Short Communication

Diversity of Bradyrhizobium in Non-Leguminous Sorghum Plants: *B. ottawaense* Isolates Unique in Genes for N$_2$O Reductase and Lack of the Type VI Secretion System

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Diverse members of *Bradyrhizobium diazoefficiens*, *B. japonicum*, and *B. ottawaense* were isolated from the roots of field-grown sorghum plants in Fukushima, and classified into “Rhizobia” with nodulated soybeans, “Free-living diazotrophs”, and “Non-diazotrophs” by nitrogen fixation and nodulation assays. Genome analyses revealed that *B. ottawaense* members possessed genes for N$_2$O reduction, but lacked those for the Type VI secretion system (T6SS). T6SS is a new bacterial weapon against microbial competitors. Since T6SS-possessing *B. diazoefficiens* and *B. japonicum* have mainly been isolated from soybean nodules in Japan, T6SS-lacking *B. ottawaense* members may be a cryptic lineage of soybean bradyrhizobia in Japan.

Key words: *Bradyrhizobium*, sorghum, nitrogen fixation, N$_2$O reductase gene, protein secretion system

*Bradyrhizobium* was initially identified as a symbiotic N$_2$-fixing bacterium in leguminous plants (Jordan, 1982). However, non-symbiotic and/or non-diazotrophic *bradyrhizobia* have frequently been found in the soil (Van Insbergh *et al.*, 2015; Jones *et al.*, 2016) and roots of non-leguminous plants (Schneijderberg *et al.*, 2018). In the soil of North American forests (Van Insbergh *et al.*, 2015), dominant *Bradyrhizobium* lacks symbiosis islands on the genome, including *nif* and *nod* genes, for N$_2$ fixation and nodulation, respectively. Recent phylogenomic studies revealed that *Bradyrhizobium* includes species with diverse lifestyle traits, including N$_2$ fixation, nodulation, and photosynthesis, across lineages (Avontuur *et al.*, 2019; Ormeño-Orrillo and Martínez-Romero, 2019).

N$_2$ fixation by endophytic *Bradyrhizobium* has been examined in non-leguminous crops for sustainable agriculture. *B. sacchari*, *Bradyrhizobium* sp. AT1, and *Bradyrhizobium* sp. SUTN9-2 were identified as diazotrophic endophytes in sugarcane (Rouws *et al.*, 2014), sweet potato (Terakado-Tonooka *et al.*, 2013), and rice (Greetatorn *et al.*, 2019), respectively. Hara *et al.* (2019) showed that the functional N$_2$-fixing bradyrhizobia (TM122 and TM124) in sorghum roots were phylogenetically close to photosynthetic *B. oligotrophicum* S58$^8$ (Okubo *et al.*, 2013) and non-nodulating *Bradyrhizobium* sp. S23321 (Okubo *et al.*, 2012). The *nif* genes of “Free-living diazotrophs” TM122, TM124, S58$^7$, and S23321 are markedly different from those on the symbiosis islands of nodule-forming *Bradyrhizobium* (“Rhizobia”: *B. diazoefficiens* and *B. japonicum*) with respect to the G+C content of the *nifDK* genes (Okubo *et al.*, 2016), *nifV* (Hara *et al.*, 2019), and possibly *nif* gene regulation (Dixon and Kahn, 2004), which have been overlooked in previous phylogenetic studies targeting the *nifH* gene alone.

The aims of the present study were (i) to examine the abilities for free-living N$_2$ fixation and nodulation by *Bradyrhizobium* isolates that inhabit the roots of non-leguminous sorghum plants and (ii) to investigate whether the isolates have other functional differences via a genome analysis. To obtain diverse sorghum bradyrhizobia, in addition to isolates by direct isolation described previously (Hara *et al.*, 2019), we applied the legume trapping method (soybean nodulation) using the roots of sorghum obtained in a previous study (Hara *et al.*, 2019).

