Bradyrhizobium ottawaense efficiently reduces nitrous oxide through high *nosZ* gene expression

Supplementary information

Sawa Wasai-Hara[1, 2], Manabu Itakura[2], Arthur Fernandes Siqueira[2], Daisaku Takemoto[1], Masayuki Sugawara[2], Hisayuki Mitsui[2], Shusei Sato[2] , Noritoshi Inagaki[1], Toshimasa Yamazaki[1], Haruko Imaizumi-Anraku[1], Yoshikazu Shimoda[1*], and Kiwamu Minamisawa[2*]

[1]National Agriculture and Food Research Organization (NARO), Tsukuba, Ibaraki 305-8604, Japan

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

*Corresponding author: Y.S., yshimoda@affrc.go.jp, K.M., kiwamu.minamisawa.e6@tohoku.ac.jp

Methods

Phylogenic analysis (Supplementary Fig. 1)

Thirty-one genes belong to AMPHORA were extracted from the information annotated by DFAST, combined, and aligned. A phylogenetic tree was generated using MEGAX (ver 10.2.6; https://www.megasoftware.net/, [1]) using the neighbor-joining method.

Measurement of N_2O reduction activity (Supplementary Fig. 2)

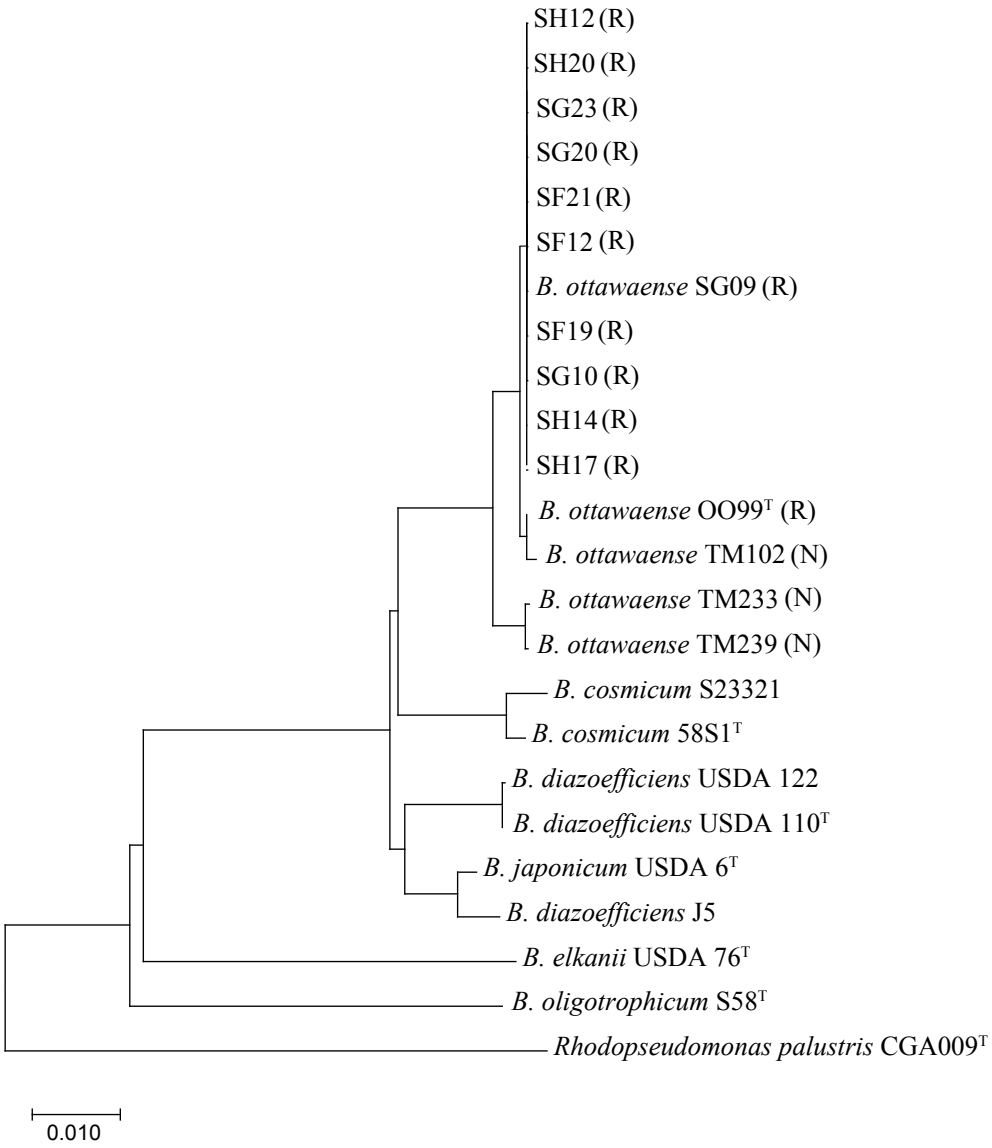
The N_2O reduction activity was performed as described in the main text and the N_2O reduction speed was normalized by optical density (OD) at 660 nm (OD_{660}); OD_{660} was measured in test tubes and calibrated to a value per 1-mL cuvette from a calibration curve.

