APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1990, p. 2587–2589
0099-2240/90/082587-03$02.00/0

Vol. 56, No. 8

Carbon Metabolism Enzymes of *Rhizobium meliloti* Cultures and Bacteroids and Their Distribution within Alfalfa Nodules†

JUAN JOSE IRIGOYEN,[1] MANUEL SANCHEZ-DIAZ,[1] AND DAVID W. EMERICH[2]*

Departamento de Fisiologia Vegetal, Universidad de Navarra, 31080 Pamplona, Spain,[1] and Department of Biochemistry, University of Missouri, Columbia, Missouri 65211[2]

Received 4 April 1990/Accepted 12 June 1990

Several carbon metabolism enzymes were measured in cultured cells and bacteroids of *Rhizobium meliloti* 102F51 and in alfalfa root nodule cytosol. The enzyme activity levels of the pentose phosphate pathway were much higher than those of the Embden-Meyerhof-Parnas or Entner-Doudoroff pathways in extracts of cultured cells. The pattern of enzyme activities in the bacteroids was different from that of cultured cells.

Bacteria of the genus *Rhizobium* can form symbioses with certain members of the family Leguminosae, resulting in the reduction of atmospheric dinitrogen to ammonium. The *Rhizobium* bacteria invade the roots via infection threads formed by the plant cells. The plant root cells undergo rapid cortical division to form a tumorlike growth referred to as a nodule. During nodule development, the bacteria undergo morphological and physiological changes that lead to a form, called a bacteroid, capable of symbiotic dinitrogen fixation. The energy to support symbiotic dinitrogen fixation is provided by carbon metabolites from the plant root nodule cells. The carbon metabolism of *Rhizobium meliloti* bacteroids is not as well characterized as that of other strains of *Rhizobium* symbionts. Here we report a comparative metabolic study of alfalfa root nodule bacteroids, free-living cultures of *R. meliloti* grown on several carbon substrates, and the plant nodule cytosol.

R. meliloti 102F51 (Nitragin Co., Milwaukee, Wis.) was grown on a minimal single-carbon-source medium that consisted of the following ingredients (per liter): 0.36 g of KH_2PO_4, 1.4 g of K_2HPO_4, 0.2 g of NaCl, 2.5 mg of H_3BO_3, 1 mg of $CuSO_4$, 2 mg of Na_2MnO_4, 0.27 g of NH_4Cl, 0.25 g of $MgSO_4$, 15 mg of disodium EDTA, 10 mg of $MnSO_4$, 1 mg of $CoCl_2$, 6.5 mg of $FeCl_3$, 1 mg of $NiCl_2$, 1 mg of $ZnSO_4$, 1 mg of thiamine, 2 mg of calcium pantothenate, 0.01 mg of biotin, and 5 mM carbon source (mannitol, glucose, sucrose, succinate, malate, or acetate). The medium was adjusted to pH 7.0 before autoclaving.

R. meliloti cultures in the mid- to late exponential phase of growth were centrifuged at 8,000 × *g* for 10 min, washed twice in 50 mM potassium phosphate buffer (pH 7.8), and suspended in 20 ml of 50 mM potassium phosphate buffer (pH 7.8) containing 0.2% (vol/vol) 2-mercaptoethanol and 0.1 mM disodium EDTA. The suspension was passed through a French pressure cell at 16,000 lb/in^2 and centrifuged at 39,000 × *g* for 20 min to remove cell debris. The supernatant was used for enzyme assays. Alcohol dehydrogenase (4), glucose-6-phosphate dehydrogenase (9), 6-phosphogluconate dehydrogenase (9), hydroxybutyrate dehydrogenase (7), isocitrate dehydrogenase (7), and malate dehydrogenase (7) were assayed spectrophotometrically by the change in A_{340}. Malate dehydrogenase from bacteria or bacteroids was measured in 100 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 8.3), and malate dehydrogenase from the plant cytosol was measured in 50 mM potassium phosphate buffer (pH 7.6). Fructose-1,6-bisphosphate aldolase was measured in 50 mM Tris buffer (pH 7.4) containing 5.7 mM D-fructose-1,6- bisphosphate, 0.3 mM NADH, and the coupling enzymes glycerophosphate dehydrogenase (2.5 U per assay) and triosephosphate isomerase (1.2 U per assay). Phosphoenolpyruvate carboxylase was assayed as described by Deroche et al. (3). Pyruvate kinase was measured in 100 mM Tris buffer (pH 8.0) containing 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 1.25 mM tri(cyclohexylammonium)phosphoenolpyruvate, 0.75 mM ATP, 0.3 mM NADH, and the coupling enzyme lactic dehydrogenase (1.2 U per assay). Glutamate-oxaloacetate transaminase was assayed as described by Bergmeyer (1). Malic enzyme was assayed in 100 mM potassium MOPS (morpholinepropanesulfonic acid) buffer (pH 7.0) containing 1 mM $MgCl_2$, 0.5 mM NADP, and 13.5 mM malate. The Entner-Doudoroff activity was assayed as described by Keele et al. (9) but with 50 mM potassium phosphate buffer (pH 7.0) and without $FeSO_4$. Protein was measured by the Bradford method (2) with bovine serum albumin as the standard.

