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Correlation of Denitrifying Capability with the Existence of *nap*, *nir*, *nor* and *nos* Genes in Diverse Strains of Soybean Bradyrhizobia

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To clarify the genetic basis to the diversity of denitrifying ability in soybean bradyrhizobia, we compared the end-products of denitrification (N_2, N_2O and NO_2^-) with the existence of denitrifying genes (*napA*, *nirK*, *norCB* and *nosZ*) of sixty phylogenetically diverse strains of *Bradyrhizobium japonicum* and *B. elkanii*. The results indicate that the existence of denitrifying genes directly determines phenotype (end-products) in most strains of *B. japonicum*. The denitrifying capability and gene set were reflected by phylogenetic position based on repeated sequences (RS)-fingerprints and 16S rRNA gene sequences. However, the denitrifying genes in HRS (highly repeated sequence-possessing) strains of *B. japonicum*, which were identified based on RS-fingerprints as having heavy hybridization, resulted in an inconsistent correlation probably because of genomic rearrangements. The evolutionary and ecological implications of the denitrifying genes and capability in soybean bradyrhizobia are discussed.

Key words: denitrification, denitrifying genes, *nosZ*, *Bradyrhizobium*

Denitrification was originally described as the loss of fixed nitrogen from the biosphere into the atmosphere as part of the global nitrogen cycle. However, in terms of bacterial physiology, denitrification is regarded as anaerobic respiration with nitrogen oxides (NO_3^-, NO_2^-, NO and N_2O) used as terminal electron acceptors in place of oxygen[30], although aerobic denitrification has been reported[23]. The denitrifiers are widely distributed among prokaryotes including proteobacteria and archaea[37,38] and have a combination of terminal reductases (nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase), which reduce specific nitrogen oxide, respectively. If the four sequential enzymatic reductions of the nitrogen oxides are activated ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$), dinitrogen gas ($N_2$) is released into the atmosphere. Because of the absence of N_2O reductase activities, some incomplete denitrifiers produce N_2O[27,28,38], which gives rise to a greenhouse effect[15] and damage to the ozone layer[35].

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To date, six genes have been reported to encode catalytic subunits of denitrifying reductases[24,38]. There are two nitrate reductase genes, *napA* (periplasmic nitrate reductase gene) and *narG* (membrane-bound nitrate reductase gene), two nitrite reductase genes, *nirK* (Cu-containing nitrite reductase gene) and *nirS* (cytochrome cd_1 nitrite reductase gene), one nitric oxide reductase gene, *norCB*, and one nitrous oxide reductase gene, *nosZ*[38]. The combinations of denitrifying genes differ among organisms. For example, *Agrobacterium tumefaciens* strain C58 is a partial denitrifier that lacks a nitrous oxide reductase gene[2].

Bradyrhizobium japonicum and *B. elkanii* are symbiotic diazotorophic bacteria associated with soybeans, which belong to the α-Proteobacteria. Strain USDA110 is an experimental representative of *B. japonicum*[13], which produces N_2 gas through denitrification in a free-living state[4,28,32]. A search of the database of the entire genomic sequence of *B. japonicum* USDA110[13] revealed that *napDABCDE*, *nirK*, *norECBQD*, and *nosRZDFYLX* gene clusters are dispersed in the genome: the products of these genes have been proved to function as denitrifying reductases[7,18,33,34]. On the

other hand, no *narG* or *nirS* gene is predicted. Furthermore, it was reported that strain USDA110 expresses the *nir*, *nor* and *nos* genes[17], and N_2O reductase activity[27] in a symbiotic state. However, the members of soybean bradyrhizobia are phylogenetically diverse[19,25,31] and the end products of denitrification (N_2, N_2O, NO_2^-) are dependent on the bradyrhizobial strain[1,4,28,32].

Previously, we compared the denitrifying capability of free-living cells of 65 field isolates in the presence of nitrate, finding that N_2 producers and N_2O producers were dominant at the Nakazawa and Tokachi field sites, respectively[28]. Similar observations have been made in standard strains and field isolates of soybean bradyrhizobia[1,4,32]. The type of bradyrhizobial denitrification was likely correlated with phenotypic and genotypic traits such as species, uptake hydrogenase, and repeated sequence (RS) fingerprints, though this has yet to be fully incorporated into a phylogenetic tree of *B. japonicum* and *B. elkanii*. The relationship between phylogeny and the existence of denitrifying genes (*napA*, *nirK*, *norCB* and *nosZ*) in soybean bradyrhizobia also remains to be solved.

The main purpose of our study is to clarify the genetic basis of the diversity of denitrifying activity in soybean bradyrhizobia. Thus, we investigated denitrifying activities and the distribution of denitrifying genes (*napA*, *nirK*, *norCB* and *nosZ*) among phylogenetically diverse *Bradyrhizobium* strains consisting of field isolates in Japan[10,20–22,26,28] and reference strains[25,31,36].

Materials and Methods

Bacterial strains and media

Bacterial strains and plasmids used in this study are listed in Table 1. For the preparation of total DNA, strains of *Bradyrhizobium* were grown aerobically at 30°C in HM medium which consists of HM salt medium[6] supplemented with 0.1% arabinose and 0.025% yeast extract (Difco, Detroit, MI, USA). *Mesorhizobium loti* MAFF303099 was grown in TY medium (0.5% Bacto-tryptone, 0.3% yeast extract, and 0.087% $CaCl_2 \cdot H_2O$), and *Sinorhizobium meliloti* 1021 was grown in Luria-Bertani medium[16].

