

# Properties of NAD<sup>-</sup> and NADP<sup>-</sup>-dependent malic enzymes of *Rhizobium (Sinorhizobium) meliloti* and differential expression of their genes in nitrogen-fixing bacteroids

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**The wild-type NAD<sup>-</sup>-dependent malic enzyme (*dme*) gene of *Rhizobium (now Sinorhizobium) meliloti* was cloned and localized to a 3.1 kb region isolated on the cosmid pTH69. This cosmid complemented the symbiotic nitrogen fixation (Fix<sup>-</sup>) phenotype of *R. meliloti dme* mutants. The *dme* gene was mapped by conjugation to between the *cys-11* and *leu-53* markers on the *R. meliloti* chromosome.  $\beta$ -Galactosidase activities measured in bacterial strains carrying either *dme-lacZ* or *tme-lacZ* gene fusions (the *tme* gene encodes NADP<sup>-</sup>-dependent malic enzyme) indicated that the *dme* gene was expressed constitutively in free-living cells and in N<sub>2</sub>-fixing bacteroids whereas expression of the *tme* gene was repressed in bacteroids. The *R. meliloti dme* gene product (DME) was overexpressed in and partially purified from *Escherichia coli*. The properties of this enzyme, together with those of the NADP<sup>-</sup>-dependent malic enzyme (TME) partially purified from *R. meliloti dme* mutants, were determined. Acetyl-CoA inhibited DME but not TME activity. This result supports the hypothesis that DME, together with pyruvate dehydrogenase, forms a pathway in which malate is converted to acetyl-CoA.**

Keywords: *Rhizobium (Sinorhizobium) meliloti*, malic enzyme, malate dehydrogenase, pyruvate

## INTRODUCTION

Rhizobia induce root nodules on leguminous plants. There is much evidence that the nitrogen-fixing bacteria (called bacteroids) within nodules are supplied with C<sub>4</sub>-dicarboxylic acids as their primary energy source. For example, transport (*dct*) mutants from several *Rhizobium* species which fail to transport C<sub>4</sub>-dicarboxylic acid as bacteroids also fail to fix nitrogen (Fix<sup>-</sup> phenotype) (Ronson *et al.*, 1981; Finan *et al.*, 1983; Arwas *et al.*, 1985; Bolton *et al.*, 1986; Engelke *et al.*, 1987; Yarosh *et al.*, 1989; Van Slooten *et al.*, 1992).

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**Abbreviations:** DME, NAD<sup>+</sup>-dependent malic enzyme (EC 1.1.1.39); LDH, lactate dehydrogenase; MDH, malate dehydrogenase; OAA, oxaloacetic acid; PCK, phosphoenolpyruvate carboxykinase; PDH, pyruvate dehydrogenase; PEP, phosphoenolpyruvate; POD, pyruvate orthophosphate dikinase; TCA, tricarboxylic acid; TME, NADP<sup>+</sup>-dependent malic enzyme (EC 1.1.1.40).

Metabolism of C<sub>4</sub>-dicarboxylic acids, such as malate, via the tricarboxylic acid (TCA) cycle (Stoval & Cole, 1978; Streeter, 1991), requires synthesis of equimolar amounts of oxaloacetate (OAA) and acetyl-CoA. OAA is the product of the oxidation of malate via malate dehydrogenase (MDH), and acetyl-CoA is formed from pyruvate via pyruvate dehydrogenase (PDH).

In *Rhizobium (now Sinorhizobium) meliloti* bacteroids the first step in the synthesis of acetyl-CoA from C<sub>4</sub>-dicarboxylic acids appears to take place via the NAD<sup>+</sup>-dependent malic enzyme. Malic enzymes convert malate to pyruvate plus CO<sub>2</sub>, and simultaneously reduce either NAD<sup>+</sup> or NADP<sup>+</sup> to NADH or NADPH, respectively. The alfalfa symbiont, *R. meliloti* produces two distinct malic enzymes. The NAD<sup>+</sup>-dependent malic enzyme, (designated DME for diphosphopyridine-nucleotide-dependent malic enzyme) shows maximal activity with NAD<sup>+</sup> as cofactor and this enzyme has reduced (> 20%) activity when NADP<sup>+</sup> is used as cofactor. *R. meliloti dme* mutants, lacking DME fail to fix nitrogen in alfalfa nodules (Driscoll & Finan, 1993). The second

**Table 1.** Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics*	Source
<b><i>R. meliloti</i></b>		
Rm1021	SU47, <i>str-21</i>	Driscoll & Finan (1993)
Rm5000	SU47, <i>rif-5</i>	Finan <i>et al.</i> (1988)
RmG212	Rm1021, Lac <sup>-</sup>	Jane Glazebrook, MIT, USA
RmG243	Rm1021, <i>pckA3::Tn3HoHo1Sp pod-1</i>	Driscoll & Finan (1993)
RmG450	RmG243, <i>dme-1::Tn5</i>	Driscoll & Finan (1993)
RmG443	RmG243, <i>dme-2::Tn5</i>	Driscoll & Finan (1993)
RmG456	Rm1021, <i>dme-1::Tn5</i>	Driscoll & Finan (1993)
RmG454	Rm1021, <i>dme-2::Tn5</i>	Driscoll & Finan (1993)
RmG492	Rm5000, <i>dme-2::Tn5</i>	Driscoll & Finan (1993)
RmG494	Rm5000, <i>dme-1::Tn5</i>	Driscoll & Finan (1993)
RmG699	Rm1021, $\Omega 5285::Tn5-233$	This study
RmG700	Rm1021, $\Omega 5286::Tn5-233$	This study
RmH182	RmG212 (pTH226)	This study
RmH201	RmG212 (pTH233)	This study
RmH239	RmG212, <i>dme-7::Tn5-B20</i>	This study
RmH240	RmG212 (pTH69)	This study
RmH242	RmG212 (pTH241)	This study
<b><i>E. coli</i></b>		
EJ1321	<i>galK2 pck dme tme</i> , Str <sup>r</sup>	Hansen & Juni (1975)
G312	MT607 $\Omega 5::Tn5-B20$	Driscoll & Finan (1993)
<b>Plasmids</b>		
pLAFR1	Broad-host-range cosmid vector, Tc <sup>r</sup>	Friedman <i>et al.</i> (1982)
pRmT100	pLAFR1 clone, carries the <i>ntrA</i> and <i>tme</i> genes	Finan <i>et al.</i> (1988)
pTH69	pLAFR1 clone, complements <i>dme</i> mutants	This study
pTH226	pTH69, <i>dme-7::Tn5-B20</i>	This study
pTH233	pRmT100, <i>tme-7::Tn5-B20</i>	Driscoll & Finan (1996)
pTH241	pTH69, <i>dme-8::Tn5-B20</i>	This study
pRK7813	Broad-host-range cloning vector, Tc <sup>r</sup>	Jones & Gutterson (1978)
pTH109	pRK7813, 3.1 kb <i>HindIII</i> fragment of pTH69	This study
pTH111	pRK7813, 3.1 kb <i>HindIII</i> fragment of pTH69	This study
pTH113	pRK7813, 8.5 kb <i>EcoRI</i> fragment of pTH69	This study
pTH114	pRK7813, 8.5 kb <i>EcoRI</i> fragment of pTH69	This study
pTH138	pUC119, 3.1 kb <i>HindIII</i> fragment of pTH69	This study
pTH139	pUC119, 3.1 kb <i>HindIII</i> fragment of pTH69	This study

\* Sp, spectinomycin; Str, streptomycin; Rif, rifampin; Tc, tetracycline.

