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Anaerobic Growth, Nitrate Reduction and Denitrification in 46 Rhizobium Strains

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A total of 46 rhizobial strains were assessed for anaerobic growth in the presence of nitrate, and, using the criteria of nitrate utilization and nitrous oxide and nitrogen production, for their ability to denitrify. Nitrite production was also measured. Half of the strains were denitrifiers: these included all five strains of *R. meliloti* tested which produced N_2 from nitrate and most of the slow-growing rhizobia, but none of the 14 strains of *R. trifolii*.

INTRODUCTION

Most research on rhizobia has as its aim the better understanding of the symbiotic nitrogen fixation carried out by the rhizobium–legume symbiosis, and the agricultural benefits of this process. Although rhizobial nitrate reduction and denitrification have for some time been known to occur (e.g. Rajagopalan, 1938; Ishizawa, 1939; Evans, 1954) relatively little research has been carried out until recently. This is surprising, since strains possessing this ability (e.g. Zablotowicz *et al.*, 1978; Daniel *et al.*, 1980*b*) are clearly potentially capable of removing fixed nitrogen from the soil. More recently, this removal of fixed nitrogen from soil by rhizobial denitrification has been demonstrated (Daniel *et al.*, 1980*a*). The net rhizobial contribution to soil fixed nitrogen may therefore depend as much upon denitrification ability as upon nitrogen fixation ability. The agricultural significance of denitrification is not yet clear. Attempts to correlate nitrate reduction with symbiotic efficiency have given conflicting results (e.g. Antoun *et al.*, 1980; Vasconcelos *et al.*, 1980). The agricultural significance may well depend upon whether the enhanced rhizobial survival which may be conferred by denitrification ability under anaerobic conditions (Zablotowicz & Focht, 1979) outweighs the disadvantages of the nitrogen loss from soils shown to occur by Daniel *et al.* (1980*a*). The ability to grow anaerobically (Daniel & Appleby, 1972; Daniel *et al.*, 1980*b*), as opposed merely to survive, may also be a factor although it is not known whether this is well correlated with denitrification ability.

Zablotowicz *et al.* (1978) surveyed 33 rhizobia, mostly *Rhizobium japonicum* and cowpea strains, for denitrification and found considerable variation between strains. This paper extends this survey, with some emphasis upon the *R. lupini* and *R. trifolii* strains which are important in New Zealand agriculture. In addition, we have attempted to correlate denitrification with rhizobial strain and with anaerobic growth.

METHODS

Bacterial strains and growth. Rhizobium japonicum 505 (Wisconsin) was obtained as CC 705 from the Division of Plant Industry, CSIRO, Canberra, Australia; PDD culture numbers 4771, 2751, 2752, 2153, 2163, 2666, 2668, 2121, 4144, 5938, and CC 814S and NZP 5301 were received from Dr P. Bonish, Ruakura Agricultural Research Centre, Hamilton, New Zealand; PDD culture numbers 4683, 1326, 3153, and NZP

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Table 1. *Anaerobic growth and end products of nitrate reduction of 46 rhizobium strains*

After addition of a 5% inoculum, fast-growing rhizobia were incubated for 14 d and slow strains for 21 d, before assay. Tubes initially contained 60 μmol KNO_3.

