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Malonate Catabolism Does Not Drive N_2 Fixation in Legume Nodules

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Malonyl-coenzyme A (CoA) decarboxylase, malonyl-CoA synthetase, and malonate transporter mutants of *Rhizobium leguminosarum* bv. viciae and trifolii fixed N_2 at wild-type rates on pea and clover, respectively. Thus, malonate does not drive N_2 fixation in legume nodules.

The C_3-dicarboxylic acid malonate is abundant in legumes, accounting for up to 4% of the weight of plants (dry weight) and up to 50% of total acidity (1–3). Despite its abundance, a role for malonate as a significant carbon source for bacteroid metabolism was initially ruled out by studies on malonate utilization and metabolite uptake studies with intact symbiosomes (4–6). However, because the level of malonate is elevated in nodules during symbiosis, it has been suggested that it may be an important carbon source during N_2 fixation (7, 8). The malonate catabolic operon has been characterized well in *Rhizobium leguminosarum* bv. trifolii, where *matA*, *matB*, and *matC* encode malonyl-coenzyme A (CoA) decarboxylase, malonyl-CoA synthetase, and a presumed malonate transporter, respectively (9). Furthermore, it was reported that a *matB* mutant of *R. leguminosarum* bv. trifolii ATCC 14479 did not fix N_2 on white clover and had nodules filled with vacuoles rather than bacteroids (10). It is possible that the paradigm that C_4-dicarboxylic acids are the principal carbon sources that fuel N_2 fixation is incorrect (11, 12). However, malonate is a competitive inhibitor of succinate dehydrogenase, and its presence in legumes may require it to be rendered nontoxic by rhizobia (7). More recently, the *Sinorhizobium meliloti* malonate catabolic operon (*matPQMAB*) was characterized (13). A separate TRAP (tripartite ATP-independent permease) system (Sma0151, Sma0155, and Sma0157) was also shown to be induced by malonate in *S. meliloti* (14) and was subsequently characterized as a malonate transporter (13). This contrasts with MatC, which is a major facilitator system (MFS) present in *R. leguminosarum* bv. trifolii. In stark contrast to the results in clover, strains with mutations in each gene of the *matPQMAB* operon of *S. meliloti* did not decrease N_2 fixation by alfalfa (13). To resolve this paradox, we examined the symbiotic role of malonate catabolism in two biovars of *R. leguminosarum*, the same clover-nodulating *R. leguminosarum* bv. trifolii TCC 14479 already used (10) and the pea-nodulating *R. leguminosarum* bv. viciae Rlv3841.

***matA*, *matB*, and *matC* mutants do not grow on malonate.** The chromosomal *matABC* operon (RL0990-RL0992) of *R. leguminosarum* bv. viciae Rlv3841 has the same structure as that of *R. leguminosarum* bv. trifolii ATCC 14479, where *matA* encodes malonyl-CoA decarboxylase, *matB* encodes malonyl CoA synthetase, and *matC* encodes a transport system for malonate. The *matA*, *matB*, and *matC* genes from strains Rlv3841 and ATCC 14479 were mutated, forming RU4053, LMB557, RU4054, LMB134, LMB510, and LMB136 (see supplemental material).

Growth of both *R. leguminosarum* Rlv3841 and ATCC 14479 was very poor on agar plates containing malonate as the sole carbon source, with concentrations above 5 mM preventing growth (Table 1; see Fig. S1 in the supplemental material). Strain Rlv3841 grew in liquid culture on malonate (5 mM) or succinate (20 mM) as the sole carbon source with generation times of 19 h ± 1.5 h (mean ± standard error of the mean [SEM]) and 5 ± 0.5 h, respectively. Mutation of *matA*, *matB*, or *matC* prevented growth of strains derived from Rlv3841 and ATCC 14479 on malonate (5 mM) (Table 1 and Fig. S1). The addition of malonate (5 mM) to Rlv3841 or ATCC 14479 prevented growth on succinate as a carbon source (Table 1). Thus, malonate (5 mM), which by itself enables slow growth, prevents growth in the presence of a pre-

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TABLE 1 Growth of *R. leguminosarum* bv. viciae and *R. leguminosarum* bv. trifolii strains on different carbon sources

Sole carbon source(s)	Growth of the following strain on the indicated carbon source[a]:							
	Rlv3841 (WT)	RU4053 (*matA*)	LMB557 (*matB*)	RU4054 (*matC*)	ATCC 14479 (WT)	LMB134 (*matA*)	LMB510 (*matB*)	LMB136 (*matC*)
None	−	−	−	−	−	−	−	−
5 mM malonate	+	−	−	−	+	−	−	−
10 mM malonate	−	−	−	−	−	−	−	−
20 mM succinate	++	++	++	++	++	++	++	++
5 mM malonate and 20 mM succinate	−	−	−	−	−	−	−	−
10 mM malonate and 20 mM succinate	−	−	−	−	−	−	−	−

[a] Growth is scored as shown in Fig. S1 in the supplemental material: −, no growth; +, poor growth; ++, good growth. WT, wild type.

