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Cloning and Characterization of the *Lactococcus lactis* subsp. *lactis* ATCC 7962 *ptsHI* Operon

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Abstract The *ptsH* and *ptsI* genes of *Lactococcus lactis* subsp. *lactis* ATCC 7962 (*L. lactis* 7962), encoding the general proteins of phosphotransferase system (PTS) components, HPr and enzyme I, respectively, were cloned and characterized. A 1.3 kb PCR product was obtained using a primer set that was hybridized to the internal region of the *L. lactis* 7962 *ptsHI* genes and then subcloned into a low-copy number vector, pACYC184. The 5' upstream and 3' downstream regions from the 1.3 kb fragment were subsequently cloned using the chromosome walking method. The complete *ptsHI* operon was constructed and the nucleotide sequences determined. Two ORFs corresponding to HPr (88 amino acids) and enzyme I (575 amino acids) were located. The *ptsHI* genes of *L. lactis* 7962 showed a very high homology (84–90%) with those genes from other Gram-positive bacteria. A primer extension analysis showed that the transcription started at either one of two adjacent bases upstream of the start codon. Using a Northern analysis, two transcripts were detected; the first, a 0.3 kb transcript corresponding to *ptsH* and the second, a 2 kb transcript corresponding to *ptsH* and *ptsI*. The transcription level of *ptsH* was higher than that of *ptsI*. The concentration of the *ptsH* transcript in cells grown on glucose was similar to that in cells grown on lactose, yet higher than that in cells grown on galactose. The *ptsI* transcript was scarcely detected in cells grown on lactose or galactose. The results of a sequence analysis and Northern blot confirmed that the *ptsH* and *ptsI* genes of *L. lactis* 7962 were arranged in an operon like other known *ptsHI* genes and the expression of the *ptsHI* genes was regulated at the transcriptional level in response to the carbon source.

Key words: PTS system, *ptsHI* operon, HPr, Enzyme I, carbon utilization

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In bacteria, carbohydrates are transported into cells via different mechanisms. One of these transport systems, the phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS), is responsible for the concomitant transport and phosphorylation of a variety of sugars at the expense of the high-energy phosphoryl bond of PEP [1, 17, 21]. In the PEP-PTS system, three components, enzyme I, HPr, and enzyme II, are involved and the transfer of the phosphate moiety of PEP to sugars is catalyzed by the general non-sugar-specific proteins, enzyme I and HPr, in addition to the sugar-specific enzyme II protein. In addition to sugar transport, the PEP:PTS system is known to be involved in the global regulation of carbohydrate metabolism, and HPr, a phosphocarrier protein of the PTS, plays the central role [9, 11]. Although the precise mechanisms through which HPr exerts these regulatory effects are still unknown, two highly conserved residues in HPr, His-15 and Ser-46, have been shown to be necessary for it to function and have also been shown to serve as sites for phosphorylation [20]. The genes encoding HPr and Enzyme I, *ptsH* and *ptsI*, respectively, have been cloned from several Gram-positive bacteria and are organized in an operon with the gene order of *ptsHI* [3, 5, 15, 18]. The *ptsH* and *ptsI* mutants of *Lactobacillus sake* [18] and *Lactococcus lactis* [15], however, are unable to take up PTS sugars, such as sucrose, fructose, and mannose. It has also been reported that sugar transports via PTS and non-PTS systems in bacteria are affected by the phosphorylation status of HPr [15]. HPr has two different phosphorylation sites: His-15 phosphorylated by enzyme I and Ser-46 phosphorylated by an ATP-dependent, metabolite-activated protein kinase [6]. In an experiment using the membrane vesicle from *L. lactis*, Ye *et al.* [19] showed that the phosphorylation of HPr at Ser-46 reduces the uptake of TMG (thiomethyl-beta-galactoside), a lactose

