Differentiation of *Rhizobium japonicum*, I. Enzymatic Comparison of Nitrogenase Repressed and Derepressed Free Living Cells and of Bacteroids

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Z. Naturforsch. **33 c**, 245–252 (1978) ; received February 9, 1978

Rhizobium japonicum, Bacteroids, Derepressed Nitrogenase, Differentiation, N-Metabolism

Derepressed free living cells of *Rhizobium japonicum* strain 61-A-101 with leucine as single nitrogen source develop a maximum nitrogenase activity of 180 nmol $C_2H_4 \cdot mg\ protein^{-1} \cdot h^{-1}$ in liquid culture under 2% O_2 in the gas phase. Only 10% of this activity is found with no oxygen in the gas phase during a 90 min incubation period. The maximum activity under 2% oxygen in the gas phase is unaffected by addition of 1–100 mM NH_4^+ and by addition of low concentrations of glutamine (0.36–1.44 mM).

Specific activities of alanine dehydrogenase (E.C. 1.4.1.1.) aspartate aminotransferase (E.C. 2.6.1.1.) and, with much lower activities, of GOGAT (E.C. 1.4.1.13) in nitrogenase active free living cells are more similar to bacteroids than to nitrogenase repressed free living cells from liquid culture. The activities in nitrogenase repressed cells were about 50% lower. Glutamine synthetase (E.C. 6.3.1.2.) activity in bacteroids and in nitrogenase active cells were also similar, but only about 25–30% of that found in nitrogenase repressed *Rhizobium japonicum* cells.

Introduction

The differentiation of free living bacteria of the genus *Rhizobium* to bacteroids in the cells of legume nodules is characterized by several changes: an increased cell size [1], and DNA content [2], alterations in the cytochrome pattern [3], cell wall composition [4], uptake of metabolites [5], and variations in enzyme activities [6, 7].

Since nitrogenase derepressed free living cells of well defined strains of *Rhizobium japonicum* are available [8–10] it became possible to study the various nutritional factors, increasing or reducing nitrogenase activity [11] separated from the plant metabolism. Furthermore we can find out with these cells, which of the described changes and alterations of bacteroids are compulsorily linked to a derepressed nitrogenase, or which may be merely effects of the location of the bacteroids in the vesicles of the legume cell. The enzymes of N-metabolism are of special interest, since *Rhizobium* bacteroids and other diazotrophic bacteria are perhaps regulated differently, *e. g.* in the function of glutamine synthetase in nitrogenase activity [7].

Steps in the differentiation of *Rhizobium japonicum* cells to bacteroids during the development of nodules of *Glycine max* are summarized in Table I.

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Materials and Methods

Cultivation of Rhizobium japonicum str. 61-A-101

a) Suspension cultures – derepressed cells

A single colony (with $1-10 \times 10^6$ living cells from agar plates) of the *Rhizobium* strain was transfered to 100 ml of the complex medium 20 E [9, 17] and grown for 5–7 d at 28 °C on a gyro-rotary shaker (120 rpm). This culture (700–1000 nephelometer units) was centrifuged (38 000 $\times g$, 10 min), resuspended in 100 ml water and mixed with 500 ml nutrient solution called medium 20 N with 1.2× medium 20 P-concentration to give 600 ml of the following medium 20 P:

In 1000 ml medium:

308	mg	$MgSO_4 \times 7\ H_2O$	$(1.25 \times 10^{-3}$ M)
61.25	mg	$CaCl_2 \times 2\ H_2O$	$(4.16 \times 10^{-4}$ M)
4.033	mg	$Na_2MoO_4 \times 2\ H_2O$	$(1.66 \times 10^{-5}$ M)
5.791	mg	$FeSO_4 \times 7\ H_2O$	$(2.08 \times 10^{-5}$ M)
7.750	mg	Na_2-EDTA in 1 ml previously dissolved	$(2.08 \times 10^{-5}$ M)
3.833	g	glycerol (= 3.04 ml)	$(4.16 \times 10^{-2}$ M)
1.516	g	mannitol	$(8.33 \times 10^{-3}$ M)
1.091	g	L-leucine	$(8.33 \times 10^{-3}$ M)
0.315	g	D-arabinose	$(2.09 \times 10^{-3}$ M)
2.250	g	Na-succinate $\times 6\ H_2O$	$(8.33 \times 10^{-3}$ M)
5.233	g	$NaH_2PO_4 \times H_2O$	$(3.80 \times 10^{-2}$ M)
0.652	g	K_2HPO_4	$(3.74 \times 10^{-3}$ M)

