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# Identification of a Phosphoenolpyruvate:Fructose Phosphotransferase System (Fructose-1-Phosphate Forming) in *Listeria monocytogenes*

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***Listeria monocytogenes*** **is a gram-positive bacterium whose carbohydrate metabolic pathways are poorly understood. We provide evidence for an inducible phosphoenolpyruvate (PEP):fructose phosphotransferase system (PTS) in this pathogen. The system consists of enzyme I, HPr, and a fructose-specific enzyme II complex which generates fructose-1-phosphate as the cytoplasmic product of the PTS-catalyzed vectorial phosphorylation reaction. Fructose-1-phosphate kinase then converts the product of the PTS reaction to fructose-1,6-bisphosphate. HPr was shown to be phosphorylated by [$^{32}$P]PEP and enzyme I as well as by [$^{32}$P]ATP and a fructose-1,6-bisphosphate-activated HPr kinase like those found in other gram-positive bacteria. Enzyme I, HPr, and the enzyme II complex of the *Listeria* PTS exhibit enzymatic cross-reactivity with PTS enzyme constituents from *Bacillus subtilis* and *Staphylococcus aureus*.**

Many strictly and facultatively anaerobic bacteria initiate carbohydrate metabolism employing the phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS). This system is found in a wide variety of gram-negative and gram-positive bacteria (15, 18), including most of the major pathogens and opportunistic pathogens of humans and other animals (8).

In recent years, the incidence of serious *Listeria monocytogenes* infections in humans has increased dramatically, particularly in immunocompromised individuals and pregnant women (3, 10). The organism has been shown to be capable of utilizing carbohydrates for growth (9), but little information concerning the transport mechanisms and metabolic pathways responsible for carbohydrate utilization is available. In order to understand the ability of *L. monocytogenes* to grow and survive in a variety of extracellular and intracellular environments, it is essential to know the mechanisms by which nutrients are taken up and metabolized. To this end, we have examined *L. monocytogenes* for the presence of the energy-coupling proteins of the PTS and the most widely disseminated enzyme II complex, that specific for the ketohexose fructose. We demonstrate that such a system is present on the basis of the following criteria. (i) [$^{14}$C]fructose is phosphorylated at the expense of PEP in a reaction dependent on enzyme I, HPr, and a fructose-inducible, fructose-specific enzyme II complex. (ii) The energy-coupling PTS proteins of *L. monocytogenes* complemented *ptsI* and *ptsH* mutants of *Staphylococcus aureus* and could be replaced by the corresponding purified proteins from *Bacillus subtilis*. (iii) The membranal enzyme II complex alone catalyzed fructose-1-phosphate (fructose-1-P):[$^{14}$C]fructose transphosphorylation, and no other sugar phosphate could serve as a phosphoryl donor. (iv) [$^{32}$P]PEP labeling of crude extracts revealed three radioactive products. Two had approximate apparent molecular masses of 60 and 14 kDa, corresponding to the molecular sizes of enzyme I and HPr, respectively. These radiolabeled proteins, as well as an uncharacterized 20-kDa [$^{32}$P]phosphoprotein, disappeared upon incubation of the extracts with fructose. (v) [$^{32}$P]ATP phosphorylated HPr on an acid-stable, base-labile residue (presumably serine) in a fructose-1,6-bisphosphate-activated, kinase-dependent process, as occurs in other gram-positive bacteria. An HPr(ser-P) phosphatase was also demonstrated.

The strain of *L. monocytogenes* used, 10403S, was obtained from Daniel Portnoy (University of Pennsylvania). Cultures were grown aerobically at 37°C in Luria-Bertani (LB) broth supplemented where appropriate with sugar at a concentration of 0.5% (wt/vol). Cells were harvested by centrifugation (10,000 × *g* for 10 min at 4°C) and washed twice in 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride. Listerial cells were ruptured by freeze-thawing followed by three passages through a French pressure cell at 10,000 lb in$^{-2}$. Cell extracts were centrifuged for 15 min at 10,000 × *g* to remove whole cells and cell debris, and the supernatants were used in assays of enzyme activities. Membranes were removed from crude extracts by centrifugation at 200,000 × *g* for 2 h at 4°C, washed, and resuspended in 0.1 volume of buffer. The supernatant from this centrifugation is referred to as high-speed supernatant.

