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Regulatory Functions of Serine-46-Phosphorylated HPr in *Lactococcus lactis*

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In most low-G+C gram-positive bacteria, the phosphoryl carrier protein HPr of the phosphoenolpyruvate: sugar phosphotransferase system (PTS) becomes phosphorylated at Ser-46. This ATP-dependent reaction is catalyzed by the bifunctional HPr kinase/P-Ser-HPr phosphatase. We found that serine-phosphorylated HPr (P-Ser-HPr) of *Lactococcus lactis* participates not only in carbon catabolite repression of an operon encoding a β-glucoside-specific EII and a 6-P-β-glucosidase but also in inducer exclusion of the non-PTS carbohydrates maltose and ribose. In a wild-type strain, transport of these non-PTS carbohydrates is strongly inhibited by the presence of glucose, whereas in a *ptsH1* mutant, in which Ser-46 of HPr is replaced with an alanine, glucose had lost its inhibitory effect. In vitro experiments carried out with *L. lactis* vesicles had suggested that P-Ser-HPr is also implicated in inducer expulsion of nonmetabolizable homologues of PTS sugars, such as *methyl* β-D-thiogalactoside (TMG) and 2-deoxy-D-glucose (2-DG). In vivo experiments with the *ptsH1* mutant established that P-Ser-HPr is not necessary for inducer expulsion. Glucose-activated 2-DG expulsion occurred at similar rates in wild-type and *ptsH1* mutant strains, whereas TMG expulsion was slowed in the *ptsH1* mutant. It therefore seems that P-Ser-HPr is not essential for inducer expulsion but that in certain cases it can play an indirect role in this regulatory process.

HPr is one of the four proteins (or domains) forming the phosphorylation cascade of the phosphoenolpyruvate (PEP): sugar phosphotransferase system (PTS), which in gram-positive and gram-negative bacteria catalyzes the uptake and phosphorylation of numerous carbohydrates (for a review, see reference 28). During PTS-mediated carbohydrate uptake and phosphorylation, HPr becomes phosphorylated by PEP and enzyme I at the Nδ1 position of His-15. P-His-HPr transfers its phosphoryl group to one of several sugar-specific EIIAs usually present in bacterial cells. P∼EIIAs donate their phosphoryl group to the corresponding EIIB, from where the phosphoryl group is finally transferred to the carbohydrate bound to the membrane-integrated EIIC. After phosphorylation by P∼EIIB, the phosphorylated sugar is released into the cytoplasm. In enzyme I and EIIAs, the phosphoryl group is attached to the Nε2 position of a histidyl residue, whereas in EIIBs the phosphoryl group can be bound either to the Nδ1 position of a histidyl residue or to a cysteyl residue (28).

In gram-positive bacteria, HPr functions not only as a phosphoryl carrier within the PTS phosphorylation cascade but also as the central regulator of carbohydrate metabolism. For example, P∼His-HPr phosphorylates not only EIIAs but also histidyl residues in non-PTS proteins such as glycerol kinase (6), antiterminators, transcriptional activators (32), and non-PTS transporters (containing an $EIIA^{Glc}$ domain) (17). In most cases, P∼His-HPr-mediated phosphorylation of non-PTS proteins leads to a stimulation of their activity. In addition, HPr of gram-positive bacteria is also phosphorylated at the regulatory serine-46 (9, 11). This reaction requires ATP and is catalyzed by the metabolite-controlled bifunctional HPr kinase/P-Ser-HPr phosphatase (5, 12, 14, 19, 21, 29). The resulting P-Ser-HPr functions as a corepressor in carbon catabolite repression (CCR) or as a coactivator in carbon catabolite activation (CCA) by interacting with catabolite control protein A (CcpA) (8, 20), a member of the LacI/GalR repressor family (18). The P-Ser-HPr/CcpA complex binds to specific operator sites (13) called catabolite response elements (*cre*) (39). The *cre*'s of catabolite-activated genes or operons are located in front of the promoter. By contrast, in the case of catabolite-repressed genes and operons, the *cre*'s either overlap the promoter or are located downstream of it (for reviews see references 7 and 33).

Based on in vitro results, P-Ser-HPr has been suggested to participate also in inducer exclusion in *Lactobacillus brevis* (44, 47). This concept was further supported by in vivo experiments with a *Streptococcus salivarius* Ile47Thr *ptsH* mutant (16), which had lost the preferential uptake and metabolism of glucose over lactose. The participation of P-Ser-HPr in inducer exclusion has been established by in vivo experiments with *Lactobacillus casei ptsH1* and *hprK* mutants, which are not able to form P-Ser-HPr. Maltose uptake, which was completely inhibited by glucose in a wild-type strain, was not affected by glucose in the *ptsH1* (37) and *hprK* mutants (12).

In vitro results had suggested that P-Ser-HPr would also participate in inducer expulsion in *Lactococcus lactis* (42, 43). Addition of glucose to cells which had accumulated the nonmetabolizable *methyl* β-D-thiogalactoside (TMG) or 2-deoxy-D-glucose (2-DG) caused rapid expulsion of the nonmetabolizable sugar analogues from *L. lactis* wild-type cells (35) or

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vesicles (43). Both sugar analogues are taken up by the PTS and are therefore accumulated as 6-P derivatives. In the first step of inducer expulsion, an intracellular sugar-P phosphohydrolase dephosphorylates the accumulated P-sugars before the unphosphorylated sugars are expelled from the cells in the second step (30). In several gram-positive bacteria, including *L. lactis*, a sugar-P phosphohydrolase has been described which was activated by P-Ser-HPr (41, 45) and which was thought to catalyze the first step of inducer expulsion. In addition, electroporation of *Bacillus subtilis* Ser46Asp mutant HPr, which structurally resembles P-Ser-HPr (40), into *L. lactis* vesicles was reported to lead to stronger inducer expulsion than electroporation of Ser46Ala mutant HPr (42). It was therefore concluded that P-Ser-HPr would participate in inducer expulsion. However, recent in vivo experiments with *L. casei ptsH1* and *hprK* mutants had established that in this organism inducer expulsion does not require P-Ser-HPr. Following the addition of glucose, *ptsH1* and *hprK* mutants, which are not able to form P-Ser-HPr, expelled preaccumulated TMG at a rate similar to that observed with an *L. casei* wild-type strain (12, 37).

