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Biochemical Characterization of a Fructokinase Mutant of *Rhizobium meliloti*

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A double mutant strain (UR3) of *Rhizobium meliloti* L5-30 was isolated from a phosphoglucose isomerase mutant (UR1) on the basis of its resistance to fructose inhibition when grown on fructose-rich medium. UR3 lacked both phosphoglucose isomerase and fructokinase activity. A mutant strain (UR4) lacking only the fructokinase activity was derived from UR3; it grew on the same carbon sources as the parent strain, but not on fructose, mannitol, or sorbitol. A spontaneous revertant (UR5) of normal growth phenotype contained fructokinase activity. A fructose transport system was found in L5-30, UR4, and UR5 grown in arabinose-fructose minimal medium. No fructose uptake activity was detected when L5-30 and UR5 were grown on arabinose minimal medium, but this activity was present in strain UR4. Free fructose was concentrated intracellularly by UR4 >200-fold above the external level. A partial transformation of fructose into mannitol and sorbitol was detected by enzymatic analysis of the uptake products. Polyol dehydrogenase activity was detected in UR4 grown in arabinose-fructose minimal medium. The induction pattern of polyol dehydrogenase activities in this strain might be due to slight intracellular fructose accumulation.

The Entner-Doudoroff pathway, present in all *Rhizobium* strains studied (7-9, 12, 13, 16), may be the principal route of carbohydrate degradation for these strains. The role of other pathways, such as the Embden-Meyerhof-Parnas and pentose phosphate pathways, is uncertain (7, 12-14). Analysis of *Rhizobium* carbohydrate pathways would be aided by studying mutants. Recently, we reported a pleiotropic mutant of *Rhizobium meliloti* L5-30 which lacked phosphoglucose isomerase (1). It has since proven useful for selection of other mutational blocks in sugar pathways, and in this paper we present the results obtained with a fructokinase-negative derivative.

Several other carbohydrate mutants have recently become available, such as those for glucokinase, fructose uptake, and pyruvate carboxylase in *Rhizobium trifolii* (16), and those for α-ketoglutarate dehydrogenase in *R. meliloti* (4).

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *Rhizobium meliloti* L5-30 (Str^r), the parent strain of the mutants isolated, was kindly supplied by J. Dénarié, Station de Pathologie Végétale, Centre National de la Recherche Agronomique, Versailles, France. UR1 (1) was a phosphoglucose isomerase mutant obtained from L5-30.

Minimal medium and growth conditions have been described earlier (1). Rich medium contained, in milligrams per milliliter: K_2HPO_4, 0.5; NaCl, 0.1; yeast extract, 1; Casamino Acids, 1; streptomycin sulfate, 0.1; and a carbon source, 5. Solid media contained 2% agar. The final pH was 7.2. All sugars, with the exception of L-arabinose, were of the D-configuration.

Viable cell counts were made on minimal medium containing 1% sugar and 2% agar.

Isolation of mutant strains. Strain UR1 (Pgi^-) is sensitive to fructose (1). The mutant strain, UR3, was obtained from UR1, previously grown on arabinose minimal medium, by plating about 5×10^6 cells on fructose-rich medium.

Translucent colonies with the same appearance as the parent growing on rich medium without fructose were selected as presumptive (Pgi^- Frk^-). UR3 was plated on xylose minimal agar, and spontaneous revertants (Xyl^+ [presumptive Pgi^+ Frk^-]) which were unable to grow on fructose, mannitol, and sorbitol were selected. Several strains with this phenotype were found, and one of them (UR4) was selected for further studies. Reversion of strain UR4 on mannitol occurred at a frequency of $<10^{-10}$; such revertants (e.g., strain UR5) also grew again on fructose and sorbitol.

Cell-free extract preparation. Cell-free extracts were prepared by French press treatment as previously described (1), except that 24 mM Tris buffer (pH 7.6) containing 5 mM EDTA and 12 mM 2-mercaptoethanol was used to resuspend the cell pellet before pressing.