0.550 mg	KI	(3.33×10^{-6} M)
0.020 mg	$CuSO_4 \times 5\ H_2O$	(8.33×10^{-8} M)
0.020 mg	$CoCl_2 \times 6\ H_2O$	(8.33×10^{-8} M)
0.239 mg	$ZnSO_4 \times 7\ H_2O$	(8.33×10^{-7} M)
0.140 mg	$MnSO_4 \times H_2O$	(8.28×10^{-7} M)
0.508 mg	H_3BO_3	(8.33×10^{-6} M)
0.228 mg	*p*-aminobenzoic acid	(1.66×10^{-6} M)
0.858 mg	pyridoxin-HCl	(4.16×10^{-6} M)
15 mg	meso-inositol	(8.33×10^{-5} M)
5.616 mg	thiamine-HCl	(1.66×10^{-5} M)
0.791 mg	calcium-D-pantothenate	(1.66×10^{-6} M)
0.608 mg	D(+)-biotin	(2.49×10^{-6} M)
1.025 mg	nicotinic acid	(8.33×10^{-6} M)
0.941 mg	riboflavin	(2.49×10^{-6} M)

pH: 5.8.

After 10 – 12 days of cultivation the pH of the medium increased, despite the 42 mM phosphate buffer concentration to 6.8.

Additions to this medium were prepared, by mixing 1 ml of a special component with 5 ml of the nutrient solution with 1.2× the medium 20 P concentration.

The cultures in a 1 l flask were continuously sparged with a gas mixture of 2% O_2, 10% CO_2 and 88% N_2 or with 1% O_2, 1% CO_2 in 98% N_2 with a rate of 12 ml·min^{-1} and additionally the suspension stirred on a magnetic stirrer. Between 5 and 12 days after inoculation, the optical density of this culture increased 2 – 4 times to an O.D. of 300 to 800 nephelometer units, corresponding to 0.35 and 1.0 mg protein·ml^{-1}.

b) Suspension cultures – repressed cells

A single colony of *Rhizobium japonicum* str. 61-A-101 was transferred to 100 ml Medium 20 E [9, 17] in a 300 ml Erlenmeyer flask, and grown for 5 – 7 d at 28 °C on a gyrorotary shaker (120 rpm).

Cultivation of Glycine max var. Caloria

Seeds of *Glycine max* var. Caloria (K. Behm Comp., Hamburg) were surface sterilized in 1% $CaOCl_2$ solution, washed with sterile tap water and germinated in "Agriperl-Pflanzenperlite" (Perlite Dämmstoff GmbH, Dortmund) for 14 d. During this time the seedlings were watered with tap water. These seedlings were infected for 10 min in a suspension of 5 ml *Rhizobium japonicum* culture in log phase in Medium 20 E (between 10^8 and 10^9 cells·ml^{-1}) and 5 ml 0.15% Agar (Noble Agar, Difco). The *Rhizobium japonicum* culture was inoculated from a single colony developed according the scheme from ref. [9]. Two infected plants in each plantpot were grown in "Agriperl" as described [17].

Assay of nitrogenase activity

The acetylene reduction assay was used as previously described [10]. 5 ml samples from liquid culture of *Rhizobium japonicum* were taken under anaerobic conditions and incubated for 90 to 120 min in 21 ml bottles under an atmosphere as indicated in the figures with 10% C_2H_2 in the gas phase at 28 °C on a gyrorotary shaker. The activity in these samples was linear during a 2 h period. For comparison with the nitrogenase activity of bacteroids in nodules, tested under air, the free living cells were also treated with nitrogen gas instead of argon, though the activity under argon was higher.

Nitrogenase activity in nodules of plants of *Glycine max* was determined by incubating the whole root system of a plant in 60 ml reagent tubes with 8.3% acetylene in air for 1 h in the dark (28 °C). After a 5 min lag period the activity proceeds linearly for 1 h and is only slightly reduced after several hours in this system. The average activity of ten plants was taken (Fig. 1, Table II). The activity was determined about 4 h after the beginning of the light period in the 14 : 10 h light dark regime. After estimation of the nodule fresh weight per plant, the nodules were stored at – 80 °C until used for enzymatic assays, without loss of enzyme activity during several months.

Microbiological control tests

Routinely single colonies from agar plates for colony counting at the end of the pure culture experiments were tested for infectivity on seedlings of *Glycine max* var. Caloria as described [9]. With 10 control plants, without nodules, the average number of nodules per plant infected with 2×10^4 viable rhizobia for 10 min was 21, infected with 3×10^3 viable bacteria it was 16 nodules.

