

## NADP<sup>+</sup>-Dependent Malic Enzyme of *Rhizobium meliloti*

BRIAN T. DRISCOLL AND TURLOUGH M. FINAN\*

*Department of Biology, McMaster University, Hamilton, Ontario L8S 4K1, Canada*

Received 26 October 1995/Accepted 1 February 1996

**The bacterium *Rhizobium meliloti*, which forms N<sub>2</sub>-fixing root nodules on alfalfa, has two distinct malic enzymes; one is NADP<sup>+</sup> dependent, while a second has maximal activity when NAD<sup>+</sup> is the coenzyme. The diphosphopyridine nucleotide (NAD<sup>+</sup>)-dependent malic enzyme (DME) is required for symbiotic N<sub>2</sub> fixation, likely as part of a pathway for the conversion of C<sub>4</sub>-dicarboxylic acids to acetyl coenzyme A in N<sub>2</sub>-fixing bacteroids. Here, we report the cloning and localization of the *tme* gene (encoding the triphosphopyridine nucleotide [NADP<sup>+</sup>]-dependent malic enzyme) to a 3.7-kb region. We constructed strains carrying insertions within the *tme* gene region and showed that the NADP<sup>+</sup>-dependent malic enzyme activity peak was absent when extracts from these strains were eluted from a DEAE-cellulose chromatography column. We found that NADP<sup>+</sup>-dependent malic enzyme activity was not required for N<sub>2</sub> fixation, as *tme* mutants induced N<sub>2</sub>-fixing root nodules on alfalfa. Moreover, the apparent NADP<sup>+</sup>-dependent malic enzyme activity detected in wild-type (N<sub>2</sub>-fixing) bacteroids was only 20% of the level detected in free-living cells. Much of that residual bacteroid activity appeared to be due to utilization of NADP<sup>+</sup> by DME. The functions of DME and the NADP<sup>+</sup>-dependent malic enzyme are discussed in light of the above results and the growth phenotypes of various *tme* and *dme* mutants.**

Malic enzymes convert malate to pyruvate and CO<sub>2</sub> with the simultaneous reduction of NAD<sup>+</sup> (P<sup>+</sup>). This enzyme was first described in extracts from pigeon liver in 1947 (35). Malic enzymes have been reported in many bacterial species including *Bacillus subtilis* (8, 36, 37), *Clostridium thermocellum* (29), *Pseudomonas putida* (19), and *Sulfolobus solfataricus* (2). Few reports have distinguished whether the activities result from one or two distinct enzymes. Biochemical studies have clearly shown separate NAD<sup>+</sup>- and NADP<sup>+</sup>-dependent malic enzymes (DME and TME, respectively) in *Escherichia coli* (25, 44), and Hansen and Juni (21) have isolated mutants of *E. coli* which lack either DME or both DME and TME. Kobayashi et al. (27) have reported the properties of TME together with the nucleotide sequence of the corresponding gene from *Bacillus stearothermophilus*.

A TME has been partially purified from *Bradyrhizobium japonicum* bacteroids (26), which have both DME and TME activities (7, 28). Both malic enzyme activities have also been reported in *Rhizobium* sp. strain NGR234 free-living cells (39) and in both free-living cells and bacteroids of *Rhizobium leguminosarum* (30).

We have been studying the role(s) of the bacterial malic enzymes in symbiotic nitrogen fixation within the alfalfa-*Rhizobium meliloti* symbiosis (9, 10). C<sub>4</sub>-dicarboxylic acids appear to be the principal source of carbon and energy supplied by the plant to N<sub>2</sub>-fixing bacteria (bacteroids) within root nodules (1, 17, 41). Bacteroids appear to metabolize C<sub>4</sub>-dicarboxylic acids directly via the citric acid cycle (31, 47, 48). In bacteroids, acetyl coenzyme A appears to be synthesized via the DME and pyruvate dehydrogenase (9, 30), and *R. meliloti* *dme* mutants (lacking DME) induce root nodules which contain bacteria but which fail to fix nitrogen (9).

To isolate *R. meliloti* *dme* mutants, we constructed a strain within which *dme* mutations generated a succinate-negative

growth phenotype. *R. meliloti* *pckA* mutants lack phosphoenolpyruvate carboxykinase (PCK) activity and grow poorly on minimal media with succinate or tricarboxylic acid (TCA) cycle intermediates as the sole carbon source. Second-site suppressor mutations, designated *pod*, allow *pckA* mutants to grow on succinate minimal media by increasing the activity of the enzyme pyruvate P<sub>i</sub> dikinase, thus increasing the conversion of pyruvate to phosphoenolpyruvate (PEP) (Fig. 1) (12, 38). In a *pckA pod-1* background (RmG243), *dme* mutants were unable to grow on succinate minimal medium (9). The *dme* succinate growth phenotype was conditional, as the introduction of the *dme* mutations into a wild-type background did not appear to alter the wild-type carbon utilization phenotype.

Here we describe the isolation of the *R. meliloti* NADP<sup>+</sup> malic enzyme gene (*tme*) and the genetic, biochemical, and symbiotic characterizations of *tme* mutants.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** The *R. meliloti* strains and plasmids used in this study are listed in Table 1. The complex and defined media (LBmc and M9, respectively), growth conditions, and antibiotic conditions used have been described previously (13, 14, 49).

**Genetic techniques.** Transductions, conjugal matings, and Tn5 mutagenesis were performed as previously described (6, 14, 16). Tn5-B20 mutagenesis of pRmT100 was performed as previously described with Tn5, except that the donor strain was *E. coli* G312. Strains in which plasmid-borne transposon and ΩSp insertions were recombined into the *R. meliloti* chromosome were identified by plasmid incompatibility, as previously described (13).

**DNA manipulations.** Plasmid DNA preparations were made and DNA restrictions were done according to standard protocols (43). The ΩSp fragment (40) was cloned into the *Eco*RI site of pTH115 following the partial *Eco*RI digestion of pTH115. Total genomic DNA was isolated from *R. meliloti* cells as previously described (32). Deletion derivatives of pRmT100 were obtained by complete digestion of pRmT100 DNA with the indicated enzyme followed by religation and transformation into *E. coli*. Deletions were screened and characterized by restriction analysis of isolated plasmid DNA. Restricted *R. meliloti* genomic DNA was transferred to an ICN BioTrans nylon membrane by using a standard Southern blot protocol (43). Probes were labelled with digoxigenin-dUTP and detected with anti-digoxigenin antibody coupled to alkaline phosphatase (Boehringer Mannheim DNA labelling and detection kit). Hybridization was carried out at 42°C.

**Biochemical techniques.** Cell growth, bacteroid isolation, and the preparation of sonicated cell extracts were performed as described previously (9, 15, 16). Buffer 1, used to resuspend the cells prior to sonication, consisted of 20 mM Tris

\* Corresponding author. Mailing address: Department of Biology, McMaster University, 1280 Main St. W., Hamilton, Ontario L8S 4K1, Canada. Phone: (905) 525-9140, ext. 22932. Fax: (905) 522-6066. Electronic mail address: finan@McMaster.CA.

