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Potential of Rice Stubble as a Reservoir of Bradyrhizobial Inoculum in Rice-Legume Crop Rotation

Pongdet Piromyou,[a] Teerana Greetatorn,[a] Kamonluck Teamtisong,[b] Panlada Tittabutr,[a] Nantakorn Boonkerd,[a] Neung Teaumroong[a]

School of Biotechnology, Institute of Agricultural Technology, Suranaree, University of Technology, Nakhon Ratchasima, Thailand[a]; The Center for Scientific and Technological Equipment, Suranaree University of Technology, Nakhon Ratchasima, Thailand[b]

ABSTRACT *Bradyrhizobium* encompasses a variety of bacteria that can live in symbiotic and endophytic associations with leguminous and nonleguminous plants, such as rice. Therefore, it can be expected that rice endophytic bradyrhizobia can be applied in the rice-legume crop rotation system. Some endophytic bradyrhizobial strains were isolated from rice (*Oryza sativa* L.) tissues. The rice biomass could be enhanced when supplementing bradyrhizobial strain inoculation with KNO_3, NH_4NO_3, or urea, especially in *Bradyrhizobium* sp. strain SUTN9-2. In contrast, the strains which suppressed rice growth were photosynthetic bradyrhizobia and were found to produce nitric oxide (NO) in the rice root. The expression of genes involved in NO production was conducted using a quantitative reverse transcription-PCR (qRT-PCR) technique. The *nirK* gene expression level in *Bradyrhizobium* sp. strain SUT-PR48 with nitrate was higher than that of the *norB* gene. In contrast, the inoculation of SUTN9-2 resulted in a lower expression of the *nirK* gene than that of the *norB* gene. These results suggest that SUT-PR48 may accumulate NO more than SUTN9-2 does. Furthermore, the *nifH* expression of SUTN9-2 was induced in treatment without nitrogen supplementation in an endophytic association with rice. The indole-3-acetic acid (IAA) and 1-amino-cyclopropane-1-carboxylic acid (ACC) deaminase produced *in planta* by SUTN9-2 were also detected. Enumeration of rice endophytic bradyrhizobia from rice tissues revealed that SUTN9-2 persisted in rice tissues until rice-harvesting season. The mung bean (*Vigna radiata*) can be nodulated after rice stubbles were decomposed. Therefore, it is possible that rice stubbles can be used as an inoculum in the rice-legume crop rotation system under both low- and high-organic-matter soil conditions.

IMPORTANCE This study shows that some rice endophytic bradyrhizobia could produce IAA and ACC deaminase and have a nitrogen fixation ability during symbiosis inside rice tissues. These characteristics may play an important role in rice growth promotion by endophytic bradyrhizobia. However, the NO-producing strains should be of concern due to a possible deleterious effect of NO on rice growth. In addition, this study reports the application of endophytic bradyrhizobia in rice stubbles, and the rice stubbles were used directly as an inoculum for a leguminous plant (mung bean). The degradation of rice stubbles leads to an increased number of SUTN9-2 in the soil and may result in increased mung bean nodulation. Therefore, the persistence of endophytic bradyrhizobia in rice tissues can be developed to use rice stubbles as an inoculum for mung bean in a rice-legume crop rotation system.

KEYWORDS rice endophytic bradyrhizobia, mung bean, rice-legume crop rotation

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Address correspondence to Neung Teaumroong, neung@sut.ac.th.

Nitrogen-fixing root nodule formation is the result of a symbiotic relationship between rhizobia and their legume host plants. Rhizobia are able to infect the specific leguminous host roots and form nodules through complex interactions between plants and microbes (1). It is now well established that in addition to symbiotic association with legumes, rhizobia may occur as an endophyte (is colonized in intercellular spaces) of the root in nonlegumes, such as rice (*Oryza sativa* L. [2, 3], *Oryza breviligulata* [4], or *O. sativa* L. cv. Pelde [5]), wheat (*Triticum aestivum*) (6), sugarcane (*Saccharum officinarum*), and maize (*Zea mays*) (7), and some rhizobia can promote plant growth and productivity (2–7). The genus *Bradyrhizobium* also encompasses a variety of bacteria that can live in symbiotic and endophytic associations with legumes and nonlegumes (3).

In fact, legumes are suitable rotational crops with rice and can be planted before or after rice-harvesting season (8). For example, precultivation of rice with mung bean crop (*Vigna radiata*) significantly increases rice dry weight and also provides the advantage of marketable mung bean grain (8). For the inoculum carrier, peat that is exhaustible natural resources has been commonly used as a carrier for rhizobia (9). However, in cases where peat is not available, other carriers have also been utilized, such as s liquid carrier, plant residues, and wastewater sludge, but these resources are exhaustible and have some limitations, such as their unstable availability, sterilization method, and shelf-life (10). Therefore, if we can select endophytic bradyrhizobia that can nodulate mung bean and establish themselves in rice tissues, it is possible that rice stubbles can be used as an inoculum in field-grown legumes to reduce the use of bacterial inoculum. In this study, endophytic bradyrhizobia with the ability to establish symbiosis with mung bean and that have the potential to promote rice growth were selected based on the response of bacteria to different types of N chemical fertilizers and the plant growth-promoting characteristics.

RESULTS

Characterization of rice endophytic bradyrhizobia and their symbiotic properties with mung bean. Among bradyrhizobial strains, *Bradyrhizobium* sp. strain SUT-PR9 produced the greatest amount of indole-3-acetic acid (IAA), followed by *Bradyrhizobium* sp. strains SUT-R74 and SUT-PR48 (29.44, 10.04, and 5.41 mg · mg of protein^{-1}, respectively) at a free-living stage. On the other hand, the lowest production of IAA was found in *Bradyrhizobium* sp. strain SUT-R55 (0.44 mg · mg of protein^{-1}). SUT-PR9 also had the highest 1-amino-cyclopropane-1-carboxylic acid (ACC) deaminase activity (5.34 μmol · h^{-1} · mg of protein^{-1}), and the lowest activity was detected in SUT-PR48 (2.18 μmol · h^{-1} · mg of protein^{-1}). All strains in free-living form were also assessed for their nitrogenase activity. The highest nitrogenase activity was observed in SUT-R74 (0.93 nmol · h^{-1} · mg of protein^{-1}). However, nitrogenase activity was not detected in *Bradyrhizobium* sp. strain SUT-R3, ORS285, or PRC008 (Table 1).