Isolation of bacteroids

Nodules of 10 plants were washed with water at 4 °C and crushed in 0.05 M TES-buffer with 0.01 M glutathione (reduced) (pH 7.5) (5 ml buffer for

1 g nodules) with addition of about 300 mg insoluble, acid washed polyvinylpyrrolidone per g nodules. The homogenate was filtered through 10 μm cheesecloth, the remaining particles crushed once again in a mortar with addition of buffer and the second filtrate added to the first. The filtrate was centrifuged for 4 min at $300 \times g$ to remove most starch particles and large cell organelles. The supernatant was centrifuged for 15 min at $4500 \times g$, the bacteroids in the sediment were washed two times in 0.05 M TES-buffer pH 7.5. Finally the washed bacteroids were resuspended in 2.5 ml buffer with 0.01 M glutathione.

Preparation of cell free extracts from bacteroids and from free living cell

The bacteroid suspension was disrupted in a French pressure cell (American Instrument Comp., Silver Spring, Maryland) two times with 20 000 psi and centrifuged two times for 10 min at $38\,000 \times g$ at 4 °C. The supernatant was immediately used for the enzyme assays. Rhizobia from liquid cultures (nitrogenase repressed and derepressed cells) were centrifuged for 10 min at $38\,000 \times g$ at 4 °C, washed once with water and stored as sediment at −80 °C. For preparation of the cell free extracts, the free living cells were disrupted as described for the bacteroids.

Protein determination

The protein content in the cell free extracts from bacteroids was determined according to the method of Bradford [25], which is not disturbed by glutathione and phenolic compounds as the method of Lowry [26] (Stripf and Werner, in preparation) though the coomassie brilliant blue dye binding method also has some problems [27, 28].

The protein content in cultured *Rhizobium* cells and extracts from these cells was determined according to [26]. Bovine serum albumin (Boehringer, Mannheim) was used as a standard for both methods.

Enzyme assays

a) Aspartate aminotransferase (E.C. 2.6.1.1.), modified from ref. [12]: 2.4 ml HEPES-buffer (N-2-hydroxyethyl-piperazine-N-2 ethane sulfonic acid), 0.05 M, pH 7.8 with 20 mg sodium aspartate and 0.01 mg pyridoxalphosphate per ml; 0.05 ml malatedehydrogenase (0.5 mg·ml^{-1}, NH_4^+-free); 0.05 ml NADH (10 mg NADH − Na_2·ml^{-1}); 0.05 ml cell free extract; after 5 min: start with 0.05 ml 2-oxoglutarate (47 mg·ml^{-1}, pH 6.5 – 7.0).

b) Alanine aminotransferase (E.C. 2.6.1.2.), modified from ref. [13]: 2.0 ml Tricine-buffer (0.05 M, pH 8.3) with 0.5 M l-alanine; 0.05 ml lactate dehydrogenase (0.5 mg·ml^{-1}, NH_4^+ free); 0.05 ml NADH (10 mg NADH − Na_2·ml^{-1}); 0.05 ml cell free extract; after 5 min: start with 0.05 ml 2-oxoglutarate (47 mg·ml^{-1}, pH 6.5 – 7.0).

c) Glutamate dehydrogenase (E.C. 1.4.1.2.), modified from ref. [14]: 1.0 ml Tris-buffer (0.1 M, pH 8.4); 0.75 ml water; 0.05 ml 2-oxoglutarate (0.2 M; pH 6.5 – 7.0); 0.05 ml NADH (4 mM); 0.05 – 0.1 ml cell free extract; after 4 min: start with 0.05 ml NH_4Cl (0.4 M).

d) Alanine dehydrogenase (E.C. 1.4.1.1.), modified from ref. [14]: 1.0 ml Tris-buffer (0.1 M, pH 8.6); 0.75 ml water; 0.05 ml sodiumpyruvate (0.2 M); 0.05 ml NADH (4 mM); 0.05 – 0.1 ml cell free extract; after 4 min: start with 0.05 ml NH_4Cl (0.4 M).

e) Glutamine amide 2-oxoglutarate aminotransferase (E.C. 1.4.1.13.), modified from ref. [14]: 1.0 ml TES (N-Tris-hydroxymethyl-methyl-2-aminoethane sulfonic acid) buffer (0.1 M, pH 7.6); 0.78 ml water; 0.02 ml 2-oxoglutarate (0.1 M; pH 6.5 – 7.0); 0.05 ml NADH (4 mM); 0.05 – 0.1 ml cell free extract; after 4 min: start with 0.05 ml glutamine (0.2 M).

f) 3-Hydroxybutyrate dehydrogenase (E.C. 1.1.1.30.), modified from ref. [15]: 2.8 ml Tris-buffer (0.036 M, pH 8.0); 0.05 ml $MgCl_2 \times 6\,H_2O$ (0.06 M); 0.025 ml NAD (48 mM); 0.05 – 0.1 ml cell free extract; after 4 min: start with 0.025 ml DL-3-hydroxybutyrate, sodium salt (0.8 M).

