Fructose and mannose metabolism in *Aeromonas hydrophila*: identification of transport systems and catabolic pathways

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***Aeromonas hydrophila* was examined for fructose and mannose transport systems. *A. hydrophila* was shown to possess a phosphoenolpyruvate (PEP):fructose phosphotransferase system (fructose-PTS) and a mannose-specific PTS, both induced by fructose and mannose. The mannose-PTS of *A. hydrophila* exhibited cross-reactivity with *Escherichia coli* mannose-PTS proteins. The fructose-PTS proteins exhibited cross-reactivities with *E. coli* and *Xanthomonas campestris* fructose-PTS proteins. In *A. hydrophila* grown on mannose as well as on fructose, the phosphorylated derivative accumulated from fructose was fructose 1-phosphate. Identification of fructose 1-phosphate was confirmed by ¹³C-NMR spectroscopy. 1-Phosphofructokinase (1-PFK), which converts the product of the PTS reaction to fructose 1,6-diphosphate, was present in *A. hydrophila* grown with fructose but not on mannose. An inducible phosphofructomutase (PFM) activity, an unusual enzyme converting fructose 1-phosphate to fructose 6-phosphate, was detected in extracts induced by mannose or fructose. These results suggest that in cells grown on fructose, fructose 1-phosphate could be converted to fructose 1,6-diphosphate either directly by the 1-PFK activity or via fructose 6-phosphate by the PFM and 6-phosphofructokinase activities. In cells grown on mannose, the degradation of fructose 1-phosphate via PFM and the Embden–Meyerhof pathway appeared to be a unique route.**

Keywords: phosphotransferase system, *Aeromonas hydrophila*, fructose, mannose, catabolic pathways

INTRODUCTION

The genus *Aeromonas* used to belong to the family *Vibrionaceae* (Farmer, 1992). Colwell *et al.* (1986) classified the genus *Aeromonas* in a new family, the *Aeromonadaceae*. The phylogenetic position of the *Aeromonadaceae* is in the gamma group of Proteobacteria with other families such as *Enterobacteriaceae*, *Vibrionaceae* and *Pasteurellaceae* and genera *Pseudomonas* and *Xanthomonas* (Woese, 1987).

Aeromonas hydrophila causes disease in fish, frogs and several other animals (Farmer *et al.*, 1992). It is now recognized as an opportunistic pathogen of humans (Thornley *et al.*, 1997). To understand the ability of *A.*

hydrophila to grow and survive in its environment, it is essential to know the mechanisms by which nutrients are taken up and metabolized.

In many Gram-negative bacteria, the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) mediates active transport and concomitant phosphorylation of sugars and hexitols (Postma & Lengeler, 1985; Postma *et al.*, 1993). It consists of two general energy-coupling proteins (enzyme I and HPr) and a number of sugar-specific transmembrane permeases (enzymes II). In *Escherichia coli*, two enzyme II systems mediating glucose transport have been described (Curtis & Epstein, 1975). They can be distinguished by their different sugar specificities (Rephaeli & Saier, 1980; Scholte & Postma, 1981). The II^{Glc} system (glucose-PTS), of narrow specificity for glucose and α -methylglucoside, comprises a membrane-bound permease (enzyme IICB^{Glc}) and a soluble protein (enzyme IIA^{Glc}).

Abbreviations: KDPG, 2-keto-3-deoxy-6-phosphogluconate; PEP, phosphoenolpyruvate; 1-PFK, 1-phosphofructokinase; 6-PFK, 6-phosphofructokinase; PFM, phosphofructomutase; PTS, phosphotransferase system.

The second system (mannose-PTS), of broader specificity (including mannose, glucose, 2-deoxyglucose, N-acetylglucosamine, glucosamine and fructose with decreasing affinity in that order), comprises a IIA^{Man} protein (a hydrophilic protein) (Stolz *et al.*, 1993) which is phosphorylated during the phosphate transfer whereas IIC^{Man} and IID^{Man} (integral membrane proteins) are not (Erni & Zanolari, 1985; Williams *et al.*, 1986). In the family *Vibrionaceae*, 15 species studied contained a PTS analogous to the glucose-PTS in the *Enterobacteriaceae* (Meadow *et al.*, 1987). Four components (enzyme I, HPr, enzyme IIA^{Glc} and enzyme IICB^{Glc}) of the glucose-PTS were separated and purified in *Vibrio parahaemolyticus* (Kubota *et al.*, 1979). Glucose and mannose are also PTS sugars in *Vibrio furnissii* (Bouma & Roseman, 1996a, b).

For PTS-mediated fructose transport in *E. coli* (Hanson & Anderson, 1968; Saier *et al.*, 1976), a protein called FPr (fructose-specific HPr) was identified which combines the functions of the IIA^{Fru} domain and of an HPr-like protein (Geerse *et al.*, 1989). In this case, it is FPr rather than HPr that is phosphorylated by enzyme I. At a low concentration (less than 2 mM), fructose enters *E. coli* via the membrane-bound permease (enzyme IIBC^{Fru}) and appears inside the cell as fructose 1-phosphate; at higher concentrations, it can also enter via the membrane-bound permease (enzyme IID^{Man}) and appears inside the cell as fructose 6-phosphate. In *Rhodobacter capsulatus* (Saier *et al.*, 1971; Wu *et al.*, 1990) and *Xanthomonas campestris* (De Crécy-Lagard *et al.*, 1991a, b, 1995), a high-molecular-mass soluble protein called MTP (multiphosphoryl transfer protein) was identified which combines the functions of the enzyme IIA^{Fru}, HPr and enzyme I domains. During the process, fructose is phosphorylated to fructose 1-phosphate, which is further metabolized to fructose 1,6-diphosphate by 1-phosphofructokinase (1-PFK). In the present study, we report the presence of a mannose-specific PTS and a fructose-specific PTS both induced by fructose and mannose, and we propose a new fructose catabolic pathway in *A. hydrophila* grown with mannose.

