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Catabolite Repression Resistance of *gnt* Operon Expression in *Bacillus subtilis* Conferred by Mutation of His-15, the Site of Phosphoenolpyruvate-Dependent Phosphorylation of the Phosphocarrier Protein HPr

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Carbon catabolite repression of the *gnt* operon of *Bacillus subtilis* is mediated by the catabolite control protein CcpA and by HPr, a phosphocarrier protein of the phosphotransferase system. ATP-dependent phosphorylation of HPr at Ser-46 is required for carbon catabolite repression as *ptsH1* mutants in which Ser-46 of HPr is replaced with an unphosphorylatable alanyl residue are resistant to carbon catabolite repression. We here demonstrate that mutation of His-15 of HPr, the site of phosphoenolpyruvate-dependent phosphorylation, also prevents carbon catabolite repression of the *gnt* operon. A strain which expressed two mutant HPrs (one in which Ser-46 is replaced by Ala [S46A HPr] and one in which His-15 is replaced by Ala [H15A HPr]) on the chromosome was barely sensitive to carbon catabolite repression, although the H15A mutant HPr can be phosphorylated at Ser-46 by the ATP-dependent HPr kinase in vitro and in vivo. The S46D mutant HPr which structurally resembles seryl-phosphorylated HPr has a repressive effect on *gnt* expression even in the absence of a repressing sugar. By contrast, the doubly mutated H15E,S46D HPr, which resembles the doubly phosphorylated HPr because of the negative charges introduced by the mutations at both phosphorylation sites, had no such effect. In vitro assays substantiated these findings and demonstrated that in contrast to the wild-type seryl-phosphorylated HPr and the S46D mutant HPr, seryl-phosphorylated H15A mutant HPr and H15E,S46D doubly mutated HPr did not interact with CcpA. These results suggest that His-15 of HPr is important for carbon catabolite repression and that either mutation or phosphorylation at His-15 can prevent carbon catabolite repression.

The bacterial phosphotransferase system (PTS) consists of a number of phosphocarrier proteins that utilize the phosphoryl group of phosphoenolpyruvate (PEP) to phosphorylate incoming sugars. The phosphoryl transfer process is PEP→enzyme I→HPr→enzyme IIA→enzyme IIBC→sugar-P (24).

HPr, the second phosphocarrier protein of the PTS, is phosphorylated on histidyl residue 15 via this phosphorylation chain, thus generating histidyl-phosphorylated HPr (P-His HPr) (17). In addition, a metabolite-activated ATP-dependent protein kinase in gram-positive bacteria is known to phosphorylate HPr on Ser-46 giving rise to seryl-phosphorylated HPr (P-Ser HPr) (6, 10, 12, 25, 26). We have recently shown that P-Ser HPr and a DNA-binding protein of the LacI-GalR family, CcpA (19, 35), interact to mediate carbon catabolite repression of the *gnt* operon in *Bacillus subtilis* (9, 11, 16). Further, data have demonstrated that GntR, the repressor of the *B. subtilis gnt* operon, is not involved in carbon catabolite repression of the *gnt* operon (15) and that neither inducer exclusion nor inducer expulsion is operative (11, 15).

The ATP-dependent HPr kinase is allosterically activated by cytoplasmic phosphorylated metabolites such as fructose-1,6-bisphosphate (FBP) (6, 26), and it specifically phosphorylates HPr at Ser-46 (10). P-Ser HPr binds to CcpA in the presence of glycolytic intermediates such as FBP, glucose-6-P or fructose-1-P (9). The CcpA/P-Ser HPr complex has recently been demonstrated to interact with an operator-like sequence located in the *gnt* operon of *B. subtilis* (16). This operator-like sequence was first described for the α-amylase gene (22) and was named the catabolite-responsive element (*cre*). The nucleoprotein complex is presumed either to block transcriptional initiation (catabolite repression) at some catabolite-sensitive promoters or to activate transcriptional initiation (catabolite activation) at others (3, 19, 20).

In contrast to wild-type *B. subtilis*, the *ptsH1* or *ccpA* mutant strains exhibited insensitivity to carbon catabolite repression of several catabolic operons (11). In *ptsH1* mutants, Ser-46 of HPr is replaced with an alanyl residue, and consequently, the mutant HPr cannot be phosphorylated by the metabolite-activated ATP-dependent HPr kinase (14, 28). We have generated site-directed mutants of *B. subtilis* HPr at His-15 or at both His-15 and Ser-46, and we here present data demonstrating that mutation or phosphorylation of His-15 blocks the interaction of seryl-phosphorylated HPr with CcpA, thereby presumably conferring carbon catabolite repression resistance to the *gnt* operon.