Under symbiotic conditions with mung bean, strain SUTN9-2 had the highest nitrogenase activity, while the nitrogenase activities of SUT-R3, SUT-PR9, SUT-PR48, SUT-PR64, and SUT-R74 were not significantly different from that of the control. The highest number and dry weights of nodules were produced by commercial strain PRC008, followed by SUTN9-2 and SUT-R55, while SUT-PR9, SUT-PR48, SUT-PR64, and SUT-R74 did not form nodules after 1 month of inoculation. Maximum plant dry weight was obtained from the treatment inoculated with commercial strain PRC008. Bradyrhizobial strain SUTN9-2 did not produce a significantly different plant dry weight from that of PRC008. In addition, strain PRC008 was not a rice endophyte (data not shown). The results suggested that SUTN9-2 is more effective than other rice endophytic bradyrhizobial strains (Table 2).

Effects of different nitrogen sources on rice growth promotion when inoculated with rice endophytic bradyrhizobia. The nitrogen sources for plants at 0.1 and 1 mM KNO_3, urea, and NH_4NO_3 were applied into N-free Hoagland's medium (11). The uninoculated control with a nitrogen source from 1 mM urea and NH_4NO_3 produced a significantly different plant dry weight compared to with that of the KNO_3 and N-free

TABLE 1 Plant growth promotion characteristics of the rice endophytic bradyrhizobial strains[a]

Bacterial strain	mg of IAA · mg of protein^{-1}	μmol alpha ketobutyrate · h^{-1} · mg of protein^{-1}	nmol ARA · h^{-1} · mg of protein^{-1} under free-living conditions
SUT-R3	1.89 ± 1.25 B	4.29 ± 0.57 AB	0 ± 0.00 D
SUT-PR9	29.44 ± 15.29 A	5.34 ± 0.26 A	0.01 ± 0.00 D
SUT-PR48	5.41 ± 2.20 B	2.18 ± 0.10 D	0.11 ± 0.07 CD
SUT-R55	0.44 ± 0.00 B	3.29 ± 0.29 BCD	0.08 ± 0.06 CD
SUT-PR64	2.76 ± 1.25 B	3.73 ± 0.52 BC	0.43 ± 0.12 B
SUT-R74	10.04 ± 1.38 B	3.56 ± 0.46 BC	0.93 ± 0.24 A
SUTN9-2	0.75 ± 0.36 B	3.48 ± 0.10 BC	0.23 ± 0.11 C
ORS285	4.79 ± 2.60 B	2.63 ± 0.87 CD	0 ± 0.00 D
PRC008	0.52 ± 0.16 B	2.55 ± 0.62 CD	0 ± 0.00 D

[a]Different letters in the same column indicate significant differences between treatments ($P \leq 0.05$) ($n = 3$).

treatment (Fig. 1A). Among eight bradyrhizobial strains, only the inoculation of non-photosynthetic *Bradyrhizobium* (SUT-R3, SUT-PR9, SUT-R55, SUT-R74, and SUTN9-2) with 1 mM KNO_3 (Fig. 1B) and NH_4NO_3 obviously increased the rice dry weight (Fig. 1C). All bradyrhizobial inoculations significantly increased rice biomass with 0.1 mM urea supplementation (Fig. 1D). In contrast, the inoculation of photosynthetic *Bradyrhizobium* (SUT-PR48, SUT-PR64, and ORS285) with 0.1 and 1 mM urea showed significantly higher total plant dry weight than that with KNO_3, NH_4NO_3, and N-free treatment (Fig. 1B to D). To understand the mechanism of endophytic bradyrhizobia on rice growth promotion and suppression, SUTN9-2 (representing the mung bean-nodulating and growth-promoting rice strain) and SUT-PR48 (representing nonnodulating mung bean and growth-suppressing rice strain) were selected for further experiments.

Localization of endophytic bradyrhizobia in rice. To confirm that bradyrhizobia are rice endophytes, strains SUTN9-2 and SUT-PR48 in rice roots were investigated at 3 and 7 days after inoculation (DAI) by using scanning electron microscopy (SEM). Rod-shaped bacterial cells were observed and were mostly located in groups or distributed so as to be covering the root surface (Fig. S1A and B). Such damage of the epidermal surface on heavily colonized areas suggests an active invasion mechanism probably associated with a high-density bacterial population. Fig. S1A and B show that bradyrhizobia invaded the inner tissues through the epidermal cells, eventually migrating to the cortex cells after 3 days of inoculation (Fig. S1C and D). At 7 days, bradyrhizobia were observed entering the intercellular and intracellular spaces (Fig. S1E and F). Strains SUTN9-2 and SUT-PR48 showed a similar invasion at 3 and 7 days (Fig. S1A to F). In this experiment, we demonstrated that *Bradyrhizobium* spp. can invade rice roots, spreading rapidly and systematically through the plant tissues.

NO production according to different nitrogen sources and bradyrhizobial strains. Since some bradyrhizobia are known to have denitrification activity, it is possible that the intermediate nitric oxide (NO) gas, which is toxic to plant cells,

TABLE 2 Effects of rice endophytic bradyrhizobia on growth, nodulation, and nitrogen fixation on mung bean (cv. SUT4)[a]

Bacterial strain	ARA (nmol ethylene · h^{-1} · nodule dry weight^{-1})	No. of nodules per plant	Nodule (mg [dry wt] · plant^{-1})	Plant (g [dry wt] · plant^{-1})
Control	0.00 ± 0.00 D	0.00 ± 0.00 C	0.00 ± 0.00 C	0.42 ± 0.02 CD
SUT-R3	7.07 ± 0.15 D	6.00 ± 1.53 C	2.63 ± 2.01 BC	0.42 ± 0.07 CD
SUT-PR9	0.00 ± 0.00 D	0.00 ± 0.00 C	0.00 ± 0.00 C	0.58 ± 0.16 BC
SUT-PR48	0.00 ± 0.00 D	0.00 ± 0.00 C	0.00 ± 0.00 C	0.32 ± 0.10 D
SUT-R55	44.88 ± 9.99 B	16.00 ± 0.57 B	8.66 ± 0.50 BC	0.62 ± 0.19 BC
SUT-PR64	0.00 ± 0.00 D	0.00 ± 0.00 C	0.00 ± 0.00 C	0.45 ± 0.07 CD
SUT-R74	0.00 ± 0.00 D	0.00 ± 0.00 C	0.00 ± 0.00 C	0.40 ± 0.07 CD
SUTN9-2	81.11 ± 3.11 A	17.00 ± 4.50 B	12.13 ± 2.73 B	0.80 ± 0.10 AB
ORS285	0.39 ± 0.01 D	5.00 ± 2.00 C	5.00 ± 2.00 BC	0.43 ± 0.04 CD
PRC008	17.50 ± 1.33 C	60.00 ± 14.90 A	49.83 ± 17.40 A	0.94 ± 0.19 A

[a]Different letters in the same column indicate significant differences between treatments ($P \leq 0.05$) ($n = 3$).

