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The HPr Protein of the Phosphotransferase System Links Induction and Catabolite Repression of the *Bacillus subtilis* Levanase Operon

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The LevR protein is the activator of expression of the levanase operon of *Bacillus subtilis*. The promoter of this operon is recognized by RNA polymerase containing the sigma 54-like factor σ^L. One domain of the LevR protein is homologous to activators of the NtrC family, and another resembles antiterminator proteins of the BglG family. It has been proposed that the domain which is similar to antiterminators is a target of phosphoenolpyruvate:sugar phosphotransferase system (PTS)-dependent regulation of LevR activity. We show that the LevR protein is not only negatively regulated by the fructose-specific enzyme IIA/B of the phosphotransferase system encoded by the levanase operon (*lev*-PTS) but also positively controlled by the histidine-containing phosphocarrier protein (HPr) of the PTS. This second type of control of LevR activity depends on phosphoenolpyruvate-dependent phosphorylation of HPr at histidine 15, as demonstrated with point mutations in the *ptsH* gene encoding HPr. In vitro phosphorylation of partially purified LevR was obtained in the presence of phosphoenolpyruvate, enzyme I, and HPr. The dependence of truncated LevR polypeptides on stimulation by HPr indicated that the domain homologous to antiterminators is the target of HPr-dependent regulation of LevR activity. This domain appears to be duplicated in the LevR protein. The first antiterminator-like domain seems to be the target of enzyme I and HPr-dependent phosphorylation and the site of LevR activation, whereas the carboxy-terminal antiterminator-like domain could be the target for negative regulation by the *lev*-PTS.

In bacteria, many sugars are taken up and concomitantly phosphorylated by the phosphoenolpyruvate:sugar phosphotransferase system (PTS). The PTS is also involved in chemotaxis and in the regulation of many catabolic pathways (see reference 43 for a review).

The phosphorylation cascade from phosphoenolpyruvate (PEP) to the sugar proceeds via enzyme I (EI), a histidine-containing phosphocarrier protein (HPr), and a sugar-specific multidomain enzyme II (EII), which may exist either as individual polypeptides or as a fused protein (48). While the HPr protein of gram-negative bacteria is phosphorylated at only one site (His-15), its gram-positive counterpart is subject to a second ATP-dependent phosphorylation at Ser-46 (12, 14, 56). Phosphorylation at His-15 is dependent on PEP and EI and is necessary for phosphotransfer to EII and subsequent sugar uptake and phosphorylation (44). In the presence of carbon sources metabolized via glycolysis, the HPr protein is phosphorylated at Ser-46 by an ATP-dependent kinase. Phosphorylation of HPr at Ser-46 inhibits the phosphorylation at His-15 about 600-fold (10). The activity of the HPr kinase is inhibited by P_i and stimulated by 2-phosphoglycerate and fructose-1,6-bisphosphate, the latter being an intracellular signal indicating a high glucose concentration (14, 33).

The PTS is involved not only in sugar uptake but also in regulation of catabolism. In *Escherichia coli*, the glucose-specific EIIA is central to carbon catabolite repression (CCR). In the absence of glucose, phosphorylated $EIIA^{Glc}$ binds to the adenylate cyclase and stimulates its activity. Cyclic AMP then forms a complex with the catabolite activator protein, which activates transcription of catabolic operons. In the presence of glucose, the unphosphorylated form of $EIIA^{Glc}$ binds to other catabolic enzymes, such as lactose permease and glycerol kinase, preventing them from being active (47).

In *Bacillus subtilis*, HPr rather than the glucose-specific EIIA is involved in CCR. Mutants in which the Ser-46 of HPr is replaced by an alanine residue are resistant to CCR of lichenase, gluconate kinase, glucitol dehydrogenase, and mannitol degradation enzymes (13, 26), suggesting that phosphorylation of HPr at Ser-46 by the HPr-kinase is required for CCR. A second *trans*-acting factor, CcpA, which is similar to the LacI/GalR repressor family, is pleiotropically involved in CCR (13, 17, 23, 26, 32). The CcpA regulator is able to form a complex with HPr(Ser-P) in the presence of fructose-1,6-bisphosphate, and this complex may bind to the targets of CCR in response to the availability of glycolytically metabolized carbon sources (11).

The PTS also regulates utilization of non-PTS carbon sources. Mutants of *B. subtilis* affected in *ptsI* and *ptsH* are not able to grow on glycerol as the only carbon source (18). The $EIIA^{Glc}$ is not necessary for glycerol utilization (21). It has been proposed that glycerol kinase or the glycerol facilitator may be the target for PTS-mediated regulation of glycerol utilization (4), a possibility substantiated by the finding that the activity of dihydroxyacetone/glycerol kinase of *Enterococcus faecalis* is stimulated 10-fold by HPr-dependent phosphorylation (15). The presence of a functional PTS is also required for maltose uptake in *B. subtilis* (54).

The PTS in *B. subtilis* is involved in three different steps of sucrose and β-glucoside utilization. (i) Both compounds are

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taken up via the PTS. (ii) The regulation of the *sacPA* and the *bglPH* operons involves the antiterminator proteins SacT and LicT, respectively, which depend on the general proteins of the PTS for activity (3, 25, 30, 53). (iii) The activity of the antiterminator proteins is probably negatively regulated by the respective sugar-specific components of the PTS, as shown for LicT (30).

The levanase operon of *B. subtilis* (*levDEFG sacC*) encodes a fructose-specific PTS (*lev*-PTS) and the extracellular enzyme levanase, which is capable of hydrolyzing fructose polymers and sucrose (34, 37). The operon is induced by fructose and repressed in the presence of glucose and other glycolytically metabolized carbon sources (35, 40). The promoter of the operon is recognized by an RNA polymerase associated with the sigma 54-like factor σ^L (9). Induction of the operon depends on an activator protein, LevR. This protein contains two domains, one homologous to the NtrC/NifA family of activators (domain A) and the other to a family of transcriptional antiterminators including BglG of *E. coli* and SacT, SacY, and LicT of *B. subtilis* (domain B) (8). In addition, the LevD and LevE proteins of the *lev*-PTS are negative regulators of expression of the levanase operon, probably by phosphorylating the activator protein LevR in the absence of fructose, thereby rendering it inactive (37). The *levR8* mutation, leading to constitutive expression of the levanase operon, is a stop mutation in the *levR* gene encoding a truncated LevR protein (LevR798) (8).

CCR of the levanase operon involves a *cis*-acting carbon-responsive element, the CcpA protein, and the HPr protein of the PTS (40). According to the model proposed recently (11), a complex of CcpA, HPr(Ser-P), and fructose-1,6-bisphosphate might bind to the carbon-responsive element sequence and prevent transcription activation by the LevR protein.

