Studies on Denitrification Process in Soybean Bradyrhizobia

Summary of Contents in this Ph.D. Thesis

Studies on Denitrification Process in Soybean Bradyrhizobia

（ダイズ根粒菌の脱窒過程に関する研究）

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Chapter I: Introduction

The nitrogen gas (N_2) composes around 78% of the atmosphere and is a necessary nutrient for all organisms. The nitrogen cycle, is composed of many steps in witch microorganisms plays a key role. The nitrogen fixation is a process in with microorganisms (plant symbionts or free-living in soil or water) are able to capture N_2 and convert into usable forms like ammonia (NH_4^+). Also, decomposers such fungi and bacteria contribute to maintain NH_4^+ levels by breaking down the cells from dead animals and plants. Nitrifying bacteria reduce the NH_4^+ to nitrate (NO_3^-) and nitrite (NO_2^-) in a process called nitrification. The NO_3^- can be directly absorbed by plants (assimilation) or reduced until nitrous oxide (N_2O) or N_2 gas, returning to the atmosphere by denitrification.

The soil is a biological, chemical and physical complex containing solid, liquid and gaseous phases. Many microorganisms are present in soil and largely concentrated in plants rhizosphere. Despite the atmosphere be mostly composed by N_2, many eukaryotic organisms, are unable to absorb and use N_2 because this molecule carries a strong and stable triple bond. In this scenario, N_2 fixing bacteria take an important role because its capability of converting atmospheric N_2 into NH_4^+. The soybean (*Glycine max* (L.) Merrill) is a leguminous and requires large amount of nitrogen to develop properly. An interesting feature of soybean plant is the capability in establish symbiosis with gram-negative α-proteobacteria N_2 fixing bacteria present in soil, known collectively as Rhizobia. The biological N_2 fixation is limited to organism that possess nitrogenage. The nitrogenase is irreversibly deactivated by oxygen, thus, requiring anoxic conditions. When rhizobia meet the soybean plant, there is an interactive and connected activity that lead to the development of special structures at the root, called nodules. Inside the nodules the biological N_2 fixation occurs.

Microorganisms known as denitrifiers sustain the denitrification, defined as the sequential reduction of $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$ mediated by nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase (encoded by *napA, nirK, norCB* and *nosZ* genes), respectively. This process in bacteria is triggered by environmental characteristics such as nitrogen oxide availability and oxygen tension. *Bradyrhizobium diazoefficiens* (formely classified as *Bradyrhizobium japonicum*) is a facultative anaerobic bacteria, belonging to Rhizobia group. It was already described that *B. diazoefficiens* can

reduce NO_3^- into N_2 when growing with nitrate anaerobically. In *B. diazoefficiens, napEDABC*, *nirK*, *norCBQD* and *nosRZDYFLX* encode the periplasmic nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase, respectively, that are responsible for maintain the denitrification process.

Chapter II: Lower Performance of Anaerobic Reduction of Nitrate to Nitrous Oxide in *Bradyrhizobium japonicum* compared to *Bradyrhizobium diazoefficiens*

When soil oxygen level drops, some bradyrhizobia can use denitrification as alternative respiration. *Bradyrhizobium diazoefficiens* (nos+) completely denitrifies nitrate (NO_3^-) to dinitrogen, but *Bradyrhizobium japonicum* (nos−) is unable to reduce nitrous oxide to dinitrogen. It was demonstrated that the occurrence of bradyrhizobia with *nosZ* (*nosZ*$^+$) or without *nosZ* (*nosZ*$^-$) in Japanese soybean fields largely depends on the soil type, suggesting that *B. diazoefficiens* (*nosZ*$^+$) is predominant in Gleysol soils, whereas *B. japonicum* (*nosZ*$^-$) is predominat in Andosols. Thus, the aim of the present study was to identify key physiological traits in denitrification that differentiate the distribution of *B. diazoefficiens* and *B. japonicum* in soybean fields in Japan.

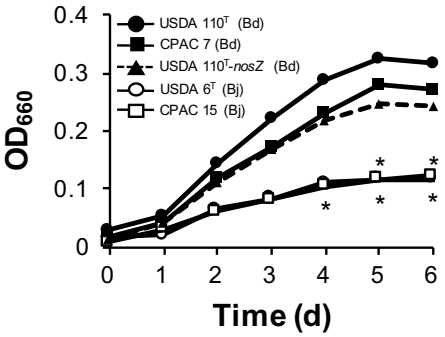

Figure 1. Anaerobic growth of *B. japonicum* (USDA 6^T and CPAC 15) and *B. diazoefficiens* (USDA 110^T, CPAC 7, and USDA 110^T-*nosZ* mutant) in HMMN medium. *Values significantly different from those of *B. diazoefficiens* USDA 110^T (*t*-test, $P<0.05$; $n=3$).

