Insertion of Transposon Tn5tac1 in the *Sinorhizobium meliloti* Malate Dehydrogenase (*mdh*) Gene Results in Conditional Polar Effects on Downstream TCA Cycle Genes

Sergiy I. Dymov, David J. J. Meek, Blaire Steven, and Brian T. Driscoll

Department of Natural Resource Sciences (Microbiology unit), McGill University, Macdonald Campus, 21,111 Lakeshore Rd, Ste-Anne-de-Bellevue, QC, H9X 3V9, Canada

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To isolate *Sinorhizobium meliloti* mutants deficient in malate dehydrogenase (MDH) activity, random transposon Tn5tac1 insertion mutants were screened for conditional lethal phenotypes on complex medium. Tn5tac1 has an outward-oriented isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible promoter (P_{tac}). The insertion in strain Rm30049 was mapped to the *mdh* gene, which was found to lie directly upstream of the genes encoding succinyl-CoA synthetase (*sucCD*) and 2-oxoglutarate dehydrogenase (*sucAB* and *lpdA*). Rm30049 required IPTG for wild-type growth in complex media, and had a complex growth phenotype in minimal media with different carbon sources. The *mdh::Tn5tac1* insertion eliminated MDH activity under all growth conditions, and activities of succinyl-CoA synthetase, 2-oxoglutarate dehydrogenase, and succinate dehydrogenase were affected by the addition of IPTG. Reverse-transcriptase polymerase chain reaction (RT-PCR) studies confirmed that expression from P_{tac} was induced by IPTG and leaky in its absence. Alfalfa plants inoculated with Rm30049 were chlorotic and stunted, with small white root nodules, and had shoot dry weight and percent-N content values similar to those of uninoculated plants. Cosmid clone pDS15 restored MDH activity to Rm30049, complemented both the mutant growth and symbiotic phenotypes, and was found to carry six complete (*sdhB*, *mdh*, *sucCDAB*) and two partial (*lpdA*, *sdhA*) tricarboxylic acid cycle genes.

Additional keywords: *Rhizobium leguminosarum*, symbiotic nitrogen fixation.

The tricarboxylic acid (TCA) cycle is a central metabolic pathway in the rhizobia (Dunn 1998). Rhizobia are gram-negative aerobic bacteria from several related genera (including *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, and *Bradyrhizobium*) that form N_2 -fixing symbioses with legume plants. Oxidation of compounds via the TCA cycle yields reducing equivalents, metabolic energy in the form of ATP, and metabolites that are exchanged with many other metabolic pathways. The exchange of metabolites between legumes and rhizobia, within

nodules, is central to their symbiosis. In return for an energy source, differentiated rhizobia (bacteroids) supply the host with a source of nitrogen. These processes, and those of related anapleurotic pathways, have been extensively reviewed in recent years (Dunn 1998; McDermott et al. 1989; Poole and Allaway 2000; Streeter 1991; Tajima and Kouchi 1996; Udvardi and Day 1997; Vance and Heichel 1991).

C_4 -dicarboxylic acid (C_4 -DCA) intermediates of the TCA cycle, such as malate, are the principal source of energy supplied by the plant to N_2 -fixing bacteroids. In *S. meliloti* bacteroids, because of the absence of phosphoenolpyruvate carboxykinase (PCK) activity (Finan et al. 1991), malate must be channeled through both malate dehydrogenase (MDH), and the NAD^+ malic enzyme (DME)–pyruvate dehydrogenase (PDH) pathway, to produce the oxaloacetate (OAA) and acetyl-CoA, respectively, required for synthesis of citrate, the first intermediate of the TCA cycle (Cabanes et al. 2000; Driscoll and Finan 1993). MDH catalyses the (reversible) oxidation of malate to OAA, whereas DME oxidizes malate to pyruvate and PDH oxidizes pyruvate to acetyl-CoA.

The exchange of amino acids, such as aspartate, glutamate, and possibly alanine, between rhizobia and their host plants also appears to be critical for successful symbiosis. In a recent paper, Ludwig and associates (2003) demonstrated that *Rhizobium leguminosarum* mutants deficient in two ABC-type amino acid transporters formed root nodules with the ability to fix nitrogen, but which failed to supply pea plants with a source of fixed nitrogen. The exchange of amino acids in symbiosis is proposed to function by shutting down bacteroid ammonium assimilation and allowing fixed nitrogen to flow to the plant. This cycle includes the TCA cycle intermediate oxaloacetate, via aspartate aminotransferase, an enzyme that is required for symbiotic nitrogen fixation in both *R. leguminosarum* and in *Sinorhizobium meliloti* (Ludwig et al. 2003; Watson and Rastogi 1993).

The enzymes of the TCA cycle have been detected in several rhizobia, and they appear to be highly active in bacteroids (Dunn 1998; McDermott et al. 1989; Poole and Allaway 2000; Streeter 1991; Tajima and Kouchi 1996; Udvardi and Day 1997; Vance and Heichel 1991). Although enzymatic characterization of the TCA cycle in rhizobia has been relatively thorough, genetic studies with well-defined mutants have been limited. Most mutants that completely lack a TCA cycle enzyme form root nodules that do not fix N_2 (Duncan and Fraenkel 1979; Gardiol et al. 1982; Hernández-Lucas et al. 1995; McDermott and Kahn 1992; Mortimer et al. 1999; Walshaw et al. 1997). In contrast, *Bradyrhizobium japonicum* *sucA* mutants retain the ability to fix some

Corresponding author: B. T. Driscoll; Telephone: (514) 398-7887; Fax: (514) 398-7990; E-mail: driscoll@nrs.mcgill.ca

Complete nucleotide sequences for *mdh*, *sucC*, and *sucA* are available in the GenBank database under accession numbers AF322647, AF326913, and AY026040, respectively.

N₂, possibly due to a metabolic bypass of 2-oxoglutarate dehydrogenase (OGD) via succinic semialdehyde through the activities of 2-ketoglutarate decarboxylase and succinate semialdehyde dehydrogenase (Green and Emerich 1997; Green et al. 2000, 2003). This may be because inhibition of the OGD complex under low oxygen concentrations, which can occur in the root nodule, can result from an increase in NADH concentration when aerobic respiration is limited by oxygen availability (Salminen and Streeter 1990; Streeter 1991). This situation would require cells to have an alternate pathway from 2-oxoglutarate to succinate to allow the TCA cycle to continue to function. Inhibition of OGD and a subsequent increase in 2-oxoglutarate also can result in glutamate excretion, and there is evidence from *R. leguminosarum* that OGD can be bypassed via the γ -aminobutyrate (GABA) shunt, and that these cells excrete glutamate (Lodwig et al 2003; Prell et al. 2002).

If *mdh* mutants were, like those of *Escherichia coli* (Hansen and Juni 1979), simply unable to utilize certain TCA cycle intermediates as sole carbon source, they likely would have been isolated previously via Tn5 mutagenesis in *S. meliloti* (Finan et al. 1988) or *R. leguminosarum* (McKay et al. 1988; Walshaw et al. 1997). Due to these findings, we hypothesized that *mdh* was the first gene in an operon, and that polar effects of *mdh*::Tn5 insertions on downstream genes caused strains carrying such mutations to be nonviable under culture conditions used. Indeed, the *mdh* gene recently was shown to be part of an operon with the genes encoding succinyl-CoA synthetase (*sucCD*) and subunits of 2-oxoglutarate dehydrogenase (*sucAB*) in *R. leguminosarum* (Poole et al. 1999; Walshaw et al. 1997).

To isolate viable *S. meliloti mdh* insertion mutants, a transposon that does not produce polar effects was required. Tn5tac1 is a derivative of Tn5 that carries an outward-oriented *tac* promoter (*P*_{tac}) and the *lacI*^q repressor (Chow and Berg 1988). In the presence of isopropyl- β -D-thiogalactopyranoside (IPTG), genes downstream of the insertion may be expressed via *P*_{tac}. In the absence of IPTG, there is a low level of expression (leakage) from *P*_{tac} in *E. coli* (Llosa et al. 1991). In this study, we report the isolation of an *S. meliloti mdh* mutant, the characterization of its complex conditional phenotype, and the location of *mdh* in a cluster of TCA cycle genes. To the best of our knowledge, with respect to rhizobia, this is the first report of an *mdh* mutant, and the first demonstration of the use of transposon Tn5tac1 to generate a mutant with a conditional phenotype in this system.

RESULTS

Isolation of an *S. meliloti mdh* mutant.

