The role of glutaminase in *Rhizobium etli*: studies with a new mutant

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In order to examine the role of glutaminase in *Rhizobium etli*, we isolated and characterized a *R. etli* glutaminase mutant (LM16). This mutant was selected for its impaired ability to grow on glutamine as nitrogen and carbon source while retaining the ability to grow on other nitrogen and carbon sources. The mutant showed very low levels of glutaminase activity under various growth conditions in comparison with the wild-type strain. With glutamine as the only nitrogen and carbon source, LM16 showed poor growth, with a very high content of glutamine, low glutamate content, and reduced ammonium excretion and $^{14}\text{CO}_2$ evolution from $[\text{U-}^{14}\text{C}]\text{glutamine}$ compared to the wild-type strain. This indicates that the main role of *R. etli* glutaminase is in the use of glutamine as carbon source. *R. etli* glutaminase also plays a role in maintaining the balance between glutamate and glutamine, as shown by the accumulation of glutamine and the low glutamate content of the mutant under different growth conditions. These results also indicate that glutaminase participates in a glutamine cycle in which it degrades glutamine which is then resynthesized by glutamine synthetase. The higher glutamine and lower glutamate content found in bacteroids of LM16 in comparison with bacteroids of the wild-type strain indicate that glutamine degradation by glutaminase plays an important role during the symbiosis between *R. etli* and *Phaseolus vulgaris*.

Keywords: *Rhizobium etli*, mutant, glutaminase, catabolism, symbiosis

INTRODUCTION

Glutamine plays a central role in the nitrogen metabolism of micro-organisms. It is a nitrogen donor for biosynthetic reactions (Stadtman, 1973), and has been proposed as the nitrogen metabolite responsible for nitrogen catabolite repression in several micro-organisms (Halpern, 1988). It is synthesized by glutamine synthetase (GS) from ammonium and glutamate. In rhizobia, ammonium assimilation proceeds mainly through the GS–glutamate synthase pathway (Bravo & Mora, 1988). The family *Rhizobiaceae*, which includes genera such as *Rhizobium*, *Bradyrhizobium* and *Agrobacterium*, is distinct from other bacteria in that its members have at least two forms of GS (Darrow & Knotts, 1977; Fuchs & Keister, 1980; Kumar & Rao, 1986; Tsupurun *et al.*, 1987), with the exception of *Azorhizobium caulinodans* (Donald & Ludwig, 1984). The

form termed GSI is structurally and enzymically typical of prokaryotic GSs, its activity is post-translationally regulated by reversible adenylation and it appears to be synthesized constitutively (Bravo & Mora, 1988; Rossi *et al.*, 1989). In contrast, GSII is distinct from all other known prokaryotic GSs and is not subject to adenylation; GSII levels are dependent on the nitrogen source present in the medium and it is not expressed in *ntrA* or *ntrC* mutants (Bravo & Mora, 1988; de Bruijn *et al.*, 1989; Martin *et al.*, 1988; Rossbach *et al.*, 1987; Rossi *et al.*, 1989; Shatters *et al.*, 1989). A third locus, termed *glnT*, which encodes a GSIII, has been identified in *Rhizobium meliloti*, *Agrobacterium tumefaciens* and *Rhizobium leguminosarum* bv. *phaseoli* (Chiurazzi *et al.*, 1992; de Bruijn *et al.*, 1989; Espín *et al.*, 1990; Shatters *et al.*, 1993).

In *Rhizobium etli*, glutamine is assimilated by transamidation reactions and, in addition, it is converted to 2-oxoglutarate and ammonium by the enzymes of the glutamine transaminase- ω -amidase pathway (Durán & Calderón, 1995). In this pathway, glutamine is trans-

Abbreviation: GS, glutamine synthetase.