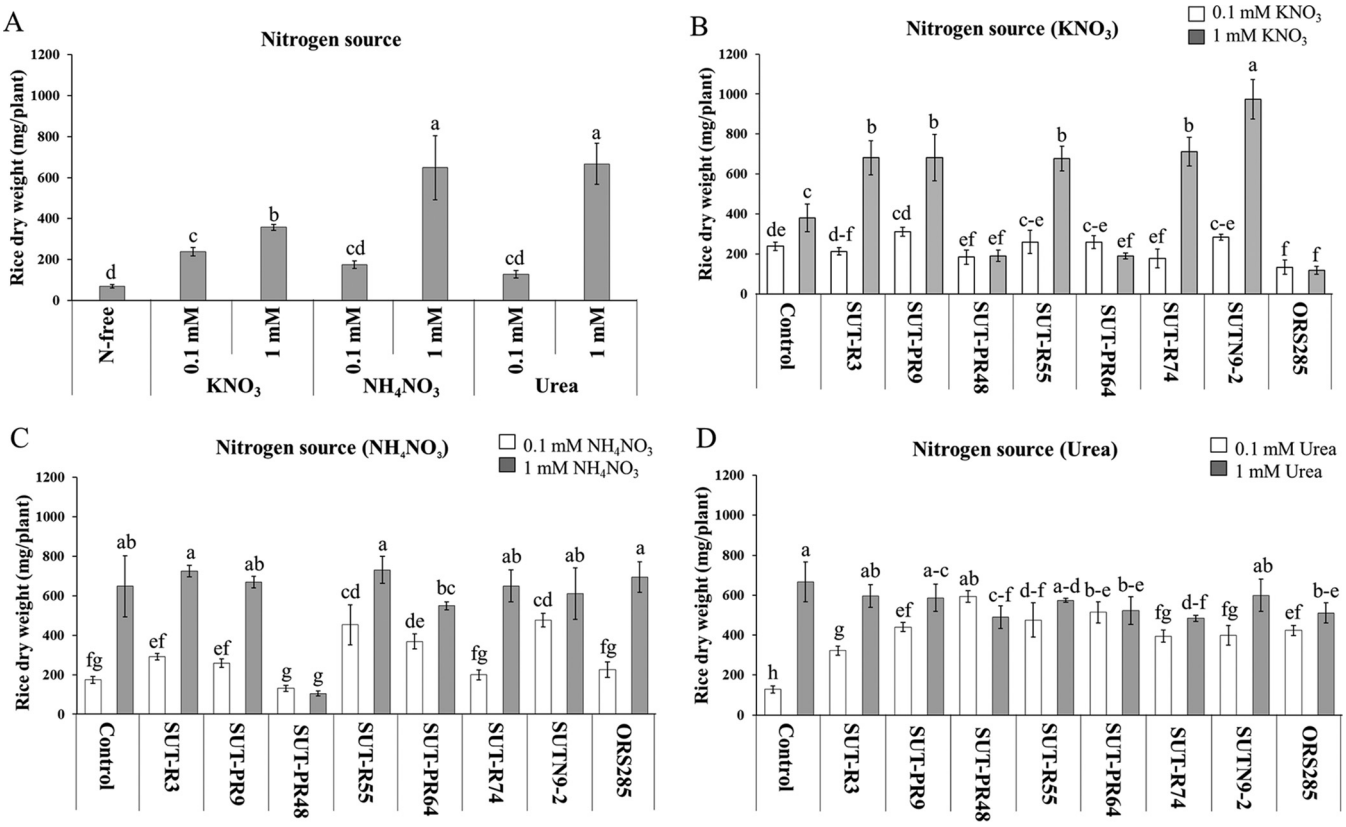

FIG 1 Effect of different nitrogen sources and rice endophytic bradyrhizobial strains on rice growth; uninoculated control (A), KNO_3 (B), NH_3NO_3 (C), and urea (D) supplementations. Within each N supplementation, means labeled with different letters are statistically different at a *P* value of ≤0.05 ($n = 3$).

accumulates in plant cells and inhibits plant growth. The NO produced by plant cells without bacterial inoculation was not significantly different from those in N-supplied (KNO_3, HH_4NO_3, and urea) and N-free nutrient solutions (Fig. S3A). Thus, the NO production in rice root inoculated with different bradyrhizobia under supplementation of different N sources was determined. Distinct fluorescence indicating NO production was detected when the rice roots were inoculated with bradyrhizobial strains SUT-PR48 and SUTN9-2 in the treatments supplied with KNO_3 and NH_4NO_3 (Fig. 2). In contrast, fluorescence was not observed when those rice plants were inoculated with SUT-PR48 and SUTN9-2 in the treatment supplied with urea (Fig. 2B and C). Fluorescence was found at 14 DAI with photosynthetic bradyrhizobial strain SUT-PR48 in both treatments of KNO_3 and NH_4NO_3 amendments (Fig. 2C). However, some fluorescence was also observed in nonphotosynthetic *Bradyrhizobium* SUT-R3 (see Fig. S2 in the supplemental material). For NO production in free-living cells, NO production of SUT-PR48 was significantly higher than that of SUTN9-2 in both the KNO_3 and NH_4NO_3 supplementations (Fig. S3B). It seems that high-level NO production from strain SUT-PR48 (Fig. 2 and S3B) correlated with the significant suppression of rice growth (Fig. 1B and C).

Relative expression of genes involved in nitric oxide production of endophytic bradyrhizobia SUT-PR48 and SUTN9-2 in rice roots. The endophytic bradyrhizobium-related genes were selected from their mode of nitric oxide production, including the Cu-containing nitrite reductase gene (*nirK*) and nitric oxide reductase gene (*norB*). The expression levels of the *nirK* and *norB* genes of SUT-PR48 and SUTN9-2 are presented in Fig. 3. The inoculation of SUT-PR48 with KNO_3 demonstrated the highest *nirK* gene expression level (0.82-fold), followed by that with inoculation with NH_4NO_3 (0.69-fold). However, the *nirK* gene expression level of SUT-PR48 was significantly the lowest when treated with urea (0.31-fold). In contrast, the highest relative expression level of *norB* was detected in the inoculation of SUT-PR48 with urea (0.93-fold), followed by that with inoculation with NH_4NO_3 (0.46-fold) and KNO_3 (0.39-fold), respectively (Fig. 3A).

