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Transcriptional Regulation of the *Bacillus subtilis* Glucitol Dehydrogenase Gene

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The regulatory region of the *Bacillus subtilis* glucitol dehydrogenase (*gutB*) gene was divided into three subregions: a promoter, an upstream positive regulatory region, and a downstream negative regulatory region. Data from primer extension, deletion, and site-directed mutagenesis analyses were consistent with two possible models for the *gutB* promoter. It is either a σ^A-type promoter with an unusually short spacer region (15 bp) or a special σ^A promoter which requires only the hexameric −10 sequence for its function. Sequence carrying just the promoter region (from −48 to +6) failed to direct transcription in vivo. An upstream regulatory sequence was essential for glucitol induction. When this sequence was inserted in a high-copy-number plasmid, an effect characteristic of titration of a transcriptional activator was seen. Downstream from the promoter, there is an imperfect, AT-rich inverted repeat sequence. Deletion of this element did not lead to constitutive expression of *gutB*. However, the induced *gutB* expression level was enhanced three- to fourfold.

Genetic and biochemical studies demonstrated that *Bacillus subtilis* has a unique pathway for glucitol catabolism (2, 4, 11). In the presence of glucitol (also known as sorbitol) in the medium, two genes, *gutA* and *gutB*, are selectively induced. *gutA* encodes a glucitol permease which transports glucitol into the cell without any chemical modification while *gutB* encodes a glucitol dehydrogenase which oxidizes glucitol to fructose. Fructose generated through this pathway can then be sequentially phosphorylated by fructokinase and phosphofructokinase to generate fructose-1,6-diphosphate (20, 40). Both *gutB* and *gutA* were mapped to 50° on the *B. subtilis* chromosome and are likely arranged in an operon (11). Expression of these two genes is also subject to catabolite repression (22). A regulatory mutant, *gutR1*, was isolated (11). This mutant expresses *gutB* and *gutA* constitutively without glucitol. To determine molecular mechanisms controlling glucitol induction and catabolite regulation, we cloned *gutB* by screening a subgenomic library with a set of degenerate oligonucleotide probes designed according to the N-terminal amino acid sequence of the purified *B. subtilis* glucitol dehydrogenase (29). In this report, we demonstrate that transcriptional regulation is one of the mechanisms controlling glucitol induction. The regulatory region of *gutB* can be divided into three subregions. Potential functional roles for each subregion were examined.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. subtilis* 168 (*trpC2*) and *Escherichia coli* DH5α [(φ80 d*lacZ*Δ*M15*) *endA1 recA1 hsdR17* ($r^- m^-$) *supE44 thi-1* λ^- *gyrA relA1* F^- Δ(*lacZYA-argF*)*U169*] were used for routine transformations. Competent cells for these bacteria were prepared as described previously (45). *B. subtilis* plasmid pUB18 (43, 46) and *E. coli* Bluescribe plasmid (pBS from Stratagene) were used for routine subcloning. Integration vector pDH32 (10, 33, 39) was used for the construction of transcriptional fusions.

Culture and induction conditions. For glucitol induction and catabolite repression studies, cells carrying the integrated *gutB-lacZ* fusions were cultured in SMMYT medium with or without extra carbon sources as specified under the following conditions: (i) no supplement, (ii) 2% glucitol, (iii) 2% glucose, and (iv) 2% glucitol–2% glucose. SMMYT medium is the Spizizen minimal medium (1) containing 0.05% yeast extract and 40 μg of tryptophan per ml. To prepare RNA for the primer extension analysis, cells were cultured in modified superrich medium (Bacto tryptose, 2.5 g/liter; Bacto yeast extract, 2 g/liter; monobasic potassium phosphate, 3 g/liter; pH 7.5). When the cell density reached 100 Klett units, cells were divided into two sets. Glucitol was added to one set of the culture at the final concentration of 2% (wt/vol). Cells were cultured for 30 min after induction and were collected for RNA isolation. The other set of culture serves as the noninduced control. To prepare RNA for hybridization studies, cells were cultured in modified superrich medium under four different conditions specified in the legend to Fig. 1. For routine plasmid isolation from both *B. subtilis* and *E. coli*, Luria-Bertani medium was used. Kanamycin concentration was adjusted to 10 μg/ml for *B. subtilis* carrying pUB18 or its derivatives. For *E. coli* carrying Bluescribe plasmid, ampicillin at a concentration of 75 μg/ml was added to the culture medium.