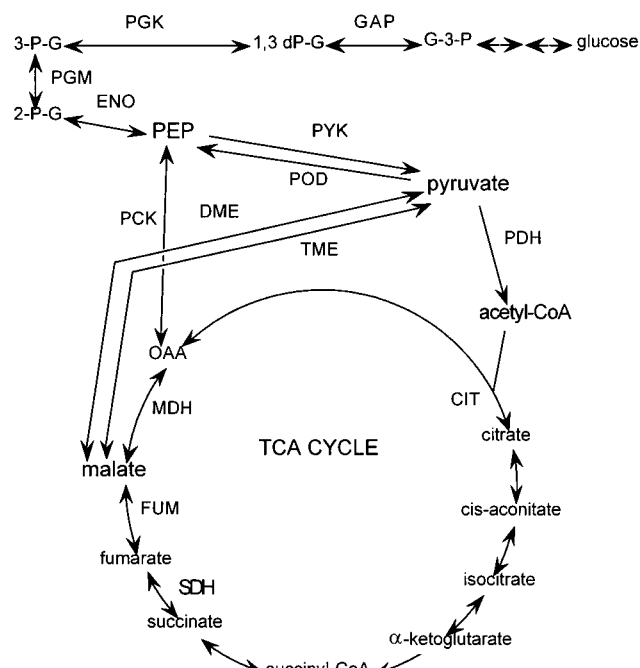


FIG. 1. TCA cycle and metabolic pathways. POD, pyruvate P<sub>1</sub> dikinase; PYK, pyruvate kinase; ENO, enolase; PGM, phosphoglycerate mutase; PGK, 3-phosphoglycerate kinase; GAP, glyceraldehyde-3-phosphate dehydrogenase; PDH, pyruvate dehydrogenase; CIT, citrate synthetase; SDH, succinate dehydrogenase; FUM, fumarase; OAA, oxaloacetic acid; 2-P-G, 2-phosphoglycerate; 3-P-G, 3-phosphoglycerate; 1,3 dP-G, 1,3 diphosphoglycerate; G-3-P, glyceraldehyde-3-phosphate; CoA, coenzyme A.

(pH 8.4), 1 mM MgCl<sub>2</sub>, 10 mM KCl, 20% glycerol, and 10 mM β-mercaptoethanol. All cell extracts were centrifuged (15 min, 4°C, 14,000 × g) to reduce NADH oxidase activity and to ensure that the extracts were free of cell debris. Malate dehydrogenase (MDH) activity was determined by the method of England and Siegal (11). PCK activity was determined by the method of Hansen et al. (22). DME and TME activities were determined as previously described (9). The protein concentration was determined with Bio-Rad protein assay dye by the Bradford method (5). Bovine serum albumin was the protein standard.

The β-galactosidase activity of permeabilized cells from triplicate *R. meliloti* cultures was determined according to the method of Miller (33). Cells were washed twice with 0.85% NaCl, subcultured into various minimal media, and grown overnight. The cultures were diluted into Z buffer, and the cell density ( $A_{675}$ ) was determined. The diluted cells (1 ml per sample) were permeabilized by the addition of 5 μl of 0.1% sodium dodecyl sulfate and 10 μl of chloroform and subsequently equilibrated in a 30°C water bath for 10 min before the addition of 200 μl of a 4-mg/ml ONPG (*o*-nitrophenyl-β-D-galactopyranoside) solution. The reaction was stopped with 0.5 ml of 1 M Na<sub>2</sub>CO<sub>3</sub>. The cells were pelleted by centrifugation (2,100 × g, 5 min), and the  $A_{420}$  of the supernatant was determined. β-Galactosidase activity (expressed in Miller units) was calculated according to the following formula:  $A_{420} \times 1,000/\text{time (in minutes)} \times A_{675}$ .

For the determination of β-galactosidase specific activity, extracts added to 2 ml of Z buffer were equilibrated in a 30°C water bath for 10 min; 0.4 ml of a 4-mg/ml ONPG solution was then added, and after sufficient time for color development, the reaction was stopped with 1 ml of 1 M Na<sub>2</sub>CO<sub>3</sub> (3.4 ml, final volume).  $A_{420}$  was measured, and the specific activity (nanomoles per minute per milligram of protein) was calculated as follows:  $(A_{420} \times 3.4 \text{ ml}) \div (\text{time [in minutes]} \times \text{mg of protein added} \times 0.0045)$ . The 0.0045 term is derived from the extinction coefficient for *o*-nitrophenol and is included in the calculation to convert the  $A_{420}$  value to nanomoles (33).

**DEAE-cellulose chromatography.** Crude cell extracts were initially centrifuged (20 min, 4°C, 8,600 × g) to remove cell debris and subjected to ultracentrifugation (90 min, 21,000 × g 4°C) to remove the membrane fraction, prior to application (4.5 mg of protein per ml of DEAE-cellulose) to the DEAE-cellulose column. All chromatography steps were performed as previously described (9). The eluted fractions were assayed for protein level and DME, TME, PCK, and MDH activities.

**Plant growth conditions.** *Medicago sativa* var. Iroquois seeds were surface sterilized, germinated, planted, and inoculated in Leonard assemblies as previously described (49). The growth conditions used and the method for determining the dry weight of the shoots have been previously described (15, 49).

## RESULTS

### Isolation of *tme* gene by complementation of *dme* mutants.

We previously identified three DME (*dme*) mutations in *R. meliloti* by screening Tn5 insertion mutants of strain RmG243 (*pckA pod-1*) for those unable to grow on succinate minimal medium (9). We showed that the *dme* succinate growth phenotype was conditional, as the introduction of the *dme* mutations into a wild-type background did not appear to alter the wild-type carbon utilization phenotype. No mutants which lacked TME activity were isolated in the above-described study.

While screening for plasmids which complement the succinate growth phenotype of the *pckA pod-1 dme* triple mutant (RmG443), we examined previously identified clones (pRmT100, pRmT102, pRmT103, pRmT105, and pRmT105 [Table 1]) known to carry genes necessary for growth on succinate (16). As expected, we found that the cosmid clone, pRmT103, which carries the wild-type *pckA* gene, allowed the *pckA pod-1 dme* triple mutant to grow on succinate. Unexpectedly however, we observed that the cosmid pRmT100, which carries the *R. meliloti rpoN* gene region, allowed the triple mutant to grow slowly on succinate. Our subsequent analysis (described below) has shown that in addition to *rpoN*, pRmT100 also carries the *R. meliloti tme* gene.

To delineate the region of pRmT100 necessary for growth of the *dme pckA pod-1* mutant on succinate, derivatives of pRmT100 in which sections of the insert DNA were deleted were tested for complementation (Fig. 2). These analyses limited the complementing DNA to a 7-kb region which lay approximately 10 kb from *rpoN* gene (42). The plasmid complementation appeared to result from increased TME activity, as the levels of TME activity in extracts from various *dme* pTH240(pRmT100ΔC) (Fig. 2) transconjugants were 1.5- to 1.8-fold higher than the activities in the *dme* mutants lacking the plasmid. Only low background levels of DME activity were detected in all of these extracts. The elevated TME activity indicated that pTH240 carried the gene encoding TME, which is designated *tme* after an earlier use in *E. coli* (20).

We subcloned the 6.8-kb *Bam*HI fragment from pTH240, in both orientations, into the *Bam*HI site of the broad-host-range plasmid pRK7813. The resulting plasmids, pTH115 and pTH116, complemented the conditional succinate growth phenotype of *dme* mutants, as defined above, indicating that the entire *tme* gene was present on the 6.8-kb *Bam*HI fragment.

