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^{31}P-NMR and ^{13}C-NMR studies of mannose metabolism in *Plesiomonas shigelloides*

Toxic effect of mannose on growth

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The metabolism of mannose was examined in resting cells *in vivo* using ^{13}C-NMR and ^{31}P-NMR spectroscopy, in cell-free extracts *in vitro* using ^{31}P-NMR spectroscopy, and by enzyme assays. *Plesiomonas shigelloides* was shown to transport mannose by a phospho*enol*pyruvate-dependent phosphotransferase system producing mannose 6-phosphate. However, a toxic effect was observed when *P. shigelloides* was grown in the presence of mannose.

Investigation of mannose metabolism using *in vivo* ^{13}C NMR showed mannose 6-phosphate accumulation without further metabolism. In contrast, glucose was quickly metabolized under the same conditions to lactate, ethanol, acetate and succinate. Extracts of *P. shigelloides* exhibited no mannose-6-phosphate isomerase activity whereas the key enzyme of the Embden–Meyerhof pathway (6-phosphofructokinase) was found. This result explains the mannose 6-phosphate accumulation observed in cells grown on mannose. The levels of phospho*enol*pyruvate and P_i were estimated by *in vivo* ^{31}P-NMR spectroscopy. The intracellular concentrations of phospho*enol*pyruvate and P_i were relatively constant in both starved cells and mannose-metabolizing cells. In glucose-metabolizing cells, the phospho*enol*pyruvate concentration was lower, and about 80% of the P_i was used during the first 10 min. It thus appears that the toxic effect of mannose on growth is not due to energy depletion but probably to a toxic effect of mannose 6-phosphate.

Keywords: mannose; metabolism; microbial diversity; NMR; *Plesiomonas shigelloides*.

The genus *Plesiomonas* appears to occupy a position between the families Enterobacteriaceae and Vibrionaceae in the gamma group of Proteobacteria [1]. *Plesiomonas shigelloides* is a facultatively anaerobic chemo-organotrophic Gram-negative bacterium. It has been implicated as the causative agent of gastroenteritis as well as of extraintestinal infections, primaly septicemia and meningitis [2]. To understand the ability of *P. shigelloides* to 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 specific phospho*enol*-pyruvate-dependent phosphotransferase system (PTS) mediates active transport and concomitant phosphorylation of sugars and hexitols [3,4]. The PTS uses phospho*enol*pyruvate as the phosphoryl donor in a transfer chain that involves two general energy-coupling enzymes, Enzyme I and HPr, and the sugar-specific membrane-bound Enzyme II complex across the cell membrane.

In order to study the metabolic diversity in Gram-negative bacteria, we have investigated sugar metabolism in *P. shigelloides*. In the present study, we show that mannose is transferred into the cells by a PTS producing mannose 6-phosphate. Curiously, when *P. shigelloides* was grown in nutrient broth supplemented with mannose, a lower growth yield than on nutrient broth was observed. Mannose and glucose catabolism were investigated *in vivo*, in resting cells using ^{13}C-NMR spectroscopy and *in vitro*, in cell-free extracts using ^{31}P-NMR spectroscopy and by enzyme assays.

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Abbreviation: PTS, phospho*enol*pyruvate-dependent phosphotransferase system.

Enzymes: ATP–D-fructose-6-phosphate-1-phosphotransferase (6-phosphofructokinase; EC 2.7.1.11); mannose-6-phosphate isomerase (EC 5.3.1.8).

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METHODS

Bacterial strains and growth conditions

Twenty-two *P. shigelloides* strains, *Proteus vulgaris* CDC 1404-81 and *Serratia marcescens* 504 were obtained from the Unité des Entérobactéries. They were grown aerobically at 30 °C in nutrient broth or nutrient broth containing 0.2% (w/v) glucose or mannose. The cells were collected at late-exponential phase by centrifugation at 4 °C and washed twice in 100 mM phosphate buffer (K_2HPO_4/NaH_2PO_4, 6 : 4, v/v) at pH 7.0. *Escherichia coli* TP 2811: F^-, *xyl*, *arg* H1, *ilvA*, *aroB*, Δ*lac*X74, Δ(*pts*H, *pts*I, *crr*) Km^r (Lévy, 1990) was grown in Luria–Bertani broth containing 0.2% (w/v) glucose.