The growth conditions and preparation of extracts for *S. aureus* (strains S797A [*ptsH*] and S710A [*ptsI*]) and *B. subtilis* have been described previously (12, 14). PTS complementation assays, sugar transphosphorylation assays, and detection of HPr(ser) phosphorylation with [γ-$^{32}$P]ATP on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels were performed by previously published procedures (12, 14, 19). [$^{32}$P]PEP was prepared by the method of Mattoo and Waygood (7). Proteins labeled with [$^{32}$P]PEP were separated by SDS-PAGE and detected by autoradiography as described previously (12, 14). Fructose-1-P kinase and fructose-6-P kinase activities were assayed by the method of Baumann and Baumann (1).

Table 1 shows that a crude extract of *L. monocytogenes*

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TABLE 1. Presence of enzyme I and HPr in *L. monocytogenes*, demonstrated by PEP-dependent sugar phosphorylation assays

| Source of extract[a] | [$^{14}$C]TMG-phosphate formed (nmol/30 min) |
|---|---|
| *L. monocytogenes* | 0.04 |
| *S. aureus ptsH* | 0.01 |
| *S. aureus ptsI* | 0.04 |
| *L. monocytogenes* + *S. aureus ptsH* | 0.93 |
| *L. monocytogenes* + *S. aureus ptsI* | 2.72 |

[a] Preparation of extracts and assay conditions were as described previously (14). The indicated extracts were present at the following concentrations (in micrograms of protein per 0.1 ml of reaction mixture): *L. monocytogenes*, 235; *S. aureus ptsH*, 80; and *S. aureus ptsI*, 110.

complemented *ptsI* and *ptsH* mutant extracts of *S. aureus*. For these assays, the lactose PTS of *S. aureus* was monitored by using [$^{14}$C]methylthio-β-galactoside ([$^{14}$C]TMG) as a substrate; the *L. monocytogenes* extract was derived from glucose-grown cells, and no lactose PTS was observed in extracts derived from *L. monocytogenes* cells grown in the presence of either glucose or lactose (see below). The stimulation of [$^{14}$C]TMG phosphorylation by enzyme I and HPr of *L. monocytogenes* was comparable to that observed with extracts of other gram-positive bacteria (data not shown).

Table 2 reveals that the PEP-dependent [$^{14}$C]fructose phosphorylation activity was dependent on pure enzyme I and HPr from *B. subtilis* when washed membranes of fructose-grown *L. monocytogenes* were used as a source of the enzyme II complex. The reaction rate was dependent on the concentrations of enzyme I and HPr, as expected. The

TABLE 2. Phosphorylation of [$^{14}$C]fructose by *L. monocytogenes* membranes in the presence of PTS proteins and different phosphate donors[a]

| Addition[b] | Phosphate donor[c] | [$^{14}$C]fructose-P formed (nmol/20 min) |
|---|---|---|
| None | None | 0.01 |
| | PEP | 0.03 |
| Enzyme I (4 μg) + HPr at (μg): | | |
| 0.06 | PEP | 0.05 |
| 0.6 | PEP | 0.11 |
| 6 | PEP | 0.26 |
| HPr (6 μg) + enzyme I at (μg): | | |
| 0.08 | PEP | 0.14 |
| 0.4 | PEP | 0.19 |
| 4 | PEP | 0.25 |
| 4 | ATP | 0.10 |
| None | Fructose-1-P | 0.12 |
| | Fructose-6-P | 0.01 |

[a] *L. monocytogenes* membranes were prepared from cells grown in the presence of fructose and used directly in assays at a concentration of 120 μg of membrane protein per 0.1 ml of reaction mixture.

[b] Assay conditions were as described previously (14). Purified PTS proteins were prepared from *B. subtilis* as described elsewhere (16, 17).