Growth experiment (Supplementary Fig. 3)

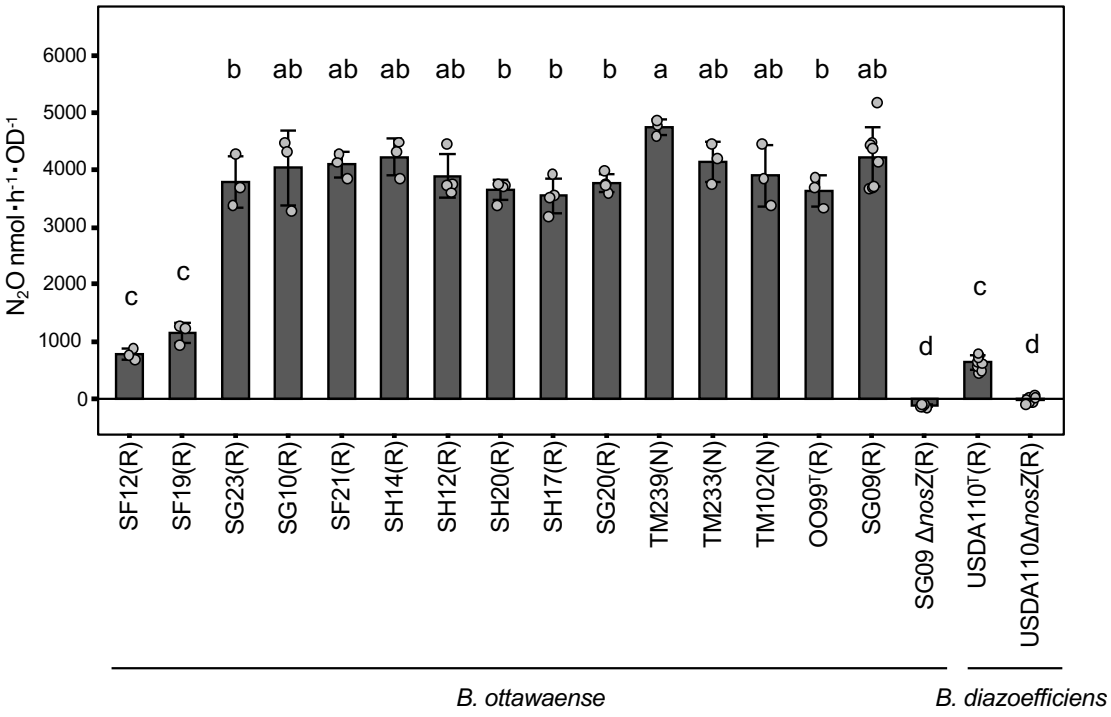
B. ottawaense SG09 and OO99^T and *B. diazoefficiens* USDA110^T were cultured under N_2O-respiring conditions. The strains were cultured HM liquid medium and initial N_2O concentration was adjusted to 30%.

Comparison of gene cluster (Supplementary Fig. 5)

