4.5)	This work
pJPM10/11	*bla cat* (4.7)	This work
pJPM15	*bla erm* (11.6)	This work
pSGMU2	*bla cat* (3.7)	J. Errington; 11
pSGMU32	*bla cat lacZ* (7.8)	J. Errington; 9
pSK[±]	*bla* (3.0)	E. Elliott
pSP64	*bla* (3.0)	Promega, Inc.
pTV20	*bla cat erm* (15.3)	T. Henkin; 52

[a] The *comPΔK1* mutation is an in-frame deletion that is linked by transformation to a silent kanamycin resistance gene (14).
[b] An arrow indicates construction by transformation.
[c] SPβ-mediated transduction.

ent exhaustion-induced transcription of *gsiA* required the products of *comP* and *comA*. We propose that *gsiA* and *gsiB* are members of an adaptive pathway of genes whose products are involved in nonsporulation responses to nutrient deprivation. In the accompanying report (28), we show that the product of *gsiA* acts, directly or indirectly, as a negative regulator of the sporulation pathway.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1. Strains MB258 (*mecA42*) and MB259 (*mecB31*) were constructed by congression in the following fashion. Chromosomal DNAs isolated from *mecA42* and *mecB31* mutant strains (BD1692 and BD1697), which also carried the *comA124*::pTV55Δ2*cat* and *comG12*::Tn*917-lacZ* mutations, were used to transform strain MB39 (*gsiA-lacZ*), with selection for Cm[r] (i.e., for introduction of the *comA* mutation). Since *gsiA-lacZ* is entirely dependent on *comA* for its expression (see Results), the transformants were then scored for expression of β-galactosidase on nutrient broth sporulation medium (DS) (42) plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (120 μg/ml). For the cross with *mecB31* DNA, transformants were also screened for the Spo[−] phenotype exhibited by *mecB* mutants (37). To confirm that the *comA mec* strains did not possess the *comG12-lacZ* fusion, the *gsiA* fusion was reintroduced into strain SMY by transformation with selection for Erm[r]. Erm[r] transformants were scored for β-galactosidase production on DS plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and screened for the Amy[−] phenotype. Both the *gsiA* and *comG* fusions carry the *ermC* gene from Tn*917*, but only the *gsiA* fusion is expressed in complex medium and is integrated

within the *amyE* locus. All of the Erm^r transformants generated with DNAs from strains MB258 and MB259 exhibited a LacZ$^+$ Amy$^-$ phenotype on DS plates.

The plasmids used in this study were the following. Integration plasmid pJPM1 was constructed by substituting the 322-bp *Pvu*II fragment of pSGMU2 (11) with the 382-bp *Pvu*II fragment of plasmid pBS$^-$ (Stratagene, Inc.). Plasmid pJPM1 and its derivatives are unable to replicate in *B. subtilis* but, upon integration into the chromosome, confer selectable chloramphenicol resistance. pJPM1 also contains T3-T7 promoter-primer sequences for plasmid sequencing and in vitro synthesis of uniformly labeled RNA probes. Integrative *lacZ* transcriptional fusion vector pJPM3, which contains a single *Bam*HI site in the polylinker upstream of *lacZ*, was constructed by subcloning the 5.0-kb *Bam*HI-*Bgl*II *lacZ-cat* cassette from pSGMU32 (9) into the unique *Bam*HI site of pSP64 (Promega, Inc.). Plasmids pJPM8 and pJPM9 are derivatives of pSK$^-$ (Stratagene, Inc.) that carry the erythromycin resistance (Erm^r) gene from plasmid pTV20 (52) in the *Eco*RV site in opposite orientations. pJPM11 and pJPM12 carry the *cat* gene from pC194 cloned in the *Eco*RV site of plasmid pSK$^-$ in opposite orientations. Plasmid pJPM15 is an Erm^r derivative of pAF1 (12). It is a single-copy integrative vector which allows construction of transcriptional fusions to *lacZ* and insertion into the *amyE* gene. Integration of the insert in pJPM15 occurs by double crossover into the chromosome within the α-amylase gene, whose inactivation was confirmed by absence of a halo of starch hydrolysis on TBAB (Difco Laboratories)-starch plates stained with a solution of 1.0% (wt/vol) iodine. Plasmids pJPM67 and pJPM74 were rescued from the chromosome by using conventional cloning techniques (52) following recombinational integration of *gsiA*- or *gsiB*-containing plasmids derived from pJPM1.

T3-T7 transcription plasmids. The following plasmids are derivatives of T3-T7 transcription vector pBS$^-$ or pSK$^-$ (Stratagene, Inc.). Plasmid pCM213, containing the *dciA* promoter region, was described by Slack et al. (44). pJLB10, a derivative of pSK$^-$, contains the *gsiA* promoter region as a 0.65-kb *Pst*I-*Acc*I fragment (Fig. 1A). Plasmid pJPM17 contains the *gsiB* promoter region in pSK$^-$, as shown schematically in Fig. 1B and 2. pJLB4 contains the *spo0H* 5′ region in pSK$^-$ as a 500-bp *Hin*dIII fragment from pJOH25 (16). Plasmid pJLB7, which contains the *veg* promoter region, was constructed by subcloning the 334-bp *Eco*RI-*Bam*HI fragment of pPH9 (19) into the compatible sites of pSK$^-$. pBScitB is a derivative of pBS$^-$ which was created by subcloning the 350-bp *Eco*RI-*Hin*dIII fragment from plasmid pMR41 (38).

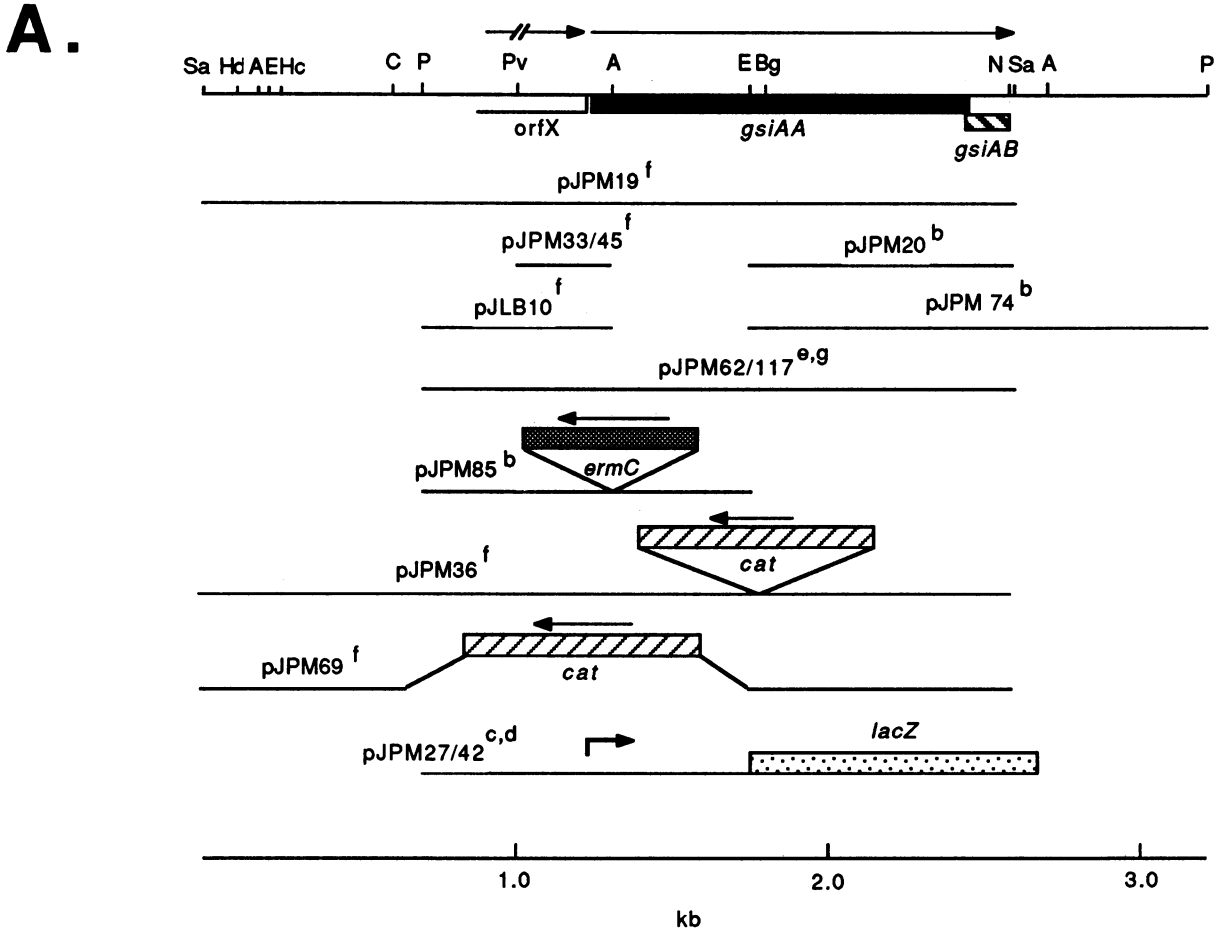
General methods. *B. subtilis* cells were made competent and transformed by the method of Piggot et al. (35). Selection for drug resistance was on DS medium plates containing chloramphenicol (2.5 μg/ml), neomycin (5.0 μg/ml), or erythromycin and lincomycin (0.5 and 12.5 μg/ml, respectively). Strains of *Escherichia coli* were made competent and transformed by the method of Hanahan (15). Plasmid DNA was isolated from *E. coli* by a method based on the alkaline lysis method of Birnboim and Doly (1). Restriction endonucleases and DNA modification enzymes were obtained from New England BioLabs, Inc., and used as recommended by the supplier.

