ROLES OF MALIC ENZYMES OF R. MELILOTI IN SYMBIOSIS

THE ROLES OF THE MALIC ENZYMES OF *RHIZOBIUM* (*SINORHIZOBIUM*)

MELILOTI IN SYMBIOTIC NITROGEN FIXATION

By

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ABSTRACT

The genome of *R. meliloti* contains two genes for malic enzymes. One uses NAD$^+$ as a cofactor (*dme*) and one utilizes NADP$^+$ (*tme*). The two enzymes have been purified and the genes cloned and sequenced. Loss of TME enzyme function gives no detectable phenotype in either *R. meliloti* grown in culture or in bacteroids. Loss of DME function gives no detectable phenotype in *R. meliloti* grown in culture but does result in bacteroids that are unable to fix nitrogen (Fix⁻). Expression of *tme* is reduced in bacteroids whereas *dme* expression remains unchanged.

In order to overexpress *tme* in bacteroids a fusion gene was constructed with the *dme* promoter driving expression of the *tme* structural gene (*dtme*). The *dtme* gene was expressed and functional in *R. meliloti* cells grown in culture, but alfalfa plants inoculated with strains expressing only the *dtme* gene were Fix⁻. In addition the NAD$^+$-dependent malic enzyme gene from *Streptococcus bovis* (*maeE*) was similarly cloned downstream of the *dme* promoter. The fusion gene (*dmaeE*) was expressed in *R. meliloti* cells grown in culture, surprisingly plants inoculated with strains expressing only the *dmaeE* gene showed a Fix⁻ phenotype. A truncated *dme* gene was constructed which contained only the N-terminal, malic enzyme domain of the protein (*dmeΔPst*). The truncated enzyme was expressed and active in *R. meliloti* cells grown in culture and gave a Fix$^+$ phenotype when inoculated onto alfalfa plants.

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ABBREVIATIONS

DME	diphosphopyridine nucleotide dependent malic enzyme.
TME	triphosphopyridine nucleotide dependent malic enzyme.
NAD^+	nicotinamide adenine dinucleotide.
NADH	nicotinamide adenine dinucleotide reduced form.
$NADP^+$	nicotinamide adenine dinucleotide phosphate.
NADPH	nicotinamide adenine dinucleotide phosphate reduced form.
Gm	gentamycin.
Sp	spectinomycin.
Nm	neomycin.
DMSO	dimethyl sulfoxide.
DEPC	diethyl pyrocarbonate.
ECL	enhanced chemiluminescence.
PCR	polymerase chain reaction.
ATP	adenosine triphosphate.
dNTPs	deoxyribonucleotide triphosphates.
rRNA	ribosomal RNA.
PAGE	polyacrylamide gel electrophoresis.
TBE	Tris-Borate-EDTA buffer.
MOPS	3-[N-Morpholino] propane sulfonic acid
HRP	horseradish peroxidase

CHAPTER 1

Introduction

Rhizobiaceae and nodulation.

Rhizobium (Sinorhizobium) meliloti is a Gram negative bacterium found in soil. It is classified as a fast-growing Rhizobium species based on generation time, compared to the Bradyrhizobium species which are slow growing. In addition Rhizobium species produce acid in mineral salts-mannitol medium whereas the Bradyrhizobium species produce an alkaline reaction in the same medium. The Rhizobiaceae form nitrogen-fixing nodules on the roots of legumes. For most Rhizobium species this interaction is specific for only certain legumes, for example Rhizobium meliloti will form nodules on alfalfa (Medicago), sweet clover (Melilotus) and Trigonella spp. Recognition of the plant host and nodule formation is a multi-step process involving both the plant and the bacterium. The bacteria are attracted to the rhizosphere by flavonoids and possibly also amino acids and dicarboxylic acids secreted by the plant roots (reviewed in van Rhijn and Vanderleyden, 1995). Once in close proximity to the roots the bacteria become attached to the root hairs and induce a series of morphological changes which include root hair branching, deformation and curling. These changes are brought about by factors produced by the rhizobia called Nod factors, which are expressed in response to the flavonoids produced by the plant (see van Rhijn and Vanderleyden, 1995 and references

therein). The root hairs curl around and trap the rhizobia, an indentation is produced in the cell wall of the root cells and the bacteria enter the plant cell by formation of a tube-like structure known as the infection thread. The infection thread is surrounded by plant cell wall and as the bacteria divide it elongates into the root tissue. Concomitantly the cortical cells of the root begin to divide forming the nodule primordium towards which the infection thread grows. Rhizobia are released from the infection thread into the plant cells, but remain surrounded by a peribacteroid membrane (derived from the plant cell) and are now termed "bacteroids". Cell proliferation ceases in the bacteroid state and the genes required for nitrogen fixation, such as the *nif* genes which encode the nitrogenase enzyme and other components for nitrogen fixation and the *fix* genes, which form components that are specific for symbiotic nitrogen fixing bacteria, are expressed (reviewed by Long, 1989).

Different legumes produce different types of nodules. *Rhizobium meliloti* on alfalfa, for example, produces elongated indeterminate nodules. The apical meristem is maintained as the infection thread grows allowing the nodule to continue to elongate. The central portion of the nodule contains the differentiated bacteroids actively fixing nitrogen, whilst the base of the nodule contains a zone of senescence. Other legumes produce determinate nodules which do not continue to grow once the rhizobia have differentiated and are fixing nitrogen. The type of nodule produced is dependent on the plant host and is not a function of the infecting rhizobia (reviewed by van Rhijn and Vanderleyden, 1995).

Most evidence suggests that C_4-dicarboxylic acids, produced from

photosynthate, are supplied to the bacteroids by the plant to generate the energy required to fix atmospheric nitrogen. The nitrogenase reaction is thought to produce ammonia, which is then passed back to the plant host. The differentiated bacteroids actively take up C_4-dicarboxylic acids via the Dct (dicarboxylic acid transport) system. The importance of the specific transport of dicarboxylic acids is known because null mutants, whilst still able to produce infected nodules, are no longer able to fix nitrogen (Ronson et al, 1981; Finan et al, 1983; Yarosh et al,1989). In addition malate and succinate are found at high levels in root nodules and are thought to be the main carbon source supplied to the bacteroids (reviewed by Vance and Heichel, 1991). These compounds are presumed to be metabolized via the enzymes of the tricarboxylic acid cycle (TCA cycle), which are known to be present in the bacteroids, to generate the energy and possibly the reducing power required for the nitrogenase reactions (McKay et al, 1988).

To maintain the TCA cycle when malate and succinate are the sole carbon sources it is necessary to provide a source of acetyl CoA for the synthesis of citrate from oxaloacetic acid (see Figure 1). This is thought to occur in bacteroids by the action of malic enzymes, which catalyze the conversion of malate to pyruvate and CO_2 with the concomitant reduction of a nicotinamide cofactor. Together with pyruvate dehydrogenase this will produce acetyl CoA for maintenance of the TCA cycle (Figure 1). In addition there is some evidence from B. japonicum that reduced nitrogen is transported to the plant in the form of alanine (Waters et al, 1998), the synthesis of which would require pyruvate (see Figure 1). This may be an indication of another important role for malic enzymes in bacteroids.

Figure 1: TCA cycle and related pathways

glucose ←→ G-3-P $\overset{GAP}{\longleftrightarrow}$ 1,3-P-G $\overset{PGK}{\longleftrightarrow}$ 3-P-G $\overset{PGM}{\longleftrightarrow}$ 2-P-G

alanine ← [ADH] ← NH₃ ← pyruvate $\underset{PYK}{\overset{POD}{\rightleftharpoons}}$ PEP

acetyl CoA (PDH)

DME / TME

malate ←[MDH]→ OAA ← [CIT] → citrate

FUM
fumarate
SDH
succinate

cis-aconitate

isocitrate

succinyl CoA ←→ αketoglutarate

PCK

ENO
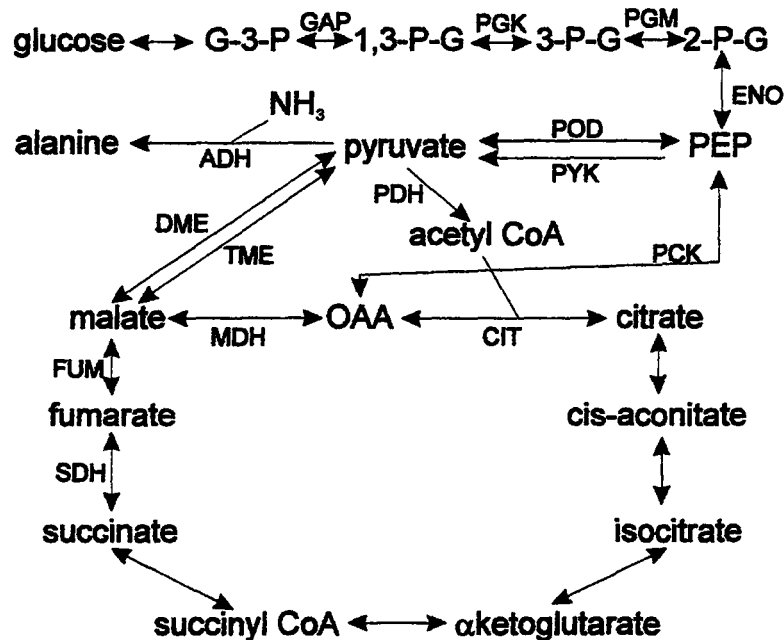
Enzyme designations: DME, NAD⁺- dependent malic enzyme; TME, NADP⁺-dependent malic enzyme; PCK, phosphoenolpyruvate carboxykinase; POD, pyruvate orthophosphate dikinase; PYK, pyruvate kinase; ENO, enolase; PGM, phosphoglycerate mutase; PGK, 3-phosphoglycerate kinase; GAP, glyceraldehyde -3-phosphate dehydrogenase; PDH, pyruvate dehydrogenase; ADH, alanine dehydrogenase; CIT, citrate synthase; SDH, succinate dehydrogenase; FUM, fumarase; MDH, malate dehydrogenase.

Abbreviations: OAA, oxaloacetic acid; PEP, phosphoenolpyruvate; 2-P-G, 2-phospho-glycerate; 3-P-G, 3-phosphoglycerate; 1,3 dP-G, 1,3 diphosphoglycerate, G-3-P, glyceraldehyde-3-phosphate.

Malic Enzymes

Malic enzyme activity was first identified in extracts of pigeon liver (Ochoa et al, 1948). Since then malic enzymes have been purified, cloned and extensively studied from a wide variety of eukaryotic organisms including plants but to a lesser extent from prokaryotic organisms. Malic enzymes have been classified by the International Union of Biochemistry into three groups based on cofactor requirement and their ability to decarboxylate oxaloacetic acid (OAA). Group EC1.1.1.38 uses NAD^+ as cofactor and can decarboxylate OAA; EC1.1.1.39 uses NAD^+ preferentially but can also utilize $NADP^+$ and cannot decarboxylate OAA; the third group, EC1.1.1.40, uses $NADP^+$ as cofactor and will also decarboxylate OAA (reviewed by Wedding, 1989). In addition, malic enzymes from eukaryotic organisms are further characterized based on localization within the cell. An $NADP^+$-dependent isoform of malic enzyme is strictly cytosolic and is thought to be involved in providing reducing power for fatty acid synthesis, another $NADP^+$-dependent isoform is mitochondrial and may be involved in the cycling of NADPH into the mitochondrion and a third isoform is also mitochondrial in origin but uses NAD^+ as cofactor and is found predominantly in cells with high rates of cell division, most notably tumour cells where high levels of expression are linked to progression of the tumour (see Wedding, 1989, and references therein). Most eukaryotic malic enzymes consist of multimers of a single subunit, usually in the 50kD - 60kD size range, but some plant malic enzymes appear to consist of two different subunits (Winning et al, 1994). All show regions of homology characteristic of malic enzymes such as cofactor binding domains, malate binding domain, divalent cation domain and other

regions of homology that have not been ascribed function.

Prokaryotic malic enzymes

Malic enzymes in prokaryotes, with the notable exception of E. coli, have been less well studied. Two malic enzyme activities have been identified in E. coli, one of which utilizes NAD^+ as cofactor and another that requires $NADP^+$ as cofactor (Katsuki et al, 1967). The NAD^+-dependent malic enzyme was further characterized as belonging to the EC1.1.1.38 class (Yamaguchi et al, 1973) and the $NADP^+$-dependent enzyme as EC1.1.1.40 (Sanwal and Smando, 1969). In addition the $NADP^+$-dependent malic enzyme is allosterically inhibited by acetyl CoA and oxaloacetic acid (Sanwal et al, 1968), whereas the NAD^+-dependent malic enzyme is not. However the NAD^+-dependent malic enzyme is inhibited by CoA and activated by aspartate (Sanwal, 1970). Molecular weight determinations of the two enzymes from E. coli indicate that the NAD^+-dependent enzyme is a tetramer of 55kD subunits, a size similar to the eukaryotic malic enzymes (Yamaguchi et al, 1973), whereas the $NADP^+$-dependent enzyme is a hexamer of ~80kD subunits (Iwakura et al, 1979). Sequencing of the complete E. coli genome has subsequently confirmed the presence of two malic enzyme genes (Blattner et al, 1997). One is the previously identified sfcA gene which, based on the predicted size of the protein, corresponds to the NAD^+-dependent malic enzyme (Mahajan et al, 1990; Stohls and Donnelly, 1997), the other is an uncharacterized open reading frame designated f759 which, again based on the predicted size of the protein, corresponds to the $NADP^+$-dependent malic enzyme. Malic enzymes have also

been reported to be present in other bacterial species such as *Bacillus stearothermophilus* which has an NAD⁺-dependent enzyme, EC1.1.1.38 (Kobayashi et al, 1989), *Clostridium thermocellum* (Lamed and Zeikus, 1981), *Pseudomonas putida* which has an NADP⁺-dependent malic enzyme, EC1.1.1.40 (Garrido-Pertierra et al, 1983), *Pseudomonas diminuta* which has an NAD⁺-dependent malic enzyme, EC1.1.1.39 (Suye et al, 1992) and the archaebacterium *Sulfolobus solfataricus* which also has an EC1.1.1.40 NADP⁺-dependent malic enzyme (Bartolucci et al, 1987). In addition an NAD⁺-dependent malic enzyme (EC1.1.1.39) has recently been cloned and characterized from the Gram positive bacterium *Streptococcus bovis* (Kawai et al, 1996). The *S. bovis* enzyme (*maeE*) is similar in size to the enzymes reported for *B. stereothermophilus*, *C. thermocellum* and *Solfolobus* having a subunit size of only 40kD and the holoenzyme exists as a dimer as determined by native gel electrophoresis (Kawai et al, 1996). In addition the *S. bovis* enzyme exhibits malate decarboxylating activity with both NAD⁺ and NADP⁺ as cofactor but with a 10 fold difference in affinity (K_m for NAD⁺ = 0.083mM, for NADP⁺ = 0.7mM; Kawai et al, 1996).

Recent genome sequencing projects have revealed the presence of genes corresponding to malic enzymes in *B. subtilis* (Kunst et al, 1997), *Synecocystis* (Kaneko et al, 1996), *Rickettsia prowazekii* (S. Andersson, personal communication) and *H. influenza* (Fleishman et al,1995). Although there is no data on the functioning of these genes the predicted malic enzymes of *R. prowazekii* and *H. influenza* are of particular interest as they show an especially high degree of

homology to the malic enzyme genes of R. meliloti (Mitsch et al, 1998).

Malic enzymes in Rhizobium

In soybean nodules produced by Bradyrhizobium japonicum two malic enzyme activities of bacteroid origin have been identified, an NAD^+-dependent and an $NADP^+$-dependent activity (Kouchi et al, 1988; Copeland et al, 1988). Both enzymes have been partially purified from bacteroids to determine kinetic parameters and it was found that the K_m of the NAD^+-dependent malic enzyme for malate was 1.9mM whereas as that of the $NADP^+$-dependent enzyme for malate was 0.1mM (Copeland et al, 1988). Purification of the $NADP^+$-dependent malic enzyme from Bradyrhizobium japonicum in culture revealed a protein of 77.6kD which exists as either a dimer or a tetramer depending on pH (Chen et al, 1997). This enzyme is also inhibited by acetyl CoA and OAA as is the $NADP^+$-dependent enzyme from E. coli (Chen et al, 1997). However, neither gene has been cloned or mutagenized so there is no genetic data as to the possible physiological roles of the two enzymes in either free living B. japonicum or bacteroids.

Two malic enzyme activities have also been shown to be present in Rhizobium leguminosarum bacteroids isolated from pea nodules (McKay et al, 1988). Again, one is an NAD^+-dependent activity and the other is $NADP^+$ dependent, but there is no genetic evidence for the possible roles of two malic enzymes in either the free living or bacteroid state for R. leguminosarum.