Assay of phospho*enol*pyruvate-dependent phosphorylating activity of [^{14}C]mannose (PTS activity for mannose), mannose-6-phosphate isomerase and 6-phosphofructokinase

Phospho*enol*pyruvate-dependent and ATP-dependent phosphorylation of [^{14}C]mannose (2 mM, 10.7 GBq·mmol^{-1}) and 2-deoxy[^{14}C]glucose (0.4 mM, 10.7 GBq·mmol^{-1}) were assayed in toluenized cells of *P. shigelloides* grown on nutrient broth,

nutrient broth + mannose or nutrient broth + glucose as described previously [5]. For enzyme assays, pellets were suspended to ≈ 0.15 g (wet weight) per mL in 10 mM Tris buffer, pH 7.5, with 10 mM $MgCl_2$ and 1 mM dithiothreitol. 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. After centrifugation (1 h at 12 000 ***g***), enzyme activities were assayed in the cell-free extract. For mannose-6-phosphate isomerase assay, the reaction mixture contained 0.05 M Tris/HCl (pH 8), 5 mM $MgCl_2$, 1.2 $U \cdot mL^{-1}$ phosphoglucoisomerase, 1.2 $U \cdot mL^{-1}$ glucose-6-phosphate dehydrogenase, 0.5 mM NADP and cell-free extract in a total volume of 2 mL. A_{340} was monitored at room temperature. Subsequently, mannose 6-phosphate (100 mM) was added and the linear increase in absorbance was measured. The assay for 6-phosphofructokinase was as described previously [6]. Specific activities were expressed as nmol substrate consumed$\cdot min^{-1} \cdot (mg\ protein)^{-1}$ and were means of at least three determinations. Protein concentrations were determined with the Coomassie blue reagent [7].

Complementation assays

Membrane and cytoplasmic fractions were prepared as described previously [5]. PTS activities were assayed by measuring the phospho*enol*pyruvate-dependent phosphorylation of α-[^{14}C]methylglucose. Under appropriate assay conditions (low concentration), α-methylglucose is the specific substrate for the II^{Glc} system. The following reaction mixture was incubated at 30 °C: 10 mM $MgCl_2$, 1 mM dithiothreitol, 12.5 mM KF, crude extract, cytoplasmic fraction or membranes in 50 mM sodium phosphate buffer, pH 7, with or without 10 mM phospho*enol*pyruvate. The reaction was initiated by adding α-[^{14}C]methylglucose to a final concentration of 0.4 mM (10.7 GBq$\cdot mmol^{-1}$). The reaction was stopped by flash freezing, and phosphorylated sugars were separated from sugars as previously described [5].

Preparation of whole cells for ^{13}C-NMR studies

The pellets were suspended in an equal volume of the 100 mM phosphate buffer at pH 7.0 to ≈ 5×10^{10} cells$\cdot mL^{-1}$. The cell suspension was then incubated in an NMR spectrometer at 30 °C. At time zero, [^{13}C]glucose labeled at C1 ([1-^{13}C]glucose > 99 atom% ^{13}C) or [^{13}C]mannose labeled at C6 ([6-^{13}C]mannose > 99 atom% ^{13}C) were added to give a final concentration of 50 mM and 10 mM, respectively.

