Denitrifying ability of indigenous strains of Bradyrhizobium japonicum isolated from fields under paddy-upland rotation.

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Summary. In Japan some paddy fields are used for upland crops for

several years and returned to paddy fields (paddy-upland rotation). Soybean (Glycine max L.) is an important summer crop. The ability and some characteristics of denitrification by isolated strains of Bradyrhizobium japonicum were investigated to clarify the frequency of denitrifiers in indigenous populations of B. japonicum in fields under paddy-upland rotation. Eight field plots with different cropping systems in the fields at two sites were used. The fields consisted of a Gray Lowland Soil and soybean or paddy rice (Oryza sativa L.) were grown as a summer crop, and barley (Hordeum distichum L.) or wheat (Triticum aestivum L.) were grown as a winter crop. All B. japonicum strains present in the plots were able to denitrify. Isolated strains fell into two main groups(group I and II) according to the rate of denitrification. Strains of group I evolved N_2O with C_2H_2 at a rate comparable to that of Alcaligenes denitrificans IAM 12370, whereas strains of group II had 100 times lower denitrification activity than strains of group I. Both group I and II strains occurred in each plot. Amounts of N_2O produced by indigenous strains with and without C_2H_2 suggest that strains of group I and II evolved N_2 or N_2O, respectively, as the end product of denitrification. One strain (S107) was isolated which had the highest denitrifying ability with an end product of N_2O. These results indicate that indigenous bradyrhizobia may partly contribute to denitrification of field soil under a paddy-upland rotation.

Key words: Bradyrhizobium japonicum, Denitrification, Paddy-upland rotation, Indigenous populations, Soybean

Denitrification is common in some species of Rhizobium and Bradyrhizobium (Ishizawa 1953; O'Hara and Daniel 1985; Tiedje 1988). O'Hara et

al. (1984) reported that rhizobia in soil were able to denitrify significant amounts of nitrogen under field conditions. However, Breitenbeck and Bremner (1989) showed that denitrifying capacity of B. japonicum did not significantly influence the quantity and quality of soil denitrification. On the other hand, denitrification by intact soybean (Glycine max L.) nodules inoculated with denitrifying strains of B. japonicum also has been observed (Bryan et al. 1985; Smith and Smith 1986).

These studies were carried out using limited numbers of strains. Little is known about the occurrence or frequency of denitrifying rhizobia and bradyrhizobia in natural populations, although such information is necessary to understand denitrification in soil and nodules under field condition. The first and sole report was made on the frequency of denitrifiers in indigenous populations of R. meliloti by Chan et al. (1989).

In Japan, some paddy fields are used for upland crops for several years and returned to paddy fields (paddy-upland rotation). Soybean is an important summer crop used in this rotation. Indigenous bradyrhizobia are subjected to periodic flooded and nonflooded conditions in these fields. In a previous paper, indigenous bradyrhizobia survived well under flooded conditions (Asakawa and Ikeda 1990). However, the occurrence and frequency of denitrifying bradyrhizobia was not reported.

The objective of this study was to clarify the frequency of denitrifiers in indigenous populations of B. japonicum in fields under a paddy-upland rotation. I examined the ability and some characteristics of denitrification by indigenous strains of B. japonicum isolated from the fields.

Materials and methods

Experimental fields and bacterial strains

Experimental fields and soil samples were the same as those described previously, which were used for measuring the population of soybean-nodulating bacteria (Asakawa and Ikeda 1990). The fields used in this study consisted of a Gray Lowland Soil. Table 1 shows the cropping systems of the fields. The dates of soil sampling for isolation were 21 July 1988 (A~D) and 4 September 1987 (E~H). Strains of soybean-nodulating bacteria were isolated from soybean nodules(cv. Fukuyutaka) used for enumeration by nodulation-dilution frequency method. Several plants, which were inoculated with a few serial dilutions of soil, were used for isolation from each plot. The nodules were washed in distilled water, surface sterilized by a 3-min exposure to 70% ethanol and rinsed in sterile water. The crushed nodule suspension was streaked onto yeast extract-mannitol agar (YMA: mannitol, 10.0g; KH_2PO_4, 0.5g; $MgSO_4 \cdot 7H_2O$, 0.2g; NaCl, 0.1g; $FeCl_3 \cdot 6H_2O$, 0.002g; yeast extract (Difco), 0.4g; agar, 15.0g; and distilled water, 1 liter; pH 6.8) plates. Isolated colonies were restreaked onto YMA plates containing congo red (0.025 g per liter) to ensure purity. Isolates were checked for nodule formation on soybean, pH change in yeast extract-mannitol broth (YMB), and growth rate on YMA. Isolates were maintained on YMA slants.