We hypothesized that *S. meliloti mdh*::Tn5tac1 mutants would have a conditional lethal phenotype and, therefore, would grow on complex media in the presence of IPTG, but not in its ab-

sence. Approximately 10,000 random Tn5tac1 insertion mutants, maintained in the presence of IPTG, were screened for growth on LB plates with and without IPTG. Although several strains with putative IPTG-dependent phenotypes were isolated during the initial screening, only one strain, Rm30049 (Table 1), was found to have a marked IPTG-dependent phenotype on LB plates upon purification. Rm30049 was the only strain to be affected in TCA cycle enzyme activities. Seven other Tn5tac1 mutants have been isolated, and these strains showed various IPTG-dependent growth phenotypes on minimal media with glucose, arabinose, or glutamate as sole carbon sources; however, the genes in which the transposon is inserted have not been identified to date. Including Rm30049, half of the mutants isolated showed better growth in the presence of IPTG, and half showed better growth in its absence (data not shown). Although Rm30049 did grow on LB plates without IPTG, growth was poor. Plasmid pSI01 was constructed by ligating a *SacI* restriction digest of Rm30049 DNA into vector pBSKS+, and selecting for kanamycin-resistant transformants. There are no *SacI* sites in Tn5tac1; therefore, the entire transposon plus flanking *S. meliloti* DNA regions are carried on pSI01. DNA sequence analysis of pSI01 revealed that the transposon was inserted into a gene with a high degree of homology to the *R. leguminosarum mdh*. Thus, Rm30049 appeared likely to be an *S. meliloti mdh* mutant. The Tn5tac1 insertion mapped to nucleotide 448 (of 963) in the coding region of *mdh*; therefore, the allele was designated *mdh448*::Tn5tac1.

Growth phenotype of Rm30049.

Growth of Rm30049 was slow compared with that of the wild-type strain Rm1021 in all media (Fig. 1). In liquid minimal media, Rm30049 grew best with glucose (Fig. 1A) and slowest with either arabinose or glutamate (Fig. 1B). In *S. meliloti*, both arabinose and glutamate enter the TCA cycle at 2-oxoglutarate (Duncan and Fraenkel 1979; Encarnación et al. 1995). Rm1021 grew more rapidly with arabinose than with glutamate, but this was not observed with Rm30049. With all carbon sources, Rm30049 cultures ultimately attained final optical density (OD) values close (94 to 100%) to those of Rm1021, although this took 170 h for the arabinose and glutamate cultures (data not shown). Plate growth phenotypes mirrored those of liquid culture. In LBmc (Luria-Bertani broth supplemented with 2.5 mM MgSO₄ and 2 mM CaCl₂) (Fig. 1C), the addition of IPTG caused the doubling time of Rm30049 to decrease to 4.25 h, from 6.19 h without IPTG. The doubling time of Rm1021 (2.17 h) was not affected by IPTG treatment. The addition of IPTG to LBmc increased the final OD of the Rm30049 cultures to 91% of the wild-type value, from 78% in LBmc alone.

The plate growth phenotype of Rm30049 was compared with that of Rm1021, using media with and without IPTG (data not shown). Similar to the growth curves (Fig 1B), the

Table 1. Bacterial strains and plasmids

Strain, plasmid	Relevant characteristics ^a	Reference, source
<i>Sinorhizobium meliloti</i>		
Rm1021	SU47 <i>str</i> -21	Meade et al. 1982
MD1	L5-30 <i>kgd</i>	Duncan and Fraenkel 1979
Rm30049	Rm1021 <i>mdh448</i> ::Tn5tac1	This study
<i>Rhizobium leguminosarum</i>		
RU116	3841 <i>sucD</i> ::Tn5	Walshaw et al. 1997
RU156	3841 <i>sucA</i> ::Tn5	Walshaw et al. 1997
Plasmids		
pLAFR1	IncP cosmid cloning vector, Tc ^r	Friedman et al. 1982
pTC303	pRK600 Ω Tn5tac1, Cm ^r Km ^r	T. C. Charles
pDS15	pLAFR1 clone, carrying <i>sdhB</i> , <i>mdh</i> , <i>sucCDAB</i>	This study
pBSKS+	Bluescript cloning vector, Ap ^r	Stratagene
pSI01	pBSKS+, 9.4-kb <i>SacI</i> fragment, <i>mdh448</i> ::Tn5tac1	This study

^a Tc^r, Cm^r, Km^r, and Ap^r = tetracycline-, chloramphenical-, kanamycin-, and ampicillin-resistant, respectively.

poorest growth for Rm30049 was observed on M9 plates containing either glutamate or arabinose as sole carbon sources (without IPTG). When Rm30049 was grown on the same media but with IPTG, growth was visibly better, but poorer than that of Rm1021. Rm30049 also grew poorly on M9 plates containing either glucose or succinate, without IPTG, and, as observed in the growth curves (Fig 1A), growth was better than for glutamate or arabinose. On glucose or succinate plates to which IPTG had been added, growth was visibly better, nearly to the level of Rm1021. The same results were obtained for Rm30049 when L-malate was used in place of succinate as sole carbon source in M9 plates (data not shown).

Isolation of the *S. meliloti mdh* gene.

Prior to the confirmation that Rm30049 was an *mdh* mutant, the *S. meliloti mdh* gene was isolated via heterologous complementation. Attempts to complement *E. coli mdh* mutant EJ1477 (Hansen and Juni 1979) with an *S. meliloti* clone bank

(Friedman et al. 1982) were unsuccessful, even though a similar approach was used successfully to isolate several alfalfa *mdh* cDNA clones (Miller et al. 1998). Several clones were isolated via complementation of *R. leguminosarum sucA* mutant RU156 (Walshaw et al. 1997). All clones were found to fall into one class, based upon restriction digestion patterns, and a representative plasmid, pDS15, was chosen for subsequent experiments. pDS15 also complemented the growth phenotypes of *R. leguminosarum sucD* mutant RU116, *S. meliloti* 2-oxoglutarate dehydrogenase mutant MD1 (Duncan and Fraenkel 1979), and Rm30049. Southern blots revealed that pDS15 carries *S. meliloti* DNA homologous to the region disrupted by Tn5tac1 in Rm30049 (data not shown).

Enzymatic characterization.

The activities of succinyl-CoA synthetase (SCS), OGD, and succinate dehydrogenase (SDH) were reduced in Rm30049 compared with the wild type (Table 2). Addition of IPTG to

Fig. 1. Growth phenotype of the *Sinorhizobium meliloti mdh* mutant in liquid culture. Cultures of Rm30049 and Rm1021 were grown in minimal media with different carbon sources **A**, glucose or succinate, **B**, arabinose or glutamate, and **C**, in a complex medium with or without isopropyl- β -D-thiogalactopyranoside (IPTG). Values are means of optical density determinations from triplicate cultures, and the bars represent standard errors.

the culture resulted in increased SCS and OGD, and wild-type SDH activities. MDH activity was reduced to background levels in extracts of Rm30049 cells grown in the presence or absence of IPTG. Background activity, due to the presence of NAD⁺-dependent malic enzyme, was expected (Driscoll and Finan 1997). In extracts of Rm30049 cells carrying pDS15, OGD, SDH, and isocitrate dehydrogenase (ICD), activities were comparable with the wild-type values, but MDH and SCS activities were increased 2-fold and 4.7-fold, respectively. Although SDH and ICD activities were measured as controls, their levels changed under the different conditions. ICD activity increased when MDH activity was absent, and SDH activity decreased when SCS and OGD levels were below wild-type levels.

Symbiotic phenotype.

Alfalfa seedlings inoculated with Rm30049 had a shoot dry weight value and appearance similar to the uninoculated control (Table 3). The plant tops were chlorotic and stunted, and small white root nodules were present. Plants inoculated with Rm30049 carrying pDS15 were green, and had pink-colored root nodules and higher shoot dry weight values than plants inoculated with Rm30049 without the plasmid. Although the shoot dry weight values were much less than those of the wild type, only 13% of colonies isolated from Rm30049 (pDS15) nodules were resistant to tetracycline, indicating the presence of pDS15. A high rate of loss of pLAFRI plasmids under unselected conditions in bacteroids was observed in a previous study (Driscoll and Finan 1997). Plants inoculated with

Rm1021 carrying pDS15 had lower shoot dry weight values than those of plants inoculated with Rm1021. This difference does not appear to be significant and, in replicate experiments using the same strains and conditions, plants inoculated with Rm1021 (pDS15) have been found to have shoot dry weights closer to and even slightly higher than those inoculated with Rm1021 (data not shown).

