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Molecular Characterization of the *Lactococcus lactis ptsHI* Operon and Analysis of the Regulatory Role of HPr

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The *Lactococcus lactis ptsH* and *ptsI* genes, encoding the general proteins of the phosphoenolpyruvate-dependent phosphotransferase system, HPr and enzyme I, respectively, were cloned, and the regulatory role of HPr was studied by mutational analysis of its gene. A promoter sequence was identified upstream of the *ptsHI* operon, and the transcription start site was mapped by primer extension. The results of Northern analyses showed the presence of two glucose-inducible transcripts, one of 0.3 kb containing *ptsH* and a second of 2.0 kb containing both *ptsH* and *ptsI*. Disruption of the *ptsH* and *ptsI* genes in strain NZ9800 resulted in a reduced growth rate at the expense of glucose, but no growth at the expense of sucrose and fructose, confirming the dominant role of the phosphotransferase system in the uptake of these sugars in *L. lactis*. Complementation of the *ptsH* and *ptsI* mutants with the intact genes under the control of a regulated promoter resulted in the restoration of the wild-type phenotype. The role of HPr(Ser-P) in the recently established CcpA-mediated control of galactose metabolism as well as glycolysis was analyzed by producing an HPr mutant carrying an aspartic acid on residue 46 which mimicks a phosphorylated serine. The results of these experiments demonstrated the role of HPr(Ser-P) as corepressor in the catabolite repression of the *gal* operon. Furthermore, we show for the first time that HPr(Ser-P) functions as a coactivator in the CcpA-mediated catabolite activation of the pyruvate kinase and L-lactate dehydrogenase genes.

The main sugar uptake system in many bacteria is the phosphoenolpyruvate:sugar phosphotransferase system (PEP-PTS), which mediates the uptake and phosphorylation of carbohydrates (32). The PTS is a group translocation process in which the transfer of the phosphate moiety of PEP to carbohydrates is catalyzed by the general non-sugar-specific proteins enzyme I and HPr in combination with sugar-specific enzyme II (EII) proteins. After autophosphorylation of enzyme I at the expense of PEP, enzyme I catalyzes the phosphorylation of HPr at histidine 15, resulting in HPr(His-P). The phosphate group from HPr(His-P) is then transferred to the sugar substrate via a two-step phosphorylation reaction mediated by a dedicated EII protein. EII proteins can consist of one or more proteins and are composed of three domains: the EIIA and EIIB domains, which are involved in the phosphotransfer, and the membrane-located EIIC domain, which is most likely involved in the translocation of the sugar substrate (32). The genes encoding HPr and enzyme I, *ptsH* and *ptsI*, respectively, have been cloned from several bacteria and found often to be organized in an operon structure with the gene order *ptsHI* (13, 18, 22). Expression of the *Escherichia coli* and *Bacillus subtilis ptsHI* operons appears to be regulated at the transcriptional level, since mRNA levels were found to be higher in glucose-grown cells than in cells grown on non-PTS sugars (6, 38).

Apart from its function in the uptake of sugars, the PTS also plays a regulatory role. In *E. coli* and other gram-negative bacteria, the PTS regulates the concentration of cyclic AMP (cAMP) via activation of adenylate cyclase by the phosphorylated form of the glucose-specific EIIA, the concentration of which increases in the absence of PTS substrates (32). Elevated concentrations of cAMP lead to transcriptional activation of several genes via the binding of the cAMP receptor protein complexed with cAMP to operator sites located in the promoter regions of affected genes. Furthermore, the unphosphorylated form of the glucose-specific EIIA reduces the uptake of several non-PTS sugars via an interaction with the uptake protein (32). Regulatory functions for the PTS have also been described for gram-positive bacteria. The HPr(His-P)-mediated phosphorylation of two glycerol kinases results in an increased activity of both enzymes in *Enterococcus* spp. (4). In contrast, enzyme I/HPr(His-P)-mediated phosphorylation of the lactose permease in *Streptococcus thermophilus* results in a reduced permease activity leading to a decreased uptake of sugar (31). In *B. subtilis*, the PTS regulates the expression of the levanase operon by HPr(His-P) mediated phosphorylation of the transcriptional regulator LevR, resulting in activation of transcription (37).

Apart from phosphorylation at residue His-15, a second phosphorylation site has been identified in HPr, the function of which has been shown only in gram-positive bacteria. Phosphorylation of HPr at residue Ser-46 is catalyzed by an ATP-dependent protein kinase that is activated by fructose-1,6-diphosphate (8, 35). Recently, the genes encoding the HPr(Ser) kinase and the HPr(Ser) phosphatase were cloned from *B. subtilis*, and their involvement in the phosphorylation of HPr at residue Ser-46 was established (16, 33). The seryl-phosphorylated form of HPr, designated HPr(Ser-P), interacts with several PTS and non-PTS sugar permeases, and this process, termed inducer exclusion, results in a reduced uptake of sugars (35). In addition, HPr(Ser-P) allosterically activates sugar-phosphate phosphatases in *Lactococcus lactis*, *Enterococcus faecalis*, and *Streptococcus pyogenes* that catalyze the dephosphorylation of various phosphorylated sugars, resulting in an efflux of the sugar from the cell, a process known as inducer expulsion (44, 47). Apart from these allosteric control systems, HPr(Ser-P) can also negatively regulate the transcription of genes by an interaction with the catabolite control protein, CcpA (10, 20). It has been reported that the in vitro binding of CcpA to a *cis*-acting *cre* (catabolite-respon-

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TABLE 1. *L. lactis* strains and plasmids used in this study