Denitrifying capability

Denitrifying capabilities of bradyrhizobia were characterized based on the end-products of denitrification using the ^{15}N/TCD method described in a previous paper[28]. Strains were grown anaerobically at 30°C with 2 mM ^{15}N-KNO_3 (99.6 atom%) (Shoko Co., Ltd., Tokyo, Japan) as the sole electron accepter in HMM medium which consists of HM medium supplemented with 0.55 μM $Na_2MoO_4 \cdot 2H_2O$, 1 μM $FeCl_2$, and 1 μM $CuSO_4 \cdot 2H_2O$. To quantify the final products of denitrification, gas chromatgraphic assay for N_2O and N_2 were performed as described previously[28]. NO_3^- [5] and NO_2^- [14] were measured colorimetrically.

Isolation of DNA

Total genomic DNA of *Bradyrhizobium* and *Mesorhizobium* was isolated as described previously[21]. Total genomic DNA of *Sinorhizobium* and *Mesorhizobium* was kindly provided by H. Mitsui (Tohoku University) and M. Itakura (Tohoku University), respectively.

Determination of 16S rRNA gene sequences

Almost full-length 16S rRNA genes were amplified from the total genomic DNA by using a eubacterial primer set, pA and 1492r[8]. After the amplified fragments were purified by precipitation with 40% (w/v) polyethylene glycol (PEG8000), 3.3 mM $MgCl_2$, and 0.4 M CH_3COONa, the DNA sequences were determined by automated fluorescent dye primer sequencing with an SQ5500 sequencer (Hitachi Instruments service Co., Ltd., Tokyo, Japan). The forward sequencing primer f3L spanned positions 1094 to 1112 and the reverse primers r1L, r2L', and r4L spanned positions 536 to 518, 805 to 786, 1111 to 1093, and 1406 to 1389, respectively (numbered as for the *E. coli* 16S rRNA gene).

Southern hybridization with RS probes

Southern hybridization was carried out as described previously[21] using 3 μg of total genomic DNA digested with *Xho*I. Full-length fragments of RSα and RSβ were prepared for probes as described[26]. Hybridization probes were labeled using a DIG DNA labeling kit (Roche Diagnostics).

Southern hybridization with denitrifying gene probes

Southern hybridization was carried out as described previously[21] using 300 ng of total genomic DNA digested with *Bam* HI. Hybridization with each probe was done at 42°C to achieve low stringent conditions (the Tm of probes of denitrifying genes were 75–76°C) in which fragments possessing 73–78% identity could be detected, logically. Four primer sets were designed from sequences of the denitrifying genes *napA* (blr7038), *nirK* (blr7089), *norCB* (blr3214, blr3215), and *nosZ* (blr0315), which were recognized in the database of the genome sequence of *Bradyrhizobium japonicum* USDA110 (http://www.kazusa.or.jp/rhizobase/Bradyrhizobium/) to amplify partial fragments of each gene. The primer set napA-1 5'-CGAGGTGAGCGAATCTGAAATGGGACAAGG-3' and

Table 1. Bacterial strains and plasmids used in this study

Strain and plasmid	Relevant characteristics	Source or reference
Strain		
B. japonicum		
USDA6[abcd], USDA38[bc], USDA110[abcd], USDA122[abcd], USDA123*[acd], USDA124[abcd], USDA129*[acd], USDA135*[acd]	*B. japonicum* strains from the USDA ARS National *Rhizobium* Germplasm Collection	31
NC2[bcd], NC3*[cd], NC4[bcd], NC5[b], NC6[bcd], NC8[bcd], NC10[bcd], NC13[b], NC15[bcd], NC17[b], NC18[b], NC19[bcd], NC21[b], NC27[b], NC28[b], NC29[b], NC32*[cd], NC33[bcd], NC34[b], NC35[b], NC36[b], NC37[b], NC38[b], NC39[b], NC41[b]	Field isolates from Nakazawa, Niigata, Japan	10, 20, 21, 22, 26, 28
T1[b], T2*[cd], T7[bcd], T8[b], T9[bcd], T10[b], T12[b], T20[b], T27[b], T29[b], T37[b], T39[b],	Field isolates from Tokachi, Hokkaido, Japan	10, 20, 22, 26, 28
NK2[abcd], NK5*[acd], NK6*[acd]	Field isolates from Nagakura, Niigata, Japan	10, 20, 22, 26
2281*[acd]	Isolate from Heilongjiang Province, China. Species name "liaoningense" was proposed though most features are consistent with *B. japonicum* USDA135.	31, 36
B. elkanii		
USDA61[abcd], USDA76[abcd], USDA94[abcd]	*B. elkanii* strains from the USDA ARS National *Rhizobium* Germplasm Collection	31
NC7[b], NC25[b], NC31[b], NC42[b], NC43[b], NC44[b], NC45[b], NC46[b]	Field isolates from Nakazawa, Niigata, Japan	10, 20, 21, 22, 26, 28
Sinorhizobium meliloti		
Sm1021[cd]	Denitrifying genes *napA*, *nirK*, *norCB*, and *nosZ* are located at pSymA.	9
Mesorhizobium loti		
MAFF303099[cd]	No homologue of a denitrifying gene is located in the genome.	12
Plasmid		
pαHD7	pCNTR containing RS α fragment obtained from *B. japonicum* NK5	10
pT14HD4	pCNTR containing RS β fragment obtained from *B. japonicum* T2	10
pCR2.1	Vector for TA cloning (3.9 kb)	Invitrogen
pCR110nap, pCR110nir, pCR110nor, pCR110nos	pCR2.1 derivatives containing *napA nirK norCB* or *nosZ* fragment obtained from *B. japonicum* USDA110 respectively	This study