In this report, we present genetic evidence that the activity of the LevR activator protein is not only negatively regulated by the *lev*-PTS but is also stimulated by the HPr protein. Studies with mutants affected in phosphorylation sites of the HPr protein indicate that LevR can be phosphorylated by PEP via HPr(His-15-P). Biochemical evidence for this phosphorylation is presented. This finding has implications for induction and catabolite repression of the levanase operon.

MATERIALS AND METHODS

Bacterial strains and culture media. The *B. subtilis* and *E. coli* strains used in this work are listed in Table 1. *E. coli* TGI was used for cloning experiments.

E. coli and *B. subtilis* were grown as described in the accompanying paper (40).

Transformation and characterization of phenotype. Standard procedures were used to transform *E. coli* (49), and transformants were selected on LB plates supplemented with ampicillin (100 μg/ml). *B. subtilis* was transformed with plasmid or chromosomal DNA by the two-step protocol described previously (27). Strains MZ303 (*ptsH*::*cat*) and QB5350 (*ptsH*-H15A) of *B. subtilis* were transformed with glucitol (20 g/liter) instead of glucose (20 g/liter) as a carbon source. Transformants were selected on SP plates containing chloramphenicol (Cm; 5 μg/ml), kanamycin (Km; 5 μg/ml), tetracycline (Tc; 15 μg/ml), spectinomycin (Spc; 100 μg/ml), or erythromycin (Em) plus lincomycin (Lin) (1 and 10 μg/ml, respectively). The *cat* cassette present in the *ptsH* gene of MZ303 was selected on SP plates containing only 3 μg of chloramphenicol per 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.

Doubling times were determined by growing the bacteria in C minimal medium supplemented to 1% (wt/vol) with the carbon source. C medium containing 1 g of glucitol per liter was used for the precultures.

Quantitative studies of *lacZ* expression in *B. subtilis* in liquid medium were performed as described in the accompanying paper (40). For the determination of β-galactosidase activities in *E. coli*, cells were grown in LB supplemented with sugars as indicated. At an optical density of 600 nm (OD_{600}) of 1.0, cells were harvested, and enzyme activity was measured as previously described (42). 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 values indicated represent averages of at least three independent experiments.

DNA manipulations. Standard procedures were used to extract plasmids from *E. coli* (49). Restriction enzymes and phage T4 DNA ligase were used as recommended by the manufacturers. DNA fragments were purified from agarose gels with the Prep-A-Gene kit (Bio-Rad Laboratories, Richmond, Calif.) or the Jetsorb kit (Genomed GmbH, Bad Oeynhausen, Germany). *Thermus aquaticus* DNA polymerase was used for PCR as previously described (38). DNA sequences were determined by the dideoxy chain termination method (50).

Plasmid constructions. Vector pHT304 contains an origin of replication from a *Bacillus thuringiensis* resident plasmid cloned in pUC19 (2). Plasmid pHT304Δ1 is a derivative of pHT304 obtained by deletion of an *Eco*RV DNA fragment from pHT304. This plasmid lacks the origin of replication from *B. thuringiensis* and is an integrative plasmid in *B. subtilis* containing an *erm* gene (erythromycin resistance).

Vector pAC7 (57) carries the *aphA3* gene (kanamycin resistance) (55) and a promoterless *lacZ* gene between two fragments of the *B. subtilis amyE* gene. Plasmid pAC21 contains a pΔB (−148, +189) *levD′-′lacZ* translational fusion (38). Plasmid pAC57 was constructed by cloning a 388-bp *Eco*RI-*Bam*HI fragment of pAC21 containing the levanase promoter (ΔB) and the first 54 codons of *levD* into pAC7, which was linearized with the same enzymes.

Plasmid pAC68 was obtained as follows. A 395-bp *Pst*I-*Sac*I DNA fragment carrying the part of the *ptsH* gene containing the S46D mutation and the 3′ region of *ptsH* and 5′ region of *ptsI* was purified from plasmid pJRS46D (a gift from J. Reizer) and cloned between the *Pst*I and *Sac*I restriction sites of plasmid pHT304Δ1. Plasmid pAC70 was obtained by cloning an 877-bp *Pst*I DNA fragment from pTS20 (22) containing the 3′ end of *ptsG* and the 5′ end of *ptsH* into the unique *Pst*I restriction site of pAC68.

Plasmid pLUM1104 was constructed by cloning a 105-bp *Nhe*I-*Hin*dIII fragment of pJRH15A (a gift from J. Reizer) containing the *ptsH* gene with the H15A mutation into the large *Nhe*I-*Hin*dIII fragment of pTS22 (22). The presence of the mutation was verified by sequencing the *ptsH* allele in pLUM1104.

In plasmid pRL58, the *levR*, *levD*, *levE*, *levF*, and *levG* genes are deleted and replaced by an *aphA3* cassette (55). A *Cla*I DNA fragment containing this cassette was cloned between the two *Bst*BI sites of pRL2 (8).

Plasmids pRL5, pRL6, and pRL7 containing *levD′-′lacZ* fusions and various alleles of *levR* have been described previously (8). In plasmid pRL40 (39), the *levR* gene is deleted and replaced by an *aphA3* cassette.

Construction of *B. subtilis* strains containing point mutations in the *ptsH* gene. Strain MZ303 (*ptsH*::*cat*) was transformed with pAC70 containing the *ptsH*-S46D allele, and Em^r transformants corresponding to the integration of this plasmid by a single crossover event at the *ptsH* locus were selected. The Em^r transformants were grown for 60 generations in the absence of selection. About 300 resulting clones were screened for sensitivity to chloramphenicol and erythromycin: Em^s Cm^s clones result from a second crossover event, which replaces the *cat* cassette present in the *ptsH* gene with an intact copy of *ptsH* containing the S46D point mutation. One Em^s Cm^s clone was found.

This approach was not successful for the *ptsH*-H15A mutation. The frequency of double crossover events can be enhanced by cotransformation with another marker. Therefore, competent cells of *B. subtilis* 168 were transformed with chromosomal DNA of strain QB5187 [*amyE*::(*levD′-′lacZ aphA3*)] and the integrative plasmid pLUM1104, which contains the *ptsH*-H15A allele. The transformants selected for Km^r are white on plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as long as the PTS is functional but blue if the PTS is inactive. This loss of activity can occur only after a double crossover between the *ptsH* alleles, resulting in replacement of His-15 by an alanine. Blue transformants were identified on plates containing kanamycin and X-Gal. Glucose utilization deficiency and Cm^s resulting from the loss of the vector part of pLUM1104 were verified in these transformants.

The chromosomal copies of the *ptsH* alleles of strains QB5262 (*ptsH*-S46D) and QB5350 (*ptsH*-H15A) were amplified by PCR, and the presence of the mutations was confirmed by sequencing.

