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Two Different Mechanisms Mediate Catabolite Repression of the *Bacillus subtilis* Levanase Operon

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There are two levels of control of the expression of the levanase operon in *Bacillus subtilis*: induction by fructose, which involves a positive regulator, LevR, and the fructose phosphotransferase system encoded by this operon (*lev*-PTS), and a global regulation, catabolite repression. The LevR activator interacts with its target, the upstream activating sequence (UAS), to stimulate the transcription of the $E\sigma^L$ complex bound at the "−12, −24" promoter. Levanase operon expression in the presence of glucose was tested in strains carrying a *ccpA* gene disruption or a *ptsH1* mutation in which Ser-46 of HPr is replaced by Ala. In a *levR*$^+$ inducible genetic background, the expression of the levanase operon was partially resistant to catabolite repression in both mutants, indicating that the CcpA repressor and the HPr-SerP protein are involved in the glucose control of this operon. In addition, a *cis*-acting catabolite-responsive element (CRE) of the levanase operon was identified and investigated by site-directed mutagenesis. The CRE sequence TGAAAACGCTT(a)ACA is located between positions −50 and −36 from the transcriptional start site, between the UAS and the −12, −24 promoter. However, in a background constitutive for levanase, neither HPr, CcpA, nor CRE is involved in glucose repression, suggesting the existence of a different pathway of glucose regulation. Using truncated LevR proteins, we showed that this CcpA-independent pathway required the presence of the domain of LevR (amino acids 411 to 689) homologous to the BglG family of bacterial antiterminators.

Many catabolic enzymes in *Bacillus subtilis* are subject to carbon catabolite repression by glucose and other rapidly metabolizable carbon sources (12). The mechanisms by which gene expression is controlled in response to nutrient availability in *B. subtilis* are not yet understood but are known to differ from the catabolite repression system in *Escherichia coli*. In *E. coli*, a positive regulatory protein, catabolite activator protein, in the presence of high intracellular levels of cyclic AMP (cAMP), activates transcription of a variety of catabolite-responsive operons (24). In addition, $EIIA^{Glc}$ of the phosphoenolpyruvate:sugar phosphotransferase system (PTS) is involved in the glucose effect by inhibiting the activity of some sugar permeases, resulting in failure to transport the inducer (inducer exclusion). By contrast, the phosphorylated form of $EIIA^{Glc}$ appears to activate adenylate cyclase, the cAMP biosynthetic enzyme (35, 38). *B. subtilis* does not contain detectable cAMP or adenyl cyclase (3).

Recently, *trans*-active mutations that led to glucose-resistant synthesis of one or several catabolic enzymes have been isolated in *B. subtilis*. The corresponding genes were identified as the σ^{43} gene *rpoD* (43); *ptsH*, encoding the histidine-containing phosphocarrier protein (HPr) of the PTS (11); and *ccpA*, encoding a putative catabolite repressor (14).

The *ptsH* gene is involved in glucose regulation in *B. subtilis*. HPr can be phosphorylated both at a histidine residue (His-15) by enzyme I in the presence of phosphoenolpyruvate (PEP) and at a serine residue (Ser-46) by an ATP-dependent protein kinase (36). The mutation *ptsH1* changes the regulatory seryl residue to alanine (HPr-S46A), so that ATP-dependent phosphorylation is not possible. This mutant is defective for catabolite repression by glucose of gluconate kinase, glucitol dehydrogenase, mannitol-1-phosphate dehydrogenase, and the mannitol-specific PTS permease (9). By contrast, the introduction of a negative charge on residue 46 of HPr (HPr-S46D) strongly inhibits the PEP-dependent phosphoryl transfer reaction in vitro (37).

A transposon insertion mutation (*gra26*::Tn*917*) in the *ccpA* gene has been obtained and causes the loss of glucose repression of α-amylase synthesis. The CcpA protein exhibits similarities to the LacI/GalR repressor family (14). A *cis*-active DNA sequence which overlaps the translational start site of the *amyE* gene has also been identified as a target of carbon catabolite repression (33). This sequence is called the catabolite-responsive element (CRE). Both the CcpA protein and the CRE sequence are involved in glucose control of several systems, including the xylose operon, the gluconate operon, the β-glucanase gene, and the histidine utilization operon (17–19, 31, 46, 47). The consensus sequence (T/A)GNAA(C/G)CGN (T/A)(T/A)NCA has been proposed for the CRE (16). This sequence is similar to the operator sites for the LacI and GalR repressors, suggesting that CcpA represses the controlled genes by binding to the CRE DNA sequence (4).

The levanase operon of *B. subtilis* (*levD*, *levE*, *levF*, *levG*, and *sacC*) encodes the proteins of a fructose-specific PTS and a levanase able to hydrolyze fructose polymers and sucrose (25, 27). The expression of this operon is induced by fructose and repressed by glucose (21, 26). The transcription of the levanase operon is controlled by (i) the RNA polymerase associated with a specific sigma factor, σ^L, which is an equivalent of the σ^{54} in gram-negative bacteria (7); (ii) an activator, LevR, which is a member of the NifA/NtrC family of regulators (6); and (iii) the LevD and LevE polypeptides, which are part of a fructose-specific PTS (27). A current model for induction is that LevR is a positive regulator inactivated by PEP-dependent phosphorylation via the LevD and LevE polypeptides when fructose

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is limiting. In the presence of fructose, LevD and LevE are involved in fructose uptake and LevR is present in an active unphosphorylated form. Interestingly, the carboxy-terminal part of LevR contains a domain (domain B) with similarities to the BglG family of bacterial antiterminators, whose activity is also controlled by the PTS (1, 2, 5, 6, 22). By testing the ability of truncated LevR activators to stimulate transcription of the levanase operon, it has recently been shown that the carboxy-terminal part of this activator, including domain B, is involved in the modulation of LevR activity by the inducer, whereas the amino-terminal part of the protein plays a role in DNA binding and transcriptional activation (29).

The mechanism by which glucose regulates the expression of the levanase operon has been partially explored (26). In this work, we report the involvement of CcpA and HPr in the glucose-mediated repression of this operon in a wild-type genetic background. A CRE-like sequence located upstream from the "−12, −24" promoter was also characterized. We also supply evidence for a different pathway of glucose regulation in a genetic background allowing constitutive expression of the levanase operon.

