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Characterization of *ptsHI* Operon from *Leuconostoc mesenteroides* SY1, a Strain Isolated from *Kimchi*

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Abstract The *ptsHI* operon from *Leuconostoc mesenteroides* ssp. *mesenteroides* SY1 (*L. mesenteroides* SY1), a strain isolated from *kimchi*, was cloned and characterized. The *ptsH* open reading frame (ORF) was 273 bp in size, which can encode a protein of 90 amino acid residues with a molecular weight of 9,212 Da. The *ptsI* ORF was 1,719 bp in size, which was capable of encoding a protein of 572 amino acids with a molecular mass of 62,549 Da. *ptsH* and *ptsI* genes were transcribed as a single transcript of 2.0 kb in size regardless of carbon sources, supporting the operon structure. Although the deduced amino acid sequences of the HPr and EI were highly homologous with those of other Gram-positive bacteria, an additional amino acid (glutamine at the 3rd amino acid) was present in HPr from *L. mesenteroides* SY1. Phosphorylation sites of HPr included the histidine residue (16th) and serine residue (47th). Mutant HPrs, in which each phosphorylation site was mutated into alanine, were obtained, and phosphorylation with HPr and mutated HPrs showed that HPr was phosphorylated at the serine residue (47th) by HPr kinase/phosphorylase (HPr K/P).

Key words: *ptsHI* operon, *Leuconostoc mesenteroides*, Kimchi

In bacteria, carbon sources are transported into cells via different mechanisms. One of the transport systems, the phosphoenolpyruvate:sugar phosphotransferase system (PEP-PTS), is responsible for the concomitant transport and phosphorylation of a variety of sugars at the expense of the high-energy phosphoryl bond of phosphoenolpyruvate (PEP) [13]. The PTS system uses PEP in a group translocation process to phosphorylate incoming mono- and disaccharides via a phosphoryl-transfer cascade involving the non-sugar-specific proteins, Enzyme I (EI) and histidine containing phosphorcarrier protein (HPr), and a family of sugar-specific enzyme II complexes (EII) [13]. In Gram-positive bacteria, the PTS controls sugar metabolism by regulating transporter activities and gene transcription via the HPr [14]. These proteins can be phosphorylated by EI at the expense of PEP on the position 15 histidine, generating P-His-HPr, and by an ATP-dependent protein kinase/phosphorylase, called HPr K/P, on the position 46 serine, generating P-Ser-HPr [4, 14]. Both P-His-HPr and P-Ser-HPr possess regulatory functions. P-His-HPr accomplishes its regulatory functions by reversibly phosphorylating its targets, and P-Ser-HPr accomplishes its regulatory functions by protein-protein interactions [3, 5]. The genes encoding HPr and EI, *ptsH* and *ptsI*, respectively, have been cloned from several bacteria and found to form an operon with the gene order of *ptsHI* [1, 2, 8].

In this note, we report the cloning and transcriptional analysis of the *ptsHI* operon from *L. mesenteroides* SY1, a strain isolated from *Kimchi* [7], and show that the regulatory role of HPr is carried out by phosphorylation of a serine residue by HPr K/P. As far as we are aware of, this is the first report on the functional characterization of the *ptsHI* operon and the regulatory role of HPr K/P from *L. mesenteroides*.

L. mesenteroides SY1 was grown in MRS broth containing different carbon sources (1%, w/v) or on MRS plates (1.5% agar, w/v) at 30°C [6]. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth at 37°C with vigorous agitation. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal: Sigma, U.S.A.) was supplemented at a concentration of 40 μg/ml, and isopropyl-β-D-thiogalactopyranoside (IPTG:

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Sigma, U.S.A.) was added to the final concentration of 0.5 mM when necessary. Antibiotics were used at the following concentrations: ampicillin (Ap: Sigma, U.S.A.), 100 μg/ml; kanamycin (Km: Sigma, U.S.A.), 30 μg/ml for the *E. coli*. The *ptsHI* operon was amplified with *L. mesenteroides* SY1 genomic DNA as the template by using PCR. A primer pair of ptsHI[BamH1]-F (5'-CGCGGATCCATTC-GTACTGAAGCGGACCCGCT-3') and ptsHI[BamH1]-R (5'-CGCGGATCCCCTCACCAGGATGAGCAGCAATC-3') was synthesized based on the sequence of the *ptsHI* operon in the database (GneBank accession number: NZ_AABH02000026) [9]. A BamHI site introduced is underlined. The amplified 3,499-bp fragment was digested with BamHI and ligated with pBluescript KS (+) (Stratagene, CA, U.S.A.), resulting in a recombinant plasmid, pLPTS35 (6.5 kb). The DNA sequence of the 3.5-kb fragment including the *ptsHI* operon reported here was deposited in the GenBank database under accession number AY750960. DNA sequencing of the 3.5-kb fragment showed that the sequence was very similar to that of the *ptsHI* operon of *L. mesenteroides* ATCC 8293 (99%, nucleotide sequence level), and the presence of two complete open reading frames, OFR1 and ORF2, and two partial ORFs (Fig. 1). ORF1 was 273 bp in size and capable of encoding HPr protein with 90 amino acids with a calculated molecular weight of 9,212 Da. The pI value for HPr was calculated to be 4.34. ORF2 was 1,719 bp in size and capable of encoding EI of 572 amino acids with a calculated molecular mass of 62,549 Da. The pI value for the Enzyme I was calculated to be 4.46. The 5' part of a putative metal-dependent protease gene and the 3' part of a Zn-dependent carboxylpeptidase gene were present downstream of *ptsI* and upstream of *ptsH*, respectively (Fig. 1).

ptsH started with an ATG codon at 719 nt (nucleotide) and a potential RBS (GGAGAA) was located 9 bp upstream of the start codon (Fig. 1). A putative promoter region with characteristic features, such as AT-rich region and potential -10 (GATAAA, 669 nt) and -35 (TTGATA, 648 nt) regions, were also found (Fig. 1). The *ptsH* stop codon (TAA) and *ptsI* start codon (ATG) were located 986 nt and 990 nt, respectively. A potential RBS of *ptsI* (GCAGAA) was located 5 bp upstream of the start codon. A putative rho-independent transcription terminator (AAAAAGCGCC-ATTGGCGTATTTTTT) was found at eight nt downstream from the stop codon of *ptsI* (2,703 nt) with ΔG of -6.6 kcal/mol.

The *ptsH* and *ptsI* sequences from *L. mesenteroides* SY1 were compared with those of other *ptsH* and *ptsI* genes in the DNA database. The *ptsH* and *ptsI* from *L. mesenteroides* SY1 was quite similar to those of other known *ptsH* and *ptsI* at the amino acid sequence level. The identity scores of the amino acid sequence of HPr were 80%, 78%, and