METHODS

Bacterial strains and growth conditions. Strains used were: *Aeromonas hydrophila* CIP 7614^T, obtained from the Collection de l'Institut Pasteur; *Xanthomonas campestris* pathovar *campestris* NRRLB 1459 (Jeanes *et al.*, 1961); and *Escherichia coli* TP2811, F⁻ *xyl argH1 ilvA aroB ΔlacX74 Δ(ptsH ptsI crr)* Km^r (Lévy *et al.*, 1990).

For PTS assays and the preparation of crude extract and cytoplasmic and membrane fractions, *A. hydrophila* was grown in minimal medium (Tanaka *et al.*, 1967) supplemented with 0.03% (w/v) Casamino acids and 0.2% (w/v) fructose, mannose or Casamino acids at 30 °C with shaking for 12–15 h. *X. campestris* and *E. coli* TP2811 were grown in Luria-Bertani broth (Maniatis *et al.*, 1982) containing 0.2% (w/v) fructose or mannose at 30 °C with shaking for 12–15 h. Cells were harvested by centrifugation in the late exponential phase of growth.

Preparation of membrane and cytoplasmic fractions. Cells were washed three times with 20 mM Tris/HCl buffer (pH 7.5) containing 1 mM DTT and 1 mM EDTA and frozen at -20 °C. Cells were resuspended in the same buffer to approximately 0.2 g (wet wt) ml⁻¹ and were sonicated (Branson sonifier 450 tip 3) three times for 1 min in an ethanol/ice bath with 1 min allowed for cooling between sonications. The homogenate was centrifuged for 15 min at 5000 g to remove intact cells and cell debris. The resulting cell-free extract (crude extract) was centrifuged at 200 000 g for 3 h at 4 °C. The clear supernatant was removed and the membrane pellet was washed with 20 mM Tris/HCl buffer (pH 7.5) containing 1 mM DTT and 1 mM EDTA, and resuspended in the same buffer. Cytoplasmic fractions were concentrated three- to fivefold on Centricon 3 (Amicon). The membrane pellet, the cytoplasmic fraction and the crude extract were stored at 4 °C.

Assays for PEP:sugar PTS activity and ATP:sugar phosphorylation activity in toluene-treated cells. Cells were washed three times in M63 minimal medium (Miller, 1972) and then suspended in M63 minimal medium to 0.15 g wet weight ml⁻¹. Phosphorylation of [¹⁴C]fructose or [¹⁴C]mannose (250 μM; 290 mCi mmol⁻¹, 10.7 GBq mmol⁻¹) was assayed in cells which were treated with toluene. The optimal amount of toluene was determined previously using 0.5–10 μl toluene. A 1 ml aliquot of bacterial suspension was mixed with 2 μl toluene and incubated for 25 min at 30 °C. The reaction was monitored as described by Bouvet & Grimont (1987) at 30 °C. PEP or ATP was used as phosphate donor at a final concentration of 5 mM or 10 mM (respectively) and the reaction was stopped at different times by flash-freezing. The results are presented as the amount (nmol) min⁻¹ (mg protein)⁻¹ of labelled fructose or mannose converted to phosphorylated [¹⁴C]fructose or [¹⁴C]mannose in the presence or the absence of PEP or ATP and represent a mean of at least three determinations. For competition assays, the substrate specificity of membrane-bound permeases was determined by measuring the extent to which unlabelled sugars (fructose, mannose, 2-deoxyglucose and N-acetylglucosamine at 10 mM) reduced the rate of PEP-dependent phosphorylation of [¹⁴C]-fructose or [¹⁴C]mannose at 500 μM when added simultaneously to the reaction mixture. The results are expressed as percentage inhibition of the [¹⁴C]fructose or [¹⁴C]mannose phosphorylation.

Assays for PEP:sugar PTS activity in cell extracts. PTS activities were assayed by measuring the PEP-dependent phosphorylation of ¹⁴C-labelled sugars. The following reaction mixture was incubated at 30 °C: 10 mM MgCl₂, 1 mM DTT, 12.5 mM KF, 140 μl crude extract, cytoplasmic fraction or membranes in 50 mM sodium phosphate buffer (pH 7) with or without 10 mM PEP. The reaction was initiated by adding [¹⁴C]fructose or [¹⁴C]mannose to a final concentration of 250 μM (290 mCi mmol⁻¹, 10.7 GBq mmol⁻¹) or 2-deoxyglucose to a final concentration of 400 μM (10.7 GBq mmol⁻¹). The reaction was stopped at different times by flash-freezing and phosphorylated sugars were separated from sugar as previously described (Bouvet & Grimont, 1987). The results represent a mean of at least three determinations and were linear over 5 min.

Determination of kinetic constants of mannose- and fructose-PTS by uptakes of [¹⁴C]fructose and [¹⁴C]mannose in intact cells. To determine kinetic constants (*K_m* and *V_{max}*), the cell density was adjusted to OD₆₀₀ 0.15 and concentrations of [¹⁴C]fructose and [¹⁴C]mannose varying from 0.1 to 5 μM were added. The reaction mixture was incubated at 30 °C and

aliquots were taken at different times (each 10 s for 2 min), deposited on GF/C filters (Whatman; 2.5 cm diameter), then washed with M63 medium to remove unincorporated [^{14}C] fructose and [^{14}C]mannose. Each filter was dried and the radioactivity was measured in a scintillation counter. Results obtained were calculated as nmol ^{14}C -labelled sugars transported min^{-1} (mg protein^{-1}). Rates were linear for 20–30 s and kinetic constants were determined by Lineweaver–Burk plots.