MATERIALS AND METHODS

Bacterial strains and culture media. The *B. subtilis* strains used in this work are listed in Table 1. *E. coli* TGI [K-12 Δ(*lac pro*) *supE thi hsd5*/F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ* ΔM15] was used for cloning experiments. *E. coli* CJ236 (*dut ung thi relA*/pCJ105 [Cm^r]) was used for oligonucleotide-directed mutagenesis.

E. coli was grown in LB broth (39), and *B. subtilis* was grown in SP medium (2), C minimal medium (27), or MM minimal medium [60 mM K_2HPO_4, 44 mM KH_2PO_4, 15 mM $(NH_4)_2SO_4$, 3 mM trisodium citrate, 2 mM $MgSO_4$, 2.2 mg of ferric ammonium citrate per liter] supplemented with carbon sources and auxotrophic requirements (at 100 mg/liter). CSK is C medium supplemented with potassium succinate (6 g/liter) and potassium glutamate (8 g/liter) (27), and C Glc is C medium supplemented with glucose (4 g/liter). LB, SP, and MM plates were prepared by the addition of 17 g of Bacto agar (Difco) per liter.

Transformation and phenotype characterization. Standard procedures were used to transform *E. coli* (39), and transformants were selected on LB plates supplemented with ampicillin (100 μg/ml). *B. subtilis* was transformed as previously described with plasmid or chromosomal DNA (27), and transformants were selected on SP plates containing chloramphenicol (Cm; 5 μg/ml), kanamycin (Km; 5 μg/ml), erythromycin (Em; 1 μg/ml) plus lincomycin (Lin; 10 μg/ml), or spectinomycin (Spc; 60 μg/ml).

In *B. subtilis*, amylase activity was detected after growth on tryptose blood agar base (TBAB; Difco) supplemented with 10 g of hydrolyzed starch per liter (Connaught). Starch degradation was detected by sublimating iodine onto the plates.

lacZ expression in liquid medium was studied as follows. Cells were grown in CSK medium supplemented with either 2 g of fructose, 10 g of glucose, 10 g of glycerol, or 10 g of glucitol per liter. Cells were harvested at an optical density at 600 nm (OD_{600}) of 0.6 to 0.8 for cultures grown in CSK and an OD_{600} of 0.8 to 1 for cultures grown in CSK with sugar. β-Galactosidase specific activities were determined by the method of Miller (30) with cell extracts obtained by lysozyme treatment. One unit of β-galactosidase activity is defined as the amount of enzyme which produces 1 nmol of *o*-nitrophenol per min at 28°C. The specific activities are expressed as averages for three to five experiments.

DNA manipulations. Standard procedures were used to extract plasmids from *E. coli* (39). Restriction enzymes, phage T4 DNA polymerase, phage T4 DNA ligase, and T4 polynucleotide kinase were used as recommended by the manufacturers. DNA fragments were purified from the agarose gel with a Prep-A-Gene kit (Bio-Rad Laboratories, Richmond, Calif.). Oligonucleotide primers were synthesized by the β-cyanoethylphosphoramidite method by using a Milligen/Biosearch Cyclone Plus synthesizer (Millipore Inc., Burlington, Mass.).

The PCR technique (32) with *Thermus aquaticus* DNA polymerase was used for amplification as previously described (28). The oligonucleotide primers used included mismatches allowing the creation of *Eco*RI and *Bam*HI restriction sites.

To perform site-directed mutagenesis, the Muta-Gene M13 in vitro mutagenesis kit (Bio-Rad) was used, and the method was based on the procedure described previously (20). M13mp18 A5 phage DNA (28), containing the pΔB (−148, +189) promoter region of the levanase operon, was used to introduce point mutations into the CRE-like sequence. The presence of the mutations was confirmed by DNA sequencing by the dideoxy chain termination method (40).

Plasmid constructions. Vector pAC5 (28), a derivative of pAF1 (13), carries the pC194 chloramphenicol resistance gene *cat* and a *lacZ* gene between two fragments of the *B. subtilis amyE* gene. pAC21 (28) is a pAC5 derivative containing a pΔB (−148, +189) *levD′-′lacZ* translational fusion.

Plasmid pRL12 is a pBQ200 derivative containing the *levR8* gene under the control of the *degQ36* promoter (29).

Plasmid pJC32 was constructed in two steps as follows. Plasmid pJC7 (27) was digested with *Eco*RV and *Sca*I. The large fragment was recircularized to give plasmid pJC31. The kanamycin resistance gene *aphA3* (45) was then introduced at the single *Cla*I site of pJC31 to give pJC32. In this plasmid, the 3′ end of the *levR* gene is disrupted by the kanamycin cassette.

Point mutations were introduced in the CRE-like sequence of the levanase operon (see above). To test the effect of these mutations on the expression of *levD′-′lacZ* fusions in *B. subtilis*, the following constructions were performed. M13 replicative forms corresponding to phage carrying each mutation were extracted and digested with *Eco*RI and *Bam*HI. The corresponding *Eco*RI-*Bam*HI fragments were purified and cloned between the *Eco*RI and *Bam*HI sites of pAC5, creating *levD′-′lacZ* fusions containing point mutations in the promoter region. These constructs were integrated into the *B. subtilis* chromosome at the *amyE* locus.

Random point mutations were obtained in the promoter region of the levanase operon by PCR (10), with pAC29 [pΔF (−48, +30)] as a template (28). The PCR product was treated with *Bam*HI and *Eco*RI and cloned into pAC5. About 400 colonies obtained after transformation of *E. coli* TGI were pooled. Plasmid DNA was extracted and used to transform *B. subtilis* 168 and QB169 (*levR8*). The resulting *B. subtilis* transformants were screened on MM plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) plus fructose and glucose or glucose, depending on the inducibility (strain 168) or constitutivity (QB169) of the strain. Isolates forming blue colonies on X-Gal plates in the presence of glucose were obtained. Chromosomal DNA from these isolates was purified, and the levanase promoter integrated at the *amyE* locus was amplified by PCR. The mutations present were then identified by direct sequencing of the PCR product.

Construction of a *B. subtilis* strain containing the *ptsH1* mutation by transduction. PBS1 transduction was performed as previously described (44). A PBS1 transducing lysate of strain SA003 (*ptsH1*) was used to transduce strain GM329 (Δ*ptsGHI*::*erm metC3 trpC2*). Met$^+$ recombinants were selected on MM plates containing glucitol (20 g/liter), glutamate (2 g/liter), and tryptophan (100 μg/ml). Met$^+$ Em^S transductants resulting from replacement of the Δ*ptsGHI*::*erm* cassette by the *ptsH1* mutation were isolated to give strain QB5223. The presence of the *ptsH1* mutation was checked by DNA sequencing.