**Insertion mutagenesis of *tme* gene.** The *tme* gene region was localized following the isolation of Tn5, Tn5-B20, and Ω insertions within the cloned gene regions in pTH115 and pRmT100 (Fig. 3). The Ω fragment carrying spectinomycin and streptomycin resistance genes (40) was cloned into the central *Eco*RI site of pTH115. These plasmids were transferred to the *dme pckA pod-1* mutant (strain RmG443), and the resulting transconjugants were screened for growth in M9-succinate. Transconjugants carrying pTH115 with Tn5 insertions 1, 2, and 3 and the ΩSp insertion failed to grow, whereas those carrying Tn5 insertions Ω5295 and Ω5296 grew. All insertions were recombined into the *R. meliloti* genome, and their precise recombination into the genome was verified by Southern hybridization with labelled pTH240 as the probe (data not shown). Malic enzyme and MDH activities were determined for strains carrying Tn5 and ΩSp insertions (Table 2). Only 13 to 26% of the wild-type TME activity was detected in extracts of cells carrying the homogenized mutations which failed to complement the conditional succinate growth phenotype of the *dme* mutants when present on pTH115. The level of DME activity in these strains, however, was not decreased.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s) <sup>a</sup>	Source or reference
<i>Rhizobium meliloti</i>		
Rm1021	SU47, <i>str-21</i>	32
RmF361	Rm1021, <i>pckA-1::Tn5-132 pod-1</i>	9
RmG212	Rm1021, Lac <sup>-</sup>	Jane Glazebrook
RmG243	Rm1021, <i>pckA-3::Tn3HoHo1Sp pod-1</i>	9
RmG443	RmG243, <i>dme-2::Tn5</i>	9
RmG456	Rm1021, <i>dme-1::Tn5</i>	9
RmG894	Rm1021, <i>tme-1::Tn5</i> (pPH1J1)	This study
RmG896	Rm1021, <i>tme-4::ΩSp</i> (pR751)	This study
RmG916	Rm1021, <i>tme-2::Tn5</i> (pPH1J1)	This study
RmG917	Rm1021, Ω5296::Tn5 <i>tme</i> linked (pPH1J1)	This study
RmG918	Rm1021, <i>tme-3::Tn5</i> (pPH1J1)	This study
RmG926	Rm1021, Ω5295::Tn5 <i>tme</i> linked (pPH1J1)	This study
RmG927	Rm1021, <i>tme-1::Tn5</i> (φRmG894→Rm1021)	This study
RmG994	Rm1021, <i>dme-3::Tn5 tme-4::ΩSp</i>	This study
RmG995	Rm1021, <i>tme-4::ΩSp</i> (φRmG896→Rm1021)	This study
RmH183	RmG456 (pRmT100)	This study
RmH185	RmG927 (pRmT100)	This study
RmH187	RmF361, <i>tme-4::ΩSp</i>	This study
RmH188	RmF361, <i>dme-1::Tn5</i>	This study
RmH194	RmH187, <i>dme-1::Tn5</i>	This study
RmH215	Rm1021, <i>tme-2::Tn5</i> (φRmG916→Rm1021)	This study
RmH216	Rm1021, Ω5296::Tn5 (φRmG917→Rm1021)	This study
RmH217	Rm1021, <i>tme-3::Tn5</i> (φRmG918→Rm1021)	This study
RmH218	Rm1021, Ω5295::Tn5 (φRmG926→Rm1021)	This study
RmH234	RmG212, <i>tme-5::Tn5-B20</i>	This study
RmH235	RmG212, <i>tme-6::Tn5-B20</i>	This study
RmH236	RmG212, <i>tme-7::Tn5-B20</i>	This study
RmH237	RmG212, <i>tme-8::Tn5-B20</i>	This study
RmH238	RmG212, <i>tme-9::Tn5-B20</i>	This study
<i>Escherichia coli</i>		
MT607	<i>pro-82 thi-1 hsdR17 supE44 recA56</i>	Laboratory collection
G312	MT607, Ω5::Tn5-B20	This study
Plasmids		
pLAFR1	Broad-host-range cosmid vector, Tc <sup>r</sup>	18
pRK7813	Broad-host-range cloning vector, Tc <sup>r</sup>	24
pPH1J1	Broad-host-range IncP plasmid, Gm <sup>r</sup> Sp <sup>r</sup>	4
pR751	Broad-host-range IncP plasmid, Tp <sup>r</sup>	23
pRmT100	pLAFR1 clone, complements <i>ntrA</i> and <i>tme</i> mutants	16
pRmT102	pLAFR1 clone, complements <i>eno</i> mutants	16
pRmT103	pLAFR1 clone, complements <i>pckA</i> mutants	16
pRmT104	pLAFR1 clone, complements <i>pgk</i> and <i>gap</i> mutants	16
pRmT105	pLAFR1 clone, complements <i>dct</i> mutants	16
pTH115	7.1-kb <i>Bam</i> HI fragment of pTH240 in pRK7813 (OR2)	This study
pTH116	7.1-kb <i>Bam</i> HI fragment of pTH240 in pRK7813 (OR1)	This study
pTH228	pRmT100, <i>tme-5::Tn5-B20</i>	This study
pTH230	pRmT100, <i>tme-6::Tn5-B20</i>	This study
pTH233	pRmT100, <i>tme-7::Tn5-B20</i>	This study
pTH235	pRmT100, <i>tme-8::Tn5-B20</i>	This study
pTH237	pRmT100, <i>tme-9::Tn5-B20</i>	This study
pTH240	<i>Clal</i> deletion of pRmT100 (pRmT100ΔC)	T. Finan

<sup>a</sup> Transposons Tn5 (Nm<sup>r</sup>) and Tn5-132 (Ot<sup>r</sup>) (3), Tn5-B20 (Nm<sup>r</sup>) 45, and Tn3HoHo1Sp (Sp<sup>r</sup> Sm<sup>r</sup>) (46) have been described previously. The ΩSp fragment (40) encodes Sp<sup>r</sup> Sm<sup>r</sup>. The *pod* alleles were previously designated *spk*, for suppression of the Pck<sup>-</sup> growth phenotype (9, 10). Orientation designations for pTH115 and pTH116 are relative to the *lac* promoter of pRK7813 and are arbitrary (see the text). The *pod* alleles encode pyruvate, orthophosphate dikinase, the *dme* alleles encode diphosphopyridine nucleotide (NAD<sup>+</sup>)-dependent malic enzyme, the *tme* alleles encode triphosphopyridine nucleotide (NADP<sup>+</sup>)-dependent malic enzyme, and the *per* alleles encode phosphoenolpyruvate carboxykinase. Or, orientation.

Because of the large reduction in TME activity, the above insertion mutations were labelled *tme-1*, *tme-2*, *tme-3*, and *tme-4*. Extracts of cells bearing Ω5295::Tn5 and Ω5296::Tn5, which did not affect pTH115 complementation, were found to have wild-type levels of all enzymes tested. High levels (>376 nmol/min/mg of protein) of MDH activity were detected in the extracts of all strains.

#### DEAE-cellulose chromatography of extracts lacking TME.

In a previous study, we used DEAE-cellulose column chromatography to demonstrate that two separate enzymes produce the DME and TME activities detected in *R. meliloti* extracts. The DME activity peak was absent, but the TME activity peak was present in elution profiles of cell extracts prepared from strains carrying *dme::Tn5* insertions. In the absence of DME, TME was shown to have a strict cofactor specificity for NADP<sup>+</sup> (9).