Plants inoculated with Rm30049 had shoot percent-N content and C:N values very close to those of the uninoculated control plants, confirming that these plants were deficient in N₂-fixation. The shoot percent-N content and C:N values also supported the observation from shoot dry weight analysis that pDS15 complemented the symbiotic phenotype of Rm30049. The range of C:N ratios of Fix⁺ plants was 55.9 to 60, while Fix⁺ plants ranged from 36.7 to 25.6. The lowest C:N ratio was observed for wild-type inoculated plants.

Genetic characterization of pDS15.

Transposon mutagenesis of pDS15 with Tn5 and Tn5-B20 yielded nine insertions that disrupted complementation of the Rm30049 arabinose growth phenotype. Approximate positions of transposon insertions in pDS15 were determined by restriction analysis. Noncomplementing insertions were subcloned into vector pBSKS+, selecting for the transposon resistance marker, and their insertion sites were determined by sequencing DNA outward from the transposon. The noncomplementing insertions mapped to *mdh*, *sucC*, *sucD*, and *sucA* (Fig. 2). This revealed that genes encoding MDH, SCS, and OGD were in the same complementation group. Insertions that did not

Table 2. Effects of complementing plasmid or isopropyl-β-D-thiogalactopyranoside (IPTG) in growth medium on enzyme activities in Rm30049

Strain	1 mM IPTG	Enzyme specific activity ^a				
		Malate dehydrogenase	Succinyl-CoA synthetase	2-oxoglutarate dehydrogenase	Succinate dehydrogenase	Isocitrate dehydrogenase
Rm1021	–	591 ± 24	76.2 ± 13.1	35.9 ± 0.5	191 ± 0	383 ± 21
Rm30049	–	5.3 ± 0.5	22.2 ± 1.3	14.5 ± 1.6	66.7 ± 19.3	495 ± 16
Rm30049	+	8.5 ± 0.5	30.3 ± 8.1	23.0 ± 1.4	256 ± 15	593 ± 23
Rm30049 (pDS15)	–	1,239 ± 11	359 ± 14	29.9 ± 0.5	229 ± 33	329 ± 11

^a Specific activities (nmol per minute per mg protein) expressed as mean ± standard error of triplicate assays of each sample.

Table 3. Shoot dry weight (DW) and N-content analysis

Inoculated strain	Shoot DW ^a	WT (%) ^b	N content (%) ^c	C content (%) ^c	C:N
Rm1021	80 ± 6	100	2.27 ± 0.03	58.1 ± 1.4	25.6
Uninoculated	15 ± 2	19	1.00 ± 0.02	56.0 ± 0.9	55.9
Rm30049	11 ± 1	14	0.92 ± 0.00	55.2 ± 0.5	60.0
Rm30049 (pDS15)	36 ± 2	45	1.59 ± 0.03	58.5 ± 0.4	36.7
Rm1021 (pDS15)	56 ± 18	70	1.95 ± 0.02	57.6 ± 0.9	29.5

^a Shoot DW expressed in milligrams per plant as mean ± standard error of triplicate samples. Each sample consisted of 8 to 12 shoots (29 to 31 total) harvested 28 days after inoculation.

^b Percentage of the wild-type (WT; Rm1021) value.

^c Percentage of N and C content expressed as mean percentage of dry matter ± standard error of triplicate samples. The samples were the same as used for shoot DW determinations.

Fig. 2. Physical and genetic map of tricarboxylic acid (TCA) cycle genes on pDS15. TCA cycle genes are indicated by shaded bars oriented in direction of transcription (left to right). Transposon insertions are indicated by filled circles (Tn5), filled arrowheads (Tn5-B20), or an open arrowhead (*mdh448::Tn5tac1*), and *EcoRI* restriction sites are indicated (R). The direction of the arrowheads for Tn5-B20 insertions indicate orientation of the promoterless *lacZ* gene of the transposon with respect to the *Sinorhizobium meliloti* gene. The direction of the arrowhead for Tn5tac1 indicates the direction of transcription from *P*_{tac}.

disrupt complementation mapped to other regions of pDS15 (data not shown). Attempts to recombine transposon insertions in *mdh*, *sucC*, and *sucA* by plasmid incompatibility and *sacB* selection methods failed. Apparently, recombinants carrying strongly polar insertions in these genes were nonviable. Thus, we failed to isolate any nonconditional mutants. In control experiments, insertions that did not disrupt complementation were readily recombined into the *S. meliloti* genome.

TCA cycle genes.

DNA sequence data for TCA cycle genes carried by pDS15 were generated from subcloned transposon insertions, *EcoRI* subclones of pDS15, and pDS15 itself. Gaps were filled by sequencing using custom primers or by using data from the 1× coverage of the *S. meliloti* genome. Sequences finally were verified against the completed *S. meliloti* chromosomal sequence (Capela et al. 2001). Complete nucleotide sequences determined for the *mdh*, *sucC*, and *sucA* genes were deposited in GenBank. Partial sequences were generated for other TCA cycle genes on pDS15. BLAST comparisons and CLUSTAL W alignments with homologous sequences from *S. meliloti* (Capela et al. 2001), *R. leguminosarum* (Walshaw et al. 1997), *Mesorhizobium loti* (Kaneko et al. 2000), *B. japonicum* (Green and Emerich 1997; Westenberg and Guerinot 1999), and *Agrobac-*

terium tumefaciens (Goodner et al. 2001) were used to identify the genes shown in Figure 2. Putative ribosomal binding sites were identified upstream of each gene (data not shown). The left border of pDS15 is an *EcoRI* site at nucleotide 754 of the 1,842-nucleotide (nt) *sdhA* coding sequence, and the right border is an *EcoRI* site at nucleotide 617 of the 1,407-nt *lpdA* coding sequence. This *lpdA* allele was annotated as *lpdA2* in the *S. meliloti* genome sequence, and there appear to be several open reading frames with unknown function between *sucB* and *lpdA*. The pDS15 sequence was determined to be 23,811 nt long, spanning nucleotides 3,332,718 to 3,308,908 of the *S. meliloti* chromosome.

An amino acid alignment of homologous MDH sequences (Fig. 3) revealed that the *S. meliloti* MDH has high identity with those of *R. leguminosarum* (93%), *M. loti* (84%), and *A. tumefaciens* (90%). The *S. meliloti* and *R. leguminosarum* *mdh* nucleotide sequences had 87% identity (not shown). The region upstream of the *S. meliloti* *mdh*, including the putative promoter, also appeared to be similar to that reported in *R. leguminosarum* (Poole et al. 1999). Although not similar to known consensus promoter sequences (Thöny and Hennecke 1989), this region has high AT content and an apparent hairpin loop centered at position -101 relative to the *mdh* start codon (not shown). This element was similar to one in *R. legumino-*

