Symbiosis, 9 (1990) 229–232
Balaban Publishers, Philadelphia/Rehovot

Nitrate Utilization by *Bradyrhizobium japonicum* Bacteroids*

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Abstract

The activity of nitrate reductase (NR) in bacteroids of *Bradyrhizobium japonicum* strain L-236 incubated with nitrate under anaerobiosis declined during incubation for 15 h, while that of bacteroids incubated aerobically remained constant. In both cases, after a 15 h incubation, there was an increase in NR activity. This increase was prevented by chloramphenicol. Constitutive NR activity was located in the soluble fraction of L-236 bacteroids.

1. Introduction

A constitutive NR was first demonstrated in the soybean root noduls by Evans (1954). Since then assimilatory and dissimilatory nitrate reduction has been shown in *B. japonicum* bacteroids (Becana and Sprent, 1987 and references therein). Whether the constitutive NR enzyme from *Bradyrhizobium* bacteroids is of assimilatory or dissimilatory type is not clear. This study reports on the utilization of nitrate by isolated bacteroids of *B. japonicum* strain L-236 incubated under aerobic and anaerobic conditions.

2. Materials and Methods

Seeds of *Glycine max* L. (Merr.) cv. Williams were inoculated with *B. japonicum* strain L-236. Plant growth conditions and bacteroid isolation procedure have been described previously (Delgado et al., 1989). Bacteroids (1 mg protein ml^{-1}) were incubated aerobically and anaerobically (argon atmosphere) in the medium described by Streeter and Devine (1983), except that 3-(N-morpholino) propane sulfonic acid (MOPS)/KOH buffer, (pH 7.5) was used. Incubation was for 35 h at 28°C with and

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without 100 μg/ml chloramphenicol. Aliquots (2 ml) were taken at regular intervals and centrifuged at 8000 × g for 10 min. The supernatant was used to measure nitrite (Snell and Snell, 1949) and nitrate (Cawse, 1967) contents. The pelleted bacteroids were washed with MOPS/KOH buffer and resuspended in the same buffer. NR activity was determined *in situ* using the reaction mixture described by Delgado et al. (1989) with alkyltrimethyl ammonium bromide (MTAB) to permeabilize the cells. Assays were run aerobically or anaerobically according to the incubation conditions. Nitrite reductase (NiR) activity was determined as for NR, except that nitrite (5 mM) was substituted for nitrate.

To study the location of NR in freshly isolated bacteroids, cells were disrupted with an ultrasonic probe for 5 min, in 30-s periods at 80 W and 4°C. Unbroken cells were removed by centrifugation. The particulate and soluble fractions were prepared by further centrifugation of the supernatant at 250 000 × g for 1 h. The pellet was washed with 50 mM Tris/HCl buffer (pH 7.5) and the membrane vesicles were dispersed by suspension in the same buffer containing 4% (w/v) Triton X-100 and incubation on ice for 5 min. Triton X-100 insoluble material was removed by further centrifugation.

In vitro soluble and membrane vesicles NR activity were assayed as indicated above, except that MTAB was omitted from the reaction mixtures. Protein was measured by the procedure of Markwell et al. (1978) using BSA as a standard protein. Data presented represent experiments that have been repeated at least twice. NR assays were run in duplicate.

3. Results and Discussion

As previously reported for *B. japonicum*, bacteroids of strain L-236 had constitutive NR activity (Fig. 1). Rates of activity under anaerobiosis were higher than those found when the assays were made under aerobic conditions, presumably because oxygen affected NR activity (Figs. 1A and 1C).

When bacteroids were incubated anaerobically with nitrate a rapid decline in NR activity was observed (Fig. 1A). Nitrate accumulation in the medium and nitrate uptake were detected shortly after incubation and inversely correlated with each other (Fig. 1A). After a 15 h incubation there was an increase in NR activity and a continous disappearance of nitrate and of nitrite (Fig. 1A). Inclusion of chloramphenicol in the incubation medium prevented the development of the NR activity, nitrite was not removed and nitrate uptake ceased (Fig. 1B). Rigaud (1976) and Giannakis et al. (1988) have also demonstrated an induction of NR in anaerobic preparations of *R. phaseoli* and *B. japonicum* bacteroids.

When bacteroids were incubated under aerobic conditions, NR activity remained constant for 15 h and increased afterwards (Fig. 1C). Nitrite accumulation was much lower than in bacteroids incubated anaerobically (Figs. 1A and 1C), probably because