Identification of fructose-PTS products by TLC and ^{13}C -NMR spectroscopy. PEP: sugar phosphorylation assays in toluene-treated cells of *A. hydrophila* induced by fructose or by mannose were performed as described above except that reactions were started by adding 500 μM [^{14}C]fructose (290 mCi mmol^{-1} , 10.7 GBq mmol^{-1}) or [$1\text{-}^{13}\text{C}$]fructose (EUR-ISO-top) in the presence of PEP (final concentration of 5 mM) or ATP (final concentration of 10 mM). The reaction was stopped after 5 min at 30 °C by flash-freezing. Aliquots (10 μl) were deposited on thin layer plates of MN300 cellulose (Merck) with reference compounds in the same way. Chromatography was performed as described by Ferenci & Kornberg (1973). Chromatograms were developed twice in ammonium acetate (pH 5), 95% (v/v) ethanol, 0.1 M disodium EDTA (30:70:1, by vol.) at 30 °C. The positions of free [^{14}C]fructose and the labelled reaction products were located by placing the plates in contact with Amersham Hyperfilm-MP. The position of reference compounds (fructose 1-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate) on the chromatograms was revealed using a spray composed of 4% (w/v) ammonium molybdate, butanol and 3 M sulfuric acid (10:25:10, by vol.) (Wawszkiewicz, 1961) and was used to identify labelled reaction products. The identification of products accumulated in toluene-treated cells grown on fructose and on mannose was confirmed by a ^{13}C -NMR experiment. The area containing the $1\text{-}^{13}\text{C}$ -labelled reaction product was located by autoradiography. After that, the zone was scraped off and the sorbent was collected. The $1\text{-}^{13}\text{C}$ -labelled reaction product was extracted with distilled water and analysed by ^{13}C -NMR spectroscopy.

NMR spectroscopy. All NMR data were recorded at 303 K on a Bruker AM250 spectrometer using a 10 mm broadband probe. The sample was introduced in an 8 mm NMR tube, itself inserted in a 10 mm NMR tube containing $^2\text{H}_2\text{O}$.

^{13}C -NMR spectra were acquired with composite pulse decoupling. The instrumental parameters were: operating frequency, 62.9 MHz; spectral width, 15 625 Hz; memory size, 32 K; recycling time, 2.05 s; number of transients, 108 000; and pulse angle, 40°. Exponential filtering of 3 Hz was applied prior to Fourier transformation. Chemical shifts were referred to tetramethylsilane through the use of the $\alpha\text{-C1}$ glucose resonance at 93.0 p.p.m. as an internal reference. Metabolite peaks were identified from their chemical shifts. The assignments were confirmed by adding authentic samples of each metabolite to the medium.

^{31}P -NMR spectra were acquired with composite pulse decoupling. The instrumental parameters were: operating frequency, 101.2 MHz; spectral width, 4065 Hz; memory size, 16 K; recycling time, 2.015 s; number of transients, 280; and pulse angle, 45°. Exponential filtering of 1 Hz was applied prior to Fourier transformation. Chemical shifts were given relative to external triethyl phosphate (0 p.p.m.), which was added to $^2\text{H}_2\text{O}$. Metabolites were identified as described before.

Preparation of cell-free extracts for enzyme assays. Bacteria were harvested in the late exponential growth phase, washed

three times in M63 minimal medium and resuspended to approximately 0.15 g (wet wt) ml^{-1} in 50 mM Tris buffer (pH 7.5) with 5 mM MgCl_2 , 2.5 mM DTT, 12.5 mM KF and 1% KCl.

This bacterial suspension was added to glass beads (0.10–0.11 mm diameter) and the mixture was vigorously shaken five times for 30 s at 4 °C (Bouvet *et al.*, 1994). After centrifugation (1 h at 12 000 g), the supernatant was assayed for enzyme activity. Supernatants were stored at –20 °C.

Enzyme assays. All assays were performed at room temperature and measured by spectrophotometric determination (at 340 nm) of the rate of NADH oxidation or the rate of NAD/NADP reduction. Specific activities were expressed as nmol substrate consumed min^{-1} (mg protein^{-1}) and represented means of at least three determinations. Activities of the following enzymes were assayed as described in previous publications: 6-phosphofructokinase (6-PFK) and 1-PFK (Baumann & Baumann, 1975), 6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (Lessie & Neidhart, 1967), phosphofructomutase (PFM), phosphoglucose isomerase, fructose-1,6-bisphosphatase and fructose-1,6-bisphosphate aldolase (Conrad & Schlegel, 1977), and glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Conrad & Schlegel, 1977).

Glucokinases and fructokinases were determined at 30 °C by a radioactive method (Postma & Stock, 1980) with or without 6 mM ATP. Sugar phosphates were separated from unphosphorylated sugars by ion exchange chromatography (DE81; Whatman). [^{14}C]Glucose and [^{14}C]fructose were used at a final concentration of 10 mM and 1 mM, respectively. To study substrate specificity, different substrates were added to the reaction mixture at final concentrations five- to tenfold higher than the radioactive substrate. For glucokinase activity, glucose, mannose and fructose (50 mM) were used. For fructokinase activities, glucose, fructose, mannose, fructose 1-phosphate, fructose 6-phosphate and fructose 1,6-diphosphate (10 mM) were used. Specific activities were expressed as nmol sugar utilized min^{-1} (mg protein^{-1}) and represented means of at least three determinations.

Protein assay. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as standard.

