Ammonia Assimilation in *Rhizobium japonicum* Colonial Derivatives Differing in Nitrogen-fixing Efficiency

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Ammonia assimilatory activities were investigated in cultures of small, efficient nitrogen-fixing derivatives (110-I, 76-NS) and large, inefficient nitrogen-fixing derivatives (110-L₁, 76-s) of *Rhizobium japonicum* strains 311b110 and 61A76. Specific activities of the key ammonia assimilatory enzymes, glutamine synthetase (EC 6.3.1.2), glutamate synthase (EC 2.6.1.53) and glutamate dehydrogenase (EC 1.4.1.2), were determined in aerobic, microaerophilic and bacteroid cell-free extracts. In aerobic culture, 110-L₁ and 76-s assimilated more ammonia than 110-I and 76-NS because of apparent twofold or greater assimilatory enzyme activities. Specific ammonia assimilatory enzyme activities were 10- to 100-fold lower for all derivatives in nitrogen-fixing microaerophilic and bacteroid cultures. In addition to these already low ammonia assimilatory activities, the assimilatory activities of 110-I and 76-NS were twofold lower than those of 110-L₁ and 76-s. The small colony types also excreted ammonia under nitrogen-fixing conditions.

These findings support the idea that rhizobia can simultaneously derepress nitrogenase biosynthesis whilst repressing ammonia assimilatory enzyme biosynthesis. This investigation has also linked the efficiency of ammonia assimilation with the efficiency of nitrogenase activity as an inverse function, i.e. those *R. japonicum* derivatives that fix greater amounts of nitrogen also assimilate less fixed nitrogen.

INTRODUCTION

Recent studies concerned with the regulation of symbiotic nitrogen fixation in the Rhizobium-legume association have focused on the fate of the fixed-nitrogen product, ammonia, and the key enzymes of its assimilation. Brown & Dilworth (1975), Robertson, Warburton & Farnden (1975) and Kurz, Rokosh & LaRue (1975) have shown that several enzymes involved in ammonia assimilation have low specific activities during nitrogen fixation. These three studies together with the earlier report of Bergersen & Turner (1967) indicated that fixed nitrogen was excreted as ammonia from the nodule bacteroids.

Using the recently developed technique of free-living nitrogen fixation (Keister, 1975; Keister & Evans, 1976; Kurz & LaRue, 1975; McComb, Elliot & Dilworth, 1975; Pagan *et al.*, 1975; Tjepkema & Evans, 1975) with ¹⁵N analysis, O'Gara & Shanmugam (1976) demonstrated that most fixed nitrogen in liquid cultures of rhizobia was exported into the cell supernatant as NH₄⁺. This observation led to a model of symbiotic nitrogen fixation in which rhizobia export fixed nitrogen as NH₄⁺ by simultaneously derepressing their *nif* genes whilst repressing genes of NH₄⁺ assimilation.

Previous reports from our laboratory (Kuykendall & Elkan, 1976; Upchurch & Elkan, 1977) have described the isolation and partial characterization of colony type derivatives from pure strains of *Rhizobium japonicum*. These clones, which differ in morphology and carbohydrate metabolism, also differ in symbiotic and non-associative nitrogen fixation. In

view of the reports (Bishop *et al.*, 1976; Brill, 1975; O'Gara & Shanmugam, 1976) linking the reactions of ammonia assimilation to the control of nitrogenase activity, we have studied the activities of glutamine synthetase [EC 6.3.1.2; L-glutamate:ammonia ligase (ADP-forming)], glutamate synthase [EC 2.6.1.53; L-glutamine:2-oxoglutarate aminotransferase (NADPH-oxidizing)] and glutamate dehydrogenase [EC 1.4.1.2; L-glutamate:NAD⁺ oxidoreductase (deaminating)] in cell-free extracts of these colony derivatives. In this paper we describe the patterns of ammonia assimilation for cultures of two previously described sets of colony derivatives of strains 31b110 and 61A76 grown under aerobic or under micro-aerophilic conditions. Comparisons are made with enzyme activities found in bacteroid extracts from appropriately infected soybean root nodules.

METHODS

Bacterial strains. *Rhizobium japonicum* colony types of strain 31b110 were obtained from Dr L. D. Kuykendall (U.S. Department of Agriculture, Beltsville, Maryland, U.S.A.). *Rhizobium japonicum* strain 61A76 was obtained from Dr J. C. Burton (Nitragin Co., Milwaukee, Wisconsin, U.S.A.). Colony types from this strain were isolated as described by Upchurch & Elkan (1977).