Enzyme assays. All enzyme assays were done at 25°C. Enzyme specific activities were expressed as nanomoles of substrate consumed or product formed per minute per milligram of extract protein. Control

assays were done by omitting the individual substrates in the test assay. Fructokinase was measured by following the reduction of $NADP^+$ at 340 nm in a Beckman spectrophotometer (model DU). The incubation mixture (1.0 ml) contained, in micromoles: fructose, 4; ATP, 2; $MgCl_2$, 2; $NADP^+$, 0.2; Tris buffer (pH 7.6), 50; excess glucose-6-phosphate dehydrogenase and phosphoglucose isomerase (Sigma Chemical Co., St. Louis, Mo.); and cell-free extract. To detect the presence of phosphofructomutase, fructose-1-phosphate was substituted for fructose and ATP in the incubation mixture. Phosphoenolpyruvate-dependent phosphorylation was assayed by substituting phosphoenolpyruvate (2 μmol) for ATP in the reaction mixture.

Previously described spectrophotometric methods were used to assay the following enzyme activities: phosphoglucose isomerase (EC 5.3.1.9) (1); $NADP^+$-linked glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (1); $NADP^+$-linked phosphogluconate dehydrogenase (EC 1.1.1.43) (1); combined activity of 6-phosphogluconate dehydratase (EC 4.2.1.12) and 2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14) (5); and polyol dehydrogenase (11).

Protein was measured by the method of Bücher (3) which was standardized with bovine serum albumin.

Uptake of [^{14}C]fructose and [^{14}C]mannitol. Freshly harvested cells were washed once in cold minimal medium and suspended in the same medium containing 100 μg of chloramphenicol per ml to 3 to 3.6 mg (dry weight) of cells per ml of suspension. The resuspended cells were incubated at 30°C for 15 min in a shaker.

[*U*-^{14}C]fructose uptake was measured by adding 0.5 ml of cell suspension to 0.45 ml of minimal medium containing 0.1 μmol of fructose and 0.02 μmol of [*U*-^{14}C]fructose (approximately 6,000 cpm/nmol). Samples (0.1 ml) were removed, and the reaction was stopped by dilution with 2.0 ml of minimal medium. Samples were then immediately filtered on a 0.45-μm-porosity membrane filter (Millipore Corp., Bedford, Mass.). Filters were washed once in 2 ml of the same medium. They were dried under a heat lamp and radioactivity was determined.

[*U*-^{14}C]mannitol uptake was measured as described above, using 0.02 μmol of mannitol and 0.004 μmol of [*U*-^{14}C]mannitol (approximately 35,000 cpm/nmol). The radioactivity was determined by a thin-window gas flow Geiger counter.

Analysis of fructose uptake products. The cells to be used for obtaining the radioactive pool were incubated, filtered, and washed as described for the uptake assay. The bacteria were immediately extracted with 2 ml of boiling water for 4 min, and the debris was removed by centrifugation. The extracted pool was evaporated under vacuum and spotted for descending chromatography on Whatman no. 1 filter paper. The solvent system contained 1-butanol–pyridine–water (6:4:3, vol/vol/vol). Chromatograms were run at room temperature for 38 h. [*U*-^{14}C]fructose and [*U*-^{14}C]mannitol were run simultaneously with the experimental material. After drying, the chromatograms were cut into 0.5- or 1-cm-wide strips, and these were counted. In addition, the location of individual sugars was established by spraying dried chromatograms with ammoniacal silver nitrate (18). The acid-soluble pool was prepared by the method of Böck and Neidhart (2). The extracted pool was spotted for descending chromatography as described above. Fructose, mannitol, and sorbitol were run as controls. Fructose was determined by the method of Roe et al. (15). Mannitol was assayed by an enzymatic method (10), using cell-free extract from *Lactobacillus brevis* ATCC 367 to supply mannitol dehydrogenase. For the sorbitol assay the same test was used, substituting sorbitol dehydrogenase (Sigma Chemical Co.) in excess for *L. brevis* extract.

DEAE-cellulose filter assay for fructose phosphorylation. Radioactive pool aliquots were pipetted into the center of Whatman DE81, 2.3-cm disks (Reeve Angel Co., Clifton, N.J.) and allowed to dry for 30 min at room temperature. The filters were dipped briefly in a beaker with water and washed on a filter apparatus with 10 ml of water. They were dried under a heat lamp, and their radioactivity was determined.

