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Abstract

N$_2$O is the major greenhouse gases influencing global warming, and agricultural land is the predominant (anthropogenic) source of N$_2$O emissions. Here, we report the high N$_2$O-reducing activity of *Bradyrhizobium ottawaense*, suggesting the potential for efficiently mitigating N$_2$O emission from agricultural lands. Among the 15 *B. ottawaense* isolates examined, the N$_2$O-reducing activities of most (13) strains were approximately 5-fold higher than that of *Bradyrhizobium diazoefficiens* USDA110$^T$ under anaerobic free-living conditions. This robust N$_2$O-reducing activity of *B. ottawaense* was confirmed by N$_2$O reductase (NosZ) protein levels and in the soybean rhizosphere after nodule decomposition. While the NosZ of *B. ottawaense* and *B. diazoefficiens* showed high homology, nosZ gene expression in *B. ottawaense* was over 150-fold higher than that in *B. diazoefficiens* USDA110$^T$, suggesting the high N$_2$O-reducing activity of *B. ottawaense* is achieved by high nos expression. Furthermore, we examined the nos operon transcription start sites and found that, unlike *B. diazoefficiens*, *B. ottawaense* has two transcription start sites under N$_2$O-respiring conditions, which may contribute to the high nosZ expression. Our study proposes the potential of *B. ottawaense* for effective N$_2$O reduction and unique regulation of nos gene expression that contributes to the high performance of N$_2$O mitigation in the soil.
Introduction

The expansion of human activities is triggering irreversible environmental damage, including global warming and stratospheric ozone depletion. N$_2$O is a long-lived greenhouse gas (GHG) whose atmospheric lifetime is an estimated 116 ± 9 years [1]. Moreover, N$_2$O has a stratospheric ozone-depleting effect. Although N$_2$O concentration in the atmosphere is still low compared with other GHG such as CO$_2$ and CH$_4$, N$_2$O is an alarming GHG due to its high global warming potential per unit [2]. Agricultural land is the primary source of N$_2$O, accounting for 52% of anthropogenic origin emissions [3]. N$_2$O is markedly emitted from nitrogen-rich environments, such as agricultural fields in which excess N fertilizers are applied and crop residues, including nodulated legume roots [4, 5]. Biochemically, microbial nitrification and denitrification are the two major processes of N$_2$O generation [6, 7]. During nitrification, N$_2$O is produced as a byproduct when ammonia is oxidized to nitrite via hydroxylamine. N$_2$O is also generated from NO during incomplete denitrification, which intricately involves diverse soil bacteria, fungi, and archaea [8]. However, to date, only one microbial enzyme, N$_2$O reductase (encoded by the nosZ gene), reportedly reduces N$_2$O to N$_2$ [7].

Since some rhizobial species possess the nosZ gene, strategies to reduce N$_2$O emissions from agricultural fields using rhizobia have been studied. In particular, soybeans are grown globally, and the amount of N$_2$O emitted from soybean fields is higher than that from corn or wheat. For example, N$_2$O emissions from soybean fields in Argentina are estimated to reach 5.1 kg N ha$^{-1}$ yr$^{-1}$ [9]. The use of rhizobia is, therefore, an effective approach to reducing global GHG emissions.

*Bradyrhizobium* nodulates various legumes, including soybean, and has been studied as a model denitrification microorganism. Soybean roots nodulated with *Bradyrhizobium diazoefficiens* USDA110$^T$ scavenges exogenous N$_2$O, even in ambient air containing a low concentration of N$_2$O (0.34 ppm) [10]. Moreover, N$_2$O fluxes from soybean fields have been mitigated by inoculation with *B. diazoefficiens* mutants with high N$_2$O reductase activity (Nos$^{++}$ mutants) [11]. The utility of *B. diazoefficiens* in N$_2$O mitigation has also been verified in soybean ecosystems in Japan [12], France [13], and South America [14].

On the other hand, rhizobial strains carrying nos genes are uncommon; nos genes and N$_2$O-reducing activity have been observed only in *B. diazoefficiens*, soybean rhizobia [10], and *Ensifer melliloti*, an alfalfa endosymbiont [15]. Several soybean rhizobia species, including *B. diazoefficiens*, *B. japonicum*, *B. elkanii*, and *Ensifer fredii*, have been identified, but most soybean rhizobia in Japan and the world are non-nos-possessing (nos$^-$) species [16]. However, nos gene clusters have been recently found in *Bradyrhizobium ottawaense* [17] and *Rhizobium leguminosarum* [13], suggesting
that the strategy for mitigating N₂O emissions from the legume rhizosphere using rhizobia could be expanded to various legume and rhizobial species.

The highest N₂O emissions from the rhizosphere occur after soybean harvest during nodule decomposition [11]. Nitrification is promoted when the accumulated N and rhizobia are released from decomposed nodules into the soil: N₂O is released by denitrification around nos⁻ or even nos⁺ strains due to the balance between the generation and reduction of N₂O. Therefore, to effectively prevent N₂O release, using rhizobia with high N₂O-reducing activity in the free-living state is necessary.

