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Regulation of the *Bacillus subtilis* GlcT Antiterminator Protein by Components of the Phosphotransferase System

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***Bacillus subtilis* utilizes glucose as the preferred source of carbon and energy. The sugar is transported into the cell by a specific permease of the phosphoenolpyruvate:sugar phosphotransferase system (PTS) encoded by the *ptsGHI* operon. Expression of this operon is induced by glucose and requires the action of a positive transcription factor, the GlcT antiterminator protein. Glucose availability is sensed by glucose-specific enzyme II (EII^{Glc}), the product of *ptsG*. In the absence of inducer, the glucose permease negatively controls the activity of the antiterminator. The GlcT antiterminator has a modular structure. The isolated N-terminal part contains the RNA-binding protein and acts as a constitutively acting antiterminator. GlcT contains two PTS regulation domains (PRDs) at the C terminus. One (PRD-I) is the target of negative control exerted by EII^{Glc}. A conserved His residue (His-104 in GlcT) is involved in inactivation of GlcT in the absence of glucose. It was previously proposed that PRD-containing transcriptional antiterminators are phosphorylated and concomitantly inactivated in the absence of the substrate by their corresponding PTS permeases. The results obtained with *B. subtilis* glucose permease with site-specific mutations suggest, however, that the permease might modulate the phosphorylation reaction without being the phosphate donor.**

Bacteria are capable of utilizing many different carbohydrates as their only source of carbon and energy. The expression of the genes encoding the catabolic enzymes is in most cases induced by the specific substrate and repressed by glucose and other preferred carbon sources. Both regulatory processes are integrated by the bacterial phosphoenolpyruvate: carbohydrate phosphotransferase system (PTS) which transports and phosphorylates its sugar substrates concomitantly. Moreover, the PTS is involved in chemotaxis and a variety of regulatory mechanisms (see reference 35 for a review).

The PTS is composed of two general energy-coupling proteins, enzyme I (EI) and HPr, that serve to transfer the phosphate moiety derived from phosphoenolpyruvate to sugar-specific permeases. The specific permeases (also called enzymes II [EII]) consist of three to four domains which may exist as individual polypeptides or as a fused protein (38). While HPr from enteric bacteria is phosphorylated only by EI at the catalytically active His-15, its counterpart from gram-positive bacteria is subject to an ATP-dependent regulatory phosphorylation at Ser-46. The HPr kinase, the product of the *ptsK* gene, is stimulated by glycolytic intermediates such as fructose-1,6-bisphosphate and 2-phosphoglycerate (36). Phosphorylation of HPr at Ser-46 inhibits EI-dependent phosphorylation at His-15 about 600-fold, thus resulting in negative regulation of phosphotransferase activity of HPr (14).

The expression of several catabolic operons in bacteria is positively controlled by regulators that contain an evolutionarily conserved structural motif, called a PTS regulation domain (PRD) (see reference 46 for a recent review). These regulators act as transcriptional activators or as antiterminators. For both classes, the activity is modulated by phosphorylation of the PRDs. Interestingly, all of these regulators contain two PRD copies which are differently involved in the control of the protein's activity. The best-studied examples of this family are the activator protein LevR and the antiterminators SacY, SacT, and LicT from *Bacillus subtilis* and the *Escherichia coli* antiterminator BglG (12, 13, 41, 42, 53). The LevR activator controls expression of the *B. subtilis* levanase operon. Inducer-specific regulation is achieved by phosphorylation of a His residue strongly conserved in all PRDs by the $EIIB^{Lev}$ encoded by the levanase operon (33). In the presence of fructose, EII^{Lev} transfers its phosphate to the sugar whereas LevR is phosphorylated and thereby inactivated in its absence. In the absence of glucose, LevR is directly phosphorylated by HPr and its activity is thus stimulated (33, 47). It has been demonstrated that a single histidine (His-585) is the target of HPr-dependent phosphorylation while His-869 is phosphorylated by $EIIB^{Lev}$ (33). PRD-containing antiterminators were also shown to be phosphorylated by PTS components. As LevR, they are all negatively controlled by their corresponding EII, and some are also subject to positive control by direct HPr-dependent phosphorylation. HPr-dependent positive control was observed for the *B. subtilis* antiterminators SacT and LicT, while the SacY and GlcT antiterminators are active irrespective of the presence or absence of HPr (3, 11, 27, 48). Positive control of the PRD-containing regulators LevR and LicT is a novel mechanism of carbon catabolite repression in *B. subtilis* (25, 32, 47). Phosphorylation by HPr has been demonstrated for SacT and LicT (4, 16). Interestingly, SacY, which does not depend on HPr for activity, is also directly phosphorylated by HPr (51). Both LicT and SacY are multiply phosphorylated by HPr. The phosphorylation sites have been identified by biochemical or genetic approaches. LicT is phosphorylated twice in each PRD at the four conserved His residues. SacY is phosphorylated by HPr on three His residues, once in PRD-I and twice in PRD-II (16, 51). The consequences of mutations of the phosphorylation sites in SacY have been studied. While the sites in PRD-II were not required for activity or regulation, a replacement of His-99, the phosphoryla-