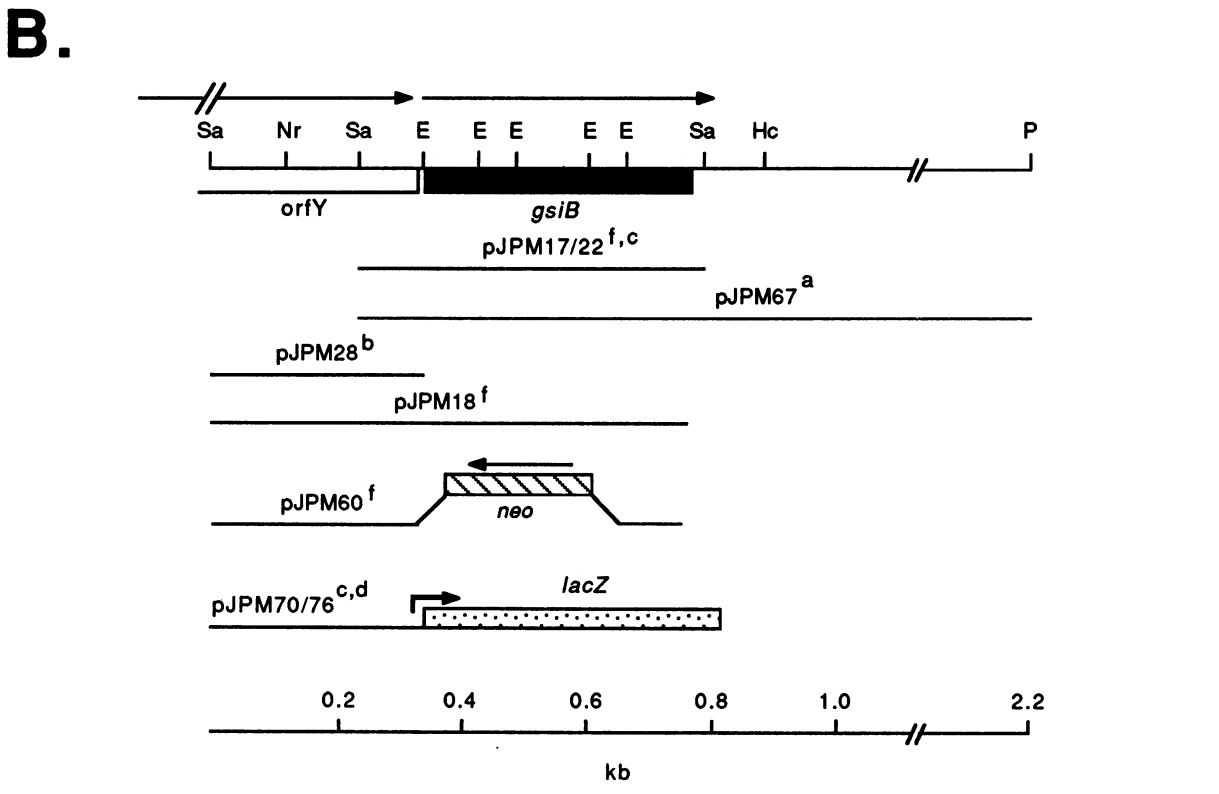
Growth and sporulation conditions. *B. subtilis* cells were induced to sporulate either by medium replacement (resuspension) or by nutrient exhaustion. For resuspension experiments, cells in the exponential growth phase (~100 Klett units) in medium 121J (Tris-buffered glucose-glutamate medium; 27) were harvested and resuspended in medium that lacked glucose, NH_4Cl, or K_2HPO_4 for carbon, nitrogen, or phosphate starvation, respectively. Medium 121CG differed from 121J in that glucose was replaced with 1.0% Casamino Acids. Medium 121F was prepared as described by Slack et al. (44). Time zero (T_0) is defined as the time of resuspension in starvation medium. For exhaustion experiments, an exponential-phase culture in DS medium was diluted to give an A_{600} of 0.05. Culture growth at 37°C was monitored by measuring A_{600}; T_0 was defined as the end of exponential growth. Induction of sporulation with decoyinine has already been described (44). The number of heat-resistant spores was determined after 12 to 24 h at 37°C. Samples were plated either before or after heat treatment (80°C, 20 min) to measure the number of viable or heat-resistant CFU, respectively.

RNA isolation and RNase protection analysis. Isolation of total RNA from *B. subtilis* was done by the guanidinium isothiocyanate method (44). Cells were harvested during exponential growth in 121J medium and 1 h after starvation for glucose (T_1) or at 5-min intervals after addition of decoyinine (500 μg/ml) to 121F medium. Antisense RNA probes were synthesized by using the Stratagene riboprobe system. RNA hybrids were treated with RNase T2 as described by Slack et al. (44). Nuclease-resistant hybrids were separated by electrophoresis in 5% polyacrylamide–7 M urea sequencing gels and visualized by autoradiography.

Identification of the *gsiA* and *gsiB* transcription start sites. Primer extension reactions were performed as described by Sambrook et al. (40). The primers were synthetic 19-nucleotide DNA oligonucleotides complementary to the 5′ terminal region of *gsiA* or *gsiB* mRNA (Fig. 3). Sixty picomoles of oligomer was end labeled by incubation with 150 μCi of [γ-^{32}P]ATP (5,000 Ci/mmol) and T4 polynucleotide kinase as described previously (40). Free nucleotides were separated from labeled oligonucleotide by three precipitations with ammonium acetate-ethanol. For primer extension reactions, 6 ng of oligomer (5×10^4 cpm) was annealed with 20 μg of RNA in a buffer containing 80% formamide, 0.5 M NaCl, 1 mM EDTA, and 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.8). The mixture was incubated at 80°C for 3 min and then cooled gradually to 30°C. The hybridized nucleic acids were precipitated with ethanol, dissolved in 20 μl of reverse transcriptase buffer (50 mM KCl; 1 mM EDTA; 55 mM Tris-HCl [pH 8.0]; 5 mM dithiothreitol; 8 mM $MgCl_2$; dATP, TTP, dGTP, and dCTP each at 2.5 mM) and incubated with 20 U of avian myeloblastosis virus reverse transcriptase (Life Sciences; St. Petersburg, Fla.) at 42°C for 45 min. Four microliters of a solution containing 0.25 M EDTA and 10 μg of RNase A per ml was added, and the incubation was continued at 37°C for 30 min. The reaction products were extracted with phenol-chloroform, precipitated with ethanol, solubilized in 5 μl of loading buffer, and separated by electrophoresis in a 5% polyacrylamide–7 M urea gel.