Strain or plasmid	Relevant properties	Reference
Strains		
NZ9800		26
NZ9880	NZ9800 derivative; Δ*ptsH*	This work
NZ9881	NZ9800 derivative: Δ*ptsI*	This work
Plasmids		
pUC19E	pUC19 derivative containing an Erm^r gene	28
pNZ8030	Lactococcal cloning and expression vector	7
pNZ9279	pGEM-T containing a 0.8-kb PCR product derived from the *L. lactis* ptsHI operon	This work
pNZ9280	pUC19 containing a 4.5-kb *Sst*I-*Cla*I fragment carrying the *ptsH* gene and part of the *ptsI* gene	This work
pNZ9281	pUC19 containing a 3.5-kb *Eco*RV-*Bam*HI fragment carrying the *ptsH* and *ptsI* genes	This work
pNZ9282	pNZ8030 containing the *L. lactis* ptsH gene translationally fused to the *nisA* promoter	This work
pNZ9283	pNZ8030 containing the *B. subtilis* ptsH gene translationally fused to the *nisA* promoter	This work
pNZ9284	pNZ8030 containing the S46A *ptsH* gene translationally fused to the *nisA* promoter	This work
pNZ9285	pNZ8030 containing the S46D *ptsH* gene translationally fused to the *nisA* promoter	This work
pNZ9286	pNZ8030 containing the *ptsH* and *ptsI* genes translationally fused to the *nisA* promoter	This work
pNZ9287	pNZ9286 derivative containing the *ptsI* gene transcriptionally fused to the *nisA* promoter	This work
pNZ9288	pNZ8030 containing S46A *ptsH* and *ptsI* translationally fused to the *nisA* promoter	This work
pNZ9289	pNZ8030 containing S46D *ptsH* and *ptsI* translationally fused to the *nisA* promoter	This work
pNZ9290	*ptsH* disruption construct	This work
pNZ9291	pUC19E derivative containing an internal *Cla*I-*Pst*I fragment of *ptsI*	This work

sive element) operator site, located in the promoter region of genes controlled by CcpA, is enhanced by an interaction with HPr(Ser-P), glucose-6-P, and NADP (12, 17, 23). Deutscher et al. (9) demonstrated that expression of the gene encoding S46D HPr, which is structurally similar to HPr(Ser-P), leads to catabolite repression of the *B. subtilis* gluconate kinase gene even in the absence of glucose. In addition, they showed that the replacement of HPr with S46A HPr has the same effect as a mutation in the *ccpA* gene, rendering the gluconate kinase gene insensitive to catabolite repression.

Recently, we have demonstrated (i) the involvement of the *L. lactis* CcpA in the negative regulation of the expression of the genes involved in galactose metabolism and (ii) the positive control of the *las* operon encoding phosphofructokinase, pyruvate kinase, and L-lactate dehydrogenase (29). Here, we report the cloning and analysis of the *L. lactis* ptsH and ptsI genes and the involvement of HPr in the catabolite repression of galactose metabolism. Furthermore, we show for the first time the participation of HPr(Ser-P) in the CcpA-mediated transcriptional activation of the *las* operon.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and transformation procedure. The *L. lactis* strains and plasmids used in this study are described in Table 1. The strains were cultivated without aeration at 30°C in M17 broth supplemented with different carbon sources. *L. lactis* was transformed by electroporation as described by Holo and Nes (22). *E. coli* MC1061 was used as a host for cloning experiments and grown in L-broth-based medium with aeration at 37°C. Antibiotics were used in the following concentrations: ampicillin, 50 μg/ml (for selection of pUC19-based plasmids in *E. coli*); chloramphenicol, 5 μg/ml (for selection of pNZ8030-based plasmids in *E. coli* and *L. lactis*); and erythromycin, 2.5 μg/ml (for selection for the integration of the erythromycin resistance [Erm^r] gene into the chromosome of *L. lactis*). Bacterial growth was monitored spectrophotometrically at 600 nm.

DNA techniques and DNA sequence analysis. All manipulations with recombinant DNA were carried out according to standard procedures (36) and as specified by the enzyme manufacturer (Gibco/BRL Life Technologies [Breda, The Netherlands] or United States Biochemical Corp. [Cleveland, Ohio]). Plasmid and chromosomal DNA of *L. lactis* was isolated as described previously (41). The nucleotide sequence of the *ptsHI* genes was determined on both strands, using an ALF DNA sequencer (Pharmacia LKB Biotechnology, Uppsala, Sweden). PCR was performed in a total volume of 50 μl containing 10 mM Tris-HCl (pH 8.8), 50 mM NaCl, 2 mM MgCl$_2$, 10 μg of gelatin, 200 μM each deoxynucleoside triphosphate, 1 U of *Taq* polymerase (Gibco/BRL), 10 pmol of each primer, and 10 to 100 ng of template DNA. A small volume of mineral oil was added to prevent evaporation. PCR amplifications were performed in 25 cycles, each cycle consisting of a denaturation step at 95°C for 1 min, a primer annealing step at the appropriate temperature for 1 min, and a primer extension step at 72°C for 2.5 min, carried out in a DNA Thermocycler 480 (Perkin Elmer, Norwalk, Conn.).

Construction of plasmids. Primers PTS1 (5′-AACWGGWATTCATGCWM GWCCWGC-3′) and PTS2 (5′-GGTACCWCCAATATTWGTWACAAAW GC-3′) were used in a PCR with chromosomal DNA from *L. lactis* NZ9800 to amplify part of the *ptsHI* operon. The PCR product was cloned in the pGEM-T vector (Promega, Madison, Wis.), yielding pNZ9279. The insert from pNZ9279 was used as a probe to clone a 4.5-kb *Sst*I-*Cla*I fragment from the chromosomal DNA of *L. lactis* into *Sst*I-*Acc*I-digested pUC19 (43), resulting in pNZ9280. The same fragment was used as a probe to clone a 3.5-kb *Eco*RV-*Bam*HI fragment from the chromosomal DNA of *L. lactis* in *Sma*I-*Bam*HI-digested pUC19, resulting in pNZ9281.