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

Strain	Relevant genotype	Source, reference, or construction[a]
168	*trpC2*	Laboratory stock
GM1221	*trpC2 pheA1* Δ*bgaX pts*$^+$ *cat amyE*::(*gntRK'-lacZ phl*)	15
QB5335	*trpC2 amyE*::(*bglP-lacZ phl*)	48
QB5330	*trpC2 levR8 ptsG56 amyE*::(ΔLA *ptsG'-'lacZ cat*)	48
QB5445	*trpC2 ptsG*::pHT181	pGP59 → 168
QB5448	*trpC2 amyE*::(ΔLA *ptsG'-'lacZ aphA3*)	48
GP100	*trpC2 ptsG*-C461D *amyE*::(*bglP-lacZ phl*)	6
GP101	*trpC2 ptsG*-H620D *amyE*::(ΔLA *ptsG'-'lacZ aphA3*)	QB5448 → GP104
GP102	*trpC2 ptsG*-C461D *amyE*::(ΔLA *ptsG'-'lacZ aphA3*)	QB5448 → GP100
GP103	*trpC2 glcT1 amyE*::(ΔLA *ptsG'-'lacZ aphA3*)	pGP102 + QB5448 → 168[b]
GP104	*trpC2 ptsG*-H620D *amyE*::(*bglP-lacZ phl*)	6
GP106	*trpC2 glcT2 amyE*::(ΔLA *ptsG'-'lacZ aphA3*)	See Results
GP108	*trpC2 glcT3 amyE*::(ΔLA *ptsG'-'lacZ aphA3*)	See Results
GP109	*trpC2* Δ*glcT8 amyE*::(ΔLA *ptsG'-'lacZ aphA3*)	See Results
GP111	*trpC2 ptsG*-ΔEIIB *amyE*::(ΔLA *ptsG'-'lacZ aphA3*)	pGP112 + QB5448 → 168[b]
GP113	*trpC2 ptsG*-ΔEIIBA *amyE*::(ΔLA *ptsG'-'lacZ aphA3*)	pGP111 + QB5448 → 168[b]
GP116	*trpC2 glcT*-T102S D103T I109Q *amyE*::(ΔLA *ptsG'-'lacZ aphA3*)	pGP116 + QB5448 → 168[b]
GP117	*trpC2 glcT*-T102S D103T *amyE*::(ΔLA *ptsG'-'lacZ aphA3*)	pGP115 + QB5448 → 168[b]
GP118	*trpC2 glcT*-I109Q *amyE*::(ΔLA *ptsG'-'lacZ aphA3*)	pGP113 + QB5448 → 168[b]
GP119	*trpC2 ptsG*-C461A *amyE*::(*bglP-lacZ phl*)	pGP119 + QB5335 → 168[b]
GP120	*trpC2 ptsG*-H620A *amyE*::(*bglP-lacZ phl*)	pGP120 + QB5335 → 168[b]
GP121	*trpC2 ptsG*-C461A *amyE*::(ΔLA *ptsG'-'lacZ aphA3*)	QB5448 → GP119
GP122	*trpC2 ptsG*-H620A *amyE*::(ΔLA *ptsG'-'lacZ aphA3*)	QB5448 → GP120

[a] Arrows indicate construction by transformation.
[b] These strains were constructed by congression as described in Materials and Methods.

tion site in PRD-I, resulted in constitutive activity of SacY (11, 51). Similarly, mutations of the corresponding His residue in PRD-I cause constitutive activity of SacT (12). Thus, the question of how negative regulation by the specific EII might be achieved arises. Phosphorylation of antiterminators in the absence of their corresponding sugar was reported for BglG from *E. coli* and SacY from *B. subtilis* (1, 24). BglG is phosphorylated on histidine residues, and phosphorylation was localized to His-208 (2, 9). The IIB domain of the β-glucoside permease was shown to directly phosphorylate BglG, while HPr was not capable of phosphorylating BglG in these experiments directly (8). Given the high degree of conservation of the PRDs and the conflicting results obtained with very similar and even functionally exchangeable antiterminators (BglG and LicT [41]), a more complex mode of inducer-specific control of the antiterminators seems to be operative.

We are interested in the regulation of glucose transport in *B. subtilis*. This sugar, which is the preferred source of carbon and energy in *B. subtilis*, is transported by the PTS. The glucose permease is encoded by the *ptsG* gene which is located upstream of the *ptsHI* operon (21, 22). Expression of the *ptsGHI* operon is induced by glucose and under control of the GlcT antiterminator (48). Regulation of glucose transport by an antiterminator might be a common regulatory mechanism in gram-positive bacteria, since a protein very similar to GlcT is also present in *Staphylococcus carnosus* (10). GlcT activity is negatively controlled by PTS components. The present study was aimed at the elucidation of negative inducer-specific control of a transcriptional antiterminator by the PTS.

MATERIALS AND METHODS

Bacterial strains and culture media. The *Bacillus subtilis* strains used in this work are listed in Table 1. *E. coli* DH5α (39) was used for cloning experiments. *E. coli* was grown in Luria broth (LB), and *B. subtilis* was grown in SP medium or C minimal medium (30) supplemented with carbon sources and auxotrophic requirements (at 100 mg/liter). CSE is C medium supplemented with potassium succinate (6 g/liter) and potassium glutamate (8 g/liter). LB or SP plates were prepared by the addition of 17 g of Bacto Agar/liter (Difco) to the medium.

Transformation, transduction, and phenotypic analysis. Standard procedures were used to transform *E. coli* (39), and transformants were selected on LB plates containing ampicillin (100 μg/ml). *B. subtilis* was transformed with plasmid or chromosomal DNA by the two-step protocol described previously (26). Transduction was performed with phage PBS1 (49). Transformants and transductants were selected on SP plates containing chloramphenicol (5 μg/ml), kanamycin (5 μg/ml), phleomycin (6 μg/ml), or erythromycin (1 μg/ml) plus lincomycin (10 μg/ml).