The activity of these enzymes (a – f) was recorded at 340 nm every minute for five minutes in two parallels and the arithmetic average taken (substracting a blank without substrate). The specific activities are given as nM substrate·min^{-1}·mg protein^{-1} (at 25 °C) (m-units).

g) Glutamine synthetase (E.C. 6.3.1.2.), modified according to ref. [16, 7]: 15 ml 0.1 M glutamine (pH 7.15); 2 ml imidazole-HCl buffer (1.0 M, pH 7.15); 1 ml ADP-Na_2 (0.01 M; pH 7.15); 1.5 ml sodium arsenate (1.0 M; pH 7.15); 1.5 ml hydroxylamine-HCl (2.0 M; pH 7.0); 1.5 ml $MnCl_2 \times 4\,H_2O$ (0.01 M): altogether brought up to a volume of 25 ml assay mixture (pH 7.15); 0.5 ml

of this assay mixture and 0.5ml cell free extract (diluted several times with TES-buffer (0.05 M; pH 7.15) were incubated for 10 min at 25 °C then stopped by addition of 2 ml of a solution with the following composition: 40 ml $FeCl_3$ (10% w/w); 10 ml trichloracetic acid (24% w/w); 5 ml HCl (6 M); 65 ml water.

After the development of the colour we centrifuged for 5 min at $1300 \times g$ and measured the absorption at 540 nm against a blank, which was set up without ADP and arsenate in the assay mixture. The magnesium dependent assay included 60 mM $MgCl_2 \cdot 6\,H_2O$ in the assay mixture. The standard curve was made with glutamyl-hydroxamate (Sigma, Munich).

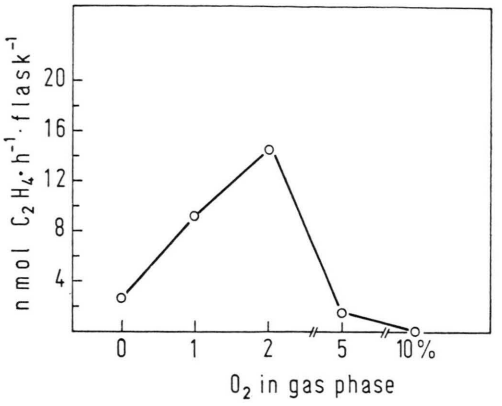

Fig. 2. Oxygen dependent nitrogenase activity in pure liquid culture of *Rhizobium japonicum* 61-A-101 cultivated under an atmosphere of 1% O_2, 1% CO_2 and 98% N_2 in medium 20 P, tested for 90 min under increasing concentrations of O_2 in nitrogen gas. Protein content per test-flask is 2.3 mg (in 5 ml suspension).

Results

The development of nitrogenase activity in intact nodules of *Glycine max* (Fig. 1) shows a sharp increase between 15 and 20 days after infection of the 14 day old seedlings. Using a phytotron in which temperature, light intensity, light period, and moisture were controlled, with an artificial soil system ("Perlite") and a synthetic nitrogen free medium for cultivation of the plants, there was only a variation of not more than two days for this sharp rise in nitrogenase activity. Even with an other variety (Chippewa [64]) of *Glycine max*, the maximum activity was found only 2 days later. 24 d after inoculation, the activity starts to drop. Together, we find a 20 day period of active nodules under these conditions. Taking an average figure of 6 nmol $C_2H_4 \cdot h^{-1} \cdot$ mg nodule^{-1}, 15% nodule dry weight per fresh weight and 30% protein per dry weight with 16% N in the protein, we find a production of about 2 μg ammonia-N during the 20 d period fixed by 1 μg protein-N in the nodules.