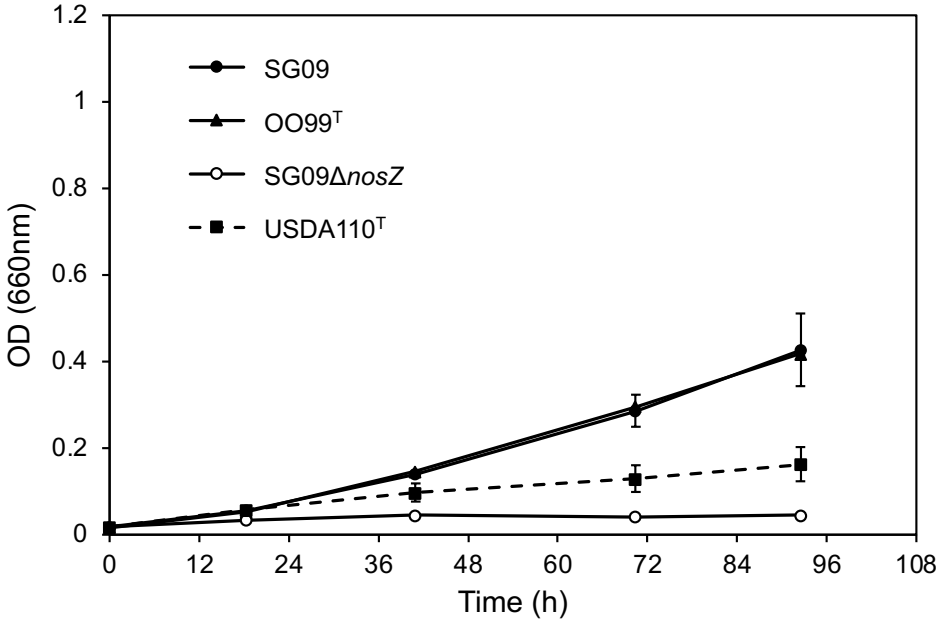
Comparison of gene cluster organization and homology was performed using GenomeMatcher (ver 3.04, [2]).



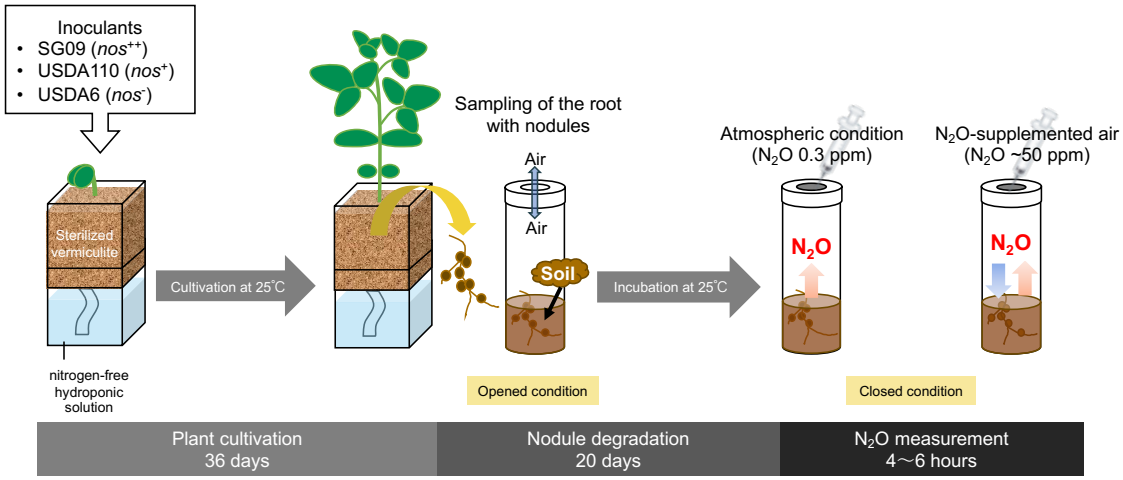
Supplementary Fig. 1. Phylogenic tree based on AMPHORA genes. Parentheses after the strain name indicate nodule-forming ability. R = nodule-forming strain (rhizobia), N = non-nodulation and non-diazotroph.



Supplementary Fig. 2. N_2O-reducing activity of *B. ottawaense* isolates, type strain OO99^T, *B. diazoefficiens* stain USDA110^T, and the *nosZ*-deficient strain (Δ*nosZ*) normalized by the value of OD_{660}. 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.



Supplementary Fig. 3. Growth curve of *Bradyrhizobium ottawaense* SG09, OO99^T, SG09 Δ*nosZ*, and *B. diazoefficiens* USDA110^T under N_2O-respiring conditions. Error bars denote standard deviation (n = 3).