TABLE 2 Complementation of ATCC 14479-derived malonate mutants with different complementing plasmids

Complementing plasmid	Growth of the following mutant with the indicated complementing plasmid[a]:		
	matA mutant (LMB134)	*matB* mutant (LMB510)	*matC* mutant (LMB136)
pLMB605 (p*matA*)	−	−	−
pLMB610 (p*matAB*)	−	−	ND
pLMB606 (p*matC*)	ND	ND	−
pLMB628 (p*matABC*)	−	−	−
pLMB654 (p*matRABC*)	+	+	+
pLMB509 (pTau vector)[b]	ND	ND	−
pLMB653 (pTau::*matC*)[b]	ND	ND	+

[a] Growth of mutant strains derived from *R. leguminosarum* ATCC 14479 is scored as shown in Fig. S1 in the supplemental material: −, no growth; +, poor growth; ND, not determined. Growth was measured on UMS agar (see detailed methods in the supplemental material) supplemented with 5 mM malonate as the sole carbon source.
[b] Grown on 5 mM malonate plus 0.1 mM taurine.

ferred carbon source such as succinate, consistent with malonate being an inhibitor of succinate dehydrogenase (7). Succinate dehydrogenase mutants of *R. leguminosarum* also grow slowly on succinate, although this was overcome by growth on other dicarboxylates such as malate (15). Growth on succinate may be particularly sensitive to malonate because the first step in its catabolism is blocked. Malonate also prevented growth on succinate when the gene encoding the malonate transporter (*matC*) was mutated, suggesting the presence of an alternative low-affinity malonate transporter.

Complementation of malonate catabolic and transport mutants. The promoter for the putative *matABC* operon has been shown to be immediately upstream of *matA* in strain ATCC 14479 by footprinting (16). However, while *matA* and *matB* overlap by 3 bp, suggesting translational coupling, there is an intergenic region of 96 bp between *matB* and *matC* in both Rlv3841 and ATCC 14479. In order to determine whether there is a separate promoter for *matC* from strain ATCC 14479, this 96-bp intergenic region, together with the whole *matC* gene, was cloned into pRU1097 (promoterless vector) to form pLMB606. Plasmid pLMB606 did not complement strain LMB136 (ATCC 14479 *matC*) for growth on malonate (5 mM) as the sole carbon source (Table 2). Thus, *matC* lacks its own promoter. For a control, *matC* was cloned into the taurine-inducible broad-host-range vector pLMB509 (17), forming pLMB653. The complemented strain [LMB136(pLMB653)], but not the *matC* strain containing an empty vector [LMB136 (pLMB509)], grew on malonate (5 mM) plus taurine (0. 1 mM) (Table 2). To further investigate the promoter of the *mat* operon, clones of *matA*, *matAB*, and *matABC* were made in pRU1097. These clones contain the complete intergenic region between *matR* and *matA*, but none complemented growth of *matA* or *matB* or *matC* mutants on malonate (Table 2). However, the entire *matRABC* region did complement *matA*, *matB*, and *matC* strains for growth on malonate (Table 2 and see Fig. S1 in the supplemental material). This is consistent with *matR* being a positive regulator of *matABC*, which form a single operon. When *matABC* is cloned without *matR*, the chromosomal copy of *matR* may result in insufficient MatR for productive binding across multiple operator sites contained on several copies of the plasmid.

