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Carbon Metabolism Enzymes of *Rhizobium tropici* Cultures and Bacteroids

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We determined the activities of selected enzymes involved in carbon metabolism in free-living cells of *Rhizobium tropici* CFN299 grown in minimal medium with different carbon sources and in bacteroids of the same strain. The set of enzymatic activities in sucrose-grown cells suggests that the pentose phosphate pathway, with the participation of the Entner-Doudoroff pathway, is probably the primary route for sugar catabolism. In glutamate- and malate-grown cells, high activities of the gluconeogenic enzymes (phosphoenolpyruvate carboxykinase, fructose-6-phosphate aldolase, and fructose bisphosphatase) were detected. In bacteroids, isolated in Percoll gradients, the levels of activity for many of the enzymes measured were similar to those of malate-grown cells, except that higher activities of glucokinase, glucose-6-phosphate dehydrogenase, and NAD-dependent phosphogluconate dehydrogenase were detected. Phosphoglucomutase and UDP glucose pyrophosphorylase showed high and constant levels under all growth conditions and in bacteroids.

Nitrogen fixation in legume root nodules is driven by photosynthates, mainly in the form of sucrose, that are produced in the shoots. The reducing power and ATP indispensable for nitrogenase function are produced in bacteroids by the oxidation of these host plant-provided carbon compounds (2, 26, 30).

One of the approaches used to gain insight into the C-metabolic pathways in bacteroids has been to determine the activities of key enzymes in different biochemical pathways and to compare the activities obtained from bacteroids with those of free-living bacteria grown under different conditions. The general conclusion from this type of work, which has been performed on *Rhizobium* sp. strain NGR234, (25), *Rhizobium leguminosarum* bv. viciae (16), and *R. meliloti* (4, 9), is that carbohydrate-catabolic enzymes in bacteroids have low activities whereas tricarboxylic acid cycle enzymes have high activities. This conclusion supports the generally accepted concept that the major carbon source for bacteroids are the C_4-dicarboxylic acids: malate, succinate, and fumarate (2, 30).

R. tropici was recently identified as a new species on the basis of DNA-DNA hybridization, ribosomal gene sequences, electrophoretic mobilities of metabolic enzymes, and phenotypic characteristics. Strains of *R. tropici* are capable of nodulating *Phaseolus vulgaris* beans as well as several other legumes. The bacteria are highly tolerant to stress conditions such as high temperature, acidity, and the presence of heavy metals, and they have highly stable symbiotic properties (14). These characteristics make *R. tropici* an attractive subject for research.

Here we report the results of comparative studies of carbon-metabolic enzymes by using *R. tropici* bacteroids isolated from *P. vulgaris* root nodules and free-living cells of *R. tropici* grown on several carbon sources.

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MATERIALS AND METHODS

Bacterial cultures and growth conditions. A spectinomycin-resistant derivative of *R. tropici* CFN299 was used. Cultures grown for 1 day on PY agar medium plates (20) were used to inoculate liquid minimal medium (MM). The basal MM had the following composition (per liter of demineralized water): K_2HPO_4, 0.25 g; KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NH_4Cl, 0.375 g; $CaCl_2$, 0.2 g; $FeCl_3$, 0.01 g; biotin, 1 mg; H_3BO_3, 3.17 mg; Na_2MoO_4, 1.0 mg; $MnSO_4 \cdot 4H_2O$, 1.52 mg; $ZnSO_4 \cdot 7H_2O$, 0.25 mg; and $CuSO_4 \cdot 5H_2O$, 0.087 mg. The pH was adjusted to 6.8 with 1 N NaOH. The basal MM was supplemented with 2 g of carbon source per liter, and NH_4Cl was omitted from the MM containing glutamate. Cultures were grown at 30°C with shaking at 220 rpm.