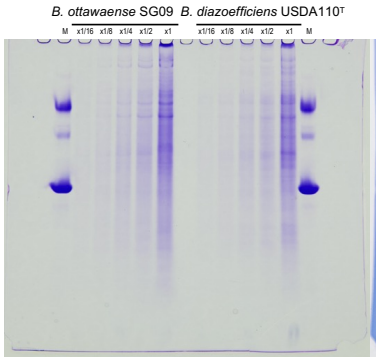


Supplementary Fig. 4

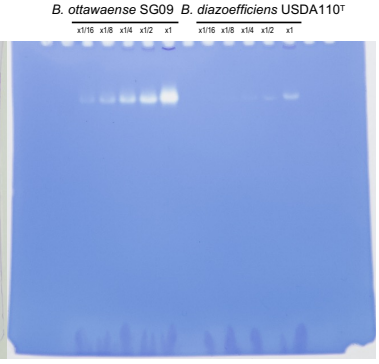
Overview of N_2O flux experiment in the nodule decomposed soybean rhizosphere shown in Fig. 2. Four pots were prepared per each strain, and each strain was inoculated 1 mL cell suspension of 1 x 10^8 cells/mL per pot. Plant cultivation was conducted in a growth chamber at 25°C with 18 hours of light and 6 hours of dark. N_2O flux was determined by measuring the change in N_2O concentration in the vials with sealing for 4-6 hours by using gas chromatograph.