DNA sequencing. Fragments of DNA from plasmids pJPM18 and pJPM19 were cloned into phagemids pSK$^+$ and pSK$^-$ for sequencing by the dideoxy-chain termination method of Sanger et al. (41) with modified T7 DNA polymerase (Sequenase, Version 2.0; U.S. Biochemical Corp.). When necessary, reactions using *Taq* DNA polymerase (Cetus) and dGTP analogs were used to resolve sequence compressions. Single-stranded DNA templates were prepared from R408 helper phage lysates of superinfected *E. coli* JM107 cells harboring recombinant pSK plasmids, as recommended by Russel et al. (39). The nucleotide sequence


A.
Sa Hd AEHc
C P
Pv
A
EBg
N Sa A
P
orfX
gsiAA
gsiAB
pJPM19 f
pJPM33/45 f
pJPM20 b
pJLB10 f
pJPM 74 b
pJPM62/117 e,g
pJPM85 b
ermC
pJPM36 f
cat
pJPM69 f
cat
pJPM27/42 c,d
lacZ
1.0
2.0
3.0
kb


B.
Sa
Nr
Sa
E
E
E
E
E
Sa
Hc
P
orfY
gsiB
pJPM17/22 f,c
pJPM67 a
pJPM28 b
pJPM18 f
pJPM60 f
neo
pJPM70/76 c,d
lacZ
0.2
0.4
0.6
0.8
1.0
2.2
kb

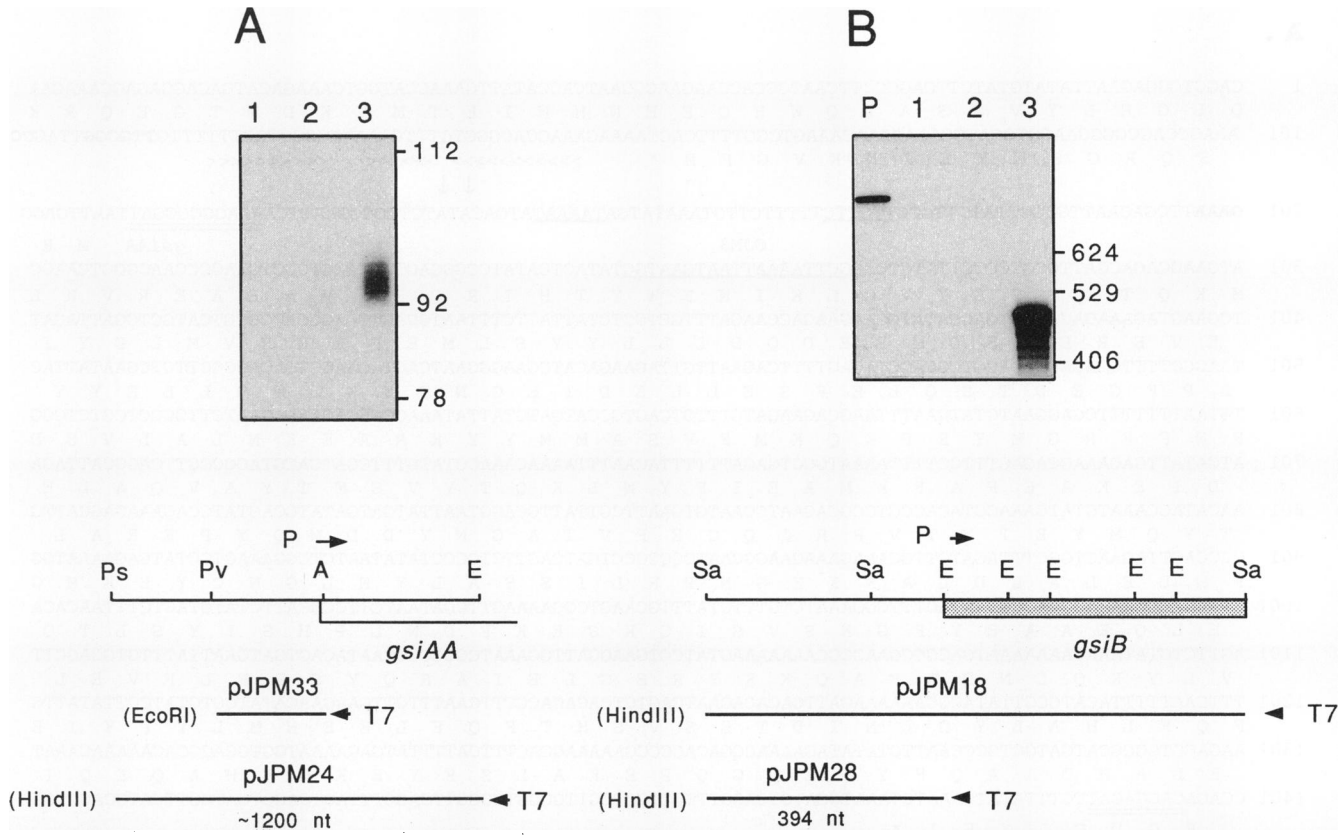


FIG. 2. RNase T2 mapping of the 5′ termini of *gsiA* (A) and *gsiB* (B) mRNAs and appearance of *gsiA* and *gsiB* mRNAs in response to glucose deprivation. The 351-bp *Pvu*II-*Acc*I *gsiA* fragment and the 521-bp *Sau*3A *gsiB* fragment were used for synthesis, in the direction indicated, of uniformly labelled antisense RNA probes specific for each sequence. Labeled *gsi* probes were separately hybridized with 20-μg samples of total *B. subtilis* RNA from cells collected at T_0 in 121J medium (lane 2) or T_1 in 121J lacking glucose (lane 3) or with RNA from *Saccharomyces cerevisiae* (lane 1). P, untreated probe (5,000 cpm). The hybridization products were treated with RNase T2, and the nuclease-resistant products were resolved on a 5% polyacrylamide–7 M urea sequencing gel. The samples were run alongside end-labeled *Hpa*II fragments of pBR322, and the sizes are shown in base pairs on the right. nt, nucleotides. For restriction site abbreviations, see the legend to Fig. 1. Ps, *Pst*I.

of both strands was determined by using a series of synthetic oligonucleotides that prime at intervals of approximately 350 nucleotides. Oligonucleotides were synthesized by using an Applied Biosystems 380B DNA synthesizer at the Tufts Protein Chemistry Facility. Sequence analysis was performed by using the University of Wisconsin Genetics Computer Group package (4).

Construction of *gsiA-lacZ* and *gsiB-lacZ* transcriptional fusions. Plasmid pJPM27 is a *gsiA-lacZ* transcriptional fusion. It was constructed by cloning the 1.2-kb *Pst*I-*Bgl*II fragment carrying the *gsiA* promoter in front of the *lacZ* gene of vector pJPM3 (Fig. 1A). Plasmid pJPM70 is a *gsiB-lacZ* transcriptional fusion constructed by cloning the 0.36-kb *Sau*3A-*Eco*RI fragment carrying the *gsiB* promoter (purified from a multiple cloning site as a *Hin*dIII-*Bam*HI fragment) into *Hin*dIII-*Bam*HI-digested pJPM3 (Fig. 1B). The *gsiA-lacZ* and *gsiB-lacZ* fusions were inserted into the chromosome by single-reciprocal recombination by transformation of competent cells of strain SMY with plasmids pJPM27 and pJPM70 and selection for chloramphenicol resistance (Cm^r). Chromosomal DNA from one chloramphenicol-resistant transformant was purified from each cross and subjected to Southern hybridization analysis to confirm that pJPM27 and pJPM70 had integrated in a single copy at the chromosomal *gsiA* and *gsiB* loci, respectively (data not shown).

Plasmid pJPM42 was constructed by subcloning the 3.2-kb *Hin*dIII-*Sac*I fragment of pJPM27 into the *Hin*dIII-*Sac*I backbone of pJPM15. pJPM42 was linearized with *Nru*I and used to transform SMY to Erm^r. Since the constructs carried homology to the *amyE* locus, this transformation resulted in integration of the fusion constructs by replacement recombination at this locus.

