Simultaneous Assimilation and Denitrification of Nitrate by *Bradyrhizobium japonicum*

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(Received 8 August 1988; accepted 25 August 1988)

Bradyrhizobium japonicum strain CB 1809 assimilated and denitrified $^{15}\text{NO}_3^-$ simultaneously to $^{15}\text{NH}_4^+$ and $^{15}\text{N}_2$, respectively, when incubated under anaerobiosis. Washed cells from cultures grown in air in the presence of 5 mM-nitrate, assimilated 70% of the $^{15}\text{NO}_3^-$ into cell-nitrogen but only after a lag of 10 h during which time NO_2^- accumulated. In washed cells grown at 2% (v/v) O_2 with 5 mM-nitrate, $^{15}\text{NO}_3^-$ was denitrified to $^{15}\text{N}_2$ (95%) in 1–2 h. The low O_2 tension in the culture medium resulted in increased activity of nitrate and nitrite reductases in the cells. When 10 mM-glutamate was included with 5 mM-nitrate at 2% (v/v) O_2 , washed cells from these cultures had less nitrate and nitrite reductase activities and when $^{15}\text{NO}_3^-$ was supplied to these cells there was a transient accumulation of NO_2^- (1 h) prior to maximum denitrification. Washed cells prepared from cultures grown at 2% (v/v) O_2 with glutamate alone utilized $^{15}\text{NO}_3^-$, only after a lag period of 20–40 h, assimilation by (60%) and denitrification (40%).

INTRODUCTION

The metabolism of nitrate may occur by two pathways: assimilation via ammonium into cell-nitrogen or denitrification of N_2O and N_2 . In both cases nitrite is an intermediate but the interrelationship between the pathways has not been studied. Many strains of rhizobia utilize nitrate for growth (Bergersen, 1977) and the slow-growing strains (bradyrhizobia) can also denitrify nitrate (Zablotowicz *et al.*, 1978; Daniel *et al.*, 1982; O'Hara & Daniel, 1985).

In *Bradyrhizobium japonicum*, denitrification is derepressed by anaerobic conditions (O'Hara *et al.*, 1983), as in other denitrifiers (Knowles, 1982). In some micro-organisms nitrate is required for the production of dissimilatory nitrate reductase (NR) (Kapralék *et al.*, 1982), but not in *B. japonicum* (O'Hara *et al.*, 1983).

The aim of this study was to determine whether assimilation and denitrification of ^{15}N nitrate in washed cells of *B. japonicum* strain CB 1809 occurred concurrently, and to consider the influence of O_2 tension and nitrogen supply in the culture medium on the operation of these pathways. Strain CB 1809 is a strain used commercially in Australia for the inoculation of soybean.

METHODS

Bacteria and growth conditions. *Bradyrhizobium japonicum* strain CB 1809 was supplied by Dr A. H. Gibson from the CSIRO Division of Plant Industry, Canberra, ACT, Australia. Cells were cultured at 28 °C in defined liquid medium (1 l in 2 l Erlenmeyer flasks) containing mineral salts and 15 mM-glucose (Dalton, 1980). The nitrogen source was either KNO_3 or glutamate at the concentrations indicated in the text. Aerobically grown cells were shaken on a rotary reciprocator while cells grown under an atmosphere of 2% O_2 /98% N_2 (v/v) were flushed continuously through glass sintered discs with this gas mixture. The final concentration of each gas in the mixture

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Abbreviations: NR, nitrate reductase; NiR, nitrite reductase.

was monitored by measuring individual flow rates through two rotameters. Cells were harvested at the late exponential phase of growth in a Sorvall RC-2B centrifuge, fitted with a GSA rotor (Sorvall), at 100 *g* for 10 min (4 °C) followed by centrifugation at 10000 *g* for 10 min (4 °C). They were washed with 100 mM-potassium phosphate buffer (pH 7.5) and resuspended in the same buffer. Anaerobic conditions were maintained by sparging the buffer with Ar and centrifuging under Ar (99.9% pure; Commonwealth Industrial Gases, Cowandilla, S. Australia).