Driscoll and Finan (1993) showed that R. meliloti in culture contains two

malic enzyme activities; one NAD$^+$-dependent the other NADP$^+$- dependent, which could be separated by ion exchange chromatography of cell extracts. Using an *R. meliloti* strain in which the *pck* gene was disrupted to block gluconeogenesis via OAA and PEP (see Figure 1) but which carried a second site mutation which restored growth on succinate it was possible to isolate transposon insertion mutants which lacked malic enzyme activity. Interestingly only mutations within the NAD$^+$-dependent malic enzyme gene (designated *dme* for diphosphopyridine nucleotide dependent malic enzyme) were obtained. But consistent with the proposed role of malic enzymes in the metabolism of dicarboxylic acids in bacteroids, plants infected with Dme$^-$ *R. meliloti* produced nodules that contained bacteroids that were Fix$^-$ (Driscoll and Finan, 1993). Subsequently the genes encoding both malic enzymes were isolated from *R. meliloti* but when insertion mutants of the NADP$^+$-dependent enzyme (*tme*: triphosphopyridine nucleotide dependent malic enzyme) were made it was found that the resulting Tme$^-$ strains still produced Fix$^+$ nodules on alfalfa (Driscoll and Finan, 1996; Driscoll and Finan, 1997).

dme and *tme* genes of *R. meliloti*

The genes for the TME and DME proteins have been sequenced and reveal an unusual feature in the primary structure of the predicted polypeptides. Both proteins have a predicted molecular weight of ~80kD, significantly larger than eukaryotic malic enzymes and many prokaryotic malic enzymes, but similar to that

of the *E. coli* NADP⁺-dependent malic enzyme (Iwakura et al, 1979) and the *B. japonicum* NADP⁺-dependent malic enzyme (Chen et al, 1997). Sequence analysis shows that only the NH_2-terminal 450 amino acids of both DME and TME are homologous to other malic enzymes, the remaining 320 amino acids show limited homology (20 - 30% identity) to phosphotransacetylase (PTA) enzymes (Mitsch et al, 1998). With the advent of complete genome sequences putative malic enzymes in *Haemophilus influenza*, *Rickettsia* and the f759 open reading frame of *E. coli* also show this similar chimeric structure (Mitsch et al, 1998). The malic enzyme regions of these chimeric proteins show a high degree of homology (60 - 70% identity) but this level of homology does not extend to the C-terminal region of the proteins (20 -30% identity).

The DME and TME holoenzymes have been purified to homogeneity from *E. coli* strains over-expressing the genes from the *lac* promoter of pUC119 (Voegele et al, in press). Electrophoresis of the purified enzymes through non-denaturing polyacrylamide gels suggests that they both exist as octamers. Interestingly, deletion of the C-terminal 320 amino acids produced stable polypeptides that formed dimers, as determined with non-denaturing gels, but still retained malic enzyme activity (Mitsch et al, 1998). Kinetic data reveals that the K_m for malate of DME is 9.4mM and for TME is 2.6mM (Voegele et al, in press). DME utilizes NAD⁺ as co-factor (K_m = 89μM) but can also use NADP⁺ but with a K_m of 1.56mM, whereas TME is strictly dependent on NADP⁺. Therefore, based on substrate specificity DME is classified as an EC1.1.1.39 malic enzyme and TME as EC1.1.1.40,

although no OAA decarboxylating activity with TME could be detected (Voegele et al, in press). Additional biochemical characterization revealed that DME is allosterically inhibited by acetyl CoA and stimulated by fumarate and succinate. In contrast TME showed little change in activity in the presence of these or other compounds (Voegele et al, in press).

This work

The object of this work is to extend our understanding of the different roles of DME and TME in *R. meliloti*, especially in regard to their function in nitrogen-fixing bacteroids. In particular the role of TME is unclear. Studies on the levels of *dme* and *tme* expression in bacteroids using *lacZ* transcriptional gene fusions had indicated that whereas *dme* expression remained constant in either free-living bacteria or in bacteroids, *tme* expression was reduced 5 to 10 fold in bacteroids (Driscoll and Finan, 1996). Therefore, if *tme* expression could be increased to that of *dme* would it functionally replace DME in a Dme⁻ mutant strain?

Secondly, the function of the C-terminal 320 amino acids needs further elucidation. The C-terminal regions of both enzymes appear to be involved in multimerization but apparently are not required for dimerization or catalysis of the malate to pyruvate reaction (Mitsch et al, 1998). Could DME be functionally replaced by a smaller NAD^+-dependent malic enzyme that lacks this C-terminal extension such as the enzyme isolated from *Streptococcus bovis*, that has a subunit size of only 40kD (Kawai et al, 1996)? In addition, will the truncated DME protein,

which retains some malic enzyme activity when over expressed in *E. coli*, functionally replace full length DME in *R. meliloti* and produce nitrogen-fixing bacteroids?

To address these questions a hybrid gene was constructed in which the *tme* coding region was expressed from the *dme* promoter in a plasmid which could be transferred to the *R. meliloti* chromosome. Secondly, the coding region from the *S. bovis* NAD$^+$-dependent malic enzyme was similarly expressed from the *dme* promoter and transferred to the *R. meliloti* chromosome. Thirdly, a truncated *dme* gene that contained only the NH$_2$-terminal malic enzyme domain was constructed and also recombined into the *R. meliloti* chromosome.

It was found that the TME protein expressed from the *dme* promoter was present and active at levels equal to that of DME in bacteroids but in a Dme$^-$ background the nodules remained Fix$^-$. The *S. bovis* enzyme was expressed and active in free-living *R. meliloti* cells but activity was greatly reduced in bacteroids and so when expressed in a *dme*, *tme* null strain the nodules were again Fix$^-$. The truncated *dme* gene was expressed as well as the full length gene and showed activity levels similar to the full length protein and produced Fix$^+$ nodules on alfalfa indistinguishable from wild type *R. meliloti* nodules.

CHAPTER 2

Materials and Methods

Bacterial strains

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in LB medium: 10 gm/l Bacto Tryptone, 5 gm/l Yeast Extract, 5 gm/l NaCl, with the addition of 4 ml/l 1M NaOH in LB broth and 1 ml/l 1M NaOH in LB agar. *R. meliloti* strains were grown in LBmc: LB with the addition of 2.5mM MgSO$_4$, 2.5mM CaCl$_2$. Minimal media (M9 salts) was prepared from a commercially available premixed powder (Gibco-BRL). Glucose and succinate were added to a final concentration of 15mM. 2.5mM MgSO$_4$, 1.25mM CaCl$_2$ and 1µg/ml biotin were added when growing *R. meliloti* in M9 (M9mc). Solid media was prepared by the addition of 15 gm/l Difco Bacto Agar. Antibiotics were added when required.

Transductions

Transductions were carried out using bacteriophage ΦM12 (Finan et al,1984). A 5 ml culture of the donor strain was grown to an OD$_{600}$ of ~0.5, 50µl M12 phage was added and the culture incubated overnight. A few drops of chloroform was added to the lysate and the cell debris spun down at 4000rpm for 5 min. Lysates were stored in screw cap tubes at +4°C. Phage lysates were diluted 1/20 or 1/30 in sterile saline before use, 0.5 ml diluted phage was added to 0.5 ml recipient culture (at OD$_{600}$ ~1) and incubated for 15 min at room temperature. The

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cells were spun down at 3000rpm for 5 min, washed twice with 3 ml 0.85% NaCl, resupended in 0.5 ml 0.85% NaCl and 0.1 ml aliquots were spread onto selective media. Selected colonies were then purified three times by repeated streaking on selective media.

Conjugal transfer of plasmids

Constructs in the plasmid pUCP30T (Schweizer et al, 1997) which carries *oriT* and the gene for gentamycin resistance, were transferred to *R. meliloti* using the helper plasmid pRK600 in *E. coli* MT616. 100µl each of overnight cultures of the donor *E. coli* strain carrying the plasmid to be transferred, the *R. meliloti* recipient strain and the helper strain MT616 were mixed in a microfuge tube, spun down briefly, resuspended in 50µl LB and spotted onto LB plates and incubated overnight at 30°C. The mating spot was scooped up in a loop and streaked onto selective medium. Single cross-over events were selected for using 60µg/ml gentamycin and other antibiotics appropriate for the recipient strain. Selected colonies were purified three times by repeated streaking on selective media, then stored as a frozen culture in LB containing 7% DMSO at -80°C.

Table 1: Bacterial strains and plasmids

Rhizobium meliloti strains

Strain	Genotype	Reference
Rm1021	SU47, *str 21*	Meade et al, 1982
RmF642	Rm1021, *dctA14::Tn5*	Yarosh et al, 1989
RmF647	Rm1021, *dctA26::TnphoA*	Yarosh et al, 1989
RmG212	Rm1021, Lac⁻	Jane Glazebrook
RmG454	Rm1021, *dme-2::Tn5*	Driscoll & Finan, 1993
RmG455	Rm1021, *dme-3::Tn5*	Driscoll & Finan, 1993
RmG456	Rm1021, *dme-1::Tn5*	Driscoll & Finan, 1993
RmG492	Rm5000, *dme-2::Tn5*	Driscoll & Finan, 1993
RmG927	Rm1021, *tme-1::Tn5*	Driscoll & Finan, 1996
RmG994	Rm1021, *dme-3::Tn5, tme-4ΩSp*	Driscoll & Finan, 1996
RmG995	Rm1021, *tme-4::ΩSp*	Driscoll & Finan, 1996
RmH194	Rm1021, *pckA1::Tn3HoHo, pod-1,dme-1::Tn5,tme-4::ΩSp*	Driscoll & Finan, 1996
RmH240	RmG212 (pTH69)	Driscoll & Finan, 1997
RmH897	Rm1021, *dme-3::Tn5, tme-4::ΩSp::pTH433*	This work
RmH898	Rm1021, *tme-4::ΩSp::pTH433*	This work
RmH899	Rm1021, *dme-3::Tn5,tme-4::ΩSp::pTH433*	This work
RmH900	Rm1021, *tme-4::ΩSp::pTH433*	This work
RmH911	Rm1021, *tme-4::ΩSp, dme-3::Tn5::pTH442*, isolate #2	This work
RmH912	Rm1021, *tme-4::ΩSp, dme-3::Tn5::pTH442*, isolate #6	This work
RmH915	RmH194, *dme-1::Tn5::pTH442*	This work
RmH917	Rm1021, *dme::pTH442*, isolate #1	This work
RmH918	Rm1021, *dme::pTH442*, isolate #2	This work

RmH947	Rm1021, *dctA14::Tn5::pTH461*, isolate #1	This work
RmH948	Rm1021, *dctA14::Tn5::pTH461*, isolate #2	This work
RmH949	Rm1021, *dctA26::TnphoA::pTH461*, isolate #1	This work
RmH950	Rm1021, *dctA26::TnphoA::pTH461*, isolate #2	This work
RmH996	Rm1021, *dme-2::Tn5::pTH458*, isolate #2	This work
RmH998	Rm1021, *dme-2::Tn5::pTH458*, isolate #20	This work
RmH999	Rm1021, *dme-1::Tn5::pTH458*, isolate #2	This work
RmH1000	Rm1021, *dme-1::Tn5::pTH458*, *isolate* #9	This work
RmK120	Rm1021, *dme-2::Tn5::pTH458*, *tme-4::ΩSp* #1	This work
RmK121	Rm1021, *dme-2::Tn5::pTH458*, *tme-4::ΩSp* #3	This work
RmK143	Rm1021, *dme-3::Tn5::pTH473*, *tme-4::ΩSp*, #6	This work
RmK144	Rm1021, *dme-3::Tn5::pTH473*, *tme-4::ΩSp*, #8	This work
RmK145	Rm1021, *dme-3::Tn5::pTH473*, *tme-4::ΩSp*, #12	This work

Eschericia coli strains

Strain	Genotype	Reference
CJ236	*dut1 ung1 thi-1 relA1/pCJ105 (cam^r F')*	Kunkel et al, 1987
MT616	MT607 (pRK600)	Finan et al, 1986
EJ1321	*galK2, pck, dme, tme*, Sm^r	Hansen & Juni, 1975
EcJ217	EJ1321 (pTH407B)	This work
EcJ218	EJ1321 (pTH408)	This work
EcJ220	EJ1321 (pTH409)	This work
EcJ271	EJ1321 (pTH434)	This work

Plasmids

Name	Comments	Reference
pSK2	*S. bovis maeE* gene in pUC	Kawai et al, 1996
pSK4	*S. bovis maeP* gene in pUC	Kawai et al, 1997
pUCP30T	transfer vector, Gm^r, oriT	Schweizer et al,1997
pTH69	pLAFR1 clone, complements *dme*	Driscoll & Finan, 1997
pTH139	3kb *HindIII* fragment of pTH69 in pUC119	Driscoll & Finan, 1997
pTH251	*7.1kb BamHI* fragment of pT100 in pUC118	Mitsch et al, 1998
pTH392	2.7kb *HindIII* fragment of *tme* in pUC119	Mitsch et al, 1998
pTH398	*SalI* deletion of pTH139	This work
pTH399	*BspHI-EcoRI tme* promoter in pUC119	This work
pTH400	pTH398 with *SphI* site at ATG	This work
pTH401B	pTH399 with *SphI* site at ATG	This work
pTH407B	*tme* gene with *SphI* site at ATG	This work
pTH408	*dme* gene with *SphI* site at ATG	This work
pTH409	*dme* promoter-*tme* gene fusion in pUC119	This work
pTH433	*HindIII-KpnI dtme* fragment in pUCP30T	This work
pTH434	*S. bovis maeE* PCR product in pTH400	This work
pTH442	*dmaeE* gene fusion in pUCP30T	This work
pTH449	*dmaeP* gene fusion in pUC119	This work
pTH452	*PstI* deletion of pTH139	This work
pTH458	*HindIII-XbaI* fragment of pTH452 in pUCP30T	This work
pTH461	*dmaeP* gene fusion in pUCP30T	This work
pTH473	*PstI-KpnI* deletion of pTH433	This work

Preparation of cell extracts

R. meliloti strains were inoculated into 5 ml LBmc and incubated overnight at 30°C. Cultures were transferred to 15 ml conical, plastic tubes and spun at 4000rpm, +4°C, 5 min in a Beckman bench top centrifuge. The cell pellet was washed with 3 ml cold 10mM Tris.HCl pH 7.8, 1mM MgCl$_2$ and spun as before. The cell pellet was resuspended in 200 µl cold Sonication Buffer (10mM Tris.HCl pH 8, 1mM MgCl$_2$, 10mM KCl, 20% glycerol, 10mM 2-mercaptoethanol). Cells were disrupted using a Heat Systems Ultrasonic Processor XL. Sonication was for 30 seconds on, 30 seconds off at setting #8, for 45 minutes total time, at +4°C. The cell lysate was then spun at 12,000 rpm for 5 min, +4°C to pellet cell debris. The supernatent was aliquoted into 0.5 ml microfuge tubes (75µl each), flash frozen in liquid nitrogen and stored at -80°C.

Bacteroids were isolated by picking the nodules from the roots of 30 plants and placing them in cold MMS buffer (40mM MOPS, 20mM KOH, 2mM MgSO$_4$, 0.3M sucrose, pH7.0). All steps and reagents were kept cold. Nodules were transferred to a cold mortar and pestle and all but about 1 ml of buffer was removed, the nodules were then ground to a paste. The slurry was passed through 4 layers of cheesecloth into a 15 ml conical tube and the mortar and pestle rinsed with 5 ml MMS buffer which was also passed through the cheesecloth. The slurry was spun at 700rpm for 5 min to remove plant material, the supernatent was then spun at 4000rpm, 5 min to pellet the bacteroids. The pellet could now be stored frozen at -80°C. To prepare protein extracts the bacteroids were washed as above and

resuspended in 200μl Sonication Buffer for Fix⁺ bacteroids, Fix⁻ bacteroids from 30

plants were resuspended in 50 - 100 μl Sonication Buffer. Bacteroids required only

25 min sonication time at setting #8, 30 seconds on, 30 seconds off, +4°C. The

extracts were then treated as above and stored at -80°C.

Protein concentrations were measured using the mini protocol for the

Bradford colour reagent (BioRad) and a standard curve of 0 - 15μg/ml bovine serum

albumin (Bradford,1976)

DNA manipulations

Restriction endonucleases and modifying enzymes were used according to

manufacturers recommendations. Preparative digests for cloning purposes typically

contained 2 - 5μg plasmid DNA in a final digestion volume of 50μl. Following

digestion samples were electrophoresed through 1% agarose gels in TAE buffer

(Sambrook et al, 1989), visualized with ethidium bromide and desired bands excised

and eluted in 20μl TE (10mM Tris.HCl pH 7.5, 1mM EDTA) using a Geneclean kit

(BIO101). Ligations were carried out in 20μl final volume containing 2μl vector DNA

and 4μl insert DNA with 0.5 - 1μl T4 DNA ligase (MBI Fermentas) at room

temperature overnight. Following ligation plasmid constructs were transformed into

competent DH5α cells (Ausubel et al, 1989). Plasmid DNA was isolated by the

alkaline lysis method for both small scale diagnostic purposes and large scale

preparations (Sambrook et al, 1989). Details of plasmid constructions will be given

in the relevant chapters.

Polymerase Chain Reaction

Primers were resupended in TE (10mM Tris.HCl pH 7.5, 1mM EDTA) at

100pmoles/µl. Reactions were carried out in 100µl final volume containing 10ng

plasmid DNA, 100pmoles each primer, 4.5mM MgCl$_2$, 0.8mM dNTPs in the buffer

supplied with the enzyme and 1µl Taq DNA polymerase (Perkin-Elmer). Tubes

were incubated for 5 min at 94°C before commencing the cycle of 94°C for 1 min,

47°C for 30 sec, 72°C for 30 sec, for 25 cycles. The reactions were completed by

incubation at 72°C for 7 min. 10µl from the reaction was run on a 1% agarose gel

to verify DNA synthesis had occurred. The rest of the sample was precipitated with

ethanol then digested with appropriate restriction enzymes and the DNA fragment

isolated from 1% agarose gel by Geneclean (Bio101).

RNA isolation

RNA was isolated from nodules using the method of Corbin et al, 1983.

