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Transport and Catabolism of D-Mannose in *Rhizobium meliloti*

A. ARIAS, A. GARDIOL, AND G. MARTÍNEZ-DRETS*

Division of Biochemistry, Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay

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Rhizobium meliloti L5-30 grows on D-mannose as the sole carbon source. The catabolic pathway of D-mannose was characterized. The following activities were present: mannose transport system, mannokinase, and mannosephosphate isomerase. Several mannose-negative mutants were selected; they were classified into three functional groups: group I, mannokinase and mannosephosphate isomerase defective; group II, mannokinase defective; and group III, mannosephosphate isomerase defective. Mannose uptake was an active process, since it was inhibited by azide, dinitrophenol, and cyanide, but not by fluoride or arsenate. Growth on succinate repressed mannose uptake activity. The mannose transport system was present in all the mutants. Uptake studies showed that mannose-negative mutants did not metabolize this sugar.

Rhizobia are able to utilize a variety of carbon sources (2, 5). The characterization of some hexose-catabolizing enzymes in cell-free extracts of rhizobia grown on different carbon sources has been previously reported (9, 10, 13, 14), but relatively little is known of hexose transport systems. Glucose transport in *Rhizobium leguminosarum* (7; G. de Vries, Ph. D. thesis, University of Leiden, Leiden, Holland, 1980) and fructose uptake in *Rhizobium trifolii* (14) and in *Rhizobium meliloti* (4) have been reported; isolation and analysis of mutants impaired in carbohydrate metabolism have also been reported recently (1, 3, 4, 14).

In this report, we present the results of a biochemical study of D-mannose transport and catabolism in *R. meliloti* L5-30 and the characterization of three functional groups of mutants which were unable to grow on mannose.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The wild-type strain was *R. meliloti* L5-30 (Str^r). UR1 (1) was a phosphoglucose isomerase mutant obtained from strain L5-30.

Minimal medium, rich medium, and growth conditions have been described previously (1, 4). Mannose-tetrazolium plates contained rich medium supplemented with 10 mg of mannose, 0.05 mg of 2,3,5-triphenyltetrazolium, and 20 mg of agar per ml. All sugars, with the exception of L-arabinose, were of the D configuration.

Isolation of mutant strains. Mutant strains were obtained by two different methods. Method 1 was based on the inability of the phosphoglucose isomerase mutant strain UR1 to grow on mannose-rich medium. Mutant strains were obtained from UR1 by spreading about 5×10^6 cells on mannose-tetrazolium plates. Colonies resembling the parent strain growing on the same medium lacking mannose were selected as presumptive mannose-negative mutants. The *pgi* lesion was subsequently reverted on fructose (as in reference 4). Thus, several mannose-negative mutants in a phosphoglucose isomerase-positive background were obtained, and four of them were selected (UR8, UR9, UR10, and UR11) for further study.

In method 2, wild strain *R. meliloti* L5-30 was grown to logarithmic phase, washed in 0.05 M Tris-maleate buffer (pH 6.5), and suspended in the same buffer. The cells were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (200 μg/ml) at 30°C for 30 min. The mutagenized cells (25% survival) were washed in the same buffer and grown overnight in arabinose minimal medium. A subculture was inoculated onto mannose minimal medium, and penicillin G (2,000 U/ml) was added in the early logarithmic phase. The surviving cells (0.5%) were cloned on arabinose minimal medium, and ca. 1,000 colonies were patched onto mannose minimal medium. Of the 50 presumptive mannose-negative mutants isolated, 11 grew on all the sugars tested (glucose, fructose, galactose, ribose, xylose, and glycerol) except mannose. According to growth on rich medium with mannose, two mutants (UR12 and UR13) were uninhibited, and the other nine, including UR14 and UR15, were inhibited. Revertants were isolated for all the mutants in mannose minimal medium.

Preparation of cell-free extracts. Cell-free extracts were prepared by French press treatment as previously described (1), except that 50 mM Tris-hydrochloride buffer (pH 7.6) containing 10 mM 2-mercaptoethanol was used.