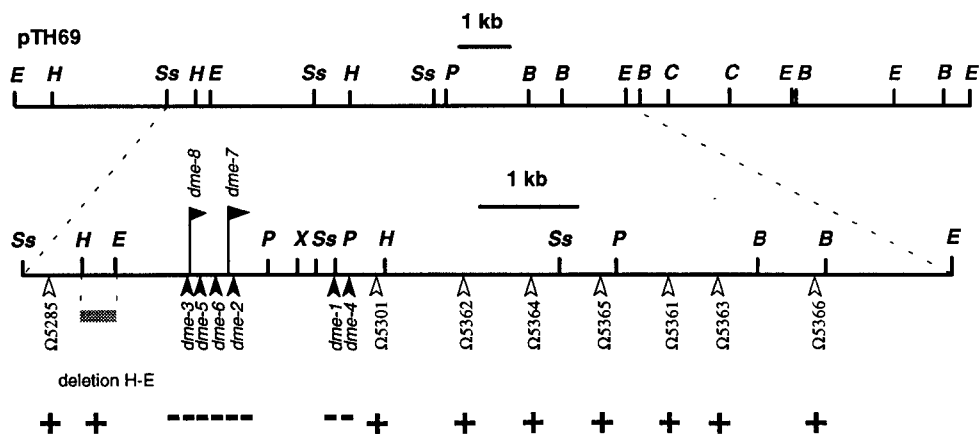
malic enzyme (TME: triphosphopyridine-nucleotide-dependent malic enzyme), only shows activity with NADP<sup>+</sup> as cofactor. This enzyme is not required for symbiotic N<sub>2</sub>-fixation as *tme* mutants induce wild-type N<sub>2</sub>-fixing root nodules (Driscoll & Finan, 1996).

As part of a continuing study of malic enzymes and nodule metabolism we have isolated and characterized the *dme* gene, and have partially characterized the DME and TME enzymes from *R. meliloti*. Our findings, in particular the inhibition of DME, but not TME, by acetyl-CoA, indicate that the end-product of the metabolic pathway to which DME belongs is acetyl-CoA. We therefore propose that DME is required for symbiotic N<sub>2</sub>-fixation because it is part of the metabolic pathway which converts C<sub>4</sub>-dicarboxylic acids to acetyl-CoA in bacteroids.

## METHODS

**Bacterial strains, media and genetic techniques.** *R. meliloti* and *Escherichia coli* strains and plasmids used in this study are listed in Table 1. Bacterial media and culture conditions, plant growth, shoot dry weight determinations and acetylene reduction assays were done as previously described (Yarosh *et al.*, 1989).

Phage  $\phi$ M12 transduction, conjugal mating, Tn5 and Tn5-B20 mutagenesis, and recombination of markers into the *R. meliloti* genome were performed as previously described (Charles & Finan, 1990; Driscoll & Finan, 1996; Finan *et al.*, 1988; Yarosh *et al.*, 1989). The *R. meliloti dme* gene was isolated following transfer of the pLAFR1 library (Friedman *et al.*, 1982) into the *dme pck pod-1* strain RmG450. Complementing plasmids were selected on M9 minimal media containing succinate and tetracycline (2 mg ml<sup>-1</sup>).



**Fig. 1.** Physical map of pTH69 and the *dme* gene region. *H*, *Hind*III; *B*, *Bam*HI; *P*, *Pst*I; *Ss*, *Sst*I; *E*, *Eco*RI; *X*, *Xho*I; *C*, *Cl*aI. Tn5 insertions within the *dme* gene are indicated by black arrowheads, Tn5 insertions outside the *dme* gene are indicated by white arrowheads (insertion  $\Omega$ 5285 is Tn5-233). The *dme*-7::Tn5-B20 and *dme*-8::Tn5-B20 insertions are indicated by black flags which point in the direction of transcription of the promoterless *lacZ* gene. The *dme*-1, *dme*-2 and *dme*-3 Tn5 insertions were mapped by Southern hybridization employing pTH69 as probe. The grey bar represents a deletion of 0.4 kb between the indicated *Hind*III and *Eco*RI sites. The + and – signs indicate insertions which produce Dme<sup>+</sup> and Dme<sup>-</sup> phenotypes, respectively.

To map the *dme* gene we first employed a standard transduction procedure to isolate the Tn5-233 insertions  $\Omega$ 5285 and  $\Omega$ 5286 in strains RmG699 and RmG700 which are 100% and 30% linked, respectively, by co-transduction to *dme* (Charles & Finan, 1990). The Tn5-*mob* insertions  $\Omega$ 601,  $\Omega$ 602,  $\Omega$ 611,  $\Omega$ 612,  $\Omega$ 614,  $\Omega$ 615 and  $\Omega$ 637 (Klein *et al.*, 1992) were transduced into strains RmG699 and RmG700, and the mobilizing plasmid pGMI102 (Finan *et al.*, 1988) was then transferred from *E. coli* into one of each of the above transductants. Each of the resulting strains carrying Tn5-*mob*, Tn5-233 and pGMI102 was mated overnight with Rm5000. The mating mixtures, together with donor and recipient controls, were then plated on LB containing rifampicin, spectinomycin and gentamycin.

Plasmid DNA isolation, transformation, DNA restrictions, agarose gel electrophoresis and Southern hybridizations were done according to standard protocols (Sambrook *et al.*, 1989). In plasmids pTH109, pTH113 and pTH139, the vector *lac* promoter is positioned to the left of the insert fragment (as depicted in Fig. 1). In plasmids pTH111, pTH114 and pTH138, the vector *lac* promoter is positioned to the right of the fragment (as depicted in Fig. 1).