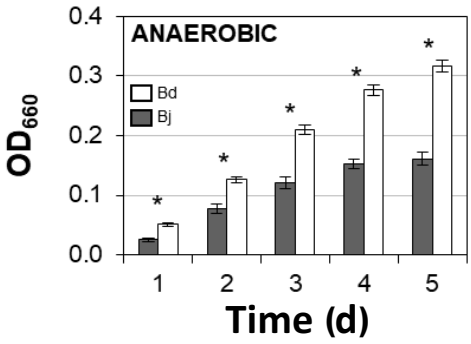

Figure 2. Average growth profiles of *B. diazoefficiens* (15 strains) and *B. japonicum* (11 strains) the indicated conditions in HMMN medium; error bars indicate SE. *Values significantly different between *B. diazoefficiens* and *B. japonicum* (*t*-test, $P<0.001$; $n=3$). Bd, *B. diazoefficiens*; Bj, *B. japonicum*.

Cells were precultured for 72 h at 30°C in HM salt medium (8) supplemented with 0.1% L-(+)-arabinose and 0.25% (w/v) yeast extract. HM medium supplemented with trace metals (16) and 10 mM KNO_3 (HMMN medium) was employed for denitrification assays. Foam stoppers were used for aerobic treatment, whereas butyl rubber stoppers were used for anaerobic (100% N_2) and microaerobic (2% O_2 [vol/vol], N_2 balance) treatments.

Anaerobic growth of *B. japonicum* USDA 6^T and CPAC 15 with NO_3^- as the electron acceptor was significantly lower than that of *B.*

diazoefficiens USDA 110^T and CPAC 7 in means of optical density and cell number (Fig. 1). However, no differences were observed between the growth of *B. japonicum* and *B. diazoefficiens* strains under aerobic and microaerobic conditions in the presence of NO_3^-. The growth of the *nosZ* mutant USDA 110^T (USDA 110^T-*nosZ*) was similar to that of wild-type USDA 110^T, indicating that the N_2O reduction step did not markedly contribute to the difference in growth between *B. japonicum* and *B. diazoefficiens* under anaerobic NO_3^--respiring conditions (Fig. 1). To examine whether the difference in growth is extended to the species level, 11 strains of *B. japonicum* and 15 strains of *B. diazoefficiens* were selected randomly. In the presence of NO_3^-, the mean growth of *B. japonicum* strains was significantly lower than that of *B. diazoefficiens* strains under anaerobiosis (Fig.2), but not under aerobiosis and microaerobiosis. NO_3^- and N_2O concentrations in batch cultures of *B. japonicum* USDA 6^T and CPAC 15 and *B. diazoefficiens* USDA 110^T and CPAC 7 and *nosZ*- mutant was monitored under anaerobic NO_3^--respiring conditions. *B. japonicum* strains consumed less NO_3^- and produced less N_2O than did the USDA 110^T-*nosZ* mutant. Also, periplasmic nitrate reductase (Nap) activity was markedly lower in *B. japonicum* (USDA6^T and CPAC 15) than in *B. diazoefficiens* (USDA 110^T, CPAC 7 and USDA 110^T-*nosZ*) under anaerobic NO_3^--respiring conditions. However, *napA* expression was not significantly different between *B. diazoefficiens* and *B. japonicum* suggesting that the low efficiency in NO_3^- reduction in *B. japonicum* relies on posttranscriptional events.

(Siqueira *at al.* 2017 - Microbe and Enviroments, 32: 398-401)

Chapter III: Evaluations of Overexpression of Nap in *Bradyrhizobium japonicum*

In bradyrhizobia, Nap responsible for the dissimilatory reduction of NO_3^- to nitrite (NO_2^-) is encoded by *napEDABC* genes. A mutant of *B. japonicum* that overexpresses the *napEDABC* cluster by strong *rrn* promoter was constructed to access if the overexpression of Nap would restore the NO_3^- reduction efficiency in *B. japonicum* to *B. diazoefficiens* level. The anaerobic with KNO_3^- of *B. japonicum* USDA 6 mutants was similar to that of the wild-type USDA 6^T, suggesting that the high level of transcripts does not contribute to improve the growth of USDA 6 under an anaerobic NO_3^--respiring conditions. However, *napA* expression of AS mutants was inferior to wild-type USDA 6^T (data not shown) and the same results were obtained with *napC* expression (data not shown) probably due a promoter incompatibility. To clarify the objectives new mutants will be constructed using different candidate promoters for *B. japonicum* USDA 6.