MDH_Sme	1	MARN KIALIGSGMIGGTLAHL AGLKGELGDIVLFDIADGIPQGKGLDIAQS	50
MDH_Rle	1	MARN KIALIGSGMIGGTLAHL AGLKGELGDIVLFDIADGIPQGKGLDISQS	50
MDH_Mlo	1	MARN KIALIGSGMIGGTLA HMIGLKDLDGVVLFDAEGIPQGKGLDIAQS	50
MDH_Atu	1	MARK KIALIGSGMIGGTLAHL ASLKGELGDIVLFDIADGIPQGKGLDIAQS	50
		*** *****. **.*.*****.*****.***	
MDH_Sme	51	SPVEGFDA SLTGASD YSAIEGADVCI VTAGVPRKPGMSR DDLGINLKVM	100
MDH_Rle	51	SPVEGF DVNL TGASD YSAIEGADVCI VTAGV ARKPGMSR DDLGINLKVM	100
MDH_Mlo	51	SPVDGFDSRLTG VNDYAGIEGADVCI VTAGV PRKPGMSR DDLGINLKVM	100
MDH_Atu	51	GPVEGFNAKL SGASD YAAIEGADVCI VTAGVARKPGMSR DDLGINLKVM	100
		.*. *.*. *. *****.*****.*****.*****	
MDH_Sme	101	EQVGAGIKKYAPNAFVICITNPLDAMVWALQKFSGLPKNKVVMAGV LD S	150
MDH_Rle	101	EQVGAGIKKYAPNAFVICITNPLDAMVWALQKFSGLPANKVVMAGV LD S	150
MDH_Mlo	101	EQVGAGLKKYAPKAFVICITNPLDAMVWALQKFSGLPKTHVVMAGV LD S	150
MDH_Atu	101	EQVGAGIKKYAPNAFVICITNPLDAMVWALQKFSGLPKNKVVMAGV LD S	150
		*****.*****.*****.*****.*****.*****.*****	
MDH_Sme	151	SRFRLFLAEEFNVSVDITAFVLGGHGD TMVPLARYSTVAGIPL PLDIQM	200
MDH_Rle	151	SRFRLFLAKEFNVSVD ITAFVLGGHGD TMVPLARYSTVGGIPLTDLV TM	200
MDH_Mlo	151	ARFRYFLAEEFKVSVD ITAFVLGGHGD SMVPMIRYSTVSGIPLPLDV KM	200
MDH_Atu	151	ARFRLFLAEEFNVSVD ITAFVLGGHGD TMVPLARYSTVGGVPLTDLV KM	200
		..***.***.***.*****.*****.*****.***.***.***	
MDH_Sme	201	GWTTKEKLDQIIQRTRDGGAEIVGLLKTGS AYY APAASA IE MAEAYLKDK	250
MDH_Rle	201	GWVTKERLEEIIQRTRDGGAEIVGLLKTGS AYY APAASA IE MAESYLKDK	250
MDH_Mlo	201	GWTSKEKLDQIVQRTRDGGAEIVGLLKTGS AYY APAASA IA MAESYLKDK	250
MDH_Atu	201	GWLTAERLEQIIQRTRDGGAEIVGLLKTGS AYY APAASA IE MAESYLKDK	250
		.*.*.***.*****.*****.*****.*****.***.*****	
MDH_Sme	251	KRVLP CA AHL SGQYGV KDMYVGVP TVIGAGG IERIEIDLNK GEKEAF DK	300
MDH_Rle	251	KRVLP CA AHL SGQYGV KDMYVGVP TVIGAGG VERIEIDLNK TEKEAF DK	300
MDH_Mlo	251	KRVLP CA AHL SGQYGV KGT YVGV PV TVIGAGG VERIEIDLNK SEQKMF ES	300
MDH_Atu	251	KRVLP CA AHL SGQYGV DDMYVGVP TVIGAGG IERVIEIDLNK EEEEAF QK	300
		*****.*****.*****.*****.***.***.***.***.***.***	
MDH_Sme	301	SVAAVAGLCEACINIAPSLK	320
MDH_Rle	301	SVGAVAGLCEACINIAPALK	320
MDH_Mlo	301	SVATVQGLTEACVKIAPQLASK	322
MDH_Atu	301	SVGAVAGLCEACINIAPSLKN	321
		.*.*.***.***.***.***.***.***.***	

Fig. 3. Amino acid alignment of malate dehydrogenase (MDH) proteins. The *Sinorhizobium meliloti* MDH (MDH_Sme) is aligned with sequences of closely related species *Rhizobium leguminosarum* (MDH_Rle), *Mesorhizobium loti* (MDH_Mlo), and *Agrobacterium tumefaciens* (MDH_Atu). The nucleotide binding motif between positions 5 and 13 (Wierenga and Hol 1983), and six amino acids (Arg-83, Arg-89, Asp-149, Arg-152, His-176, Ala-231) predicted to be important for substrate binding and catalysis (Poole et al. 1999) are shown in bold. Identical amino acids are indicated by asterisks, and similar amino acids are indicated by dots.

sarum described as the *rho*-independent transcription terminator of a gene upstream of *mdh* (Poole et al. 1999). With respect to the other *S. meliloti* TCA cycle genes, the *mdh-sucC*, *sucC-sucD*, *sucA-sucB*, and *sdhA-sdhB* intergenic regions do not appear to be large enough to encode promoters. In addition to *mdh*, putative promoters exist upstream of *sucA* and *lpdA*. The promoter of a putative *sdhCDAB* operon, and a second putative promoter upstream of *sdhA*, lie outside of the pDS15 region.

Reverse-transcriptase polymerase chain reaction analysis.

Reverse-transcriptase polymerase chain reaction (RT-PCR) was used to characterize expression of the P_{tac} promoter of Tn5tac1 downstream from its insertion site within the *mdh* gene. Growth kinetic (Fig. 1) and enzyme assay (Table 2) studies indicated that expression of genes (*sucCDAB*) downstream of *mdh* was occurring in the absence of IPTG, and that this expression was higher in the presence of IPTG. RT-PCR revealed that, although some RNA from downstream of the *mdh::Tn5tac1* insertion (*mdh-sucC*) could be detected from Rm30049 cells grown without IPTG, the amount was greater from cells grown with IPTG (Fig. 4). The amount of *mdh-sucC* RT-PCR product was much greater in Rm1021 samples. This result also demonstrated that *mdh* and *sucC* are cotranscribed. The amount of RT-PCR product obtained for the *icd* gene product was similar for all strains and conditions. For this reason, and because ICD activity was found to vary the least under the conditions tested and between Rm30049 and Rm1021 (Table 2), the *icd* gene was chosen as control for this experiment. Similar results were obtained when both sets of primers were amplified in the same tubes (data not shown).

DISCUSSION

Transposon Tn5tac1 proved invaluable in the isolation of an *S. meliloti mdh* mutant. Tn5tac1 has been used to isolate conditional mutants in *E. coli*, *Bordetella pertussis*, *Klebsiella aerogenes*, and *Erwinia chrysanthemi* (Alfano et al 1995; Chow and Berg 1988; Cookson et al. 1990; Schwacha and Bender 1993), but it was not known if either the *tac* promoter or the *lacI^q* would function in *S. meliloti*. We hypothesized that *mdh* was the first gene in a TCA cycle operon, and that polar effects of Tn5 insertions in the *mdh* gene would prevent transcription of downstream genes, resulting in a lethal phenotype. Indeed, we were unable to isolate insertions in any

of the TCA cycle genes depicted in Figure 2 via site-directed mutagenesis of pDS15 with transposons that produce strong polar effects. These results, combined with analysis of complementation of Rm30049, enzymatic assays, RT-PCR, and DNA sequencing, indicated that *mdh* is the first gene in an operon including *sucCDAB*.

This arrangement is the same as that reported in *R. leguminosarum* (Poole et al. 1999; Walshaw et al. 1997). In *Bradyrhizobium japonicum*, the *mdh* gene is located upstream of the *sucCD*, *sucAB*, *scdA* and *lpdA*, genes (Green et al. 2003). In this organism, the latter four genes are cotranscribed; however *mdh* does not appear to be part of this operon. In *Escherichia coli*, the *mdh* is neither near to nor coordinately regulated with the *sucABCD* genes (Guest et al. 1989). Our results differ from findings from *R. leguminosarum* (Walshaw et al. 1997) and *B. japonicum* (Green and Emerich 1997; Green et al. 2000) studies, in that insertions in the *S. meliloti sucA* and *sucC* genes resulted in apparently lethal phenotypes in *S. meliloti*. This may be due to the existence of functional OGD bypass pathways in *R. leguminosarum* and *B. japonicum*, as has been hypothesized, but not in *S. meliloti*.

Bypass of OGD by the activities of 2-ketoglutarate decarboxylase and succinate semialdehyde dehydrogenase (Green et al. 2000) appears to be the reason *B. japonicum sucA* mutants are able to fix N_2 with soybean plants, and seems to be linked to a need to compensate for the inhibition of the OGD complex under low oxygen conditions (Salimen and Streeter 1990; Streeter 1991) and the lack of a complete glyoxylate shunt in *B. japonicum* bacteroids (Green et al. 1998). In *R. leguminosarum*, bypass of OGD could be accomplished via the GABA shunt or the glyoxylate pathway, but neither appear to be required for symbiotic N_2 fixation (García-de los Santos et al. 2002; Prell et al. 2002). The glyoxylate pathway seems to be present in free-living cells of *S. meliloti*, *B. japonicum*, and *R. leguminosarum*; however, it was found that the form of malate synthase present in *R. leguminosarum* is the malate synthase B, not the malate synthase A associated with the glyoxylate cycle in *E. coli* (Duncan and Fraenkel 1979; Green et al. 1998; García-de los Santos et al. 2002). Evidently, much work needs to be done concerning anapleurotic pathways related to the TCA cycle in rhizobia (Dunn 1998).