¹⁵N studies. Suspensions of cultured cells were incubated, for the time period indicated, in 3.5 ml sealed tubes fitted with suba-seals at 25 °C in an assay mixture (2 ml) which contained 100 mmol potassium phosphate buffer (pH 7.5), 4 μmol K¹⁵NO₃ (97 atom % excess), 40 μmol sodium succinate and 2 mg cell protein. The assay mixture (minus KNO₃) was degassed with a water pump for 2–3 min and then flushed with Ar for 10 min. The assay was started by the addition of the K¹⁵NO₃ solution which had also been degassed and flushed with Ar. Samples (20–50 μl) were removed with a microsyringe at regular intervals for the determination of nitrate and nitrite. Gas samples (100 μl) were analysed for ¹⁵N₂O and ¹⁵N₂ in a GC/mass spectrometer (model 592B; Hewlett-Packard) fitted with a glass column (183 cm length, 6 mm outer diameter, 2 mm inner diameter) packed with Porapak Q (80–100 mesh). Helium flow through the column was 5 ml min⁻¹ at 25 °C and the electron multiplier voltage varied between 1400 and 2000 V. The data was assembled by means of a selective Ion Monitor programme (Hewlett-Packard) which allowed the simultaneous monitoring of six specified atomic-mass-unit values (28, 29, 30, 40, 44, 46). Ar was used as an internal standard to allow comparison between injections when sample size may have varied. ¹⁵N₂O was calibrated against a standard curve of N₂O with the solubility of N₂O in liquid taken into account (Byrne & Nicholas, 1986). ¹⁵N₂ was calibrated against a standard curve of a gas mixture of ¹⁵N₂/¹⁴N₂, prepared by adding hypobromite solution to (¹⁵NH₄)₂SO₄ (37.5 atom % excess). ¹⁵NH₄⁺ was determined by first liberating ¹⁵NH₃ from the assay mixture (Conway, 1962) and then measuring ¹⁵N enrichment of samples in an Isomass 602E mass spectrometer (Middlewich, Cheshire, UK), according to the procedure of Brownell & Nicholas (1967). ¹⁵N incorporated into cell-nitrogen was also determined by mass spectrometry after digestion and distillation of samples (Markham, 1942).

Nitrate reductase (NR) and nitrite reductase (NiR) activities. These were determined for washed cells using the same assay mixture as for ¹⁵N studies, except that unlabelled KNO₃ (2 μmol ml⁻¹; NR) or NaNO₂ (2 μmol ml⁻¹; NiR) were used. Samples (20–50 ml) were removed after appropriate intervals (5–60 min) for nitrite determination. Sodium diethyl dithiocarbamate (1 mM) was included in the assay mixture to inhibit NiR when determining NR activities.

Nitrate and nitrite determination. Nitrate was determined by nitration of salicylic acid (Cataldo *et al.*, 1975) and nitrite by the method of Nicholas & Nason (1957).

Protein determination. Protein in washed cells was determined by the microbiuret method (Itzhaki & Gill, 1964) using bovine serum albumin (BSA) as a standard protein.

RESULTS

Washed cells of *B. japonicum* strain CB 1809 grown at 20% (v/v) O₂ with 5 mM-nitrate were supplied with ¹⁵NO₃ under anaerobic conditions. About 70% of the ¹⁵NO₃ was reduced to ¹⁵NH₄⁺ over a 20 h period while the remainder was denitrified to ¹⁵N₂ (Fig. 1*a*). Under the same conditions, cells which had been grown under 2% (v/v) O₂ denitrified 95% of the ¹⁵NO₃ (Fig. 1*b*); the remainder was reduced to ¹⁵NH₄⁺. In this case the reduction of ¹⁵NO₃ to ¹⁵N₂ and ¹⁵NH₄⁺ was complete within 2 h. Washed cells which had been cultured under 2% (v/v) O₂ exhibited a more rapid utilization of ¹⁵NO₃ under anaerobic conditions than those grown in air (Fig. 1*a, b*). In contrast to a transient accumulation (<1 h) of a low level of NO₂⁻ by cells grown at 2% (v/v) O₂ (Fig. 1*b*), the aerobically grown cells exhibited a prolonged accumulation (10 h) of a higher concentration of NO₂⁻ under anaerobiosis (Fig. 1*a*) and then it was utilized rapidly.

These observations on ¹⁵NO₃ utilization are correlated with the activities of NR and NiR (Table 1). Cells grown at 2% (v/v) O₂ had higher NR and NiR activities than those from cultures grown in air. A much larger increase in NiR activity was observed.

Cells which had been cultured at 2% (v/v) O₂ with glutamate and nitrate showed a more pronounced accumulation of NO₂⁻, when incubated anaerobically with ¹⁵NO₃, than those grown with nitrate alone (Fig. 1*b, c*). The former had a lower NiR activity (Table 1). In the nitrate plus glutamate grown cells, denitrification to ¹⁵N₂ in washed cells increased markedly after a lag period of 1 h reaching a final value of 80% of the ¹⁵NO₃ supplied (Fig. 1*c*) compared to 95% for nitrate grown cells (Fig. 1*b*).