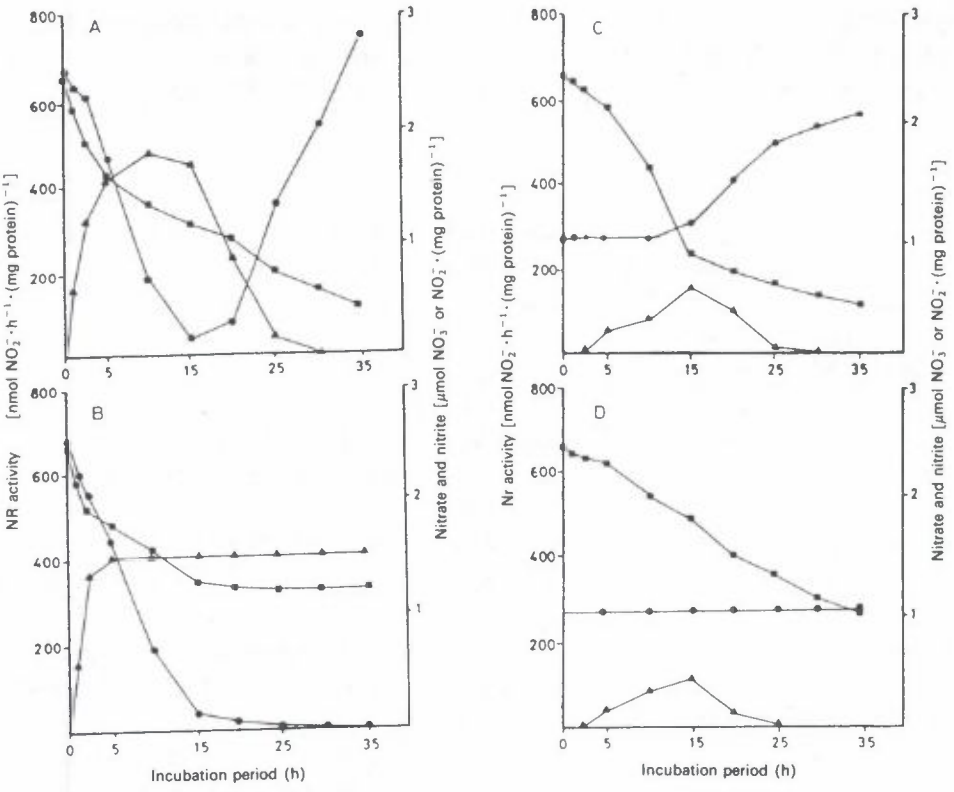

Figure 1 (A–D). Nitrate utilization by *B. japonicum* L-236 bacteroids. Bacteroids were incubated anaerobically (A, B) and aerobically (C, D) without (A, C) or with (B, D) 100 μg/ml chloramphenicol. ●—● NR activity ■—■ Nitrate ▲—▲ Nitrate.

of the higher NiR activity in bacteroids incubated under aerobic conditions [392 and 548 nmol $NO_2^- h^{-1}$ (mg protein)$^{-1}$, respectively, after incubation for 15 h]. The presence of chloramphenicol did not affect the rate of activity during incubation for 15 h and prevented the development of NR after that time (Fig. 1D). No increase in protein content was observed after incubations of the bacteroids for 35 h.

Freshly isolated bacteroids of strain L-236 showed NR activity only in the soluble fraction [65 nmol $NO_2^- h^{-1}$ (mg protein)$^{-1}$]. No activity could be detected in the membrane vesicles. In contrast, Kennedy et al. (1975) reported NR activity in both soluble and particulate fractions of *B. japonicum* strain CC705 bacteroids.

The oxygen concentration in the bacteroids infected tissue of the nodules is very low, and thus it would favour the expression of a dissimilatory NR. However, our results indicate that the constitutive NR of L-236 bacteroids seems to be of assimilatory

type since chloramphenicol did not inhibit activity under aerobic conditions and that this activity was located in the soluble fraction of the bacteroids.

This research was supported by C.I.C.Y.T., Grant No BT 87/0003.

REFERENCES

Becana, M. and Sprent, J.I. 1987. Nitrogen fixation and nitrate reduction in the root nodules of legumes. *Physiol. Plant.* **70:** 757–765.

Cawse, P.A. 1967. The determination of nitrate in acid solution by ultraviolet spectrophotometry. *Analyst.* **92:** 311–313.

Delgado, M.J., Olivares, J., and Bedmar, E.J. 1989. Nitrate reductase activity of free-living and symbiotic uptake hydrogenase-positive and uptake hydrogenase-negative strains of *Bradyrhizobium japonicum*. *Arch. Microbiol.* **151:** 166–170.

Evans, H.J. 1954. Diphosphopyridine nucleotide-nitrate reductase from soybean nodules. *Plant Physiol.* **29:** 238–301.

Giannakis, C., Nicholas, D.J.D., and Wallace, W. 1988. Utilization of nitrate by bacteroids of *Bradyrhizobium japonicum* in the soybean root nodules. *Planta.* **174:** 51–58.

Kennedy, J.R., Rigaud, J., and Trinchant, J.R. 1975. Nitrate reductase from bacteroids of *Rhizobium japonicum*: enzyme assay and characteristics and possible interaction with nitrogen fixation. *Biochim. Biophys. Acta.* **397:** 24–35.

Markwell, M.A.K., Hass, S.M., Bieber, L.L., and Tolbert, N.E. 1978. A modification of the Lowry procedure to simplify protein determination. *Anal. Biochem.* **87:** 206–210.

Rigaud, J. 1976. Effect des nitrates sur la fixation d'azote par les nodules de haricots (*Phaseolus vulgaris L.*). *Physiol. Veg.* **14:** 294–308.

Snell, F.D. and Snell, C.T. 1949. Colorimetric Methods of Analysis. D. Van Nostrand, Co. pp. 802–807. New York.

Streeter, J.G. and Devine, P.J. 1983. Evaluation of nitrate reductase activity in *Rhizobium japonicum*. *App. Environ. Microbiol.* **46:** 521–524.