Preparation of cell extracts. *R. tropici* CFN299 cultures in the mid- to late exponential phase of growth were centrifuged at 15,000 × *g* for 10 min at 4°C. The pellets were washed twice in 50 mM potassium phosphate buffer (pH 7.0) and were resuspended in a small volume of the same buffer. The cells were sonicated on ice six times for 45-s intervals with 1-min rest periods by using a Soniprep (MSE). The lysates were centrifuged at 36,000 × *g* for 30 min at 4°C to remove cell debris. The supernatants were divided into two samples. The first sample was immediately used for the citrate synthase, invertase, and maltase assays. To the second sample were added 2-mercaptoethanol (0.2% vol/vol) and 0.1 mM disodium EDTA (9), and it was stored on ice and used for the other enzymatic assays within 5 h after preparation.

The protein concentration in crude extracts (including bacteroid extracts [see below]) ranged from 0.9 to 2.6 mg/ml. The final protein concentration in the assay mixtures ranged from 5 to 13 μg/ml for malate dehydrogenase and 200 to 500 μg/ml for invertase. For other enzymes, 20 to 260 μg of protein per ml was used as appropriate to give similar rates of change in A_{340} in all assays.

Bacteroid isolation. *P. vulgaris* seeds (cv. Negro Xamapa) were surface disinfected and germinated as previously described (13). Three-day-old seedlings were inoculated with *R. tropici* CFN299, planted in sterile vermiculite, and grown aseptically in a greenhouse. The pots were treated with a

nitrogen-free nutrient solution twice per week and with water once per week (13).

The identities of the bacteria in nodules were determined as described previously (13). Pink nodules (3.5 g) from 24-day-old plants were removed from the roots and gently crushed in a chilled mortar containing 2 ml of phosphate-buffered saline (PBS; 0.15 M NaCl in 0.05 M KH_2PO_4 [pH 7.6]). The homogenate was filtered through four layers of cheesecloth, and bacteroids were isolated by centrifugation through self-generating Percoll gradients as described by Reibach et al. (21), except that a Sorvall RC-SB centrifuge with a SS-34 rotor was used at 45,000 × *g* for 55 min.

The bacteroid fraction was collected, washed with PBS, and centrifuged at 25,000 × *g* for 15 min. The pellet was washed twice with 50 mM potassium phosphate buffer (pH 7.0) with centrifugations at 15,000 × *g* for 15 min. The bacteroid pellet was suspended in 4 ml of the same buffer, and the crude extract was obtained as described above for bacterial cultures.

Enzyme assays. Invertase (EC 3.2.1.26) and maltase (EC 3.2.1.20) were assayed as described by Hoelzle and Streeter (8), but the reaction mixture was incubated for 20 min at 30°C. Reactions were terminated by boiling for 5 min, and glucose production was measured with a Boehringer Mannheim kit. Boiled extracts were used as controls, and the glucose values obtained were subtracted from the values obtained with the experimental samples. Malic enzymes (NAD dependent [EC 1.1.1.38] and NADP dependent [EC 1.1.1.40]) were assayed by measuring the production of pyruvate from L-malate as described by McKay et al. (15), but the final absorbance was measured at 445 nm (3). Other enzyme assays were carried out with reaction volumes of 1 ml and a Perkin-Elmer Lambda 3A spectrophotometer at room temperature.

Citrate synthase (EC 4.1.3.7) was assayed in a reaction mixture containing 50 μmol of Tris-HCl buffer (pH 8.0), 0.1 μmol of acetylcoenzyme A, 0.25 μmol of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and 0.2 μmol of oxalacetate. The reaction was initiated by the addition of oxalacetate, and enzyme activity was determined by monitoring the increase in A_{412}.

Malate dehydrogenase (EC 1.1.1.37) (25), β-hydroxybutyrate dehydrogenase (EC 1.1.1.30) (25), isocitrate dehydrogenase (EC 1.1.1.41) (19), phosphoenolpyruvate (PEP) carboxykinase (EC 4.1.1.49) (7), phosphoglucomutase (EC 1.7.5.1) (12), UDP glucose pyrophosphorylase (EC 2.7.7.9) (24), and sucrose phosphorylase (EC 2.4.1.7) (18) were assayed spectrophotometricaly by monitoring the change in A_{340}.