analogue, via the PTS and enhances the efflux of TMG-phosphate, a phenomena known as inducer exclusion and inducer expulsion, respectively. HPr phosphorylated at Ser-46, HPr (Ser-P), also plays a regulatory role for genes under catabolite repression. HPr (Ser-P) enhances the binding of CcpA (catabolite control protein) to a *cis*-acting *cre* (catabolite-responsive element) site, located in the promoter region of genes controlled by CcpA, through interaction with CcpA [4, 10, 12]. Luesink *et al.* [16] showed that CcpA acts as a negative regulator for the transcription of the *gal* operon in *L. lactis* subsp. *cremoris* NZ9800 (*L. lactis* NZ9800). In most *L. lactis* strains, including NZ9800, the mechanism responsible for the uptake of lactose is PTS with the exception of *L. lactis* 7962 where a non-PTS, the *lac* permease system, is the major mechanism [13-14]. We have been investigating the organization and transcription of the *gal/lac* operon genes in *L. lactis* 7962 and are interested in the *ptsHI* genes since HPr very likely affects the expression of the *gal/lac* operon genes. This study reports on the cloning and characterization of the *ptsHI* operon genes of *L. lactis* 7962.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

L. lactis 7962 was cultured statically in M17 broth (Difco Lab, Detroit, Michigan, U.S.A.) with 1% (w/v) glucose, galactose or lactose at 30°C. *E. coli* DH5α cells and recombinant *E. coli* cells were cultured in LB containing appropriate antibiotics (ampicillin, 50 μg/ml; tetracycline, 10 μg/ml) at 37°C.

Cloning of *ptsHI* Genes

The PCR reaction was performed with *L. lactis* 7962 chromosomal DNA as the template using a GeneAmp PCR system 2400 (Perkin Elmer, Foster city, CA, U.S.A.). A primer set of HPrI (5'-GCATGCCAGAAACAGGAA-TTCATGCAC-3'; hybridizing to 519-545 nt in Fig. 2) and EnzI (5'-GCATGCGGGTTCATTTCTTTAGGAAG-3'; hybridizing to 1,810-1,835 nt in Fig. 2) was used to amplify the internal region of the *ptsHI* operon and the primer sequences were identical to those used for cloning the *ptsHI* genes of *Listeria monocytogenes* Scott A [5]. The *Sph*I site introduced is underlined. Ex-*Taq* polymerase (Takara Shuzo Co., Shiga, Japan) was used and the denaturation, annealing, and extension conditions were 94°C for 1.5 min, 54°C for 1.5 min, and 72°C for 1.5 min, respectively. A total of 30 cycles followed by a final 10 min extension at 72°C was employed. The upstream and downstream regions of the 1.3 kb PCR fragment were further cloned using the chromosome walking method [2]. The DNA sequencing was carried out by the dideoxynucleotide chain termination method using an ABI prism bigdye terminator cycle sequencing ready reaction kit (Perkin-Elmer Biosystems, Foster city, U.S.A.). Both strands were sequenced and the sequence homology was examined using the BLAST program at the NCBI (National Center for Biotechnology Information, Bethesda, MD, U.S.A.) GenBank database.

RNA Preparation

The total RNA was isolated from *L. lactis* 7962 cells grown on M17 broth containing either glucose or galactose or lactose according to the published procedure [2]. The cells were harvested during the exponential phase of growth (OD_{600}, 0.6-0.8) and resuspended in a lysis buffer (30 mM Tris-HCl pH 7.4, 10 mM NaCl, 5 mM EDTA, 1% SDS, proteinase K 100 μg/ml). The cells were disrupted by sonication; three cycles of 10 s of sonication using a microtip sonicator (Bandelin electronic, Sonopuls HD60) on ice water followed by 10 s standing on ice water. The cell lysate was repeatedly extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v) until the white protein clot layer could no longer be seen. To the aqueous phase, NaCl was added to a final concentration of 0.25 M plus 2 vol of cold ethanol. The precipitated pellet was dissolved in a DNase digestion buffer (20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$), treated with 10 unit RNase free DNase I (Promega, Madison, U.S.A.) and incubated for 60 min at 37°C. After one more extraction with phenol, the RNAs were precipitated and dissolved in DEPC (diethyl pyrocarbonate) treated ddH_2O and stored at -70°C.