By the use of oligonucleotides HPRNCOI (5′-GAACCATAACCATGGCAT CTAAAG-3′) and HPRHINDIII (5′-GACAAGCAAGCTTGCCTTAGCTA CTG-3′) the *ptsH* gene was amplified by PCR with chromosomal DNA from *L. lactis* NZ9800 as the template. Bases that were changed to introduce the *Nco*I and *Hind*III restriction sites are underlined. These sites were used to clone the PCR product in *Nco*I-*Hind*III-digested pNZ8030, resulting in plasmid pNZ9282.

The *B. subtilis* ptsH gene (18) was amplified by PCR with oligonucleotides BSHPRNCO (5′-GGAGAATGATAACCATGGCACAAAAAAC-3′) and BSH PRHIND (5′-GCAGAGATGTTAAGCTTTTCAACC-3′) and chromosomal DNA from *B. subtilis* 168 as the template. The PCR product was digested with *Nco*I and *Hind*III and cloned into *Nco*I-*Hind*III-digested pNZ8030, yielding pNZ9283.

Residue serine 46 of *L. lactis* HPr was changed to an alanine and an aspartic acid by PCR-mediated megaprimer mutagenesis of the *ptsH* gene, as described previously (25), with the mutagenic primers HPR46A (5′-GTAAACCTTAAA GCAATCATGGGTGTTATGTC-3′) and HPR46D (5′-GTAAACCTTAAAG ATATCATGGGTGTTATGTC-3′), respectively. The substitutions needed to change codon 46 are underlined. Two amplification rounds were used: the first with the mutagenic primers and the antiparallel primer PTSHINDIII; the second with the purified PCR products from the first round as megaprimer, together with primer PTSHNCOI. The PCR products were digested with *Nco*I and *Hind*III and cloned into *Nco*I-*Hind*III-digested pNZ8030, yielding pNZ9284 and pNZ9285, carrying the genes encoding S46A HPr and S46D HPr, respectively.

For the construction of a translational fusion between the *nisA* promoter and the entire *ptsHI* operon encoding wild-type, S46A, or S46D HPr together with enzyme I, a 3.2-kb *Dra*III-*Bam*HI fragment was isolated from pNZ9281. After treatment with Klenow DNA polymerase, this fragment was cloned into pNZ9282, pNZ9284, or pNZ9285 digested with *Dra*III and *Hind*III. The latter site was made blunt by treatment with Klenow DNA polymerase, and the resulting plasmids were designated pNZ9286, pNZ9288, and pNZ9289, respectively.

Plasmid pNZ9287 was constructed by digesting plasmid pNZ9286 with *Nco*I and *Dra*III to remove the 5′ end of the *ptsH* gene and then treating the ends with Klenow enzyme and T4 DNA polymerase, followed by self-ligation of the resulting DNA. To disrupt the *ptsH* gene, a *Bam*HI site was introduced in the *ptsH* open reading frame by PCR using primers HPRBAM2 (5′-GTGTTGGATC CCTCGGTGTTGGTCAAG-3′), corresponding to nucleotides 403 to 419, and
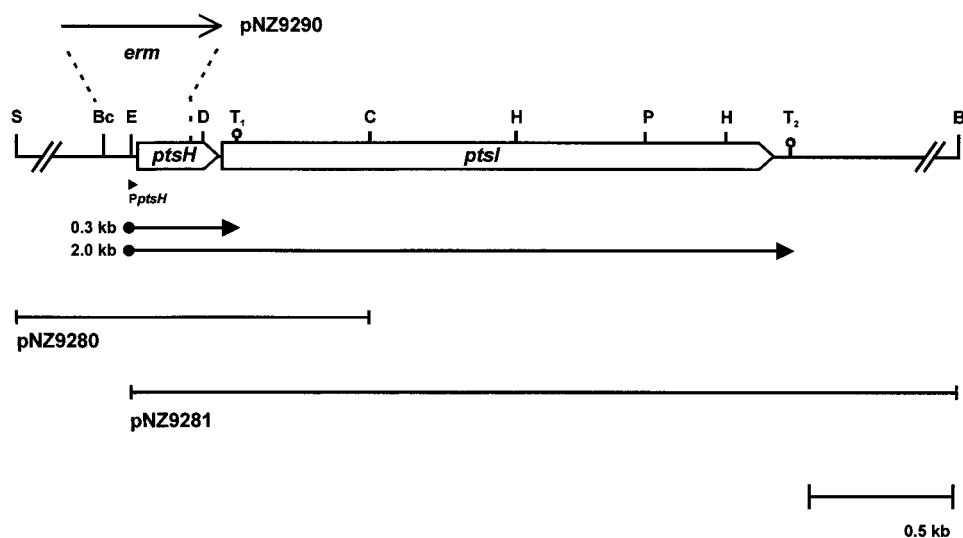
FIG. 1. Transcriptional organization of the *ptsHI* operon of *L. lactis* NZ9800. The genes (open arrows) connected by intergenic regions (thin lines) are shown with their products. The orientation of the Erm[r] gene (*erm*) in plasmid pNZ9290 is indicated. The arrows below the genes denote the promoter and transcripts mapped with primer extension and Northern analysis, respectively. The putative terminators downstream of *ptsH* and *ptsI*, (T_1 and T_2, respectively) are indicated. Relevant restriction sites: S, *Sst*I; Bc, *Bcl*I; E, *Eco*RV; D, *Dra*III; C, *Cla*I; H, *Hin*dIII; P, *Pst*I; B, *Bam*HI.