Denitrification reactions involving N₂O reduction occur under anaerobic conditions. In bradyrhizobia, N₂O reductase (nos) genes are regulated by three different two-component regulatory systems [18]. The FixLJK₂ cascade is the primary oxygen-sensing regulator for nos operons. Under moderate low oxygen concentration conditions (<5%), FixLJK₂ recognizes the FixK box [TTG(A/C)-N6-(T/G)CAA] located upstream of nosR and promotes nos operon expression [19, 20]. It has also been shown that the NasST two-component regulatory system, which senses NO₃⁻ concentrations and regulates the NO₃⁻ assimilation gene (nas) operon, is also responsible for regulating the nos operon [21]. NasT act as activators of the nas/nos operons and NasS acts on NasT, inhibiting its function: in the absence of NO₃⁻/NO₂⁻, NasS and NasT bind to each other, and transcription is arrested by the terminator structure upstream of the nas/nos operon. On the other hand, in the presence of NO₃⁻/NO₂⁻, NasT is released from NasS and binds to the mRNA upstream of the nos operon, resulting in a conformational change in the hairpin termination structure of the mRNA and read-through transcription of the nos genes [18]. In nasS deletion mutants, transcription of the nos operon is activated independently of NO₃⁻. Itakura et al. [11] developed nos⁺⁺ strains from naturally occurring nasS mutants and verified their utility in N₂O reduction in laboratory and field experiments. Additionally, the RegSR two-component regulatory system presumably controls nosR expression via the NifA protein [22].

In this study, we characterized nos-possessing B. ottawaense strains isolated from sorghum roots based on their genome sequence and activity. Most B. ottawaense strains showed significantly higher N₂O-reducing activity than that of B. diazoefficiens USDA110ᵀ. Gene expression and promoter analyses showed that B. ottawaense strongly expressed the nosZ gene under both N₂O- and NO₃⁻-reducing conditions, and its high-level expression is thought to be achieved by different nos operon transcription start sites and not by already known regulation systems. Our study proposes the potential of B. ottawaense in N₂O mitigation and the unique regulation of nos gene expression that
contributes to the high performance of N₂O reduction.

Results

**B. ottawaense N₂O-reducing activity**

The *B. ottawaense* strains used in this study are listed in Supplementary Table 1. Among them, the phylogenetic relationships and gene conservation of the denitrification pathway of four strains (SG09, TM102, TM233, and TM239) have been reported [17]. To confirm species classification and gene organization, we determined the draft genome sequence of 10 strains, including 3 strains reported by Wasai-Hara et al. [17] (see Supplementary Table 1). All isolates showed more than 95.0% average nucleotide identity (ANI) values with the type strain *B. ottawaense* OO99^T [23], indicating that the isolates were classified into *B. ottawaense* (see Supplementary Table 2). Furthermore, phylogenetic analysis based on multiple housekeeping genes (AMPHORA [24]) supported this classification, as shown in Supplementary Fig. 1.

The N₂O-reducing activity of the *B. ottawaense* strains was determined under free-living, N₂O-respiring conditions (Fig. 1a, see also Supplementary Fig. 2). Almost all isolates and the type strain *B. ottawaense* OO99^T showed activity in the range of 1,387–1,855 nmol h⁻¹ OD⁻¹, which was 5.5–7.4-fold higher than that of *B. diazoefficiens* USDA110^T (252 nmol h⁻¹ OD⁻¹). Also, growth under N₂O-respiring conditions was better in *B. ottawaense* strains than in *B. diazoefficiens* USDA110^T (Supplementary Fig. 3). Conversely, two strains (SF12 and SF19) showed relatively low activity, with values of 309 and 454 nmol h⁻¹ OD⁻¹, respectively, comparable to that of *B. diazoefficiens* USDA110^T (Fig. 1a). We also analyzed the N₂O-reducing activity of TM102, TM233, and TM239, which lacked nodulation and nitrogen-fixing ability [17], but no significant difference was observed from the other nodulating strains of *B. ottawaense* (p<0.05, Tukey’s test). Monitoring N₂O concentrations over time showed a rapid decrease in *B. ottawaense* (SG09, OO99, TM102, and TM233), while *B. diazoefficiens* USDA110^T exhibited a slow decrease (Fig. 1b).

Next, we examined the effects of *B. ottawaense* inoculation on the N₂O flux associated with nodule degradation in a soybean rhizosphere in a laboratory system (Fig. 2). Under atmospheric conditions (approximately 340 ppb of N₂O in the gas phase), N₂O flux from soybean rhizospheres inoculated with the non-nos possessing strain *B. japonicum* USDA 6^T and *B. ottawaense* SG09 was 29.2 and 2.3 nmol h⁻¹ plant⁻¹, respectively, indicating that N₂O flux following SG09 inoculation significantly decreased relative to that after *B. diazoefficiens* USDA 6^T (nos⁻) and USDA110^T (nos⁺) inoculation (Fig. 2a). In N₂O-supplemented air (50 ppm N₂O), *B. ottawaense* SG09 inoculation
exclusively showed negative N₂O flux. However, such negative flux was not observed with *B. diazoefficiens* USDA 110ᵀ and USDA 6ᵀ inoculation (Fig. 2b). These results demonstrate the effectiveness of *B. ottawaense* inoculation in reducing N₂O emissions from the soybean rhizosphere.

**nosZ gene expression and protein activity of wild-type *B. ottawaense* strains**

nosZ expression in *B. ottawaense* strains was evaluated by RT-qPCR under both N₂O- and NO₃⁻-respiring conditions based on *B. diazoefficiens* USDA110ᵀ (Table 1). Under N₂O-respiring conditions, wild-type (WT) *B. ottawaense* SG09 and OO99ᵀ strains showed 211.5- and 163.5-fold higher expression levels than WT *B. diazoefficiens* USDA110ᵀ, respectively. Under NO₃⁻-respiring conditions, the nosZ expression of WT strains was upregulated in both *B. ottawaense* and *B. diazoefficiens* being 29.6-fold (USDA110ᵀ), 9.2-fold (SG09), and 15.1-fold (OO99ᵀ) higher than that under N₂O-respiring conditions. In the comparison among strains, *B. ottawaense* SG09 and OO99ᵀ showed 66.3- and 83.9-fold higher nosZ expression than that of USDA110ᵀ respectively, even under NO₃⁻-respiring conditions. On the other hand, the two strains with low N₂O-reducing activity (SF12 and SF19; Fig. 1a) showed low expression levels that were 31.2- and 40.6-fold higher than those of USDA110ᵀ, respectively, and less than 1/5 those of SG09 under N₂O-respiring conditions.