aminated to yield mainly glycine, alanine and 2-oxoglutarate through the participation of a glutamine transaminase. Subsequently, 2-oxoglutarate is hydrolysed to 2-oxoglutarate and ammonium by the action of an ω -amidase. Although glutamine transaminase is reversible, the presence of ω -amidase activity ensures that the reaction proceeds in the direction of glutamine utilization. Durán & Calderón (1995) showed that glutamine transaminase activity was similar during the different growth stages and under several nutritional conditions, except when a PY-rich medium was used, in which case its activity was low. It has been proposed that the *R. etli* glutamine transaminase may play a role in the irreversible synthesis of glycine, alanine and other amino acids, and the transamination of these amino acids may contribute to the synthesis of other amino acids to preserve the amino acid balance and to prevent loss of essential carbon chains (Durán & Calderón, 1995). The glutamine transaminase- ω -amidase pathway has also been found in other micro-organisms (Calderón *et al.*, 1985; Soberón & González, 1987; Osorio *et al.*, 1993).

In *R. etli*, glutamine is also degraded by a glutaminase which catalyses its hydrolytic deamidation to yield glutamate and ammonium (Durán & Calderón, 1995). Glutaminase activity was found to be positively regulated by glutamine, and negatively regulated by ammonium and by the carbon source, and it is high in bacteroids. It has been proposed that glutaminase plays a catabolic role in the degradation of glutamine to carbon skeletons and may also play a role in maintaining the balance between glutamate and glutamine, the universal nitrogen donors of the cell (Durán & Calderón, 1995).

In *R. etli*, the ammonium released from glutamine is assimilated by GS thus leading to the operation of a glutamine cycle where glutamine is synthesized and degraded at the expense of ATP (Durán & Calderón, 1995). This cycle operates at lower rates during growth on glutamine as carbon source, thus allowing the use of glutamine as carbon source for growth (Durán & Calderón, 1995). In *Neurospora crassa*, the glutamine transaminase- ω -amidase pathway has been found to participate in a glutamine cycle in which this amino acid is continually degraded and resynthesized (Calderón *et al.*, 1989; Calderón & Mora, 1985, 1989). It has been proposed that the glutamine cycle is necessary for carbon utilization by this micro-organism (Mora, 1990).

Since *R. etli* glutaminase is an important enzyme in glutamine catabolism, linking nitrogen metabolism with carbon metabolism, we describe here the isolation and characterization of an *R. etli* mutant with altered glutaminase activity.

METHODS

Strains and plasmid. The *Rhizobium etli* CFN42 wild-type strain, previously classified as *Rhizobium leguminosarum* bv. *phaseoli* (Segovia *et al.*, 1993), and *Escherichia coli* strain S17-1 carrying the *tra* gene region of RP4 integrated in the chromosome and the suicide vector pSUP5011 containing the Tn5-Mob (Simon, 1984), were used in this study.

Growth conditions. Batch cultures of *R. etli* were grown at 30 °C and with shaking at 200 r.p.m. For growth on minimal medium (MM) (Beringer, 1974), cells previously grown overnight on a rich medium (PY) containing 0.5 % peptone, 0.3 % yeast extract and 7 mM CaCl₂ were washed and used as inoculum. The initial OD₅₄₀ of the medium was adjusted to 0.05. The nitrogen and carbon sources in MM were used at 10 mM. Growth was monitored by measurement of the OD₅₄₀ and by protein determination using the Lowry method.

Tn5 mutagenesis. The *E. coli* strain S17-1 was used to transfer the suicide vector pSUP5011 carrying transposon Tn5-Mob to *R. etli* strain CFN42. Kanamycin-resistant transconjugants were isolated on PY medium containing this antibiotic (30 µg ml⁻¹) plus nalidixic acid (20 µg ml⁻¹).

Determination of glutaminase activity. Cell extracts were prepared by sonicating whole cells in extraction buffer (100 mM KH₂PO₄, pH 8) with a Soniprep 150 ultrasonic disintegrator; the homogenates were centrifuged for 2 min at room temperature in a microcentrifuge. The activity was assayed in a 0.25 ml reaction mixture containing 30 mM KH₂PO₄, pH 8, 10 mM glutamine and 0.05 ml cell extract. Ammonium formation was measured as described elsewhere by Chaney & Marbach (1962) and Durán & Calderón (1995).

