

## Oxydation of substrates in organic acids utilization negative mutants and the wild type *Rhizobium meliloti* strain S<sub>14</sub>

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### Abstract

Two mutants defective in succinate utilization were isolated by NTG mutagenesis of the effective wild type *Rhizobium meliloti* strain S<sub>14</sub>. The mutants used carbon sources in a fashion similar to strain S<sub>14</sub>, but they were not able to grow on succinate, fumarate or malate. The mutants nodulated alfalfa plants but did not exhibit any nitrogenase activity. The mutants oxidized glucose and fructose, but were not able to oxidize organic acids. Cultured free-living bacteria of strain S<sub>14</sub> appeared to have an inducible C<sub>4</sub>-dicarboxylic acid uptake system and a constitutive glucose uptake system. When S<sub>14</sub> cells were grown on glucose in the presence of 5 mM or more succinate or malate, the rate of glucose-dependent O<sub>2</sub> consumption significantly decreased suggesting the presence of a catabolite repression like phenomenon.

### Introduction

The supply of carbon to the bacteroid by the plant is a vital event in symbiosis and is believed to be one of the major factors limiting nitrogen fixation (Pate, 1977). The efficiency of energy derivation from carbon-source utilization by rhizobia is also probably a determining factor in the efficiency of symbiotic nitrogen fixation (Elkan and Kuykendall, 1982). The exact nature of the carbon source supplied by the plant bacteroids is not yet known, however several facts suggest that the energy sources of *Rhizobium* in symbiotic state are organic acids (Elkan and Kuykendall, 1982). In *Rhizobium meliloti*, the effective strains used better than the ineffective strains, the tricarboxylic acid cycle inter-

mediates and related compounds (Antoun *et al.*, 1984), and mutants defective in the uptake of dicarboxylic acids were unable to fix nitrogen (Bolton *et al.*, 1986). In the present paper, we described the isolation of ineffective organic acids utilization negative mutants, from a wild type effective *R. meliloti* strain S<sub>14</sub> (Antoun *et al.*, 1984). We also report on the oxidation of organic acids, glucose and fructose in strain S<sub>14</sub>, its mutants and revertants.

### Materials and methods

#### Bacteria

*Rhizobium meliloti* S<sub>14</sub> a wild type strain effective on alfalfa (*Medicago sativa* L. Cv. Saranac) was used. This strain was able to use most of the tricarboxylic acid (TCA) cycle intermediates as sole carbon source (Antoun *et al.*, 1984).

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*Media*

BM<sub>1</sub> was a basal medium consisting of (mg per liter of distilled water): CaCl<sub>2</sub>·2H<sub>2</sub>O, 50; MgSO<sub>4</sub>·7H<sub>2</sub>O, 200; NaCl, 100; KNO<sub>3</sub>, 600; K<sub>2</sub>HPO<sub>4</sub>, 520; KH<sub>2</sub>PO<sub>4</sub>, 410; biotin,  $5 \times 10^{-2}$ ; thiamine,  $5 \times 10^{-2}$ ; ferric citrate, 5; CoCl<sub>2</sub>·6H<sub>2</sub>O,  $4 \times 10^{-3}$ ; CuSO<sub>4</sub>·5H<sub>2</sub>O,  $8 \times 10^{-3}$ ; H<sub>3</sub>BO<sub>3</sub>, 2.86; H<sub>2</sub>MoO<sub>4</sub>·H<sub>2</sub>O,  $9 \times 10^{-2}$ ; MnCl<sub>2</sub>·4H<sub>2</sub>O, 1.81; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.22. Phosphates were autoclaved separately and the pH of the medium was 6.9. BM<sub>1</sub>-succinate and BM<sub>1</sub>-mannitol were prepared by adding to BM<sub>1</sub>, 10 mM succinate or 1% mannitol. Medium BM<sub>2</sub> was similar to BM<sub>1</sub>, but additionally buffered to pH 7 (by adding the following filter sterilized (0.22 µm pore size Nalgene Co. Rochester, N.Y.) ingredients: 3-[N-Morpholino]propanesulfonic acid (MOPS), 40 mM; KOH, 20 mM and MgSO<sub>4</sub>·7H<sub>2</sub>O, 4 mM.