Media. Cultures were maintained on slopes of yeast extract/mannitol (YEM) (Lillich & Elkan, 1968). The HEPES/MES buffered medium (HMG) of Upchurch & Elkan (1977) was used for aerobic growth [HEPES: *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; and MES: 2-(*N*-morpholino)ethanesulphonic acid were obtained from Sigma]; this medium contained 0.5% (w/v) potassium gluconate and 0.025% (w/v) yeast extract.

The M-HMG medium for free-living microaerophilic culture of *R. japonicum* was identical to HMG medium except that it did not contain NH₄Cl and 30 μ M-Na₂MoO₄ and 0.1 mM-EDTA were added.

Bacterial growth. Mid-exponential phase cells grown in HMG medium were harvested by centrifuging at 10000 *g* for 10 min. The cells were washed once in HEPES/MES salts and resuspended in an equal volume of sterile HEPES/MES mineral salts. One ml of the suspension was inoculated into 50 ml of fresh HMG medium containing 260 μ g NH₄Cl ml⁻¹ in 300 ml side-arm flasks. At intervals, culture densities were determined using a Klett-Summerson colorimeter (red filter), and 1 ml culture samples were aseptically removed for ammonia determinations. Fifty ml cultures were harvested after about 120 h of growth (late-exponential phase) for analysis of the enzymes of ammonia assimilation.

Microaerophilic growth and measurement of nitrogenase activity. Colony types were grown under micro-aerophilic conditions in the presence of nitrogen and acetylene in liquid medium using modifications (Upchurch & Elkan, 1977) of the procedures of Tjepkema & Evans (1975) and Keister (1975). For nitrogenase derepression studies, triplicate test-tube cultures were removed from the incubator at time zero and at daily intervals throughout the 7 day induction period. Cultures were analysed for nitrogenase activity (acetylene reduction), cell density (Klett-Summerson colorimeter) and extracellular ammonia.

To assay the enzymes of ammonia assimilation in microaerophilic nitrogen-fixing cultures, 10 identical test-tube cultures of each colony type were incubated for 120 h and then analysed individually for nitrogenase activity as described above. The mean nitrogenase activity was determined from the 10 replicates and expressed as nmol acetylene reduced per tube. After the nitrogenase assays were completed, the cultures were combined and injected into serum stoppered 50 ml Erlenmeyer flasks which were flushed with nitrogen and then placed in an ice bath until cell extracts were made.

Symbiotic nitrogen fixing activity. Growth chamber experiments using the Lee variety of soybean and the colony types of *R. japonicum* strains 31b110 and 61A76 were performed over 35 d as described by Upchurch & Elkan (1977). Symbiotic nitrogenase activity was expressed as nmol acetylene reduced h⁻¹ (mg dry wt nodule tissue)⁻¹. The nodules used were the same as those used to prepare extracts for ammonia assimilatory enzymes.

Preparation of bacteroids. Soybean root nodules (approx. 1 g) were collected from plants infected with each colony type and bacteroids were prepared from these nodules by the method of Bishop *et al.* (1976) except that 0.1 M-HEPES/0.2 M-sucrose buffer was used instead of the phosphate/sucrose (pH 7.6) buffer. The supernatant fractions from the nodule macerates were frozen for subsequent ammonia analyses. Approximately 100 mg of the bacteroid samples were suspended in 2.5 ml of the HEPES/sucrose buffer. These suspensions were injected into serum-stoppered 10 ml Erlenmeyer flasks, flushed with nitrogen and then placed in an ice bath until cell-free extracts were made.

Preparation of cell-free extracts. *Rhizobium japonicum*, whether from aerobic, microaerophilic or bacteroid cultures, was harvested by centrifugation at 10000 *g* for 10 min, washed twice and then resuspended in 2.5 ml of 0.05 M-K⁺-HEPES buffer (pH 7.5). These cells were then disrupted by sonication three times for

Table 1. *Summary of properties characteristic of the derivatives used in this investigation*

The isolation of these colony types and their properties have been described by Kuykendall & Elkan (1976) and Upchurch & Elkan (1977).

Property	<i>Rhizobium japonicum</i> derivative			
	110-L ₁	110-I	76-s	76-NS
Relative colony size (mm diam.)	4-5	0.5-1	5-6	1-2
Basis of colony morphology on YEM/HM medium	Utilizes D-mannitol	Does not utilize D-mannitol	Produces extracellular polysaccharides	Does not produce extracellular polysaccharides
Ability to nodulate Lee soybeans	+	+	+	+
Symbiotic nitrogen fixation	Low	High	Low	High
Free-living nitrogen fixation	Low	High	Low	High

1 min (Branson Sonifier) with 30 s intervals during which the probe was chilled in an ice-water bath. Extracts were cleared of cell debris by centrifuging at 20000 *g* for 20 min at 4 °C. The clear, slightly amber fluid was used within 8 h for enzyme assays.