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

Strain	Genotype	HPr[a]	Reference or source[b]
GM122	*sacB'-'lacZ trpC2*		32
GM273	*sacB'-'lacZ trpC2* Δ(*ptsGHI*)		4
SA003	*sacB'-'lacZ trpC2 ptsH1*	S46A (O)	11
SA013	*sacB'-'lacZ trpC2 ptsH1* (pHPr-wt)	S46A (O); Wild type (P)	Tf SA003 × pHPr-wt
SA023	*sacB'-'lacZ trpC2 ptsH1* (pHPr-H15A)	S46A (O); H15A (P)	Tf SA003 × pHPr-H15A
SA033	*sacB'-'lacZ trpC2 ptsH1* (pHPr-S46D)	S46A (O); S46D (P)	Tf SA003 × pHPr-S46D
SA043	*sacB'-'lacZ trpC2 ptsH1* (pHPr-H15E,S46D)	S46A (O); H15E,S46D (P)	Tf SA003 × pHPr-H15E,S46D
SA053	*sacB'-'lacZ trpC2 ptsH1 amyE*::*ptsH*$^+$ *neo*	S46A (O); Wild type (E)	Tf SA003 × pAC7-wt
SA063	*sacB'-'lacZ trpC2 ptsH1 amyE*::*ptsH2 neo*	S46A (O); H15A (E)	Tf SA003 × pAC7-H15A
SA163	*sacB'-'lacZ trpC2 ptsH1 amyE*::*ptsH2 cat neo*	S46A (O); H15A (E)	Tf SA063 × pDG491
SA200	*sacB'-'lacZ trpC2 ptsH1 cat*	S46A (O)	Tf GM122 × SA163
HA273	*sacB'-'lacZ trpC2* Δ(*ptsGHI*) *amyE*::*ptsH2 neo*	H15A (E)	Tf GM373 × SA163
QB5262	*ptsH*-S46D	S46D (O)	33
QB5350	*trpC2 ptsH-H15A amyE*::(*levD'-'lacZ aphA3*)	H15A (O)	33

[a] The wild-type and mutant HPr proteins were expressed from the corresponding HPr-encoding gene present either in the *pts* operon (O) or in the pHPr plasmids (P) or incorporated into the *amyE* locus (E).
[b] Tf, transformation of the indicated strain with the indicated plasmid or chromosomal DNA wt, wild type.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains used in this study are listed in Table 1. Cells were grown in Luria-Bertani medium or in C mineral medium (1) containing the appropriate antibiotic (7 μg of neomycin ml^{-1} or 5 μg of chloramphenicol ml^{-1}) and 1% gluconate or 1% gluconate plus 1% glucose to an optical density at 600 nm of 0.7 to 0.9.

The previously described pHPr plasmids (11, 28) were used for the expression of wild-type HPr, S46D (Ser-46 replaced by Asp) mutant HPr or doubly mutated H15E,S46D HPr in *B. subtilis* SA003. These plasmids are derivatives of the vector pCPP (2) carrying a pUC19 polylinker in which a *Hin*dIII-*Sac*I fragment containing the *B. subtilis* wild-type *ptsH* gene or one of the genes encoding HPr with the indicated mutations have been incorporated. The genes encoding HPr or its various mutant forms are expressed in these plasmids from the heterologous, constitutive *spac* promoter. The integration plasmid pAC7 (36) was used to integrate the wild-type *ptsH* gene or the H15A *ptsH* allele (*ptsH2*) into the *amyE* locus of the *ptsH1* mutant strain SA003. The pHPr plasmid containing the wild-type *ptsH* gene or the *ptsH2* allele was cut with *Eco*RI and *Sac*I, and the resulting DNA fragment bearing *ptsH* or *ptsH2* was incorporated into pAC7 cut with the same enzymes. After transformation of SA003 with the resulting plasmids, neomycin-resistant transformants were isolated which had the wild-type *ptsH* gene or the *ptsH2* allele incorporated into the *amyE* locus of the recipient strain and expressed under control of the *spac* promoter.

Plasmid pDG491 was used to confirm the presence of the *ptsH1* gene in strain SA063. It is a derivative of pJH101 carrying a 400-bp *Stu*I-*Bgl*II *B. subtilis* DNA fragment disrupted by a chloramphenicol resistance cassette which allows its integration about 6 kb downstream of the *ptsH* 3′ end (see strain MO481 in reference 11). The *cat* gene present in pDG491 was integrated downstream of the *ptsH1* gene of strain SA063 and conferred chloramphenicol resistance to the integrants. DNA obtained from one of these integrants (SA163) was used to transform strain GM122, and chloramphenicol-resistant clones were isolated. When grown on solid C mineral medium containing 1% mannitol, 9 of 14 clones showed a reduced colony size, as has been described previously for *ptsH1* mutant strains (11). In three of these mutants (SA200), the *ptsH1* phenotype was confirmed by measuring gluconate kinase activity under inducing and repressing conditions (11). To confirm conservation of the *ptsH2* gene incorporated into the *amyE* locus of strain SA063 together with a neomycin resistance cassette, we transformed DNA isolated from SA063 into the Δ*ptsGHI* mutant strain GM273, providing the neomycin-resistant strain HA273. A crude extract of this strain was heated to 75°C for 5 min, and denatured proteins were removed by centrifugation. HPr, which is resistant to heat treatment, remains in the supernatant and was phosphorylated for 5 min at 37°C in reaction mixtures that contained 5 mM $MgCl_2$, 50 mM Tris-HCl buffer (pH 7.4), and either [^{32}P]PEP (0.1 μCi, 2 μM) and enzyme I or [γ-^{32}P]ATP (0.1 μCi, 5 μM) and the *Enterococcus faecalis* HPr kinase (6). Proteins were separated on a sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gel, and phosphorylated proteins were detected by autoradiography. Control experiments were carried out with purified wild-type HPr and H15A mutant HPr.

