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Loss of Protein Kinase-Catalyzed Phosphorylation of HPr, a Phosphocarrier Protein of the Phosphotransferase System, by Mutation of the *ptsH* Gene Confers Catabolite Repression Resistance to Several Catabolic Genes of *Bacillus subtilis*

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In gram-positive bacteria, HPr, a phosphocarrier protein of the phosphoenolpyruvate:sugar phosphotransferase system (PTS), is phosphorylated by an ATP-dependent, metabolite-activated protein kinase on seryl residue 46. In a *Bacillus subtilis* mutant strain in which Ser-46 of HPr was replaced with a nonphosphorylatable alanyl residue (*ptsH1* mutation), synthesis of gluconate kinase, glucitol dehydrogenase, mannitol-1-P dehydrogenase and the mannitol-specific PTS permease was completely relieved from repression by glucose, fructose, or mannitol, whereas synthesis of inositol dehydrogenase was partially relieved from catabolite repression and synthesis of α-glucosidase and glycerol kinase was still subject to catabolite repression. When the S46A mutation in HPr was reverted to give S46 wild-type HPr, expression of gluconate kinase and glucitol dehydrogenase regained full sensitivity to repression by PTS sugars. These results suggest that phosphorylation of HPr at Ser-46 is directly or indirectly involved in catabolite repression. A strain deleted for the *ptsGHI* genes was transformed with plasmids expressing either the wild-type *ptsH* gene or various S46 mutant *ptsH* genes (S46A or S46D). Expression of the gene encoding S46D HPr, having a structure similar to that of P-ser-HPr according to nuclear magnetic resonance data, caused significant reduction of gluconate kinase activity, whereas expression of the genes encoding wild-type or S46A HPr had no effect on this enzyme activity. When the promoterless *lacZ* gene was put under the control of the *gnt* promoter and was subsequently incorporated into the *amyE* gene on the *B. subtilis* chromosome, expression of β-galactosidase was inducible by gluconate and repressed by glucose. However, we observed no repression of β-galactosidase activity in a strain carrying the *ptsH1* mutation. Additionally, we investigated a *ccpA* mutant strain and observed that all of the enzymes which we found to be relieved from carbon catabolite repression in the *ptsH1* mutant strain were also insensitive to catabolite repression in the *ccpA* mutant. Enzymes that were repressed in the *ptsH1* mutant were also repressed in the *ccpA* mutant.

Catabolite-repressible genes in the gram-positive bacterium *Bacillus subtilis* are controlled by multiple mechanisms rather than by a single global regulatory system. These mechanisms are known to differ from those operating in enteric bacteria such as *Escherichia coli*, but the molecular details are poorly understood (15, 40, 46). A mutant (*ccpA*) has been isolated (20), defective in a presumed repressor homologous to LacI and GalR, which exhibited resistance to catabolite repression of several genes including the gluconate kinase gene *gntK* (19). A *cis*-acting sequence involved in catabolite repression and sharing homology with the consensus sequence of catabolite responsive elements (CREs) of *B. subtilis* catabolite-repressed genes (48) has been identified within the gluconate (*gnt*) operon (29). It has previously been reported that expression of the inducible *gnt* and *iol* (inositol) operons in *B. subtilis* is repressed only by metabolizable carbohydrates. Studies by Dowds et al. (12) and later by Nihashi and Fujita (30) have implicated certain glycolytic intermediates, including fructose-1,6-bisphosphate (FBP) in catabolite repression of the *gnt* and *iol* operons. Since the ATP-dependent HPr kinase of gram-positive bacteria (10) which phosphorylates seryl residue 46 in HPr (8) is allosterically regulated by glycolytic intermediates including FBP (7, 33, 35, 37), we investigated the potential role of this phosphorylation reaction in catabolite repression.

HPr, a phosphocarrier protein of the phosphotransferase system (PTS) is phosphorylated at two different sites: (i) at the catalytic His-15 by enzyme I of the PTS in a phosphoenolpyruvate-dependent reaction and (ii) at the regulatory Ser-46 by an ATP-dependent, metabolite-activated protein kinase (8). To study a potential involvement of seryl-phosphorylated HPr in catabolite repression, we introduced the serine 46-to-alanine (*ptsH1*) mutation into the *B. subtilis* chromosomal *ptsH* gene (encoding HPr). We found that this mutation had no effect on the uptake either of the PTS substrates glucose and mannitol or of the non-PTS substrates gluconate and glucitol. However, the mutation abolished the catabolite repressive effects of glucose on gluconate kinase, glucitol dehydrogenase, and the mannitol-specific catabolic enzymes. It partially relieved the

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

Strain	Genotype[a]	Reference or source[b]
JH642	*trpC2 pheA1*	1
MO481	*trpC2 pheA1* (Cam^r)	P. Stragier
GM122	*sacB'-'lacZ trpC2*	44
GM273[c]	*sacB'-'lacZ trpC2 Δ(ptsGHI)*	pTS224 (13) tf GM122
GM803	*sacB'-'lacZ trpC2 Δ(ptsGHI) iol*	pTS224 (13) tf SA003a
GM808	*sacB'-'lacZ trpC2 ptsH$^+$ iol*	pTS22 (13) tf GM803
GM766	*trpC2 pheA1 Δ(bgaX)*	43
GM1096	*trpC2 leuB6 sacB'-'lacZ ccpA*::Tn*917*Δ(*lacZ-erm*)	45
GM1212	*sacB'-'lacZ trpC2 ptsH1*	MO481 chromosomal DNA tf SA003
GM1199	*trpC2 pheA1 Δ(bgaX) amyE*::(*gntRK'-lacZ*)	This work
GM1221	*trpC2 pheA1 Δ(bgaX) amyE*::(*gntRK'-lacZ*) *ptsH$^+$* (Cam^r)	GM1212 chromosomal DNA tf GM1199
GM1222	*trpC2 pheA1 Δ(bgaX) amyE*::(*gntRK'-lacZ*) *ptsH1* (Cam^r)	GM1212 chromosomal DNA tf GM1199
GM1225	*trpC2 pheA1 Δ(bgaX) amyE*::(*gntRK'-lacZ*) *ccpA*::Tn*917*Δ(*lacZ-erm*)	GM1096 chromosomal DNA tf GM1199
SA003	*sacB'-'lacZ trpC2 ptsH1*	pTS223 (13) tf GM273
SA003a	*sacB'-'lacZ trpC2 ptsH1 iol*	This work
YF160	*gntK10 trpC2 metC7*	17