**Biochemical techniques.** DME was isolated from 3 l of *E. coli* EJ1321(pTH139) cells which were grown in LB medium (plus 50 mg ml<sup>-1</sup> ampicillin) at 37 °C. Cells were centrifuged, re-suspended in buffer 1 [20 mM Tris pH 8.4, 1 mM MgCl<sub>2</sub>, 10 mM KCl, 20% (v/v) glycerol, 10 mM  $\beta$ -mercaptoethanol] and disrupted by sonication. Following centrifugation, the protein extracts were concentrated by ultrafiltration using an Amicon stirred cell with a YM30 (M, 30000 exclusion) membrane. The resulting protein solution was then washed by ultrafiltration with several volumes of degassed buffer 1. Fractions containing the DME protein were isolated from this protein solution by DEAE-cellulose column chromatography as previously described (Driscoll & Finan, 1993, 1996). TME was isolated from cell-free extracts of two different *dme* mutant strains. Following DEAE-cellulose chromatography (see Driscoll & Finan, 1993), elution fractions between 230 ml and 285 ml for RmG492 protein and between 195 ml and 250 ml for RmG494 protein were pooled, concentrated and

washed by ultrafiltration with several volumes of buffer 1. Neither lactate dehydrogenase (LDH) nor pyruvate dehydrogenase activities were detected in the DME preparation.

Protein concentrations were determined using the BioRad protein assay dye and BSA (bovine serum albumin) as a standard. Malic enzyme specific activity [nmol min<sup>-1</sup> (mg protein)<sup>-1</sup>] of extracts was determined from the rate of pyruvate formation (Driscoll & Finan, 1993). The assay solution contained 100  $\mu$ mol Tris pH 7.8, 30  $\mu$ mol potassium L-malate, 3  $\mu$ mol MnCl<sub>2</sub>, 50  $\mu$ mol KCl and 0.25  $\mu$ mol NAD(P)<sup>+</sup> in a final volume of 1 ml. For most assays, the malate solution was made by adjusting the pH of the malate (free acid) to 7.8 with KOH (potassium L-malate). To study the effects of monovalent cations (in the absence of excess potassium) the pH of the malate solution was adjusted to 7.8 with NaOH (sodium L-malate). For partially purified DME and TME preparations, malic enzyme activity was determined from the rate of reduction of NAD(P)<sup>+</sup> to NAD(P)H, measured by absorption at 340 nm (Uvikon 930 spectrophotometer). When using NAD<sup>+</sup> reduction, the concentration of NAD<sup>+</sup> used was increased from 0.25 mM to 2.5 mM. Malate dehydrogenase activity was determined by the method of England & Siegal (1969).  $\beta$ -Galactosidase activities (as Miller units or as specific activities [nmol min<sup>-1</sup> (mg protein)<sup>-1</sup>] were determined by the method of Miller (1972), as previously described (Driscoll & Finan, 1996).

SDS-polyacrylamide (10% total acrylamide, 3.3% cross-linked) gel electrophoresis of proteins was done according to standard procedures (Sambrook *et al.*, 1989), using broad-range molecular mass standards (Pharmacia). Enzyme kinetic data were analysed using the Biosoft Fig.P (version 6.0c) program.

## RESULTS

### Isolation of a cosmid clone carrying the wild-type *dme* gene

Mutants of *R. meliloti* which lack the enzyme phosphoenolpyruvate carboxykinase (PCK) grow poorly on minimal medium with succinate as carbon source (Finan

*et al.*, 1988). We have isolated suppressor mutations (e.g. *pod-1*) which allow  $Pck^-$  mutants to grow like the wild-type on succinate minimal medium. The suppressor mutations result in increased pyruvate orthophosphate dikinase (POD) activity, which in turn allows enhanced production of phosphoenolpyruvate (PEP) from pyruvate (M. Østerås, B. T. Driscoll & T. M. Finan, unpublished). Mutant strains which lack  $NAD^+$ -dependent malic enzyme activity (*dme*) grow like the wild-type on succinate (Driscoll & Finan, 1993). However, when *dme* mutations are transferred into strains such as RmG243 that are  $Pck^-$  and carry the *pod-1* mutation, the resulting strains grow poorly on succinate minimal medium.

The *dme* structural gene was isolated by complementation of the succinate-growth phenotype of *dme pckA pod-1* mutants (RmG450 and RmG443) with an *R. meliloti* clone bank. Two classes of complementing plasmids were identified by restriction enzyme analysis. As expected, one class of plasmids carried the *pckA* structural gene (Østerås *et al.*, 1995). Strains carrying the second class of complementing plasmids, such as pTH69 (Fig. 1) showed increased  $NAD^+$ -dependent malic enzyme activity and thus were putatively identified as carrying the *dme* gene.

As *dme* mutants induce root nodules which fail to fix  $N_2$  (Driscoll & Finan, 1993), we tested pTH69 for complementation of this symbiotic phenotype. Plants inoculated with RmG454 and RmG456 transconjugants bearing pTH69 showed acetylene reduction values 66–81% of the wild-type values, whereas the *dme* mutant controls, RmG454 and RmG456, showed values of only 1.2–1.3%, approximately equal to the activity determined for uninoculated root systems. Thus pTH69 restores  $N_2$ -fixing ability to *dme* mutants.

### Genetic characterization of the *dme* gene

The 3.1 kb *Hind*III and 8.5 kb *Eco*RI fragments of pTH69 (Fig. 1) were sub-cloned in both orientations into pRK7813. The resulting plasmids (pTH109, pTH111, pTH113 and pTH114, see Table 1) complemented the growth of *dme pckA pod-1* strains (RmG443 or RmG450) on succinate indicating that the *dme* gene was present. A derivative of pTH111, in which the 0.4 kb sequence between the *Hind*III and *Eco*RI sites had been deleted, retained the ability to complement RmG450, indicating that this region was not required for expression of *dme* (Fig. 1). Plasmids pTH138 and pTH139 were constructed by sub-cloning the 3.1 kb *Hind*III restriction fragment in both orientations into vector pUC119.

Growth of *E. coli* EJ1321 (*dme tme pck*) on succinate was complemented by plasmids pTH109, pTH113 and pTH139, but not by plasmids pTH111, pTH114 and pTH138, carrying the same fragments in the opposite orientation. Moreover, high levels of malic enzyme activity were detected in extracts from *E. coli* EJ1321(pTH139), but not in extracts from EJ1321(pTH138) cells (data not shown). Together, the

*R. meliloti* and *E. coli* data indicate that complementation in *E. coli* EJ1321 is due to *dme* transcription from the vector *lac* promoter, that *dme* transcription is from left to right with respect to Fig. 1, and that the *R. meliloti dme* gene promoter is non-functional in *E. coli*.

Three derivatives carrying Tn5 insertions in the 3.1 kb *Hind*III fragment of pTH113 (*dme-4::Tn5*, *dme-5::Tn5*, and *dme-6::Tn5* see Fig. 1) failed to complement growth of *R. meliloti* RmG450 (*dme pckA pod-1*) on succinate. The *dme::Tn5* insertions were recombined into the *R. meliloti* genome and were then transduced into different *R. meliloti* strains. The transductants had identical phenotypes to strains carrying *dme* mutations (Driscoll & Finan, 1993). Tn5 insertions, such as  $\Omega 5301::Tn5$ , which mapped outside the *dme* gene region, did not affect malic enzyme activity, nor did they affect  $N_2$ -fixation (data not shown).