Rhizobium species and strain no.	Host	Growth speed*	Anaerobic growth†	NO_3^- removal‡	NO_2^- production§	N_2O production‖	N_2 production	Major end product
R. japonicum								
CC 705 (Wisconsin 505)	*Glycine max*	S	√√	++	–	++	Tr	N_2O
PDD 2864 (ATCC 10324, B. E. Caldwell 311B6)	*Glycine max*	S	√√	++	–	++	–	N_2O
PDD 1312 (L. W. Erdman 311 B46)	*Glycine max*	S	–	–	+	Tr	Tr	NO_2
PDD 2094 (L. Diatloff CB 1809)	*Glycine max*	S	√√	++	–	–	+	N_2
R. lupini								
PDD 4681 (CC 814S)	*Lotus* sp.	S	√√	++	–	++	–	N_2O
PDD 4683 (NZP 2257)	*Lotus* sp.	S	√√	++	–	++	–	N_2O
PDD 3905 (R. M. Greenwood, 5024, AIRCS WU425)	Lupin, *Ornithopus*	S	?	–	?	–	ND	?
PDD 3152 (NZP 5042)	*Ulex europaeus*	S	–	?	–	Tr	Tr	N_2
PDD 4771 (NZP 2076)	*L. hispidus*, lupin, *Ornithopus*	F/S	√√	+	–	–	+	N_2
PDD 4682 (NZP 2213)	*L. tenuis*	F	?	+	–	–	?	?
PDD 1314 (A. Hastings AH 17)	*Ornithopus sativus*	F	–	?	–	Tr	–	N_2O
PDD 3153 (NZP 2238)	*L. corniculatus*	F	–	?	–	Tr	–	N_2O
PDD 3663 (NZP 2196, AIRCS SU434)	*L. corniculatus*, lupin	F	√	+	?	–	?	?
PDD 1326 (NZP 2037)	*L. divaricatus*	F	?	–	–	–	–	–
NZP 2048	*L. pedunculatus*	F	–	–	–	–	–	–
R. meliloti								
PDD 4384 (CC 2165)	*Medicago tornata*	F	?	+	–	Tr	+	N_2
PDD 2752 (D. O. Norris SU47)	*M. sativa*	F	?	+	–	+	+	N_2
PDD 1666 (A. Hastings AH 31)	*M. sativa*	F	–	+	?	–	+	N_2
PDD 1300 (J. M. Vincent U45)	*M. sativa*	F	?	+	+	–	+	N_2
PDD 2751	*M. sativa*	F	–	+	–	–	+	N_2
R. phaseoli								
PDD 3305 (CC 511)	*Phaseolus* sp.	S	?	–	?	–	–	–
PDD 2672	*P. vulgaris*	ND	?	–	–	–	ND	–
PDD 2781	*P. coccineus*	ND	–	–	–	–	–	–
R. leguminosarum								
PDD 1298 (J. M. Vincent SU 364)	*Pisum sativum*	F	–	?	–	Tr	–	N_2O
PDD 1299 (J. M. Vincent SU 391)	*P. sativum*, *Vicia sativa*	F	√	–	+	Tr	–	NO_2
R. trifolii								
PDD 2940 (ATCC 10328, D. Weber 3D1K5)	*Trifolium pratense*	F	?	–	?	–	–	–
NZP 550	*T. vesiculosum*	F	–	–	?	–	–	–
NZP 514	*T. subterraneum*	F	–	–	–	–	–	–
PDD 5938 (NZP 545, C. A. Parker WU 95)	*T. subterraneum*	F	–	–	–	–	–	–
PDD 2121 (TAI via N. Gibbins)	*T. subterraneum*, *Trifolium* sp.	F	–	–	?	–	–	–
PDD 4144 (TAI via R. Roughly)	*T. subterraneum*	F	–	–	–	–	–	–
PDD 2153 (A. Hastings TARA-H)	*T. repens*	F	–	–	?	?	–	?
PDD 2163 (CC 275E)	*T. repens*	F	–	–	–	–	ND	–
PDD 2666 (A. Hastings T280)	*T. repens*	F	–	–	–	–	–	–
PDD 2668 (A. Hastings T282)	*T. repens*	F	–	–	–	–	–	–
NZP 564	*T. repens*	F	–	–	?	–	–	–
NZP 565	*T. repens*	F	–	–	?	–	–	–
NZP 566	*T. repens*	F	–	–	–	–	–	–
NZP 567	*T. repens*	F	–	–	–	–	–	–