Fig. 1. Assimilation and denitrification of $^{15}\text{NO}_3^-$ in washed cells grown with nitrate and glutamate. Cells were grown in air with 5 mM- KNO_3 (a), and in 2% $\text{O}_2/98\%$ (v/v) N_2 with 5 mM- KNO_3 (b), 5 mM- KNO_3 plus 10 mM-glutamate (c) and 10 mM-glutamate (d). Washed cells were incubated under anaerobic conditions with $^{15}\text{NO}_3^-$ and assayed for the products of nitrate reduction as described in Methods. $\square$, NO_3^- ; $\bullet$, NO_2^- ; $\blacktriangle$, $^{15}\text{NH}_4^+$; $\circ$, $^{15}\text{N}_2\text{O}$; $\triangle$, $^{15}\text{N}_2$.

Table 1. *Influence of O₂ tension and nitrogen source on NR and NiR activities*

Cells were grown both aerobically and under 2% (v/v) O₂/98% (v/v) N₂ in defined medium containing 5 mM-KNO₃ and 10 mM-glutamate as indicated. Activities are expressed in μmol nitrite produced (NR) or utilized (NiR) h⁻¹ (mg protein)⁻¹.

O ₂ tension (% v/v) during growth	Nitrogen source for growth (mM)		NR activity	NiR activity
	Nitrate	Glutamate		
20	5	0	6.90	0.24
2	5	0	14.40	4.20
2	5	10	14.70	1.40
2	0	10	2.50	0.12

Cells grown with glutamate at 2% (v/v) O₂ utilized ¹⁵NO₃⁻, under anaerobic conditions, at a considerably slower rate than those grown on nitrate at the same O₂ tension (Fig. 1*d*). There was also a pronounced and prolonged accumulation of NO₂⁻; ¹⁵NH₄⁺ and ¹⁵N₂, were only detected in washed cells after a 20–40 h incubation with ¹⁵NO₃⁻. Only 40% of the ¹⁵NO₃⁻ supplied was denitrified and the remainder reduced to ¹⁵NH₄⁺. The glutamate grown cells had relatively low activities of NR and NiR compared to those cultured with nitrate (Table 1).

DISCUSSION

Anaerobic growth supported by denitrification has been demonstrated in several strains of bradyrhizobia (Van Berkum & Keyser, 1985), but hitherto it has not been established in rhizobia or other bacteria whether denitrification and assimilation of nitrate into cell-nitrogen can occur concurrently. In this study on *B. japonicum* the simultaneous reduction of ¹⁵NO₃⁻ to ¹⁵NH₄⁺ and denitrification of ¹⁵NO₃⁻ to ¹⁵N₂ is demonstrated.

The ratio of denitrification to assimilation was influenced by the conditions under which the *B. japonicum* cells were grown. Even though the anaerobic conditions used to follow the utilization of ¹⁵NO₃⁻ would have favoured the production of the denitrifying enzymes (O'Hara *et al.*, 1983), cells grown in air still assimilated 70% of the ¹⁵NO₃⁻ supplied to the washed cells. When cells were cultured under 2% (v/v) O₂ about 95% of the ¹⁵NO₃⁻ was denitrified by washed cells. An increase in the capacity of cells to denitrify nitrate at low O₂ tensions is in accord with the results of O'Hara *et al.* (1983) who demonstrated an inhibition of denitrification by O₂ in *B. japonicum*.

The greater capacity of the cells to denitrify when grown under 2% (v/v) O₂ correlates well with the increased activities of NR and in particular NiR. In many denitrifying bacteria, NiR is more sensitive to O₂ inhibition than NR, especially when the NiR has a cytochrome oxidase activity associated with a cytochrome *d* component (Lam & Nicholas, 1969; Krul & Veeningen, 1977; Meiberg *et al.*, 1980). There is good evidence that the nitrogen oxide reductases in denitrifying bacteria are derepressed at low O₂ tensions (Knowles, 1982) and that there are separate assimilatory and dissimilatory NR and NiR enzymes (Payne 1981).

It is not clear whether decreased O₂ tension alone fully derepresses denitrification or whether nitrate is also involved. O'Hara *et al.* (1983) claimed that the synthesis of the denitrifying enzymes in bradyrhizobia was independent of nitrate. However, based on results of the present study it is clear that the high rate of denitrification in cells of *B. japonicum* grown at 2% (v/v) O₂ is dependent on the presence of nitrate. Cells grown with glutamate alone utilized nitrate and especially nitrite at much slower rates and nitrate was assimilated to ammonium to a greater extent than its denitrification to N₂ gas.

The award of a postgraduate scholarship from the Wheat Industry Research Council of Australia (FV) is gratefully acknowledged and the authors thank David Hein, Mike Byrne and Richard Batt for their technical assistance.

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