

Utilization of the tricarboxylic acid cycle intermediates and symbiotic effectiveness in *Rhizobium meliloti**

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Summary The utilization of the tricarboxylic acid cycle intermediates and related compounds was studied in strains of *Rhizobium meliloti* having different symbiotic effectiveness. In general, the very effective (VE) strains used these compounds as sole carbon source better than the ineffective (I) strains. However, a significant difference was observed between VE and I strains in their ability to use acetate or oxaloacetate for growth. In fact, at a concentration of 2 mM, 80% of the VE strains used acetate or oxaloacetate while 50% of the I strains used acetate and none was able to grow on oxaloacetate. No correlation was found between the symbiotic effectiveness of the strains and their ATP content, when grown on mannitol. The highest ATP content ($9.21 \text{ nM} \times \mu\text{g protein}^{-1}$) was found in the I strain S₂₀ and the lowest ($0.69 \text{ nM} \times \mu\text{g protein}^{-1}$) was found in the effective strain S₈. Numerical analysis of the patterns of utilization of the TCA cycle intermediates and related compounds indicated that the 49 strains tested formed 11 distinct groups at 86% similarity, according to Jaccard's coefficient. Several strains showed unique patterns of utilization and can be clearly identified under laboratory conditions.

Introduction

In the legume-Rhizobium symbiosis, the energy required for nodule function is derived from products of photosynthesis. The carbon source supplied by the plant to bacteroids has yet to be fully identified¹³, however several investigations indicate that bacteroids probably receive a supply of tricarboxylic acid cycle (TCA) intermediates from plant cytosols as major source of ATP and reductant for nitrogen fixation^{4,14,20}.

It was also reported that the TCA cycle pathway was operating less efficiently in non-nodulating *Rhizobium meliloti* mutants, suggesting that this pathway may be essential for the nodulation process⁵.

The fast and slow growing rhizobia have an apparently defective TCA cycle¹⁸, and the utilization of the TCA cycle intermediates and carbohydrates have been used to differentiate between fast and slow growing strains⁷.

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In the present paper, the patterns of utilization of the TCA cycle intermediates and related compounds were determined in different strains of *R. meliloti* with the aim of correlating these patterns with the symbiotic effectiveness of the strains. The possibility of using these patterns for strain identification under laboratory conditions was also studied. As the TCA cycle is an important source of ATP for nitrogenase activity, and that an effective strain accumulated more ATP in its cells than an ineffective strain¹², the relationship between ATP content of *R. meliloti* strains grown on mannitol and their symbiotic effectiveness was investigated. If this observation made with a few strains¹² could be applied to a larger number of isolates, then ATP accumulation in *R. meliloti* can be used as a rapid test for the selection of very effective strains.

Materials and methods

Symbiotic effectiveness

The symbiotic effectiveness of the 49 strains of *R. meliloti* was previously described². However, because of the instability of the symbiotic effectiveness of Rhizobia^{3,15} and because symbiotic effectiveness is estimated better in the greenhouse in the presence of nitrate in the nutrient solution^{8,16}, the symbiotic effectiveness of 46 *R. meliloti* strains was assessed by the method previously described² modified as follows. Seeds of the cultivar Saranac of lucerne (*Medicago sativa* L.) were surface sterilized¹⁹ and germinated on sterile agar (1.5%) for 36 h in the dark. Fifteen germinated seeds were sown in 'Riviera' pots (Manufacture provençale de matières plastiques de Marseille, France) sterilized with a 0.5% Oakite solution (Sanitizer no. 1, Oakite Products of Canada, Bramalea, Ontario) and containing 2.3 l of an autoclaved mixture of 50% (v/v) vermiculite and 50% sand. The pots reservoirs were filled with a Hoagland's nutrient solution² containing 30 µg/ml N as KNO₃, and the pots were covered with transparent plastic bags. Seven days after sowing, the plants were inoculated by adding to each pot 110 ml of the nutrient solution containing approximately 10⁷ Rhizobium cells/ml. When the plants formed their third leaf, the plastic bags were removed and the pots were thinned to seven uniform plants per pot. The first harvest was taken seven weeks after sowing and the second harvest three weeks later. In the growth room, plants were grown under a 16 h light period (1.6 Klx) at 18–20°C and 8 h darkness at 11–12°C. The experimental design was a randomized complete block with 3 replicates. A strain was arbitrarily rated very effective (VE) when its dry matter yield with lucerne was higher than the total mean of a harvest plus the standard deviation, effective (E) when its yield was between that of the mean ± the standard deviation and ineffective (I) when its yield was smaller than the mean minus the standard deviation².