*Isolation of mutant and revertant strains*

Log phase *R. meliloti* S<sub>14</sub> cultures grown in BM<sub>1</sub>-mannitol, were treated with N-Methyl-N'-nitro-N-nitrosoguanidine (NTG) 200 µg/ml for 30 min. at 25°C. The cells then were washed twice with phosphate buffer saline (PBS; 3 mM phosphate buffer in 0.7% NaCl, pH 6.8), suspended in BM<sub>1</sub>-mannitol broth and incubated for 24 h. Mutant enrichment was carried out by adding penicillin G (2000 IU/ml) to the washed (twice in PBS) NTG-treated cells in BM<sub>1</sub>-succinate broth and incubating for 48 h at 30°C. Enriched cultures were washed and plated for single colonies on BM<sub>1</sub>-mannitol plates (solidified with 1.5% agar). Mutants defective in the utilization of dicarboxylic acids were isolated by screening (replica plating) for colonies unable to grow on BM<sub>1</sub>-succinate plates and growing on yeast extract mannitol agar (Vincent, 1970). Revertants were selected by plating a dense washed culture on BM<sub>1</sub>-succinate plates. Mutants and revertants were preserved at -80°C in yeast extract mannitol broth containing 10% glycerol.

*Determination of growth*

Growth on different carbon sources was determined by using an indicator plate method (Bochner

and Savageau, 1977). Plates contained medium BM<sub>2</sub> modified by using 0.6 g.l<sup>-1</sup> proteose peptone (Difco) as nitrogen source and by adding filter sterilized 2, 3, 5-triphenyl tetrazolium chloride (TTC, Sigma) to a final concentration of 25 mg.l<sup>-1</sup>. Carbon sources (0.5% sugar of D-configuration or 10 mM TCA cycle intermediates and related compounds as sodium salts) were filter sterilized and added to the modified BM<sub>2</sub> medium containing 2% agar and cooled to 45°C. Plates were inoculated with washed log phase bacteria, growing on BM<sub>1</sub>-mannitol, by using a multiple inoculator (Antoun *et al.*, 1984). Growth was recorded after 5 d incubation at 30°C.

*Nitrogenase activity*

Nodulated alfalfa plants were obtained in growth pouches as previously described (Bordeleau *et al.*, 1981; Sirois and Peterson, 1982) and nitrogenase activity was assessed on 26 days old plants, incubated for 1 h in 30 ml test tubes capped with Subba Seal (William Freeman and Co., Barnsley, England) and containing 10% acetylene. Ethylene produced was measured with a Perkin Elmer Sigma 3 b dual FID gas chromatograph equipped with a stainless steel column packed with Porapak R (80–100 mesh) and operated at 40°C. The flame ionization detector was kept at 125°C.

*Measurement of O<sub>2</sub> consumption*

Bacteria grown on BM<sub>2</sub> medium supplemented with the appropriate carbon source (0.5% sugar or 10 mM organic acid) were harvested at an A<sub>600</sub><sup>cm</sup> of 0.8–1.0, washed twice with cold PBS (4°C), resuspended to the same cell density in BM<sub>2</sub> without carbon source and kept on ice until assayed. Substrate-dependent O<sub>2</sub> consumption was measured (Bordeleau *et al.*, 1980) at 25°C on 5 ml samples, by using a biological oxygen monitor (Yellow Spring Instrument, Ohio), as follow. O<sub>2</sub> consumption was measured during 5 min with 4.8 ml cell suspension (prewarmed to 25°C for 5 min) and for 10 min following the addition of 200 µl carbon substrate (to a final concentration of 10 mM organic acid or 0.5% sugar). Results are expressed as n moles O<sub>2</sub> min<sup>-1</sup> (mg protein)<sup>-1</sup>. Data are averages of at least 2 replications.

Table 1. Growth of *R. meliloti* S<sub>14</sub> and its mutant and revertant strains on different carbon sources<sup>a</sup>

Carbon source	Strain				
	Wild type	Mutants		Revertants	
	S <sub>14</sub>	S <sub>14</sub> S <sub>7</sub>	S <sub>14</sub> S <sub>9</sub>	S <sub>14</sub> S <sub>7</sub> R <sub>1</sub>	S <sub>14</sub> S <sub>9</sub> R <sub>1</sub>
Acetate, arabinose fructose, galactose glucose, glycerol lactate, lactose maltose, mannitol mannose, pyruvate ribose, sucrose and xylose	+++ <sup>b</sup>	+++	+++	+++	+++
Fumarate, malate and succinate	+++	+	+	+++	+++
Citrate, gluconate $\alpha$ -ketoglutarate and oxaloacetate	-	-	-	-	-

<sup>a</sup> Observations are from six replicates.