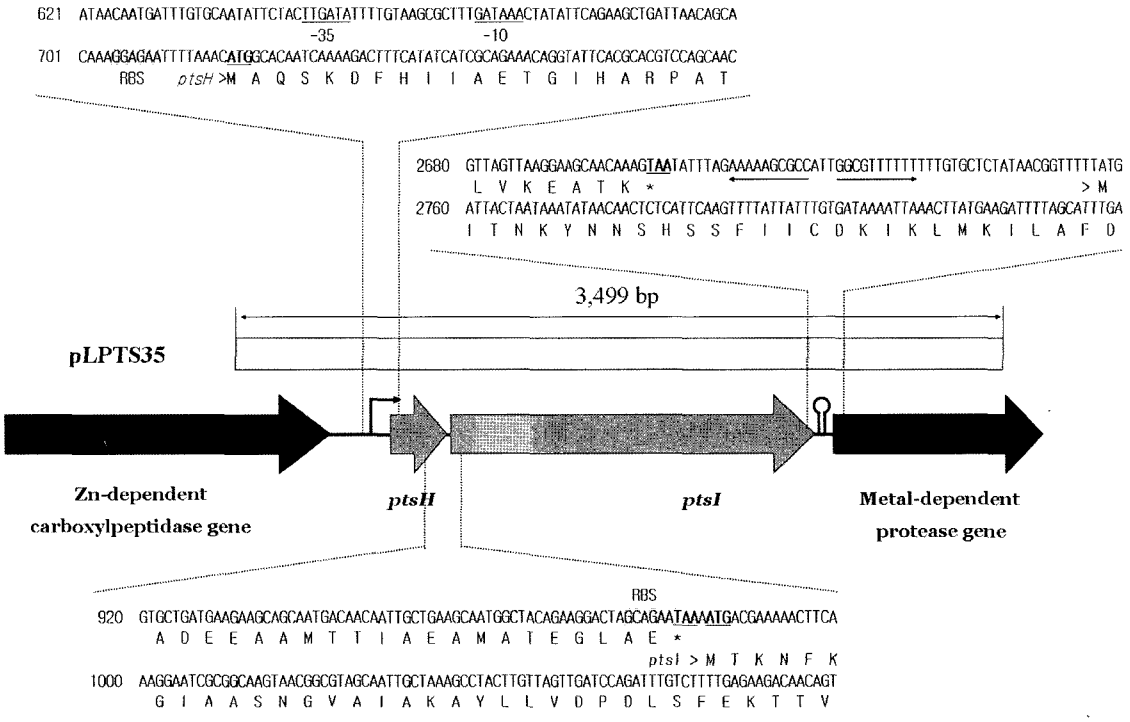

Fig. 1. Genetic organization of the *ptsHI* operon and surrounding genes of *L. mesenteroides* SY1.
The size and orientation of ORFs were deduced from the nucleotide sequence. Nucleotide sequence of the upstream regulatory region of *ptsH* and the downstream region of *ptsI* are shown. Putative RBS and RNA polymerase binding sites (-10 and -35 regions) in the sequence are underlined. Translational start and stop codons are underlined, too.

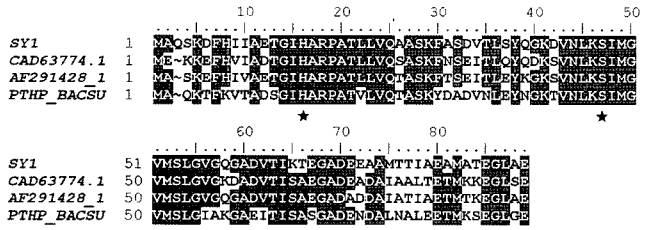

Fig. 2. Multiple sequence alignment of HPrs from various Gram-positive bacteria.
Phosphorylation sites are marked by asterisks. The HPr sequence of *L. mesenteroides* SY1 (SY1) was compared with those of *Lactobacillus plantarum* (CAD63774), *Lactococcus lactis* subsp. *lactis* ATCC 7962 (AF291420), and *Bacillus subtilis* (PTHP_BACSU).

77% with *Streptococcus pyogenes* (accession number: YP_060413), *S. agalactiae* (YP_329567), and *S. pneumoniae* (AAK99867), respectively. As shown in Fig. 2. the HPr from *L. mesenteroides* SY1 exhibited a high level of identity with other HPrs from Gram-positive bacteria, except one noticeable difference: In HPr from *L. mesenteroides* SY1, one additional amino acid (glutamine at position 3) was inserted near the N-terminus compared with other HPrs in the protein database. The presence of this additional glutamine was also confirmed for HPr from *L. mesenteroides* ATCC 8293, indicating a unique feature among *L. mesenteroides* strains. The amino acid sequence surrounding phosphorylation sites (histidine at position 16 and serine at position 47) was conserved. The identity scores of the amino acid sequence of EI were 68%, 67%, and 66% for *Lactobacillus plantarum* (NP_784928), *Pediococcus pentosaceus* (ZP_00322576), and *Oenococcus oeni* (ZP_00318838), respectively.

