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New Method of Denitrification Analysis of *Bradyrhizobium* Field Isolates by Gas Chromatographic Determination of ^{15}N-Labeled N_2

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To evaluate the denitrification abilities of many *Bradyrhizobium* field isolates, we developed a new ^{15}N-labeled N_2 detection methodology, which is free from interference from atmospheric N_2 contamination. $^{30}N_2$ ($^{15}N^{15}N$) and $^{29}N_2$ ($^{15}N^{14}N$) were detected as an apparent peak by a gas chromatograph equipped with a thermal conductivity detector with N_2 gas having natural abundance of ^{15}N (0.366 atom%) as a carrier gas. The detection limit was 0.04% $^{30}N_2$, and the linearity extended at least to 40% $^{30}N_2$. When *Bradyrhizobium japonicum* USDA110 was grown in cultures anaerobically with $^{15}NO_3^-$, denitrification product ($^{30}N_2$) was detected stoichiometrically. A total of 65 isolates of soybean bradyrhizobia from two field sites in Japan were assayed by this method. The denitrification abilities were partly correlated with filed sites, *Bradyrhizobium* species, and the *hup* genotype.

Denitrification is anaerobic respiration using nitrate (NO_3^-), nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O) as terminal electron acceptors. Denitrifying species are distributed over a broad variety of bacteria, including *Proteobacteria* spp., gram-positive bacteria, and archaea (31). Most denitrifying bacteria reduce the electron acceptors sequentially as $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$, and each step of these reactions is catalyzed by specific respective reductases (31). To evaluate denitrification capability, the acetylene inhibition assay (30) has been widely used in pure cultures (13) as well as for environmental samples such as soils (8, 14, 27) and water (24) because of sensitivity and atmospheric N_2 contamination (13). In this assay, the excess of N_2O emission in the presence of acetylene over the level of N_2O emission in the absence of acetylene is regarded as the N_2 evolution (because acetylene inhibits N_2O reductase). N_2O can be detected by a gas chromatograph (GC) equipped with a ^{63}Ni electron-capture detector possessing high-level sensitivity for N_2O. The amount of N_2O is calculated on the basis of the measured headspace concentration and corrected for dissolved gas by using the Bunsen coefficient (29).

However, the acetylene inhibition assay has some drawbacks for evaluating bacterial denitrification. The most serious disadvantage is that it is impossible to measure N_2O reductase activity directly. For this reason, the activities and kinetic parameters of N_2O reductase have been studied directly by measurement of the N_2O decrease (4) or by spectrophotometric assay of reduced benzyl viologen for nitrous oxide reductase activity in vitro (12, 23). Moreover, the underestimation of denitrification capability may occur because of incomplete blockage of N_2O reductase at a low nitrate concentration or in the presence of sulfide (24). In practice, two determinations of N_2O concentration per sample—in the presence and absence of acetylene—are required for estimation of the amount of N_2 evolved by N_2O reductase.

Acetylene inhibition assays have revealed the diversity of the denitrification activities of *Bradyrhizobium* species (1, 2, 28). *Bradyrhizobium japonicum* USDA110, which possesses a full set of denitrification genes on the chromosome (11), consistently shows denitrification from nitrate to N_2 (2, 28). However, soybean bradyrhizobia often show diverse denitrification patterns. Their denitrification end products are N_2O and NO_2^- as well as N_2 when nitrate is supplied to media under anaerobic conditions, while a few strains are unable to denitrify at all (1, 2, 28). This polymorphism is probably due to truncation of the processes of anaerobic nitrate respiration (31). It has been shown that indigenous populations of soybean bradyrhizobia show significant field site variation in terms of DNA fingerprints according to insertion sequences, species level, and hydrogen uptake phenotype and genotype (7, 16, 17, 21). Similarly, we wanted to determine whether the denitrification patterns of indigenous populations of soybean bradyrhizobia differed in accordance with their field sites.

We therefore developed a new methodology for determination of $^{30}N_2$ levels by using a GC equipped with a thermal conductivity detector (TCD). This method, which we refer to as the ^{15}N/TCD method, enables ^{15}N tracer analysis without the need for isotope ratio determination by mass spectrometry and will overcome some of the problems of the acetylene inhibition method when large numbers of field isolates are analyzed.

