Glutamine synthetase, glutamate synthase and glutamate dehydrogenase in *Rhizobium japonicum* strains grown in cultures and in bacteroids from root nodules of *Glycine max*

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**Abstract.** The growth yields of three strains of *Rhizobium japonicum* (CB 1809, CC 723, CC 705) in culture solutions containing L-glutamate were about twice those grown with ammonium. The activities of glutamine synthetase (GS; EC 6.3.1.2) and glutamate dehydrogenase (GDH; EC 1.4.1.4) were dependent on the nitrogen source in the medium and also varied with growth. Both NADPH- and NADH-dependent glutamate synthase (GOGAT; EC 1.4.1.13) and NADPH-dependent GDH were found in strains grown with either glutamate or ammonium but NADH-linked GDH was only detected in glutamate-grown cells. Glutamine synthetase was adenylylated in cells grown with NH$_4^+$ (90%) and to a lesser extent in those grown with L-glutamate (50%). In root nodules produced by the three strains in *Glycine max* (L.) Merr., the bulk of GS was located in the nodule cytosol (60–85%). The enzyme was adenylylated in bacteroids (43–75%) and in the nodule tissues (52–68%). The enzyme in cell-free extracts of *Rh. japonicum* (CC 705) grown in culture solutions containing glutamate and in bacteroids (CC 705) was deadenylylated by snake-venom phosphodiesterase. L-methionine-DL-sulfoximine restricted the incorporation of 15N-labelled (NH$_4$)$_2$SO$_4$ into cells of strains CB 1809 and CC 705, as well as in bacteroids of strain CC 705. It is noteworthy that appreciable activities for GDH were found in the free-living rhizobia grown on glutamate. Thus the presence of an enzyme does not necessarily imply that a particular pathway is operative in assimilating ammonium into cell nitrogen. Based on 15N studies, the GS-GOGAT pathway of rhizobia (strains CB 1809 and CC 705) is important when grown in culture solutions as well as in bacteroids from root nodules of *G. max*.

**Key words:** Adenylylation (glutamine synthetase) – Ammonium assimilation – Bacteroid – Glutamate dehydrogenase, synthase – Glutamine synthetase – *Glycine* (nitrogen assimilation) – *Rhizobium* strains.

**Introduction**

For a long time glutamate dehydrogenase (GDH; EC 1.4.1.4) has been considered a key enzyme in the assimilation of ammonium in microorganisms and plants. An alternative route involves glutamine synthetase (GS; EC 6.3.1.2) but the nitrogen assimilated by this enzyme was only considered to contribute to the non-$\varepsilon$-amino-N of tryptophan, arginine and histidine (for a review, see Meister 1962). However, the work of Tempest et al. (1970a, b) showed that, under certain conditions, the nitrogen assimilated by bacteria proceeds through glutamine. The presence of glutamate synthase (GOGAT; EC 1.4.1.13), which transfers the amide-amino group of glutamine to 2-oxoglutarate to form glutamate, supported the importance of this route.

Brown and Dilworth (1975) have shown that the assimilation of ammonia in strains of rhizobia proceeds by either the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway or by GDH, depending on the nitrogen source in the culture medium. However, other authors do not consider that GDH is an important enzyme in ammonium assimilation in bacteria and higher plants (Miflin and Lea 1977; Scott 1978).

Dilworth and Brown (1976), Duke and Ham (1976) and Duke et al. (1979) consider that the ab-
sence of GOGAT in bacteroids indicates that the GS-GOGAT pathway may not be important for the fixation of dinitrogen in root nodules. Upchurch and Elkan (1978) have shown that ammonium is transported from bacteroids to the nodule cytosol where, as shown by Ohyama and Kumazawa (1980), it is assimilated via the GS-GOGAT system. Duke and Ham (1976) found sufficient GDH activity in both bacteroids and plant tissues (nodules and roots) of soybeans, inoculated with various strains of *Rhizobium japonicum*, to assimilate all the ammonium produced by dinitrogen fixation.

In *Klebsiella pneumoniae* (Streicher et al. 1974), GS activity is regulated by ammonium. High concentrations of ammonium adenylylated the enzyme, resulting in its inhibition, and when ammonium had been utilized deadenylylation occurred, restoring activity. Glutamine synthetase is also regulated in this way in rhizobia grown in culture solutions with either L-glutamate (Bishop et al. 1976) or ammonium (Ludwig and Signer 1977) as a sole source of nitrogen, or in a medium containing organic nitrogen compounds (Tronick et al. 1973).

The aim of the present study was to determine the route whereby ammonium is incorporated into nitrogenous compounds of the cells in three strains of rhizobia grown in cultures with ammonium or L-glutamate as well as in bacteroids derived from *Glycine max*. We have determined the activities of GS, GOGAT and GDH in cell-free extracts of the three strains grown in culture solutions containing either L-glutamate (Bisho et al. 1976) or ammonium (Ludwig and Signer 1977) as a sole source of nitrogen, or in a medium containing organic nitrogen compounds (Tronick et al. 1973).

Cell-free extracts of rhizobia and bacteroids. The cultured rhizobia were centrifuged at 15,000 g for 10 min at 4 °C. They were washed with 100 mM K-phosphate buffer (pH 7.6) and then centrifuged at 40,000 g for 20 min at 4 °C in a RSCE centrifuge (Sorvall, Norwalk, Conn., USA).

Bacteroids separated from the nodule cytosol were extracted according to the method of Bishop et al. (1976).

