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Glucose-6-Phosphate Dehydrogenase Deficiency in Pleiotropic Carbohydrate-Negative Mutant Strains of *Rhizobium meliloti*

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Several mutant strains of *Rhizobium meliloti* isolated after nitrosoguanidine mutagenesis were selected as unable to grow on mannose. Some of them also failed to grow on glucose, fructose, ribose, and xylose but grew on L-arabinose, galactose, and many other carbon sources. Biochemical analysis demonstrated that the mutants lacked NAD- and NADP-linked glucose-6-phosphate dehydrogenase activities that reside on a single enzyme species. One such mutant was found to accumulate glucose-6-phosphate, and this could partially explain the inhibition of growth observed on mixtures of permissive and nonpermissive carbon sources. Symbiotic properties remained unaffected in all these mutants.

The isolation and biochemical characterization of mutants imparied in specific degradative pathways has proven useful in studies of sugar transport and metabolism in *Rhizobium meliloti* (1, 2, 6, 7, 9, 10). Such studies also suggest that the major route of carbohydrate metabolism in this organism is the Entner-Doudoroff pathway and not the Embden-Meyerhof pathway (1, 11).

Specific mannose-negative mutants were recently characterized (2). Mutants which failed to grow on other sugars as well were also obtained. In the present study, we report on a group of six mutants that are unable to grow on mannose, glucose, fructose, xylose, ribose, and other sugars and that lack glucose-6-phosphate dehydrogenase (EC 1.1.1.49) activity.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The wild-type strain was *R. meliloti* L5-30 (Str^r) reisolated from alfalfa (*Medicago sativa* var. Du Puits) nodules. The minimal medium (MM) and growth conditions used have been described previously (1). All sugars were of the D configuration, except for L-arabinose. The parental and mutant strains were maintained on galactose- or L-arabinose-containing MM. Growth rates were determined by turbidimetric measurements at 660 nm in sidearm flasks with a Coleman spectrophotometer. One absorbance value unit was found to correspond to 1.6 mg of wet cells per ml.

Isolation of mutant and revertant strains. Strains UR16, UR17, UR18, UR19, UR20, and UR21 were those incapable of growing on MM containing glucose, fructose, mannose, xylose, or ribose out of the 50 presumptive mannose-negative mutants isolated after nitrosoguanidine treatment as previously described (2).

Spontaneous revertants of strains UR16, UR17, and UR18 were isolated from MM plates supplemented with fructose, xylose, or ribose.

Cell-free extract preparation. Cell-free extracts were prepared by French press treatment as described elsewhere (1), except that 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM 2-mercaptoethanol was used.

Enzyme assays. 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.

Previously published methods were used for assaying NADP-linked glucose-6-phosphate dehydrogenase (1), NADP-linked gluconate-6-phosphate dehydrogenase (phosphogluconate dehydrogenase; EC 1.1.1.43) (1), and phosphoglucose isomerase (glucosephosphate isomerase; EC 5.3.1.9) (1). The combined activity of 6-phosphogluconate dehydratase (phosphogluconate dehydratase; EC 4.2.1.12) and 2-keto-3-deoxy-6-phosphogluconate aldolase (phospho-2-keto-3-deoxy-gluconate aldolase; EC 4.1.2.14) was determined by the method of Fradkin and Fraenkel (8) but with 30 min of incubation. NAD-linked glucose-6-phosphate dehydrogenase was assayed by substituting NAD^+ (1 μmol) for $NADP^+$ in the reaction mixture (1).

Protein was measured by the method of Bücher (5) with bovine serum albumin as the standard.

Determination of G6P. The acid-soluble pool was prepared by a slight modification of the Böck and Neidhardt method (4) described previously (1). The glucose-6-phosphate (G6P) assay mixture (1 ml) contained the following: glycylglycine buffer (pH 8.0), 70 μmol; $NADP^+$, 0.3 μmol; $MgCl_2$, 5 μmol; glucose-6-phosphate dehydrogenase (catalog no. G-6378; Sigma Chemical Co.), 1 μg; and sample. The amount of G6P was calculated by using the molar absorbtion value of NADPH, $6.22 \times 10^3\ M^{-1}\ cm^{-1}$.