Utilization of the TCA cycle intermediates

Media Strains were maintained as slant cultures on yeast extract mannitol agar¹⁹. Bacterial growth with the various compounds tested were performed on a basal medium consisting of (mg per liter of distilled water): CaCl₂·2H₂O, 50; MgSO₄·7H₂O, 200; NaCl, 100; (NH₄)₂SO₄, 300; K₂HPO₄, 520; KH₂PO₄, 410; biotin, 0.25; CoCl₂·6H₂O, 4 × 10⁻³; CuSO₄·5H₂O, 8 × 10⁻³; H₃BO₃, 2.86; H₂MoO₄·H₂O, 9 × 10⁻²; MnCl₂·4H₂O, 1.81; ZnSO₄·7H₂O, 0.22. Phosphates were autoclaved separately and the pH of the medium was 6.9. The TCA cycle intermediates and related compounds as sodium salts (Sigma Chem. Co., St. Louis, Mo) were used at concentrations of 2 or 20

mM in the presence or absence of 55 mM mannitol. All carbon sources were filter sterilized (0.2 μm pore, Nalgene Co., Rochester, N. Y.) and mixed to the sterile basal medium containing 1.5% agar cooled to 45°C.

Preparation of inocula The inocula were prepared by growing the cultures in 250 ml Erlenmeyer flasks containing 50 ml of yeast extract mannitol broth¹⁹. The flasks were incubated at 30°C for 3 days on a rotary shaker operating at 150 rev. min⁻¹. The cells from 10 ml cultures were washed twice in phosphate buffered saline (3 mM phosphate buffer in 0.7% NaCl, pH 6.8) and resuspended in 10 ml buffer. This final cell suspension was used as inoculum.

Plate inoculation Each of the 28 wells matching an aluminum multiple inoculator¹⁰ received 0.1 ml of the inoculum. It was estimated that each prong of the inoculator transferred about 10⁴ bacteria. For each strain tested 3 wells were chosen randomly and 2 plates from each medium were inoculated. In each test, the strain A₂ was used to test the reproducibility of the results.

Growth Plates containing the basal medium without a carbon source and the basal medium with mannitol were used as controls. Growth was recorded after 7 days incubation at 30°C. The presence of growth was scored 1 and the absence of growth was scored 0.

Numerical analysis Tests in which all cultures gave the same result were disregarded for computer analysis. Cluster analysis was carried out by using Clustran computer program (Wishart D., Edinburgh University, Scotland, 1978). Jaccard's coefficient was calculated and the results are shown as a dendrogram prepared by the unweighted pair group method using arithmetic averages¹⁷.