Nodules from 30 plants were picked and placed immediately into liquid nitrogen,

ground to a powder in a mortar and pestle, transferred to a Dounce homogenizer

in 2ml Buffer A (0.5M mannitol, 20mM NaOAc, 1mM EDTA, 0.1% DEPC) and

disrupted with 5 strokes of the homogenizer. This slurry was added to 5 ml Buffer

B (0.5M mannitol, 20mM NaOAc, 1mM EDTA, 3% SDS) in a 15 ml conical tube in

a boiling water bath and boiled for 3 min. The homogenizer was washed out with

a further 2 ml Buffer B which was added to the tube. The lysate was transferred to

two 12ml snap cap tubes and extracted twice with buffer saturated phenol, once with

chloroform, ammonium acetate was added to the aqueous phase to 0.5M followed by 2.5 volumes ethanol to precipitate nucleic acids. The resulting pellet was dissolved in 400μl RNase-free water, Tris.HCl pH7.5 was added to 10mM, MgCl$_2$ was added to 10mM and 10μl RNase-free DNaseI (Boehringer Mannheim) was added. Incubation was for 30 mins at 37°C, the sample was then extracted with buffer saturated phenol, ammonium acetate added to 0.5M and 2.5 volumes ethanol added to precipitate the RNA. The pellet was dissolved in 200μl RNase-free water and the OD$_{240}$ measured on a 1/100 dilution to determine the RNA concentration. Approximately 2μg RNA was run on a 1.2% agarose gel to verify the integrity of the RNA. The resultant RNA contained ~50% bacteroid RNA 50% plant derived RNA as judged by the rRNA bands on agarose gels.

RNA was purified from 50ml logarithmically growing *R. meliloti* cells in culture using the RNeasy Midi kit from Qiagen. Integrity of the RNA was verified by agarose gel electrophoresis using autoclaved agarose and buffers. Typically about 200 μg RNA was obtained from 50 ml of logarithmic culture.

Northern blot

Northern blots were performed as described in Ausubel et al, 1989 using formaldehyde gels. All solutions were made up in DEPC treated water. 1.2gms agarose was boiled in 87 mls DEPC treated water, once cooled to 60°C 10 mls 10x MOPS buffer (0.2M MOPS pH 7, 50mM sodium acetate, 10mM EDTA) and 3mls 37% formaldehyde was added and the gel was poured. RNA samples were

prepared by adding 11µl RNA to a tube containing 5µl 10x MOPS buffer, 8.75µl 37% formaldehyde, 25µl formamide (final volume = 50µl), and heating at 55°C for 15 min. Gels were run in 1x MOPS buffer at 5V/cm until the bromophenol blue dye had migrated ~2/3rds the length of the gel. 10µl of each sample was run on one gel and stained with ethidium bromide, 40µl of each sample was run on a second gel to be blotted.

Gels were blotted by capillary action onto Biotrans nylon membranes (ICN) as described in Ausubel et al, 1989. The membranes were baked at 80°C for 1hr then prehybridized at 42°C for 2 - 3hrs in 25 ml Prehybridization solution: 25mM KPO_4, pH 7.4, 5x SSC, 5x Denhardts solution, 50µg/ml salmon sperm DNA, 50% formamide. ^{32}P-labeled DNA probe was denatured by boiling for 5 min and added to 25 ml Prehybridization solution and then added to the membrane and hybridized overnight at 42°C. The membrane was washed 2 x 15 min in 1 x SSC, 0.1% SDS at room temperature, 2 x 15 min 0.25 x SSC, 0.1% SDS at room temperature. The membrane was then wrapped in SaranWrap and exposed to Kodak XAR film.

Random primed labelling of DNA.

A DNA fragment containing the *dme* promoter and untranslated leader sequence was excised from 10µg plasmid pTH400 with *Hind*III and *Sph*I. The fragment was isolated from an agarose gel in 20µl TE and 2µl run on another gel to determine the amount of DNA present. 2µl purified DNA fragment was labelled according to the Random Primed Labeling Kit of Boehringer Mannheim with α-^{32}P-

dCTP (3000Ci/mmol, NEN). After the labeling reaction the DNA was precipitated by addition of 200µl 0.5M ammonium acetate, 10mM EDTA, 10µgs tRNA carrier and 2.5 volumes ethanol. The pellet was dissolved in 200µl 0.5M ammonium acetate, 10mM EDTA and reprecipitated. The pellet was then dissolved in 100µl TE, 1µl was spotted onto a 2.4 cm Whatman GF-C filter and counted in a scintillation counter to determine the amount of radioactivity incorporated.

Primer extension

The protocol followed was that of Ausubel et al, 1989. An oligonucleotide primer complementary to a sequence near the 5'-end of the *dme* coding region was synthesised: 5'-ATCGATGTCTCCGCTGGCGG-3' (nt 58 to 38, ATG = 1, Mitsch et al, 1998). 10 pmoles of primer was radio-labeled using 50µCi ^{33}P-ATP (3000Ci/mmol, NEN) and 10U polynucleotide kinase (NEB) in a final volume of 20µl for 60 min at 37°C. 180µl 0.5M NH_4OAc, 10mM EDTA, 10µg tRNA as carrier and 2.5 volumes ethanol was added to precipitate the labeled oligonucleotide. The sample was dissolved in 200µl 0.5M NH_4OAc, 10mM EDTA and reprecipitated. The labeled oligonucleotide was dissolved in 50µl TE, 1µl was spotted onto a 2.4 cm Whatman GF-C filter and counted in a scintillation counter to determine the amount of radioactivity incorporated. 10^6 cpm primer was added to 30µg bacterial RNA, 60µg nodule RNA or 25µg tRNA for control, NaOAc was added to 0.3M and the samples were then precipitated with 2.5 volumes ethanol. The pellets were washed with 70% ethanol and air dried then dissolved in 30µl 3x aqueous hybridization

buffer (3M NaCl, 0.5M Hepes pH 7.5, 1mM EDTA) and annealed overnight at 30°C.

170μl 0.3M NaOAc was added and the samples precipitated with ethanol, the

pellets washed with 70% ethanol and air dried. The pellets were dissolved in 25μl

of a mix containing 3.5μl 4mM dNTPs, 5μl 5x RT buffer, 1μl RNase Inhibitor

(Boehringer Mannheim), 1μl 0.1M DTT, 14.5 μl H_2O. 1.5μl MoMLV reverse

transcriptase (MBI Fermentas) was added and the reaction incubated at 37°C, 2 hrs.

1μl 0.5M EDTA was added to stop the reaction and 0.5μl 2mg/ml boiled RNase A

was added and incubated for 30 mins at room temperature. 175μl 0.5M NH4OAc

was added, the samples extracted with buffer saturated phenol and ethanol

precipitated. The pellets were dissolved in 2μl TE, 3μl formamide dye mix was

added, heated at 85°C for 5 min then electrophoresed on a 7% acrylamide-

bisacrylamide, 50% urea, 1xTBE sequencing type gel (Sambrook et al, 1989). The

same labeled primer was used in standard dideoxy sequencing reactions with

plasmid pTH139 using the Sequenase 2.0 kit (Amersham). The sequencing

reactions were run alongside the primer extension reactions so as to identify the

position(s) of any resulting band(s).

Oligonucleotide-directed mutagenesis

The method of Kunkel et al, 1987 as described in Sambrook et al, 1989 was

used. Plasmids into which the target region had been subcloned (see Chapter 4,

Figure 3) were transformed into the *ung, dut* strain of *E. coli* CJ236. Single-

stranded DNA was isolated following infection with bacteriophage M13K07

(Sambrook et al, 1989). Oligonucleotides corresponding to the ATG and surrounding sequence were synthesised to contain an *Sph*I restriction enzyme recognition site (CCATGG) at the ATG. For *dme* the following oligonucleotide was synthesized: 5'-CGCCCGTGT**GCATGCC**TTTGC-3', for *tme* the oligonucleotide synthesized was: 5'-GATACCCGGCATGCTTCCTCAACCTC-3'. 100 pmoles oligonucleotide was phosphorylated with ATP (0.5mM) and polynucleotide kinase (NEB) in 20μl reaction volume, 37°C, 45 min followed by 10 min, 65°C to inactivate the enzyme. The reaction was then diluted with H_2O to 2 pmoles/μl oligonucleotide. 2 pmoles phosphorylated oligonucleotide was mixed with 0.2μg single-stranded plasmid DNA in 10μl final volume containing 20mM Tris.HCl pH 7.4, 2mM $MgCl_2$, 50mM NaCl, heated to 70°C then allowed to slowly cool to room temperature to anneal. 1μl 10x synthesis buffer (5mM dNTPs, 10mM ATP, 100mM Tris.HCl pH 7.4, 50mM $MgCl_2$, 20mM DTT), 1μl T4 DNA polymerase (NEB) and 0.5μl T4 DNA ligase (NEB) was added. The reaction was incubated for 5 min on ice, 5 min room temperature then 90 min 37°C. 2μl was run on a 1% agarose gel, then 40μl TE added to the remainder and 5μl transformed into competent DH5α cells and plated onto LB agar containing 100μg/ml ampicillin. Colonies were picked and plasmid DNA was isolated by alkaline lysis (Sambrook et al, 1989) and analyzed for the presence of the *Sph*I site by digestion with *Sph*I (NEB). Selected clones were then sequenced (Mobix Central Facility) to confirm the mutation and to ensure no other mutations had occured.

Malic enzyme assays

Pyruvate formation was measured by a colorimetric assay utilizing 2,4-dinitrophenylhydrazine in a modification of the method in Driscoll (1995). 495µl reaction mix containing 100mM Tris.HCl pH 7.8, 30mM K-L-malate pH 7.8, 3mM MnCl$_2$, 50mM KCl, 0.25mM NAD(P)$^+$ was dispensed into microfuge tubes and the tubes placed on ice. 5 or 10µl crude cell or bacteroid extract was added (~50 - 100µg protein) and the tubes incubated at 30°C for 10 min then returned to ice. 165µl 0.1% 2,4-dinitrophenyl hydrazine (DPH) in 2.25M HCl was added and the tubes incubated for 10 min at room temperature. 835µl 2.5M NaOH was added drop-wise and the tubes mixed thoroughly then incubated 10 min, room temperature. The tubes were spun 5 mins, 13,000 rpm and 1ml transferred to plastic semi-micro cuvettes and the absorbance at 445nm was read against reagent blanks. A standard curve of pyruvate concentrations between 0 - 200nmoles was prepared in the same way. All samples were done in triplicate and specific activity was calculated as nmoles pyruvate produced/minute/mg protein.

Malate dehydrogenase assays

Malate dehydrogenase activity was measured in crude cell extracts as the change in absorbance at 340nm due to the reduction of NAD$^+$ to NADH. 1 ml reaction mix containing 100mM glycine-NaOH pH 10, 85mM L-malate pH 7.5, 2.5mM NAD$^+$ was dispensed into plastic semi-micro cuvettes. 2µl sonicated cell or bacteroid extract was added to the side of the cuvette and the reaction started by

inverting the cuvette to mix. The reaction kinetics for 3 minutes was measured using the Cary Varian spectrophotometer with the slope over 1 minute calculated. Specific activity was calculated as nmoles NADH produced/min/mg protein with the equation: (slope/0.00622)/[protein].

SDS PAGE and Western blot analysis

Buffer recipes for SDS PAGE can be found in Sambrook et al,1989 and will not be included here. 1 - 2μgs protein extract was diluted into 10μl 1xSDS Loading dye, heated at 100°C for 5 min then loaded onto a 7% (30% acrylamide:0.8% bisacrylamide) SDS polyacrylamide gel (Laemmli,1970) using the BioRad Mini Protean gel system. Gels were run at 100 volts for ~2 hours, until the bromophenol blue dye reached the bottom, in Tris/glycine/SDS buffer. Gels were then transferred to Millipore Imobilon P membranes electrophoretically at 100 volts for 1 hour at +4°C in Tris/glycine /methanol Transfer buffer.

Membranes were blocked with Post blot buffer (Pbb: 0.15M NaCl, 10mM Tris.HCl pH 7.4) containing 5% milk powder for 60 min room temperature. The membranes were then incubated with the primary antibody (rabbit anti-DME or anti-TME serum) diluted 1/10,000 in Pbb + 0.05% NP40 + 5% milk powder overnight at +4°C. After washing 3 x 10 min with Pbb + 0.05% NP40 the membranes were incubated with the secondary antibody (goat anti-rabbit HRP conjugated, Sigma) diluted 1/1000 in Pbb + 0.05% NP40 + 5% milk powder for 60 min room temperature. After washing 2 x 10 min with Pbb + 0.05% NP40, 2 x 10 min 10mM

Tris.HCl pH 7.4 the membranes were developed for 1 minute with ECL (Enhanced ChemiLuminescence) Reagent (Amersham) and immediately exposed to Kodak XAR X-Ray film for between 10 seconds and 2 minutes depending on the signal.

Native gel electrophoresis and staining

Solutions and buffers for running non-denaturing polyacrylamide gels can be found in the Sigma technical bulletin MKR-137 and will not be reproduced here. 7% separating polyacrylamide gels for the BioRad Mini Protean gel system were prepared using 1.5 ml separating gel buffer, 3 ml acrylamide solution (separating), 6.75 ml sucrose solution and 0.75 ml, 1% ammonium persulfate and placed in a 37°C incubator to speed polymerization. The stacking gel consisted of 1 ml stacking gel buffer, 2 ml acrylamide solution (stacking), 4 ml sucrose solution, 0.5 ml riboflavin, 0.5 mls 1% ammonium persulfate. Samples contained 30μg protein in 20μl total volume sample buffer and were separated by electrophoresis at 4mA per gel until the bromophenol dye had passed through the stacking gel and then at 7mA/gel until the dye was close to the bottom. Gels were run at +4°C to avoid protein denaturation due to overheating.

To stain for malic enzyme activity the gels were washed for 10mins with 100mM Tris.HCl pH 7.8, 30mM K-L-malate pH 7.8, 3mM $MnCl_2$, 50mM KCl then incubated in the same buffer containing 0.3mg/ml 3-(4,5-dimethly thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 0.04mg/ml phenazine methosulphate (PMS) and NAD^+ or $NADP^+$ at 0.3mg/ml at 37°C in the dark, without shaking until bands

could be seen (approximately 30 min). Gels were dried and stored in the dark.

Plant assays

Leonard assembly pots were set up with a cotton wool wick between the beaker and the pot. The pot was filled with a 1:1 (w:w) mix of vermiculite and sand and 200 ml Jensens medium (see Driscoll, 1995 for components) was poured on. The join between the pot and the beaker was sealed with foil and autoclave tape, the top was covered with foil and the whole assembly was autoclaved for 30 min. Alfalfa seeds (Iroquois variety) were surface sterilized with 95% ethanol for 5 mins, 2.5% sodium hypochlorite for 10 min then washed repeatedly with sterile water for 30 min. The seeds were then placed on 1.5% water agar plates and placed in the dark for 2 days to germinate. Ten seedlings, approximately 1 - 2 cm in length, were planted at ~1cm depth in each pot, placed in a Conviron growth chamber but kept covered by the foil for 2 days. Fresh overnight cultures of the test strains were grown, diluted 0.1 ml culture into 10 ml sterile water and added to the pots. The seedlings were then uncovered and grown for 28 days with 16 hours light, 8 hours dark; daytime temperature of 23°C and night-time temperature of 18°C and watered with sterile distilled water when required.

Nodules were picked to make bacteroid extracts or RNA as required. Plant shoots were cut off at the root-stem boundary and dried for 2 weeks at 70°C before measuring dry weights.

CHAPTER 3

Construction and analysis of a *dme-tme* hybrid gene

Identification of the promoter region of *dme*

The gene for *dme* was localized to a 3.2kb *Hind*III fragment subcloned from a pLAFR1 cosmid clone (Driscoll and Finan, 1997). This 3.2kb fragment has now been sequenced and contains one continuous open reading frame of 2313 bp corresponding to the coding region of the NAD^+- dependent malic enzyme (DME, Mitsch et al, 1998). Little is known about the regulation of *dme* in *R. meliloti*: expression appears to be constitutive in both free-living cells and in bacteroids (Driscoll and Finan, 1997). Analysis of the DNA sequence upstream of the translational start codon did not reveal any obvious promoter sequences or transcription factor binding sites. As a prerequisite for using the *dme* promoter to construct chimeric genes it was necessary to first determine the site of transcriptional initiation and identify any potential upstream regulatory elements.

To map the RNA start sites primer extension reactions were carried out. RNA was isolated from wild type *R. meliloti* (Rm1021) cells grown to logarithmic phase in LBmc, strain RmH240 (wild type RmG212 carrying the stable plasmid pTH69, Driscoll and Finan, 1997) which over expresses DME and from alfalfa root nodules produced by Rm1021, 28 days post infection. The amount of RNA isolated was measured by OD_{260} and the integrity of the RNA verified by agarose gel

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electrophoresis (data not shown). As judged by the relative intensity of the bacterial rRNA bands compared to the eukaryotic rRNA bands in the nodule RNA preparation it appeared to be approximately 50% bacteroid RNA, therefore twice as much nodule RNA was used in the primer extension reactions.

An oligonucleotide primer was synthesized complementary to a region downstream of the ATG initiation codon (corresponding to nucleotide 38 to 58 when the ATG is #1). The DNA sequences of *dme* and *tme* share a high degree of homology in this region of the gene so care was taken to design a primer with minimum chance of cross-hybridization to *tme* RNA and that would still produce a predicted product in the 50 to 100 nt size range. The resulting primer contained only 8 nucleotides complementary to the corresponding sequence in *tme* and they were non-contiguous (5'-ATCGATGTCTCCGCTGGCGG-3').