We here report the construction of an *L. lactis ptsH1* mutant strain synthesizing Ser46Ala mutant HPr with the aim to test whether P-Ser-HPr-dependent inducer exclusion is a widespread phenomenon in gram-positive bacteria and is present also in *L. lactis*. In addition, we carried out in vivo expulsion experiments with TMG and 2-DG in order to test whether P-Ser-HPr participates in this regulatory process, as has been proposed based on the reported stimulation of a sugar-P phosphohydrolase by P-Ser-HPr (45) and on in vitro expulsion experiments carried out with *L. lactis* vesicles, into which purified *B. subtilis* wild-type and mutant HPrs had been electroporated (42, 43).

MATERIALS AND METHODS

Strains, culture conditions, and transformation procedures. *L. lactis* MG5267, an MG1363 derivative (15) carrying the lactose operon integrated in the chromosome (36), was used in this study. MG5267 and its derivatives were grown at 30°C under static conditions in M17 medium supplemented with either 0.5 or 0.8% of the indicated carbohydrates. *Escherichia coli* NM522 (Stratagene) was used as a host for the cloning experiments. It was grown in Luria-Bertani medium at 37°C under agitation. The antibiotics chloramphenicol and erythromycin were used at a concentration of 5 μg/ml for *L. lactis*. For *E. coli*, chloramphenicol was used at 10 μg/ml and ampicillin was used at 100 μg/ml. Solid media were prepared by adding 1.5% agar to the liquid media. For α-complementation in *E. coli*, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside was used at a concentration of 20 μg/ml. For the electroporation experiments with *L. lactis* cells, strains were grown in M17 medium supplemented with 0.5% glucose, 0.5 M sucrose, and 1% glycine to an optical density at 600 nm (OD_{600}) of 0.5. The cells were subsequently washed twice with cold 0.5 M sucrose containing 10% glycerol and finally resuspended in this solution (1% of initial volume). A 50-μl aliquot was electroporated at 2.5 kV, 200 Ω, and 25 μF in cuvettes with a 0.2-cm distance between the electrodes (Bio-Rad Gene-Pulser). Five milliliters of M17 medium containing 0.5% glucose, 0.5 M sucrose, 20 mM $MgCl_2$, and 2 mM $CaCl_2$ was rapidly added to the electroporated cells, which were subsequently incubated for 2 h at 30°C before aliquots were plated on selective media.

Construction of plasmids and strains. Chromosomal DNA from *L. lactis* MG5267 was isolated as previously described (24) and was used to amplify by PCR a 3-kb DNA fragment containing the complete *ptsH*I operon and its upstream sequence. The PCR was carried out with *Pfu* DNA polymerase (Promega) and oligonucleotides PTS3 (5′-GACCTGCAGTACAAAGTTATC-3′) and PTS4 (5′-TAA*GGATCC*TATTATAGCTAAACAG-3′) as primers. The resulting 3-kb DNA fragment was digested with *Bam*HI (restriction site indicated in italics in PTS4) and cloned into *Sma*I-*Bam*HI-digested pNEB193 (New England Biolabs). In order to replace serine 46 in HPr with an alanine, the obtained plasmid pTSHI was used as a template in a PCR amplification together with the two divergent primers S46A (5′-CCTTAAAGCAATCATGGGTGT-3′) and S46A2 (5′-TTTACTGATTTACCTTTGTAT-3′). The altered codon 46 leading to the Ser46Ala replacement in *ptsH* (TCA to GCA) is underlined in the sequence of primer S46A. The resulting PCR product was phosphorylated with T4 polynucleotide kinase, ligated, and used to transform *E. coli* NM522. The presence of the *ptsH1* mutation (Ser46Ala replacement) and the absence of other mutations were verified by sequencing the insert of plasmids isolated from several clones using a Perkin-Elmer Abiprism 373 automated sequencer. One plasmid, called pTS46A, carried the correct mutant *ptsH1* allele and wild-type *pts*I and was used for further experiments. Plasmid pTS46A was digested with *Kpn*I and made blunt ended with Klenow DNA polymerase. After digestion with *Bam*HI, the 3-kb fragment obtained was cloned into the thermosensitive pGhostCm vector (2) digested with *Eco*RV and *Bam*HI, thus providing pGhostS46A.

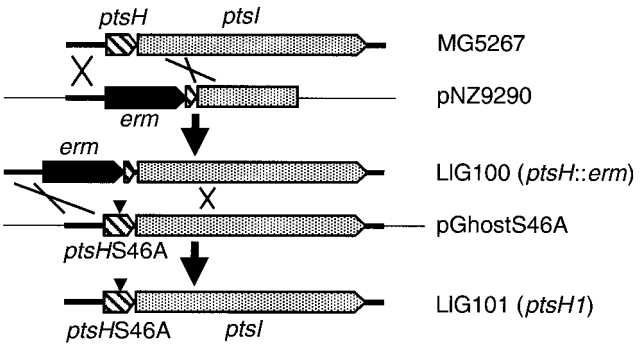

FIG. 1. Construction of an *L. lactis* strain expressing Ser46Ala mutant HPr. The wild-type strain MG5267 was transformed with plasmid pNZ9290 (26), which contains the 5′ part of the *L. lactis ptsI* gene and several hundred base pairs of the upstream region of the *ptsHI* operon. The *ptsH* gene located in front of *pts*I is partly deleted and the deleted region is replaced with an erythromycin resistance cassette. After a double-crossover recombination, a *ptsH*::*erm* strain (LIG100) was obtained. This strain exhibited a *pts*-negative phenotype and was transformed with the thermosensitive plasmid pGhostS46A, which carries the *ptsH1* allele (the position of the Ser46Ala mutation is indicated with a triangle). After two successive recombination events, a pts^+ strain carrying the *ptsH1* allele (LIG101) was obtained.