Two strains of B. japonicum and one strain of Alcaligenes denitrificans from culture collections were also used in this work. B. japonicum strains MAFF 03-03160 and MAFF 03-03161 (formerly NIAES 3160 and NIAES 3161, respectively) were obtained from the National Institute of Agrobiological Resources, Ministry of Agriculture, Forestry and Fisheries, Japan. These strains were isolated from soil at Menda, Kumamoto, Japan (Sawada et al. 1989). They were maintained on YMA slants. A. denitrificans strain IAM 12370 was obtained from the Institute of Applied Microbiology, the University of Tokyo, Japan. This strain

was maintained on peptone-yeast extract agar (peptone, 5g; yeast extract (Difco), 3g; beef-extract (Difco), 3g; glucose, 10g; agar, 18g; and distilled water, 1 liter; pH 7.0~7.2) slants.

Denitrification assay

Rhizobial strains were grown in 18mm o.d.x180mm test tubes containing 5ml YMB with 1.0g KNO_3 per liter. The tubes were inoculated and incubated aerobically with Silicosen® (Shin-etu Polymer Co., Ltd.) at 28°C for 7 days (late log-early stationary phase). The plugs were then removed and the tubes were sealed with butyl rubber stoppers (Sanshin Industry Co., Japan) aseptically. The tubes were evacuated (1 min) and flushed with Ar (152 kPa) containing 10% C_2H_2 and 300ppm CO_2 (0.5 min); this was repeated three times (Sato and Wada 1988). In some experiments the tubes were also flushed with Ar (152 kPa). Headspace samples were removed with a syringe for determination of N_2O and growth was estimated turbidimetrically after incubation at 28°C for prescribed periods, as described below. N_2O was measured with a gas chromatograph (Shimadzu GC-15A) equipped with a ^{63}Ni electron capture detector. The gas sample (0.2ml) was injected into the gas chromatograph using a gas sampler (Shimadzu MGS-5). A 3.0mm i.d.x2.0m glass column packed with Porapak Q (80-100 mesh) was used. The carrier gas was 95% Ar-5%CH_4 and the flow rate was 60ml·min^{-1}. Detector, column, and injection port were kept at 345, 50, and 150°C, respectively. N_2O standard gases (0.98, 12.0 and 127 ppmv, Takachiho Trading Co., Ltd.)were used for calibration. The absorbance at 660nm of culture solutions was used to determine dry weights of cells from a standard curve. The results were expressed

as n mol N_2O per hour per mg dry cell. The A. denitrificans strain was grown in nutrient broth (beef extract (Difco), 10g; peptone, 10g; NaCl, 5g; and distilled water, 1 liter; pH 7.2) with 1.0g KNO_3 per liter at 28°C for 24h (late log-early stationary phase) for the denitrification assay.

For gas production in Durham tubes, rhizobial strains were grown in 15mm o.d.x150mm test tubes containing 7ml YMB with 1.0g KNO_3 per liter or 7ml Giltay's medium (KNO_3, 1g; l-asparagine, 1g; sodium citrate, 8.5g; $MgSO_4 \cdot 7H_2O$, 1g; $FeCl_3 \cdot 6H_2O$, 0.05g; KH_2PO_4, 1g; $CaCl_2 \cdot 2H_2O$, 0.2g; bromothymol blue, 0.05g; and distilled water, 1 liter; pH 7.0~7.2) and an inverted Durham tube. The tubes were covered with aluminum caps and incubated at 28°C for 7 days. Gas production was determined by visual observation.