ATP assay

R. meliloti strains were grown in 250 ml Erlenmeyer flasks containing 50 ml of the basal medium with mannitol. The flasks were incubated at 20°C on a rotary shaker operating at 160 r.p.m. After 4 days, the cells from 10 ml cultures were washed twice in sterile saline (0.85% NaCl). ATP was extracted by the addition of 2.4 ml dimethyl sulfoxide⁹, and the suspension was thoroughly mixed for 15 sec on a Vortex mixer. After 2 min, 3.6 ml of a sterile Mops-Mg buffer (10 mM Mops, 33 mM Mg SO₄·7H₂O, pH adjusted to 7.4 with 1N NaOH) were added and the mixtures were kept at -20°C overnight, and centrifugated at 12,000 × *g* for 10 min. The supernatant fluid was assayed for ATP by a modification of the luciferin-luciferase method⁹: 0.5 ml of a freshly prepared luciferin-luciferase reagent (10 mg in 5 ml Mops buffer without Mg) were added to 0.3 ml of the supernatant with immediate shaking. Luminescence was measured in an LKB luminometer (Wallac 1250). ATP standards were assayed concurrently. All reagents were obtained from Sigma Chemical Co. (St. Louis, Mo). For protein measurements, washed cells from 10 ml cultures were digested for 15 min in 1 ml 1N NaOH at 90°C and protein was assayed with the Folin phenol reagent¹¹. All assays were performed in duplicate.

Results and discussion

The strain A₂ of *R. meliloti* used as a check, always gave very good reproducibility. None of the compounds tested were readily used as sole carbon source by all the 49 strains tested (Table 1). Most of the strains used acetate, fumarate, lactate, pyruvate and succinate as the only carbon source at concentrations of 2 and 20 mM. The concentration of acetate, glyoxylate, lactate and pyruvate in the culture medium appeared to be the growth limiting factor for some strains, as indicated by an increase in the number of strains showing growth when the

Table 1. Growth of the 49 strains of *R. meliloti* on TCA cycle intermediates and related compounds.

Group no.	Subgroup no.	Strain	Without mannitol																							
			2 mM							20 mM ^b																
			AC	CI	FU	GL	KG	LA	MA	OA	PY	SU	AC	FU	GL	LA	MA	OA	PY	SU	LA	AC	CI	FU	LA	OA
1		S ₁₄	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		E ₂	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		A ₄ , S ₁₁ , D ₃	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		S ₁₅	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		V ₅	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		S ₂₁ , V ₅ , D ₂	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		54032	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	1	A ₃ , S ₁	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	2	3Doo8	0	+	+	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	0	+	+	+	+	+
	3	V ₂ , A ₅	0	+	+	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	1	A ₂ , S ₂₂ , I ₃ , Y ₆	+	+	+	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	2	D ₁ , Alfalfa D	+	+	+	0	0	+	+	+	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	3	S ₈	+	+	+	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	4	V ₁	0	+	+	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	1	S ₂ , S ₁₂ , I ₂	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	2	S ₄ , Z3A	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	3	S ₃	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	4	V ₇	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	5	S ₇	+	+	+	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	6	S ₁₃ , S ₁₆ , S ₁₉ , R ₁	+	+	+	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	7	S ₉ , S ₂₀	0	+	+	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	8	3Doo20a	0	+	+	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	1	S ₅	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	2	V ₄	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	1	54033	0	+	+	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	2	C ₁	0	+	+	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	7	S ₆	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8		S ₁₀	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9		A ₁	0	+	+	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10		E ₁	0	+	+	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11		I ₁	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

^a Abbreviations: AC, acetate; CI, citrate; FU, fumarate; GL, glyoxylate; KG, α -Ketoglutarate; LA, lactate; MA, malate; OA, oxaloacetate; PY, pyruvate; SU, succinate; + = growth; 0 = no growth.

^b All the strains scored 0 with CI, KG and MA.

^c All the strains scored + with AC, CI, FU, GL, KG, MA, OA, PY and SU.

^d All the strains scored + with GL and SU and 0 with KG and MA.

concentration of these compounds was increased from 2 to 20 mM in the culture medium (Table 2). Growth of all strains was inhibited by 20 mM citrate, α -ketoglutarate and malate. The addition of 55 mM mannitol, eliminated only the inhibitory effect of citrate in 28 strains (Table 1). In general, the TCA cycle intermediates and related compounds had little effect on the growth of the *R. meliloti* strains, when mannitol was added to the culture media.