Strain LIG100 (*ptsH*::*erm*) was constructed by transforming *L. lactis* MG5267 with pNZ9290 (26) and selecting for erythromycin-resistant clones (Fig. 1). Strains carrying the plasmid inserted by a double crossover, which leads to the inactivation of *ptsH*, were identified by their inability to grow on PTS sugars, and in one such strain, LIG100, the insertion of the erythromycin resistance cassette into *ptsH* was confirmed by PCR amplification with appropriate primers.

For the construction of the chromosomal *ptsH1* mutant LIG101, LIG100 was transformed with pGhostS46A (Fig. 1). One chloramphenicol-resistant transformant was grown overnight at 38°C in the absence of antibiotics and subsequently plated on chloramphenicol-containing solid M17 medium and incubated at 38°C. Since pGhost is not replicated at 38°C, this procedure forced the integration of pGhostS46A into the chromosome of LIG100 by homologous recombination. One strain resistant to chloramphenicol and erythromycin during growth at 38°C was subsequently grown for several generations at 30°C in the absence of antibiotics and then plated on M17 medium, incubated at 30°C, and replica plated on M17 medium containing chloramphenicol and erythromycin. LIG101 was selected as a chloramphenicol- and erythromycin-sensitive strain in which the disrupted *ptsH* gene was replaced with the *ptsH1* mutant allele by a second crossover event. The presence of this mutation in LIG101 was confirmed by sequencing appropriate PCR products.

Western blotting. *L. lactis* strains were grown in 25 ml of glucose-containing M17 medium to an OD_{600} between 0.6 and 0.7 before the pH of the culture was rapidly lowered to 4.5 by adding concentrated HCl. After centrifugation at 4°C, the cell pellets were resuspended in 1 ml of 20 mM sodium acetate, pH 4.5, and cells were broken in a Fast-prep apparatus (Biospec) using 0.1-mm glass beads and three cycles of 30 s at maximum speed. The low pH and temperature were used to minimize changes in the HPr phosphorylation state potentially caused by enzyme I and HPr kinase/P-Ser-HPr phosphatase present in the extracts. The cell lysates were clarified by centrifugation, and proteins were separated on a 15% nondenaturing polyacrylamide gel. After electrophoretic transfer of the proteins

onto nitrocellulose membranes, the blots were probed with rabbit polyclonal antibodies raised against *B. subtilis* HPr and developed by using anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (Promega) as a second antibody.

β-Glucoside transport and 6-P-β-glucosidase assays. Strains MG5267 and LlG101 were grown in 20 ml of M17 medium supplemented with either 0.8% salicin or 0.8% salicin plus 0.8% glucose to an OD_{600} between 0.4 and 0.5. Cells grown in medium containing only salicin exhibited an about 1.8-fold slower growth rate than cells grown on salicin plus glucose. The cells were washed twice with 50 mM Tris-HCl buffer, pH 7.4, and resuspended in 200 μl of the same buffer. 6-P-β-Glucosidase activities were determined in 50-μl assay mixtures containing 50 mM Tris-HCl, pH 7.4, 40 μl of the cell suspension, 5 mM *p*-nitrophenyl-β-D-glucopyranoside, and 5 mM $MgCl_2$. The assay mixtures were incubated for 30 min at 30°C before the reaction was stopped by adding 800 μl of 10% sodium carbonate. After centrifugation, the OD_{405} was measured in the samples. Control experiments carried out with salicin-grown wild-type cells confirmed that there was a linear correlation between the measured OD_{405} and either the incubation time or the amount of cells used for the assay. Enzyme activities are expressed in nanomoles of *p*-nitrophenol formed per minute per milliliter of cell culture exhibiting an OD_{600} of 0.5. To determine whether glucose exerts an exclusion effect on *p*-nitrophenyl-β-D-glucopyranoside uptake, the above-described assay was carried out with salicin-grown wild-type and *ptsH1* mutant cells in the presence of 10 mM glucose.

Sugar transport, inducer exclusion, and inducer expulsion. Sugar transport studies and inducer exclusion experiments in the presence of 10 mM glucose were performed using the rapid-filtration method (37). Cells used for transport studies were grown in M17 medium containing different carbohydrates (at a concentration of 0.5%). Glucose-promoted expulsion experiments with cells which had accumulated the lactose analogue TMG or the glucose analogue 2-DG were carried out as previously described (12). ^{14}C-radiolabeled sugars were purchased from Isotopchim (Ganagobie-Peyrus, France) and used at a final concentration of 1 mM (at a specific radioactivity of 0.5 mCi/mmol).

Thin-layer chromatography was used to separate phosphorylated and nonphosphorylated [^{14}C]TMG. After cells had taken up [^{14}C]TMG, they were washed twice with 1 ml of transport buffer before 10 mM glucose was added to one-half of the suspension (500 μl) and expulsion was allowed to proceed for 5 min. Subsequently, cells were kept for 10 min at 100°C and clarified by centrifugation, and 10-μl aliquots were separated by thin-layer chromatography on Silica Gel 60 plates (Merck) using a mixture of 1 M ammonium acetate, pH 5, 98% ethanol, and 0.1 M EDTA, pH 8 (70:29:1), as the solvent. The approximate amounts of TMG and TMG-6-P were determined by autoradiography (4 days of exposure with a Biomax MR film [Kodak]). The migration position of TMG was determined with untreated [^{14}C]TMG.