Polyacrylamide gel isoelectric focusing. The general procedures and preparation of polyacrylamide gel rods (0.5 by 7.5 cm) containing 2% Pharmalyte (pH 3 to 10) were done as recommended by Pharmacia Fine Chemicals (14). The electrode solutions used were 0.2% aqueous sulfuric acid for the anode (upper chamber) and 0.4% aqueous ethanolamine for the cathode. Isoelectric focusing was done for 3 h at 4°C with an initial current of 1.5 mA per gel rod and a voltage output set to a limiting value of 350 V.

Zones of glucose-6-phosphate dehydrogenase activity were made visible by incubation of the gels for 10 to 15 min in the dark at 30°C in 5 ml of an assay mixture containing the following: glycylglycine buffer (pH 8.0), 500 μmol; sodium G6P, 30 μmol; $MgCl_2$, 50 μmol; phenazine methosulfate, 0.4 mg; *p*-Nitro Blue Tetrazolium chloride, 0.7 mg; and $NADP^+$, 1.5 μmol, or NAD^+, 4.5 μmol, or both. The reactions were stopped by the addition of 0.5 ml of glacial acetic acid, and then the gels were rinsed with distilled water and stored in 7% (vol/vol) acetic acid.

The pH gradient was determined by cutting a control gel

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TABLE 1. Enzymes associated with glucose catabolism

Strain[a]	Sp act (nmol/min per mg of protein) of[b]:			
	PGI	G6PD	6PGD	EDD/EDA
L5-30	310	220	69	20
UR16	410	0	46	17
UR17	308	0	45	15
UR18	372	0	25	<1
UR19	457	0	44	12
UR20	280	0	42	17
UR21	505	0	43	<1

[a] Cells were grown in MM containing 0.5% galactose.

[b] Abbreviations: PGI, phosphoglucose isomerase; G6PD, NADP-linked glucose-6-phosphate dehydrogenase; 6PGD, NADP-linked gluconate-6-phosphate dehydrogenase; and EDD/EDA, 6-phosphogluconate dehydratase/2-keto-3-deoxy-6-phosphogluconate aldolase (Entner-Doudoroff enzymes).

rod into small pieces, which were then homogenized in a small volume of distilled water; pH values were directly measured in each tube at 20°C.

Enzyme band activities in control runs of parental strain L5-30 samples (some of them also mixed with mutant UR16 cell extract) were shown to be totally dependent on G6P and oxidized pyridine nucleotides.

All cell extracts used in isoelectric focusing experiments were prepared from galactose-grown cells.

Nitrogen fixation assays. Alfalfa plants (*M. sativa*) grown on 15 ml of nitrogen-free nutrient agar enclosed in test tubes (200 by 25 mm) and maintained at 20°C in a light-controlled room were inoculated with the proper rhizobium culture to ca. 10^5 cells per plant. Nitrogenase activity was measured on whole plants 4 weeks after inoculation by acetylene reduction as previously described (1).