Replication 1

a CBB staining

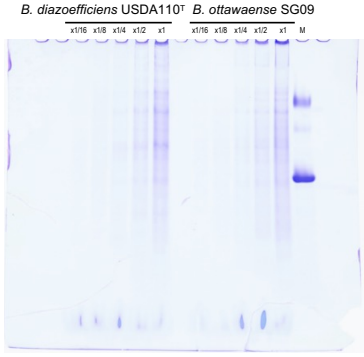


b NosZ activity staining

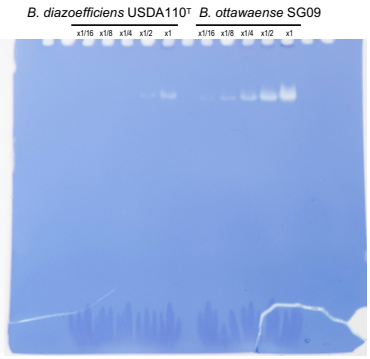


Replication 2

c CBB staining

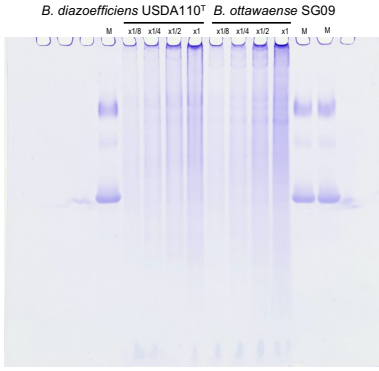


d NosZ activity staining

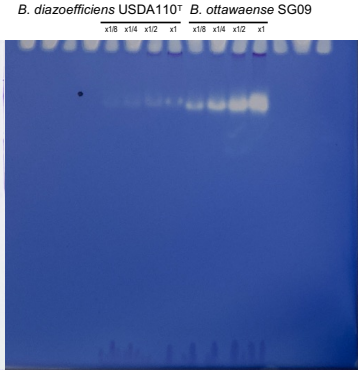


Replication 3

e CBB staining

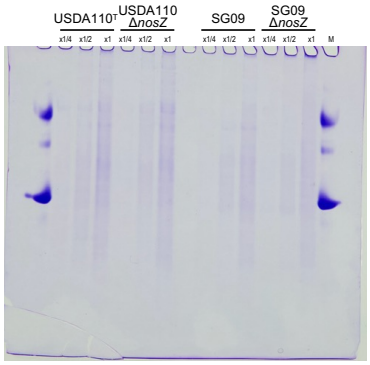


f NosZ activity staining

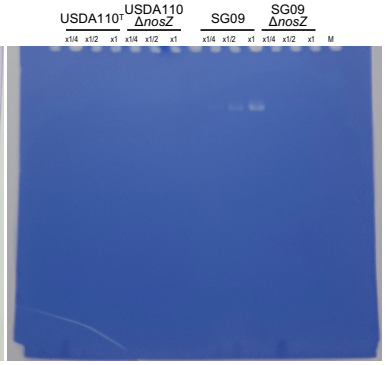


Δ*nosZ* strains

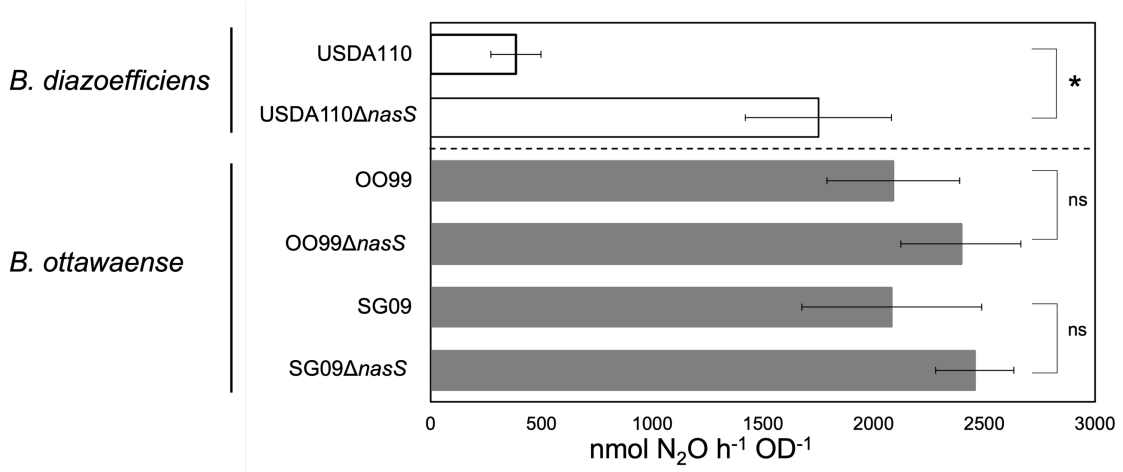
g CBB staining



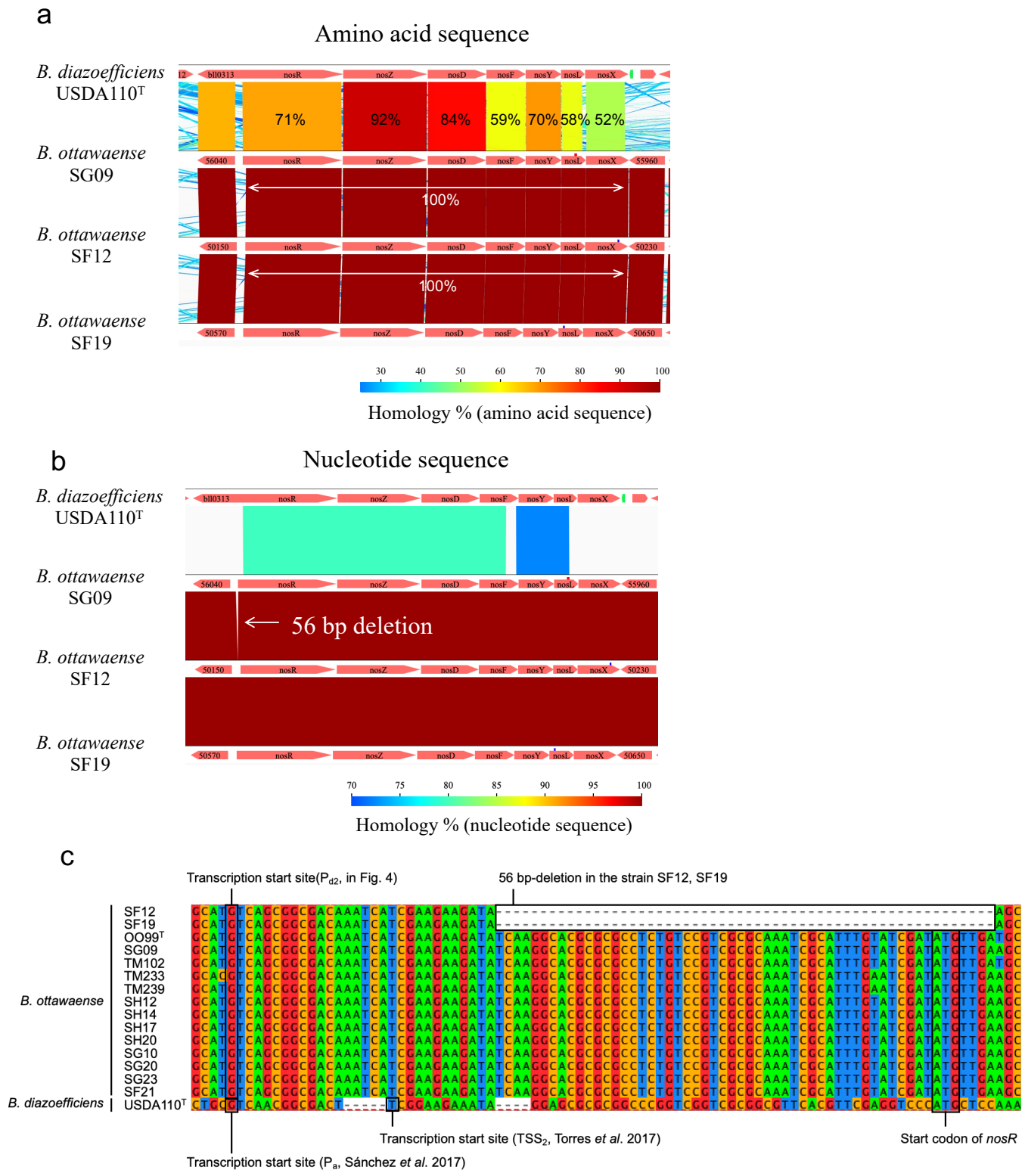