Enzyme assays. Glutamine synthetase was assayed by the γ-glutamyl transferase method (Shapiro and Stadtman 1970), except that 0.3 mM Mn2+ was used. Glutamate synthase and GDH were assayed at 30 °C by measuring the initial rates of oxidation of NAD(P)H in a 1-cm cuvette at 340 nm in a recording spectrophotometer (model 635; Varian, Melbourne, Vic., Australia). In each case 0.5 mM NAD(P)H and 100 mM K-phosphate buffer (pH 7.6) were used. In addition, the reaction mixture, for the GOGAT assay, contained in 3 ml, 5 mM glutamine and α-ketoglutarate, and for the GDH assay, either 160 mM L-sodium glutamate as a nitrogen source. The culture solutions were inoculated with a 1% (v/v) inoculum of the appropriate strain and grown for specified periods at 30 °C.

For the determination of the extent of adenylylation, GS was assayed with and without MgCl2·6H2O at 60 mM. The values obtained were then used in the following formula proposed by Shapiro and Stadtman (1970):

\[ n = 12 - \frac{0.3 \text{ mM Mn}^2+ + 60 \text{ mM Mg}^{2+}}{0.3 \text{ mM Mn}^2+} \]

Per cent adenylylation was determined by dividing n by 12, then multiplying by 100. The effects of SVD on the adenylylation of GS in cell-free extracts of rhizobia, grown in cultures, and in those of bacteroids, prepared from root nodules, were also determined by this method. In each case, 50-μl samples were obtained from Dr. F.J. Bergersen, Division of Plant Industry, CSIRO, Canberra, Australia. They were maintained on yeast extract-mannitol slopes at 30 °C (Dalton 1980). The culture solutions (1 l) for growing the rhizobia were as described by Brown and Dilworth (1975), with either ammonium chloride or L-sodium glutamate as a nitrogen source. The culture solutions were inoculated with a 1% (v/v) inoculum of the appropriate strain and grown for specified periods at 30 °C. Growth was monitored at 540 nm in a spectrophotometer (model 635; Varian, Melbourne, Vic., Australia). Strain CB 1809 was slow growing. CC 705 was fast growing and CC 723 was intermediate, thus representing a range of growth patterns.

Seeds of *Glycine max* (L.) Merr. (cv. Clark 63) were supplied by D.L. Chase, Agricultural Research Station, Leeton, N.S.W., Australia. The seeds were sterilized by soaking in 0.05% (w/v) HgCl2 for 2 min, followed by immersion for 10 s in 95% (v/v) ethanol. The seeds were then washed with distilled water before sowing in sterile sand in 20-cm pots. The plants were grown in 0.25 strength Hoagland's solution (Hoagland and Arnon 1950), minus nitrogen, containing 0.23 μM Fe 138 (CIBA Geigy, Basle, Switzerland) and 0.5 μM Na2MoO4. This was supplied every third day, after inoculation, and distilled water was given in between times to keep the soil moist.

At the time of sowing, nutrient solution containing 1 mM KNO3 was supplied. Up to the time of inoculation the seedlings were given distilled water only.

Nodules were sampled seven weeks after inoculation.

Materials and methods

**Chemicals.** Snake venom phosphodiesterase (SVD), NADH, NADPH, ADP, α-ketoglutarate γ-glutamylhydroxamate, imidazole, L-glutamine, azaaserine and MSX were obtained from Sigma Chemical Co., St. Louis, Mo., USA; [14C]ATP and Phase combining System (PCS) scintillation fluid were purchased from Amersham Co., Sydney, Australia; and [15NH4]2SO4 (32.55 A%N) was obtained from Office National Industriel de l'Azote (ONIA), Marseille, France. All other chemicals were of the highest purity available.

Culturing rhizobia and inoculation of *Glycine max*. The three strains (CB 1809, CC 723 and CC 705) of *Rhizobium japonicum* were obtained from Dr. F.J. Bergersen, Division of Plant Industry, CSIRO, Canberra, Australia. They were maintained on yeast extract-mannitol slopes at 30 °C (Dalton 1980). The culture solutions (1 l) for growing the rhizobia were as described by Brown and Dilworth (1975), with either ammonium chloride or L-sodium glutamate as a nitrogen source. The culture solutions were inoculated with a 1% (v/v) inoculum of the appropriate strain and grown for specified periods at 30 °C. Growth was monitored at 540 nm in a spectrophotometer (model 635; Varian, Melbourne, Vic., Australia). Strain CB 1809 was slow growing. CC 705 was fast growing and CC 723 was intermediate, thus representing a range of growth patterns.

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**Cell-free extracts of rhizobia and bacteroids.** The cultured rhizobia were centrifuged at 15,000 g for 10 min at 4 °C. They were washed with 100 mM K-phosphate buffer (pH 7.6) and then suspended in the same buffer. The bacterial cells were then disrupted (using an ultrasonic titanium probe, 20 kc/s; MSE, London, UK) for 4-6 min at 4 °C and centrifuged at 40,000 g for 20 min at 4 °C in a RC5B centrifuge (Sorvall, Norwalk, Conn., USA).

Bacteroids separated from the nodule cytosol were extracted according to the method of Bishop et al. (1976).

**Enzyme assays.** Glutamine synthetase was assayed by the γ-glutamyl transferase method (Shapiro and Stadtman 1970), except that 0.3 mM Mn2+ was used. Glutamate synthase and GDH were assayed at 30 °C by measuring the initial rates of oxidation of NAD(P)H in a 1-cm cuvette at 340 nm in a recording spectrophotometer (model 635; Varian, Melbourne Vic., Australia). In each case 0.35 mM NAD(P)H and 100 mM K-phosphate buffer (pH 7.6) were used. In addition, the reaction mixture, for the GOGAT assay, contained in 3 ml, 5 mM glutamine and α-ketoglutarate, and for the GDH assay, either 160 mM NH4Cl (extracts of rhizobia), or 100 mM extracts of bacteroids (or nodule cytosol). The reactions were started by adding α-ketoglutarate.