Deletion of the chromosomal *gsiA* and *gsiB* genes. Plasmids containing in vitro-derived deletions and insertions of *gsiA*

FIG. 1. Cloned DNA from the *gsiA* (A) and *gsiB* (B) regions of the *B. subtilis* chromosome. Physical maps of the DNA inserts in the plasmids used are shown below the abbreviated restriction maps. The locations of the *gsi* genes are indicated. The positions and structures of various insertions in the chromosome are also indicated. Restriction sites: E, *Eco*RI; Hd, *Hin*dIII; Pv, *Pvu*II; B, *Bgl*II; A, *Acc*I; Hc, *Hin*cII; C, *Cla*I; N, *Nru*I; Nr, *Nar*I; Sa, *Sau*3A; P, *Pst*I. Vectors are indicated by the following superscripts: a, pBS; b, pJPM1; c, pJPM3; d, pJPM15; f, pSK^-; g, pBEST501.

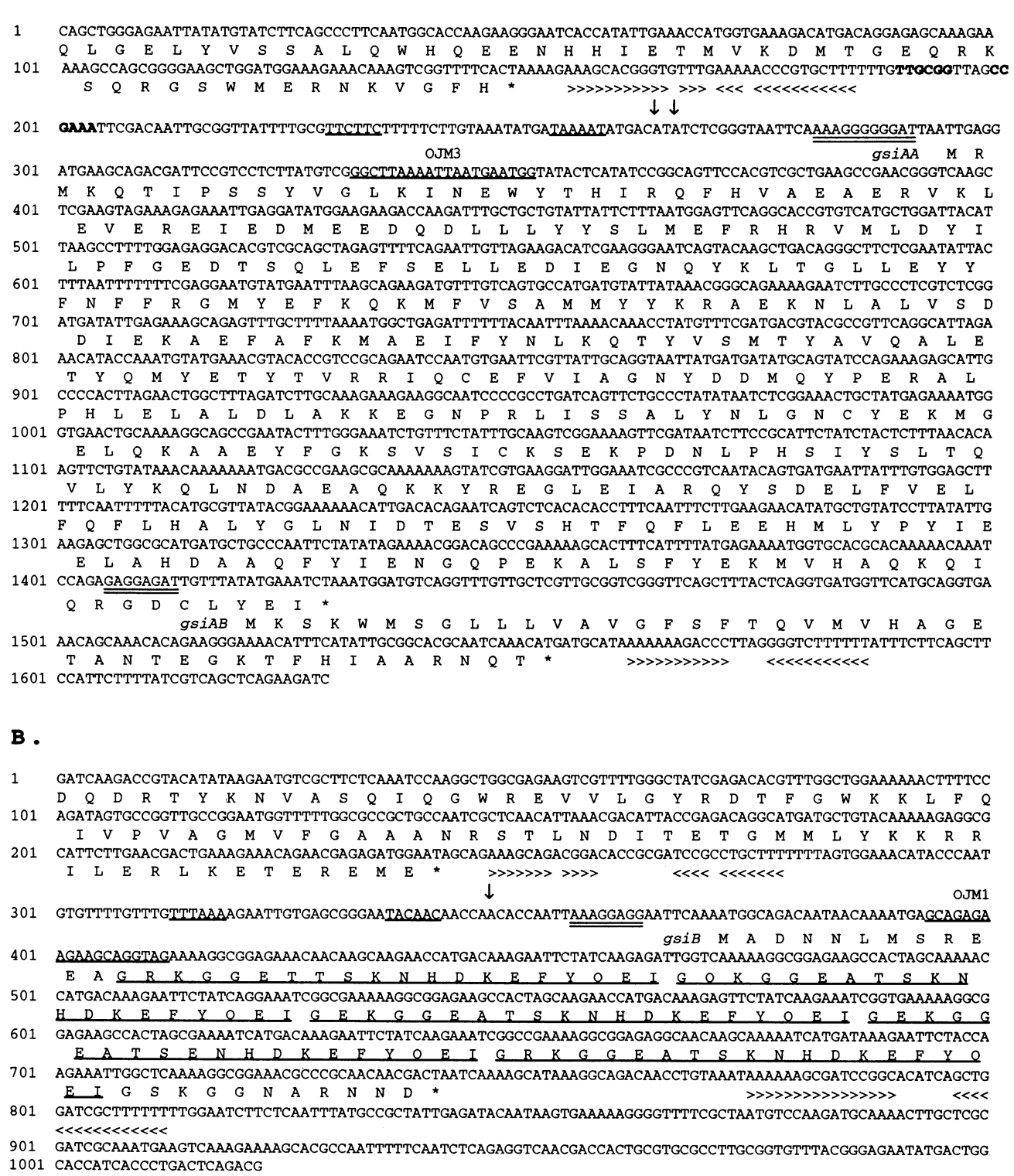


FIG. 3. DNA sequences of the nontemplate strands of *gsiA* (A) and *gsiB* (B). The derived amino acid sequences of the *gsi* genes are shown below the nucleotide sequences. Single underlining identifies the nucleotide sequences complementary to oligonucleotides OJM3 and OJM1, which were used for primer extension mapping. The vertical arrows identify the proposed 5′ ends of the *gsi* transcripts. The presumed −10 and −35 regions are shown underlined. The putative ribosome-binding sites are doubly underlined. The proposed ComA box upstream of the *gsiA* promoter is shown in boldface. The stems of putative transcription terminator sequences are shown as converging arrowheads. The 20-amino-acid repeats of the proposed GsiB protein are shown by single underlinings.

and *gsiB* (Fig. 1A and B) were linearized and used to transform competent cells of strain SMY to antibiotic resistance. Mutations of *gsiA* and *gsiB* constructed by gene replacement were confirmed by Southern blot analysis (data not shown).

Insertion of *gsiA* at the SPβ locus. Plasmid pJPM117 (Fig. 1A) carries the complete *gsiA* operon and all of the control sequences of the *gsiA* promoter. This plasmid was constructed by ligating the 2.0-kb fragment of pJPM19 into the *Hin*dIII and *Pst*I sites of pBEST501 (17). Plasmid pJPM117 was used to transform competent cells of strain ZB307A to neomycin resistance (Neo^r) (32). The Neo^r transformants were pooled, and an SPβ transducing lysate was generated that contained a bacteriophage mixture, some of which carried pJPM117. To obtain a pure SPβ*gsiA*$^+$ lysogen, the lysate was used to infect exponential-phase cells of SPβ-sensitive strain MB186 with selection for neomycin resistance (53). A Neo^r lysogen of MB186 was used as a source of an SPβ*gsiA*$^+$ specialized transducing phage.

Measurement of β-galactosidase activity. β-Galactosidase was assayed as previously described (22, 44).

Nucleotide sequence accession numbers. The DNA sequences presented in this report have been forwarded to the EMBL, GenBank, and DDJL nucleotide sequences data libraries under accession numbers X56679 (*gsiA*) and X56680 (*gsiB*).

RESULTS

Analysis of *gsiA* and *gsiB* transcription units. We previously described the isolation of DNA segments designated *gsiA* and *gsiB* from a λZAP library of *B. subtilis* chromosomal DNA by use of cDNA probes enriched for glucose starvation-induced transcripts (27). To define the relevant transcription units more precisely, total RNA was prepared from cells growing exponentially in a medium containing 0.5% glucose and from cells harvested 1.0 h after resuspension in the same medium lacking glucose. The RNA was annealed to uniformly labeled RNA probes spanning the cloned *gsiA* and *gsiB* regions in both orientations, and hybrid formation was tested by treatment with RNase T2. The direction of transcription of *gsiA* and *gsiB* was judged by the pattern of protection.