[c] Where indicated, PEP or ATP was added to a final concentration of 2.5 mM, while sugar phosphates were added to a final concentration of 10 mM. The following sugar phosphates were tested and found to be unable to support phosphorylation of [$^{14}$C]fructose by *L. monocytogenes* membranes: fructose-1,6-bisphosphate, galactose-6-P, glucose-6-P, glucosamine-6-P, mannose-6-P, mannitol-1-P, *N*-acetylglucosamine-6-P, and sorbitol-6-P.

TABLE 3. Fructose-P kinase activities in high-speed supernatant extracts of *L. monocytogenes*

| Growth medium | Activity[a] | |
|---|---|---|
| | Fructose-1-P kinase | Fructose-6-P kinase |
| LB | 11 | 8 |
| LB + fructose | 190 | 9 |
| LB + glucose | 26 | 87 |

[a] Expressed as nanomoles of substrate phosphorylated per minute per milligram of protein at 25°C.

PEP-dependent reaction rate exceeded that observed when ATP served as a potential phosphoryl donor, showing that the fructose-specific PTS exhibited greater activity than fructokinase in these extracts. Only one additional phosphoryl donor was active, namely, fructose-1-P, which effectively phosphorylated [$^{14}$C]fructose in the presence of *L. monocytogenes* membranes but in the absence of the PTS energy-coupling proteins (Table 2). Other sugar phosphates, including fructose-6-P, could not replace fructose-1-P as a phosphoryl donor, as would be predicted for a transphosphorylation reaction catalyzed by a fructose-1-P-forming PTS (19). Neither PEP- nor fructose-1-P-dependent activity was exhibited by membranes prepared from *L. monocytogenes* cells grown in the absence of fructose, indicating that the enzyme II complex of the fructose PTS is inducible.

Extracts prepared from fructose-induced cells of *L. monocytogenes* were found to contain high levels of fructose-1-P kinase activity and 20-fold-lower levels of fructose-6-P kinase activity (Table 3). The former activity was comparable to activities found in fructose-grown *B. subtilis* (5) and *Streptococcus mutans* (4). The results establish that *L. monocytogenes* contains a fructose-1-P-forming PEP:fructose PTS as well as fructose-1-P kinase. Interestingly, the results in Table 3 also demonstrate that phosphofructokinase (fructose-6-P kinase) activity is specifically induced by growth in glucose-containing medium.

*L. monocytogenes* is capable of growth in media containing high salt concentrations (2, 6). We therefore examined the sensitivity of the fructose PTS to NaCl. The fructose-1-P-dependent transphosphorylation of fructose by the listerial PTS was found to be sensitive to NaCl (data not shown). The rate of phosphorylation decreased linearly with increasing salt concentrations up to 1 M, at which point around 10% of the activity remained. The transphosphorylation of fructose catalyzed by *B. subtilis* membranes exhibited similar salt sensitivity. Thus, despite the ability of *L. monocytogenes* to grow under conditions of high salt concentration, the results show that sugar transport by the PTS is not salt resistant and suggest a requirement for an effective mechanism of ion homeostasis in this organism.

[$^{14}$C]fructose uptake measurements for intact cells of *L. monocytogenes* revealed that this sugar was taken up rapidly when the cells had previously been grown in a fructose-containing medium. Uptake was not inhibited by uncouplers of oxidative phosphorylation such as CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) and its more potent analog, FCCP, both used at a concentration of up to 50 μM, consistent with a PTS being the route of accumulation of the substrate.

