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Mutants of *Rhizobium meliloti* Defective in Succinate Metabolism

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We characterized mutants of *Rhizobium meliloti* SU47 that were unable to grow on succinate as the carbon source. The mutants fell into five groups based on complementation of the succinate mutations by individual recombinant plasmids isolated from a *R. meliloti* clone bank. Enzyme analysis showed that mutants in the following groups lacked the indicated common enzyme activities: group II, enolase (Eno); group III, phosphoenolpyruvate carboxykinase (Pck); group IV, glyceraldehyde-3-phosphate dehydrogenase (Gap), and 3-phosphoglycerate kinase (Pgk). Mutants in groups I and V lacked C_4-dicarboxylate transport (Dct^-) activity. Wild-type cells grown on succinate as the carbon source had high Pck activity, whereas no Pck activity was detected in cells that were grown on glucose as the carbon source. It was found that in free-living cells, Pck is required for the synthesis of phosphoenolpyruvate during gluconeogenesis. In addition, the enzymes of the lower half of the Embden-Meyerhoff-Parnas pathway were absolutely required for gluconeogenesis. Eno, Gap, Pck, and one of the Dct loci (*ntrA*) mapped to different regions of the chromosome; the other Dct locus was tightly linked to a previously mapped *thi* locus, which was located on the megaplasmid pRmeSU47b.

Two lines of evidence strongly suggest that a C_4-dicarboxylic acid(s) (succinate, fumarate, or malate) is the primary carbon and energy source supplied by the plant to nitrogen-fixing bacteroids in leguminous root nodules. First, these acids stimulate nitrogenase activity in isolated bacteroids (6, 51) and, second, C_4-dicarboxylate transport mutants of *Rhizobium trifolii*, *Rhizobium leguminosarum*, and *Rhizobium meliloti* form nodules which fail to fix nitrogen (i.e., ineffective nodules) (3, 8, 15, 21, 47). Ronson and co-workers (44–46) have shown that in free-living cells, regulation of C_4-dicarboxylate transport involves interaction of two C_4-dicarboxylate-specific regulatory genes and the *ntrA* (*rpoN*) gene product, the latter of which is also required for *nifH* expression and nitrate assimilation.

Succinate metabolism has been implicated in the regulation of a number of metabolic activities in *Rhizobium* species (51, 52); however, unlike hexose and pentose metabolism, the pathways of succinate metabolism in *Rhizobium* species have been addressed in only a few reports. Mutant and enzyme analyses have shown that the Entner-Doudoroff and pentose phosphate pathways play central roles in hexose and pentose metabolism in *R. meliloti* L5-30 (1, 2, 11) and other fast-growing *Rhizobium* strains (3, 48; for a review, see reference 51). In addition, the tricarboxylic acid (TCA) cycle mutants of *R. meliloti* L5-30, which are deficient in α-ketoglutarate dehydrogenase and succinate dehydrogenase activities, have been described previously (14, 25). Glenn and co-workers (3, 37) have demonstrated that phosphoenolpyruvate carboxykinase is required for growth on gluconeogenic substrates such as succinate in *R. leguminosarum*.

To obtain more information on succinate transport and metabolism in *R. meliloti* cells and, ultimately, in bacteroids, we assayed many of the enzymes that are involved in carbon metabolism in free-living cells and biochemically and genetically characterized 17 independent mutants of *R. meliloti* which were defective in succinate metabolism. The mutants fell into five groups (I through V), three of which were deficient in enolase, phosphoenolpyruvate carboxykinase, and glyceraldehyde-3-phosphate dehydrogenase or phosphoglycerate kinase activities and two of which were defective in C_4-dicarboxylate transport.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. Complex (LB) and defined (M9) media, antibiotic concentrations, and routine growth conditions were as described previously (18, 19).

Genetic techniques. Transduction, plasmid conjugation, transposon mutagenesis, and transposon replacements were done as described previously (13, 18, 19). For Tn*V* replacements, *Escherichia coli* MT607(pRK600) was used to mobilize plasmid pTF1 to *Rhizobium meliloti* Rm5065 and Rm5073 in overnight matings at 30°C. Streptomycin- and neomycin-resistant colonies were selected and screened for oxytetracycline sensitivity as described previously (19) for Tn*5-oriT* replacements of Tn*5*-132. *R. meliloti* conjugal mapping experiments with Tn*5-mob* and Tn*5-oriT* donor strains were performed by mixing equal volumes of overnight cultures (optical density at 675 nm of ca. 1.0) of the *R. meliloti* donor strains and *E. coli* mobilizing strain MM294A(pGMI102) or MT607(pRK2013). The mixture was added to equal volumes of overnight recipient cultures, and 100-μl portions were spotted onto LB plates. Control spots containing only donor or recipient cultures were included in all experiments. After overnight incubation at 30°C, the cells were suspended in 5 ml of 0.85% NaCl, diluted, and plated onto M9 medium containing 15 mM succinate.