Enzyme assays. Gluconate kinase activity was determined in crude extracts of *B. subtilis* cells grown under inducing or repressing conditions in C mineral medium as previously described (11, 23). Measurement of gluconate kinase activity in cells grown in Luria-Bertani medium containing gluconate or gluconate plus glucose gave values similar to those determined in cells grown in C mineral medium (data not shown). Crude extracts of the various strains in which gluconate kinase activity was measured were prepared as described previously (11). Enzyme I and HPr activities were determined by the mutant complementation assay (18) with *Staphylococcus aureus* S710A (*lacR ptsI*) and S797A (*lacR ptsH*), respectively. Uptake of [^{14}C]mannitol was determined as described previously (27).

Protein purification. Wild-type *B. subtilis* HPr was purified as described previously (14). The different mutant HPrs were expressed from the corresponding pHPr plasmids and purified as described previously (28). Enzyme I from *B. subtilis* was synthesized with a His tag at its N terminus after a 1,661-bp *Bam*HI-*Sal*I fragment obtained by PCR was cloned into the expression vector pQE30 (Diagen, Hilden, Germany) cut with the same enzymes. The modified enzyme I carrying 10 additional amino acids at its N terminus was purified in a one-step procedure on a Ni-nitrilotriacetic acid (NTA) column (Diagen) and was found to phosphorylate HPr and to be active in the *S. aureus* mutant complementation assay (18). The ATP-dependent HPr kinase was partially purified from *E. faecalis* (6). A membrane preparation of *E. faecalis* was used as the source of P-Ser HPr phosphatase (8). CcpA from *Bacillus megaterium* was expressed with a His tag and an enterokinase cleavage site at the N terminus and was purified as described previously (9).

Electrophoretic analysis of phosphorylated forms of HPr in crude extracts. Strains GM122, SA003, SA053, and SA063 were grown in 40 ml of C mineral medium containing 1% glucose to an optical density at 600 nm of 0.7 to 0.9. Cells were harvested by centrifugation, washed twice with 20 mM Tris-HCl (pH 7.4) containing 1 mM EDTA and 1 mM dithiothreitol, and resuspended in 300 μl of the same buffer containing 0.5% glucose. The cell suspension was incubated for 1 min at 37°C to allow the uptake and metabolism of glucose, thereby leading to high intracellular concentrations of glycolytic intermediates which activate the HPr kinase (12, 26, 34). Subsequently, most of the proteins were precipitated by keeping the cell suspension for 10 min at 80°C. At this temperature, the phosphoamidate bond in P-His HPr and in doubly phosphorylated P-His,P-Ser HPr is hydrolyzed. The heat-stable HPr and its seryl-phosphorylated derivative remain in solution, and after centrifugation HPr activity can be demonstrated in the supernatant by the mutant complementation assay (18). Aliquots (30 μl) of the supernatant were loaded onto 15% nondenaturing polyacrylamide gels. PEP-dependent phosphorylation of HPr in aliquots (30 μl) of the heat-treated extracts was carried out at 37°C for 10 min in the presence of PEP (5 mM)–$MgCl_2$ (10 mM)–6 μg of *B. subtilis* enzyme I. An identical phosphorylation experiment was carried out with a mixture of purified HPr and P-Ser HPr, thereby generating standards of P-His HPr and doubly phosphorylated HPr (Fig. 1, lane n). Dephosphorylation of P-Ser HPr in aliquots (30 μl) of heat-treated extracts of strains SA053 and SA063 was carried out by using a membrane-associated P-Ser HPr phosphatase of *E. faecalis* as described previously (8). After 20 min of incubation at 37°C, the assay mixture was heated at 80°C for 5 min and centrifuged to remove precipitated proteins, and the supernatant was subsequently loaded onto a 15% nondenaturing polyacrylamide gel.

Elution retardation experiments with various forms of HPr and immobilized CcpA. CcpA from *B. megaterium* carrying a His tag and an enterokinase cleavage site was immobilized on a Ni-NTA column (9), and mixtures of wild-type HPr with either P-Ser HPr, S46D HPr, or H15E,S46D doubly mutated HPr, or a mixture of H15A HPr and H15A P-Ser HPr were passed over a 130-μl CcpA column. The HPr proteins were dissolved in 50 μl of 100 mM Tris-HCl buffer (pH 7.4) containing 20 mM FBP. The same buffer was used for the elution of the HPr proteins from the CcpA column. Aliquots (15 to 40 μl) of the collected fractions (fraction size, 80 μl) were separated on nondenaturing polyacrylamide gels which were stained with Coomassie Brilliant Blue to detect the retardation of HPr or its phosphorylated derivatives as previously described (9). Control

