Linked expression of nap and nos genes in a Bradyrhizobium japonicum mutant with increased \( N_2O \) reductase activity

Cristina Sánchez, Manabu Itakura, Hisayuki Mitsui and Kiwamu Minamisawa$^a$

Graduate School of Life Sciences, Tohoku University, 2-1-1 Katahira, Aoba-ku, Sendai 980-8577, Japan

Running title: Linked expression of nap and nos genes in \( B. \ japonicum \)

$^a$Corresponding author: kiwamu@ige.tohoku.ac.jp

Abstract

To understand the mechanisms underlying the increased \( N_2O \) reductase activity in the \( B. \ japonicum \) 5M09 mutant from enrichment culture under \( N_2O \) respiration, we analyzed the expression of genes encoding denitrification reductases and regulators. Our results suggest a common regulation of nap (encoding periplasmic nitrate reductase) and nos (encoding \( N_2O \) reductase).

Nitrous oxide (\( N_2O \)) is a key greenhouse gas that also damages the ozone layer. Soybean nodules emit \( N_2O \) under field conditions in the late growth period (1–3). While \( N_2O \) generation is due to a diversity of microbial enzymes, \( N_2O \) consumption appears to be due exclusively to \( N_2O \) reductase (Nos) (4–6). Nos catalyzes the two-electron reduction of \( N_2O \) to \( N_2 \). In strain USDA110 of the soybean symbiont \( B. \ japonicum \), the complete reduction of nitrate to \( N_2 \) depends on napEDABC (which encode periplasmic nitrate
reductase: Nap), nirK (copper-containing nitrite reductase: NirK), norCBQD (c-type nitric oxide reductase: cNor), and nosRZDYFLX (Nos) (7).

A *B. japonicum* mutant, 5M09, with increased N₂O reductase activity (Nos++) was isolated through the impairment of proofreading activity and the use of enrichment culture under selection pressure for N₂O respiration (8). Recently, it has been shown that postharvest N₂O emissions from soybean fields can be mitigated by inoculation with 5M09 at the field scale (9). Because the mechanisms underlying the increased Nos activity in 5M09 remain unclear, the main objective of this work was to evaluate the expression of genes encoding denitrification reductases and regulators in 5M09. A *B. japonicum* mutant (PRNOS) that overexpresses the nos genes as controlled by the rRNA gene promoter (9) was used as a Nos++ reference.

*B. japonicum* USDA110 (United States Department of Agriculture, Beltsville, MD, USA), and 5M09 (8) and PRNOS (9) mutant derivative strains were used in this study. The bacterial strains and plasmids used in this work are listed in Supplemental Table S1. Cells were routinely cultured at 30 °C in HM salt medium (10) supplemented with 0.1% arabinose, 0.025% (w/v) yeast extract, and trace metals (HMM medium) (11). For β-galactosidase assay, methyl viologen (MV)-dependent nitrate reductase assay, and RNA isolation, aerobically grown cells were collected and washed twice. They were then adjusted to an optical density at 660 nm of about 0.1 by the addition of HMM medium. Cells (10 mL) were incubated for 24 h in 120-mL airtight vials with air (aerobic conditions) or N₂ (anaerobic conditions). Where appropriate, either N₂O (1%, 5%, 20%, or 25% [v/v]) or KNO₃ (10 or 20 mM) was added. For β-galactosidase assays, we used chromosomally integrated transcriptional lacZ fusions with the napE, nirK, norC, and nosZ promoters. The plasmids pBG0614 (12), pRJ2498 (13), pRJ2499 (13), and pNOSLZch were integrated by homologous recombination into the chromosome of each of USDA110 and 5M09. β-Galactosidase activity was
determined with permeabilized cells as described (14). Total RNA was prepared and 
quantitative reverse-transcription PCR (qRT-PCR) was performed as described (9). MV-
dependent nitrate reductase assay was performed as described (15).