* The HRS strains which have a high copy number and many kinds of insertion sequences[10,20–22,26,28].
[a] The strains used for determination of final products of denitrification.
[b] The strains used for phylogenic analysis by RS fingerprintings.
[c] The strains used for phylogenic analysis with the sequences of the 16S rRNA gene.
[d] The strains used for Southern hybridization with denitrifying genes, *napA*, *nirK*, *norCB*, and *nosZ*.

napA-2 5'-TAGGCCGCGCTCTTTGTGATAGGCGTC-GAA-3' amplify a 1988 bp fragment of *napA*, the primer set nirK-1 5'-GGATGCTTCAGATGTTCACCCGCA-3' and nirK-2 5'-CCTTGTCGCGTCTAGTTGGTGTTG-3' amplify a 1117 bp fragment of *nirK*, and the primer set norCB-1 5'-CTACGGCGGCTCGGCTTTTTCTTCG-3' and norCB-2 5'-GCCGGACCCAGTAGAACATAGCGAGC-3' amplify a 1683 bp fragment of *norCB*. Primer3 5'-GAG-GATCGTTTCGCATGAGCGACAGCGACAACAT-3' and Primer4 5'-CGCTCCCGATCAGACCGATTT-3' amplify the entire *nosZ* gene (1962 bp). The amplified fragments were cloned into pCR2.1 using a TA cloning kit (Invitrogen) and the fragments were confirmed as each denitrifying gene by determining 400–500 bp sequences from both ends of the cloned fragments using the M13 reverse primer and the M13 forward (−20) primer, a Big dye primer v3.0 cycle sequencing kit and an ABI prism 310 genetic analyzer (ABI). Plasmids containing the genes were digested with *Bam*HI and *Xba*I and the fragments of genes were separated from the vector by agarose electrophoresis and extracted using a QIAEX II DNA extraction kit from agarose gels (QIAGEN). The genomic DNA of *S. meliloti* 1021 and *M.*

loti MAFF303099 was used as a positive and negative control of hybridization respectively. The similarities between each probe of the denitrifying genes and the *S. melilotii* 1021 genomic sequence were calculated using FASTA (DDBJ, Mishima, http://www.ddbj.nig.ac.jp/search/fasta-j.html).

Construction of phylogenetic trees

Two phylogenetic trees were constructed from the 16S rRNA gene sequences and the relatedness of RS-fingerprints, respectively. Sequences of 16S rRNA genes were unified in length (1396–1401 bp). Multiple sequence alignments, calculations of genetic distance with Kimura's two-parameter method, bootstrap resamplings, and the construction of phylogenetic trees by the neighbor-joining method were performed using Clustal W (DDBJ, Mishima, Japan, http://www.ddbj.nig.ac.jp/). The 16S rRNA genes in the genomic sequences of *Sinorhizobium meliloti* Sm1021[9] and *Mesorhizobium loti* MAFF303011[12] were used as the outgroup.

To evaluate the relatedness of RS-fingerprints, similarity coefficients (S_{AB}) were calculated from RSα- and RSβ-specific hybridization profiles. Cluster analyses were carried out using a matrix of S_{AB} as described previously[21].

Analyzing GC content and codon usage

In order to examine the features of the individual denitrifying gene clusters, (*napDABCDE*, *nirK*, *norECBQD*, and *nosRZDFYLX*) in strain USDA110, we compared the GC content and codon usage using the Codon Usage Database (http://www.Kazusa.or.jp/codon/).

Nucleotide sequence accession numbers

The sequences of the 16S rRNA gene determined in this study have been deposited in the DDBJ database under the following accession numbers: AB231916 (USDA61), AB231917 (NC4), AB231918 (NC6), AB231919 (NC10), AB231920 (NC15), AB231921 (NC2), AB231922 (NC8), AB231923 (NC19), AB231924 (NC33), AB231925 (NC3), AB231926 (NC32), AB231927 (USDA6), AB231928 (USDA38), AB231929 (T7), and AB231930 (T9).

Results

Denitrifying capability

The denitrifying capability of indigenous strains from soybean fields in Japan has already been evaluated using the ^{15}N-TCD method[28]. For comparisons with phylogenetic positions, we continued to determine the denitrifying capability of additional reference strains; 11 strains from the USDA Germplasm Collection (*B. japonicum* USDA6, USDA38, USDA110, USDA122, USDA123, USDA124, USDA129 and USDA135, *B. elkanii* USDA61, USDA76 and USDA94), 3 field isolates NK2, NK5, NK6, aud 2281 (Table 1).