Fructose bisphosphatase (EC 3.1.3.11) was assayed as described by Stowers and Elkan (27) but with 50 mM Tris-HCl buffer (pH 8.0). PEP carboxylase (EC 4.1.1.31) was measured as described by Gordon and Kessler (6) but with 50 mM MOPS (morpholinepropanesulfonic acid [pH 8.0]) or 50 mM Tris-HCl buffer (pH 7.8). Entner-Doudoroff (ED) enzymes (6-phosphogluconate dehydratase [EC 4.2.1.12] and 2-keto-3-deoxy-6-phosphogluconate aldolase [EC 4.1.2.14]) were assayed by two methods as described by Lessie and Vander Wyk (11). In the first method, the rate of the reaction was monitored spectrophotometrically with lactate dehydrogenase and NADH (11). In the second method, pyruvate formation was measured by using 2,4-dinitrophenylhydrazine (11), with modifications as described above for malic enzymes (15). Both methods gave similar results.

Glucokinase (EC 2.7.1.2) was assayed in a reaction mixture containing 50 μmol of Tris-HCl buffer (pH 8.5), 5 μmol of $MgCl_2$, 0.5 μmol of NADP, 2 μmol of ATP, 50 μmol of glucose, and 7 U of glucose-6-phosphate dehydrogenase. The reaction was initiated by the addition of glucose. Fructokinase (EC 2.7.1.4) was assayed in the same mixture, but glucose was replaced by fructose and 7 U of phosphoglucose isomerase was added. Fructose bisphosphate aldolase (EC 4.1.2.13) was assayed in a mixture containing 50 μmol of Tris-HCl buffer (pH 8.0), 3 μmol of fructose 1,6-bisphosphate, 1 μmol of NAD, 5 μmol of $MgCl_2$, 1 μmol of Na_2HAsO_4, 8 U of glyceraldehyde-3-phosphate dehydrogenase, and 5 U of triosephosphate isomerase. Fructose 1,6-bisphosphate was added to start the reaction.

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) were assayed in a reaction with 50 μmol of Tris-HCl buffer (pH 8.5); 5 μmol of $MgCl_2$, 0.5 μmol of NADP, and 1 μmol of glucose 6-phosphate or 6-phosphogluconate, respectively. NAD-dependent 6-phosphogluconate dehydrogenase (EC 1.1.1.43) was assayed in the same mixture but with 50 mM potassium phosphate buffer (pH 7.6). All reactions were initiated by the addition of substrates.

Corrections were made for background NADH-oxidase or NAD- and NADP-reductase activities in all assays. Leghemoglobin was determined as described by LaRue and Child (10) with an SLM Aminco fluorimeter. Bovine hemoglobin was used as the standard. The excitation wavelength was 405 nm, and the fluorescence was measured at 600 nm. Protein was determined by using a Bio-Rad kit with bovine serum albumin as the standard.

Chemicals. ATP, ADP, DTNB, EDTA, PEP, pyruvic acid, isocitric acid, oxaloacetic acid, malic acid, glutathione (reduced form), and bovine hemoglobin were from Sigma. All other chemicals and enzymes were from Boehringer Mannheim.

RESULTS AND DISCUSSION

Carbon metabolism enzymes in free-living cells. Invertase activity was 3.0- to 4.5-fold higher in *R. tropici* CFN299 cells grown in sucrose than in those grown in glutamate or malate (Table 1). There was no detectable sucrose phosphorylase activity in extracts of sucrose grown-cells, suggesting that invertase is the sole enzyme responsible for sucrose cleavage. Similar results were reported for *Rhizobium* sp. strain NGR234 (25).

In sucrose-grown cells, high activities of the key enzymes of the pentose phosphate pathway (glucose-phosphate dehydrogenase and phosphogluconate dehydrogenase), high activities of the ED enzymes, and very low activities of a key enzyme of the Embden-Meyerhof-Parnas pathway (phosphofructokinase) were found. There were no significant differences between enzyme activities of glucose- and fructose-grown cells and the data for sucrose-grown cells listed in Table 1, except for fructokinase (low when grown in glucose) and invertase and maltase (in glucose and fructose, these activities were similar to those found in malate). In general, the results in Table 1 agree with those obtained with other species of fast-growing rhizobia, *R. leguminosarum* bv. trifolii (23) and bv. viciae (16), *R. meliloti* (5, 9), and *Rhizobium* sp. strain NGR234 (25). Taken together, these results indicate that the pentose phosphate pathway, with the participation of the ED pathway, is the primary route for sugar catabolism in *Rhizobium* spp.