MATERIALS AND METHODS

Bacterial strains. Strain USDA110 was used as a standard strain of *B. japonicum* (11). A total of 65 field isolates of *Bradyrhizobium* were isolated from the soils of the Nakazawa fields at the Niigata Agricultural Experiment Station (Nagaoka, Niigata, Japan) and the Tokachi field at the Tokachi Agricultural Station (Memuro, Tokachi, Hokkaido, Japan) as described previously (9, 15, 18).

Standard gases. A gas mixture composed of 40% (vol/vol) $^{30}N_2$ ($^{15}N^{15}N$) (^{15}N, 99.7 atom%), 40% (vol/vol) Ar, and 20% (vol/vol) O_2 (Shoko Co., Ltd., Tokyo,

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Japan) was used as a $^{30}N_2$ original standard gas. A standard gas of $^{29}N_2$ ($^{15}N^{14}N$) was prepared by using codenitrification of *Fusarium solani* IFO9425 anaerobically grown in cultures under He with $Na^{15}NO_2$ according to the method of Shoun et al. (22) and Tanimoto et al. (26). The amount of $^{29}N_2$ was determined by using a GC-mass spectrometer (GC-MS QP5050; Shimadzu, Kyoto, Japan).

Growth media and growth conditions. *Bradyrhizobium* cells were aerobically grown to full growth at 30°C in 15-ml HM salts medium (5) supplemented with 0.1% arabinose and 0.025% (wt/vol) yeast extract (Difco, Detroit, Mich.). For anaerobic growth for denitrification, HM medium was further supplemented with 0.55 μM $Na_2MoO_4 2H_2O$, 1 μM $FeCl_2$, and 1 μM $CuSO_4 2H_2O$, which were required for nitrate, nitrite, and nitrous oxide reductase activities, respectively (31). Filter-sterilized $K^{15}NO_3$ (^{15}N, 99.6 atom%) (Shoko Co., Ltd.) or $K^{14}NO_3$ was added to the medium at a final concentration of 2 mM.

We used two bottle systems for denitrification experiments: 70 ml of medium in a 123-ml airtight specimen vial with a butyl rubber stopper and an aluminum seal (Maruemu, Osaka, Japan), and 15 ml of medium in a 27-ml test tube with a double butyl rubber stopper. Full-grown cells of bradyrhizobia were inoculated to the media to achieve an initial optical density of 0.05 at 660 nm. Just after the vials and test tubes were sealed, the overhead space gas was replaced several times with N_2 or He in a vacuum line. Gas samples were collected 10 days after incubation at 30°C. For the acetylene inhibition assay, $^{14}NO_3^-$ was used as a substrate of denitrification in the presence and absence of 10% (vol/vol) acetylene.

Gas chromatography. For $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ analyses, gas samples (1 ml or 0.5 ml) were injected into a GC (GC-7A; Shimadzu) equipped with a TCD and a Molecular Sieve 5A column (80/100 mesh, 0.3-mm diameter by 2-m length). The carrier gas used was He or commercially available N_2 gas that has a natural abundance of ^{15}N (0.366 atom%). The flow rate of the carrier gas was 30 ml/min. Throughout the TCD GC analyses, the temperatures of injection, column, and detector were 100, 50, and 100°C, respectively. Determination of N_2O levels was carried out by the TCD GC system described above except for use of a PorapakQ column (80/100 mesh, 0.3-mm diameter by 2-m length) and He carrier gas (flow rate, 65 ml/min). The amount of N_2O emission was calculated on the basis of the measured headspace concentration and corrected for the presence of dissolved gas by using the Bunsen coefficient (29). Peak areas were calculated from chromatograms by an integrator (ChromatoPack C-R18; Shimadzu).

Nitrate assay. The concentration of nitrate in the culture was measured colorimetrically (3).

Statistics. Regression analysis was carried out with Microsoft Excel X for Mac software (Microsoft Co., Redmond, Wash.).