Rhizobium species and strain no.	Host	Growth speed*	Anaerobic growth†	NO_3^- removal‡	NO_2^- production§	N_2O production‖	N_2 production	Major end product
Rhizobium sp.								
PDD 1292 (J. M. Vincent CB 756)	*Vigna* sp.	S	✓	++	–	++	–	N_2O
PDD 2095 (L. Diatloff CB 756)	Tropical legumes	S	✓	++	–	++	–	N_2O
PDD 1310 (ATCC 10316, L. W. Erdman 3CIAI)	*Crotalaria sagittalis*	S	–	–	+	Tr	Tr	NO_2
PDD 1317 (ATCC 10317, L. W. Erdman 312BI)	*Erythrina variegata*	F	?	+	–	+	–	N_2O
PDD 4764 (CC401, R. Close NA 725-1)	*Coronilla*	F	–	–	–	–	–	–
PDD 1304 (J. M. Vincent QA 638)	*Muncuna deeringianum*, *Vigna* sp.	F	–	?	–	–	–	–
NZP 5301	Sainfoin	F	–	–	–	–	–	–

ND, Not determined; –, absent; Tr, trace only; ?, small and variable between runs and/or duplicates.
* S. slow: F. fast.
† ✓✓, as for CC 705 (Daniel *et al.*, 1980*b*); ✓, poorer than CC 705.
‡ +, <30 μmol NO_3^- removed; ++, >30 μmol NO_3^- removed.
§ +, <10 μmol NO_2^- produced; ++, >10 μmol NO_2^- produced.
‖ +, <30 μmol N_2O produced; ++, >30 μmol N_2O produced; Tr, <3 μmol N_2O produced.

culture numbers 2048, 2213, 514, 550, 564, 565, 566, 567 were received from Dr B. Scott, Division of Applied Biochemistry, DSIR, Palmerston North, New Zealand. All other cultures were received from Drs A. Hastings and D. Dye of Plant Diseases Division, DSIR, Mount Albert, Auckland, New Zealand.

Rhizobia were maintained on yeast extract/mannitol (Daniel & Appleby, 1972) agar slopes. Inocula from cultures grown aerobically in the same yeast extract/mannitol medium in a gyrotary shaker at 26 °C were transferred to 150 mm × 12 mm test tubes containing 9·5 ml yeast extract/mannitol medium supplemented with separately sterilized 6 mM-KNO_3. Sterile liquid paraffin (0·5 ml) was layered over the liquid and the 1·5 ml headspace closed with a sterile rubber seal. All strains were grown in this way on four separate occasions and on one of these in duplicate. On two of these occasions duplicate cultures were grown in medium lacking 6 mM-KNO_3; at 3 d intervals, anaerobic growth was assessed turbidometrically at 680 nm by comparison between cultures grown with and without nitrate.

Flushing the headspace with N_2 had no significant effect on any of the parameters examined.

The relative growth rate of different strains was assessed by visual estimation of colony size on agar plates over a 9 d period. Rhizobia from agar slopes were streaked on yeast extract/mannitol agar both with and without 6 mM-KNO_3, and after appreciable growth had occurred a single colony was re-streaked on solid media, and colony size was again estimated after 9 d. Each strain examined gave a similar growth rate on all four plates (with and without 6 mM-KNO_3, first streak and single colony re-streak).

Determination of nitrate, nitrite, nitrous oxide and ammonium. Ammonium and nitrate plus nitrite were determined by autoanalyser (Brown, 1973; Kamphake *et al.*, 1967). This method, including the correction for nitrite when necessary, and the dilution step necessary when little nitrate was utilized, may not be sufficiently accurate to detect reproducibly utilization of less than 5% of the total nitrate present.

Nitrite concentration was estimated approximately using the Merckoquant nitrite test strips (Merck) and comparing the colour with standards. Where necessary, samples were diluted to bring the nitrite concentration into the range 0·1–0·5 mM. This method is sufficiently sensitive to detect the presence of 0·5 μmol nitrite in our system (i.e. about 50 μM-nitrite).

N_2O in the culture headspace was determined by gas chromatography as described previously (Daniel *et al.*, 1980*b*). This method is very sensitive and will reproducibly detect the production of less than 50 nmol N_2O.