Primer Extension

The oligonucleotide primer, HPrUP3 (5'-TGGACGTGC-ATGGATACC-3'; 533-550 nt in Fig. 2), was 5'end labeled with [γ-^{32}P]dATP (Amersham Pharmacia Biotech) using a T4 DNA polynucleotide kinase (Promega). The oligonucleotide was hybridized with 50 μg of total cellular RNA extracted from *L. lactis* 7962 cells grown on glucose, and the annealed primer was extended with an AMV reverse transcriptase (Promega), as described by the manufacturer. The extended product was analyzed on a sequencing gel adjacent to a DNA sequencing ladder generated from the HPrUP3 primer with pUhts as the template. pUhts is a pUC19 harboring 200 bp PCR product corresponding to the 5' region of *ptsH* including a tentative transcription start site and promoter sequences. The fragment was obtained by a PCR using a primer set of HD2 (5'-CAAAGTCTAGCATGCATTC-3'; 338-356 nt in Fig. 2) and HPrUP3.

Northern Analysis

Twenty micrograms of total RNA was resolved on a 2.2 M formaldehyde agarose gel in a 1× MOPS running buffer (0.4 M MOPS, pH 7.0, 0.1 M sodium acetate, 0.01 M EDTA), blotted onto a nylon membrane, UV-crosslinked (150 mJ, BioRad GS Gene Linker), and hybridized at 42°C in a formamide hybridization solution (5 × SSC, 5× Denhardt

solution, 50% formamide, 1% SDS, 100 μg/ml denatured, fragmented salmon sperm DNA) with a probe radioactively labeled using [α-^{32}P]dATP and a random primer labeling kit (Promega, Madison, WI, U.S.A.). The probes for the Northern blot were obtained by a PCR. HPrI and 2EnzI (5'-GAAACAGCCCAAAGTTG-3'; 1,417–1,433 nt in Fig. 2) were employed for amplifying a ca. 900 bp fragment as a *ptsH* probe. 3EnzI (5'-GACAATTGCTGATAC-3'; 861–875 nt in Fig. 2) and EnzI were used for the amplification of ca. 970 bp as a *ptsI*-specific probe.

Nucleotide Sequence Accession Number

The DNA sequence reported here was deposited in the GenBank database under accession number AF291428.

Results and Discussion

Cloning of the *ptsHI* Operon Genes

When the PCR was performed with *L. lactis* 7962 chromosomal DNA as the template and the primer set of HPrI and EnzI, as described in Materials and Methods, a 1.3 kb fragment was amplified. The DNA sequencing of the fragment confirmed that the fragment contained part of the *ptsH* and *ptsI* genes. The sequences of the primers were initially designed by Christensen *et al.* [5] based on the nucleotide sequences of the *ptsH* and *ptsI* coding regions of *B. subtilis*, *Streptococcus salivarius*, *Streptococcus mutans*, and *Enterococcus faecalis*. The 1.3 kb fragment was cloned into a low-copy number vector, pACYC184, after being digested with *Sph*I, and the resulting recombinant plasmid was named pTYH-1 (Fig. 1). DNA sequencing indicated that the 1.3 kb fragement lacked the ca. 150 bp 5' region including the start codon and promoter sequence of *ptsH* and the ca. 700 bp 3' region corresponding to the C-terminal end of enzyme I. The chromosome-walking method was

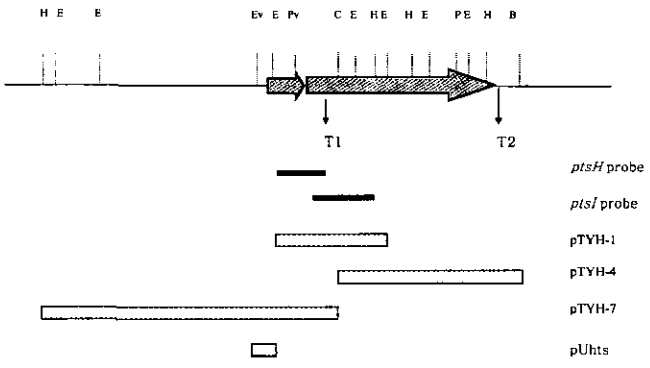

Fig. 1. Restriction map of the *ptsHI* operon of *L. lactis* 7962.
The positions of insert in pTYH-1, pTYH-4, and pTYH-7 are shown under the restriction map. The insert in pUhts, used as a sequencing size ladder for primer extension, is shown. The locations of possible transcription terminators, T1 and T2, are also indicated. Restriction sites: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II.