After transfer to the defined medium 20 P under 2% oxygen (+10% CO_2, 88 N_2) free living cells of *Rhizobium japonicum* develop, within 5 to 8 days, a maximum nitrogenase activity of 180 nmol $C_2H_4 \cdot h^{-1} \cdot$ mg bacterial protein^{-1} (Fig. 1). This is about 30% of the activity we find in the intact nodules (per bacteroid protein). Nitrogenase activity in the derepressed cells is oxygen dependent and increases 7 fold from a 100% nitrogen gas atmosphere to a 2% oxygen concentration (Fig. 2), when the cells were cultivated under 1% O_2 with 1% CO_2 and 98% N_2. This increase is even bigger, about 10 fold, when the rhizobia come from a con-

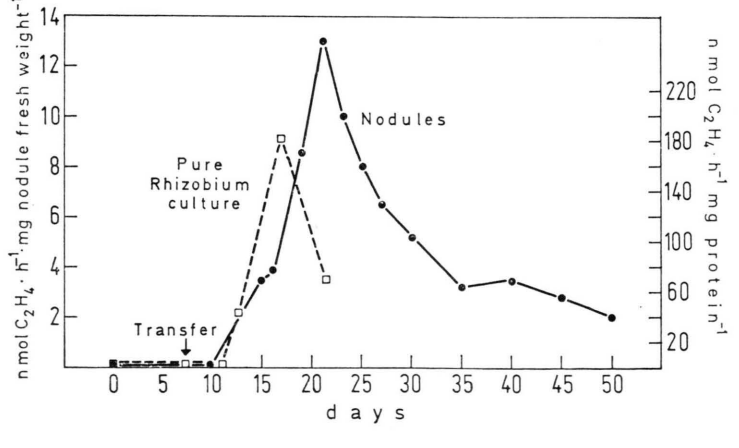

Fig. 1. Nitrogenase activity in nodules of *Glycine max* var. Caloria in nmol $C_2H_4 \cdot h^{-1} \cdot$ mg nodule fresh weight^{-1} against days after infection of 14 d old seedlings with cells of a single colony of *Rhizobium japonicum* 61-A-101; and in pure liquid culture of the same strain, inoculated from a single colony at zero time in medium 20 E under air and transfered after 7 d in medium 20 P under 2% oxygen, 10% CO_2 and 88% N_2. At peak activity the O.D. of the culture has 370 nephelometer units with 0.400 mg protein · ml^{-1}.

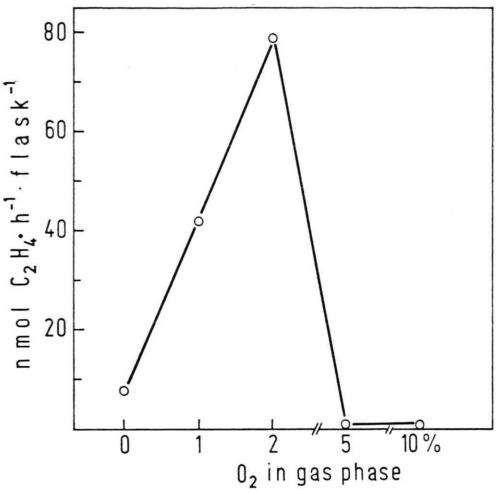

Fig. 3. Oxygen dependent nitrogenase activity of pure liquid culture of *Rhizobium japonicum* 61-A-101, cultivated under an atmosphere of 2% O_2, 10% CO_2 and 88% N_2 in medium 20 P, tested for 90 min under increasing concentrations of O_2 in nitrogen gas. Protein content per test-flask is 2.6 mg (in 5 ml suspension).

tainer under 2% O_2, 10% CO_2 and 88% N_2 (Fig. 3). A further increase to 5% oxygen results in a fall to zero (Fig. 3) or only 10% activity (Fig. 2).

Nitrogenase activity in the derepressed cells is not affected by addition of concentrations of 1 and 10 mM ammonia during a 2 h incubation period under 2% oxygen (Fig. 4). Even 10^{-1} M ammonia does not reduce significantly this activity.

As we will show in another publication [11], small differences in the concentration of glutamine in the range of 0.5 to 2.0 mM effects very much the development (derepression) of nitrogenase in

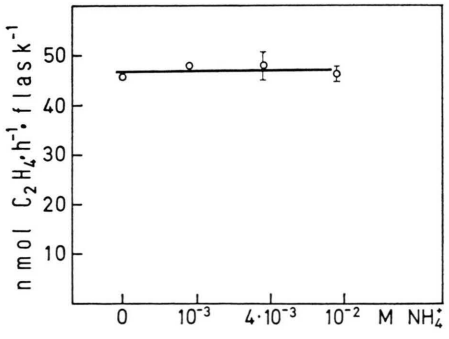

Fig. 4. Effect of 1—10 mM NH_4^+Cl on nitrogenase activity in pure liquid culture of *Rhizobium japonicum* 61-A-101 in medium 20 P under 2% oxygen in the gas phase. Protein content per test flask is 3.8 mg (in 5 ml suspension). Verticals: 1 s deviation.