[a] MO481 contains a pJH101 derivative (conferring CAM resistance) inserted downstream from *ptsHI*. Δ(*ptsGHI*) confers resistance to ERY. Δ(*bgaX*) is a deletion of a cryptic β-galactosidase gene. Tn*917*Δ(*lacZ-erm*) is a Tn*917* derivative conferring resistance to SPC. The *gntRK'-lacZ* fusion is associated with a PHL resistance gene.

[b] tf, the indicated plasmid or chromosomal DNA was used to transform the indicated recipient strain.

[c] GM273-S46A, -S46D, -S46T, and -S46Y are transformants of GM273 containing plasmids pHPr-S46A, -S46D, -S46T, or -S46Y, respectively.

repressive effect on inositol dehydrogenase, whereas repression of glycerol kinase and α-glucosidase was not affected. At least in the case of gluconate kinase, this effect occurs at the level of gene expression.

MATERIALS AND METHODS

Strains and growth conditions. The *B. subtilis* strains used in this study are listed in Table 1. Strains GM273 and SA003 were constructed from GM122 by using plasmids pTS224 and pTS223 (13), following the procedure described previously (13). GM273(pHPr) transformants were selected and tested as described below. Strain SA003a, derived from strain SA003, had acquired a spontaneous *iol* mutation (9). SA003a exhibited the same sensitivity to catabolite repression of the enzymes gluconate kinase and glucitol dehydrogenase as was observed for the parental strain SA003 (9). The S46A *ptsH* gene in SA003a was replaced by the wild-type *ptsH* gene by using plasmids pTS224 and pTS22 (13), which provided first strain GM803 (Δ*ptsGHI*) and subsequently strain GM808. The different strains were generally grown in Luria Bertani (LB) medium, or, when indicated, in C mineral medium (2). These media contained the indicated carbon sources (1% for LB medium and 0.5% final concentration for C mineral medium); chloramphenicol (CAM, 5 μg/ml) was added for GM273(pHPr) transformants. The growth rates of the various strains examined were the same in either LB (2.1 h^{-1}) or minimal medium (1.1 to 0.7 h^{-1}, depending on the carbon source used) with deviations not exceeding ±10%. However, GM273(pHPr) transformants showed a 1.5-fold slower growth rate in LB medium (containing 5 μg of CAM ml^{-1}) compared with the untransformed GM273 (grown in LB medium without CAM). The pHPr plasmids are previously described derivatives of plasmid pCCP (39). These replicative plasmids, conferring resistance to CAM in *B. subtilis*, contain the *B. subtilis* wild-type *ptsH* gene or the S46A, S46Y, S46T, or S46D mutant *ptsH* gene expressed under the control of a heterologous, constitutive promoter (39). Competent cells of GM273 were prepared as described previously (14), and GM273(pHPr) transformants were selected on LB plates containing 5 μg of CAM ml^{-1}. Transformants were tested for expression of the different HPr proteins by immunoblot analysis after aliquots of crude extracts of the different transformants were separated by nondenaturing polyacrylamide gel electrophoresis. A stained protein band was detected in the different GM273(pHPr) transformants but not in untransformed GM273. Transformants expressing the S46D HPr exhibited a stained HPr band on Western blots (immunoblots) which, because of the additional negative charge, migrated ahead of the other HPr mutant proteins during electrophoretic separation, as has been shown previously (39). The immunospecific signal in extracts of GM273(pHPr) transformants was two- to three-fold stronger than the signal observed in extracts of GM122, in which the *ptsH* wild-type gene is expressed from its natural promoter (9).

The *ccpA*::Tn*917*Δ(*lacZ-erm*) insertion present in the chromosome of strain GM1096 confers spectinomycin resistance (45); it is a derivative of the original *ccpA*::Tn*917lac* insertion (20).

Construction of GM1199 and its derivatives is described below.