Nucleic acid manipulations. RNA was isolated by the method of Gilman et al. (12) from *B. subtilis* 168. For RNA slot blot hybridization, RNA was denatured at 68°C for 15 min in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 50% formamide and 7% formaldehyde and then cooled on ice. Five and ten micrograms of denatured RNA were loaded to each slot of the manifold mounted with a piece of nitrocellulose filter. The washing, baking, prehybridization, and hybridization conditions were the same as described by Sambrook et al. (36). A 0.8-kb *Eco*RI-*Hin*dIII fragment carrying the 5′ end portion of *gutB* from pSDH1 (29) was labeled and used as the hybridization probe. Excess probes were used for the hybridization studies. The autoradiogram of the RNA slot blot was semiquantified with an LKB Ultrascan XL enhanced-laser densitometer. For primer extension, regular DNA sequencing, PCR amplification, DNA labeling with hexameric random primers, and subcloning, procedures described previously (21) were used. The 30-nucleotide (nt)-long

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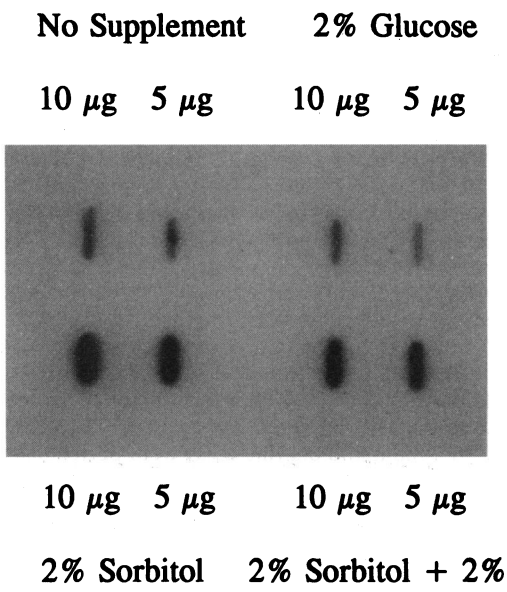


FIG. 1. RNA slot blot analysis of the *gutB* transcript. Total RNA was prepared from *B. subtilis* 168 cultured under four different conditions. Five and ten micrograms of denatured total RNA from each sample were loaded onto individual slots and hybridized with excess *gutB*-specific probe.

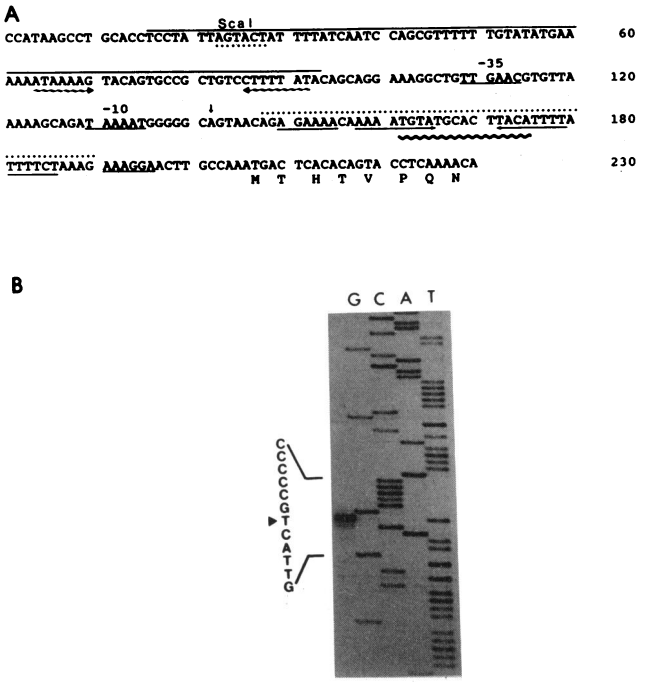


FIG. 2. Features of the *gutB* regulatory region. (A) Sequence of the *gutB* promoter region. The apparent transcription start site is indicated by an arrow. The −35 and −10 regions of the putative σ^A-type promoter and the ribosome-binding site of *gutB* are underlined. Inverted repeat sequences located upstream and downstream of the putative promoter are marked by arrows with wavy and straight lines, respectively. The first eight amino acid residues of GutB are translated, and the sequences corresponding to the *Sca*I site and the putative catabolite regulatory sequence are marked by a dotted line and a wavy line, respectively. The upstream (nt 16 to 93) and downstream (nt 148 to 190) regulatory sequences are overlined with a straight line and a dotted line, respectively. The promoter region (nt 94 to 147) is located between these upstream and downstream regulatory regions. (B) Primer extension analysis of the *gutB* transcription start site. The sequence flanking the start site is shown, and the apparent transcription start site is marked by an arrowhead.

oligonucleotide probe used in the primer extension analysis has the following sequence: 5′GTGCATAACAGCCGCTT TCATGTTTTGAGG3′. The last 10 nt of this sequence are complementary to the last 10 nt (nt 221 to 230) shown in Fig. 2A.