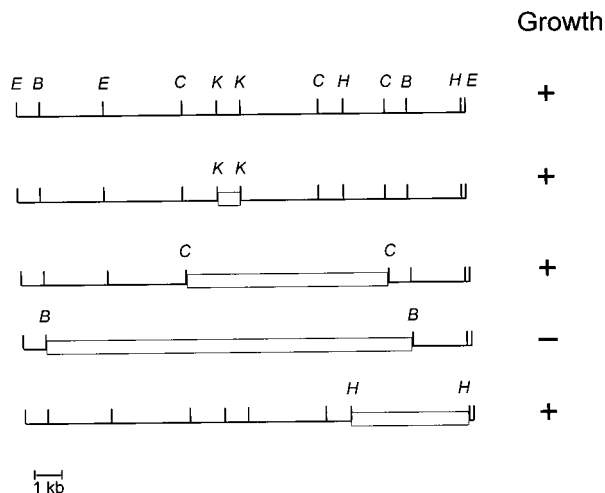


FIG. 2. Complementation by deletion derivatives of pRmT100. In the top line, the restriction map of pRmT100, the cosmid clone carrying the *tme* and *ntrA* genes of *R. meliloti*, is shown. The sizes and positions of deletions are indicated by open boxes. The plasmid designations (for the lines from top to bottom) were pRmT100, pRmT100ΔK, pRmT100ΔC, pRmT100ΔB, and pRmT100ΔH, respectively. E, *Eco*RI; B, *Bam*HI; C, *Cla*I; K, *Kpn*I; H, *Hind*III. On the right, growth of the *dme pckA pod-1* mutant on M9-succinate is indicated by a plus and no growth is indicated by a minus sign.

An extract of RmG927 (*tme-1::Tn5*) cells was applied to a DEAE-cellulose chromatography column, and the proteins were eluted with a linear 0 to 0.3 M KCl gradient. No TME peak was observed (Fig. 4B), whereas a TME peak was present in the elution profile of the wild-type *R. meliloti* strain, Rm1021 (Fig. 4A, 250 to 400 ml). The position of the DME peak was similar in the elution profiles of the wild type and the *tme-1::Tn5* mutant (Fig. 4A and B). The only TME activity detected in the elution profile of *tme-1* extract coeluted with the DME peak, at 375 ml. This small peak resulted from the reduction of NADP<sup>+</sup> by DME. Neither of the malic enzyme activity peaks was observed when an extract of the *tme dme* double mutant RmG994 was eluted from the DEAE-cellulose chromatography column (Fig. 4C). Elution of MDH (125 to 225 ml) and PCK activities (data not shown) were monitored as controls. A low level of apparent DME activity was detected

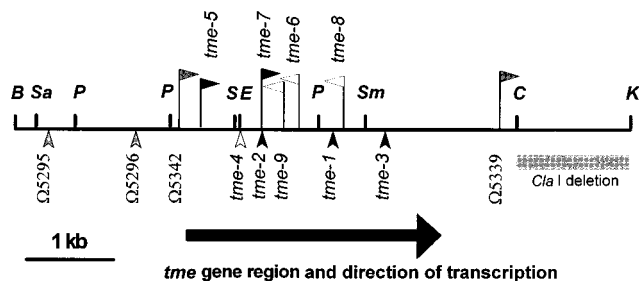


FIG. 3. Delineation of *tme* gene region. Tn5 insertions within the *tme* gene are indicated by closed arrowheads, Tn5 insertions outside of the *tme* gene are indicated by stippled arrowheads, and the omega ( $\Omega$ ) fragment cloned into the *Eco*RI site within the *tme* gene is indicated by an open arrowhead. Black flags indicate Tn5-B20 insertions within the *tme* gene producing considerable levels of  $\beta$ -galactosidase activity, open flags indicate Tn5-B20 insertions producing negligible levels of  $\beta$ -galactosidase activity, and stippled flags indicate Tn5-B20 insertions outside of the *tme* gene. The flags point in the direction of transcription. The large closed arrow indicates the net *tme* gene region to date and the deduced direction of transcription. E, *Eco*RI; B, *Bam*HI; C, *Cla*I; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sm, *Sma*I.

in fractions coeluting with MDH activity (Fig. 4B and C). This activity represents cross-reactivity of oxaloacetic acid with 2,4-dinitrophenylhydrazine used to detect pyruvate in the malic enzyme assay (9).

**Symbiotic and growth phenotypes of *tme* mutants.** The symbiotic phenotype of Rm1021 *tme* mutants was examined by inoculating alfalfa seedlings growing under nitrogen-deficient conditions. After growth for 4 to 6 weeks, the dry weights of the plant shoots were determined. The dry weight is a measure of the cumulative amount of nitrogen fixation. The dry weights of plants inoculated with the wild type and four *tme* mutants were similar (Table 3), showing that the *tme* mutations had no effect on N<sub>2</sub> fixation. As expected, the dry weights of plants inoculated with the *dme* mutant RmG456, which is Fix<sup>-</sup> (9), were similar to those of the uninoculated control. As pRmT100 partially complemented the conditional succinate growth phenotype of *dme* mutants, we investigated whether pRmT100 could complement the *dme* Fix<sup>-</sup> phenotype. Although the mean dry weight of plants inoculated with RmH183 [RmG456(pRmT100)] was somewhat higher than that of plants inoculated with RmG456, plants inoculated with either strain appeared chlorotic and stunted. Moreover, the dry weights of plants inoculated with RmH183 were similar to those of RmG994-inoculated plants. We thus conclude that pRmT100 does not complement the symbiotic phenotype of *dme* mutants.

To investigate whether the *tme* mutations generated a carbon utilization phenotype, the growth of Rm1021 *tme* mutants in minimal medium containing various carbon sources (15 mM glucose, succinate, or lactate or 0.1% potassium acetate) was compared with that of the wild-type parent strain, Rm1021. No differences were observed. When the *tme* mutations were transduced into the RmG243 (*pckA pod-1*) background, the transductants (*tme pckA pod-1*) grew as well on minimal medium plates with succinate as the sole carbon source as RmG243 (data not shown). When the *tme* mutations were transduced into a Pck<sup>-</sup> strain, the transductants grew more slowly on succinate plates than did the strains carrying the *pckA* mutation alone (not shown).