We compared the growth ability of USDA110 with that of 5M09 and PRNOS (both 
Nos++ strains), and a USDA110ΔnosZ mutant (16) as a negative control. *B. japonicum*
USDA110 was able to grow anaerobically, using exogenous N₂O as the sole electron acceptor 
(Fig. 1). This is unlike *Pseudomonas aeruginosa* PAO1, which cannot grow on exogenous 
N₂O as the only electron acceptor due to a regulatory effect caused by a lack of nitric oxide as 
the inducer for nos expression (17–19). The growth ability of 5M09 and PRNOS was 
significantly higher than that of USDA110 (Fig. 1). This likely resulted from higher Nos 
activity in the Nos++ mutants. Previously, Nos activities of 5M09 and PRNOS were reported 
to be approximately 5 and 9.5 times the USDA110 values, respectively (9). We observed no 
significant differences in growth ability of any of the strains cultured anaerobically with NO₃⁻ 
as the final electron acceptor (Fig. S1).

As in many other denitrifiers, the expression of denitrification genes in USDA110 
requires both oxygen limitation and the presence of a nitrogen oxide (NOₓ) (20). We analyzed 
β-galactosidase activity in 5M09 cells carrying the PnosZ-lacZ fusion. Cells were incubated 
aerobically or anaerobically with or without 10 mM KNO₃ or (anaerobic only) 5% N₂O (Fig. 
2). β-Galactosidase activities under aerobic (None), anaerobic (None), and anaerobic + N₂O 
conditions in 5M09 were about double those in USDA110 (Fig. 2). As previously reported 
(21), expression of PnosZ-lacZ was greatest in cells grown anaerobically with NO₃⁻ as an 
electron acceptor, indicating that not only anaerobiosis but also an NOₓ is required for 
maximal induction of nosZ. When NO₃⁻ was added to both aerobic and anaerobic incubations, 
USDA110 and 5M09 exhibited similar levels of β-galactosidase activity (Fig. 2), suggesting 
that the mechanism for nosZ regulation in 5M09 was not effective in the presence of NO₃⁻.
Anaerobic levels of β-galactosidase activity did not change (in USDA110 or 5M09) regardless of the presence of N₂O (Figs. 2, S2). These results would exclude N₂O as a regulator of nosZ transcription in *B. japonicum*. Consistent with this, it has been reported that N₂O does not appear to regulate the expression of any denitrification gene (5). In *B. japonicum* USDA110, the involvement of the FixLJ/FixK₂ regulatory cascade in the microaerobic induction of nap, nirK, and nor is well established (20). The NnrR protein expands control by the FixLJ/FixK₂ regulatory cascade in response to NOₓ (20). In addition, the involvement of the RegSR/NifA cascade in the full expression of nap, nirK, and nor has recently been proposed (20). We analyzed the expression of denitrification reductases (napA, nirK, norC, nosR, nosZ, nosD) and regulators (fixL, fixK₂, nmrR, regS, and nifA) in USDA110, 5M09 and PRNOS by qRT-PCR. Since 5M09 was previously isolated under N₂O-respiring conditions (5% N₂O) (8), we used this condition for the qRT-PCR study. We compared the change of expression (relative to USDA110) in 5M09 with that in PRNOS (Table 1). Expression of nosZ, nosD, and nosR genes was enhanced in both strains. In PRNOS, nosR expression was 9× that in 5M09, and nosZ and nosD expression was 40× that in 5M09. Since nosR has been suggested to be a Nos expression regulator (17), the positive regulation of nosZ and nosD in PRNOS was likely due to overexpression of nosR in this strain (9). The expression of genes encoding regulators (fixL, fixK₂, nmrR, regS, nifA), NirK (nirK), and Nor subunit C (norC) showed similar responses between 5M09 and PRNOS (Table 1). This regulation may be a consequence of the Nos⁺⁺ phenotype of 5M09 and PRNOS. Only napA (which encodes the Nap large subunit precursor) was differentially regulated: it showed a 2.6× change in 5M09 but no significant change in PRNOS (Table 1).