The products from the primer extension reactions were resolved on a 7% acrylamide, 50% urea sequencing gel alongside sequencing reactions using the same labeled primer and pTH139 plasmid DNA (Figure 2A). Transcription of *dme* appears to initiate at two positions: A at -78 and A at -80 from the ATG (Figure 2, panel A, lanes 1 and 2). The same start sites are also utilized in bacteroids confirming the hypothesis that *dme* is not differently regulated in bacteroids (Figure 2, panel A, lane 3). The sequence upstream of the transcriptional start sites bears little homology to other *R. meliloti* promoters that are thought to transcribed by the vegetative sigma factor (SigA in *R. meliloti*, Figure 2, panel B)

Panel A: Lanes G,A,T,C are sequencing reactions of pTH139 DNA. Lane 1, primer extension using 30μg RNA from Rm1021 cells grown in Lbmc. Lane 2, primer extension using 30μg RNA from strain RmH240 (wild type *R. meliloti* carrying plasmid pTH69) grown in LBmc. Lane 3, primer extension using 60μg RNA isolated from 28 day wild type alfalfa root nodules inoculated with Rm1021. Lane 4, primer extension with 25μg tRNA.

Panel B: Sequence of the *dme* promoter region compared to that of several other *Rhizobium sp.* promoters. Sequences at -35 and -10 and start sites of transcription are shown in bold. Sequence of the *tme* promoter from Mitsch et al, 1998; the other sequences are taken from Osteras et al, 1995 and Bardin et al, 1998.