The activity of eight carbon metabolism enzymes was measured in cell extracts of *R. meliloti* 102F51 grown on the six carbon sources (Table 1). In general, the results in Table 1 agree with those obtained with other species of fast-growing rhizobia (5, 8, 10–12), indicating that the pentose phosphate pathway may be the primary route of sugar catabolism. The pentose phosphate pathway enzymes showed appreciable activity when the bacteria were grown on the dicarboxylic acids succinate or malate or on acetate, although the activity levels were lower than those in bacteria grown on sugars or mannitol. Glucose-6-phosphate dehydrogenase activity levels were 20 to 40% lower when the bacteria were grown on succinate, malate, or acetate than when they were grown on sugars or mannitol. The Embden-Meyerhof-Parnas pathway did not appear to operate, as phosphofructokinase activity was not detected (data not shown) and the fructose-1,6-bisphosphate aldolase activity level was significantly lower than the glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activity levels.

The activity level of the Entner-Doudoroff pathway in cultures grown on organic acids was 40 to 50% of those found in cultures grown on sugars (Table 1), showing the same trend as was reported for *Rhizobium leguminosarum* (5, 11). Malate dehydrogenase and isocitrate dehydrogenase

* Corresponding author.

† Contribution no. 11,215 of the Agricultural Experiment Station of the University of Missouri.

TABLE 1. Specific activities of the several carbon metabolism enzymes in free-living cells growing in different carbon sources and in bacteroids of *R. meliloti* 102F51

Enzyme or pathway[a]	Sp act[b] in:						
	Free-living cells grown in:						Bacteroids
	Mannitol	Glucose	Sucrose	Succinate	Malate	Acetate	
MDH	406 ± 60	611 ± 110	622 ± 40	903 ± 40	528 ± 50	659 ± 6	1,074 ± 110
ICDH	534 ± 60	579 ± 22	612 ± 30	678 ± 20	758 ± 80	697 ± 80	642 ± 90
GPDH	442 ± 50	436 ± 40	526 ± 34	193 ± 30	128 ± 20	99 ± 30	16 ± 2
PGDH	204 ± 10	219 ± 20	244 ± 15	176 ± 10	169 ± 14	151 ± 7	19 ± 2
ADH	268 ± 14	309 ± 80	343 ± 44	695 ± 131	448 ± 70	194 ± 7	7 ± 2
ALD	83 ± 4	38 ± 4	66 ± 6	47 ± 2	25 ± 1	26 ± 11	6 ± 1
PK	261 ± 50	184 ± 10	306 ± 45	221 ± 20	137 ± 7	103 ± 20	19 ± 2
ED	62 ± 7	63 ± 3	66 ± 9	39 ± 3	39 ± 2	33 ± 10	<0.1

[a] Abbreviations: MDH, malate dehydrogenase; ICDH, isocitrate dehydrogenase; GPDH, glucose-6-phosphate dehydrogenase; PGDH, 6-phosphogluconate dehydrogenase; ADH, alcohol dehydrogenase; ALD, aldolase; PK, pyruvate kinase; ED, Entner-Doudoroff pathway.

[b] Activity is expressed in nanomoles of product per minute per milligram of protein. Each value is the mean ± the standard deviation of three independent experiments.

were usually the most active of all of the enzymes measured, regardless of the carbon source. Malate dehydrogenase activity was greatest in succinate-grown cultures and varied by a factor of about two over the range of carbon substrates used, whereas isocitrate dehydrogenase activity was greatest in malate-grown cultures and varied by 30% or less over the carbon substrate range.