RESULTS

Sugar phosphorylation in toluene-treated cells and extracts

To identify PTS activities towards fructose and mannose in *A. hydrophila*, PEP-dependent phosphorylation experiments were conducted both in cell extracts and in toluene-treated cells (Tables 1 and 2). In toluene-treated cells (Table 1), *A. hydrophila* exhibited PEP-dependent phosphorylation of [^{14}C]fructose and [^{14}C]mannose (and its analogue 2-deoxy[^{14}C]glucose). Fructose-PTS activity was inducible by addition of fructose or mannose in the culture (Table 1). However, the mannose-PTS activity was induced to a low level during growth in the presence of fructose. When the same experiment was done using ATP instead of PEP, fructose was phosphorylated only in fructose-grown cells, mannose was weakly phosphorylated and 2-deoxyglucose was never phosphorylated by ATP (data not shown).

Table 1. PEP-PTS activity for different ^{14}C -labelled sugars in toluene-treated cells of *A. hydrophila*

Inducer*	^{14}C -labelled sugar†	PEP-dependent phosphorylation activity‡
Casamino acids	Fructose	0.4 (0.4)
	Mannose	0.3 (0.2)
Fructose	Fructose	10.1 (0.8)
	Mannose	1.5 (0.7)
	2-Deoxyglucose	1.0 (0.5)
Mannose	Mannose	8.6 (1.2)
	2-Deoxyglucose	1.7 (0.4)
	Fructose	9.6 (1.5)

* Inducer substrates were used at 0.2% in the culture.

† [^{14}C]Fructose and [^{14}C]mannose were used at a final concentration of 250 μM ; 2-deoxy[^{14}C]glucose was used at a final concentration of 400 μM .

‡ PEP-dependent phosphorylation was monitored at 30 °C as described in Methods. Results are expressed as nmol ^{14}C -labelled sugar phosphorylated $\text{min}^{-1} \text{mg}^{-1}$. Values in parentheses correspond to the phosphorylation activity without PEP.

Whatever induction was used (fructose or mannose), extracts of *A. hydrophila* exhibited PEP-dependent phosphorylation of [^{14}C]fructose and [^{14}C]mannose (Table 2). Separation of extracts into soluble and membrane fractions resulted in loss of activities (data

not shown), which were restored on recombining the two preparations (Table 2). These results suggested the presence of two PEP-dependent PTSs which transported and phosphorylated fructose and mannose. As for PTSs from other bacteria, these systems required the presence of cytoplasmic and membrane fractions. The detection of an ATP-dependent phosphorylation activity of fructose in fructose-grown cells suggested the presence of fructokinases in *A. hydrophila*.

When using heterologous complementation assays between membranes of *A. hydrophila* grown with mannose and the cytoplasmic fraction of *A. hydrophila* grown with fructose and inversely, a PEP-dependent phosphorylation of [^{14}C]fructose and [^{14}C]mannose (Table 2) was observed. These results demonstrated that each system is expressed in cells grown on fructose or mannose.

The specificity of membrane-bound permeases was determined by PEP-dependent phosphorylation of [^{14}C]fructose and [^{14}C]mannose (500 μM) and by competition with different sugars added (fructose, glucose, mannose and N-acetylglucosamine at 10 mM). In toluene-treated cells of *A. hydrophila* grown with fructose, the [^{14}C]fructose phosphorylation was inhibited by fructose (92%), while mannose, glucose and N-acetylglucosamine did not significantly inhibit the activity (data not shown). In the same way, in toluene-treated cells of *A. hydrophila* grown with mannose, the [^{14}C]mannose phosphorylation was strongly inhibited by mannose (86%), while fructose, glucose and N-acetylglucosamine

Table 2. Functional homology between *A. hydrophila* and *E. coli* mannose-PTSs and between *A. hydrophila*, *E. coli* and *X. campestris* fructose-PTSs by complementation assays

Aero M, *A. hydrophila* induced by mannose during the culture; Aero F, *A. hydrophila* induced by fructose; *E. coli* TP2811 F or M, *E. coli* TP2811 mutant ($\Delta ptsH ptsI crr$) induced by fructose or by mannose during the culture; XC F, *X. campestris* induced by fructose. The amount of protein introduced in the reaction mixture was between 0.8 and 1.2 mg.

Cytoplasmic fraction	Membrane fraction	^{14}C -labelled sugar*	Phosphorylation activity†
Aero M	Aero M	Mannose	1.14
Aero M	Aero F	Mannose	0.68
Aero F	Aero M	Mannose	0.75
Aero F	<i>E. coli</i> TP2811 M	2-Deoxyglucose	2.97
Aero M	<i>E. coli</i> TP2811 M	2-Deoxyglucose	3.74
Aero F	Aero F	Fructose	0.78
Aero F	Aero M	Fructose	0.81
Aero M	Aero F	Fructose	0.51
Aero F	<i>E. coli</i> TP2811 F	Fructose	0.85
Aero F	XC F	Fructose	0.13
Aero M	<i>E. coli</i> TP2811 F	Fructose	1.00
Aero M	XC F	Fructose	0.20

* [^{14}C]Fructose and [^{14}C]mannose were used at 250 μM and 2-deoxy[^{14}C]glucose was used at 400 μM .

† Results are expressed as nmol ^{14}C -labelled sugars phosphorylated $\text{min}^{-1} \text{mg}^{-1}$. Activities in experiments without PEP were from 0.04 to 0.06 nmol $\text{min}^{-1} (\text{mg protein})^{-1}$.

Fig. 1. Identification of [^{14}C]fructose-PTS products by TLC. The figure shows products of fructose phosphorylation in *A. hydrophila* induced by fructose, in the presence of PEP (lane 1) or ATP (lane 2) for 5 min, in *A. hydrophila* induced by fructose or by mannose without phosphate donor (lane 3), and in *A. hydrophila* induced by mannose in the presence of PEP (lane 4) for 5 min.

did not significantly inhibit the activity ($< 5\%$, data not shown).