Protein purification. EI of the PTS was purified from *Staphylococcus carnosus* and HPr was purified from *B. subtilis* as described before (16, 24). For the purification of LevR, the *levR* gene was cloned under the control of the strong *degQ36* promoter (39). The resulting plasmid, pRL11, allows overproduction of LevR in *B. subtilis* strains in the stationary phase. LevR was partially purified from crude extracts by ammonium sulfate precipitation. The LevR polypeptide made up about 20% of the total protein in the pellets (39).

In vitro phosphorylation of LevR. [^{32}P]PEP was prepared from [γ-^{32}P]ATP as described earlier (45). [^{32}P]PEP was separated from [γ-^{32}P]ATP by ion-exchange chromatography (41). LevR (17 μg of protein extract) was phosphorylated by incubating a reaction mixture containing EI (2 μg), HPr (1 μg), $MgCl_2$ (10 mM), Tris-HCl (40 mM; pH 7.4) and 1 μM [^{32}P]PEP (0.5 μCi) in a final volume of 25 μl. The reaction was finished by adding electrophoresis sample buffer containing 0.1% sodium dodecyl sulfate (SDS). Electrophoresis of proteins was carried out on 12.5% polyacrylamide–SDS gels as described earlier (39). Gels were dried and exposed for 2 h to autoradiography (Kodak Biomax).

RESULTS

Expression of the levanase operon in mutants with deletions in the *pts* operon. The expression of the levanase operon is constitutive in mutants affected in the general and *lev*-specific

TABLE 1. Strains used

Strain	Genotype	Source[a] or reference
E. coli		
TG1	K-12 Δ(*lac-pro*) *supE thi hsd5* (F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15)	20
TP2111	F$^-$ *xyl argH1* Δ*lacZ74 aroB ilvA*	46
TP2811	F$^-$ *xyl argH1* Δ*lacZ74 aroB ilvA* Δ(*ptsHI crr*) Km^r	31
B. subtilis		
168	*trpC2*	Laboratory stock
QB168	*trpC2 levE7*	28
QB169	*trpC2 levR8*	28
QB2018	*leuA8 hisA1 sacA321 levD6*	28
QB5075	*trpC2 levE7 levR*::*aphA3 sacC*′-*lacZ*$^+$ *erm*	40
QB5081	*trpC2 amyE*::(*levD*′-′*lacZ cat*)	38
QB5173	*trpC2* Δ*levR*::*aphA3*	39
QB5176	*trpC2* Δ*levR*::*aphA3 amyE*::(*levD*′-′*lacZ cat*)	39
QB5187	*trpC2 amyE*::(*levD*′-′*lacZ aphA3*)	pAC57→168
QB5232	*trpC2 ptsH*::*cat amyE*::(*levD*′-′*lacZ aphA3*)	MZ303→QB5187
QB5242	*trpC2* Δ*levR*::*aphA3 sacC*′-*lacZ*$^+$ *erm amyE*::(*levR E824* stop Δ*lacZ cat*)	39
QB5243	*trpC2* Δ*levR*::*aphA3 sacC*′-*lacZ*$^+$ *erm amyE*::(*levR K777* stop Δ(*lacZ cat*)	39
QB5245	*trpC2* Δ*levR*::*aphA3 sacC*′-*lacZ*$^+$ *erm amyE*::(*levR G723* stop Δ*lacZ cat*)	39
QB5253	*trpC2* Δ*levR*::*aphA3 sacC*′-*lacZ*$^+$ *erm amyE*::(*levR E407* stop Δ*lacZ cat*)	39
QB5255	*trpC2* Δ*levR*::*aphA3 sacC*′-*lacZ*$^+$ *erm amyE*::(*levR* Δ*lacZ cat*)	39
QB5262	*ptsH*-S46D	See Results
QB5263	*ptsH*-S46D *amyE*::(*levD*′-′*lacZ aphA3*)	QB5187→QB5262
QB5271	*ptsH*-S46D Δ*levR*::*aphA3*	pRL40→QB5262
QB5273	*trpC2* Δ*levR*::*aphA3 amyE*::(*levR8 levD*′-′*lacZ cat*)	pRL7→QB5173
QB5275	*ptsH*-S46D Δ*levR*::*aphA3 amyE*::(*levR8 levD*′-′*lacZ cat*)	pRL7→QB5271
QB5276	*trpC2* Δ(*levR levDEFG*)::*aphA3 amyE*::(*levD*′-′*lacZ cat*)	pRL58→QB5081
QB5277	*trpC2* Δ(*levR levDEFG*)::*aphA3* Δ*ptsGHI*::*spc amyE*::(*levD*′-′*lacZ cat*)	QB6096→QB5276
QB5340	*leuA8 hisA1 sacA321 levD6 amyE*::(*levD*′-′*lacZ aphA3*)	QB5232→QB2018
QB5341	*leuA8 hisA1 sacA321 levD6 ptsH*::*cat amyE*::(*levD*′-′*lacZ aphA3*)	QB5232→QB2018
QB5342	*trpC2 levE7 amyE*::(*levD*′-′*lacZ aphA3*)	QB5232→QB168
QB5343	*trpC2 levE7 ptsH*::*cat amyE*::(*levD*′-′*lacZ aphA3*)	QB5232→QB168
QB5345	*trpC2 levR8 amyE*::(*levD*′-′*lacZ aphA3*)	QB5232→QB169
QB5346	*trpC2 levR8 ptsH*::*cat amyE*::(*levD*′-′*lacZ aphA3*)	QB5232→QB169
QB5350	*trpC2 ptsH*-H15A *amyE*::(*levD*′-′*lacZ aphA3*)	See Results
QB5363	*trpC2 ptsH*-H15A *cat amyE*::(*levD*′-′*lacZ aphA3*)	GM1221→QB5350
QB5364	*trpC2 ptsH*$^+$ *cat amyE*::(*levD*′-′*lacZ aphA3*)	GM1221→QB5350
QB5365	*trpC2* Δ*levR*::*aphA3 amyE*::(*levD*′-′*lacZ cat*) Δ*ptsGHI*::*tet*	GM1220→QB5176
QB5366	*trpC2 ptsH*$^+$ *cat levR8 amyE*::(*levD*′-′*lacZ aphA3*)	QB5364→QB5345
QB5367	*trpC2 ptsH*-H15A *cat levR8 amyE*::(*levD*′-′*lacZ aphA3*)	QB5363→QB5345
QB5373	*trpC2* Δ*levR*::*aphA3 sacC*′-*lacZ*$^+$ *erm amyE*::(*levR E824* stop Δ*lacZ cat*) Δ*ptsGHI*::*tet*	GM1220→QB5242
QB5374	*trpC2* Δ*levR*::*aphA3 sacC*′-*lacZ*$^+$ *erm amyE*::(*levR E407* stop Δ*lacZ cat*) Δ*ptsGHI*::*tet*	GM1220→QB5253
QB5375	*trpC2* Δ*levR*::*aphA3 sacC*′-*lacZ*$^+$ *erm amyE*::(*levR* Δ*lacZ cat*) Δ*ptsGHI*::*tet*	GM1220→QB5255
QB5378	*trpC2 levE7 levR*::*aphA3 sacC*′-*lacZ*$^+$ *erm amyE*::(*levR* Δ*lacZ cat*)	QB5255→QB5075
QB5379	*trpC2 levE7 levR*::*aphA3 sacC*′-*lacZ*$^+$ *erm amyE*::(*levR E824* stop Δ*lacZ cat*)	QB5242→QB5075
QB5380	*trpC2 levE7 levR*::*aphA3 sacC*′-*lacZ*$^+$ *erm amyE*::(*levR E407* stop Δ*lacZ cat*)	QB5253→QB5075
QB5381	*trpC2* Δ*levR*::*aphA3 sacC-lacZ erm amyE*::(*levR G723* stop Δ*lacZ cat*) Δ*ptsGHI*::*tet*	GM1220→QB5245
QB5382	*trpC2* Δ*levR*::*aphA3 sacC-lacZ erm amyE*::(*levR K777* stop Δ*lacZ cat*) Δ*ptsGHI*::*tet*	GM1220→QB5243
QB5383	*leuA8 hisA1 sacA321 levD6 ptsG*::*cat amyE*::(*levD*′-′*lacZ aphA3*)	QB6046→QB5340
QB5384	*trpC2 levE7 ptsG*::*cat amyE*::(*levD*′-′*lacZ aphA3*)	QB6046→QB5342
QB5385	*trpC2 ptsG*::*cat amyE*::(*levD*′-′*lacZ aphA3*)	QB6046→QB5187
QB6046	*sacT30 ptsG*::*cat amyE*::(*sacP*′-′*lacZ aphA3*)	3
QB6096	*trpC2* Δ*ptsGHI*::*spc*	M. Arnaud
GM1220	Δ*bgaX* Δ*sacB* Δ*sacT* Δ*sacY* Δ*ptsGHI*::*tet* SPβ$^-$	M. Steinmetz
GM1221	*trpC2 pheA1* Δ*bgaX amyE*::(*gntRK*′-′*lacZ phl*) *ptsH*$^+$ *cat*	13
MZ303	*ptsH*::*cat*	3