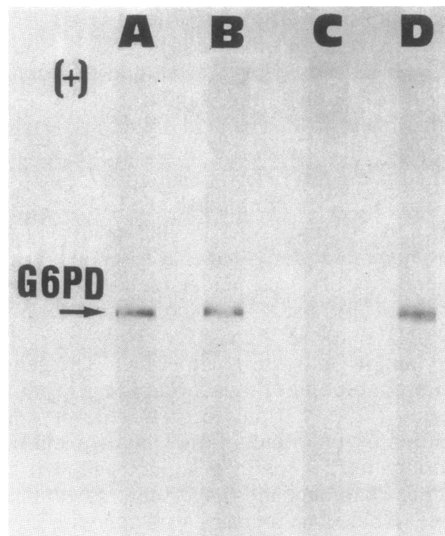

FIG. 1. Analytical isoelectric focusing of glucose-6-phosphate dehydrogenase (G6PD) from *R. meliloti*. Samples (0.1 ml) containing 100 μg (lanes A, B, and D) or 500 μg (lane C) of cell-extract protein in a 1/15 dilution of Pharmalyte (pH 3 to 10) containing 15% sucrose were layered onto four polyacrylamide gel rods (0.5 by 7.5 cm). After isoelectric focusing for 3 h at 4°C, glucose-6-phosphate dehydrogenase was detected by the specific staining method described in the text. Lanes: A, L5-30 cell-extract sample incubated with NAD^+; B, L5-30 cell-extract sample incubated with $NADP^+$; C, UR16 cell-extract sample incubated with NAD^+ and $NADP^+$; and D, UR22 cell-extract sample incubated with NAD^+ and $NADP^+$. A pI of 5.9 (at 20°C) was estimated for the single band detected.

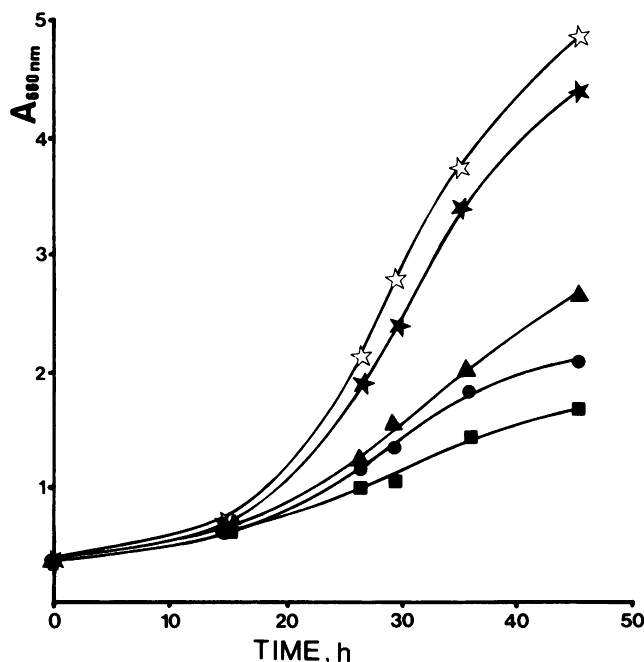

FIG. 2. Growth of *R. meliloti* L5-30 and UR16 in MM containing selected carbon sources. Growth was determined by turbidimetric measurements and plotted as a function of time for parallel cultures. Symbols: ☆, strain L5-30, 30 mM galactose; ★, strain UR16, 30 mM galactose; ▲, strain UR16, 30 mM galactose and 30 mM xylose; ●, strain UR16, 30 mM galactose and 30 mM ribose; and ■, strain UR16, 30 mM galactose and 15 mM fructose. A_{660nm}, Absorbance at 660 nm.

RESULTS

Growth characteristics and enzyme activities of mutant strains. The six strains in question (UR16, UR17, UR18, UR19, UR20, and UR21) from the original group of 50 mannose-negative mutants did not grow on MM containing glucose, fructose, mannose, mannitol, sorbitol, sucrose, maltose, ribose, or xylose but did grow on several other carbon sources, such as galactose, glycerol, succinate, and L-arabinose.

Table 1 shows the results of assays of several enzymes of intermediary metabolism. NADP-linked glucose-6-phosphate dehydrogenase activity was absent in extracts of all the mutants. These extracts also lacked NAD-linked glucose-6-phosphate dehydrogenase activity (data not shown). Both dehydrogenase activities were present in the parental strain, irrespective of the carbon source used for growing the cells. In addition, strains UR18 and UR21 also lacked Entner-Doudoroff enzyme activities. The growth of strain L5-30 on carbon sources whose metabolism is expected to be via G6P resulted in increased activities of these enzymes (60 and 7 nmol/min per mg of protein in cells grown on fructose and succinate, respectively).

