Short Communication

Levels of Periplasmic Nitrate Reductase during Denitrification are Lower in *Bradyrhizobium japonicum* than in *Bradyrhizobium diazoefficiens*

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Soybean is a globally important leguminous crop that generally hosts endosymbiotic dinitrogen (N₂)-fixing bacteria from the genus *Bradyrhizobium*. Under oxygen-limiting conditions, *Bradyrhizobium* strains may perform denitrification as an alternative respiratory process and sequentially reduce nitrate (NO₃⁻) to nitrite (NO₂⁻) to nitric oxide (NO), nitrous oxide (N₂O), and N₂ gases. Denitrification is functional in free-living bradyrhizobia at the soybean rhizosphere and in symbiotic bradyrhizobia inside the root nodules (Mesa et al., 2004; Sameshima-Saito et al., 2006b; Sánchez et al., 2011; Inaba et al., 2012).

*Bradyrhizobium diazoefficiens* (reclassified from *Bradyrhizobium japonicum* [Delamuta et al., 2013]; type strain USDA 110) may completely reduce NO₃⁻ to N₂. These reactions depend on napEDABC, which encodes periplasmic NO₃⁻ reductase (Nap); nirK, which encodes copper-containing NO₂⁻ reductase (NirK); norCBQD, which encodes c-type NO reductase (Nor); and nosRZDYLFX, which encodes N₂O reductase (Nos) (Sánchez et al., 2011). While *B. diazoefficiens* is a complete denitrifier, *B. japonicum* (type strain USDA 6) lacks the nos gene cluster and is, thus, unable to reduce NO₂⁻ to N₂ (Sameshima-Saito et al., 2006b; Itakura et al., 2009; Kaneko et al., 2011; Siqueira et al., 2014).

We previously screened the growth of 11 strains of *B. japonicum* and 15 strains of *B. diazoefficiens* and found that anaerobic (≤90% O₂) growth by *B. japonicum* with NO₃⁻ as the electron acceptor was significantly lower than that by *B. diazoefficiens*; however, no significant differences were observed between the growth rates of *B. japonicum* and *B. diazoefficiens* strains under microaerobic (≤2% O₂) and aerobic (≤12% O₂) growth conditions in the presence of NO₃⁻ (Siqueira et al., 2017). The lower growth rate of *B. japonicum* in anaerobiosis was not explained by the absence of nos, but by markedly impaired Nap activity that had a negative impact on the reduction of NO₃⁻ to N₂O (Siqueira et al., 2017). *B. japonicum* conserved the FixLJ–FixK regulatory cascade, which mediates the response of the nap operon to low oxygen and NO₂⁻; the binding sites for FixK/FNR (fumarate and nitrate reductase) regulators upstream of napE were also conserved (Robles et al., 2006; Bueno et al., 2017; Siqueira et al., 2017). Additionally, napA transcript levels were similar in *B. japonicum* and *B. diazoefficiens* (Siqueira et al., 2017). Thus, we proposed that impaired Nap activity in *B. japonicum* may be due to posttranscriptional events (Siqueira et al., 2017).

In the present study, we used the type strains *B. diazoefficiens* USDA 110 (Bd-USDA 110) and *B. japonicum* USDA 6 (Bj-USDA 6) to expand on our earlier research on the mechanisms responsible for impaired Nap activity in *B. japonicum* under denitrifying conditions. *Bradyrhizobium* cells were precultured at 30°C in HM salt medium (Cole and Elkan, 1973) supplemented with 0.1% L- (+)-arabinose and 0.25% (w/v) yeast extract. HM medium supplemented with trace metals (Sameshima-Saito et al., 2006a) and 10 mM KNO₃ (HMNN medium) was used in all assays. *Escherichia coli* cells were grown at 37°C in Luria–Bertani medium (Miller, 1972). The following antibiotics were used for the *Bradyrhizobium* culture: tetracycline (Tc, 100 μg mL⁻¹), spectinomycin (Sp, 100 μg mL⁻¹), streptomycin (Sm, 100 μg mL⁻¹), and polymixin B (50 μg mL⁻¹). Tc (50 μg mL⁻¹), Sp (50 μg mL⁻¹), and Sm (50 μg mL⁻¹) were used for the *E. coli* culture.