Determination of GS activity. GS activity was assayed with cell extracts prepared by sonicating the cells in 10 mM imidazole hydrochloride and 0.5 mM EDTA (pH 7.0). GS was measured by its synthetase activity as described by Bender *et al.* (1977). GSI and II were distinguished by their different heat stabilities on heating at 50 °C for 1 h as described by Darrow & Knotts (1977).

Determination of nitrogenase activity. Nitrogenase activity was determined by measuring acetylene reduction. The flasks containing the nodules were sealed with rubber stoppers, acetylene was injected at a final concentration of 10 % of the gas phase, and ethylene production was determined by gas chromatography (Bravo *et al.*, 1988).

Ammonium determination. Ammonium concentration in samples of media was measured with an Orion electrode (Calderón & Mora, 1989).

Measurement of ¹⁴CO₂ from [U-¹⁴C]glutamine. Samples of cultures were incubated with [U-¹⁴C]glutamine [0.2 µCi ml⁻¹ (7.5 kBq ml⁻¹)]. Released CO₂ was absorbed in 2.5 M NaOH and radioactivity was measured as described elsewhere by Mora *et al.* (1972).

Determination of amino acid pools. Cells were harvested by centrifugation, suspended in 5 ml of 80 % (v/v) ethanol and heated at 90 °C for 10 min. They were then removed by centrifugation and the supernatant containing the free amino acids was lyophilized and then resuspended in distilled water. The amino acids were determined with a System Gold Liquid chromatographic system (Beckman) and a Gilson fluorometer (model 121) after coupling with *o*-phthalaldehyde (Calderón *et al.*, 1985).

Plant test. The seeds were surface-sterilized in 5 % (v/v) hypochlorite and germinated on moist sterile paper for 3 d. The seedlings were transferred to plastic growth pots containing vermiculite and Jensen's medium (Gibson, 1980). After 6 d, the seedlings were inoculated with bacteria grown on PY medium with appropriate antibiotics and washed with distilled water. Bacteria were reisolated from the surface-sterilized nodule and individual nodules were crushed and spread onto PY plates. Individual colonies were scored for antibiotic resistance and nutritional phenotype.

Bacteroid isolation. This was done as described by Durán & Calderón (1995).

Reproducibility of results. The experiments reported were each repeated at least once; representative results are shown.

RESULTS

Isolation of the LM16 mutant

Mutants of *R. etli* were obtained by general Tn5 mutagenesis with plasmid pSUP5011. Approximately 100 000 kanamycin-resistant transconjugants were obtained, then collected into PY medium. To enrich for mutants unable to grow on glutamine as nitrogen and carbon source, this suspension was used to inoculate MM medium supplemented with glutamine and with 200 μg ampicillin ml^{-1} and the cells grown for 48 h changing the medium every 12 h. The cells were then spread on PY-kanamycin plates and 2500 colonies which appeared after 3 d were replica-plated to MM agar containing ammonium plus succinate, glutamine plus succinate or glutamine. Forty-three mutants which failed to grow on glutamine as nitrogen and carbon source were purified

and replica-plated again in the same medium and on MM plates containing glutamate plus succinate and glutamate as nitrogen and carbon source. The LM16 mutant was the only one unable to grow on glutamine as nitrogen and carbon source and able to grow on the other media tested.

Growth and glutaminase activity

The LM16 mutant grew very poorly on glutamine as nitrogen and carbon source, whereas on glutamine as nitrogen source and succinate as carbon source or in other media such as PY, ammonium plus succinate, and glutamate or asparagine with or without succinate, it grew as well as the wild-type strain (Fig. 1).

Since we have previously found that glutaminase activity was the highest degradative enzyme activity when *R. etli* was grown on glutamine as nitrogen and carbon source (Durán & Calderón, 1995), we examined the LM16 glutaminase activity under different nutritional conditions. It was found that in comparison with the wild-type strain, the mutant had very low levels of glutaminase activity under the different conditions tested being about

Fig. 1. Growth curves of *R. etli* wild-type strain (filled symbols) and LM16 mutant (open symbols) on: (a) glutamine plus succinate (●, ▽) and glutamine (▼, □); (b) PY (●, ▽) and ammonium plus succinate (▼, □); (c) glutamate plus succinate (●, ▽) and glutamate (▼, □); (d) asparagine plus succinate (●, ▽) and asparagine (▼, □).