$^{15}N_2$ production. Cells were grown in duplicate in test tubes as described above but with 6 mM-$K^{15}NO_3$ instead of 6 mM-KNO_3. The $^{15}N_2$ content of the headspace gas was determined on a Micromass 602C mass spectrometer equipped with a twin inlet system and dual collector plates. A working standard of atmospheric N_2 was used.

RESULTS AND DISCUSSION

As judged by the data for nitrate removal (Table 1), 7 of the 46 rhizobium strains tested were active denitrifiers, which converted more than half of the supplied nitrate to N_2O or N_2; the end product in 6 of these strains was N_2O. All of these 7 were slow-growing. Another 9 strains were moderately active denitrifiers, but most of these were fast growers yielding N_2 as the end product. Judged by the more sensitive criteria of N_2O production, or $^{15}N_2$ production from $K^{15}NO_3$, an additional 7 strains catalysed denitrification at low rates. Thus a total of 23 of the 46 strains tested were capable of some denitrification. Zablotowicz *et al.* (1978) found that 7 of the 33 organisms they tested were able to reduce nitrate but were not denitrifiers. We have not found this to be the case for any of the strains we tested, although a few did accumulate nitrite, suggesting a relatively low nitrite reductase activity.

Although many rhizobial strains will nodulate more than one type of host plant, the taxonomy of rhizobial strains is still based on the ability to nodulate a particular plant species, and is of uncertain validity (Buchanan & Gibbons, 1974; Vincent *et al.*, 1979). Thus any attempt to correlate a physiological property such as denitrification with rhizobial species is difficult. With that proviso, the most significant species correlation which can be drawn from the data in Table 1 is the lack of denitrification ability among any of the *R. trifolii* strains tested. None of the three *R. trifolii* strains tested by Zablotowicz *et al.* (1978) were capable of denitrification. Although the ability is also absent from all the *R. phaseoli* strains and present at low rates among all *R. leguminosarum* strains tested, the number of strains involved is small. However, these three species have been proposed on the basis of DNA homology (Jarvis *et al.*, 1980) and other criteria (e.g. Roberts *et al.*, 1980; Vincent *et al.*, 1979) to be more closely related to one another than to the fourth species of fast-growing rhizobia, *R. meliloti*. It is therefore interesting that among the former three species there are no good or moderately good denitrifiers, whereas all the *R. meliloti* tested denitrify moderately well and all produce the same end product, N_2. Among *R. lupini* and *Rhizobium* sp. there is a tendency for the slow-growing strains to be the more vigorous denitrifiers, and for denitrification ability to be completely absent only from fast growers.

Although the number of strains tested here is small, if further work confirms that *R. meliloti* are the only fast-growing rhizobia which reduce nitrate rapidly to N_2 and that no *R. trifolii* are capable of any denitrification, these could be taxonomically useful characteristics.

One of the currently most reliable taxonomic characteristics among rhizobia is growth speed. Overall, of the 21 strains which definitely do not denitrify, all but one are fast growers (although the converse is not the case). The exception, *R. phaseoli* PDD3305, would normally be regarded as a fast grower, and its slow growth in our hands may have been a response to conditions rather than an intrinsic property. Apart from this strain, and *R. lupini* PDD3905 which responded inconsistently, all the slow-growing strains are capable of some denitrification. Although Zablotowicz *et al.* (1978) also found, by the criteria of nitrate removal and gas production in Durham tubes, that none of their 6 strains of fast-growing rhizobia would denitrify, nor would 5 of their 16 *R. japonicum* strains. However, as seen from Table 1, these techniques may not be sufficiently sensitive to detect low denitrification activity.

Earlier work (Daniel *et al.*, 1980*b*) suggests that the simple turbidometric method of assessing anaerobic growth used here may not give a positive result with rhizobia which display poor anaerobic growth. Bearing this in mind there is a reasonable correlation between the ability to grow anaerobically and the capacity for denitrification. No strains grew anaerobically in the absence of nitrate. Although it is difficult to be sure that the strains tested here are representative of all rhizobia, we believe these results indicate that a substantial proportion of slow-growing rhizobia are capable of anaerobic growth in the presence of nitrate, and most, if not all, are capable of some denitrification; and that denitrification is rare among fast-growing rhizobia other than *R. meliloti*.