Figure 2: Primer extension of *dme* RNA

A

B

```
dme:      TCGAAACTGTACGTGCCGGGC--GATTCATGCCAAAAGGCACGACA
              -35                              -10
tme:      AAAGAGATGTCACACTATCC---GCAAGCATTTATAGACGACAACA
pck:      GCTATCTTGTCTTGGGTCAG---CCTTGCCGGTATGTTCCGACGA
trpE:     TCCCGCTTGCGCGCCAGCGC---AAGCCGCGCTAACACTTCCGCCA
nodD1:    CGCACCTTGATTCCATTAACTTCAGGGTTCTCTAATAGGACTC
hemA:     CCGCAATTGCTTGACTTCGATCGATGTTCGGGAGAATGAAGTTTTG
hemA:     CGGGGTTGACCACTGATCG---CTTTGAAGGAAGAAAGGCGACA
ntrA:     CCACGCTTGACCAAATTCCA---GTAATAAGCAATTTTTGGGCCA
orfA-pit: AACTTTCCCCGAGCGCG---CAAGTACAATATGACAACTGCG
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Generation of SphI sites at the ATG of dme and tme

To construct a chimeric gene with the dme promoter driving the tme coding region it is necessary to have complementary restriction enzyme sites somewhere in the non-translated leader sequence of each gene. There were no such naturally occurring sites so it was decided to create restriction enzyme sites at the ATG of each gene by site-directed mutagenesis. The restriction enzyme SphI was chosen because the recognition sequence includes ATG (GCATGC) and only one site exists in tme and no sites in dme. The method of Kunkel et al (1987) was used where the plasmid DNA is first transformed into an ung, dut strain of E. coli so that the resulting single-stranded plasmid DNA, isolated after infection with M13 phage, contains randomly incorporated uracil. This reduces the efficiency of replication of that DNA strand when transferred back to DH5α, thereby increasing selection for the mutated DNA strand. Typically greater than 50% of the resulting colonies contain the mutation.

Before carrying out the mutagenesis is was necessary to subclone a smaller target area from both dme and tme to reduce the chance of other mutations being introduced. To do this for dme plasmid pTH139, which contains the 3.2kb HindIII fragment encompassing the entire gene, was cut with SalI and religated to give the plasmid pTH398 containing nt 1 to 865 of the HindIII fragment (see Figure 3). For tme the plasmid pTH251, containing a 7.1kb BamHI fragment (Mitsch et al, 1998) was cut first with BspHI, treated with Klenow polymerase to fill in the 5' overhang and then cut with EcoRI. The resulting 480bp fragment was isolated from an

agarose gel and ligated into pUC119 cut with *Sph*l treated with Klenow polymerase
to remove the 3' overhang and recut with *Eco*RI to give the plasmid pTH399. These
plasmids were then transformed into *E. coli* CJ236 (*ung, dut*) and single-stranded
DNA was produced by infection with M13K07 bacteriophage (Sambrook et al,
1989). The mutagenesis was carried out and the resulting DNA transformed into
DH5α cells. Colonies were picked, plasmid DNA isolated and checked for the
presence of a new *Sph*l site by digestion with the enzyme. Positive clones
(designated pTH400 and pTH401B, see Figure 3) were isolated and the nucleotide
sequence of one strand of the entire fragment determined to ensure no other
mutations had been introduced.

Both the full length *dme* gene and *tme* gene were reconstructed to contain
the new *Sph*l sites. To reconstruct *dme* the *Sal*l fragment deleted from pTH139 was
reintroduced to give pTH408, for *tme* an *Eco*RI fragment from pTH392 (Mitsch et al,
1998) was inserted at the *Eco*RI site to give pTH407B. To determine if the
reconstructed enzymes were still functional pTH407B and pTH408 were
transformed into *E. coli* strain EJ1321, a malic enzyme deficient strain that cannot
grow with succinate as the sole carbon source (Hanson and Juni, 1975), to test if
growth on M9 succinate was restored. Both plasmids allowed EJ1321 to grow on
M9 succinate indicating that both mutagenized genes were producing functional
malic enzymes.

Mutagenesis and cloning steps for both *dme* and *tme* are shown in parallel. Restriction enzyme sites relevant to the constructions are indicated. The introduced *SphI* site is shown in bold. Steps in the constructions are indicated alongside the arrows.

Figure 3: Diagram of the cloning scheme used to construct the *dme-tme* fusion gene
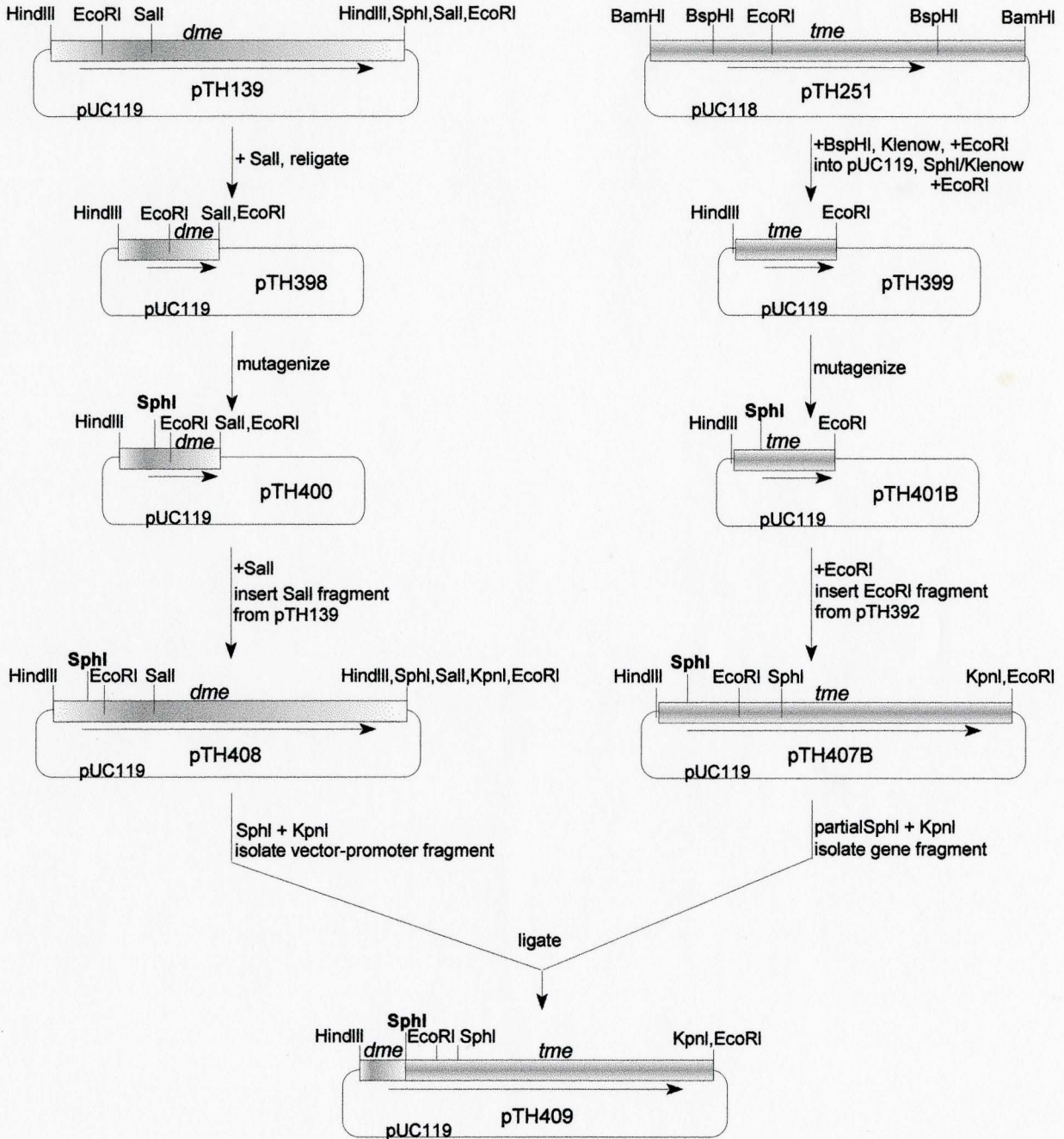
Construction of the hybrid *dtme* gene containing the *dme* promoter and the *tme* structural gene

The chimeric gene (designated *dtme*) was constructed by digesting pTH408 with *Sph*I and *Kpn*I and gel purifying the 3.8kb vector-promoter fragment, digesting pTH407B with *Sph*I and *Kpn*I and isolating the 2.7kb gene fragment and ligating them together (see Figure 3). One positive clone, pTH409, was then transformed into EJ1321 and it also fully restored growth on M9 succinate. These constructs were made in the plasmid pUC119; this is not a suitable vector for the transfer of genes into *R. meliloti* as it is not mobilizable and ampicillin resistance is not a suitable antibiotic selection in *R. meliloti*. It was therefore necessary to clone the *dtme* chimeric gene into a transferable plasmid such as the vector pUCP30T (Schweizer et al,1996). This 4kb plasmid carries gentamycin resistance, multiple cloning sites and the origin of transfer from pRK2 (*oriT*). The *dtme* gene was excised as a *Hind*III - *Kpn*I fragment from pTH409 and cloned into the corresponding sites of pUCP30T to give the plasmid pTH433. The vector pUCP30T contains the *lac* promoter adjacent to the multiple cloning sites so the *dtme* gene fragment was cloned in the opposite orientation to avoid interference from it.

Plasmid pTH433 was transferred to *R. meliloti* strain RmG994 (*dme-3::Tn5, tme-4::ΩSp*, Driscoll and Finan, 1996) in a triparental mating with the helper plasmid pRK600. Mating spots were streaked onto LB agar containing spectinomycin 100μg/ml and gentamycin 50μg/ml to select for single recombination events. Two colonies were selected and purified three times on the same medium and given the

designations RmH897 and RmH899. The *dtme* hybrid gene in plasmid pTH433 carries approximately 500bp from the *dme* gene and approximately 2.5kb from the *tme* gene therefore the single cross-over event selected for could have occurred in either gene. In order to determine which was the case the *dtme* region was transduced from RmH897 and RmH899 with the general transducing phage ΦM12 (Finan et al, 1984) into *R. meliloti* 1021 wild type strain, selecting on LB containing only gentamycin. Colonies were then patched onto LB neomycin at 200μg/ml, LB spectinomycin at 100μg/ml and LB gentamycin at 50μg/ml. All colonies were spectinomycin and gentamycin resistant indicating that the cross-over event in both strains had occurred into the *tme-4::ΩSp* gene. One colony from each transduction was further purified and saved as strains RmH898 and RmH900 respectively.

Malic enzyme activity of the *dtme* hybrid gene in crude cell extracts

To confirm that the *dtme* gene was functional in *R. meliloti* sonicated cell extracts were prepared from cultures grown overnight in LBmc. Both NAD^+ and $NADP^+$-dependent malic enzyme activity was assayed using a colorimetric assay for pyruvate formation. The results are shown in Table 2. In addition malate dehydrogenase activity, measured as the change in absorbance at 340nm due to reduction of NAD^+, was also measured as a control for the integrity of the extracts.

Table 2: Malic enzyme assays on *R. meliloti* cell extracts

Strain	Genotype	NAD$^+$ activity*	NADP$^+$ activity*	MDH activity**
Rm1021	wild type	58.2 +/- 4.0	74.5 +/- 6.2	738.7+/- 15
RmG455	dme3	21.2 +/- 2.2	61.4 +/- 2.4	610.9+/- 9
RmG995	tme4	71.7 +/- 5	0	782.2+/-19.3
RmG994	dme3, tme4	20.8 +/- 1.1	0	863.6+/-25.0
RmH897	dme3, tme4,dtme$^+$	23.7 +/- 1.1	104 +/- 7.8	684.2+/-12
RmH899	dme3, tme4,dtme$^+$	14 +/- 0.9	67.0 +/- 4.6	695.3+/-15.5
RmH898	tme4,dtme$^+$	70.4 +/- 2.5	134.2 +/- 4.6	703.5+/-11.4
RmH900	tme4,dtme$^+$	47.4 +/- 0.6	86.0 +/- 4.5	1061.8+/-10.7

*Specific activity as nmoles pyruvate formed/minute/mg protein is shown as the mean +/- SE of triplicate samples.
**Malate dehydrogenase activity measured as nmoles NADH formed /min/mg protein is given as the mean +/- SE of triplicate samples.

As can be seen in Table 2 the *dtme* gene was fully active producing NADP$^+$-dependent malic enzyme activity equal to or exceeding the normal wild type levels.

Plant growth assays with *dtme R. meliloti* strains.

Alfalfa seedlings were inoculated with strains RmH897 and RmH899 (*dme3, tme4, dtme$^+$*) which only express dTME, as well as strains RmH898 and RmH900 (*tme4, dtme$^+$*) which also express DME, together with wild type *R. meliloti* and uninoculated plants as controls and after 28 days plants were collected. The root nodules from all plants were picked, bacteroids isolated and sonicated extracts prepared from them. The plant shoots were dried for two weeks to determine dry weights and these results are shown in Table 3.

Table 3: Plant dry weight determinations

Strain	Genotype	Phenotype	# plants	Wt/plant(mgs)	%WT
Rm1021	wild type	Fix$^+$	29	28 +/- 1.5	100
RmG455	dme3	Fix$^-$	30	8 +/- 0.6	28
RmG995	tme4	Fix$^+$	30	17 +/- 1.7	61
RmG994	dme3, tme4	Fix$^-$	28	7.2 +/- 0.2	25
RmH897	dme3, tme4, dtme$^+$	Fix$^-$	29	9.6 +/- 0.9	34
RmH899	dme3, tme4, dtme$^+$	Fix$^-$	30	8 +/- 0	28
RmH898	tme4,dtme$^+$	Fix$^+$	29	22.4 +/- 0.9	80
RmH900	tme4,dtme$^+$	Fix$^+$	30	24 +/- 0.6	86
control	none	Fix$^-$	40	5.25 +/- 0.6	19

Dry weights expressed as a mean +/- SE of 3 pots of 8 - 10 plants each.
Fix+ represents large pink nodules on large green plants.
Fix- represents small whitish nodules on small yellow plants

Only plants inoculated with *R. meliloti* strains expressing *dme* were green and healthy indicating that the nodules were Fix$^+$, while the plants inoculated with the strains expressing *dtme* were indistinguishable in appearance from uninoculated controls. Both NAD$^+$ and NADP$^+$ dependent malic enzyme activity was measured in the bacteroid extracts where possible, however, many of the Fix$^-$ samples contained too little protein to assay. The results are shown in Table 4.

Table 4: Assays on bacteroid extracts

Strain	Genotype	NAD$^+$ activity*	NADP$^+$ activity*	MDH activity**
Rm1021	wild type	44.8+/-0.6	18.9+/-0.8	2607.7+/-58.5
RmG455	dme3	ND	ND	2511.6+/-189.8
RmG995	tme4	49.6+/-1.6	7.7+/-0.6	3269.0+/-84.8
RmG994	dme3, tme4	ND	ND	3769.2+/-135.5
RmH897	dme3, tme4, dtme$^+$	0***	88.7+/-1.7***	2864.2+/-63.0
RmH898	tme4, dtme$^+$	52.0+/-2.8	59.5+/-0.9	3298.6+/-72.4

*Specific activity measured as nmoles pyruvate formed/minute/mg protein, is expressed as the mean +/- SE of in triplicate samples.
**Malate dehydrogenase activity measured as nmoles NADH formed /min/mg protein, expressed as the mean +/- SE of triplicate samples.
***Reactions were incubated for 30 minutes at 30°C
ND - not determined

As reported previously (Driscoll and Finan,1997) the activity of TME in bacteroids is greatly reduced and that is confirmed here with the Rm1021 bacteroid extract, but activity is high in the bacteroids expressing TME from the dtme gene. The expression of dtme was further analyzed in both the Fix⁻ and Fix⁺ bacteroids by SDS-PAGE and Western blotting. 2μg protein was run on 7% SDS polyacrylamide gels, transferred to Imobilon P membranes (Millipore) and probed with either rabbit antiserum against DME or TME, followed by goat anti-rabbit IgG coupled to HRP (horseradish peroxidase) and detected using the ECL system (Figure 4). As reflected in the enzyme assays (Table 4), the amount of TME protein in Rm1021 and RmG455 bacteroids is significantly reduced (Figure 4, panel B, lanes 3 and 4) from that of cells grown in LBmc (Figure 4, panel B, lane 1). However, the amount of TME protein produced from the dtme gene is elevated to that of free-living cell

Figure 4: Western blots on bacteroid extracts
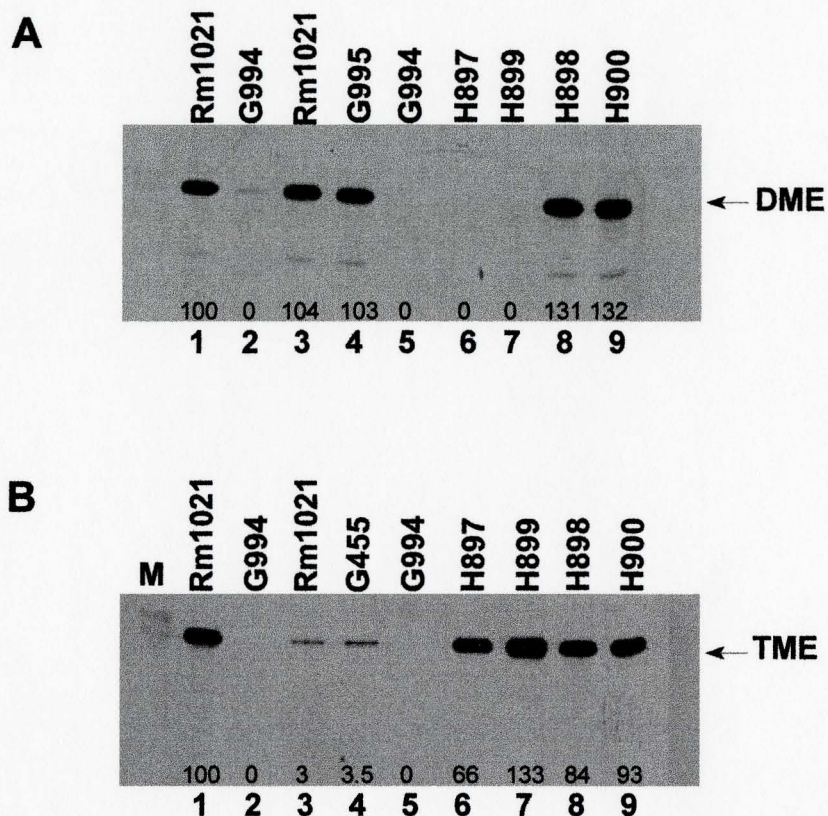
Lanes I and 2, extracts from *R. meliloti* cells grown in Lbmc. Lanes 3 - 9 bacteroid extracts, 2µg protein loaded per lane. Panel A was probed with anti-DME serum, panel B with anti-TME serum. Blots were developed using an ECL reagent and exposed to X-ray film for 20 seconds. Lane headings indicate strain number, small numbers at the bottom of each lane indicates relative intensity of the band compared to Rm1021 in lanes 1. Genotypes: Rm1021, wild type; RmG994, *dme3 tme4*; RmG455, *dme3*; RmG995, *tme4*; RmH897,RmH899, *dme3 tme4 dtme*[+]; RmH898, RmH900, *tme4 dtme*[+].

extracts (Figure 4, panel B, lanes 6,7,8 and 9) and appears to be similar to the level of DME in bacteroids (Figure 4, panel A, lanes 3, 4, 8 and 9). The X-ray film was scanned by a laser densitometer to determine the relative intensities of the bands. These values are shown in Figure 4 as the small numbers at the bottom of each lane with 100% being the intensity of the band in Lane 1 (ie Rm1021 cells grown in LBmc) of each blot.

From these results it appears that TME does not functionally replace DME to produce nodules competent for nitrogen-fixation. Increasing the level of *tme* expression to that of *dme* had no effect on the Fix⁻ phenotype of Dme⁻ *R. meliloti* strains.

CHAPTER 4

Expression of the *Streptococcus bovis* malic enzyme gene in *R. meliloti*

Construction of the *dme-maeE* fusion gene

An NAD$^+$-dependent malic enzyme has been cloned, sequenced and characterized from the Gram positive bacterium *Streptococcus bovis* and given the designation *maeE* (Kawai et al, 1996). A plasmid subclone containing a 1.6kb fragment harbouring the complete open reading frame for *maeE*, which encodes a protein of 40kD, was obtained from Dr. Kawai, Kyoto, Japan. This gene is part of an operon and the promoter was not included in the 1.6kb fragment. In order to express the *maeE* gene in *R. meliloti* in both the free-living and bacteroid state it was decided to reconstruct the *maeE* coding region behind the *dme* promoter. PCR (polymerase chain reaction) was used to generate a DNA fragment of the *maeE* gene with an *SphI* restriction enzyme site at the ATG and a *BamHI* site at the putative transcriptional terminator (Kawai et al, 1996). Two primers were synthesized; for the 5' end of the gene the sequence 5'-GCGC**GCATGCC** AACAAAAGATGTAAAAG-3' was made, and for the 3' end the sequence 5'-**GCGGATCC**GGACAACCTAACTCAAGAGG-3' was used (restriction enzyme recognition sequences shown in bold). After amplification by PCR the DNA fragment was precipitated with ethanol, digested overnight with *SphI* and *BamHI*, isolated from a 1% agarose gel and ligated into plasmid pTH400 (containing the

dme promoter with the *SphI* site at the ATG in pUC119, see Figure 3) also digested

with *SphI* and *BamHI*. Plasmid DNA from one positive clone (pTH434) was

transformed into the *E. coli* malic enzyme deficient strain EJ1321 (Hansen and Juni,

1975) and one resulting transformant (designated EcJ271) was used to test if

growth on M9 agar with succinate as sole carbon source was restored.

EcJ271(pTH434) did grow on M9 succinate suggesting that the *S. bovis maeE* gene

was being functionally expressed. Although the plasmid pTH434 contains the *dme*

promoter transcription of the *dme-maeE* fusion gene in *E. coli* is probably driven

from the *lac* promoter of pUC119 (Driscoll and Finan, 1996)

To transfer the gene into *R. meliloti* it was necessary to clone the *dme*

promoter-*maeE* gene fragment (designated *dmaeE* for the gene and MAE for the

protein) into the gentamycin resistant transfer vector pUCP30T (Schweizer et al,

1996). Plasmid pTH434 was digested with *HindIII* and *BamHI*, the gene fragment

isolated from a 1% agarose gel and ligated into pUCP30T digested with the same

enzymes. One positive clone was further purified and the associated plasmid

designated pTH443.

Transfer of the *dmaeE* fusion gene to *R. meliloti*

Plasmid pTH443, in *E. coli* DH5α, was mated into *R. meliloti* strain RmG994

(*dme-3::Tn5, tme-4::ΩSp*) in a triparental mating with pRK600 in *E. coli* MT616, as

helper plasmid and single recombination events selected for by plating on LB with

60μg/ml gentamycin, 200μg/ml neomycin and 100μg/ml spectinomycin. Two