To localize the *dme* gene on the *R. meliloti* chromosome we mapped the gentamycin and spectinomycin resistance markers of the Tn5-233 insertions  $\Omega 5285$  (see Fig. 1) and  $\Omega 5286$  which co-transduce at 100% and 30%, respectively, with the *dme* gene (see Methods). Two groups of seven rifampicin-sensitive donor strains each carrying one of seven Tn5-*mob* insertions, the mobilizing plasmid, pGMI102, and  $\Omega 5285::Tn5-233$  or  $\Omega 5286::Tn5-233$  were constructed. The Tn5-*mob* strains transfer large regions of the *R. meliloti* chromosome in a polar fashion as in the case of *E. coli* Hfr strains (Finan *et al.*, 1988; Klein *et al.*, 1992). These were then mated with the rifampicin-resistant recipient strain Rm5000 and recombinants were selected on LB medium containing rifampicin, gentamycin and spectinomycin. The highest frequencies of recombinants resulted from the  $\Omega 637::Tn5-mob$  insertion (linked to *cys-11*), followed by  $\Omega 601::Tn5-mob$  (linked to *leu-53*) and lastly  $\Omega 614::Tn5-mob$  (linked to *pyr-49*). From the Tn5-*mob* position and direction of transfer (Klein *et al.*, 1992), we conclude that the *dme* gene mapped to the *R. meliloti* chromosome in the region between *cys-11* and *leu-53*, but closer to *cys-11* than *leu-53*.

### Regulation of *dme* gene expression in free-living cells

Expression of *dme* was examined using transcriptional *lacZ* gene fusions generated with the transposon Tn5-B20 (Simon *et al.*, 1989). Two pTH69::Tn5-B20 derivatives, pTH226 and pTH241, which failed to complement RmG450 for growth on succinate were isolated. Both Tn5-B20 insertions were mapped by restriction analysis to within the *dme* gene (see Fig. 1) such that expression of *dme* would result in *lacZ* expression and hence production of the  $\beta$ -galactosidase protein. The *dme::Tn5-B20* insertions were recombined into the  $Lac^-$  *R. meliloti* strain RmG212 and as expected only low levels of  $NAD^+$ , but normal  $NADP^+$ -dependent malic enzyme activities were detected in cell extracts from these recombinants.

Strains carrying the *dme-7::Tn5-B20* insertion on the chromosome or plasmid (pTH226) showed less than a

**Table 2.** Regulation of *dme* expression in *R. meliloti* under different growth conditions

Cells were grown as described (see Methods) in minimal media with either succinate or glucose as sole carbon source, or in LBmc. Data presented are means  $\pm$  standard error of triplicate measurements of each sample.

Strain*	Relevant characteristics	$\beta$ -Galactosidase specific activity (Miller units)		
		Succinate	Glucose	LBmc
RmG212	Lac <sup>-</sup>	9 $\pm$ 4	14 $\pm$ 3	9 $\pm$ 1
RmH182	pTH69 <i>dme-7::Tn5-B20</i>	861 $\pm$ 4	744 $\pm$ 3	1278 $\pm$ 15
RmH239	<i>dme-7::Tn5-B20</i>	244 $\pm$ 8	230 $\pm$ 3	288 $\pm$ 5
RmH239 O/N	<i>dme-7::Tn5-B20</i>	268 $\pm$ 3	125 $\pm$ 2	207 $\pm$ 3
RmH239 O/2N	<i>dme-7::Tn5-B20</i>	253 $\pm$ 3	144 $\pm$ 3	303 $\pm$ 15

\* O/N, overnight; O/2N, over two nights.

**Table 3.** Expression of the *dme* and *tme* genes in *R. meliloti* bacteroids

Bacteroids were isolated from plants harvested 27 d after inoculation with the bacterial strains. Free-living cells were grown in LBmc with tetracycline (5 mg ml<sup>-1</sup>) to select for plasmids (except for RmG212). Data presented are means  $\pm$  standard error of triplicate measurements.

Strain	Relevant characteristics	$\beta$ -Galactosidase specific activity [nmol min <sup>-1</sup> (mg protein) <sup>-1</sup> ]		
		Free-living cells	Bacteroids	Percentage of free-living cells*
RmG212	Lac <sup>-</sup>	8 $\pm$ 1	6 $\pm$ 1	–
RmH240	Lac <sup>-</sup> (pTH69)	6 $\pm$ 2	ND	–
RmH182	pTH69 <i>dme-7::Tn5-B20</i>	3498 $\pm$ 190	1334 $\pm$ 102	87
RmH201	pRmT100 <i>tme-7::Tn5-B20</i>	2465 $\pm$ 135	215 $\pm$ 7	20

ND, Not determined; –, not applicable.

\* For comparison with free-living cells values for bacteroids were multiplied by a factor of 2.27 (to 3028 for RmH182 and to 488 for RmH201) (see Results).

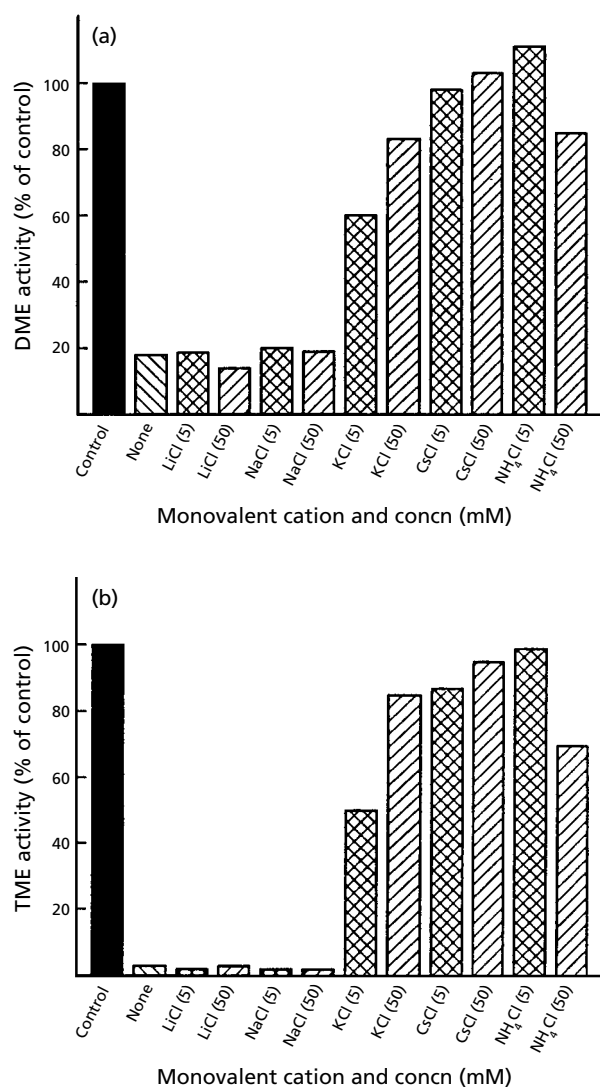
threefold difference in  $\beta$ -galactosidase activity following growth in minimal medium with glucose or succinate as carbon source or in complex LBmc medium [10 g tryptone (Difco), 5 g yeast extract, 5 g NaCl, 4 ml 1 M NaOH, 1 l H<sub>2</sub>O; MgSO<sub>4</sub> and CaCl<sub>2</sub> each added after autoclaving to final concentrations of 2.5 mM] (Table 2). Cells bearing the plasmid fusion (pTH226) showed 3.4 to 4-fold greater activity than cells with chromosomal *dme-7::Tn5-B20* insertions. This is presumably due to the higher copy number of the plasmid-carried fusion. In summary, the level of *dme* gene expression, like that of the *tme* gene (Driscoll & Finan, 1996), showed very little response to differences in growth conditions.