Construction of various single-copy *gutB-lacZ* transcription fusions. To dissect the regulatory region of *gutB*, different portions of the regulatory region were inserted into pDH32 to construct the *gutB-lacZ* transcription fusions (Fig. 3). The integration vector pDH32 (33) carries the promoterless *spoVG-lacZ* flanked by both the front and back pieces of the *B. subtilis amyE* sequence with the *cat* gene as the selection marker (Fig. 3). To construct WB1000 (Table 1), pDH32 was linearized by *Pst*I and transformed into *B. subtilis* 168. Chloramphenicol-resistant clones with the amylase-deficient phenotype were selected for further characterization. PCR amplification was performed with a pair of *amyE*-specific primers to confirm the proper integration at the *amyE* locus. To construct WB1001 to WB1006, DNA fragments covering different regulatory regions were amplified (Table 1). All these fragments have an *Eco*RI site at the 5′ end and a *Bam*HI site at the 3′ end. These fragments were electroeluted, digested with both *Eco*RI and *Bam*HI, and inserted into pDH32 to generate plasmids pGUT1 to pGUT6. Integration of these vectors into *B. subtilis* 168 generated WB1001 to WB1006.

Construction of pIGUTB and WB1007. Plasmid pIGUTB is an integration vector that allows the integration of a *gutB-lacZ* construct into the *gutB* locus. This construct is composed of three elements. (i) One is a 911-bp *Hin*dIII-*Bam*HI fragment carrying part of *gutR* (47), a regulatory gene located upstream of *gutB*, and the entire regulatory region of *gutB*. This fragment is generated by PCR amplification with a pair of primers (PRV and PIR). Chromosomal DNA from *B. subtilis* 168 was used as the template. The 3′ end of this fragment is identical to that of the fragments applied to construct pGUT1 and pGUT2. (ii) Another is a 3,160-bp *Bam*HI-*Kpn*I fragment carrying the *lacZ* reporter cassette from pDH32. This fragment is also generated by PCR amplification with the PPT and PLKPN primers. Plasmid pDH32 was used as the template. (iii) The other is a 1,628-bp *Kpn*I-*Sst*I fragment carrying the 3′ end of *gutB* (begin from nt 1052 reported in reference 29) and part of *gutA*, which is located downstream from *gutB* (48). The *Kpn*I and *Sst*I sites are introduced to the fragment by PCR with a pair of primers (PD1 and PD2). Chromosomal DNA from *B. subtilis* 168 was used as the template. Each of these fragments was inserted sequentially to the *E. coli* Bluescribe plasmid to generate a final construct, pIGUTB. To avoid any potential PCR errors in these amplified DNA fragments, each of the inserted fragments in pIGUTB was exchanged with the corresponding authentic fragments (either from the genomic clone or from pDH32) by selection of the appropriate internal restriction enzyme sites. Sequences not exchanged were then confirmed to be correct by DNA sequencing. Plasmid pIGUTB was then linearized by *Hin*dIII and cotransformed with pE194 to *B. subtilis* 168. Cells were plated on tryptose blood agar base plates containing 2% glucitol, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), erythromycin, and lincomycin (each of the antibiotics was at a final concentration of 5 μg/ml). Cells showing blue were selected, and the proper integration of this *gutB-lacZ* construct at the *gutB* locus was confirmed by

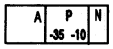
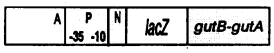
Strains	Constructs	β-gal Activity			
		N.S.	Sorb	Glu	Glu+Sorb
WB1000		0.86	0.59	0.63	0.74
WB1001		1.2	345	0.21	154
WB1002		2.3	332	0.61	178
WB1003		1.5	1254	0.70	722
WB1004		0.73	0.23	0.21	0.19
WB1005		0.95	0.18	0.27	0.27
WB1006		2.93	363	0.67	183
WB1007		1.1	356	0.24	157

FIG. 3. Dissection of the *gutB* regulatory region. WB1000 carries the *lacZ* cassette from pDH32 in the *amyE* locus. No promoter was inserted. Strains WB1001 to WB1006 carry different regions of the *gutB* regulatory sequence inserted upstream of the *lacZ* cassette. A, P, and N represent the upstream activator binding site, the promoter sequence, and the downstream negative regulatory element, respectively. In WB1005, the −10 region of the *gutB* promoter was deleted. In WB1006, part of the activator binding site was deleted. WB1007 carries the *gutB-lacZ* transcriptional fusion integrated at the *gutB* locus. The β-galactosidase activity was expressed in Miller units. Abbreviations: β-gal, β-galactosidase; N.S., no supplement; Sorb, sorbitol (glucitol); Glu, glucose.