RESULTS

Construction of an *L. lactis ptsH1* mutant strain. To study the role of P-Ser-HPr in the regulation of carbon metabolism in *L. lactis*, we constructed a *ptsH1* mutant strain in which the phosphorylatable Ser-46 of HPr was replaced with an alanine. The *L. lactis ptsHI* operon encoding enzyme I and HPr of the PTS has recently been cloned and characterized (26). After insertion of an erythromycin resistance gene at the 3′ end of the *ptsH* gene of strain MG5267, the antibiotic resistance cassette and the wild-type *ptsH* were replaced with the Ser46Ala *ptsH* allele (*ptsH1*) present on the integrative plasmid pGhostS46A as described in Materials and Methods (Fig. 1). The expected absence of P-Ser-HPr in the resulting *ptsH1* mutant strain LlG101 was confirmed by Western blotting. Polyacrylamide gel electrophoresis was performed under nondenaturing conditions with crude extracts prepared from the *L. lactis* wild-type and the *ptsH1* mutant strains grown in glucose-containing M17 medium. This allowed us to separate HPr, P~His-HPr/P-Ser-HPr, and doubly phosphorylated HPr and to get an estimate of their ratios in the cell. The approximate amounts of the different forms of HPr were detected with polyclonal antibodies directed against *B. subtilis* HPr. Heating an aliquot of the crude extract to 65°C allowed us to distinguish between P~His-HPr and P-Ser-HPr, which migrate to nearly identical positions. P~His-HPr is rapidly hydrolyzed at 65°C (38), whereas P-Ser-HPr is stable under these conditions. According to the results presented in lanes 1 and 2 of Fig. 2, glucose-grown *L. lactis* wild-type cells were estimated to contain a considerable amount of P-Ser-HPr and somewhat less HPr and P~His-HPr, whereas only a small amount of doubly phosphorylated HPr was present. By contrast, glucose-grown *ptsH1* mutant cells contained neither P-Ser-HPr nor doubly phosphorylated HPr and the major part of HPr was present as P~His-HPr (Fig. 2, lanes 3 and 4).

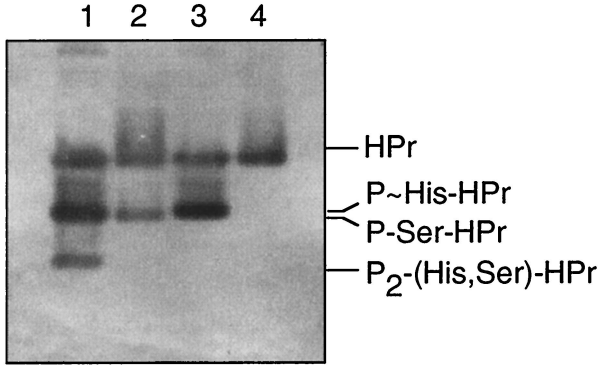

FIG. 2. Western blot with *L. lactis* crude extracts prepared from glucose-grown wild-type and *ptsH1* mutant strains and separated on a nondenaturing polyacrylamide gel. The various forms of HPr were detected with antibodies raised against *B. subtilis* HPr. Crude extracts from *L. lactis* wild-type MG5267 (lanes 1 and 2) and *L. lactis ptsH1* mutant LlG101 (lanes 3 and 4) are shown. Extracts separated in lanes 2 and 4 were heated for 10 min at 65°C before they were loaded onto the gel.

Glucose transport in *L. lactis* wild-type and *ptsH1* mutant strains and glucose-mediated exclusion of PTS sugars. Since the main function of HPr is to act as phosphocarrier protein during PTS-catalyzed sugar transport and phosphorylation, we tested whether the *ptsH1* mutation would influence PTS-catalyzed sugar uptake. Glucose transport activities were found to be very similar for the wild-type and the *ptsH1* mutant strains (Fig. 3). By contrast, the *ptsH* disruption strain LlG100 had completely lost the capacity to transport glucose at the concentration used in the transport assay (1 mM). However, strain LlG100 was able to slowly grow in M17 medium containing 25 mM glucose, indicating the presence of a non-PTS transporter capable of transporting glucose with low affinity. Slow growth of an *L. lactis ptsH* strain on glucose has also been reported by Luesink et al. (26). In *L. lactis* MG5267, TMG is taken up by a lactose-specific chromosome-encoded PTS and accumulated as TMG-6-P (see Fig. 6). Compared to the wild-type strain, PTS-catalyzed TMG uptake by the *ptsH1* mutant was slightly slower (Fig. 4A). In addition, the inhibition exerted by glucose on TMG uptake in the wild-type strain (sevenfold) was much weaker in the *ptsH1* mutant (only about twofold).