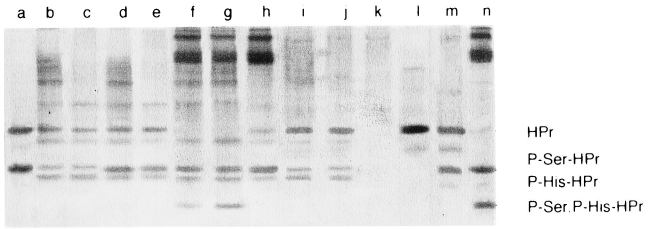

FIG. 1. Presence of HPr, H15A mutant HPr, and their seryl-phosphorylated derivatives in strains GM122, SA003, SA053, and SA063 as demonstrated by PEP-dependent phosphorylation and P-Ser HPr phosphatase-catalyzed dephosphorylation followed by nondenaturing polyacrylamide gel electrophoresis. The following samples were loaded onto a 15% polyacrylamide gel: a mixture of 3 μg of HPr and 3 μg of P-Ser HPr (lane a), 30-μl aliquots of heat-treated extracts of strains GM122, SA003, SA053, and SA063 (lanes b to e, respectively), PEP-dependent phosphorylation of HPr and P-Ser HPr catalyzed by enzyme I (7 μg) in 30-μl aliquots of heat-treated extracts of strains GM122, SA053, and SA063 (lane f to h, respectively), dephosphorylation of seryl-phosphorylated HPr in 30-μl aliquots of heat-treated extracts of strains SA053 and SA063 with 5 μl of a partially purified membrane associated P-Ser HPr phosphatase (lanes i and j, respectively), 5 μl of P-Ser HPr phosphatase (lane k), dephosphorylation of P-Ser HPr in a mixture of 3 μg of HPr and 3 μg of P-Ser HPr (as in lane a) by the partially purified membrane-associated P-Ser HPr phosphatase (5 μl) (lane l), and PEP-dependent phosphorylation of a mixture of 6 μg of HPr and 6 μg of P-Ser HPr, with half of the reaction mixture kept for 10 min at 80°C prior to electrophoresis (lane m) and the other half loaded directly onto the gel (lane n).

experiments, demonstrating that neither HPr nor P-Ser HPr is retarded on Ni-NTA resin devoid of CcpA, have been described previously (9).

RESULTS

Wild-type HPr, but not H15A HPr, restores carbon catabolite repression in a *B. subtilis ptsH1* mutant strain. The *ptsH1* mutant strain SA003, expressing HPr in which Ser-46 has been replaced with an alanyl residue and which, therefore, cannot be phosphorylated by the ATP-dependent HPr kinase (14, 28), has previously been shown to be resistant to carbon catabolite repression of several catabolic operons including the *gnt* operon (11). Reversion of this mutation, providing the wild-type *ptsH* gene, restored carbon catabolite repression (11). Similarly, transformation of the *ptsH1* mutant strain with a pHPr plasmid expressing wild-type HPr also restored carbon catabolite repression (Table 2, strain SA013). We expected that transformation of SA003 with a pHPr plasmid containing the *ptsH2* gene (expressing H15A mutant HPr) would also restore carbon catabolite repression, as this mutant HPr can be readily phosphorylated at Ser-46 (28). However, such transformants (SA023) were found to take up mannitol very slowly (about sixfold more slowly than did the wild-type strain GM122), and they grew poorly on C mineral medium containing the PTS sugar glucose or mannitol. Consequently, this transformant could not be used to measure carbon catabolite repression. Nevertheless, SA023 grew normally on non-PTS sugars such as glucitol or gluconate. The reduced PTS transport activity of strain SA023 is most likely due to high-level expression of the plasmid-encoded H15A mutant HPr, which has previously been demonstrated to exhibit an eightfold-higher affinity for enzyme I than did wild-type HPr; it competitively inhibits the PEP-dependent phosphorylation of wild-type HPr (29).

We, therefore, integrated the wild-type *ptsH* gene or the *ptsH2* gene into the chromosome at the *amyE* locus of the *ptsH1* mutant strain SA003, providing strains SA053 and SA063, respectively. In contrast to SA023, SA063 grew normally on solid C mineral medium containing the PTS sugar glucose or mannitol, and it transported mannitol at rates similar to those observed for SA003 (transport activity, expressed in nanomoles of [^{14}C]mannitol taken up per minute per milligram of cellular protein, was 3.34 for wild-type strain GM122, 3.42 for *ptsH1* mutant SA003, 3.16 for strain SA063, and 0.54 for strain SA023). Synthesis of the wild-type HPr from the *ptsH* gene integrated into the *amyE* locus of SA003 was found to restore carbon catabolite repression (Table 2). Surprisingly, strain SA063, synthesizing two mutant HPrs, each of which can be phosphorylated only once (the H15A mutant HPr at Ser-46 and the S46A mutant HPr at His-15), exhibited an eightfold-higher resistance to carbon catabolite repression of the *gnt* operon (Table 2) than did strain SA053 (synthesizing wild-type and S46A HPr). As the H15A mutant HPr can be phosphorylated in vitro at Ser-46, the mutation at His-15 of this phosphocarrier protein was expected to be responsible for its failure to restore carbon catabolite repression of the *gnt* operon in the *ptsH1* mutant strain. No repression of *gntK* expression by glucose was observed in a mutant strain expressing only H15A mutant HPr (Table 2, strain QB5350), which is most likely due the inability of this strain to take up PTS sugars.