[a] Arrows indicate construction by transformation.

components of the PTS (37, 40). However, the lower expression in the *ptsI6* and *ptsH* mutants than in the *levD6* and *levE7* mutants was not in agreement with the model of a single negatively acting regulatory mechanism of LevR activity by the *lev*-PTS. The influence of mutations in the *pts* operon on the expression of the levanase operon was therefore analyzed in more detail. To test the relationships between mutations in the general enzymes of the PTS and those in specific components of the *lev*-PTS that lead to constitutive expression of the levanase operon, a series of strains containing a *levD*′-′*lacZ* translational fusion and the *levD6* or *levE7* constitutive mutation combined with deletions of either *ptsH* or the EIIA^{Glc} domain of *ptsG* were constructed. The strains were grown in CSK medium, and the β-galactosidase activities were determined (Table 2). The *ptsG* deletion did not modify the expression of the *levD*′-′*lacZ* fusion either in the wild-type strain or in the

TABLE 2. Effect of deletions of *ptsG* and *ptsH* on inducible and constitutive expression of the levanase operon[a]

Strain	Relevant genotype	β-Galactosidase sp act[b] (U/mg of protein)
QB5187		11 (180)
QB5232	*ptsH*::*cat*	490
QB5385	*ptsG*::*cat*	10 (230)
QB5340	*levD6*	2,027
QB5341	*levD6 ptsH*::*cat*	257
QB5383	*levD6 ptsG*::*cat*	3,654
QB5342	*levE7*	2,346
QB5343	*levE7 ptsH*::*cat*	140
QB5384	*levE7 ptsG*::*cat*	3,932
QB5345	*levR8*	2,648
QB5346	*levR8 ptsH*::*cat*	3

[a] Cells were grown at 37°C in CSK medium. The β-galactosidase specific activity of the different strains was measured in extracts prepared from exponentially growing cells (OD_{600}, 0.7 to 1). All strains contain a pΔB (−148, +189) *levD'-'lacZ* translational fusion (38) integrated at the *amyE* locus.

[b] β-Galactosidase activity is expressed in units per milligram of protein. Values in parentheses indicate activity after induction with 0.2% fructose.

constitutive *levD6* and *levE7* mutants. Thus, the $EIIA^{Glc}$ is not involved in the regulation of the levanase operon. In contrast, an intact *ptsH* gene was necessary for full constitutive expression of the operon in the constitutive *levD6* and *levE7* backgrounds. Deletion of the *ptsH* gene reduced the expression of the levanase operon 8- and 16-fold in the constitutive *levD6* and *levE7* backgrounds, respectively. The high expression of the levanase operon in *levD6* and *levE7* mutants reflects the high activity of the LevR protein in the absence of negative regulation by the *lev*-PTS. It is possible that LevR is less active in the absence of the HPr protein, the *ptsH* gene product. This possibility is reinforced by the observation that a *ptsH* gene deletion in the *levR8* mutant causes a 900-fold decrease in the expression of the levanase operon (Table 2). Thus, the activity of the truncated LevR798 polypeptide encoded by the *levR8* allele is fully dependent on the presence of HPr.

The dependence of the antiterminator protein LicT on the general proteins of the PTS can be overcome by expressing the *licT* gene on a multicopy plasmid (25). Therefore, we tested the PTS dependence of a *levD'-'lacZ* fusion in the presence of the *levR8* gene expressed from the strong *degQ36* promoter on the multicopy plasmid pRL12 (39). The expression of the fusion was determined in a pts^+ strain (QB5176) and its isogenic Δ*ptsGHI* counterpart (QB5365), each containing pRL12. The β-galactosidase activity was 1,640 U/mg of protein in the pts^+ strain and 195 U/mg of protein in the Δ*ptsGHI* mutant. The difference (which could be described as the dependence of the overexpressed LevR798 on the PTS) was thus 8-fold, whereas it was about 900-fold when a single copy of *levR8* was expressed from its own promoter. This indicates that overexpression of the *levR8* allele partially suppresses the dependence of the LevR798 protein on stimulation by the general proteins of the PTS.