colonies were further purified for analysis and designated strains RmH911 and RmH912. Because of only limited homology between *maeE* and *dme* (<30% identity at the nucleotide level) the recombination events should have occurred in the *dme* promoter region. To confirm this gentamycin resistance was transduced from RmH911 into Rm1021 using the general transducing phage ΦM12 (Finan et al, 1984) and selecting on LB with 60μg/ml gentamycin and 200μg/ml streptomycin. Ten colonies were then patched onto LB, 200μg/ml neomycin and LB, 100μg/ml spectinomycin. All colonies were found to be neomycin resistant due to co-transduction of the Tn5 insertion in the *dme* gene. Plasmid pTH443 was simlarly mated into wild type *R. meliloti* strain 1021 and single cross-over events selected for by plating onto LB with 60μgs/ml gentamycin and 200μgs/ml streptomycin. This resulted in strains still expressing DME and TME as well as the *S. bovis* malic enzyme (MAE). Again two colonies were purified for analysis and the resulting strains were given the designation RmH917 and RmH918.

Plasmid pTH443 was also mated into *R. meliloti* strain H194 (*pckA-1::Tn3HoHoSp, pod-1, dme-1::Tn5, tme-4::ΩSp*) which is unable to grow on M9 with succinate as sole carbon source because the two known pathways for glucose production from succinate are blocked (see Figure 1). After selection on LB with 60μg/ml gentamycin, 200μg/ml neomycin and 100μg/ml spectinomycin resulting colonies were streaked onto M9mc with either succinate or glucose as sole carbon source. All grew equally well on either succinate or glucose indicating that the *dmaeE* gene was being expressed and was functional in *R. meliloti*.

Sonicated cell extracts were prepared from strains RmH911, RmH912, RmH917 and RmH918 grown in LBmc, along with appropriate controls and assayed for malic enzyme activity using the pyruvate colorimetric assay. The results are shown in Table 5.

Table 5: Enzyme activity of _R. meliloti_ strains carrying the _S. bovis_ malic enzyme

Strain	Genotype	NAD$^+$ activity*	NADP$^+$ activity*	MDH activity**
Rm1021	wild type	51 +/- 6.2	43.6 +/- 1.9	1025.7 +/- 6.1
RmG994	_dme3 tme4_	24 +/- 1.9	0.4 +/- 0.4	1113.8 +/- 37
RmH911	_dme3 tme4 dmae$^+$_	51 +/- 5.1	21.5 +/- 1.3	773.1 +/- 45.2
RmH912	_dme3 tme4 dmae$^+$_	56.7 +/- 7.4	35.6 +/- 1.9	1221.4 +/- 7.8
RmH917	_dmae$^+$_	62.6 +/- 7.1	42.7+/- 3.1	941.8 +/- 21.8
RmH918	_dmae$^+$_	51.7 +/- 3.8	34.5 +/- 4.4	846.9 +/- 22.5

*specific activity determined as nmoles pyruvate formed /min/mg protein, expressed as the mean +/- SE of triplicate samples.
**specific activity determined as nmoles NADH formed /min/mg protein, expressed as the mean +/- SE of triplicate samples.

The _S. bovis_ enzyme appears to be active in these assays at specific activities similar to that of DME. As previously reported (Kawai et al, 1996) and unlike DME, it also has significant activity with NADP as cofactor in the assay as performed here. Interestingly there does not appear to be an additive effect in the strains expressing DME, TME and the _S. bovis_ enzyme.

Plant growth assays with _dmaeE_.

Alfalfa seedlings were planted in Leonard assemblies and inoculated with the strains listed above in Table 5. After 28 days the plants were collected, nodules

were picked, bacteroids isolated and sonicated extracts prepared from them. The plant shoots were then dried for 2 weeks and dry weights determined. Somewhat surprisingly the plants inoculated with strains RmH911 and RmH912, expressing only the *dmaeE* gene, were Fix⁻, being indistinguishable from uninoculated and control plants as can be seen from the plant dry weight values in Table 6.

Table 6: Plant dry weights

Strain	Genotype	phenotype	#plants	wt (mgs)/plant	% wild type
Rm1021	wild type	Fix⁺	29	28 +/- 1.5	100
RmG994	dme3 tme4	Fix⁻	28	7.2 +/- 0.2	25
RmH911	dme3 tme4 dmae⁺	Fix⁻	30	9 +/- 1	32
RmH912	dme3 tme4 dmae⁺	Fix⁻	28	8.9 +/- 0.03	32
RmH917	dmae⁺	Fix⁺	29	22.1 +/- 0.7	79
RmH918	dmae⁺	Fix⁺	28	22.8 +/- 3.7	81
control	none	Fix⁻	40	5.25 +/- 0.6	19

dry weights are expressed as the mean +/- SE of three pots of ~10 plants
Fix⁺ describes large green plants with large pink root nodules.
Fix⁻ describes small yellow plants with small whitish root nodules.

The cause for the Fix⁻ phenotype of the *dme tme dmae⁺* strains RmH911 and RmH912 may be due to either the failure of the *S. bovis* enzyme to complement the function of the DME protein or to the lack of expression of *dmaeE* in bacteroids. To determine if the MAE protein was active malic enzyme assays were carried out. Because only a small amount of protein can be extracted from Fix⁻ nodules, bacteroid extracts from only the Fix⁺ nodules were assayed and the results are shown in Table 7.

Table 7: Malic enzyme activities in bacteroid extracts

Strain	Genotype	NAD$^+$ activity*	NADP$^+$ activity*
Rm1021	wild type	42.6 +/- 2.9	18.7 +/- 0.6
RmG994	dme3 tme4	ND	ND
RmH911	dme3 tme4 dmae$^+$	ND	ND
RmH912	dme3 tme4 dmae$^+$	ND	ND
RmH917	dmae$^+$	32.8 +/- 1.1	10.7 +/- 0.9
RmH918	dmae$^+$	47 +/- 0.9	12.7 +/- 0.9

* specific activity expressed as nmoles pyruvate formed /min/mg protein, expressed as the mean +/-
SE of triplicate samples.
ND = not determined

The Fix⁻ nodules from RmH911 and RmH912 did not provide enough protein

to assay for enzyme activity in the pyruvate assay used for crude extracts and it was

not possible to tell from the Fix$^+$ nodule extracts, RmH917 and RmH918, if there was

any activity due to the MAE enzyme or just from the DME enzyme. Antibodies for

the S. bovis enzyme are not available so it was not possible to use Western blot

analysis to determine if the MAE protein was present in the bacteroid extracts.

However, because of the significant difference in the sizes of the DME and MAE

holoenzymes (8 x 80kD and 2 x 40kD respectively) they are easily separated on

non-denaturing acrylamide gels which can then be stained in situ for malic enzyme

activity.

Non-denaturing gel electrophoresis of MAE extracts.

The gel system described by Sigma for protein molecular weight

determinations was used (Mitsch et al, 1998). 30μg total protein for each sample

Figure 5: Non-denaturing gel of *R. meliloti* strains expressing *dmae*.
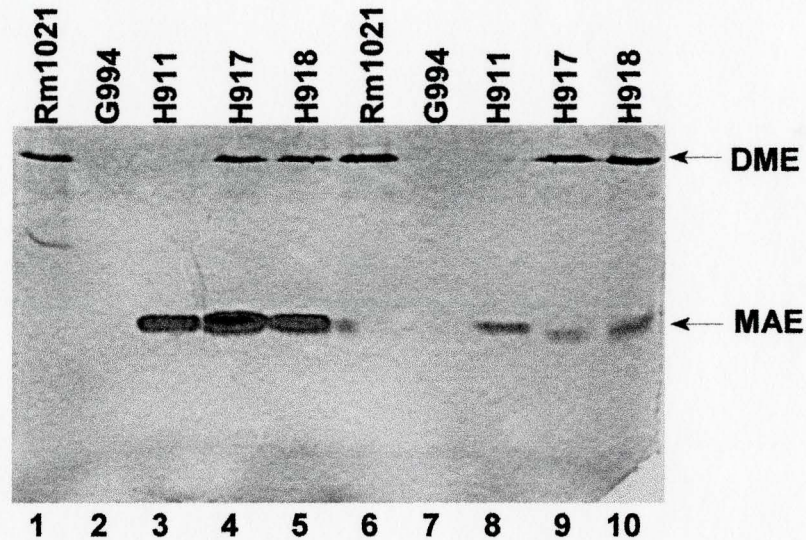
30μgs protein per lane was run on a 7% acrylamide gel. Gels were then stained for the presence of NAD$^+$-dependent malic enzyme activity. Lanes 1 - 5 contain extracts from cells grown in LBmc, lanes 6 -10 contain bacteroid cell extracts.

Lane headings indicate *R. meliloti* strains.

Genotypes: Rm1021, wild type; RmG994, *dme3 tme4*;

RmH911, *dme3 tme4 dmae$^+$*, RmH917 and RmH918, *dmae$^+$*.

was electrophoresed through 7% non-denaturing gels until the bromophenol blue dye was near the bottom. The gels were then washed and stained for NAD$^+$-dependent malic enzyme activity *in situ*. After staining the gels were dried which increased the intensity of the band staining considerably. The resulting gel as seen in Figure 5 shows how the two enzyme activities are resolved. A slowly migrating band corresponding to DME is visible in extracts from Rm1021, RmH917 and RmH918 grown in LBmc (lanes 1, 4 and 5) and occurs at a similar intensity in bacteroid extracts from the same strains (lanes 6, 9 and 10). A faster migrating band, corresponding to MAE, is detected in RmH911, RmH917 and RmH918 grown in LBmc (lanes 3, 4 and 5) but is greatly reduced in intensity in the bacteroid extracts from those strains (lanes 8, 9 and 10). This suggests that the MAE protein is either not expressed efficiently in bacteroids, is present but is inactivate, or is expressed but selectively degraded or unstable in bacteroids.

Northern blot analysis of *dmae* expression.

The low level of activity of MAE seen in bacteroid extracts may be because of low expression of the *dmae* gene. It may not be transcribed efficiently in bacteroids even though transcription is being driven by the *dme* promoter, or the RNA may be unstable. To address this question RNA was prepared from bacteroids and cells grown in LBmc in order to perform Northern blot analysis. Approximately 10 - 20µgs of each RNA was electrophoresed in a 1.2% agarose-formaldehyde gel in MOPS buffer (Ausubel et al, 1989), transferred to Biotrans

nylon membrane (NEN) and probed with a ^{32}P-labeled 500bp DNA fragment of the *dme* promoter and untranslated leader sequence. In addition 2 - 4µgs of each RNA was run on a parallel gel and stained with ethidium bromide to determine the integrity of the RNA preparations. As can be seen in Figure 6 the RNA prepared from free living cells is of good quality (panel A, lanes 1 - 5) and on the blot a band corresponding to the *dmae* transcript is clearly visible (panel B, lanes 2 - 5). The endogenous *dme* transcript should also have been detected with the probe used. The predicted size of the *dme* transcript is approximately 3kb, however distortion or blocking of the membrane by the 23S rRNA which is the same size (ie 2.9kb) may account for the lack of signal, or alternatively the *dme* transcript may be more rapidly degraded. The RNA prepared from root nodules was somewhat degraded (panel A, lanes 6,7 and 8) and in Figure 6 panel B no bands of any kind are visible on the blot for those samples. Faint bands corresponding to the *dmae* transcript were visible on a longer exposure of the membrane suggesting that the RNA from the *dmae* gene is present. Although inconclusive the fact that bands corresponding to the *dmae* transcript are detected in nodule RNA when even the background smearing seen in the RNA from free living cells is absent suggests that transcription is occurring as expected.

Panel A: 2 - 4µg RNA were electrophoresed on a 1.2% agarose-formaldehyde gel in MOPS buffer at 3V/cm. The gel was stained in MOPS buffer containing ethidium bromide then photographed. Lanes 1 - 5 contain RNA from free living cells, lanes 6 - 8 contain RNA from root nodules. The positions of the bacterial 23S and 16S rRNA are indicated. M is a 1kb size marker, bright band = 3kb (MBI Fermentas).

Panel B: 10 - 20µg of the same RNA samples were run on a similar gel and then transferred to a membrane and probed with a [32]P-DNA fragment of the *dme* promoter. Lane designations are as in panel A. The position of the 23S rRNA band is indicated and the position of the *dmae* transcript. Lane headings indicate *R. meliloti* strains

Genotypes: Rm1021, wild type; RmH911 and RmH912, *dme3 tme4 dmae*[+]; RmH917 and RmH918, *dmae*[+].

Figure 6: Gel and northern blot on RNA from *dmae* strains.
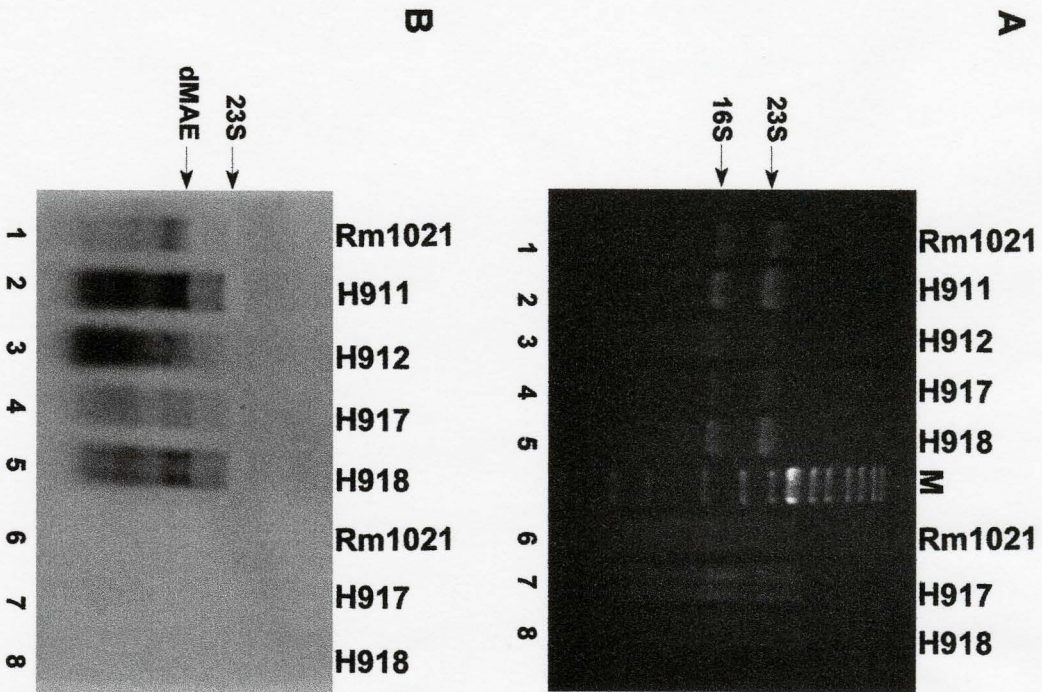
CHAPTER 5

Expression of a truncated DME protein in *R. meliloti.*

Construction of a truncated *dme* gene.

Analyses of the deduced amino acid sequences of the *R. meliloti* DME and TME proteins revealed that unlike previously characterized malic enzymes they appear to have a chimeric structure. The amino terminal 420 amino acids showed strong homology to other malic enzymes but the carboxyl terminal 350 amino acids diverged significantly from that of known malic enzymes. However this region did show limited homology to phosphotransacetylase (PTA) enzyme (26% identity, 46% similarity between DME and the phosphotransacetylase from *Methanosarcina thermophila,* Mitsch et al, 1998). Mitsch et al, 1998, also showed that deletion of the C-terminal region of both DME and TME reduced but did not abolish malic enzyme activity when assayed *in situ* in non-denaturing acrylamide gels. The oligomeric state of the holoenzymes was affected in that both truncated enzymes appeared to migrate as dimers instead of octamers on the non-denaturing gels. However, for these experiments only the enzymes over-expressed in *E. coli* were analyzed, how the truncated enzymes might function in *Rhizobium* is not known.

A single *Pstl* restriction enzyme site exists in the *dme* structural gene, another *Pstl* site is present in the pUC119 polylinker at the 3'-end of the *dme* gene in plasmid pTH139. Cleavage of pTH139 DNA with *Pstl* followed by religation resulted

in a *dme* gene truncated at amino acid 449 with 3 additional amino acids from the pUC119 polylinker before translation terminates at the *Xba*I site in the polylinker. The resulting plasmid was sequenced to confirm the deletion had occurred as predicted and was designated pTH452. This plasmid was transformed into the malic enzyme/*pck* deficient *E. coli* strain, EJ1321 (Hansen and Juni, 1975) which is unable to grow on M9 plates with succinate as sole carbon source. Growth was fully restored with EJ1321(pTH452) transformants indicating that the truncated DME protein (designated DMEΔPst) was expressed and functional.

Transfer of *dmeΔPst* to *R. meliloti*.

The *dmeΔPst* gene was excised from pTH452 as a *Hind*III - *Xba*I fragment and inserted into the same sites of the transferable vector pUCP30T (Schweizer et al, 1996) to give the plasmid pTH458. This was then transferred into two different *R. meliloti* strains carrying two different *dme* mutant alleles, RmG454 (*dme-2::Tn5*) and RmG456 (*dme-1::Tn5*), in triparental matings as described previously. Single recombination events were selected for by plating on LB with 60μg/ml gentamycin, 200μg/ml neomycin. These recipient strains were chosen because the positions of the Tn5 insertions have been mapped as being either in the middle (*dme2*) or in the C-terminal (*dme1*) region of the *dme* gene (Driscoll and Finan, 1997). This maximizes the probability that the recombination event between the truncated *dme* gene in the plasmid and the interrupted gene in the chromosome occurs in the 5'-region of the gene, 5' of the transposon insertion. This was important because

recombination 3' of the transposon would result in regeneration of an intact full length *dme* gene. Two colonies from each mating were further purified for analysis and given the designations RmH996, RmH998 (from RmG454) and RmH999, RmH1000 (from RmG456).

Sonicated cell extracts were prepared from 5 ml overnight cultures, grown in LBmc, to assay for the presence of the truncated protein. $2\mu g$ total protein was electrophoresed on 7% SDS polyacrylamide gels, transferred to Imobilon P nylon membranes and probed with rabbit anti-DME serum, followed by goat anti-rabbit HRP conjugated antibody and visualized using the ECL system, the results are shown in Figure 7. Neither parental strain showed detectable full length DME protein (Figure 7, lanes 2 and 6), all four recombinant strains showed the presence of a band migrating at approximately 50kD corresponding to the truncated DME protein (Figure 7, lanes 3, 4, 7 and 8).

The sonicated cell extracts were assayed for both NAD^+- and $NADP^+$- dependent malic enzyme activity using the colorimetric assay for pyruvate formation. The results of these assays are shown below in Table 8.

Figure 7: Western blot analysis of extracts of dme△Pst strains
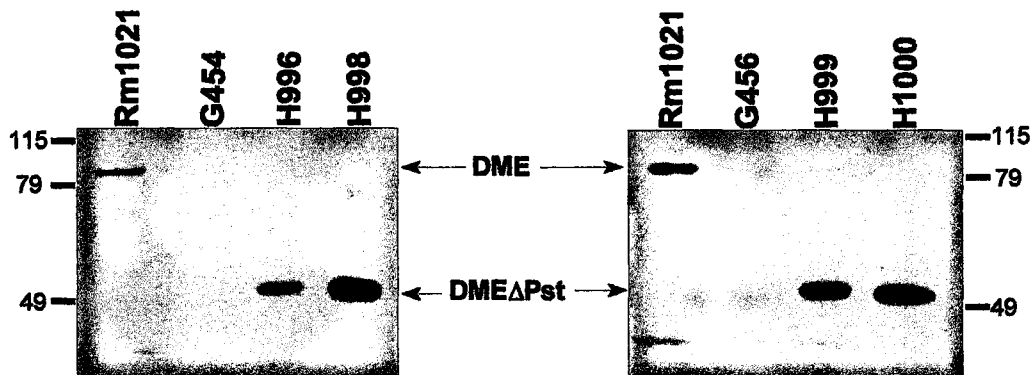
2μgs total protein was run in each lane on a 7% SDS polyacrylamide gel, transferred to membranes and probed with anti-DME serum and developed using the ECL system. Membranes were exposed to X-ray film for 1 minute. Positions and sizes in kD of the Biorad prestained markers are indicated at the sides. The positions of the full length and truncated DME proteins are indicated by arrows. Lane headings refer to R. meliloti strain number.

Genotypes: Rm1021, wild type; RmG454, dme2; RmG456, dme1;

RmH996 and H998, dme2 dme△Pst⁺; RmH999 and H1000, dme1 dme△Pst⁺.

Table 8: Malic enzyme activities of dmeΔPst cell extracts

Strain	Genotype	NAD⁺ activity*	NADP⁺ activity*	MDH activity**
Rm1021	wild type	57.3 +/- 5.4	81.7 +/- 2.7	244.3 +/- 4.8
RmG454	dme2	15.1 +/- 0.3	71.6 +/- 4.2	231.7 +/- 4.4
RmH996	dme2,dmeΔPst⁺	66.1 +/- 7.1	76.3 +/- 3.6	250.3 +/- 3.3
RmH998	dme2,dmeΔPst⁺	104.7 +/- 6.1	103.6 +/- 4.3	223.0 +/- 4.0
RmG456	dme1	5.5 +/- 2.3	93.4 +/- 3.7	294.0 +/- 2.5
RmH999	dme1,dmeΔPst⁺	75.6 +/- 2.8	69.8 +/- 3.8	202.3 +/- 9.6
RmH1000	dme1,dmeΔPst⁺	95.2 +/- 13.5	79.3 +/- 2.6	225.0 +/- 5.1

* specific activity measured as nmoles pyruvate formed /min/mg protein expressed as the mean +/- SE of triplicate samples
** specific activity as nmoles NADH formed/min/mg protein, mean +/- SE of triplicate samples

Plant growth assays with dmeΔPst strains