The only genetic background in which *tme* mutations produced a distinct plate growth phenotype was strain RmH188, which is both Dme<sup>-</sup> and Pck<sup>-</sup> and which carries the *pod-1* suppressor allele. RmH188 cells grew poorly on succinate plates (a leaky succinate growth phenotype). When the *tme-4::ΩSp* insertion was transduced into RmH188, the resulting transductants (i.e., RmH194) showed no growth at all on succinate-minimal medium plates. RmH194 was fully complemented for growth on succinate plates by plasmids carrying the

TABLE 2. TME activity abolished by Tn5 and  $\Omega$ Sp insertions recombined into *R. meliloti* genome

Strain	Relevant characteristic	Sp act <sup>a</sup>		
		DME	TME	MDH
Rm1021	Wild type	121 ± 11	93.4 ± 11.1	656 ± 29
RmG927	<i>tme-1::Tn5</i>	113 ± 8	13.9 ± 5.3	560 ± 31
RmH215	<i>tme-2::Tn5</i>	136 ± 8	13.3 ± 4.3	442 ± 6
RmH218	$\Omega$ 5295::Tn5	117 ± 5	101 ± 15	376 ± 2
RmH216	$\Omega$ 5296::Tn5	103 ± 5	95.4 ± 10.3	514 ± 7
RmH217	<i>tme-3::Tn5</i>	130 ± 11	22.1 ± 7.6	401 ± 13
RmG896	<i>tme-4::ΩSp</i>	119 ± 2	25.6 ± 5.8	614 ± 31

<sup>a</sup> Cells were grown in LBmc liquid medium, and crude cell extracts were prepared as described in Materials and Methods. Specific activity values (nanomoles per minute per milligram of protein) are expressed as the means of triplicate assays of each extract ± standard errors.

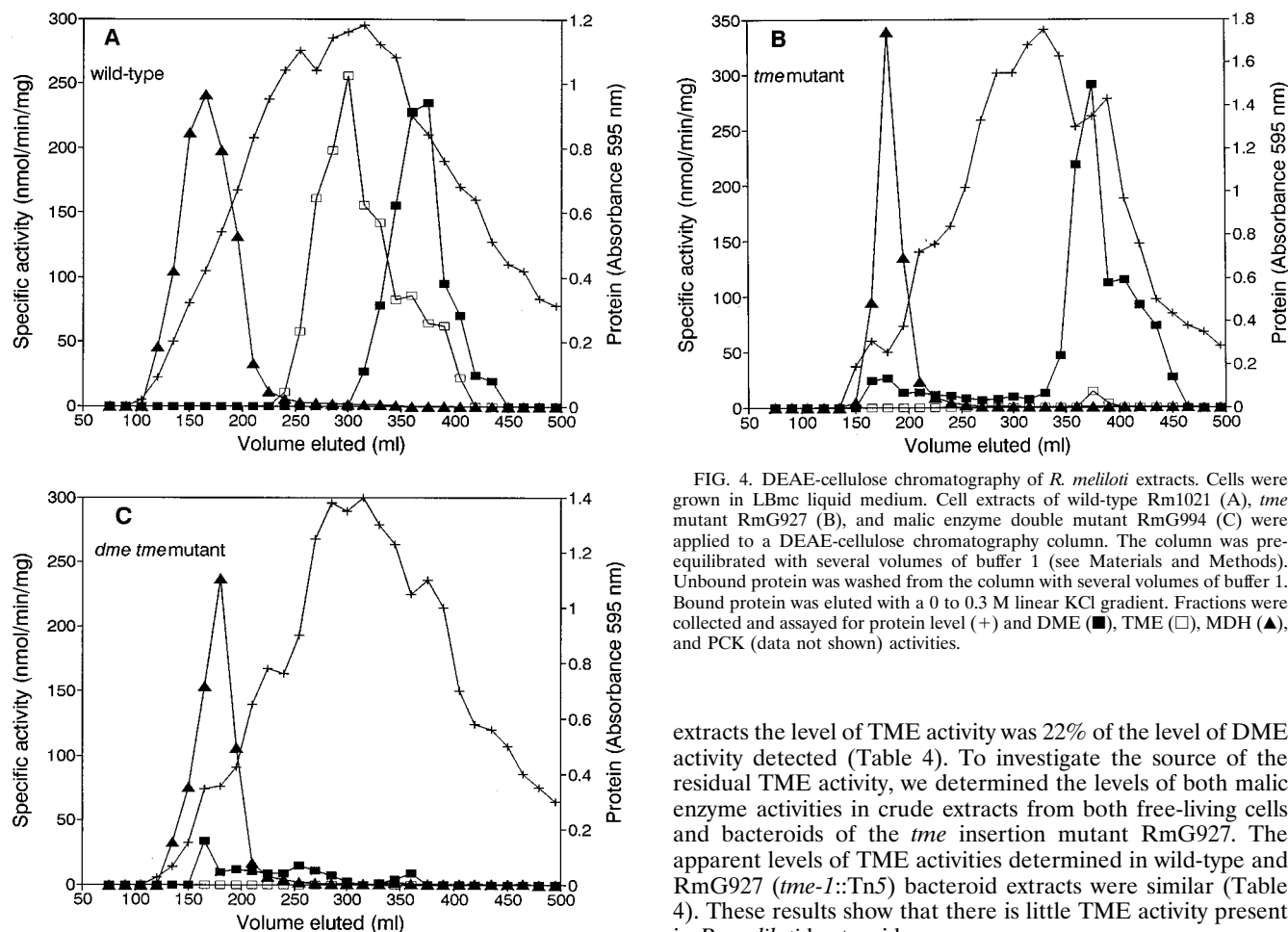


FIG. 4. DEAE-cellulose chromatography of *R. meliloti* extracts. Cells were grown in LBmc liquid medium. Cell extracts of wild-type Rm1021 (A), *tme* mutant RmG927 (B), and malic enzyme double mutant RmG994 (C) were applied to a DEAE-cellulose chromatography column. The column was pre-equilibrated with several volumes of buffer 1 (see Materials and Methods). Unbound protein was washed from the column with several volumes of buffer 1. Bound protein was eluted with a 0 to 0.3 M linear KCl gradient. Fractions were collected and assayed for protein level (+) and DME (■), TME (□), MDH (▲), and PCK (data not shown) activities.

extracts the level of TME activity was 22% of the level of DME activity detected (Table 4). To investigate the source of the residual TME activity, we determined the levels of both malic enzyme activities in crude extracts from both free-living cells and bacteroids of the *tme* insertion mutant RmG927. The apparent levels of TME activities determined in wild-type and RmG927 (*tme-1::Tn5*) bacteroid extracts were similar (Table 4). These results show that there is little TME activity present in *R. meliloti* bacteroids.

DME, MDH, and PCK activities were unaffected by the *tme* mutation. These enzyme activities have been previously reported for bacteroids and free-living cells of *R. meliloti* (9, 15). Bacteroids had high levels of MDH and very low levels of PCK activity compared with those in free-living cells, whereas the levels of DME activity were similar in both cell types.

**Regulation of *tme* gene expression.** To monitor *tme* expression we used Tn5-B20 to generate gene fusions in which transcription of the *lacZ* gene was directed by the *tme* promoter. Five independent Tn5-B20 insertions in pRmT100 which failed

genes encoding either DME or PCK and partially complemented (as described above) by plasmids carrying the *tme* gene.