In vivo phosphorylation at Ser-46 in HPr and H15A HPr. The H15A mutant HPr can be phosphorylated in vitro at Ser-46 at a rate similar to that of wild-type HPr (28). To insure that the H15A mutant HPr is also phosphorylated in vivo by the ATP-dependent HPr kinase, and to exclude the possibility that the reduced sensitivity towards carbon catabolite repression in strain SA063 is due to inefficient seryl phosphorylation of H15A HPr, we compared the amounts of P-Ser HPr and H15A P-Ser HPr present in strains SA053 and SA063, respectively. The two strains were grown in C mineral medium containing 1% glucose. Cells were lysed, and most of the proteins were precipitated by heat treatment as described in Materials and Methods. Although HPr was resistant to the heat treatment and its activity could be detected in the supernatant, the phosphate bond in His-phosphorylated HPr was hydrolyzed, whereas the phosphate bond in Ser-phosphorylated HPr was not affected by the heat treatment (12).

TABLE 2. Gluconate kinase activity in strain SA003 expressing the chromosomally located *ptsH1* gene and in different transformants or integrants of SA003 expressing, in addition, the wild-type *ptsH* (SA013 and SA053), *ptsH2* (SA063), or the gene encoding S46D mutant HPr (SA033) or doubly mutated H15E,S46D HPr (SA043) and in strain QB5350 and QB5262 carrying the H15A or S46D *ptsH* allele, respectively

Strain	HPr[a]	Gluconate kinase activity after growth in C medium supplemented with[b]:	
		Gluconate	Gluconate + glucose
GM122	wt, O	28.4 ± 3.7	2.3 ± 0.9
SA003	S46A, O	31.3 ± 3.8	33.4 ± 3.7
SA013	S46A, O; wt, P	32.5 ± 4.4	1.8 ± 0.8
SA033	S46A, O; S46D, P	8.2 ± 1.7	6.9 ± 1.9
SA043	S46A, O; H15E,S46D, P	28.8 ± 4.2	23.2 ± 3.6
SA053	S46A, O; wt, E	35.9 ± 4.7	2.1 ± 0.9
SA063	S46A, O; H15A, E	33.8 ± 4.5	18.9 ± 3.3
QB5350	H15A, O	34.7 ± 3.9	32.2 ± 4.2
QB5262	S46D, O	31.6 ± 4.4	32.1 ± 3.9

[a] Wild-type (wt) and/or mutant Hpr expressed in each strain is indicated. O, P, and E, corresponding HPr expressed from the *ptsH* gene located in the *pts* operon, the pHPr plasmid, or the *amyE* locus, respectively. See Table 1.

[b] The mean enzyme activity ± standard deviation, expressed as nanomoles of product formed per minute per milligram of protein, for three independent experiments is given.

The identities of HPr, P-Ser HPr, and P-Ser,P-His HPr were verified by a series of in vitro experiments summarized in Fig. 1. They included PEP-dependent, enzyme I-catalyzed phosphorylation of HPr at His-15 and P-Ser HPr phosphatase-catalyzed dephosphorylation of P-Ser HPr. Identification of the various forms of HPr was based on the different electrophoretic mobilities exhibited by HPr, singly phosphorylated HPr (P-His HPr or P-Ser HPr), and doubly phosphorylated HPr on nondenaturing polyacrylamide gels (Fig. 1). In control experiments, a mixture of HPr and P-Ser HPr was incubated with PEP and enzyme I, producing P-His HPr and P-Ser,P-His HPr (lane n). A portion of the reaction mixture was heated to demonstrate the thermosensitivity of the phosphoamidate bond in histidine phosphates (lane m). In addition, P-Ser HPr present in a mixture of HPr and P-Ser HPr (lane a) was dephosphorylated in vitro by incubation with P-Ser HPr phosphatase (lane l).

Heat-treated extracts of strains SA053 and SA063 contained approximately equal amounts of a protein with the mobilities of P-Ser HPr and P-His HPr (Fig. 1, lanes d and e, respectively). Since histidine phosphorylation of HPr is removed by the experimental procedure, the presence of this protein indicates that H15A HPr can be phosphorylated at Ser-46 to approximately the same extent as can wild-type HPr. A less abundant protein with similar mobility was also seen in extracts of GM122 and SA003 (lanes b and c). This protein cannot be P-Ser HPr since S46A HPr is the only form of HPr found in strain SA003 and cannot be phosphorylated at Ser-46. This unknown protein presumably contributes a small background to all of the bands with the same mobilities found in the extracts.

When extracts of strains GM122 and SA053 were subjected to PEP-dependent phosphorylation, the band corresponding to unphosphorylated HPr disappeared in favor of bands that comigrated with P-His HPr and P-Ser,P-His HPr (lanes f and g). By contrast, similar treatment of an extract of strain SA063 did not convert all of the HPr to a phosphorylated form (indicating the presence of H15A HPr) and produced no P-Ser,P-His HPr (lane h). The presence of P-Ser HPr in extracts of strains SA053 and SA063 was confirmed by the effects of incubation with P-Ser HPr phosphatase (lanes i and j).