Alfalfa seedlings were planted in Leonard assembly pots as described previously and inoculated with the R. meliloti strains listed in Table 8. After 28 days growth the plants were collected and the plant shoots dried to determine dry weight. The nodules were picked, bacteroids isolated and sonicated extracts prepared. The plants inoculated with the strains expressing the dmeΔPst gene (RmH996, RmH998, RmH999, RmH1000) were all green and healthy and indistinguishable from plants inoculated with wild type R. meliloti (Figure 8), indicating that the nodules were fixing nitrogen. This observation was confirmed by the plant dry weights as seen in Table 9.

Figure 8: Alfalfa plants inoculated with the *dme∆Pst R. meliloti* strains

| CONTROL | Rm1021 | RmG454 | RmH996 | RmH998 |

| CONTROL | Rm1021 | RmG456 | RmH999 | RmH1000 |

Seedlings were planted in Leonard assemblies with Jensens medium at 10 plants/pot and watered with sterile distilled water as required. Photographs taken after 24 days incubation in a Conviron growth chamber with 16 hours light at 23°C. Genotypes: Rm1021, wild type; Rm G454, *dme2*; RmH996 and RmH998, *dme2*, *dme∆Pst⁺*; RmG456, *dme1*; RmH999 and RmH1000, *dme1*, *dme∆Pst⁺*.

Table 9: Plant dry weights with *dmeΔPst R. meliloti* strains

Strain	Genotype	Phenotype	# plants	wt/plant (mg)	% wild type
Rm1021	wild type	Fix⁺	30	57.3 +/- 6.7	100
RmG454	*dme2*	Fix⁻	30	7.3	13
RmH996	*dme2,dmeΔPst⁺*	Fix⁺	29	56.2 +/- 4.7	98
RmH998	*dme2,dmeΔPst⁺*	Fix⁺	29	44.1 +/- 0.7	77
RmG456	*dme1*	Fix⁻	29	7.6	13
RmH999	*dme1,dmeΔPst⁺*	Fix⁺	33	45.1 +/- 1.7	79
RmH1000	*dme1,dmeΔPst⁺*	Fix⁺	30	45.0 +/- 3.5	79
control	none	Fix⁻	20	7.0	12

Wt/plant determined as the mean +/- SE of three pots of ~10 plants/pot.
Fix+ refers to large, green plants with large, pink nodules,
Fix- is determined as small, yellow plants with small, whitish nodules.

Assays for the presence of the truncated DME protein in bacteroids

Sonicated extracts prepared from the the Fix⁺ bacteroids were assayed for

NAD⁺- and NADP⁺- dependent malic enzyme activity using the colorimetric assay

for pyruvate formation. The results are shown below in Table 10.

Table 10: Malic enzyme activities of *dmeΔPst* bacteroid extracts

Strain	Genotype	NAD⁺ activity*	NADP⁺ activity*	MDH activity**
Rm1021	wild type	70.8 +/- 1.5	22.9 +/- 1.7	3383 +/- 42
RmH996	*dme2,dmeΔPst⁺*	51.8 +/- 3.9	17.8 +/- 1.0	4521 +/- 115
RmH998	*dme2,dmeΔPst⁺*	57.3 +/- 1.9	10.9 +/- 1.3	2067 +/- 132
RmH999	*dme1,dmeΔPst⁺*	92.3 +/- 4.6	14.0 +/- 2.7	4394 +/- 202
RmH1000	*dme1,dmeΔPst⁺*	112.8 +/- 1.0	12.0 +/- 2.6	4992 +/- 126

* specific activity measured as nmoles pyruvate formed /min/mg protein expressed as the mean +/- SE of triplicate samples
** specific activity as nmoles NADH formed/min/mg protein, mean +/- SE of triplicate samples.

The level of NAD$^+$-dependent malic enzyme activity in the bacteroids expressing the truncated DME protein (ie: RmH996, 998, 999 and 1000) were approximately equal to that of the wild type full length enzyme. The lower malate dehydrogenase activity observed for the RmH998 extract seen in Table 10 suggests that some inactivation or denaturation of proteins in this extract may have occured during preparation, which may account for the lower values in the malic enzyme assays. All the strains tested retain the normal *tme* gene and as expected for bacteroid extracts the level of NADP$^+$-dependent malic enzyme is reduced. This indicates that the truncated DME protein is not affecting *tme* expression and that the Fix$^+$ phenotype observed is not a result of altered *tme* expression.

To confirm that only the truncated DME protein was present in the bacteroids the extracts were subjected to SDS PAGE and Western blot analysis. 2µgs protein per lane was electrophoresed on 7% polyacrylamide SDS gels then transferred to membranes. The blots were probed with either rabbit anti-DME (Figure 9, panel A) or rabbit anti-TME serum (Figure 9, panel B), followed by goat anti-rabbit IgG conjugated to HRP and detected with the ECL system. As can be seen in Figure 9, panel A only the truncated protein was present in the bacteroids from RmH996, RmH998, RmH999 and RmH1000. Figure 9, panel B confirms the data from the NADP$^+$-dependent malic enzyme assays that the level of TME is unchanged.

Transfer of the *dme2 dmeΔPst* allele to a Tme$^-$ strain.

To rule out the possible involvement of TME in restoring nitrogen fixing

Figure 9: Western blot analysis of *dme△Pst* bacteroid extracts.

A

DME →

← DME△Pst

B

TME →
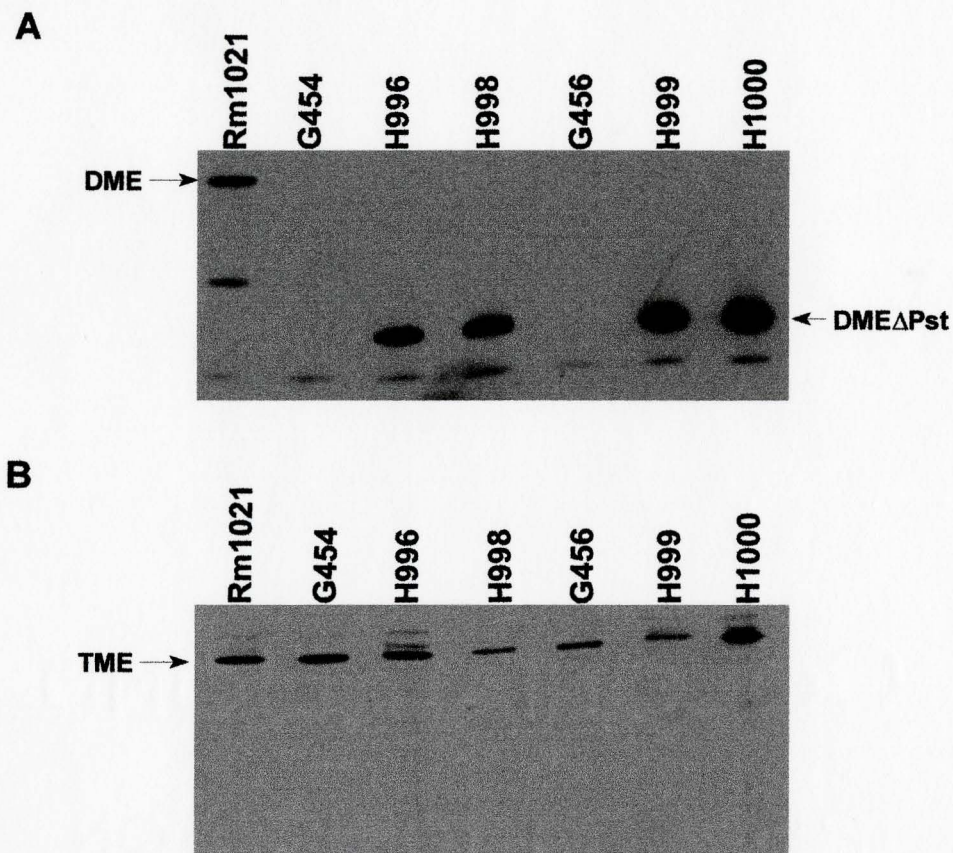
2μgs total protein/lane was run on 7% SDS polyacrylamide gels, transferred to membranes and probed with either anti-DME serum (panel A) or anti-TME serum (panel B), developed with the ECL system and exposed to X-ray film for 1 minute (panel A) or 2 minutes (panel B). Positions of TME, DME and the truncated DME protein are indicated by arrows. Lane headings represent *R. meliloti* strains.

Genotypes: Rm1021, wild type; RmG454, *dme2*; RmG456, *dme1*; RmH996 and H998, *dme2 dme△Pst$^+$*; RmH999 and H1000, *dme1 dme△Pst$^+$*.

activity to the *dme-, dmeΔPst+* strains, the *dme-2::Tn5::dmeΔPst* gene region was

transduced from RmH998 into the Tme⁻ strain RmG995 (*tme-4::ΩSp*). Two resulting

Gm^r, Nm^r, Sp^r colonies were purified and designated RmK120 and RmK121.

Sonicated extracts were prepared from cells grown in LBmc and analyzed by

western blot to ensure that only the DMEΔPst protein was present (Figure 10, panel

A, lanes 3, 4, 5). Assays for both NAD^+- dependent and NADP^+-dependent malic

enzyme activity were also performed to ensure no TME activity was present, Table

11.

Table 11: Malic enzyme activities of *dmeΔPst*, *tme⁻* cell extracts

Strain	Genotype	NAD^+ activity*	NADP^+ activity*	MDH activity**
Rm1021	wild type	64.6 +/- 2.7	62.7 +/- 5.5	828.4 +/- 17.4
RmH998	*dme2 dmeΔPst*	55.1 +/- 0.5	47.6 +/- 3.5	654.2 +/- 14.7
RmK120	*dme2 tme4 dmeΔPst*	39.0 +/- 1.5	0	820.3 +/- 67.0
RmK121	*dme2 tme4 dmeΔPst*	36.3 +/- 1.8	0	935.6 +/- 78.2

* specific activity measured as nmoles pyruvate formed /min/mg protein expressed as the mean +/-
SE of triplicate samples
** specific activity as nmoles NADH formed/min/mg protein, mean +/- SE of triplicate samples.

These strains were then inoculated onto alfalfa seedlings together with

appropriate controls. The phenotype of the plants inoculated with RmK120 and

RmK121 (*dme2 tme4 dmeΔPst+*) was the same as that of the original strain that

still expressed *tme* (RmH998), ie the plants were green and healthy indicative of

nitrogen-fixation competent nodules. After 28 days growth the plants were collected

and dried to determine the dry weights (see Table 12 below). The nodules were

picked from the roots, bacteroids isolated and sonicated extracts prepared.

Table 12: Plant dry weights from dmeΔPst+, tme- strains.

Strain	Genotype	Phenotype	# plants	wt/plant (mg)	%wild type
Rm1021	wild type	Fix$^+$	30	36.3 +/- 5.9	100
RmG994	dme3, tme4	Fix$^-$	32	7.5 +/- 0.3	21
RmH998	dme2 dmeΔPst+	Fix$^+$	30	27.3 +/- 0.9	76
RmK120	dme2 tme4 dmeΔPst+	Fix$^+$	27	21.2 +/- 2.5	59
RmK121	dme2 tme4 dmeΔPst+	Fix$^+$	27	24.0 +/- 0.5	67
control	none	Fix$^-$	21	6.2 +/- 0.8	17

Wt/plant determined as the mean +/- SE of three pots of ~10 plants/pot.
Fix+ refers to large, green plants with large, pink nodules,
Fix- is determined as small, yellow plants with small, whitish nodules.

The results in Table 12 indicate that the plants inoculated with strains expressing the truncated DME protein are somewhat smaller than those inoculated with wild type R. meliloti, having dry weight values of 60 - 70% of wild type. To see if this was as a result of reduced expression of the truncated protein the bacteroid extracts were subjected to SDS PAGE and analyzed by Western blot as before, Figure 10, panel A, lanes 5 - 8. Instead of expression of the truncated protein being reduced it appears to be highly expressed to a far greater degree than the full length protein. To determine if this also resulted in a large amount of malic enzyme activity the extracts were assayed for both NAD$^+$- and NADP$^+$-dependent malic enzyme activity, Table 13.

Figure 10: Western blots on cell and bacteroid extracts expressing DMEΔPst.
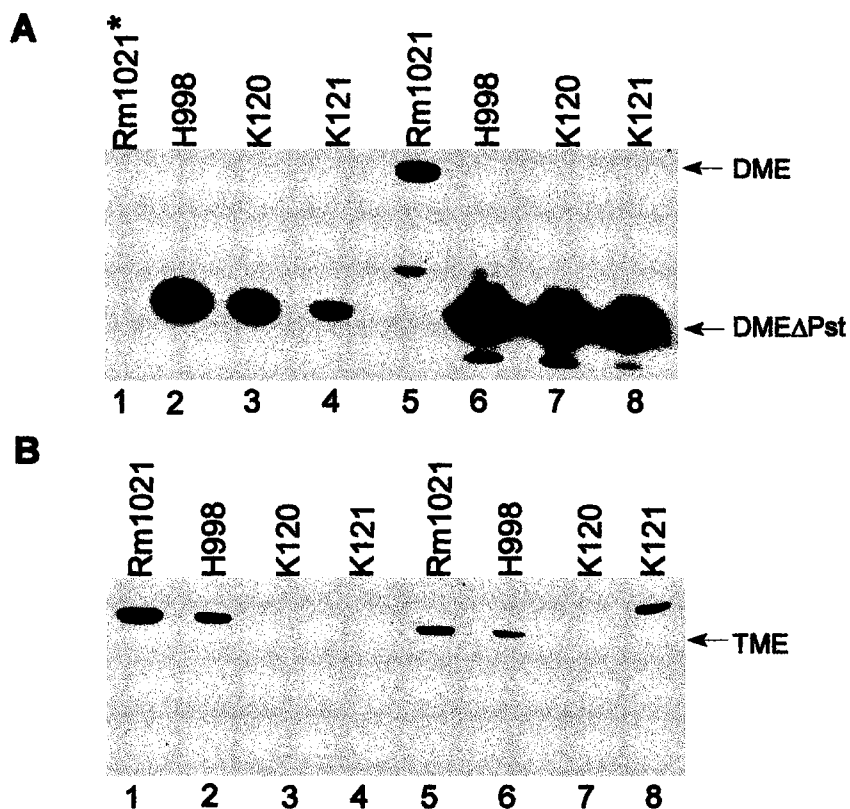
Panel A: 2μgs/lane total protein on a 7% polyacrylamide gel, transferred to membrane and probed with anti-DME serum. Lanes 1 - 4 are extracts from cells grown in Lbmc, lanes 5 - 8 are extracts from bacteroids. Panel B: same extracts as in panel A but probed with anti-TME serum. Lane headings refer to *R. meliloti* strains. * = sample lost.

Genotypes: Rm1021, wild type; RmH998, *dme2 dmeΔPst⁺*; RmK120 and RmK121, *dme2 tme4 dmeΔPst⁺*.

Table 13: Malic enzyme activities of dme∆Pst⁺, tme⁻ bacteroid extracts.

Strain	Genotype	NAD⁺ activity*	NADP⁺ activity*	MDH activity**
Rm1021	wild type	137.8 +/- 4.7	53.6 +/- 2.7	3834.2 +/- 109.8
RmH998	dme2 dme∆Pst⁺	158.4 +/- 23.9	19.9 +/- 0.9	3044.9 +/- 122.9
RmK120	dme2 tme4 dme∆Pst⁺	165.3 +/- 28.5	0	4742.7 +/- 174.3
RmK121	dme2 tme4 dme∆Pst⁺	147.7 +/- 8.8	0	4317.8 +/- 91.9

* specific activity measured as nmoles pyruvate formed /min/mg protein expressed as the mean +/- SE of triplicate samples
** specific activity as nmoles NADH formed/min/mg protein, mean +/- SE of triplicate samples.

From the results in Table 13 it appears that the large increase in protein seen in the bacteroid extracts does not result in an increase in enzyme activity as assayed *in vitro*. However the results from both the *R. meliloti* strains expressing only the truncated DME protein (RmK120, RmK121) or the strains still having a functional *tme* gene present (RmH996,998,999 and 1000), indicate that in fact the NH_2-terminal malic enzyme portion of DME is sufficient to produce nodules competent for nitrogen fixation.

CHAPTER 6

Discussion

Over-expression of TME does not complement loss of DME in bacteroids

Previous work had shown that loss of *dme* function but not *tme* resulted in a Fix⁻ phenotype when such *R. meliloti* strains were inoculated onto alfalfa (Driscoll and Finan, 1993, Driscoll and Finan, 1996). It was also shown by malic enzyme assay and analysis of *lacZ* fusions that expression of *tme* is reduced 5 to 10 fold in bacteroids (Driscoll and Finan, 1997). This has now been shown, by Western blot analysis presented here (and M. Mitsch, personal communication), to be because of a reduced amount of protein present in bacteroids (Figure 4, panel B). It was reasoned that if expression of *tme* could be increased to that of *dme* then the increased TME activity might complement for loss of DME function in bacteroids. To this end a hybrid gene was constructed in which expression of the *tme* coding region was under the control of the *dme* promoter. This hybrid gene (termed *dtme*) was recombined into the chromosome of a *dme tme* null mutant strain of *R. meliloti*. By enzyme assay and Western blot analysis of free living *R. meliloti* cell extracts it was determined that the *dtme* gene was producing functional TME protein (Table 2), however, when inoculated onto alfalfa seedlings the resulting plants, after 28 days of growth, were yellow and stunted indicating that the nodules were Fix⁻ (Table 3). Analysis of bacteroid protein extracts by enzyme assay and Western blot

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revealed that the amount of TME present, expressed from the *dtme* gene, was indeed increased to that of DME (Table 4; Figure 4, panel A).

This result suggests that the TME enzyme, although presumed to catalyze the same reaction as DME, is not effective in supporting nitrogen fixation in bacteroids. One possible reason for this may be the cofactor requirement. Concentrations of $NAD^+ + NADH$ and $NADP^+ + NADPH$ in *B. japonicum* bacteroids have been determined as being in the order of 600nmoles/gm and 40nmoles/gm respectively. However the ratio of $NAD^+/NADH$ is 2 whereas the ratio of $NADP^+/NADPH$ is 0.4 (Tajima and Kouzai, 1989) suggesting a highly active $NADP^+$ reducing system in *B. japonicum* bacteroids. Similar determinations have not been made in the case of *R. meliloti* bacteroids and although the $NADP^+$-dependent malic enzyme of *B. japonicum* is active in bacteroids no genetic evidence exists for its requirement. It is possible that the concentration of $NADP^+$ is too low for TME to be functional. Most of the enzymes of the TCA cycle that utilize nicotinamide cofactors use NAD^+, however an $NADP^+$-dependent isocitrate dehydrogenase, which catalyzes the conversion of isocitrate to α-ketoglutarate, is present and active in *R. meliloti* bacteroids and has a similar K_m for the cofactor (15μM, Chandrasekharan Nambier and Shethna, 1976) as TME (33μM, Voegele et al, in press). Although these two values are similar it does not rule out the possibility that cofactor concentration is limiting for TME in the bacteroid. Based on sequence homology between *dme*, *tme* and other prokaryotic and eukaryotic malic enzymes three regions of the protein may be involved in cofactor binding but what determines the

Figure 11: Diagram of DME,TME and MAE protein domains
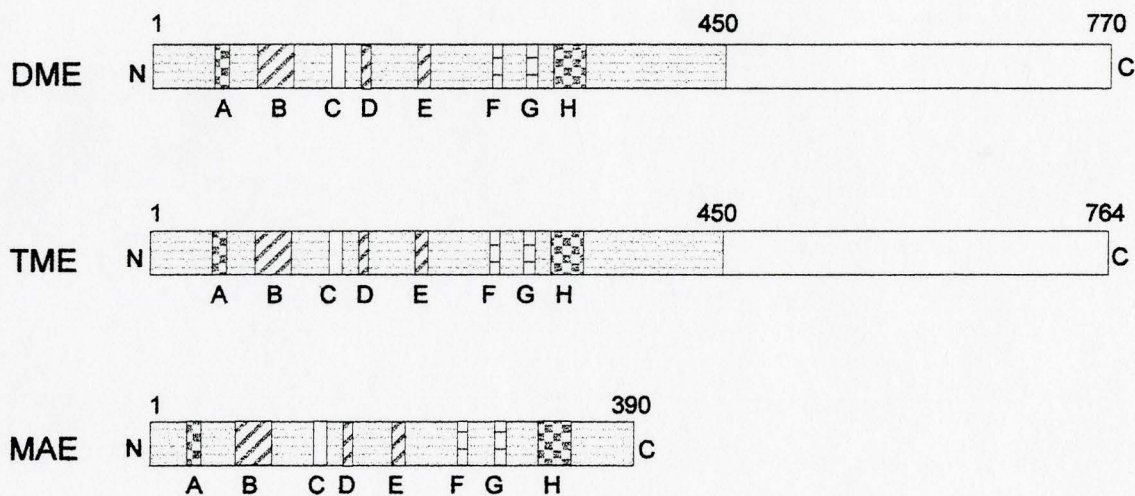
DME: NAD⁺-dependent malic enzyme from *R. meliloti*, TME: NADP⁺-dependent malic enzyme from *R. meliloti*, MAE: NAD⁺-dependent malic enzyme from *S. bovis*. Numbers above refer to amino acid number. Regions of homology are indicated by shaded areas, letter designations are as in Mitsch et al, 1998. A and H: putative substrate binding domains; B, D and E: putative cofactor binding domains; C: possible active site; F and G: unknown function.

Figure 11: Diagram of DME,TME and MAE protein domains

DME: NAD$^+$-dependent malic enzyme from *R. meliloti*, TME: NADP$^+$-dependent malic enzyme from *R. meliloti*, MAE: NAD$^+$-dependent malic enzyme from *S. bovis*. Numbers above refer to amino acid number. Regions of homology are indicated by shaded areas, letter designations are as in Mitsch et al, 1998. A and H: putative substrate binding domains; B, D and E: putative cofactor binding domains; C: possible active site; F and G: unknown function.

specificity for NAD$^+$ or NADP$^+$ is not known (Figure 11, Mitsch et al, 1998). Therefore experiments to change the cofactor specificity of either DME or TME to test this hypothesis would not be trivial.

Very little is known about the role of TME in *R. meliloti* as no phenotype can be ascribed to loss of its function. It may occupy a different location in the cell from DME and so not be able to functionally replace DME. Alternatively, evidence from studies on eukaryotic systems suggests that many metabolic enzymes, particularly those of the TCA cycle, are formed into large multi-enzyme complexes (Beekmans and Kanarek, 1981; Porpaczy et al, 1983). DME and TME may be constituents of different multi-enzyme complexes, with TME not able to form the same interactions as DME and so not able to functionally replace DME.

The malic enzyme from *Streptococcus bovis* cannot complement loss of DME in bacteroids.

The inability of TME to complement loss of DME raised the possibility that cofactor specificity was a limiting factor. The malic enzyme of *Streptococcus bovis* has been purified, characterized and the gene cloned. The enzyme is classified as an EC1.1.1.39 NAD$^+$-dependent malic enzyme, as is DME (Kawai et al, 1996). However the gene (designated *maeE*) encodes a short polypeptide of 40kD encompassing only malic enzyme domains (Figure 11), unlike *dme* which encodes a polypeptide of 80kD which includes a 320 amino acid C-terminal extension of unknown function (Mitsch et al, 1998). The *maeE* coding region was cloned

downstream of the *dme* promoter and transfered into a *dme tme* null mutant strain of *R. meliloti*. By enzyme assay and non-denaturing gel electrophoresis the MAE protein was determined to be present and active (Table 5; Figure 5). The *dme-maeE* (*dmaeE*) hybrid gene was also transfered into a wild type strain of *R. meliloti*. Interestingly, in crude sonicated extracts from these strains there was no additive effect from having two NAD$^+$-dependent malic enzymes present (Table 5). However on non-denaturing gels stained for NAD$^+$-dependent malic enzyme there was an additive effect evident (Figure 5). In the malic enzyme assays the substrate is not limiting, therefore this puzzling result suggests that some kind of inhibitor or other limiting factor is present in the crude cell extract that can be removed by electrophoretic separation.