Previous studies using cDNA probes prepared from glucose-starved cells localized the *gsiA* transcription unit to the 1.6-kb *Pvu*II-*Sau*3A fragment carried on plasmid pJPM24 (27; Fig. 1 and 2A). About 635 bases of an RNA probe transcribed from this fragment were protected following hybridization to RNA from glucose-starved cells (data not shown). This established an approximate location of the 5′ end of the *gsiA* transcriptional unit within the 0.35-kb *Pvu*II-*Acc*I fragment. The same RNA protected a segment of an antisense probe, produced by using pJPM33, that corresponded approximately to the 94 bases to the left of the *Acc*I site shown in Fig. 2A. RNA from glucose-starved cells protected approximately 440 bases of a *gsiB* probe made from plasmid pJPM18, suggesting that the 5′ end of the *gsiB* transcriptional unit lies near the leftmost *Eco*RI site (Fig. 2B). We failed to detect any signal after hybridization of labeled antisense RNA probes prepared from plasmid pJPM28. The precise location of the 5′ end of *gsiB* mRNA was resolved by nucleotide sequence analysis and primer extension experiments (see below). The *gsiA* and *gsiB* transcripts were not detectable in exponentially growing cells, and an increase in the steady-state levels of *gsiA* and *gsiB* transcripts was observed within 1.0 h after resuspension (Fig. 2A and B).

DNA sequence analysis of *gsiA* and *gsiB*. Figure 1 shows a genetic and physical map of the *gsiA* and *gsiB* regions of the *B. subtilis* chromosome. The nucleotide sequences of the 1.6-kb *Pvu*II-*Sau*3A fragment containing *gsiA* and a 1.0-kb region of the *gsiB* locus were determined on both strands (Fig. 1 and 3). The sequenced region of *gsiB* extends from the upstream *Sau*3A restriction site to 236 bp downstream of the downstream *Sau*3A restriction site (Fig. 1 and 3B). The sequence of the *gsiA* locus revealed two open reading frames, designated *gsiAA* and *gsiAB*, which are capable of encoding proteins of 378 and 44 residues, with deduced molecular weights of 44,959 and 4,793, respectively. *gsiAA* and *gsiAB* overlap by eight nucleotides and may be transcribed as an operon (Fig. 3A). The putative ATG start codons of *gsiAA* and *gsiAB* are preceded by sequences (indicated in Fig. 3) that should, in principle, serve as ribosome-binding sites. A translational fusion of *gsiAA* to *lacZ* encoded β-galactosidase activity, indicating that the *gsiAA* reading frame is used in vivo (data not shown). It is not known whether *gsiAB* actually encodes a protein.

DNA sequence analysis of the *gsiB* locus revealed a single open reading frame which could code for a protein of 123 amino acids with a calculated molecular weight of 13,789. The putative *gsiB* protein has an unusual structure consisting of multiple direct repeats organized in five tandem units of 20 amino acids (Fig. 3B). The putative start codon of *gsiB* is preceded by an apparent ribosome-binding site, and a translational fusion of *lacZ* to *gsiB* was active, indicating that this open reading frame is used in vivo (data not shown). The primary products of the *gsiA* operon and the *gsiB* gene are predicted to be markedly hydrophilic.

DNA sequence analysis also showed the presence of partial protein-coding sequences upstream of both *gsiA* and *gsiB* (Fig. 3) which had no significant homology with proteins in the GenBank data base. Furthermore, inactivation of *orfX* and *orfY* did not affect growth or sporulation.

Immediately downstream of the *gsiAB* and *gsiB* stop codons are sequences which show strong similarity to factor-independent transcription termination sites (i.e., sequences capable of coding for an RNA stem-loop structure followed by a uridine-rich sequence). The location of these sites is consistent with the apparent locations of the 3′ ends of *gsiA* and *gsiB* transcripts as determined by nuclease protection experiments (data not shown).

Mapping of the 5′ termini of *gsiA* and *gsiB* mRNAs. The results of low-resolution experiments suggested a single region of initiation for each of the transcription units and, in conjunction with DNA sequence analysis, allowed the design of chemically synthesized DNA oligonucleotides which were used to identify the mRNA start sites for *gsiA* and *gsiB* transcripts induced by glucose starvation. The glucose starvation-induced promoters for *gsiA* and *gsiB* were mapped to a region immediately upstream from GsiAA and GsiB by extension of 19-nucleotide-long synthetic primers called OJM3 and OJM1, respectively (Fig. 3). Each primer was labeled at its 5′ end, annealed to total RNA isolated from cells collected after 60 min of glucose starvation, and extended by using reverse transcriptase. The primer extension products were then electrophoresed together with dideoxynucleotide chain termination reactions carried out by using the same primer (Fig. 3A and B). The 5′ termini of *gsiA* and *gsiB* mRNAs were 37 and 21 bases upstream of their respective putative translation initiation codons (Fig. 3 and 4). No primer extension products were obtained with RNA

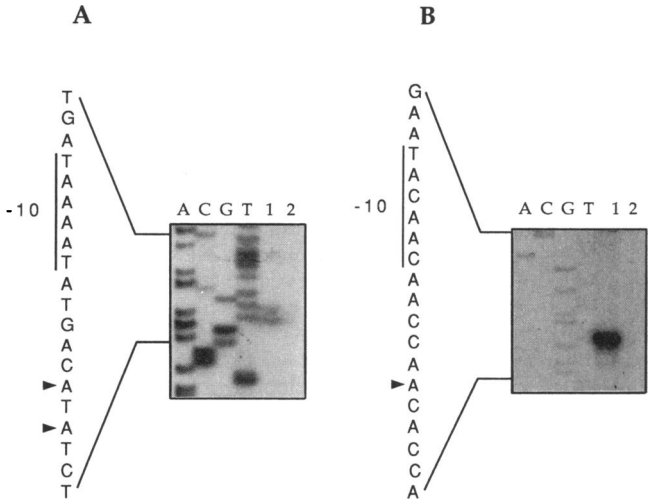


FIG. 4. Determination of the 5′ termini of the *gsiA* (A) and *gsiB* (B) transcripts by primer extension. A ^{32}P-end-labeled oligonucleotide primer was annealed to 20 μg of total cellular RNA and extended by using deoxynucleoside triphosphates and reverse transcriptase. The DNA products were separated by electrophoresis on a 5% polyacrylamide–7 M urea gel. Their mobilities were compared with a dideoxynucleotide sequencing ladder (lanes A, C, G, and T) produced with the identical primer and the sense strand of single-stranded pJPM45 (*gsiA*) or pJPM17 (*gsiB*) as the template. Strain SMY was grown in 121J medium, and RNAs were extracted from exponentially growing cells (lane 2) and at 60 min after glucose starvation (lane 1). The complement of the sequence surrounding the inferred transcription start point is indicated. The complements of the nucleotides that correspond to the start sites of *gsiA* and *gsiB* mRNAs are indicated by arrowheads.

from exponentially growing cells (Fig. 4). These findings are in accordance with the transcription start points defined by RNase protection experiments.

Figure 3 shows that the sequences upstream of the putative start sites of *gsiA* and *gsiB* mRNAs have limited homology to promoters recognized by the Eσ^A form of *B. subtilis* RNA polymerase. The −10 sequence deviated from the consensus TATAAT by one nucleotide for *gsiA* and by two nucleotides for *gsiB*. Located upstream from these possible −10 sequences are rather poor −35 sequences. Promoters that require positive regulators often have poor −35 sequences (36). We have been unable to detect transcription of *gsiA* or *gsiB* in vitro with purified Eσ^A RNA polymerase (29). The *gsiA* and *gsiB* promoter regions contain no matches to the consensus sequences for alternate forms of *B. subtilis* RNA polymerase (3, 24).

***gsiA* and *gsiB* promoters are responsive to various forms of nutrient limitation.** We monitored the transcriptional responses of the *gsiA* and *gsiB* genes during growth and sporulation by using *gsiA*- and *gsiB-lacZ* gene fusions. Fusion plasmids pJPM27 and pJPM70 (Fig. 1) were integrated into the chromosome of wild-type strain SMY, and the levels of β-galactosidase in response to various environmental conditions were measured. As expected, both genes were expressed at a low level during exponential growth in medium containing excess glucose and were induced rapidly in response to glucose limitation (Fig. 5A and D). This effect was reversed immediately by restoration of glucose to starved cells (data not shown). Expression of *gsiA* and *gsiB* was also assessed under other conditions that lead to efficient sporulation. Both promoters (in particular, *gsiA*) were induced by decoyinine (Fig. 5B and E), a purine analog that inhibits GMP synthetase (20, 23). The *gsiA-lacZ* fusion was also turned on, albeit transiently, when cells in DS medium entered the stationary phase (Fig. 5C), but under similar conditions, *gsiB*-directed β-galactosidase activity was no higher than the background level of the endogenous β-galactosidase activity normally present in *B. subtilis* (Fig. 5F).