Construction of the *gntRK'-lacZ* fusion and its insertion into the *B. subtilis* chromosome. Two oligonucleotides were synthesized to amplify, by PCR, an 800-bp fragment encompassing the *gnt* promoter region (including the AT-rich sequences upstream from the −35 box which, according to Miwa and Fujita [28], are required for full promoter strength), the complete *gntR* gene (encoding the *gnt* repressor) including a site responsible for catabolite repression of the operon (29) and the very beginning of the *gntK* gene (encoding gluconate kinase). The oligonucleotide 5'-ATTGAATTCCAATGTAC CATAATTGATCTGG-3' hybridizes to the 5' upstream region (−121 to −91 with respect to the transcriptional start site) of the *gnt* promoter (18). It contains two mismatches (underlined), generating an *Eco*RI site. The oligonucleotide with the sequence 5'-CCGATGTCGACTCCTAACATATAACTAGT CATTG-3' hybridizes within the 5' part of *gntK* (18) (+759 to +792 with respect to the transcriptional start point) and also contains two mismatches (underlined), generating a *Sal*I site. The PCR product, generated by using DNA isolated from GM122 as a template, was digested with both *Sal*I and *Eco*RI and was inserted into pIC112 digested by the same enzymes, giving pIC295. pIC112 is a pSL150 (5) derivative containing a single *Sal*I site. The PCR product was recovered from pIC295 as an *Eco*RI-*Sal*I fragment. The *Eco*RI site was converted to a blunt end by using the Klenow fragment of DNA polymerase. The PCR product was then inserted into pIC172 (see below) digested with *Sma*I and *Sal*I. Ligation of the *Sal*I extremities of the pIC172 vector and the PCR product allowed the construction of a translational in-phase fusion of the first seven codons of the *gntK* gene with the *lacZ* gene, the latter being devoid of transcriptional and translational signals. pIC172 is a vector designed to insert *lacZ* fusions into the *amyE* gene (42) in association with the pUB110 (21) phleomycin (PHL) resistance gene. pIC172 was derived from pSL150 (5) by replacing

the 1.2-kb *Eco*RI-*Sma*I fragment, containing the CAM resistance gene, with the 1.4-kb *Eco*RI-*Pvu*II PHL resistance cassette of pIC22 (45). The pIC172 derivative containing the *gntRK'* insertion was designated pIC319. Insertion of the translational *gntRK'-lacZ* fusion into the *amyE* gene of strain GM766 was screened as described previously (42), providing strain GM1199. PHL-resistant transformants were selected on LB medium containing 0.25 μg of PHL ml^{-1} (purchased from Cayla).

Cotransformation of the *ptsH1* mutation with a resistance marker. In order to construct a *ptsH1* derivative of strain GM1199, we used a *ptsH1*-linked insertion, present in the chromosome of strain MO481 (provided by P. Stragier). MO481 was constructed by using a derivative of the nonreplicative plasmid pJH101 (conferring CAM resistance [Cam^r]) (14) which carried a 0.4-kb *Stu*I-*Bgl*II fragment derived from the *B. subtilis* chromosome 6 kb downstream from the *ptsH* 3' end (1). This plasmid was inserted by homologous recombination into the chromosome of strain JH642, resulting in strain MO481. DNA extracted from MO481 was used to transform strain SA003. About 40% of the chloramphenicol-resistant transformants showed a *ptsH1* phenotype by forming small colonies on mannitol mineral medium (13). DNA extracted from one of these *ptsH1* recombinants (GM1212) was used to cotransfer *ptsH1* into GM1199. As expected, about 60% of the chloramphenicol-resistant transformants showed the *ptsH1* phenotype. This cross provided the pair of isogenic strains GM1221 (*ptsH*$^+$) and GM1222 (*ptsH1*).

Uptake of [^{14}C]glucitol from solid C mineral medium. Strains GM122 and SA003 were grown overnight on LB medium. Aliquots were spread onto solid C mineral medium (2) containing 1% glucose and 1 μM [^{14}C]glucitol (1.18×10^2 GBq/mmol) (50). Colonies grew up within 2 days. Nitrocellulose filters, type HB 80 (Schleicher and Schuell, Dassel, Germany), were used to obtain replicas. After air drying, the filters were exposed to autoradiography for 4 days.

Enzyme assays. Cells of the different *B. subtilis* strains were grown in 50 ml of LB medium (containing 5 μg of CAM if appropriate) or, when mentioned, in C mineral medium (2), to an optical density at 578 nm of 0.6 to 0.9 (exponential growth phase). Cells were harvested by centrifugation and were washed twice with 100 mM Tris-HCl buffer (pH 7.4). They were then resuspended in 1 ml of the same buffer and were incubated for 30 min at 37°C with 100 μg of lysozyme and 10 μg of DNase I. Expression of the various enzymes was induced by growth in the presence of the appropriate carbon source. Gluconate kinase, inositol dehydrogenase, and glucitol dehydrogenase activities were determined as described previously (16, 30); mannitol PTS activity was determined according to the method of Reiche et al. (32); mannitol-1-P dehydrogenase activity was measured according to the method of Novotny et al. (31); glycerol kinase activity was measured according to the method of Deutscher and Sauerwald (11); α-glucosidase activity was determined as described previously (4) by using *p*-nitrophenyl-α-D-glucopyranoside as substrate, and β-galactosidase activity was measured according to the method of Miller (27). Protein concentration was determined by following the Coomassie brilliant blue assay procedure (3).

Uptake of ^{14}C-sugars. Uptake studies were carried out according to the method of Reizer et al. (34) by taking samples at intervals of between 1 and 15 min. Cells were grown in LB medium supplemented with different carbon sources, each at a concentration of 1%. When the inhibitory effect of glucose on gluconate or mannitol transport was studied, glucose was added to the growth medium 90 min before harvesting the cells. [^{14}C]Gluconate was synthesized from [^{14}C]glucose as described previously (6). To measure inducer expulsion of gluconate in *B. subtilis*, we used strain YF160 (*gntK* mutant), described previously (17). This strain is capable of accumulating unphosphorylated gluconate because of the absence of gluconate kinase activity. Expulsion studies were carried out as described previously (36).