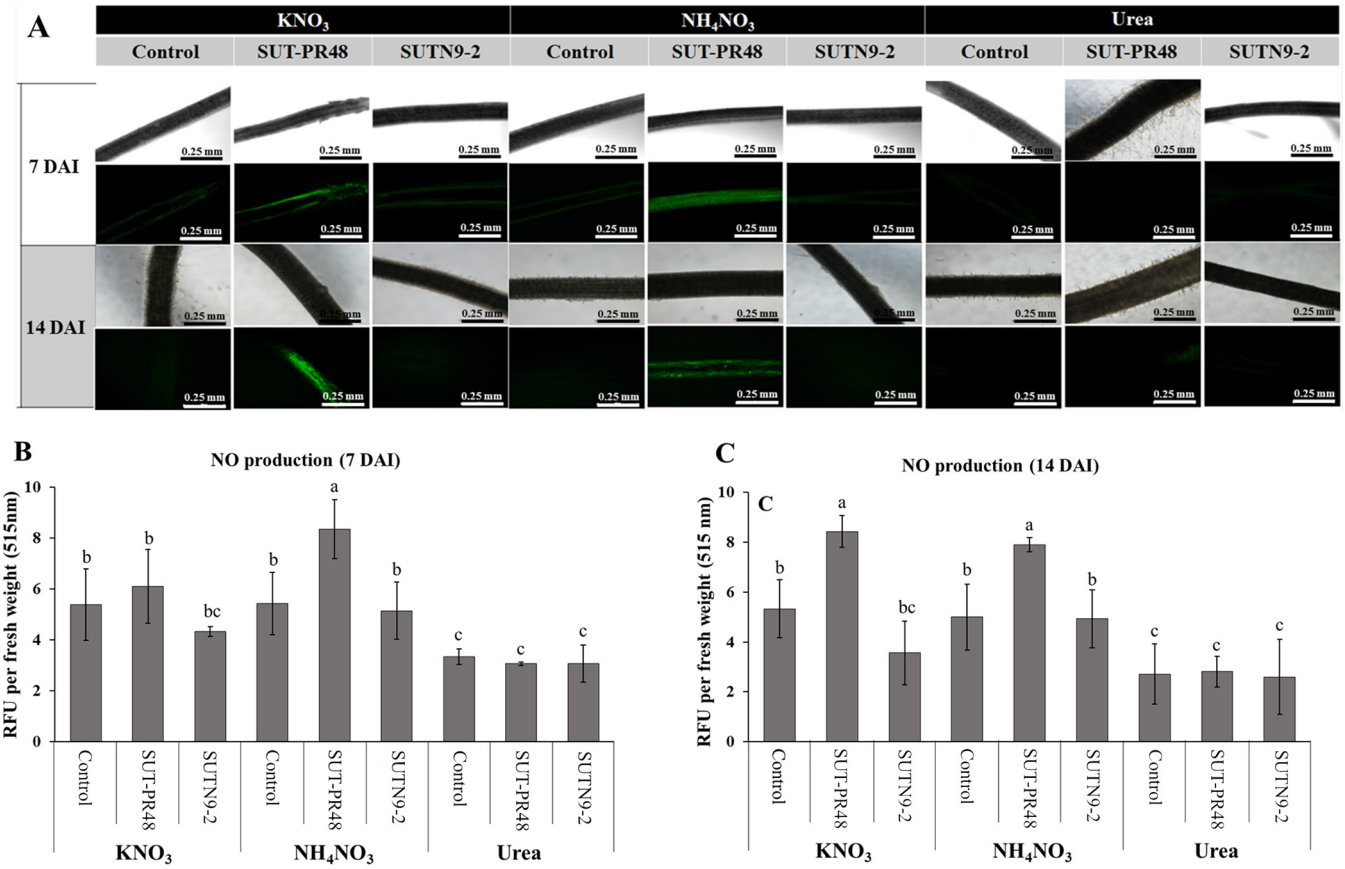

FIG 2 (A) Effect of different nitrogen sources and some rice endophytic bradyrhizobial strains, SUT-PR48 and SUTN9-2, on nitric oxide production detected by DAF-FM DA solution under confocal laser scanning microscopy. (B and C) NO production in rice roots: quantification of NO produced in rice roots (*O. sativa* PT1) at 1 week (B) and 2 weeks (C) after inoculation. Relative fluorescence unit (RFU) values per rice root fresh weight at 515 nm were estimated. Means labeled with different letters are statistically different at a *P* value of ≤0.05 ($n = 3$).

The expression levels of the *nirK* and *norB* genes of SUTN9-2 are depicted in Fig. 3B. The *nirK* gene expression level of SUTN9-2 was not detected when KNO_3 or urea was used as a source of nitrogen. However, the inoculation of SUTN9-2 with NH_4NO_3 induced the *nirK* expression level by 0.66-fold. In the case of the *norB* gene, the

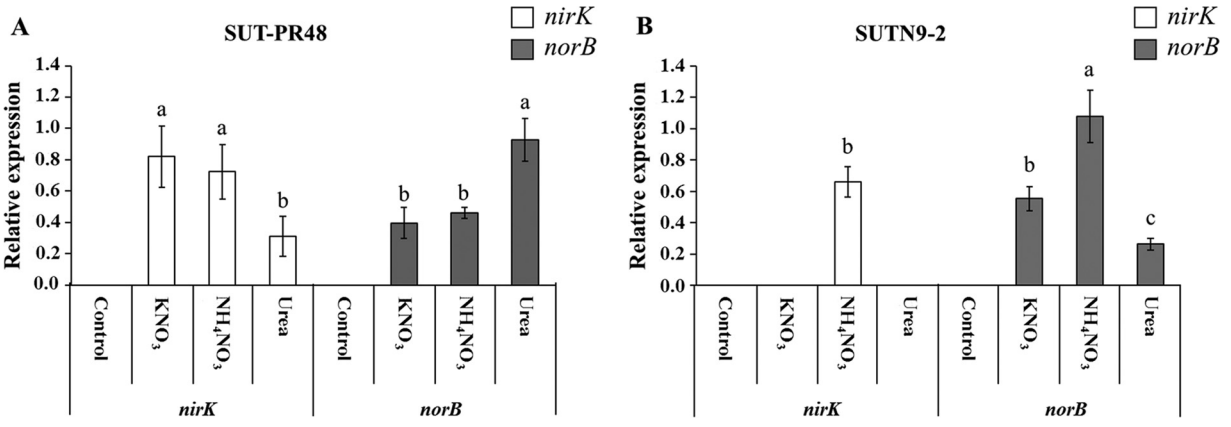

FIG 3 Relative expression of *nirK* gene and *norB* gene of *Bradyrhizobium* sp. SUT-PR48 (A) and SUTN9-2 (B) in rice roots at 7 DAI in response to different nitrogen sources. The housekeeping gene *atpD* was used as an endogenous control. Letters indicate the N-amended treatments. Control, uninoculated control; KNO_3, KNO_3 supplementation; NH_4NO_3, NH_4NO_3 supplementation; urea, urea supplementation. The statistical analysis was separately calculated between *nirK* and *norB* genes. Means labeled with different letters are statistically different at a *P* value of ≤0.05 ($n = 3$).