Kinetic constants were determined by uptake of [^{14}C]fructose and [^{14}C]mannose in intact cell assays. Apparent K_m values for fructose and mannose uptakes were $2.8\ \mu\text{M}$ and $2.3\ \mu\text{M}$, respectively, and the apparent $V_{\max}$ of fructose uptake was $60.9\ \text{nmol min}^{-1} (\text{mg protein})^{-1}$ and $26.8\ \text{nmol min}^{-1} (\text{mg protein})^{-1}$ for mannose uptake.

Identification of fructose-PTS products by TLC and ^{13}C -NMR spectroscopy

To determine the product of fructose phosphorylation in *A. hydrophila* grown with fructose or mannose, the phosphorylated derivatives of fructose accumulated in toluene-treated cells were chromatographed as described in Methods. As shown in Fig. 1, phosphorylated derivatives accumulated from fructose in the presence of PEP (lane 1) in *A. hydrophila* grown with fructose were products having the same R_F values as fructose 1-phosphate and 3-phosphoglycerate. In the presence of ATP (lane 2), accumulated phosphorylated derivatives were products having the same R_F values as fructose 6-phosphate and 3-phosphoglycerate. Other unidentified

Fig. 2. Identification of fructose-PTS products accumulated in toluene-treated cells grown on mannose by ^{13}C -NMR spectroscopy. The resonance peaks were assigned to β - (67.4 and 67.3 p.p.m.) and α - (66.9 and 66.8 p.p.m.) fructofuranose 1-phosphate.

products were visible. Only traces of fructose 1-phosphate were detectable without phosphate donor (lane 3). In *A. hydrophila* grown with mannose, the phosphorylated derivative accumulated from fructose, in the presence of PEP (lane 4), was fructose 1-phosphate too. 3-Phosphoglycerate was not detected. The identification of the fructose-PTS product and 3-phosphoglycerate accumulated in toluene-treated cells grown on fructose and mannose was confirmed by ^{13}C -NMR spectroscopy (Fig. 2). The PEP-sugar phosphorylation assay was initiated by adding [$1\text{-}^{13}\text{C}$]fructose and the phosphorylation derivative labelled at the C1 position was identified. The resonance peaks were assigned to α - and β -fructofuranose 1-phosphate.

The results suggested the presence of a PEP:fructose-specific PTS in *A. hydrophila* grown on fructose or mannose which produced fructose 1-phosphate and the presence of a PEP:mannose-specific PTS in *A. hydrophila* grown with fructose or mannose. The fact that in cells grown on mannose, the phosphorylated derivative accumulated from fructose in the presence of PEP was not fructose 6-phosphate confirms that fructose could not be transported and phosphorylated by mannose-PTS.

Interaction with other PTS systems

The functional homology between the *A. hydrophila* fructose-PTS and mannose-PTS and PTSs from other bacteria was examined in heterologous sugar phosphorylation assays by using soluble and membrane fractions from different sources (Table 2). Membranes

Table 3. Specific activities of some enzymes of carbohydrate metabolism in cell-free extracts of *A. hydrophila*

Enzyme	Specific activity [nmol (mg protein) ⁻¹ min ⁻¹] after growth in minimal medium containing:		
	Casamino acids	Fructose	Mannose
6-PFK	<20	720	650
1-PFK	<30	257	<30
Phosphoglucose isomerase	620	1579	1610
PFM	<20	47	60
Fructose-bisphosphate aldolase	<30	329	457
Fructose-bisphosphatase	<20	48	68
Glucose-6-phosphate dehydrogenase	<10	<10	<10
6-Phosphogluconate dehydrogenase	<10	<10	<10
6-Phosphogluconate dehydratase and KDPG aldolase	<20	<20	<20

of *E. coli* TP2811 $\Delta(ptsH\ ptsI\ crr)$ grown with fructose or mannose or of *X. campestris* grown with fructose exhibited negligible activities of [¹⁴C]fructose and [¹⁴C]mannose phosphorylation (data not shown). The cytoplasmic fraction from *A. hydrophila* grown with fructose or mannose was capable of complementing membranes from *E. coli* TP2811 and *X. campestris* grown with fructose for phosphorylation of fructose (Table 2). However, values obtained with the *X. campestris* extract were very low (compared with other activities). This is probably due to the instability of *X. campestris* extracts. In the same way, the cytoplasmic fraction from *A. hydrophila* grown with fructose or mannose was capable of complementing membranes from *E. coli* TP2811 grown with mannose for phosphorylation of 2-deoxyglucose (Table 2).

The fructose-PTS in *A. hydrophila* grown with fructose or mannose exhibited cross-reactivity with *E. coli* and *X. campestris* cytoplasmic fructose-PTS proteins. The mannose-PTS in *A. hydrophila* grown with fructose and mannose exhibited cross-reactivity with *E. coli* cytoplasmic mannose-PTS proteins.