Regulation of LevR activity by the PTS of *E. coli*. Both the wild-type and constitutive *levR* alleles allow efficient expression of a *levD'-'lacZ* translational fusion in *E. coli* (8). In *B. subtilis*, the LevR798 protein is not active unless HPr is also present. To test whether the regulation by the PTS is also operative in *E. coli*, strains TP2111 (wild type) and TP2811 [Δ(*ptsHI crr*)] were transformed with plasmids containing a *levD'-'lacZ* fusion and either the wild-type *levR* gene or the *levR8* allele. A plasmid containing the *levD'-'lacZ* fusion in the absence of the *levR* gene was used as a control. These strains were grown in LB with or without 1% glucose, and the β-galactosidase activities were determined (Table 3). As found in *B. subtilis*, the wild-type LevR protein was weakly dependent on the presence of the PTS, with only a threefold decrease in β-galactosidase activity when *ptsHI* and *crr* were deleted. Again, the truncated LevR798 protein was not active in the Δ(*ptsHI crr*) mutant, with a 225-fold decrease in activity compared with the pts^+ strain. The presence of glucose in the medium led to a threefold repression of *levD'-'lacZ* expression in the $levR^+$ pts^+ strain and to a 14-fold repression in the *levR8* pts^+ strain, suggesting that in *E. coli*, the LevR798 polypeptide is more sensitive to catabolite repression than the wild-type LevR protein, as has been observed in *B. subtilis* (40). In the Δ(*ptsHI crr*) mutant strain, there was no effect of glucose for any *levR* allele studied (Table 3), as expected for mutants that are unable to take up glucose.

These data indicate that in *E. coli* as in *B. subtilis*, the full activity of the wild-type LevR protein requires the presence of the PTS and that the LevR798 protein is completely dependent on the PTS.

Construction and characterization of strains carrying point mutations in the *ptsH* gene. To study the nature of the possible interaction between HPr and LevR in more detail, we constructed two mutants in which the two phosphorylation sites of HPr, His-15 and Ser-46, were replaced by other amino acids. Plasmids containing mutations in the *ptsH* gene changing the His-15 to Ala and the Ser-46 to Asp are available, and the modified proteins have been purified and characterized biochemically. In both cases, the mutations result in loss or strong decrease of PEP-dependent phosphorylation of HPr, respectively (44). However, no *B. subtilis* strains in which the chromosomal copy of the *ptsH* gene has been replaced with these alleles have been previously constructed.

As described in the Materials and Methods section, a mutant containing the *ptsH*-S46D mutation was constructed by transformation of a strain containing a *ptsH*::*cat* cassette with plasmid pAC70 (*ptsH*-S46D). Transformants were grown for several generations without selective pressure and checked for a second crossover event. A mutant encoding the *ptsH*-H15A mutation was obtained by simultaneous transformation of *B. subtilis* 168 with a chromosomal DNA containing a *levD'-'lacZ* fusion and plasmid pLUM1104 (*ptsH*-H15A) and direct screening of the transformants for the presence of the mutation.

The mutant strains QB5263 (*ptsH*-S46D) and QB5350 (*ptsH*-H15A) were characterized with respect to growth on different carbon sources by determining doubling times. The doubling times of wild-type strain QB5187 and of the isogenic *ptsH*

TABLE 3. Effect of the PTS on the expression of a *levD'-'lacZ* translational fusion in *E. coli* in the presence of either the wild-type or *levR8* allele[a]

Plasmid	β-Galactosidase sp act (Miller units)			
	TP2111 (wild type)		TP2811 [Δ(*ptsHI crr*)]	
	− Glc	+ Glc	− Glc	+ Glc
pRL5 (*levD'-'lacZ*)	12	ND[b]	8	ND
pRL6 ($levR^+$ *levD'-'lacZ*)	3,738	1,380	1,414	1,252
pRL7 (*levR8 levD'-'lacZ*)	2,263	161	10	10

[a] The *lacZ* gene is fused to codon 54 of the *levD* gene (8). Cells were grown at 37°C in LB with or without 1% glucose and harvested at an OD_{600} of 1. β-Galactosidase specific activities were determined.

[b] ND, not determined.

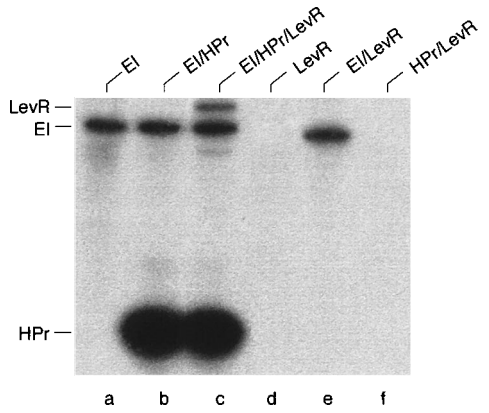

FIG. 1. In vitro [^{32}P]PEP-dependent phosphorylation of LevR. Autoradiograph of a 12.5% polyacrylamide–SDS gel on which samples containing the following proteins had been loaded: lane a, EI; lane b, EI and HPr; lane c, EI, HPr, and LevR; lane d, LevR; lane e, EI and LevR; lane f, HPr and LevR. The detailed phosphorylation conditions are described in Materials and Methods.

mutants were also determined. While all strains had about the same doubling time on glucitol (60 to 80 min), the mutant strains QB5263 and QB5350 did not grow on fructose, maltose, or glycerol as the only carbon source. Maltose and glycerol are not taken up via the PTS, so the failure of these mutant strains to grow indicates the involvement of PEP-dependent phosphorylation of HPr at His-15 in the regulation of glycerol and maltose utilization. These strains grew very slowly on glucose (doubling time, 350 to 360 min), as observed for strain QB5232 (*ptsH*::*cat*). These observations might indicate the presence of a minor PTS-independent uptake system for glucose in *B. subtilis*. The existence of such a system has recently been described for the gram-positive bacterium *Streptococcus mutans* (6).

Effect of point mutations in the *ptsH* gene encoding the HPr protein on the regulation of the levanase operon. To determine the effect of point mutations in *ptsH* on the expression of the levanase operon, a series of strains containing fusions between *lacZ* and the levanase operon promoter and either the *ptsH*-S46D or *ptsH*-H15A mutation were constructed. The strains were grown in CSK, and their β-galactosidase activities were measured (Table 4). As observed for the Δ*ptsH* mutant (Table 2), the *ptsH*-S46D and *ptsH*-H15A mutations resulted in constitutive expression of the levanase operon in a *levR*$^+$ strain. The *levD′-′lacZ* translational fusion was not expressed in a *levR8 ptsH*-H15A double mutant. Similarly, there was no expression in a *levR8 ptsI6* double mutant (data not shown). Therefore, PEP-dependent phosphorylation of HPr at His-15 is necessary to stimulate the activity of the LevR798 protein. In the *levR8 ptsH*-S46D double mutant, the expression of the levanase operon was about fivefold lower than in the *levR8* strain (Table 4). This suggests that ATP-dependent phosphorylation of HPr plays only a minor role in the regulation of LevR798 activity, as observed with the HPrS46A protein (40).