RESULTS

From strain UR1 plated on fructose-rich medium, a double mutant, UR3, was isolated on the basis of its resistance to fructose inhibition (see Materials and Methods). UR3 showed no growth on fructose, mannitol, sorbitol, mannose, ribose, xylose, or arabitol, but it did grow on other carbon sources, including sucrose, glucose, L-arabinose, glycerol, and succinate (Table 1). Thus, UR3 grew similarly to UR1 on single carbon sources.

A spontaneous revertant, Pgi^+ (UR4), selected on xylose grew like the wild type on most carbon sources but failed to grow on fructose, mannitol, or sorbitol. The phenotype of UR4 was apparently caused by a single mutation, since revertants selected on mannitol (UR5) also grew on fructose and sorbitol. Table 2 shows assays of fructokinase and phosphoglucose isomerase activities. UR3 and UR4 lacked detectable fructokinase activity and UR5 had regained it. The phosphoglucose isomerase activity of UR3 was about 1% of normal. UR4 and UR5 had regained this activity, but their level was much higher than that of the parent strain; this unusual characteristic of phosphoglucose isomerase reversion was noted earlier (1).

Table 3 shows the specific activities of several enzymes of carbohydrate intermediary metabolism. Fructokinase activity was not detected in UR4 under any growth conditions. All of the other enzymes tested were present in comparable levels in fructokinase mutants and parent strains. The level of glucose-6-phosphate dehydrogenase activity was increased when the bacteria were grown with fructose or glucose. 6-Phosphogluconate dehydratase and 2-keto-3 deoxy-6-phosphogluconate aldolase activity was lower when L5-30 and UR5 were cultured in arabinose, whereas this combined activity was

TABLE 1. *Growth of parent (L5-30), mutant (UR1, UR3, UR4), and revertant (UR5) strains on several carbon sources*

Carbon source	Growth[a]				
	L5-30	UR1	UR3	UR4	UR5
Fructose	++	−	−	−	++
Mannitol	++	−	−	−	++
Sorbitol	++	−	−	−	++
Mannose	++	−	−	++	++
Ribose	++	−	−	++	++
Xylose	++	−	−	++	++
Arabitol	++	−	−	++	++
Sucrose	++	+	+	++	++
Glucose	++	+	+	++	++
Arabinose	++	++	++	++	++
Glycerol	++	+	+	++	++
Succinate	++	+	+	++	++

[a] Cultures were spread on minimal medium plates to give ca. 50 colonies per plate. After 8 days of incubation at 30°C, growth was recorded as follows: parent strain growth (++), less than parent strain growth (+), and no growth (−).

TABLE 2. *Fructokinase and phosphoglucose isomerase activities in cells of the parent (L5-30), mutant (UR3, UR4), and revertant (UR5) strains*[a]

Enzyme	Sp act (nmol/min per mg of protein)			
	L5-30	UR3	UR4	UR5
Fructokinase	70	0	0	69
Phosphoglucose isomerase	1,440	20	15,200	22,000

[a] Cells were grown in minimal medium containing 0.5% arabinose plus 0.5% fructose.

induced when cells were grown in glucose or fructose. The specific activity of phosphoglucose isomerase in L5-30, UR4, and UR5 did not vary significantly with the nature of the carbon source.

Fructose, mannitol, and sorbitol were identified as products of fructose uptake. Mannitol and sorbitol were detected in an acid-soluble pool from UR4 cells, by using specific polyol dehydrogenases. They were also identified by descending paper chromatography, and the spots formed were coincident with fructose, mannitol, and sorbitol standards.

Table 4 shows polyol dehydrogenase activities of strains L5-30 and UR4 under different growth conditions. Strain L5-30 only induced polyol dehydrogenase activities when growing in arabinose minimal medium containing mannitol or sorbitol. These activities were very low or not detected at all when L5-30 was grown in fructose or in the other carbon sources. However, strain UR4 showed similar high levels of polyol dehydrogenase activities when the cells were grown in fructose, mannitol, or sorbitol, and low activities were detected when other carbon sources were used.