Quantitative studies of *lacZ* expression in *B. subtilis* were performed as follows: cells were grown in CSE medium supplemented with different carbon sources as indicated. Cells were harvested at optical densities at 600 nm of 0.6 to 0.8 for cultures in CSE medium and 0.8 to 1 for cultures in CSE medium with sugar. β-Galactosidase specific activities were determined by the method of Miller (34) with cell extracts obtained by lysozyme treatment. One unit of β-galactosidase is defined as the amount of enzyme which produces 1 nmol of *o*-nitrophenol per min at 28°C.

DNA manipulations. Plasmid DNA was prepared from *E. coli* by standard procedures (39). Restriction enzymes, T4 DNA ligase, and DNA polymerases were used as recommended by the manufacturers. DNA fragments were purified from agarose gels by using the Nucleotrap Gel Extraction kit (Macherey & Nagel, Düren, Germany).

Pfu DNA polymerase was used for the PCR as recommended by the manufacturer. DNA sequences were determined by the dideoxy-chain termination method (39).

Plasmid constructions and site-directed mutagenesis. A PCR-based approach was used to construct plasmids harboring point mutations in the *ptsG* or *glcT* gene. The strategy was followed as outlined previously (6). Plasmid pTS22 (20) containing the 3′ part of *ptsG*, *ptsH*, and the 5′ part of *ptsI*, served as the template to replace the phosphorylation sites in *ptsG*, Cys-461 and His-620, by alanine residues. The mutagenic primers were SB27 (5′ CTTGATGCTGCTATCACTC GTCTG [C461A] [the nonmatching nucleotides are underlined]) and SB28 (5′ ATTTTAATCGCCTTTGGTATTGA [H620A] [the nonmatching nucleotides are underlined]).

Plasmids harboring *ptsG* alleles with the phosphorylatable domains deleted were constructed as follows: pBluescript SK$^-$ (Stratagene Cloning Systems) was cut by *Eco*RV and *Hinc*II and religated to eliminate the *Hin*dIII restriction site. The resulting plasmid, pGP107, was linearized with *Eco*RI, and the 1,960-bp *Eco*RI fragment of pTS22 containing the 3′ part of *ptsG* was inserted into this vector to give pGP110. The 851-bp *Bgl*II-*Hin*dIII fragment of pGP110 was subsequently replaced by artificially generated *Bgl*II-*Hin*dIII fragments that result in in-frame deletions of the part of *ptsG* encoding domains IIB and IIBA. To produce the fragments, PCR products were generated with pTS22 as the template and primer pair SB2 (hybridizes in *ptsI* downstream of the *Hin*dIII site; 5′ CCGGTACGCTTTTGCAATCGC) and SB9 (5′ CAGAGAACAAGAAGATC TTGTGAAG [for the IIBA deletion]) and primer pair SB2 and SB10 (5′ GCT TCCGGAGATCTGGAAGTCGTCGGC [for the IIB deletion]). Both SB9 and

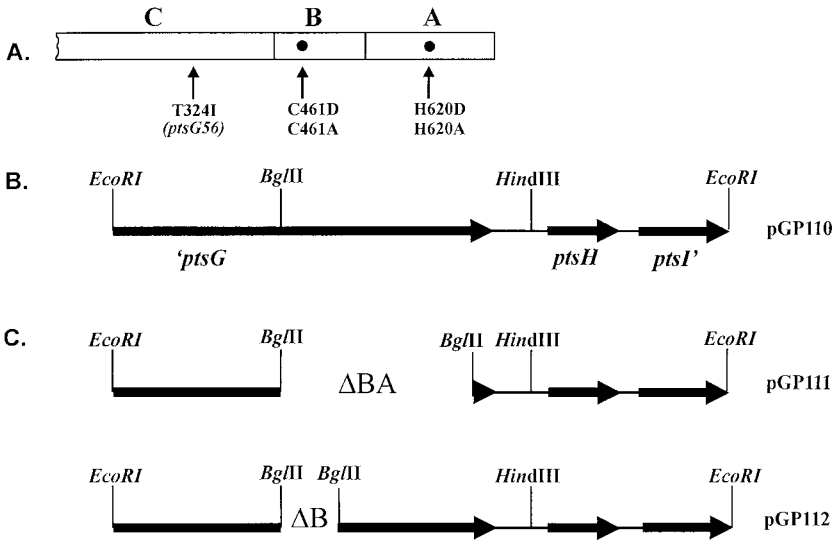

FIG. 1. Schematic presentation of mutations in *ptsG*. (A) Domain structure of EII^{Glc}. The phosphorylation sites are indicated by small solid circles, and the positions of point mutations used in this work are marked with arrows. (B) Genetic organization of the wild-type *pts* region cloned in pGP110. (C) Constructs used for the introduction of in-frame deletions in *ptsG* as described in the text.

SB10 introduced *Bgl*II sites. The PCR products were cut with *Bgl*II and *Hin*dIII and the 91- and 706-bp fragments (for the IIBA and IIB deletions, respectively, cloned into pGP110 devoid of the wild-type *Bgl*II-*Hin*dIII fragment. The resulting plasmids were pGP111 and pGP112, respectively. The genetic arrangement of the plasmids used to construct the domain deletions is outlined in Fig. 1.