The strains carrying the *dmaeE* gene were inoculated onto alfalfa seedlings, however after 28 days of growth only the strains which retained DME function appeared healthy. The strains expressing only the *dmaeE* gene produced small stunted plants indicative of Fix⁻ nodules (Table 6). Analysis of bacteroid extracts by non-denaturing gel electrophoresis revealed that the activity of the MAE enzyme was greatly reduced from that of cells grown in culture (Figure 5). Root nodules from these strains were subsequently assayed for acetylene reduction activity and were found to have ~10% the activity of nodules from wild type *R. meliloti* (M. Mitsch personal communication) which may account for the slight increase in plant dry weight from the controls (Table 6).

It is not known why the level of MAE protein as determined by activity on non-

denaturing gels is so greatly reduced. Antibodies against the MAE protein are not available and so it is not possible to determine if the reduced activity is because of reduced amount of protein or because of some inhibition of activity. Northern blot analysis of RNA isolated from both bacteroids and cells in culture was performed to determine if the *dmaeE* was being transcribed (Figure 6). On a long exposure of the blot a band corresponding to the *dmae* transcript in bacteroids was visible, but the results were inconclusive.

Streptococcus bovis is a Gram positive bacterium with a genome of low G+C content (40% in coding sequences), whereas *R. meliloti* is Gram negative with a genome of high G+C content (61% in coding sequences). It is not altogether surprising that heterologous expression of the *S. bovis* malic enzyme is problematic. What is surprising is that the protein was expressed and active in cells grown in culture but not in bacteroids. This suggests that there are previously unidentified control mechanisms in the bacteroid that are not present in the free living state. The product of the *E. coli degP* gene is a protease thought to be involved in the degradation of incorrectly folded proteins (Strauch and Beckwith, 1988). A homologous gene has been identified in *R. meliloti* (Glazebrook et al, 1996) and *B. japonicum* (Narberhaus et al, 1998) and is known to be present in *R. meliloti* bacteroids (Glazebrook et al, 1996) but whether it would be activated in this situation is unknown. In addition the *S. bovis maeE* gene is part of an operon and is co-expressed with a malate specific permease gene (Kawai et al, 1997). It is possible that correct functioning of MAE requires the permease protein to be

present, particularly in a situation where malate is possibly the sole carbon source such as in bacteroids.

Deletion of the C-terminal region of DME appears to have no effect on function.

The malic enzymes of R. meliloti and several other Gram negative bacteria have a chimeric structure, being composed of an N-terminal malic enzyme region and a C-terminal extension of unknown function (Figure 11, Mitsch et al, 1998). A convenient Psfl restriction enzyme site located at the codon for amino acid 449 in the dme gene allows easy deletion of the C-terminal non-malic enzyme domain. Previous work on this truncated protein expressed in E. coli suggested that the C-terminal region was required for formation of an octameric holoenzyme but not for dimerization or for malic enzyme activity (Mitsch et al, 1998). Therefore the function of the C-terminal region remains some thing of a mystery.

The plasmid encoding the truncated DME enzyme (designated DMEΔPst) was transfered into two R. meliloti strains carrying different mutant alleles of dme but retaining the wild type tme gene. Analysis of crude cell extracts from the strains grown in culture confirmed expression and function of the truncated enzyme (Figure 7; Table 8). The strains were inoculated onto alfalfa seedlings and after 28 days growth the plants inoculated with the strains expressing dmeΔPst looked indistinguishable from the wild type plants, indicating that the nodules were competent for nitrogen fixation (Figure 8; Table 9). The bacteroids from the root nodules were analyzed to confirm that the truncated DME was being expressed and

that no full length protein was present (Figure 9, panel A). In addition TME levels were analyzed to ensure that no alteration in *tme* expression had occurred and might be contributing to the Fix⁺ phenotype (Figure 9, panel B).

To confirm that TME was having no effect the *dme2* mutant allele containing the recombined *dmeΔPst* plasmid was transduced into a Tme⁻ strain. As before crude cell extracts were assayed for enzyme activity and for presence of the protein by Western blot (Table 11; Figure 10). From the enzyme assays the activity of the truncated DME enzyme was somewhat reduced from wild type levels (Table 11) and only the truncated protein was present on the Western blot (Figure 10). The *R. meliloti* strains expressing only the truncated DME together with the parent strain that still retained the wild type *tme* gene were inoculated onto alfalfa seedlings with appropriate controls. After 28 days growth the plants were green and healthy as expected but the plant dry weights revealed that the plants were a little smaller than those inoculated with wild type *R. meliloti* (Table 12). Interestingly analysis of the bacteroid extracts showed that a large amount of truncated DME protein was being produced (Figure 10, panel A), however there was not an increase in enzyme activity (Table 13). The increase in the amount of truncated protein was also evident in the bacteroid extracts of the first experiment using the strains still expressing TME (Figure 9) indicating that it is not a result of loss of TME.

Biochemical studies on DME enzyme purified from *E. coli* strains over expressing the protein from a plasmid showed that acetyl CoA acted as a strong inhibitor of DME activity (Voegele et al, in press). Preliminary results with the

truncated enzyme partially purified from *E. coli* indicated that it was no longer subject to inhibition by acetyl CoA (summarized in Table 14, R. T. Voegele unpublished results). If acetyl CoA acts as a regulator of DME activity this suggests that the truncated enzyme may not be regulated in the same way.

Table 14: Comparison of DME and DMEΔPst

Property	DME	DMEΔPst
K_m malate	9.4mM	36mM
K_m NAD$^+$	89µM	159µM
inhibition by acetyl CoA	yes	no
stimulation by fumarate	yes	no
multimeric state	octamer	dimer

Results with enzymes purified from E. coli strains overexpressing the cloned genes from a plasmid R. T. Voegele, personal communication.

The results seen in Figure 10 would support the hypothesis that the enzyme is not being regulated, possibly at the post- translational level. It is of interest to note that Voegele et al (in press) found that a trypsin sensitive site exists between the malic enzyme domain and the C-terminal domain of DME. Limited digestion with trypsin cleaves the protein into two peptides which are further cleaved by increasing trypsin digestion. Perhaps this protease sensitive site is important for the turnover of the mature protein and by introducing a stop codon in this region a stable peptide is produced that can no longer be regulated in this way. On the other hand TME is not cleaved by trypsin in this way and the C-terminal domain may play a less obvious role in TME functioning (Voegele et al, in press).

The result that the truncated DME enzyme functions almost as well as the full length enzyme is somewhat surprising as it suggests that over 40% of the polypeptide is dispensable. The fact that other Gram negative species, such as *E. coli* and *H. influenza*, as well as *R. meliloti* and *B. japonicum* have these larger malic enzymes would suggest that there is a reason for the C-terminal domains but so far the function, evolutionary and physiological relevance remains unclear.

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APPENDIX

Cloning and expression of the *S. bovis maeP* gene in *R. meliloti.*

Dicarboxylic acids are supplied to the bacteroid from the plant in order to provide the energy required for nitrogen fixation. Whether it is malate, fumarate or succinate that is provided by the plant is unknown. The *maeP* gene of *S. bovis* encodes a malate specific permease and is part of an operon with the *maeE* malic enzyme gene (Kawai et al. 1997). In order to resolve the question as to which dicarboxylic acid is supplied by the plant it was decided to express the *S. bovis maeP* gene in *R. meliloti* under control of the *dme* promoter. The plasmid pSK4 containing the *maeP* gene was obtained from the authors. PCR was used to subclone the coding region into the plasmid containing the *dme* promoter with the *Sph*I site at the ATG (pTH400). A primer was synthesized for the 5' end of the gene to include an *Sph*I site at the ATG: 5'-GAAATA**GCATGC**AAAAGAAATTGCCC-3'. The M13 reverse sequencing primer (5'-AGCGGATAACAATTTCACACAGGA-3') was used as the 3' primer. Following amplification the PCR product was digested with *Sph*I and *Kpn*I and the DNA fragment isolated from an agarose gel. The plasmid pTH400 was similarly digested with *Sph*I and *Kpn*I and the two fragments ligated together. The resulting pUC119 derived plasmid containing the *dme* promoter and the *maeP* coding sequence was designated pTH449. This hybrid gene (*dmaeP*) was then transfered to the vector pUCP30T (*oriT*, Gm^r) by digesting pTH449 with *Hind*III and *Kpn*I, isolating the gene fragment from an agarose gel and

ligating into pUCP30T digested with the same enzymes. The resulting plasmid was given the designation pTH461.

Plasmid pTH461 was transfered into two *R. meliloti* strains carrying two different mutant alleles of the dicarboxylic acid transport (*dct*) gene locus: RmF642 (Rm1021, *dctA14*::Tn5) and RmF647 (Rm1021, *dctA26*::TnphoA) in a triparental mating as described before. Colonies were selected for on LB containing 60μg/ml gentamycin, 200μg/ml streptomycin, 200μg/ml neomycin. Two colonies from each mating were purified for further characterization, RmH947 and RmH948 from RmF642, RmH949 and RmH950 derived from RmF647.

Growth on malate.

Single colonies from the strains listed below in Table 15 were streaked onto M9 plates containing either 15mM glucose, 15mM succinate or 15mM malate and growth was scored after 5 days at 30°C. The results are shown in Table 15.

Table 15: Growth of *dmaeP* strains on malate.

| Strain	Genotype	glucose	succinate	malate
Rm1021	wild type	+	+	+
RmF642	*dctA14*	+	-	-
RmF647	*dctA26*	+	-	-
RmH947	*dctA14, dmaeP*+	+	-	+
RmH948	*dctA14, dmaeP*+	+	-	+
RmH949	*dctA26, dmaeP*+	+	-	+
RmH950	*dctA26, dmaeP*+	+	-	+

As reported by Kawai et al (1997) the *maeP* gene product allows specific growth on malate but not succinate.

Growth of these strains was also measured in liquid culture. 5 ml cultures were grown to saturation in LBmc, the cells were pelleted and washed twice with sterile saline and resuspended in 5 ml sterile saline. Tubes containing 5 ml M9mc + 15mM glucose or 15mM malate were inoculated with 10μl washed cells. The initial OD_{600} was measured and then the tubes were incubated at 30°C. The OD_{600} was determined at various times over a 48 hour time span and the results plotted below.
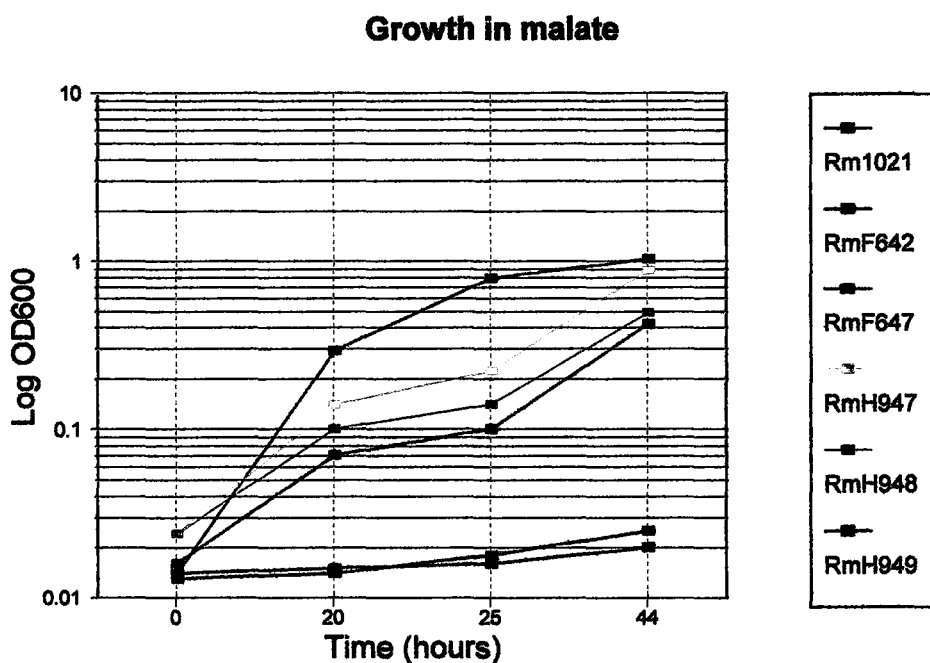
Growth in malate
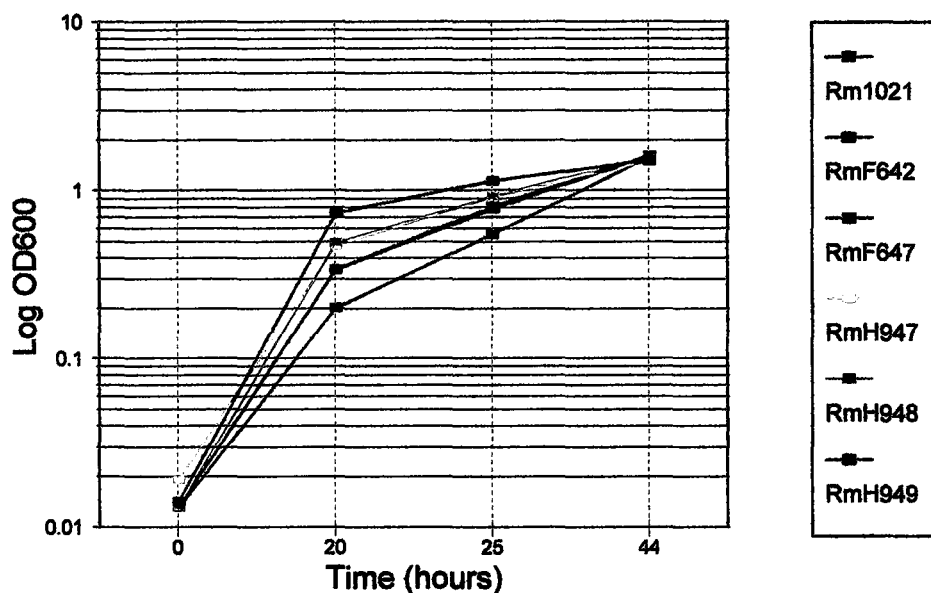
Growth in glucose

The *R. meliloti* strains expressing the *dmaeP* gene grew with malate as the sole carbon source but more slowly than wild type *R. meliloti*. Growth in glucose was basically the same for all strains tested. Assays to determine the relative rate of uptake of either malate or succinate were carried out (R. T. Voegele unpublished results: Book 9, p 2191). The results from these assays are summarized below.

Table 16: Uptake assays on *dmaeP* expressing *R. meliloti* strains.

Strain	Genotype	Succinate* (% WT)	Malate* (% WT)
Rm1021	wild type	60.66 +/- 2.81 (100)	39.38 +/- 0.2 (100)
RmF642	*dctA14*	1.89 +/- 0.14 (3)	1.15 +/- 0.11 (3)
RmH947	*dctA14, dmaeP+*	0.88 +/- 0.17 (1.5)	7.73 +/- 0.25 (20)
RmH948	*dctA14, dmaeP+*	0.81 +/- 0.16 (1.3)	6.85 +/- 0.16 (17)

Cells were grown in M9mc +glucose and malate.
*measured as nmoles/min/mg protein.

The uptake assays show that the S. bovis malate permease transports malate at around 20% the rate of the endogenous Dct system. These strains were inoculated onto alfalfa seedlings and after 28 days the plants were small and yellow indicating that the nodules were Fix⁻. No antibodies to the *S. bovis* malate permease protein are available so because of problems determining if the protein was actually being expressed no further analysis of the bacteroids was undertaken.

Cloning and expression of a truncated *tme* gene.

The *R. meliloti* TME enzyme can be truncated by removal of the C-terminal domain and still retain malic enzyme activity (Mitsch et al, 1998) but how this truncated enzyme functions in bacteroids is not known. It is possible that the C-terminal region might somehow be inhibiting TME activity in bacteroids and that truncation of the enzyme might now allow it to functionally replace DME in nitrogen fixing bacteroids. As with *dme* there is a convenient *Pst*I restriction site in *tme* between the malic enzyme domain and the C-terminal region of the gene at amino acid 440. Because of the down-regulation of *tme* expression in bacteroids it was necessary to make the deletion in the *dtme* hybrid gene in the transfer vector pUCP30T (plasmid pTH433). The plasmid was digested with *Pst*I and *Kpn*I, the ends filled in with the Klenow fragment of DNA polymerase and dNTPs and then religated and transformed into DH5α cells. The resulting plasmid (designated pTH473), carrying the gene *dtme*Δ*Pst*, was sequenced to confirm the site of deletion and the presence of an in-frame stop codon occurring 5 amino acids downstream

of the deletion end point.

The plasmid pTH473 was transferred into the R. meliloti dme tme null mutant strain RmG994. For tme expression to remain under control of the dme promoter it was necessary to select for recombination events that had occurred into the dme gene and not the tme gene. To do this the mating spots were first plated out onto LB containing 60μg/ml gentamycin, 100μg/ml spectinomycin, 200μg/ml neomycin. From a plate that had approximately 50 colonies the colonies were scraped off as a pooled mixture and resuspended in 1 ml LBmc. The suspension was diluted to an OD_{600} ~0.6, 50μl ΦM12 was added and the tube incubated overnight at 30°C to produce the phage lysate. The lysate was diluted 1/30 with sterile saline and used to infect a culture of the tme⁻ strain RmG995 which was then plated out onto LB 60μg/ml gentamycin, 200μg/ml neomycin. Several of the resulting colonies were tested by making sonicated cell extracts from 5 ml overnight cultures and analyzing by Western blot. Most of the colonies expressed the truncated TME protein (data not shown). Three colonies were further purified for analysis and given the designations RmK143, RmK144 and RmK145.

Alfalfa seedlings were inoculated with the strains expressing the TMEΔPst protein and after 28 days the plants were small and yellow indicating that the nodules were Fix⁻. Nodules were picked from some of the plants and bacteroid extracts made to determine if the truncated TME protein was being expressed and at an elevated level as measured by Western blot. For both RmK143 and RmK144 the truncated TME protein was present and at levels much increased over that of

the full length protein in wild type Rm1021 bacteroids (data not shown). Therefore, deletion of the C-terminal region of TME does not alter the fact that it will not complement for loss of DME in producing nitrogen-fixation competent bacteroids.