PCR amplification. The pE194 plasmid in transformed cells was eliminated by culturing these cells at 51°C. One of the resulting strains was designated WB1007 and used for further characterization.

Site-directed mutagenesis of the *gutB* promoter. To mutate the putative −10 and −35 regions of *gutB*, an inverse PCR-mediated site-specific mutagenesis method described by Hemsley et al. (14) was used. Two pairs of mutagenic primers (P-12F/P-12B and P-31F/P-31B) were designed to mutate the −12 and −31 positions in the *gutB* promoter. Plasmid pGUT2, which carries the intact regulatory region of *gutB* (188 bp), was selected as the template. T at position −12 was changed to either G or C, and G at position −31 was changed to T. Mutated plasmids were identified by sequencing. The 188-bp regulatory region in each class of mutants was sequenced to confirm that no extra mutation was introduced into this region. Each of the mutated plasmids was then digested with *Eco*RI-*Bam*HI, and the 188-bp fragment was cloned to pDH32 to generate p-12G, p-12C, and p-31T, respectively. These plasmids were linearized by *Pst*I and transformed to *B. subtilis* 168 to generate WB1008 (with the T-12G mutation), WB1009 (with the T-12C mutation), and WB1010 (with the G-31T mutation). Integration at the *amyE* locus was confirmed by PCR amplification with a pair of *amy*-specific primers. The presence of the expected mutation in each of these strains was confirmed by direct genomic sequencing (47) with a *lacZ*-specific sequencing primer. This primer anneals specifically to the *gutB-lacZ* integrants without any interference from the original *gutB* in the *gutB* locus.

Other methods. For the β-galactosidase assay, cells were cultured to the stationary phase and harvested at 1.5 to 3 A_{595} units. Toluene treatment and enzyme assay were performed as described by Nicholson and Setlow (30). The activity was expressed in Miller units. Results reported in Table 2 and Fig. 3 are an average of three independent measurements with a standard error of ± 10%.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to GenBank with accession number L16626.

RESULTS

Transcriptional regulation of *gutB*. To determine whether the glucitol-induced *gutB* expression is regulated at the level of transcription, total RNA was isolated from *B. subtilis* 168 cultured under different conditions. RNA slot blot analysis (Fig. 1) with the *gutB* probe demonstrated that a fivefold increase in the *gutB*-specific transcript could be detected after a 30-min induction. In the absence of glucitol or in the presence of glucose as the sole carbon source in the culture medium, only a low level of *gutB* mRNA could be detected. Addition of glucose to the glucitol-containing culture medium reduced the expression of *gutB* by 1.6-fold. These results suggest that transcriptional regulation is one of the mechanisms that control the induction and catabolite repression of *gutB*.

Mapping the *gutB* promoter. As one of the steps to determine the molecular mechanism of glucitol induction, the 5′ end of *gutB* mRNA was mapped by primer extension. Figure 2A shows the nucleotide sequence within the regulatory region of *gutB*. A 30-nt primer was used for both the primer extension analysis and the generation of sequence ladders. An apparent mRNA 5′ end was found to lie 64 bp upstream from the first nucleotide of the translation initiation codon (Fig. 2B). Poten-

TABLE 1. Features of integration vectors for the construction of the WB1000 series

Strain	Integration vector	Features of the inserted regulatory sequences in pDH32[a]	Position of the amplified region[b]	Length of the amplified fragment[c]	Primers for the amplification
WB1000	pDH32	None			
WB1001	pGUT1	A + P + N	−269–190	459	P2 + PIR
WB1002	pGUT2	A + P + N	16–190	188	PRI + PIR
WB1003	pGUT3	A + P	16–147	145	PRI + PMIR
WB1004	pGUT4	P	94–147	68	PPT + PMIR
WB1005	pGUT5	A + 1/2P	16–128	127	PRI + P10
WB1006	pGUT6	SA + P	26–147	136	PSC + PMIR

[a] A, P, and N represent the upstream activator binding site, the promoter, and the downstream negative regulatory element, respectively. 1/2P represents a partial *gutB* promoter fragment containing only the −35 region. SA indicates a shortened, incomplete activator binding site.
[b] Numbered according to the sequence shown in Fig. 2A.
[c] Restriction sites were introduced to both ends.