Table 1. Glutamine and glutamate content of *R. etli* wild-type strain and LM16 mutant grown on different substrates

Amino acid content was determined after 5 h growth and is expressed as nmol (mg protein)⁻¹.

Growth substrate	Wild-type		LM16	
	Glutamine	Glutamate	Glutamine	Glutamate
Glutamine	0.9	21.5	48.0	6.6
Glutamine + succinate	0.6	27.5	3.3	15.6
Ammonium + succinate	0.4	16.1	0.9	5.5
Peptone-yeast extract	0.4	22.3	0.8	18.8

tenfold lower when grown on glutamine with succinate [86 versus 7 nmol min⁻¹ (mg protein)⁻¹] or without succinate [85 versus 8 nmol min⁻¹ (mg protein)⁻¹] and fivefold lower when grown on ammonium plus succinate [30 versus 6 nmol min⁻¹ (mg protein)⁻¹].

Glutamine content, glutamate content and GS activities

Since the low glutaminase activity observed in the LM16 mutant should result in impairment of the catabolism of glutamine to glutamate, we measured the intracellular glutamine and glutamate pools in LM16 under different growth conditions. In comparison with the wild-type strain, LM16 showed a 53-fold higher glutamine content and a threefold lower glutamate content when grown on glutamine as nitrogen and carbon source, a sixfold higher glutamine and a twofold lower glutamate content when grown on glutamine plus succinate, a twofold higher glutamine and a threefold lower glutamate content when grown on ammonium plus succinate and a twofold higher glutamine and similar glutamate content when grown on PY-rich medium (Table 1). This indicates that the inability of LM16 to grow on glutamine as nitrogen and carbon source is not due to the lack of transport of this amino acid.

In order to know if the GSI and II activities were altered in the mutant, since it accumulates glutamine due to the decrease of glutamine degradation through glutaminase, we measured these activities under different growth conditions. We found that GSI and II activities in the mutant were similar to the wild-type strain when both strains were grown on ammonium plus succinate or on glutamine with or without succinate (data not shown).

Ammonium excretion, and the release of ¹⁴CO₂ from [U-¹⁴C]glutamine

We have found that *R. etli* excretes ammonium when grown on glutamine with or without succinate (Durán & Calderón, 1995). To test the participation of glutaminase in this excretion, we measured the ammonium released by LM16 and the wild-type strain under these conditions.

Fig. 2. Ammonium excretion by the *R. etli* wild-type strain (●) and LM16 mutant (▽) growing on glutamine plus succinate (a) or on glutamine (b).

Fig. 2 shows that the mutant excreted twofold lower amounts of ammonium than the wild-type strain after 24 h of growth on glutamine plus succinate, whereas on glutamine as nitrogen and carbon source after 24 h the mutant excreted sevenfold lower amounts of ammonium than the wild-type strain.

To evaluate the participation of glutaminase in the utilization of glutamine as carbon source, we measured the ¹⁴CO₂ released from [U-¹⁴C]glutamine by the LM16 mutant and wild-type strain under different growth conditions. Fig. 3 shows that, compared with the wild-type strain, the mutant liberated similar amounts of ¹⁴CO₂ (mg protein)⁻¹ h⁻¹ when it was grown on ammonium plus succinate or on glutamine plus succinate, whereas on

Fig. 3. $^{14}\text{CO}_2$ release by *R. etli* wild-type strain (□) and LM16 mutant (▨) under different growth conditions: ammonium and succinate; glutamine and succinate; glutamine.

glutamine as nitrogen source, the mutant liberated twofold lower amounts of $^{14}\text{CO}_2$ (mg protein) $^{-1}$ h $^{-1}$ than the wild-type strain.