Site-directed mutagenesis of *glcT* was performed by applying the same strategy as used for *ptsG* mutagenesis. The mutagenic primer was SB5 (5′ GCGTTGA CAGACGATATCGCATTTGC [H104D] [the mismatch G is underlined]), and the outer primers were JS32 (5′ GAGTGTTTGAGGCAATGG) and JS11 (48). The 1,165-bp *Eco*RI fragment of the final PCR product containing the mutated *glcT* gene was cloned into pBluescript SK^- linearized with the same enzyme to give pGP102. Mutations in the vicinity of the conserved His-104 were introduced by using oligonucleotides IL1 (5′ AAAGTCGACGTGAATGGGTCCTTCACA GTG) and JS17 (48) as outer primers and the mutagenic primers SB19 (5′ CATATCGCATTTGCGCAGAAAAGGCAG [I109Q]), SB20 (5′ GCGTTGA GTACTCATATCGCATTTGCG [T102S D103T]), and SB21 (5′ GCGTTGAG TACTCATATCGCATTTGCGCAGAAAAGGCAG [T102S D103T I109Q]). The PCR products were cut with *Eco*RI and *Sal*I, and the resulting 965-bp fragments were cloned into pBluescript SK^-. The resulting plasmids were pGP113, pGP115, and pGP116, respectively.

Plasmid pBQ200 was used for the expression of cloned genes in *B. subtilis* under control of the strong *degQ36* promoter (31). The DNA fragment encoding the putative RNA-binding domain of GlcT was amplified by PCR, using primers SB22 (5′ AAAGGATTCGAATGACAAAGGAGCTGAGGATCGTGA) and SB29 (5′ AAACTGCAGCTATTGTTCCTTCTCGTCTTTTAAAATGAAC). The *Bam*HI and *Pst*I sites that were introduced upon PCR and used to clone the fragment into pBQ200 cut with the same enzymes are underlined. A stop codon (doubly underlined) was introduced after the 60th codon of *glcT*. The resulting plasmid was pGP118.

To integrate a plasmid into the *ptsG* promoter region, pGP59 was constructed as follows. A 0.4-kb *Eco*RI fragment of pGP58 (48) was cloned into the integrative vector pHT181 (28) linearized with the same enzyme.

Construction of *B. subtilis* strains carrying mutations in *ptsG* or *glcT*. Strains containing point mutations or domain deletions in *ptsG* were constructed as described previously (6). Briefly, competent cells of *B. subtilis* were transformed simultaneously with chromosomal DNA from strain QB5335 containing a *bglP-lacZ* fusion that is linked to a phleomycin resistance gene (48) and the plasmid carrying the *ptsG* allele of interest. Transformants were selected for phleomycin resistance (Phl^r) and screened for loss of glucose repression of the *bglP-lacZ* fusion (blue colonies on SP plates containing salicin, glucose, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-Gal]). The *bglP-lacZ* fusion was subsequently replaced by a *ptsG′-′lacZ* translational fusion. Chromosomal DNA from the resulting strains was analyzed by PCR (for the domain deletions) or by DNA sequencing of the regions carrying the point mutations.

A *B. subtilis* strain containing the *glcT1* mutation replacing the presumptive regulatory histidine-104 by aspartate was constructed as follows. Competent cells of strain 168 were transformed with chromosomal DNA from strain QB5448 carrying a *ptsG′-′lacZ* fusion (48) and plasmid pGP102 harboring the *glcT1* allele. Km^r transformants were screened for altered expression of the *ptsG′-′lacZ* fusion on SP plates containing X-Gal. Blue colonies in the absence of glucose expressed the *lacZ* fusion constitutively and were further analyzed. The *glcT* allele of the presumptive mutants was amplified by PCR, and the presence of the *Eco*RV restriction site introduced upon mutagenesis was verified. All blue colonies contained the *glcT1* mutation.

All strains constructed by congression as described above were in addition tested for loss of the vector parts of the plasmids used to integrate the mutations. A PCR was performed with primers internal to the *bla* gene of pBluescript SK^- encoding ampicillin resistance using the chromosomal DNAs from the constructed strains and QB5445 (*ptsG*::pHT181) as positive control. None of the strains except QB5445 gave a positive amplification signal, confirming that the *ptsG* and *glcT* mutations were the result of double-crossover events and that the vector was lost in all cases.

RESULTS

Identification of the RNA-binding domain of GlcT. GlcT is very similar to transcriptional antiterminators of the BglG family (48). These antiterminators recognize a strongly conserved RNA sequence, the ribonucleic antiterminator (RAT) overlapping the terminators (5). The potential RAT sequences controlled by GlcT in *B. subtilis* and *S. carnosus* are, however, very different from RAT sequences recognized by other antiterminators. Genetic and structural analyses revealed recently that the N-terminal portions of the antiterminators of the BglG family are a novel class of RNA-binding domains (29, 52). We wished therefore to test whether the N terminus of GlcT would also be endowed with RNA-binding activity. A fragment of the *glcT* gene encoding the N-terminal 60 amino acids was cloned into the expression vector pBQ200 under control of the strong *degQ36* promoter. RNA-binding and antitermination activities of the expressed truncated polypeptide [GlcT(1-60)] were assayed using translational (ΔLA, −451, +63) *ptsG′-′lacZ* fusions inserted at the *amyE* locus (48). *B. subtilis* QB5448 was transformed with pGP118 and pBQ200, and the β-galactosidase activities were determined after growth in CSE minimal medium with or without glucose (Table 2). As reported previously (48), *ptsG* expression was induced by glucose in the strain containing the vector alone. The production of the N-terminal part of GlcT resulted, by contrast, in constitutive expression of the *ptsG′-′lacZ* fusion. A mutation affecting domain IIC of the glucose permease (*ptsG56*) results in loss of *ptsG* expression, since GlcT is permanently negatively regulated in this mutant

TABLE 2. Effect of expression of GlcT(1-60) on the expression of the *ptsGHI* operon in different genetic backgrounds[a]

Strain	Relevant genotype	Plasmid	β-Galactosidase activity[b]	
			CSE	CSE + Glc
QB5448	Wild type	pBQ200	28	480
		pGP118	1,025	800
QB5430	*ptsG56*	pBQ200	10	15
		pGP118	835	630
GP109	Δ*glcT8*	pBQ200	5	10
		pGP118	1,270	810

[a] All strains contain a ΔLA *ptsG′-′lacZ* translational fusion at the *amyE* locus. Cells were grown at 37°C in CSE medium with or without glucose. The β-galactosidase specific activities of the different strains were measured in extracts prepared from exponentially growing cells (optical density at 600 nm of 0.7 to 1).
[b] β-Galactosidase activity is expressed in units per milligram of protein.