For transcriptional analysis of *ptsH* and *ptsI*, Northern blot analysis was performed. Total RNA was isolated from *L. mesenteroides* SY1 cells grown on MRS broth containing 1% (w/v) each of glucose and raffinose as described previously [11]. Total RNA (20 μg) was separated on 1.2% agarose-formaldehyde gel, transferred to Hybond^{TM}-XL nylon membrane (Amersham Bioscience, Uppsala, Sweden) [12], and hybridized at 65°C with ^{32}P-labeled 263-bp *ptsH* and 322-bp *ptsI* specific probes, respectively. Northern blot results showed that only a single transcript of 2.0 kb in size was detected (Fig. 3). The size matched well with the combined sizes of *ptsH* and *ptsI*, indicating the operon structure for *ptsH* and *ptsI* genes as reported from some other Gram-positive bacteria. In the cases of *Lactococcus lactis* subsp. *lactis* ATCC 7962 and *Lb. sake*, two distinct transcripts, 0.3 kb and 2.0 kb, were detected [8, 15]. Figure 3 also indicates that the frequency of transcription of *ptsHI* operon genes from *L. mesenteroides* SY1 was independent of carbon sources in the media, as reported for the *ptsHI* operon from *Lb. sake* [15].

To replace histidine at position 16 and serine at position 47 with alanine in the HPr protein (HPrH16A and HPrS47A,

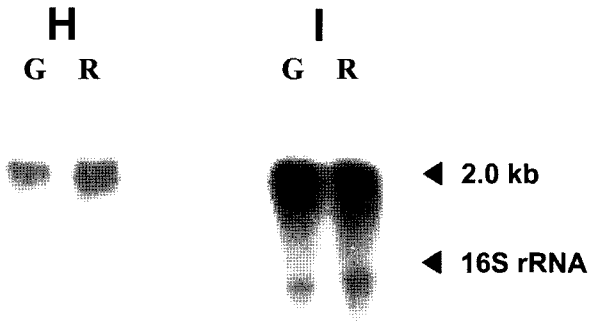

Fig. 3. Northern blot analysis of *ptsH* and *ptsI*.
Total RNA from *L. mesenteroides* SY1 cells grown on MRS medium containing 1% (w/v) each of glucose (G) and raffinose (R) were isolated and used for RNA-DNA hybridization. ^{32}P-labeled 263-bp and 322-bp sequences were used as *ptsH* (H) and *ptsI* (I) probes, respectively.

respectively), site-directed mutagenesis for the *ptsH* gene was carried out by using one-step overlap extension PCR methods [16]. To construct HPrH16A, mutated PCR amplifications were done by using universal ptsH-primers (ptsH-F1: 5'-GCGTTGAACTGATGATGTTTAACG-3' and ptsH-R1: 5'-GRGCTTGTTCAGCCGCCAC-3') and mutagenic primers (H16A-F: 5'-ACAGGTATTGCCGCACGTCCAGC-3' and H16A-R: 5'-GCTGGACGTGCGGCAATACCTGT). To construct HPrS47A, mutated PCR amplifications were done by using universal ptsH-primers and mutagenic primers (S47A-F: 5'-CTTGAAGGCAATCATGGGTG-3' and S47A-R: 5'-CACCCATGATTGCCTTCAAG-3'). Amplified DNA fragments were cloned into T-vector (pGEM-T easy vector, Promega) and sequenced to confirm the changes. The *ptsH* and mutated *ptsH* genes were cloned into pET26b (+) vector for overexpression in *E. coli*. The *ptsH* and mutated *ptsH* genes were amplified by using a primer pair of *ptsH*-expF[NdeI] (5'-GGAATTCCATATGGCACAATCAAAAGACTTC-3') and ptsH-expR[XhoI] (5'-CTACTCGAGTTCTGCTAGT-CCTTCTGTAG-3'). Amplified DNA fragments were digested with NdeI and XhoI, and ligated to pET26b(+). The resulting recombinant plasmids were introduced into *E. coli* BL21 (DE3) (Novagen, Madison, U.S.A.). When 1 mM IPTG was added into the growth media, His-tagged proteins were synthesized as soluble forms in the cytoplasm (data not shown). His-tagged proteins were purified by affinity column chromatography with a HiTrap Chelating HP column (Amersham Biosciences, Uppsala, Sweden), according to the protocol provided by the manufacturer. Figure 4 shows the results of SDS-PAGE analyses for purified His-tagged proteins. Phosphorylation experiments