RESULTS

Non-PTS catabolic enzyme activities in wild-type and *ptsH1* mutant strains. Starting with strain GM122, we constructed a *ptsH1* mutant strain, SA003 (Table 1), in which the phosphorylatable Ser-46 residue of HPr was replaced with an alanyl residue. The data shown in Table 2 demonstrate that the enzymes gluconate kinase and glucitol dehydrogenase exhibited sensitivity to catabolite repression in the *B. subtilis* wild-type strain GM122. However, neither glucose nor mannitol repressed gluconate kinase activity or glucitol dehydrogenase activity in the isogenic *ptsH1* mutant strain SA003 (Table 2). Repression of these enzymes by fructose was also no longer observed (9). Glucose was still capable of repressing glycerol kinase, α-glucosidase, and, if cells were grown in LB medium, inositol dehydrogenase activities in strain SA003 to nearly the same extent as was observed in the wild-type strain GM122 (Table 2). However, if cells were grown in C mineral medium, the repressive effect of glucose on inositol dehydrogenase activity was much less severe in the *ptsH1* mutant strain (4-fold) than in the wild-type strain (more than 120-fold). Repression of α-glucosidase and glycerol kinase was nearly the same in the two growth media (Table 2). Essentially the same results were obtained when the activities of these enzymes (except inositol dehydrogenase) in strain SA003a were examined (data not shown). A potentially causal relationship between HPr(ser) phosphorylation and catabolite repression of the two enzymes gluconate kinase and glucitol dehydrogenase was further substantiated by replacement of the *ptsH1* mutation in strain SA003a with the wild-type *ptsH* gene, yielding strain GM808. Sensitivity of gluconate kinase and glucitol dehydrogenase synthesis to catabolite repression was completely restored in this revertant (Table 2). In all of the strains tested, gluconate exerted little or no repressive effect on the synthesis of the glucitol catabolic enzymes and vice versa (Table 2). The repressive effect of glycerol on gluconate kinase and inositol dehydrogenase synthesis was not relieved by the *ptsH1* mutation (Table 2).

Mannitol PTS and mannitol-1-P dehydrogenase activities in mutant and wild-type strains. Since glucose also represses the synthesis of PTS permeases in *B. subtilis* (41), we measured PTS-dependent mannitol phosphorylation and mannitol-1-P dehydrogenase activities in strains GM122 and SA003 after growth in mannitol-containing medium with and without glucose. We observed that both mannitol PTS and mannitol-1-P dehydrogenase activities were repressed by glucose in the wild-type strain but not in the mutant strain SA003 (Table 3).

Uptake of [^{14}C]glucitol from solid C mineral medium. The failure of glucose to repress the expression of glucitol dehydrogenase in the *ptsH1* mutant strain was expected to lead to increased incorporation of radioactivity into SA003 compared with GM122 when the two strains were grown in the presence of glucose and [^{14}C]glucitol. After growth in LB medium, cells were spread on solid C mineral medium containing 1 μM [^{14}C]glucitol and 1% glucose. Colonies grew up within 2 days and were treated as described in Materials and Methods. The *ptsH1* mutant strain SA003 was indeed found to incorporate markedly more [^{14}C]glucitol than the wild-type strain GM122 under the above conditions (data not shown).

TABLE 2. Catabolic enzyme activities measured after growth under inducing and repressing conditions in the wild-type GM122, the *ptsH1* mutant SA003, and the revertant GM808

Enzyme	Growth conditions[a]	Enzyme activity[b]		
		GM122 (wt)	SA003 (*ptsH1*)	GM808 (wt, revertant)
Gluconate kinase	LB	<1	<1	<1
	LB + gluconate	24.8 ± 2.5	23.8 ± 2.9	25.3 ± 2.6
	LB + gluconate + glucose	2.4 ± 1.3	21.4 ± 3.1	2.5 ± 1.5
	LB + gluconate + mannitol	4.2 ± 1.5	20.3 ± 2.4	3.8 ± 1.7
	LB + gluconate + glucitol	22.6 ± 2.7	21.4 ± 2.8	24.0 ± 2.7
	LB + gluconate + glycerol	9.2 ± 2.2	9.7 ± 2.3	9.3 ± 2.1
Glucitol dehydrogenase	LB	2.4 ± 1.7	4.2 ± 2.1	1.4 ± 0.9
	LB + glucitol	97.2 ± 5.6	88.3 ± 4.0	106.6 ± 7.4
	LB + glucitol + glucose	9.7 ± 2.4	54.8 ± 7.6	7.3 ± 2.2
	LB + glucitol + mannitol	13.5 ± 3.1	81.8 ± 5.1	12.2 ± 3.2
	LB + glucitol + gluconate	85.0 ± 4.8	83.9 ± 5.3	85.4 ± 6.2
Inositol dehydrogenase	LB + inositol	116.6 ± 10.2	119.4 ± 11.0	
	LB + inositol + glucose	1.7 ± 0.8	5.3 ± 2.3	
	C + inositol	124.4 ± 11.2	129.0 ± 12.0	
	C + inositol + glucose	<1	30.3 ± 4.2	
	C + inositol + glycerol	30.8 ± 3.6	28.6 ± 3.9	
α-Glucosidase	LB + maltose	7.3 ± 1.1	7.7 ± 1.4	
	LB + maltose + glucose	0.7 ± 0.3	0.8 ± 0.3	
	C + maltose	7.0 ± 0.9	6.8 ± 1.1	
	C + maltose + glucose	0.2 ± 0.05	0.1 ± 0.05	
Glycerol kinase	LB + glycerol	18.4 ± 6.3	19.5 ± 6.7	19.3 ± 6.4
	LB + glycerol + glucose	1.1 ± 0.6	1.2 ± 0.5	1.3 ± 0.7
	C + glycerol	15.8 ± 4.9	16.2 ± 4.5	
	C + glycerol + glucose	<1	<1	

[a] Growth rates in LB medium or LB medium plus sugars were always around 2.1 per h. Strain SA003 (*ptsH1* mutant) grew a little more slowly than the wild-type strains. The deviation, however, never exceeded 15% of the growth rate of the wild-type strains. The growth rates (h^{-1}) in C mineral medium complemented with different sugars (C + sugar) were as follows: glucose, 1.1; glycerol, 1.0; inositol, 0.8; maltose, 0.7. The mutant strain SA003 grew more slowly (10%) than the parental strain GM122. If two sugars were present in the medium, the growth rates corresponded to the value observed for the faster metabolizable carbon source (data not shown).