As *pckA* expression in *R. meliloti* was induced in stationary phase (Østerås *et al.*, 1995), we measured  $\beta$ -galactosidase activity in RmH239 (*dme-7::Tn5-B20*) cells after prolonged incubation (Table 2). No increase in activity was detected in cells grown in LBmc or

minimal medium with succinate as sole carbon source, but reduced  $\beta$ -galactosidase activity was observed in cells grown with glucose as sole carbon source. The significance of the latter result is unclear. Our results showing little genetic regulation of *dme* expression are consistent with previous data in which similar levels of malic enzyme activities were detected in cells grown in different media (Driscoll & Finan, 1993).

#### Expression of the *dme* and *tme* genes in bacteroids

The regulation of *dme* and *tme* in nodules was examined by measuring  $\beta$ -galactosidase activities in bacteroids carrying plasmid-borne *tme* and *dme lacZ* gene fusions (Table 3). We used plasmid-borne fusions as *dme* mutants induced nodules which did not fix nitrogen. To compare free-living and bacteroid  $\beta$ -galactosidase activities, it was necessary to correct for plasmid loss which occurs during bacteroid formation. The proportion of bacteroids retaining a typical pLAFR1



**Fig. 2.** Effect of monovalent cations on (a) DME and (b) TME activities. The enzymes were equilibrated in assay mixtures containing 3 mM  $\text{MnCl}_2$ , 30 mM sodium L-malate and the indicated concentrations of monovalent cation chloride salts. Controls were assayed in the standard solution plus 50 mM KCl. Activities were initiated by the addition of coenzyme (2.5 mM  $\text{NAD}^+$  for DME, 0.25 mM  $\text{NADP}^+$  for TME). The results represent percentage of the control values [ $372 \pm 18 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$  for DME and  $63.7 \pm 8.1 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$  for TME], and were determined from the means of triplicate measurements of each sample.

plasmid (pRmT100) was determined to be 44%. Consequently, the bacteroid  $\beta$ -galactosidase specific activity multiplied by a factor of 2.27, was calculated to be 87% of the free-living cell value for the *dme-lacZ* fusion, and 20% for the *tme-lacZ* fusion. Only negligible  $\beta$ -galactosidase activity was detected in extracts of the Lac<sup>-</sup> control strains RmG212 and RmG240 (Table 3). The low level of *tme* gene expression detected in bacteroids is in agreement with our previous finding that most, if not all of the  $\text{NADP}^+$ -dependent malic enzyme activity detected in bacteroid extracts is due to utilization of

$\text{NADP}^+$  by DME (Driscoll & Finan, 1996). In summary, we conclude that the *dme* gene is expressed in bacteroids whereas the *tme* gene is repressed.

### Partial purification of the malic enzymes

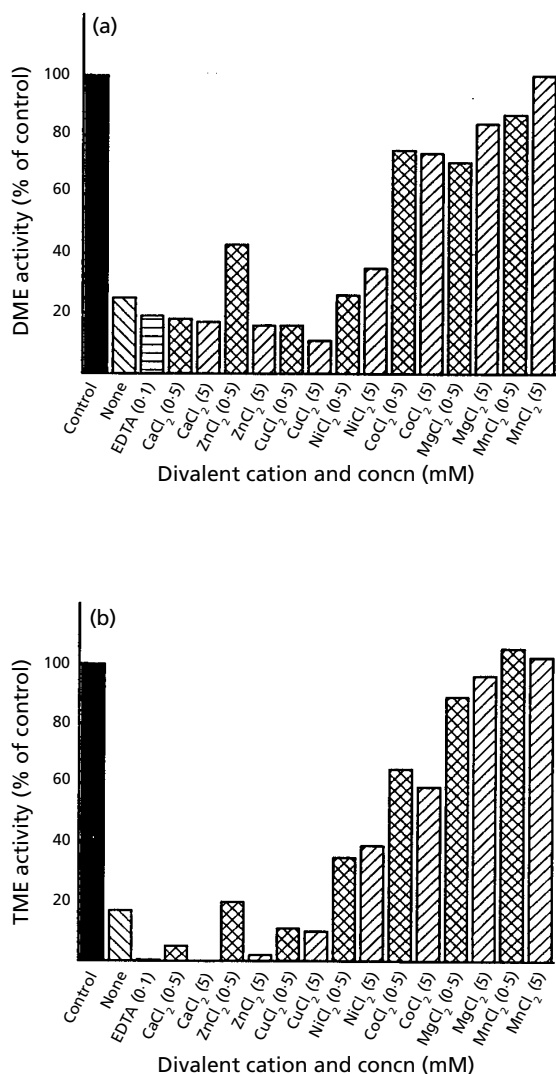
As the malic enzymes were difficult to purify while maintaining enzyme activity, we sought to determine some basic biochemical properties of the enzymes from partially purified preparations. A similar approach was used to study *Bradyrhizobium japonicum* malic enzymes (Copeland *et al.*, 1989). To study the properties of DME, we wished to isolate a DME preparation free of both TME and MDH.

The *R. meliloti* DME protein was overexpressed in *E. coli* EJ1321(pTH139) and separated from *E. coli* MDH by DEAE-cellulose chromatography (Driscoll *et al.*, 1993). In the concentrated DME preparation a 15:1 ratio of DME activity with the coenzymes  $\text{NAD}^+$  (0.25 mM) and  $\text{NADP}^+$  (0.25 mM) was observed. A prominent band of molecular mass 74000 Da, which appeared to be the over-expressed DME, was observed on SDS-polyacrylamide gels loaded with EJ1321(pTH139) extract. This band was not observed in lanes loaded with extracts of *E. coli* EJ1321(pTH138) or EJ1321 (data not shown). *E. coli* DME and TME are reported to have molecular masses of 57500 Da and 67000 Da, respectively (Spina *et al.*, 1968; Yamaguchi *et al.*, 1973).

Preparations of TME which were free of DME were isolated by DEAE-cellulose chromatography of proteins extracted from the *R. meliloti dme* mutant strains RmG492 and RmG494 (Driscoll & Finan, 1993; Driscoll *et al.*, 1993). TME had a strict coenzyme specificity for  $\text{NADP}^+$  as no  $\text{NAD}^+$ -dependent activity was detected in the TME preparations.