Alfalfa (*Medicago sativa* cv. Aragon) seedlings, 3 days old, were inoculated with *R. meliloti* 102F51 and planted in sand. Twice a week the pots were treated with a nitrogen-free nutrient solution (7), and once a week they were watered to avoid salt accumulation. The acetylene reduction activity of 12-week-old plants was 100.1 ± 1.54 μmol/g (dry weight) of nodules per h (mean ± standard deviation). Nodules were removed from the roots, added to isolation buffer (0.5 g [fresh weight] of nodules per 10 ml of 50 mM potassium phosphate buffer [pH 7.8]–0.2% [vol/vol] 2-mercaptoethanol–0.1 mM disodium EDTA–10% [wt/wt] polyvinylpyrrolidone), and ground with an ice-cold mortar and pestle. The homogenate was filtered through four layers of cheesecloth and centrifuged at 120 × *g* for 10 min at 4°C to remove plant debris. All subsequent steps were performed at 4°C. The supernatant was centrifuged at 19,000 × *g* for 10 min, and the resulting supernatant, the plant cytosol, was used for enzyme assays. The pellet, which contained the bacteroids, was suspended in 10 ml of 50 mM potassium phosphate buffer (pH 7.8) and washed three times to remove any plant material adhering to the bacteroid surface. The bacteroids were suspended in 10 ml of 50 mM potassium phosphate buffer (pH 7.8) containing 0.2% (vol/vol) 2-mercaptoethanol and 0.1 mM disodium EDTA and ruptured by passage through a French pressure cell at 16,000 lb/in^2 and centrifuged at 39,000 × *g* for 20 min to remove cell debris. The supernatant was used for enzyme assays.

Bacteroids isolated from alfalfa root nodules were found to contain all of the enzyme activities measured in free-living cultures with the exception of the Entner-Doudoroff activity. No Entner-Doudoroff activity was measured after prolonged periods of assay (1, 2, 3, 4, and 12 h) or under a variety of assay conditions. The presence of a possible endogenous inhibitor was tested by mixing cell extracts from bacteroids with extracts of cultured cells. No inhibition of the activity of cultured cells was observed. Saroso et al. (11) have reported the apparent absence of Entner-Doudoroff activity in cowpea rhizobia.

The pattern of specific activities of the enzymes measured from *R. meliloti* bacteroid extracts was not similar to any of the patterns observed from cells cultured on defined medium containing single carbon sources. The activity levels of the pentose phosphate enzymes glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were 16% or less of the activity levels found in extracts of cultured cells. The low activities of these two enzymes and aldolase, alcohol dehydrogenase, and pyruvate kinase have been noted in bacteroids from *Rhizobium* spp. (11) and *R. leguminosarum* (5). The two enzymes of the citric acid cycle, malate dehydrogenase and isocitrate dehydrogenase, were the only ones which displayed activities comparable to those in cultured cells. In snakebean bacteroids (11), these two enzyme activity levels were lower than those observed in free-living cells.

Hydroxybutyrate dehydrogenase and phosphoenolpyruvate carboxylase were used as marker enzymes for the bacteroid and plant nodule cytosol, respectively, to ensure that each fraction was not contaminated with the other corresponding fraction (Table 2). A comparison of the relative activity levels of several carbon-metabolic enzymes showed that the plant nodule cytosol had considerably

TABLE 2. Distribution of several carbon metabolism enzymes in alfalfa nodules

Enzyme[a]	Activity level[b] in fraction		Activity ratio (plant fraction: bacteroid fraction)
	Plant	Bacteroid[c]	
MDH	439 ± 36	3.1 ± 0.2	142:1
ICDH	7.4 ± 0.4	1.7 ± 0.1	4.4:1
GPDH	2.6 ± 0.1	0.19 ± 0.02	13.7:1
PGDH	3.0 ± 0.1	0.19 ± 0.02	15.8:1
ME	0.78 ± 0.04	0.07 ± 0.01	11:1
ADH	8.1 ± 0.1	0.08 ± 0.02	101:1
ALD	1.8 ± 0.1	0.03 ± 0.01	60:1
PK	7.6 ± 0.4	0.09 ± 0.01	84:1
GOT	136 ± 11	1.83 ± 0.16	74:1
HBD	ND[d]	0.08 ± 0.01	
PEPC	39.4 ± 1.7	ND	

[a] Abbreviations: ME, malic enzyme; GOT, glutamate-oxaloacetate transaminase; HBD, hydroxybutyrate dehydrogenase; PEPC, phosphoenol pyruvate carboxylase. All other enzyme abbreviations are the same as in Table 1, footnote *a*.