RESULTS

Detection of ^{15}N-N_2 by TCD gas chromatography. We have often used a gas mixture composed of 40% (vol/vol) $^{30}N_2$ (^{15}N 99.7 atom%), 40% (vol/vol) Ar, and 20% O_2 (vol/vol) to determine nitrogenase activity of rhizobia and diazotrophic endophytes associated with plants. When we analyzed, by chance, the gas mixture with a TCD GC using a Molecular Sieve 5A column and N_2 carrier gas, an obvious peak appeared at a retention time after Ar and O_2 had already eluted (data not shown). Although TCD senses any gaseous materials that have thermal conductivity different from that of carrier gas (6, 10, 20), it is surprising that chemical species identical to the carrier gas might be able to form a peak on the gas chromatogram. Thus, we wanted to examine whether the peak is really derived from ^{15}N-labeled N_2 (^{15}N-N_2) gas and whether this detection could be utilized for denitrification studies.

Three kinds of 4% (vol/vol) N_2 gas, $^{28}N_2$ ($^{14}N^{14}N$), $^{29}N_2$ ($^{15}N^{14}N$), and $^{30}N_2$ ($^{15}N^{15}N$), and their balance gases (a mixture of Ar and O_2 gas for $^{28}N_2$ and $^{30}N_2$ and He for $^{29}N_2$) were measured by using 0.5-ml injection and He or N_2 as a carrier gas (Fig. 1). When He was used as a carrier, $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ were detected at similar retention times of 4.1 min with similar peak sizes. When N_2 gas was used as a carrier, on the other hand, $^{28}N_2$ was not detected at all (Fig. 1B); however, the use of $^{30}N_2$ gave rise to an apparent peak at a retention time of 3.4 min, as expected (Fig. 1C). Moreover, $^{29}N_2$ gas injection showed a peak at the same retention time as $^{30}N_2$ gas injection. The size of the $^{29}N_2$ peak was about half (43%) of that of the $^{30}N_2$ peak on a molar basis (Fig. 1D). Since the retention times of $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ molecules were identical when He and N_2 carriers were used (Fig. 1), a Molecular Sieve 5A column did not separate these molecules. The thermal conductivity values of ^{15}N-N_2 molecules are likely different from those of normal N_2 with a natural abundance of ^{15}N, however, which enabled us to observe a peak (3.4 min) specific for ^{15}N-N_2 in the chromatogram. Interestingly, ^{15}N-N_2 behaved as if the chemical species of ^{15}N-N_2 differed from that of the N_2 carrier gas. These results demonstrated that the system of TCD gas chromatography is able to detect ^{15}N-N_2 gases including $^{30}N_2$ and $^{29}N_2$. In this work, we refer to this ^{15}N-N_2 detection system as the "^{15}N/TCD method."

Quantification of $^{30}N_2$ by the ^{15}N/TCD method. We further investigated the usefulness of this ^{15}N/TCD method for quantification. A standard gas of $^{30}N_2$ was diluted with air, N_2, or He to make concentrations of $^{30}N_2$ from 0.01 to 40% (vol/vol). A total of 1 ml of each gas sample was injected. Fig. 2 shows a standard curve between the amounts of $^{30}N_2$ and the areas of peaks. The limit of detection was 0.04% (32.7 nmol of ^{15}N). A best-fit curve without an intercept was calculated by linear regression. The equation of the resultant curve was given by $y = 1{,}031.9x$ ($r^2 = 0.9985$). From the best-fit curve, the quantitative range was 0.1% (81.8 nmol of ^{15}N) to 40% (32.7 μmol of ^{15}N). Changes in the dilution medium (air, atmospheric N_2, or He) had no effect on the measurements.

Detection of ^{15}N-N_2 generated from *B. japonicum* cells grown in cultures under denitrification conditions. After anaerobic cultivation of *B. japonicum* USDA110 for 10 days with 2 mM $^{15}NO_3^-$ or 2 mM $^{14}NO_3^-$ in a 123-ml airtight specimen vial (70 ml of culture and 53 ml of headspace), 1 ml of headspace gas was taken and introduced to the GC. When the headspace gas of the vial with $^{15}NO_3^-$ was measured, the peak of ^{15}N-N_2 was observed (Fig. 3B) in the same retention time (3.8 min) as for $^{30}N_2$ standard gas (Fig. 3A). The slight delay of retention time compared to the result shown in Fig. 1 (3.4 min) was possibly derived from a decrease in the flow rate of carrier gas by manual adjustments. In contrast, no peak was detected at this position when 2 mM $^{14}NO_3^-$ was supplied (Fig. 1C). Therefore, it is apparent that ^{15}N-N_2 was generated from $^{15}NO_3^-$ by cells of *B. japonicum* USDA110. Recently, the process of anaerobic ammonia oxidation, which converts nitrogen from NH_4^+ and NO_2^- to N_2 by the activity of a microorganism, was discovered to be another route of anaerobic N_2 generation (25). We thus examined whether anaerobic N_2 generation by *B. japonicum* occurs exclusively through classical denitrification. Because NH_4Cl is the sole nitrogen source of the HM medium, we replaced 6 mM $^{14}NH_4Cl$ with $^{15}NH_4Cl$ (99.7 atomic percentage) (Shoko Co., Ltd.) and added 2 mM $^{14}NO_3^-$ as a denitrification substrate. No ^{15}N-N_2 peak value was detected from the gas phase that was sampled from the anaerobic culture with $^{15}NH_4Cl$ and $^{14}NO_3^-$ (Fig. 3D). Therefore, the production of ^{15}N-N_2 observed in the culture of *B. japonicum* USDA110 was exclusively a consequence of classical denitrification.