then used to clone these missing fragments. Southern blot analyses were performed with *L. lactis* 7962 chromosomal DNA digested by various single or two different restriction enzymes and the 5' and 3' parts of the 1.3 kb fragments as probes. A single *Cla*I site was located in the 5' region of *ptsI* and near the middle of the 1.3 kb fragment. 700 bp *Sph*I-*Cla*I and 600 bp *Cla*I-*Sph*I fragments were used as probes for cloning the missing 5' and 3' regions, respectively. When the 700 bp *Sph*I-*Cla*I fragment (Fig. 1) was used as the probe, a 4.1 kb *Cla*I-*Hin*dIII fragment hybridized with the probe (results not shown). A *Pst*I fragment and *Sal*I fragment, larger than 20 kb in size, also hybridized with the probe. When the 600 bp *Cla*I-*Sph*I fragment was used as the probe, a 2.5 kb *Bam*HI-*Cla*I fragment was detected. Based on these results, genomic libraries of *L. lactis* 7962 were constructed using *L. lactis* 7962 chromosomal DNA digested with *Cla*I-*Hin*dIII and *Cla*I-*Bam*HI, independently, and pBlueScript KSII (+) (Stratagene, La Jolla, U.S.A.). The libraries were then screened with radioactively-labeled probes: 700 bp *Sph*I-*Cla*I fragment for cloning the 5' upstream region and 600 bp *Cla*I-*Sph*I fragment for cloning the 3' downstream region. Using colony hybridization, recombinant clones harboring the plasmid with the expected size were obtained and confirmed by DNA sequencing. A 4.1 kb *Cla*I-*Hin*dIII fragment corresponding to the 5' upstream region of *ptsH* was cloned and the recombinant plasmid was named pTYH-7 (Fig. 1). Furthermore, a 2.5 kb *Cla*I-*Bam*HI fragment corresponding to the 3' downstream of *ptsI* was cloned and this plasmid was named pTYH-4 (Fig. 1). The complete *ptsHI* operon including the upstream regulatory region was constructed by combining pTYH-1 with pTYH-7 and pTYH-4. The complete nucleotide sequence of the *ptsHI* operon was determined and compared with those of other *ptsHI* genes in the DNA data libraries. The *ptsHI* genes from *L. lactis* 7962 were found to be very similar to those of other known *ptsHI* genes in the nucleotide sequence level as well as in the amino acid sequence level. The identity scores of the nucleotide sequences were: *L. lactis* NZ9800 94%, *L. sake* 84%, *S. mutans* 80%, *S. salivarius* 83%, *Listeria monocytogenes* ScottA 89%, *Bacillus subtilis* 87%, and *E. faecalis* 89%. This result confirms that *ptsHI* genes are well conserved among bacteria, and the functions of HPr and enzyme I are identical. The successful PCR cloning of the *ptsHI* genes from *L. lactis* 7962 also reflects this fact. From the analysis of the DNA sequence of the *ptsHI* operon, two open reading frames were found, each corresponding to *ptsH* and *ptsI* and encoding a protein of 88 amino acids and 575 amino acids, respectively. The size of HPr of *L. lactis* 7962 was the same or very similar to other HPrs previously reported (Fig. 3). The HPr from *L. lactis* NZ9800 [15] consists of 88 amino acids, as do those from *L. sake* [18], *Listeria monocytogenes* [5], and *Staphylococcus carnosus* [8]. The HPr from *S. mutans* [3] has 87 amino acids. The