Table 3 shows the dry matter yield of lucerne inoculated with 46 strains of *R. meliloti* at the first and second harvest. In the following discussion, the symbiotic effectiveness of the strains was appraised from yields obtained at the second

Table 2. Utilization of the TCA cycle intermediates and related compounds by 46 strains of *R. meliloti* having different symbiotic effectiveness

Compound	Concn. mM	No. of positive strains					
		Without mannitol			55 mM mannitol		
		VE†	E	I	VE	E	I
Acetate	2	4	28	3	5	35	6
	20	5	33	4	4	33	4
Citrate	2	5	35	5	5	35	6
	20	0	0	0	4	19	2
Fumarate	2	5	35	5	5	35	6
	20	5	34	5	5	34	5
Glyoxylate	2	0	7	1	5	35	6
	20	3	32	3	5	35	6
α -Ketoglutarate	2	2	18	1	5	35	6
	20	0	0	0	0	0	0
Lactate	2	5	30	4	5	34	5
	20	5	32	4	5	34	5
Malate	2	5	35	5	5	35	6
	20	0	0	0	0	0	0
Oxaloacetate	2	4	18	0	5	35	6
	20	3	12	0	5	32	4
Pyruvate	2	3	24	5	5	35	6
	20	5	32	5	5	35	4
Succinate	2	5	35	5	5	35	6
	20	5	34	4	5	35	6

† VE = very effective, E = effective, I = ineffective. At the second harvest, 5 strains were rated VE, 35 E and 6 I.

Table 3. Symbiotic effectiveness and ATP content of strains of *Rhizobium meliloti*

Strains	Dry matter yield (g/pot)		ATP nanomoles \times $\mu\text{g protein}^{-1}$
	1st harvest	2nd harvest	
S ₁	10.79 VE†	19.01 VE	2.38
S ₂	9.06 E	16.87 E	1.63
S ₄	9.58 E	16.06 E	3.00
S ₅	10.30 VE	20.20 VE	2.57
S ₆	8.71 E	15.84 E	5.29
S ₇	7.60 E	12.06 I	1.24
S ₈	8.74 E	12.38 E	0.69
S ₁₀	5.29 I	13.00 E	1.81
S ₁₁	10.51 VE	15.54 E	3.94
S ₁₂	11.12 VE	16.31 E	1.46
S ₁₄	7.34 E	16.00 E	3.75
S ₁₅	12.45 VE	16.78 E	4.12
S ₁₆	10.17 E	16.20 E	1.30
S ₁₉	10.41 VE	14.39 E	2.21
S ₂₀	3.90 I	9.12 I	9.21
S ₂₁	8.36 E	15.34 E	1.28
S ₂₂	6.92 E	15.10 E	2.98
V ₁	8.64 E	14.88 E	1.92
V ₂	7.76 E	15.18 E	0.73
V ₃	7.39 E	16.98 E	4.85
V ₄	3.83 I	5.74 I	3.29
V ₅	5.85 I	15.84 E	1.33
V ₆	6.96 E	11.83 I	4.00
V ₇	8.89 E	18.77 VE	3.35
D ₁	8.70 E	16.96 E	1.32
D ₂	11.53 VE	16.15 E	4.18
D ₃	6.66 E	12.99 E	2.01
A ₁	6.87 E	12.97 E	2.61
A ₂	6.92 E	14.37 E	3.44
A ₃	11.41 VE	19.48 E	3.46
A ₄	10.20 E	15.96 E	3.55
A ₅	7.43 E	15.48 E	4.52
I ₁	5.26 I	11.88 I	2.98
I ₂	7.40 E	12.24 E	1.60
I ₃	7.25 E	14.43 E	1.14
I ₄	6.51 E	12.86 E	1.94
R ₁	5.63 I	15.03 E	3.09
E ₁	4.23 I	9.00 I	1.30
E ₂	11.40 VE	16.17 E	2.03
23A	7.28 E	17.49 E	0.95
3 Doa20a	8.28 E	14.69 E	3.52
3 Doa8	10.62 VE	19.33 VE	2.86
C ₁	7.92 E	17.06 E	3.31
54032	8.43 E	15.68 E	4.05
54033	7.97 E	15.05 E	4.03
Alfalfa D	5.86 I	12.46 E	4.68
Mean	8.14	14.94	
Standard deviation	2.12	2.81	