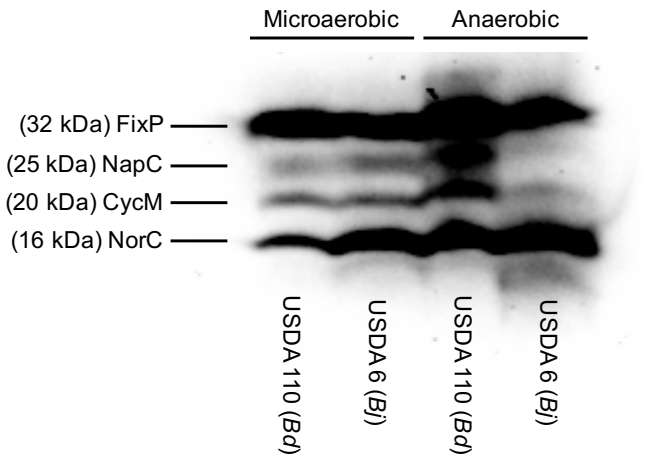

Figure 3. Haem-staining proteins from *Bradyrhizobium diazoefficiens* (USDA 110) and *Bradyrhizobium japonicum* (USDA 6) cells in microaerobic conditions (left) and anaerobic conditions (right). Each lane contains about 20 µg of protein and 15 min of signal accumulation. **B. diazoefficiens* = Bd, *B. japonicum* = Bj.

Genetic comparison of *napEDABC*, *nirK* and *norCBQD* cluster of *B. diazoefficiens* USDA 110 and *B. japonicum* USDA 6 indicate high identity at nucleotide level (≥ 85%) and NapE, NapD, NapA, NapB and NapC the shared identity was 96%, 89%, 94%, 97% and 93%, respectively. NapA and NapB (the catalytic and electron-transfer subunits of Nap, respectively) amino acid sequences shared 94-97% identity among USDA 110^T and USDA 6^T and conserved the motifs involved in catalysis, which suggest functional conservation of Nap in *B. japonicum.* The amount of Nap protein produced by *B. japonicum* and *B. diazoefficiens* under anaerobic NO_3^--respiring conditions was determined indirectly by haem-staining of NapC. Four stained bands of 32, 25 20 and 16 kDa were detected in membranes of *B. japonicum* and *B. diazoefficiens (*Fig. 3). NapC may correspond to the 25-kDa band. The 32-kDa band may correspond to FixP and FixO proteins (that are co-migrating) of the cbb_3-type, high affinity cytochrome oxidase encoded by the *fixNOQP* operon and the 20-kDa and 16-kDa bands may correspond to CycM and the NorC subunit of the nitric oxide reductase.

Under anaerobic conditions with NO_3^-, the band representing NapC (~25 kDa) was detected with high intensity in *B. diazoefficiens* but not in *B. japonicum*. This result suggests that, despite *napA* transcript levels were the same between *B. japonicum* and *B. diazoefficiens* cells, *B. japonicum* produce very low amount of NapC and consequently, Nap. These results supported my hypothesis that post-transcriptional effects are the main reason for the low efficiency in NO_3^- reduction in *B. japonicum.*

Chapter V: Competition of *Bradyrhizobium japonicum* and *Bradyrhizobium diazoefficiens* under anaerobic nitrate respiring growth

As an ecological approach, it has been suggested that *B. diazoefficiens* (*nos*+) is predominant in Gleysol soils, rich in low-oxygen conditions, whereas *B. japonicum* (*nos*-) is predominant in Andosols, rich in aerobic enviroments. To evaluate the competitiveness of *B. japonicum*, the growth of *B. diazoefficieins* USDA 110 lacking *nosZ* gene (*nosZ*-) and *B. japonicum* USDA 6 wild-type cohabiting the same culture environment under a NO_3^--respiring condition was analyzed. Results indicated that USDA 110 *nosZ*- possessed higher competitiveness than USDA 6. Taken together, results indicate that the capacity in nitrate reduction may be the main factor that drive the distribution of bradyrhizobia in soybean fields in Japan.

Chapter VI: Conclusions and General Discussion

In conclusion, when *B. japonicum* was exposed to anaerobic environment with NO_3^-, the cells had low performance of Nap enzyme and were unable to synthesize the enough amount of Nap due to post-transcription events. As a consequence, the *B. japonicum* cells are unable to use all NO_3^- for respiration, leading to less energy available for cell maintenance, growth, and competitiveness compared to *B. diazoefficiens.* These characteristics may be an important factor driving the distribution of *B. japonicum* and *B. diazoefficiens* in soybean fields in Japan.

Keywords: Denitrification, Bradyrhizobia, Periplasmic Nitrate Reductase.

Publication:

Siqueira AF, Minamisawa K, Sánchez C. 2017. Anaerobic reduction of nitrate to nitrous oxide is lower in *Bradyrhizobium japonicum* than in *Bradyrhizobium diazoefficiens*. Microbes Environ. 32: 398-401.