Transketolase and transaldolase enzyme activities were also detected in all the strains tested in this study.

In the isoelectric focusing experiment shown in Fig. 1, NADP- and NAD-linked glucose-6-phosphate dehydrogenase activities of parental strain cell extracts migrated as a single unresolved band. A representative mutant strain (UR16) lacked both activities, and a spontaneous revertant

strain (UR22, see below) regained both of them simultaneously. The isoelectric band patterns of glucose-6-phosphate dehydrogenase in cell extracts of strains L5-30 and UR22 were the same, regardless of which cofactor was included in the incubation mixture.

Characteristics of revertant strains. Spontaneous revertants of UR16, UR17, and UR18 were isolated from MM plates supplemented with fructose, glucose, mannose, ribose, or xylose. The reversion frequency was ca. 10^7 for hexoses and ca. 10^5 for pentoses.

All revertant strains isolated from hexose-supplemented plates showed a wild-type phenotype and regained NAD- and NADP-linked glucose-6-phosphate dehydrogenase activities. Strain UR22 was a spontaneous revertant isolated from a fructose-containing MM plate spread with a UR16 culture. In the case of revertant strains of UR18 selected on fructose, the Entner-Doudoroff enzyme activities were also present at normal levels.

Growth characteristics on combined carbon sources. Strain UR16 was selected for studies of growth characteristics on mixed carbon sources (permissive and nonpermissive, 0.5% each). This mutant strain formed no colonies on MM containing galactose or glycerol together with the nonpermissive carbon source glucose, fructose, ribose, or xylose. MM containing succinate or L-arabinose together with the same nonpermissive carbon sources did support colony growth, but the colonies were considerably smaller than those on control plates. The growth of the parental strain was not inhibited by any mixture tested.

The results for liquid cultures are shown in Fig. 2. When a nonpermissive carbohydrate (fructose, ribose, or xylose) was included along with galactose, significant inhibition of strain UR16 growth was observed. Both parental and mutant strains grew similarly in galactose-supplemented medium (doubling time, ca. 6 h).

TABLE 2. Levels of G6P in parental (L5-30) and mutant (UR16) strains[a]

Strain	Carbon source	Time (h)	OD_{660}	G6P[b] Inside (nmol/g)	Outside nmol/ml	Outside mmol/g
L5-30	Glycerol			231	4	0.3
	L-Arabinose			174	7	0.6
	Succinate			286	8	0.6
	Galactose			157	3	0.3
	Xylose			260	3	1.5
	Ribose			351	<2	<0.3
UR16	Glycerol			1,714	9	0.6
	L-Arabinose			737	8	0.7
	Succinate			1,994	8	0.5
	Galactose			974	10	0.8
	Galactose and fructose	14	0.76	3,186	14	11
		27	1.2	2,072	100	52
		62	1.3	1,296	379	178
	Galactose and ribose	13	1.0	4,017	16	10
		25	1.5	2,525	39	17
		60	2.1	1,901	134	40

[a] Cells were grown in MM containing 0.5% of each carbon source. For growth on permissive carbon sources, cultures were inoculated to an optical density at 660 nm (OD_{660}) of 0.25 and allowed to grow to an OD of ca. 1.2. For growth on mixtures of permissive and inhibitory carbon sources, cultures were inoculated to an OD of ca. 0.30, and G6P was assayed after the indicated incubation time.

[b] G6P was determined by an enzymatic method (see text) and was expressed on a wet-cell-weight basis or as nanomoles per milliliter of culture.

Accumulation of G6P. In certain cases, the inhibition of bacterial growth by sugars whose metabolism is blocked has been associated with the accumulation of metabolic intermediates (e.g., as in a phosphoglucose isomerase mutant [1]). In the present study, G6P levels were of interest. Table 2 shows that, by comparison with the wild-type strain, mutant strain UR16 contained 5- to 10-fold higher levels of this compound even when grown on its permissive carbon sources. These levels increased even further when an inhibitory carbon source was also present. During prolonged incubation, the internal levels decreased somewhat, with G6P appearing in the medium.