We next analyzed NosZ protein activity in *B. ottawaense* and *B. diazoefficiens* by specific activity staining with methyl viologen after sodium deoxycholate polyacrylamide gel electrophoresis (DOC-PAGE). Equal amounts of *B. ottawaense* and *B. diazoefficiens* total protein were loaded and confirmed by Coomassie brilliant blue (CBB) staining (Fig. 3a, see also Supplementary Fig. 4). On the same gel, the intensity of activity staining was clearly higher for SG09 than that for USDA110ᵀ; the intensity of the 8-fold diluted SG09 lane was comparable to that of the non-diluted USDA110ᵀ lane, indicating that NosZ protein activity in *B. ottawaense* was approximately 8-fold higher than that in *B. diazoefficiens* (Fig. 3b, c).

**nosZ expression in nasS deletion mutants**

To investigate the high nosZ expression in *B. ottawaense*, we compared the sequences of the genes involved in the expression of the nos operon, nasST, fixLJK₂, and regSR, in *B. ottawaense* SG09 and *B. diazoefficiens* USDA110ᵀ. As shown in Supplementary Table 3, all genes showed >90% identity in amino acid sequence. However, the upstream sequence of the nos operon, which is recognized by NasT to suppress transcription, showed only 48% identity in the nucleotide sequence.
Therefore, we examined whether the NasST regulatory system is also functional in \textit{B. ottawaense}, similar to \textit{B. diazoefficiens} USDA110\textsuperscript{T} [21]. To this end, we analyzed \textit{nosZ} gene expression in \textit{nasS} deletion mutants of \textit{B. ottawaense} SG09 and OO99\textsuperscript{T}. In \textit{B. diazoefficiens} USDA110\textsuperscript{T}, \textit{nosZ} expression was significantly increased (3.4-fold) in the Δ\textit{nasS} mutant. Similarly, \textit{nosZ} expression was significantly increased in the \textit{B. ottawaense} SG09 (2.03-fold) and OO99\textsuperscript{T} (2.06-fold) Δ\textit{nasS} mutants under N\textsubscript{2}O-respiring conditions. This significant expression increase in \textit{nasS} deletion mutants was not observed in the presence of NO\textsubscript{3}\textsuperscript{-} (Table 1). The effect of \textit{nasS} deletion was also observed in N\textsubscript{2}O-reducing activity, but there was no significant difference in the activity change in \textit{B. ottawaense} (See Supplementary Fig. 5).

\textbf{nos operon transcription system in \textit{B. ottawaense}}

To investigate the effect of the transcriptional regulation of the \textit{nos} operon on \textit{nosZ} expression, we determined the transcriptional start site of \textit{nosR}, which is located upstream of the operon (see Supplementary Fig. 6). The transcription start site was investigated under anaerobic conditions with NO\textsubscript{3}\textsuperscript{-} or N\textsubscript{2}O as the sole electron acceptor by 5' rapid amplification of cDNA ends (5' RACE).

Under N\textsubscript{2}O-respiring conditions, two start sites were detected in \textit{B. ottawaense} SG09 at C and G, 212 and 79 nucleotides (nt) upstream of the \textit{nosR} start codon, respectively (P\textsubscript{d1} and P\textsubscript{d2}, respectively; Fig. 4, Supplementary Fig. 7). The -35/-10 consensus sequence was predicted upstream of the two transcription start sites. In addition, the putative FixK box was predicted upstream of P\textsubscript{d1}. On the other hand, under NO\textsubscript{3}\textsuperscript{-}-respiring conditions, a single transcription start site, P\textsubscript{d1}, was observed in \textit{B. ottawaense} SG09 (Fig. 4 and Supplementary Fig. 7). We also confirmed that \textit{B. ottawaense} OO99\textsuperscript{T} has identical promoter sequences and two transcription start sites (P\textsubscript{d1} and P\textsubscript{d2}; Fig. 4 and Supplementary Fig. 7). In \textit{B. ottawaense} OO99\textsuperscript{T}, two transcription start points (P\textsubscript{d1} and P\textsubscript{d2}) were detected under NO\textsubscript{3}\textsuperscript{-}-respiring conditions, but comparing the band intensities, it was clear that P\textsubscript{d1}, which was also detected in SG09, was strongly transcribed. We additionally examined the transcription start site in USDA110\textsuperscript{T} under N\textsubscript{2}O-respiring conditions and detected a single site (G, 84 nucleotides upstream of \textit{nosR}) that was identical to the previously reported site of USDA110\textsuperscript{T} under NO\textsubscript{3}\textsuperscript{-}-respiring conditions [20, 25](see Supplementary Fig. 7). Our results indicate that, unlike \textit{B. diazoefficiens} USDA110\textsuperscript{T}, the \textit{nosR} of \textit{B. ottawaense} has two transcription start sites under N\textsubscript{2}O-respiring conditions.
Two B. ottawaense strains with low N₂O-reducing activity