PTS6 (5′-GATGGATTGTAAGGTTGATA-3′), complementary to nucleotides 1947 to 1967 of the *ptsHI* genes. The PCR product was digested with *Bam*HI and *Hin*dIII, and the 1.0-kb fragment was cloned into *Bam*HI-*Hin*dIII-digested pUC19. The resulting plasmid was digested with *Sst*I and *Bam*HI, and a 1.2-kb *Sau*3A fragment from plasmid pIL253 carrying an Erm[r] gene and a 3.5-kb *Sst*I-*Bcl*I fragment carrying the upstream region of the *ptsH* gene from plasmid pNZ9280 were cloned into these sites. The expected constructs were verified by restriction endonuclease analysis, one product, containing the Erm[r] gene in the same orientation as the *ptsI* open reading frame and designated pNZ9290, was selected for further study. Plasmid pNZ9291 was constructed by cloning a 0.9-kb *Cla*I-*Pst*I fragment from plasmid pNZ9281, the *Cla*I site of which was made blunt by Klenow DNA polymerase treatment, into *Pst*I-*Bam*HI-digested pUC19E, the *Bam*HI site of which was filled in by use of Klenow DNA polymerase. The nucleotide sequences of all DNA fragments obtained via PCR were verified and found to be correct.

RNA analysis. RNA was isolated from *L. lactis* cultures as described previously (26). RNA was denatured and size fractionated on a 1% agarose gel containing formaldehyde according to standard procedures (36). The RNA was stained by adding ethidium bromide to the sample buffer. As molecular weight markers, a 0.24- to 9.5-kb RNA ladder from Bethesda Research Laboratories was used. The gel was blotted on a nylon membrane (Gene Screen Plus nylon membranes; NEN Research Products, Wilmington, Del.) as recommended by the manufacturer. Blots were hybridized with oligonucleotides PEPTSH (for *ptsH*) and PTS6 (for *ptsI*). Following autoradiography, bands were cut out and total radioactivity was determined in a liquid scintillation counter (Beckman LKS 7500).

Primer extension analysis. The oligonucleotide used for priming cDNA synthesis was PEPTSH (5′-CTGCAACGATGTGGAATTCTTTAG-3′), complementary to nucleotides 254 to 278 in the coding strand of the *ptsH* gene. Primer extension reactions were performed by annealing 2 ng of oligonucleotide to 100 μg of total RNA as described elsewhere (26).

Enzyme assays. Pyruvate kinase and L-lactate dehydrogenase activities were determined according to standard methods (5, 21). Protein concentrations were assayed by the method of Bradford (3), with bovine serum albumin as the standard.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession no. Z97203.

RESULTS

Cloning of the *ptsHI* operon. Sequences of HPr proteins from different gram-positive bacteria were aligned. Oligonucleotide PTS1 was designed based on the conserved region TGIHARP, encompassing the catalytic phosphorylation site His-15 in several HPr sequences. The reported active-site sequence of the *L. lactis* enzyme I (1) was used to design an antisense oligonucleotide, PTS2. Assuming that the gene order

in *L. lactis* would be identical to that found in other bacteria (*ptsHI*), primers PTS1 and PTS2 were used in a PCR under nonstringent conditions with chromosomal DNA from *L. lactis* NZ9800 as the template. The PCR product had the expected size of 0.8 kb. The nucleotide sequence of this fragment was analyzed and found to contain two partial open reading frames encoding proteins with high sequence homology to HPr and enzyme I sequences. This fragment was used as a probe and hybridized to a single DNA fragment of the *L. lactis* chromosome that was cloned as two overlapping fragments, resulting in plasmids pNZ9280 and pNZ9281.

Characterization of the *ptsH* and *ptsI* genes. Analyses of the nucleotide sequences in the inserts of plasmids pNZ9280 and pNZ9281 revealed the presence of two complete open reading frames. The first, designated *ptsH* (see below), could encode a protein of 88 amino acids that shows high sequence homology to HPr proteins from different bacteria, with up to 91% identical residues compared to the *S. salivarius* HPr protein (13). A sequence (5′-AAGGAG-3′) resembling a typical lactococcal ribosome binding site was identified 10 nucleotides upstream of the ATG start codon (11). The second open reading frame, designated *ptsI* (see below), could encode a protein of 575 amino acids with high sequence similarity to enzyme I proteins of various bacteria, i.e., 83% identity compared to the *S. salivarius* enzyme I (14). The putative ATG start codon of the *ptsI* gene, which is located eight nucleotides downstream of the *ptsH* gene, is preceded by a putative lactococcal ribosome binding site (5′-AAGGA-3′) with an unusual spacing of 14 bp. Two stem-loop structures were identified in the *ptsHI* operon: one with a ΔG value of −6.4 kcal/mol located downstream of the *ptsH* gene (terminator T_1), and a second with a ΔG value of −9.0 kcal/mol followed by an AT-rich stretch of nucleotides downstream of the *ptsI* gene (T_2) (Fig. 1).