Catabolic pathways of *A. hydrophila*

An ATP-dependent phosphorylation of fructose, phosphorylating intracellular fructose to fructose 6-phosphate, was detected in *A. hydrophila* extracts. *A. hydrophila* exhibited fructokinase activity of 12.6 nmol phosphorylated [¹⁴C]fructose min⁻¹ (mg protein)⁻¹ and was 80% inhibited by fructose 1-phosphate, 69% by fructose 6-phosphate, 48% by fructose 1,6-diphosphate, 14% by mannose and 10% by glucose. This fructokinase activity was inducible by fructose and not by mannose. The activities of enzymes involved in glucidic metabolism were measured spectrophotometrically in cell-free extracts (Table 3). *A. hydrophila* grown with

fructose or mannose possessed enzymes characteristic of the Embden–Meyerhof pathway (6-PFK). Enzymes characteristic of the Entner–Doudoroff pathway (6-phosphogluconate dehydratase and KDPG aldolase) and of the pentose phosphate pathway (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) were not detected in crude extracts of *A. hydrophila*. Phosphoglucose isomerase and fructose-1,6-bisphosphate aldolase were present in fructose-grown cells as in mannose-grown cells. In addition, an inducible PFM activity (Conrad & Schlegel, 1977), an unusual enzyme converting fructose 1-phosphate to fructose 6-phosphate, was detected in extracts induced by fructose and mannose (Table 3).

The phosphorylation of fructose by the fructose-PTS of *E. coli* (Ferenci & Kornberg, 1974), *X. campestris* (De Crécy-Lagard *et al.*, 1991a) and *Streptococcus salivarius* (Bourassa & Vadeboncoeur, 1992) gives rise to fructose 1-phosphate, which is further metabolized to fructose 1,6-diphosphate by 1-PFK. The extract of *A. hydrophila* grown with fructose had detectable 1-PFK activity. No activity of this enzyme was detected in extracts prepared from *A. hydrophila* grown with mannose (Table 3). This surprising result was confirmed by ³¹P-NMR spectroscopy by incubating cell-free extracts with fructose 1-phosphate and ATP (data not shown). In the extract of *A. hydrophila* grown with fructose, resonances corresponding to ADP, P_i and fructose 1,6-diphosphate appeared during time-courses indicating 1-PFK activity. No activity could be detected in extracts of *A. hydrophila* grown with mannose.

DISCUSSION

The observations reported above demonstrate the presence of a fructose-specific PTS and a mannose-specific PTS which are both inducible by fructose and mannose.

Fig. 3. Proposed pathways for transport and metabolism of fructose and mannose in *A. hydrophila*. FK, fructokinase; MI, mannose-6-phosphate isomerase.

Each system requires both membrane and cytoplasmic fractions to be functional.

The mannose-PTS in *A. hydrophila* exhibited cross-reactivity with *E. coli* mannose-PTS proteins. The tight specificity of the mannose-PTS discovered in *A. hydrophila* contrasts with the broad specificity of the mannose-PTS of *E. coli*. Moreover, this system is present in *A. hydrophila* cells grown with mannose or fructose. Phosphorylated proteins with molecular masses similar to those of *E. coli* mannose-PTS proteins were visualized when incubated with [32 P]PEP (Mattoo & Waygood, 1983; Waygood *et al.*, 1984) (data not shown). Additional experiments are necessary to confirm identification of the labelled proteins.

In *E. coli* and other Gram-negative bacteria, enzyme IIA^{Glc}, a component of the PTS, regulates the adenylate cyclase activity (Lévy *et al.*, 1990) and plays an important regulation role in catabolic repression and in inducer exclusion depending on its phosphorylation state. The *A. hydrophila* adenylate cyclase gene (Trotot *et al.*, 1996) has been cloned and sequenced. The presence of a PTS system and of an adenylate cyclase in *A. hydrophila* could suggest a possible regulatory role of the PTS as in *E. coli* and other Gram-negative bacteria (Crasnier & Danchin, 1990; Feucht & Saier, 1980).

The transport and phosphorylation of fructose which yields fructose 1-phosphate and the substrate specificity restricted to fructose clearly demonstrates the presence of fructose-specific enzymes II. Phosphorylated proteins with molecular masses similar to those of *X. campestris* fructose-PTS proteins (enzyme IIBC^{Fr} and a high-molecular-mass protein, MTP-like) were detected using [32 P]PEP-dependent protein phosphorylation assays (data not shown). Further experiments are necessary to confirm identification of these labelled proteins. The fructose-PTS of *A. hydrophila* grown on fructose and mannose generates cytoplasmic fructose 1-phosphate.

This phosphate sugar is further phosphorylated to give fructose 1,6-diphosphate. In *A. hydrophila* grown with fructose, the presence of an inducible PEP-fructose-PTS and 1-PFK, together with a PFM activity and a 6-PFK activity, suggested that fructose 1-phosphate was directly degraded to fructose 1,6-diphosphate or via fructose 6-phosphate (Fig. 3). In mannose-grown cells, 1-PFK was not induced. Only the PFM and 6-PFK activities were present, suggesting that fructose 1-phosphate was degraded via fructose 6-phosphate. In *E. coli*, genes encoding the enzymes of the fructose-PTS and 1-PFK form an operon (Geerse *et al.*, 1989; Jones-Mortimer & Kornberg, 1974). The arrangement of these genes is not known in *A. hydrophila* but our results indicated that the regulation of the genes encoding the fructose-PTS and 1-PFK was not coordinated. To our knowledge, uncoordinated regulation of the fructose genes has never been found in Gram-negative bacteria, only in Gram-positive bacteria (Bourassa & Vadeboncoeur, 1992; Chassy & Thompson, 1983; Leblanc *et al.*, 1979).

A rare PFM activity was detected in extracts of cells induced by mannose and fructose. This prevents toxic accumulation of fructose 1-phosphate in the cells grown on mannose.

Durham & Phibbs (1982) reported that both soluble and membrane fractions of the fructose-PTS from *Pseudomonas aeruginosa* do not cross-complement the respective systems in *E. coli* and vice versa. In this study, we observed that *A. hydrophila* fructose-PTS proteins exhibit cross-reactivities and functional homologies with *E. coli* and *X. campestris* fructose-PTS proteins.