h NosZ activity staining



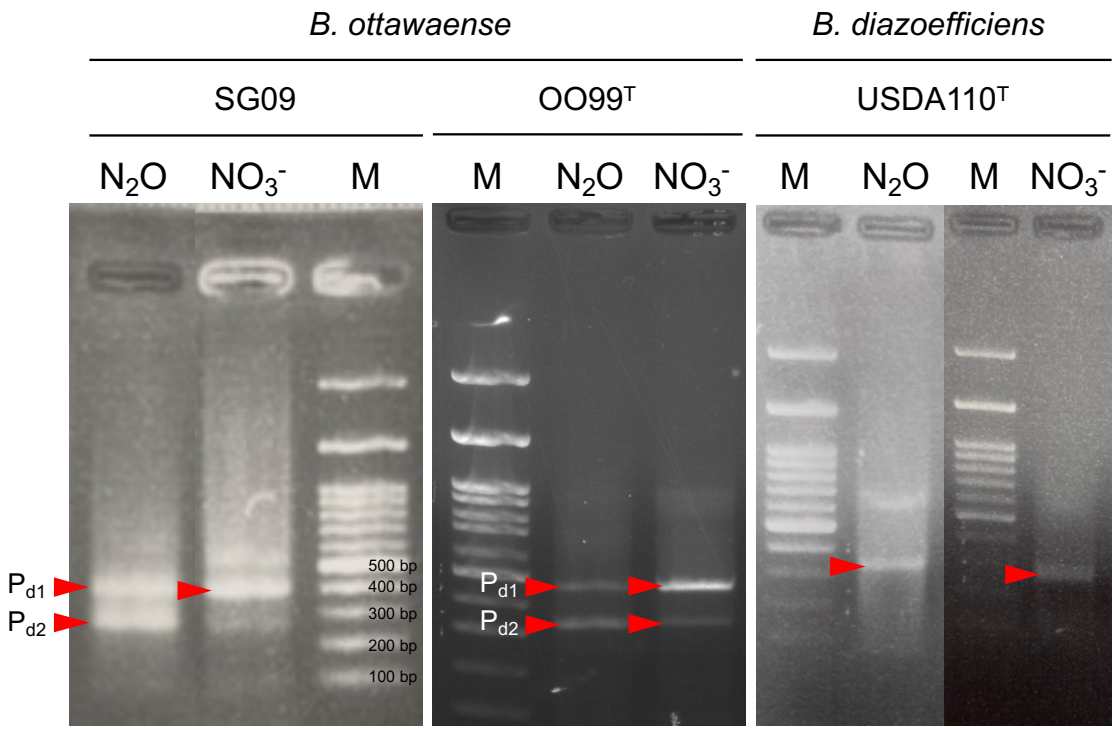
Supplementary Fig. 5. Activity of the NosZ protein of *B. ottawaense* and *B. diazoefficiens* in replicate experiments. Coomassie brilliant blue staining (**a, c, e, g**) and NosZ-specific activity staining (DOC-PAGE, **b, d, f, h**) protein extracted from *B. ottawaense* SG09 and *B. diazoefficiens* USDA110^{T}. **a** and **b** are the full size gel images of Fig. 3. **g** and **h** include the Δ*nosZ* mutant of SG09 and USDA110. 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).