Therefore, although the TCA cycle genes (Fig. 3) and gene arrangements appear quite similar among the rhizobia, significant differences appear to exist in related anapleurotic pathways

Fig. 4. Reverse transcriptase polymerase chain reaction (RT-PCR) detection of transcription downstream of the *mdh::Tn5tac1* insertion. Reactions were done with (lanes 7 to 12) and without (lanes 2 to 4) the RT step, or without RNA (lane 5). RNA was extracted from LBmc-grown cells of Rm1021 (lanes 2, 7, and 10), Rm30049 (lanes 3, 8, and 11), and Rm30049 cells grown with 1 mM isopropyl- β -D-thiogalactopyranoside in LBmc (lanes 4, 9, and 12). Primers amplified the *mdh* transcript downstream of the Tn5tac1 insertion (lanes 7, 8, and 9) and the *icd* transcript (lanes 10, 11, and 12). Molecular weight standards were run in lanes 1 and 6.

and, possibly, in the regulation of the TCA cycle genes themselves. A better understanding of the physiological processes that support symbiotic N₂ fixation in general will require study of these processes in several rhizobia.

Although Rm30049 was isolated on the basis of its apparent lack of growth on LB plates without IPTG, this strain was able to grow, at different rates, with all media tested (Fig. 1). In the absence of IPTG, Rm30049 eventually was able to overcome the absence of MDH and decreased activities of several other TCA cycle enzymes (Table 2). In minimal media, glucose would be readily metabolized to pyruvate and enter the TCA cycle as OAA via pyruvate carboxylase (PYC) activity (Dunn et al. 1996), as in wild-type cells. PYC activity was detected in Rm30049 cell extracts (not shown). Growth of Rm30049 with succinate, glutamate, or arabinose as sole carbon source would require a metabolic bypass of MDH, because there would be no production of OAA via MDH. OAA is the substrate of PCK, the major gluconeogenic pathway from TCA cycle intermediates in *S. meliloti* (Finan et al. 1988, 1991).

Bypass of MDH could be accomplished by the activities of DME and PYC, and MDH and PCK both could be bypassed via DME and pyruvate orthophosphate dikinase (POD) activities (Østerås et al. 1997). In *B. subtilis* *mdh* mutants, MDH is bypassed via malic enzyme and pyruvate carboxylase (Hederstedt 1993). Indeed, activities of both DME and PYC have been detected in extracts of Rm30049 (data not shown), meaning that this pathway is available in this mutant. The more dramatic growth rate reduction of Rm30049 with arabinose and glutamate may be due to the fact that gluconeogenesis of these compounds requires them first to be converted to succinate via OGD and SCS, both of which seem to be expressed at low levels in this strain. This implies that growth with succinate or glucose may be less dependent upon the lower half of the TCA cycle.

The activities of SCS and OGD (Table 2) in Rm30049 revealed two important things. First, although the levels of both enzymes were reduced, they both were detected at significant levels. Second, both were present at higher levels with IPTG, and both were restored to higher levels by pDS15. Although both MDH and SCS activities were very high in the Rm30049 (pDS15) extract, OGD activity was at a level comparable to the wild type. This may have been because the copy of *lpdA* on pDS15 is incomplete. Both OGD and PDH enzyme complexes contain subunits encoded by *lpdA* genes (Cabanès et al. 2000), and it would be interesting to obtain transposon insertions in the different *S. meliloti* *lpdA* loci to determine the effects on these enzymes. To date, none have been reported.

The enzyme assays also indicated that, although the *P*_{tac} of Tn5tac1 is leaky in the absence of IPTG, as was observed in *E. coli* and *Erwinia chrysanthemi* (Alfano et al. 1995; Llosa et al. 1991), the IPTG/*lacI*^q system does function to regulate expression from *P*_{tac} in *S. meliloti*. These results were supported by RT-PCR, which also demonstrated that *mdh* and *sucC* are co-transcribed (Fig. 4). Thus, we conclude that the Tn5tac1 insertion in the *S. meliloti* *mdh* gene results in different effects on expression of downstream TCA cycle genes, depending upon the presence or absence of IPTG.

In *R. leguminosarum* strains carrying mutations within the *suc* genes, expression from the *mdh* promoter was increased (Poole et al. 1999). This, and other evidence, indicated that *mdh-sucCDAB* constitute an operon in *R. leguminosarum*, and that deficiencies in *sucCDAB*-encoded enzyme levels in the mutants resulted in overexpression of the operon. Poole and associates (1999) hypothesized that cotranscription of the *mdh* and *suc* genes is important for coordinate expression of the TCA cycle under different growth conditions, including bacteroids, and that pools of amino acids, such as glutamate, that are overflow products of the TCA cycle are important for the

regulation of the TCA cycle. Recently, the same group proposed an elegant model to explain the role of amino acid cycling in the *R. leguminosarum*-pea symbiosis, and expanded on the interaction of amino acids with the TCA cycle (Lodwig et al. 2003). These findings are highly relevant to the study of *S. meliloti* physiology as well.

The low isolation frequency of conditional *mdh::Tn5tac1* insertions (1 of 10,000 mutants screened) is consistent with the large genome size of *S. meliloti* (6.68 Mb) (Capela et al. 2001) and the fact that only insertions in one orientation would produce the expected phenotype. Although it is not known how many essential genes may be found downstream of nonessential ones in operons in *S. meliloti*, and although it is possible that insertions in other operons could produce a similar phenotype to that of Rm30049, our results suggest that their number is not great.

In addition to the apparent low frequency of insertions producing the desired phenotype, it should be noted that background growth due to the leaky expression from *P*_{tac} in the absence of IPTG likely caused the phenotypes of some mutants that may have arisen to be obscured at the patch screening stage. A higher frequency of conditional insertions in the TCA cycle genes, and in other *S. meliloti* genes, likely could be isolated by employing different selection and screening procedures. Insertion of Tn5tac1 downstream of, and in an antisense orientation to, some *Escherichia coli* genes also has been shown to yield a phenotype in which growth was conditional upon the absence of IPTG (Neuwald et al. 1992, 1993). Preliminary evidence from our lab indicates that such mutants can be isolated in *S. meliloti* as well. Of the eight Tn5tac1 mutants, including Rm30049, that have IPTG-dependent phenotypes, half show better growth in the presence of IPTG and half in its absence. Thus, it would appear that this transposon works in *S. meliloti* both to relieve polar effects of insertions in operons as well as to interfere with gene expression when inserted in an antisense orientation.

The inability of Rm30049 to fix N₂ in symbiosis with alfalfa indicates that MDH activity is necessary for symbiotic N₂ fixation (Table 3). Although Rm30049 was deficient in several activities, enzymatic assays indicated that only MDH was completely absent, and growth curves indicated that Rm30049 was able to grow under all conditions tested, albeit slowly. The growth of the free-living cells likely was due, in part, to a bypass of MDH via DME and PYC, and PYC activity is absent from *R. etli* bacteroid extracts (Dunn et al. 1997); therefore, this bypass may be unavailable in Rm30049 bacteroids. MDH activity has been observed to be much higher in extracts of *S. meliloti* bacteroids than in those of cultured cells (Driscoll and Finan 1993), and this finding seems to be supported by recent proteome analysis (Natera et al. 2000). We currently are investigating the putative promoters of *mdh* and *sucA*, and the regulation of the TCA cycle genes in free-living cells and bacteroids.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Bacterial strains and plasmids are listed in Table 1. Complex (LBmc) and defined (M9) media, growth conditions, and antibiotic concentrations were as described previously (Finan et al. 1984, 1986; Yarosh et al. 1989). M9 was supplemented with 0.25 mM CaCl₂, 1 mM MgSO₄, biotin at 0.3 mg/liter, and 15 mM carbon source (Na-L-glutamate, Na-succinate, L-arabinose, or D-glucose). *R. leguminosarum* strains were cultured in complex and defined media as described (Walshaw et al. 1997). For growth curves, overnight LBmc (plus 0.5 mM IPTG for Rm30049) cultures were washed and then resuspended in 0.85% NaCl, and then 0.15 ml was subcultured into 5 ml of

each medium. Cultures were grown in 16-by-150-mm culture tubes with horizontal shaking (200 rpm), and OD at 600 nm was followed. Standard methods were used for doubling time calculations from mean OD values (Stanier et al. 1986).

Genetic techniques.