Stoichiometric conversion of NO_3^- to N_2 observed using the ^{15}N/TCD method. To examine whether the conversion of

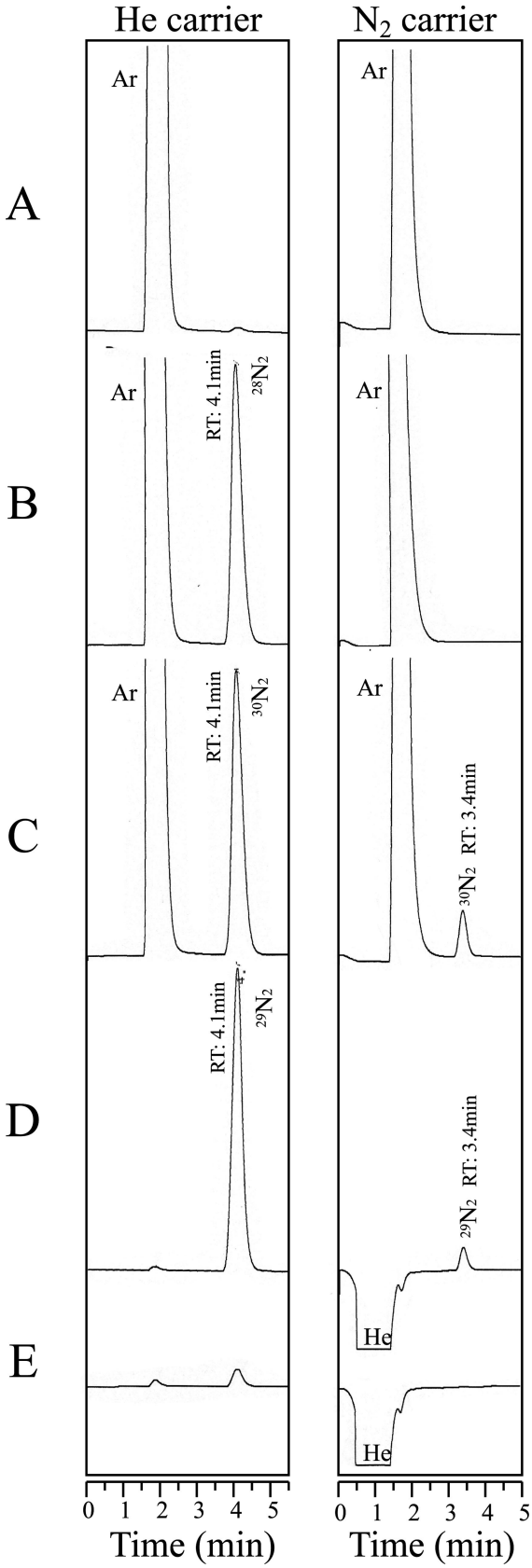

Fig. 1. Gas chromatograms of ^{15}N-N_2 standards and reference gases with He or N_2 as a carrier gas. (A) 80% Ar and 20% O_2; (B) 4.0% $^{28}N_2$, 77% Ar, and 19% O_2; (C) 4.0% $^{30}N_2$, 76% Ar, and 20% O_2; (D) 4.3% $^{29}N_2$ in He; (E) He. Gas samples (0.5 ml) were injected into a GC (GC-7A, Shimadzu) equipped with a TCD and a Molecular Sieve 5A column (80/100 mesh, 0.3-mm diameter by 2-m length). The carrier gas used was He or commercially available N_2 gas that has a natural abundance of ^{15}N of 0.366 atom%. The flow rate of the carrier gases was 30 ml/min. $^{29}N_2$ gas was generated by codenitrification culture of *F. solani* IFO9425 under He atmosphere conditions (22, 26), and the amount of evolved $^{29}N_2$ was determined by gas chromatography-mass spectrometry (GC-MS QP5050; Shimadzu). RT, retention time of the corresponding peak on gas chromatograms. The intensity of the $^{29}N_2$ peak was about half (43%) of that of the $^{30}N_2$ peak on a molar basis when the gas was eluted with N_2 carrier.

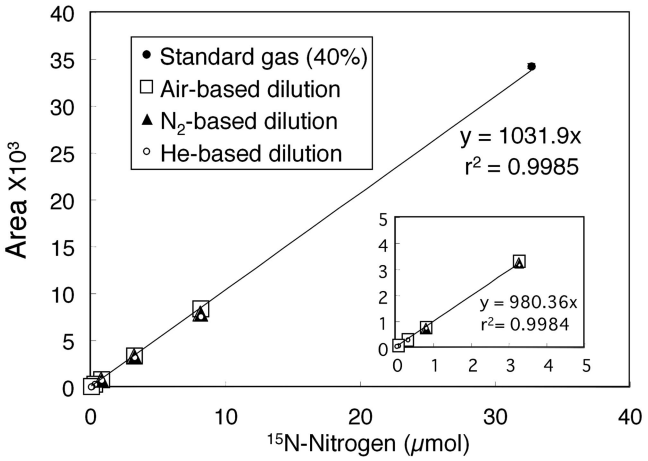

Fig. 2. Standard curve for $^{30}N_2$ quantification by the ^{15}N/TCD method. Gas samples (1 ml) were injected into the TCD GC with a Molecular Sieve 5A column and N_2 as a carrier gas. Areas were calculated from a peak specific for ^{15}N-N_2 (retention time, 3.4 min) as described for Fig. 1. Standard gas (solid circles) contains 40% $^{30}N_2$, 20% O_2, and 40% Ar. Dilution series were prepared with air (open squares), N_2 gas with a natural abundance of ^{15}N (solid triangles), or He (open circles). The best-fit curve was found by linear regression analysis ($y = 1{,}031.9x$, $r^2 = 0.9985$). The interior figure shows an enlarged area representing the results seen with 0 to 5 μmol of ^{15}N.

$^{15}NO_3^-$ occurs stoichiometrically or not, the nitrogen amounts of NO_3^-, N_2O, and ^{15}N-N_2 were monitored in an anaerobic culture of *B. japonicum* USDA110 supplemented with 2 mM $^{15}NO_3^-$ in a 123-ml airtight specimen vial (70 ml of culture and 53 ml of headspace). We periodically sampled 0.1 ml of liquid culture for NO_3^-, 0.2 ml of gas for N_2O, and 0.5 ml of gas for ^{15}N-N_2 determinations. Fig. 4A shows that the final amount of ^{15}N-N_2 was 140 μmol of N/vial, which was equal to the amount of $^{15}NO_3^-$ initially supplied. This result clearly indicates that all nitrogen supplied as $^{15}NO_3^-$ was transformed into $^{30}N_2$.

Comparison with the acetylene inhibition method. To compare the ^{15}N/TCD method with an acetylene inhibition assay, conversion of $^{14}NO_3^-$ to N_2O-N and N_2-N were observed using two anaerobic cultures of *B. japonicum* USDA110 in the presence and absence of C_2H_2. Almost no evolution of N_2O-N was detected in the culture without C_2H_2 (Fig. 4B), whereas the evolution of N_2O-N in the culture with C_2H_2 (Fig. 4C) corresponded to that of $^{30}N_2$-N in the culture without C_2H_2 (Fig. 4A). Moreover, the pattern of $^{30}N_2$ evolution (Fig. 4A) was in accordance with that of N_2O evolution (Fig. 4C). These results indicate that the ^{15}N/TCD method is compatible with