Enzyme essays. Enzyme activities were measured in a Gilford model 250 spectrophotometer at 25°C and expressed as nanomoles of substrate consumed or product formed per minute per milligram of extract protein. Mannokinase (EC 2.7.1.7) was measured as a mannose-dependent ATPase activity. The reaction mixture (1.0 ml) contained, in micromoles: Tris-hydrochloride buffer (pH 7.6), 50; KCl, 80; $MgCl_2$, 10; ATP, 1; mannose, 10; NADH, 0.2; phosphoenolpyruvate,

2.5; KCN, 2; excess of lactic dehydrogenase (Sigma Chemical Co.) containing pyruvate kinase; and cell-free extract. KCN inhibited NADH oxidase activity, but it did not affect the activity of mannokinase. The assay was ATP dependent. Mannosephosphate isomerase (EC 5.3.1.8) was assayed in a mixture (1.0 ml) containing, in micromoles: mannose 6-phosphate, 2; NADP, 0.2; $MgCl_2$, 4; Tris-hydrochloride buffer (pH 7.6), 100; excess glucose-6-phosphate dehydrogenase and phosphoglucose isomerase (Sigma); and cell-free extract. Control assays were done by omitting the individual substrates in the test assay.

Protein was measured by the method of Lowry et al. (11) with bovine serum albumin as the standard.

Uptake assays. Cells for the uptake experiments were prepared as previously described (4). [*U*-^{14}C]mannose uptake was measured by adding 0.06 ml of cell suspension to 0.06 ml of minimal medium containing 12.5 nmol of mannose and 0.4 nmol of [*U*-^{14}C]mannose (approximately 200,000 cpm/μmol). Samples (0.02 ml) were removed, filtered on a membrane filter (Millipore Corp., Bedford, Mass. [0.45 μm by 25 mm]), and washed with 2.0 ml of minimal medium. The radioactivity was determined by a thin-window gas flow Geiger counter.

RESULTS

R. meliloti L5-30 grows on several carbon sources, as shown in previous tests (1). Activities of mannokinase and mannosephosphate isomerase were present in extracts of *R. meliloti* L5-30 grown in arabinose or arabinose plus mannose (Table 1). The product of the mannokinase reaction with mannose as substrate is most likely mannose 6-phosphate, as indicated by the immediate coupling of the enzyme reaction to a system formed by mannosephosphate isomerase, glucosephosphate isomerase, glucose-6-phosphate dehydrogenase, and NADP.

All of the mutant strains exhibited the wild-type phenotype except for mannose, which did not support growth. None of the mutants could utilize mannose as the sole carbon source, and the two (UR14 and UR15) originally screened as being inhibited on a rich medium containing mannose also proved to be strongly inhibited in minimal medium plates containing 0.5% mannose and 0.5% of a permissive carbon source, such as glucose, fructose, or arabinose.

The specific activities of mannokinase and mannosephosphate isomerase in cell-free extracts of mannose-negative mutant strains are also shown in Table 1. The mutants could be placed in one of three functional groups. Group I mutants had undetectable mannokinase activity and a reduced level of mannosephosphate isomerase. These mutants were obtained from strain UR1 as spontaneous mutants resistant to mannose inhibition. Spontaneous revertants on mannose were isolated for the three mutants, and they regained wild-type levels of both enzymes (data not shown). Group II mutants had little or no mannokinase but normal levels of mannosephosphate isomerase. Spontaneous revertants of all three mutants regained normal mannokinase levels (data not shown). The mannose-inhibited mutants, UR14 and UR15 (above), formed group III, with a normal level of mannokinase but low or undetectable mannosephosphate isomerase.

Cells of *R. meliloti* L5-30 grown in mannose minimal medium were able to take up [*U*-^{14}C]mannose immediately (Fig. 1). Mannose uptake was also present when cells were grown in glucose or glycerol. The rate of uptake was very low or undetectable when mannitol or succinate was the carbon source employed for growth. The action of metabolic inhibitors on mannose uptake was tested (Table 2): azide (2 mM), dinitrophenol (1 mM), and cyanide (1 mM) inhibited mannose uptake by more than 84%. The sulfhydryl group reagent *N*-ethylmaleimide (1 mM) inhibited [*U*-^{14}C]mannose uptake (90%), and EDTA (10 mM) also caused a strong inhibition. Arsenate (5 mM) and fluoride (5 mM), on the other hand, had no significant effect on the uptake.

