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Cloning and Expression of the *Listeria monocytogenes* Scott A *ptsH* and *ptsI* Genes, Coding for HPr and Enzyme I, Respectively, of the Phosphotransferase System†

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The phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) utilizes high-energy phosphate present in PEP to drive the uptake of several different carbohydrates in bacteria. In order to examine the role of the PTS in the physiology of *Listeria monocytogenes*, we identified the *ptsH* and *ptsI* genes encoding the HPr and enzyme I proteins, respectively, of the PTS. Nucleotide sequence analysis indicated that the predicted proteins are nearly 70% similar to HPr and enzyme I proteins from other organisms. Purified *L. monocytogenes* HPr overexpressed in *Escherichia coli* was also capable of complementing an HPr defect in heterologous extracts of *Staphylococcus aureus ptsH* mutants. Additional studies of the transcriptional organization and control indicated that the *ptsH* and *ptsI* genes are organized into a transcription unit that is under the control of a consensus-like promoter and that expression of these genes is mediated by glucose availability and pH or by by-products of glucose metabolism.

Listeria monocytogenes is a ubiquitous gram-positive organism that can be found in several environments, including soil, plants, animals, and water. Under certain conditions, however, this organism can cause serious food-borne illnesses with manifestations ranging from malaise and flu-like symptoms to severe meningitis and fulminating bacteremia in immunocompromised individuals (13). While many studies have focused on the arsenal of virulence factors carried by this organism and the growth patterns of the organism in food matrices, the mechanisms through which *L. monocytogenes* transports and metabolizes nutrients have only recently been studied (6, 15, 17).

We have begun to examine glucose uptake in *L. monocytogenes* by using biochemical experiments to identify the nature of the uptake systems (6, 17). In our experiments, we observed that glucose uptake persisted in the presence of bacteriocins which collapse the proton motive force gradient of resting cells. The residual glucose uptake and phosphorylation were subsequently found to be dependent on phosphoenolpyruvate (PEP), suggesting that *L. monocytogenes* possesses two distinct systems for glucose uptake, a high-affinity PEP-dependent phosphotransferase system (PTS) and a low-affinity proton motive force-mediated system (17). In separate studies, biochemical evidence obtained by Mitchell et al. (15) also demonstrated that there is a fructose-specific PTS whose components appear to be inducible.

The PTS has been well-studied in enteric bacteria, lactic acid bacteria, and the gram-positive model organism *Bacillus subtilis* (18, 22–25) and consists of two nonspecific energy-coupling proteins, HPr and enzyme I. An additional set of three activ-

ities distributed among one or more proteins (enzyme II protein complex) provides the substrate specificity for transport. Unlike the protein found in *Escherichia coli*, HPr plays an active role in coordinating the expression of carbon catabolism genes in *B. subtilis* (20). While the precise mechanisms through which HPr exerts this regulatory effect are not known, two highly conserved residues in HPr, His-15 and Ser-46, have been shown to be necessary for it to function and have been shown to serve as sites for phosphorylation (8, 24).

In order to further study the mechanisms of sugar transport in *L. monocytogenes*, we isolated the genes encoding the HPr (*ptsH*) and enzyme I (*ptsI*) components of the *L. monocytogenes* PTS. We report here that the overexpressed and purified *ptsH* product (HPr) can complement PTS activity in *Staphylococcus aureus* extracts prepared from *ptsH* mutants, demonstrating that this protein is capable of functioning in a heterologous system. The results of studies of the expression of *ptsH* and *ptsI* in *L. monocytogenes* further suggest that these genes form an operon transcribed by the primary form of RNA polymerase, whose transcription is subject to regulation by glucose and pH or by metabolites of glucose.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in Luria broth (LB) at 37°C. *S. aureus* and *L. monocytogenes* were grown in tryptic soy broth (Difco Laboratories, Ann Arbor, Mich.) containing 0.5% yeast extract (TSBYE) and either lactose (for *S. aureus*) or glucose (for *L. monocytogenes*). Antibiotics were added as indicated below.

DNA procedures. *L. monocytogenes* Scott A chromosomal DNA was isolated as described by Byun et al. (4), with some modifications. Cells were grown in TSBYE to the late log phase and harvested by centrifugation. Lysozyme (20 mg/ml) and sodium chloride (final concentration, 300 mM) were added prior to phenol-chloroform extraction of the DNA. *E. coli* was transformed by electroporation. Plasmid DNA was harvested from 1.5 ml of an *E. coli* culture grown in LB containing either ampicillin (100 µg/ml) or tetracycline (10 µg/ml). The cell pellets were resuspended in 50 µl of H₂O prior to addition of 150 µl of phenol and 150 µl of chloroform. The mixture was placed in a microfuge tube containing 0.3 g of high-vacuum grease (Dow Corning, Midland, Mich.), and the mixture was centrifuged for 20 s at 12,000 × g (to quickly separate the aqueous and organic phases). Then 150 µl of chloroform-isoamyl alcohol (24:1) was added, and the mixture was centrifuged for 20 s. The upper aqueous phase was

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
<i>E. coli</i> strains		
ES1301	<i>mutS</i>	Promega
JM109	<i>recA</i>	Promega
E509	λ DE3, pLYSE	UN-L ^a
<i>S. aureus</i> S797A	<i>ptsH</i>	19
<i>L. monocytogenes</i> Scott A	Wild type	UN-L
Plasmids		
pGEM-T		Promega
pALTER		Promega
pRSET		Promega

^a UN-L, University of Nebraska-Lincoln.

removed, and the nucleic acids were precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of ice-cold ethanol.