Figure 1 shows the uptake of [^{14}C]fructose as a function of time in strains L5-30, UR4, and UR5. This uptake activity was induced in strains L5-30 and UR5 by fructose, and no uptake activity could be detected when the cells were grown in the absence of fructose. UR4, which is a mutant unable to grow in fructose, did have fructose transport activity when growing on either arabinose or arabinose plus fructose.

The intracellular product of the fructose transport was not metabolized by mutant strain UR4, as shown in the plateau of its uptake curve. In contrast, no plateau was observed with parent and revertant strains. UR4 cells accumulated radioactivity 150-fold above the external concentration. The transport of fructose by UR4 exhibited typical Michaelis-Menten kinetics with an apparent K_m of 2 μM. The action of metabolic inhibitors on fructose uptake was tested; sodium azide (4 mM), 2,4dinitrophenol (2 mM), and sodium *p*-chloromercuribenzoate (1 mM) decreased the plateau of incorporation to 75, 35, and 7% of the control value, respectively.

Free fructose was detected by radiochromatography in the intracellular radioactive material accumulated by UR4 after 10 min of incubation with [^{14}C]fructose. The radioactivity corresponding to free fructose was about 80% of the total radioactivity; a minor peak was not identified. The radioactive pool was also assayed for formation of anionic material from [^{14}C]fructose

TABLE 3. *Enzymes associated with fructose catabolism*

Strain	Carbon source[a]	Sp act (nmol/min per mg of protein)[b]				
		FK	PGI	G6PD	6PGD	EDD/EDA
L5-30	Ara	9	984	39	57	7
	Ara + Fru	70	1,440	184	27	32
	Glu	20	2,060	150	57	21
UR4	Ara	0	18,400	37	20	6
	Ara + Fru	0	15,190	51	30	6
	Glu	0	20,000	107	40	21
UR5	Ara	13	16,800	46	33	8
	Ara + Fru	69	22,240	138	69	38
	Glu	20	21,700	97	46	25

[a] Cells were grown in minimal medium containing 0.5% arabinose (Ara), 0.5% arabinose plus 0.5% fructose (Ara + Fru), or 0.5% glucose (Glu).

[b] Abbreviations: fructokinase (FK); phosphoglucose isomerase (PGI); $NADP^+$-linked glucose-6-phosphate dehydrogenase (G6PD); $NADP^+$-linked gluconate 6-phosphate dehydrogenase (6PGD); Entner-Doudoroff dehydratase and aldolase (EDD/EDA).

TABLE 4. *Polyol dehydrogenase activities in parent (L5-30) and mutant (UR4) strains*[a]

Carbon source	Sp act (nmol/min per mg of protein)					
	L5-30			UR4		
	MDH[b]	SDH	ADH	MDH	SDH	ADH
Arabinose-fructose	1	4	4	61	56	124
Arabinose-mannitol	31	52	66	73	72	186
Arabinose-sorbitol	24	56	84	69	44	121
Arabinose	<1	<1	<1	3	11	14
Mannose	<1	<1	<1	3	12	19
Glucose	<1	<1	<1	4	18	21
Glycerol	ND[c]	ND	ND	1	9	8
Mannitol	63	115	133	ND	ND	ND
Sorbitol	49	206	192	ND	ND	ND
Rich medium	ND	ND	ND	<1	<1	<1

[a] Cells were grown in minimal medium containing 0.5% carbon source.

[b] Abbreviations: mannitol dehydrogenase (MDH); sorbitol dehydrogenase (SDH); arabitol dehydrogenase (ADH).

[c] ND, Not done.