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REFERENCES

- Baumann, P. & Baumann, L. (1975). Catabolism of D-fructose and D-ribose by *Pseudomonas dooudoroffii*. I. Physiological studies and mutant analysis. *Arch Microbiol* **105**, 225–240.
- Bouma, C. L. & Roseman, S. (1996a). Sugar transport by the marine chitinolytic bacterium *Vibrio furnissii*. Molecular cloning and analysis of the glucose and N-acetylglucosamine permeases. *J Biol Chem* **271**, 33457–33467.
- Bouma, C. L. & Roseman, S. (1996b). Sugar transport by the marine chitinolytic bacterium *Vibrio furnissii*. Molecular cloning and analysis of the mannose/glucose permease. *J Biol Chem* **271**, 33468–33475.
- Bourassa, S. & Vadeboncoeur, C. (1992). Expression of an inducible Enzyme II fructose and activation of a cryptic Enzyme II glucose in glucose-grown cells of spontaneous mutants of *Streptococcus salivarius* lacking the low-molecular-mass form of III^{Man}, a component of the phosphoenolpyruvate:mannose phosphotransferase system. *J Gen Microbiol* **138**, 769–777.
- Bouvet, O. M. M. & Grimont, P. A. D. (1987). Diversity of the phosphoenolpyruvate:glucose phosphotransferase system in the *Enterobacteriaceae*. *Ann Inst Pasteur Microbiol* **138**, 3–13.
- Bouvet, O. M. M., Lenormand, P., Carlier, J. P. & Grimont, P. A. D. (1994). Phenotypic diversity of anaerobic glycerol dissimilation shown by seven enterobacterial species. *Res Microbiol* **145**, 129–139.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- Chassy, B. M. & Thompson, J. (1983). Regulation of phosphoenolpyruvate-dependent phosphotransferase system and β -D-phosphogalactoside galactohydrolase activities in *Lactobacillus casei*. *J Bacteriol* **154**, 1195–1203.
- Colwell, R. R., MacDonell, M. T. & DeLey, J. (1986). Proposal to recognize the family *Aeromonadaceae* fam. nov. *Int J Syst Bacteriol* **36**, 473–477.
- Conrad, R. & Schlegel, H. G. (1977). Different degradation pathways for glucose and fructose in *Rhodopseudomonas capsulata*. *Arch Microbiol* **112**, 39–48.
- Crasnier, M. & Danchin, A. (1990). Characterization of *Escherichia coli* adenylate cyclase mutants with modified regulation. *J Gen Microbiol* **136**, 1825–1831.
- Curtis, S. J. & Epstein, W. (1975). Phosphorylation of D-glucose in *Escherichia coli* mutants defective in glucosephosphotransferase, mannosephosphotransferase and glucokinase. *J Bacteriol* **122**, 1189–1199.
- De Crécy-Lagard, V., Bouvet, O. M. M., Lejeune, P. & Danchin, A. (1991a). Fructose catabolism in *Xanthomonas campestris* pv. *campestris*. *J Biol Chem* **266**, 18154–18161.
- De Crécy-Lagard, V., Bouvet, O. M. M., Lejeune, P. & Danchin, A. (1991b). Identification of two fructose transport and phosphorylation pathways in *Xanthomonas campestris* pv. *campestris*. *Mol Gen Genet* **227**, 465–472.
- De Crécy-Lagard, V., Binet, M. & Danchin, A. (1995). Fructose phosphotransferase system of *Xanthomonas campestris* pv. *campestris*: characterization of the *fruB* gene. *Microbiology* **141**, 2253–2260.
- Durham, D. R. & Pihbs, P. V. J. (1982). Fractionation and characterization of the phosphoenolpyruvate:fructose 1-phosphotransferase system from *Pseudomonas aeruginosa*. *J Bacteriol* **149**, 534–541.
- Erni, B. & Zanolari, B. (1985). The mannose-permease of the bacterial phosphotransferase system. Gene cloning and purification of the enzyme II^{Man}/III^{Man} complex of *Escherichia coli*. *J Biol Chem* **260**, 15495–15503.
- Farmer, J. J., III (1992). The family *Vibrionaceae*. In *The Prokaryotes*, 2nd edn, pp. 2938–2951. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer.
- Farmer, J. J., III, Arduino, M. J. & Hickman-Brenner, F. W. (1992). The genera *Aeromonas* and *Plesiomonas*. In *The Prokaryotes*, 2nd edn, pp. 3013–3045. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer.
- Ferenci, T. & Kornberg, H. L. (1973). The utilization of fructose by *Escherichia coli*. *Biochem J* **132**, 341–347.
- Ferenci, T. & Kornberg, H. L. (1974). Role of PTS mediated synthesis of fructose-1-P and fructose-6-P in the growth of *Escherichia coli* on fructose. *Proc R Soc Lond Ser B* **187**, 105–119.
- Feucht, B. U. & Saier, M. H., Jr (1980). Fine control of adenylate cyclase by the phosphoenolpyruvate sugar phosphotransferase systems in *Escherichia coli* and *Salmonella typhimurium*. *J Bacteriol* **141**, 603–610.
- Geerse, R. H., Izzo, F. & Postma, P. W. (1989). The PEP:fructose phosphotransferase system in *Salmonella typhimurium*: FPr combines Enzyme III^{fru} and pseudo-HPr activities. *Mol Gen Genet* **216**, 517–525.
- Hanson, T. E. & Anderson, R. L. (1968). Phosphoenolpyruvate-dependent formation of D-fructose-1-phosphate by a four-component phosphotransferase system. *Proc Natl Acad Sci USA* **61**, 269–276.
- Jeanes, A., Pittsley, J. E. & Senti, F. R. (1961). Polysaccharide B-1459: a new hydrocolloid polyelectrolyte produced from glucose by bacterial fermentation. *J Appl Polym Sci* **5**, 519–526.
- Jones-Mortimer, M. C. & Kornberg, H. L. (1974). Genetic analysis of fructose utilization by *Escherichia coli*. *Proc R Soc Lond Ser B* **187**, 121–131.
- Kubota, Y., Iuchi, S., Fujisawa, A. & Tanaka, S. (1979). Separation of four components of the phosphoenolpyruvate:glucose phosphotransferase system in *Vibrio parahaemolyticus*. *Microbiol Immunol* **23**, 131–146.
- Leblanc, D. J., Crow, V. L., Lee, L. N. & Garon, C. F. (1979). Influence of the lactose plasmid on the metabolism of galactose by *Streptococcus lactis*. *J Bacteriol* **137**, 878–884.
- Lessie, T. G. & Neidhart, F. C. (1967). Adenosine triphosphate-linked control of *Pseudomonas aeruginosa* glucose-6-phosphate dehydrogenase. *J Bacteriol* **93**, 1337–1345.
- Lévy, S., Zeng, G. Q. & Danchin, A. (1990). Cyclic AMP synthesis in *Escherichia coli* strains bearing known deletions in the *pts* phosphotransferase operon. *Gene* **86**, 27–33.
- Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Mattoo, R. L. & Waygood, E. B. (1983). An enzymatic method for [³²P]phosphoenolpyruvate synthesis. *Anal Biochem* **128**, 245–249.
- Meadow, N. D., Revuelta, R., Chen, V. N., Colwell, R. R. & Roseman, S. (1987). Phosphoenolpyruvate:glucose phosphotransferase system in species of *Vibrio*, a widely distributed marine bacterial genus. *J Bacteriol* **169**, 4893–4900.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

