JOURNAL OF BACTERIOLOGY, Sept. 1982, p. 1621–1623
0021-9193/82/091621-03$02.00/0

Vol. 151, No. 3

Succinate Dehydrogenase Mutant of *Rhizobium meliloti*

A. GARDIOL,†* A. ARIAS, C. CERVEÑANSKY, AND G. MARTÍNEZ-DRETS
Division of Biochemistry, Instituto de Investigaciones Biologicas Clemente Estable, Montevideo, Uruguay

Received 24 March 1982/Accepted 23 May 1982

A succinate dehydrogenase mutant strain of *Rhizobium meliloti* was isolated after nitrosoguanidine mutagenesis. It failed to grow on succinate, glutamate, acetate, pyruvate, or arabinose but grew on glucose, sucrose, fructose, and other carbohydrates. The mutant strain showed delayed nodulation of lucerne plants, and the nodules were white and ineffective. A spontaneous revertant strain of normal growth phenotype induced red and effective nodules.

Organic acid metabolism in *Rhizobium* species has not been extensively studied. Graham reported that tricarboxylic acid (TCA) cycle intermediates promote growth of different species of fast-growing rhizobia; however, growth of slow growing strains was much more limited (13). Oxidation of several TCA cycle intermediates has been studied both in cultured (15, 23) and in nodule-extracted *Rhizobium* species (20, 22, 23). Organic acids are present in soybean nodules in lower concentrations than carbohydrates (2). Nevertheless, they support the highest rate of oxygen respiration by bacteroid suspensions (23), and succinate in particular is the most effective substrate for supporting nitrogen fixation (3).

These observations warranted further study of succinate catabolism by *rhizobia*. The isolation of a succinate dehydrogenase mutant strain would be useful for study of this catabolism and exploration of the relationship between the TCA cycle and symbiotic nitrogen fixation by *Rhizobium* species. A mutant strain defective in α-ketoglutarate dehydrogenase has already been isolated from *Rhizobium meliloti* (7).

This paper reports the isolation, identification, and symbiotic properties of a succinate dehydrogenase mutant of *R. meliloti* L5-30.

(A preliminary report of this work has been presented [Proc. 4th Int. Symp. Nitrogen Fixation, Canberra, Australia, December, 1980, abstr. no. 240].)

The parent strain used was *R. meliloti* L5-30 (*str*^{r}) reisolated from nodules of lucerne (*Medicago sativa* var. Du Puits). The parent and mutant strains were maintained on mannitol minimal medium (MM) (1). Cells were grown in MM containing mannitol (0.5%) plus succinate (0.3%) and 3 mg of mono- and dipotassium phosphates per ml. Plates also contained 2% agar. Rich medium (RM) was described previously (10). All sugars were of the D-configuration except for L-arabinose.

Nitrosoguanidine mutagenesis (20% survival) was performed as earlier described (1) and was followed by one penicillin enrichment cycle in succinate minimal medium to enrich for mutants unable to grow on succinate. Surviving cells (0.6%) were cloned on RM containing succinate (0.3%), and the plates with isolated colonies were overlaid with nitrotetrazolium blue (NBT) reagent in the presence of phenazine methosulfate (8). Colonies which failed to reduce this dye were picked as presumptive mutant strains deficient in succinate dehydrogenase. One of these isolates (UR6) which was unable to grow on succinate MM but still grew on mannitol MM was selected for further studies. Spontaneous revertants, which were phenotypically similar to the wild-type strain, were selected on MM containing succinate (0.3%). One of them (UR7) was used in these studies. Reversion of strain UR6 on succinate occurred at a frequency of 5×10^{-6}.

Published methods were used for assaying succinate dehydrogenase (EC 1.3.99.1) (15), malate dehydrogenase (EC 1.1.1.37) (7), isocitrate dehydrogenase (EC 1.1.1.42) (7), citrate synthase (EC 4.1.3.7) (19), α-ketoglutarate dehydrogenase (EC 1.2.4.2), and pyruvate dehydrogenase (EC 1.2.4.1) (17). Protein was measured by the method of Bücher (5), with bovine serum albumin as a standard. Nitrogen fixation activity in root nodules of lucerne plants was performed by the acetylene reduction assay as previously described (1).