We found that the activities of glucose-6-phosphate dehydrogenase and of the ED enzymes in C_4 substrate-grown cells were only 30 to 38% of the activities in sucrose-grown cells. In contrast, NADP-phosphogluconate dehydrogenase activities were similar in cells grown on any of the carbon sources tested.

In comparison with sucrose-grown cells, bacteria grown in glutamate and malate had sharply increased levels of PEP carboxykinase and fructose-6-phosphate aldolase activities.

TABLE 1. Activities of selected carbon metabolism enzymes in free-living cells growing in different carbon sources and in bacteroids of *R. tropici* CFN299

Enzyme	Activity[a] (nmol/min/mg of protein) of:			
	Sucrose-grown cells	Glutamate-grown cells	Malate-grown cells	Bacteroids isolated in Percoll gradients
Invertase	87 ± 4	29 ± 3	19 ± 5	16 ± 3
Maltase	148 ± 8	61 ± 4	37 ± 6	31 ± 7
Glucokinase	467 ± 25	176 ± 22	372 ± 50	624 ± 47
Fructokinase	385 ± 40	161 ± 8	215 ± 24	144 ± 17
Glucose-6-phosphate dehydrogenase-NADP	538 ± 38	206 ± 24	184 ± 25	379 ± 29
Phosphogluconate dehydrogenase				
NADP dependent	171 ± 22	140 ± 40	179 ± 20	156 ± 15
NAD dependent	105 ± 10	43 ± 12	47 ± 6	188 ± 20
ED enzymes	88 ± 9	28 ± 4	25 ± 12	34 ± 7
Phosphofructokinase[b]	3	1	1	18
Fructose bisphosphate aldolase	19 ± 5	75 ± 10	112 ± 15	53 ± 5
Citrate synthase	161 ± 12	93 ± 9	160 ± 17	178 ± 18
Malate dehydrogenase	3,698 ± 275	5,367 ± 600	10,100 ± 1170	8,970 ± 640
Isocitrate dehydrogenase	644 ± 45	515 ± 43	811 ± 87	596 ± 55
Malic enzymes				
NAD dependent	213 ± 21	206 ± 21	293 ± 21	354 ± 39
NADP dependent	133 ± 9	166 ± 14	157 ± 16	125 ± 15
Hydroxybutyrate dehydrogenase	10 ± 3	64 ± 11	4 ± 1	901 ± 130
PEP carboxykinase	8 ± 2	211 ± 26	432 ± 130	205 ± 15
Fructose bisphosphatase	119 ± 30	90 ± 10	94 ± 11	231 ± 18
Phosphoglucomutase	341 ± 40	327 ± 20	269 ± 20	333 ± 34
UDP glucose pyrophosphorylase	352 ± 15	363 ± 37	339 ± 40	273 ± 32

[a] Each value is the mean ± standard deviation of three or four independent experiments.
[b] Data were obtained from a single experiment.

This confirms previous reports that these two enzymes play an important role in gluconeogenesis in rhizobia (16, 17). A high activity of the PEP carboxykinase in succinate- but not glucose-grown *R. meliloti* cells has also been shown (4, 5).

Phosphoglucomutase and UDP-glucose phosphorylase, which drive glucose 6-phosphate into biosynthetic pathways, does not appear to be regulated by the carbon source in *R. tropici*, because high activities were obtained in different carbon sources. Activities of the malic enzymes and the tricarboxylic acid cycle enzymes citrate synthase and isocitrate dehydrogenase showed only slight variations as a result of carbon source, but malate dehydrogenase activity was 2.7-fold higher in malate- than sucrose-grown cells.