To assess whether the *gsiA* and *gsiB* promoters were generally responsive to other forms of nutrient limitation, we examined their activities in media deficient in a single primary nutrient. The results of these experiments indicated that *gsiA-lacZ* was induced under all conditions of nutrient limitation, while the *gsiB-lacZ* fusion was induced by phosphate limitation but unaffected by nitrogen or amino acid starvation (Table 2).

Appearance of *gsiA* and *gsiB* transcripts in response to decoyinine. The rates of response of *gsiA*, *gsiB*, and other genes to decoyinine treatment were estimated by hybridization experiments. As shown in Fig. 6A and B, levels of *gsiA* and *gsiB* steady-state RNAs increased within 5 min after treatment with decoyinine. By comparison, the steady-state level of the *dciA* transcript (which encodes a dipeptide transport system) increased between 5 and 10 min after drug addition (Fig. 6A), as previously reported (21, 44). Changes in the steady-state levels of *citB* (aconitase) and *spo0H* (σ^H) RNAs were generally consistent with previous results (5, 16). The steady-state level of *veg* (a vegetative cell transcript with an unknown function) was essentially unaffected by treatment with decoyinine (Fig. 6), as expected from previous work (12a). The changes we observed reflect a very rapid and significant change in the abundance of *gsiA* and *gsiB* mRNAs in response to the environmental signal.

The *gsiA* operon is controlled by the ComP-ComA signal transduction system. Results from several laboratories are consistent with the hypothesis that the *B. subtilis* two-component sensor-regulator system *comP-comA* (49, 51) responds to the availability of the carbon and nitrogen source or to a combination of these signals. *comA* and *comP* are required for competence gene expression, surfactin production, and transcription of at least one positive regulator of degradative enzyme synthesis (26, 30, 32, 49, 51). Since *gsiA* transcription was strongly induced in response to glucose and nitrogen deprivation and since *gsiA* mutations confer a catabolite-resistant sporulation phenotype (see below), we investigated the dependence of *gsiA* expression on *comP* and *comA*. Strains MB223 (*comA124*::pTV55Δ2) and MB225 (*comPΔK1*), each of which carries a *gsiA-lacZ* transcriptional fusion at the chromosomal *amyE* locus, showed a drastic reduction in β-galactosidase activity in DS medium compared with the *com*$^+$ parent (MB39) (Fig. 7). The β-galactosidase activity directed by the *gsiA* promoter in strain MB39 increased sharply after the beginning of the stationary phase with the same induction ratio and kinetics as the corresponding fusion integrated at its normal locus, indicating that all of the *cis*-acting sequences required for *gsiA* transcription were carried by the *Pst*I-*Bgl*II fragment. Since *gsiB* is not expressed in DS medium, its dependence on ComA-ComP was not tested. The dependence of *gsiA* expression on other regulatory genes known to affect competence was also tested (6, 7, 14, 37, 50). Induction of *gsiA*-driven β-galactosidase activity in response to nutrient exhaustion was independent of *spo0A* and *abrB* (see the accompanying report [28]), *srfA*, *comQ*, *spo0KA*(*oppA*), *spo0KE*(*oppE*), *degUS*, *sin*, and *spo0H* (data not shown).

The loci *mecA* and *mecB* are defined by mutations that suppress the competence defect of *comA* mutants and allow

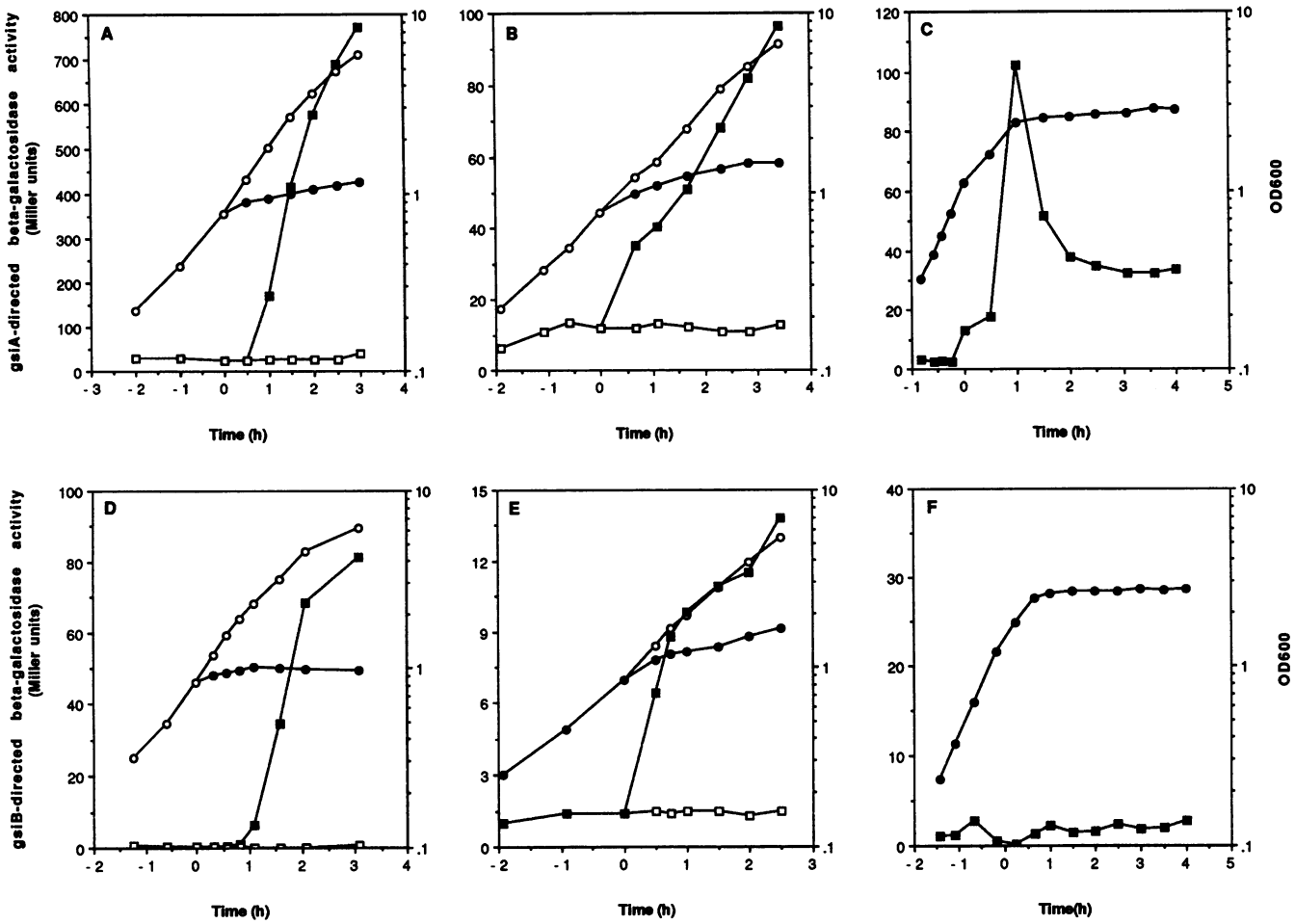


FIG. 5. Expression of β-galactosidase from *gsiA-lacZ* (A to C) and *gsiB-lacZ* (D to F) fusions in sporulating cultures of *B. subtilis* MB25 and MB60, respectively. Samples were assayed at the indicated times for growth (circles) and β-galactosidase activity (squares). Panels: A and D, 121J medium (open symbols) and 121J medium from which glucose was removed at T_0 (closed symbols); B and E, 121J medium (open symbols) and 121J medium to which decoyinine was added to 500 μg/ml at T_0 (closed symbols); C and F, DS medium (closed symbols). The abscissa is divided into hours preceding or following the end of exponential growth, which is indicated as time zero.

otherwise wild-type cells to express competence genes under inappropriate nutritional conditions (8, 37). Mutations in *mecA* suppressed the ComA dependence of *gsiA* expression and restored normal growth stage-specific regulation (Fig. 7). In a *comA mecB* mutant strain (MB259), *gsiA-lacZ* expression was partially restored (maximum activity, 15 U), compared with *comA* strain MB223 (1 to 2 U) (data not shown).