Growth properties of *R. meliloti* L5-30 and mutant strain UR6 are shown in Table 1. Strain UR6 grew on several carbon sources but produced smaller colonies than did the parent strain. The inability to grow on succinate and

† Present address: Department of Microbiology and Public Health, Michigan State University, East Lansing, MI 48824.

TABLE 1. Growth of parent (L5-30) and mutant (UR6) strains of *R. meliloti* on several carbon sources

Carbon source[a]	Colony diam (mm)[b]	
	L5-30	UR6
None	0.4	0.0
Glucose	1.9	0.5
Fructose	1.9	0.5
Mannose	2.0	0.5
Galactose	1.8	0.6
Lactose	1.8	0.4
Sucrose	1.6	0.5
Maltose	2.1	0.4
Mannitol	2.0	0.6
Sorbitol	1.8	0.6
Arabitol	2.0	0.9
Ribose	2.4	0.7
Xylose	1.5	0.4
Glycerol	1.3	0.3
Fumarate	1.2	0.5
Malate	0.8	0.5
Succinate	1.5	0.0
Glutamate	1.5	0.0
Acetate	0.5	0.0
Pyruvate	1.4	0.0
L-Arabinose	2.0	0.0

[a] Cultures were diluted to produce approximately 50 colonies and spread on minimal medium plates containing the indicated carbon source at 0.5%, except for acetate at 0.2% and malate at 0.1%.

[b] Average colony diameters were measured after 8 days of incubation at 30°C.

glutamate was expected as these substrates are channeled via the TCA cycle, which is blocked at the level of succinate dehydrogenase in UR6 (Table 2). Failure to grow on L-arabinose could be explained by similar reasoning since the parent strain metabolizes this sugar through a pathway leading to α-ketoglutarate. The glyoxylate cycle is probably used for acetate growth, and an α-ketoglutarate dehydrogenase mutant of *R. meliloti* was also unable to grow on acetate and pyruvate (7). UR6 was able to grow on fumarate and malate similarly to a mutant strain of *R. leguminosarum* with reduced succinate dehydrogenase activity (9). The nature of the mutation is not known. However, the phenotype seemed to be caused by a single mutation since revertants selected on succinate grew also on pyruvate, arabinose, acetate, and glutamate.

Table 2 shows the specific activity of several enzymes of the TCA cycle in each strain. Succinate dehydrogenase activity was not detected in UR6, and the revertant UR7 regained the activity. Succinate dehydrogenase was also detected in *R. meliloti* L5-30 grown in MM containing succinate, mannitol, mannitol plus succinate, glycerol, glucose, fructose, xylose, or L-arabinose. [^{14}C]succinate uptake activity was present in the mutant strain, although with a lower rate than in the parent strain. Ammonia assimilatory enzymes, NADH-linked glutamate dehydrogenase, NADPH-linked glutamate synthase, and glutamine synthetase were also present in cell-free extracts of parent and mutant strains (data not shown).

Nodule formation and acetylene reduction activity were assayed (Table 3). Nodulation induced by UR6 was delayed, and the nodules formed were white and unable to fix nitrogen as measured by the acetylene reduction assay. Bacteria recovered from these nodules had the same phenotype as the mutant strain. However, both *R. meliloti* L5-30 and UR7 induced the normal formation of red nodules with similar nitrogenase activities.