Synthesis of oligonucleotides. All oligonucleotides were synthesized at the University of Nebraska DNA Synthesis Core Facility. The following deoxyoligonucleotides were used as primers: HPr 1 (5'-GCA TGC CAG AAA CAG GAA TTC ATG CAC-3'), HPr 2 (5'-GCA TGC TTC AGC CAA TCC TTC TTT-3'), HPr 3 (5'-GGA TCC AAT ACC AAG AGA CAT AAC GCC-3'), 5' HPr (5'-CCG GAT CCA AAT AGT TGT AAC AAT AG-3'), 3' HPr (5'-CCG GAT CCA GAT AAG CTT TCG CAA TG-3'), Enz1 (5'-GCA TGC GGG TTC ATT TCT TTA GGA AG-3'), Enz1(B) (5'-GGA ACT CCG AAT GAT TTA GAA GG-3'), Enz1(C) (5'-CCT TAA CCA ACC ATA CAA TCC ATC C-3'), Rev HPr (5'-CTA CAA AAC TTG CTT GTT CC-3'), and pALT/Nde (5'-CTT GTT CCA TAT GCC CGC GGC-3').

PCR conditions. All PCR were performed in a Cycler Dri-Block thermocycler (Techne, Inc.). Each PCR mixture contained 10 ng of chromosomal DNA from *L. monocytogenes* Scott A as a template, each deoxynucleoside triphosphate at a concentration of 0.1 mM, 0.8 to 2.5 mM MgCl₂, 1.5 pM forward primer, 1.5 pM reverse primer, *Taq* buffer, and 0.2 U of *Taq* polymerase (Fisher Biotech) in a total volume of 30 μ l. The denaturation, annealing, and extension conditions were 94°C for 1 min, 50°C for 2 min, and 72°C for 1.5 min per kb of expected product size, respectively; a total of 30 cycles were used. For inverse PCR, the template DNA was digested with the appropriate restriction enzyme, and the fragments were self-ligated by using T4 DNA ligase (New England Biolabs), which yielded DNA concentrations of 5 to 10 μ g/ml (7). Self-ligated monomeric circular DNA was initially denatured by heating it at 99°C for 5 min and then rapidly cooling it in ice water prior to addition of *Taq* polymerase. After the initial denaturation, inverse PCR was performed as described above.

DNA sequencing and analysis. Double-stranded DNA sequencing of PCR products was performed by the dideoxy chain-terminating method of Sanger et al. (21). Sequencing reactions were performed with 18- to 26-mer synthetic oligonucleotides and a Sequenase 2.0 kit by using the PCR product sequencing protocol described by the manufacturer (United States Biochemicals). Samples were electrophoresed on 5% denaturing gels. Cloned PCR fragments were resequenced from both directions by workers at the University of Nebraska Sequencing Facility.

Northern blot analysis. Total RNA was isolated from *L. monocytogenes* Scott A cells during various stages of growth in TSBYE containing either 8 or 80 mM glucose, as previously described (16). These conditions resulted in broth cultures that had different pHs and residual glucose concentrations. Northern blot analysis was performed as described previously (12), with minor modifications. Each lane contained 10 μ g of total cellular RNA that had been denatured in glyoxal loading buffer at 55°C for 45 min. After electrophoresis, the RNA was fixed to a ZetaBind nylon membrane (Cuno Labs) by UV cross-linking. Each blot was prehybridized for 3 h at 65°C in hybridization buffer (16). An HPr-specific probe was generated by PCR by using primers HPr 1 and HPr 2. The resulting 0.2-kb fragment was sequenced to confirm specificity. An enzyme I-specific probe was also generated by PCR by using primers HPr 1 and Enz 1. In this case, the resulting 1.2-kb product was subsequently cut with *Hind*III (New England Biolabs) to generate a 0.89-kb probe internal to the *ptsI* gene, as confirmed by sequence analysis. Each probe was gel purified by using a GeneClean kit (Bio 101, Inc.) and was labeled by using the protocol supplied with a DECAprime DNA labeling kit (Ambion, Inc.). Unincorporated label was removed by using STE midi Select-D Sephadex G-50 microcentrifuge spin columns (5 Prime - 3 Prime, Inc.).

Primer extension. Oligonucleotide primer Rev HPr was 5' end labeled with [γ -³²P]ATP (Amersham) by using T4 polynucleotide kinase (New England Biolabs). The oligonucleotide was hybridized with 20 μ g of total cellular RNA extracted from *L. monocytogenes* Scott A, and the annealed primer was extended with SuperScript II reverse transcriptase (Gibco BRL) as described by the manufacturer. The resulting cDNA was analyzed on a sequencing gel adjacent to a

DNA sequencing ladder generated from the Rev HPr primer by using the 5' HPr-3' HPr PCR product as the template.