The mannose uptake system was also present in all the mutants. Figure 2 shows the uptake of [*U*-^{14}C]mannose, 0.1 μmol/ml, in *R. meliloti* L5-30 and in mutant strains representative of group I (strain UR9), group II (strain UR13), and group III (strain UR14).

TABLE 1. Enzymes of mannose catabolism in cells of the parent (L5-30) and mutants of groups I, II, and III

Strain	Carbon source[a]	Sp act (nmol/min per mg of protein)	
		Mannokinase	Mannosephosphate isomerase
R. meliloti L5-30	Arabinose	30	27
R. meliloti L5-30	Arabinose + mannose	45	53
Mutant strains			
Group I			
UR8	Arabinose + mannose	0	1.6
UR9		0	1.6
UR10		0	3.0
Group II			
UR11	Arabinose + mannose	0	27.8
UR12		1.6	30.7
UR13		0	38.9
Group III			
UR14	Arabinose	28.6	1.6
UR15		28.5	0

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

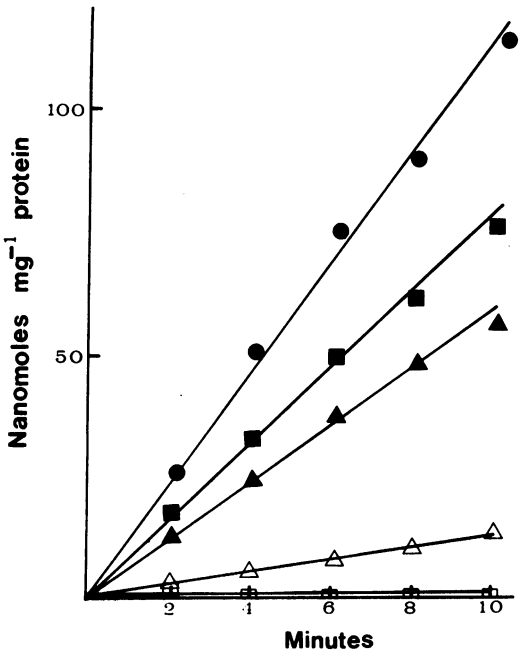

FIG. 1. Uptake of [*U*-^{14}C]mannose by *R. meliloti* L5-30 grown on minimal medium plus mannose (●), glucose (■), glycerol (▲), mannitol (△), or succinate (□).

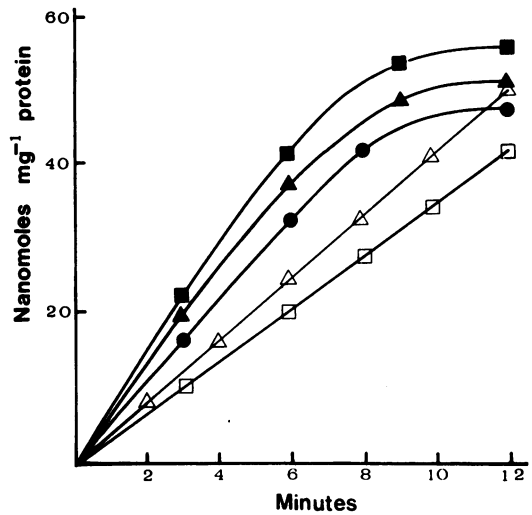

FIG. 2. Uptake of [*U*-^{14}C]mannose by *R. meliloti* L5-30 and mannose-negative mutant strains. The cells were cultured on minimal medium containing 0.5% of each of the carbon sources. Symbols: L5-30 cultured in arabinose plus mannose (△), L5-30 cultured in arabinose (□), strain UR9 (group I) cultured in arabinose plus mannose (■), strain UR13 (group II) cultured in arabinose plus mannose (●), strain UR14 (group III) cultured in arabinose (▲).

The transport of mannose by UR13 exhibited typical Michaelis-Menten kinetics with an apparent K_m of 22 μM.

The parent strain (L5-30) used more than 95% of the total [*U*-^{14}C]mannose (5 μmol/ml) in 12 h. In contrast, the mutant strain (UR13) did not consume [*U*-^{14}C]mannose over 26 h, and almost no radioactivity was associated with the cells (data not shown).