The inability of strain LlG100 to grow on mannitol suggested that this sugar might also be transported by the PTS. This assumption was supported by the finding that the genome of *L. lactis* IL1403 contains an operon (*mtlARFD*) encoding proteins with high sequence similarity to $EIICB^{Mtl}$, MtlR, $EIIA^{Mtl}$, and mannitol-1-P dehydrogenase from other organ-

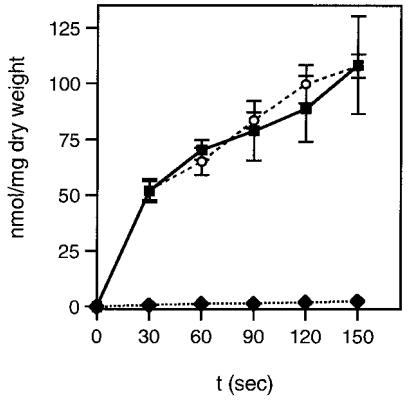

FIG. 3. Transport of [^{14}C]glucose (1 mM) by the *L. lactis* wild-type strain MG5267 (filled squares), the *ptsH*::*erm* disruption strain LlG100 (filled rhombs), and the *ptsH1* mutant LlG101 (open circles). Cells were grown in M17 medium containing 0.5% glucose.

isms (3, 4) (see also http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/framik?db=Genome&gi=171). Compared to the wild-type strain, mannitol uptake via the PTS was almost twofold greater in the *ptsH1* mutant. Interestingly, the presence of glucose completely inhibited mannitol uptake in the wild-type strain. Glucose inhibition of mannitol transport was only slightly relieved in the *ptsH1* mutant strain (Fig. 4B).

CCR of aryl-β-D-glucoside metabolism. *L. lactis* strain IL1403 was found to be capable of growing on aryl-β-D-glucosides such as esculin, salicin, and arbutin (1). Similarly, the wild-type strain MG5267 was able to grow on salicin, whereas the *ptsH*::*erm* strain LlG100 had lost this capacity, confirming that in *L. Lactis* aryl-β-D-glucosides are transported and phosphorylated by a PTS before they are split by a 6-P-β-D-glucosidase into glucose-6-P and the aglycon. The EII necessary for aryl-β-D-glucoside transport by MG5267 cells is probably encoded by the homologue of the *ptbA* gene located at kb 1482 of the *L. lactis* IL1403 chromosome (3, 4). This EIIBCA exhibits strong similarity to the EIIBCA^{Bgl} (BglP) of *B. subtilis* (23). *p*-Nitrophenyl-β-D-glucopyranoside was found to be a substrate for the PtbA of *L. lactis*, since it was taken up by salicin-grown MG5267 cells and subsequently split into glucose-6-P and *p*-nitrophenol. These activities were repressed sixfold in cells grown in the presence of salicin and glucose (Table 1). The repressive effect of glucose had disappeared in the *ptsH1* mutant strain. *p*-Nitrophenyl-β-D-glucopyranoside transport

TABLE 1. CCR of 6-P-β-glucosidase

Strain	Mean ± SD of 6-P-β-glucosidase activity[a] for cells grown in M17 medium with:		
	Sal[b]	Sal + Glc[b]	Sal (Glc)[c]
MG5267 WT[d]	98 ± 7	16 ± 5	168 ± 12
LIG101 *ptsH1*	104 ± 12	112 ± 9	177 ± 9

[a] 6-P-β-glucosidase activity is expressed in nanomoles of *p*-nitrophenol formed per minute and milliliter of cell culture with an OD_{600} of 0.5. Results from three experiments are presented.
[b] Sal, salicin; Sal + Glc, salicin plus glucose.
[c] 6-P-β-glucosidase assays were carried out with salicin-grown cells with glucose (10 mM) present in the assay mixture.
[d] WT, wild type.

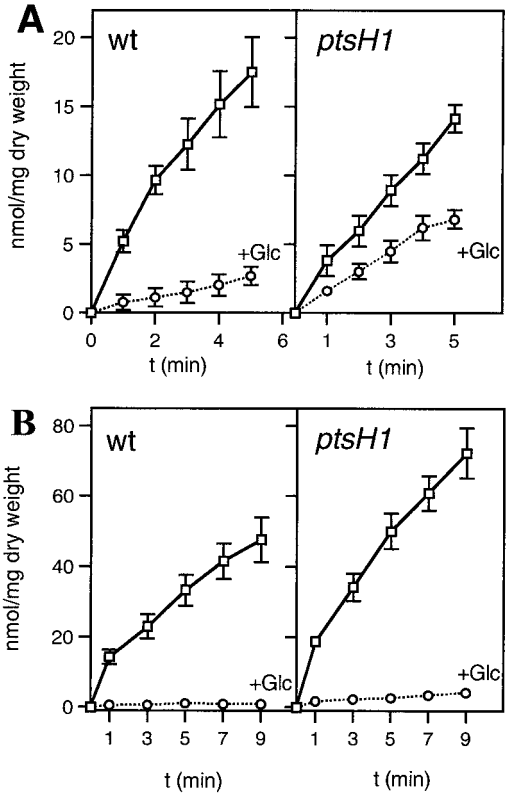

FIG. 4. Transport of ^{14}C-labeled TMG and mannitol and their exclusion by glucose in the *L. lactis* MG5267 (wild-type [wt]) and LlG101 (*ptsH1* mutant) strains. (A) TMG transport with cells grown in M17 medium containing 0.5% lactose; (B) mannitol transport with cells grown in M17 medium containing 0.5% mannitol. Transport assays were carried out in the absence of glucose (squares) or with 10 mM glucose added 1 min prior to adding the radiolabeled sugar (circles). In panel B, the error bars for the experiments carried out in the presence of glucose were too small to be drawn by the program.

and hydrolysis experiments carried out in the presence of glucose with salicin-grown wild-type and *ptsH1* mutant cells showed that glucose exerts no inducer exclusion effect on *p*-nitrophenyl-β-D-glucopyranoside uptake. The presence of glucose in the assay mixtures even stimulated *p*-nitrophenyl-β-D-glucopyranoside uptake and its subsequent hydrolysis about 1.5-fold in both wild-type and *ptsH1* mutant strains (Table 1).