Altogether, these results establish that comparable amounts of P-Ser HPr and H15A P-Ser HPr are formed in strains SA053 and SA063, respectively. Thus, the reduced sensitivity of SA063 to carbon catabolite repression is not due to inefficient phosphorylation of Ser-46 of the H15A HPr in this strain. The observed formation of relatively large amounts of P-Ser HPr in glucose-metabolizing *B. subtilis* cells is in agreement with a similar observation made previously for glucose-metabolizing oral streptococci (34).

No gene conversion had occurred in strain SA063. As strain SA063 contains two mutant *ptsH* genes (*ptsH1* in the *pts* operon and *ptsH2* in the *amyE* locus), gene conversion could have occurred, leading to the synthesis of doubly mutated H15A,S46A HPr. The doubly mutated HPr could possibly be responsible for the observed reduced sensitivity towards carbon catabolite repression in this strain. To exclude this possibility, we integrated a chloramphenicol resistance cassette about 6 kb downstream from the *ptsH1* gene into strain SA063, providing strain SA163 (see Materials and Methods). DNA isolated from strain SA163 was used to transform strain GM122, and chloramphenicol-resistant clones were isolated. About 60% of these transformants exhibited the *ptsH1* phenotype (cotransformation of the *ptsH1* gene and the *cat* gene) when grown on solid C mineral medium containing mannitol (11). In three of these mutant strains (SA200), gluconate kinase activity was determined after growth in the presence of gluconate as well as in the presence of gluconate plus glucose. No repressive effect of glucose on the expression of gluconate kinase could be observed, confirming the presence of the *ptsH1* gene in the selected SA200 mutants and in strain SA063 (data not shown).

DNA from strain SA063 was used to transform the Δ*ptsGHI* deletion strain GM273 (11), which does not synthesize HPr, and neomycin-resistant transformants were isolated. A crude extract of one of the transformants (strain HA273) was heated for 5 min at 75°C and then centrifuged. [^{32}P]PEP-dependent or [γ-^{32}P]ATP-dependent phosphorylation of HPr present in the supernatant was subsequently carried out with purified enzyme I or partially purified HPr kinase, respectively. A strong radiolabeled band comigrating with phosphorylated HPr standard was observed only for [γ-^{32}P]ATP and HPr kinase and not for [^{32}P]PEP and enzyme I (data not shown), indicating that the expressed HPr is the H15A mutant HPr. These results establish that gene conversion had not taken place in strain SA063. The synthesis of the correct wild-type and the two mutant HPrs (H15A and S46A) in strains SA053 and SA063 was also confirmed by the results presented in Fig. 1. The seryl-phosphorylated proteins can be phosphorylated by PEP and enzyme I in heat-treated extracts of strain SA053 but not in extracts of strain SA063 (Fig. 1, lanes g and h).

The H15E,S46D doubly mutated HPr cannot restore carbon catabolite repression in strain SA003. Transformation of the Δ*ptsGHI* mutant strain GM273 with a pHPr plasmid expressing S46D mutant HPr, which structurally resembles P-Ser HPr (37), was found to have a repressive effect on expression of the *gnt* operon even in the absence of a repressing sugar (11). A similar repressive effect of S46D HPr on *gnt* expression was observed when the corresponding pHPr plasmid was introduced into SA003, providing strain SA033. Induction of gluconate kinase activity in strain SA033 was reduced about threefold compared with that in SA003 (Table 2). However, expression of the doubly mutated H15E,S46D HPr from the corresponding pHPr plasmid did not lead to the permanent repression (reduced expression in the absence of glucose) of *gntK* expression observed in strain SA033 (Table 2, strain SA043). Doubly mutated H15E,S46D HPr carries negative charges at the PEP-dependent and ATP-dependent phosphorylation sites, and it is therefore considered to resemble doubly phosphorylated P-His,P-Ser HPr. It is noteworthy that in contrast to P-Ser HPr, doubly phosphorylated P-His,P-Ser HPr does not interact with CcpA (9). We therefore investigated the possibility that the failure of doubly mutated H15E,S46D HPr to repress *gnt* operon expression might be due to its inability to interact with CcpA.

S46D mutant HPr, but not H15E,S46D doubly mutated HPr, interacts with CcpA. Mixtures of purified wild-type HPr and purified S46D mutant HPr or H15E,S46D doubly mutated HPr were used in elution retardation experiments on CcpA columns as described in Materials and Methods. The experiments were carried out in the presence of 20 mM FBP. The S46D mutant HPr was significantly retarded on the CcpA column compared with wild-type HPr (Fig. 2A). However, the doubly mutated H15E,S46D HPr did not interact with CcpA, and it eluted from the CcpA column at the same position as unphosphorylated wild-type HPr (Fig. 2B). Evidently, introduction of a negative charge at the PEP-dependent phosphorylation site, His-15, of HPr prevents the interaction with CcpA.