### Characterization of the malic enzymes

The optimal pH for both DME and TME was pH 7.8. As bacterial malic enzymes are known to be stimulated by monovalent cations, we measured DME and TME activities with each of five different monovalent cation chloride salts (Fig. 2). At 30 mM potassium L-malate, the addition of 50 mM monovalent cation salts appeared to cause a general, albeit slight, decrease in DME and TME activities indicating that both enzymes were nearly maximally stimulated by the potassium added with the potassium L-malate. Thirty millimolar sodium L-malate was therefore used in assay mixtures to measure enzyme activities in the presence of various monovalent cations. Under these conditions, trace amounts of KCl (20  $\mu\text{M}$  with DME, and 50  $\mu\text{M}$  with TME) were added with the enzyme preparations. The omission of additional monovalent cation reduced DME activity to 18% and TME activity to 3% of the control values (Fig. 2). Both enzymes were stimulated by  $\text{NH}_4^+$ ,  $\text{K}^+$  and  $\text{Cs}^+$ , and neither was stimulated by  $\text{Li}^+$ . These results paralleled findings reported for other bacterial malic enzymes (Brown & Cook, 1985; Chang *et al.*, 1993; Lamed & Zeikus, 1981; Garrido-Pertierra *et al.*, 1983; Kobayashi



**Fig. 3.** Effect of divalent cations on (a) DME and (b) TME activities. The enzyme was equilibrated in assay mixtures containing 30 mM potassium L-malate, 50 mM KCl and the indicated concentrations of divalent cation salts. Controls were assayed in the standard solution plus 3 mM MnCl<sub>2</sub>. Activities were initiated by addition of coenzyme (2.5 mM NAD<sup>+</sup> for DME, 0.25 mM NADP<sup>+</sup> for TME). The results represent percentage of the control values [382 ± 31 nmol min<sup>-1</sup> (mg protein)<sup>-1</sup> for DME and 78.5 ± 9.3 nmol min<sup>-1</sup> (mg protein)<sup>-1</sup> for TME], and were determined from the means of triplicate measurements of each sample.

*et al.*, 1989). Our results were not in agreement with those of Copeland *et al.* (1989), who showed that, while both malic enzymes isolated from *B. japonicum* bacteroids were stimulated by NH<sub>4</sub><sup>+</sup>, only the NAD<sup>+</sup>-dependent malic enzyme was also stimulated by K<sup>+</sup>.

While only bacterial malic enzymes have been reported to be stimulated by monovalent cations, divalent cations are required by plant, animal and bacterial malic enzymes. The effects of seven different divalent cation chloride salts on DME and TME activities were examined (Fig. 3). In these experiments trace amounts of

**Table 4.** Summary of kinetic analyses of TME and DME in *R. meliloti*

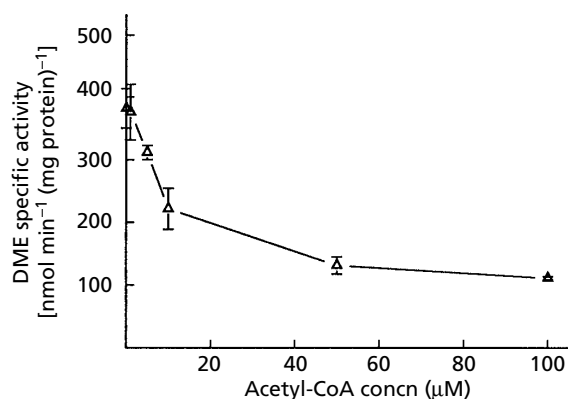
Apparent  $K_m$  and  $V_{max}$  values were calculated by linear regression analysis of the data plotted as  $[S]v^{-1}$  vs  $[S]$ . All enzyme assays were done in triplicate. The concentrations of malate, NAD<sup>+</sup> and NADP<sup>+</sup> used were 0.2–60 mM, 0.0125–5 mM and 1.25–500 μM, respectively.

Enzyme	Varied compound	Apparent $K_m$ (mM)	$V_{max}$ [nmol min <sup>-1</sup> (mg protein) <sup>-1</sup> ]
DME	Malate	8.6	545
DME	NAD <sup>+</sup>	0.142	477
TME	Malate	4.7	78
TME	NADP <sup>+</sup>	0.041	85

MgCl<sub>2</sub> in the enzyme preparation were present in each reaction (2 μM with DME and 5 μM with TME). In the absence of additional divalent cations, DME and TME activities were reduced to 25% and 17%, respectively, of control values determined in the presence of 3 mM MnCl<sub>2</sub>. The addition of 0.1 mM EDTA to these reaction mixtures reduced TME activity to less than 1% of the control and DME to 19% of the control. Addition of 1 mM EDTA did not affect DME and TME activities when 3 mM MnCl<sub>2</sub> was also added to the assays (not shown). DME and TME activities were each highly stimulated by Co<sup>2+</sup>, Mg<sup>2+</sup> and Mn<sup>2+</sup>, whereas both activities were slightly inhibited by Cu<sup>2+</sup>, Ca<sup>2+</sup> and high concentrations of Zn<sup>2+</sup>. The similar responses of both malic enzymes to the different divalent cations is consistent with reports that malic enzymes in general appear to be stimulated by Mn<sup>2+</sup>, Mg<sup>2+</sup> and Co<sup>2+</sup>, but not by Ca<sup>2+</sup> and Cu<sup>2+</sup> (Lamed & Zeikus, 1981; Garrido-Pertierra *et al.*, 1983; Bartolucci *et al.*, 1987; Kobayashi *et al.*, 1989; Drincovich *et al.*, 1991).

We reasoned that the determination of the basic kinetic properties of DME and TME would aid in the elucidation of the distinct roles of the two enzymes in metabolism (Table 4). Thus the apparent  $K_m$  of DME for malate (8.6 mM) was found to be approximately twice that of TME (4.7 mM), while the apparent  $K_m$  of DME for NAD<sup>+</sup> (142 μM) was threefold greater than the apparent  $K_m$  of TME for NADP<sup>+</sup> (41 μM).