Among the B. ottawaense analyzed in this study, two strains SF12 and SF19 showed low N₂O-reducing activity and nosZ expression (Fig. 1a, Table 1). When compared nos gene clusters (see Supplementary Fig. 6a, b), we could not identify the differences responsible for the N₂O-reducing activity as their nos genes are identical in amino acid sequence. However, when compared the genomic sequence of the upstream region of nosR, 56 bp deletion was commonly observed in the low N₂O-reducing activity strains SF12 and SF19; the deleted region includes the start codon (ATG) of nosR (see Supplementary Fig. 6c). To confirm that 56 bp deletion is the cause of low activity, the deletion mutants of SG09 and OO99Δ (SG09Δ56, OO99Δ56) were generated. In the 56 bp deletion mutants, both N₂O-reducing activity and nosZ expression levels were decreased to levels comparable to those of SF12 and SF19 (Table 2), confirming that 56 bp deletion is the cause of the low activity of SF12 and SF19.

Discussion

In this study, we demonstrated that B. ottawaense has higher N₂O-reducing activity than that of B. diazoefficiens. In a previous study, B. diazoefficiens mutants with high N₂O-reducing activity (Nos⁺⁺ mutants) were generated, and the mutants mitigated N₂O emission at the laboratory and field levels [11, 26]. One of the Nos⁺⁺ mutants (5M09) was established as a non-genetically modified organism; however, this strain has 66 mutations in the genome, raising concerns for actual agricultural use. The B. ottawaense described in this study is a WT strain that exhibits high N₂O reduction activity comparable to that of the artificially generated USDA110T Nos⁺⁺ mutant strains [21]. Furthermore, we demonstrated that SG09 inoculation resulted in almost no N₂O release in the rhizosphere due to nodule decomposition. Notably, negative N₂O flux was observed under a 50 ppm N₂O gas phase in the laboratory experiment, suggesting a system is in place to reduce high N₂O concentrations (Fig. 2). Given that GHG reduction is a current key issue, B. ottawaense is quite beneficial as it can contribute to the mitigation of N₂O in agricultural fields.

High N₂O-reducing ability is considered adaptive in environments with high N₂O concentrations. B. ottawaense was first isolated from a soybean field in Canada in 2012 as a novel species [27, 28]. Other isolates have been reported from soybean and peanut fields in China and Japan and woody legumes in Ethiopia [29-32]. B. ottawaense can form nodules in soybeans, but it is rarely detected in soybean fields in Japan [30], suggesting that it is adapted to different environments than those of conventional soybean rhizobacteria such as B. diazoefficiens, B. japonicum, and B.
elkanii. N\textsubscript{2}O reduction occurs preferentially over NO\textsubscript{3} reduction [33], and \textit{B. ottawaense} can grow better than \textit{B. diazoefficiens} under N\textsubscript{2}O-respiring conditions (see Supplementary Fig. 3). Therefore, it is possible that the ability to reduce N\textsubscript{2}O, as in \textit{B. ottawaense}, may have been important to survive in specific environments.

In the current study, we demonstrated that the N\textsubscript{2}O-reducing activity of bradyrhizobia correlated with the expression of the \textit{nosZ} gene. \textit{B. ottawaense} strains with high N\textsubscript{2}O-reducing activity (SG09 and OO99\textsuperscript{T}) strongly express the \textit{nosZ} gene under both N\textsubscript{2}O- and NO\textsubscript{3}-respiring conditions (Figs. 1 and 3, Table. 1). In addition, \textit{Bradyrhizobium} with low N\textsubscript{2}O-reducing activity (USDA110\textsuperscript{T}, SF12, SF19) showed relatively low \textit{nosZ} expression compared to that of high N\textsubscript{2}O-reducing strains (Fig. 1, Table 1). Given the relatively high homology of \textit{nosZ} between \textit{B. diazoefficiens} and \textit{B. ottawaense} (92\%, see Supplementary Fig. 6a), our results suggest that the N\textsubscript{2}O-reducing activity of \textit{Bradyrhizobium} is determined by the expression of the \textit{nosZ} gene rather than NosZ protein activity.

To investigate the cause of high \textit{nosZ} expression in \textit{B. ottawaense}, we first focused on the NasST regulatory system and examined whether it is functional in \textit{B. ottawaense}. In the \textit{nasS} deletion mutants (OO99\textDelta\textit{nasS} and SG09\textDelta\textit{nasS}), \textit{nosZ} expression levels increased under N\textsubscript{2}O-respiring condition but not increased under NO\textsubscript{3}-respiring condition (Table 1), indicating that the NasST regulatory system is functional in \textit{B. ottawaense} as in \textit{B. diazoefficiens} [21, 25]. In addition, it seems that the NasST regulatory system is not a main factor for the high expression in \textit{B. ottawaense} because the \textit{nosZ} expression of WT \textit{B. ottawaense} (163 in OO99\textsuperscript{T}, 211 in SG09) was higher than that of USDA110\textDelta\textit{nasS} (3.4) under N\textsubscript{2}O-respiring conditions (Table 1).