Recombinant plasmids which complemented succinate mutations were identified after the cosmid pLAFR1 clone bank of *R. meliloti* (23) (obtained from Fred Ausubel) was transferred into the mutants and transconjugants were selected for growth on M9 medium containing 15 mM succinate. Individual complementing plasmids were then transferred to *E. coli* MM294A or MT607. The plasmids were then introduced into all of the *R. meliloti* mutants in triparental matings (17), and selection for tetracycline-resistant transconjugants was done by using LB agar containing tetracy-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain	Relevant characteristics[a]	Origin
R. meliloti		
Rm1021	SU47 *str-21*	38
Rm3330	SU47 *trp-33 str-3 spc-1 rif-1*	39
Rm5000	SU47 *rif-5*	18
Rm5011	Rm1021, *ntrA71*::Tn*5*	This study
Rm5012	Rm1021, Eno-1::Tn*5*	This study
Rm5065	Rm1021, Pck-1::Tn*5*-132	This study
Rm5073	Rm1021, Eno-2::Tn*5*-132	This study
Rm5074	Rm1021, *ntrA72*::Tn*5*-132	This study
Rm5209	SU47, Ω5007::Tn*5*-*oriT*	19
Rm5210	SU47, Ω5007::Tn*5*-*oriT*	19
Rm5259	Rm1021, *nifH1491*::Tn*5*-*oriT*	T. M. Finan, unpublished data
Rm5234	Rm1021, Pck-2::Tn*5*-VB32	This study
Rm5308	Rm5000, *ntrA73*::Tn*5*-132	M. Williams
Rm5312	Rm1021, Dct-11:Tn*5*-*lac*	M. Williams
Rm5313	Rm1021, Gap-2::Tn*5*-*lac*	M. Williams
Rm5314	SU47, Dct-16::Tn*5*	M. Williams
Rm2.1-11	Rm5000, *recA*::Tn*5*-233 *ntrA74*::Tn*5*	G. De Vos
Rm2.2-1	Rm5000, *recA*::Tn*5*-233 Gap-1::Tn*5*	G. De Vos
Rm5418	Rm1021, Gap-1::Tn*5*	φM12(Rm2.2-1) → Rm1021, Nm^{r}[b]
Rm5419	Rm1021, *ntrA74*::Tn*5*	φM12(Rm2.1-11) → Rm1021, Nm^{r}
Rm5421	Rm1021, Dct-17::Tn*5*-233	This study
Rm5422	Rm1021, *ntrA75*::Tn*5*	This study
Rm5438	Rm1021, Eno-2::Tn*V*	Tn*V* replacement of Tn*5*-132 in Rm5073
Rm5439	Rm1021, Pck-1::Tn*V*	Tn*V* replacement of Tn*5*-132 in Rm5065
Rm6661	SU47, *his-39 trp-33 leu-53*$^{+}$ Ω601::Tn*5*-*mob* (−)	S. Klein
Rm6662	SU47, *his-39 trp-33 leu-53*$^{+}$ Ω602::Tn*5*-*mob* (+)	S. Klein
Rm6692	SU47, *his-39 leu-53 trp-33*$^{+}$ Ω611::Tn*5*-*mob* (+)	S. Klein
Rm6693	SU47, *his-39 leu-53 trp-33*$^{+}$ Ω612::Tn*5*-*mob* (−)	S. Klein
Rm6695	SU47, *his-39 trp-33 pyr-49*$^{+}$ Ω614::Tn*5*-*mob* (+)	S. Klein
Rm6696	SU47, *his-39 trp-33 pyr-49*$^{+}$ Ω615::Tn*5*-*mob* (−)	S. Klein
Rm6865	SU47, *his-39 trp-33 pyr-49 cys-11*$^{+}$ Ω637::Tn*5*-*mob* (+)	S. Klein
RmF121	Rm1021, Dct-16::Tn*5*	φM12(Rm5314) → Rm1021, Nm^{r}
RmF331	Rm1021, Pgk-11::Tn*5*	This study
RmF332	Rm1021, Dct-18::Tn*5*	This study
RmF400	SU47, *nifH1491*::Tn*5*-*oriT*	φM12(Rm5259) → SU47, Nm^{r}
E. coli		
MM294A	*pro-82 thi-1 hsdR17 supE44*	G. Walker
MT607	MM294A *recA-56*	19
Plasmids		
pRK2013	Nm^{r}, ColE1 replicon with RK2 transfer genes	17
pRK600	Cm^{r}, Nm^{s}, pRK2013 *npt*::Tn*9*	19
pTF1	Nm^{r}, pBR322::Tn*V*	24
pRmT100	pLAFR1 clone, complement group I mutants	This study
pRmT102	pLAFR1 clone, complement group II mutants	This study
pRmT103	PLAFR1 clone, complement group III mutants	This study
pRmT104	pLAFR1 clone, complement group IV mutants	This study
pRmT105	pLAFR1 clone, complement group V mutants	This study
pGMI102	Nm^{s} derivative of RP4	J. Denarie via H. Meade (38)

[a] Gene designations: Dct, C_4-dicarboxylate transport; Eno, enolase; Gap, glyceraldehyde-3-phosphate dehydrogenase; Pck, phosphoenolpyruvate carboxykinase; Pgk, 3-phosphoglycerate kinase. Tn*5* *lac* and Tn*5*-VB32 are Tn*5* promoter probes (5, 31). The + and − signs following Tn*5*-*mob* inserts indicate DNA transfer in a clockwise or counterclockwise orientation, respectively.

[b] Origins are described as follows. For example, RmF121 was constructed by phage φM12 transduction of Nm^{r} from Rm5314 to Rm1021.

cline and streptomycin or rifampin. Transconjugants were then examined for growth on succinate minimal and glucose minimal media.

Mutant isolation. Rm5011 and Rm5012 were isolated after Tn*5* mutagenesis by using the suicide vector pPH1JI (7). All other mutants were isolated after mutagenesis with Tn*5* or one of its derivatives by using vectors derived from pRK2013, as described previously (13, 19). Colonies containing transposon inserts were screened for their ability to grow on M9 medium containing 15 mM glucose but not on M9 medium containing 15 mM succinate. Strains from G. De Vos and M. Williams were also identified as mutants which grew on glucose but not on succinate minimal medium and were further characterized in this study. Revertants were isolated by spreading washed cells (ca. 3×10^8) on M9 medium containing succinate. Colonies which grew were then single colony purified 3 times on the succinate medium prior to analysis.

Biochemical techniques. Cells were grown overnight in LB broth containing 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$, washed once, and then used to inoculate 200 ml of M9 medium containing 15 mM succinate and 1 mM glucose or 200 ml of

LB broth containing 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$ in 1,000-ml flasks. Cultures were incubated overnight at 30°C in a shaker (G25 Environmental Incubator Shaker; New Brunswick Scientific Co., Inc., New Brunswick, N.J.) at 100 rpm. Before cultures were harvested, they were checked for purity by streaking them onto LB agar plates. Cell pellets were stored overnight at −20°C, thawed, washed twice in 20 mM Tris hydrochloride buffer containing 1 mM $MgCl_2$ (pH 7.4), suspended in the same buffer (4 ml/g of cell wet weight), and stored at −70°C.