**TME activity level is low in *R. meliloti* bacteroids.** The determination of TME activity levels in crude bacteroid extracts (and free-living cells) was complicated by the fact that DME, which is present in these extracts, is partially active when NADP<sup>+</sup> is supplied as the coenzyme. In wild-type bacteroid

TABLE 3. Symbiotic phenotypes of *tme* mutants

Strain inoculated	Relevant characteristic	Shoot dry wt (mg/plant) <sup>a</sup>	% of wild type	Symbiotic phenotype <sup>b</sup>
Rm1021	Wild type	12.5 ± 1.0	100	Fix <sup>+</sup>
None (uninoculated)		6.3 ± 0.3	51	Fix <sup>-</sup>
RmG927	<i>tme-1::Tn5</i>	12.6 ± 1.3	101	Fix <sup>+</sup>
RmG916	<i>tme-2::Tn5</i>	12.5 ± 0.7	100	Fix <sup>+</sup>
RmG918	<i>tme-3::Tn5</i>	13.5 ± 1.7	108	Fix <sup>+</sup>
RmG995	<i>tme-4::ΩSp</i>	15.9 ± 1.3	127	Fix <sup>+</sup>
RmG456	<i>dme-1::Tn5</i>	6.2 ± 0.5	49	Fix <sup>-</sup>
RmH183	RmG456(pRmT100)	8.4 ± 0.3	68	Fix <sup>-</sup>
RmH185	RmG927(pRmT100)	11.6 ± 1.1	93	Fix <sup>+</sup>
RmG994	<i>dme-3::Tn5 tme-4::ΩSp</i>	8.0 ± 0.9	64	Fix <sup>-</sup>

<sup>a</sup> *R. meliloti* strains were inoculated onto alfalfa seedlings as described in Materials and Methods. The plant tops were harvested 33 days postinoculation and dried, and the dry weights were determined. The values represent the means for duplicate samples (each sample consisting of 9 to 12 shoots) ± standard errors.

<sup>b</sup> The symbiotic phenotype was determined both by examination of the plants for color and by the shoot dry weight data, since the shoot dry weights for the Fix<sup>+</sup> plants are relatively low because of insufficient light in the growth chamber during the course of this experiment.

TABLE 4. Effect of *tme-1::Tn5* mutation on enzyme activities in *R. meliloti* bacteroids and free-living cells

Strain <sup>a</sup>	Relevant characteristics	Sp act (nmol/min/mg of protein) <sup>b</sup>			
		DME	TME	MDH	PCK
Rm1021					
Free-living	Wild type	72 ± 2	95 ± 8	793 ± 65	38.8 ± 2.0
Bacteroids	Wild type	113 ± 4	25 ± 4	2201 ± 55	0.3 ± 0.01
RmG927					
Free living	<i>tme-1::Tn5</i>	85 ± 11	18 ± 2	841 ± 43	80.8 ± 4.0
Bacteroids	<i>tme-1::Tn5</i>	101 ± 6	22 ± 7	3256 ± 150	0.5 ± 0.1
Bacteroids <sup>c</sup>	<i>tme-1::Tn5</i>	122 ± 6	23 ± 2	2803 ± 103	1.2 ± 0.7

<sup>a</sup> Free-living cells were grown in LBmc. Bacteroids were isolated from alfalfa root nodules, as described in Materials and Methods, 5 weeks after inoculation with the *R. meliloti* strain indicated.

<sup>b</sup> Specific activity values are expressed as the means of triplicate assays of each extract ± standard errors.

<sup>c</sup> Bacteroids were isolated from alfalfa root nodules 6 weeks after inoculation with RmG927.

to complement the conditional succinate growth phenotype of the *dme pckA pod-1* mutants were isolated (Fig. 3, insertions 5 to 9). These insertions were recombined into the *R. meliloti* genome, and the positions of the insertions in the *R. meliloti* genome were confirmed by Southern hybridization, with labelled pRmT100 as the probe (data not shown). Enzymatic assay of extracts of the recombinant strains showed that all had less than 18.1 nmol/min/mg of protein of TME activity and therefore were *tme* mutants (Table 5). These strains had levels of DME and MDH activities comparable to those in the wild-type strain, Rm1021.

The five *tme* insertions mapped to a 2.1-kb region (Fig. 3). The locations of two additional Tn5-B20 insertions in pRmT100 (Ω5342 and Ω5339) which retained the ability to complement the conditional succinate growth phenotype of the *dme* mutants were also determined. These insertions lay 3.7 kb from each other and evidently flank the *tme* gene region. Tn5-B20 insertions *tme-5* and *tme-7* lay in the same orientation, and DNA sequence analysis showed that the *lacZ* gene in these insertions is transcribed in the same orientation as the TME structural gene (12). In contrast, Tn5-B20 insertions *tme-6*, *tme-8*, and *tme-9* lay in the opposite orientation.

To investigate the regulation of *tme* gene expression, liquid cultures of strains RmH234, RmH236, and RmH237 were grown in LBmc or minimal medium with either glucose or

succinate as the sole carbon source, and the cells were assayed for β-galactosidase activity (Table 5). The strains bearing the two *tme-lacZ* fusions, RmH234 and RmH236, were found to have β-galactosidase activity levels ranging from 202 to 314 Miller units under the three different growth conditions examined. The control strains RmG212 and RmH237 had low levels of β-galactosidase activity. In summary, growth of *R. meliloti* on a glycolytic or gluconeogenic carbon source did not affect the level of *tme* expression.

## DISCUSSION

In this paper we report the isolation of the NADP<sup>+</sup> malic enzyme gene of *R. meliloti*, which we designate *tme* (triphosphopyridine nucleotide-dependent malic enzyme) on the basis of an earlier convention used in *E. coli* (20). We generated *tme* insertion mutants of *R. meliloti* and showed that chromatographic elution profiles of extracts from these strains lacked the major TME activity peak. A small TME peak was detected in these extracts; it coeluted with the DME peak and thus presumably resulted from the low level of NADP<sup>+</sup>-dependent activity of the DME enzyme. Both the major and minor TME activity peaks were absent in a chromatographic elution profile of extracts from a *tme dme* double mutant. These results, combined with preliminary analysis of nucleotide sequence obtained from plasmids carrying the Tn5-B20 insertions (12), confirmed that the mutations were within the structural gene encoding the TME.

The Fix<sup>+</sup> symbiotic phenotype of *tme* mutants clearly demonstrated that the TME was not required for symbiotic N<sub>2</sub> fixation by *R. meliloti* (Table 3). This is consistent with the very low level of TME activity detected in bacteroids (Table 4). The reduced TME activity in bacteroids is interesting as expression of the *tme* gene in free-living cells appears to be constitutive. The production of NADPH may not be advantageous to N<sub>2</sub>-fixing bacteroids, within which reduced carbon is channelled into the TCA cycle, rather than to growth. We have previously proposed that the low level of PCK activity in bacteroids may prevent uncontrolled bacterial proliferation within nodules, as PCK is required for gluconeogenesis from TCA cycle intermediates in *R. meliloti* (15). The low level of TME activity may limit growth of bacteroids, by lowering the rate of conversion of NADP<sup>+</sup> to NADPH, a form of the coenzyme required by many biosynthetic enzymes. NADPH synthesis in bacteroids could presumably occur via NADP<sup>+</sup>-dependent isocitrate dehydrogenase, which is present in *R. meliloti*. Moreover, we note

TABLE 5. Expression of *tme::lacZ* gene fusions under different free-living conditions