Symbiotic phenotype

To analyse the role of glutaminase in the degradation of glutamine in bacteroids, *R. etli* wild-type strain and LM16 mutant bacteroids were isolated using a self-generating Percoll gradient (Reibach *et al.*, 1981), and the glutamine and glutamate content was determined. The mutant contained a ninefold higher amount of glutamine [14.0 versus 1.5 nmol (g nodule) $^{-1}$] and about a twofold lower amount of glutamate [65.5 versus 112.1 nmol (g nodule) $^{-1}$]. These results indicate that glutaminase is active in bacteroids degrading glutamine to glutamate. We found that the LM16 mutant nodulates as well as the wild-type strain and that the nitrogenase level was similar to the wild-type strain (data not shown).

DISCUSSION

The LM16 mutant was selected for its inability to grow on glutamine as nitrogen and carbon source and its ability to grow on glutamine plus succinate or on ammonium plus succinate. The fact that LM16 is only affected in its capacity to grow on glutamine as nitrogen and carbon source (Fig. 1) indicates that this amino acid is transported and that the metabolic block is in the degradation of glutamine.

The LM16 mutant is altered in its glutaminase activity as shown by the low levels of activity found under different growth conditions. The low constitutive glutaminase activity found in the mutant may be explained by the

presence of other glutaminase, as has been found in *E. coli*, where two forms exist: glutaminase A, which is controlled by nitrogen metabolites and cyclic AMP, and glutaminase B, the activity of which is low constitutive (Prusiner, 1973). However, it cannot be excluded that the mutation was in a regulatory gene.

The main role of *R. etli* glutaminase is to use glutamine as a carbon source as shown by: (a) the poor growth of the mutant when glutamine was the only nitrogen and carbon source (Fig. 1), (b) the very high glutamine and low glutamate content of this mutant under these conditions (Table 1) and (c) the lower ammonium excretion (Fig. 2) and $^{14}\text{CO}_2$ evolution from [U- ^{14}C]glutamine by LM16 grown on glutamine as nitrogen and carbon source, in comparison with the wild-type strain (Fig. 3). The slow growth of LM16 on glutamine as nitrogen and carbon source (Fig. 1) as well as the fact that the excretion of ammonium (Fig. 2) and the $^{14}\text{CO}_2$ released from [U- ^{14}C]glutamine by this mutant under these conditions (Fig. 3) are not totally impaired, indicate that other pathways participate in the degradation of glutamine, but these did not compensate for the lack of glutaminase activity of the LM16 mutant under these conditions. In addition, however, glutaminase plays a role in the use of glutamine as nitrogen source. This is shown by (a) the high glutamine and low glutamate content of this mutant on glutamine plus succinate (Table 1) and (b) the decrease of the ammonium excretion of the mutant under the same conditions (Fig. 2). It may also play a role in maintaining the balance between glutamate and glutamine, as shown by the accumulated glutamine and low glutamate content of the mutant on ammonium plus succinate and PY-rich medium (Table 1). These results also indicate that under

these conditions glutaminase participates in a glutamine cycle where this amino acid is degraded by this enzyme and synthesized again by GS. We expected that the decrease of the degradation of glutamine through glutaminase might result in lower activities of the enzymes that participate in its synthesis, nevertheless the GSI and II activities were similar in the LM16 mutant and the wild-type strain. This result may be explained by the participation of other pathways in the degradation of glutamine that compensate for the lack of glutaminase activity; thus LM16 releases similar amounts of $^{14}\text{CO}_2$ from $[\text{U-}^{14}\text{C}]$ glutamine on ammonium plus succinate.

The higher glutamine content and lower glutamate content found in bacteroids of LM16 in comparison with bacteroids of the wild-type strain indicate that glutamine degradation by glutaminase plays an important role during the symbiosis between *R. etli* and *Phaseolus vulgaris*. Nevertheless the lack of glutaminase in the mutant is not reflected in the nitrogenase activity. This may be due to the presence of other glutamine-degrading enzymes that compensate for the lack of glutaminase activity (Durán & Calderón, 1995).

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