[b] Activity is expressed in micromoles of product per minute per gram (fresh weight) of nodules. Each value is the mean ± standard deviation of four to six independent determinations.

[c] Values for bacteroid enzyme activities include the three replicate experiments from Table 1, except for malic enzyme. Malic enzyme values are means ± standard deviations of three independent determinations.

[d] ND, Not detected.

higher levels of each of these enzyme activities, in terms of total enzyme activity (micromoles of product per minute per gram [fresh weight] of nodules), than the bacteroids (Table 2). The plant cytosol fraction contained approximately six times more protein than the bacteroid soluble fraction (16.5 ± 1.7 versus 2.8 ± 0.5 mg of protein per g [dry weight] of nodules). On a specific activity basis (milligrams of soluble protein), isocitrate dehydrogenase was the only enzyme whose activity level was greater in the bacteroids than in the plant cytosol. These results are in contrast to those reported by Henson et al. (6), who found that 95% of the nodule NADP-dependent isocitrate dehydrogenase was present in the plant fraction of the alfalfa nodule. Generally, in both the plant and the bacteroid, the enzymes with the highest activity levels were those which utilize citric acid cycle intermediates, followed by enzymes of the pentose phosphate pathway and pyruvate kinase. The lowest activity levels found were those of malic enzyme and aldolase. Alcohol dehydrogenase activity was low in the bacteroids, but in the plant cytosol, this activity was comparable to those of isocitrate dehydrogenase and pyruvate kinase.

We thank Jackie Kimsey for typing the manuscript.

This work was supported by the U.S.-Spain Joint Committee for Scientific and Technological Cooperation in Basic Sciences (1985 program, CCB-8509/008) and U.S. Department of Agriculture competitive grant CRCR 89-37120-4742.

LITERATURE CITED

1. **Bergmeyer, H. U.** 1974. Methods of enzymatic analysis, 3rd ed., vol. 2, p. 727–733. Verlag Chemie, Weinheim, Federal Republic of Germany.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
3. **Deroche, M. E., E. Carrayol, and E. Jolivet.** 1983. Phosphoenolpyruvate carboxylase in legume nodules. Physiol. Veg. **21:** 1075–1081.
4. **De Vries, G. E., P. T. Veld, and J. W. Kijne.** 1980. Production of organic acids in *Pisum sativum* root nodules as a result of oxygen stress. Plant Sci. Lett. **20:**115–123.
5. **Glenn, A. R., I. A. McKay, R. Arwas, and M. J. Dilworth.** 1984. Sugar metabolism and the symbiotic properties of carbohydrate mutants of Rhizobium leguminosarum. J. Gen. Microbiol. **130:** 239–245.
6. **Henson, C. A., M. Collins, and S. H. Duke.** 1982. Subcellular localization of enzymes of carbon and nitrogen metabolism in nodules of *Medicago sativa*. Plant Cell Physiol. **23:**227–235.
7. **Karr, D. B., J. K. Waters, F. Suzuki, and D. W. Emerich.** 1984. Enzymes of poly-β-hydroxybutyrate and citric acid cycles of *Rhizobium japonicum* bacteroids. Plant Physiol. **75:**1158–1162.
8. **Katznelson, H., and A. C. Zagallo.** 1957. Metabolism of Rhizobium in relation to effectiveness. Can. J. Microbiol. **3:**879–884.
9. **Keele, B. B., Jr., P. B. Hamilton, and G. H. Elkan.** 1969. Glucose catabolism in *Rhizobium japonicum*. J. Bacteriol. **97:** 1184–1191.
10. **Martinez-De Drets, G., and A. Arias.** 1972. Enzymatic basis for differentiation of *Rhizobium* into fast- and slow-growing groups. J. Bacteriol. **109:**467–470.
11. **Saroso, S., M. J. Dilworth, and A. R. Glenn.** 1986. The use of activities of carbon catabolic enzymes as a probe for the carbon nutrition of snakebean nodule bacteroids. J. Gen. Microbiol. **132:**243–249.
12. **Stowers, M. D., and G. H. Elkan.** 1985. Regulation of the hexose catabolism in *Rhizobium* sp. 32H1. FEMS Microbiol. Lett. **26:**45–48.