Enzyme assays. Glutamine synthetase was assayed by measuring the amount of γ -glutamyl hydroxamate formed in the presence of Mn^{2+} at 37 °C for 15 min as described by Shapiro & Stadtman (1970). Glutamate synthase and glutamate dehydrogenase were assayed by following the oxidation of either the reduced form of NAD or NADP as coenzymes at room temperature as described by Prusiner, Miller & Valentine (1972). The reactions were started by NADH or NADPH addition, and endogenous oxidation was determined in separate experiments. The protein concentration in the extract preparations was estimated by the absorption method of Warburg and Christian as described by Layne (1957). Extract protein was always adjusted to approx. 100 μ g protein ml⁻¹ in the assay mixtures.

Ammonia determination. Ammonia in the cell-free extract, bacteroid extract, nodule supernatant and culture supernatant was determined by the colorimetric method of Chaney & Marbach (1962). Ammonia was expressed as μ g ml⁻¹ relative to a curve relating A_{625} with NH_4Cl concentration.

RESULTS AND DISCUSSION

Previous studies (Kuykendall & Elkan, 1976; Upchurch & Elkan, 1977) in our laboratory have linked *R. japonicum* colony morphology to the level of symbiotic and free-living nitrogenase activity. The characteristics of colonies from 311b110 differed from those of colonies from 61A76 (Table 1). On YEM/HM medium (Kuykendall & Elkan, 1977; Upchurch & Elkan, 1977), derivatives of strain 311b110 either did or did not utilize D-mannitol, whereas colony morphologies of 61A76 formed on the same medium were independent of carbon source and reflected differences in polysaccharide production. A survey of several other sets of colony types indicated that the basis of colony size was due to either differences in mannitol utilization or polysaccharide production, and for that reason, the clones of 110 and 76 were used as representative cultures in the nitrogen metabolism experiments that follow. All four derivatives nodulated their soybean host plant.

Initial growth curve studies of the derivatives in aerobic HMG broth culture showed that types 110-L₁ and 76-s reached slightly higher maximum turbidities while depleting the medium of ammonia at faster rates than 110-I and 76-NS (Fig. 1). Because subtle but clear differences in aerobic ammonia utilization and growth among the colony types were observed, the specific activities of the enzymes of ammonia assimilation in aerobic, micro-aerophilic and bacteroid cultures were measured.

Fig. 1

Fig. 2

Fig. 1. Time course of (a) ammonia assimilation and (b) growth of *R. japonicum* 31b110 and 61A76 derivatives in aerobic HEPES/MES/gluconate culture (containing 5 mM-NH₄⁺). ▲, 110-1; ●, 76-NS; △, 110-L₁; ○, 76-s.

Fig. 2. Time course of (a) ammonia export, (b) nitrogenase activity (acetylene reduction) and (c) growth of *R. japonicum* 31b110 and 61A76 derivatives cultured under an atmosphere of nitrogen/acetylene (90:10, v/v). Symbols as in Fig. 1.

Ammonia assimilatory enzyme activities were measured in aerobic HMG broth cultures containing 5 mM-NH₄⁺ and 0.025% yeast extract. Highest specific enzyme activities were reached in the late-exponential phase of growth (approximately 120 h). Nitrogenase activity was not detected in these cultures (Table 2). Glutamine synthetase, glutamate synthase and glutamate dehydrogenase activities were present in all derivatives. Interestingly, the activities of the large or slimy colony types 110-L₁ and 76-s were higher for all three enzymes, i.e. twofold greater in glutamine synthetase and glutamate synthase (NADH) activities, and three to ninefold greater in glutamate dehydrogenase (NADH) activity. Although NADPH-linked glutamate synthase and dehydrogenase showed low activities (not always detected), their activity patterns followed the respective NADH activities. These enzyme data support and explain the previous utilization results (Fig. 1).

Table 2. Activities of ammonia assimilatory enzymes in aerobic cultures of *R. japonicum*

Derivative	nmol acetylene reduced per tube*	Specific activity† [nmol min ⁻¹ (mg protein) ⁻¹]				
		Glutamine synthetase	Glutamate synthase		Glutamate dehydrogenase	
			NADH	NADPH	NADH	NADPH
110-L ₁	—	1002	20	14	25	7
110-I	—	488	12	5	9	1
76-s	—	2024	18	18	28	2
76-NS	—	848	9	5	3	—

—, Activity not detected.

* Assays were performed on 2.5 ml of culture in test-tubes at 120 h.

† Assimilatory enzyme activities were determined in extracts prepared from 50 ml HMG cultures at 120 h.