Overexpression of HPr in *E. coli* E509. An *Nde*I site was generated at the *ptsH* start codon by site-directed mutagenesis by using pALTER-1 (Promega) and the pALT/Nde primer. The *ptsH* gene was removed from the resulting plasmid as an *Nde*I fragment and was ligated into pRSET(B) (Invitrogen) to create an in-frame fusion with the synthetic ribosome binding site of the vector. The pRSET vector containing the *Listeria ptsH* gene was transformed into *E. coli* E509. For overexpression, transformed cells were inoculated into LB containing 200 μ g of ampicillin per ml and grown at 37°C overnight without shaking. The culture was then diluted 1:50 with fresh medium at the same temperature and incubated until the cells reached an optical density of 0.6. Isopropyl- β -D-thiogalactopyranoside (IPTG) (final concentration, 1 mM) and an additional 100 μ g of ampicillin per ml were then added, and the culture was incubated for 2.5 h. Samples (200 μ l) were removed before and after IPTG was added and were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to confirm induction of HPr synthesis. HPr was purified from 0.2 g (dry weight) of cells by sonication (four 1-min bursts at 60% output; Vibra Cell Sonicator; Sonics and Materials, Danbury, Conn.), and the cellular debris was removed by ultracentrifugation at 40,000 \times g for 30 min. The supernatant was heated at 65°C for 20 min, and the precipitated protein (including β -galactosidase) was removed by ultracentrifugation, as described above. Ammonium sulfate (0.42 g/ml) was added to the supernatant, the preparation was incubated for 1 h at 4°C, and the precipitated protein was collected by centrifugation. The resulting pellet was resuspended in TGED buffer, dialyzed three times with 1-liter changes of TGED buffer, and applied to a Q-Sepharose ion-exchange column (ISCO, Lincoln, Nebr.). Bound proteins were eluted with a linear 50 mM to 1.0 M NaCl gradient, and the fractions were analyzed by SDS-PAGE. Fractions containing HPr were pooled, reprecipitated, and centrifuged as described above. The pellet was resuspended in 1.5 ml of TGED buffer and loaded onto a 30-ml Sephadex G-50 column. Peak fractions were pooled, dialyzed overnight in protein storage buffer (20 mM Tris [pH 7.5], 0.1 mM EDTA, 0.1 mM dithiothreitol, 50% glycerol), and stored at -70°C.

Complementation assays. To measure PTS activity, in vitro complementation assays were performed by using *S. aureus* S797A cell extracts (11). This organism lacks a functional HPr but possesses the remaining PTS proteins. Strain S797A also lacks β -galactosidase activity. Therefore, it cannot hydrolyze the chromogenic PTS substrate *ortho*-nitrophenol- β -galactosidase (ONPG). If an exogenous source of HPr is added to *S. aureus* S797A cell extracts, full PTS activity is restored and ONPG 6-phosphate is formed, which is then hydrolyzed by native phospho- β -galactosidase to form *ortho*-nitrophenol (ONP). The ONP can be measured spectrophotometrically at 420 nm. *S. aureus* S797A was grown to mid-log phase in 100 ml of TSBYE containing lactose. The cells were harvested by centrifugation (6,000 \times g, 10 min) and resuspended in 1 ml of HPr assay buffer (50 mM Tris-acetate, 5 mM MgCl₂; pH 7). The resuspended cells were transferred into a microcentrifuge tube containing 0.2 g of 0.1-mm-diameter glass beads, and the preparation was sonicated (four 20-s bursts at 80% output; Vibra Cell sonicator; Sonics and Materials). The sonicated cells were further disrupted with a mini-bead beater (two 40-s bursts; 5,000 rpm; Biospec Products, Bartlesville, Okla.). The mixture was centrifuged at 10,000 \times g for 4 min to remove the glass beads and cell debris. Supernatant samples (100 μ l) were transferred to fresh tubes to which 0.2 mM ONPG, 5 mM PEP, 5 mM MgCl₂, and 2 ng of the purified HPr fraction were added. The preparations were incubated for 1 h at 25°C, and then 1 volume of 1.0 M sodium carbonate was added to each tube to stop the reaction. Each tube was centrifuged at 14,000 \times g for 1 min before the absorbance of the supernatant at 420 nm was determined.

Nucleotide sequence accession number. The nucleotide sequence of the 1.92-kb PCR fragment containing the *ptsH* gene sequence and much of the *ptsI* gene sequence of *L. monocytogenes* Scott A has been deposited in the GenBank database under accession no. AF030824.

RESULTS

Cloning of *ptsH*. We demonstrated previously by performing a Southern blot analysis with a *ptsH* gene probe from *B. subtilis* that an *L. monocytogenes ptsH* homolog is present on a 3.2-kb *Bam*HI fragment. Size-fractionated restriction fragments of *L. monocytogenes* chromosomal DNA were then used to construct size-fractionated libraries; however, we were not able to recover the *ptsH* homolog either by colony hybridization or through complementation. After screening more than 60,000 colonies from libraries constructed in high- and low-copy-number vectors, we were unable to obtain complementing or hybridizing clones for reasons discussed below.