[b] Enzyme activities are expressed in nanomoles of product formed per minute per milligram of protein. The mean values of at least three independent experiments and their standard deviations are presented. wt, wild type.

Effect of different mutant HPrs on expression of gluconate kinase and glucitol dehydrogenase. Strain GM273 (Δ*ptsGHI*) was transformed with the plasmid expressing the wild-type *ptsH* gene or different S46 mutant *ptsH* genes (Ser-46 replaced by alanine = A, by aspartate = D, by threonine = T, and by tyrosine = Y). Expression of the wild-type protein or mutant HPr proteins was verified by Western blot analysis using antibodies against *B. subtilis* HPr (see Materials and Methods). Cells of the different transformants were grown on gluconate- or glucitol-containing medium, and the activities of gluconate kinase and glucitol dehydrogenase in the different strains were determined. Strains GM273 and GM273-S46A exhibited enzyme activities (Table 4) similar to those determined for the wild-type strain GM122 (Table 2). Similar enzyme activities were also determined for strains GM273-S46T and GM273-S46Y, as well as for GM273 transformed with a plasmid expressing the wild-type *ptsH* gene (data not shown). By contrast, the activity of gluconate kinase was reduced about threefold in strain GM273-S46D (Table 4), indicating that S46D HPr can repress the synthesis of gluconate kinase even in

TABLE 3. Mannitol-1-P dehydrogenase and mannitol PTS activities in wild-type GM122 and *ptsH1* mutant strain SA003

Enzyme	Sugar(s) present during growth in LB medium	Enzyme activity[a]	
		GM122 (wt)	SA003 (*ptsH1*)
Mannitol-1-P dehydrogenase	Mannitol	2360 ± 190	2680 ± 180
	Mannitol + glucose	530 ± 45	2790 ± 210
	Mannitol + fructose	740 ± 50	3000 ± 210
Mannitol PTS	Mannitol	1.09 ± 0.15	1.29 ± 0.23
	Mannitol + glucose	0.22 ± 0.06	1.28 ± 0.21
	Mannitol + fructose	0.27 ± 0.05	1.53 ± 0.22

[a] Enzyme activities are expressed in nanomoles of product formed per minute per milligram of protein. The mean values of three independent experiments and their standard deviations are presented.

TABLE 4. Gluconate kinase and glucitol dehydrogenase activities in strain GM273 and GM273(pHPr) transformants expressing the S46A *ptsH* gene (GM273-S46A) or the S46D *ptsH* gene (GM273-S46D)

Enzyme	Sugar present during growth in LB medium	Enzyme activity[a]		
		GM273	GM273-S46A	GM273-S46D
Gluconate kinase	Gluconate	24.9 ± 2.6	23.2 ± 2.2	7.6 ± 2.6
Glucitol dehydrogenase	Glucitol	92.3 ± 4.9	95.0 ± 5.3	77.8 ± 8.3

[a] Enzyme activities are expressed in nanomoles of product formed per minute per milligram of protein. The mean values of five independent experiments and their standard deviations are presented.

the absence of a repressing carbohydrate and in a strain lacking a functional PTS. A less pronounced reduction in the activity of glucitol dehydrogenase in this strain was also observed (Table 4). The lower repression of gluconate kinase and glucitol dehydrogenase in strain GM273-S46D compared with the repression exerted by glucose in a wild-type strain (compare Tables 2 and 4) might be due to structural differences between P-ser-HPr and S46D HPr (49).

Sugar uptake in wild-type and mutant strains. Since glucose and mannitol still repressed α-glucosidase and glycerol kinase activities in the *ptsH1* mutant strain (Table 2), it was unlikely that a reduced rate of uptake of PTS sugars was responsible for the failure of these sugars to repress gluconate kinase and glucitol dehydrogenase activities in this mutant. To completely rule out this possibility, we measured the uptake rates for mannitol and methyl-α-glucoside in the wild-type strain (GM122) and the *ptsH1* mutant strain (SA003) in cells grown in LB medium plus mannitol with glucose added 90 min before the cells were harvested. The uptake rates in the two strains were found to be similar for both carbohydrates, with the rate in the *ptsH1* mutant strain being slightly higher (10–15%) (data not shown).

To address the question of whether glucose exerts its repressive effect on the mannitol catabolic enzymes by inhibiting mannitol uptake, we measured [^{14}C]mannitol uptake in the presence of 5 mM glucose in cells grown as described above. The presence of glucose inhibited mannitol uptake in both the wild-type strain (4-fold) and the *ptsH1* mutant strain (2.5-fold) (data not shown).

To investigate whether P-ser-HPr represses the synthesis of gluconate kinase and glucitol dehydrogenase by inhibiting the corresponding permeases (inducer exclusion), as was demonstrated for unphosphorylated enzyme IIA^{glc} and the lactose, maltose, and melibiose permeases of *E. coli* (26), we studied gluconate uptake in the wild-type strain GM122 and in the *ptsH1* mutant strain SA003 in the presence of glucose. Both strains were grown on gluconate-containing medium with glucose added 90 min before harvesting. If an inducer exclusion mechanism were operative, we would have expected to see inhibition of [^{14}C]gluconate uptake by the wild-type strain in the presence of glucose. However, the two strains, in the presence of glucose, took up gluconate at similar rates (Fig. 1). Almost identical uptake rates of gluconate were observed in strains in the absence of glucose.