*L. monocytogenes* is capable of utilizing glucose and lactose, in addition to fructose, for growth (9). Attempts were made to identify PEP-dependent phosphorylation of radioactive lactose, methyl β-thiogalactoside, glucose, and

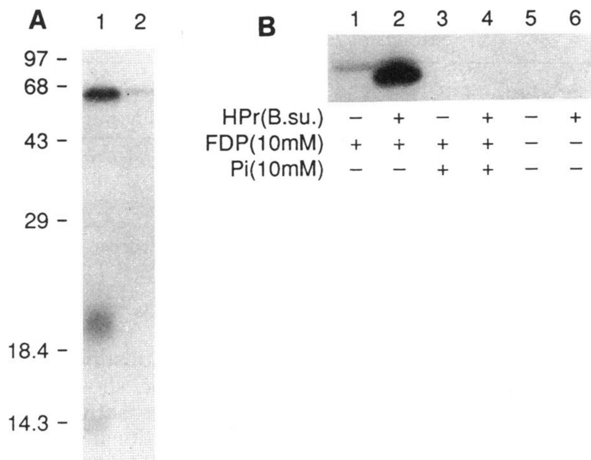


FIG. 1. In vitro phosphorylation of proteins in *L. monocytogenes* extracts by [$^{32}$P]PEP (A) and [$\gamma$-$^{32}$P]ATP (B). Extracts were prepared from cells grown in LB medium supplemented with fructose (0.5%). (A) Autoradiogram showing proteins labeled by incubation for 15 min at 37°C with [$^{32}$P]PEP (lane 1) and after a further 5-min incubation in the presence of 5 mM fructose (lane 2). Two phosphoproteins (20 and 60 kDa) are readily visible, while a third (14.3 kDa) was evident but faint in the autoradiogram. (B) Autoradiogram showing the fructose-1,6-bisphosphate-dependent, $P_i$-inhibited labeling of the *L. monocytogenes* HPr following incubation for 15 min at 37°C with [$\gamma$-$^{32}$P]ATP. Purified HPr from *B. subtilis* was added at a concentration of 2.5 μg/40 μl of reaction mixture. The single spot corresponds to a molecular mass of 14 kDa, as expected for HPr. FDP, fructose-1,6-bisphosphate.

methyl α-glucoside after growth of the cells in lactose-, glucose-, or fructose-containing media. In no case was convincing PEP-dependent phosphorylation of these sugars demonstrated. Fructose-specific PTSs in gram-negative bacteria have been reported previously (18), but this is the first report suggesting the presence of a fructose-specific PTS in a gram-positive bacterium.

As revealed by the autoradiogram shown in Fig. 1A, two proteins in the *L. monocytogenes* extract with molecular masses similar to those of enzyme I (60 kDa) and HPr (14 kDa) of other bacteria were strongly and faintly phosphorylated, respectively, when incubated with [$^{32}$P]PEP (lane 1). A third labeled band of intermediate molecular mass (~20 kDa) was also observed. The molecular size of this protein is suggestive of a IIA protein of the PTS (20). The addition of fructose to the prephosphorylated proteins resulted in nearly complete dephosphorylation of all three bands (Fig. 1A, lane 2). The 14-kDa protein could also be phosphorylated at the expense of $^{32}$P-labeled ATP (Fig. 1B) in a process which was activated by fructose-1,6-bisphosphate (compare lanes 1 and 5). Exogenously added HPr from *B. subtilis* was similarly phosphorylated by $^{32}$P-labeled ATP, and this activity was also dependent on fructose-1,6-bisphosphate (Fig. 1B, compare lanes 2 and 6). $P_i$ (lanes 3 and 4) and $PP_i$ (not shown) strongly inhibited phosphorylation of the endogenous HPr as well as the *B. subtilis* HPr. The phosphorylated protein from *L. monocytogenes* was resistant to acid treatment but labile under basic conditions (data not shown), as expected for a seryl phosphorylated protein (12, 14). These results are in accordance with expectations, assuming that *L. monocytogenes* possesses a metabolite-activated and ATP-dependent HPr kinase similar to those found in other low-G+C gram-positive bacteria (11, 13, 14, 15). HPr(ser-P) phosphatase activity was also demonstrated in extracts of *L. monocytogenes* (data not shown). These results therefore suggest not only that *L. monocytogenes* possesses a phosphotransferase system but also that it has the cyclic HPr seryl modification system presumed to function in the regulation of carbohydrate metabolism in gram-positive bacteria.

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