To further investigate this regulation, we analyzed the β-galactosidase activity of PnapE-lacZ, PnirK-lacZ, PnorC-lacZ, and PnosZ-lacZ in USDA110 and 5M09 (Table S2). Similar to the qRT-PCR results, β-galactosidase activity mediated by PnapE-lacZ and PnosZ-lacZ in
5M09 was about 2.2 and 3.2 times, respectively, that in USDA110. In addition, Nap activity in 5M09 was about 2.7× that in USDA110 and 4.0× that in PRNOS (Table S3).

These results suggest that regulation of nap and nos genes in 5M09 is not dependent on the FixLJ-FixK2-NnrR and RegSR-NifA regulatory cascades. Thus we suggest that a regulatory network, that is independent from the above mentioned and is commonly regulating nap and nos genes, may be mutated in 5M09 strain. Given the potential of 5M09 to mitigate N2O emissions in soybean fields (9), a better understanding of this regulation is an essential issue to explore.

References


Acknowledgements

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**Figure legends**

**Figure 1.** Growth of *B. japonicum* USDA110, 5M09, PRNOS, and USDA110 ΔnosZ cells in HMM medium under anaerobic conditions with 25% (v/v) N₂O as an electron acceptor. Values are means of two different starter cultures, grown in duplicate. *Values differ significantly from USDA110 (P < 0.05, t-test).*

**Figure 2.** β-Galactosidase activity mediated by a PnosZ-lacZ transcriptional fusion in *B. japonicum* strains USDA110 and 5M09 incubated under different conditions. Data, in Miller units (MU), are means ± SD of at least 3 cultures that were assayed in triplicate. Bars within the same condition labeled with the same letter do not differ significantly (P < 0.05, Tukey’s HSD test). Concentrations used were 5% (v/v) N₂O and 10 mM KNO₃.
Table 1. Expression analysis of denitrification genes in *B. japonicum* 5M09 and PRNOS.

Gray shading indicates differential regulation between 5M09 and PRNOS.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description (22)</th>
<th>Change of expression relative to USDA110&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5M09</td>
</tr>
<tr>
<td>reductases</td>
<td></td>
<td></td>
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<tr>
<td><em>napA</em></td>
<td>Periplasmic nitrate reductase large subunit precursor</td>
<td>2.64 ± 0.73*</td>
</tr>
<tr>
<td><em>nirK</em></td>
<td>Copper-containing nitrite reductase</td>
<td>0.22 ± 0.06*</td>
</tr>
<tr>
<td><em>norC</em></td>
<td>NO reductase subunit C</td>
<td>0.20 ± 0.05*</td>
</tr>
<tr>
<td><em>nosZ</em></td>
<td>N₂O reductase</td>
<td>2.59 ± 0.36*</td>
</tr>
<tr>
<td><em>nosD</em></td>
<td>Periplasmic copper-binding precursor</td>
<td>2.83 ± 1.43*</td>
</tr>
<tr>
<td><em>nosR</em></td>
<td>N₂O reductase expression regulator</td>
<td>2.50 ± 0.74*</td>
</tr>
<tr>
<td>regulators</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>fixL</em></td>
<td>Two-component oxygen-sensor histidine kinase</td>
<td>1.79 ± 0.57*</td>
</tr>
<tr>
<td><em>fixK2</em></td>
<td>Transcriptional regulatory protein Crp family</td>
<td>0.49 ± 0.10*</td>
</tr>
<tr>
<td><em>nnrR</em></td>
<td>Transcriptional regulatory protein Crp family</td>
<td>0.24 ± 0.13*</td>
</tr>
<tr>
<td><em>regS</em></td>
<td>Two-component sensor histidine kinase</td>
<td>2.97 ± 1.00*</td>
</tr>
<tr>
<td><em>nifA</em></td>
<td>nif-specific regulatory protein</td>
<td>3.01 ± 1.02*</td>
</tr>
</tbody>
</table>

*The relative expression of the target gene was calculated by the *2−ΔΔCt* method (23). Values are the ratio of gene expression (normalized to *sigA*) relative to that in USDA110. *Values differ significantly from USDA110 (*P* < 0.05, Tukey’s HSD test). Data are means of two independent RNA samples isolated from four replicates.