Transcriptional analysis of the *ptsHI* operon. Primer extension analysis was performed with total RNA isolated from strain NZ9800 grown on glucose. Two equally intense labeled primer extension products were obtained, indicating that transcription starts at two adjacent sites (Fig. 2). Upstream of these start sites, a sequence corresponding to consensus *L. lactis*
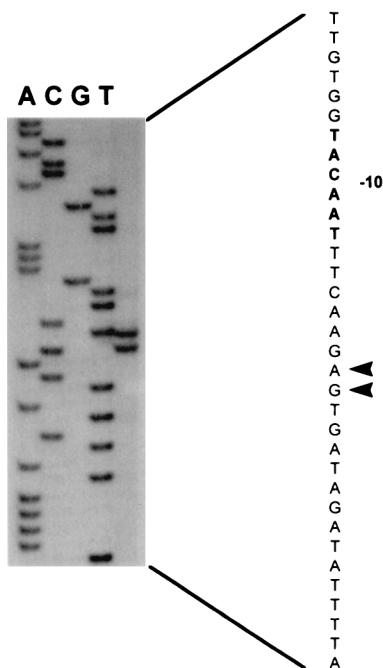
FIG. 2. Determination of the transcription start sites of the *pts* promoter. The transcription start sites are indicated with arrowheads. The putative −10 region in the coding strand is in bold. RNA was isolated from strain NZ9800 grown on glucose.
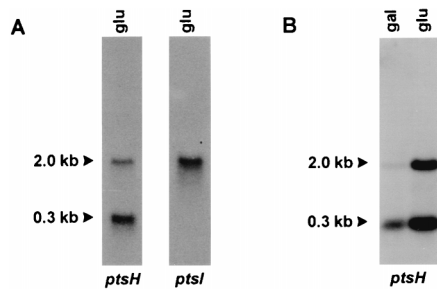

FIG. 3. (A) Northern analysis of *ptsH* and *ptsI* transcription in *L. lactis* NZ9800 grown at the expense of glucose; (B) transcriptional analysis of the *ptsH* gene in *L. lactis* strain NZ9800 grown at the expense of galactose and glucose. Estimated sizes of the transcripts are indicated with arrowheads.

promoter sequences was identified (11, 40). Northern analysis of the *ptsHI* genes after hybridization with a *ptsH*-specific probe revealed the presence of two transcripts, one of 0.3 kb and a second of 2.0 kb (Fig. 3A). When the same blot was stripped and rehybridized with a *ptsI*-specific probe, only the 2.0-kb transcript hybridized (Fig. 3A). RNA isolated from cells grown on galactose and glucose was hybridized with a *ptsH*-specific probe. The results showed that the transcription of the *ptsHI* operon varies in response to the carbon source, since transcription levels were at least 10-fold higher in glucose-grown cells than in galactose-grown cells, as determined by scintillation counting (Fig. 3B). This finding demonstrates that expression of the *L. lactis* *ptsHI* operon is regulated at the transcriptional level in response to the carbon source.

Disruption of the *ptsH* and *ptsI* genes. To analyze the functional and regulatory roles of the *L. lactis* *ptsH* and *ptsI* genes, both genes were disrupted by replacement recombination. The *ptsH* gene was disrupted by chromosomal integration of plasmid pNZ9290, which contains an Erm^r cassette flanked by upstream and downstream regions of the *ptsH* gene. Integrants were obtained and analyzed via PCR and Southern hybridization. We selected one strain in which the integration had occurred via two simultaneous crossover events resulting in the replacement of the *ptsH* promoter and the region encoding residues His-15 and Ser-46 by the Erm^r cassette. This strain, designated NZ9880, had the Erm^r cassette integrated in the same orientation as the *ptsI* gene. Disruption of the *ptsI* gene was achieved by the integration of plasmid pNZ9291 into the chromosome of strain NZ9800, resulting in a strain, named NZ9881, that contains two disrupted copies of the *ptsI* gene.

Growth characteristics of the *ptsH*-disrupted strain. Disruption of the *ptsH* or *ptsI* gene resulted in a severe reduction of the growth rate on all sugars tested (Table 2). No growth at the expense of sucrose and fructose was observed, while growth rate at the expense of glucose was reduced to approximately 1/10 of that observed in the wild-type strain. Remarkably, the growth at the expense of galactose and maltose was also affected by these mutations; the growth rate on both carbon sources was reduced to half of that observed for the wild-type strain (Table 2). Results for disruption of the *ptsI* gene were identical to those for the *ptsH* disruption, confirming the important role of the PTS in the utilization of these sugars.

TABLE 2. Maximal growth rates of strains used in this study

Strains	Nisin A induction (μg/ml)	Mean maximal growth rate (h⁻¹) ± SE[a]				
		Glucose	Sucrose	Fructose	Galactose	Maltose
NZ9800 (wt[b])		1.40 ± 0.06	1.16 ± 0.04	0.91 ± 0.02	0.59 ± 0.1	0.50 ± 0.04
NZ9880 (ΔptsH)		0.13 ± 0.03	NG	NG	0.30 ± 0.04	0.24 ± 0.06
NZ9880 + pNZ9282 (*L. lactis ptsH*)	10^{-3}	ND	0.09 ± 0.03	ND	0.35 ± 0.02	ND
NZ9880 + pNZ9283 (*B. subtilis ptsH*)	10^{-3}	ND	0.10 ± 0.06	ND	0.39 ± 0.09	ND
NZ9880 + pNZ9286 (wt *ptsHI*)	10^{-5}	1.39 ± 0.09	1.09 ± 0.06	0.90 ± 0.09	0.61 ± 0.04	0.48 ± 0.03
NZ9880 + pNZ9288 (S46A *ptsHI*)	10^{-5}	1.17 ± 0.08	0.99 ± 0.01	0.80 ± 0.05	0.48 ± 0.03	0.39 ± 0.03
NZ9880 + pNZ9289 (S46D *ptsHI*)	10^{-5}	0.12 ± 0.04	NG	NG	0.21 ± 0.04	0.20 ± 0.04
NZ9881 (ΔptsI)		0.16 ± 0.05	NG	NG	0.25 ± 0.08	0.26 ± 0.06
NZ9881 + pNZ9286 (wt *ptsHI*)	10^{-5}	1.35 ± 0.09	1.18 ± 0.09	0.84 ± 0.06	0.63 ± 0.08	0.50 ± 0.07
NZ9881 + pNZ9287 (wt *ptsI*)	10^{-3}	1.20 ± 0.03	1.02 ± 0.03	0.89 ± 0.04	0.55 ± 0.06	0.43 ± 0.07
NZ9800 + pNZ9284 (S46A *ptsH*)	10^{-4}	1.40 ± 0.11	1.18 ± 0.10	ND	0.61 ± 0.09	ND
NZ9800 + pNZ9285 (S46D *ptsH*)	10^{-4}	1.35 ± 0.02	1.02 ± 0.07	ND	0.09 ± 0.02	ND

[a] NG, no growth; ND, not determined.
[b] wt, wild type.