Because of the difficulties in using standard cloning techniques to isolate the *ptsH* gene, PCR was used. The primers used for amplifying the *ptsH* and *ptsI* genes were designed based on the nucleotide sequences of the *ptsH* and *ptsI* coding

regions of *B. subtilis*, *Streptococcus salivarius*, *Streptococcus mutans*, and *Enterococcus faecalis*. When these primers were used in PCR with *L. monocytogenes* chromosomal DNA, we obtained the expected 1.2-kb product, as determined by agarose gel electrophoresis. This fragment, which we predicted would carry the C-terminal two-thirds of the *ptsH* gene and the amino-terminal two-thirds of the *ptsI* gene, was subsequently cloned into pGEM-T and subjected to nucleotide sequence analysis. The analysis indicated that, as expected, the clone was missing some of the N terminus of *ptsH* and the C terminus of *ptsI* (data not shown).

In order to amplify the remaining portion of the gene, we designed internal primers and used them to prime inverse PCR performed with *L. monocytogenes* DNA that had been partially digested and ligated with *ClaI* or *Sau3AI* as the template. The first inverse PCR was performed with primers HPr 2 and EnzI(B) by using *ClaI*-recircularized fragments as the template. This reaction yielded a single product that was approximately 0.8 kb long. Nucleotide sequence analysis revealed that this product contained overlapping *ptsH* and *ptsI* homologs (specifically, bases 146 to 361 and 1238 to 1855) (Fig. 1). A second inverse PCR was carried out to determine the sequence of the promoter region of the *ptsH* and *ptsI* genes. In this reaction, primers HPr 3 and EnzI(C) were used in combination with DNA that had been partially digested with *Sau3AI* and recircularized. This PCR yielded a 1.0-kb product that, when cloned and sequenced to the first *Sau3AI* site in either direction, provided bases 1 to 264 and 1826 to 1914 (Fig. 1).

When the overlapping sequences were assembled, a 1,914-base contiguous sequence was constructed that contained two open reading frames whose sizes were consistent with the sizes of previously sequenced *ptsH* and *ptsI* genes of other gram-positive bacteria. The first open reading frame (bases 121 to 385) encoded an amino acid sequence which exhibited levels of similarity of >75% with other gram-positive HPr proteins (Fig. 2). This region included highly conserved histidine and serine residues at positions 15 and 46, respectively, that have been shown to be involved in regulating carbohydrate uptake and catabolite repression in *B. subtilis*. The sequence encoded by the second open reading frame (bases 387 to >1914), which included the *ptsI*/homolog, exhibited >80% amino acid similarity with other gram-positive PTS enzyme I proteins. Our nucleotide sequence, however, did not encode the C terminus of this protein.

***ptsH* and *ptsI* are cotranscribed.** In order to evaluate the transcriptional organization of the *ptsH* and *ptsI* genes, a Northern blot analysis was performed by using a variety of growth conditions. RNA was isolated from cells grown in media containing glucose at concentrations at harvest ranging from 0.4 to 73 mM. When either *ptsH*- or *ptsI*-specific probes were used, a 2.1-kb transcript was observed, suggesting that the genes are part of a single transcription unit (Fig. 3). Moreover, with each probe, the same transcript was observed at glucose concentrations ranging from 1.2 to 73 mM. The transcript was quite strong at glucose concentrations ranging from 7.4 to 73 mM; however, at concentrations below 1.2 mM the transcript was barely detectable, and it was not observed at a glucose concentration of 0.4 mM.

Like the glucose experiments described above, accumulation of the 2.1-kb transcript was affected by pH (Fig. 3). While the transcript was strong at pH values of 6.6 to 5.3, the level of the transcript was reduced at pH 4.4, and it was not detectable at pH 4.2, even though the glucose concentration was more than 50 mM. Thus, given the identical sizes of the transcripts observed with both the *ptsH* and *ptsI* probes and given the observation that transcripts hybridizing to each probe responded