The failure of mutant strain UR6 to induce effective nodules suggests that a complete TCA cycle is needed for a functional symbiosis. This supports many observations pointing to the role of the TCA cycle for uptake and metabolism of organic acids in the nitrogen fixation process of legume root nodules. Succinate, fumarate, malate, and malonate have been detected in root nodules of soybean plants in higher concentrations than in roots or leaves (21); organic acid production, mainly of malate, has been demonstrated in *Psium sativum* root nodules; and a high rate of malate and succinate uptake has been reported for isolated bacteroids of *R. leguminosarum* (6). Pea nodules which are ineffective because of the plant genotype have been incubated in the presence of succinate or pyruvate and regained the ability to reduce acetylene

TABLE 2. TCA cycle enzymes in the parent (L5-30), mutant (UR6), and revertant (UR7) strains[a]

Enzyme	Sp act (nmol/min per mg of protein)[b]		
	L5-30	UR6	UR7
Pyruvate dehydrogenase	5.1	4.0	3.9
Citrate synthase	78	156	83.3
Isocitrate dehydrogenase	241	195	135
α-Ketoglutarate dehydrogenase	7.1	3.0	2.4
Succinate dehydrogenase	38	0	15
Malate dehydrogenase	704	533	572

[a] Cells were grown in minimal medium containing 0.5% mannitol plus 0.3% succinate.

[b] Cell-free extracts were prepared by French press treatment as previously described (1), except that 0.025 M Tris-hydrochloride buffer (pH 7.6) containing 10 mM 2-mercaptoethanol and 0.1% bovine serum albumin was used to resuspend the cell pellet. All enzyme activities were measured in a Gilford spectrophotometer model 250 and expressed as nanomoles of substrate consumed or product formed per minute per milligram of extract protein. Corrections were made for the small changes in controls without substrate.

TABLE 3. Acetylene reduction by lucerne plants inoculated with parent (L5-30), mutant (UR6), and revertant (UR7) strains of *R. meliloti*[a]

Strain	Acetylene reduction activity[b] (nmol of C_2H_4 per plant per h)
L5-30	92
UR6	4
UR7	96

[a] Activity was measured on whole plants of lucerne 21 days after inoculation.

[b] Data are mean values obtained from several plants. (Three independent experiments were run.) Corrections were made for the small amounts of ethylene produced in uninoculated controls. Ethylene formation was measured by gas chromatography with the use of a column (0.5 m by 3 mm) packed 0.25 m with Porapak N and 0.25 m with Porapak G (80 to 100 mesh) at 20°C and a flame ionization detector of a Glowall 310 gas chromatograph. Ethylene peaks were measured by using an ethylene standard curve.

(14). TCA cycle intermediates have been used for nitrogenase production in free-living cultures of *Rhizobium* sp. strain 32H1, and the highest activity is with succinate (11); omitting succinate from the culture medium causes the concentration of dissolved oxygen to rise, and nitrogenase activity is lost (4). Similar to the UR6 mutant strain, *R. trifolii* mutant strains defective in C_4-dicarboxylate transport (18), *R. leguminosarum* mutant strains with decreased uptake and metabolism of succinate (12), and an α-ketoglutarate dehydrogenase mutant strain of *R. meliloti* (7) also form ineffective nodules on their respective hosts.

We thank F. Dazzo, G. Favelukes, and J. Sherwood for critical review of this manuscript.

This investigation was partially supported by project RLA 78-024 from PNUD/UNESCO and the Organization of American States (RPSDAT).