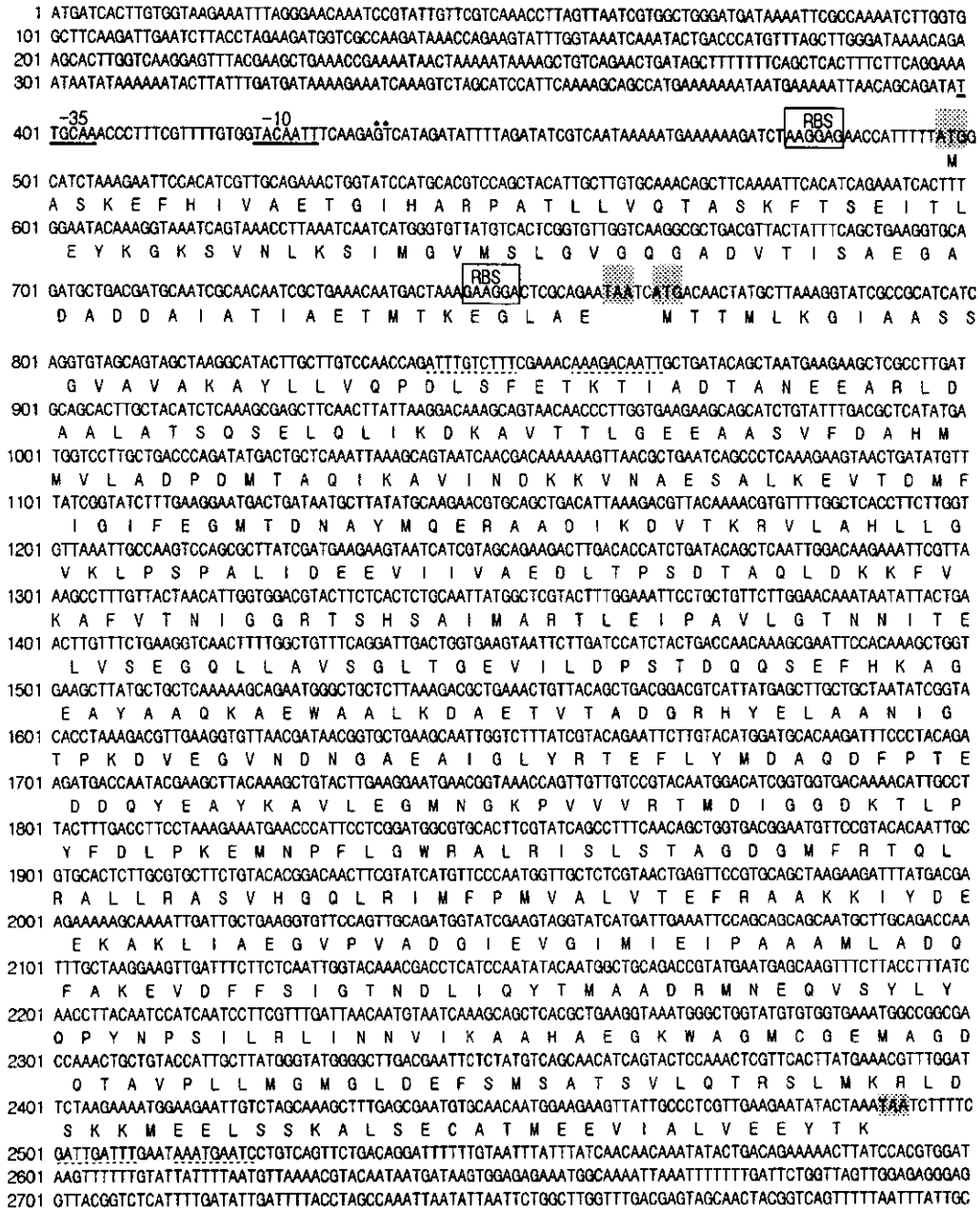

Fig. 2. Nucleotide sequence of the *ptsHI* operon of *L. lactis* 7962.
The putative -35 and -10 promoter sequences are underlined and directly indicated above the sequences. The ribosome binding sites for *ptsH* and *ptsI* are boxed. The bold and shaded letters correspond to the translation start and stop codons of *ptsH* and *ptsI*. The transcription start sites determined by primer extension are indicated by the •• shape above the letters (GT). The possible transcription terminators, T1 and T2, are underlined by a dotted line (----).