-35	-10	+1
AAATAGTTGTAACAATAGGTAACATGGTACTCTTTTAAATGAGGGATAGTCTTTTGG		60
CTGAAGTTTTTTCGGCTTAAAGGTTACAACAAATTTCTTTTACAAGGAGAAATGTAAT	RBS	120
HPF ⁺		
ATGGACAAGCAAGTTTGTAGTAATCGATGAACAGGAATTCACGCACGCCGCCAACT		180
M E Q A S F V V I D E T G I H A R P A T		
CTATTGGTGCAGGCTGCAAGTAATAACAGCTCTGACGTTCAAATTAAGTACACTGGCAAA		240
L L V Q A A S K Y S S D V Q I E Y T G K		
AAAGTAAACCTTAAATCAATCATGGCGTATTGCTCTGCTATGCTAAGGCGCTGAC		300
K V N L K S I M G V M S L G I G K G A D		
ATTACTATCTACACTGAAGGTAGCGATGAAAGAGCAATTTGAAGGACTAATCTGAAGT		360
I T I Y T E G S D E K E A I E G L T E V		
CTTAAGAAAGGATTGGCTGAATAATGGCTAAAGAGTTGAAAGGTATCGCAGCATCTG	RBS	420
L K K E G L A E *	EnzI ⁺	
ATGGGATGCCATTCGCAAGCTTATCTGCTGTAACCTGATCTTTCTATGAAAAA		480
G I A I A K A Y L L V E P D L G E V E K T		
CAGAAGTACCGATGTTGAGAGTGAAGTAAAGCGTTTGAAGCGCGCTAGACATTTCTA		540
E V T D V E S E V K R F E S A L E V S R		
GAACAGAACTTCAATGATTCGTGAGAAAGCTGCTAAGACTTAGCGAAGATAAGGCAC		600
T E L S M I R E K A A K D L G E D K A Q		
AAATTTTACCGCGCATCTCTAGTGTAAATGATCGTGAATTAACGAGCAATTTGAAG		660
I F D A H L L V L N D P E L T G P I E E		
AAAGTACAAATTTCTAAACGAATGCAGAAACAGCTTTTACAAGAAACGACACATGT		720
S I K N S K T N A E T A G E T T D M G F		
TTATTGGTATGTTGAATCTATGGACAACGATACGCTGACGCTACGCGGATATTA		780
I G M F E S M D N E Y M R E R A D A I K		
AAAGTACGTCAGCGCTCTTCTCTACTAGTGGTGAATTTCTTCTAATCCAGCTT		840
D V R K R V L S H L L G V E T I P N P A L		
TAATTGATGAGGAAGTAGTTGTTGTCAGCTGATTAAACGCTTCCGATACCGCACAC		900
I G E N D I V I I D L T P S D T A Q V		
TTAACGTAATCTTGTAAAGGTTTCGTAACGATATGGTGACGCTCTCTCTCTCCG		960
N R N F V K G F V T D I G G R T S H S A		
CTATTGGCAGCTTCTCTGAAATTCAGCAGTATGGAACAAAGATTAATCTGTA		1020
I M A R S L E I P A V V G T K E V T A S		
CGTAGCGAAAAACGATATTGTTATTATGATGGTTTGAAGGTAATGTTATCATCCACC		1080
V A K N D I V I I D L T P S D T A Q V		
CAACAGAAGCAAAATCGCTCAGTATGAAAAATTAATCTGATTTGCTTTTACAACAG		1140
T E E Q I A H Y E K I K T D F A L Q A G		
CAGAATGGGATAAACTTAAAAATGAAAAACCGTTTCTAAAGCGCGCTTCAAGTCGAG		1200
E W D K L K N E K T T V S G M D G K S V V		
TTGCAGCAAACTCGGAATCGGAATGATTAGAAAGGTTTATTTCTAACGGTGGGGAAG		1260
A A N I G T P N D L E G V I S N G G E A		
CAGTTGGACTTTATCTGACAGAAATCTGTACAGTTCGCGACATTTTCCAAACAGAA		1320
V G L Y R T E F L Y M G R D N F P T E E		
AAGAGCAATTCGAAGCTTATAAAGCAGTAGTTTCCGGAATGACGGAATCTGTGGTTG		1380
E Q F E A Y K A V V S G M D G K S V V		
TTGCTACATTAGATATCGGTGGCGATAAAACATTACACATCTAGAACTTCTCTGAAGAA		1440
R T L D I G G D G K T P Y L E L P E E M		
TGAACCAATCTTAGGATTCGCTGCAATCCGTTCTTTTGGGAATGAAGAAATTTCC		1500
N P F L G F R A I R L C F A N E L F R		
GTACACAATTCGCGCTTACTTCGCGCAAGTGATATGTAACCTTAAAAATTTATGTTCC		1560
T Q L R A L L R A S V Y G N L K I M F P		
CGATATTGCAAGCAGTAATGAATTCCTGACAGCGGTGATATTTTACTAGATGAGAAAG		1620
M I A T V N E F R Q A R D I L L D E K A		
CAAACTAAAGCTGCTGGAACAGAGTATCTGATTCGCAATGGAATTTATGATTG		1680
K I K A A G T E V S D S I C E L G I M I E		
AAATCTCTGCTGCTGCTCTTCTGCTGATCAATTTGCAAGAAAGTGTATTTCTCTCTA		1740
I P A A A V L A D Q F A K E V D F F S I		
TCGGAACAAATGACTTAATTCAGTATCAATGGCTGACAGCCGTATGATTAAGCGGTTT		1800
G T N D L I Q Y T M A A D N N E R V S		
CTTACCTTTTACCAACCATACAATCCATCCATTTTACGTTTGTAGTCAAAATGGTTATCGATG		1860
Y L L Y Q P Y N P S I L R L V K M V I D A		
CTTCTCATAAAGGCGCAATGGACTGTTGTTGCGGAAATGGCTGGAGATC		1914
S H K E G K W T G M C G E M A G D		

FIG. 1. Nucleotide and deduced amino acid sequences of the *ptsH* and *ptsI* genes of *L. monocytogenes* Scott A. The putative -35 and -10 promoter sequences and ribosome binding sites (RBS) are underlined. The transcriptional start site is indicated by +1. The *ptsH* stop codon is indicated by an asterisk.

identically to glucose concentration and pH, we hypothesized that the two genes constitute a single transcription unit whose accumulation is sensitive to glucose concentration and pH or metabolites of glucose.