Table 3. Activities of ammonia assimilatory enzymes in microaerophilic cultures of *R. japonicum*

Derivative	nmol acetylene reduced per tube*	Specific activity† [nmol min ⁻¹ (mg protein) ⁻¹]				
		Glutamine synthetase	Glutamate synthase		Glutamate dehydrogenase	
			NADH	NADPH	NADH	NADPH
110-L ₁	25	13	3	< 2	2	—
110-I	307	4	—	—	< 2	—
76-s	40	12	4	2	3	—
76-NS	275	3	< 2	< 2	< 2	—

—, Activity not detected.

* Activities were determined in 168 h microaerophilic cultures and are the mean of three independent experiments.

† Assimilatory enzyme activities were determined in cell-free extracts of 168 h microaerophilic cultures and are the mean of three independent experiments.

Table 4. Activities of ammonia assimilatory enzymes in bacteroids of *R. japonicum*

Derivative	nmol acetylene reduced h ⁻¹ (mg nodule tissue) ⁻¹ *	Specific activity† [nmol min ⁻¹ (mg protein) ⁻¹]				
		Glutamine synthetase	Glutamate synthase		Glutamate dehydrogenase	
			NADH	NADPH	NADH	NADPH
110-L ₁	27	188	11	8	7	4
110-I	119	99	6	3	3	—
76-s	18	168	8	7	10	2
76-NS	90	61	5	6	5	3

—, Activity not detected.

* Activities are the mean of two separate plant experiments.

† Assimilatory enzyme activities are the mean of two separate plant experiments and were determined by assay of bacteroid extracts.

Table 5. *The partitioning of ammonia in nodule bacteroids*

Ammonia determinations of nodule macerate supernatants and extracts are the mean of two independent experiments. Results expressed as $\mu\text{g NH}_4^+$ ml⁻¹.

Derivative	Nodule supernatant	Bacteroid extract
110-L ₁	13.50	0.23
110-I	20.50	2.80
76-s	6.30	0.05
76-NS	11.60	0.47

The fate of fixed nitrogen in microaerophilic cultures of the derivatives was investigated by time-course experiments. Results indicated that after 48 h incubation, acetylene reduction activity increased in a near linear fashion. Increases in nitrogenase activity were accompanied by the appearance of ammonia in the cell-free supernatants of these cultures (Fig. 2). Free-living cultures of the small colony types (110-I and 76-NS) were many fold more efficient and also excreted more ammonia than 110-L₁ and 76-s. Microaerophilic cultures of the derivatives grown under an argon/acetylene atmosphere also had similar patterns of acetylene reduction activity, but no appreciable ammonia in the supernatant was ever detected in cultures of 110-I and 76-NS.

Of the various enzyme activities of microaerophilic cultures, nitrogenase activity patterns in the non-associative cultures were as expected, with 110-I and 76-NS many fold more efficient than corresponding large or slimy colony types (Table 3). Assimilatory enzyme activities were much lower in these cultures. Glutamine synthetase activity was 10- to 100-fold lower in microaerophilic culture as compared to aerobic culture. Glutamate synthase and glutamate dehydrogenase specific activities were near the limit of detection; in some cases neither NADH- nor NADPH-linked enzyme activities were detected. A consistent glutamine synthetase pattern was evident, i.e. 110-L₁ and 76-s had higher while 110-I and 76-NS had lower assimilatory activities. The specific activities of glutamate synthase and glutamate dehydrogenase activity (NADH-linked), even though low, also followed a similar pattern. The levels of assimilatory activities detected in microaerophilic culture support the working model of nitrogenase regulation proposed by O'Gara & Shanmugam (1976). Important differences were also revealed about the nitrogen metabolism of the colony derivatives. Colony types 110-I and 76-NS were able to derepress their *nif* genes while concomitantly more efficiently repressing their assimilatory genes and thereby excreting more fixed NH_4^+ nitrogen. From these observations with microaerophilic cultures, it is inferred that the linkage between ammonia assimilation and nitrogenase activity is less 'tight' in derivatives of 110-L₁ and 76-s.

The nitrogenase and ammonia assimilatory activities developed in bacteroids from the nodules of appropriately infected plants were also assayed (Table 4). Nitrogenase activity patterns were similar to those observed in the microaerophilic cultures (Table 3). Bacteroid assimilatory enzyme activities were also much lower than those detected in aerobic culture. Glutamine synthetase, glutamate synthase and glutamate dehydrogenase activities exhibited similar patterns to those observed in microaerophilic cultures, i.e. the activities in 76-s and 110-L₁ were high whereas those in 76-NS and 110-I were low. The majority of soluble NH_4^+ was found in the bacteroid cytosol fraction rather than in the bacteroid enzyme extract. Ammonia was found in higher concentration in the bacteroid cytosols of 110-I and 76-NS than in the cytosols of 110-L₁ and 76-s (see Table 5). These preliminary bacteroid studies thus confirmed our earlier observations made with the non-associative nitrogen-fixing systems.

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