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Evaluation of Nitrate Reductase Activity in *Rhizobium japonicum*†

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Nitrate reductase activity was evaluated by four approaches, using four strains of *Rhizobium japonicum* and 11 chlorate-resistant mutants of the four strains. It was concluded that in vitro assays with bacteria or bacteroids provide the most simple and reliable assessment of the presence or absence of nitrate reductase. Nitrite reductase activity with methyl viologen and dithionite was found, but the enzyme activity does not confound the assay of nitrate reductase.

Since the discovery of nitrate reductase (NR) in *Rhizobium japonicum* bacteroids (7), there has been considerable interest in differences in the expression of this enzyme among *Rhizobium* species. Some of this interest is related to questions about the role of *Rhizobium* sp. as a denitrifying bacterium in soils (4, 5, 9, 18). Some of the interest in NR is related to the relationship between NR and nitrogenase activities (1, 10, 12, 14, 15). Our interest in *Rhizobium* NR is based on the utility of NR-deficient mutants in studies on the inhibition of legume nodule growth and N_2-fixing activity by nitrate (16), and this interest is shared by other groups (6, 8, 13).

de Vasconcelos et al. (6) have recently studied (i) the aerobic growth of *R. japonicum* in a defined medium with nitrate as the only N source and (ii) the accumulation of nitrite in the medium when bacteria were grown under anaerobic conditions. Their work suggests that a thorough assessment of the presence or absence of NR would require both of these approaches. However, we found that nitrite accumulation under anaerobic conditions was highly variable among strains and very erratic as a function of time for some strains. This problem led us to a comparison of approaches (i) and (ii) with direct assays of NR, and this, in turn, led to the conclusion that direct assays are a reliable index of the presence or absence of NR.

R. japonicum 61A76 (Nitragin Co.) and the chlorate-resistant derivatives 76CR1 and 76CR6 (NR1 and NR6 in reference 6) were supplied by C. A. Neyra, Rutgers University, New Brunswick, N.J. We have learned (N. Malik, personal communication) that these chlorate-resistant derivatives do not serotype as 61A76. Thus, although these isolates were derived from 61A76, they should be viewed as multiple mutants. This view is consistent with the difference between 61A76 and the derivatives in slime production (6), a difference which we have also noted. *R. japonicum* 3I1b110, 3I1b138, and 3I1b142 and chlorate-resistant mutants of these strains were supplied by D. G. Blevins, University of Missouri, Columbia, Mo.

For routine culture, bacteria were grown in a yeast extract-mannitol medium (16). The medium described by Manhart and Wong (12) was used for evaluation of aerobic growth with nitrate as the N source. For the study of nitrite accumulation under anaerobic conditions, bacteria were first grown in the defined medium of Manhart and Wong (12) with glutamate (10 mM) substituted for nitrate. This was required because of the failure of many strains to grow with nitrate as the N source. Glutamate-grown cells were collected after 6 to 8 days by centrifugation at 3,000 × *g* in sterile, capped centrifuge tubes after taking a sample for determination of the absorbance at 660 nm. Cells were suspended in anaerobic, defined medium (with nitrate only) under N_2 in a laminar-flow hood. The defined medium (10 ml in a 13- by 150-mm test tube) was made anaerobic by vigorous bubbling of N_2 gas for 1 min before autoclaving. All liquid cultures were incubated at room temperature (21 ± 1°C) on an orbital shaker at 100 rpm.

Nitrite accumulation in the medium was determined by using supernatant after centrifugal removal of cells. Supernatant (1.0 ml) was mixed with 1.0 ml of 0.02% *N*-(1-napthyl)ethylenediamine in 95% (vol/vol) ethanol and 1.0 ml of 1% sulfanilamide in 1.5 N HCl, and the absorbance at 550 nm was determined with a Gilford 250 spectrophotometer. Uninoculated medium was used as a blank and never contained nitrite.

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For the assay of NR in cultured bacteria, 100 ml of culture was centrifuged at 10,000 × g for 15 min. Medium and slime were discarded, and the cells were suspended in 15 ml of 0.2 M sodium phosphate (pH 7.5) and centrifuged a second time. The supernatant was discarded, and the cells were resuspended in 4 ml of the buffer just described. Duplicate reaction mixtures consisted of 1.0 ml of 0.2 M sodium phosphate (pH 7.5), 0.2 ml of 0.1 M KNO_3, 0.2 ml of 0.25 M succinate (pH 7.5), and 200 μl of bacterial suspension (100 to 300 μg of protein). Boiled bacteria were the control, and a standard nitrite determination (with boiled bacteria) was included with every assay. Reaction mixtures were incubated at 30°C for 1 h, and reactions were stopped by adding reagents for nitrite determination (see above). Samples (1.7 ml) of these mixtures were centrifuged at 8,000 × g for 5 min with a Beckman Microfuge before determining the absorbance at 550 nm.