Analysis of \textDelta\textit{nasS} mutants also showed little linearity between \textit{nosZ} gene expression and N\textsubscript{2}O-reducing activity. \textit{B. ottawaense} \textDelta\textit{nasS} mutants exhibited higher \textit{nosZ} gene expression than that of WT (Table 1), but N\textsubscript{2}O-reducing activity did not significantly differ between WT and \textDelta\textit{nasS} mutants (see Supplementary Fig. 5). In addition, when comparing \textit{nosZ} expression in \textDelta\textit{nasS} mutants, \textit{B. ottawaense} demonstrated a 100-fold higher expression than that of \textit{B. diazoefficiens} (Table 1), but N\textsubscript{2}O-reducing activity only slightly differed (see Supplementary Fig. 5). The lack of linearity between gene expression and N\textsubscript{2}O-reducing activity may indicate upper limits for NosZ protein activity. This may be due to translation efficiency or depletion of the components required for NosZ activity, such as copper and electrons, during N\textsubscript{2}O reduction. Moreover, NosZ protein activation requires highly complex pathways, such as sequential metal trafficking and assembly to copper sites via NosDFY [34]. The exact cause is presently unknown, but the aforementioned factors
may define the upper limit of N₂O reduction activity in bradyrhizobia.

In the current study, the single transcription start point was detected in \textit{B. diazoefficiens} USDA110\textsuperscript{T} under anaerobic conditions regardless of different electron acceptors (N₂O or NO\textsubscript{3}⁻), whereas \textit{B. ottawaense} has a variable transcription start point depending on the electron acceptors: two transcription start points were detected under N₂O respiration conditions in both SG09 and OO99\textsuperscript{T}. Changes in the transcription start site depending on two different electron acceptor have been reported in studies on \textit{Geobacter} [35]. Also, genome-wide analysis of transcription start sites in \textit{Clostridium} identified several metabolism-related genes with multiple transcription start sites that change depending on the substrate [36]. Although the importance of having multiple transcription start sites has not been fully elucidated, it is considered an important regulatory mechanism of gene expression because it largely influences transcription efficiency, translation initiation, and protein abundance [37]. Changes in the transcription start sites of \textit{B. ottawaense nosR} depend on the type of electron acceptor, which may be part of the \textit{nos} genes expression regulatory mechanism in the denitrification system.

Genome sequence comparisons of high and low N₂O-reducing activity strains revealed a novel determinant of activity. Incidentally, 56 bp deletion in the upstream region of \textit{nosR} was detected specifically in the low N₂O-reducing activity strains, SF12 and SF19, as shown in Supplementary Fig. 6, and introducing the deletion in the high N₂O-reducing activity strains (SG09 and OO99\textsuperscript{T}) reduced \textit{nosZ} gene expression and N₂O-reducing activity (Table 2). These results are consistent with previous studies where decreased \textit{nosZ} expression was observed in artificially generated \textit{nosR}-deleted \textit{Pseudomonas aeruginosa} strains in which the \textit{nos} genes were encoded in a single operon similar to that of \textit{Bradyrhizobium} [38]. Since partial gene deletion is among the driving forces for environmental adaptation or functional evolution in bacteria [39-41], the strains with natural deletion isolated in the present study may have evolved to adapt to environments with limited denitrification substrates. Accordingly, examining the distribution and abundance of high and low N₂O-reducing activity strains in various environments may reveal the importance of N₂O-reducing activity in environmental adaptation.

In summary, we demonstrated that the N₂O-reducing activity of \textit{B. ottawaense} is significantly higher than that of conventional strains, and this activity is achieved via high \textit{nosZ} expression. Since N₂O is a GHG mainly generated in agricultural lands, developing strategies for reducing N₂O emissions from agricultural lands is an urgent task. The \textit{B. ottawaense} we reported here has great potential for GHG mitigation in the rhizosphere owing to its high N₂O-reducing activity. In addition,
the regulatory mechanism of nos gene expression we elucidated in this study will be useful for developing and identifying bacteria with higher GHG-reducing ability. Further studies on the ecology of *B. ottawaense* including its compatibility with legume crops and competitiveness with other indigenous rhizobacteria are needed to improve its utility on actual agricultural land.

**Methods**

**Bacterial strains, isolation, and genome analysis**

The type strain *B. ottawaense* OO99\(^T\) was purchased from the Microbial Domain Biological Resource Centre HAMBI (Helsinki, Finland). The type strain *B. diazoefficiens* USDA110\(^T\) was provided by Dr. Michael J. Sadowsky at University of Minnesota. We used the culture stock of the nosZ deleted mutant *B. diazoefficiens* USDA110 ΔnosZ generated by Hirayama *et al.* 2011[42]. The *B. ottawaense* strains used in this study are listed in Supplementary Table 1. Eight strains (SG09, SG10, SG20, SG23, TM102, TM233, and TM239) have been reported by Wasai-Hara *et al.* [17], and the other strains were isolated by the same procedures. For whole genome sequencing, genomic DNA was extracted using a Bacteria GenomicPrep Mini Spin Kit (Cytiva, Tokyo, Japan). DNA libraries were prepared using a Nextera Sample Preparation Kit (Illumina, San Diego, CA, USA), and the 300-bp paired-end libraries were sequenced using Illumina Miseq (Illumina). Subsequently, 20 bp of the 5′ and 3′ ends were trimmed, and the genomes were assembled using CLC Genomics Workbench ver. 8.5.1(Illumina). Genome annotation was performed using DFAST [43].