Cell extracts were prepared by adding dithiothreitol (0.1 mM) to thawed cells and disrupting the cells by sonication with a sonicator (Braun-Sonic 1510). The sonic extracts were centrifuged at 500 × *g* for 15 min at 4°C, and the supernatant fractions were used for assays of malate dehydrogenase (16), succinate dehydrogenase (43), fumarase (28), α-ketoglutarate dehydrogenase (40), citrate synthase (50), isocitrate dehydrogenase (41), glucose-6-phosphate dehydrogenase (NADP dependent) (34), and 6-phosphogluconate dehydrogenase (NADP dependent) (34).

For all other assays, the extract was further centrifuged (Eppendorf 5414) at 4°C for 15 min in order to decrease levels of NADH-oxidase activity. This soluble fraction was used to assay the following enzymes by using the indicated modifications: fructose bisphosphatase (35); fructose bisphosphate aldolase (35) with 40 mM Tris hydrochloride buffer (pH 8.0)–100 mM KCl–0.5 mM $Co(NO_3)_2 \cdot 6H_2O$–0.1 mM reduced NADH–2.5 mM D-fructose-1, 6-diphosphate–1 U of 3-glycerophosphate dehydrogenase–10 U of triose phosphate isomerase–cell extract–water to a final volume of 1 ml; triose phosphate isomerase (36); glyceraldehyde-3-phosphate dehydrogenase (NADP dependent); 3-phosphoglycerate kinase; phosphoglycerate mutase and enolase (36); and phosphoenolpyruvate carboxykinase (27), all with a NADH concentration of 0.1 mM. The combined activities of 6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase (Entner-Doudoroff pathway) were assayed by measuring the amount of pyruvate formed from 6-phosphogluconate. The reaction mixture contained 40 mM HEPES (*N*-2-hydroxethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 8.0), 1.25 mM 6-phosphogluconate (sodium salt), cell extract, and water to a final volume of 1.0 ml (34). Extract and substrate were incubated in buffer for 10 min at 30°C, and the pyruvate that formed was measured by determining the rate of NADH disappearance in the presence of lactic dehydrogenase (10). Corrections were made for calculated background NADH-oxidase activity in all assays.

Specific activities are expressed as nanomoles of product formed per minute per milligram of protein. Protein was measured as described by Bradford (9) by using the Coomassie blue reagent (Bio-Rad Laboratories, Richmond, Calif.) and bovine serum albumin as standards.

Assays of succinate transport activity were done at 30°C as described previously (20). Cells were grown to the mid-log phase in LB medium containing 2.5 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 15 mM sodium succinate and washed 3 times with transport assay solution (20). Uptake of [2,3-^{14}C]succinate was a linear function of time for at least 2 min, and initial rate determinations were determined at 30-s intervals.

Chemicals. [2,3-^{14}C]succinic acid (48 Ci/mol) was obtained from New England Nuclear Corp. (Boston, Mass.). The coupling enzymes used to assay enzyme activities were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.).

TABLE 2. Growth of *R. meliloti* succinate mutants on minimal medium containing different carbon sources

Carbon source	Growth of the following mutant groups[a]:				
	I	II	III	IV	V
Glucose	++	+	++	+	++
Xylose	++	++	++	++	++
Glycerol	++	±	++	±	++
Pyruvate	++	−	±	−	++
Arabinose	++	−	±	−	++
Succinate	−	−	±	−	−
Fumarate	−	−	±	−	−
Malate	−	−	±	−	−
Citrate	++	−	±	−	++

[a] Plates were scored after incubation for 5 days at 30°C. Carbon source concentrations were 7.5 mM for fumarate and malate, 5 mM for citrate, and 15 mM for all others. Symbols: ++, same as that of wild-type Rm1021; +, and ±, slow and very slow, respectively, compared with that of Rm1021; −, no growth.

RESULTS

Mutant groups. Seventeen independent mutants that were defective in succinate metabolism were identified after transposon mutagenesis and after colonies were screened for their ability to grow on glucose minimal medium but not on succinate minimal medium. Transduction of the transposon-encoded antibiotic resistance marker from 15 of the mutants into Rm1021 resulted in the cotransfer of the inability to grow on succinate minimal medium. Strains Rm5312 and RmF332 were not examined by transduction. Recombinant plasmids which complemented individual mutations were identified by transferring a *R. meliloti* clone bank (23) into the mutant and selecting for growth on succinate minimal medium. All of the mutants were complemented by one of five recombinant plasmids (Tables 1 and 2), and mutants complemented by one plasmid were not complemented by the others. Thus, by plasmid complementation the mutants fell into five distinct groups (I through V), and the growth characteristics of mutants within a group were the same (Table 2).

Biochemical characterization. Mutants in groups I and V were unable to grow on succinate, fumarate, or malate but grew normally on all other carbon sources examined (Table 2). This growth phenotype is characteristic of C_4-dicarboxylic acid transport mutants (20, 26, 47). To examine this possibility, cultures of the mutants were grown to the mid-log phase in LB medium supplemented with 2.5 mM $CaCl_2$–2.5 mM $MgSO_4$–15 mM succinate and assayed for succinate transport activity. No activity was detected in the mutants (<0.5 nmol/min per mg of protein), while wild-type Rm1021 activity was 13 nmol/min per mg of protein. Thus, the group I and V mutants appeared to be defective in C_4-dicarboxylic acid transport.

Mutants in groups II and IV failed to grow on TCA cycle intermediates and pyruvate. The two mutants in group III had a similar phenotype, except that both mutants were leaky, as they grew slowly on nonpermissive substrates such as succinate (Table 2). Arabinose is metabolized to α-ketoglutarate in *R. meliloti* (14). Thus, the lack of growth on arabinose of mutants in groups II, III, and IV is consistent with their inability to grow on TCA cycle intermediates. Cell extracts of mutants Rm5012, Rm5065, and Rm5418 from groups II, III, and IV, respectively, were assayed for specific activities of the enzymes that are involved in carbon metabolism (Table 3). The strains specifically lacked eno-