According to the end-products of denitrification from $^{15}N\text{-}NO_3^-$ in anaerobic cultures[28], the *B. japonicum* strains tested were categorized into four types (Table 2). Strains USDA110, USDA122, and NK2 stoichiometrically reduced $^{15}N\text{-}NO_3^-$ to $^{15}N\text{-}N_2$, indicating that they possessed a full set of functional denitrifying genes. On the other hand, strains USDA6, USDA38, and USDA124 reduced NO_3^- to N_2O, indicating they apparently lacked N_2O reductase activity. *B. elkanii* strains USDA61, USDA76, and USDA94 partially reduced NO_3^- into NO_2^-. USDA129, USDA123, USDA135, NK5, NK6, and 2281 were non-denitrifiers that did not reduce NO_3^- at all. The end products determined previously and in this study have been placed after the strain names in the phylogenetic trees (Fig. 1 and Fig. 2).

Phylogenetic analysis by RS fingerprinting

To examine phylogenetic relationships with denitrification phenotypes, we carried out a fingerprinting analysis using repeated sequences (RS) of RSα and RSβ, which are distributed in the *B. japonicum* genome[11]. Since indigenous strains from soybean fields in Japan have already been examined[28], we analyzed the fingerprints of additional reference strains like in the experiment above on denitrifying capability. The identification and phylogenetic analysis of *B. japonicum* strains were based on the variation in position and copy number of RSs in the genomes of respective strains, while they are stable during cultivation or nodule formation[21]. The 51 strains used for the RS phylogenetic tree are listed in Table 1 with the superscript "b". Owing to the extremely high copy numbers of RSα and RSβ, 9 strains (USDA123, USDA129, USDA135, NC3, NC32, NK5, NK6, and 2281) were identified as HRS strains which have high copy numbers and many kinds of insertion sequences[10,20–22,26,28] and autonomously excluded from the RS fingerprinting. Strain USDA129 was found to be a HRS strain in this study in addition to the previously recognized HRS strain (data not shown). The phylogenetic tree of RS fingerprints was divided into 4 major clusters (cluster I to IV in Fig. 1). *B. japonicum* were distributed in clusters I to III, while *B. elkanii* assembled in cluster IV. *B. japonicum* USDA124 was located at an isolated position between clusters III and IV. All of the strains in clusters I and III were N_2 producers. Cluster II was further divided into cluster II-1

Table 2. Denitrifying capability and genes in *B. japonicum* and *B. elkanii* strains categorized by end product from NO_3^-

Isolates	phylogenetic position		hup[b]	HRS[c]	Denitrification end prouts (%)[a]			end	Southern hybridization			
	16S rRNA gene	RS-Finger-print			N_2	N_2O	NO_2^-	NO_3^-	*napA*	*nirK*	*norCB*	*nosZ*
Bradyrhizobium japonicum, N_2 producer												
USDA110	BJ1	I	+	–	104				+	+	+	+
NC6[d]	BJ1	I	+	–	101				+	+	+	+
NC10[d]	BJ1	I	+	–	110				+	+	+	+
NC15[d]	BJ1	I	+	–	101				+	+	+	+
NK2	BJ1		+	–	102				+	+	+	+
NC2[d]	BJ1	II-1	–	–	101				+	+	+	+
NC19[d]	BJ1	II-1	–	–	108				+	+	+	+
USDA122	BJ1	III	+	–	103				+	+	+	+
NC4[d]	BJ1	III	+	–	104				+	+	+	+
Bradyrhizobium japonicum, N_2O producer												
USDA6	BJ2	II-2	–	–	–	86			+	+	+	–
USDA38	BJ2	II-1	–	–	–	88			+	+	±[e]	–
NC33[d]	BJ2	II-2	–	–	–	87			+	+	+	–
T7[d]	BJ2	II-2	–	–	–	93			+	+	+	–
T9[d]	BJ2	II-2	–	–	–	89			+	+	+	–
NC8[d]	BJ2	II-1	–	–	–	87			+	+	+	–
USDA124			–	–	–	84			+	+	+	–
Bradyrhizobium elkanii, NO_2^- producer												
USDA61	BE	IV	–	–	–	–	88	37	–	–	–	–
USDA76	BE	IV	–	–	–	–	81	34	–	–	–	–
USDA94	BE	IV	–	–	–	–	37	72	+	–	–	–
Bradyrhizobium japonicum, Nondenitrifier												
USDA129	BJ1	HRS[c]	–	+	–	–	–	106	+	+	+	–
NC3[d]	BJ1	HRS	+	+	–	–	–	89	+	+	+	–
NC32[d]	BJ1	HRS	+	+	–	–	–	122	+	+	+	–
NK5	BJ1	HRS	+	+	–	–	–	117	+	+	+	–
NK6	BJ1	HRS	+	+	–	–	–	111	+	+	+	–
USDA123	BJ2	HRS	–	+	–	–	–	100	+	+	+	–
USDA135	BJ2	HRS	–	+	–	–	–	100	+	+	–	–
2281	BJ2	HRS	–	+	–	–	–	100	+	+	–	–
T2[d]	BJ2	HRS	–	+	–	–	–	106	+	+	+	–
Sinorhizobium meliloti												
Sm1021	out-group								+	+	+	+
Mesorhizobium loti												
MAFF 303099	out-group								–	–	–	–

The 15 strains used in this study to determine denitrification end-products are listed with the superscript "a" in Table 2.