Conjugal matings, phage Φ M12 transduction, Tn5, and Tn5-B20 (Simon et al. 1989) mutagenesis, and recombination of markers into the *S. meliloti* genome by plasmid incompatibility were done as previously described (Charles and Finan 1990; Driscoll and Finan 1996; Finan et al. 1984, 1988; Yarosh et al. 1989). Recombination of markers into the *S. meliloti* genome also was done using the *sacB* method (Quandt and Hynes 1993). Tn5tac1 (Chow and Berg 1988) mutagenesis of the *S. meliloti* genome was done by conjugal transfer of the self-mobilizable suicide plasmid pTC303 from *E. coli* into Rm1021. Transconjugants were selected on plates containing streptomycin and neomycin. Conjugation and selection were done in the presence of 1 mM IPTG. Cosmid clone pDS15 was isolated by heterologous complementation of the arabinose-growth phenotype of *R. leguminosarum* RU156 with the Rm1021 genomic library (Friedman et al. 1982).

Molecular biology techniques.

Standard techniques were used for alkaline extraction of plasmid DNA, digestion of DNA with restriction endonucleases, DNA ligations, transformation of CaCl_2 -competent *E. coli* cells, and agarose gel electrophoresis (Sambrook et al. 1989). DNA fragments were isolated from agarose gels using the QIAEX II Gel Extraction Kit (Qiagen, Mississauga, Ontario, Canada). Bacterial genomic DNA was extracted as previously described (Meade et al. 1982). Restriction endonuclease digested genomic DNA was transferred by Southern blot to positively charged nylon membranes (Boehringer Mannheim, Laval, Quebec, Canada), as described (Sambrook et al. 1989). Membranes were hybridized with pDS15 labeled using the DIG DNA Labeling and Detection Kit–Nonradioactive (Boehringer Mannheim/Roche Diagnostics, Laval, Quebec, Canada). Hybridization was detected using the colorimetric method.

DNA sequencing.

DNA sequences were determined from plasmid DNA using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Mississauga, Ontario, Canada). Reactions were run on an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). Automated DNA sequencing also was done by MOBIX (McMaster University, Hamilton, Ontario, Canada). Tn5 insertion sites were determined using the IS50 primer (5' GCC CAG TCG GCC GCA CGA TGA AGA GCA) (Charles and Nester 1993). Tn5tac1 insertion sites were sequenced using primers tac1-R1 (5' TCG ATG ATA AGC TGT CAA AC) and tac1-L1 (5' GAT AAC AAT TTC ACA CAG). Tn5-B20 insertion sites were sequenced using the IS50 primer and primer B20-L1 (5' TCA CAT GGA AGT CAG ATC CT). The ends of the insert in pDS15 were sequenced using pLAFR1 primers LAFR1-F1 (5' CGC CTC GAT CAG CTC TTG CAC) and LAFR1-R1 (5' GGT GCT GGC ATC GAC TTT CA). Additional DNA sequence data for *S. meliloti* was obtained from the 1x *S. meliloti* genome sequence, released by S. R. Long and her colleagues (Department of Biological Sciences, Howard Hughes Medical Institute, and the Stanford DNA Sequencing and Technology Center).

Sequence analysis.

Nucleotide sequences were compiled using Sequencher (v. 3.0; Gene Codes Corporation, Inc., Ann Arbor, MI, U.S.A.). Nucleotide and amino sequences were compared with the non-

redundant databases using the BLAST algorithms (blastn, blastx, blastp) (Altschul et al. 1997). Sequences were analyzed using MacVector 7.0 (Oxford Molecular Ltd., Genetics Computer Group, Madison WI, U.S.A.) and aligned using the CLUSTAL W algorithm (Thompson et al. 1994). Complete nucleotide sequences for *mdh*, *sucC*, and *sucA* were deposited in GenBank under accession numbers AF322647, AF326913, and AY026040, respectively. These sequences are identical to sequences (discussed below) from the recently reported *S. meliloti* complete chromosomal nucleotide sequence (Capela et al. 2001), accession number AL591792. Sequences for comparison also were obtained from the *M. loti* (Kaneko et al. 2000) and *A. tumefaciens* (Goodner et al. 2001) genome sequences. The following sequences (accession numbers in parentheses) were obtained from GenBank. *S. meliloti*: *mdh* (CAC47635), *sucCDAB* (CAC47634, CAC47633, CAC47632, CAC47631), *lpdA2* (CAC47627), and *sdhCDAB* (CAC47651, CAC47650, CAC47649, CAC47648); *A. tumefaciens*: *mdh* (AAK88360), *sucCDAB* (AAK88359, AAK88358, AAK88357, AAK88356), *lpdA* (AAK88353), and *sdhCDAB* (AAK88367, AAK88366, AAK88365, AAK88364); *M. loti*: *mdh* (BAB50996) *sucCDAB* (BAB50995, BAB50992, BAB50990, BAB50989), *lpdA* (BAB50985), and *sdhCDAB* (BAB50963, BAB50962, BAB50961, BAB50959); *B. japonicum*: *sucA* (AAC44748) and *sdhCDAB* (AF007569); *R. leguminosarum*: *mdh* (CAA05717).

RT-PCR.

For RT-PCR, total RNA was extracted from *S. meliloti* cultures grown overnight in LBmc using the QIAGEN RNeasy Mini Kit (Qiagen). RNA was treated two times with RNAase-free DNAase following standard protocols (Qiagen). RT-PCR was done using the QIAGEN One-step RT-PCR Kit (Qiagen) and the manufacturer's protocol. The reactions contained the following: 10 μ l of 5 $\times$ buffer, 2 μ l of dNTPs, 3 μ l of each primer, 2 μ l of enzyme mix, 1.25 μ l of RNAase inhibitor (5 U), 1 μ l of total RNA (200 ng), and 27.75 μ l of RNAase-free water. Reactions were done as follows: reverse transcription at 50°C for 30 min, and RT inactivation and DNA polymerase activation at 95°C for 15 min. The reactions were cycled 20 times as follows: denaturation at 94°C for 45 s, annealing at 60°C for 10 s, and extension at 72°C for 1.5 min. After cycling, a final extension at 72°C for 10 min was done. Control reactions were done by skipping the RT step by incubating the reactions on ice for 30 min prior to RT inactivation and by performing the reactions as described but omitting RNA. Relative band intensities were determined using the Quantity One (v. 4.2.1) software (BioRad Laboratories, Mississauga, Ontario, Canada). The primers used, followed by their positions as per the *S. meliloti* genome project, were *mdh-sucC*-F1221 (5' GCGCTTCCGGCTCTTCCTCG) (3319008–3318989), *mdh-sucC*-R1221 (5' GCGCATTGCCGTCGAAGGAG) (3317768–3317787), *icd*-F680 (5' GGGCGTCGAAAACCGTGATG) (1937220–1937239), and *icd*-R680 (5' TAGCCGCCGACCACTTCAG) (1937876–1937857). Aliquots (5 μ l) of each reaction were run on a 1% agarose gel with a 100-bp ladder molecular weight standard (Invitrogen, Burlington, Ontario, Canada).

Biochemical techniques.

Cell growth and preparation of cell-free sonicated extracts were performed essentially as described (Finan et al. 1988). Cells from late-log phase LBmc cultures were washed twice with 20 mM Tris, pH 7.8, and 1 mM MgCl_2 , resuspended (4 ml of buffer per gram of cells) in sonication buffer (20 mM Tris pH 7.8, 1 mM MgCl_2 , 10% glycerol, 10 mM β -mercaptoethanol), and then disrupted by sonication. Protein concentration was determined by the Bradford method (Bradford 1976) using the BioRad protein assay dye (BioRad Laboratories) with

bovine serum albumin as standard. Malate dehydrogenase (EC 1.1.1.37) (Englard and Siegal 1969), succinyl-CoA synthetase (EC 6.2.1.5) (Bridger et al. 1969), 2-oxoglutarate dehydrogenase (EC 1.2.4.2) (Reed and Mukherjee 1969), succinate dehydrogenase (EC 1.3.99.1) (Veeger et al. 1969), and isocitrate dehydrogenase (EC 1.1.1.42) (Reeves et al. 1971) activities were determined as described, at ambient temperature (24 to 27°C).

Plant growth conditions.

Alfalfa (*Medicago sativa* var. Iroquois) nodulation experiments in Leonard jars containing 1:1 (vol/vol) sand and vermiculite were done as previously described (Yarosh et al. 1989). *S. meliloti* strains were inoculated into a minimum of three pots with 10 seedlings each. Controls were inoculated with sterile distilled H₂O alone. Growth chamber (Conviron Inc., Winnipeg, Manitoba, Canada) conditions were 16 h of day (25°C) and 8 h of night (20°C). Shoot dry weights were determined from plant tops cut at the stem-root interface 28 days after inoculation. Shoots from each pot were pooled and dried for 3 weeks at 50°C prior to weighing. Percent nitrogen and carbon content analyses were done by applying 2-mg samples of ground-dried shoots to an NC2500 elemental analyzer (CE Instruments, Milan, Italy).