Preparation of neutralized perchloric acid extracts for ^{31}P-NMR studies

The pellets were resuspended to ≈ 5×10^{10} cells$\cdot mL^{-1}$ in a buffer containing 50 mM Mes, 100 mM Pipes, 60 mM NaCl, 1 mM Na_2HPO_4 and 1 mM KH_2PO_4, pH 7.3. Samples of cell suspension (2 mL) were incubated at 30 °C. The reaction was initiated by adding the carbon sources glucose and mannose to a final concentration of 50 mM and terminated after the required incubation time by the addition of cold perchloric acid as described by Lohmeier-Vogel *et al*. [8].

NMR spectroscopy

All NMR data were recorded at 303 K on a Bruker AM250 spectrometer using a 10-mm broad-band probe. Sample was introduced in a 8-mm NMR tube, itself inserted in a 10-mm NMR tube containing D_2O. ^{13}C-NMR spectra were obtained at 62.9 MHz and ^{31}P-NMR spectra at 101.2 MHz [9].

^{13}C-NMR data were acquired over 5 min (256 scans) with composite pulse decoupling. Exponential filtering of 1 Hz was applied before Fourier transformation. Chemical shifts were referred to the α-C1 resonance of D-glucose (93.0 p.p.m.). Metabolite peaks were identified from their chemical shifts. Their assignments were confirmed by adding authentic samples of each metabolite to the medium.

^{31}P-NMR data were acquired over 57 min (1600 scans) with composite pulse decoupling. Exponential filtering of 3 Hz was applied before Fourier transformation. Chemical shifts were given relative to an external reference (0 p.p.m., 6 mM triethyl phosphate) added to D_2O. Metabolites were identified as described above. Concentrations of mannose 6-phosphate, P_i and phospho*enol*pyruvate were determined by NMR analysis. A series of ^{13}C-NMR and ^{31}P-NMR spectra for each product at different known concentrations were recorded in the cell-free media. Metabolite concentrations were determined from the standard concentration curves obtained as described previously [9].

RESULTS

Study of mannose phenotype in *P. shigelloides*

Mannose utilization was investigated in *P. shigelloides* (22 strains) and *P. vulgaris* CDC 1404-81 using Biotype 100 carbon source utilization strips (BioMérieux, Marcy l'Etoile, France) (Table 1). *S. marcescens* 504, which is known to ferment mannose, was used as a control. This experiment shows that all strains of *P. shigelloides* and *P. vulgaris* are unable to (a) produce acid from mannose [1] and (b) use mannose as sole carbon source. However, when *P. shigelloides* was grown on nutrient broth supplemented with mannose (Table 1, Fig. 1), a lower growth yield than on nutrient broth alone was observed. Under the same conditions, *P. vulgaris* growth was not altered by addition of mannose. To explain this result, activities of enzymes involved in mannose metabolism were measured spectrophotometrically in cell-free extract (Table 1). Mannose

Table 1. Investigation of mannose metabolism in *P. shigelloides*, *P. vulgaris* and *S. marcescens*. Mannose utilization as sole carbon source was studied using Biotype 100 carbon source strips. M6P isomerase, mannose-6-phosphate isomerase; NB, nutrient broth. Enzyme activities are mean ± SD.

Species	Mannose utilization	Growth on:			Activity ($nmol \cdot min^{-1} \cdot mg^{-1}$)	
		NB	NB + mannose	NB + glucose	M6P isomerase	6-Phosphofructokinase
P. shigelloides	–	0.8	0.5	1.3	< 5	425 ± 30
P. vulgaris	–	0.8	0.8	1.4	< 5	380 ± 30
S. marcescens	+	0.9	1.4	1.5	372 ± 20	440 ± 30