TABLE 2. Effects of mutations at the −10 and −35 regions of the *gutB* promoter on the expression of *gutB-lacZ* transcription fusions

Strain	Mutation	β-Galactosidase sp act[a,b] (Miller units)
WB1002	Wild type	348.8
WB1008	T-12G	0.2
WB1009	T-12C	0.4
WB1010	G-31T	28.2

[a] Cells were cultured in SMMYT medium with 2% glucitol.
[b] Data represented an average of three independent experiments.

tial −10 (TAAAAT) and −35 (TTGAAC) hexameric sequences of a σ^A-type promoter could be found in this region. However, the resulting promoter would have an unusually short spacer sequence of 15 bp. This is about 2 to 3 nt shorter than the typical spacer. Downstream from the putative promoter, there is an AT-rich imperfect inverted repeat sequence located between the apparent transcription start site and the ribosome-binding site (Fig. 2A). Upstream of the transcription start site, another AT-rich inverted repeat sequence (Fig. 2A) and an 8-bp palindromic sequence which overlaps with the *Sca*I recognition sequence were found.

Dissecting regulatory elements flanking the *gutB* promoter region. To determine whether these flanking sequences are essential in regulating glucitol expression, six *gutB-lacZ* transcriptional fusions were constructed (Fig. 3) in pDH32, which carries the *spoVG-lacZ* cassette as the reporter gene. To ensure that all the constructs represent transcriptional fusions, none of the *gutB* fragments inserted into pDH32 carried the *gutB* ribosome-binding site or translation initiation codon (Table 1). All constructs were confirmed to have the right insert in the correct orientation by DNA sequencing before integration. Cells were cultured in SMMYT medium with or without glucitol or glucose. As shown in Fig. 3, *B. subtilis* 168 with the integrated pDH32 (WB1000) showed a very low level of basal β-galactosidase activity under all conditions. When either a 459-bp or a 188-bp *gutB* fragment containing the regulatory region was installed in pDH32 and integrated into the chromosome of *B. subtilis* 168 to generate WB1001 and WB1002, respectively, a 144-fold induction of β-galactosidase activity by glucitol was observed. In the presence of both glucitol and glucose, a 77-fold induction could be observed, indicating that glucose exerts a weak catabolite repression effect which results in a 1.9-fold reduction in *gutB* expression. When the downstream inverted repeat sequence was deleted (as in strain WB1003), a 3.8-fold-higher level of induction by glucitol was observed. There was no significant increase in basal β-galactosidase activity in the absence of inducer. When a 68-bp fragment containing the putative *gutB* promoter region was inserted in pDH32 (WB1004), no β-galactosidase activity could be detected under any of four different culture conditions. These data suggest that the putative promoter sequence by itself is insufficient to direct transcription. The upstream sequence is likely to be a *cis*-acting regulatory element for glucitol induction while the downstream sequence appears to be a site of negative regulation of *gutB* expression.

Integration of the *gutB-lacZ* fusion at the *gutB* locus. With the pDH32 vector, all the *gutB-lacZ* transcriptional fusions were integrated at the *amyE* locus. It is important to examine whether these fusions integrated at the *amyE* locus behaved in the same manner as they did when they were integrated at the *gutB* locus. To address this question, an integration vector, pIGUTB, was constructed. This vector carries a front portion and a back portion from *gutB* with the *lacZ* transcriptional reporter cassette inserted between these sequences. The 3′ end of the front portion of the *gutB* sequence in this construct is identical to that of the inserts applied to construct pGUT1 and PGUT2. Integration of this transcriptional fusion at the *gutB* locus in *B. subtilis* 168 generated WB1007. As shown in Fig. 3, WB1007 showed the same behavior as WB1001 and WB1002 under all the culture conditions including glucitol induction and catabolite repression. These results indicate that the *amyE* environment does not affect the expression of the *gutB-lacZ* transcriptional fusions.