TABLE 3. L-Lactate dehydrogenase and pyruvate kinase activities of strains used in this study

Strain	Nisin A induction (μg/ml)	Carbon source	Activity (mean ± SE)	
			Pyruvate kinase (μmol of PEP formed/mg/min)	L-Lactate dehydrogenase (μmol of NADH formed/mg/min)
NZ9800 (wild type)		Glucose	3.20 ± 0.57	14.2 ± 0.20
		Galactose	1.67 ± 0.48	9.05 ± 0.31
NZ9800/pNZ9284 (S46A ptsH)	10^{-5}	Glucose	3.19 ± 0.43	13.84 ± 0.39
	10^{-5}	Galactose	1.84 ± 0.06	9.12 ± 0.65
NZ9800/pNZ9285 (S46D ptsH)	10^{-5}	Glucose	2.95 ± 0.09	12.38 ± 0.23
	10^{-5}	Galactose	2.85 ± 0.16	12.43 ± 0.90
NZ9870 (ΔccpA)		Glucose	0.79 ± 0.78	6.32 ± 0.55
NZ9870/pNZ9285 (S46D ptsH)	10^{-3}	Glucose	0.75 ± 0.26	6.42 ± 0.54
NZ9880 (ΔptsH)		Glucose	2.14 ± 0.53	9.87 ± 0.65
		Galactose	1.45 ± 0.56	8.31 ± 0.32

Complementation of the *ptsH* and *ptsI* mutations. Since the pIL253-derived Erm[r] cassette used to construct the disruption mutants does not contain a terminator structure (27), readthrough of the Erm[r] promoter leads to expression of the *ptsI* gene in strain NZ9880 (Δ*ptsH*), albeit not to the wild-type level (data not shown). Strain NZ9880 was transformed with plasmid pNZ9282 or pNZ9283, containing the *L. lactis* or *B. subtilis ptsH* gene, respectively, under the control of the regulated *nisA* promoter. The *nisA* promoter allows inducible expression in response to the amount of nisin A added to the growth medium (7). Induction of the expression of *ptsH* of either *L. lactis* or *B. subtilis* by the addition of inducing concentrations nisin A resulted in partial complementation of the observed growth defect of strain NZ9880 grown at the expense of sucrose and galactose (Table 2). Comparable results were obtained with glucose, fructose, and maltose. Without nisin A induction, strain NZ9880 harboring either pNZ9282 or pNZ9283 did not grow at the expense of sucrose. The addition of a relatively high concentration of nisin A (10^{-3} μg/ml) restored the ability to grow on sucrose, although the maximal specific growth rate was approximately 1/10 of that observed for the wild-type strain, probably because of insufficiently high expression of *ptsI*. Transformation with plasmid pNZ9286, which contains the entire *L. lactis ptsHI* operon under control of the *nisA* promoter, almost completely restored the observed growth defect of strain NZ9880 after induction by the addition of a low concentration of nisin A (10^{-5} μg/ml). Similarly, the *ptsI* mutation in strain NZ9881 could be complemented with plasmid pNZ9286 (containing the entire *ptsHI* operon) or plasmid pNZ9287 (containing the *ptsI* gene transcriptionally fused to the *nisA* promoter), although the latter strain required higher concentrations of nisin A to reach optimal induction of *ptsI* (Table 2).

Analysis of the effects of overproduction of altered HPr proteins on galactose utilization. Two mutated *ptsH* genes that encode proteins affected in the phosphorylation on residue serine 46 were constructed. One mutated *ptsH* gene encodes S46D HPr, where residue serine 46 was changed to an aspartic acid that mimics a phosphate group on the seryl residue; another encodes S46A HPr, carrying an alanine on residue 46 that can no longer be phosphorylated. Both mutated genes were tested for the ability to complement the *ptsH* mutation in strain NZ9880 in comparison to the wild-type *ptsH* gene (Table 2). This was done by expressing either the wild-type or the mutated gene together with the wild-type *ptsI* gene, using plasmid pNZ9288 and pNZ9289, respectively. Plasmid pNZ9288 (coding for S46A HPr) but not plasmid pNZ9289 (coding for S46D HPr) was, after induction with low concentrations of

nisin A, able to restore the growth defect in the *ptsH* mutant strain. The observed maximal growth rate after complementation with pNZ9288 was approximately 80% of that observed after complementation with pNZ9286 carrying the wild-type *ptsHI* genes.

Recently, we showed that CcpA is involved in the catabolite repression of the galactose operon in *L. lactis*, most likely through an interaction with a *cre* site located in the promoter region of the *gal* operon (29). To investigate the role of HPr(Ser-P) in this regulatory process, the mutant *ptsH* genes were also overexpressed without the coexpression of *ptsI* in the wild-type strain NZ9800. Moderate overexpression of mutant *ptsH* genes encoding S46A HPr and S46D HPr, by the addition of 10^{-5} μg of nisin A/ml, had no effect on the growth rate of the cells (data not shown). However, high overexpression of the mutant gene encoding S46D HPr, by the addition of 10^{-3} μg of nisin A/ml, resulted in a severe reduction of the growth on galactose, whereas the high overexpression of the gene encoding S46A HPr did not affect growth on galactose. The high overexpression of genes encoding S46A HPr or S46D HPr did not influence the rates of growth on glucose and sucrose (Table 2).