RESULTS

Expression of the levanase operon in strains without the *levR* gene and carrying constitutive mutations in the genes encoding the *lev*-PTS. Two groups of regulatory genes are involved in induction of the levanase operon: the *levR* gene, which encodes a positive regulator, and *ptsI*, *levD*, and *levE*, whose products are involved in a fructose-PTS uptake and which act as negative regulators (27). To test the relationship between these two types of regulators, a series of strains carrying a *sacC′-lacZ*$^+$ transcriptional fusion, a *levR* gene disruption, and various constitutive mutations in the *lev*-PTS were constructed (Table 1). The strains were grown in CSK medium in the presence or absence of fructose as the inducer, and the β-galactosidase activities were measured (Table 2). The *levD6*, *levE7*, *ptsI6*, and *ptsH*::*cat* mutations allowed constitutive expression of the levanase operon, but constitutive expression was abolished in the strain with a *levR* gene disruption, indicating that the *levR* gene is epistatic over the genes *levD*, *levE*, *ptsH*, and *ptsI*.

Carbon catabolite repression of expression of the levanase operon by various sugars. The repression exerted by carbon sources (glucose, glycerol, fructose, or glucitol) on the expression of a pΔB (−148, +189) *levD′-′lacZ* translational fusion was determined in an inducible (QB5081) and in a *levR8* constitutive (QB5091) strain. The mutation *levR8* leads to the synthesis of a truncated LevR polypeptide (LevR798) (6). β-Galactosidase activities were measured after growth of strains QB5081 and QB5091 in CSK medium supplemented with sugars at a final concentration of 1% (Table 3). The expression of the levanase operon in strain QB5081 is inducible by fructose, so 0.2% fructose was also added to the culture medium as indicated in Table 3. The addition of glucose resulted in the strongest repression, with 5% residual activity in a wild-type

TABLE 1. Strains used

Strain	Genotype or description[a]	Source or reference
168	*trpC2*	Laboratory stock
QB168	*trpC2 levE7*	21
QB169	*trpC2 levR8*	21
QB2018	*leuA8 hisA1 sacA321 levD6*	21
QB5030	*trpC2 sacC'-lacZ*$^+$ *erm*	26
QB5031	*leuA8 hisA1 sacA321 levD6 sacC'-lacZ*$^+$ *erm*	26
QB5051	*trpC2 metC3 ptsI6 sacC'-lacZ*$^+$ *erm*	27
QB5072	*trpC2 levE7 sacC'-lacZ*$^+$ *erm*	QB5030 → QB168
QB5073	*leuA8 hisA1 sacA321 levD6 levR*::*aphA3 sacC'-lacZ*$^+$ *erm*	pJC32 → QB5031
QB5075	*trpC2 levE7 levR*::*aphA3 sacC'-lacZ*$^+$ *erm*	pJC32 → QB5072
QB5076	*trpC2 metC3 ptsI6 levR*::*aphA3 sacC'-lacZ*$^+$ *erm*	pJC32 → QB5051
QB5081	*trpC2 amyE*::(pΔB *levD'-'lacZ cat*)	28
QB5091	*trpC2 levR8 amyE*::(pΔB *levD'-'lacZ cat*)	28
QB5176	*trpC2* Δ*levR*::*aphA3 amyE*::(pΔB *levD'-'lacZ cat*)	29
QB5182	*trpC2* Δ*levR*::*aphA3 sacC'-lacZ*$^+$ *erm*	29
QB5185	*hisA1 leuA8 sacA321 levD6 amyE*::(pΔB *levD'-'lacZ cat*)	pAC21 → QB2018
QB5186	*trpC2 levE7 amyE*::(pΔB *levD'-'lacZ cat*)	pAC21 → QB168
QB5223[b]	*trpC2 ptsH1*	SA003 → GM329
QB5224	*trpC2 ptsH1 amyE*::(pΔB *levD'-'lacZ cat*)	pAC21 → QB5223
QB5226	*trpC2 ptsH1 levD6 amyE*::(pΔB *levD'-'lacZ cat*)	QB5185 → QB5223
QB5228	*trpC2 ptsH1 ccpA*::Tn*917*	WLN29 → QB5223
QB5229	*trpC2 ptsH1 levR8 amyE*::(pΔB *levD'-'lacZ cat*)	QB5091 → QB5223
QB5230	*trpC2 ptsH1 levE7 amyE*::(pΔB *levD'-'lacZ cat*)	QB5186 → QB5223
QB5233	*trpC2 ptsH1 ccpA*::Tn*917 amyE*::(pΔB *levD'-'lacZ cat*)	pAC21 → QB5228
QB5234	*trpC2 ccpA*::Tn*917 amyE*::(pΔB *levD'-'lacZ cat*)	WLN29 → QB5081
QB5236	*trpC2 ccpA*::Tn*917 levR8 amyE*::(pΔB *levD'-'lacZ cat*)	WLN29 → QB5091
QB5242	*trpC2* Δ*levR*::*aphA3 sacC'-lacZ*$^+$ *erm amyE*::(*levRE824* stop Δ*lacZ cat*)	29
QB5245	*trpC2* Δ*levR*::*aphA3 sacC'-lacZ*$^+$ *erm amyE*::(*levRG723* stop Δ*lacZ cat*)	29
QB5253	*trpC2* Δ*levR*::*aphA3 sacC'-lacZ*$^+$ *erm amyE*::(*levRE407* stop Δ*lacZ cat*)	29
QB5254	*trpC2* Δ*levR*::*aphA3 sacC'-lacZ*$^+$ *erm amyE*::(*levR8* Δ*lacZ cat*)	29
QB5255	*trpC2* Δ*levR*::*aphA3 sacC'-lacZ*$^+$ *erm amyE*::(*levR* Δ*lacZ cat*)	29
QB5267	*trpC2 ccpA*::Tn*917 levE7 amyE*::(pΔB *levD'-lacZ*$^+$ *cat*)	WLN29 → QB5186
QB5280	*ptsH*::*cat sacC'-lacZ*$^+$ *erm*	QB5030 → MZ303
QB5281	*ptsH*::*cat levR*::*aphA3 sacC'-lacZ*$^+$ *erm*	QB5500 → MZ303
QB5287	*trpC2* Δ*levR*::*aphA3 ccpA*::Tn*917 spc sacC'-lacZ*$^+$ *erm amyE*::(*levRE824* stop Δ*lacZ cat*)	GM1225 → QB5242
QB5288	*trpC2* Δ*levR*::*aphA3 ccpA*::Tn*917 spc sacC'-lacZ*$^+$ *erm amyE*::(*levRG723* stop Δ*lacZ cat*)	GM1225 → QB5245
QB5289	*trpC2* Δ*levR*::*aphA3 ccpA*::Tn*917 spc sacC'-lacZ*$^+$ *erm amyE*::(*levR407* stop Δ*lacZ cat*)	GM1225 → QB5253
QB5290	*trpC2* Δ*levR*::*aphA3 ccpA*::Tn*917 spc sacC'-lacZ*$^+$ *erm amyE*::(*levR8* Δ*lacZ cat*)	GM1225 → QB5254
QB5500	*trpC2 levR*::*aphA3 sacC'-lacZ*$^+$ *erm*	6
QB7000	*trpC2 amyE*::[(pΔB *levD'-'lacZ* C → A −44) *cat*]	pAC73 → 168
QB7001	*trpC2 amyE*::[(pΔB *levD'-'lacZ* C → A −37) *cat*]	pAC74 → 168
QB7002	*trpC2 amyE*::[(pΔB *levD'-'lacZ* C → T −37) *cat*]	pAC75 → 168
QB7003	*trpC2 amyE*::[(pΔB *levD'-'lacZ* ΔA −39, +A −36) *cat*]	pAC76 → 168
QB7004	*trpC2 levR8 amyE*::[(pΔB *levD'-'lacZ* C → A −44) *cat*]	pAC73 → QB169
QB7005	*trpC2 levR8 amyE*::[(pΔB *levD'-'lacZ* C → A −37) *cat*]	pAC74 → QB169
QB7006	*trpC2 levR8 amyE*::[(pΔB *levD'-'lacZ* C → T −37) *cat*]	pAC75 → QB169
QB7007	*trpC2 levR8 amyE*::[(pΔB *levD'-'lacZ* ΔA −39, +A −36) *cat*]	pAC76 → QB169
QB7008	*trpC2 levR8 amyE*::[(pΔF *levD'-'lacZ* A → G −48) *cat*]	pAC77 → QB169
QB7009	*trpC2 levR8 amyE*::[(pΔF *levD'-'lacZ* A → G −38) *cat*]	pAC78 → QB169
QB7010	*trpC2 amyE*::[(pΔF *levD'-'lacZ* A → G −48) *cat*]	pAC77 → QB168
QB7011	*trpC2 amyE*::[(pΔF *levD'-'lacZ* A → G −38) *cat*]	pAC78 → QB168
QB7012	*trpC2 amyE*::[(pΔF *levD'-'lacZ* A → G −46) *cat*]	pAC79 → QB168
QB7015	*trpC2 amyE*::[(pΔF *levD'-'lacZ* T → A −40) *cat*]	pAC80 → QB168
GM329	*trpC2 metC3* Δ*ptsGHI*::*erm*	11
GM1225	*trpC2 pheA1* Δ(*bgaX*) *amyE*::(*gnfRK'-'lacZ*) *ccpA*::Tn*917 spc*	9
MZ303	*ptsH*::*cat*	2
SA003	*trpC2 sacB'-'lacZ ptsH1*	9
WLN29	*trpC2 aroG932 ccpA*::Tn*917*	14