In growth experiments, precultured cells were inoculated into 34-mL test tubes containing 5 mL of HMNN medium. The initial optical density at 660 nm (OD₆₆₀) was adjusted to 0.01. In the competition experiment, initial cell numbers...
were adjusted to 3×10⁷ cells mL⁻¹ and cells were used at a 1:1 ratio. Aerobic (≈12% O₂), microaerobic (≈2% O₂), and anaerobic (≈0% O₂) treatments were prepared and monitored as previously described (Siqueira et al., 2017). Cells were grown at 30°C with reciprocal shaking at 300 rpm. Growth was monitored daily by measuring the OD₆₆₀ of the cultures, and the number of cells was directly counted using a 20-µm-deep hemocytometer (Sunlead Glass) and BX51 Fluorescence Microscope (Olympus).

To construct Bd-USDA 110 tagged with GFP and Bj-USDA 6 tagged with DsRed, pRJPaph-gfp (Ledermann et al., 2015) and pBjGroEL4::DsRed2 (Hayashi et al., 2014) plasmids were transferred by triparental mating using pRK2013 as a helper plasmid (Figurski and Helinski, 1979). Single recombination events, indicating the integration of pRJPaph-gfp into the Bd-USDA 110 chromosome or pBjGroEL4::DsRed2 into the Bj-USDA 6 chromosome, were selected based on their acquisition of resistance to Tc or Sp/Sm, respectively. Single recombinant strains were further confirmed using the BX51 Fluorescence Microscope (Olympus).

In immunoblotting and heme-staining analyses, precultured Bradyrhizobium cells were inoculated into 500-mL flasks containing 100 mL of HMNN (OD₆₆₀≈0.03) and incubated microaerobically (≈0.57 times; Fig. 1A) and anaerobically (≈0.57 times; Fig. 1A and Table S1). This result suggested that Bj-USDA 6 NapA band was >85% identity at the nucleotide level (Fig. S1). The amino acid sequences of Bj-USDA 6 NapEDABC were conserved, with 89–97% identity with Bd-USDA 110 (Fig. S1). In NapA of Bj-USDA 6, the twin arginine motif, the residues involved in the binding of the Mobis-MGD cofactor, and the [4Fe4S] cluster were conserved, except in some cases in which there was a substitution with a similar residue. In Bj-USDA 6 NapB, both the cytochrome c-binding sites and motif for translocation via the general secretory pathway were conserved. The structural similarity of the Bj-USDA 6 and Bd-USDA 110 Nap proteins was high, with a TM-score of 0.81, which indicated the same type of folding (Fig. S1). These results suggested that the structure and function of Nap were conserved in Bj-USDA 6.