The roots of the sorghum line KM2 (102 d after transplant) stored at –80°C (Hara *et al.*, 2019) were surface-sterilized with 2.5% NaOCl at room temperature for 10 min, and washed ten times with sterilized water. Approximately 30 g of the root tissues were powdered with liquid N$_2$, using a mortar and pestle and 200 mL of 50 mM Tris-HCl buffer (pH 7.5) was thoroughly mixed and passed through a Miracloth (Millipore) to remove plant residues. Filtered samples were centrifuged at 9,876×g for 10 min. The pellet was suspended in 10 mL of 50 mM Tris-HCl buffer (pH 7.5), which was then inoculated into surface-sterilized seeds of the soybean cultivar Enrei in a Leonard jar assembly (Inaba *et al.*, 2012). After growing the soybean plants at 23°C for 3 weeks, the bacteroid cells of the nodule section were streaked on 1/100 strength NA agar medium (Difco™ Nutrient Broth, Becton, Dickinson and Company). After 10 d of incubation at 28°C, bacterial colonies were further purified twice by single colony isolation. Their 16S–23S rRNA gene internal transcribed spacer (ITS) sequences were
elucidated to examine whether the isolates belonged to the genus *Bradyrhizobium* (Willems et al., 2003; Saeki et al., 2013; Shiina et al., 2014).

Acetylene reduction activity (ARA) under free-living conditions was evaluated as described previously (Hara et al., 2019). Briefly, isolates were pre-cultured in HM broth medium and inoculated into test tubes containing Rennie semi-solid medium (Rennie, 1981). After 3 d of cultivation, acetylene was introduced at a final concentration of 10% (v/v) in the headspace of the test tube. The resultant ethylene concentration was assessed by gas chromatography (Hara et al., 2019). Nodulation was evaluated by performing inoculation tests on three leguminous plants: soybean (*Glycine max* cv. Enrei), cowpea (*Vigna unguiculata* cv. California black eye), and siratro (*Macroptilium atropurpureum*). *Bradyrhizobium* cells were inoculated into the surface-sterilized seeds of the three plants (10^6^ cells seed^-1^) (Hara et al., 2019). Root nodulation and plant growth were observed after cultivation under a daily light cycle of 16 h of light and 8 h of dark at 25°C for 3 (soybean) or 5 (cowpea and siratro) weeks in a growth cabinet (Koitō Electric Industries).

DNA was extracted from bacterial isolates using the Illus-tr™ Bacteria Genomic Prep Mini Spin kit (GE Healthcare). Draft genome sequences were elucidated using MiSeq (Hara et al., 2019). To analyze phylogenetic relationships, 31 single-copy genes were extracted from the draft genome using AMPHORA2 (Wu and Scott, 2012). A phylogenetic tree was constructed based on the concatenated amporha gene by MEGA v. 7.0 (Tamura et al., 2011) and the neighbor-joining method (Saitou and Nei, 1987). The completion patterns of the KEGG modules for metabolic and physiological functions were examined using the new MAPLE system v. 2.3.1 (Takami et al., 2016; Arai et al., 2018). We used GHOSTX as a homology search engine because it is markedly faster than BLAST (Arai et al., 2018). The total DNAs of SG09 and TM102 were described as previously (Minamisawa, 1990; Minamisawa et al., 1992; Rouws et al., 2014) to obtain high quality samples for a complete genome analysis. Complete genomes were elucidated using the PacBio RSII (Pacific Biosciences) platform. Island viewer4 based on the SIGI-HMM and IslandPath-DIMOB programs was used to detect genomic islands (Bertelli et al., 2017). The comparison of gene clusters and blastseq was performed using GenomeMatcher (Ohitsu et al., 2008).

We obtained 38 *Bradyrhizobium* isolates from different nodules of 20 soybean plants inoculated with the macerate of the surface-sterilized roots of field-grown sorghum, termed the “Trapping” method (Table 1 and S1). We also used 7 bradyrhizobial isolates in oligotrophic agar media from the same plant materials of field-grown sorghum roots as described previously (Hara et al., 2019) (Table 1 and S1). Based on the ITS sequence, 45 isolates from sorghum roots were grouped into 6 operational taxonomic units (OTUs), which were phylogenetically close to *B. diazoefficiens*, *B. japonicum*, *B. ottawaense*, and *Bradyrhizobium* sp. S23321 (Fig. S1).