[a] *cat*, pC194 chloramphenicol acetyltransferase gene; *erm*, Tn*917* erythromycin resistance gene; *aphA3*, *Streptococcus faecalis* kanamycin resistance gene. The *levD6*, *levE7*, and *levR8* mutations correspond to the former *sacL6*, *sacL7*, and *sacL8* mutations (21), which are located in the *levD*, *levE*, and *levR* genes, respectively. Transcriptional fusions are indicated by *sacC'-lacZ*$^+$, and translational fusions are indicated by *levD'-'lacZ*. For clarity, the genotype of strain GM1225 was renamed *ccpA*::Tn*917 spc* instead of *ccpA*::Tn*917* Δ(*lacZ erm*). +A indicates the presence of an additional A at position −36.

[b] This strain was constructed by transduction as indicated in Materials and Methods.

inducible background and 0.8% residual activity in the *levR8* constitutive mutant. Similarly, the repression by glucitol was 3-fold in the wild-type and 30-fold in the *levR8* background. Two other constitutive mutants, *levD6* (QB5185) and *levE7* (QB5186), were also sensitive to catabolite repression, with 18 and 4% residual activity, respectively (Table 4). Glycerol affected the constitutive expression of the levanase operon (six-fold decrease) (Table 3) but not expression of the operon in

TABLE 2. Effect of a *levR* gene disruption on constitutive expression of the *sacC'-lacZ*$^+$ transcriptional fusion in *lev*-PTS mutants[a]

Strain	Relevant genotype	β-Galactosidase sp act (U/mg of protein)	
		CSK	CSK-fructose
QB5031	*sacC'-lacZ*$^+$ *levD6*	2,200	340
QB5073	*sacC'-lacZ*$^+$ *levD6 levR*::*aphA3*	2.5	0.5
QB5072	*sacC'-lacZ*$^+$ *levE7*	1,700	115
QB5075	*sacC'-lacZ*$^+$ *levE7 levR*::*aphA3*	4	0.8
QB5051	*sacC'-lacZ*$^+$ *ptsI6*	720	670
QB5076	*sacC'-lacZ*$^+$ *ptsI6 levR*::*aphA3*	2.5	2
QB5280	*sacC'-lacZ*$^+$ *ptsH*::*cat*	710	540
QB5281	*sacC'-lacZ*$^+$ *ptsH*::*cat levR*::*aphA3*	2.5	2.5

[a] Cultures were grown at 37°C in CSK medium in the presence and absence of 0.2% fructose. β-Galactosidase specific activities were determined in extracts obtained from exponentially growing cells (A_{600} of 0.6 to 0.8 for CSK and 1 for CSK with sugar).

the wild-type background. As previously observed (26), fructose seems to play a dual role in the expression of the levanase operon, as an inducer at low concentrations in the wild-type background and as a corepressor at high concentrations in the wild-type context or in constitutive mutants.