Analysis of the role of HPr(Ser-P) in regulation of the *las* operon. Previously, we showed that the activities of pyruvate kinase and L-lactate dehydrogenase vary in response to the carbon source provided (29). Cells grown on glucose show higher activities of both enzymes than cells grown on galactose (Table 3). Furthermore, we found that the disruption of the *L. lactis ccpA* gene resulted in a reduction of the expression of the *las* operon, which encodes phosphofructokinase, pyruvate kinase, and L-lactate dehydrogenase, when the cells were grown on glucose. These results indicated that CcpA acts as an activator of the expression of the *las* operon most likely by an interaction with a *cre* site located in the promoter region of the *las* operon (29). Expression of the pNZ9285-borne gene encoding S46D HPr in galactose-grown cells resulted in pyruvate kinase and L-lactate dehydrogenase activities higher than those in wild-type cells (Table 3). In contrast, the overproduction of S46A HPr did not affect both activities. When either of the mutant *ptsH* genes was expressed in cells grown on glucose, the activities of pyruvate kinase and L-lactate dehydrogenase were not altered. Higher levels of expression of the mutant genes achieved by the addition of higher amounts of nisin A to the growth medium resulted in lower pyruvate kinase and L-lactate dehydrogenase activities (data not shown). The disruption of the *ptsH* gene in strain NZ9880 resulted in a reduction of both pyruvate kinase and L-lactate dehydrogenase activities to 70% of the values measured in wild-type cells. Higher pyruvate

kinase and L-lactate dehydrogenase activities were measured in strain NZ9880 (Δ*ptsH*) grown on glucose than in cells grown on galactose, suggesting that regulation of the *las* operon expression is not dependent on the presence of the *ptsH* gene. To assess the role of HPr(Ser-P) in the CcpA-mediated activation of the *las* operon, we expressed the S46D HPr gene in strain NZ9870, which carries a disrupted copy of the *ccpA* gene. Since strain NZ9870 does not grow on galactose, the effects of the overproduction of S46D HPr could be analyzed only in cells grown on glucose. Overexpression of the S46D HPr gene in strain NZ9870 (Δ*ccpA*) did not result in an increase of the activities to the levels measured in the wild-type strain NZ9800 grown on glucose (Table 3). The results of these experiments indicate the requirement of an intact CcpA for the observed HPr(Ser-P)-mediated activation of pyruvate kinase and L-lactate dehydrogenase.

DISCUSSION

Using inactivation, mutation, and overexpression studies, we have cloned and analyzed the *L. lactis* *ptsH* and *ptsI* genes in order to assess their roles in the phosphorylation cascade of the PTS and the global regulation of carbohydrate metabolism. The genes are located in an operon structure with the gene order *ptsHI*. Two equally strong, adjacent transcriptional start sites were mapped and found to be located downstream of a typical lactococcal promoter sequence (11, 40). The results of the transcriptional analysis revealed that the *ptsH* gene was located on two transcripts: a small transcript of approximately 0.3 kb and a larger transcript of 2 kb. Probing with a *ptsI*-specific probe indicated that this gene is located only on the 2-kb transcript. These results indicate that the *ptsHI* genes are transcribed as a single mRNA species of 2 kb that terminates at the rho-independent terminator structure (T_2) located downstream of *ptsI* and that the stem-loop structure (T_1) located downstream of *ptsH* functions as a limited transcriptional terminator. This transcriptional organization most likely results in a higher level of expression of the *ptsH* gene, in accordance with the observation that higher amounts of HPr protein than of enzyme I are found in several bacteria, including *Staphylococcus carnosus* (24).

Expression of the *L. lactis* *ptsHI* operon is regulated at the transcriptional level, since mRNA levels, as determined by scintillation counting, were 10-fold higher in glucose-grown cells than in galactose-grown cells. Induction of *ptsHI* gene expression by glucose has also been reported for *E. coli* and *B. subtilis* (6, 34, 38). The induction by glucose of the *B. subtilis* *ptsGHI* operon, which also contains the gene encoding the glucose-specific EII (*ptsG*), is mediated via the antiterminator protein GlcT (38). No *cis*-acting sequences like *cre* sites (42), involved in catabolite repression, or ribonucleic antiterminator sequences (2), which play a role in antitermination mediated by proteins of the BglG/SacY family, were detected at relevant positions in the DNA sequence of the *L. lactis* *ptsHI* operon.

The disruption of both the *ptsH* and *ptsI* genes resulted in strains that were severely affected in growth at the expense of sucrose and fructose, indicating that these sugars are taken up exclusively via the PTS. Growth at the expense of glucose was affected but not completely abolished, in accordance with a previous report which indicated that *L. lactis* can take up glucose via two transport systems, a permease and a PTS (39). Surprisingly, growth at the expense of galactose and maltose was also affected by the *ptsH* and *ptsI* mutations since the growth rates were reduced to half of those observed with the wild-type strain. Both sugars are presumed to be taken up via non-PTS permeases in *L. lactis* (19, 27). Our results suggest

that a functional PTS is required for the efficient utilization of galactose and maltose in *L. lactis*. An activating role for the PTS component HPr(His-P) has been reported before in the case of the *B. subtilis* levanase regulator LevR (37) and the enterococcal GlpK proteins (4). HPr(His-P) is absent in both NZ9880 (Δ*ptsH*) and NZ9881 (Δ*ptsI*) since phosphorylation at His-15 requires the presence of both HPr and enzyme I. A role for either HPr(Ser-P) or enzyme I in the observed reduction of the growth rate on non-PTS sugars in the PTS-negative strains can be ruled out since these proteins are still present in strains NZ9881 (Δ*ptsI*) and NZ9880 (Δ*ptsH*), respectively. This finding suggests that HPr(His-P), the concentration of which increases in the absence of a PTS substrate, stimulates the utilization of galactose and maltose in *L. lactis*.