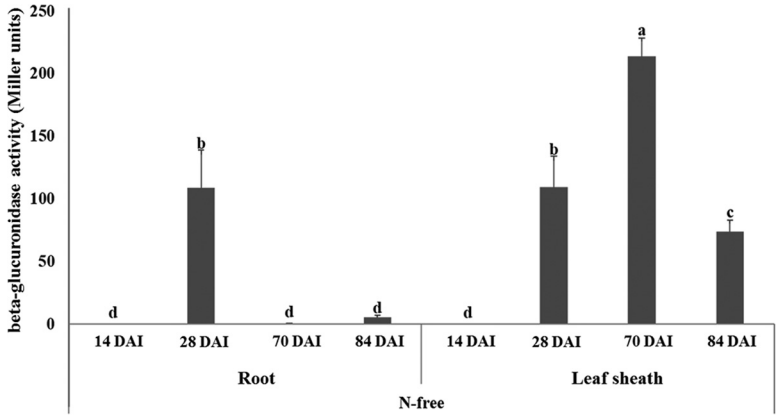

FIG 4 Expression of *nifH* gene of *Bradyrhizobium* sp. SUTN9-2 *nifH-GUS* fusion labeled strain in rice leaf sheaths and roots at 14, 28, 70, and 84 DAI in response to N-free solution compared to uninoculated control. Means labeled with different letters are statistically different at a *P* value of ≤0.05 ($n = 3$).

expression level in NH_4NO_3 (1.08-fold) was also significantly higher than that of the other nitrogen sources, KNO_3 (0.55-fold) and urea (0.26-fold), respectively.

Expression of genes involved in nitrogen fixation of endophytic *Bradyrhizobium* SUTN9-2 in rice tissues. In a filtrate of rice tissues, the SUTN9-2 *nifH-GUS*-labeled strain was separated from the rice root and leaf sheath at 14, 28, 70, and 84 DAI. The lowest *nifH* gene expression of SUTN9-2 was produced in the leaf sheath (0.035 ± 0.009 Miller units) and root (0.23 ± 0.05 Miller units) at 14 DAI in an N-free solution. The highest *nifH* gene expression in a root was detected at 28 DAI (109.16 ± 30.04 Miller units). However, the *nifH* gene expression in a root was clearly decreased at 70 and 84 DAI, respectively. In contrast, the highest *nifH* gene expression in a leaf sheath was detected at 70 DAI (213.97 ± 14.55 Miller units) but slightly decreased at 84 DAI (Fig. 4).

IAA and ACC deaminase production by *Bradyrhizobium* sp. SUTN9-2 in rice tissues. SUTN9-2 produced a greater amount of IAA than the uninoculated control. The IAA production by SUTN9-2 in the rice root and leaf sheath increased from 26.46 ± 3.75 to 37.04 ± 2.80 and 92.42 ± 1.01 to 131.17 ± 7.77 mg of IAA · g (dry weight) of plant^{-1}, respectively, compared with the uninoculated control. SUTN9-2 was also able to produce ACC deaminase, and the ACC deaminase activity of SUTN9-2 in the rice root and leaf sheath was significantly higher than that of the uninoculated control (Table 3).

Enumeration of rice SUTN9-2 in rice tissues. The population densities and the persistence of SUTN9-2 (SUTN9-2GUS) in rice tissues were determined by plant most probable number (MPN) count. The population size of SUTN9-2 was found in both root and leaf sheath tissues, and the population densities varied from 10^4 to 10^6 (MPN/g of inoculant/g [fresh weight] of rice). The population densities of the leaf sheath and root significantly decreased after 14 and 21 DAI, respectively. However, no significant differences in population densities were observed in leaf sheath (at 14 to 28 DAI) or root

TABLE 3 IAA and ACC deaminase production by *Bradyrhizobium* sp. SUTN9-2 in rice tissues[a]

Treatment	mg of IAA · g^{-1} (dry wt) of plant	ACC deaminase (μmol alpha ketobutyrate · g^{-1} [dry wt] of plant)
Control (without SUTN9-2)		
Leaf sheath	92.42 ± 1.01 B	368.98 ± 6.99 B
Root	26.46 ± 3.75 D	79.63 ± 8.63 D
SUTN9-2		
Leaf sheath	131.17 ± 7.77 A	414.81 ± 13.45 A
Root	37.04 ± 2.80 C	210.00 ± 8.38 C

[a]Different letters in the same column indicate significant differences between treatments ($P \leq 0.05$) ($n = 3$).

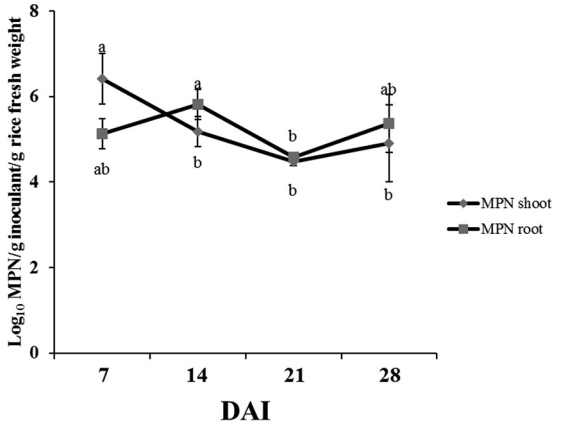

FIG 5 Enumeration of rice endophytic *Bradyrhizobium* sp. SUTN9-2 in rice tissues at different times using mung bean most probable number (MPN). Means labeled with different letters are statistically different at a P value of ≤ 0.05 ($n = 3$).

(at 7, 14, and 28 DAI) tissues. In addition, SUTN9-2 still persisted in rice tissues from 7 to 28 DAI (Fig. 5).

Investigation of the persistence of SUTN9-2 in the rice plant under pot trial conditions. The rice inoculated with of SUTN9-2 (SUTN9-2GUS) planted in both soil containing low nutrients and high organic matter (paddy soil) showed no significant differences between the number of plants per hill and panicles per hill compared to the uninoculated control (Fig. S5). The persistence of SUTN9-2 in rice grown in pots under greenhouse conditions was evaluated by plate count of the CFU. The numbers of the blue colonies were counted to display the SUTN9-2 population densities in different rice tissues. The results revealed that the population density of SUTN9-2 was determined in all tissues of rice and was similar in the low-organic-matter soil and the high-organic-matter soil (paddy soil). The highest population densities were observed in leaf sheaths, and the other plant tissues (stubbles and roots) had slightly lower population densities (10^3 to 10^4 CFU/g [fresh weight]). However, the population densities were not significantly different in the tissues. On the other hand, the lowest population densities were observed in seed and leaf tissues (10^1 to 10^2 CFU/g [fresh weight]) (Fig. S6).