Gene inactivation of the *gsiA* and *gsiB* loci. We constructed deletion-insertion mutations in the cloned *gsiA* and *gsiB* genes in vitro (Fig. 1), which were then used to replace the wild-type alleles in the chromosome of strain SMY with a gene that confers antibiotic resistance by homologous recombination. Chromosomal deletion of *gsiA* or *gsiB* or both yielded no obvious effects on bacterial growth or viability in complex or minimal media containing various fermentable or nonfermentable carbon substrates. We saw no significant differences in the abilities of the mutant cultures to form spores (Table 3 and data not shown), establish competency, develop motility, or survive glucose starvation. The lack of a phenotype associated with deletion of *gsiA* or *gsiB* or both might result from the existence of other genes with similar or overlapping functions.

Because both *gsiA* and *gsiB* are induced by glucose deprivation, we considered a role for these genes in catabolite repression. We discovered that *gsiA* (but not *gsiB*) mutants have a defect in catabolite repression of sporulation (Crs^- phenotype). Under conditions which normally repress sporulation (2.0% glucose), a *gsiA* mutant was extremely efficient in the formation of heat-resistant endospores (Table 3). To demonstrate that the Crs^- phenotype did not result from a polar effect of the chloramphenicol resistance gene, $gsiA^+$ was restored to the Δ*gsiA* strain on a specialized tranducing phage. As shown in Table 3, the Crs^- phenotype was abolished when a wild-type copy of the *gsiA* operon was provided in *trans* at the SPβ locus. Disruption of *gsiA* did not relieve glucose repression of extracellular protease or amylase production, however, indicating that it does not cause a general release of catabolite repression. Transcription of *gsiA* was induced in stationary-phase wild-type cells in nutrient broth sporulation medium containing 1% glucose,

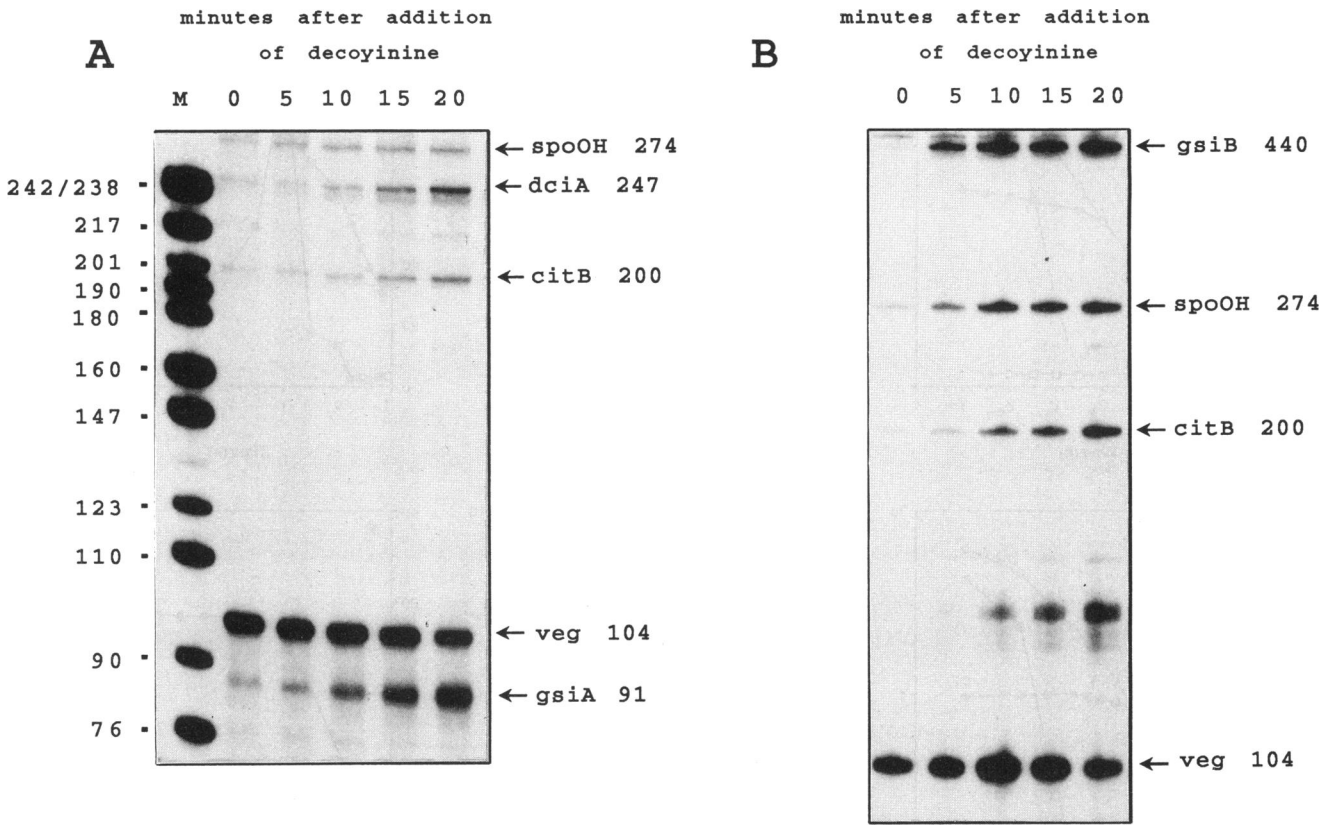


FIG. 6. Time course of appearance of sporulation-associated transcripts in response to decoyinine. Labeled antisense probes were hybridized to 20 μg of *B. subtilis* RNA purified from SMY cells in 121F medium harvested at the onset (0) and at 5-min intervals after addition of decoyinine (500 μg/ml). Hybrids were treated with RNase T2 and subjected to electrophoresis in a 5% polyacrylamide–7 M urea gel alongside end-labeled *Hpa*II fragments of plasmid pBR322 (lane M). The probes were synthesized from pBScitB (*citB*, 434 nucleotides), pCM213 (*dciA*, 738 nucleotides), pJLB4 (*spo0H*, 589 nucleotides), pJLB7 (*veg*, 390 nucleotides), pJLB10 (*gsiA*, 713 nucleotides), and pJPM17 (*gsiB*, 634 nucleotides). The numbers in parentheses designate the sizes of the antisense RNA probes. Arrowheads indicate the positions (in bases) of the protected portions of the labeled antisense RNA probes. Lane M contained molecular size markers.

but the time of peak activity was delayed by approximately 1 h (data not shown).

DISCUSSION

The *gsiA* and *gsiB* loci of *B. subtilis* were initially identified and isolated because they are induced by glucose deprivation (27). The *gsiA* locus is required for normal catabolite control of sporulation, suggesting that it plays some role in sensing or responding to carbon sources. In fact, *gsiA* and *gsiB* are induced by many different changes in nutritional status. They may respond, therefore, to a common signal generated by multiple forms of nutritional deprivation or to multiple signals specific to different stresses.

TABLE 2. Responsiveness of the *gsiA* and *gsiB* promoters to various conditions of nutrient limitation[a]

Limiting nutrient	β-Galactosidase activity (Miller units)			
	gsiA-lacZ		*gsiB-lacZ*	
	Exponential	Stationary	Exponential	Stationary
Glucose	8	771	1	74
Nitrogen	5	105	1	1
Phosphate	6	515	1	190
Casamino Acids	8	95	1	1

[a] *B. subtilis* MB25 (*gsiA-lacZ*) and MB60 (*gsiB-lacZ*) were grown in 121J or 121CG medium. Exponential-phase samples were collected when the turbidity of the culture gave an A_{600} of 0.7. Stationary-phase samples were collected approximately 3 h after resuspension of cells in medium lacking the indicated nutrient. Stationary-phase activities were normalized by subtracting the activity in control cultures. The activities shown are averages of two experiments.