Seryl-phosphorylated H15A mutant HPr does not interact with the CcpA protein. As His-15 of HPr appears to be important for the interaction of seryl-phosphorylated HPr with CcpA, we tested whether the H15A mutation might prevent

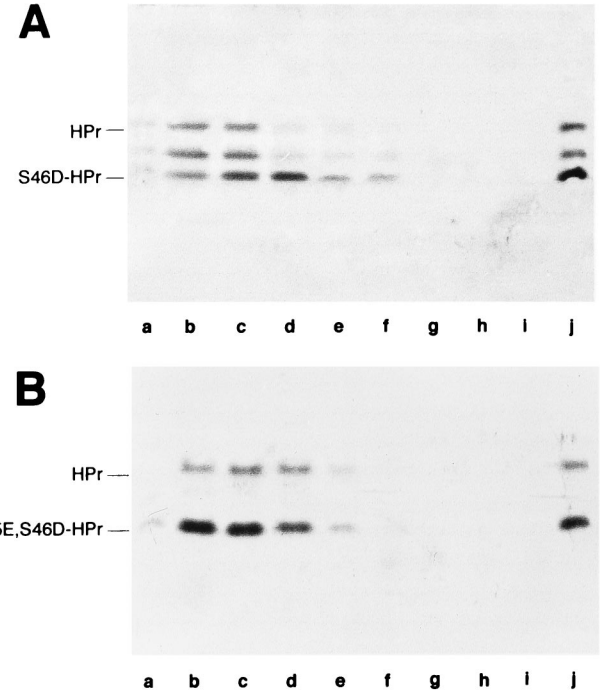

FIG. 2. Elution retardation experiments with CcpA immobilized on a Ni-NTA column and mixtures of HPr (25 μg) and S46D mutant HPr (50 μg) (A) or HPr (25 μg) and H15E,S46D doubly mutated HPr (50 μg) (B). Solutions of 50 μl of the HPr proteins in 100 mM Tris-HCl buffer (pH 7.4) were passed over a CcpA column (130-μl bed volume) in the presence of 20 mM FBP, and fractions (80 μl) were collected. Aliquots (15 μl) (A) or (30 μl) (B) were separated on 12.5% nondenaturing polyacrylamide gels, and protein bands were stained with Coomassie brilliant blue. Standards of mixtures of *B. subtilis* wild-type HPr with the two different mutant HPrs (S46D and H15E,S46D) are shown in lanes j in panels A and B, respectively. The band migrating between HPr and S46D HPr is an impurity present in the S46D HPr preparation.

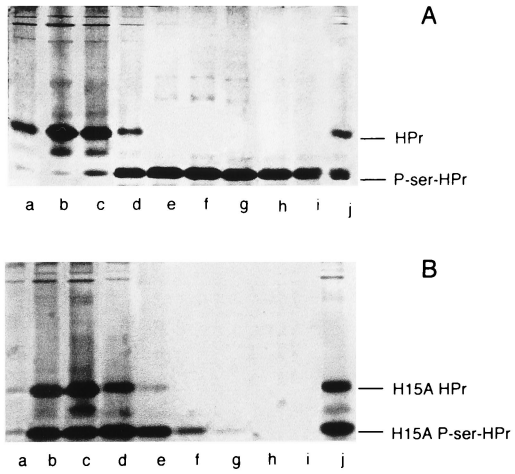

FIG. 3. Elution retardation experiments with CcpA immobilized on a Ni-NTA column and mixtures of HPr and P-Ser HPr (each 50 μg) (A) or H15A HPr and seryl-phosphorylated H15A HPr (each 50 μg) (B). Elution retardation experiments were carried out in the presence of 20 mM FBP as described in the legend to Fig. 2. Aliquots (40 μl) of the collected fractions (80 μl) were separated on 12.5% nondenaturing polyacrylamide gels, and proteins were visualized by staining with Coomassie brilliant blue. Standards of *B. subtilis* wild-type HPr and P-Ser HPr or of H15A HPr and H15A P-Ser HPr are shown in lanes j in panels A and B, respectively.

the binding of P-Ser HPr to CcpA. The mutant HPr was purified as described for the wild-type protein (29), and ATP-dependent phosphorylation at Ser-46 was carried out with *E. faecalis* HPr kinase (6). A mixture of wild-type HPr and P-Ser HPr and a mixture of H15A mutant HPr and seryl-phosphorylated H15A mutant HPr were passed over a CcpA column as described above. Wild-type P-Ser HPr was strongly retarded on a CcpA column in the presence of 20 mM FBP (Fig. 3A). However, when a mixture of H15A mutant HPr and the seryl-phosphorylated derivative of this protein was passed over the CcpA column in the presence of FBP, only weak retardation of the phosphorylated mutant HPr was observed (Fig. 3B).