Rhizobium japonicum during a 8 – 14 days period in another experimental system (agar surfaces). Similar concentration of glutamine (0.36 – 1.44 mM) added to the bacteria with an already developed high or low nitrogenase activity have no significant effect in the presence of oxygen (2%) in the gas phase (Fig. 5) and in the absence (Fig. 6). Also no effect of glutamine on nitro-

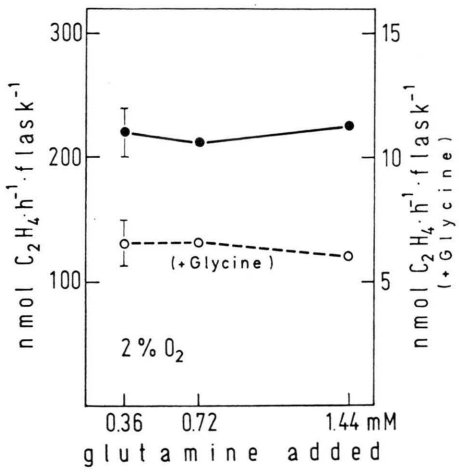

Fig. 5. Effect of 0.36 to 1.44 mM glutamine on nitrogenase activity in pure liquid culture of *Rhizobium japonicum* 61-A-101 in medium 20 P under 2% oxygen in the gas phase (—●—). Protein content per test flask is 1.75 mg (in 5 ml suspension). (—○—) 1 mM glycine added to the culture 7 d before testing. Verticals: 1 s deviation.

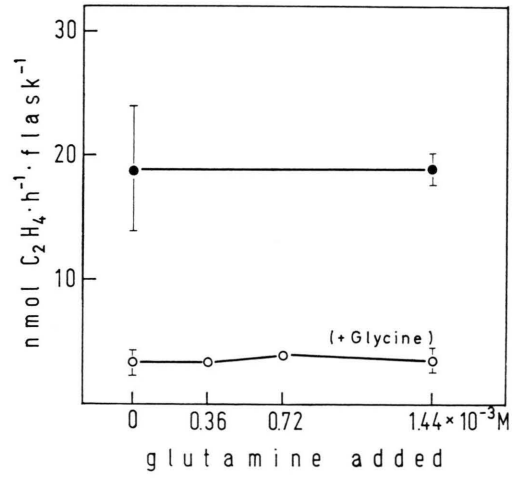

Fig. 6. Effect of 0.36 to 1.44 mM glutamine on nitrogenase activity in pure liquid culture of *Rhizobium japonicum* 61-A-101 in medium 20 P under nitrogen gas in the gas phase (—●—). Protein content per test flask is 1.5 mg (in 5 ml suspension). (—○—) 1 mM glycine added to the culture 4 d before testing. Verticals: 1 s deviation.

genase activity was observed under the same conditions, when 1 mM glycine was added to the cultures 7 d before testing, thus reducing the activity of the cells to about 3% (6 nmol $C_2H_4 \cdot h^{-1} \cdot flask^{-1}$ versus 220 nmol, Fig. 5) under 2% oxygen or to about 15% (3 nmol versus 19 nmol, Fig. 6) with no oxygen in the gas phase.

These free living cells of nitrogenase active rhizobia were now compared with bacteroids isolated from nodules of *Glycine max*, infected with the same strain (*Rhizobium japonicum* 61-A-101) with regard to the specific activities of several enzymes of N-metabolism (Table II). Specific activity of alanine dehydrogenase in the nitrogenase active free living cells is about four times higher compared to the repressed cells, but is comparable to that found in bacteroids. These bacteroids were isolated from nodules with maximum nitrogenase activity (Fig. 1), 22 d after infection. Activity of aspartate aminotransferase is about twice as high in nitrogenase active free living cells as in the non active rhizobia with 400 nmol substrate·mg protein^{-1}·min^{-1} and is also very close to the value found in the bacteroids. A third enzyme with a similar activity in nitrogenase active, free-living cells and in bacteroids and significantly reduced in repressed cells, is the glutamine amide 2-oxoglutarate aminotransferase (GOGAT). But the specific activities found in all three types of cells are much smaller than those for the first two enzymes described.

Table II. Specific activities (nmol substrate·mg protein^{-1}·min^{-1}) of enzymes of N-metabolism from nitrogenase derepressed and repressed free living cells of *Rhizobium japonicum* 61-A-101 and of bacteroids from *Glycine max* var. Caloria, infected with the same strain. The deviations in repeated experiments were about 10% or less, of the figures given.