† VE = very effective; E = effective; I = ineffective.

harvest, because it appeared to give the most necessary informations for the exact evaluation of effectiveness². At the second harvest 5 strains were rated VE, 35, E and 6, I. The VE strains, in general, used the TCA cycle intermediates and related compounds better than the I strains. For example, all the 5 VE strains used 2 mM citrate, fumarate, lactate, malate and succinate as sole carbon source while none of the compounds tested were readily used as sole carbon source by all the I strains (Table 2). These results indicate as previously reported^{4,5,14,20}, that the TCA cycle pathway may play a role in the nodulation process and in the activity of bacteroids in the nodule. Although differences in growth (colony size) were observed with several compounds, no attempt was made to score growth objectively or to correlate the differences in growth with symbiotic effectiveness, because these differences might only be due to a stimulation of extracellular polysaccharide production. However, significant differences were observed between VE and I strains in their ability to use acetate or oxaloacetate. In fact, at 2 mM, 80% of the VE and 80% of the E strains used acetate as the sole carbon source but only 50% of the I strains were able to grow on acetate. Moreover, all the I strains were not able to use 2 mM oxaloacetate while 80% of the VE and 51% of the E strains indicated growth (Table 2). Similar results were observed with the 10 strains rated VE and the 8 strains rated I at the first harvest (Tables 1 and 3). At the first harvest 90% and 80% of the VE strains used 2 mM of acetate or oxaloacetate for growth but only 63% and 25% of the I strains used these 2 compounds as carbon source. Results obtained with 20 mM acetate or oxaloacetate are comparable to those observed at 2 mM, but at this concentration oxaloacetate showed an inhibitory effect on 1 VE strain (V₇) and on 6 E strains (S₂, S₁₀, S₁₂, I₂, 54032 and 54033). These results suggest that the ability of *R. meliloti* to use acetate or oxaloacetate *in vitro* is linked to some extent to its ability to establish an efficient symbiotic association with lucerne. Acetate and oxaloacetate may be two major substances supplied to the bacteroids by plant cytosols. These two compounds can influence several metabolic pathways and they have a direct effect on citrate synthase, the enzyme controlling the functioning of the TCA cycle⁶, the pathway yielding energy for nitrogenase activity.

The growth patterns of the 49 strains tested on TCA cycle intermediates and related compounds is shown in Table 1. At a level of 86% similarity, the 49 strains formed 11 groups including 31 distinct clusters (Fig. 1). Group 4 is the largest group and it contains 8 clusters formed by 15 strains including the VE strain V₇ and the I strains S₇ and S₂₀. Group 2 included 3 of the VE strains and the I strains E₁ and I₁. The E strains S₆, S₁₀ and A₁ formed each a distinct group and had an apparent very defective TCA cycle.

When grown on mannitol, the I strain S₂₀ had the highest ATP content (9.21 nM × μg protein⁻¹) and the E strain S₈ the lowest one (0.69 nM × μg protein⁻¹) (Table 3). No significant correlations were observed between strains ATP content and the dry matter yields obtained with lucerne at the first ($r = -0.11$) and