We compared the amount of Nap in Bj-USDA 6 and Bd-USDA 110 cells incubated under microaerobic and anaerobic conditions in the presence of NO₃⁻. An immunoblotting analysis of soluble fractions revealed a bradyrhizobial NapA protein band at ≈100 kDa (Fig. 1A), which was consistent with the expected molecular weight of 94.5 kDa (Delgado et al., 2003). This was further confirmed by the absence of this band in soluble fractions of the B. diazoefficiens ΔnapA mutant (Fig. 1A). The intensity of the NapA band was weaker in Bj-USDA 6 than in Bd-USDA 110, in cells incubated microaerobically (≈0.57 times; Fig. 1A and Table S1), and particularly in cells incubated anaerobically (≈0.33 times; Fig. 1A and Table S1). This result suggested that Bj-USDA 6 produced less NapA than Bd-USDA 110 under low-oxygen conditions (i.e., ≤2% O₂) in the presence of NO₃⁻. Additionally, we detected NapC in the membrane fractions of bradyrhizobial cells by heme staining; the staining of heme covalently bound to c-type cytochromes (Vargas et al., 1993). Bj-USDA 6 and Bd-USDA 110 showed the typical profile of five stained bands previously identified in membrane fractions of Bd-USDA 110 (Fig. 1B) (Preisig et al., 1993; Mesa et al., 2002; Delgado et al., 2003; Bueno et al., 2008; Torres et al., 2014a). The 32-kDa and 28-kDa bands, almost co-migrating, corresponded to the FixP and FixO proteins, respectively, of cbb₃-type high affinity cytochrome oxidase, encoded by the fixNOQP operon (Preisig et al., 1993, 1996); the 25-kDa band corre-
conditions (Fig. 1B and Table S1). This result suggested that microaerobic conditions and ≈0.8-fold under anaerobic conditions; the amounts of FixP and FixO were lower in Bj-USDA 6 than in Bd-USDA 110 by ≈0.45-fold under anaerobic conditions; the amounts of FixP and FixO were lower in Bj-USDA 6 than in Bd-USDA 110 (Fig. 1B and Table S1). Thus, immunoblotting and heme-staining results indicated that Bj-USDA 6 produced lower levels of Nap under low-oxygen conditions in the presence of NO₃⁻, which was reported previously (Delgado et al., 2003). Under anaerobic conditions, the intensity of the NapC band was ≈0.3-fold weaker in Bj-USDA 6 than in Bd-USDA 110 (Fig. 1B and Table S1). Therefore, impaired Nap activity in *B. japonicum* during NO₃⁻-dependent anaerobic growth, which was reported previously (Siqueira et al., 2017), may be caused by a low level of Nap.

Based on the results of heme staining, the amount of NorC was higher in Bj-USDA 6 than in Bd-USDA 110 under microaerobic and anaerobic conditions (≈1.3- and 1.6-fold, respectively) in the absence of NO₃⁻ (Fig. 1B and Table S1). This result was supported by a previous finding showing that norB transcript levels were higher in Bj-USDA 6 than in Bd-USDA 110 under anaerobic NO₃⁻-dependent growth (Siqueira et al., 2017). Heme staining also revealed that besides NapC, FixP and FixO were the only other cytochromes affected in Bj-USDA 6 under low-oxygen conditions; the amounts of FixP and FixO were lower in Bj-USDA 6 than in Bd-USDA 110 by ≈0.45-fold under microaerobic conditions and ≈0.8-fold under anaerobic conditions (Fig. 1B and Table S1). This result suggested that cbb₃ oxidase is a critical enzyme for the adaptation process to NO₃⁻ respiration under low-oxygen conditions in *Bradyrhizobium*. Accordingly, the maximal expression of Nap does not appear to occur until the oxygen concentration becomes very low, and this is only observed after cbb₃ oxidase has consumed the oxygen present in the growth medium (Bueno et al., 2008). Since the transcription of nap genes appeared to be unaffected in *B. japonicum* cells grown anaerobically in the presence of NO₃⁻ (Siqueira et al., 2017), the remaining oxygen in the growth medium may affect the amount of the Nap protein at the posttranscriptional level by inhibiting the translation of nap messenger RNA or inducing the degradation of Nap proteins.

The mechanisms responsible for low levels of cbb₃ oxidase in *B. japonicum* under low-oxygen conditions in the presence of NO₃⁻ may be related to *B. japonicum* being unable to make an effective switch to denitrification in the absence of oxygen, similar to other bacteria (Aida et al., 1986; Bergaust et al., 2011; Torres et al., 2014b; Siqueira et al., 2017). We tested the NO₃⁻-dependent anaerobic growth of bradyrhizobial cells preincubated microaerobically, instead of aerobically as previously reported (Siqueira et al., 2017). The microaerobic preincubation resulted in a reduced growth rate of Bj-USDA 6, to a similar extent as the aerobic preincubation (Fig. S2), indicating the absence of a significant difference between a rapid (aerobic preincubation) and gradual (microaerobic preincubation) transition from aerobic to anaerobic growth. However, further studies are needed to elucidate the mechanisms responsible for the changes observed in cbb₃ oxidase levels in *B. japonicum* under denitrifying conditions.