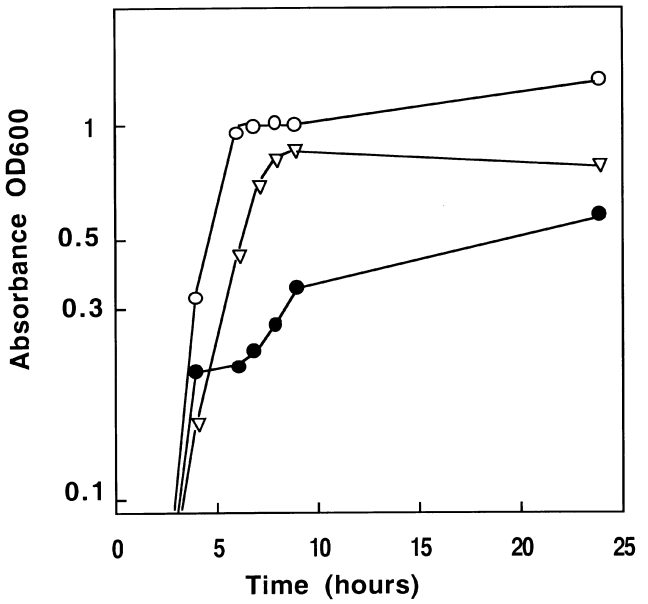

Fig. 1. Growth of *P. shigelloides* 27-77 cells grown in nutrient broth (▽), nutrient broth + glucose (○) or nutrient broth + mannose (●), as a function of time.

6-phosphate isomerase was not detected in extracts of *P. shigelloides* and *P. vulgaris*. Extracts of *P. shigelloides* had levels of 6-phosphofructokinase equivalent to those of *P. vulgaris* and *S. marcescens*.

Mannose PTS activity

In toluenized cells, *P. shigelloides* strains grown on mannose or glucose exhibited phospho*enol*pyruvate-dependent phosphorylation of [^{14}C]mannose (15 ± 2 nmol·min^{-1}) and 2-deoxy[^{14}C]glucose (9 ± 2 nmol·min^{-1}). When the same experiment was performed using ATP instead of phospho*enol*pyruvate, mannose was very weakly phosphorylated (2 nmol·min^{-1}) and 2-deoxyglucose was never phosphorylated. ATP did not serve as an alternative phosphate donor. Phosphorylation activities without phospho*enol*pyruvate or ATP were 1 nmol·min^{-1}. These phospho*enol*pyruvate-dependent phosphorylation activities were also detected in strains of *P. shigelloides* grown without mannose or glucose. The detection of a phospho*enol*pyruvate-dependent phosphorylation activity of [^{14}C]mannose and its analog 2-deoxy[^{14}C]glucose suggested the presence of a PTS activity for mannose in *P. shigelloides*. Evidence for the presence of Enzyme I and HPr, two components of PTS, was obtained with complementation assays (Table 2). The *E. coli* TP2811 Δ(*ptsH,ptsI,crr*) extract or the *P. shigelloides* 27-77 cytoplasmic fraction exhibited negligible phosphotransferase activity. When these two extracts were mixed, phospho*enol*pyruvate-dependent phosphorylation of the glucose analog α-[^{14}C]methylglucose was recovered; *P. shigelloides* was able to complement the *E. coli* Δ(*ptsH,ptsI,crr*) mutant extract for α-[^{14}C]methylglucose uptake.

The utilization of *E. coli* TP2811 membranes mixed with the *P. shigelloides* cytoplasmic fraction showed a phospho*enol*pyruvate-dependent phosphorylation analogous to that of *E. coli* TP 2811 crude extract mixed with *P. shigelloides* cytoplasmic fraction. *P. shigelloides* appears therefore to