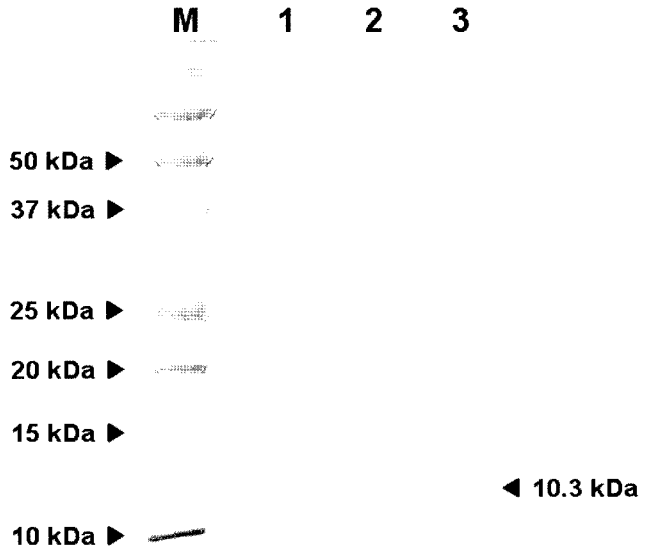

Fig. 4. SDS-PAGE of purified His-tagged proteins.
M. Precision Plus Protein™ standard (BioRad, Hercules, U.S.A.); 1. His-tagged HPr (10,277.5 Da); 2. His-tagged HPrH16A (10,211.5 Da); 3. His-tagged HPrS47A (10,261.5 Da). The gel was stained with Coomassie blue R-250 after electrophoresis. The molecular weight of HPr is 9,212.43 Da.

using these purified proteins and HPr K/P were done. It was reported that HPr and phosphorylated HPr could be easily distinguished on Coomassie blue-stained 12.5% nondenaturing acrylamide gels, as phosphorylated HPr runs considerably faster than HPr [10]. However, we could not separate HPr from phosphorylated HPr, when we used 12.5% nondenaturing gels. Separation of HPr and phosphorylated HPr, however, was achieved when the concentration of acrylamide was increased to 18%. After treatment with HPr K/P, wild-type HPr and HPrH16A migrated much faster than the proteins that were not treated. However, HPrS47A did not show any change by HPr K/P treatment (Fig. 5). The results confirm that HPr from *L. mesenteroides* SY1 is phosphorylated at the serine residue (47^{th} for *L. mesenteroides*, 46^{th} for other Gram-positive bacteria) like other Gram-positive bacteria. It also strongly indicates that HPr K/P must play important regulatory

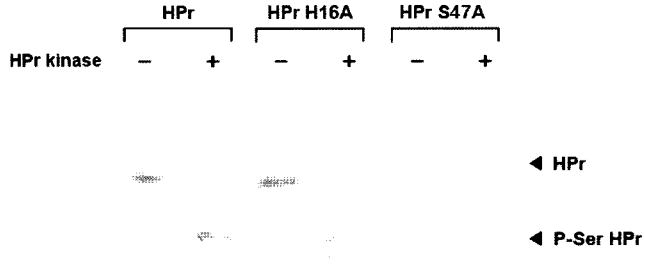

Fig. 5. Phosphorylation of HPr by HPr K/P.
Phosphorylated HPr migrated much faster than unphosphorylated HPr on nondenaturing 18% polyacrylamide gel. The gel was stained with Coomassie blue R-250 after electrophoresis.

roles in the carbon catabolite repression (CCR) system in *L. mesenteroides* SY1.

In conclusion, the *ptsH* and *ptsI* genes of *L. mesenteroides* SY1 are highly homologous to other known *ptsH* and *ptsI* genes from Gram-positive bacteria at the amino acid sequence level. The *ptsH* and *ptsI* genes of *L. mesenteroides* SY1 constitute the same operon, and a single 2.0-kb transcript (*ptsHI*) is synthesized independent of carbon sources. HPr K/P specifically phosphorylates HPr at its serine residue.

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