<sup>b</sup> + + +, growth as good as wild type and TTC reduced; +, very poor growth and TTC not reduced; -, no growth,

#### Protein determination

Protein was determined by the Folin phenol method (Lowry *et al.*, 1952) using bovine serum albumin as a standard.

## Results and discussion

#### Isolation of organic acids-utilization mutants and revertants

After NTG mutagenesis, mutants defective in succinate utilization were isolated at a frequency of  $3.3 \times 10^{-3}$ . The two selected mutants S<sub>14</sub>S<sub>7</sub> and S<sub>14</sub>S<sub>9</sub> showed a very poor growth with succinate, malate and fumarate but did not reduce TTC, which is an indication of their failure to catabolize these substrates (Bochner and Savageau, 1977). The mutants were able to use all other carbon sources in a fashion similar to the wild type strain S<sub>14</sub> (Table 1). The good growth of the mutants on arabinose and other sugars indicates that they have a functional TCA cycle (Bolton *et al.*, 1986; Duncan and Fraenkel, 1979), thus they are probably altered in their dicarboxylic acid transport system. The spontaneous revertants S<sub>14</sub>S<sub>7</sub>R<sub>1</sub> and S<sub>14</sub>S<sub>9</sub>R<sub>1</sub> were isolated on succinate at frequencies of  $1.6 \times 10^{-7}$  and  $9.5 \times 10^{-7}$  respectively. The pattern of utilization of the different carbon sources tested was similar

with the revertants and the wild type strain S<sub>14</sub> (Table 1).

#### Symbiotic properties

All mutants and revertants nodulated alfalfa plants (Table 2). However, mutants formed white ineffective nodules which did not show any nitrogenase activity as measured by acetylene reduction (Table 2). Revertants formed effective nodules and had a nitrogenase activity not statistically different from the activity of the wild type effective

Table 2. Symbiotic properties of *R. meliloti* S<sub>14</sub> and its mutant and revertant strains

Strain	Nodulation	N <sub>2</sub> fixation n moles C <sub>2</sub> H <sub>4</sub> h <sup>-1</sup> plant <sup>-1</sup>
<i>Wild type</i>		
S <sub>14</sub>	+	374.70 a <sup>a</sup>
<i>Mutants</i>		
S <sub>14</sub> S <sub>7</sub>	+	2.73 b
S <sub>14</sub> S <sub>9</sub>	+	2.42 b
<i>Revertants</i>		
S <sub>14</sub> S <sub>7</sub> R <sub>1</sub>	+	353.42 a
S <sub>14</sub> S <sub>9</sub> R <sub>1</sub>	+	399.12 a
<i>Uninoculated</i>	-	2.93 b

<sup>a</sup> Means followed by the same letter are not significantly different ( $P \leq 0.05$ ) according to Duncan's multiple range test. Means are from 9 replicates.

Table 3. Substrate-dependent O<sub>2</sub> consumption by free-living cells of *R. meliloti* S<sub>14</sub>

Carbon source in growth medium <sup>a</sup>	Rate of consumption in n moles O <sub>2</sub> min <sup>-1</sup> (mg protein) <sup>-1</sup> in the presence of:				
	Succinate (10 mM)	Fumarate (10 mM)	Malate (10 mM)	Glucose (0.5%)	Fructose (0.5%)
Succinate	657.0	262.0	267.0	122.7	21.3
Fumarate	348.3	268.3	303.0	30.5	1.6
Malate	630.3	320.7	267.3	106.3	29.8
Glucose	0.0	8.7	1.4	512.3	202.3
Fructose	1.2	2.9	13.1	306.3	347.0

<sup>a</sup> Cells were grown on BM<sub>2</sub> medium supplemented with 10 mM organic acid or 0.5% sugar as carbon source.

strain S<sub>14</sub>. These results are consistent with the previous observations (Antoun *et al.*, 1984; Bolton *et al.*, 1986; Gardiol *et al.*, 1982; Gardiol *et al.*, 1984) indicating that in *R. meliloti* as in other rhizobia a functional dicarboxylic acid transport system and the metabolism of organic acids through a complete TCA cycle are necessary to maintain an efficient symbiotic nitrogen fixation system in the *R. meliloti*-alfalfa symbiosis.