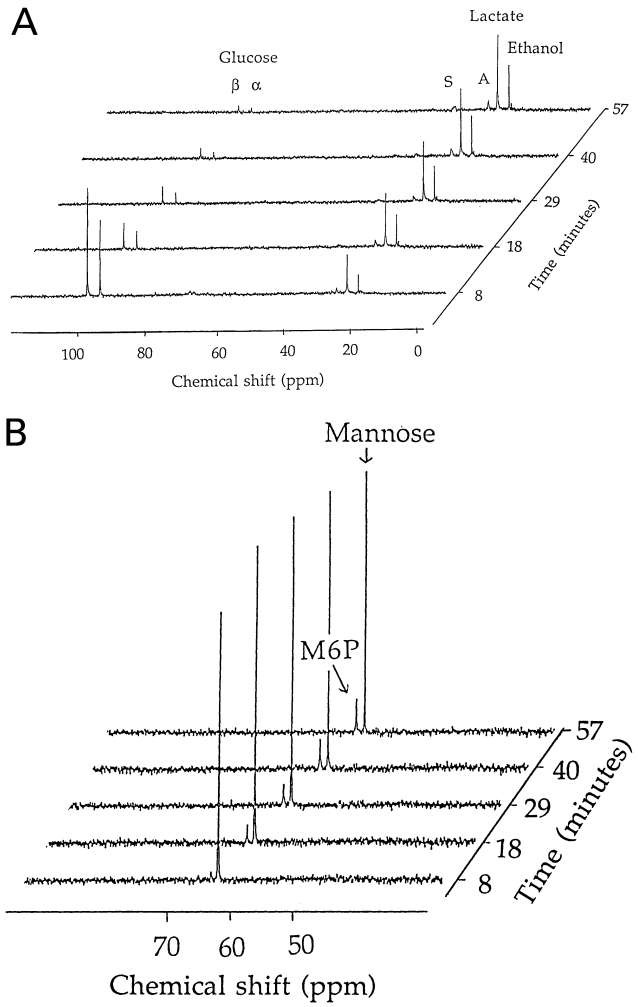

Fig. 2. ^{13}C-NMR spectra of *P. shigelloides* whole cells at 30 °C as a function of time after addition of (A) [1-^{13}C]glucose or (B) [6-^{13}C]mannose. The cells were suspended in 100 mM phosphate buffer at pH 7.0. [1-^{13}C]glucose or [6-^{13}C]mannose was added to a final concentration of 50 mM in the NMR sample at time 0. In (A) α and β anomers are visible as well as four end products of glucose metabolism [lactate, ethanol, succinate (S), acetate (A)]. In (B) mannose 6-phosphate (M6P) can be seen. The time given for each spectrum indicates the middle of the accumulation period, referred to glucose addition.

Table 2. Presence of Enzyme I and HPr in *P. shigelloides* demonstrated by complementation assays. [^{14}C]α-MG, α-[^{14}C]methylglucose used at final concentration of 0.4 mM. Values in parentheses are the amount of protein present in the reaction mixture. PEP, phospho*enol*pyruvate.

P. shigelloides cytoplasmic fraction	*E. coli* Tp 2811 Δ(ptsH,ptsI,crr)	PEP	Phosphorylation activity (nmol [^{14}C]αMG-P·min^{-1})
None	Crude extract (642 mg)	–	0.2
		+	0.3
+ (100 mg)	None	–	0.2
		+	0.3
+ (100 mg)	Crude extract (451 mg)	–	0.2
		+	1.6
+ (100 mg)	Membranes (286 mg)	–	0.5
		+	7.5