**N\(_2\)O-reducing activity**

N\(_2\)O-reducing activity was determined by culturing the bacteria under anaerobic conditions with 1% N\(_2\)O supplemented as the sole electron acceptor. The N\(_2\)O concentration was measured using a gas chromatograph (GC2014; Shimadzu, Kyoto, Japan) equipped with a thermal conductivity detector and Porapak Q column (GL Sciences, Tokyo, Japan). Bacterial strains were aerobically cultured for over 6 h in a 75-mL test tube with an air-permeable plug containing 10 mL HM liquid medium [44] supplemented with 0.1% (w/v) arabinose and 0.025% (w/v) yeast extract at 28 °C with shaking at 200 rpm. Thereafter, the appropriate volume of bacterial culture was added to new tubes containing 10 mL HM medium to reach an optical density (OD) at 660 nm (OD\(_{660}\)) of 0.05. The OD was measured using a \(\phi = 25\) mm test tube (TEST25NP; AGC Techno Glass Co., Ltd., Shizuoka, Japan). The test tube was closed with a butyl rubber cap, and the gas phase was replaced with 4.98% N\(_2\)O + 95.02% N\(_2\) gas following overnight (12–14 h) culture to induce N\(_2\)O reduction
metabolism. Subsequently, the gas phase was again replaced with 100% N\textsubscript{2} gas, after which 100% N\textsubscript{2}O was supplemented to adjust to a final concentration of 1%. Finally, the test tube was incubated at 28 °C with shaking at 200 rpm, and 100 µL of gas phase was withdrawn every 1–3 h and subjected to the gas chromatography.

*N\textsubscript{2}O flux experiment*

N\textsubscript{2}O flux in the soybean rhizosphere was measured using a previously described method with modifications [11]. Briefly, bacterial strains were aerobically cultured in HM liquid medium at 30 °C for 1 week, after which the prepared bacterial suspension was adjusted to 1 × 10\textsuperscript{8} cells mL\textsuperscript{-1} using sterilized water. Soybean seeds (*Glycine max*, cv. Enrei (GmJMC025) seeds acquired from Genebank Project NARO, Japan) were sterilized using 0.5% sodium hypochlorite were sown in Leonardo Jar pots—at three seeds per pot—containing sterilized vermiculite and were inoculated with 1 mL of bacterial suspension. The seeds were cultivated in a growth chamber at 25 °C for 16 h in light and 8 h in the dark. Thinning was performed on the third day after sowing, leaving an individual plant that was in the best germination state, and cultivation continued for another 27 days. A nitrogen-free hydroponic solution was periodically added to the pot during cultivation. After cultivation, the root system from each plant was transferred to a 100-mL glass vial containing 30 mL of soil obtained from the Kashimadai field (38°27′36.0″N 141°05′24.0″E, at the permission of Tohoku University, Japan). Thereafter, the vials with the roots and soil were incubated at 25 °C for 20 days to induce nodule degradation. N\textsubscript{2}O flux was determined by measuring the concentration of N\textsubscript{2}O in the gas phase in the vials with the roots and soil using a gas chromatograph (GC2014; Shimadzu) equipped with a 63\textsuperscript{Ni} electron capture detector and tandem Porapak Q columns (GL Sciences; 80/100 mesh; 3.0 mm × 1.0 m and 3.0 mm × 2.0 m).

*Expression analysis*

*nosZ* gene expression levels were measured under N\textsubscript{2}O- and NO\textsubscript{3}-respiring conditions. For N\textsubscript{2}O-respiring conditions, cells were prepared the same as described above. Three hours after exposure to 1% N\textsubscript{2}O conditions, a 1 mL phenol solution (10% phenol in ethanol) was added to the 1-mL culture to stop metabolism. After centrifugation, the pellets were stored at -80 °C until further processing. For NO\textsubscript{3} respiring conditions, cells were anaerobically grown in 20 mL HM medium supplemented with 10 mM KNO\textsubscript{3} in a 75-mL test tube. The OD\textsubscript{660} was initially adjusted to 0.05 and monitored to induce the exponential growth phase of cells. When OD\textsubscript{660} reached 0.1, the cells were
collected as described above. Subsequently, total RNA was isolated using the hot-phenol method as described previously [35], followed by DNase I treatment (RQ1; Promega, Madison, WI, USA) and further purification using RNA Clean & Concentrator-5 (Zymo Research, Irvine, CA, USA). First-strand cDNA was synthesized using 500 ng RNA as a template and SuperScript IV Reverse Transcriptase (Invitrogen, Waltham, MA, USA) according to the manufacturer’s instructions. RT-qPCR was performed using a LightCycler Nano Instrument (Roche, Basel, Switzerland), LightCycler® FastStart DNA MasterPLUS SYBR® Green I (Roche), and specific primers for sigA (sigAf/sigAr) and nosZ (nosZ_qPCR_F/ nosZ_qPCR_R) (see Supplementary Table 4) at an annealing temperature of 60 ºC for 50 cycles. Relative expression calculated using the $2^{-\Delta\Delta Ct}$ method [45] was normalized to sigA expression.

**nosZ activity staining**

*B. ottawaense* SG09 and *B. diazoefficiens* USDA110T were cultured overnight under N$_2$O-respiring conditions. After centrifugation of the cultured cells, total protein was extracted using lysis buffer (CelLytic B; Sigma-Aldrich, St. Louis, MO, USA) and sonication (BIORUPTOR; BM Equipment Co., Ltd., Tokyo, Japan). Protein content in the supernatants was measured using a DC-protein assay (Bio-Rad Laboratories, Hercules, CA, USA). For DOC-PAGE, approximately 9.6 µg of total protein was used as the non-diluted sample (1×, Fig. 3). After electrophoresis, each gel was immersed in the buffer containing 25 mM Tris, 192 mM glycine, and 1 mM methyl viologen (pH 8.3). Subsequently, Ti(III)-citrate was used to reduce methyl viologen, and N$_2$O-saturated H$_2$O was added for the in-gel N$_2$O-reducing enzymatic reaction. Band signal intensity was determined using an ImageJ macro, Band/Peak Quantification [46]. All experiments except for protein quantification were performed under an N$_2$ atmosphere. CBB staining was performed after N$_2$O-reducing activity staining to confirm protein content.