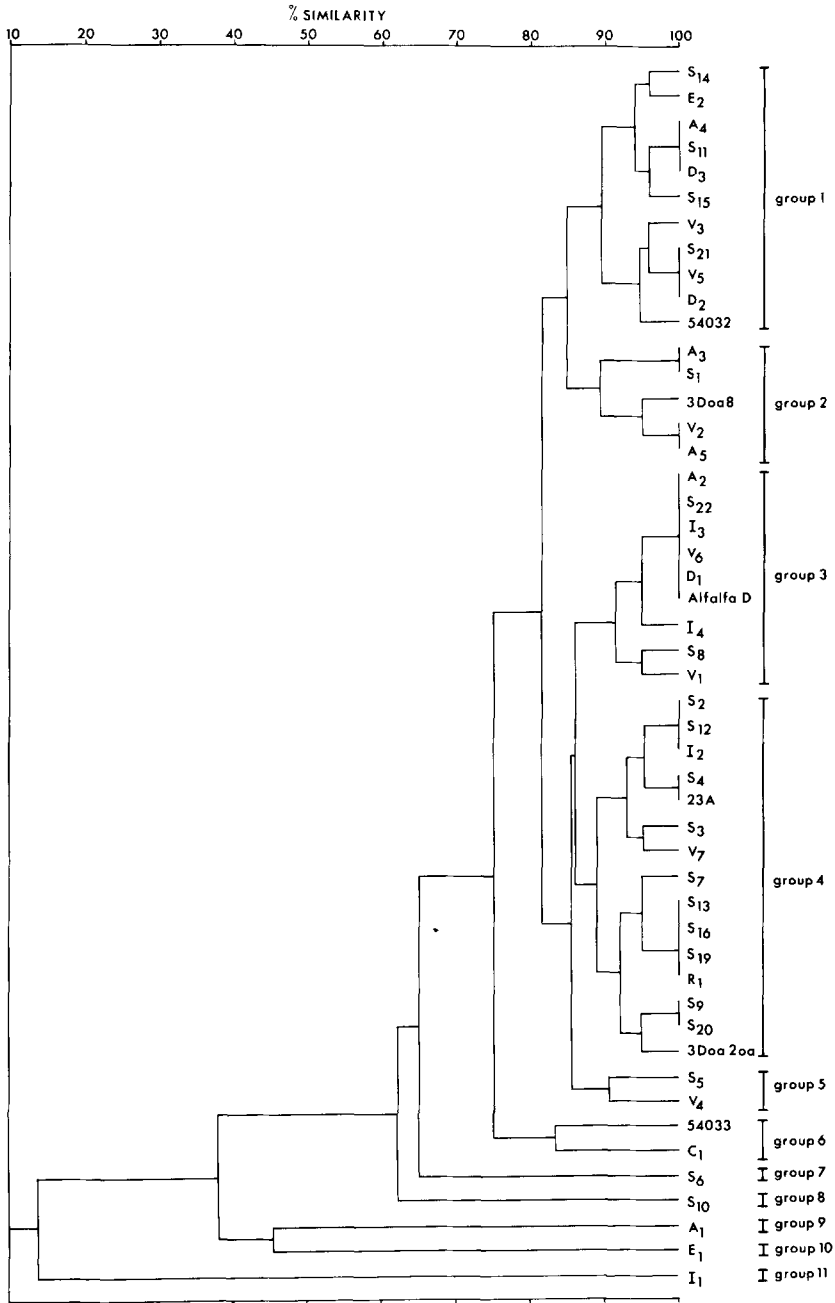


Fig. 1. Dendrogram, based on Jaccard's coefficient of the *R. meliloti* strains according to their utilization of the TCA cycle intermediates and related compounds.

the second ($r = -0.10$) harvest. The ability of a *R. meliloti* strain, grown on mannitol, to accumulate ATP in its cells under laboratory conditions cannot be used as a rapid test for the selection of VE strains. However, as the bacteroids probably receive a TCA cycle intermediate as an energy source^{4,14,20}, it will be relevant to investigate the effect of the TCA cycle intermediates and related compounds on ATP accumulation in *R. meliloti*.

The present work shows that under laboratory conditions, some *R. meliloti* strains can be clearly identified according to their patterns of utilization of the TCA cycle intermediates and related compounds as previously reported with the intrinsic antibiotic resistance of the strains¹. The results also indicate that the TCA pathway is operating more efficiently in VE strains. As the TCA cycle is an important source of ATP, further work is required to correlate the efficiency of this pathway with nitrogenase activity.

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