No inhibitory effect of S46D HPr on the uptake of [^{14}C]gluconate was observed, although this mutant protein was found to lower the expression of gluconate kinase (Table 4). GM273(pHPr) transformants expressing S46D HPr exhibited elevated gluconate uptake activity compared with GM273(pHPr) transformants expressing S46A HPr (Fig. 1).

Elimination of [^{14}C]gluconate expulsion as a mechanism for *gnt* operon catabolite repression. To determine whether P-ser-HPr can influence the expression of *gntK* by an inducer expulsion mechanism (36), we examined *B. subtilis* strains GM122 (wild type) and YF160 (*gntK*). The latter strain was expected and demonstrated to accumulate unphosphorylated gluconate because of the absence of gluconate kinase activity. As shown in Fig. 2, the addition of glucose to the wild-type strain did not result in appreciable expulsion. However, gluconate transport in the wild-type strain is probably rate limiting for gluconate utilization, so that the amount of free, cytoplasmatic gluconate may be vanishingly small. Expulsion of the meager amount of inducer present might not have been noticed. The experiment was therefore repeated with a gluconate kinase-negative (*gntK*) mutant in which gluconate could not be metabolized. Accumulation by this strain of [^{14}C]gluconate was found to be poor in the absence of a metabolizable carbon source. When glucose was added to [^{14}C]gluconate-preloaded cells, it did not trigger expulsion. Instead, it caused further uptake of the radioactive sugar acid (Fig. 2). Similar stimulation of [^{14}C]gluconate uptake by the addition of glucose was observed in YF160 cells which had accumulated a larger amount of [^{14}C]gluconate in the presence of glucitol (data not shown).

Expression of β-galactosidase activity under control of the *B. subtilis gnt* promoter. A *B. subtilis ptsH1* mutant strain (GM1222) and a wild-type strain (GM1221) were constructed from strain GM1199. These isogenic strains carried a *gntRK'-lacZ* translational fusion inserted into the chromosomal *amyE* gene (see Materials and Methods). In both strains expression of β-galactosidase activity was inducible by growth on glu-

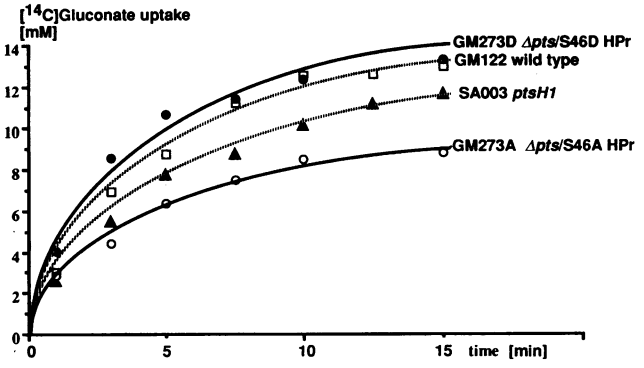

FIG. 1. Uptake of [^{14}C]gluconate by various *B. subtilis* strains. Cells were grown on gluconate-containing medium with glucose added 90 min before harvesting the cells. The uptake experiments with strains GM122 and SA003 were carried out in the presence of 50 mM glucose. The different symbols indicate uptake experiments with the following strains: rectangles, GM122; triangles, SA003; filled circles, GM273D; and open circles, GM273A.

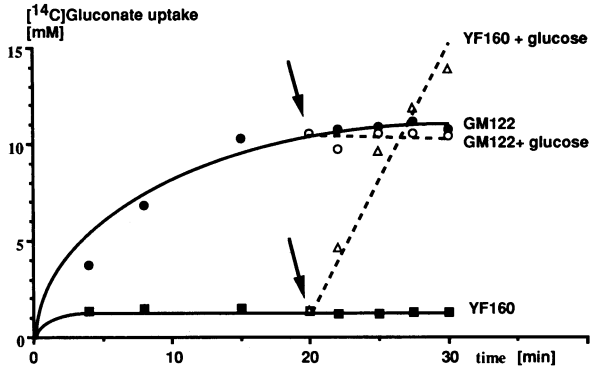

FIG. 2. Inducer expulsion experiments in a *B. subtilis* wild-type strain (GM122) and a *gntK* (YF160) mutant strain. Cells were allowed to accumulate [^{14}C]gluconate for 20 min. Glucose was then added to half of the cells. The concentrations of intracellular [^{14}C]gluconate were measured for an additional 10 min in the control cells without glucose (closed circles, GM122; and rectangles, YF160) and in the cells to which glucose had been added (open circles, GM122; and triangles, YF160).

conate-containing medium. However, repression of β-galactosidase expression by glucose occurred only in the wild-type strain GM1221 and not in the *ptsH1* mutant strain GM1222 (Table 5). The gluconate kinase activities measured in GM1221 and GM1222 after growth in medium containing gluconate or gluconate plus glucose correlated with the β-galactosidase activities in these strains measured under the same growth conditions (Table 5).

Catabolic enzyme activities in a *ccpA* mutant strain. To investigate a potential correlation between the enzymes relieved from catabolite repression by the *ptsH1* mutation and those relieved from catabolite repression by the *ccpA* mutation, we constructed a *ccpA* mutant strain (GM1225) from strain GM1199 as described in Materials and Methods. The activities of gluconate kinase, glucitol dehydrogenase, mannitol-1-P dehydrogenase, inositol dehydrogenase, α-glucosidase, and glycerol kinase were measured in the wild-type strain GM1199 and in its *ccpA* mutant derivative GM1225 (Table 6). From comparison of the results shown in Tables 2, 3, and 6 it is clear that the same enzymes are relieved from catabolite repression in the *ptsH1* and *ccpA* mutant strains. In addition, the repressive effect of glycerol on inositol dehydrogenase and gluconate kinase, as well as sensitivity to glucose-promoted catabolite repression of α-glucosidase and glycerol kinase, persisted in both mutant strains.