PEP-dependent, EI- and HPr-catalyzed phosphorylation of LevR. The data presented above suggest that PEP-dependent phosphorylation of HPr is a prerequisite for the stimulation of LevR activity. We asked therefore whether HPr might phosphorylate LevR. Partially purified LevR was used to demonstrate PEP-dependent, PTS-catalyzed phosphorylation. To avoid any phosphorylation in the cell prior to purification, the LevR protein was purified from a strain in which the genes encoding the general and *lev*-specific components of the PTS are deleted (QB5277). No phosphorylation of LevR was observed in the presence of [^{32}P]PEP (Fig. 1, lane d). Addition of only EI or HPr was not sufficient to allow phosphorylation of LevR (Fig. 1, lanes e and f, respectively). LevR was phosphorylated only when both PTS proteins, EI and HPr, were present together (Fig. 1, lane c). The identity of the additional radiolabeled band in Fig. 1, lane c, with the transcriptional activator LevR was confirmed by experiments in which extracts from strains containing the empty vector without the *levR* gene or partially purified LevR798, a truncated polypeptide encoded by the *levR8* allele, were used instead of wild-type LevR. In these cases, the additional radiolabeled band was absent or migrated markedly faster than wild-type LevR, respectively (data not shown).

TABLE 4. Effect of point mutations in *ptsH* on expression of the levanase operon in wild-type and constitutive *levR8* strains[a]

Strain	Relevant genotype	β-Galactosidase sp act (U/mg of protein) in medium:	
		CSK	CSK-Fru
QB5187	*amyE*::(*levD′-′lacZ*)	11	177
QB5263	*ptsH*-S46D *amyE*::(*levD′-′lacZ*)	450	600
QB5350	*ptsH*-H15A *amyE*::(*levD′-′lacZ*)	466	610
QB5366	*levR8 pts*$^+$ *amyE*::(*levD′-′lacZ*)	2,400	ND[b]
QB5367	*levR8 ptsH*-H15A *amyE*::(*levD′-′lacZ*)	2.5	ND
QB5273	*amyE*::(*levR8 levD′-′lacZ*)	3,232	ND
QB5275	*ptsH*-S46D *amyE*::(*levR8 levD′-′lacZ*)	614	ND

[a] All strains contain a pΔB (−148, +189) *levD′-′lacZ* translational fusion (38) integrated at the *amyE* locus. β-Galactosidase activities were determined as described in Table 2, footnote *a*.
[b] ND, not determined.

Modulation of the activity of truncated LevR polypeptides by PTS components. Several truncated LevR proteins lacking the carboxy-terminal part of the protein retain activity (Table 5 and Fig. 2) (39). To determine the target in LevR of positive regulation by HPr, the activity of truncated LevR proteins was studied in Δ*ptsGHI* mutants. The activity of the truncated LevR proteins was monitored by assaying the expression of a *sacC′-lacZ*$^+$ transcriptional fusion. The strains were grown in

TABLE 5. Effect of a deletion of the *pts* operon on the expression of a *sacC′-lacZ*$^+$ transcriptional fusion in strains encoding truncated LevR polypeptides[a]

Strain	Relevant genotype	β-Galactosidase sp act[b] (U/mg of protein)
QB5255	*levR*$^+$	7 (114)
QB5375	*levR*$^+$ Δ*ptsGHI*::*tet*	225
QB5378	*levR*$^+$ *levE7*	988
QB5242	*levR824*	123
QB5373	*levR824* Δ*ptsGHI*::*tet*	5.5
QB5379	*levR824 levE7*	27
QB5243	*levR777*	127
QB5382	*levR777* Δ*ptsGHI*::*tet*	2.3
QB5245	*levR723*	133
QB5381	*levR723* Δ*ptsGHI*::*tet*	2.5
QB5253	*levR407*	122
QB5374	*levR407*Δ *ptsGHI*::*tet*	107
QB5380	*levR407 levE7*	46

[a] All strains are deleted for the wild-type copy of *levR* and carry a *sacC′-lacZ*$^+$ transcriptional fusion (35) at the *lev-sacC* locus. The *levR* alleles analyzed are inserted at the *amyE* locus. See Fig. 2 for a map of the truncated LevR polypeptides. Bacteria were grown in CSK.
[b] Activity after induction with 0.2% fructose is shown in parentheses.

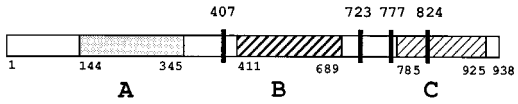

FIG. 2. Map of the truncated LevR polypeptides. The locations of the domains of the LevR protein are indicated. Domain A is similar to the NtrC/NifA-like regulators. Domains B and C both resemble a family of bacterial antiterminator proteins. Bars indicate the location of stop codons in LevR.

CSK, and the β-galactosidase activities were measured (Table 5). Truncated LevR polypeptides LevR824, LevR777, and LevR723 containing both domains A and B (see the introduction and Fig. 2) were strongly dependent on the presence of the PTS, as was the LevR798 protein (see Table 2). This suggests that the target of HPr-dependent positive regulation of LevR activity is located in the N-terminal 723 amino acids. In contrast, the activity of the truncated LevR407 polypeptide, in which domain B is deleted, was independent of the presence of the PTS and was not subject to any direct regulation by PTS components. Domain B of the LevR protein, which is similar to other antiterminators of the BglG family, may thus be the target for this regulation.

To study the negative regulation of the activity of truncated LevR polypeptides by the *lev*-PTS, the expression of the *sacC'-lacZ*$^+$ fusion was determined for the different *levR* alleles in a *levE7* mutant. This latter mutation had only minor effects on the activity of the truncated LevR proteins (Table 5). In contrast, the *levE7* mutation led to strong constitutive expression of the levanase operon in a *levR*$^+$ strain (QB5378 [Table 5]). Thus, the *lev*-PTS might interact with the carboxy-terminal part of the LevR protein downstream from amino acid 824.

