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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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School of Biological Sciences, 1 and Department of Food Science and Technology, 2 University of Nebraska-Lincoln, Lincoln, Nebraska 68583-0919

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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 performed in the presence of bacteriocins which collapse the proton motive force gradient (17). The residual glucose uptake and phosphorylation were shown to be independent of 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 activities 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 mg/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 microcentrifuge 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-isooamyl alcohol (24:1) was added, and the mixture was centrifuged for 20 s. The upper aqueous phase was
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 to a purity of >95% at CCA GAT AAG CTT TCG CAA TG-3

E. coli strains

Strains

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ES1301</td>
<td>mutS</td>
<td>Promega</td>
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<tr>
<td>JM109</td>
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<td>Promega</td>
</tr>
<tr>
<td>E509</td>
<td>Ade3, PLYSE</td>
<td>UN-L*</td>
</tr>
<tr>
<td>S. aureus S797A</td>
<td>Wild type</td>
<td>UN-L</td>
</tr>
<tr>
<td>L. monocytogenes Scott A</td>
<td></td>
<td></td>
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<tr>
<td>Plasmids</td>
<td></td>
<td></td>
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<tr>
<td>pGEM-T</td>
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</tr>
<tr>
<td>pALTER</td>
<td></td>
<td>Promega</td>
</tr>
<tr>
<td>pRSET</td>
<td></td>
<td>Promega</td>
</tr>
</tbody>
</table>

* UN-L, University of Nebraska-Lincoln.

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 NdeI site was generated at the psiH start codon by site-directed mutagenesis by using pALTER-1 (Promega) and the pALT-Nde primer. The psiH gene was inserted in-frame and was removed from the resulting plasmid as an NdeI fragment and was ligated into pRSET(B) (Invitrogen) to create an in-frame fusion with the synthetic ribosome binding site of the vector. The psiR vector containing the Listeria psiH gene was transformed into E. coli E509. For overexpression, transformed cells were inoculated into 200 μl 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 μg of ampicillin per ml were then added, and the culture was incubated for 2.5 h. Samples (200 μl) were removed before and after IPTG was added and were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to confirm induction of the psiH gene. 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 × g for 30 min. The supernatant was heated at 65°C for 20 min, and the precipitated protein (including β-galactosidase) was removed by ultrafiltration as described above. Ammonium sulfate (0.4 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 to 1.0 M NaCl gradient, and fractions were analyzed by SDS-PAGE. Fractions containing HPr were pooled, precipitated, 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-D-galactoside (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-galactoside 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 TSBY containing lactose. The cells were harvested by centrifugation (6,000 × g, 10 min) and resuspended in 1 ml of HPr assay buffer (50 mM Tris-ace tate, 5 mM MgCl2; pH 7.5). 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 × g for 4 min to remove the glass beads and cell debris. Supernatant samples (100 μl) were transferred to fresh tubes to which 0.2 mM ONPG, 5 mM PEP, 5 mM MgCl2, and 2 μg 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 × g for 1 min before the absorbance of the supernatant was determined.

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

RESULTS

Cloning of psiH. We demonstrated previously by performing a Southern blot analysis with a psiH gene probe from B. subtilis that an L. monocytogenes psiH homolog is present on a 3.2-kb BamHI fragment. Size-fractioned restriction fragments of L. monocytogenes chromosomal DNA were then used to construct size-fractioned libraries; however, we were not able to recover the psiH 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 psiH gene, PCR was used. The primers used for amplifying the psiH and psiI genes were designed based on the nucleotide sequences of the psiH and psiI 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 804) was similar to other gram-positive HPr proteins (Fig. 2). The sequence encoded by the second open reading frame (bases 146 to 361 and 1238 to 1855) (Fig. 1) is conserved 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 this second open reading frame frame (bases 146 to 361 and 1238 to 1855) (Fig. 1) also has similarity with other gram-positive HPr proteins (Fig. 2).

*ptsH* and *ptsI* are cotranscribed. In order to evaluate the transcriptional organization of the *ptsH* and *ptsI* genes, a Northern blot analysis was performed with RNA isolated from *L. monocytogenes* cells grown in TSB, pH 4.2, even though the glucose concentration was more than 0.4 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 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 TSB, pH 4.2, and electrophoresed next to a sequencing ladder primed with the primer. The transcriptional start site is indicated by +1. The *ptsH* stop codon is indicated by an asterisk.

![Fig. 1](https://example.com/fig1.png)  
**Fig. 1.** Northern blot analysis of transcriptional start site of the putative operon. A 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.
other gram-positive bacteria (GenBank accession no. X12832) (9). Putative moter 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 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 pSET(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-
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 \textit{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 \textit{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 \textit{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 \textit{Aspergillus fumigatus} (1), a eukaryotic mold lacking a PTS. This region is also present in the HPr protein of a \textit{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 \textit{L. monocytogenes} \textit{ptsH} and \textit{ptsI} genes are organized into a single transcription unit. Primer extension analyses revealed a single transcription start site having nucleotide sequences upstream of the start site similar to the nucleotide sequences of other housekeeping promoters of gram-positive bacteria and the consensus promoter of \textit{E. coli}. However, we observed only a single transcript in the \textit{ptsHI} region in \textit{L. monocytogenes}. Other organisms, including \textit{E. coli}, \textit{Salmonella typhimurium}, \textit{B. subtilis}, and \textit{S. salivarius}, have multiple promoters and/or multiple terminators which give rise to both full-length transcripts and transcripts containing only \textit{ptsH} (10). In \textit{S. salivarius}, the \textit{ptsH}-specific transcript accounts for at least 50% of the \textit{ptsH}-containing RNA that accumulates. Although \textit{ptsH} transcription appears to originate from a single promoter in gram-positive organisms, regulation of \textit{ptsH} 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>
<thead>
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<th>Reaction components</th>
<th>PTS activityb</th>
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<tr>
<td>\textit{S. aureus} extract only</td>
<td>NDa</td>
</tr>
<tr>
<td>\textit{L. monocytogenes} HPr only</td>
<td>ND</td>
</tr>
<tr>
<td>\textit{S. aureus} extract plus \textit{E. coli}-expressed \textit{L. monocytogenes} HPr</td>
<td>618.5</td>
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\textsuperscript{a}All reaction mixtures contained 5 mM PEP, 5 mM MgCl\textsubscript{2}, and 0.2 mM ONPG.

\textsuperscript{b}Activity is expressed as micromoles of ONP formed per minute per milligram of HPr protein.

\textsuperscript{c}ND, not detected.
ther an open reading frame proceeding in the direction of the direction of the \textit{L. monocytogenes ptsH} gene or terminator sequences in the C-terminal end of the \textit{ptsI} gene. The fact that only \textit{ptsH} transcripts were found and the fact that there are no terminator-like sequences in the \textit{ptsI} region suggest that regulation of HPr and enzyme I in \textit{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 \textit{ptsH} 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 \textit{ptsH} 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 \textit{ptsH} 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 accomplished or a product of the growth state of the cells remains to be determined. In addition, the results did not distinguish whether \textit{ptsH} gene expression in \textit{L. monocytogenes} may differ from regulation of HPr activity in other gram-positive and gram-negative organisms, suggests that regulation of HPr activity in \textit{L. monocytogenes} may differ from regulation of HPr activity in other organisms. Further research is required to assess the degree to which the \textit{L. monocytogenes} PTS is similar to or different from other PTSs.

**ACKNOWLEDGMENTS**

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