size of enzyme I would thus appear to be more variable than HPr among different bacteria: The enzyme I of *L. lactis* 7962 consists of 575 amino acids as does that from *L. lactis* NZ9800. However, the enzyme I of *S. mutans* has 2 additional amino acids and that of *L. sake* lacks one amino acid. As shown in Fig. 3, the HPr from *L. lactis* 7962 exhibited a high level of identity with other HPrs from Gram-positive bacteria. The residues His-15 and Ser-46, as well as the flanking amino acids, were also conserved. This suggests that the HPr of *L. lactis* 7962 can exert regulatory functions after being phosphorylated at the Ser-46 residue, as in other Gram-positive bacteria. The two conserved phosphorylation sites are highlighted as shaded boxes in the consensus residues in Fig. 3. In particular, the amino acid sequence of the HPr of *L. lactis* 7962 was identical to that of *L. lactis* NZ9800. The nucleotide sequences of the two *ptsH* genes from *L. lactis* were exactly the same except in one point. The codon of the

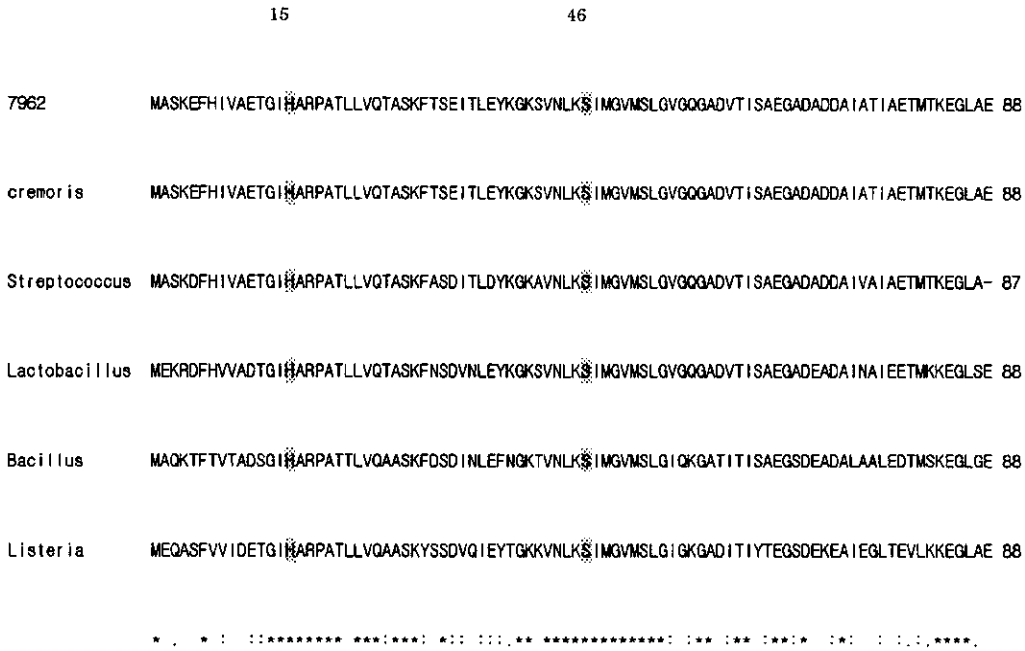

Fig. 3. Multiple alignment of the amino acid sequences of HPrs using ClustalW.
The *ptsH* genes from various Gram-positive bacteria and their GenBank accession numbers are as follows: 7962, *Lactococcus lactis* subsp. *lactis* ATCC 7962 (AF291428); cremoris, *Lactococcus lactis* subsp. *cremoris* NZ9800 (Z97203); Streptococcus, *Streptococcus salivarius* (M81756); Lactobacillus, *Lactobacillus sake* (U82366); Bacillus, *Bacillus subtilis* (M98359); Listeria, *Listeria monocytogenes* (AF030824). *, :, and . indicate the presence of identical amino acids, conservative amino acids, and one different type of amino acid, respectively. The phosphorylation sites, His-15 and Ser-46 residues (shaded-bold letters) are conserved in all HPrs.