Mapping of the *ptsH* promoter. To identify the transcriptional start site of the putative operon, a primer extension analysis was performed with RNA isolated from *L. monocytogenes* cells grown in TSBYE (Fig. 4). The extension product was electrophoresed next to a sequencing ladder primed with the same primer. The extension product aligned with nucleotides T and G of the sequence shown in Fig. 4 and lies 77 to 78 bases upstream of *ptsH*. Interestingly, the position of the transcriptional start site is consistent with the position observed in

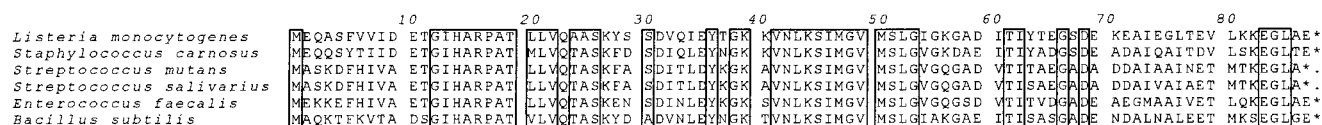


FIG. 2. Amino acid alignment comparing the HPr protein of *L. monocytogenes* to the HPr proteins of other gram-positive bacteria. Areas of 100% conservation are enclosed in boxes. The GenBank accession numbers for the nucleotide sequences encoding the proteins are as follows: *S. carnosus*, X60766; *S. mutans*, L15191; *S. salivarius*, Z17217; *E. faecalis*, Z19137; and *B. subtilis*, X12832.

other gram-positive bacteria (GenBank accession no. X12832) (9). Putative -10 (TTT AAT) and -35 (TTG TAA) promoter boxes similar to other *L. monocytogenes* promoter boxes were also identified. The -35 box is similar to the boxes observed for *L. monocytogenes* superoxide dismutase (TTG AAA) (3) and the TnpR gene (TTG ACT) (14). The -10 box is also consistent with other *L. monocytogenes* promoter boxes (5). The 23-bp space between the two putative promoter boxes is uncharacteristic compared with the normal 12- to 18-bp space observed in most promoter regions. However, extended promoter box spacing has been observed previously in *L. monocytogenes*, including 23 bp between the -10 and -35 promoter boxes of the *actA* promoter region (GenBank accession no. X59723).

Cloning of the contiguous *ptsH* (HPr) gene. In order to perform genetic and biochemical analyses of the putative *ptsH* gene and its product, PCR primers (5' HPr and 3' HPr) were designed to flank the *ptsH* promoter and coding region. A 0.4-kb PCR product was readily obtained, and its identity was confirmed by DNA sequence analysis. Cloning of the PCR product, however, was not readily achieved. After several unsuccessful attempts to clone the product into both high-copy-number (pUC18 and pTRKH2) and low-copy-number (pACYC184 and pTRKL2) vectors, the product was finally cloned directly into pGEM-T. During the nucleotide sequence analysis of the cloned PCR product, we found that the 5' end of the clone began at the *ptsH* initiation codon and therefore lacked its cognate promoter. This finding is consistent with our previous unsuccessful efforts to clone the intact gene and its promoter. It is also consistent with the observation that PCR products lacking the intact *ptsH* gene were readily clonable. These results suggest that the gene and/or promoter is toxic to the *E. coli* host. Toxicity of intact heterologous *ptsH* genes in *E. coli* has been reported previously by Gagnon et al. (9).

Function of the *ptsH* (HPr) protein. Due to the difficulties in constructing the plasmids necessary for genetic analysis of *ptsH*, we took a biochemical approach. To facilitate purification of the *L. monocytogenes* *ptsH* gene product, the gene was overexpressed in a T7 expression system (Fig. 5). The cloned *ptsH* PCR product was first moved into the site-directed mutagenesis vector pALTER-1, and an *NdeI* restriction site was engineered at the putative initiation codon by using the mutagenic oligonucleotide pALT/Nde. Cloning of the 0.3-kb *NdeI* fragment from the resulting plasmid into the *NdeI* site of the T7 expression vector pRSET(B) thus created a +1 translational fusion of the *ptsH* gene to the synthetic ribosome binding site of the vector. The entire *ptsH* region of the resulting plasmid was subjected to nucleotide sequence analysis to confirm its integrity.

To test the function of the *L. monocytogenes* Scott A HPr protein, in vitro complementation assays were performed. Cell extracts obtained from HPr-deficient *S. aureus* S797A lacked PTS activity and were unable to convert ONPG to ONPG 6-phosphate, as indicated by the lack of ONP formation by native phospho- β -galactosidase. When *Listeria* HPr was added, conversion of ONPG to ONPG 6-phosphate and finally to ONP

occurred (Table 2), indicating that the *L. monocytogenes* HPr was active and capable of reconstituting PTS activity in heterologous *S. aureus* extracts.