The *ptsH1* mutation prevents inducer exclusion of non-PTS sugars maltose and ribose. In *L. lactis*, maltose and ribose are probably taken up by ATP-binding cassette (ABC) transport systems (MalE, MalF, and MalG; and RbsA, RbsC, and RbsD, respectively) (3, 4). For unknown reasons, the *ptsH* disruption strain LlG100 was unable to grow on ribose, although it grew normally on maltose. In the wild-type strain, the uptake of both carbohydrates was strongly inhibited when glucose was present during the transport reaction (Fig. 5A and B), suggesting that an inducer exclusion mechanism was operative. Interestingly, the inhibitory effect of glucose on the uptake of non-PTS carbohydrates ribose and maltose had disappeared in the *ptsH1* mutant LlG101 (Fig. 5A and B), although it transported glucose at a rate identical to that observed with the wild-type strain (Fig. 3).

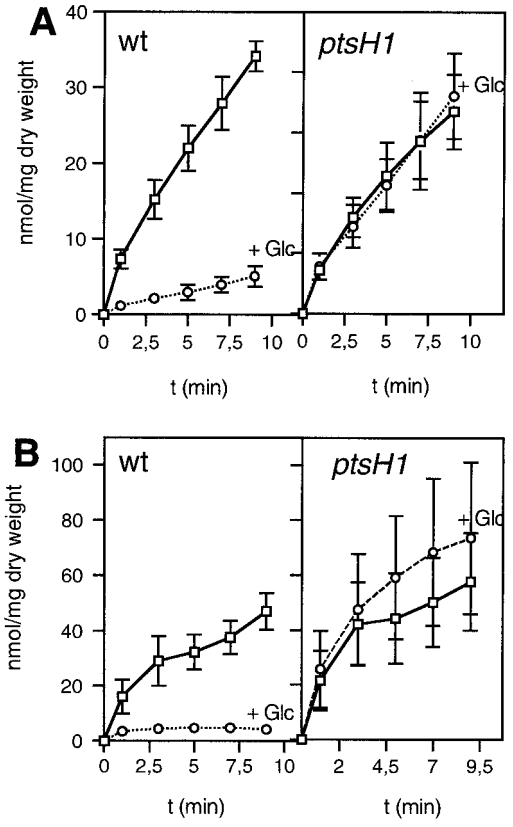

FIG. 5. Transport of the ^{14}C-labeled non-PTS sugars ribose (A) and maltose (B) and their exclusion by 10 mM glucose in *L. lactis* MG5267 (wild-type) and LIG101 (*ptsH1* mutant) strains. Transport assays were carried out in the absence of glucose (squares) or with 10 mM glucose added 1 min prior to adding the radiolabeled sugar (circles). Cells were grown in M17 medium containing 0.5% ribose (A) or 0.5% maltose (B).

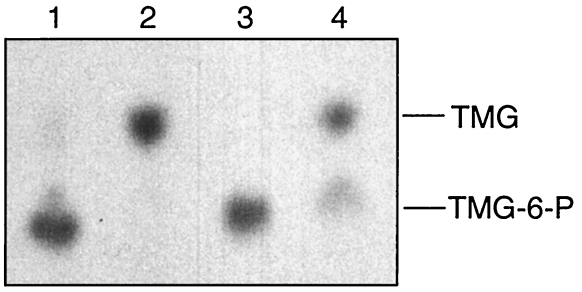

FIG. 6. Autoradiogram showing the amounts of [^{14}C]TMG and [^{14}C]TMG-6-P present in *L. lactis* cells and in the medium before and after inducer expulsion. [^{14}C]TMG and [^{14}C]TMG-6-P were separated by thin-layer chromatography. Lanes 1 and 3, [^{14}C]TMG-6-P accumulated in wild-type and *ptsH1* mutant cells; lanes 2 and 4, [^{14}C]TMG present in cells and in the medium after 5 min of expulsion. Expulsion experiments were carried out with the wild-type strain MG5267 (lanes 1 and 2) and the *ptsH1* mutant LIG101 (lanes 3 and 4). The cells were grown in 0.5% lactose-containing M17 medium.

P-Ser-HPr is not essential for inducer expulsion in *L. lactis*. In vitro results obtained with *L. lactis* vesicles had suggested that P-Ser-HPr participates in inducer expulsion by stimulating the activity of a sugar-P phosphohydrolase catalyzing the first step of inducer expulsion (42, 43). To test whether P-Ser-HPr is indeed implicated in this regulatory process, we measured TMG expulsion in an *L. lactis* wild-type strain and a *ptsH1* mutant strain. *L. lactis* strain MG5267 takes up [^{14}C]TMG via the lactose-specific PTS and accumulates it as [^{14}C]TMG-6-P, which cannot be further metabolized (Fig. 6, lane 1). When glucose was added to MG5267 cells preloaded with [^{14}C]TMG-6-P, the nonmetabolizable sugar was rapidly expelled from the cells (Fig. 7A). In the first step, the presence of glucose has been shown to initiate the intracellular dephosphorylation of TMG-6-P (30) before TMG is expelled from the cells in the second step, probably via EIICB^{Lac} (31). In agreement with this model, only TMG and no TMG-6-P was found to be expelled from MG5267 cells preloaded with [^{14}C]TMG-6-P (Fig. 6, lane 2). The *ptsH1* mutant was also capable of accumulating [^{14}C]TMG-6-P (Fig. 6, lane 3). However, expulsion of [^{14}C]TMG occurred at a significantly slower rate and was not yet completed after 5 min (Fig. 7A). Similar to what was observed with the wild-type strain, [^{14}C]TMG-6-P was dephosphorylated during the expulsion process (Fig. 6, lane 4). After 5 min of incubation in the presence of glucose, about two-thirds of the accumulated [^{14}C]TMG-6-P was expelled from the *ptsH1* mutant and dephosphorylated, whereas one-third remained in the cell as [^{14}C]TMG-6-P (Fig. 7A).