Inducible expression of the levanase operon in *ccpA* and *ptsH1* mutants. A transposon insertion in the *ccpA* gene, which codes for a protein with similarities to the LacI/GalR repressor family, and the *ptsH1* mutation, which changes the seryl 46 residue of HPr to alanine, preventing phosphorylation of the protein by an ATP-dependent protein kinase, cause the loss of glucose repression of several catabolic genes (9, 14). To investigate the role of CcpA and HPr(Ser-P) in catabolite repression of the levanase operon, a *ptsH1* mutation and a *ccpA* gene disruption (*ccpA*::Tn*917*) were introduced into strains carrying a translational *levD'-'lacZ* fusion in a wild-type inducible *levR*$^+$ background. A double *ccpA ptsH1* mutant was also obtained (Table 1). The β-galactosidase activity was measured after growth of the strains in CSK, CSK-fructose, and CSK-fructose-glucose (Table 4).

While the *ptsH*::*cat* gene disruption led to constitutive expression of the levanase operon (Table 2), the *levD'-'lacZ* fusion remains inducible in strain QB5224 (*levR*$^+$ *ptsH1*), suggesting that ATP-dependent phosphorylation of HPr is not involved in the specific regulation by fructose of the LevR activator mediated by the *lev*-PTS. This is in agreement with the active fructose uptake via the *lev*-PTS observed in the *ptsH1* mutant (data not shown).

The effect of glucose on the inducible expression of the *levD'-'lacZ* fusion was also tested in strains QB5081 (*ccpA*$^+$ *ptsH*$^+$), QB5224 (*ccpA*$^+$ *ptsH1*), and QB5234 (*ccpA*::Tn*917* *ptsH*$^+$). The β-galactosidase activity was decreased about three- and twofold by the addition of glucose in the *ptsH1* and *ccpA*::Tn*917* mutants, respectively. The corresponding decrease was 17-fold in the *ptsH*$^+$ *ccpA*$^+$ isogenic wild-type strain and 1.4-fold in the *ptsH1 ccpA* double mutant QB5233 (Table 4). The *ptsH1* mutant encoding the modified HPr-S46A and the *ccpA* mutant are partially resistant to glucose repression of the levanase operon. Therefore, HPr(Ser-P) and the CcpA repressor play a major role in carbon catabolite repression of the levanase system in the wild-type inducible background.

Glucose repression independent of CcpA and HPr(Ser-P) in constitutive mutants of the levanase operon. The role of CcpA and HPr(Ser-P) in glucose control was investigated more precisely by testing the effect of *ptsH1* and *ccpA* mutations on the regulation of the levanase operon in *levD6*, *levE7*, and *levR8* constitutive mutants. Strains containing the *ptsH1* or *ccpA* mutation, a *levD'-'lacZ* translational fusion, and *levD6*, *levE7*, or *levR8* mutations were constructed as indicated in Table 1. The doubling time in C medium in the presence of glucose was 110 min for the *ptsH1* mutants, compared with 65 min for the wild-type *ptsH*$^+$ strain. As a control, we also measured fructose uptake by the *lev*-PTS in the *levR8 ptsH*$^+$ and *levR8 ptsH1* strains. The *levR8* constitutive mutant was used to avoid uptake by the other fructose-PTS of *B. subtilis*, whose expression is inducible by fructose (27). In strain QB5229 (*levR8 ptsH1*), the uptake rate was increased twofold compared with that of QB5091 (*levR8*) (data not shown). The *ptsH1* mutant has a slightly increased uptake rate for other carbohydrates also (9, 37), and the reason for this phenomenon remains to be determined.

The effect of glucose and fructose on the constitutive expression of the levanase operon in the *ptsH1* and *ccpA* mutants was tested by comparing the β-galactosidase activity measured after growth on CSK and CSK-glucose or CSK-fructose (Table 4). The *ptsH1* mutation corresponding to the modified HPr-S46A had no effect on glucose repression of a *levD'-'lacZ* fusion in the constitutive *levR8* (QB5229), *levE7* (QB5230), and *levD6* (QB5226) backgrounds compared with that in their isogenic wild-type strains (QB5091, QB5186, and QB5185, respectively). Furthermore, the *ccpA* gene disruption did not affect glucose or fructose control of the levanase operon in the *levR8* constitutive background (QB5236). In a *levE7* mutant (QB5267), glucose repression was only partially relieved by the *ccpA* gene disruption (8-fold instead of 27-fold repression in the *ccpA*$^+$ strain) (Table 4). These results indicate that repression by glucose and fructose of the levanase operon in the *levD6*, *levE7*, and *levR8* constitutive mutants is not or is only weakly affected by the CcpA repressor and HPr(Ser-P). The existence of other pathways of regulation will be considered in the Discussion.

Characterization of the CRE sequence involved in glucose repression of expression of the levanase operon. A DNA sequence, TGAAAACGCTT(a)ACA, located between positions −50 and −36 from the transcriptional start site of the levanase operon, has similarities to the CRE involved in glucose repression of several genes in *B. subtilis* (4, 16). An additional A at position −39 is present in the levanase promoter region compared with the CRE consensus sequence (Fig. 1).

Specific deletion of the CRE-like sequence is difficult because the spacing between the −12, −24 promoter and the

TABLE 3. Effects of various carbon sources on the expression of a *levD'-'lacZ* translational fusion in wild-type and constitutive *levR8* strains[a]

Sugar(s) added to CSK	β-Galactosidase sp act (U/mg of protein)	
	QB5081 (*levD'-'lacZ*)	QB5091 (*levD'-'lacZ levR8*)
None	10	2,500
Fructose	320	60
Fructose + glucose	15	10
Glucose	8	20
Fructose + glycerol	270	17
Glycerol	5	400
Fructose + glucitol	100	10
Glucitol	8	80

[a] Cultures were grown at 37°C in CSK medium in the presence of 0.2% fructose and either glucose, glycerol, or glucitol (1%). β-Galactosidase specific activities were determined as indicated in Table 2, footnote *a*.