[a] The denitrification end-products were recovered from $^{15}NO_3^-$ (2 mM) after 7 days of cultivation.

[b] Genotype and phenotype of uptake hydrogenase[20–22,25].

[c] HRS, highly reiterated sequence-possessing strains carrying high copy numbers of insertion sequences, which were determined by RS fingerprinting[10,20–22,26,28].

[d] The strains determined in a previous study.

[e] Weak hybridization.

ND: not detected.

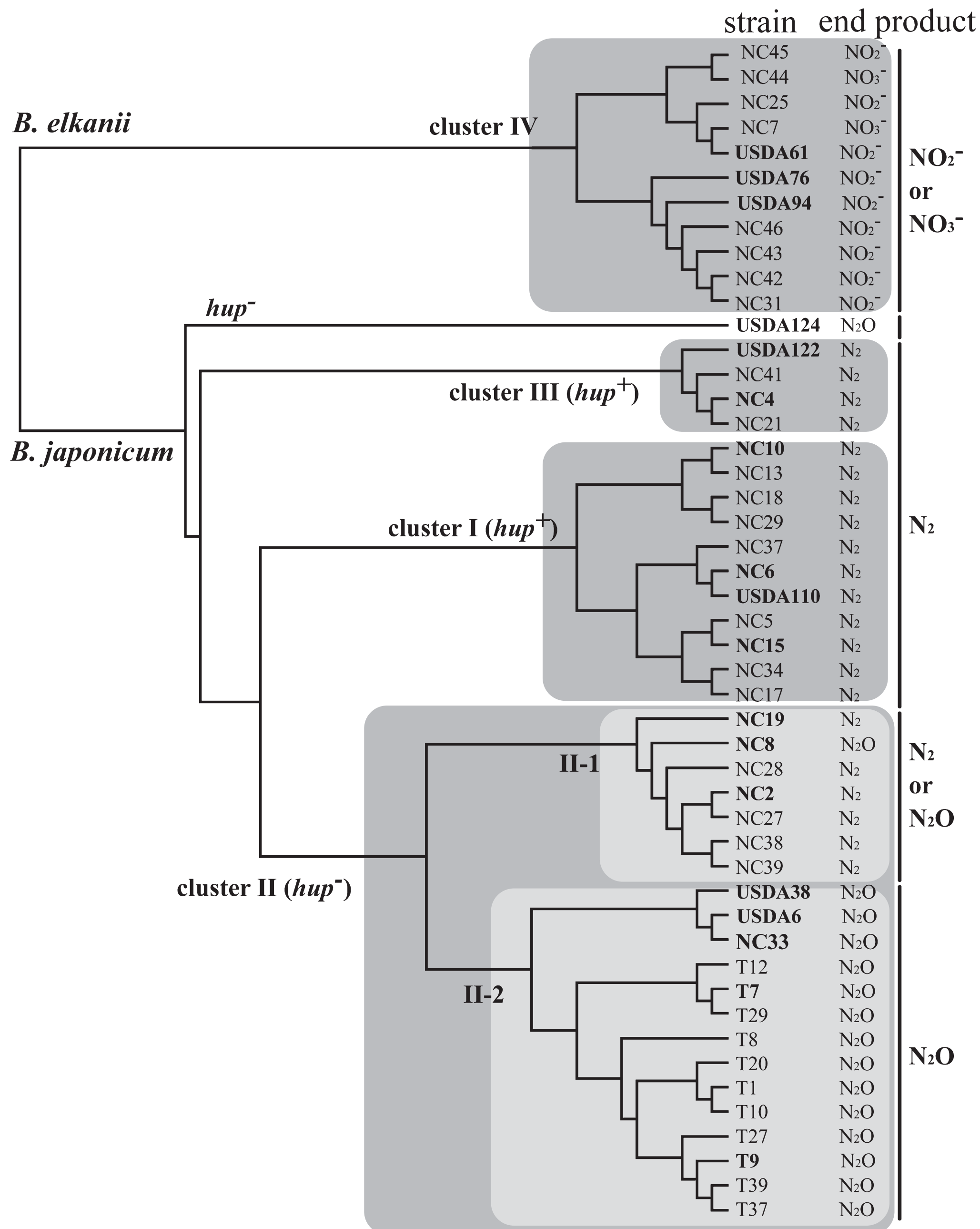

Fig. 1. Phylogenetic tree of RS-fingerprints. The 51 strains used are listed in Table 1 with the superscript "b". Because of extremely high copy numbers of RSα and RSβ, 9 HRS strains were autonomously excluded from the analysis. The tree of RS fingerprints was divided into 4 clusters (I–IV, see text). On the basis of these clusters, denitrification phenotypes were well correlated with genotype (see text).

and cluster II-2. Cluster II-1 consisted of six N_2 producers and one N_2O producer (NC8). All strains in cluster II-2 were N_2O producers. The *B. elkanii* members of cluster IV produced NO_2^- except for non-denitrifiers (NC7). The above results indicate that denitrification phenotypes were well correlated with the phylogenetic tree of RS-fingerprints. Interestingly, *hup*-positive strains of *B. japonicum* carrying uptake hydrogenase were always full denitrifiers (from NO_3^- to N_2).