Expulsion experiments were also carried out with cells which had taken up [^{14}C]2-DG. Like TMG, [^{14}C]2-DG is accumulated by *L. lactis* cells as the phospho derivative. After glucose was added, [^{14}C]2-DG-6-P was first intracellularly dephosphorylated and subsequently expelled as unphosphorylated [^{14}C]2-DG (34). Compared to TMG expulsion, glucose-activated expulsion of [^{14}C]2-DG occurred at a slower rate (Fig. 7B). But almost no difference of [^{14}C]2-DG expulsion could be observed between *L. lactis* wild-type and *ptsH1* mutant strains.

DISCUSSION

HPr is the major regulator of carbon metabolism in gram-positive bacteria. The implication of P-Ser-HPr in CCR and CCA has been well established for *B. subtilis* (7), *Staphylococcus xylosus* (19), and *L. casei* (12, 37) and has also been suggested for *L. lactis*. Expression of the *L. lactis las* operon encoding several glycolytic enzymes was stimulated by the presence of glucose, whereas activation of *las* operon expression was absent in a *ccpA* mutant (27) as well as a *ptsH* disruption mutant transformed with a plasmid containing the *ptsH1* allele (encoding Ser46Ala HPr) (26). The participation of P-Ser-HPr in catabolite regulation was confirmed by constructing a chromosomal *ptsH1* mutant. Like *B. subtilis, L. lactis* possesses an aryl-β-glucoside-specific EII (PtbA) and a 6-P-β-glucosidase (BglH) (1, 4). In the wild-type strain, the synthesis of these enzymes was strongly repressed by glucose, but it was relieved from CCR in the *ptsH1* mutant. In *B. subtilis*, the *bgl* operon is regulated by two CCR mechanisms (22): one involves P-Ser-HPr/CcpA and a *cre* present in the promoter region, and the other involves the antiterminator LicT, which is activated by P~His-HPr-mediated phosphorylation (25). BglR, a homologue of LicT, is controlling β-glucoside metabolism in *L. lactis* (1), and a potential *cre* is preceding the *ptbA* gene encoding EIICBA^{Bgl} (4), suggesting that

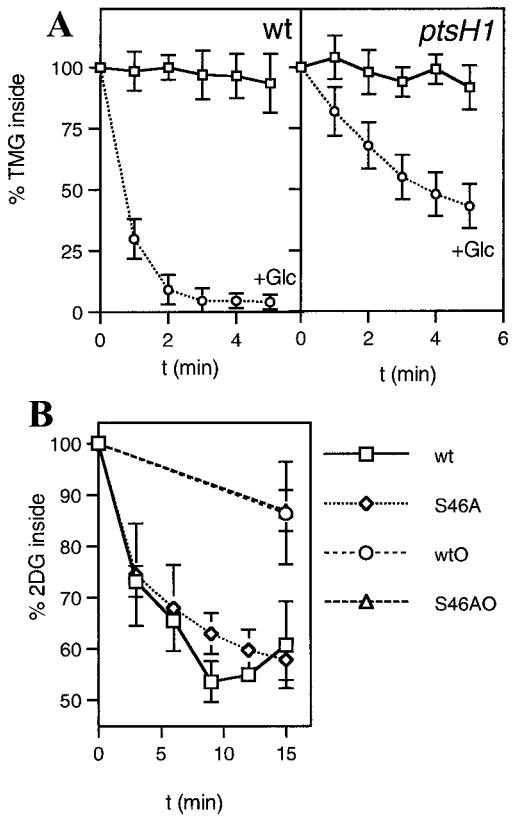

FIG. 7. Expulsion of accumulated [^{14}C]TMG-6-P (A) and [^{14}C]2-DG-6-P (B) in the *L. lactis* wild-type strain MG5267 and the *ptsH1* mutant LlG101. Cells grown in the presence of 0.5% lactose or 0.5% glucose were preloaded with [^{14}C]TMG or [^{14}C]2-DG, respectively. The amount of labeled sugar remaining inside the cells during a 5-min (for TMG) or 15-min (for 2-DG) incubation period at 37°C in the presence or absence of glucose was determined by withdrawing aliquots at the indicated time intervals and analyzing them by the rapid-filtration method (37). Squares, no sugar added; circles, 10 mM glucose added at time zero. S46AO and wtO, no glucose added to cells preloaded with [^{14}C]2-DG-6-P. For the latter samples, aliquots were withdrawn only at the beginning and at the end of the experiments. Leakage levels of [^{14}C]2-DG-6-P from the cells were found to be nearly identical for the wild-type and the *ptsH1* mutant strains, explaining why the lines for the two strains coincide.

CCR mechanisms similar to those described for the *B. subtilis bgl* operon might be operative for the *ptbA-bglH* operon in *L. lactis*.

We observed that in *L. lactis* glucose exerted a strong exclusion or expulsion effect on other PTS carbohydrates. The uptake of TMG and mannitol was almost completely inhibited when glucose was present. A much weaker inhibitory effect of glucose or fructose on mannitol uptake has been observed with *B. subtilis* (10, 46). In the latter organism, competition for the common phosphoryl donor P~His-HPr seemed to be the reason for this inhibitory effect, since inhibition of mannitol transport was almost completely relieved in a *B. subtilis ptsH1* mutant (10). Since Ser46A mutant HPr cannot be phosphorylated by HprK/P, a *ptsH1* mutant contains more P~His-HPr for the phosphoryl group transfer within the PTS phosphorylation cascade than does a wild-type strain (Fig. 2). TMG uptake in *L. lactis* seems to be regulated in a manner similar to that of mannitol uptake in *B. subtilis*, since the inhibitory effect of glucose on TMG uptake was much weaker in a *ptsH1* mutant. The slowed TMG expulsion in the *ptsH1* mutant (Fig. 7A) could also be responsible for the weaker inhibitory effect of glucose on TMG-6-P accumulation. Glucose-mediated inhibition of mannitol transport in *L. lactis* follows a different mechanism, since glucose exerted similarly strong inhibitory effects on mannitol transport in both wild-type strain MG5267 and *ptsH1* mutant LlG101.