**nasS, nosZ, and 56-bp deletion mutants**

*nasS* deletion mutants were generated using the in-frame markerless method. The deletion region was determined as in the *B. diazoefficiens* USDA110T *nasS* mutant strain (5M09) reported by Sánchez et al. [21]. Briefly, pK18mobsacB-Ω was created by replacing the kanamycin resistance gene coding region of the suicide vector pK18mobsacB with a streptomycin-spectinomycin resistance gene (*aadA*). pK18mobsacB was digested with NcoI and BglII to obtain 5.1 kb of linear DNA with 0.6 kb of the kanamycin resistance gene partially deleted. The aadA fragment to be
introduced was amplified by PCR using primers aadA_F_IF and aadA_R_IF and Prime STAR® Max DNA Polymerase (Takara Bio Inc., Shiga, Japan) (details on the primers are shown in Supplementary Table 4) with pHP45Ω as a template. Thereafter, 1.1 kb of amplified DNA was extracted using Wizard® SV Gel and a PCR Clean-Up System (Promega). The resulting linear pK18mobsacB and aadA were combined using an In-fusion HD cloning kit (Takara Bio Inc.) and transformed E. coli DH5α according to the manufacturer’s instructions.

The up- and downstream regions of the nasS gene were amplified by PCR using primers Bo_nasSdel_F1/R1 and Bo_nasSdel_F2/R2 (see Supplementary Table 4 for details on primers) and Prime STAR® Max DNA Polymerase (Takara Bio Inc.). Amplified fragments were combined by overlap extension PCR and inserted into the SmaI site of pK18mobsacB-Ω using an In-fusion HD Cloning Kit (Takara Bio Inc.). The sequence of the introduced fragment was confirmed by sequencing, and the resultant plasmid was designated pMS187. Transmission of pMS187 to B. ottawaense strains and homologous recombination of the nasS region were performed by triparental mating with a mobilizing E. coli HB101 strain harboring the pRK2013 helper plasmid. Next, B. ottawaense SG09 or OO99Δ, E. coli DH5α harboring pMS187, and E. coli HB101 harboring pRK2013 were mixed and cultured for mating. Transconjugants were selected by resistance to streptomycin (Sp, 100 µg/mL), spectinomycin (Sm, 100 µg/mL), and polymyxin (Px, 50 µg/mL) and sensitivity to sucrose (10%). The single crossover strains were further cultured in HM medium without antibiotics, and deletion mutants that showed Sp/Sm sensitivity and sucrose resistance—SG09ΔnasS and OO99ΔnasS—were obtained.

ΔnosZ and 56 bp deletion mutants were generated using the same methods as for the nasS mutants with the pK18mobsacB-Ω vector. Briefly, the up- and downstream regions of the nosZ gene were amplified by PCR using primers SG09_nos-1F/1R and SG09_nos-2F/2R (see Supplementary Table 4 for details on the primers) and Prime STAR® max DNA Polymerase (Takara Bio Inc.). The amplified fragments were combined by overlap extension PCR using primers SG09_nos-1F/1R and SG09_nos-2F/2R (see Supplementary Table 4) and Prime STAR® max DNA Polymerase (Takara Bio Inc.). The PCR fragments and pK18mobsacB-Ω were digested with EcoRI and HindIII and then ligated using a DNA Ligation Kit (<Mighty Mix>; Takara Bio Inc.). Thereafter, triparental mating was performed using the sequence-introduced vector as described above.

For 56-bp deletion mutants, the up- and downstream regions of the 56-bp region were amplified by PCR using primers 56del_F1/R1 and 56del_F2/R2 (see Supplementary Table 4) and then combined and inserted into the Smal site of pK18mobsacB-Ω using an In-fusion HD Cloning
Kit (Takara Bio Inc.). Thereafter, triparental mating was performed using the sequence-introduced vector as described above. The generated 56 bp deletion mutants were designated SG09 Δ56 and OO99 Δ56.

5′ RACE

5′ RACE experiments were performed using a 5′/3′ RACE kit, 2nd Generation (Roche). Briefly, the total RNA of *B. ottawaense* and *B. diazoefficiens* strains were isolated from cells grown under N$_2$O- and NO$_3$- respiring conditions using the hot-phenol method as described above. cDNA synthesis and amplification of the 5′-region of nosR were conducted according to the manufacturer’s instructions using the primers listed in Supplementary Table 4 (Bw_SP1, SP2, and SP3 for *B. ottawaense* strains, R_SP1, SP2, and SP3 for *B. diazoefficiens* strains). The amplified fragments were sequenced to determine the transcription start site.

Statistical analysis

Differences in N$_2$O reducing activities between all strains tested were evaluated using Tukey’s test after ANOVA analysis. Differences in N$_2$O flux and nosZ gene expression between the two strains were evaluated using Student’s *t* tests at a significance level of 0.05.

Data availability

Genome data are available in NCBI (https://www.ncbi.nlm.nih.gov/), and accession numbers are detailed in the Supplementary Information files.

References

16 Shiina, Y. et al. Relationship between soil type and N₂O reductase genotype (nosZ) of indigenous soybean bradyrhizobia: nosZ-minus populations are dominant in Andosols. Microbes Environ. 29, 420-6 (2014)


**Author contributions**

S.W-H carried out experiments, data analysis, and genome analysis and drafted the original manuscript. M.I., A.F.S., and D.T. designed and performed experiments and contributed to manuscript writing. M.S. contributed to the generation of gene deletion mutants and interpretation of data. N.I. and T.Y. supervised protein experiments and contributed to the interpretation of data. H.M., S.S., H.I-A, Y.S., and K.M. supervised the conduct of this study. Y.S. and K.M. contributed to manuscript finalization and revision. All authors reviewed the results and approved the final version of the manuscript.