- Postma, P. W. & Lengeler, J. W. (1985). Phosphoenolpyruvate: carbohydrate phosphotransferase system of bacteria. *Microbiol Rev* 49, 232–269.
- Postma, P. W. & Stock, J. B. (1980). Enzymes II of the phosphotransferase system do not catalyse sugar transport in the absence of phosphorylation. *J Bacteriol* 141, 476–484.
- Postma, P. W., Lengeler, J. W. & Jacobson, G. R. (1993). Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. *Microbiol Rev* 57, 543–594.
- Rephaeli, A. W. & Saier, M. H., Jr (1980). Substrate specificity and kinetic characterization of sugar uptake and phosphorylation, catalyzed by the mannose enzyme II of the phosphotransferase system in *Salmonella typhimurium*. *J Biol Chem* 255, 8585–8591.
- Saier, M. H., Jr, Feucht, B. U. & Roseman, S. (1971). Phosphoenolpyruvate-dependent fructose phosphorylation in photosynthetic bacteria. *J Biol Chem* 246, 7819–7821.
- Saier, M. H., Jr, Simoni, R. D. & Roseman, S. (1976). Sugar transport. Properties of mutant bacteria defective in proteins of the phosphoenolpyruvate:sugar phosphotransferase system. *J Biol Chem* 251, 6584–6597.
- Scholte, B. J. & Postma, P. W. (1981). Competition between two pathways for sugar uptake by the phosphoenolpyruvate-dependent sugar phosphotransferase system in *Salmonella typhimurium*. *Eur J Biochem* 114, 51–58.
- Stolz, B., Huber, M., Markovic-Housley, Z. & Erni, B. (1993). The mannose transporter of *Escherichia coli*. Structure and function of the IIAB^{Man} subunit. *J Biol Chem* 268, 27094–27099.
- Tanaka, S., Lerner, S. A. & Lin, E. (1967). Replacement of a phosphoenolpyruvate-dependent phosphotransferase by a nicotinamide adenine dinucleotide-linked dehydrogenase for the utilization of mannitol. *J Bacteriol* 93, 642–648.
- Thornley, J. P., Shaw, J. G., Gryllos, I. A. & Eley, A. (1997). Virulence properties of clinically significant *Aeromonas* species: evidence for pathogenicity. *Rev Med Microbiol* 8, 61–72.
- Trotot, P., Sismeiro, O., Vivarès, C., Glaser, P., Bresson-Roy, A. & Danchin, A. (1996). Comparative analysis of the *cya* locus in enterobacteria and related Gram-negative facultative anaerobes. *Biochimie* 78, 277–287.
- Wawszkiewicz, E. J. (1961). A two dimensional system of paper chromatography of some sugar phosphates. *Anal Chem* 33, 252–254.
- Waygood, E. B., Mattoo, R. L. & Peri, K. G. (1984). Phosphoproteins and the phosphoenolpyruvate:sugar phosphotransferase system in *Salmonella typhimurium* and *Escherichia coli*: evidence for II^{mannose}, III^{fructose}, III^{glucitol}, and the phosphorylation of Enzyme II^{mannitol} and Enzyme II^{N-acetylglucosamine}. *J Cell Biochem* 25, 139–159.
- Williams, N. D., Fox, K., Shea, C. & Roseman, S. (1986). Pel, the protein that permits λ DNA penetration of *E. coli*, is encoded by a gene in *pts M* and is required for mannose utilization by the phosphotransferase system. *Proc Natl Acad Sci USA* 83, 8934–8938.
- Woese, C. R. (1987). Bacterial evolution. *Microbiol Rev* 51, 221–271.
- Wu, L. F., Tomich, J. M. & Saier, M. H., Jr (1990). Structure and evolution of a multidomain multiphosphoryl transfer protein. Nucleotide sequence of the *fruB* (HI) gene in *Rhodobacter capsulatus* and comparisons with homologous genes from other organisms. *J Mol Biol* 213, 687–703.

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