All the determinations were duplicated.

Results

A total of 103 strains were isolated. The number of strains in each plot was as follows: A, 9; B, 16; C, 14; D, 11; E, 15; F, 12; G, 13; H, 13. All the strains nodulated soybean (cv. Fukuyutaka), produced alkaline reactions in YMB after incubation for 5 days, and all expressed the features of slow-growing rhizobia on YMA. These results indicate that all the strains belong to B. japonicum. All the strains had denitrifying ability. Isolates fell into two main groups (group I and II) according to the rate of denitrification. Fig. 1 shows the relationships between incubation time after gas treatment and amounts of N_2O produced by two strains (0001 and 1067, isolated from plot E and F, respectively) of B. japonicum representative of the groups. Strain 0001 actively denitrified and N_2O production increased linearly for up to 1 h, whereas strain 1067 evolved a detectable amount

of N_2O only after 12 h of incubation. Therefore, N_2O was determined after 0.5-1 h of incubation (strains of group I) or 12-24 h of incubation (strains of group II).

Table 2 shows denitrifying activity and gas production in Durham tubes of B. japonicum strains and A. denitrificans IAM 12370. Two strains of B. japonicum obtained from the culture collection were also able to denitrify. Group I strains had 100 times higher denitrification activity than group II strains. That level was comparable to the level of A. denitrificans IAM 12370. Strain S107 showed the highest activity. The activity of MAFF 03-03160 and 03-03161 was similar to the levels of group I strains and group II strains, respectively. Group I strains and MAFF 03-03160 evolved gas bubbles in Durham tubes with YMB, whereas group II strains, strain S107 and MAFF 03-03161 showed no gas production in Durham tubes with YMB. All the indigenous strains produced no gas bubbles in Durham tubes with Giltay's medium.

Fig. 2 shows the proportion of group I and II strains isolated from each plot. Both strains of group I and II occurred in each plot. Strains of group I, which denitrified with high activity, were mainly isolated from plot E without a history of soybean cultivation, whereas the proportions of strains of group II, which had low denitrifying activities, were relatively low in the plots with a history of soybean, especially in plot G. The differences in the proportion of group I and II strains among plots were significant ($P<0.01$).

Fig. 3 and 4 show the effect of C_2H_2 addition on amounts of N_2O produced by indigenous strains and strains obtained from culture collections. Most of the strains of group I, MAFF 03-03160 and A. denitrificans IAM 12370 evolved 10-100 times smaller amounts of N_2O without C_2H_2 than with C_2H_2 (Fig. 3). On the other

hand, N_2O production by strains of group II and MAFF 03-03161 was little affected by C_2H_2 (Fig. 4). Addition of C_2H_2 had little influence on N_2O production of strain S107, although it evolved larger amount of N_2O than strains of group II (data not shown).

Discussion

All the indigenous strains of B. japonicum isolated from two field sites with a paddy-upland rotation were capable of denitrification (Table 2). This is the first report on the occurrence and frequency of indigenous denitrifying B. japonicum in field soils. Chan et al. (1989) first reported the frequency of denitrifiers in indigenous populations of R. meliloti isolated from nodules of field-grown plants, and reported that 57 of 120 isolates of R. meliloti were able to denitrify. Denitrification is common in B. japonicum (Tiedje, 1988). Ishizawa (1953) reported that possible denitrification occurred widely in soybean rhizobia according to the ability of nitrate reduction and nitrite accumulation. Zablotowicz et al. (1978), and van Berkum and Keyser (1985) investigated B. japonicum strains originating from culture collections, and showed that most of the B. japonicum strains tested were able to denitrify; they also reported the occurrence of nondenitrifying strains of B. japonicum. However strains not capable of denitrification were not isolated from the fields in the present study. The difference can be ascribed in part to the assay method for denitrification. Zablotowicz et al. (1978) observed gas production in Durham tubes in liquid culture. van Berkum and Keyser (1985) measured N_2O using a gas chromatograph equipped with a thermal conductivity detector by the acetylene inhibition method. In the present study N_2O was determined with a gas chromatograph equipped with an electron capture detector, which is highly

sensitive to N_2O. Strains of group II evolved detectable amount of N_2O only after 12 h of incubation (Fig. 1) and did not evolve gas bubbles in Durham tubes with YMB (Table 2). Surveying denitrifying ability with a highly sensitive method for N_2O may increase the detection of denitrification among B. japonicum.