TABLE 3. Enzyme activities in wild-type and succinate mutants of *R. meliloti*

Enzyme	Sp act (nmol/min per mg of protein) of the following strains (mutant group)[a]			
	RM1021 (wild type)	Rm5012 (II)	Rm5065 (III)	Rm5418 (IV)
Fructose-1,6-P_2 phosphatase	6	13	6	7
Fructose-1,6-P_2 aldolase	60	78	86	83
Triose-phosphate isomerase	83	168	150	45
Glyceraldehyde-3-phosphate dehydrogenase	132	58	109	2
3-phosphoglycerate kinase	158	192	70	15
Phosphoglycerate mutase	73	113	37	60
Enolase	28	0	60	29
Pyruvate kinase	60	20	ND[b]	37
Phosphoenolpyruvate carboxykinase	456	201	0	329
Citrate synthase	43	79	101	97
Isocitrate dehydrogenase	237	250	428	159
Ketoglutarate dehydrogenase	8	18	ND	13
Succinate dehydrogenase	3	8	12	7
Malate dehydrogenase	324	453	430	442
Fumarase	165	459	589	158

[a] TCA cycle enzymes were assayed in extracts of cells grown in LB medium. Other assays were performed on extracts of cells grown in minimal media containing succinate (15 mM) and glucose (1 mM).
[b] ND, Not determined.

lase, phosphoenolpyruvate carboxykinase, and NADP-dependent glyceraldehyde-3-phosphate dehydrogenase activities, respectively. (No-NAD dependent glyceraldehyde-3-phosphate dehydrogenase activity was detected in cell extracts of Rm1021.) Analysis of the other strains in each group showed that group II mutants lacked enolase activity and that group III mutants lacked phosphoenolpyruvate carboxykinase activity (Tables 3 and 4).

The group IV mutants showed a more complex profile of enzyme activities (Table 4). Extracts from group IV strains Rm5418 and Rm5313 had less than 2% of the wild-type glyceraldehyde-3-phosphate dehydrogenase activity and approximately 10% of the wild-type 3-phosphoglycerate kinase activity. However, the remaining group IV mutant RmF331 had normal glyceraldehyde-3-phosphate activity but had only 5% of the wild-type 3-phosphoglycerate kinase activity. Further work is required to determine the reason for the low 3-phosphoglycerate kinase activity in Rm5418 and Rm5313. It is possible that glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase lie within an operon such that the promoter-proximal polar insertions in strains Rm5418 and Rm5313 affect both activities, while the downstream insertion in RmF331 affects only 3-phosphoglycerate kinase activity. Consistent with the proposition given above is the recent finding that in *Zymomonas mobilis* the *pgk* gene is located immediately downstream from the *gap* gene and both appear to be under the control of a common promoter (12).

TABLE 4. Enzyme activities in mutants and revertants of *R. meliloti*

Strain	Group	Sp act (nmol/min per mg of protein) of the following enzymes[a]:				
		Gap	Pgk	Pgm	Eno	Pck
Rm1021		131	158	76	28	456
Rm5438	II	50	126		1	
Rm5234	III	57	93		23	0
Rm5313	IV	0	17	50	27	
RmF331	IV	140	7	67	34	
RmF307	Rm5438 (II)[b]	80			7	
RmF308	Rm5438 (II)	90			16	
RmF362	Rm5065 (III)	92				110
RmF363	Rm5065 (III)	93				164
RmF311	Rm5418 (IV)	68			20	
RmF312	Rm5418 (IV)	71			20	
RmF384	RmF331 (IV)	96	98			

[a] Enzymes were assayed in extracts of cells grown in minimal medium containing succinate (15 mM) plus glucose (1 mM). Abbreviations: Gap, glyceraldehyde-3-phosphate dehydrogenase; Pgk, 3-phosphoglycerate kinase; Pgm, phosphoglycerate mutase; Eno, enolase; Pck, phosphoenolpyruvate carboxykinase.
[b] Revertants of the indicated strains. Mutant groups are given in parentheses.

The addition of cell extracts from the mutant strain to the wild-type extracts did not alter the enzyme activities. Thus, the absence of enzyme activity in extracts from the mutant strains was not due to the presence of inhibitors.

Revertants of representative mutants were isolated on succinate minimal medium; and two independent revertants from strains Rm5438, Rm5065, and Rm5418 and one revertant from strain RmF331 were found to have lost their respective transposon-encoded resistance markers, confirming that the mutant phenotype is linked to the transposon insertion. In addition, the seven revertants regained wild-type enzyme activities (Table 4), indicating that the growth phenotype of the mutants is due to their respective enzyme deficiencies.

Enzyme levels in cells grown on glucose and succinate. To integrate the data obtained from the analysis of the succinate mutants with general carbon metabolism in wild-type cells, we assayed various enzyme levels in cells grown on glucose and those grown on the gluconeogenic substrate succinate. Results of these experiments are summarized in Table 5. Cells grown on succinate showed high phosphoenolpyruvate carboxykinase activity, whereas cells grown on glucose had no such activity. This observation, coupled with the phenotype of the mutants that lacked phosphoenolpyruvate carboxykinase activity, suggests that this enzyme has a key role in (the synthesis of phosphoenolpyruvate during) gluconeogenesis. Conversely, the low activity of the Entner-Doudoroff pathway in succinate versus glucose-grown cells is consistent with the primary use of the Entner-Doudoroff pathway in glucose catabolism.

TABLE 5. Enzyme activities in succinate- and glucose-grown cells of *R. meliloti* Rm1021

Enzyme	Sp act (nmol/min per mg of protein) of cells grown on:	
	Succinate	Glucose
Entner-Doudoroff pathway[a]	1	30
Glucose-6-phosphate dehydrogenase (NADP-linked)	19	96
6-phosphogluconate dehydrogenase (NADP)	44	60
Fructose-1,6-P_2 phosphatase	3	4
Fructose-1,6-P_2 aldolase	7	20
Triose-phosphate isomerase	173	341
Glyceraldehyde-3-phosphate dehydrogenase	79	125
3-Phosphoglycerate kinase	143	159
Phosphoglycerate mutase	41	27
Enolase	32	32
Isocitrate dehydrogenase	354	386
Succinate dehydrogenase	9	7
Malate dehydrogenase	759	475
Phosphoenolpyruvate carboxykinase	393	0

[a] The Entner-Doudoroff pathway represents the combined activities of 6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase.