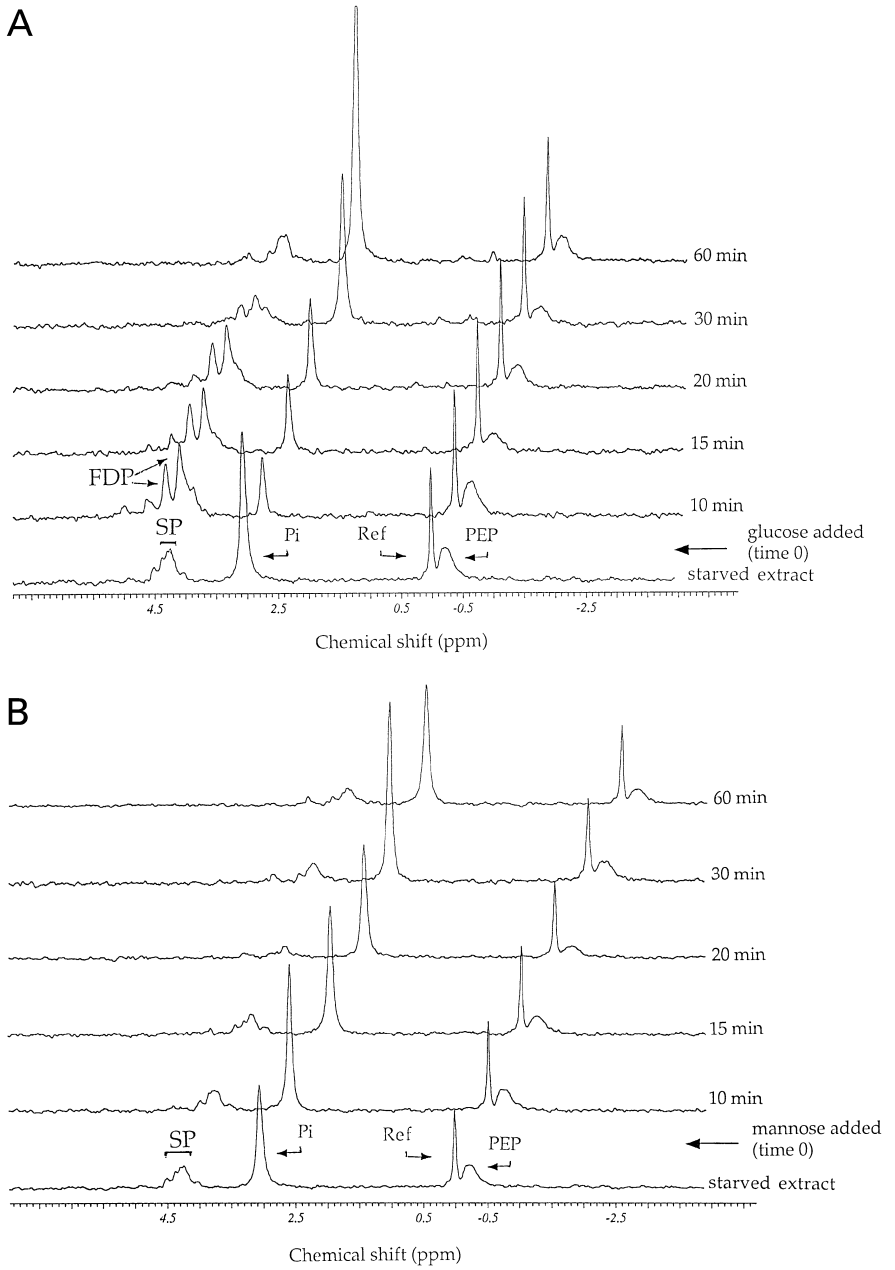

Fig. 3. ^{31}P-NMR spectra of perchloric acid extracts of *P. shigelloides* as a function of time after addition of (A) glucose or (B) mannose. SP, sugar phosphate; FDP, fructose 1,6-bisphosphate; PEP, phospho*enol*pyruvate.

possess cytoplasmic proteins analogous to EI, HPr and IIA of *E. coli* and exhibited cross-reactivity with *E. coli* PTS proteins.

Surprisingly, it appears that *P. shigelloides* is able to transport mannose but is unable to utilize it as a carbon source. So the mannose catabolism was investiged using *in vivo* ^{13}C-NMR spectroscopy.