72nd amino acid (Asp) was GAU in 7962 whereas the corresponding codon in NZ9800 is GAC. The amino acid sequence of the enzyme I in *L. lactis* 7962 was different from that in *L. lactis* NZ9800 at 4 residues. The 236, 246, 415, and 426th amino acid residues of the 7962 enzyme were Asp, Glu, Ala, and Asp, respectively, whereas the corresponding residues in the NZ9800 enzyme were Glu, Asp, Ser, and Glu, respectively. Since these changes are only conservative substitutions, it would seem that the functions of enzyme I are the same in both strains. The putative ribosome binding site, 5'-AAGGAG-3', was located 10 nucleotides upstream of the ATG start codon of *ptsH*. The ribosome binding site of *ptsI*, 5'-GAAGGA-3', was located 14 nucleotides upstream of the start codon. Accordingly, the spacing between the RBS and the start codon of *ptsI* was longer than the commonly observed spacing (10 bp). No *cre*-like sequences were found in front of the *ptsH* gene.

Transcription Start Site of the *ptsHI* Operon

A primer extension analysis was carried out to determine the transcription start site (TSS) of *ptsHI*, using the total RNA isolated from *L. lactis* 7962 cells grown on glucose as the template. Two equally labeled extended products (Fig. 4) were obtained, indicating that the transcription started at either one of two adjacent nucleotides, GT, at 436-437 nt in Fig. 2, and lying 61 and 60 bases upstream of *ptsH*, respectively. The sequence of the complementary strand obtained using the same primer, HPrUP3, is shown on the right side of Fig. 4 together with GT. Luesink *et al.* [15] reported that the transcription of the *ptsHI* genes of *L. lactis* NZ9800 started at either one of two adjacent bases, A and G, corresponding to 435 and 436 nt, respectively, as shown in Fig. 2. Considering this together with the fact that most TSSs reported so far are purines, the TSS of the *ptsHI* genes of *L. lactis* 7962 would most likely be G at 436 nt. Upstream of G, putative promoter sequences similar to the *E. coli* σ^{70} or *B. subtilis* σ^{43} consensus sequence were identified. A -10 promoter sequence (TACAAT) deviated from the consensus sequence in one base and a TG doublet, often found one bp upstream of the -10 sequences of many lactococcal genes [7], was also present at the expected position. A -35 promoter sequence (TTGCAA) deviated from the consensus sequence at two bases was also found 17 bp upstream of the -10 sequence.

Transcriptional Analysis of the *ptsHI* Operon

Northern hybridization experiments were performed as described in Materials and Methods. When a 900 bp PCR fragment containing *ptsH* and the 5' region of *ptsI* (obtained using primers HPrI and 2EnzI) was used as a probe, two transcripts, 0.3 kb and 2.0 kb in size, were detected. When the Northern analysis was repeated with a *ptsI*-specific probe (containing no *ptsH* sequence, obtained by a PCR using primers 3EnzI and EnzI), only a 2.0 kb transcript

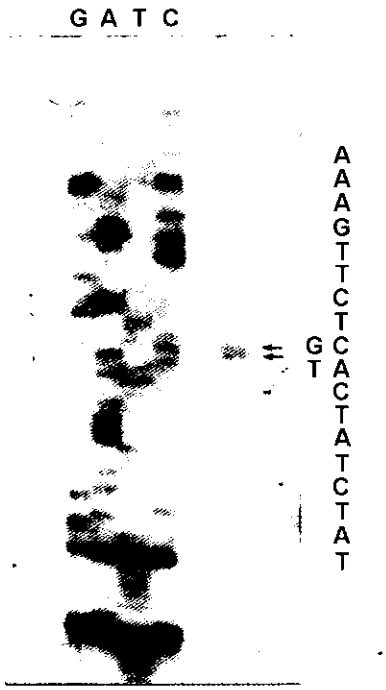

Fig. 4. Primer extension of the *ptsHI* operon.
The arrows on the right-side indicate two extended products. The sequences of the complementary strand were determined using pUhts as the template and HPrUP3 as the primer, and are shown together with two nucleotides (GT), which correspond to the transcription start sites.