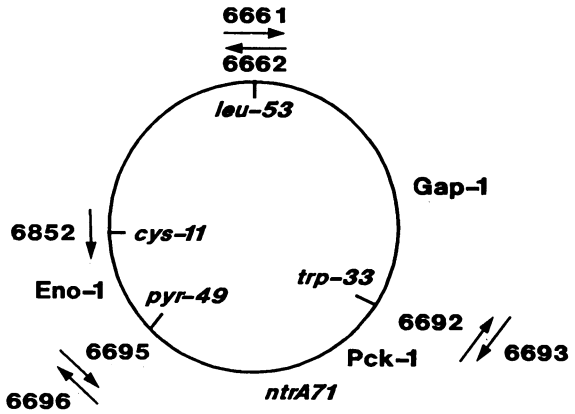

FIG. 1. Chromosomal map of *R. meliloti* SU47 showing locations of Eno-1, *ntrA71*, Pck-1, and Gap-1. Locations of *cys-11*, *pyr-49*, *trp-33*, and *leu-53* are from Meade and Signer (39). Arrows and four-digit numbers indicate positions of Tn*5-mob* insertions in the strain with the given number. The arrowhead is the origin of transfer, and the arrow tail is the direction of transfer.

Genetic mapping. To determine the location of the succinate mutations relative to those of known markers on the chromosome of *R. meliloti* SU47 (39), we used a set of donor strains that mobilized the entire *R. meliloti* chromosome in segments (S. Klein and K. Lohman, personal communication). Table 6 summarizes the data obtained from mating experiments in which the Tn*5-mob* (49) donor strains were crossed with one mutant from each genetic group, with selection for recombinants which grew on succinate minimal medium. The data are consistent with the following gene order: *trp-33*, Pck-1 (group III), *ntrA71* (group I), *pyr-49*, Eno-1 (group II), *cys-11*, *leu-53*, Gap-1 (group IV), *trp-33* (Fig. 1). The group V mutation in RmF121 showed no apparent chromosomal linkage, suggesting that Dct-16::Tn*5* was located on one of the two megaplasmids present in this strain. Thus, RmF121 was crossed with pRmeSU47a and pRmeSU47b donor strains RmF400 and Rm5209, respectively (Table 1), and recombinants which grew on succinate minimal medium containing streptomycin were obtained only with the pRmeSU47b donor. The position of Dct-16::Tn*5* on pRmeSU47b was determined by measuring the frequency of succinate recombinants obtained with the donor strains Rm5209 and Rm5210, which transfer pRmeSU47b in clockwise and counterclockwise orientations, respectively (19). The results suggest that the group V locus mapped close to the previously located (19) *thi-501*::Tn*5* mutation (data not shown). In subsequent experiments, Nm^r was transduced from RmF121 to Rm5300 (*thi*-502::Tn*5*-11), and of 182 transductants examined, 175 lost the Tn*5*-11-encoded resistance to gentamicin and spectinomycin. Thus, the *thi* and C_4-dicarboxylate transport loci are tightly linked.

DISCUSSION

The pathways for carbon metabolism outlined in Fig. 2 are based primarily on results of experiments with fast-growing *Rhizobium* species (1–3, 11, 14, 48). The data presented here suggest that these pathways operate in *R. meliloti* SU47. The growth characteristics of enolase, phosphoenolpyruvate carboxykinase, and glyceraldehyde-3-phosphate dehydrogense mutants (mutant groups II, III, IV; Table 2) and the comparison of enzyme levels in cells grown in succinate with those grown in glucose (Table 5) suggests that during gluconeogenesis, glyceraldehyde-3-phosphate synthesis from oxaloacetate occurs via the enzymes phosphoenolpyruvate carboxykinase, enolase (phosphoglycerate mutase), phosphoglycerate

TABLE 6. Conjugal mapping of succinate mutations in *R. meliloti*

Recipient strain[a]	No. of recombinants/10^8 donor cells in the following donor strains[b]:						
	6662 [(+) *leu*]	6661 [(−) *leu*]	6692 [(+) *trp*]	6693 [(−) *trp*]	6695 [(+) *pyr*]	6696 [(−) *pyr*]	6865 [(+) *cys*]
Rm5011 (Dct-1)	0	0	144	0	0	0	0
Rm5012 (Eno-1)	0	3	0	2	130	0	0
Rm5436 (Pck-1)	0	0[c]	7,230	0	0	0	ND[d]
Rm5418 (Gap-1)	339	0	0	847	6	6	ND
RmF121 (Dct-16)	0	0	5	1	0	2	0

[a] Designations in parentheses indicate the mutant alleles present in the respective recipient strains. Abbreviations: Dct, C_4-dicarboxylate transport; Eno, enolase; Pck, phosphoenolpyruvate carboxykinase; Gap, glyceraldehyde-3-phosphate dehydrogenase.

[b] Recombinants were selected on minimal medium with succinate as the carbon source. Donor strains are identified by strain number; for example, 6662 is Rm6662. The direction of transfer and the approximate origin of transfer is indicated; for example, in Rm6662 clockwise (+) transfer occurred from *leu*, while in strain Rm6696 counterclockwise (−) transfer occurred from *pyr* (see Fig. 1 legend).

[c] The mobilizing plasmid was pRK2013. In all other matings pGMI102 was the mobilizing plasmid.

[d] ND, Not determined.