Supplementary Fig. 6. N_2O-reducing activity of the Δ*nasS* mutants of *B. ottawaense* SG09, OO99^T, and *B. diazoefficiens* USDA110^T. Asterisk represents significant difference at $p < 0.05$, $n = 4$, by *t*-test. ns = not significant.



Supplementary Fig. 7. Homology of *nos* gene cluster amino acid (**a**) and nucleotide sequences (**b**). The pink arrow indicates gene configuration, and the colors among these genes indicate the homology between the upper and lower strains. Nucleotide sequence upstream of *nosR* and the 56 bp deletion site (**c**).



Supplementary Fig. 8. Electrophoresis images of 5′ RACE analysis in *Bradyrhizobium ottawaense* SG09, OO99^T, and *B. diazoefficiens* USDA110^T. The "N_2O" and "NO_3" lanes indicate samples obtained under N_2O- and NO_3-reducing conditions. Lane M is a size maker (FastGene 100 bp DNA Marker; Nippon Genetics, Tokyo, Japan). Red arrows indicate bands for which the sequence was determined.

Supplementary Table 1. Bacterial strains used in this study.

Strain	Accession no.	Reference for genome sequence
USDA110^{T}(R)	BA000040.2	[3]
OO99^{T}(R)	CP029425.1	[4]
SG09(R)	AP021854.1	[5]
TM102(N)	AP021855.1	[5]
TM233(N)	BTIG01000001-BTIG01000413	[5]
TM239(N)	BTIR01000001-BTIR01000077	[5]
SG10(R)	BTIQ01000001-BTIQ01000335	This study
SG20(R)	BTIH01000001-BTIH01000340	This study
SG23(R)	BTII01000001-BTII01000335	This study
SF12(R)	BTIN01000001-BTIN01000328	This study
SF19(R)	BTIO01000001-BTIO01000828	This study
SF21(R)	BTIP01000001-BTIP01000318	This study
SH12(R)	BTIJ01000001-BTIJ01000507	This study
SH14(R)	BTIK01000001-BTIK01000358	This study
SH17(R)	BTIL01000001-BTIL01000343	This study
SH20(R)	BTIM01000001-BTIM01000619	This study

Parentheses after the strain name indicate nodule-forming ability. R = nodule forming strain (rhizobia), N = non-nodulation and non-diazotroph.

Supplementary Table 2. Average nucleotide identity between *Bradyrhizobium ottawaense* isolates and type strain OO99^{T}

	OO99^{T}(R)	SF12(R)	SF19(R)	SF21(R)	SG09(R)	SG10(R)	SG20(R)	SG23(R)	SH12(R)	SH14(R)	SH17(R)	SH20(R)	TM102(N)	TM233(N)	TM239(N)
OO99^{T}(R)		99.04	99.01	99.07	99.08	99.05	99.07	99.07	99.07	99.05	99.05	99.07	98.99	95.30	95.29
SF12(R)	99.04		99.95	99.93	99.92	99.99	99.94	99.95	99.94	99.93	99.92	99.94	99.15	95.33	95.27
SF19(R)	99.01	99.95		99.92	99.90	99.96	99.93	99.92	99.91	99.91	99.90	99.89	99.13	95.35	95.26
SF21(R)	99.07	99.93	99.92		99.96	99.94	99.96	99.96	99.96	99.96	99.97	99.95	99.09	95.36	95.25
SG09(R)	99.08	99.92	99.90	99.96		99.92	99.94	99.94	99.94	99.95	99.95	99.93	99.10	95.35	95.27
SG10(R)	99.05	99.99	99.96	99.94	99.92		99.95	99.95	99.94	99.94	99.93	99.94	99.14	95.37	95.28
SG20(R)	99.07	99.94	99.93	99.96	99.94	99.95		99.98	99.97	99.95	99.96	99.96	99.12	95.43	95.30
SG23(R)	99.07	99.95	99.92	99.96	99.94	99.95	99.98		99.99	99.95	99.95	99.99	99.11	95.45	95.30
SH12(R)	99.07	99.94	99.91	99.96	99.94	99.94	99.97	99.99		99.95	99.96	99.98	99.10	95.45	95.29
SH14(R)	99.05	99.93	99.91	99.96	99.95	99.94	99.95	99.95	99.95		99.99	99.94	99.08	95.34	95.25
SH17(R)	99.05	99.92	99.90	99.97	99.95	99.93	99.96	99.95	99.96	99.99		99.95	99.10	95.35	95.29
SH20(R)	99.07	99.94	99.89	99.95	99.93	99.94	99.96	99.99	99.98	99.94	99.95		99.10	95.47	95.30
TM102(N)	98.99	99.15	99.13	99.09	99.10	99.14	99.12	99.11	99.10	99.08	99.10	99.10		95.34	95.30
TM233(N)	95.30	95.33	95.35	95.36	95.35	95.37	95.43	95.45	95.45	95.34	95.35	95.47	95.34		98.90
TM239(N)	95.29	95.27	95.26	95.25	95.27	95.28	95.30	95.30	95.29	95.25	95.29	95.30	95.30	98.90	