Further evidence for the functionality of the *ptsH* gene was obtained by the complementation of the *ptsH* mutation in strain NZ9880 by the expression of either the *L. lactis* or the *B. subtilis* *ptsH* gene under the control of the inducible *nisA* promoter. Induction of the *nisA* promoter with increasing concentrations of nisin A resulted in only a partial restoration of the growth defect observed in the *ptsH* mutant strain when grown on the PTS sugar sucrose, while growth at the expense of galactose was not affected. The readthrough from the promoter of the Erm[r] gene was most likely not sufficient to allow full functional expression of the *ptsI* gene, since coexpression of the *ptsH* and *ptsI* genes did result in complete complementation of the growth defect. This result also demonstrates the dependence of *ptsI* expression on the promoter located upstream of the *ptsH* gene.

The *ptsI* mutation in strain NZ9881 was complemented by the expression of either the entire *ptsHI* operon or the *ptsI* gene alone, transcriptionally fused to the *nisA* promoter on plasmid pNZ9287. These results primarily show the functionality of the *ptsI* gene but also establish the functionality of the ribosome binding site located upstream of the *ptsI* gene, despite its unusual spacing (14 bp) relative to the putative ATG start codon of the *ptsI* open reading frame.

To analyze the regulatory role of HPr in the metabolism of *L. lactis*, we constructed in the *L. lactis* *ptsH* gene site-specific mutations encoding HPr proteins that were affected in the phosphorylation of residue Ser-46. Replacement of residue Ser-46 with alanine (S46A HPr) has been shown to inactivate HPr as a regulatory molecule (9), while the introduction of aspartate at residue 46 (S46D HPr) results in a permanently negatively charged residue 46 that resembles a phosphorylated serine. Production of S46D HPr in the wild-type strain NZ9800 resulted in a strong reduction of the growth rate on galactose, whereas no effects were observed when S46A HPr was overproduced. This can be explained by an enhanced repression of *gal* gene expression by CcpA, in the presence of S46D HPr, but not of S46A HPr. S46DHPr has been shown to stimulate the binding of CcpA to *cre* sites, thus mediating catabolite repression (12, 17). The role of CcpA in this mechanism could not be further substantiated since a *ccpA*-negative strain is impaired in the utilization of galactose (29). Moreover, the reduced growth on galactose after overexpression of the S46D HPr gene could be explained by the S46D HPr-mediated activation of the inducer exclusion and expulsion mechanisms. These mechanisms, which result in a reduction of inducer concentration, have been described for nongrowing *L. lactis*, and their activities are stimulated by the introduction of *B. subtilis* S46D HPr protein in *L. lactis* vesicles via electroporation (45, 46). The production of *L. lactis* HPr mutants in growing cells is a nonartificial system to study the inducer exclusion and expulsion mechanisms. Production of S46D HPr could result in a reduced uptake of galactose via an interaction with the galac-

tose permease. In addition, S46D HPr-mediated activation of the recently purified sugar-phosphate phosphatase (47) could lead to an increased dephosphorylation of galactose intermediates, resulting in a reduced growth rate. In conclusion, we have shown that varying the expression level of HPr mutants influences the growth rate, *gal* gene expression, and pyruvate kinase and lactate dehydrogenase activities. Moreover, we show that *L. lactis* regulates the expression level of *ptsH* as a function of the carbon source used (Fig. 3B), which stresses the physiological importance of the regulation.

Since it has been reported that CcpA can form a complex with HPr phosphorylated at residue serine 46 and that this complex enhances the binding of CcpA to *cre* sites (12, 17, 20), we were interested in determining if this complex also plays a role in the regulation of the *las* operon, known to be transcriptionally activated by CcpA (29). Production of S46D HPr but not S46A HPr in wild-type cells grown on galactose resulted in pyruvate kinase and L-lactate dehydrogenase activities which were elevated to the same level as found in the wild-type grown on glucose, suggesting that HPr(Ser-P) acts as a signal molecule that modulates the expression level of the pyruvate kinase and L-lactate dehydrogenase genes in response to the carbon source provided. To establish the role of CcpA in this mechanism, the gene encoding S46D HPr was also expressed in strain NZ9870 (Δ*ccpA*). Due to the inactivation of the *ccpA* gene, the activities of both pyruvate kinase and L-lactate dehydrogenase were no longer activated by CcpA and therefore lower than those measured in the wild-type strain (29). The fact that these no longer responded to the production of S46D HPr indicated that CcpA is required for the observed S46D HPr-mediated activation of the activities in the wild-type strain. These results indicate that HPr(Ser-P) acts as a signal molecule involved in the CcpA-mediated transcriptional activation of the *las* operon, providing an activation mechanism that has not been described before for a gram-positive microorganism. Remarkably, disruption of the *ptsH* gene did not result in a strong reduction of the activities of pyruvate kinase and L-lactate dehydrogenase, nor did it affect carbon source-dependent regulation. This finding suggests that additional signal molecules may be involved in the CcpA-dependent regulation of the *las* operon. Possible candidates are glucose-6-P and NADP, which have been shown to enhance the binding of CcpA to *cre* sites (17, 23), or a lactococcal analogue of Crh, an HPr-like protein that was recently identified in *B. subtilis* and that functions as a signal molecule in the catabolite repression of several genes (15).

The results presented here show the predominant role of the PTS in the uptake of various sugars in *L. lactis*. Furthermore, they show the regulatory functions of HPr in establishing a hierarchy in the preference for different sugars in *L. lactis*. Finally, we provide experimental evidence for the involvement of HPr(Ser-P) in the CcpA-mediated transcriptional activation of genes encoding glycolytic enzymes in *L. lactis*.

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