Soybean bradyrhizobia (“Rhizobia”) often do not exhibit any N2-fixing activity under free-living conditions, but show symbiotic N2 fixation in nodule bacteroids (Kuykendall, 2005), whereas diazotrophic bacteria from non-leguminous plants and soils (“Free-living diazotrophs”) exhibit N2-fixing activity under free-living conditions (Okubo et al., 2012; Okubo et al., 2013; Terakado-Tonooka et al., 2013; Rouws et al., 2014; de Matos et al., 2017; Hara et al., 2019). To test the differential N2-fixing capability between “Rhizobia” and “Free-living diazotrophs”, the ARA of the reference strains were assessed in Rennie semi-solid medium. ARA (<0.01 nmol C2H4 h^-1^ tube^-1^) was not detected in the culture of the soybean bradyrhizobia of *B. diazoefficiens* USDA110^T^ or *B. japonicum* USDA 6^T^, irrespective of pellitice formation by cell growth (Fig. S2A and B). This is most likely due to the rhizobial *nif* gene cluster lacking *nifV*—an essential gene for the production of homocitrate, a necessary component of the FeMo cofactor present in nitrogenase—which is found in “Free-living diazotrophs” (Hakoyama et al., 2009; Okubo et al., 2016; Hara et al., 2019). In contrast, significant ARA (5–33 nmol C2H4 h^-1^ tube^-1^) was observed in the “Free-living diazotrophs” of *B. oligotrophicum* SS8^T^, *Bradyrhizobium* sp. S23321, and *Bradyrhizobium* sp. BTA11 (Fig. S2C, D, and E). This result confirmed the above criteria of N2-fixing activity between “Rhizobia” and “Free-living diazotrophs” under free-living conditions (Kuykendall, 2005). Thus, we adopted this method for the 45 isolates (Table 1 and S1). ARA was not observed among the 38 isolates with prefixes SF, SG, and SH by the trapping method or the 4 isolates obtained by direct isolation from sorghum roots (TM220, TM102, TM233, and TM239) (Table 1 and S1), whereas ARA was detected in TM221 under free-living conditions (Fig. S2F), in addition to previously reported TM122 and TM124 (Hara et al., 2019).

TM220 nodulated the leguminous plants of soybean, cowpea, and siratro, whereas the other 6 isolates (TM102, TM122, TM224, TM221, TM233, and TM239) did not (Fig. 2A).
S3). The ARA of the soybean nodules formed by TM220 was 24.3 μmol h⁻¹ plant⁻¹ (Fig. S3), suggesting that TM220 fixed N₂ symbiotically and fell into the category of “Rhizobia” (Table 1 and S1).

Based on the ARA and nodulation assays, 45 isolates of sorghum bradyrhizobia were categorized into “Rhizobia”, “Free-living diazotrophs”, or “Non-diazotrophs”: “Rhizobia” showed nodulation and symbiotic N₂ fixation abilities, but no N₂ fixation in free-living cells. “Free-living diazotrophs” lacked the ability to nodulate legumes, but possessed the capability to fix N₂ under free-living conditions. “Non-diazotrophs” lacked any potential to fix nitrogen or for nodulation (Table 1).

Draft genomes were elucidated for 16 representative isolates, including 6 OTUs (Table S1, Fig. S1) from the 38 nodule isolates and 4 direct isolates (TM220, TM221, TM233, and TM239) (Hara et al., 2019). According to the phylogenetic relationships obtained using AMPHORA, we defined three major taxonomic groups: groups D, J, and W, which corresponded to B. diazoefficiens, B. japonicum, and B. ottawaense, respectively (Fig. 1A). “Rhizobia” with the prefixes SF/SG/SH belonged to group D, J, or W. “Free-living diazotrophs” TM221, TM124, and TM122 were not included in group D, J, or W. The non-diazotrophic isolates TM102, TM233, and TM239 fell exclusively into the group W. Thus, group W members included “Non-diazotrophs” (TM102, TM233, and TM239) and “Rhizobia” (SG09 and SG11), which contained a new species, B. ottawaense (group D) in soybean bradyrhizobia (Itakura et al., 2014; Andrews et al., 2019). The existence of protein secretion systems was markedly different among the isolates or their lineages (Fig. 1B). Denitrification genes were consistently detected in members of groups D, J, and W (Fig. 1B). Denitrification generally requires four enzymes: nitrate reductase (Nap), nitrite reductase (Nir), nitric oxide reductase (Nor), and N₂O reductase (Nos) (Zumft, 1997; Jang et al., 2018; Sánchez et al., 2019). An examination of the genes of these four enzymes indicated that nos gene clusters encoding N₂O reductase were consistently found in group D and W members (black circle in Fig. 1B and S5). Although soybean bradyrhizobia carrying nosRZDYFLXL genes are able to mitigate the emission of N₂O, a greenhouse gas, from soil (Itakura et al., 2013; Akiyama et al., 2016; Saeki et al., 2017), the existence of nos genes appears to be confined within B. diazoefficiens (group D) in soybean bradyrhizobia (Itakura et al., 2013; Shina et al., 2014; Akiyama et al., 2016; Saeki et al., 2017). Thus, group W members may mitigate N₂O emission from soil as well as B. diazoefficiens. Bradyrhizobial isolates phylogenetically close to B. ottawaense from African woody legumes were recently confirmed to reduce N₂O to N₂ (Mania et al., 2019).