Our previous findings prompted the hypothesis that *B. japonicum* may be less competitive than *B. diazoefficiens* due to energy depletion under anaerobic denitrifying growth (Siqueira et al., 2017). We examined the competitive growth of Bd-USDA 110 and Bj-USDA 6 tagged with GFP and DsRed proteins, respectively. The tagged strains were tested to exclude the possible effects of tagging on growth rates (Fig. S3). Under microaerobic and anaerobic conditions, in the presence of NO₃⁻, the growth of DsRed-tagged Bj-USDA 6 was lower than that of GFP-tagged Bd-USDA 110, whereas the aerobic growth rates of these two species were similar (Fig. 2). This result indicated that Bd-USDA 110 is more competitive than Bj-USDA 6 at low-oxygen levels in the presence of NO₃⁻. In *Bradyrhizobium*, growth at low-oxygen levels in the presence of NO₃⁻ is supported by both cbb₃ oxidase, which is active during free-living microaerobic growth and in N₂-fixing bacteroids, and the denitrification pathway (Preisig et al., 1993, 1996; Bueno et al., 2008). The weaker competitiveness of *B. japonicum* USDA 6 under low-oxygen conditions in the presence of NO₃⁻ may correlate with low levels of Nap (Fig. 1 and Table S1).

In conclusion, the present study demonstrated that the level of Nap under low-oxygen conditions in the presence of NO₃⁻ was lower in *B. japonicum* USDA 6 than in *B. diazoefficiens* USDA 110 and suggests that the capacity to maintain a sufficient quantity of Nap is an advantage under denitrifying conditions. The lower levels of Nap in *B. japonicum* USDA 6 presumably resulted in a competitive disadvantage against *B. diazoefficiens* USDA 110 due to energy depletion during denitrifying growth. Ecologically, this advantage of *B. diazoefficiens* over *B. japonicum* in soils exposed to low-oxygen conditions (Shiina et al., 2014).
Fig. 2. Average number of cells in co-cultures (1:1) of Bradyrhizobium diazoefficiens USDA 110 tagged with GFP (white bars) and Bradyrhizobium japonicum USDA 6 tagged with DsRed (grey bars) under anaerobic, microaerobic, and aerobic conditions in HMMN medium. Error bars indicate SE. * Values significantly different between USDA 110 and USDA 6 (t-test, grey bars) under anaerobic, microaerobic, and aerobic conditions in Argaw, A. (2014) Symbiotic effectiveness of inoculation with Bradyrhizobium japonicum USDA 110 tagged with GFP (white bars) and Bradyrhizobium japonicum USDA 6 tagged with DsRed (grey bars) under anaerobic, microaerobic, and aerobic conditions in HMMN medium. Error bars indicate SE. * Values significantly different between USDA 110 and USDA 6 (t-test, grey bars) under anaerobic, microaerobic, and aerobic conditions in Bradyrhizobium japonicum. Antimicrob Agents Chemother 4: 248–253. Delamata, J.R., Ribeiro, R.A., Ormeño-Orrillo, E., Melo, I.S., Martínez-Romero, E., and Hungria, M. (2013) Polyphasic evidence supporting the reclassification of Bradyrhizobium japonicum group Ia strains as Bradyrhizobium diazoefficiens sp. nov. Int J Syst Evol Microbiol 63: 3342–3351. Delgado, M.J., Bonnard, N., Tresierra-Ayala, A., Bedmar, E.J., and Müller, P. (2003) The Bradyrhizobium japonicum napEDABC genes encoding the periplasmic nitrate reductase are essential for nitrate respiration. Microbiology 