^{13}C-NMR studies of whole cells

Metabolism of glucose and mannose was investigated using glucose labeled at C1 ([1-^{13}C]glucose) and mannose labeled at C6 ([6-^{13}C]mannose). When [1-^{13}C]glucose was added to whole cells of *P. shigelloides* 27-77, the α-anomer (93 p.p.m.) and β-anomer (96.8 p.p.m.) of glucose were rapidly consumed (Fig. 2A). Consumption of glucose was accompanied by the appearance of the resonance peaks of [2-^{13}C]acetate (24.1 p.p.m.), [3-^{13}C]lactate (21.1 p.p.m.) and [2-^{13}C]ethanol (17.8 p.p.m.). Later, the resonance peak of [3-^{13}C]succinate (34.9 p.p.m.) emerged.

When [6-^{13}C] mannose (61.9 p.p.m.) was added to whole cells of *P. shigelloides* (Fig. 2B), all the label ended up in [6-^{13}C]mannose 6-phosphate (64.4 p.p.m.) (M6P). The identity of the product was confirmed by adding a sample of mannose 6-phosphate to the extract. Only 15% of the added mannose was consumed, and the mannose 6-phosphate content was 2.8 (12.1 mM) mmol per 100 mg (dry weight) of cells. Calculation of the intracellular concentration is based on the assumption that 1 g (dry weight) of bacteria corresponds to 2.33 g of intracellular water [10]. End products of glucose metabolism (lactate, ethanol, acetate and succinate) were not detected. It appears that, in *P. shigelloides*, a smaller quantity of mannose is transported and phosphorylated to mannose 6-phosphate without further metabolism. To study the energetic and metabolic

changes brought about by the mannose transport, intracellular levels of phospho*enol*pyruvate and P_i were estimated by *in vivo* ^{31}P-NMR spectroscopy.

^{31}P-NMR studies of extracts

^{31}P-NMR spectra of cell extracts of *P. shigelloides* at different times after glucose or mannose addition are shown in Fig. 3. The spectra obtained from resting cells prepared before glucose addition (named starved extract) are shown in Fig. 3A. There is a very broad peak (4–4.5 p.p.m.) due to sugar phosphate (SP), the P_i peak at 3 p.p.m., the reference peak at 0 p.p.m. and the phospho*enol*pyruvate (PEP) peak at −0.22 p.p.m. The major components found in the SP region were 3-phosphoglyceric acid and fructose-1,6-bisphosphate. After addition of glucose to resting cells, as seen at 10 min, the broad SP peak was split into a typical fructose-1,6-bisphosphate pattern (two major peaks at 4.5 and 4.7 p.p.m.). At the same time, the P_i peak decreased. These results are in good agreement with those of Lohmeier-Vogel *et al*. [8] and Thompson & Torchia [11], who reported that the FDP increase and the P_i decrease were correlated events. At the end of the glycolysis, in the 30 min spectrum, the P_i increased and a steady-state was attained.

The spectra obtained after addition of mannose to resting cells (Fig. 3B) were very similar to those obtained from starved cells. No change appeared in the SP region of the spectrum. The intracellular concentration of P_i was relatively constant (10–13 mM), whereas, in glucose-metabolizing cells, about 80% of the P_i was used in the first 10 min. These results agree well with the reported values in *E. coli* [12]. The intracellular concentration of phospho*enol*pyruvate was relatively constant in both starved and mannose-metabolizing cells (28 and 22 mM, respectively). Similar values for the intracellular concentration of phospho*enol*pyruvate (30 mM) were recorded in *E. coli* by Chen *et al*. [13]. In glucose-metabolizing cells, about 50% of the phospho*enol*pyruvate was used during the first 15 min.

DISCUSSION

Species of the genus *Plesiomonas* are known to be unable to use mannose as carbon source [1]. However, the observations reported above demonstrate that, in *P. shigelloides*, mannose is transferred into the cells by a PTS producing mannose 6-phosphate. This system exhibited cross-reactivity with *E. coli* mannose-PTS proteins. Investigation of mannose metabolism by *in vivo* ^{13}C NMR, shows mannose 6-phosphate accumulation without further metabolism. Only 15% of the added mannose was transported. In contrast, glucose was quickly metabolized under the same conditions to four end products (lactate, ethanol, acetate, succinate). Lactate and ethanol are the major end products of glucose metabolism.