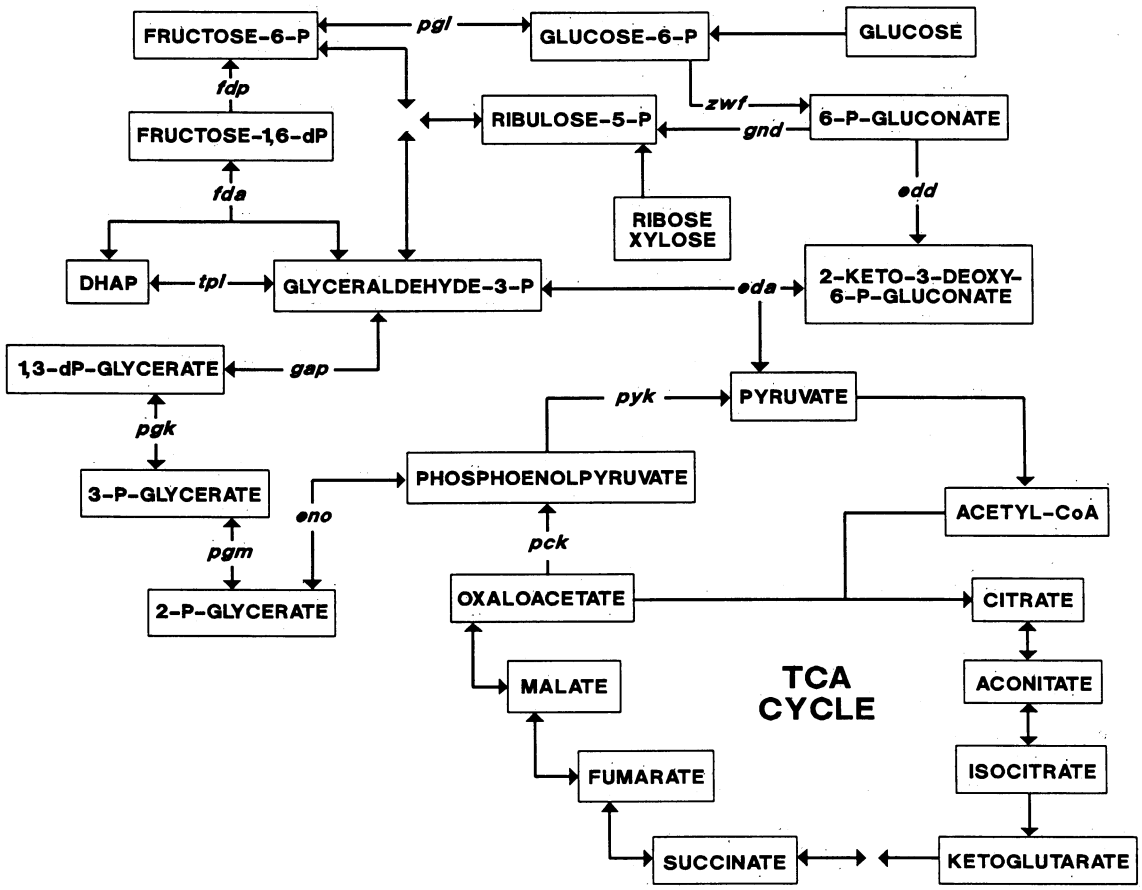

FIG. 2. Glycolytic and gluconeogenic pathways in *R. meliloti*. Abbreviations: DHAP, dihydroxyacetone phosphate; *pgi*, phosphoglucose isomerase; *zwf*, glucose-6-phosphate dehydrogenase; *gnd*, 6-phosphogluconate dehydrogenase; *edd*, 6-phosphogluconate dehydratase; *eda*, 2-keto-3-deoxy-6-phosphogluconate aldolase; *fdp*, fructose-1,6-P_2 phosphatase; *fda*, fructose-1-6-P_2 aldolase; *tpi*, triose-phosphate isomerase *gap*, glyceraldehyde-3-phosphate dehydrogenase; *pgk*, 3-phosphoglycerate kinase; *pgm*, phosphoglycerate mutase; *eno* enolase; *pyk*, pyruvate kinase; *pck*, phosphoenolpyruvate carboxykinase; dP, diphosphate; CoA, coenzyme A.

kinase, and glyceraldehyde-3-phosphate dehydrogenase. We assumed that glyceraldehyde-3-phosphate was metabolized via triose phosphate isomerase, fructose biphosphate aldolase, fructose biphosphatase, and phosphoglucose isomerase to yield glucose-6-phosphate, as outlined in Fig. 2. We found that the addition of low concentrations (1 mM) of glucose or glycerol (but not pyruvate) to succinate (15 mM) minimal medium stimulated the growth of representative enolase, phosphoenolpyruvate, and glyceraldehyde-3-phosphate dehydrogenase mutants. These data are consistent with the metabolic defects indicated through enzyme analysis and support the metabolic scheme outlined in Fig. 2. In addition, our observations concerning the regulation of phosphoenolpyruvate carboxykinase (Table 5) and the growth characteristics of phosphoenolpyruvate carboxykinase mutants (group III; Table 2) are similar to those described previously in *R. leguminosarum* (37).

Mutant phenotypes and enzyme analysis (Tables 2 and 5) suggest that glucose catabolism occurs via the Entner-Doudoroff pathway to form glyceraldehyde-3-phosphate and pyruvate. Thus, the *gap* and *eno* mutants grow on glucose because the formation of pyruvate results in an effective bypass of the metabolic lesions in the mutants. However, the absence of enolase or glyceraldehyde-3-phosphate dehydrogenase resulted in a large decrease in the growth yield on glucose (data not shown), suggesting that the lower half of the Embden-Meyerhof-Parnas pathway functions catabolically as well as anabolically in *R. meliloti*. Growth of the *gap* and *eno* mutants on xylose correlates with the operation of the pentose-phosphate pathway, a major product of which, fructose-6-phosphate, can enter the Entner-Doudoroff pathway via phosphoglucose isomerase and glucose-6-phosphate dehydrogenase.

A C_4-dicarboxylic acid transport system has been described previously for *R. meliloti* (8, 15, 53) and other *Rhizobium* species (20, 26, 42, 48). Ronson et al. (45) have recently described the isolation of *ntrA* mutants of *R. meliloti* SU47 and showed that the *ntrA* gene product is required for diverse metabolic functions, including C_4-dicarboxylate transport and nitrate assimilation. Group I mutants failed to grow on C_4-dicarboxylates (Table 2), and in experiments to be described elsewhere we show that group I (and not group V) mutants are identical to the *ntrA* mutants described by Ronson et al. (45). Engelke et al. (15) recently identified at least two C_4-dicarboxylate transport loci in *R. meliloti* SU47, and it appears that the two loci correspond to the group I and V mutations identified in this study. In addition, the C_4-dicarboxylate transport locus identified by the group V mutants appears similar to a C_4-dicarboxylate transport locus in *R. meliloti* JJc10, which was also localized to megaplasmid pRmeSU47b (53). The location of C_4-dicarboxylate transport genes and their tight linkage to one of two

thi loci on megaplasmid pRmeSU47b is interesting. Genes that are involved in the synthesis of an exopolysaccharide required for effective nodulation (32) are also located on megaplasmid pRmeSU47b (19, 29). The evolutionary or biological significance of the megaplasmid location of the *thi*, exopolysaccharide, and C_4-dicarboxylate transport genes is not apparent.