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LITERATURE CITED

Alfano, J. R., Ham, J. H., and Collmer, A. 1995. Use of Tn5tac1 to clone a *pel* gene encoding a highly alkaline, asparagine-rich pectate lyase isozyme from an *Erwinia chrysanthemi* EC16 mutant with deletions affecting the major pectate lyase isozymes. *J. Bacteriol.* 177:4553-4555.

Altschul, S. F., Madden, T. L., Schäffer, A., Zhang, Z., Miller, W., and Lipman, D. 1997. Gapped BLAST and PSI-BLAST: new generation of protein database search programs. *Nucleic Acids Res.* 25:3389-3402.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.

Bridger, W. A., Ramaley, R. F., and Boyer, P. D. 1969. Succinate coenzyme A synthetase from *E. coli*. *Methods Enzymol.* 13:70-75.

Cabanes, D., Boistard, P., and Batut, J. 2000. Symbiotic induction of pyruvate dehydrogenase genes from *Sinorhizobium meliloti*. *Mol. Plant-Microbe Interact.* 13:483-493.

Capela, D., Barloy-Hubler, F., Gouzy, J., Bothe, G., Ampe, F., Batut, J., Boistard, P., Becker, A., Boutry, M., Cadieu, E., Dreano, S., Gloux, S., Godrie, T., Goffeau, A., Kahn, D., Kiss, E., Lelaure, V., Masuy, D., Pohl, T., Portetelle, D., Pühler, A., Purnelle, B., Ramsperger, U., Renard, C., Thebault, P., Vandenbol, M., Weidner, S., and Galibert, F. 2001. Analysis of the chromosome sequence of the legume symbiont *Sinorhizobium meliloti* strain 1021. *Proc. Natl. Acad. Sci. U.S.A.* 98:9877-9882.

Charles, T. C., and Finan, T. M. 1990. Genetic map of *Rhizobium meliloti* megaplasmid pRmeSU47b. *J. Bacteriol.* 172:2469-2476.

Charles, T. C., and Nester, E. W. 1993. A chromosomally encoded two-component sensory transduction system is required for virulence of *Agrobacterium tumefaciens*. *J. Bacteriol.* 175:6614-6625.

Chow, W. -Y., and Berg, D. E. 1988. Tn5tac1, a derivative of transposon Tn5 that generates conditional mutations. *Proc. Natl. Acad. Sci. U.S.A.* 85:6468-6472.

Cookson, B. T., Berg, D. E., and Goldman, W. E. 1990. Mutagenesis of *Bordetella pertussis* with transposon Tn5tac1: conditional expression of virulence-associated genes. *J. Bacteriol.* 172:1681-1687.

Driscoll, B. T., and Finan, T. M. 1993. NAD⁺-dependent malic enzyme of *Rhizobium meliloti* is required for symbiotic nitrogen fixation. *Mol. Microbiol.* 7:865-873.

Driscoll, B. T., and Finan, T. M. 1996. NADP⁺-dependent malic enzyme of *Rhizobium meliloti*. *J. Bacteriol.* 178:2224-2231.

Driscoll, B. T., and Finan, T. M. 1997. Properties of NAD⁺- and NADP⁺-dependent malic enzymes of *Rhizobium (Sinorhizobium) meliloti* and differential expression of their genes in nitrogen-fixing bacteroids. *Microbiology* 143:489-498.

Duncan, M. J., and Fraenkel, D. G. 1979. α -Ketoglutarate dehydrogenase mutant of *Rhizobium meliloti*. *J. Bacteriol.* 37:415-419.

Dunn, M. F. 1998. Tricarboxylic acid cycle and anaplerotic enzymes in rhizobia. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Rev.* 22:105-123.

Dunn, M. F., Araña, G., Cevallos, M. A., and Mora, J. 1997. Regulation of pyruvate carboxylase in *Rhizobium etli*. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett.* 157:301-306.

Dunn, M. F., Encarnación, S., Araña, G., Vargas, M. C., Dávalos, A., Peralta, H., Mora, Y., and Mora, J. 1996. Pyruvate carboxylase from *Rhizobium etli*: mutant characterization, nucleotide sequence, and physiological role. *J. Bacteriol.* 178:5960-5970.

Encarnación, S., Dunn, M., Willms, K., and Mora, J. 1995. Fermentative and aerobic metabolism in *Rhizobium etli*. *J. Bacteriol.* 177:3058-3066.

Englard, S., and Siegal, L. 1969. Mitochondrial L-malate dehydrogenase of beef heart. *Methods Enzymol.* 13:99-100.

Finan, T. M., Hartwig, E. K., LeMieux, K., Bergman, K., Walker, G. C., and Signer, E. R. 1984. General transduction in *Rhizobium meliloti*. *J. Bacteriol.* 159:120-124.

Finan, T. M., Kunkel, B., DeVos, G. F., and Signer, E. R. 1986. Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. *J. Bacteriol.* 167:66-72.

Finan, T. M., McWhinnie, E., Driscoll, B., and Watson, R. J. 1991. Complex symbiotic phenotypes result from gluconeogenic mutations in *Rhizobium meliloti*. *Mol. Plant-Microbe Interact.* 4:386-392.

Finan, T. M., Oresnik, I., and Bottacin, A. 1988. Mutants of *Rhizobium meliloti* defective in succinate metabolism. *J. Bacteriol.* 170:3396-3403.

Friedman, A. M., Long, S. R., Brown, S. E., Buikema, W. J., and Ausubel, F. M. 1982. Construction of a broad host range cloning vector and its use in the genetic analysis of *Rhizobium* mutants. *Gene* 18:289-296.

García-de los Santos, A., Morales, A., Baldomá, L., Reid, S. R. D., Brom, S., Yost, C. K., Hernández-Lucas, I., Aguilar, J., and Hynes, M. F. 2002. The *glcB* locus of *Rhizobium leguminosarum* VF39 encodes and arabinose-inducible malate synthase. *Can. J. Microbiol.* 48:922-932.

Gardioli, A., Arias, A., Cervenansky, C., and Martinez-Drets, G. 1982. Succinate dehydrogenase mutant of *Rhizobium meliloti*. *J. Bacteriol.* 151:1621-1623.

Goodner, B., Hinkle, G., Gattung, S., Miller, N., Blanchard, M., Qurollo, B., Goldman, B. S., Cao, Y., Askenazi, M., Halling, C., Mullin, L., Houmiel, K., Gordon, J., Vaudin, M., Iartchouk, O., Epp, A., Liu, F., Wollam, C., Allinger, M., Doughty, D., Scott, C., Lappas, C., Markelz, B., Flanagan, C., Crowell, C., Gurson, J., Lomo, C., Sear, C., Strub, G., Cielo, and C., Slater, S. 2001. Genome sequence of the plant pathogen and biotechnology agent *Agrobacterium tumefaciens* C58. *Science* 294:2323-2328.

Green, L. S., and Emerich, D. W. 1997. *Bradyrhizobium japonicum* does not require α -ketoglutarate dehydrogenase for growth on succinate or malate. *J. Bacteriol.* 179:194-201.

Green, L. S., Karr, D. B., and Emerich, D. W. 1998. Isocitrate dehydrogenase and glyoxylate cycle enzyme activities in *Bradyrhizobium japonicum* under various growth conditions. *Arch. Microbiol.* 169:445-451.

Green, L. S., Li, Y., Emerich, D. W., Bergersen, F. J., and Day, D. A. 2000. Catabolism of α -ketoglutarate by a *sucA* mutant of *Bradyrhizobium japonicum*: evidence for an alternative tricarboxylic acid cycle. *J. Bacteriol.* 182:2838-2844.

Green, L. S., Waters, J. K., Ko, S., and Emerich, D. W. 2003. Comparative analysis of the *Bradyrhizobium japonicum* *sucA* region. *Can. J. Microbiol.* 49:237-243.

Guest, J. R., Angier, S. J., and Russell, G. C. 1989. Structure, expression, and protein engineering of the pyruvate dehydrogenase complex of *Escherichia coli*. *Ann. N. Y. Acad. Sci.* 573:76-79.

Hansen, E. J., and Juni, E. 1979. Properties of mutants of *Escherichia coli* lacking malic dehydrogenase and their revertants. *J. Biol. Chem.* 254:3570-3575.