Bacteroids were isolated from soybean (*Glycine max* (L.) Merr) nodules as described previously (16). NR in bacteroids was assayed as described previously (16), using 25 to 50 μl of bacteroid suspension (150 to 300 μg of protein). The assay for nitrite reductase in bacteroids was patterned after the assay recommended by Vega et al. (17). The reaction mixture contained 55 μmol of sodium phosphate buffer (pH 7.5), 0.70 μmol of KNO_2, 0.75 μmol of methyl viologen (Sigma Chemical Co.), 21.5 μmol of sodium dithionite, and 300 μl of bacteroid suspension in a total volume of 1.0 ml. Dithionite was added last to start the reaction. The control was boiled bacteroids, and a blank lacking nitrite was included with each assay. The mixtures were incubated for 1 h at 30°C, and the reaction was stopped by vigorously stirring the mixtures to oxidize the dithionite. Duplicate 0.10-ml samples were diluted with 1.0 ml of water, and the nitrite concentration was determined as described above. The reduction of nitrite was approximately linear with time up to 75 min and with the amount of bacteroid suspension up to 300 μl.

Protein content of bacteria and bacteroids was determined by the method of Lowry et al. (11) after boiling a sample of cells in 1.0 N NaOH for 10 min and neutralizing the mixture with HCl. Acetylene reduction activity was determined on samples of 5 to 7 nodulated roots (16).

Most of the data in Table 1 are single observations from single experiments. However, for NR activity in bacteria and bacteroids, we have many analyses of many of the strains, and all of the results are completely consistent. Strains 61A76, 3I1b110, 110CR4, 110CR5, 3I1b138,

TABLE 1. Evaluation of NR activity in *R. japonicum*, using enzyme assays and response of bacteria to culture conditions[a]

R. japonicum strain	NR assay[b]		Defined medium with nitrate as N source		
			Aerobic		
	Bacteroids[c]	Cultured bacteria[d]	Growth (A_{660})[e]	Nitrite in medium	Anaerobic nitrite in medium[f]
61A76	1,920	39	+++	+	+
76CR1	0	0	−	−	−
76CR6	0	0	−	−	−
3I1b110	770	39	+	−	+
110CR1	0	0	−	−	−
110CR2	0	0	−	−	−
110CR4	770	33	−	−	+
110CR5	180	123	+	−	+
3I1b138	1,120	104	++	+	+
138CR1	0	0	−	−	−
138CR2	0	0	−	−	−
138CR4	0	0	−	−	−
138CR5	1,200	14	−	−	+
3I1b142	1,530	205	+	−	+
142CR2	1,340	195	±	−	+

[a] All strains were effective as judged by acetylene reduction assays of nodulated roots.
[b] Nanomoles per milligram of protein per hour.
[c] Isolated from nodules from plants supplied with nitrate.
[d] Grown in yeast extract-mannitol; aerobic assay.
[e] A_{660}, Absorbance at 660 nm.
[f] Several different patterns of nitrite accumulation are shown in Fig. 1.

138CR5, 3I1b142, and 142CR2 all have NR activity, based on direct assays of the enzyme in bacteroids and cultured bacteria. The presence of NR in some chlorate-resistant isolates has been observed before (6) and may be related to the inability of some isolates to absorb chlorate. Differences in enzyme activity among NR-positive strains should be interpreted with caution for two reasons. First, the data shown are single observations. Second, the rate of nitrite formation for some strains did not appear to be linear with protein concentration (i.e., quantity of cells per assay). We have not pursued this anomaly. We recommend that, when the presence of NR is uncertain, several concentrations of cells should be assayed.

NR activity is higher in bacteroids than in cultured bacteria (Table 1; 2, 3, 12). Daniel et al. (2, 3) found no increase in NR in *R. japonicum* upon the addition of NO_3^- to aerobic cultures. Also, we have found that bacteroids of 61A76, 3I1b110, nd 3I1b138 from nodules supplied with high nitrate concentrations (5 to 7 mM NO_3^-) have no higher NR activity than bacteroids from nodules supplied with no nitrate. All of these results are consistent with results showing that expression of NR in *R. japonicum* is controlled by oxygen and not by nitrate (3).

An important question is whether the regulation of $[O_2]$ during the culture of *R. japonicum* is required to assure the expression of NR. We neither controlled nor measured $[O_2]$ during *R. japonicum* culture because NR activity is expressed over a wide $[O_2]$ range (3). NR activity was severalfold greater at $[O_2]$ of 0 to 3% than at $[O_2] > 7\%$, but activity was still readily detected at $[O_2]$ of 21% (3). A comparison of our results (Table 1) with those of Daniel and Gray (3) suggests that our cultures were grown with an $[O_2]$ of 7 to 21%.

We also assayed NR in several strains of cultured bacteria by an anaerobic assay and found two- to threefold greater enzyme activity relative to the results under aerobic conditions. Anaerobic assays of 76CR1 and 76CR6 indicated no NR activity. Although anaerobic NR activity is higher (12), these assays are cumbersome and, if an indication of the presence or absence of the enzyme is all that is desired, anaerobic assays are not required.