We have shown that a ComA-dependent mechanism activates *gsiA* expression in response to an undefined signal associated with exhaustion of nutrient broth. Expression of *gsiA* in DS medium is transient and seems to represent a special branch of the ComP-ComA pathway, since transcription of *gsiA* is independent of the products of intermediate and late competence genes (37). ComA also appears to be necessary for induction of *gsiA* gene expression by other forms of nutrient deprivation (e.g., glucose, nitrogen, and phosphate), as well as by treatment with decoyinine (43). The similarity between various transcriptional activators and the ComA protein and the requirement for ComA in the transcription of *gsiA-lacZ* raise the possibility that ComA functions directly as a positive regulator of *gsiA* transcription as cells enter the stationary phase. Indirect mechanisms cannot be ruled out, however (see below).

The regulation and *comP-comA* dependency of *gsiA-lacZ* expression resemble those of the *degQ* gene, which codes for a putative positive regulatory protein for degradative

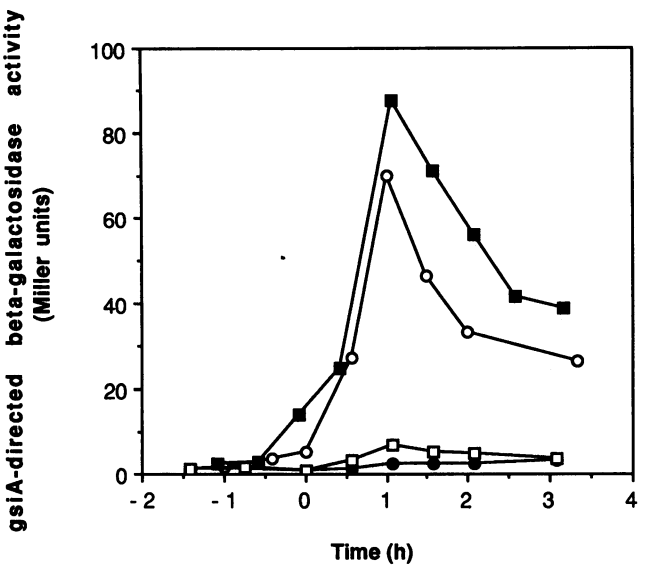


FIG. 7. Dependence of *gsiA*-directed β-galactosidase synthesis on *comA* and *comP*. Production of β-galactosidase in DS medium as a function of growth stage in wild-type, *comA124*::pTV55Δ2, and *comPΔK1* cells carrying *gsiA-lacZ*. Symbols: ○, MB39 (*com*$^+$ *ΔamyE*::*ΦgsiA-lacZ ermC*); □, MB223 (*comA124*::pTV55Δ2); ●, MB225 (*comPΔK1*); ■, MB258 (*comA124*::pTV55Δ2 *mecA42*). The abscissa is divided into hours preceding or following the end of exponential growth, which is indicated as time zero.

enzyme synthesis (25, 26). Another *B. subtilis* transcription unit whose expression is dependent on *comP-comA* is *srfA*, an operon required for production of the antibiotic surfactin. In this case, at least one gene required for establishment of competence is encoded within this large operon (18, 30, 31, 32, 48). Transcription of *srfA*, *degQ*, and *gsiA* is responsive to deprivation of glucose, nitrogen, or phosphate and to decoyinine treatment (18, 25, 26, 32). A proposed target site for ComA-dependent regulation of *degQ* and *srfA* was reported to be located in a dyad symmetry element just upstream of each promoter region (31). The sequence contains a 6-bp inverted repeat typical of sequences recognized by dimeric DNA-binding proteins containing helix-turn-helix motifs. An imperfect inverted repeat upstream of the *gsiA* promoter region (TTGCGG-N_4-CCGAAA) is homologous to the symmetrical sequence in the *degQ* and *srfA* promoter regions (Table 4). Directed mutagenesis studies have revealed that alteration of positions 3 and 4 of the CCGCAA

TABLE 3. Catabolite-resistant sporulation of a *gsiA* mutant

Medium	Relevant genotype	CFU/ml		Frequency[a]
		Total	Heat resistant	
DS	*gsiA*$^+$	4.0×10^8	2.6×10^8	0.65
	ΔgsiA69::*cat*	3.9×10^8	2.8×10^8	0.72
	ΔgsiA69::*cat* SP*βgsiA*$^+$	3.6×10^8	2.4×10^8	0.67
DS–2% glucose	*gsiA*$^+$	1.2×10^9	1.7×10^6	1.4×10^{-3}
	ΔgsiA69::*cat*	8.6×10^8	4.9×10^8	0.57
	ΔgsiA69::*cat* SP*βgsiA*$^+$	1.0×10^9	1.2×10^6	1.2×10^{-3}

[a] Heat-resistant CFU divided by total CFU. The values given are averages of at least two independent experiments.

TABLE 4. Comparison of some ComA-dependent promoter sequences

Gene[a]	Sequence	Position[b]
srfA	TTGCGGCATCCCGCAA-N_6-TTGCTGTAAATAAACT	−117
srfA	TTTCGGCATCCCGCAT-N_6-TTTCACCCATTTTTCG	−73
degQ	TTGCGGTGTCACGCAG-N_7-TTGCATACTTTTCGGT	−70
gsiA	TTGCGGTTAGCCGAAA-N_6-TTGCGGTTATTTTGCG	−75
ComA box	TTGCGGnnnnCCGCAA-N_n-TTGCnnnnATTT	
Conserved frequency	443444 344342 4434 3343	

[a] The sequence information was taken from the following sources: *degQ*, Msadek et al. (25); *srfA*, Nakano et al. (30).
[b] Location of the 5′ base relative to the start point of transcription.

motif prevent ComA-dependent transcription of *srfA* (31). These nucleotides are invariant in the putative ComA boxes identified upstream of the *srfA*, *degQ*, and *gsiA* promoter regions (Table 4). This reinforces the notion that this sequence functions as a *cis*-acting target site for activation by a ComA-dependent mechanism.

The *comP*, *comQ*, *spo0K*, and *comA* products appear to function as components of a signal transduction system that results in phosphorylation of ComA by ComP and that in turn causes increased expression of *srfA* with subsequent activation of late genes in the competence cascade (14, 33, 50). This cannot be the only pathway for phosphorylation of ComA or the only pathway derived from the activity of ComA, however. Transcription of *gsiA* and *degQ* (26), while dependent on ComA and ComP, is independent of *srfA*. Moreover, mutations in *degQ* or *gsiA* have no obvious effect on competence development (26). For *gsiA*, the necessity for positive regulation mediated by ComP-ComA can be efficiently bypassed by a *mecA* mutation. If the effect of the *mecA* mutation on *gsiA* is direct and if the allele of *mecA* tested has a gain-of-function mutation, such as a mutation that causes constitutive synthesis or activation of MecA, this result might imply either that MecA normally interacts with the *gsiA* promoter region or that MecA can acquire by mutation the ability to activate *gsiA* transcription. If the *mecA42* allele is a loss-of-function mutation, MecA might normally be a negative regulator of *gsiA* transcription whose activity must be counteracted by ComA to allow gene expression. Direct interaction between the *gsiA* promoter and ComA or MecA will be demonstrated only when it is possible to do DNA-binding experiments by using purified proteins. Regulation of the *gsiA*, *com*, and *deg* systems clearly has overlapping elements, although each system has unique features as well. For example, *com* and *deg* genes respond to both the ComA-ComP and DegU-DegS systems (albeit in different ways), while *gsiA* responds to only ComA-ComP. Thus, transcriptional control of adaptive responses in *B. subtilis* appears to involve coordination of multiple regulatory pathways.

We have little information on the precise function of GsiA; however, in the accompanying report (28) we show that the turnoff of *gsiA* transcription after the first hour of the stationary phase fails to occur in certain early-blocked sporulation (*spo0A* and *kinA*) mutants. The sporulation defect in *kinA* mutants results from overexpression of *gsiA* and indicates that a product of the *gsiA* operon is a negative regulator of an early developmental event.

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