The soil condition of the fields could also affect the frequency of denitrifying ability of indigenous strains of B. japonicum. The fields used in the present work had been paddy fields since before 1980. Before 1980 only lowland rice had been grown as a summer crop. Flooding and cultivation of rice causes an anaerobic condition, which is favorable for denitrification. Denitrifying ability of rhizobia should enhance survival under anaerobic conditions (Zablotowicz et al. 1978; O'Hara and Daniel 1985). The populations of indigenous B. japonicum in the plots except for plot E were 10^4~10^5 cells g^{-1} dry soil and this level was maintained during 3 years of rice cropping (Asakawa and Ikeda 1990). Further investigation is needed to know whether denitrifying ability of the indigenous strains is important in their survival under flooded condition. Denitrifying strains (group I) of bradyrhizobia which had comparable activities to that of the common denitrifier A. denitrificans inhabited all the plots (Table 2, Fig. 2). Assuming that half of the indigenous bradyrhizobia consisted of strains of group I, each plot except plot E had 10^4~10^5 cells g^{-1} dry soil of actively denitrifying bradyrhizobia. I also enumerated the numbers of denitrifiers by the most-probable number (MPN) method using Giltay's medium in the same soil samples as those used for isolation of bradyrhizobial strains and found that the populations of denitrifiers were 10^4~10^5 cells g^{-1} dry soil (data not shown). These results indicate that the number of actively denitrifying bradyrhizobia in soil was comparable with that of total soil denitrifiers, however,

the MPN method using Giltay's medium is not precise (Tiedje 1982) and indigenous strains of B. japonicum were not able to denitrify in Giltay's medium (Table 2). Concerning number and activity, indigenous bradyrhizobia in the fields may contribute to denitrification under flooded conditions. O'Hara et al. (1984) reported significant amounts of nitrogen were lossed from soils because of Rhizobium-dependent denitrification. Denitrification activity of indigenous strains of B. japonicum was determined only in liquid culture as free-living cells in this study. To assess the contribution of bradyrhizobia to denitrification in field soil, the ability of indigenous strains to denitrify in soil requires investigation. On the other hand, the fields used were under paddy-upland rotations and sometimes cropped with soybean (Table 1). Further investigation of denitrification in nodules or nodulated plants infected by these strains will elucidate symbiotic denitrification by indigenous B. japonicum.

Nitrous oxide and dinitrogen gas have been reported as products of bradyrhizobial denitrification (O'Hara and Daniel 1985). Most of the strains of group I of indigenous bradyrhizobia, B. japonicum MAFF 03-03160, and A. denitrificans IAM 12370 evolved 10-100 times larger amount of N_2O with C_2H_2 than without C_2H_2 (Fig. 3), which indicates that the end product of these strains is N_2. On the other hand, addition of C_2H_2 had little influence on N_2O production of group II strains of indigenous bradyrhizobia, and B. japonicum MAFF 03-03161, which may indicate that their end product is N_2O. Strains of group II and MAFF 03-03161 had about 2 order of magnitude lower denitrification activities than group I strains, MAFF 03-03160 and A. denitrificans IAM 12370 (Table 2). Little influence of C_2H_2 on N_2O production and low denitrification activities suggest that strains of

group II and MAFF 03-03161 belong to "nonrespiratory denitrifiers" described by Tiedje (1988). Nonrespiratory denitrifiers have lower rates of N_2O production and convert smaller percentage of NO_3-N to N_2O-N than respiratory denitrifiers (Tiedje 1988). Nonrespiratory denitrifiers produce only N_2O as the end product and N_2O production is not increased by C_2H_2 (Smith and Zimmerman 1981; Kaspar 1982). Nonrespiratory N_2O production by Bradyrhizobium has not been reported. Stoichiometric examination is required to elucidate the denitrifying property of group II strains and MAFF 03-03161 as "nonrespiratory denitrifiers".