(48). We have tested whether the *ptsG56* mutation present in QB5430 affects the activity of the RNA-binding domain. As shown in Table 2, no *ptsG* expression was observed when QB5430 was transformed with the empty vector pBQ200. The overproduction of GlcT(1-60) resulted in constitutive expression even in the *ptsG56* genetic background. These findings suggest that GlcT(1-60) is the RNA-binding domain of GlcT and that the RNA-binding and antitermination activities of this domain are no longer regulated by PTS components. Thus, the PRDs that have been deleted from GlcT(1-60) might control the activity of wild-type GlcT in response to the presence of glucose.

His-104 is the target of negative regulation of GlcT activity. PRD-containing transcriptional antiterminators are inactivated in the absence of the inducers of the controlled operons presumably by phosphorylation of conserved His residues. However, the target of negative control has not yet been unequivocally identified for the different antiterminators (see introduction). To address the target of glucose-specific regulation in GlcT, a mutant strain (GP103) containing the *glcT1* allele (*glcT*-H104D) was constructed by congression as described in Materials and Methods. The activity of the mutant antiterminator was assayed by determining the expression of a *ptsG′-′lacZ* fusion integrated at the *amyE* locus. As shown in Table 3, the mutant allele resulted in constitutive synthesis of β-galactosidase, suggesting that His-104 in GlcT is required for negative regulation of the antiterminator's activity.

To further analyze the interaction of PRD-I and the PTS components mediating negative regulation of GlcT in the absence of glucose, mutations affecting conserved amino acids in PRD-I around the site of negative regulation (His-104) were constructed. The active-site histidine residue is preceded by an aspartate in all PRD-I of transcriptional antiterminators. Moreover, an isoleucine is often found at the position equivalent to Ile-109 in GlcT (Fig. 2). In light of the high similarity between PRD-I and PRD-II and their functional specialization (46, 51) (Fig. 2), these amino acids might be involved in the interaction with regulatory partners or be required for the regulatory action of the PRD. We therefore constructed mutations replacing Thr-102 and Asp-103 or Ile-109 of GlcT as described in Materials and Methods. The expression of the *ptsG′-′lacZ* fusions present in the mutants was assayed after growth of the strains in CSE with or without glucose (Table 3). While Ile-109 was dispensable for negative control of GlcT activity (GP118), the residues preceding His-104 were necessary for negative regulation of GlcT (GP117). Similarly, strain GP116 that contained a triple exchange exhibited constitutive synthesis of β-galactosidase.

TABLE 3. Effects of different *glcT* mutations on the expression of the *ptsGHI* operon[a]

Strain	Relevant *glcT* allele	β-Galactosidase activity[b]	
		CSE	CSE + Glc
QB5448	Wild type	15	490
GP103	*glcT1*	895	420
GP116	*glcT*-T102S D103T I109Q	850	640
GP117	*glcT*-T102S D103T	1,030	675
GP118	*glcT*-I109Q	30	413

[a] All strains contain a ΔLA *ptsG′-′lacZ* translational fusion at the *amyE* locus. The β-galactosidase specific activities were determined as described in footnote *a* of Table 2.
[b] β-Galactosidase activity is expressed in units per milligram of protein.

Isolation and characterization of *glcT* mutations resulting in loss of *ptsG* expression. The *glcT1* mutant strain GP103 formed blue colonies on SP plates containing X-Gal irrespective of the presence or absence of glucose. On plates without glucose, white colonies were occasionally observed. For three of these mutants (GP106, GP108, GP109 [Table 1]), β-galactosidase activities were assayed after growth of the strains in CSE with or without glucose. The activities were very low in all mutant strains and not inducible by glucose (data not shown). Since β-galactosidase expression levels obtained with GP103 are not known to be toxic for *B. subtilis* and since white colonies were obtained only in the absence of glucose, it was concluded that constitutive expression of *ptsG* might be deleterious for *B. subtilis* cells in the absence of the substrate. Based on the information available about *ptsG* expression, only mutations in *glcT* or *ptsG* would prevent *ptsG* expression. Transduction experiments with PBS1 lysates of *B. subtilis* GM1221 containing a *cat* gene downstream of the *ptsGHI* operon revealed more than 90% linkage between either of the three mutations tested and the *cat* marker. Since GP103 encodes the constitutively active GlcT1 protein, mutations in *ptsG* would not affect the expression of the *ptsG′-′lacZ* fusion at the *amyE* locus and therefore not result in white colonies. The *glcT* alleles of the three mutants were amplified by PCR and sequenced. In one mutant (GP106), the *glcT* gene contained a G-to-T exchange at position 139 of the 1,169-bp *Eco*RI fragment (the numbers are as in EMBL entry Y11193). This mutation changes the proposed ribosomal binding site of *glcT*. In strain GP108, the A at position 884 was replaced by a C, causing a T245P mutation in the GlcT protein. The third mutant had an in-frame deletion from positions 323 to 797 in the *glcT* gene (corresponding to amino acids 58 to 215 in the protein). The *glcT* in-frame deletion in *B. subtilis* GP109 was complemented for *ptsG* antitermination by expressing GlcT(1-60). While no expression of *ptsG* was observed in the presence of the vector pBQ200, constitutive synthesis of β-galactosidase was found if GP109 was transformed with pGP118 (Table 2).