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REFERENCES

ANTOUN, H., BORDELEAU, L. M., PREVOST, D. & LACHANCE, R. A. (1980). Absence of a correlation between nitrate reductase and symbiotic nitrogen fixation efficiency in *Rhizobium meliloti*. *Canadian Journal of Plant Science* **60**, 209–212.

BROWN, M. W. (1973). A highly sensitive automated technique for the determination of ammonia nitrogen. *Journal of the Science of Food and Agriculture* **24**, 1119–1123.

BUCHANAN, R. E. & GIBBONS, N. E. (editors) (1974). *Bergey's Manual of Determinative Bacteriology*, 8th edn. Baltimore: Williams & Wilkins.

DANIEL, R. M. & APPLEBY, C. A. (1972). Anaerobic-nitrate, symbiotic, and aerobic growth of *Rhizobium japonicum*. Effects on cytochrome P-450, other haemoproteins, nitrate and nitrite reductases. *Biochimica et biophysica acta* **275**, 347–354.

DANIEL, R. M., STEELE, K. W. & LIMMER, A. W. (1980*a*). Denitrification by rhizobia. A possible factor contributing to nitrogen losses from soils. *New Zealand Agricultural Science* **14**, 109–112.

DANIEL, R. M., SMITH, I. M., PHILLIP, J. A. D., RATCLIFFE, H. D., DROZD, J. W. & BULL, A. T. (1980*b*). Anaerobic growth and denitrification by *Rhizobium japonicum* and other rhizobia. *Journal of General Microbiology* **120**, 517–521.

EVANS, H. J. (1954). Diphosphopyridine nucleotide-nitrate reductase from soybean nodules. *Plant Physiology* **29**, 298–301.

ISHIZAWA, S. (1939). On the consumption of inorganic nitrogen by root nodule bacteria of leguminous plants. *Journal of Soil Science and Manure, Japan* **13**, 135–143.

JARVIS, B. D., DICK, A. G. & GREENWOOD, R. M. (1980). DNA homology among strains of *Rhizobium trifolii* and related species. *International Journal of Systematic Bacteriology* **30**, 42–57.

KAMPHAKE, L. J., HANNAL, S. A. & COHEN, J. M. (1967). Automated analyses for nitrate by hydrazine reduction. *Water Research* **1**, 205–216.

RAJAGOPALAN, T. (1938). Studies on ground nut nodule organism. IV. Physiology of the organism: intermediary metabolism. *Indian Journal of Agricultural Science* **8**, 379–402.

ROBERTS, G. P., LEPS, W. T., SILVER, L. E. & BRILL, W. J. (1980). Use of two-dimensional polyacrylamide gel electrophoresis to identify and classify rhizobium strains. *Applied and Environmental Microbiology* **39**, 414–422.

VASCONCELOS, L. DE, MILLER, L. & NEYRA, C. A. (1980). Free-living and symbiotic characteristics of chlorate-resistant mutants of *Rhizobium japonicum*. *Canadian Journal of Microbiology* **26**, 338–342.

VINCENT, J. M., NUTMAN, P. S. & SKINNER, F. A. (1979). The identification and classification of *Rhizobium*. In *Identification Methods for Microbiologists*, 2nd edn, pp. 49–69. Edited by F. A. Skinner & D. W. Lovelock. London: Academic Press.

ZABLOTOWICZ, R. M., ESKEW, D. L. & FOCHT, D. D. (1978). Denitrification in Rhizobium. *Canadian Journal of Microbiology* **24**, 757–760.

ZABLOTOWICZ, R. M. & FOCHT, D. D. (1979). Denitrification and anaerobic, nitrate-dependent acetylene reduction in cowpea rhizobium. *Journal of General Microbiology* **111**, 445–448.