Phylogenetic analysis using the 16S rRNA gene sequence

We also used 16S rRNA gene sequences to confirm the

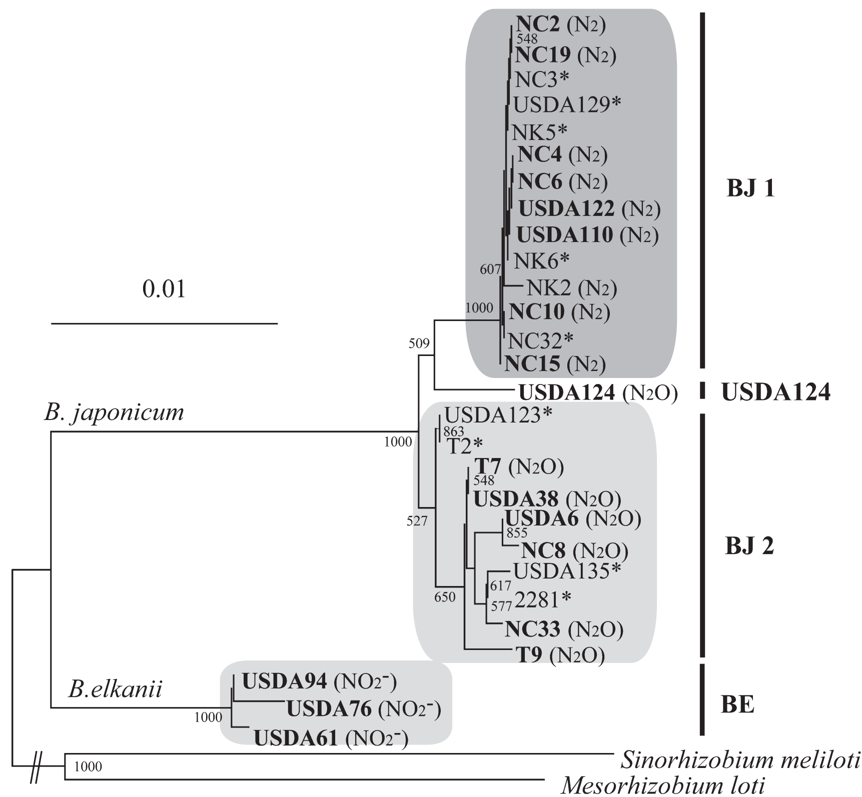

Fig. 2. Phylogenetic tree based on the 16S rRNA gene. The HRS strains are indicated with the superscript "*". We chose representatives from each cluster in the RS phylogenetic tree (Fig. 1), reference USDA strains, and HRS strains. The types of denitrification end products corresponded with each cluster (BE, BJ1 and BJ2, see text), except for non-denitrifying HRS strains. Except for the HRS strains, all members of the BJ1 cluster were N_2 producers, whereas all BJ2 members were N_2O producers. *B. elkanii* strains in the BE cluster were NO_2^- producers.

validity of the phylogeny and to analyze phylogenetic positions of the HRS strains. We chose reference USDA strains, representatives from each cluster (cluster I, II-1, II-2, III, and IV) in the RS phylogenetic tree, and HRS strains. The phylogenetic analysis showed 3 major clusters; BE (*B. elkani*), BJ1 (*B. japonicum*) and BJ2 (*B. japonicum*) (Fig. 2). *B. japonicum* USDA124 was located at an isolated position based on the 16S rRNA gene sequence (Fig. 2) as well as the RS phylogenetic tree (Fig. 1). The types of denitrification end products corresponded strictly to the three clusters, except for non-denitrifying HRS strains (Asterisked in Fig. 2). Except for the HRS strains, all members of the BJ1 cluster were N_2 producers, whereas all BJ2 members were N_2O producers. The *B. elkanii* strains in the BE cluster were NO_2^- producers.

Distribution of denitrifying genes

To compare the denitrifying activities with the presence of denitrifying genes, Southern hybridization was performed with probes for four denitrifying genes, *napA*, *nirK*, *norCB*, and *nosZ*, which were prepared from the genome of USDA110. A representative hybridization sheet is shown in Fig. 3. Hybridization was carried out under low stringent conditions so that the homologues in *S. meliloti* were detectable (Fig. 3), where the sequence similarity of *napA*, *nirK* *norCB* and *nosZ* between USDA110 probes and the *S. melilotii* 1021 genome was 74%, 75%, 84% and 77%, respectively.

The results of hybridization are all listed in Table 2. N_2-producing strains possessed four denitrifying genes (*napA*, *nirK norCB*, and *nosZ*). On the other hand, N_2O-producing strains consistently lacked the *nosZ* gene which encodes N_2O reductase, but possessed the other denitrifying genes (*napA*, *nirK* and *norCB*) though strain USDA38 showed weak hybridization with the *norCB* gene. Thus, the absence of *nosZ* probably gives rise to partial denitrification from NO_3^- to N_2O in N_2O-producers (Table 2).