Clover and pea infected with *mat* mutants are fixation positive. Since malonate catabolism has been reported to be essential for N_2 fixation by white clover infected with *R. leguminosarum* bv. trifolii (10), our principal aim was to examine the symbiotic phenotypes of *mat* mutants of *R. leguminosarum* nodulating pea and clover. Pea (*Pisum sativum* cv. Avola) or red and white clover (*Trifolium pretense* and *Trifolium repens*, respectively) inoculated with *R. leguminosarum* bv. viciae or *R. leguminosarum* bv. trifolii strains, respectively, were grown on nitrogen-free medium, and acetylene reduction was measured at 4 and 5 weeks as previously described (18). Peas nodulated by *matA*, *matB*, or *matC* mutants produced normal red nodules, and plants were green and healthy, unlike uninoculated plants, which were yellow and stunted (data not shown). Furthermore, peas nodulated by *mat* mutants were not altered in acetylene reduction relative to strain Rlv3841 (Table 3), indicating no impairment of N_2 fixation. From this, we conclude that as for alfalfa, malonate is not an important carbon source required for N_2 fixation in pea. However, *mat* mutants of strain ATCC 14479 inoculated on red and white clover also pro-

TABLE 3 Acetylene reduction by strains of *R. leguminosarum* on pea (*P. sativum*) and red and white clover (*T. pretense* and *T. repens*)

Plant	Time postinoculation (wk)	Strain	Relevant genotype or phenotype[a]	Amt of acetylene reduced (μmol/plant/h) (mean ± SEM) (*n*)[b]
Pea	3	Rlv3841	WT	2.65 ± 0.17 (6)
		RU4053	*matA*::pK19	2.82 ± 0.15 (6)
		RU4054	*matC*::pK19	2.87 ± 0.16 (6)
	4	Rlv3841	WT	6.9 ± 0.35 (6)
		LMB557	*matB*::ΩSp^r	7.2 ± 0.50 (6)
Red clover[c]	4	ATCC 14479	WT	0.118 ± 0.020 (30)
		LMB134	*matA*::ΩTc^r	0.099 ± 0.009 (30)
		LMB136	*matC*::ΩTc^r	0.117 ± 0.023 (30)
	5	ATCC 14479	WT	0.431 ± 0.01 (12)
		LMB510	*matB*::ΩSp^r	0.470 ± 0.015 (24)
White clover[c]	5	ATCC 14479	WT	0.407 ± 0.019 (24)
		LMB510	*matB*::ΩSp^r	0.446 ± 0.044 (36)

[a] Abbrevations used: WT, wild type; Sp^r, spectinomycin resistance; Tc^r, tetracycline resistance.
[b] The number of plants tested is shown in parentheses.
[c] Plants shown in Fig. S2 in the supplemental material.

duced green healthy plants with red nodules, which were indistinguishable from plants inoculated with ATCC 14479. This contrasts with uninoculated control plants that were stunted and yellow (see Fig. S2 in the supplemental material). Light and electron micrographs of nodules infected with *matA* or *matC* strains were indistinguishable from nodules infected with ATCC 14479 (data not shown). Furthermore, the rates of acetylene reduction were unaltered in red clover inoculated with *matA*, *matB*, and *matC* mutants relative to those inoculated with ATCC 14479 (Table 3). Likewise, white clover inoculated with a *matB* mutant was unaltered relative to plants inoculated with ATCC 14479. Bacteria recovered from nodules from peas and from both red and white clover showed the expected resistance markers and gene insertions, indicating that the mutations were stably maintained throughout the experiments. In agreement with results for alfalfa (13), malonate cannot be an essential carbon source for N_2 fixation in white clover, red clover, or pea.

An important question is why our results for red and white clover are in stark contradiction to those previously published (10). In the two studies, the same parent strain of *R. leguminosarum* bv. trifolii ATCC 14479 was used. One possibility is that malonate catabolism in nodules is principally a detoxification reaction. The malonate mutants characterized previously (10) did not form mature bacteroids, indicating that their development had been impaired. This can be contrasted with dicarboxylate transport mutants (*dct*) mutants of *R. leguminosarum* biovars trifolii and viciae which develop normally to form morphologically branched cells (19, 20), although they senesce early (11). Thus, high concentrations of malonate in the nodule might poison carbon metabolism and potentially prevent bacteroid development and N_2 fixation. The levels of malonate reported in legumes vary widely, and this may be due to cultivation conditions or the plant species or cultivar. For example, *matABC* in strain Rlv3841 was induced in the rhizosphere of alfalfa but not pea, suggesting that differing levels of malonate were encountered (21). This might explain why *mat* mutants used in different studies could lead to different N_2 fixation phenotypes in clover. We conclude that malonate does not drive N_2 fixation in legume nodules but instead is a poor carbon source that can also act as a metabolic poison.

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