**Additional information**

**Competing interests:** The authors declare no competing interests.

**Acknowledgments**

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Fig. 1 N₂O-reducing activity of *B. ottawaense*. (a) N₂O-reducing activity of *B. ottawaense* isolates, type strain OO99ᵀ, *B. diazoefficiens* strain USDA110ᵀ, and the nosZ-deficient strain (ΔnosZ). Different letters above the bars represent significant differences between inoculation treatments analyzed using Tukey’s test after analysis of variance (ANOVA; *p* < 0.05). Parentheses after the strain name indicate nodule-forming ability. R = nodule forming strain (rhizobia), N = non-nodulation and non-diazotroph. (b) N₂O-reducing activity in representative strains of *B. ottawaense* and *B. diazoefficiens*. The graph shows the changes in N₂O concentration over time in the gas phase in the test tube.
Fig. 2 N₂O flux in rhizosphere inoculated with either *Bradyrhizobium ottawaense* B. *diazoefficiens* and *B. japonicum*. N₂O flux from the rhizosphere of soybean plants inoculated with *B. ottawaense* SG09 (*nosZ*+), *B. diazoefficiens* USDA110⁷ (*nosZ*+), and *B. japonicum* USDA 6⁷ (*nosZ*) under (a) an atmospheric concentration (approximately 340 ppb) of N₂O and (b) N₂O-supplemented air (50 ppm). Asterisks represent significant differences at *p* < 0.05 by the *t*-test.
Fig. 3 Activity of the NosZ protein of *Bradyrhizobium ottawaense* and *B. diazoefficiens*.
Coomassie brilliant blue staining(a) and NosZ-specific activity staining (DOC-PAGE, b) protein extracted from *B. ottawaense* SG09 and *B. diazoefficiens* USDA110T. The numbers in each lane indicate the concentration (x) rate of extracted protein samples. ‘M’ indicates the protein size marker (60, 120, and 240 kDa were indicated). The gel images are cropped; full images are shown in Supplemental Fig. 4. Panel c shows the signal intensity of the NosZ-specific activity staining.
Fig. 4 Transcriptional organization of *nosR* in *Bradyrhizobium ottawaense* SG09 and OO99ᵀ.

P₁(c) is the transcription start site under N₂O- and NO₃⁻-respiring conditions. P₂(g) is the transcription start site under N₂O-respiring conditions. -35/-10 consensus sequences preceding each transcription start site are indicated by underlining. Putative FixK box located upstream of P₁ is shown in the box. The translational start codon (ATG) of *nosR* is shown in bold case. The promoter sequences of *B. ottawaense* SG09 and OO99ᵀ are completely identical. The dotted underlined region indicates the 56 bp deleted in strains SF12 and SF19 (see Supplementary Fig. 6 for details).
### Tables

**Table 1.** Relative expression of *nosZ* under N$_2$O- and NO$_3$-respiring conditions

<table>
<thead>
<tr>
<th></th>
<th>N$_2$O-respiring conditions</th>
<th>NO$_3$-respiring conditions</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
<td>ΔnasS</td>
</tr>
<tr>
<td><em>B. diazoefficiens</em> USDA110$^T$</td>
<td>1.0 ± 0.48</td>
<td>3.4 ± 1.7</td>
</tr>
<tr>
<td><em>B. ottawaense</em> SG09</td>
<td>211.5 ± 58</td>
<td>430.2 ± 164</td>
</tr>
<tr>
<td><em>B. ottawaense</em> OO99$^T$</td>
<td>163.5 ± 81</td>
<td>337.4 ± 132</td>
</tr>
<tr>
<td><em>B. ottawaense</em> SF12</td>
<td>31.2 ± 8.3</td>
<td>nm</td>
</tr>
<tr>
<td><em>B. ottawaense</em> SF19</td>
<td>40.6 ± 8.2</td>
<td>nm</td>
</tr>
</tbody>
</table>

Expression is shown relative to *Bradyrhizobium diazoefficiens* USDA110$^T$, which is set to 1.0 and normalized to *sigA* gene expression. Data are presented as the mean values ± standard error of three independent experiments. *Significant difference, $p < 0.05$, $n = 4$-6, t-test, nm = not measured.
Table 2. Relative expression of nosZ and N₂O-reducing activity in the 56 bp deletion mutant of B. ottawaense

<table>
<thead>
<tr>
<th></th>
<th>Relative expression</th>
<th>N₂O-reducing activity (nmol/h/OD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. ottawaense SG09Δ56</td>
<td>24.0 ± 7.9</td>
<td>303 ± 17.2</td>
</tr>
<tr>
<td>B. ottawaense OO99Δ56</td>
<td>38.4 ± 5.0</td>
<td>588 ± 44.8</td>
</tr>
<tr>
<td>B. ottawaense SG09*</td>
<td>211.5 ± 58</td>
<td>1645 ± 202</td>
</tr>
<tr>
<td>B. ottawaense OO99*</td>
<td>163.5 ± 81</td>
<td>1418 ± 106</td>
</tr>
<tr>
<td>B. diazoefficiens USDA110*</td>
<td>1.0 ± 0.48</td>
<td>252 ± 47.1</td>
</tr>
</tbody>
</table>

*The wild-type data were obtained from Fig. 1a and Table 1.
Supplementary Files

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