Effects of mutations in *ptsG* on the activity of GlcT. As observed for other antiterminators and PRD-containing transcriptional regulators, GlcT is negatively controlled by its corresponding EII, the glucose permease (48). Three classes of *ptsG* mutations with respect to GlcT activity were observed: a mutation inactivating *ptsG* resulted in constitutive expression of a *ptsG′-′lacZ* fusion, the *ptsG56* mutation affecting EIIC led to loss of *ptsG* expression, and a point mutation affecting EIIB (*ptsG21*) gave rise to weak constitutive expression of the *ptsG′-′lacZ* fusion (48). If GlcT were negatively regulated by EII^{Glc}-dependent phosphorylation as proposed for BglG from *E. coli*

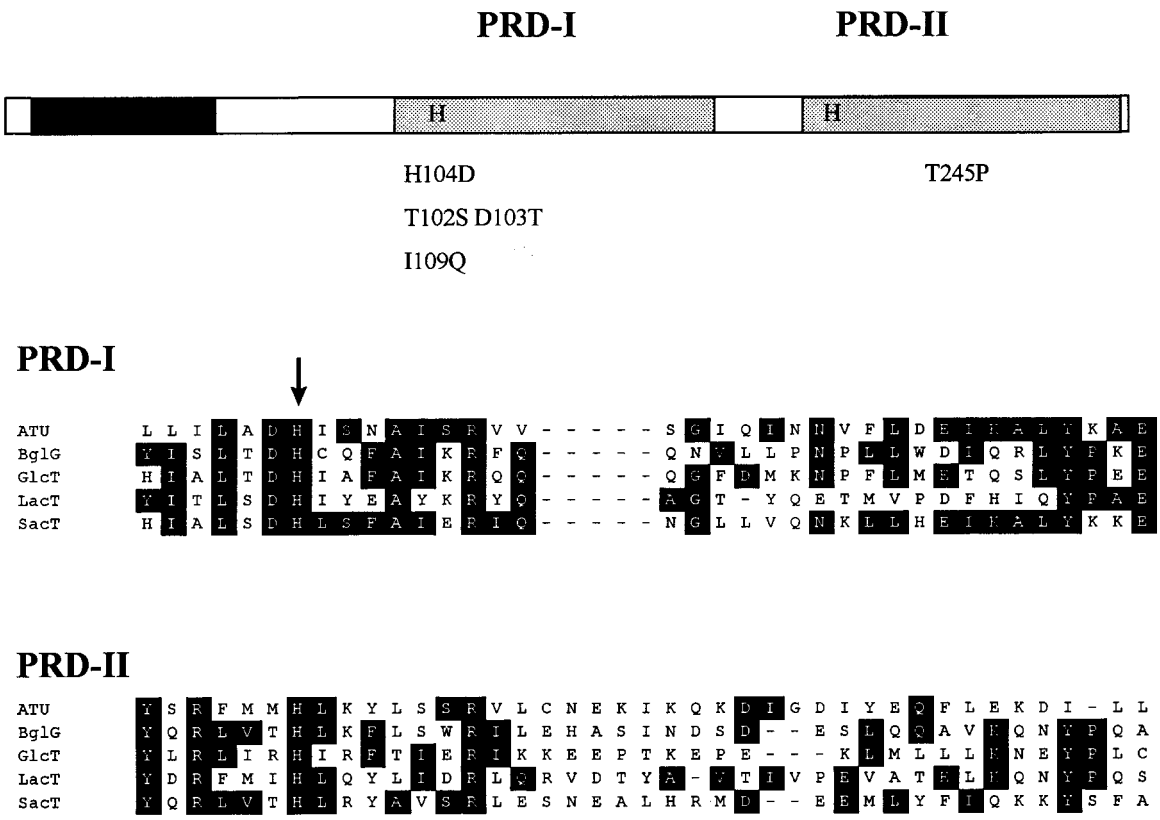

FIG. 2. Mutations in GlcT. (Top) Domain structure of GlcT with conserved histidine residues. The N-terminal RNA-binding domain is black, and the duplicated PTS regulation domains (PRD-I and PRD-II) are stippled. The positions of constructed or isolated point mutations in GlcT are indicated below the bar. (Bottom) Alignment of the regions around the conserved histidine residues in PRD-I and PRD-II. The *B. subtilis* GlcT sequence was compared to those of other known and putative antiterminator proteins with different specificities (ATU, *E. coli* antiterminator of unknown function; BglG, *E. coli* BglG; GlcT, *B. subtilis* GlcT; LacT, *Lactobacillus casei* LacT; SacT, *B. subtilis* SacT [see reference 46 for detailed references]). His residues which represent the target of negative regulation in PRD-I are indicated by an arrow. Conserved residues are boxed. Gaps introduced to maximize alignment are indicated by dashes.