The −10 region is essential for *gutB* promoter activity. Besides the σ^A-type promoter sequence, a σ^L promoter-like sequence (nt 98 to 113 [Fig. 2A]) was found around the apparent *gutB* transcription start site (25). If this promoter is indeed functional, the transcription start site should be close to but different from the one observed experimentally (Fig. 2B). To determine which promoter is more likely to be responsible for directing transcription, the −10 region of the putative σ^A-type promoter was deleted from pGUT3 to generate pGUT5 (Fig. 3 and Table 1). In the new construct, both the upstream positive regulatory sequence and the intact −24 and −12 regions for the putative σ^L promoter remain. As shown in Fig. 3, strain WB1005 carrying integrated pGUT5 showed no β-galactosidase activity above the basal level under four different culture conditions. These data confirmed that the −10 region of the putative σ^A-type promoter sequence is essential for *gutB* expression.

Site-directed mutagenesis of the −10 and −35 regions of the *gutB* promoter. To further examine the roles of putative −10 and −35 regions in *gutB* expression, site-directed mutagenesis was performed. T (italicized) at the −12 position of the conserved hexameric sequence (*T*AAAAT) was changed to either G or C. G (italicized) at the −31 position of the TT*G*AAC sequence was changed to T. These T and G nucleotides at the −10 and −35 regions were selected as targets for site-directed mutagenesis because these nucleotides in two promoters, *tms* (6) and *lacBS* (17), were shown to be important for interaction with σ^A (17). Insertion of these mutated *gutB* promoters into pDH32 and integration at the *amyE* locus generated WB1008 (T-12C), WB1009 (T-12G), and WB1010 (G-31T). WB1008 and WB1009 showed essentially no β-galactosidase activity while WB1010 had only 8% of the β-galactosidase activity produced by WB1002 (Table 2). These data support the idea that the putative −10 and −35 regions are indeed important sequences required for the expression of *gutB*.

Titration effect mediated by the upstream regulatory element inserted in a multicopy plasmid. To determine whether the upstream regulatory sequence serves as a binding site for a positive regulatory factor, two sequences which correspond to the −126 to −41 region (86 bp from nt 16 to 101) and the −126 to −14 region (nt 16 to 128), respectively, in Fig. 2A were amplified. An *Eco*RI site and a *Bam*HI site were added to the 5′ and 3′ ends of each fragment to facilitate the cloning of these fragments into pUB18, a pUB110 derivative with a copy number of 80 per cell during the logarithmic phase (32). The resulting plasmid which carries the 86-bp insert was designated pUB-ARS while the plasmid with the 113-bp insert was designated pUB-ARL. As shown in Table 3, both WB1002 [pUB-ARS] and WB1002[pUB-ARL] cultured in SMMYT medium containing 2% glucitol showed a 50% reduction in β-galactosidase activity in comparison with that of WB1002 or WB1002[pUB18]. The same degree of reduction was observed when WB1003 was used as the host. These data suggest that the upstream regulatory sequence in pUB-ARS titrates a positive regulatory factor which is present in a limited quantity.

TABLE 3. Titration of a transcription activator by introducing the activator binding site in a multicopy plasmid

Strain	β-Galactosidase activity (Miller units)	% of β-galactosidase activity
WB1002	215	99
WB1002[pUB18]	217	100
WB1002[pUB-ARS]	113	52
WB1002[pUB-ARL]	115	53
WB1003	918	97
WB1003[pUB18]	942	100
WB1003[pUB-ARS]	375	40
WB1003[pUB-ARL]	392	42

Upper boundary of the upstream regulatory sequence. The 78-bp upstream regulatory sequence in pGUT2 and pGUT3 (Fig. 3 and Table 1) was able to facilitate transcription from the *gutB* promoter. Addition of extra sequences upstream of this 78-bp region (WB1001 in Fig. 3) did not result in any further enhancement of the β-galactosidase activity under the induction condition. To define the upper boundary for a functional upstream regulatory sequence, 10 bp from the 5′ end of this sequence was deleted from pGUT3 by a *Sca*I digestion to generate pGUT6 (Table 1). Integration of pGUT6 to *B. subtilis* 168 generated WB1006. The β-galactosidase activity from WB1006 under the glucitol induction condition was only 25% of that from WB1003.