149: 3395–3405. Figurski, D.H., and Helinski, D.R. (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci U S A 76: 1648–1652. Fischer, H.M., Velasco, L., Delgado, M.J., Bedmar, E.J., Schären, S., Zingg, D., et al. (2001) One of two hvm genes in Bradyrhizobium japonicum is functional during anaerobic growth and in symbiosis. J Bacteriol 183: 1300–1311. Hayashi, M., Shiro, S., Kanamori, H., Mori-Hosokawa, S., Sasaki-Yamagata, H., Sayama, T., et al. (2014) A thaumatin-like protein, Rj4, controls nodule symbiotic specificity in soybean. Plant Cell Physiol 55: 1679–1689. Inaba, S., Ikenishi, F., Itakura, M., Kikuchi, M., Eda, S., Chiha, N., et al. (2012) N2O emission from degraded soybean nodules depends on denitrification by Bradyrhizobium japonicum and other microbes in the rhizosphere. Microbes Environ 24: 470–476. Itakura, M., Saecki, K., Omori, H., Yokoyama, T., Kaneko, T., Tabata, S., et al. (2009) Genomic comparison of Bradyrhizobium japonicum strains with different symbiotic nitrogen-fixing capabilities and other Bradyrhizobaceae members. OMBrJ 13: 326–339. Kaneko, T., Maeta, H., Hirokawa, H., Uchiike, N., Minamisawa, K., Watanabe, A., and Sato, S. (2011) Complete genome sequence of the soybean symbiont Bradyrhizobium japonicum strain USDA65. Genes 2: 763–787. Ledermann, R., Bartsch, I., Remus-Emmermann, M.N., Vorholt, J.A., and Fischer, H.M. (2015) Stable fluorescent and enzymatic tagging of Bradyrhizobium japonicum diazoefficiens to analyze host-plant infection and colonization. Mol Plant Microbe Interact 28: 959–967. Mesu, S., Velasco, L., Manzanera, M.E., Delgado, M.J., and Bedmar, E.J. (2002) Characterization and regulation of the nitric oxide reductase-encoding region of Bradyrhizobium japonicum. Microbiology 148: 3553–3560. Mesu, S., Alech, J.D., Bedmar, E.J., and Delgado, M.J. (2004) Expression of nir, nor and nos denitrification genes from Bradyrhizobium japonicum in soybean root nodules. Physiol Plant 120: 205–212. Miller, J.H. (1972) Experiments in Molecular Genetics. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. Ohtsubo, Y., Ikeda-Ohtsubo, W., Nagata, Y., and Tsuda, M. (2008) Genome-Matcher: a graphical user interface for DNA sequence comparison. BMC Bioinformatics 16: 9: 376. Preissig, O., Anthamatten, D., and Hennecke H. (1993) Genes for a microaerobically induced oxidase complex in Bradyrhizobium japonicum are essential for a nitrogen-fixing endosymbiosis. Proc Natl Acad Sci U S A 90: 3309–3313. Preissig, O., Zafferey, R., Thiény-Meyer, L., Appleby, C.A., and Hennecke, H. (1996) A high-affinity cbb-type cytochrome oxidase terminates the symbiosis-specific respiratory chain of Bradyrhizobium japonicum. J Bacteriol 178: 1532–1538. Robles, E.F., Sánchez, C., Bonnard, N., Delgado, M.J., and Bedmar, E.J. (2006) The Bradyrhizobium japonicum napEDABC genes are controlled by the FixL-J-FixK2-NirR regulatory cascade. Biochem Biophys Res Commun 343: 108–110. Sameshima-Saito, R., Chiha, K., Hirayama, J., Itakura, M., Mitsui, H., Eda, S., and Minamisawa, K. (2006a) Symbiotic Bradyrhizobium japonicum reduces N2O surrounding the soybean root system via nitrous oxide reductase. Appl Environ Microbiol 72: 2526–2532.

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