The growth characteristics of the *R. meliloti gap* and *pgk* mutants is similar to those of analogous mutants of *Pseudomonas aeruginosa* (4, 33). In contrast, *E. coli eno*, *pgk*, and *gap* mutants require two carbon sources for growth, one above and one below the metabolic block (22, 30). Possible explanations for the different carbon requirements have been discussed recently (4).

This study was carried out to determine the enzymatic pathways for succinate metabolism in free-living cells of *R. meliloti* SU47. To understand the role of these pathways in nodule formation and function, we are currently measuring enzyme levels in bacteroids and are determining the detailed symbiotic phenotypes of the mutants that were isolated in this study. Preliminary results indicate that mutants from groups I, II, and IV induce ineffective nodules and that the acetylene-reducing activity of plants inoculated with the group III phosphoenolpyruvate carboxykinase mutant Rm5065 is ca. half the wild-type activity. The group V mutants consist of at least two symbiotic types, one of which is similar to Rm5065, while the other induces nodules with less than 5% of the wild-type acetylene-reducing activity.

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LITERATURE CITED

1. **Arias, A., C. Cervenansky, A. Gardiol, and G. Martinez-Drets.** 1979. Phosphoglucose isomerase mutant of *Rhizobium meliloti*. J. Bacteriol. **137**:409–414.
2. **Arias, A., A. Gardiol, and G. Martinez-Drets.** 1982. Transport and catabolism of d-mannose in *Rhizobium meliloti*. J. Bacteriol. **151**:1069–1072.
3. **Arwas, R., I. A. McKay, F. R. Rowney, M. J. Dilworth, and A. R. Glenn.** 1985. Properties of organic acid utilization mutants of *Rhizobium leguminosarum* strain 300. J. Gen. Microbiol. **131**: 2059–2066.
4. **Banerjee, P. C., A. Darzins, and P. K. Maitre.** 1987. Gluconeogenic mutations in *Pseudomonas aeruginosa*: genetic linkage between fructose-bisphosphate aldolase and phosphoglycerate kinase. J. Gen. Microbiol. **133**:1099–1107.
5. **Bellofatto, V., L. Shapiro, and D. A. Hodgson.** 1984. Generation of a Tn*5* promoter probe and its use in the study of gene expression in *Caulobacter crescentus*. Proc. Natl. Acad. Sci. USA **81**:1035–1039.
6. **Bergersen, F. J., and G. L. Turner.** 1967. Nitrogen fixation by the fraction of breis of soybean root nodules. Biochim. Biophys. Acta **141**:507–515.
7. **Beringer, J. E., J. L. Beynon, A. V. Buchanan-Wollaston, and A. W. B. Johnston.** 1978. Transfer of the drug resistance transposon Tn*5* to *Rhizobium*. Nature (London) **276**:633–634.
8. **Bolton, E., B. Higgisson, A. Harrington, and F. O'Gara.** 1986. Dicarboxylic acid transport in *Rhizobium meliloti*: isolation of mutants and cloning of dicarboxylic acid transport genes. Arch. Microbiol. **144**:142–146.
9. **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.
10. **Bucher, T., R. Czok, W. Lamprecht, and E. Latzko.** 1965. Pyruvate, p. 253–259. *In* H.-U. Bergmeyer (ed.), Methods of enzyme analysis. Academic Press, Inc. New York.
11. **Cervenansky, C., and A. Arias.** 1984. Glucose-6-phosphate dehydrogenase deficiency in pleiotropic carbohydrate-negative mutant strains of *Rhizobium meliloti*. J. Bacteriol. **160**:1027–1030.
12. **Conway, T., and L. O. Ingram.** 1988. Phosphoglycerate kinase gene from *Zymomonas mobilis*: cloning, sequencing, and localization within the *gap* operon. J. Bacteriol. **170**:1926–1933.
13. **De Vos, G. F., G. Walker, and E. R. Signer.** 1986. Genetic manipulations in *Rhizobium meliloti* utilizing two new transposon Tn*5* derivatives. Mol. Gen. Genet. **204**:485–491.
14. **Duncan, M. J., and D. G. Fraenkel.** 1979. α-Ketoglutarate dehydrogenase mutant of *Rhizobium meliloti*. J. Bacteriol. **137**: 415–419.
15. **Engelke, T. H., M. N. Jagadish, and A. Puhler.** 1987. Biochemical and genetic analysis of *Rhizobium meliloti* mutants defective in C_4-dicarboxylate transport. J. Gen. Microbiol. **133**:3019–3029.
16. **Englard, S., and L. Seigal.** 1969. Mitochondrial L-malate dehydrogenase of beef heart. Methods Enzymol. **13**:99–106.
17. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a function provided in *trans*. Proc. Natl. Acad. Sci. USA **76**:1648–1652.
18. **Finan, T. M., E. K. Hartwieg, K. LeMieux, K. Bergman, G. C. Walker, and E. R. Signer.** 1984. General transduction in *Rhizobium meliloti*. J. Bacteriol. **159**:120–124.
19. **Finan, T. M., B. Kunkel, G. F. De Vos, and E. R. Signer.** 1986. Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. J. Bacteriol. **167**:66–72.
20. **Finan, T. M., J. M. Wood, and D. C. Jordan.** 1981. Succinate transport in *Rhizobium leguminosarum*. J. Bacteriol. **148**:193–202.
21. **Finan, T. M., J. M. Wood, and D. C. Jordan.** 1983. Symbiotic properties of C_4-dicarboxylic acid transport mutants of *Rhizobium leguminosarum*. J. Bacteriol. **154**:1403–1413.
22. **Fraenkel, D. G.** 1986. Mutants in glucose metabolism. Annu. Rev. Biochem. **55**:317–337.
23. **Friedman, A. M., S. R. Long, S. E. Brown, W. J. Buikema, and F. M. Ausubel.** 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. Gene **18**:289–296.
24. **Furuichi, T., M. Inouye, and S. Inouye.** 1985. Novel one-step cloning vector with a transposable element: application to the *Myxococcus xanthus* genome. J. Bacteriol. **164**:270–275.
25. **Gardiol, A., A. Arias, C. Cervenansky, and G. Martinez-Drets.** 1982. Succinate dehydrogenase mutant of *Rhizobium meliloti*. J. Bacteriol. **151**:1621–1623.
26. **Glenn, A. R., and N. J. Brewin.** 1981. Succinate resistant mutants of *Rhizobium leguminosarum*. J. Gen. Microbiol. **126**: 237–242.
27. **Hansen, R. J., H. Hinze, and H. Holzer.** 1976. Assay of phosphoenolpyruvate carboxykinase in crude yeast extracts. Anal. Biochem. **74**:576–584.
28. **Hill, R. L., and R. A. Bradshaw.** 1969. Fumarase. Methods Enzymol. **13**:91–99.
29. **Hynes, M. F., R. Simon, P. Muller, K. Niehaus, M. Labes, and A. Puhler.** 1986. The two megaplasmids of *Rhizobium meliloti* are involved in the effective nodulation of alfalfa. Mol. Gen. Genet. **202**:356–362.
30. **Irani, M. H., and P. K. Maitra.** 1977. Properties of *Escherichia coli* mutants deficient in enzymes of glycolysis. J. Bacteriol. **132**:398–410.
31. **Kroos, L., and D. Kaiser.** 1984. Construction of Tn*5* *lac*, a transposon that fuses *lacz* expression to exogenous promoters