DISCUSSION

Addition of glucitol to the *B. subtilis* culture is known to induce the expression of both *gutB* and *gutA*. Expression of these genes is also subject to catabolite repression. As a first step in understanding the regulatory mechanism controlling the expression of *gutB*, the apparent transcription start site for *gutB* was mapped by primer extension. No typical promoter sequences of any kind were found in the vicinity of the transcription start site. A sequence at nt 98 to 113 (Fig. 2A) has some similarity to the consensus σ^L-type promoter (CTGGY RYR-N_4-TTGGA). The σ^L form of RNA polymerase is known to direct transcription of the *B. subtilis* levanase operon (3, 24, 25). If the above-mentioned sequence indeed functions as the −24 and −12 region of a σ^L-type promoter, the transcript start site from this promoter would be located 19 to 20 nt upstream from the mRNA 5′ end actually observed. Furthermore, insertion of this putative promoter sequence (including the upstream positive regulatory sequence) into pDH32 failed to lead to expression of *lacZ* (WB1005 in Fig. 3). Therefore, this sequence does not function as a promoter in vivo.

A hexameric sequence, TAAAAT (nt 130 to 135 in Fig. 2A), located just upstream of the apparent transcription start site, is very similar to the consensus −10 sequence of the σ^A-type promoter. However, the best match to the consensus −35 sequence of a σ^A-type promoter can only be found at nt 109 to 114 in Fig. 2A. This generates a σ^A-type promoter with a 15-bp spacer between the −10 and −35 regions. Deletion of the −10 region and site-directed mutagenesis of the −10 and −35 regions of the putative σ^A-type promoter confirmed that these sequences were indeed important for expression of *gutB*. If these sequences really constitute the *gutB* promoter, this *gutB* promoter by itself fails to direct any transcription (WB1004 in Fig. 3). The unusually short spacer is likely to be the limiting factor restricting the function of this promoter, since most σ^A-type promoters have a spacer of 17 to 18 bp (28). Presence of a short spacer sequence would require the use of activators to modulate the topology of DNA so that the promoter can be recognized by RNA polymerase. Regulation of gene expression by promoters with the unusually long or short spacer regions has been reported in *B. subtilis*, *E. coli*, and *Haemophilus influenzae*. In *Bacillus* sp. RC607 and transposon Tn*21* of *E. coli*, promoters for the mercuric ion resistance operon (*mer*) have a spacer of 20 and 19 bp, respectively (13, 23, 31). To activate these operons, the binding of Hg-MerR, a positive metalloregulatory factor, to an activator binding site located within the promoter (i.e., between the −35 and −10 regions) is required (31, 38). The Hg-MerR activator induces a DNA distortion through allosteric unwinding of the promoter DNA. In *H. influenzae*, the length of the spacer for both the *hifA* and *hifB* promoters was also suggested to vary from 14 to 18 bp to turn on and off these genes which are involved in fimbria synthesis and assembly (42).

In a second model for *gutB* transcription, the −10 region is the only sequence with which RNA polymerase interacts strongly. Upstream sequences may form the binding site for a transcriptional activator. The binding of this transcriptional activator can possibly compensate for the absence of a functional, appropriately distanced −35 sequence. In *B. subtilis*, the promoters for the *spoIIE* and *spoIIG* operons (16, 18, 49) are recognized by σ^A-associated RNA polymerase. These promoters have σ^A-type consensus −10 and −35 sequences with a 21- or 22-bp spacer region. Detailed characterization of these promoters demonstrated that the σ^A −35 recognition hexamer in these promoters actually serves as the binding site for the phosphorylated Spo0A protein rather than as a recognition site for σ^A-associated RNA polymerase (15, 37, 49). To identify the correct model, it would be necessary to attempt to suppress the deleterious effects of the −10 and −35 mutations (shown in Table 2) in an allele-specific manner by specific mutations in *sigA* (17). Although it is possible that the observed *gutB* transcription start site may be an artifact (i.e., mapping of an RNA processing site instead of the transcription start site) and the actual promoter sequence may be located further upstream, results from a deletion mutant (WB1005 in Fig. 3) and site-directed mutagenesis (Table 2) analyses are not consistent with this idea.

Both models require a positive regulatory factor which binds to an activator binding site to facilitate transcription. Deletion studies (WB1004 in Fig. 3) suggest the presence of a 78-bp *cis*-acting regulatory sequence (−126 to −47) located upstream of the *gutB* promoter. This sequence is required for glucitol induction. Introduction of a multicopy plasmid, pUB-ARS, carrying the upstream regulatory region (nt 16 to 101 in Fig. 2A) in WB1002 showed a titration effect which led to a 50% reduction in the *gutB-lacZ* expression. The observed titration effect (Table 3) is relatively weak since there should be about 80 copies of pUB-ARS per cell during the logarithmic phase of growth. Perhaps the topology of this binding site in a plasmid is not the same as that in the chromosome. Since glucitol is required for the induction, it may directly or indirectly activate the positive regulatory factor. Genetic studies demonstrate that a regulatory gene, *gutR*, is located upstream of *gutB*. A mutation in this gene results in the constitutive expression of *gutB* (11). Our recent studies suggest that GutR is a transcriptional activator (see the accompanying paper [47]) which binds specifically to the upstream activator binding site (34).