Addition of [1-^{13}C]glucose to whole cells of *P. shigelloides* resulted in the formation of C2-labeled ethanol, C3-labeled lactate, C2-labeled acetate and C2-labeled and C3-labeled succinate. This labeling pattern confirms that glucose is converted via the Embden–Meyerhof pathway [9].

Enzymological data indicated that extracts of *P. shigelloides* exhibited no mannose-6-phosphate isomerase activity whereas the key enzyme of the Embden–Meyerhof pathway (6-phosphofructokinase) was found (Fig. 4). This result explains the mannose 6-phosphate accumulation observed in cells grown on mannose. Toxic utilization of carbohydrate by accumulation of hexose phosphate is usually reported in mutant strains. For example, toxic accumulation of hexose phosphate has been demonstrated in mutants of *E. coli* deficient in phosphoglucoisomerase and glucose-6-phosphate dehydrogenase [14] and in mutants of *Pseudomonas aeruginosa* deficient in phosphoglucoisomerase [15]. In the latter, the toxic effect of mannitol on growth is due to accumulation of high intracellular concentrations of fructose 6-phosphate.

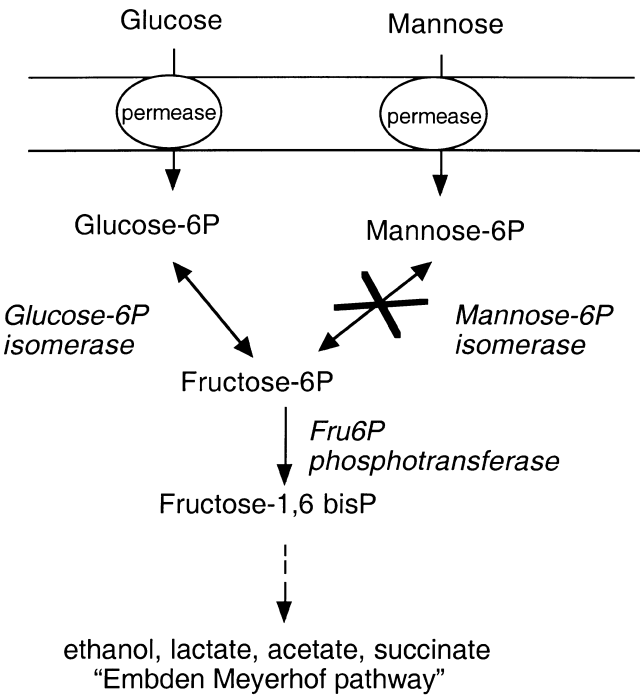

Fig. 4. Glucose and mannose metabolism in *P. shigelloides*.

In this study, the mannose 6-phosphate accumulation has a toxic effect on cells grown in nutrient broth containing mannose. This effect was observed in the 22 strains of *P. shigelloides*. The level of phospho*enol*pyruvate in mannose-metabolizing cells was estimated by ^{31}P-NMR spectroscopy. We show that the intracellular concentration of phospho*enol*pyruvate is higher in starved and mannose-metabolizing cells than in glucose-metabolizing cells. It thus appears that the toxic effect of mannose on growth is not due to energy depletion but probably to a toxic effect of mannose 6-phosphate. In *P. shigelloides*, the mannose permease probably permits the transport of nutrients other than mannose.

In the Enterobacteriaceae, the species of genera *Proteus, Leminorella* and *Budvicia aquatica* are known to be unable to use mannose as carbon source [16]. These species lack the mannose membrane-bound permease (II^{Man}) [17] and the mannose-6-phosphate isomerase (not shown). This pattern is different in *P. shigelloides*. To our knowledge, it is the first time that a transport system for a sugar has been found in a bacterium that is unable to use it as a carbon source.

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