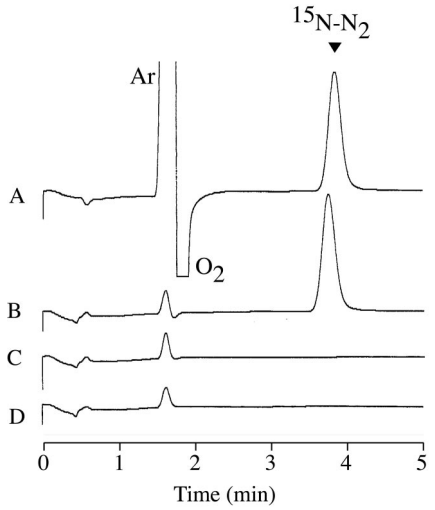

Fig. 3. Gas chromatograms of ^{15}N-N_2 produced in the *B. japonicum* USDA110 culture cultivated under denitrifying conditions in the presence of 2 mM $^{15}NO_3^-$ (B), 2 mM $^{14}NO_3^-$ (C), or 2 mM $^{14}NO_3^-$ and 6 mM $^{15}NH_4Cl$ (D). (A) To identify the position of $^{30}N_2$ elution, a gas mixture of 4% $^{30}N_2$, 21% O_2, and 5% Ar was analyzed. The arrowhead indicates the position of a ^{15}N-N_2 peak. The retention time of the $^{30}N_2$ peak was slightly longer than that seen in a previous analysis of TCD gas chromatography (Fig. 1), probably because the flow rate of N_2 gas was slightly reduced compared to the value of 30 ml/min obtained earlier. O_2 was partially coeluted with Ar and showed a peak in the minus direction at a retention time of about 1.8 min, because the gas contained as much as 21% of O_2. Gas samples (1 ml) were injected into the TCD GC with a Molecular Sieve 5A column and N_2 as a carrier gas.

the conventional denitrification assay by acetylene inhibition. In addition, ^{15}N/TCD method is a useful assay for direct and simple analysis for bacterial denitrification.

Denitrification abilities of indigenous soybean bradyrhizobia. We used 65 *Bradyrhizobium* isolates from soybeans inoculated with the soils from the Nakazawa and Tokachi field sites. The species, *hup* genotype, and insertion sequence accumulation of the isolates had been previously characterized (9, 15, 18). The production of $^{30}N_2$ from anaerobic culture of the isolates with $^{15}NO_3^-$ was examined by the ^{15}N/TCD method. A total of 28 isolates from the Nakazawa field emitted an amount of $^{30}N_2$ equivalent to the amount of $^{15}NO_3^-$ supplied in the medium, whereas no evolution of $^{30}N_2$ was detected from the other isolates (Table 1). To examine whether these were truncated variants lacking N_2O reductase (2, 28, 31), we determined the N_2O concentration 7 days after inoculation in the medium supplemented with $^{14}NO_3^-$ (2 mM). N_2O accumulated from 3 isolates from the Nakazawa field and from 18 isolates from the Tokachi field (Table 1). The amount of N_2O-N was approximately equivalent to that of the added NO_3^--N (even in the test tube assay).

DISCUSSION

We developed a new method of GC measurement of $^{30}N_2$ generated through denitrification by bacterial pure cultures. As far as we know, this is a first report of ^{15}N-N_2-specific determination by TCD GCs (Fig. 1). The sensitivity of TCD is directly proportional to the differences in thermal conductivity between the sample and the carrier gases (20). The elute profiles of $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ (Fig. 1) showed that a ^{15}N-N_2 peak appeared in the direction opposite from that of the peak of He with a higher thermal conductivity in the chromatogram eluted with N_2 carrier (20). This suggests that the thermal conductivities of $^{30}N_2$ and $^{29}N_2$ are slightly lower than that of $^{28}N_2$. Recently, it has been shown that the absolute response of TCD to the eluting component cannot be interpreted simply but is involved in a number of heat-transfer factors such as flow rate, molar heat capacity, convection, and radiation (6, 10). Thus, it