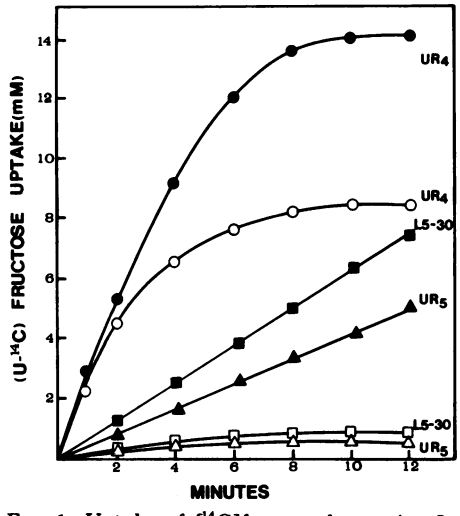

FIG. 1. *Uptake of [^{14}C]fructose by strains L5-30, UR4, and UR5. The cells were cultured in minimal medium containing 0.5% each of the carbon sources. Symbols: L5-30 cultured in arabinose plus fructose, (■); L5-30 cultured in arabinose (□); UR4 cultured in arabinose plus fructose, (●); UR4 cultured in arabinose (○); UR5 cultured in arabinose plus fructose, (▲); UR5 cultured in arabinose (△).*

by using DEAE-cellulose filters (6). No incorporation of counts above that of the control or the fructose standard was found in the pool sample tested after washing the filters with distilled water, indicating the absence of phosphorylated products.

DISCUSSION

We have shown that a mutant lacking fructokinase, UR4, fails to grow on fructose, mannitol, or sorbitol. No activity was detected in any of several media assayed. Considering the selection of UR4 as resistant to fructose toxicity in a phosphoglucose isomerase mutant and the fact that no phosphofructomutase activity was observed even in the parental strain, we conclude that fructokinase makes fructose-6-phosphate, and that this enzyme is used both for catabolism of exogenously supplied fructose and for metabolism of mannitol and sorbitol. In addition, the fact that the mutant is unaffected in growth on glucose or mannose shows that fructokinase is specific for fructose.

These experiments, combined with what was previously known about fructose metabolism in *Rhizobium* spp. (11, 16) and the failure to find phosphofructokinase or fructose biphosphate aldolase activity (1; unpublished data) fit the general pattern of carbohydrate metabolism in *R. meliloti* shown in Fig. 2.

We also present evidence for penetration of fructose into *R. meliloti* by an active transport system of high affinity (K_m about 2 μM), which in the mutant allows considerable accumulation of free fructose. No evidence for anionic material, such as would be formed by a phosphoenolpyruvate phosphotransferase (17), was obtained. Interestingly, some mannitol and sorbitol also accumulated from fructose, evidently because of induction of polyol dehydrogenase activities in the mutant even when it was grown on unrelated carbon sources. In the parental strain, as shown earlier for *R. meliloti* 9930 (11), the dehydrogenases were induced only by mannitol and sorbitol. Perhaps minimal formation of fructose in the fructokinase mutant is sufficient to cause the induction. A relationship between fructokinase

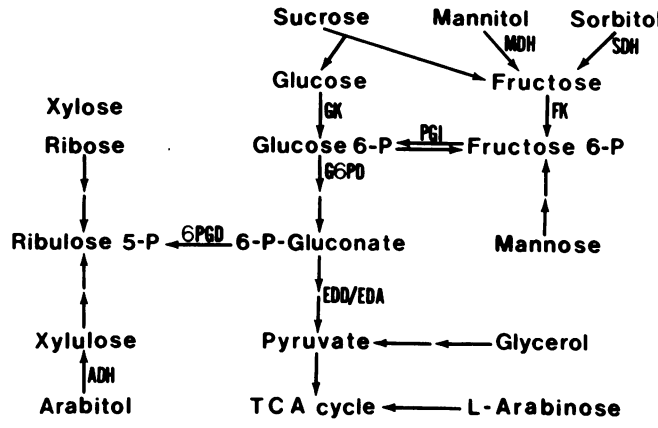

FIG. 2. *Possible pathways of carbohydrate metabolism in R. meliloti. Pathway intermediates and enzymes are abbreviated as follows: 6-phosphogluconate dehydrogenase (6PGD); glucose-6-phosphate dehydrogenase (G6PD); phosphoglucose isomerase (PGI), glucokinase (GK), fructokinase (FK), refers to the Entner-Doudoroff dehydratase and aldolase (EDD/EDA); tricarboxylic acid (TCA); arabitol dehydrogenase (ADH), mannitol dehydrogenase (MDH); sorbitol dehydrogenase (SDH).*

activity and the induction of polyol dehydrogenases seems to exist.

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