Investigation of the nodulation of SUTN9-2 from rice stubbles in mung bean. At 1 week after rice stubbles were incorporated into soil, mung bean nodulation from rice stubbles was not observed. However, the nodulation was observed at 2 weeks with around 3 to 4 nodules (data not shown). An increase in the number of mung bean nodules was obtained at 5 weeks after rice stubbles were incorporated into the low-organic-matter soil and high-organic-matter soil, with nodule numbers around 60 and 35 nodules, respectively. No significant differences in the nodule dry weight, plant dry weight, and acetylene reduction activity were observed between low- and high-organic-matter soils, and the activity was significantly higher than that of the uninoculated control (Table 4). In addition, the population of SUTN9-2 (SUTN9-2GUS) in the soil was also determined by the plant infection (MPN) method, and the nodules formed by SUTN9-2 were confirmed by β-glucuronidase (GUS) staining. The results revealed that a small amount of SUTN9-2 remained in the soil (8 MPN/g of stubble/g of soil [dry

TABLE 4 Nodulation of SUTN9-2 in mung bean 5 weeks after incorporated rice stubbles into the soil[a]

Soil type	ARA (nmol ethylene · h^{-1} · nodule dry $weight^{-1}$)	No. of nodules per plant	Nodule (mg [dry wt] · $plant^{-1}$)	Plant (g [dry wt] · $plant^{-1}$)
Control (without SUT9-2)	0.00 ± 0.00 B	0.00 ± 0.00 C	0.00 ± 0.00 B	0.13 ± 0.006 B
Low-organic-matter soil	26.30 ± 6.20 A	60.00 ± 5.00 A	0.58 ± 0.16 A	0.26 ± 0.07 A
High-organic-matter soil	25.90 ± 5.17 A	35.00 ± 11.00 B	0.70 ± 0.35 A	0.22 ± 0.04 A

[a]Different letters in the same column indicate significant differences between treatments ($P \leq 0.05$) ($n = 3$).

weight]) before the step of rice incorporation, and then the population densities of SUTN9-2 increased at 1 week after incorporating the rice stubbles into the soil (50 MPN/g of stubble/g of soil [dry weight]) (data not shown).

DISCUSSION

Investigation of the influence of N sources on rice growth demonstrated that rice growth was differently affected by the supplementation of urea, NH_4NO_3, and KNO_3 (Fig. 1A). Jang et al. (12) reported that rice (*O. sativa* L.) growth was also higher in ammonium-based N fertilizers than in nitrate-based fertilizers. In rice, the application of NH_4^+ is preferred over NO_3^- as a nitrogen source because NH_4^+ metabolism requires less energy than that of NO_3^- (12, 13), since the absorption of NH_4^+ occurs faster than the absorption of NO_3^- (14). While NH_4^+ can be assimilated directly into amino acid, NO_3^- must first be reduced into NO_2^- and then NH_4^+ via nitrate reductase and nitrite reductase (15). Thus, NH_4^+ is the main form of N available to rice. In contrast, NO_3^- is the dominant form of nitrogen for plant uptake in aerobic soil. Fageria et al. (16) reported that plants supplemented with equal proportions of NH_4^+ and NO_3^- grew as well as those supplemented with any single amount of an N form. In addition, plants can also absorb both forms of N equally, and the N form absorbed is mainly determined by what form is abundant and assessable at any given time (17, 18).

The rice dry weights in the treatments of KNO_3 and NH_4NO_3 after inoculation with nonphotosynthetic bradyrhizobia were higher than those in the treatments of urea. On the other hand, the rice dry weights from inoculation with photosynthetic bradyrhizobia were lower in the treatments of KNO_3 and NH_4NO_3 than in urea (Fig. 1). *Bradyrhizobium* has the capability of denitrification in the dissimilatory reduction of nitrate (NO_3^-) to N_2 via the gaseous intermediates nitric oxide (NO) and nitrous oxide (N_2O) (19). NO is an inorganic free radical that can become very toxic for the plant cells (20–22). The results suggested that some endophytic bradyrhizobial strains may produce nitric oxide, resulting in rice growth suppression (Fig. 2). Therefore, to select the endophytic bacteria as a biofertilizer inoculum, the NO production from bacterial strains must first be considered.

The production of NO by bradyrhizobia has been previously reported, especially in *Bradyrhizobium japonicum* (15, 23, 24). Our results revealed that NO in the rice root was produced from the treatments of NH_4NO_3 and KNO_3 inoculated with photosynthetic bradyrhizobium (SUT-PR48) (Fig. 2). This result implied that some photosynthetic bradyrhizobia accumulated NO using NO_3^- from NH_4NO_3 and KNO_3 via denitrification. On the other hand, an associated function of denitrification is the detoxification of cytotoxic compounds, such as NO_2^- and NO produced as intermediates during denitrification reactions (18). This function was found in some strains (SUT-R3, SUT-R55, SUT-R74, and SUTN9-2) because NO was not observed from the treatments of NH_4NO_3 and KNO_3 inoculated with nonphotosynthetic bradyrhizobia, especially strain SUTN9-2 (Fig. 2). Thus, it can be assumed that inoculation with bradyrhizobia producing NO in the rice roots may also inhibit rice growth. In addition, NO production was detected only in rice roots inoculated with SUT-PR48 (Fig. 2). In this study, although the fluorescent signals representing NO production were detected in free-living cells (Fig. S3), it could be the artifact from the N supplementation, since the control medium (without bacterial cells) produced a high signal level (approximately 5 relative fluorescence units [RFU] per optical density [OD]) (Fig. S3B). The decrease in fluorescence signal in medium containing bacterial cells may be because a certain amount of the N source was consumed by bacteria, resulting in that signal reduction (approximately 0.5 RFU per OD) (Fig. S3B) compared with the control medium. In addition, since this experiment was performed under aerobic conditions, denitrification in bradyrhizobia may not have occurred. The verification of NO production in free-living bradyrhizobia remains to be further examined under anaerobic conditions.

Several studies have clearly shown that the production of NO occurred in early stages of rhizobium-legume symbiosis (25–28). Thus, the *nirK* genes of *Bradyrhizobium* were found to be essential for denitrification; then, *norB* catalyzes the two-electron

reduction of NO to the greenhouse gas N_2O in the detoxification process (25). The results of this study revealed that the *nirK* gene expression was activated by nitrogen source containing nitrate, especially in *Bradyrhizobium* sp. SUT-PR48 (Fig. 3). In contrast, nitrate did not activate the expression of the *norB* gene, which is required for NO detoxification and led to the accumulation of NO in rice plants. This may be the reason why SUT-PR48 showed a suppression in rice growth when nitrate was used as a nitrogen source. However, NO accumulation in the treatment of NH_4NO_3 was significantly higher than that of KNO_3 (Fig. 2A), whereas *nirK* expression of NH_4NO_3 treatment was not significantly different from that with KNO_3 supplementation (Fig. 3A). It seems that the amount of transcripts does not always correspond to NO activity levels. Perhaps, plant root tissues also contribute to NO production (and to NO reduction as well) (29). Likewise, bradyrhizobial genes other than *nirK* and *norB* may contribute to NO production and detoxification in the oxygen-limited host environment (30, 31).