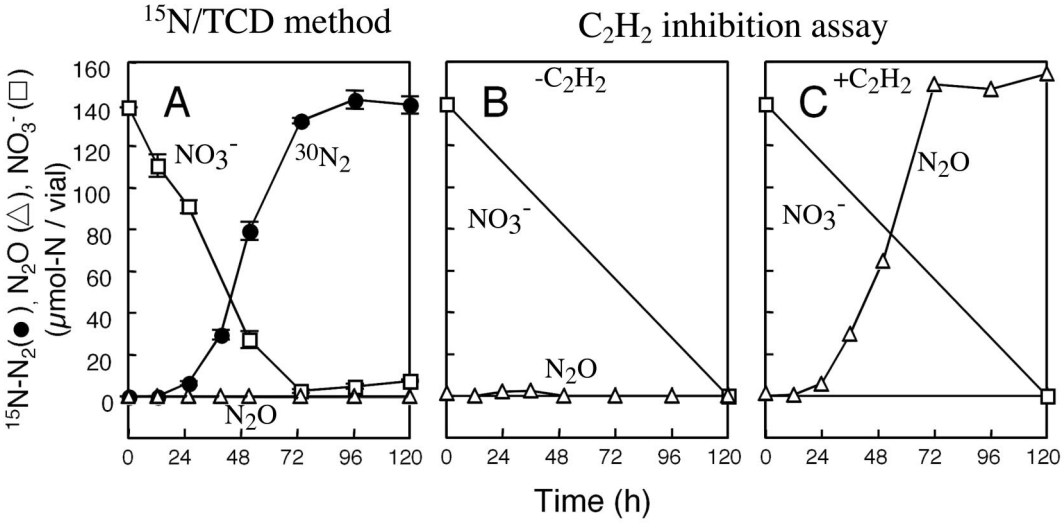

Fig. 4. Comparison of the ^{15}N/TCD method (A) and the acetylene inhibition assay (B and C) in the detection of NO_3^- conversion via denitrification by *B. japonicum* USDA110. Concentrations of ^{15}N-N_2 (solid circles), N_2O (open triangles), and NO_3^- (open squares) in culture were determined periodically as described in the text. *B. japonicum* USDA110 was cultivated anaerobically in the presence of 2 mM $^{15}NO_3^-$ (A) or 2 mM $^{14}NO_3^-$ with (C) or without (B) 10% (vol/vol) acetylene. Results are shown as means of triplicate determinations. Bars indicate standard deviations.

TABLE 1. Denitrification capability of soybean bradyrhizobia isolated from two field sites in Japan by the ^{15}N/GC method[a]

Field site (no. of isolates)	Denitrification end product[b]		Species	*hup*[c]	HRS[d]	No. of isolates
	N_2	N_2O				
Nakazaka (n = 42)	+	−	*B. japonicum*	+	−	22[e]
	+	−	*B. japonicum*	−	−	6[f]
	−	+	*B. japonicum*	−	−	3[g]
	−	−	*B. japonicum*	+	+	2[h]
	−	−	*B. elkanii*	−	−	9[i]
Tokachi (n = 23)	−	+	*B. japonicum*	−	−	17[j]
	−	+	*B. japonicum*	−	+	1[k]
	−	−	*B. japonicum*	−	−	1[l]
	−	−	*B. japonicum*	−	+	4[m]

[a] Soybean bradyrhizobia previously isolated from Nakazawa and Tokachi field soils were characterized for species, uptake hydrogenase, and repeated sequence fingerprints (9, 18).

[b] The N_2 column indicates completion of the full process of denitrification from $^{15}NO_3^-$ (2 mM) to $^{30}N_2$ with from 98 to 110% recovery after 7 days (+) except for a low recovery (62%) of isolate NC34a. −, the isolate evolved no $^{30}N_2$ (<0.03% [vol/vol]). The N_2O column indicates positive (+) and negative (−) N_2O accumulation in the presence of $^{14}NO_3^-$ (2 mM) after 7 days. $^{30}N_2$ and N_2O were determined at least in duplicate.

[c] Genotype and phenotype of uptake hydrogenase (16, 18).

[d] HRS, highly reiterated sequence-possessing strains carrying high copy numbers of insertion sequences (9, 15, 18, 21).

[e] Isolates NC4a, NC5a, NC6a, NC10a, NC11a, NC12a, NC13a, NC14a, NC15a, NC16a, NC17a, NC18a, NC20a, NC21a, NC22a, NC24a, NC26a, NC29a, NC34a, NC35a, NC37a, and NC41a.

[f] Isolates NC2a, NC19b, NC27a, NC28a, NC38a, and NC39a.