Symbiotic properties of mutant strains. All mutant strains induced nodule formation and plant response similar to those induced in parental strain-inoculated controls. No significant differences in acetylene reduction levels were detected (Table 3). In each case, bacteria recovered from nodules induced by mutant strains were revertant free.

DISCUSSION

Glucose-6-phosphate dehydrogenase activity in *R. meliloti* L5-30 uses both NAD^+ and $NADP^+$, as previously shown for other rhizobia (11, 12). The mutant strains described in this paper lacked both activities, and their spontaneous reversion frequency suggests that a single mutation was responsible. Together with the isoelectric focusing analysis, these results point to a single enzyme responsible for the two activities, as is also the case in other procaryotes (13, 15).

The growth characteristics of the mutant strains show that glucose-6-phosphate dehydrogenase is an essential enzyme for the catabolism of mannose, glucose, fructose, ribose, and xylose. Their normal growth on L-arabinose and galactose is in accord with the knowledge that the major routes of metabolism of these two sugars are not via G6P (7, 17). NAD- and NADP-linked galactose dehydrogenase activities were detected in parental and mutant strains grown on galactose (data not shown). These results, therefore, confirm the general pattern of carbohydrate metabolism previously suggested for *R. meliloti* (1, 2, 9).

Measurements of G6P yielded results in accord with the assignment of the metabolic block of mutant UR16 being at glucose-6-phosphate dehydrogenase. Even in cells grown on permissive carbon sources, this metabolite was present at substantially higher concentrations than normal. This result might be expected, in that even though the major metabolism of the permissive carbon sources proceeds via other pathways, the cells must have an available biosynthetic route to hexose phosphate, presumably via fructose-1,6-bisphosphate. The considerable accumulation of G6P under these

TABLE 3. Specific activities of nitrogenase in alfalfa root nodules induced by different *R. meliloti* strains[a]

Strain	Acetylene reduction activity (nmol of C_2H_2 per plant per h)[b]
L5-30	50 ± 14
UR16	45 ± 16
UR17	48 ± 12
UR18	38 ± 9
UR19	47 ± 6
UR20	44 ± 7
UR21	37 ± 9
UR22	42 ± 8

[a] Activity was measured on whole plants 4 weeks after inoculation.

[b] Values represent the average of 10 assays ± standard deviations.

permissive conditions, however, shows that the cells are not particularly sensitive to such a perturbation in the level of this intermediate. However, when a major carbon source whose metabolism is normally via G6P was also present, the accumulation of G6P was even greater, and growth was inhibited. It is conceivable that it is the increased accumulation which is directly responsible for the growth inhibition, but this question cannot be resolved from the present data.

Two other matters deserve brief consideration. First, the symbiotic performance of mutant strains may confirm previous findings with a *R. trifolii* mutant incapable of growing on glucose, fructose, or sucrose but still able to develop effective nodules (17). It is not clear whether the same catabolic pathways are used by bacteroids and free-living bacteria, but it seems that hexoses are not important carbon sources for bacteroids, as may be the case with tricarboxylic acid cycle intermediates (10).

Another finding is the lack of Entner-Doudoroff enzyme activities in two of the mutants. One possible explanation might be that, as known for *Escherichia coli* (3) and recently shown for *Pseudomonas aeruginosa* (16), glucose-6-phosphate dehydrogenase and the two genes for the Entner-Doudoroff pathway are closely linked, in which case a reversible point mutation might affect two of the activities. Perhaps more likely would be an explanation related to the yet unknown mechanism of induction of the Entner-Doudoroff pathway enzymes in *R. meliloti*. For example, different degrees of "leakiness" of the glucose-6-phosphate dehydrogenase lesions might affect induction if 6-phosphogluconate were the inducer.

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