The NO_2^- producing strains had no denitrifying genes except for a *napA* homologue in strain USDA94. Non-denitrifying HRS strains appeared to posses *nap*, *nir* and *nor* ho-

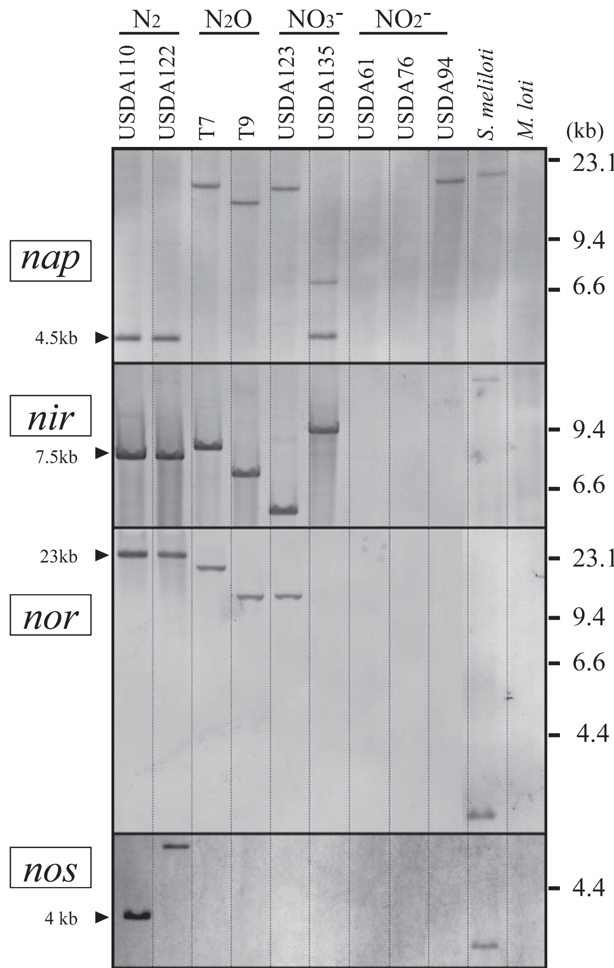

Fig. 3. A representative result of Southern hybridization with probes for the four denitrifying genes *napA*, *nirK*, *norCB*, and *nosZ* of strain USDA110. Hybridization was carried out under low stringent conditions so that the homologues in *S. meliloti* were detectable, where the sequence similarity of *napA*, *nirK norCB* and *nosZ* between the USDA110 probes and the *S. melilotii* 1021 genome was 74%, 75%, 84% and 77%, respectively.

mologues, but they consistently lacked the *nosZ* gene (Table 2, Fig. 3). These results indicate that the existence of denitrifying genes directly determines phenotype in non-HRS strains.

GC content and codon usage

The GC content of each gene cluster (*napDABCE*: 65.4%, *nirK*: 63.4%, *norECBQD*: 66.2% and *nosRZDFYLX*: 64.2%) was similar to that of the genome overall (64.7%) (column "Triplet codon" in Table 3). However, the GC content of the 3rd base of the *nosRZDFYLX* cluster (81.8%) was lower than that of the other clusters (*napDABCE*: 89.7%, *nirK*: 88.0%, *norECBQD*: 87.1%), and was similar to that of the entire genome (82.4%) (column "3rd" in Table 3). The *nap*, *nir* and *nor* clusters also differed from the *nos* cluster and entire genome in terms of frequency profiles of codon usage (data not shown).

Table 3. GC content of whole genome and denitrifying genes in *B. japonicum* USDA110 (%)

	Triplet codon	1st	2nd	3rd
Whole genome	64.7	64.8	46.7	82.4
napDABCE	65.4	60.9	45.5	89.7
nirK	63.4	61.4	40.8	88.0
norECBQD	66.2	65.9	45.5	87.1
nosRZDFYLX	64.2	63.8	47.0	81.8

In order to examine the features of the individual denitrifying gene clusters, (*napDABCDE*, *nirK*, *norECBQD*, and *nosRZDFYLX*) in strain USDA110, we compared GC content and codon usage using the Codon Usage Database.

Discussion

Correlation among denitrifying genes, phenotype and phylogeny

The results clearly demonstrated that most strains of *B. japonicum* possess the denitrifying genes *napA*, *nirK*, and *norCB* as core genes, with *nosZ* seeming to be optional (Table 2). The denitrifying capability (N_2-producing or N_2O-producing ability) of non-HRS strains in *B. japonicum* coincided well with the presence and absence of *nosZ* (Table 2). Moreover, the *nosZ* genotype well correlated with the phylogenetic positions of non-HRS strains of *B. japonicum* (Fig. 1, Fig. 2 and Table 2). The presence of HRS strains confuses the relationship between the denitrifying genes and phenotypes in *B. japonicum*.

Although HRS strains were dispersed in both the BJ1 and BJ2 clusters based on the 16S rRNA gene sequence, they all lacked *nosZ*. Two HRS strains (USDA 135 and 2281) lacked *norCB* as well. All HRS strains possessed *napA* and *nirK*. The data suggested that the HRS strains lost the function of at least nitrate reductase, as they did not show the activity (Table 2). Previous reports indicate that genomic rearrangement and shuffling occurred in HRS strains by insertion sequences[22,26,28]. Therefore, the denitrifying genes in HRS strains were probably inactivated or deleted by the insertion sequences.

In this study, a RS-fingerprint analysis autonomously identified and excluded HRS strains, which revealed the hidden and original relationships among the denitrifying genes, phenotype, and phylogeny. Therefore, *B. japonicum*

must have gained or lost *nosZ* during evolution, although the other denitrifying genes, *napA*, *nirK*, and *norBC*, were conserved.