and SacY from *B. subtilis* (8, 24), a mutation of the phosphorylation site active in GlcT phosphorylation would result in constitutive GlcT activity and, therefore, constitutive *ptsG* expression. In order to investigate the roles of the different domains of EII^{Glc} in the control of GlcT activity in more detail, we constructed a set of mutant strains in which the phosphorylation sites of the glucose permease were replaced by nonphosphorylatable amino acids (Fig. 1) and that contained a *ptsG'-'lacZ* fusion to assay the activity of GlcT in the mutants. The strains were grown in CSE minimal medium with or without glucose, and their β-galactosidase activities were determined (Table 4). Strains with mutations affecting the phosphorylation site of domain IIA (H620, see strains GP101 and GP122 in Table 4) exhibited inducible synthesis of β-galactosidase. Expression in the absence of glucose was, however, somewhat increased over that of the isogenic wild-type QB5448. Similarly, the replacement of the active-site cysteine of EIIB, C461, by an aspartate residue, led to inducible expression of the *ptsG'-'lacZ* fusion. In contrast, the *ptsG*-C461A mutation or in-frame deletions encompassing the part of *ptsG* which encodes EIIB resulted in nearly complete loss of regulation of GlcT activity (GP121, GP111, and GP113 [Table 4]) (Fig. 1). These findings suggest that $EIIB^{Glc}$ might be involved directly or indirectly in negative control of GlcT activity in the absence of glucose (discussed below).

DISCUSSION

Many catabolic genes in bacteria are controlled by positively acting transcriptional regulators. Positive regulators can be grouped according to their regulatory target and the mechanism of regulation they mediate as transcriptional activators and antiterminators (37, 44). Alternatively, they can be classified according to the mechanism used for sensing of the catabolic substrate. Activators such as AraC from *E. coli* or anti-

TABLE 4. Effects of different *ptsG* mutations on the expression of the *ptsGHI* operon[a]

Strain	Relevant genotype	β-Galactosidase activity[b]	
		CSE	CSE + Glc
QB5448	Wild type	15	665
GP101	*ptsG*-H620D	65	1,025
GP122	*ptsG*-H620A	140	1,255
GP102	*ptsG*-C461D	80	530
GP121	*ptsG*-C461A	200	495
GP111	*ptsG*-ΔEIIB	230	605
GP113	*ptsG*-ΔEIIBA	360	720

[a] All strains contain a ΔLA *ptsG'-'lacZ* translational fusion at the *amyE* locus. The β-galactosidase specific activities were determined as described in footnote *a* of Table 2.

[b] β-Galactosidase activity is expressed in units per milligram of protein.

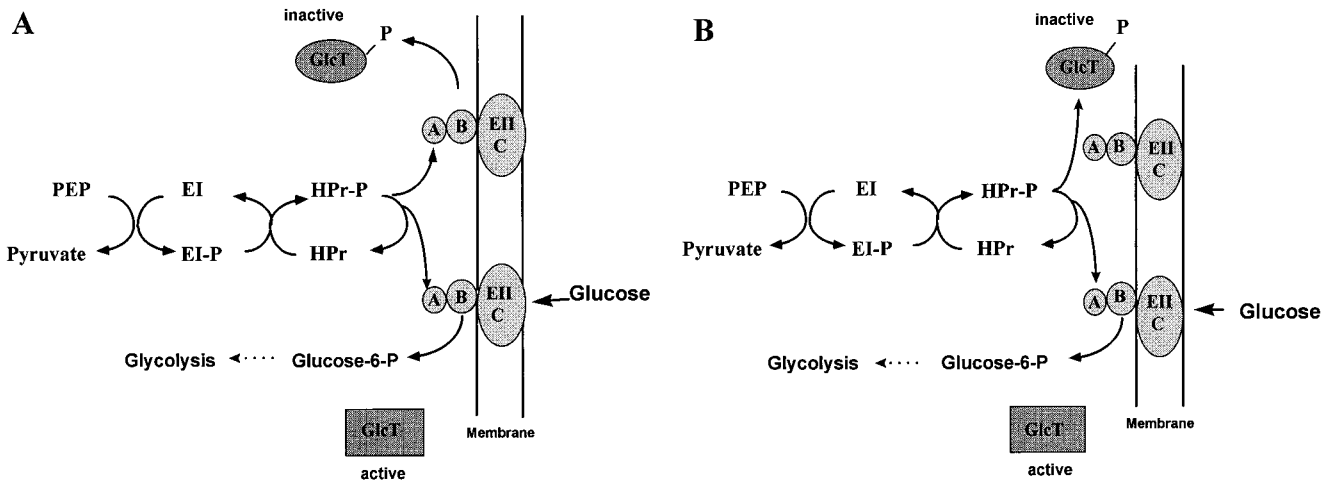

FIG. 3. Proposed models of the regulation of GlcT activity (see Discussion). (A) In the presence of glucose, a phosphate residue (P) is transferred from phosphoenolpyruvate (PEP) via EI, HPr, and EIIAB to the sugar which enters the cell upon phosphorylation. GlcT is phosphorylated by EII^{Glc} in the absence of glucose and thereby inactivated. EII^{Glc} is able to phosphorylate two different substrates, a sugar and a protein. (B) As proved in this study, HPr–His-15–P is the phosphate donor for GlcT in absence of the substrate. The presence of EII^{Glc} is essential for a successful phosphate transfer. In the absence of domain B, GlcT is constitutively active, whereas GlcT still is regulated to a certain extent in strains containing point mutations of the phosphorylation sites of PtsG.

terminators such as *B. subtilis* GlpP interact directly with their inducer (19, 23). In contrast, another class of positive regulators contains an evolutionary conserved regulatory domain, PRD, and depends on the activity of a protein kinase for inducer-specific control of activity (46). The BglG antiterminator of *E. coli* and the *B. subtilis* activator proteins LevR and LicR are the prototypes of the different PRD-containing regulators (8, 9, 33, 50).