In order to compare the carbon metabolism in the mutants and the wild type strain, we further investigated their ability to oxidize organic acids, glucose and fructose.

#### Oxidation of substrates by *R. meliloti* S<sub>14</sub>

When grown on succinate, fumarate or malate, strain S<sub>14</sub> was capable of oxidizing the other two C<sub>4</sub>-dicarboxylic acids (Table 3). However, cells grown on glucose or fructose were not able to oxidize readily the three organic acids tested. In fact, malate-dependent O<sub>2</sub> consumption of fructose-grown cells [13.1 n moles O<sub>2</sub> min<sup>-1</sup> (mg protein)<sup>-1</sup>] was the highest organic acid-dependent O<sub>2</sub> consumption value observed, and was only 5% that of the malate-grown cells (Table 3). These observations show that as reported for some strains of rhizobia (Finan *et al.*, 1981; McAllister and Lepo, 1983) *R. meliloti* S<sub>14</sub> possess a C<sub>4</sub>-dicarboxylic acid uptake system which is inducible and mediates the uptake of succinate, fumarate and malate. With cells grown on organic acids, fructose-dependent O<sub>2</sub> consumption was very low (less than 10% of the rates obtained with cells grown on fructose). When grown on glucose, the cells of strain S<sub>14</sub> oxidized fructose at a rate approximately 60% that of the fructose grown cells. These data suggest that the

free living cells of the wild type *R. meliloti* S<sub>14</sub> possess an inducible fructose uptake system, as reported for another strain (Gardiol *et al.*, 1980), which might be induced by glucose or some products of its metabolism or that the fructose uptake system is repressed by organic acids. Studies with the organic acids utilization mutants will show some evidence supporting the repression hypothesis. Cells grown on any substrate had glucose-dependent O<sub>2</sub> consumption, which support the previous observation indicating the presence of a constitutive glucose transport system in *R. meliloti* (Theodoropoulos *et al.*, 1985), however as compared to the glucose grown cells, the glucose-dependent O<sub>2</sub> consumption of organic acids grown cells did not exceed 25% of the maximum activity recorded (Table 3). Catabolic repression like phenomenon mediated by succinate or malate were previously observed in *R. meliloti* (Ucker and Signer, 1978) and other rhizobia (Röhm and Werner, 1985; De Vries *et al.*, 1982). In order to investigate the presence of such repression in strain S<sub>14</sub>, bacteria were grown in the presence of both glucose and succinate or malate. In the presence of glucose the addition of increasing amounts of succinate, increased the succinate-dependent O<sub>2</sub> consumption up to a concentration of 15 mM (Table 4). A similar tendency was also observed by adding up to 10 mM malate. These observations confirm the presence of an inducible C<sub>4</sub>-dicarboxylic acid uptake system in strain S<sub>14</sub>. The addition of 5 mM or more succinate or malate significantly decreased the rate of glucose-dependent O<sub>2</sub> consumption suggesting the presence of a catabolite repression like phenomenon caused by succinate (or malate). In fact, strain S<sub>14</sub> metabolize succinate more rapidly than glucose. The mean generation time on BM<sub>2</sub> medium is 2.8 h and 2.4 h with glucose and suc-

Table 4. Substrate-dependent O<sub>2</sub> consumption by free-living cells of *R. meliloti* S<sub>14</sub> grown on BM<sub>2</sub> medium containing 0.5% glucose and supplemented with different concentrations of organic acid

Concentration mM	Rate of consumption in n moles O <sub>2</sub> min <sup>-1</sup> (mg protein) <sup>-1</sup> in the presence of:	
	Glucose (0.5%)	Succinate (10 mM)
<i>Supplemented with succinate</i>		
0.0	554.0	7.5
2.5	272.5	65.5
5.0	212.0	237.0
10.0	76.0	285.0
15.0	81.0	369.5
20.0	69.5	316.0
<i>Supplemented with malate</i>		
0.0	306.5	0.0
2.5	359.0	19.5
5.0	145.0	238.0
10.0	84.0	342.0
15.0	70.5	289.5
20.0	16.5	264.0

cinate respectively. Moreover, as shown later such repression appears to be absent in the organic acids utilization mutants.