and its introduction into *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA **81**:5816–5820.
32. **Leigh, J. A., E. R. Signer, and G. C. Walker.** 1985. Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules. Proc. Natl. Acad. Sci. USA **82**:6231–6235.
33. **Lessie, T. G., and P. V. Phibbs, Jr.** 1984. Alternative pathways of carbohydrate utilization in pseudomonads. Annu. Rev. Microbiol. **38**:359–387.
34. **Lessie, T. G., and J. C. Vander Wyk.** 1972. Multiple forms of *Pseudomonas multivorans* glucose-6-phosphate and 6-phosphogluconate dehydrogenases: differences in size, pyridine nucleotide specificity, and susceptibility to inhibition by adenosine 5′-triphosphate. J. Bacteriol. **110**:1107–1117.
35. **Lynch, W. H., J. Macleod, and M. Franklin.** 1975. Effect of temperature on the activity and synthesis of glucose-catabolizing enzymes in *Pseudomonas fluorescens*. Can. J. Microbiol. **21**:1560–1572.
36. **Maitra, P. K., and Z. Lobo.** 1971. A kinetic study of glycolytic enzyme synthesis in yeast. J. Biol. Chem. **246**:475–488.
37. **McKay, I. A., A. R. Glenn, and M. J. Dilworth.** 1985. Gluconeogenesis in *Rhizobium leguminosarum* MNF3841. J. Gen. Microbiol. **131**:2067–2073.
38. **Meade, H. M., S. R. Long, G. B. Ruvkun, S. E. Brown, and F. M. Ausubel.** 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. J. Bacteriol. **149**:114–122.
39. **Meade, H. M., and E. R. Signer.** 1977. Genetic mapping of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **74**:2076–2078.
40. **Reed, L. J., and B. B. Mukherjee.** 1969. Ketoglutarate dehydrogenase complex from *Escherichia coli*. Methods Enzymol. **13**: 55–61.
41. **Reeves, H. C., R. Rabin, W. S. Wegener, and S. J. Ajl.** 1971. Assays of enzymes of the tricarboxylic acid and glyoxylate cycles, p. 435–436. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology. Academic Press, Inc., New York.
42. **Reibach, P. H., and J. G. Streeter.** 1984. Evaluation of active versus passive uptake of metabolites by *Rhizobium japonicum* bacteroids. J. Bacteriol. **159**:47–52.
43. **Rogers, H. J., D. A. Reaveley, and I. D. J. Burdett.** 1967. The membrane systems of *Bacillus licheniformis*, p. 303–313. *In* Protides of the biological fluids, vol. 15. Elsevier/North-Holland Publishing Co., Amsterdam.
44. **Ronson, C. W., P. M. Astwood, and J. A. Downie.** 1984. Molecular cloning and genetic organization of C_4-dicarboxylate transport genes from *Rhizobium leguminosarum*. J. Bacteriol. **160**:903–909.
45. **Ronson, C. W., P. M. Astwood, B. T. Nixon, and F. M. Ausubel.** 1987. *Rhizobium meliloti ntrA* (*rpoN*) gene is required for diverse metabolic functions. J. Bacteriol. **169**:2424–2431.
46. **Ronson, C. W., P. M. Astwood, B. T. Nixon, and F. M. Ausubel.** 1987. Deduced products of C_4-dicarboxylate transport regulatory genes of *Rhizobium leguminosarum* are homologous to nitrogen regulatory gene products. Nucleic Acids Res. **15**:7921–7934.
47. **Ronson, C. W., P. Lyttleton, and J. G. Robertson.** 1981. C_4-dicarboxylate transport mutants of *Rhizobium trifolii* form ineffective nodules on *Trifolium repens*. Proc. Natl. Acad. Sci. USA **82**:6231–6245.
48. **Ronson, C. W., and S. R. Primrose.** 1979. Carbohydrate metabolism in *Rhizobium trifolii*: identification and symbiotic properties of mutants. J. Gen. Microbiol. **112**:77–88.
49. **Simon, R.** 1984. High frequency mobilization of gram-negative bacterial replicons by the in vitro constructed Tn*5*-mob transposon. Mol. Gen. Genet. **196**:413–420.
50. **Srere, P. A.** 1969. Citrate synthase. Methods Enzymol. **13**:3–11.
51. **Stowers, M. D.** 1985. Carbon metabolism in *Rhizobium* species. Annu. Rev. Microbiol. **39**:89–108.
52. **Ucker, D. S., and E. R. Signer.** 1978. Catabolite-repression-like phenomenon in *Rhizobium meliloti*. J. Bacteriol. **136**:1197–2000.
53. **Watson, R. J., Y. Chan, R. Wheatcroft, A. Yang, and S. Han.** 1988. *Rhizobium meliloti* genes required for C_4-dicarboxylate transport and symbiotic nitrogen fixation are located on a megaplasmid. J. Bacteriol. **170**:927–934.