DISCUSSION

The PTS plays a primary role in carbohydrate uptake and catabolite repression in many gram-negative and gram-positive bacteria (18). In gram-positive bacteria, regulation of PTS activity is associated most directly with the phosphorylation state of the general PTS protein HPr (10, 19). We describe here identification and characterization of the *L. monocytogenes* *ptsH* and *ptsI* genes encoding the HPr and enzyme I proteins, respectively. The predicted amino acid sequence of *L. monocytogenes* HPr is highly homologous to the amino acid sequences of other gram-positive HPr proteins, including a C-terminal region that is shared by both gram-positive and gram-negative organisms and two regions at positions 22 to 29 and 42 to 56 (Fig. 2) unique to gram-positive organisms. Like all HPr proteins that have been identified thus far, the *L. monocytogenes* homolog contains a highly conserved histidine at position 15 that has been identified as the site of PEP-dependent phosphorylation by enzyme I in *S. aureus* (2). A second site of phosphorylation, serine 46, is also found in all gram-positive HPr proteins, including the *L. monocytogenes* sequence described here. This serine residue is a site of ATP-dependent phosphorylation that potentiates catabolite repression by enhancing the affinity of HPr for the CcpA protein (20). It has been postulated that binding of the HPr-CcpA heterodimer in the proximity of ca-

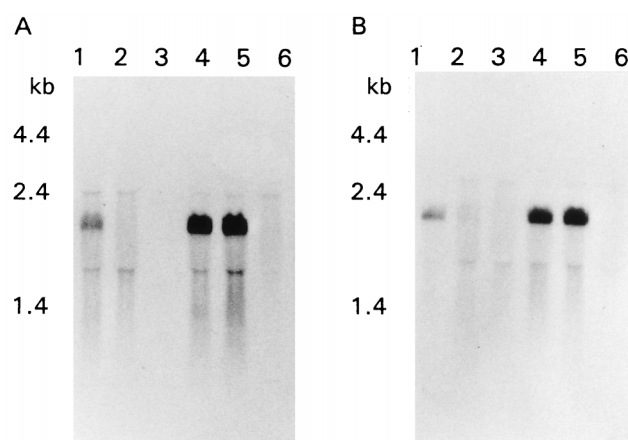


FIG. 3. Northern blot analysis of *L. monocytogenes* Scott A RNA performed with probes specific for *ptsH* (A) and *ptsI* (B). (A) The lanes contained total RNA from cells harvested under the following conditions: lane 1, pH 5.4, 1.2 mM glucose; lane 2, pH 5.3, 0.4 mM glucose; lane 4, pH 6.2, 70.6 mM glucose; lane 5, pH 5.34, 64.2 mM glucose; lane 6, pH 4.4, 54.9 mM glucose. Lane 3 contained no RNA. (B) The lanes contained total RNA from cells harvested under the following conditions: lane 1, pH 6.6, 7.4 mM glucose; lane 2, pH 5.4, 1.2 mM glucose; lane 3, pH 5.3, 0.4 mM glucose; lane 4, pH 6.6, 72.9 mM glucose; lane 5, pH 6.2, 70.6 mM glucose; lane 6, pH 4.4, 54.9 mM glucose.

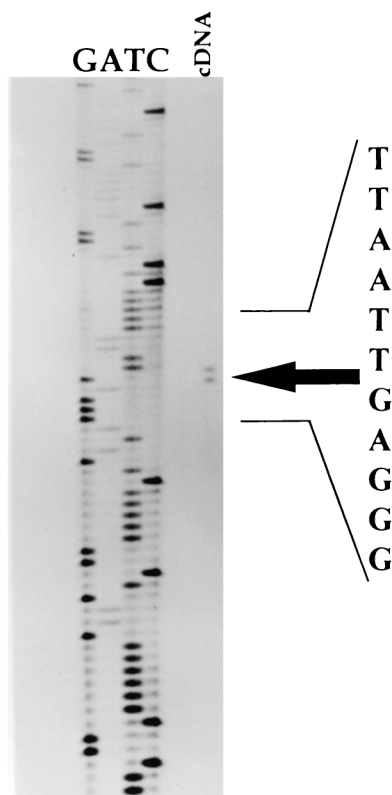


FIG. 4. Primer extension of the *ptsHI* transcriptional start site. The position of the cDNA primer extension product is indicated by an arrow.

tabolite-repressed promoters is directly responsible for inhibiting the transcription of the promoters in the presence of glucose (20).

The effects of phosphorylation at both histidine 15 and serine 46 of the *B. subtilis* HPr protein have recently been demonstrated in biochemical experiments in which it was observed that HPr phosphorylated at amino acid position 46 interacted with CcpA but only if histidine 15 was unaltered and in a non-phosphorylated state (20). These analyses were performed with mutant *ptsH* alleles coding for either unphosphorylatable HPr proteins or HPr proteins which permanently mimic the phosphorylated state. The results suggest that the sensitivity of HPr (serine 46-phosphate)-CcpA complex formation to PEP-dependent phosphorylation of histidine 15 provides a mechanism for coupling catabolite repression to carbohydrate uptake (20). The results of preliminary experiments performed with the H15A, H15D, S46T, and S46D mutants of the *L. monocytogenes* HPr protein that we constructed also support the hypothesis that these residues play similar roles (6a).

The central region of conserved residues (positions 22 to 29) (Fig. 2) is also observed in the HPr proteins of other gram-positive organisms. One of the most interesting occurrences of this sequence is found in *Aspergillus fumigatus* (1), a eukaryotic mold lacking a PTS. This region is also present in the HPr protein of a *Lactobacillus brevis* strain which has been shown to lack a functional PTS (19). Together, these findings suggest that this region may be involved in interactions with non-PTS proteins.