Strain	Relevant characteristic	Mean ± SE <sup>a</sup>					
		β-Galactosidase activity (Miller units) in:			Enzyme activity (nmol/min/mg of protein)		
		LBmc	Succinate	Glucose	DME	TME	MDH
RmG212	Lac <sup>-</sup>	7.1 ± 0.9	8.1 ± 1.6	3 ± 1.6	—	—	—
RmH234 <sup>b</sup>	<i>tme-5::Tn5-B20</i>	220 ± 23	202 ± 21	224 ± 15	54.7 ± 2.1	18.1 ± 1.4	811 ± 84
RmH236 <sup>b</sup>	<i>tme-7::Tn5-B20</i>	312 ± 10	314 ± 41	282 ± 44	68.3 ± 3.2	12.8 ± 0.9	616 ± 52
RmH237 <sup>c</sup>	<i>tme-8::Tn5-B20</i>	33.3 ± 0.6	11 ± 3	7 ± 1.9	63.0 ± 2.3	13.9 ± 1.4	767 ± 85
RmH235 <sup>c</sup>	<i>tme-6::Tn5-B20</i>	14.0 ± 1.0	ND	ND	53.4 ± 0.5	12.8 ± 1.6	529 ± 14
RmH238 <sup>c</sup>	<i>tme-9::Tn5-B20</i>	10.6 ± 0.3	ND	ND	61.4 ± 1.9	14.4 ± 0.9	593 ± 3
Rm1021	Wild type	—	—	—	62.4 ± 0.9	75.8 ± 2.3	816 ± 61

<sup>a</sup> Data are mean values of triplicate assays of samples ± standard errors. β-Galactosidase activity (see Materials and Methods) was determined for cells grown in LBmc or in minimal medium with succinate or glucose as the sole carbon source, as indicated. Enzyme activity was determined from crude cell extracts prepared as described in Materials and Methods. ND, not determined; —, not applicable.

<sup>b</sup> *lac* in the same orientation as *tme*.

<sup>c</sup> *lac* in the opposite orientation from *tme*.

that *Bradyrhizobium japonicum* bacteroids contain substantial TME activity (7, 26, 28), suggesting that the regulation of *tme* expression and/or TME activity in *B. japonicum* may differ from that in *R. meliloti*.

The very low level of TME activity detected in bacteroids may explain the failure of pRmT100 to complement the symbiotic phenotype of Dme<sup>-</sup> mutants. Assuming that the low level of TME activity results from low levels of *tme* expression, the ability of TME to functionally replace DME in bacteroids will require further experiments in which expression of the *tme* gene is directed from a promoter which is active in bacteroids. Further characterization of the regulation of the *tme* gene in bacteroids may lead to the identification of a bacteroid-specific system regulating carbon metabolism.

The growth phenotypes of strains carrying *dme* or *tme* mutations in a *pckA* or *pckA pod-1* background suggest that DME, and to lesser extent TME, functions in vivo in the conversion of malate to pyruvate. However, *R. meliloti tme* or *dme* mutants and *tme dme* double mutants grew at rates similar to that of the wild type when 15 mM succinate was supplied as the sole carbon source (this work; 9). Pyruvate formation in these mutants presumably results from the combined activities of succinate dehydrogenase, MDH, PCK, and pyruvate kinase, respectively, which convert succinate to malate, malate to oxaloacetate, oxaloacetate to PEP, and PEP to pyruvate. NADH is formed in the MDH reaction, and ATP which is consumed in the PCK reaction is regenerated by pyruvate kinase. In wild-type cells, formation of pyruvate from malate catalyzed by malic enzyme generates NAD(P)H; thus, on energetic grounds, whether bacteria convert malate to pyruvate via malic enzyme or via the PCK enzyme route should have no effect on cellular growth with succinate or other TCA cycle intermediates as the carbon source.

*E. coli* has been shown to have two distinct malic enzymes (25), and much work has been done on the biochemical characterization of these enzymes. Sanwal (44) proposed that, in *E. coli*, TME may function in a primarily biosynthetic capacity and that DME has a primarily catabolic function. This hypothesis was partially validated by Murai et al. (34), who showed that production of both enzymes was repressed in glucose-grown cells and induced in malate-grown cells. Reversal of the repression of DME activity by glucose was proportional to the malate concentration, indicating that this enzyme was important for catabolism of malate. The same response was not observed for TME. In addition to glucose, TME was repressed by glycerol, lactate, and acetate, supporting a role for this enzyme in a pathway producing acetyl coenzyme A for lipid biosynthesis. Studies with <sup>14</sup>C-labelled succinate indicated that succinate is likely metabolized via the malic enzyme during biosynthesis of fatty acids (34). The requirement in *R. meliloti* for DME as part of the pathway converting malate to acetyl coenzyme A represents a catabolic role for this enzyme. Moreover, the low level of TME activity in bacteroids, which are nondividing cells, is consistent with the hypothesis of a biosynthetic role for TME.

The presence of the two malic enzymes in many bacteria suggests that these enzymes have fundamental roles in bacterial metabolism. From the foregoing discussion, it is evident that the precise role(s) of these enzymes in bacterial metabolism requires clarification. It is likely that physiological studies on the single and double mutants generated in this study will give insight into why these enzymes appear to be conserved among diverse bacterial species.

## ACKNOWLEDGMENTS

We thank Magne Østerås, Asif Khattak, Jason Marsh, and Sylvie Bardin for technical help and for critical reading of the manuscript.

This study was supported by an operating grant to T.M.F. from the Natural Sciences and Engineering Research Council of Canada.