Supplementary Table 3. Homology of nos-regulating genes between *Bradyrhizobium ottawaense* SG09 and *B. diazoefficiens* USDA110^{T}

	FixL	FixJ	$FixK_2$	FixK box	NasS	NasT (ANTAR region)	*nosR* upstream*	RegS	RegR
nucleotide	88%	92%	92%	SG09 **ATG**CGCTAGCG**CAA** USDA110:**TTG**ATCCAGCG**CAA**	88%	96% (96%)	48%	93%	93%
amino acid	91%	96%	93%	-	90%	99% (100%)	-	97%	99%

*The sequence between the *nosR* gene and the upstream hypothetical gene (reverse oriented) was compared (see Supplementary Fig. 5).

Homologies of the NasT-ANTR region [8] are shown in parentheses.

Supplementary Table 4. Primers used in this study.

Primer mane	purpose	sequence (5′→3′)	Reference
nosZ_qPCR_F	qPCR	ACCCGCGAATTCCTCAAGAA	This study
nosZ_qPCR_R	qPCR	CTGTTGGCCTTGTCGTTCATG	This study
sigAf	qPCR	GAGAACCAGATGTCGCTTGC	[6]
sigAr	qPCR	TGGATGTCCTGCTCCTGAAG	[6]
Bo_nasSdel_F1	nasS deletion	TCGAGCTCGGTACCCCGGAATCCACCAATGCCTTG	This study
Bo_nasSdel_R1	nasS deletion	GCAGCTCCGAATCAGGCCTTGTTGGACCAGGACACCTCG	This study
Bo_nasSdel_F2	nasS deletion	CGAGGTGTCCTGGTCCAACAAGGCCTGATTCGGAGCTGC	This study
Bo_nasSdel_R2	nasS deletion	CTCTAGAGGATCCCCGGTCCTGGATACCCTCGATC	This study
aadA_F_IF	nasS deletion	CAGGGGATCAAGATCGTGGACATAAGCCTGTTCGG	This study
aadA_R_IF	nasS deletion	GCAGGCATCGCCATGAGTGCATCTAACGCTTGAGT	This study
56del_F1	56 bp deletion	TCGAGCTCGGTACCCGTCGAGGTAGCCGTCGACCA	This study
56del_R1	56 bp deletion	GGTATCGTCATCCGGTCGCTTATCTTCTTCGATGATTTGTCGCCG	This study
56del_F2	56 bp deletion	CGGCGACAAATCATCGAAGAAGATAAGCGACCGGATGACGATACC	This study
56del_R2	56 bp deletion	CTCTAGAGGATCCCCCGGCTTCAGGCTGTCCTTCA	This study
SG09_nos-1F	nosZ deletion	CTCGAATTCCGCTGATCTTCATTCTGTGG	This study
SG09_nos-1R	nosZ deletion	CAGATCGTCCCCGGGCGCTCGAGAAGAACACGTAG	This study
SG09_nos-2F	nosZ deletion	TCTCGAGCGCCCGGGGACGATCTGTTCGACGACAA	This study
SG09_nos-2R	nosZ deletion	CTCAAGCTTCGGAAACATGCGTAAGAGAA	This study
Bw_SP1	5' RACE	GAGGAAGATGAATCCCTGGAGCTG	This study
Bw_SP2	5' RACE	GCGGTACACTGGTACGATGG	This study
Bw_SP3	5' RACE	GGCGTAATCTTCGGCAGGTAT	This study
R_SP1	5' RACE	CAGGTACACGAAGCCCTGCAATTG	[7]
R_SP2	5' RACE	GCGATAGGCCGGTATGATAG	[7]
R_SP3	5' RACE	GGGCAATCTTCGACAGGTAA	[7]

References

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