In accordance with our results in SUTN9-2, *nirK* gene expression was detected only in the treatment of NH_4NO_3. It is possible that the NH_4^+ from this source of fertilizer could be assimilated more easily than NO_3^-. The remaining NO_3^- may be accumulated at high levels and may induce the expression of *nirK* when rice is supplemented with NH_4NO_3 (Fig. 3B). However, the *norB* gene of SUTN9-2 showed a higher expression level than the *nirK* gene. This result implied that SUTN9-2 may perform the NO detoxifications which contribute to the rice growth promotion.

In this study, the physiological characteristics of rice endophytic bradyrhizobia, such as IAA and ACC production (Table 1), were also investigated. The strains which promoted rice growth had the capability to produce IAA and ACC deaminase. Thus, these characteristics may affect plant growth promotion. For the nitrogenase activity (determined by acetylene reduction assay [ARA]), bradyrhizobial strains are known to fix nitrogen under free-living conditions (32). This feature may provide bradyrhizobia an advantage to survive under oligotrophic conditions. However, this experiment aimed to explore the possibility of plant growth promotion characteristics derived from those plant growth-promoting rhizobial (PGPR) characteristics which may facilitate rice growth promotion. Although it could not be concluded that the low nitrogen fixation activity detected from rice endophytic bradyrhizobial strains would support their efficiency in plant growth promotion, SUTN9-2 in rice tissues showed the ability to produce both IAA and ACC deaminase at 1 month after inoculation. Bhattacharjee et al. (33) found that *Rhizobium leguminosarum* bv. trifolii SN10 was able to synthesize ACC deaminase and IAA and could enhance the growth of various varieties of rice grown in the subcontinent. Therefore, we can speculate that SUTN9-2 cells or IAA and ACC deaminase produced by SUTN9-2 may account for the increasing rice growth promotion. Nitrogen accumulation in rice may be caused by biological nitrogen fixation of endophytic bradyrhizobia. This reaction occurs by the activity of the nitrogenase enzyme and the gene that encodes the nitrogenase structural component is *nifH*. During nitrogen fixation, the *nifH* gene was expressed in the treatment without nitrogen source (N-free) at 14, 28, 70, and 84 DAI (Fig. 4 and S4). This indicated that this gene was induced under the treatment without a nitrogen source. Similarly, Terakado-Tonooka et al. (34) suggested that bradyrhizobia colonize and express the *nifH* gene not only in the root nodules of leguminous plants but also in sweet potatoes as diazotrophic endophytes. However, it has been observed that *R. leguminosarum* bv. trifolii does not fix nitrogen in association with rice (2, 35). Thus, the nitrogen fixation ability of endophytic bacteria is also dependent on the variety of rice and diazotrophic endophytes. Thus, the nitrogen fixation of SUTN9-2 may be suspected as one of the factors involved in rice growth promotion.

Chi et al. (36) examined the persistence of viable populations of endophytic rhizobia within rice plants and found that rhizobia inoculated into the rhizosphere of rice were recovered from within surface-sterilized leaf sheaths, leaves, and roots. In addition, *R. leguminosarum* bv. trifolii utilizes a dynamic infection process that permits them to migrate endophytically upward into the stem base, leaf base, leaf sheaths, and some leaves of rice (37). However, our results also revealed that SUTN9-2 persisted in rice

TABLE 5 Bradyrhizobial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics and source of isolation[a]	Source or reference
Strains		
PB strains[b]		
SUT-PR48	Rice root isolate (rice with crop rotation)	3
SUT-PR64	Rice root isolate (rice with crop rotation)	3
ORS285	*Aeschynomene afraspera* root or stem nodule isolate	63
Non-PB strains		
SUT-PR9	Rice root isolate (rice with crop rotation)	3
SUT-R3	Rice root isolate (rice with crop rotation)	3
SUT-R55	Rice root isolate (rice with crop rotation)	3
SUT-R74	Rice root isolate/paddy soil isolate (rice with crop rotation)	3
SUTN9-2	*Aeschynomene americana* nodule isolate (paddy crop)	62
SUTN9-2GUS	SUTN9-2 marked with mTn*5SSgusA20* (pCAM120); Sm^r Sp^r	54
SUTN9-2*nifH*-GUS	Transcriptional *nifH*::*gus* reporter construct (pVO155nifHpm9-2gus)	This work
PRC008	Recommended for mung bean/non-rice endophyte	Department of Agriculture (DOA), Thailand
Escherichia coli S17-1	*pro recA* RP4-2 (Tc^s::Mu) (Km^s::Tn*7*), Mob^+	64
Plasmids		
pCAM120	mTn*5SSgusA20* in pUT/mini-Tn*5*	S. Okazaki[c]; 65
pVO155nifHpm9-2gus	*nifH*::*gus* reporter construct into pVO155-npt2-cefo-npt2-gfp; Km^r $Cefo^r$	This work

[a]Sm^r, spectinomycin resistance; Sp^r, streptomycin resistance; Tc^s, tetracycline susceptibility; Km^s, kanamycin susceptibility; Km^r, kanamycin resistance; $Cefo^r$, cefotaxime resistance.
[b]PB, photosynthetic *Bradyrhizobium*.
[c]Department of International Environmental and Agricultural Science, Graduate School of Agriculture, Tokyo University of Agriculture and Technology, Tokyo, Japan.

tissues until rice-harvesting season (Fig. S6). In addition, endophytic rhizobia persisted in rice throughout the vegetative and into the reproductive phases of development (36). These results indicated that it is possible to use endophytic bradyrhizobia in the rice-legume cropping system.

The maximum viable number of rhizobia per seed of mung bean was 10^7 to 10^8 rhizobia/seed (38). Perkins (39) found that increasing the inoculum level above 100 rhizobia/seed did not increase the nodule number. However, smaller amounts of inoculum resulted in abundant nodulation on the lateral roots of soybeans grown in the growth chamber, but smaller amounts of inoculum failed to produce good nodulation in the field (40). The results of this study indicated that small and large amounts of SUTN9-2 were released from rice stubbles harvested after 1 week and 1 month, respectively. This implied that the nodulation of mung bean using rice stubbles as an inoculum may be affected by the degradation of rice stubbles which release SUTN9-2 into the soil. Therefore, the persistence of endophytic bradyrhizobia in rice tissue can be developed for using rice stubbles as the inoculum for mung bean in the rice-legume crop rotation system. Further investigation of rice stubble inoculum will be performed under field conditions to compare it with the normal inoculum to assess the ability of nitrogen fixation in legume plants as well as frequency of normal inoculum application.