Strain S107 isolated from plot B evolved the largest amount of N_2O, but did not produce gas bubbles in Durham tubes (Table 2). Production of N_2O by this strain was little affected by C_2H_2 (data not shown). These results suggest that N_2O is the end product of denitrification by this strain. N_2O is very water soluble and will not form a bubble in Durham tubes (Tiedje 1982). This must be the reason why strain S107 did not produce gas bubbles. Although only one strain of B. japonicum was isolated from the fields which produced N_2O actively as the end product, bradyrhizobial denitrification might contribute to N_2O emission from soil according to the extent of predominance of this strain in indigenous populations of B. japonicum.

There were significant ($P<0.01$) differences in the proportion of group I and II strains among plots (Fig. 2). Chan et al. (1989) showed that frequency of occurrence of denitrifying R. meliloti at a site without a history of Medicago sativa cultivation was significantly greater than at a site with a history of the host plant. The legume might select rhizobia with low denitrifying activity for symbiotic N_2

fixation from indigenous populations. Further investigation would be necessary to clarify the relationship between history of soybean cultivation and the proportion of strains with different activity of denitrification.

The results obtained in this work indicate that indigenous strains of B. japonicum may partly contribute to denitrification of field soil under a paddy-upland rotation. Further investigation is needed on the ecology of denitrifying bradyrhizobia in paddy-upland rotational fields in order to understand denitrification by bradyrhizobia in soils and nodules.

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Table 1. Cropping systems of

fields[a]

Site	Plot	1980	1981	1982	1983	1984	1985	1986	1987	1988
Saga	A	R-B	R-B	R-B	R-B	R-B	R-B	S-B	S-B	S-B
	B	R-B	R-B	R-B	R-B	R-B	R-B	R-B	S-B	R-B
	C	R-B	R-B	R-B	S-B	R-B	R-B	S-B	R-B	R-B
	D	R-B	R-B	R-B	R-B	R-B	S-B	R-B	R-B	R-B
Chikugo	E	R-W	R-W	R-W	R-W	R-W	R-W	R-W	R-W	R-W
	F	S-W	R-W	S-W	R-W	S-W	R-W	S-W	R-W	S-W
	G	S-W	S-W	R-W	S-W	S-W	R-W	S-W	S-W	R-W
	H	S-W	S-W	S-W	R-W	S-W	S-W	S-W	R-W	S-W

[a]Summer crop: R, rice; S, soybean; winter crop: B, barley; W, wheat

Table 2. Denitrifying activity and gas production in Durham tubes of strains of B. japonicum and A. denitrificans IAM 12370

Strain		Denitrifying activity	Gas production in Durham tube[a] in
		n mol N_2O/hr·mg cell	
		YMB	Giltay's
Strains of group I	(44)[b]	18.6 ±8.07[c]	+
Strains of group II	(58)[b]	0.0740±0.0306[c]	-
Strain S107		419	-
MAFF 03-03160		9.68	-
MAFF 03-03161		0.160	N.D.

-	N.D.
A. denitrificans IAM 12370	50.8
	N.D.
	N.D.

[a]+, positive; -, negative; N.D., not determined

[b]Number of strains

[c]Mean±standard error

Legends

Fig. 1. Relationship between incubation time and amounts of N_2O produced by B. japonicum strains isolated from Chikugo soil. I, strain 0001 isolated from plot E; II, strain 1067 isolated from plot F.

Fig. 2. Proportion of strains of group I and II in strains isolated from each plot. The number of strains in each plot: A, 9; B, 16; C, 14; D, 11; E, 15; F, 12; G, 13; H, 13. See Discussion about the denitrifying property of strain S107.

Fig. 3. Effect of C_2H_2 addition on amounts of N_2O produced by strains of group I(o), MAFF 03-03160(Δ) and A. denitrificans IAM 12370( ).■

Fig. 4. Effect of C_2H_2 addition on amounts of N_2O produced by strains of group

II(o) and MAFF 03-03161(Δ).