Hederstedt, L. 1993. The Krebs citric acid cycle. Pages 181-197 in: *Bacillus subtilis* and Other Gram-Positive Bacteria. A. L. Sonnenschein, J. A. Hoch, and R. Losick, eds. American Society for Microbiology, Washington, DC.

- Hernández-Lucas, I., Pardo, M. A., Segovia, L., Miranda, J., and Martínez-Romero, E. 1995. *Rhizobium tropici* chromosomal citrate synthase gene. *Appl. Environ. Microbiol.* 61:3992-3997.
- Kaneko, T., Nakamura, Y., Sato, S., Asamizu, E., Kato, T., Sasamoto, S., Watanabe, A., Idesawa, K., Ishikawa, A., Kawashima, K., Kimura, T., Kishida, Y., Kiyokawa, C., Kohara, M., Matsumoto, M., Matsuno, A., Mochizuki, Y., Nakayama, S., Nakazaki, N., Shimpo, S., Sugimoto, M., Takeuchi, C., Yamada, M., and Tabata, S. 2000. Complete genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*. *DNA Res.* 7:331-338.
- Llosa, M., Jubete, Y., and de la Cruz, F. 1991. Tn5tac1 insertion polarity in *Escherichia coli*. *Plasmid* 26:222-224.
- Lodwig, E. M., Hosie, A. H., Bourdes, A., Findlay, K., Allaway, D., Karunakaran, R., Downie, J. A., and Poole, P. S. 2003. Amino-acid cycling drives nitrogen fixation in the legume-Rhizobium symbiosis. *Nature* 422:722-726.
- McDermott, T. R., Griffith, S. M., Vance, C. P., and Graham, P. H. 1989. Carbon metabolism in *Bradyrhizobium japonicum* bacteroids. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Rev.* 63:327-340.
- McDermott, T. R., and Kahn, M. L. 1992. Cloning and mutagenesis of the *Rhizobium meliloti* isocitrate dehydrogenase gene. *J. Bacteriol.* 174:4790-4797.
- McKay, I. A., Dilworth, M. J., and Glenn, A. R. 1988. C₄-dicarboxylate metabolism in free-living and bacteroid forms of *Rhizobium leguminosarum* MNF3841. *J. Gen. Microbiol.* 134:1433-1440.
- Meade, H. M., Long, S. R., Ruvkin, G. B., Brown, S. E., and Ausubel, F. M. 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon mutagenesis. *J. Bacteriol.* 149:114-122.
- Miller, S. S., Driscoll, B. T., Gregerson, R. G., Gantt, J. S., and Vance, C. P. 1998. Alfalfa malate dehydrogenase (MDH): molecular cloning and characterization of five different forms reveals a unique nodule-enhanced MDH. *Plant J.* 15:101-112.
- Mortimer, M. W., McDermott, T. R., York, G. M., Walker, G. C., and Kahn, M. L. 1999. Citrate synthase mutants of *Sinorhizobium meliloti* are ineffective and have altered cell surface polysaccharides. *J. Bacteriol.* 181:7608-7613.
- Natera, S. H. A., Guerreiro, N., and Djordjevic, M. A. 2000. Proteome analysis of differentially displayed proteins as a tool for the investigation of symbiosis. *Mol. Plant-Microbe Interact.* 13:995-1009.
- Neuwald, A. F., Krishnan, B. R., Ahrweiler, P. M., Frieden, C., and Berg, D. E. 1993. Conditional dihydrofolate reductase deficiency due to transposon Tn5tac1 insertion downstream from the *folA* gene in *Escherichia coli*. *Gene* 125:69-73.
- Neuwald, A. F., Krishnan, B. R., Brikun, I., Kulakauskas, S., Suziedelis, K., Tomcsanyi, T., Leyh, T. S., and Berg, D. E. 1992. *cysQ*, a gene needed for cysteine synthesis in *Escherichia coli* K-12 only during aerobic growth. *J. Bacteriol.* 174:415-425.
- Østerås, M., Driscoll, B. T., and Finan, T. M. 1997. Increased pyruvate orthophosphate dikinase activity results in an alternative gluconeogenic pathway in *Rhizobium (Sinorhizobium) meliloti*. *Microbiology* 143:1639-48.
- Poole, P. S., and Allaway, D. 2000. Carbon and nitrogen metabolism in *Rhizobium*. *Adv. Microbial Physiol.* 43:117-163.
- Poole, P., Reid, C., East, A. K., Allaway, D., Day, M., and Leonard, M. 1999. Regulation of the *mdh-sucDAB* operon in *Rhizobium leguminosarum*. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett.* 176:247-255.
- Prell, J., Boetsen, B., Poole, P., and Priefer, U. B. 2002. The *Rhizobium leguminosarum* bv. *viciae* VF39 γ -aminobutyrate (GABA) aminotransferase gene (*gabT*) is induced by GABA and highly expressed in bacteroids. *Microbiology* 148: 615-623.
- Quandt, J., and Hynes, M. F. 1993. Versatile suicide vectors which allow direct selection for gene replacement in Gram-negative bacteria. *Gene* 127:15-21.
- Reed, L. J., and Mukherjee, B. B. 1969. α -Ketoglutarate dehydrogenase complex from *Escherichia coli*. *Methods Enzymol.* 13:55-61.
- Reeves, H. C., Rabin, R., Wegener, W. S., and Ajl, S. J. 1971. Assay of enzymes of the tricarboxylic acid and glyoxylate cycles. *Methods Microbiol.* 6A:435-436.
- Salminen, S. O., and Streeter, J. G. 1990. Factors contributing to the accumulation of glutamate in *Bradyrhizobium japonicum* bacteroids under microaerobic conditions. *J. Gen. Microbiol.* 136:2119-2126.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Schwacha, A., and Bender, R. A. 1993. The product of the *Klebsiella aerogenes nac* (nitrogen assimilation control) gene is sufficient for activation of the *hut* operons and repression of the *gdh* operon. *J. Bacteriol.* 175:2116-2124.
- Simon, R., Quandt, J., and Klipp, W. 1989. New derivatives of transposon Tn5 suitable for mobilization of replicons, generation of operon fusions and induction of genes in gram-negative bacteria. *Gene* 80:161-169.
- Stanier, R. Y., Ingraham, J. L., Wheelis, M. L., and Painter, P. R. 1986. *The Microbial World*, 5th ed. Prentice-Hall, Englewood Cliffs, NJ.
- Streeter, J. G. 1991. Transport and metabolism of carbon and nitrogen in legume nodules. *Adv. Bot. Res.* 18:129-187.
- Tajima, S., and Kouchi, H. 1996. Metabolism and compartmentation of carbon and nitrogen in legume nodules. Pages 27-60 in: *Plant-Microbe Interactions*, Vol. 2. G. Stacey, and N. T. Keen, eds. Kluwer Academic Publishers, Boston.
- Thompson, J. D., Higgins, D. G., and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22:4673-4680.
- Thöny, B., and Hennecke, H. 1989. The -24/-12 promoter comes of age. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Rev.* 63:341-357.
- Udvardi, M. K., and Day, D. A. 1997. Metabolite transport across symbiotic membranes of legume nodules. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48:493-523.
- Vance, C. P., and Heichel, G. H. 1991. Carbon in N₂ fixation: limitation or exquisite adaptation. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 42:373-392.
- Veeger, C., DerVartanian, D. V., and Zeylemaker, W. P. 1969. Succinate dehydrogenase. *Methods Enzymol.* 13:81-90.
- Walshaw, D. L., Wilkinson, A., Mundy, M., Smith, M., and Poole, P. S. 1997. Regulation of the TCA cycle and general amino acid permease by overflow metabolism in *Rhizobium leguminosarum*. *Microbiology* 143:2209-2221.
- Watson, R. J., and Rastogi, V. K. 1993. Cloning and nucleotide sequencing of *Rhizobium meliloti* aminotransferase genes: an aspartate aminotransferase required for symbiotic nitrogen fixation is atypical. *J. Bacteriol.* 175:1919-1928.
- Westenberg, D. J., and Gueriot, M. L. 1999. Succinate dehydrogenase (Sdh) from *Bradyrhizobium japonicum* is closely related to mitochondrial Sdh. *J. Bacteriol.* 181:4676-4679.
- Wierenga, R. K., and Hol, W. G. 1983. Predicted nucleotide-binding properties of p21 protein and its cancer-associated variant. *Nature* 302:824-844.
- Yarosh, O. K., Charles, T. C., and Finan, T. M. 1989. Analysis of C₄-dicarboxylate transport genes in *Rhizobium meliloti*. *Mol. Microbiol.* 3:813-823.