Similarity of the carboxy-terminal part of LevR to antiterminator proteins. The sequence of the carboxy-terminal part of the LevR protein was used to screen sequences in the databases (19). This region is similar to the same family of antiterminators as domain B of the protein and to domain B itself (Fig. 3). Therefore, there has probably been a duplication of this regulatory domain. We refer to this part of the protein, which extends from amino acid residues 785 to 925, as domain C (Fig. 2 and 3). Moreover, the sequence of the CelR protein (29) is also suggestive of a domain structure with a duplication of the domain homologous to the antiterminators. CelR is a putative regulatory protein of cellobiose catabolism in *Bacillus stearothermophilus* (29). The gene encoding this protein has not yet been completely sequenced. However, the known sequence of its domain B was 34% identical with residues in the corresponding part of domain B of LevR. Interestingly, the region around the conserved histidine residue (His-101 in

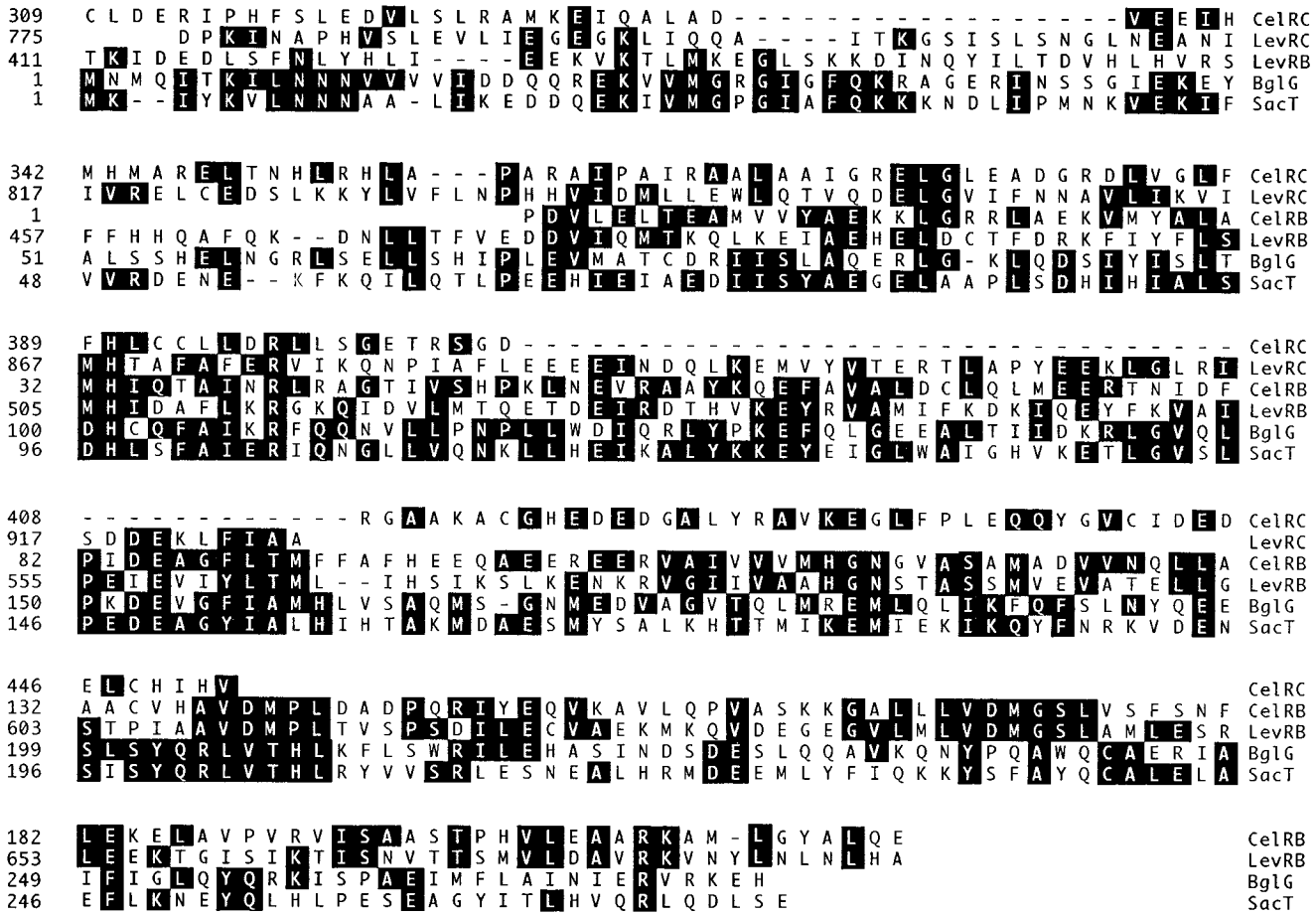

FIG. 3. Comparison of the amino acid sequences of SacT from *B. subtilis* and BglG from *E. coli* (7, 52) with domains B and C of the LevR protein and CelR of *B. stearothermophilus* (8, 29). Residues shared with either BglG or SacT are boxed. The percentages of identity between domain B of LevR and BglG, SacT, domain B of CelR, and domain C of LevR are 12, 13, 34, and 14%, respectively. The percentages of identity between domain C of LevR and BglG and SacT are 17 and 18%, respectively.

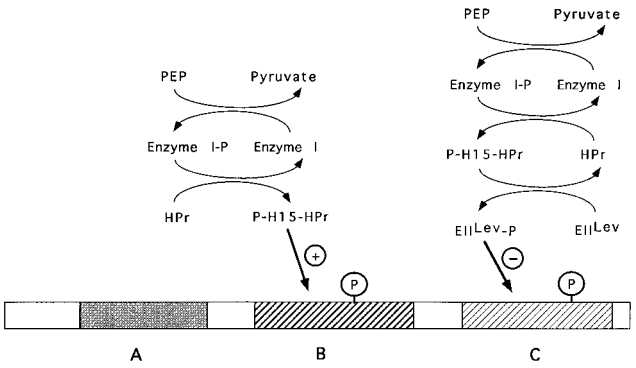

FIG. 4. Model for the regulation of LevR activity by the PTS. In the presence of HPr(His-15-P), HPr phosphorylates domain B of LevR and stimulates its activity. In the absence of fructose, the LevD and LevE proteins of the *lev*-PTS phosphorylate domain C of LevR. This phosphorylation results in inactivation of the protein regardless of the phosphorylation status of domain B. In the presence of fructose, we may suppose that neither domain B nor domain C is phosphorylated, leading to activation of transcription of the levanase operon. In *levD*, *levE*, and *levR8* mutants, domain C of LevR can never be phosphorylated, but domain B can be phosphorylated by HPr, explaining the high constitutive expression of the levanase operon in these mutants. This second phosphorylation by HPr could result from the availability of carbon sources.

BglG of *E. coli*) is much more conserved in domain C than in domain B of LevR. Mutations replacing this His residue or an adjacent amino acid lead to constitutive activity of the SacY and SacT antiterminators, respectively (7). This histidine residue is a good candidate for phosphorylation by the *lev*-PTS.

DISCUSSION

The expression of the levanase operon in *B. subtilis* is regulated by both induction in the presence of fructose and CCR by glucose, fructose, and other glycolytically metabolized sugars (35, 40). We present data indicating that both types of regulation involve the HPr protein of the PTS and the activator protein LevR.