## REFERENCES

- Arwas, R., I. A. McKay, F. R. P. 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.
- Bartolucci, S., R. Rella, A. Guagliardi, C. Raia, A. Gambacorda, M. De Rosa, and M. Rossi. 1987. Malic enzyme from archaebacterium *Sulfolobus solfataricus*: purification, structure, and kinetic properties. *J. Biol. Chem.* **262**:7725–7731.
- Berg, C. M., and D. E. Berg. 1987. Uses of transposable elements and maps of known insertions, p. 1071–1109. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
- Beringer, J. E., J. L. Beynon, A. V. Buchanan-Wollaston, and A. W. B. Johnston. 1978. Transfer of the drug-resistance transposon Tn5 to *Rhizobium*. *Nature (London)* **276**:633–634.
- 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.
- Charles, T. C., and T. M. Finan. 1990. Genetic map of *Rhizobium meliloti* megaplasmid pRmeSU47b. *J. Bacteriol.* **172**:2469–2476.
- Copeland, L., R. G. Quinnell, and D. A. Day. 1989. Malic enzyme in bacteroids from soybean nodules. *J. Gen. Microbiol.* **135**:2005–2011.
- Diesterhaft, M. D., and E. Freese. 1973. Role of pyruvate carboxylase, phosphoenolpyruvate carboxykinase, and malic enzyme during growth and sporulation of *Bacillus subtilis*. *J. Biol. Chem.* **248**:6062–6070.
- Driscoll, B. T., and T. M. Finan. 1993. NAD<sup>+</sup>-dependent malic enzyme of *Rhizobium meliloti* is required for symbiotic nitrogen fixation. *Mol. Microbiol.* **7**:865–873.
- Driscoll, B. T., M. Østerås, and T. M. Finan. 1993. Succinate metabolism in *Rhizobium meliloti*, p. 517–522. *In* New horizons in nitrogen fixation, Kluwer Academic Publishers, Boston.
- Englard, S., and L. Siegal. 1969. Mitochondrial L-malate dehydrogenase of beef heart. *Methods Enzymol.* **13**:99–100.
- Finan, T. M., B. Driscoll, and M. Østerås. Unpublished data.
- Finan, T. M., E. K. Hartwig, K. LeMieux, K. Bergman, G. C. Walker, and E. Signer. 1984. General transduction in *Rhizobium meliloti*. *J. Bacteriol.* **159**:120–124.
- Finan, T. M., B. Kunkel, G. F. DeVos, and E. R. Signer. 1986. Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. *J. Bacteriol.* **167**:66–72.
- Finan, T. M., E. McWhinnie, B. Driscoll, and R. J. Watson. 1991. Complex symbiotic phenotypes result from gluconeogenic mutations in *Rhizobium meliloti*. *Mol. Plant-Microbe Interact.* **4**:386–392.
- Finan, T. M., I. Oresnik, and A. Bottacin. 1988. Mutants of *Rhizobium meliloti* defective in succinate metabolism. *J. Bacteriol.* **170**:3396–3403.
- Finan, T. M., J. M. Wood, and D. C. Jordan. 1983. Symbiotic properties of C<sub>4</sub>-dicarboxylate acid transport mutants of *Rhizobium leguminosarum*. *J. Bacteriol.* **154**:1403–1413.
- 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.
- Garrido-Pertierra, A., C. Martinez Marcos, M. Martin Fernandez, and M. Ruiz-Amil. 1983. Properties and function of malate enzyme from *Pseudomonas putida*. *Biochimie* **65**:629–635.
- Goldie, A. H. 1979. The regulation of phosphoenolpyruvate carboxykinase and other gluconeogenic enzymes in *Escherichia coli*, p. 12–44. Ph.D. thesis. University of Western Ontario, London, Ontario, Canada.
- Hansen, E. J., and E. Juni. 1975. Isolation of mutants of *Escherichia coli* lacking NAD- and NADP-linked malic enzyme activities. *Biochem. Biophys. Res. Commun.* **65**:559–566.
- Hansen, R. J., H. Hinze, and H. Holzer. 1976. Assay of phosphoenolpyruvate carboxykinase in crude yeast extracts. *Anal. Biochem.* **74**:576–584.
- Jobanputra, R. S., and N. J. Datta. 1974. Trimethoprim R factors in enterobacteria from clinical specimens. *J. Med. Microbiol.* **7**:169–177.
- Jones, J. D. J., and N. Gutterson. 1987. An efficient mobilizable cosmid vector, pRK7813, and its use in a rapid method for marker exchange in *Pseudomonas fluorescens* strain HV37a. *Gene* **61**:299–306.
- Katsuki, H., K. Takeo, K. Kameda, and S. Tanaka. 1967. Existence of two malic enzymes in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **27**:331–336.
- Kimura, I., and S. Tajima. 1989. Presence and characteristics of NADP-malic enzyme in soybean nodules. *Soil Sci. Plant Nutr.* **35**:271–279.
- Kobayashi, K., S. Doi, S. Negoro, I. Urabe, and H. Okada. 1989. Structure and properties of malic enzyme from *Bacillus stearothermophilus*. *J. Biol. Chem.* **264**:3200–3205.

28. **Kouchi, H., K. Fukai, H. Katagiri, K. Minamisawa, and S. Tajima.** 1988. Isolation and enzymological characterization of infected and uninfected cell protoplasts from root nodules of *Glycine max*. *Physiol. Plant.* **73**:327–334.
29. **Lamed, R., and J. G. Zeikus.** 1981. Thermostable, ammonium-activated malic enzyme of *Clostridium thermocellum*. *Biochim. Biophys. Acta* **660**:251–255.
30. **McKay, I. A., M. J. Dilworth, and A. R. Glenn.** 1988. C<sub>4</sub>-dicarboxylate metabolism in free-living and bacteroid forms of *Rhizobium leguminosarum* MNF3841. *J. Gen. Microbiol.* **134**:1433–1440.
31. **McKay, I. A., A. R. Glenn, and M. J. Dilworth.** 1985. Gluconeogenesis in *Rhizobium leguminosarum* MNF3841. *J. Gen. Microbiol.* **131**:2067–2073.
32. **Meade, H. M., S. R. Long, G. B. Ruvkin, S. E. Brown, and F. M. Ausubel.** 1982. Physical and genetical characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn5 mutagenesis. *J. Bacteriol.* **149**:114–122.
33. **Miller, J. H.** 1972. *Experiments in molecular genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
34. **Murai, T., M. Tokushige, J. Nagai, and H. Katsuki.** 1971. Physiological functions of NAD- and NADP-linked malic enzymes in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **43**:875–881.
35. **Ochoa, S., A. Mehler, and A. Kornberg.** 1947. Reversible oxidative decarboxylation of malic acid. *J. Biol. Chem.* **167**:871–872.
36. **Ohné, M.** 1975. Regulation of the dicarboxylic acid part of the citric acid cycle in *Bacillus subtilis*. *J. Bacteriol.* **122**:224–234.
37. **Ohné, M., and B. Rutberg.** 1976. Repression of sporulation in *Bacillus subtilis* by L-malate. *J. Bacteriol.* **125**:453–460.
38. **Østerås, M., B. T. Driscoll, and T. M. Finan.** 1995. Molecular and expression analysis of the *Rhizobium meliloti* phosphoenolpyruvate carboxykinase (*pckA*) gene. *J. Bacteriol.* **177**:1452–1460.
39. **Østerås, M., T. M. Finan, and J. Stanley.** 1991. Site-directed mutagenesis and DNA sequence of *pckA* of *Rhizobium* NGR234, encoding phosphoenolpyruvate carboxykinase: gluconeogenesis and host-dependent symbiotic phenotype. *Mol. Gen. Genet.* **230**:257–269.
40. **Prentki, P., and H. M. Krisch.** 1984. *In vitro* insertional mutagenesis with a selectable DNA fragment. *Gene* **29**:303–313.
41. **Ronson, C. W., P. Lyttleton, and J. G. Robertson.** 1981. C<sub>4</sub>-dicarboxylate transport mutants of *Rhizobium trifolii* form ineffective nodules on *Trifolium repens*. *Proc. Natl. Acad. Sci. USA* **78**:4284–4288.
42. **Ronson, C. W., T. B. Nixon, L. M. Albright, and F. M. Ausubel.** 1987. *Rhizobium meliloti ntrA (rpoN)* is required for diverse metabolic functions. *J. Bacteriol.* **169**:2424–2431.
43. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
44. **Sanwal, B. D.** 1970. Allosteric controls of amphibolic pathways in bacteria. *Bacteriol. Rev.* **34**:20–39.
45. **Simon, R., J. Quandt, and W. Klipp.** 1989. New derivatives of transposon Tn5 suitable for mobilizations of replicons, generation of operon fusions and induction of genes in Gram-negative bacteria. *Gene* **80**:161–169.
46. **Stachel, S. E., G. An, C. Flores, and E. W. Nester.** 1985. A Tn3lacZ transposon for the random generation of β-galactosidase gene fusions: application to the analysis of gene expression in *Agrobacterium*. *EMBO J.* **4**:891–898.
47. **Stoval, I., and M. Cole.** 1978. Organic acid metabolism by isolated *Rhizobium japonicum*. *Plant Physiol.* **61**:787–790.
48. **Streeter, J. G.** 1991. Transport and metabolism of carbon and nitrogen in legume nodules. *Adv. Bot. Res.* **18**:129–187.
49. **Yarosh, O. K., T. C. Charles, and T. M. Finan.** 1989. Analysis of C<sub>4</sub>-dicarboxylic acid transport genes in *Rhizobium meliloti*. *Mol. Microbiol.* **3**:813–823.