Downstream from the *gutB* transcription start site, there is an inverted repeat (region N). Deletion of this sequence results in a fourfold increase in the *gutB* expression. This sequence can potentially regulate the expression of *gutB*

through at least three possible mechanisms. It can affect the stability of the *gutB* mRNA since the 5′ end segment in mRNA has been shown to affect the stability of mRNA in both *E. coli* and *B. subtilis* (7, 26). It can also function as a transcription terminator or an operator. However, the sequence of this imperfect inverted repeat sequence is very AT rich. It is unlikely to form any stable secondary structure which allows *gutB* to be regulated by an antitermination mechanism as observed in *B. subtilis sacA* and *sacB* (19, 40). Further characterization of this sequence may provide insights for the physiological role of this sequence.

Under inducing conditions, glucitol dehydrogenase activity was reported to increase 172 times above the basal level (2). The observed 144-fold induction of *gutB-lacZ* (WB1002 in Fig. 3) expression is in close agreement with the induced glucitol dehydrogenase level (2). However, the induced *gutB* mRNA increases only fivefold as determined by the slot blot hybridization (Fig. 1). This discrepancy is mainly due to the differences in the culture media used in these studies and the timing of sample collection. For RNA preparation, cells were cultured in modified superrich medium and harvested for RNA isolation after a 30-min induction. For determining the *gutB-lacZ* expression, cells were cultured in SMMYT medium with 2% glucitol to the stationary phase and then collected for the β-galactosidase assay. To normalize these differences, we cultured WB1002 in modified superrich medium under the same induction condition as that applied for RNA isolation (i.e., a 30-min induction). The induced culture showed a sixfold increase in β-galactosidase activity (data not shown). These data were consistent with the observed increase at the RNA level. If cells were cultured to the stationary phase in modified superrich medium, the induced β-galactosidase level was found to be 80-fold higher than that of the noninduced culture. These data suggest that a higher induction level can be achieved by culturing WB1002 in SMMYT medium. It also takes longer than 30 min for the cell to achieve the full induction level. Therefore, the data from the RNA slot blot study and the *gutB-lacZ* expression analysis are, in fact, consistent with each other and indicate that glucitol induction operates at the transcriptional level.

Glucose was reported to repress glucitol dehydrogenase production to 25% of the fully induced level (22). However, *gutB-lacZ* fusions (WB1001-WB1003 and WB1007) showed a 50% (twofold) reduction in β-galactosidase activity in the presence of both glucose and glucitol. The variation of *gutB* expression under the condition of catabolite repression can be explained by two possibilities. (i) It is possible that some unknown regulatory sequences required for catabolite repression may be present within the coding region of *gutB*. Since the *gutB-lacZ* fusions do not carry any *gutB* coding sequence, one may not observe the full impact of catabolite repression. (ii) Differences in the bacterial strains and culture media used in these studies may account for the discrepancy. By direct measurement of the glucitol dehydrogenase activity in WB1002 cultured in SMMYT medium with glucitol alone or with both glucitol and glucose, a twofold reduction in glucitol dehydrogenase activity was observed (data not shown). This suggests that the second explanation may be valid. Therefore, catabolite repression in this case seems to be quite inefficient. In *B. subtilis*, there is more than one mechanism to regulate catabolite repression (8, 20, 41). At least three different sets of *cis*-acting sequences for glucose repression are found in *amyE*, *gnt*, and *citB* operons (9, 27, 44). An *amyE*-like catabolite regulatory element can be found within the promoter region of *gutB*. This sequence (TGTATGCACTTACA, nt 162 to 175 in Fig. 2A) overlaps with the inverted repeat sequence found in the negative regulatory region. However, deletion of this sequence (WB1003) did not abolish catabolite repression (Fig. 3). Since putative catabolite regulatory sequences can also be found within *gutR* (47), it is possible that catabolite regulation of *gutR* may indirectly affect *gutB* expression. It is interesting to note that ATP-dependent phosphorylation of HPr at seryl residue 46 appears to control catabolite repression of glucitol, mannitol, and gluconate operons in *B. subtilis* (35). Current data suggest that this specific phosphorylation process does not affect the transport activity of glucitol (5). Therefore, inducer exclusion is unlikely to be the mechanism responsible for the observed catabolite repression.

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