We hypothesized that if DME and TME activities showed different patterns of regulation in response to metabolic effectors, the distinct roles of these enzymes in cellular metabolism could be more readily determined. The compounds tested for allosteric regulation of DME and TME activities were: acetyl-CoA (1, 10 and 100 μM), 0.25 mM NADH, 0.25 mM NADPH, 1 mM aspartate, 1 mM CoA, 50 mM NaHCO<sub>3</sub>, 1 mM pyridoxal 5'-phosphate, 1 mM AMP, 1 mM ADP and 1 mM ATP (data not shown). Of these compounds, acetyl-CoA produced the most dramatic effect; 100 μM acetyl-CoA reduced DME activity by 68%. Although NADH



**Fig. 4.** Effect of varied acetyl-CoA concentrations on DME activity. DME activity was initiated by the addition of enzyme (0.014 mg total protein) to the complete malic enzyme assay mixture containing the indicated concentration of acetyl-CoA. DME specific activity was determined by the rate of reduction of NAD<sup>+</sup>. Error bars represent the standard error of the mean of triplicate measurements.

also appeared to slightly inhibit DME, the rest of the compounds tested were found to have no significant effects. None of the compounds tested appeared to greatly affect TME activity, with the possible exception of NaCO<sub>3</sub> (50 mM), which reduced TME activity by 27%. Although acetyl-CoA clearly inhibited DME, even 100 μM acetyl-CoA had no effect on TME activity.

#### Inhibition of DME activity by acetyl-CoA

Inhibition of DME activity was analysed by initiating complete malic enzyme assays, containing 1, 5, 10, 50 or 100 μM acetyl-CoA, with a fixed amount of enzyme (Fig. 4). Acetyl-CoA concentrations of 5 and 10 μM reduced DME activity by 18% and 42%, respectively. Maximal inhibition was observed at concentrations between 50 μM and 100 μM. In additional experiments, we measured DME activity at varying malate concentrations in assay mixtures containing 0, 10 or 50 μM acetyl-CoA. Analysis of the resulting data using double reciprocal plots did not allow us to determine the type of inhibition, suggesting that the response of DME to acetyl-CoA is complex.

#### DISCUSSION

*R. meliloti* has two malic enzymes: DME, which shows maximal activity with NAD<sup>+</sup> as co-factor and TME, which employs only NADP<sup>+</sup> as co-factor. We have previously reported the cloning and regulation of the *tme* gene (Driscoll & Finan, 1996). Here, we have cloned and localized the *dme* gene to a fragment of 3.1 kb and used this fragment to overproduce the DME protein in *E. coli*. To aid in defining the metabolic roles of the two malic enzymes, we characterized partially purified preparations of the overproduced DME and partially purified TME preparations isolated from *R. meliloti dme* mutants. The enzymes had many similarities,

including pH optima, sensitivity to various metabolic effectors and activities in the presence of various monovalent and divalent cations. The  $K_m$  of DME for malate (8.6 mM) was slightly higher than that of TME (4.7 mM).

The most striking difference between the two proteins lay in their differential sensitivity to acetyl-CoA, which strongly inhibited DME activity without having an observable effect on TME. Results from kinetic experiments did not allow us to define the type of acetyl-CoA inhibition of DME; however, inhibition was clearly evident at acetyl-CoA concentrations as low as 10 μM. We and others (McKay *et al.*, 1988; Kouchi *et al.*, 1988; Copeland *et al.*, 1989) have hypothesized that malic enzyme, with PDH, is important for the conversion of malate to acetyl-CoA in N<sub>2</sub>-fixing bacteroids. A pathway for the conversion of C<sub>4</sub>-dicarboxylates to acetyl-CoA is necessary if the TCA cycle is to operate in cells, such as bacteroids, which are supplied with C<sub>4</sub>-dicarboxylic acids as sole energy source. Thus the acetyl-CoA inhibition of DME, together with the Fix<sup>-</sup> phenotype of the *R. meliloti dme* mutants (Driscoll & Finan, 1993), supports the hypothesis that acetyl-CoA is the end-product of a metabolic pathway to which DME belongs.

Although the *R. meliloti* DME appears to have a catabolic role in bacteroids, its role, and the role of TME, in free-living cells is less clear. TME may be primarily an anapleurotic enzyme, however, Sanwal (1970) hypothesized that, in *E. coli*, it may also be involved in the biosynthesis of amino acids and/or lipids from TCA cycle intermediates. Our data suggest that in *R. meliloti* TME would be active in circumstances where acetyl-CoA and presumably TCA cycle intermediates which are precursors in many biosynthetic pathways accumulate. As NADP<sup>+</sup>-dependent malic enzymes have been partially purified from *B. japonicum* bacteroids (Copeland *et al.*, 1989; Kimura & Tajima, 1989), this activity may be important in these cells. *B. japonicum* bacteroids store poly-β-hydroxybutyrate, while *R. meliloti* bacteroids do not (McRae *et al.*, 1989). In *B. japonicum* bacteroids, therefore, NADP<sup>+</sup>-dependent malic enzyme activity may be involved in a pathway (with PDH) that converts malate to acetyl-CoA, which can then be converted to poly-β-hydroxybutyrate. The NADPH generated via this pathway may also be important for biosynthetic processes within these cells.

The location of *dme* on the chromosome is far from the previously mapped position of *tme* (Driscoll & Finan, 1996). Many symbiosis-specific genes map to the *nod-nif* megaplasmid in *R. meliloti*. While *dme* is essential for symbiotic nitrogen fixation (Driscoll & Finan, 1993), it does not appear to be symbiosis-specific; it is expressed in free-living cells, and in addition NAD<sup>+</sup>-dependent malic enzymes are found in many other organisms.

We observed little variation in *dme* expression in free-living cells cultured under different growth conditions (Table 2). These data parallel the levels of DME enzyme activity detected in crude extracts of cells grown under

similar conditions (Driscoll & Finan, 1993). Our results do not show the same degree of regulation as observed for *E. coli* where NAD<sup>+</sup>-dependent malic enzyme activity is repressed by glucose and induced by malate (Murai *et al.*, 1971).

Our results strongly suggest that *tme* is repressed in bacteroids (Table 3). This supports previous data indicating that most, if not all, of the TME activity detected in bacteroids was due to the utilization of NADP<sup>+</sup> by DME *in vitro* (Driscoll & Finan, 1996). In contrast to *tme*, we estimated that the level of *dme* expression in bacteroids is similar to that found in free-living cells (Table 3). In view of these results, it will be interesting to identify and compare the *tme* and *dme* promoters, and further to determine whether the same *dme* promoter is used in free-living cells and bacteroids.