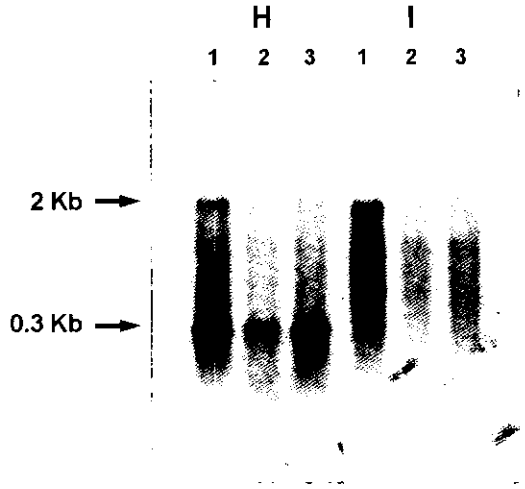

Fig. 5. Northern blot of the *ptsHI* operon.
The arrows on the left-side indicate the positions of the transcripts, 2 kb and 0.3 kb in size. H represents a Northern analysis using a *ptsH* probe and I represents a Northern analysis using a *ptsI*-specific probe. 1, RNA extracted from *L. lactis* 7962 cells grown on glucose; 2, RNA extracted from cells grown on galactose; 3, RNA extracted from cells grown on lactose.

was detected (Fig. 5). Therefore, this 2.0 kb transcript must carry both *ptsH* and *ptsI* sequences judging from its size and the absence of any other large-sized transcript, except for 2.0 kb. Figure 5 also indicates that the transcription of the *ptsHI* operon genes of *L. lactis* 7962 varied in response to the carbon source. The transcription levels of *ptsHI* were several-fold higher in glucose-grown cells than that in galactose- or lactose-grown cells. As shown in Fig. 5, the concentration of the 0.3 kb transcript (*ptsH*) was much higher than that of the 2.0 kb transcript (*ptsHI*). This indicates that the frequency of the complete transcription of *ptsI* was much lower than that of *ptsH*. The apparent concentration of 0.3 kb transcripts in cells grown on glucose was almost the same as that in cells grown on lactose, yet higher than that in cells grown on galactose. The concentration of 2.0 kb transcripts (*ptsHI*) in cells grown on glucose was much higher than that in cells grown on either lactose or galactose. In the case of *L. sake*, carbon sources used for growth do not have a significant effect on the levels of the *ptsHI* and *ptsH* transcripts [18]. However, the carbon sources tested (glucose, sucrose, fructose, mannose, and ribose) did not include lactose and galactose. In the case of *L. lactis* NZ9800, Luesink *et al.* [15] observed the same phenomena; the intensities of the hybridization signals of 2.0 kb and 0.3 kb were much lower in cells grown on galactose than those in cells grown on glucose. However, the level of *ptsHI* transcripts on lactose was not examined in NZ9800. Since 7962 and NZ9800 belong to the same species, the same transcriptional regulatory mechanism would be expected. In agreement with the observation of two different sized transcripts, two stem-loop structures were identified, which may function as transcription terminators. Terminator T2 (5'-GATTGATTTGAATAAATGAATC-3'; 2,601–2,622 nt in Fig. 2) responsible for the release of the 2 kb transcript was located downstream of the *ptsI*, whereas terminator T1 (5'-ATTTGTCTTTCGAAACAAAGACA-ATT-3'; 842–867 nt in Fig. 2) was located downstream of *ptsH* and seemed to be responsible for the release of small-sized transcripts. Luesink *et al.* [15] also reported on the presence of two stem-loop structures, one downstream of *ptsH* and the other downstream of *ptsI*. When the concentrations of the 2.0 kb transcripts in cells grown on different carbon sources were compared, it would seem that T1 was not fully functional in a glucose medium, yet became more functional in a galactose or lactose medium, thereby causing a more frequent termination of transcription at T1. Thus, the *ptsH* gene was always transcribed whereas the *ptsI* gene was only transcribed efficiently in the presence of glucose. In a galactose or lactose medium, the *ptsI* gene was not transcribed efficiently. Consequently, it can be assumed that the *ptsI* gene product, enzyme I, is necessary to maintain catabolite repression in the PTS system. Although the organization and transcription pattern of the *ptsHI* operon genes from the two *L. lactis* strains seem the same, the specific regulatory roles of these *ptsHI* genes on the utilization of a specific carbon source such as lactose or galactose may be different. Unlike NZ9800, 7962 cells utilize lactose via β-galactosidase coupled with the Leloir pathway enzymes [13]. Further studies

to elucidate the exact role of *ptsHI* on the expression of *gal/lac* operon genes in *L. lactis* 7962 are necessary.

Acknowledgments

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