DISCUSSION

The data presented in previous communications demonstrated that ATP-dependent phosphorylation of HPr at seryl residue 46 plays a role in carbon catabolite repression in *B. subtilis* (9, 11, 16). The results described in this communication suggest that the PEP-dependent phosphorylation site in HPr, i.e., His-15, is also important for HPr-mediated carbon catabolite repression of the *B. subtilis gnt* operon. Several lines of evidence support this conclusion: (i) S46D mutant HPr leads to permanent carbon catabolite repression in a *ptsH1* mutant strain independent of the presence of a repressing sugar, whereas the doubly mutated H15E,S46D HPr has no such effect; (ii) synthesis of the wild-type HPr, but not of the H15A mutant HPr, restores carbon catabolite repression in a *ptsH1* mutant strain; (iii) S46D mutant HPr interacts with CcpA in elution retardation experiments, whereas the doubly mutated H15E,S46D HPr does not; and (iv) only wild-type P-Ser HPr but not H15A P-Ser HPr interacts with CcpA in elution retardation experiments. The repressive effect of S46D HPr is observed only when the mutant HPr is expressed from a multicopy plasmid (compare strains SA003, SA033, and QB5262 [Table 2]). By contrast, the doubly mutated H15E,S46D HPr exhibited no similar repressive effect, even when expressed from a multicopy plasmid (Table 2, strain SA043).

A specific interaction of CcpA with P-Ser HPr, but not with HPr, has been demonstrated by gel retardation experiments (9) and by nuclear magnetic resonance measurements (21). The interaction with P-Ser HPr allows CcpA to bind to *cre*, an operator-like sequence located in the 5′ region of the *gntR* gene (16). Our data suggest that His-15, the site of PEP-dependent phosphorylation of HPr, is required for metabolite-activated formation of the complex between P-Ser HPr and CcpA and consequently for carbon catabolite repression. Conceivably, His-15 is directly involved in binding of P-Ser HPr to CcpA, or alterations at His-15 (mutation or phosphorylation) might cause structural changes that prevent the high-affinity binding of the modified P-Ser HPr to CcpA. It is interesting to note that the *Escherichia coli* glycerol kinase and the *B. subtilis* CcpA protein both appear to possess two regulatory binding sites, one for FBP and another for a PTS protein (unphosphorylated enzyme IIA^{Glc} in the case of glycerol kinase [5] and seryl-phosphorylated HPr in the case of CcpA [9]).

The observed sensitivity of the P-Ser HPr/CcpA interaction to PEP-dependent, enzyme I-catalyzed phosphorylation at His-15 suggests a link between carbon catabolite repression and PTS-mediated sugar transport. A similar link in members of the family *Enterobacteriaceae* has been reported, in which the phosphorylation state of a histidine in enzyme IIA^{Glc} is sensed during carbon catabolite repression and inducer exclusion (5, 30, 31). Since only P-Ser HPr has been shown to bind to CcpA, whereas unphosphorylated, His-15-phosphorylated or doubly phosphorylated HPr did not interact with this DNA

binding protein (9), we suggest that only P-Ser HPr plays a role in carbon catabolite repression in *B. subtilis*. The cellular concentration of P-Ser HPr is determined by the activities of four different enzymes: phosphorylation of HPr by the ATP-dependent HPr kinase and dephosphorylation of doubly phosphorylated HPr by one of the various enzymes IIA lead to the formation of P-Ser HPr, whereas dephosphorylation of P-Ser HPr by the P-Ser HPr phosphatase and phosphorylation of P-Ser HPr by the PEP-dependent enzyme I lower the concentration of P-Ser HPr. The uptake of a PTS sugar leads to dephosphorylation of histidyl-phosphorylated PTS proteins (24) and to high concentrations of glycolytic intermediates that activate the HPr kinase (6, 26). Under these conditions, large amounts of P-Ser HPr are expected to be present in the cell (34), (see also Fig. 1), correlating with strong carbon catabolite repression in *B. subtilis*. By contrast, during the uptake of non-PTS sugars, PTS proteins have been shown to be present in the cell as the histidyl-phosphorylated derivatives (24). Consequently, during the uptake of a non-PTS sugar by gram-positive bacteria, mainly the doubly phosphorylated or the histidyl phosphorylated HPr are expected to be present in the cell (28). Neither of these two forms of HPr interacts in vitro with CcpA (9). The catabolite-repressive effects of non-PTS sugars, such as glycerol, may therefore be exerted by a different mechanism. This conclusion is supported by the finding which demonstrated that the repressive effect of glycerol was not relieved in the *ptsH1* or *ccpA* mutant (11, 19).

P-Ser HPr of gram-positive bacteria has previously been reported to be involved in regulation of (i) PTS sugar phosphorylation (7), (ii) PTS sugar transport (40, 41), (iii) non-PTS sugar transport (38, 39), and (iv) sugar phosphate phosphatase activity (40, 41). Direct binding of P-Ser HPr to non-PTS sugar permeases and to a sugar-phosphate phosphatase has been demonstrated (42–44). In *E. faecalis*, unphosphorylated HPr as well as P-Ser HPr can regulate glycerol kinase activity by PEP-dependent protein phosphorylation (13). The results presented here and in previous publications (9, 11, 16), strongly suggest that P-Ser HPr plays a role in carbon catabolite repression and that His-15 of HPr is important for this regulation. His-15 is apparently not required for the regulation of sugar transport or sugar phosphate phosphatase activity (45). Regardless of the dependence on His-15, P-Ser HPr clearly plays a pivotal role in several processes involved in the regulation of carbon metabolism in gram-positive bacteria.

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