P-Ser-HPr has recently been shown to participate in inducer exclusion of non-PTS carbohydrates in gram-positive bacteria. The strong inhibitory effect of glucose on the uptake of the non-PTS sugar maltose observed with *L. casei* wild-type cells was absent in *ptsH1* and *hprK* mutants (12, 37). In order to find out whether this recently established mechanism of inducer exclusion of non-PTS sugars, which so far has not been detected in *B. subtilis*, is widespread within gram-positive bacteria, we tested whether it was also operative in *L. lactis*. We found that in *L. lactis* the uptake of maltose and ribose was subject to inducer exclusion. The two corresponding transport activities were strongly inhibited by the presence of glucose. Similar to what is observed for *L. casei*, inducer exclusion in *L. lactis* is mediated via P-Ser-HPr, since the strong repressive effect of glucose had completely disappeared in the *ptsH1* mutant. Interestingly, both non-PTS transport systems submitted to inducer exclusion in *L. lactis* are ABC transporters (3, 4). Since it is likely that in *L. casei* maltose is also taken up by an ABC transporter, there arises the question of whether P-Ser-HPr-mediated inducer exclusion in gram-positive bacteria affects only ABC transporters.

In previous reports, a participation of P-Ser-HPr in inducer expulsion by *L. lactis* has been suggested, whereas the results obtained during this study argue against an implication of P-Ser-HPr in this regulatory process. In the previous inducer expulsion studies, *B. subtilis* wild-type or Ser46Asp mutant HPr and glycolytic intermediates were electroporated into *L. lactis* vesicles, and the results suggested an involvement of P-Ser-HPr in the expulsion of both TMG and 2-DG (42, 43). P-Ser-HPr has been proposed to stimulate the first step of inducer expulsion, the dephosphorylation of accumulated nonmetabolizable PTS sugars, since it has been reported to activate in vitro a P-sugar phosphohydrolase (45). In contrast, in vivo experiments carried out with *L. casei* wild-type, *ptsH1*, and *hprK* mutant strains had allowed us to establish that in this organism P-Ser-HPr does not participate in inducer expulsion (12, 37). An almost identical expulsion of TMG was observed in the *L. casei* wild-type strain and the two mutant strains unable to form P-Ser-HPr. Constructing a *ptsH1* mutant was expected to allow us to either confirm or refute the proposed participation of P-Ser-HPr in inducer expulsion of *L. lactis*. Since in the *L. lactis ptsH1* mutant, 2-DG expulsion by glucose was not affected and TMG expulsion was still operative, although at a lower rate than in the wild-type strain, P-Ser-HPr is not necessary for inducer expulsion. A P-Ser-HPr-independent inducer expulsion mechanism must be operative in *L. lactis* and *L. casei* (12, 37) and probably in other gram-positive organisms. In the studies suggesting a role of P-Ser-HPr in inducer expulsion, the use of *L. lactis* vesicles, the electroporation of HPr and metabolites into these vesicles, and the use of a heterologous system (*B. subtilis* wild-type or Ser46Asp

mutant HPr instead of *L. lactis* HPr or P-Ser-HPr) could have yielded misleading results. In comparison, the results obtained in this study seem to be more reliable, since the experiments were carried out in vivo with intact cells of an *L. lactis* wild-type strain and a *ptsH1* mutant. The latter strain synthesized HPr, which was active in PTS transport but which, due to the replacement of Ser-46 with an alanine, could not be phosphorylated by HprK/P. Nevertheless, according to the Western blots (Fig. 2), the total amounts of the various forms of HPr present in the two strains were very similar.

What could have been the reason for the results of the vesicle studies suggesting an involvement of P-Ser-HPr in inducer expulsion? Ser46Asp mutant HPr might not exactly mimic P-Ser-HPr, and by using electroporation, the amount of HPr present in the vesicles was probably difficult to control. In addition, the reduced rate of TMG expulsion observed with the *L. lactis ptsH1* mutant suggested an indirect role of HPr in this regulatory process. Evidence has previously been provided that expulsion of TMG in *Streptococcus pyogenes* is catalyzed by $EIICB^{Lac}$ (31). In a wild-type strain growing in glucose-containing medium, only about 25% of the HPr is present as P~His-HPr. Under these conditions, $EIICB^{Lac}$ might be unphosphorylated or only slightly phosphorylated. By contrast, in a *ptsH1* mutant growing in glucose-containing medium, the about threefold-greater amount of P~His-HPr might allow a more efficient phosphorylation of $EIICB^{Lac}$. Phosphorylated $EIICB^{Lac}$ probably does not expel TMG from the cells but rather transports and rephosphorylates it and might therefore be responsible for the slowed TMG expulsion observed with the *ptsH1* mutant. Elevated phosphorylation of $EIICB^{Lac}$ in vesicles, into which Ser46Ala mutant HPr had been electroporated, might also be the reason why these vesicles exhibited reduced inducer expulsion. By contrast, coelectroporation of glycolytic intermediates with either wild-type HPr, which under the experimental conditions employed was partly converted to P-Ser-HPr in the vesicles (42), or Ser46Asp mutant HPr, which like P-Ser-HPr is very slowly phosphorylated at His-15 by enzyme I and PEP, probably led to inefficient phosphorylation of $EIICB^{Lac}$ and therefore caused no reduction or only a slight reduction of TMG expulsion.

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