Carbon metabolism enzymes in bacteroids. We confirmed that the bacteria occupying the nodules used for bacteroid isolation were only *R. tropici* CFN299 by identifying individual colonies from nodules for their antibiotic resistance and by observing their growth characteristics in selective media. Bacteroids were isolated from nodules in four different experiments, and the cell extracts obtained were completely free from leghemoglobin and had no activity of PEP carboxylase.

Bacteroids contained carbohydrate-catabolizing enzymes at similar or even higher levels compared with free-living bacteria grown in malate. It should be mentioned that the activity of the NAD-dependent phosphogluconate dehydrogenase in bacteroids was higher than that found in free-living bacteria grown on any of the carbon sources tested. The product of these enzymes in rhizobia is not clear (reviewed in reference 26), but the activity found in bacteroids leads us to suggest that it has a role in the symbiotic form of *R. tropici*. An unexpected finding was the presence of invertase activity in bacteroids, since it has been shown that bacteroids from soybean nodules have maltase activity but not invertase activity (29). This is why invertase is considered to be an enzymatic marker for the plant cytosol (21). Very low invertase activity has been reported in *Rhizobium* sp. strain NGR234 bacteroids (25). It is not clear if invertase plays some role in *R. tropici* bacteroids, but we believe that invertase activity should not be considered a marker enzyme of the plant cytosol in the legume root nodules formed by *Rhizobium* spp.

It has been shown that gluconeogenesis probably does not occur in *R. leguminosarum* bv. viciae (17) and in *R. meliloti* (4, 5) bacteroids. As shown in Table 1, *R. tropici* bacteroids have high activities of PEP carboxykinase, fructose bisphosphate aldolase, and fructose bisphosphatase. This suggests that the gluconeogenic pathway is operating in *R. tropici* bacteroids.

We found high activities of tricarboxylic acid cycle enzymes, as has been reported before for *Rhizobium* bacteroids (2, 4, 5, 9, 16), with the exception of strain NGR234 (25). In addition, we found high activities of malic enzyme, in agreement with other reports (3, 15).

The activity of one of the key enzymes involved in poly-β-hydroxybutyrate degradation, β-hydroxybutyrate dehydrogenase, was dramatically higher in bacteroids than in free-living cells. This increased activity in bacteroids ranged from 14-fold when compared with glutamate-grown cells to 220-fold when compared with malate-grown cells. This fact leads us to postulate that in *R. tropici* bacteroids the poly-β-hydroxybutyrate cycle is actively operating, as has been shown in *Bradyrhizobium* bacteroids (1, 22).

General remarks. The regulation of carbon-metabolizing enzymes in rhizobia by carbon source is species dependent. In succinate-grown cells of *Bradyrhizobium* strain 32H1, none of the carbohydrate-catabolizing enzymes assayed for were detectable (28). In *Rhizobium* sp. strain NGR234, key enzymes of the pentose phosphate and ED pathways in glucose-grown cells had 3- to 18-fold-greater activity than in succinate-grown cells (25), and similar data have been obtained with *R. meliloti* (4, 5). For another *R. meliloti* strain (9) and for *R. leguminosarum* bv. viciae (16), it has been shown that the response of

the sugar-catabolizing enzymes, in relation to growth on organic acids, was weak and more selective. Considering the data presented in Table 1, we suggest that in *R. tropici* CFN299, many enzymes involved in sugar metabolism are probably not highly regulated by the carbon source.

In bacteroids, the high activities of the malic and tricarboxylic acid cycle enzymes, in concert with the high activities of the gluconeogenic enzymes (PEP carboxykinase, fructose-6-phosphate aldolase, and fructose bisphosphate), support the general point of view that *R. tropici* bacteroids assimilate C_4 dicarboxylates. In addition, bacteroids contained high activities of glucokinase, glucose-6-phosphate-dehydrogenase, and phosphogluconate dehydrogenases (NADP and NAD dependent). This fact makes it possible to speculate that *R. tropici* bacteroids can simultaneously assimilate organic acids as well as some sugars. This proposal has also been made for *R. leguminosarum* bv. viciae bacteroids (17). To clarify this point, more work should be done with isolated bacteroids and with *R. tropici* mutants defective in carbon transport and metabolism.

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