LITERATURE CITED

1. **Arias, A., C. Cerveñansky, A. Gardiol, and G. Martínez-Drets.** 1979. Phosphoglucose isomerase mutant of *Rhizobium meliloti*. J. Bacteriol. **137**:409–414.
2. **Bach, M. K., W. E. Magee, and R. H. Burris.** 1958. Translocation of photosynthetic products to soybean nodules and their role in nitrogen fixation. Plant Physiol. **33**:118–124.
3. **Bergersen, F. J.** 1977. Physiological chemistry of dinitrogen fixation by legumes p. 519–555. *In* R. W. F. Hardy and W. S. Silver (ed.), A treatise on dinitrogen fixation, section III. John Wiley and Sons, New York.
4. **Bergersen, F. J., G. L. Turner, A. H. Gibson, and W. F. Dudman.** 1976. Nitrogenase activity and respiration of cultures of *Rhizobium* spp. with special reference to concentration of dissolved oxygen. Biochim. Biophys. Acta **444**:164–174.
5. **Bücher, T.** 1947. Uber ein Phosphat uber tragendes Garungsferment. Biochim. Biophys. Acta **1**:292–314.
6. **DeVries, G. E., P. In't Veld, 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.
7. **Duncan, M. J., and D. G. Fraenkel.** 1979. α-Ketoglutarate dehydrogenase mutant of *Rhizobium meliloti*. J. Bacteriol. **137**:415–419.
8. **Edwards, D. L., D. M. Belsole, H. J. Guzik, and B. W. Unger.** 1979. Selection of succinic dehydrogenase mutants of *Neurospora crassa*. J. Bacteriol. **137**:900–904.
9. **Finan, T. M., J. M. Wood, and D. C. Jordan.** 1981. Succinate transport in *Rhizobium leguminosarum*. J. Bacteriol. **148**:193–202.
10. **Gardiol, A., A. Arias, C. Cerveñansky, C. Gaggero, and G. Martínez-Drets.** 1980. Biochemical characterization of a fructokinase mutant of *Rhizobium meliloti*. J. Bacteriol. **144**:12–16.
11. **Gibson, A. H., W. R. Scowcroft, J. J. Child, and J. D. Pagan.** 1976. Nitrogenase activity in cultured *Rhizobium* sp. strain 32H7. Arch. Microbiol. **108**:45–54.
12. **Glenn, A. R., and N. J. Brewin.** 1981. Succinate-resistant mutants of *Rhizobium leguminosarum*. J. Gen. Microbiol. **126**:237–241.
13. **Graham, P. H.** 1964. Studies on the utilization of carbohydrates and Krebs cycle intermediates by *Rhizobia*, using an agar plate method. Antonie van Leeuwenhoek J. Microbiol. Serol. **30**:68–72.
14. **Holl, F. B., and T. A. La Rue.** 1976. Genetics of legume plants hosts, p. 391–399. *In* W. E. Newton and C. J. Nyman (ed.), Proceedings of the First International Symposium on Nitrogen Fixation. Washington State University Press, Pullman.
15. **Katznelson, H., and A. C. Zagallo.** 1957. Metabolism of *Rhizobia* in relation to effectiveness. Can. J. Microbiol. **3**:879–884.
16. **Khouw, B. T., and H. D. McCurdy.** 1969. Tricarboxylic acid cycle enzymes and morphogenesis in *Blastocladiella emersonii*. J. Bacteriol. **99**:197–205.
17. **Reed, L. J., and B. B. Mukherjee.** 1969. α-Ketoglutarate dehydrogenase complex from *Escherichia coli*. Methods Enzymol. **13**:55–61.
18. **Ronson, C. W., P. Lyttleton, and J. G. Robertson.** 1981. C_4-dicarboxylate transport mutants of *Rhizobium trifolii* from ineffective nodules on *Trifolium repens*. Proc. Natl. Acad. Sci. U.S.A. **78**:4284–4288.
19. **Srere, P. A., and G. W. Kosicki.** 1961. The purification of citrate-condensing enzyme. J. Biol. Chem. **236**:2557–2559.
20. **Stovall, I., and M. Cole.** 1978. Organic acid metabolism by isolated *Rhizobium japonicum* bacteroids. Plant Physiol. **61**:787–790.
21. **Stumpf, D. K., and R. H. Burris.** 1979. The organic acid content of soybeans: nodules, roots, stems and leaves. Anal. Biochem. **95**:311–315.
22. **Trinchant, J. C., and J. Rigaud.** 1979. Sur les substrats énergétiques utilisés, lors de la réduction de C_2H_2, par les bactéroides extraits des nodosités de Phaseolus vulgaris. L. Physiol. Vég. **17**:547–556.
23. **Tuzimura, K., and H. Meguro.** 1960. Respiration substrate of *Rhizobium* in the nodules. J. Biochem. **47**:391–397.