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## REFERENCES

- Arwas, R., McKay, I. A., Rowney, F. R. P., Dilworth, M. J. & Glenn, A. R. (1985). Properties of organic acid utilization mutants of *Rhizobium leguminosarum* strain 300. *J Gen Microbiol* **131**, 2059–2066.
- Bartolucci, S., Rella, R., Guagliardi, A., Raia, C. A., Gambacorta, A., De Rosa, M. & Rossi, M. (1987). Malic enzyme from archaeobacterium *Sulfolobus solfataricus*: purification, structure and kinetic properties. *J Biol Chem* **262**, 7725–7731.
- Bolton, E., Higginson, B., Harrington, A. & O'Gara, F. (1986). Dicarboxylic acid transport in *Rhizobium meliloti*: isolation of mutants and cloning of dicarboxylic acid transport genes. *Arch Microbiol* **144**, 142–146.
- Brown, D. A. & Cook, R. A. (1985). Regulatory effects of potassium and inorganic anions on the NADP-specific malic enzyme of *Escherichia coli*. *Can J Biochem Cell Biol* **63**, 128–136.
- Chang, G.-G., Satterlee, J. & Hsu, R. Y. (1993). Essential sulphydryl group of malic enzyme from *Escherichia coli*. *J Protein Chem* **12**, 7–10.
- Charles, T. C. & Finan, T. M. (1990). Genetic map of *Rhizobium meliloti* megaplasmid pRmeSU47b. *J Bacteriol* **172**, 2469–2476.
- Copeland, L., Quinell, R. G. & Day, D. A. (1989). Malic enzyme activity in bacteroids from soybean nodules. *J Gen Microbiol* **135**, 2005–2011.
- Drincovich, M. F., Iglesias, A. A. & Andreo, C. S. (1991). Interaction of divalent metal cations with the NADP<sup>+</sup>-malic enzyme from maize leaves. *Physiol Plant* **81**, 462–466.
- Driscoll, B. T. & Finan, T. M. (1993). NAD<sup>+</sup>-dependent malic enzyme of *Rhizobium meliloti* is required for symbiotic nitrogen fixation. *Mol Microbiol* **7**, 865–873.
- Driscoll, B. T. & Finan, T. M. (1996). NADP<sup>+</sup> malic enzyme of *Rhizobium meliloti*. *J Bacteriol* **178**, 2224–2231.
- Driscoll, B. T., Østerås, M. & Finan, T. M. (1993). Succinate metabolism in *Rhizobium meliloti*. In *New Horizons in Nitrogen Fixation*, pp. 517–522. Edited by R. Palacios, J. Mora & W. Newton. Boston: Kluwer.
- Engelke, T. H., Jagadish, M. N. & Pühler, A. (1987). Biochemical and genetical analysis of *Rhizobium meliloti* mutants defective in C<sub>4</sub>-dicarboxylate transport. *J Gen Microbiol* **133**, 3019–3029.
- Englard, S. & Siegal, L. (1969). Mitochondrial L-malate dehydrogenase of beef heart. *Methods Enzymol* **13**, 99–100.
- Finan, T. M., Wood, J. M. & Jordan, D. C. (1983). Symbiotic properties of C<sub>4</sub>-dicarboxylate acid transport mutants of *Rhizobium leguminosarum*. *J Bacteriol* **154**, 1403–1413.
- Finan, T. M., Oresnik, I. & Bottacin, A. (1988). Mutants of *Rhizobium meliloti* defective in succinate metabolism. *J Bacteriol* **170**, 3396–3403.
- Friedman, A. M., Long, S. R., Brown, S. E., Buikema, W. J. & Ausubel, F. M. (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., Martínez Marcos, C., Martín Fernández, M. & Ruiz-Amil, M. (1983). Properties and function of malate enzyme from *Pseudomonas putida*. *Biochimie* **65**, 629–635.
- Hansen, E. J. & Juni, E. (1975). Isolation of mutants of *Escherichia coli* lacking NAD- and NADP-linked malic enzyme activities. *Biochem Biophys Res Commun* **65**, 559–566.
- Jones, J. D. J. & Guttererson, N. (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.
- Kimura, I. & Tajima, S. (1989). Presence and characteristics of NADP-malic enzyme in soybean nodules. *Soil Sci Plant Nutr* **35**, 271–279.
- Klein, S., Lohman, K., Clover, R., Walker, G. C. & Signer, E. R. (1992). A directional, high frequency chromosomal localization system for genetic mapping in *Rhizobium meliloti*. *J Bacteriol* **174**, 324–326.
- Kobayashi, K., Doi, S., Negoro, S., Urabe, I. & Okada, H. (1989). Structure and properties of malic enzyme from *Bacillus stearo-thermophilus*. *J Biol Chem* **264**, 3200–3205.
- Kouchi, H., Fukai, K., Katagiri, H., Minamisawa, K. & Tajima, S. (1988). Isolation and enzymological characterization of infected and uninfected cell protoplasts from root nodules of *Glycine max*. *Physiol Plant* **73**, 327–334.
- Lamed, R. & Zeikus, J. G. (1981). Thermostable, ammonium-activated malic enzyme of *Clostridium thermocellum*. *Biochim Biophys Acta* **660**, 251–255.
- McKay, I. A., Dilworth, M. J. & Glenn, A. R. (1988). C<sub>4</sub>-dicarboxylate metabolism in free-living and bacteroid forms of *Rhizobium leguminosarum* MNF3841. *J Gen Microbiol* **134**, 1433–1440.
- McRae, D. G., Miller, R. W. & Berndt, W. B. (1989). Viability of alfalfa nodule bacteroids isolated by density gradient centrifugation. *Symbiosis* **7**, 67–80.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Murai, T., Tokushige, M., Nagai, J. & Katsuki, H. (1971). Physiological functions of NAD- and NADP-linked malic enzymes in *Escherichia coli*. *Biochem Biophys Res Commun* **43**, 875–881.

- Østerås, M., Driscoll, B. T. & Finan, T. M. (1995).** Molecular and expression analysis of the *Rhizobium meliloti* phosphoenolpyruvate carboxykinase (*pckA*) gene. *J Bacteriol* **177**, 1452–1460.
- Ronson, C. W., Lyttleton, P. & Robertson, J. G. (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.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanwal, B. D. (1970).** Allosteric controls of amphibolic pathways in bacteria. *Bacteriol Rev* **34**, 20–39.
- Simon, R., Quandt, J. & Klipp, W. (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.
- Spina, J., Jr, Bright, H. J. & Rosenbloom, J. (1968).** Purification and properties of L-malic enzyme from *Escherichia coli*. *Biochemistry* **9**, 3794–3801.
- Stoval, I. & Cole, M. (1978).** Organic acid metabolism by isolated *Rhizobium japonicum* bacteroids. *Plant Physiol* **61**, 787–790.
- Streeter, J. G. (1991).** Transport and metabolism of carbon and nitrogen in legume nodules. *Adv Bot Res* **18**, 129–187.
- Van Slooten, J. C., Bhuvanavari, T. V., Bardin, S. & Stanley, J. (1992).** Two C<sub>4</sub>-dicarboxylate transport systems in *Rhizobium* sp. NGR234: rhizobial dicarboxylate transport is essential for nitrogen fixation in tropical legume symbiosis. *Mol Plant-Microbe Interact* **5**, 179–186.
- Yamaguchi, M., Tokushige, M. & Katsuki, H. (1973).** Studies on regulatory functions of malic enzymes. II. Purification and molecular properties of nicotinamide adenine dinucleotide-linked malic enzyme from *Escherichia coli*. *J Biochem* **73**, 169–180.
- Yarosh, O. K., Charles, T. C. & Finan, T. M. (1989).** Analysis of C<sub>4</sub>-dicarboxylic acid transport genes in *Rhizobium meliloti*. *Mol Microbiol* **3**, 813–823.
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