TABLE 5. Gluconate kinase and β-galactosidase activities in a wild-type strain (GM1221) and a *ptsH1* mutant strain (GM1222) both expressing the *E. coli lacZ* gene under control of the *gnt* promoter

Enzyme	Sugar(s) present during growth in LB medium	Enzyme activity[a]	
		GM1221 (wt)	GM1222 (*ptsH1*)
Gluconate kinase	None	<1	<1
	Gluconate	37.3 ± 4.2	34.9 ± 3.6
	Gluconate + glucose	4.8 ± 1.5	38.2 ± 4.5
β-Galactosidase	None	<1	<1
	Gluconate	20.4 ± 4.7	27.6 ± 5.7
	Gluconate + glucose	3.3 ± 1.2	28.2 ± 6.2

[a] Enzyme activities are expressed in nanomoles of product formed per minute per milligram of protein. The mean values of three independent experiments and their standard deviations are presented. wt, wild type.

DISCUSSION

Several mechanisms exist whereby expression of catabolic genes can be regulated in gram-positive bacteria (15, 40, 46). Studies on acetoin dehydrogenase and on the gluconate and inositol operons indicated that an early intermediate of glycolysis acts as a catabolite-repressing metabolite of certain catabolic genes of *B. subtilis* (12, 23, 30). The formation of seryl-phosphorylated HPr is a well documented intracellular signal caused by the uptake of metabolizable, repressing, carbon sources such as glucose, fructose, and mannitol into gram-positive bacteria (47). Since the ATP-dependent phosphorylation of HPr at Ser-46 in *B. subtilis* and other gram-positive bacteria is stimulated by FBP and other glycolytic intermediates (7, 33, 35, 38), we constructed the *ptsH1* mutant strain SA003 in order to investigate whether P-ser-HPr formation and catabolite repression might be causally related.

In an earlier study (13), a *ptsH1* mutant strain (GM3291) was constructed, and it was reported to exhibit altered growth behavior on PTS sugars relative to the wild-type strain (13). Specifically, it attained half of the cell mass on a given amount of a PTS sugar compared with the wild-type strain (13). We repeated these experiments by growing the two pairs of *ptsH*$^+$ and *ptsH1* strains used in this study (GM122/SA003 and GM1221/GM1222) in C mineral medium containing 0.2%, 0.4%, or 1% glucose or mannitol. In contrast to the results reported by Eisermann et al. (13), no difference in growth behavior between wild-type strains and *ptsH1* mutant strains was observed (9). A possible explanation for this discrepancy is provided by the finding that strain GM3291 not only contained a *ptsH1* mutation but also was devoid of gluconate kinase and inositol dehydrogenase activities, both of which were normally expressed in the isogenic wild-type strain GM3292 (9). Fujita and Fujita (16) previously described a *B. subtilis* deletion strain, Δ*igf*, in which these two enzyme activities, as well as fructose-bisphosphatase activity, were lacking. Δ*igf* proved to be an extended deletion mutation which arose with a high frequency. It is probable that this mutation is responsible for the observed abnormal growth of strain GM3291. The additional mutations acquired by the *ptsH1* mutant GM3291 prompted us to construct strain SA003.

This new *ptsH1* mutant strain exhibited insensitivity to carbon catabolite repression of several catabolic genes (Table 2). In at least one case, relief from carbon catabolite repression in the *ptsH1* mutant strain depended on the growth medium employed. When cells were grown in rich (LB) medium, inositol dehydrogenase activity was found to be repressed in the *ptsH1* mutant strain almost to the same extent as in the wild-type strain GM122. However, if cells were grown in C mineral medium, inositol dehydrogenase activity was only 4-fold repressed in the *ptsH1* mutant strain, whereas this activity was more than 120-fold repressed in the wild-type strain (Table 2). The repressive effects of glycerol on gluconate kinase and inositol dehydrogenase were not relieved by the *ptsH1* mutation. This observation will be discussed later in connection with the *ccpA* mutation.

We did not observe the strong repressive effect of glycerol on inositol dehydrogenase synthesis published previously (30). The repressive effect of glycerol was reported to be as strong as the repressive effect of glucose (more than 300-fold), although we observed only 4-fold repression (Table 2). This discrepancy may be due to the use of nonisogenic strains or different media.

TABLE 6. Catabolic enzyme activities measured in the wild-type GM1199 and the *ccpA* mutant strain GM1225 after growth under inducing and repressing conditions