TABLE 4. Effects of the *ccpA*::Tn*917* and *ptsH1* (HPr-S46A) mutations on the inducible and constitutive expression of a *levD'-'lacZ* translational fusion[a]

Strain	Relevant genotype	β-Galactosidase sp act (U/mg of protein)			
		CSK	CSK-Fru	CSK-Fru-Glc	CSK-Glc
QB5081	*levD'-'lacZ*	10	300	18	7
QB5224	*levD'-'lacZ ptsH1*	14	550	200	40
QB5234	*levD'-'lacZ ccpA*::Tn*917*	15	450	250	38
QB5233	*levD'-'lacZ pstH1 ccpA*::Tn*917*	20	600	450	ND[b]
QB5185	*levD'-'lacZ levD6*	2,700	780	270	500
QB5226	*levD'-'lacZ levD6 ptsH1*	3,000	760	340	350
QB5186	*levD'-'lacZ levE7*	2,250	190	45	80
QB5230	*levD'-'lacZ levE7 ptsH1*	2,200	140	40	70
QB5267	*levD'-'lacZ levE7 ccpA*::Tn*917*	2,400	330	190	300
QB5091	*levD'-'lacZ levR8*	2,360	50	10	20
QB5229	*levD'-'lacZ levR8 pstH1*	2,700	20	7	10
QB5236	*levD'-'lacZ levR8 ccpA*::Tn*917*	2,600	30	14	25

[a] Cultures were grown at 37°C in CSK medium in the presence and absence of 0.2% fructose (CSK-Fru) and 1% glucose (CSK-Glc). β-Galactosidase specific activities were determined in extracts prepared from exponentially growing cells (OD_{600}, 0.6 to 0.8 for CSK and 0.8 to 1 for CSK with sugars).
[b] ND, not determined.

upstream activating sequence (UAS) is crucial for the activation of transcription by LevR. To test the involvement of the CRE-like sequence in catabolite repression, point mutations were therefore introduced by site-directed mutagenesis: the conserved C residues at positions −37 and −44 were substituted (Fig. 1). A CRE-like sequence lacking the additional base at position −39 was also constructed. To avoid the modification of the spacing between the promoter and the UAS, a base was added at position −36 in pAC76 (Fig. 1). The phenotypes of the corresponding mutants were analyzed after integration of the mutated pΔB (−148, +189) *levD'-'lacZ* fusion at the *amyE* locus of the wild-type and of the constitutive *levR8* strains. The β-galactosidase activities of the different CRE mutants were assayed after growth in CSK medium in the presence or absence of 0.2% fructose and 1% glucose (Table 5).

As observed for the *ccpA* gene disruption and the *ptsH1* mutation, the introduction of point mutations in the CRE-like sequence in a *levR8* mutant has no significant effect on glucose repression (Table 5). In a *levR*$^+$ background, the C → A modifications at positions −44 and −37 and a C → T change at position −37 decreased glucose regulation (3-, 6-, and 4-fold repression, respectively) compared with the wild-type CRE (16-fold). Thus, the CRE-like sequence is a *cis*-acting element in catabolite repression of the levanase operon. In addition, the induced level of β-galactosidase synthesis was increased twofold for the C → A (−44) and C → T (−37) modifications. As proposed previously (26), fructose is probably involved in two independent phenomena, induction and repression. Mutations in the CRE-like sequence may partially derepress the fructose-induced expression of the levanase operon. This dual effect of the inducer is also a possible explanation for the absence of induction when an improved CRE-like sequence lacking the additional A at position −39 is present.

Mutants were also obtained by random mutagenesis by PCR amplification of the pΔF (−148, +30) promoter region. The transformants obtained after reintroduction of the pΔF *levD'-'lacZ* fusions into *B. subtilis* were screened for increased β-galactosidase activity in the presence of glucose and X-Gal in both wild-type and constitutive *levR8* strains. Mutations at positions −48, −46, −40, and −38 were isolated (Table 5). All mutations mapped in the CRE-like sequence, confirming the involvement of CRE in the glucose repression of the levanase operon. Interestingly, A → G modifications at positions −48 and −38 were isolated in a *levR8* background, leading to increased β-galactosidase activity in both CSK and CSK-glucose. However, the glucose repression factor remained high: 90 for the A → G mutation at −48 and 77 for the A → G substitution at position −38. In the *levR*$^+$ background, all the mutations isolated led to partial derepression of the glucose effect (Table 5).

The CRE sequence between positions −50 and −36 is there-

A

CRE consensus sequence	T/A G N A A G/C C G N T/A T/A N C A
levanase promoter region	-50 T G A A A A C G C T TaA C A -36

B

Plasmid	Fusion	CRE-like sequence
pAC21	ΔB (-148,+189)	5' T G A A A A C G C T TaA C A3'
pAC73	ΔB (-148,+189)	5' T G A A A A **A** G C T TaA C A3'
pAC74	ΔB (-148,+189)	5' T G A A A A C G C T TaA **A** A3'
pAC75	ΔB (-148,+189)	5' T G A A A A C G C T TaA **T** A3'
pAC76	ΔB (-148,+189)	5' T G A A A A C G C T T A C Aa3
pAC77	ΔF (-148,+30)	5' T G **G** A A A C G C T TaA C A3'
pAC78	ΔF (-148,+30)	5' T G A A A A C G C T Ta**G** C A3'
pAC79	ΔF (-148,+30)	5' T G A A **G** A C G C T TaA C A3'
pAC80	ΔF (-148,+30)	5' T G A A A A C G C T **A**aA C A3'

FIG. 1. Catabolite repression-like element of the levanase promoter region. (A) Similarity between the levanase promoter region and the CRE consensus sequence (16, 46). The end positions of the DNA sequence of the levanase promoter region are indicated. The lowercase letter indicates the presence of an additional base. (B) Positions of point mutations in the CRE-like sequence. The nucleotide modified in the CRE-like sequence is indicated in boldface. The names of plasmids carrying pΔB *levD'-'lacZ* or pΔF *levD'-'lacZ* fusions with point mutations in the CRE sequence are indicated.

TABLE 5. Effect of mutations in the CRE-like sequence[a]

Point mutation(s) in CRE sequence	β-Galactosidase sp act (U/mg of protein)				
	Wild type			*levR8*	
	CSK	CSK-Fru	CSK-Fru-Glc	CSK	CSK-Glc
pΔB *levD'-'lacZ* wild type	9	390	25	1,800	10
C → A (−44)	8	890	290	1,650	25
C → A (−37)	8	450	80	1,830	16
C → T (−37)	11	800	200	1,600	25
ΔA (−39), +A (−36)	6	10	1	370	3
pΔF *levD'-'lacZ* wild type	5	120	7	4,700	15
A → G (−48)	13	960	211	23,700	260
A → G (−46)	14	780	260	ND[b]	ND
T → A (−40)	18	900	250	ND	ND
A → G (−38)	17	1,600	320	7,000	90

[a] The mutated pΔB *levD'-'lacZ* and pΔF *levD'-'lacZ* translational fusions were introduced at the *amyE* locus of the wild-type and the constitutive *levR8* strains. The mutants obtained are listed in Table 1. β-Galactosidase activities were determined as described in Table 4, footnote *a*.
[b] ND, not determined.

fore a *cis*-acting element involved in glucose repression of the levanase operon. All the mutations isolated after random mutagenesis mapped in this CRE sequence even when screened in a *levR8* mutant. It is thus unlikely that another *cis*-acting sequence is present in the pΔF (−148, +30) fragment of the levanase promoter.