Enzyme	*R. japonicum*, liquid culture, derepressed nitrogenase	*R. japonicum*, liquid culture, repressed nitrogenase	Bacteroids, 22 d after infection
	m-units		
alanine dehydrogenase	319	75	245
aspartate aminotransferase	400	220	442
glutamine amide-2-oxoglutarate aminotransferase (GOGAT)	42	20	41
3-hydroxybutyrate dehydrogenase	358	239	159
alanine aminotransferase	59	51	76
glutamine synthetase (+Mn^{2+})	477	1585	390
glutamate dehydrogenase	0	0	29
nitrogenase activity	130 [a]	0	14—18 [b]

[a] nmol C_2H_4·mg protein^{-1}·h^{-1}.
[b] nmol C_2H_4·mg nodule freshweight^{-1}·h^{-1}.

Table I. Differentiation of *Rhizobium japonicum* to bacteroids in the development of nodules.

- — Binding of *Rhizobium* to root hair surface proteins
- — Penetration in the root hairs
- — Infection-Thread formation and migration of the rhizobia in the growing thread
- — Plant cell division stimulation in the root cortex by kinetin and auxin from the rhizobia
- — Membrane-envelope (vacuole)-formation in the cytoplasm
- — Leghaemoglobin-synthesis
- — Cytochrome changes : reduction in Cyt a, a_3, increase in Cyt c, P 420, P 450
- — Increased DNA content per cell
- — Cell wall differentiation
- — Enlargement and deformation of the rhizobia to "bacteroid" forms
- — Osmotic sensitivity increase
- — Accumulation of reserve substances (PHBA)
- — Viability-loss as free living cells
- — Reduced uptake of 2-oxo-glutarate and 1-glutamate (K_0)
- — Derepressed nitrogenase
- — Differentiation in enzyme activities of N-metabolism

For the other three enzymes in Table II no such correlation was found, 3-hydroxybutyrate dehydrogenase has the lowest activity in the bacteroids 22 d after infection, an intermediate activity in the nitrogenase repressed rhizobia and the highest in the nitrogenase active cells. However the specific activity, of this enzyme in bacteroids continues to increase between 3 and 4 weeks after infection.

The alanine aminotransferase has only 15 to 20% of the specific activity of the aspartate aminotransferase in all three types of cells. The small difference between nitrogenase repressed and derepressed cells is not significant.

Glutamine synthetase has also about the same specific activity in the partially derepressed cells and in the bacteroids, but is four times more active in the cells from liquid culture with no nitrogenase activity. No activity of glutamate dehydrogenase

was found in the free living cells, but also only 29 m units in the bacteroid fraction.

Discussion

The differentiation of a free living soil bacterium of the genus *Rhizobium* to a bacteroid in the cells of legume nodules is, perhaps with the exception of the extensively studied morphological and physiological events associated with the sporulation of a *Bacillus* cell [20], one of the most pronounced differentiations in bacterial cells. From the steps, listed in Table I, the relation between the last two aspects, the derepressed nitrogenase an the differentiation in enzymes of N-metabolism is compared in free living cells and in bacteroids in this study. As shown by several authors for the cowpea *Rhizobium* strains 32 H 1 [29] and CB 756 [22] oxygen concentration also influences nitrogenase activity in *Rhizobium japonicum* 61-A-101 markedly, with a sharp maximum rate at 2% oxygen in the gas phase (Fig. 2, 3). This activity is about ten times higher than under a pure nitrogen atmosphere in cells from a batch culture, supplied with 2% oxygen, 10% CO_2 and 88% N_2. The specific nitrogenase activity of these cells tested under 2% O_2 is between 13 nmol and 180 nmol $C_2H_4 \cdot mg\ protein^{-1} \cdot h^{-1}$ (Fig. 1). This is comparable to the activities found with very low dissolved oxygen concentration with strain 32 H 1 [21] (12 – 62 nmol $C_2H_4 \cdot mg\ dry\ weight^{-1} \cdot h^{-1}$, equivalent to 25 – 125 $C_2H_4 \cdot mg\ protein^{-1} \cdot h^{-1}$), but less than the maximum figure of 314 nmol $C_2H_4 \cdot mg\ dry\ weight^{-1} \cdot h^{-1}$ for the cowpea strain CB 756 [22].