There is an absolute requirement for the *levR* gene for the expression of the levanase operon even in the presence of constitutive mutations in several components involved in fructose uptake via the *lev*-PTS (*levD6*, *levE7*, *ptsI6*, or *ptsH* gene disruption), suggesting that these mutations affect the activity of the LevR protein (40). The specific components of the *lev*-PTS, i.e., LevD and LevE, might be involved either in uptake and phosphorylation of fructose or, in the absence of this substrate, in a PEP-dependent phosphorylation of LevR, thereby inactivating it (Fig. 4). The interruption of the phosphotransfer cascade of EI, HPr, LevD, or LevE prevents the inactivation of LevR and results in constitutive expression of the levanase operon (37) (Table 2). Moreover, deletions of either *levF* or *levG*, which encode EIIC and EIID, respectively, abolish the expression of the levanase operon, probably because the phosphate residues carried by LevD/LevE in these mutants are transferred not to fructose but to LevR (37). Similarly, a replacement of the active His in the EIIB domain of BglF (EII^{Bgl}) prevents the expression of the *E. coli bgl* operon (51).

We show that the PTS not only is involved in induction of the levanase operon but also stimulates the activity of the regulator, the LevR protein. Interestingly, the truncated LevR798 protein encoded by the *levR8* allele is not active in either *B. subtilis* or *E. coli* unless it is stimulated by HPr (Tables 2 and 3). To study the nature of the effect of HPr on LevR in more detail, we constructed mutant strains in which phosphorylation of HPr is impaired. The replacement of His-15 by Ala or of Ser-46 by Asp results in a phenotype which was indistinguishable from that caused by a deletion of the *ptsH* gene with respect to the utilization of various sugars. This indicates that PEP-dependent phosphorylation of HPr is necessary not only for sugar uptake via the PTS but also for the stimulation of glycerol and maltose catabolism. As observed with a deletion of the *ptsH* gene, the mutant proteins HPr-H15A and HPr-S46D lead to constitutive expression of the levanase operon in the $levR^+$ background (Table 4). These results are in agreement with the current model of regulation by the PTS. The conversion of Ser-46 in HPr to the permanently charged Asp residue results in strong inhibition of PEP-dependent phosphoryl transfer in vitro (44). For both HPr-H15A and HPr-S46D, the *lev*-PTS-dependent phosphorylation of LevR is absent, leading to constitutive expression of the levanase operon. Moreover, the stimulation of LevR by HPr depends on PEP-dependent phosphorylation of HPr. Indeed, both a *ptsI6* and a *ptsH*-H15A mutation abolished the activity of the truncated LevR798 polypeptide.

There are two possibilities for the interaction of HPr with LevR. (i) HPr(His-P) might bind directly to LevR, as proposed for the interaction of HPr(Ser-P) with CcpA (11). (ii) HPr might phosphorylate LevR in a PEP-dependent manner, as has been proposed and demonstrated for the antiterminator protein SacT (3, 43a). To distinguish between these possibilities, phosphorylation of LevR by the general proteins of the PTS was assayed in vitro. The second hypothesis is favored by the demonstration of PEP-dependent phosphorylation of LevR involving EI and HPr (Fig. 1).

Using truncated LevR proteins, we have shown that domain B (amino acids 411 to 689) is necessary for the stimulation of LevR activity in the presence of HPr, suggesting that this domain is the target for phosphorylation by HPr (Table 5). This domain B of LevR is homologous to the transcriptional antiterminators BglG, LicT, SacT, and SacY, whose activity is also controlled by the PTS (1, 3, 5, 7, 8, 25, 30, 52, 53, 58). Interestingly, this antiterminator-like domain is duplicated in LevR (Fig. 3). The CelR protein of *B. stearothermophilus* appears to have a similar arrangement, with a duplication of this domain (29) (Fig. 3). Domain C of LevR (amino acids 785 to 925) may be the target for negative regulation by the *lev*-PTS, presumably by a second phosphorylation involved in induction. This hypothesis is reinforced by the finding that replacement of the conserved His-869 in LevR results in constitutive expression of the levanase operon (36).

The expression of the levanase operon is subject to CCR. This repression is very strong in *levR8* mutants even in the presence of concentrations of fructose that are inducing in the wild type (40). Moreover, mutations that result in glucose-resistant expression of the levanase operon in a $levR^+$ strain, such as *ccpA* and *ptsH1*, do not relieve glucose repression in a *levR8* strain (40). In all strains producing truncated LevR proteins containing domain B, CCR of the levanase operon is unaffected by deletion of the *ccpA* gene. In contrast, in a strain encoding the LevR407 polypeptide, containing only domain A, with similarities with the NifA/NtrC family of transcription activators, CCR of the levanase operon is relieved in a *ccpA* mutant (40). Therefore, all the truncated LevR proteins with glucose repression mediated independently from the CcpA regulator are completely dependent on HPr for their activity. The phosphorylation of LevR by HPr may thus be involved in CCR. PEP-dependent phosphorylation of HPr is strongly inhibited by ATP-dependent phosphorylation of Ser-46 by the HPr-kinase, whose activity is stimulated by glycolytic intermediates (10, 14). Therefore, the phosphorylation status of HPr

and the stimulation of LevR activity might depend on the carbon source present in the medium. Interestingly, growth on maltose and glycerol requires the presence of a functional PTS and PEP-dependent phosphorylation of HPr, and CCR of glycerol kinase and α-glucosidase synthesis are not abolished in *ccpA* and *ptsH1* mutants (13).

Positive regulation by HPr of the activity of proteins which are involved in catabolic processes may be an additional level of catabolite control in gram-positive bacteria. This would be a functional equivalent of the well-documented phenomenon of inducer exclusion in *E. coli*. In *E. coli*, unphosphorylated $EIIA^{Glc}$ binds to catabolic enzymes such as glycerol kinase and lactose permease and inactivates them. Moreover, phosphorylated $EIIA^{Glc}$ activates the adenylate cyclase and thus allows the expression of catabolic operons (for a review, see reference 47). In *B. subtilis*, in contrast, both mechanisms seem to involve HPr. HPr(H15-P) stimulates catabolic enzymes and regulatory proteins in the absence of glucose, while HPr(S46-P) is involved in repression of catabolic operons in the presence of glucose.

The HPr protein is thus involved in three different pathways of regulation of the levanase operon. (i) Phosphorylation of HPr at Ser-46 is necessary for CCR in $levR^+$ strains. (ii) HPr is part of the phosphotransfer cascade of the *lev*-PTS, which is a negative regulator of LevR activity in the absence of fructose. (iii) HPr phosphorylated at His-15 stimulates the activity of the LevR protein in response to sugar availability in the growth medium. Since the latter two activities of HPr are mutually exclusive, it is not surprising that the wild-type LevR protein is not completely dependent on stimulation.

Further work is required to elucidate the complex mechanisms of the regulatory interactions between PTS components and the LevR protein.

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