CcpA requirement for catabolite repression in strains containing truncated LevR polypeptides. Regulation by glucose of the levanase operon was affected by a *ccpA* gene disruption in the presence of a wild-type LevR but not in the presence of the truncated LevR798 polypeptide (*levR8*). Truncated LevR proteins which are active when present in monocopy, leading to constitutive expression of the levanase operon, have been identified (29). A *ccpA* gene disruption by a cassette conferring resistance to spectinomycin was introduced into strains containing a *sacC'-lacZ*$^+$ transcriptional fusion and various single-copy *levR* alleles at the *amyE* locus. β-Galactosidase activities were measured in CSK medium in the presence and absence of 1% glucose (Table 6). In a *ccpA*$^+$ background, the expression of the levanase operon in the presence of LevR824, LevR798, LevR723, and LevR407 (Fig. 2) was repressed by glucose about 50-, 160-, 100-, and 35-fold, respectively. The *ccpA* gene disruption had no effect on glucose repression in strains containing LevR824, LevR798, or LevR723 (Table 6). The expression of the levanase operon in the presence of LevR407 was repressed only 2-fold in a strain carrying the *ccpA* gene disruption but 35-fold in a *ccpA*$^+$ isogenic strain. Both the constitutive LevR407 polypeptide containing only domain A and the wild-type LevR$^+$ polypeptide were sensitive to the *ccpA* pathway for catabolite repression. Thus, the carboxy-terminal part of LevR, including domain B, is necessary for catabolite repression independent of CcpA.

We tested the effect of overproduction of the LevR798 polypeptide on glucose repression. The *levR8* gene was expressed under the control of the strong promoter *degQ36* on a multicopy plasmid (pRL12) in strain QB5176 containing a *levR* gene disruption and a *levD'-'lacZ* translational fusion. The β-galactosidase activity measured after growth in CSK was 1,200 U/mg of protein in the absence of glucose and 250 U/mg of protein in the presence of glucose. The repression by glucose was therefore lower when LevR798 was overproduced (5-fold reduction) than when it was produced from a single copy (160-fold) (Table 6). This phenomenon was also observed for LevR$^+$ (data not shown).

TABLE 6. Effect of a *ccpA* gene disruption on the expression of a *sacC'-lacZ*$^+$ transcriptional fusion in strains encoding truncated LevR polypeptides[a]

Truncated LevR polypeptide	*ccpA* allele	β-Galactosidase sp act (U/mg of protein)	
		CSK	CSK-Glc
LevR824	*ccpA*$^+$	95	2
	ccpA::Tn*917 spc*	85	2.5
LevR798	*ccpA*$^+$	1,025	6.5
	ccpA::Tn*917 spc*	830	4
LevR723	*ccpA*$^+$	260	2.5
	ccpA::Tn*917 spc*	235	3
LevR407	*ccpA*$^+$	180	5
	ccpA::Tn*917 spc*	80	42

[a] Growth conditions and β-galactosidase determinations are described in Table 4, footnote *a*. The strains used are described in Table 1. A map of the truncated LevR polypeptides is presented in Fig. 2.

DISCUSSION

In gram-positive bacteria, the HPr protein is phosphorylated twice, at a histidine residue (His-15) by EI in the presence of PEP, and at a serine residue (Ser-46) by an ATP-dependent kinase. Induction of the levanase operon in the presence of fructose may involve PEP-dependent phosphorylation via the *lev*-PTS (27, 42). EI, HPr, LevD, and LevE may contribute

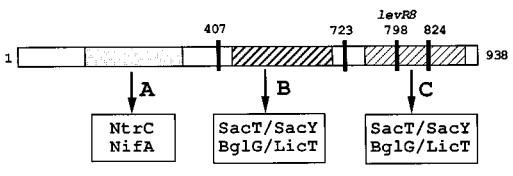

FIG. 2. Map of truncated LevR polypeptides. The 201-amino-acid domain A, which has homology with the NifA-type regulators, is shown as a stippled box. The 278-amino-acid domain B and the 148-amino-acid domain C are probably duplications of a domain which has similarities with the SacT/SacY/BglG/LicT family of antiterminators, as proposed in the accompanying paper (42). They are represented as cross-hatched boxes. Bars indicate the positions of the stop codons in *levR*.

either to uptake and phosphorylation of fructose or to the phosphorylation and thus inactivation of the transcriptional regulator LevR when fructose is present in limiting amounts. As shown in Table 2, there is an absolute requirement for the *levR* gene even in the presence of constitutive mutations in the *lev*-PTS. ATP-dependent phosphorylation of HPr is not involved in specific regulation of the LevR activator by fructose. Indeed, in the *ptsH1* (HPr-S46A) mutant, fructose is transported via the *lev*-PTS, with a twofold increase in the uptake rate (data not shown), and the levanase operon is inducible (Table 4). In the *ptsH1* mutant, serine 46 of HPr is replaced by a nonphosphorylatable alanyl residue, and synthesis of gluconate kinase, glucitol dehydrogenase, mannitol-1-phosphate dehydrogenase, the β-glucanase enzyme, and the mannitol-specific PTS permease were all released from repression by glucose, whereas synthesis of inositol dehydrogenase was only partially resistant to glucose repression (9, 19). The expression of the levanase operon was also partially insensitive (with a residual threefold repression factor) to the glucose effect in a *ptsH1* mutant (Table 4). This indicates that ATP-dependent phosphorylation at Ser-46 is directly or indirectly involved in catabolite repression of the levanase operon. Glycolytic intermediates, including fructose 1,6-bisphosphate, seem to control both the ATP-dependent phosphorylation of HPr by the serine kinase (36) and catabolite repression of gluconate and inositol operons in *B. subtilis* (23, 34).