For glutamine limited cultures of *Rhizobium* 32 H 1 the addition of 5 mM NH_4^+ reduced the nitrogenase activity by 50% with an apparent half life of 90 min [21]. In agar grown cultures of the same strain, an 80% inhibition of nitrogenase activity after 90 min incubation with 3 mM NH_4 was noticed, but this could be relieved partially by raising the pO_2 from 0.20 to 0.35 atm [31]. In the cowpea strain CB 756, in oxygen-limited cultures, an increase of added ammonia from 1.5 mM to 34 mM reduced nitrogenase only by about 20 – 30% [22]. During a 90 min incubation period, no such effect was observed by us in *Rhizobium japonicum* 61-A-101 with leucine as single nitrogen source by addition of 10 (Fig. 4) and 100 mM NH_4^+.

A marked reduction of nitrogenase activity in nodules of *Glycine max* by addition of 30 mM NH_4^+ was reported [7] but only after 6 d duration. Since no effect was found by the authors during a 2 and 4 d treatment, no direct effect on nitrogenase activity in the intact symbiosis was assumed, moreover an effect on the nodule development (nodule weight) as shown in the same laboratory earlier [30]. More than 90% of the fixed N_2 was shown to be exported as NH_4^+ by *Rhizobium japonicum* [19], though the rate of only 0.09 nmol N fixed per mg protein per h is only 1 – 2% of the maximum rate in our experiments (Figs 1 and 5). Large quantities of ammonia (up to 20 µmol per ml) were produced by *Rhizobium trifolii* [19] from l-histidine. Both types of experiments show a great difference between rhizobia and free living diazotrophic bacteria, that do not export ammonia. A similar independence of the nitrogenase activity of *Rhizobium japonicum* towards addition of glutamine (Figs 5 and 6) may indicate, that the symbiotic bacteria inside the plant cells can also tolerate higher concentrations of organic amino compounds. Asparagine, used with the same conditions and concentrations as glutamine, does similarly not reduce nitrogenase activity of our *Rhizobium* strain.

Alanine dehydrogenase activities are, of all enzymes tested (Table II), most similar in bacteroids and in nitrogenase derepressed free living *Rhizobium japonicum* cells and very different in nitrogenase repressed cells. This enzyme was recently intensively studied in *Anabaena cylindrica* [23]. The specific activity in N_2-fixing cultures was about 3 times higher in heterocysts than in vegetative cells. But, on the other hand, in nitrogen starved cultures, the activity in vegetative cells was higher than in heterocysts. The authors suggest, that newly formed ammonia in nitrogen fixing cultures is not incorporated into organic compounds by this enzyme. Since specific activity in the heterocysts of *Anabaena cylindrica* is only about 20% of that found in bacteroids and in nitrogenase active free living *Rhizobium japonicum* cells we do not assume this for *Rhizobium*.

As shown in a previous study [18], for the unfractionated nodules of *Glycine max* (var. Chippewa, infected with *Rhizobium japonicum* 3I1b85), compared to roots, the specific activity of aspartate aminotransferase is significantly higher in bacteroids than in free living cells of *Rhizobium japonicum*

(Table II). In the nitrogenase active free living cells we now find almost the same activity as in the bacteroids. This enzyme can be considered as specifically correlated with a derepressed nitrogenase. Glutamine amide 2-oxoglutarate aminotransferase has also a similar activity in bacteroids and in cells with nitrogenase activity and only half the activity in nitrogenase repressed cells. However the specific activities are only about 10% of that for the aspartate aminotransferase, and may be of less physiological importance for the function of the symbiosis. A low activity of 41 m-units for GOGAT in bacteroids was also found by Dunn and Klucas [14]. We find 6 times higher activities for alanine dehydrogenase in our bacteroids and about half the activity of glutamine synthetase as these authors. This can be explained by the different ages of the nodules, since we find considerable variation in the specific activities of these enzymes during the nodule development (Stripf and Werner, in preparation).

No glutamate dehydrogenase in several conditions of nitrogenase active free living cells was found for the cowpea *Rhizobium* strain [22] and rather low activities in bacteroids [14].

The activity and function of glutamine synthetase was studied and discussed recently in several laboratories [7, 14, 24]. The activity in the bacteroids (Table II) from a defined age (22 d after infection) is about 25% of that found in free living cells with no nitrogenase activity, compared to 5 – 20% found in the other studies. But also in cells with nitrogenase activity we find almost the same (reduced) activity as in bacteroids. But, as we will show and discuss in a forthcoming publication, the activity of this enzyme is developing very differently from nitrogenase activity in bacteroids. This is also the case for the specific activity of 3-hydroxybutyrate-dehydrogenase (Stripf and Werner, in preparation).

We thank Mrs. B. Kalkowski for excellent technical assistance, Dr. John Wilcockson for some corrections of the English manuscript and the Deutsche Forschungsgemeinschaft for the grant A 9 in the SFB 103 "Zellenergetik und Zelldifferenzierung".

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