Enzyme	Growth conditions[a]	Enzyme activity[b]	
		GM1199	GM1225
Gluconate kinase	LB	<1	<1
	LB + gluconate	25.3 ± 2.9	26.6 ± 2.8
	LB + gluconate + glucose	2.7 ± 1.4	29.5 ± 2.9
	LB + gluconate + glycerol	8.5 ± 3.3	12.6 ± 4.2
Glucitol dehydrogenase	LB + glucitol	85.3 ± 7.6	87.7 ± 8.1
	LB + glucitol + glucose	7.9 ± 2.7	95.5 ± 7.9
Inositol dehydrogenase	LB + inositol	111.3 ± 10.4	116.4 ± 10.9
	LB + inositol + glucose	2.3 ± 0.9	125.3 ± 11.3
	C + inositol	109.7 ± 12.6	138.5 ± 14.6
	C + inositol + glucose	1.5 ± 0.4	131.8 ± 13.2
	LB + inositol + glycerol	39.8 ± 5.1	48.7 ± 6.8
Mannitol-1-P dehydrogenase	LB	93 ± 10	147 ± 13
	LB + mannitol	844 ± 75	1974 ± 210
	LB + mannitol + glucose	148 ± 14	730 ± 64
α-Glucosidase	LB + maltose	6.3 ± 0.8	6.8 ± 0.8
	LB + maltose + glucose	0.9 ± 0.3	1.5 ± 0.5
Glycerol kinase	LB + glycerol	21.3 ± 5.8	25.4 ± 6.1
	LB + glycerol + glucose	<1	<1

[a] Cells were grown on either rich medium (LB) or C mineral medium (C).
[b] Enzyme activities are expressed in nanomoles of product formed per minute per milligram of protein. The mean values of three independent experiments and their standard deviations are presented.

Synthesis of the two enzymes α-glucosidase and glycerol kinase was not relieved from catabolite repression in the *ptsH1* mutant strain, when grown in either C mineral medium or LB medium. Relevant to the repression of glycerol kinase, it has been demonstrated that *B. subtilis ptsI* mutants grow poorly on glycerol as the sole carbon source and that they exhibit low glycerol uptake activities (34). This is most likely due to the HPr-catalyzed phosphorylation-dephosphorylation of glycerol kinase demonstrated in *Enterococcus faecalis* (6). The absence of an effect of the *ptsH1* mutation on glycerol kinase regulation suggests that phosphorylation of HPr at Ser-46 does not play a role in this kind of regulation.

Repression of mannitol-1-P dehydrogenase and mannitol PTS activities was also relieved in the *ptsH1* mutant strain. Glucose was found to inhibit uptake of mannitol in both the wild-type strain and in the *ptsH1* mutant strain when it was present during mannitol uptake (9). This inhibitory effect was slightly (1.6-fold) relieved by the *ptsH1* mutation (9). It is unlikely that this slight effect on mannitol transport activity is responsible for the observed insensitivity to catabolite repression of mannitol-1-P dehydrogenase and the mannitol PTS in the *ptsH1* mutant.

Our results argue against an involvement of inducer exclusion and inducer expulsion in the P-ser-HPr-dependent regulation of gluconate kinase, glucitol dehydrogenase, and inositol dehydrogenase syntheses, since gluconate uptake was not inhibited by the presence of glucose in a wild-type strain and since inducer expulsion could not be observed in a *gntK* mutant strain. Direct inhibition of gluconate kinase and glucitol dehydrogenase activities by allosteric interaction with P-ser-HPr can be ruled out, since P-ser-HPr or the S46D mutant HPr at concentrations up to 1 mM had no effect on either of these two enzyme activities when they were measured in crude extracts (9). This conclusion was further substantiated by the finding that expression of β-galactosidase under the control of the *gnt* promoter (translational fusion) was insensitive to glucose repression in the *ptsH1* mutant strain. This latter result suggests an involvement of P-ser-HPr in regulation of the expression of the *gntK* gene. Furthermore, it eliminates the possibility that a P-ser-HPr-triggered posttranslational modification of gluconate kinase mediates catabolite repression of this enzyme in *B. subtilis*.

Our results suggest that phosphorylation of seryl residue 46 in HPr is involved in catabolite repression of the *gntK*, *gutB*, and *idh* genes as well as the *mtl* operon in *B. subtilis*. The *gnt* operon has been shown to contain a *cis*-acting element (29) similar to the proposed catabolite CRE of the *amyE* gene of *B. subtilis* (48). A *B. subtilis* mutant (*ccpA*) has been isolated in which the expression of the *amyE* gene was found to be resistant to catabolite repression (20). The *ccpA* gene encodes a protein homologous to the *E. coli lacI*, *galR*, and *fruR* repressors, and the gene product may bind to the above mentioned CREs. However, in no case has an interaction of CcpA with the CRE been described. We therefore considered that P-ser-HPr might interact with CcpA and that this interaction might allow CcpA to bind to the CRE. In agreement with this possibility, the four enzymes which were insensitive to catabolite repression in the *ptsH1* mutant strain (gluconate kinase, glucitol dehydrogenase, inositol dehydrogenase, and mannitol-1-P dehydrogenase) were similarly insensitive in the *ccpA* mutant strain (compare Tables 2, 3, and 6). Relief of the *gnt* operon from catabolite repression in a *ccpA* mutant strain has recently been reported in an independent study (19). In addition, β-glucanase is relieved from catabolite repression in both the *ptsH1* mutant strain and a *ccpA* mutant strain (22). Insensitivity to catabolite repression has also been reported for levanase in both a *ccpA* mutant strain (24) and a *ptsH1* mutant strain (25).

The repressive effects of glycerol on gluconate kinase and inositol dehydrogenase were not relieved by a *ptsH1* or *ccpA* mutation. Finally, α-glucosidase and glycerol kinase remained sensitive to repression in the *ptsH1* and *ccpA* mutant strains (Tables 2 and 6). Since the same six enzymes were found to be relieved from catabolite repression, a relationship between P-ser-HPr and CcpA is implied. However, the observations that in the *ptsH1* mutant strain, inositol dehydrogenase is almost completely repressed by glucose when grown in rich medium and is partially repressed when grown in C mineral medium, whereas this enzyme was found to be completely relieved from catabolite repression in a *ccpA* mutant strain in both growth media, may indicate that CcpA is the target of more than one signalling pathway.

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