#### Oxidation of substrates by the mutants

With cells grown on glucose the organic acids-dependent O<sub>2</sub> consumption was absent or very low (Table 5). The two ineffective mutants S<sub>14</sub>S<sub>7</sub> and S<sub>14</sub>S<sub>9</sub> oxidized glucose and fructose at rates similar to those observed with the wild type strain S<sub>14</sub> or the two revertants S<sub>14</sub>S<sub>7</sub>R<sub>1</sub> and S<sub>14</sub>S<sub>9</sub>R<sub>1</sub>. The two mutants were not able to grow on medium BM<sub>2</sub> with succinate as sole carbon source and to study the effect of succinate grown cells on the oxidation of glucose, the mutants were grown on BM<sub>2</sub> with glucose and transferred on BM<sub>2</sub> with succinate for 15 h. Preliminary assays with strain S<sub>14</sub>, indicated that this time was enough to induce succinate uptake with glucose grown cells. Organic acids-dependent O<sub>2</sub> consumption by the two ineffective mutants were absent or very low. However even when exposed during 15 h to succinate, the mutants were able to oxidize glucose (at high rates) and fructose. In the wild type strain S<sub>14</sub> and the two revertants, glucose and fructose-dependent O<sub>2</sub> consumption were very low or absent when the cells were grown

Table 5. Substrate-dependent O<sub>2</sub> consumption by free-living cells of *R. meliloti* S<sub>14</sub> and its mutant and revertant strains grown on medium BM<sub>2</sub> with 0.5% glucose (Experiment I) or 10 mM succinate (Experiment II) as carbon sources

Strain	Rate of consumption in n moles O <sub>2</sub> min <sup>-1</sup> (mg protein) <sup>-1</sup> in the presence of:				
	Succinate (10 mM)	Fumarate (10 mM)	Malate (10 mM)	Glucose (0.5%)	Fructose (0.5%)
<i>Experiment I—Glucose</i>					
S <sub>14</sub>	0.0	0.0	0.0	446.5	184.5
S <sub>14</sub> S <sub>7</sub>	0.0	0.0	10.4	317.7	151.3
S <sub>14</sub> S <sub>9</sub>	7.4	0.0	0.0	249.4	209.8
S <sub>14</sub> S <sub>7</sub> R <sub>1</sub>	7.4	0.0	0.0	334.0	170.4
S <sub>14</sub> S <sub>9</sub> R <sub>1</sub>	0.0	0.6	0.0	314.3	152.5
<i>Experiment II—Succinate</i>					
S <sub>14</sub>	419.0	264.0	218.0	74.2	19.7
S <sub>14</sub> S <sub>7</sub> <sup>a</sup>	6.2	0.0	0.0	535.7	114.7
S <sub>14</sub> S <sub>9</sub> <sup>a</sup>	3.1	0.0	14.8	662.4	337.7
S <sub>14</sub> S <sub>7</sub> R <sub>1</sub>	440.4	318.6	313.7	13.6	0.0
S <sub>14</sub> S <sub>9</sub> R <sub>1</sub>	425.6	307.5	272.5	12.9	0.0

<sup>a</sup> Mutants were grown on medium BM<sub>2</sub> with 0.5% glucose and transferred on medium BM<sub>2</sub> with 10 mM succinate for 15 h prior to O<sub>2</sub> measurements.

on succinate. This study shows that the two ineffective mutants of *R. meliloti* S<sub>14</sub> are not able to utilize succinate, fumarate and malate, but are able to utilize glucose and fructose. The work also indicates that as reported with other rhizobia (Arwas *et al.*, 1985; Finan *et al.*, 1983; De Vries *et al.*, 1982) a functional C<sub>4</sub>-dicarboxylic acid uptake system is essential for the occurrence of an efficient nitrogen fixation system in alfalfa nodulated with strain S<sub>14</sub>. However, as *R. lupini* bacteroids are able to use glucose (Kretovich *et al.*, 1985) and that glucose and sucrose provide energy for acetylene reduction by bacteroids under low O<sub>2</sub> tensions (Trinchant *et al.*, 1981; Trinchant *et al.*, 1983), the inefficiency of the organic acids utilization mutants which also oxidize glucose, could be the result of an unknown mechanism or a repression like phenomenon occurring in nodules. Further investigations are required to elucidate the nature played by that mechanism in nodules.

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