Two isolates (110CR4, 138CR5) which have assayable NR activity failed to grow in a defined medium with nitrate as the N source (Table 1). This suggests that the NR activity which was assayed in bacteroids and bacteria represented dissimilatory reduction, and this suggestion was supported by the accumulation of nitrite in the medium under anaerobic conditions. Nitrite accumulation was measured after 2, 4, 6, or 8 days of anaerobic incubation. Although a continuous accumulation of nitrite was observed for one strain (61A76), nitrite accumulation was not continuous for other strains and was erratic for some strains (Fig. 1). Thus, a plus in the rightmost column of Table 1 only indicates the detection of nitrite at one or more of the sampling times during the 8-day anaerobic incubations. The fluctuations in medium $[NO_2^-]$ for some strains (Fig. 1) might be due to changes in the nitrite reductase activity which is expressed in *R. japonicum* grown anaerobically with nitrate (2).

It is important to note that some strains (3I1b110, 110CR5, 3I1b142) which grew aerobically with nitrate did not excrete detectable levels of nitrite into the medium after 9 days of growth. Thus, an assay for nitrite in the medium under aerobic conditions would be an unacceptable indicator of the presence of absence of NR. Differences among strains in the amount of

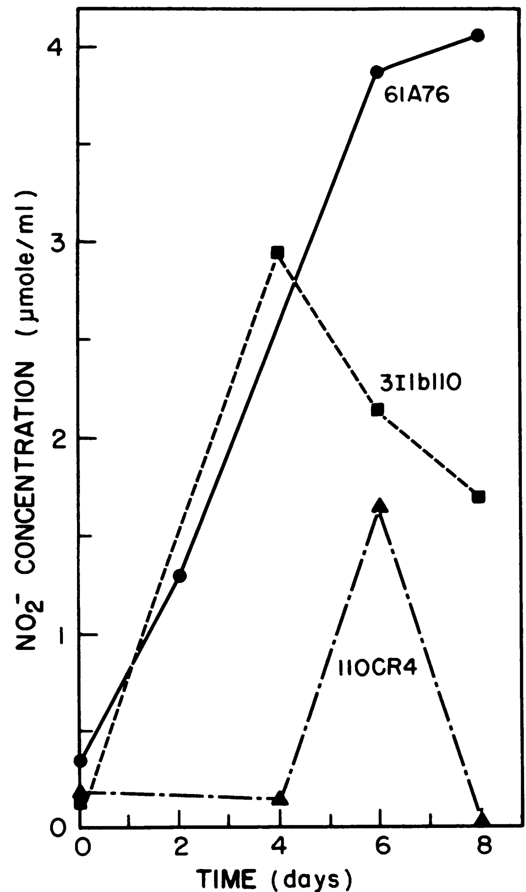

FIG. 1. Accumulation of nitrite in anaerobic, defined medium containing nitrate and inoculated with various strains of *R. japonicum*.

nitrite reductase did not appear to explain the failure of some strains, which grew on nitrate, to excrete nitrite. With the exception of 110CR2, which appeared to have very low nitrite reductase activity, there was little difference among strains (data not shown). The mean nitrite reductase activity in bacteroids across all strains (110CR2 excluded) was 68 ± 5 (standard error) nmol/mg of protein per h. Nitrite reductase was clearly present only in bacteroids from nodules supplied with nitrate. We could not detect nitrite reductase when succinate was substituted for methyl viologen plus dithionite. Cultured *R. japonicum* also lacks succinate-dependent nitrite reductase (2). Thus, the evaluation of NR with in vitro assays is not confounded by the presence of nitrite reductase as long as succinate is used as a source of reducing power.

Direct assays of NR in bacteria grown in yeast extract-mannitol medium or in bacteroids of *R. japonicum* were simple, relative to assessments attempted in defined media with nitrate. Completely anaerobic conditions in cultures (judged by the detection of O_2 with 0.1% resazurin) were found to be difficult to establish. Also, since *R. japonicum* is slow growing, culture periods of up to 9 days were required to give meaningful assessment of growth under aerobic conditions. Most important, a single NR assay was found to be a reliable index for the presence of NR (Table 1) and an acceptable substitute for two relatively tedious culture experiments.

R. japonicum bacteroids have much higher NR activity than bacteria grown aerobically with nitrate or yeast extract (Table 1; 2, 12). Thus, an assessment of the presence or absence of NR would be strengthened by an enzyme assay with bacteroids. This approach has the disadvantage of requiring facilities and additional time for growing plants. Our results indicate that assays of NR in bacteria grown in yeast extract-mannitol are a reliable indicator in themselves. Assays of NR in bacteroids provide useful confirmation of results with cultured bacteria, but anaerobic assays of cultured bacteria, which give higher NR activity than aerobic assays, would be an acceptable substitute for NR assays with bacteroids.

Finally, our results are in agreement with recent reports which indicate that there is no relationship between NR activity and symbiotic competence of *Rhizobium* spp. (1, 10, 12, 14). Effective nodules were formed on soybean plants by all of the *R. japonicum* strains studied, and these strains showed a wide range of NR activity (Table 1). There were no large differences among strains in nodule weight per plant, and there was no correlation of NR activity in bacteroids with acetylene reduction activity of nodulated roots. The mean acetylene reduction activity of nodules formed by the strains shown in Table 1 was 12.0 μmol/g of fresh weight per h.

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