[g] Isolates NC8a, NC33b, and NC36a.

[h] Isolates NC3a and NC32a.

[i] Isolates NC7a, NC23a, NC25a, NC31b, NC42a, NC43a, NC44a, NC45a, and NC46a.

[j] Isolates T1, T3, T4, T5, T6, T7, T8a, T9, T10a, T11, T12, T13, T20, T27, T29, T37, and T39.

[k] Isolate T25.

[l] Isolate T40.

[m] Isolates T2, T15, T22, and T31.

has so far been difficult to predict theoretically molar response factors of $^{30}N_2$ and $^{29}N_2$ for TCD.

The advantages of this ^{15}N/TCD method are (i) use of a more commonly available piece of equipment (a TCD GC) rather than a mass spectrometer, (ii) no interference with $^{30}N_2$ determination by atmospheric N_2 contamination, (iii) simultaneous analysis of many cultures, and (iv) presumptive application to N_2O reductase activity assay by product measurement. Although field isolates of soybean bradyrhizobia were analyzed in this work, the ^{15}N/TCD method is equally applicable to other bacteria in their corresponding media by using compounds highly enriched with ^{15}N such as NO_3^-, NO_2^-, and N_2O.

Use of the ^{15}N/TCD method rapidly demonstrated that the *Bradyrhizobium* isolates can apparently be categorized into three denitrification types: (a) full denitrifier (up to N_2), (b) truncated denitrifier (up to N_2O), and (c) nondenitrifier. No full denitrifiers was found among the Tokachi isolates, interestingly, whereas the majority of Nakazawa isolates (67%) showed full denitrification from nitrate to N_2 (Table 1). In contrast, truncated denitrifiers (up to N_2O) accounted for 78% of the Tokachi isolates (Table 1). If soybean bradyrhizobia contribute to N_2O emission from field soils, then an indigenous population dominated by *Bradyrhizobium* full denitrifiers would reduce N_2O emission from agricultural soils. When the three types of denitrification were compared with previously characterized traits, we found some relationships among the tested isolates (Table 1). All of the isolates with the ability to take up hydrogen (hydrogen uptake-positive isolates) fell into the full-denitrifier category. All of the *B. elkanii* isolates were found to be nondenitrifiers by this assay. Highly reiterated sequence-possessing isolates carrying high copy numbers of insertion sequences tended to belong to the nondenitrifiers. To confirm these relationships, examination of additional field isolates and appropriate standard strains of *Bradyrhizobium* spp. (together with analyses of their denitrification genes) would be required.

To apply this method to environmental samples such as soil and water we must consider $^{29}N_2$ ($^{15}N^{14}N$) evolution, because denitrification produces $^{30}N_2$ ($^{15}N^{15}N$), $^{29}N_2$($^{15}N^{14}N$), and $^{28}N_2$ ($^{14}N^{14}N$) through random isotope pairing (19) and because anaerobic ammonia oxidation and codenitrification contribute to $^{29}N_2$($^{15}N^{14}N$) production (22, 25, 26). Since $^{29}N_2$ gave rise to a level of signal intensity about half of that seen with $^{30}N_2$ (Fig. 1C and D), ^{15}N incorporation into N_2 molecule can be monitored by the ^{15}N/TCD method as a first screening assay for environmental samples. Afterwards, mass spectrometry analysis would be required to specify $^{30}N_2$ ($^{15}N^{15}N$) and $^{29}N_2$($^{15}N^{14}N$) molecules or their ratios for the environmental samples.

Finally, we summarize the recommended procedure of the ^{15}N/TCD method for denitrification assays of microbial pure cultures. A gas mixture of 4% (vol/vol) $^{30}N_2$ (^{15}N, more than 99 atom%) and 96% (vol/vol) Ar was recommended as an ideal standard gas, because the Ar peak was oriented in the same direction as the $^{30}N_2$ peak and because the Ar peak is smaller than the He peak (Fig. 1). Commercially available N_2 gas that has a natural abundance of ^{15}N (0.366 atom%) should be used as a carrier gas. The recommended conditions of TCD GC are as follows: flow rate of carrier gas, 30 ml/min; column, Molecular Sieve 5A (80/100 mesh, 0.3-mm diameter by 2-m length); detector temperature, 100°C: column temperature, 50°C; injection volume, 0.5 to 1.0 ml.

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