DISCUSSION

The results presented in this paper, combined with what was previously known about hexose metabolism in *Rhizobium* spp. (4, 14), demonstrate that *R. meliloti* L5-30 employs the mannose catabolic sequence shown in Fig. 3. Catabolism of D-mannose involves the transport of the hexose into the cell, phosphorylation to mannose 6-phosphate, and the isomerization to fructose 6-phosphate.

Mannokinase and mannosephosphate isomerase are present in cell-free extracts from the parent strain. The requirement of these enzymatic systems for mannose catabolism was proved directly, since mutants deficient in these

TABLE 2. Effect of metabolic inhibitors on [*U*-^{14}C]mannose uptake by *R. meliloti* L5-30[a]

Inhibitor	% Inhibition
Azide (2 mM)	84.5
Dinitrophenol (1 mM)	85.0
Potassium cyanide (1 mM)	97.0
N-Ethylmaleimide (1 mM)	90.0
EDTA (10 mM)	90.0
Arsenate (5 mM)	15.0
Fluoride (5 mM)	4.5

[a] Metabolic inhibitors were added 1 min before the addition of radioactivity. Uptake was measured for 5 min. The control rate of uptake was 14 nmol/min per mg of protein.

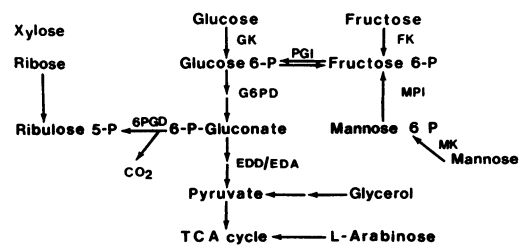

FIG. 3. Possible catabolic pathway in *R. meliloti*. Abbreviations: GK, glucokinase; FK, fructokinase; G6PD, glucose-6-phosphate dehydrogenase; PGI, phosphoglucose isomerase; 6PGD, 6-phosphogluconate dehydrogenase; MK, mannokinase; MPI, mannosephosphate isomerase; EDD/EDA, Entner-Doudoroff pathway; TCA, tricarboxylic acid cycle.

activities have been isolated. The properties of the mannose-negative mutants show that *R. meliloti* L5-30 is wholly dependent on mannokinase and mannosephosphate isomerase for growth on mannose. Mannokinase-negative mutants (group II) were found to grow normally on glucose or fructose, which suggests that mannokinase is specific for mannose as fructokinase is for fructose (4). The toxic effect of mannose on the growth of mannosephosphate isomerase-negative mutants (group III) might be due to a high intracellular concentration of mannose 6-phosphate, analogous to the fructose 6-phosphate accumulation and toxicity in the phosphoglucose isomerase mutant (1). Phosphomannose isomerase mutants are known in several microbes (6, 12, 15), and inhibition by mannose has been observed (6, 16).

Uptake of mannose was detected in *R. meliloti* L5-30 cells grown in glucose, mannose, or glycerol, but it was very low when mannitol was used as the carbon source. Growth in succinate represses mannose uptake activity, and similar results have been described for the glucose transport in *R. leguminosarum* (7) and in *Pseudomonas aeruginosa* (8). Uncouplers and inhibitors of oxidative phosphorylation show marked inhibition of the mannose uptake, whereas arsenate or fluoride, inhibitors of the glycolysis, do not interfere in mannose uptake. These results indicate that mannose accumulation in *R. meliloti* L5-30 is probably via an active process. Mannose transport activity was severely affected by the thiol-reactive agent *N*-ethylmaleimide, which suggests that it contains an essential sulfhydryl group. Strong inhibition by EDTA suggests a role for divalent cations. All of the isolated mutants were able to take up mannose at rates similar to those of the parent strain, but gave no indication of mannose utilization, suggesting the absence of alternative metabolic routes for mannose catabolism. Evidence for metabolic bypasses in ribose and xylose catabolism in mutants of the same strain has been described recently (3). The genetic nature of the mutants is not known. The simplest possibility is that groups II and III are in the structural genes for the affected enzymes. Perhaps the group I mutants indicate coregulation of expression, or perhaps the structural genes are linked and the group I mutants are polar.

The isolated mutants may be useful to obtain a better knowledge of the catabolic repression phenomena in *Rhizobium*, which might establish the order of preference in the utilization of carbon substrates.

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