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The response of *Rhizobium meliloti* to L-methionine DL-sulphoximine

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Abstract. A strain of *Rhizobium meliloti* has been shown to be capable of growth in the presence of methionine sulphoximine concentrations at least two orders of magnitude higher than that required for the complete inhibition of glutamine synthetase activity. Neither the specific growth rate, nor the nutritional requirements of the organism were affected by methionine sulphoximine in the medium. *Rhizobium meliloti* appeared to assimilate ammonia *via* the glutamate dehydrogenase pathway during growth in the presence of methionine sulphoximine. This suggests that *Rhizobium meliloti* may have some regulatory mechanism controlling ammonia assimilation that is not present in other enterobacteria possessing similar enzymatic machinery.

Keywords. *Rhizobium meliloti;* methionine sulphoximine; glutamine synthetase; glutamate dehydrogenase.

Introduction

The rhizobia have two enzyme systems for the assimilation of ammonia, the glutamate dehydrogenase (GDH) pathway, and the glutamine synthetase (GS)/glutamate synthase (GOGAT) pathway. The rhizobia, however, possess two forms of GS (Darrow and Knotts, 1977). One form, GSI, is similar to *Escherichia coli* GS, while the other form, GSII, is unique to the Rhizobiaceae (Fuchs and Keister, 1980a, b). The exact role of GSII in rhizobial metabolism is unclear (Fuchs and Keister, 1980b; Ludwig, 1980). Methionine sulphoximine (MSX) is a potent inhibitor of GS (Meister, 1980). Most enterobacteria are inhibited by 1 mM MSX in the growth medium under conditions where the GS/GOGAT pathway is operative (Dendinger *et al*., 1980; Steimer-Veale and Brenchley, 1974) but does not affect the growth of organisms using the GDH pathway (Brenchley, 1973). MSX resistance has been usually linked with glutamine auxotrophy (Masters and Hong, 1981) or bradytrophy (Miller and Brenchley, 1981). MSX resistance may also result from alterations in the GS protein (Miller and Brenchley, 1981). We have found that one strain of *Rhizobium meliloti*, is intrinsically resistant to MSX even under conditions where the GS/GOGAT pathway is generally operative. This resistance and its biochemical basis are described below.

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Abbreviations used: GDH, Glutamate dehydrogenase; GS, glutamine synthetase; MSX, methionine sulphoximine.

Materials and methods

Organism

R. meliloti 03 was isolated from effective nodules of fenugreek (*Trigonella foenum-graecum* L)

Media

The organism was routinely maintained on medium containing 10 g mannitol, 400 mg K_2HPO_4, 200 mg $MgSO_4 \cdot 7H_2O$ and 100 mg NaCl per liter, and where necessary, 15 g agar. The nitrogen source (nitrate, ammonia, or glutamate) was added to give 1 or 10 mM concentrations while glutamine was used at 0·5 or 5 mM concentrations. Glutamine was filter sterilized and added to sterile medium; MSX was added to sterile medium to give a final concentration equimolar to the nitrogen source.

Cell-free extract

Cells used for enzyme assays or for preparation of cell free extracts were washed three times in 0·05 M Tris-HCl buffer (pH 7·2) containing 0·5 mM β-mercaptoethanol. For the preparation of cell free extracts, cells were frozen and then disrupted by sonication in the same buffer. The supernatant, obtained after centrifugation at 20,000 *g* for 20 min, was used for enzyme assays.

Enzyme assays

Glutamine synthetase was assayed by the γ-glutamyltransferase assay (Shapiro and Stadtman, 1970) using cetyltrimethyl ammonium bromide in the assay mixture (Smith *et al*., 1980). Where used MSX was added to give 10–50 μmol/ml assay mixture. GSI and GSII were assayed as the fractions stable and unstable at 50°C for 15 min respectively.

Glutamate dehydrogenase was assayed by the oxidation of NADH in cell free extracts.

Units of enzyme activity are defined as μmol γ-glutamyl hydroxamate formed or NADH oxidized/min/mg protein. Nitrate was estimated by the method of Garrett and Nason (1969). Ammonia was estimated by Nesslerization (Wriston, 1970). Protein was assayed by the method of Lowry *et al*. (1951).

Gel electrophoresis

Polyacrylamide gel electrophoresis was performed on 5% polyacrylamide slabs of 1.5 mm thickness (Davis, 1964). Gels were stained for γ-glutamyl transferase activity.

Results and discussion

Two forms of GS exist in *R. meliloti* 03, as in *R. meliloti* 41 (Fuchs and Keister, 1980b). These isoforms are separable on polyacrylamide gels (table 1). GSI, the heat stable isoform, has a lower electrophoretic mobility than the heat labile GSII, and its sensitivity to 60 mM Mg^{2+} varies, reflecting variations in the degree of adenylylation

Table 1. Separation of *R. meliloti,* GSI and GSII on 5% polyacrylamide gel.

Sample treatment	Effect of GS activity band with relative electrophoretic mobility 0·18	0·46
None	+	+
50°C (15 min)	+	–
60 mM Mg^{2+}[a]	+/–	–
50 μM MSX	–	–

+ Presence or – absence of activity band.
[a] Incorporated into assay mixture.