The *B. subtilis ptsGHI* operon is induced by glucose and controlled by the transcriptional antiterminator GlcT. GlcT is a PRD-containing transcriptional regulator whose activity is regulated by the PTS in response to the availability of glucose (48). While the sequence similarity suggests that GlcT is a member of the BglG family of transcriptional antiterminators, the presumptive RNA target of GlcT in the *ptsG* mRNA leader is not similar to the RATs of other antiterminators of this family which are highly conserved (5, 37). In contrast, a potential RAT sequence very similar to that in the *B. subtilis ptsG* control region is present upstream of the *S. carnosus ptsG* gene, which seems to be regulated by an equivalent of GlcT (10). The data presented here establish that the N-terminal 60 amino acids of GlcT are sufficient for antitermination activity. Moreover, activity of the truncated polypeptide is not longer regulated by glucose. The part of GlcT that follows the RNA-binding domain might therefore serve exclusively regulatory functions.

Substrate-specific control of PRD-containing regulators is mediated by PTS-dependent phosphorylation. However, conflicting results regarding the phosphorylation site have been published. Genetic and biochemical evidence demonstrated that a conserved histidine residue in PRD-II is phosphorylated by $EIIB^{Lev}$ (LevE) in the *B. subtilis* activator protein LevR in the absence of the inducer fructose (33). Mutations of the conserved histidine residues in the antiterminators revealed that mutations in PRD-I resulted in constitutive activity of BglG, SacY, and SacT, while mutations in PRD-II had no effect (SacY) or resulted in loss of activity (SacT) (1, 11, 12, 46, 51). Thus, the genetic evidence suggests that PRD-I is the target of EII-dependent negative regulation of the antiterminators in the absence of their substrates. However, a conserved His in PRD-II was recently identified as the phosphorylation site in the *E. coli* BglG antiterminator (9). The presence of a factor Xa cleavage site close to the conserved His in PRD-I might, however, have prevented detection of phosphorylation at this site (9). The results obtained here with GlcT clearly reinforce the hypothesis that His-104 (in PRD-I) is the site of negative regulation of the antiterminator's activity.

In addition to the target of glucose-specific negative regulation of GlcT activity, it is of interest to determine the source of negative control. Studies with different *pts* mutants have shown that mutations affecting both the general (HPr) and the glucose-specific (EII^{Glc}) PTS components resulted in constitutive GlcT activity (48). To address the involvement of the glucose permease more specifically, we have analyzed the consequences of mutations of the phosphorylation sites of EII^{Glc} for the activity of GlcT. If GlcT were phosphorylated directly by $EIIA^{Glc}$ or $EIIB^{Glc}$, we would expect constitutive activity of GlcT in strains carrying the corresponding mutations. Surprisingly, both phosphorylation sites could be replaced by an aspartate without loss of negative control of GlcT in the absence of glucose. This result could be explained in two different ways. (i) The glucose permease might be involved in GlcT inactivation but not directly phosphorylate GlcT. (ii) Permeases different from the one encoded by *ptsG* might negatively control GlcT in the absence of glucose. To differentiate between these possibilities, we constructed in-frame deletions of *ptsG* resulting in loss of domain IIB or both domains IIBA. Both mutant strains exhibited constitutive activity of GlcT as monitored by the expression of a *ptsG'-'lacZ* fusion, indirectly suggesting a role of the glucose permease in control of GlcT. However, since a slight residual control of GlcT by glucose was observed even in the deletion mutants, another EII of the glucose family of the PTS might affect GlcT activity. Negative control of the *E. coli* antiterminator BglG, the prototype of PRD-containing regulators, has been extensively studied. $EIIA^{Bgl}$ and $EIIB^{Bgl}$ have both been proposed to phosphorylate and thereby inactivate BglG (8, 40). The *B. subtilis* homolog of BglG, LicT, is phosphorylated by HPr at all four conserved histidine residues in the PRDs (16). Similarly, SacY from *B. subtilis* was found to be phosphorylated by HPr on three sites, among them the conserved His in PRD-I which was identified as the target of negative regulation (51). These findings are in agreement with

the proposal that EII does not directly phosphorylate and thereby inactivate the PRD-containing antiterminator; instead, it modifies the phosphorylation activity of another factor, namely, HPr, toward the site of negative regulation in the antiterminators. In addition to the antiterminators, negative regulation of the *B. subtilis* transcriptional activator LevR has been intensively studied. This protein is inactivated in the absence of the inducer fructose by the *lev*-PTS encoded by the levanase operon. Genetic and biochemical evidence demonstrated that $EIIB^{Lev}$ phosphorylates PRD-II of LevR (7, 33). There is, however, a major difference between $EIIB^{Lev}$ and the EIIB domains of the permeases that are negatively regulating the transcriptional antiterminators: $EIIB^{Lev}$ is phosphorylated on a His residue (7), whereas the other EIIB, including $EIIB^{Glc}$ are phosphorylated on Cys residues (6, 35). Moreover, the structure of EIIB of the mannose family ($EIIB^{Man}$ and $EIIB^{Lev}$) differs substantially from the structure of $EIIB^{Glc}$ from *E. coli* (17, 18, 43, 45). Therefore, we cannot assume that a single mechanism for negative control of PRD-containing regulators is operative.

More work is required to study the molecular details of protein-protein interactions that result in inactivation of GlcT activity. Biochemical experiments to verify or falsify the models depicted in Fig. 3 are under way.

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