In addition to the conserved amino acid sequences in HPr and enzyme I homologs, we also observed that the *L. monocytogenes* *ptsH* and *ptsI* genes are organized into a single transcription unit. Primer extension analyses revealed a single tran-

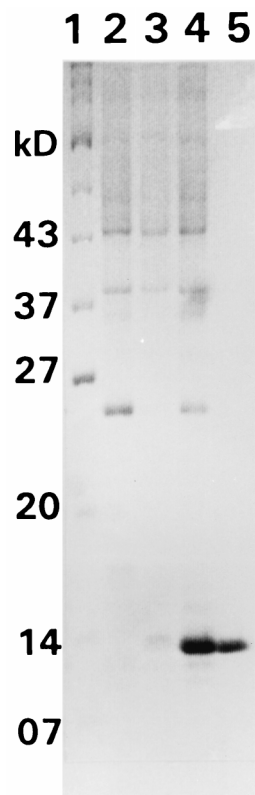


FIG. 5. SDS-PAGE. Lane 1, protein molecular weight standard; lane 2, *E. coli* E509 containing pRSET; lane 3, uninduced *E. coli* E509 containing pRSET-*ptsH* gene construct; lane 4, IPTG-induced *E. coli* E509 containing pRSET-*ptsH* gene construct; lane 5, purified *L. monocytogenes* HPr. kD, kilodaltons.

scription start site having nucleotide sequences upstream of the start site similar to the nucleotide sequences of other house-keeping promoters of gram-positive bacteria and the consensus promoter of *E. coli*. However, we observed only a single transcript in the *ptsHI* region in *L. monocytogenes*. Other organisms, including *E. coli*, *Salmonella typhimurium*, *B. subtilis*, and *S. salivarius*, have multiple promoters and/or multiple terminators which give rise to both full-length transcripts and transcripts containing only *ptsH* (10). In *S. salivarius*, the *ptsH*-specific transcript accounts for at least 50% of the *ptsH*-containing RNA that accumulates. Although *ptsHI* transcription appears to originate from a single promoter in gram-positive organisms, regulation of *ptsHI* expression is complex, and it has been proposed that both attenuation and antisense RNAs play regulatory roles (10). We were unable to locate ei-

TABLE 2. PTS activity of *S. aureus* S797A extracts complemented with *L. monocytogenes* Scott A HPr

Reaction components ^a	PTS activity ^b
<i>S. aureus</i> extract only.....	ND ^c
<i>L. monocytogenes</i> HPr only.....	ND
<i>S. aureus</i> extract plus <i>E. coli</i> -expressed <i>L. monocytogenes</i> HPr	618.5

^a All reaction mixtures contained 5 mM PEP, 5 mM MgCl₂, and 0.2 mM ONPG.

^b Activity is expressed as micromoles of ONP formed per minute per milligram of HPr protein.

^c ND, not detected.

ther an open reading frame proceeding in the direction opposite the direction of the *L. monocytogenes ptsH* gene or terminator sequences in the C-terminal end of the *ptsI* gene. The fact that only *ptsHI* transcripts were found and the fact that there are no terminator-like sequences in the *ptsI* region suggest that regulation of HPr and enzyme I in *L. monocytogenes* is fundamentally different than regulation of HPr and enzyme I in other gram-positive and gram-negative organisms. Whether the regulatory differences correspond to functional differences in the role of PTS proteins, however, remains to be determined.

When we measured the pattern of *ptsHI* RNA accumulation in Northern blots, we initially observed decreasing amounts of transcript as the cells approached the stationary phase and depletion of the glucose in the medium (Fig. 3). Whether this pattern of accumulation was the consequence of glucose depletion or a product of the growth state of the cells remains to be determined. The results of experiments in which the glucose concentration was maintained above 40 mM, however, suggest that products of glucose metabolism, not glucose concentration per se, may be the regulatory signal. Although we observed decreasing levels of *ptsHI* mRNA accumulation as the pH of the medium decreased in these experiments, our results did not distinguish whether pH itself or another signal was responsible for this observation. In addition, the results did not exclude the possibility that multiple signals contributed to *ptsHI* transcript accumulation. We therefore propose that glucose and pH or a metabolite produced during glucose catabolism serves as a regulatory signal. How this regulation is imposed is unclear at this point. We are currently cloning the *L. monocytogenes ptsHI* promoter region, including sequences well upstream of the -35 element, in order to construct transcriptional fusions to reporter genes to determine if any regulatory elements of *ptsHI* transcription exist.

The physical identification of a PTS and information regarding the transcriptional regulation of this PTS in *L. monocytogenes* contribute to the overall understanding of the physiology of this pathogen. The similarity between the *L. monocytogenes ptsHI* operon identified here and the *ptsHI* operons of other gram-positive organisms suggests that these operons have very similar functions. The lack of a terminator sequence in the C-terminal end of *ptsI*, a characteristic of other gram-positive organisms, suggests that regulation of HPr activity in *L. monocytogenes* may differ from regulation of HPr activity in other organisms. Further research is required to assess the degree to which the *L. monocytogenes* PTS is similar to or different from other PTSs.

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