Table 2. The effect of 50 μM MSX on GSI and GSII in cell free extracts of *R. meliloti* 03 grown on 10 mM nitrate.

Enzyme	MSX	IU/ml GS	Inhibition (%)
GSI	–	0·0105	96
	+	0·0004	
GSII	–	0·1517	97
	+	0·0039	

(Darrow and Knotts, 1977). GSII in cell free extracts is completely inhibited by 60 mM Mg^{2+}.

Both forms of GS in cell free extracts from 1 mM nitrate grown *R. meliloti* 03 are inactivated by MSX (table 1); over 95% of the activity being lost at 50 μM concentration of MSX (table 2). This is similar to results obtained for *E. coli* GS (which is similar to GSI) by Ronzio *et al*. (1969). At lower MSX concentrations there is a slight, but reproducible, difference between the two enzymes (figure 1).

Although rhizobial GS is inhibited by such low concentrations of MSX, growth of this organism is not stopped even at 10 mM MSX. A hundred colonies of *R. meliloti* 03

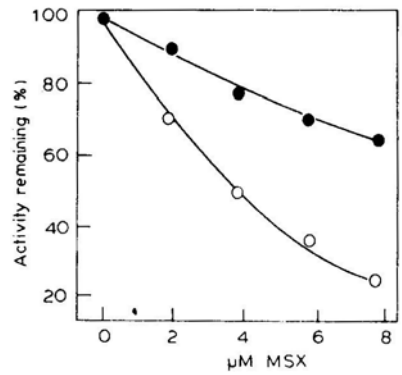

Figure 1. Effect of MSX on GSI (O) and GSII (●) in cell free extracts of *R. meliloti* 03.

chosen at random, were transferred to a grid on a plate containing medium with 10 Mm nitrate as nitrogen source. After the colonies were grown, they were replicated onto plates containing isonitrogenous nitrate, ammonia, glutamate or glutamine, with and without equimolar MSX, and the growth observed after 24 and 48 h (table 2). There was no difference in growth in the presence or absence of MSX, when glutamate or glutamine was the source of nitrogen. Glutamine is the product of the GS catalyzed reaction, while glutamate can compete with MSX for binding to the enzyme (Meister, 1980).

Every colony replicated onto medium containing MSX and nitrate or ammonia grew 24 h after colonies had developed on similar plates without MSX. Since every colony grew, this resistance to MSX is unlikely to be the result of mutation, and the 'lag' period probably reflects the time required for a change in the ammonia assimilation machinery. This hypothesis was supported by the fact, that once cells are grown on nitrate or ammonia and MSX, on further transfer to fresh medium, there is no difference in growth rate regardless of the nitrogen source or the presence or absence of MSX (data not shown). Cells transfered from MSX-containing medium to MSX-free medium showed normal GS activity and no delay in growth or alteration in growth rate (data not shown). It was noted that washed whole cells, grown on any nitrogen source in the presence of MSX did not possess detectable GS activity but did show GS activity when grown in the absence of MSX (data not shown).

Cell free extracts from washed cells grown on glutamate or nitrate, with or without equimolar MSX were assayed for GS and GDH; the results are shown in table 3. In glutamate grown cells there was GDH activity as well as GS activity in the absence of MSX and only GDH in the presence of MSX (table 4), while in nitrate grown cells GDH activity was observed only in the presence of MSX (table 4). The necessity for the induction of GDH may be one reason for the 'lag' observed on nitrate and ammonia but not on glutamate. In the enteric bacteria GDH is considered to be the major ammonia assimilatory enzyme during growth in excess ammonia (Smith *et al*., 1975). However, in our organism, at growth temperatures below 30°C and at ammonia concentrations between 1–10 mM, most of the enzyme activity appears to be GSII (S. P. Rao, U. George and V. V. Modi, unpublished observations). Assays of other enzymes like asparagine synthetase and alanine dehydrogenase gave no significant activity.

Table 3. Growth of *R. meliloti* 03 on various nitrogen sources in the presence or absence of MSX (unadapted cells).

Nitrogen source	MSX	Growth on plates after	
		24 h	48 h
Nitrate	−	++	+++
Nitrate	+	−	++
Ammonia	−	++	+++
Ammonia	+	−	++
Glutamate	−	++	+++
Glutamate	+	++	+++
Glutamine	−	++	+++
Glutamine	+	++	+++

Table 4. Levels of ammonia assimilatory in cell free extracts of *R. meliloti* 03 grown in the presence or absence of MSX.