The existence of protein secretion systems was markedly different among the isolates or their lineages (Fig. 1B). Denitrification generally requires four enzymes: nitrate reductase (Nap), nitrite reductase (Nir), nitric oxide reductase (Nor), and N₂O reductase (Nos) (Zumft, 1997; Jang et al., 2018; Sánchez et al., 2019). An examination of the genes of these four enzymes indicated that nos gene clusters encoding N₂O reductase were consistently found in group D and W members (black circle in Fig. 1B and S5). Although soybean bradyrhizobia carrying nosRZDYFLXL genes are able to mitigate the emission of N₂O, a greenhouse gas, from soil (Itakura et al., 2013; Akiyama et al., 2016; Saeki et al., 2017), the existence of nos genes appears to be confined within B. diazoefficiens (group D) in soybean bradyrhizobia (Itakura et al., 2013; Shina et al., 2014; Akiyama et al., 2016; Saeki et al., 2017). Thus, group W members may mitigate N₂O emission from soil as well as B. diazoefficiens. Bradyrhizobial isolates phylogenetically close to B. ottawaense from African woody legumes were recently confirmed to reduce N₂O to N₂ (Mania et al., 2019).
Fig. 1. Phylogenetic relationship and functional potential of bradyrhizobial isolates from sorghum roots. (A) The phylogenetic relationship based on 31 single copies of AMPHORA housekeeping genes (Wu and Scott, 2012). The strains shaded in gray denote “Free-living diazotroph” (F). Strains framed with a black square denote “Non-diazotroph” (N). The other strains denote “Rhizobia” (R). The plant genera (italics) or soil types on the right-hand side shows the origins of the isolates. In sorghum isolates, the asterisk (*) and double asterisks (**) denote the isolates in the present study and those from previous isolates (Hara et al., 2019), respectively. (B) Functional potentials of nitrogen fixation (M00175), nodulation (M00664), denitrification (M00529), and secretion systems (M00332, M00333, and M00334) evaluated by MAPLE v. 2.3.1 (Takami et al., 2016; Arai et al., 2018). The closed and open circles in the denitrification column denote the isolates possessing genes for the denitrification steps from nitrate to dinitrogen, and nitrate to nitrous oxide, respectively. ☆ in the Type III secretion system and ★ in the Type IV secretion system denote the isolates possessing different gene organizations from the other strains, which are described in detail in panel C. † in the Type IV secretion system shows that the gene cluster was partially conserved. (C) Detailed gene organization of the Type III (T3SS), IV (T4SS), and VI (T6SS) secretion systems of the isolates. Hypothetical genes were expressed as white arrows. The genomic positions of separate T3SS gene clusters were unknown due to different contigs by the draft genome assembly of MiSeq sequences.
genes were annotated to the trb genes for conjugation (Wallden et al., 2010; Sugawara et al., 2013), while T4SS genes in SF01 were highly homologous to the vir genes for the T-DNA transfer of Agrobacterium (Wallden et al., 2010; Sugawara et al., 2013) and effector secretion of Sinorhizobium (Sugawara et al., 2013) (SF01 in Fig. 1C).

The Type III secretion system (T3SS) was well conserved in all isolates and reference strains of “Rhizobia”, which corresponded to the conventional rhc gene cluster responsible for host specificities (SF01 in Fig. 1C) (Viprey et al., 1998; Tsukui et al., 2013; Tampakaki, 2014; Sugawara et al., 2018). TM233 and TM239 (“Non-diazotrophs”) in group W possessed T3SS that resembled ysc genes encoding pathogenic T3SS of Versinia species (“Non-diazotrophs”) and Toruloides sp. nov. (2012) (Viprey et al., 1998; Cornelis, 2002; Tampakaki, 2014). To the best of our knowledge, this is the first example of pathogenic type T3SS genes existing in the genus Bradyrhizobium.

In conclusion, the usage of non-leguminous sorghum plants revealed the greater diversity of Bradyrhizobium than previously considered. Members of group W of Bradyrhizobium (B. ottawaense), with and without symbiosis islands, were indigenous to Japan. Relative to conventional soybean bradyrhizobia, their genomes possessed unique traits for the presence of nos genes and the absence of T6SS, related to their presumptive differences in competition and plant associations.

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