Most of the enzymes whose synthesis is insensitive to catabolite repression in the *ptsH1* mutant are also controlled by the CcpA repressor (9, 19, 31). We confirmed the role of the CcpA regulator in glucose repression of the levanase operon in a wild-type inducible context (Table 4). HPr(Ser-P) and CcpA participate in a common pathway controlling the synthesis of several carbohydrate catabolic enzymes in the presence of glucose, including those encoded by the levanase operon. CcpA specifically interacts with HPr(Ser-P), and this interaction required the presence of fructose 1,6-bisphosphate, providing a link between glycolytic activity and carbon catabolite repression (8). The formation of the CcpA-HPr(Ser-P) complex might allow the repressor to bind to the CRE DNA sequence associated with most of the regulated genes (4, 15, 16). In the case of the levanase operon, we cannot assume that the regulation takes place at the transcriptional level, since we used a *levD'-'lacZ* translational fusion. However, it seems likely that the *ccpA* pathway modulates the expression of the levanase operon at the transcriptional level, as proposed for the other systems controlled by CcpA (17, 31, 33, 47).

The *cis*-active CRE of the levanase operon, which is located in the pΔB (−148, +189) fragment of the promoter, conferred catabolite repression on the *levD'-'lacZ* translational fusion (Table 3). A unique DNA sequence, TGAAAACGCTT(a)ACA (Fig. 1), located between positions −50 and −36, has similarities to the CRE consensus sequence but has an additional A residue. Carbon catabolite repression was decreased by point mutations in the CRE-like sequence, with 3- to 5-fold repression instead of 16-fold in the presence of the wild-type sequence. Thus, the DNA sequence TGAAAACGCTT(a)ACA is involved in carbon catabolite repression of the levanase operon, probably as the target for the CcpA-HPr(Ser-P) complex (Fig. 3). Most of the CRE sequences characterized to date are located in the coding sequence of the corresponding gene or close to the transcriptional start site (16). In the case of the levanase operon, the *cis*-acting CRE sequence is upstream from the −12, −24 promoter. The molecular mechanism mediating repression in the presence of the CRE sequence and the CcpA protein remains unknown. However, a mechanism involving direct competition for binding of the RNA polymerase-σ^{54} complex seems unlikely because mutations in the CRE-like sequence are not active in the presence of a truncated LevR798 activator (Table 5). The binding of CcpA in the promoter region between the LevR target (UAS) and the −12, −24 promoter may modify the conditions of protein-protein contact between LevR and the RNA polymerase associated with σ^{54} that is necessary for the melting of DNA and the activation of transcription (Fig. 3).

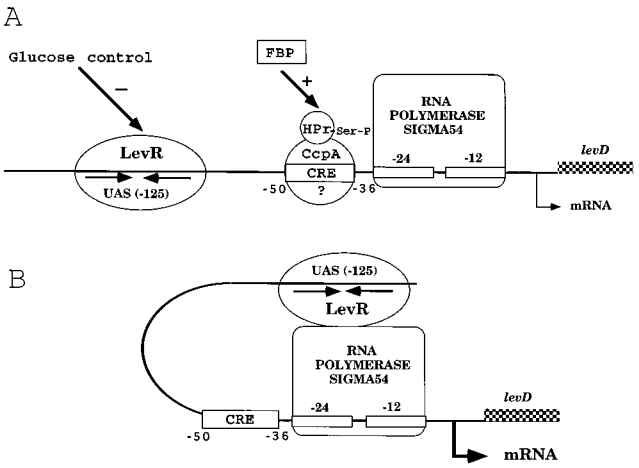

FIG. 3. Model of carbon catabolite repression of the levanase operon of *B. subtilis*. Proposed regulation of the levanase operon in the presence of glucose (A) and in the absence of glucose (B). The interaction of HPr(Ser-P) with the CcpA regulator and fructose 1,6-bisphosphate (FBP) was proposed by Deutscher et al. (8). The putative target for the CcpA-HPr(Ser-P) complex, the CRE sequence (−50 to −36), is indicated by a stippled box. An additional control via the LevR regulator is also indicated.

Several lines of evidence suggest that the catabolite repression of the levanase operon is complex, with various different pathways: (i) in *ptsH1* and *ccpA*::Tn*917* mutants, there was residual repression by glucose; (ii) the repression by various sugars differed in wild-type and *levR8* strains; (iii) fructose acts as either an inducer or a corepressor, depending on the concentration and on the inducibility or constitutivity of the *lev* system; and (iv) neither HPr(Ser-P), CcpA, nor the CRE sequence is involved in glucose repression in a background constitutive for the levanase operon. In particular, in a *levR8* strain encoding a truncated LevR798 polypeptide, repression by glucose is extremely strong compared with that observed in the wild-type or the *levD6* and *levE7* constitutive mutants and is independent of the CcpA pathway (Table 4). These results suggest that the phosphorylation state and the structure of the LevR regulator are important to the response to catabolite repression. We therefore tested the dependence of LevR on the CcpA catabolite repression pathway by using several truncated LevR polypeptides (29) (Table 6, Fig. 2). The LevR407 regulator containing domain A, homologous to that found in the NifA/NtrC family of regulators, was dependent on the CcpA pathway. All the truncated LevR proteins containing the region between amino acids 407 and 723 were CcpA independent. Domain B of LevR (411 to 689) is homologous to the BglG/SacY/SacT family of bacterial antiterminators and may play a role in catabolite repression. The possibility of an involvement of LevR in catabolite repression is supported by the partial insensitivity to glucose when the LevR798 polypeptide was overproduced. The involvement of a specific regulator in glucose control has been proposed for the xylose operon of *Bacillus licheniformis* (41). Glucose regulation of this operon is mediated via an anti-inducer effect of glucose on the XylR

repressor (41). *xylR*-dependent repression by glucose has also been shown for the xylose operon of *B. subtilis* (18). The LevR activator could be the target of other regulators important for glucose control. The isolation of mutations affecting genes other than those of the CcpA pathway could help in the identification of such regulators. However, one possibility is the direct involvement of HPr. Indeed, in addition to the negative regulation exerted by the *lev*-PTS, the LevR activator requires the presence of HPr to be fully active. This requirement is particularly strong in the presence of LevR798 (42). In vitro phosphorylation of LevR by EI and HPr has been obtained (42), suggesting the existence of a second PEP-dependent phosphorylation site, as already proposed for the SacT regulator (2). Interestingly, the part of LevR necessary for HPr and CcpA dependence is the same, domain B (Table 6) (42). This second PEP-dependent phosphorylation of LevR by EI and HPr could be involved in the transduction of information to this activator about the availability of metabolizable sugars in the cell. The complex role of fructose, which could modify the phosphorylation state of LevR at both sites, is discussed in the accompanying paper (42). The demonstration of a link between the phosphorylation of LevR by HPr and the glucose regulation of the levanase operon deserves further investigation.

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