Nitrogen source	Specific activity in extracts from cells grown					
	Without MSX			With MSX		
	GSI	GSII	GDH	GSI	GSII	GDH
Glutamate (10 mM)	0·149	0·289	0·047	—	—	0·103
Nitrate (1 mM)	0·009	0·329	—	—	—	0·095

Brenchley (1973) found that wild type *Klebsiella aerogenes* was resistant to 100 mM MSX during growth in high ammonia concentrations (35 mM), whereas GD H negative mutants were sensitive to 0·1 mM MSX. *Salmonella typhimurium* did not show similar MSX resistance (Steimer-Veale and Brenchley, 1974), although GDH was present in the organism (Dendinger *et al*., 1980). G D H from most systems is reported to have a lower affinity for ammonia than GS (Stewart *et al*., 1980). It was of interest therefore, to see if the rate of ammonia uptake increased during growth on MSX. Figure 2 shows that after the 'lag' period, neither growth nor ammonia uptake is markedly affected by MSX. This is particularly puzzling in that GDH is considered to be induced only in the presence of high intracellular ammonia concentrations. The uptake of ammonia in the presence of MSX is another difference between rhizobia and several other organisms. Many free living nitrogen fixers, including *Azotobacter vinelandii*, *Klebsiella pneumoniae* (Gordon and Brill, 1974) and several cyanobacteria (Orr and Haselkorn, 1982) export ammonia when fixing nitrogen in the presence of MSX. On the other hand, during the growth of *R. meliloti* 03 on nitrate, there was little difference in growth or extracellular ammonia levels (figure 3). In fact, when log phase cells, grown on nitrate, with or without MSX, were washed three times and resuspended in medium without $MgSO_4 \cdot 7H_2O$ (non-permissive for growth), more extracellular nitrite and ammonia were detected in medium containing cells grown in the absence of MSX (figure 4A,B). The presence or absence of MSX in the non-proliferating medium made no significant difference. The

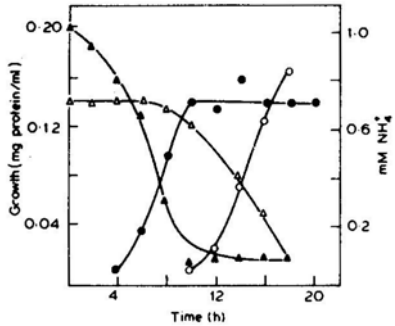

Figure 2. Growth (O, ●) and ammonia uptake (Δ, ▲) by *R. meliloti* 03 growing on 1 mM NH_4Cl with (O),(Δ) or without (●), (▲) 1 mM MSX in the medium (unadapted cells).

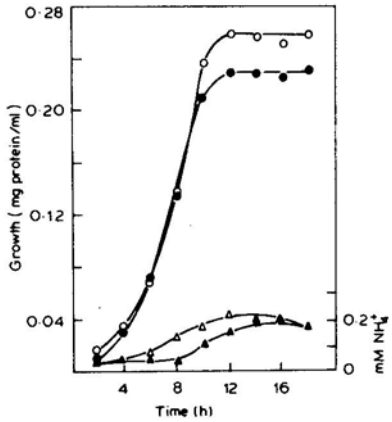

Figure 3. Growth (O, ●) and extracellular ammonia levels (Δ, ▲) during growth of *R. meliloti* 03 growing on 1 mM KNO_3 with (O, Δ) or without (●, ▲) 1 mM MSX (adapted cells).

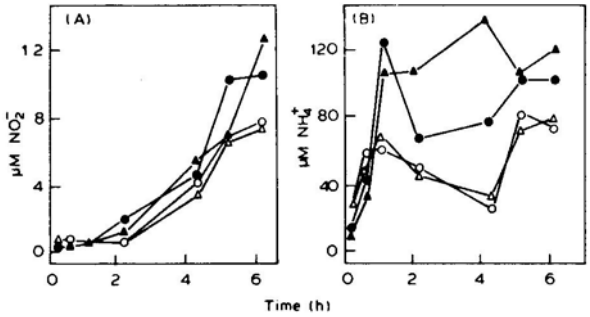

Figure 4. Nitrite (A) and ammonia (B) formation by resting cells of *R. meliloti* 03 growth with (O) or without (●) 1 mM MSX in 1 mM KNO_3. Cells were incubated in the non-proliferating medium with (Δ, ▲) or without (O, ●) 1 mM MSX.

effect of MSX on nitrogen fixation was not determined since very poor asymbiotic nitrogen fixation has been obtained in this strain.

Apart from the presence of GSII in *R. meliloti* there appears to be little difference in the type of ammonia assimilatory enzymes present in this organism and in other enterobacteria (Dendinger *et al*., 1980). GSII should make little difference to MSX sensitivity since this enzyme is also inhibited. Generally, MSX resistance is the result of changes in the enzyme such that the biosynthetic activity, but not the transferase activity of the enzyme is MSX insensitive (Miller and Brenchley, 1981), or organisms are mutated in other respects (Dendinger *et al*., 1980). *R. meliloti* 03 shows no change in growth rates or growth requirements in the presence of MSX. The innate resistance of the organism to MSX, the apparent switch to GDH in the presence of MSX without corresponding increase in rates of ammonia uptake, and the lack of ammonia export even in resting cells, suggest that this MSX resistance of *R. meliloti* 03 is not the result of any special enzymatic capacity, but is the result of some special regulatory mechanism. The nature of this regulatory mechanism is yet to be determined.

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