Evolutionary consideration of denitrifying genes in B. japonicum

There are some clues of evolutional consideration (gained or lost) regarding *nosZ* in the genomic sequence of *B. japonicum* USDA110. The genome has many genomic islands termed "*trn* elements", where external DNA elements have been inserted into tRNA genes with target duplication[13]. Because a ser-tRNA gene lies adjacent to the *nos* gene cluster in the USDA110 genome[13,27], it is possible that the *nos* gene cluster had been horizontally transferred into the genome of the BJ1 lineage as a genomic island, although a target duplication of the ser-tRNA gene was not apparent. On the other hand, the analyses of GC contents and codon usage indicated that the *nos* cluster is likely adapted to the *B. japonicum* genome in terms of these parameters (Table 3, see text). From these parameters, the BJ2 lineage may have lost the *nos* cluster during the evolution of *B. japonicum*.

In each phylogenetic tree of each denitrifying gene constructed by Philippot[24], the *nir*, *nor*, and *nos* genes in strain USDA110 were all placed within the α-Proteobacteria and closest to *Rhodopseudomonas palustris*[24], which is one of the bacteria most closely related to *B. japonicum* in terms of 16S rRNA phylogeny. It is considered that *nirK*, *norCB* and *nosZ* at least probably existed from ancient times including in an ancestor of *Bradyrhizobium*[24]. Given this global phylogeny of the denitrifying genes, it is possible that some genera or groups of α-Proteobacteria have lost denitrifying genes during evolution; for example, BJ2 strains of *B. japonicum* lost *nos*, and *B. elkanii* lost *nir*, *nor* and *nos*.

On the other hand, there are also examples of denitrifying genes being "gained" through lateral gene transfer. Recently, it has emerged that the nitrite-oxidizing bacterium *Nitrobacter winogradskii* Nb-255 which is close to the genus *Bradyrhizobium* has no distinct homologues of the *nap*, *nor* and *nos* genes of *B. japonicum* USDA110, but an ortholog of *nirK* which is possessed by an ammonia oxidizeing bacterium, *Nitrosomonas europaea*[29]. Interestingly, the *nirK* of *N. europaea* belongs to the outer cluster of the proteobacteria cluster in the phylogenetic tree constructed by Philippot[24], and the lateral gene transfer of *nirK* in the nitrifying community could be considered[29]. In some organisms including a plant pathogen, *A. tumefaciens*[2], and a fast-growing rhizobia, *S. meliloti*[9], a large gene cluster (50–60 kb) carrying denitrifying genes was observed. The existence of such large clusters favors the hypothesis of a "denitrification island" that could propagate denitrifying capacity through lateral gene transfer[24]. Although the denitrifying genes *nap*, *nir*, *nor* and *nos* in *B. japonicum* USDA110 are dispersed in the genome[13], the GC content of the 3rd base and frequency profiles of codon usage of these "core" denitrifying genes are different from those of the genome overall, which might indicate exoticness. More experimental data is needed for further evolutionary consideration of the denitrifying genes in *B. japonicum*.

Ecological significance of the existence of denitrifying genes in B. japonicum

Under anaerobic conditions, for example, in paddy field soils, nitrate respiration plays an important role in growth and survival. If the supply of nitrogen oxides as terminal electron acceptors is limited in anoxic soil, N_2 producers would have an advantage over N_2O-producers in terms of energy acquisition. The theoretical energy generated by complete denitrification from 1 M of NO_3^- ($\Delta G^{0\prime}$=–560.45 kJ/mol) is 1.4 times larger than that generated by N_2O-producing partial denitrification ($\Delta G^{0\prime}$=–389.75 kJ/mol)[3]. Therefore, BJ1 type N_2-producers of *B. japonicum* may have adapted to anaerobic environments in terms of energy cost.

It is possible that the NO_2^- reductase and/or NO reductase of plant-associating *B. japonicum* are part of the system for evading the defense mechanisms of the plant NO production during formation of symbiosis, since *B. japonicum* strains basically possess the denitrifying genes *nir* and *nor*. In *A. tumefacience* C58, *nor* genes are activated by plant-derived NO in infiltrated plant cells[2]. In some organisms, it is considered that the *nir* and *nor* genes function in the detoxification of and tolerance to nitrite and nitric oxide[38]. Indeed, it has been reported that both *nir* and *nor* mutants of *B. japonicum* strain USDA110 reduce the number of nodules, plant dry weight, and nitrogen content in the presence of NO_3^-[17].

Basis for agricultural applications and environmental conservation

In a previous study, N_2O reductase activity and a high affinity for N_2O were observed in soybean roots that were nodulated by *B. japonicum* strain USDA110 carrying the *nosZ* gene[27]. On the other hand, *nosZ* mutant-inoculated root systems do not show any reduction of N_2O reduction. One promising approach to reducing the concentration of N_2O around the soybean root system during the cultivation of soybean is to use *nosZ*-possessing strains of *B. japonicum*

as field inoculants. Therefore, the screening of inoculants using the *nosZ* gene or induction of N_2O reductase activity in BJ2 strains, which are sometimes more adaptive to soil than BJ1 strains, is important to prevent increases of N_2O in the atmosphere. The findings regarding the relationship between phylogeny and denitrification could contribute to the study of indigenous populations of soybean bradyrhizobia and help to reduce N_2O emissions from soybean fields.

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