MATERIALS AND METHODS

Plants and bacterial strains. Rice (*Oryza sativa* L.) cv. Pathum Thani 1 and mung bean (*Vigna radiata* L.) cv. SUT4 were used in this study. The bradyrhizobial strains, including photosynthetic and nonphotosynthetic bradyrhizobial strains (PB and non-PB strains, respectively) are listed in Table 5. Bradyrhizobial strains were cultured and maintained on yeast extract-mannitol (YEM) medium (41). *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 37°C. When required, the media were supplemented with the appropriate antibiotics at the following concentrations: kanamycin, 100 μg/ml; nalidixic acid, 25 μg/ml; and cefotaxime, 20 μg/ml.

Physiological characteristics of rice endophytic bradyrhizobia. The physiological characteristics of selected bradyrhizobial strains on nitrogenase activity in free-living form, indole acetic acid (IAA) production, and 1-amino-cyclopropane-1-carboxylic acid (ACC) deaminase activity were assayed. Each isolate was grown in YEM medium (41) at 28°C with agitation (125 rpm) for 5 days prior to characterization, as follows.

(i) ARA. The nitrogenase enzyme activities of rice endophytic bradyrhizobia in free-living form were investigated on the basis of the acetylene reduction assay (ARA) (4). The reactions were carried out in a 21-ml test tube containing a bacterial culture in LG medium (10 g of glucose, 0.41 g of KH_2PO_4, 0.52 g of K_2HPO_4, 0.2 g of $CaCl_2$, 0.05 g of Na_2SO_4, 0.1 g of $MgSO_4{\cdot}7H_2O$, 0.005 g of $FeSO_4{\cdot}7H_2O$, 0.0025 g of

$Na_2MoO_4 \cdot 2H_2O$ per liter) (42) and incubated at 28 ± 2°C for 7 days. Ten percent (vol/vol) of gas phase in the headspace was replaced with acetylene and further incubated at 28 ± 2°C for 24 h. After incubation, the gas from the vessel was injected into a gas chromatograph (GC) with a flame ionization detector equipped with polyethylene (PE)-alumina packed column (50 m by 0.32 mm by 0.25 μm) (PerkinElmer, USA). The standard curve of ethylene was constructed using various concentrations of pure ethylene (43). The total protein concentrations of cell suspension were determined using Lowry's method (44).

(ii) IAA production assay. The IAA production was colorimetrically determined according to the method of Costacurta et al. (45). The bradyrhizobial strains were grown in YEM broth medium supplemented with L-tryptophan (100 mg · liter^{-1}) at 28°C. The supernatant of stationary-phase culture was obtained by centrifugation at 10,000 × *g* for 15 min. The amount of IAA produced per mg of protein was detected as described by Costacurta et al. (45). Pure IAA (Sigma, USA) was used as a standard.

(iii) ACC deaminase activity assay. The bacterial cells were collected by centrifugation at 4,000 × *g* for 5 min and washed twice with minimal medium (46). Cell pellets were suspended in 15 ml of minimal medium supplemented with 1 mM ACC and further incubated at 28°C for 40 h with shaking at 125 rpm to induce ACC deaminase enzyme production. ACC deaminase activity was measured according to protocol of Tittabutr and coworkers (47).

Determination of rice endophytic bradyrhizobia on nodulation and growth promotion of mung bean. Mung bean seeds were surface-sterilized (48) prior to being put into sterilized petri dishes containing wet sterilized tissue paper and kept at room temperature for 2 days. The germinated seeds were then transplanted into the Leonard's jar containing sterilized vermiculite and N-free solution under aseptic conditions. One milliliter of 5 × 10^8 CFU · ml^{-1} rice endophytic bradyrhizobial inoculum was applied to each seedling at 2 days after transplanting. Plants were grown under controlled environmental conditions of 28 ± 2°C on 16/8-h day/night cycle (full light, 639 microeinsteins [μE] · m^{-2} · S^{-1}). After 28 DAI, plants were harvested, and the root part was used for analysis of nitrogenase activity by measurement of acetylene-reducing activity. After the ARA assay, nodules were detached from the roots, and the number of the nodules was scored. The plant and nodule dry weights were determined after drying at 70°C for 72 h.

Investigation of rice growth promotion by endophytic bradyrhizobia inoculation under different N sources. The Leonard's jar was filled with 1:3 (wt/wt) sterilized sand and vermiculite. The N-free nutrient solution contained 7 mM $CaSO_4 \cdot 2H_2O$, 17.8 mM Fe-EDTA, 1.0 mM K_2SO_4, 0.25 mM KH_2PO_4, 0.625 mM K_2HPO_4, and 2.0 mM $MgSO_4 \cdot 7H_2O$, and micronutrients adjusted pH to 6.8 (49) were added into the Leonard's jar. The different N sources of 0.1 and 1 mM KNO_3, urea, and NH_4NO_3 were added separately into the N-free solution and applied through a wick to provide nutrients to the plants. The whole apparatus was autoclaved (90 min at 121°C) prior to applying the rice seedlings. Surface-disinfected rice seeds were germinated on 0.85% agar of YEM medium for 1 day. Then, three replicates of germinated seeds were soaked overnight in broth containing various bacterial isolates (5 ml of 10^8 CFU · ml^{-1}) and then transplanted into the Leonard's jar under aseptic conditions. This was conducted as three replicates per single bradyrhizobial isolate as well as the bradyrhizobial reference strains (SUTN9-2 and ORS285). Rice was grown under controlled environmental conditions of 28 ± 2°C on 16/8-h day/night cycle (full light, 639 μE · m^{-2} · S^{-1}). The rice plants were harvested after 1 month of planting, and the dry weight was determined.

Investigation of rice endophytic bradyrhizobia inside plant tissues using SEM. The roots from rice after inoculation with *Bradyrhizobium* strains SUT-PR48 and SUTN9-2 at 3 and 7 days of culture were fixed with 2.5% (vol/vol) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 2 h and postfixed in 1% (wt/vol) osmium tetroxide in the same buffer for 2 h. The fixed roots were dehydrated in a graded ethanol series. Then, the samples were treated with CO_2, mounted on an aluminum cylinder (stub), and covered with a steam of carbon and ionized gold (49, 50). The samples were examined under an SEM (JSM 7800F; JEOL Ltd., Tokyo, Japan).

Detection of NO production. The rice plants inoculated with bradyrhizobial strains were grown in the test tube containing N-free nutrient solution with different N sources of 1 mM KNO_3, urea, and NH_4NO_3. One- and two-week-old-plants were collected for diaminofluorescein-FM diacetate (DAF-FM DA) detection. A stock solution of 5 mM DAF-FM DA in dimethyl sulfoxide was diluted 500-fold (10 μM DAF-FM final concentration) in water before use (51). The rice roots were placed for 30 min on filter paper soaked with the DAF-FM DA solution, and the fluorescence images of the roots were then observed under confocal laser scanning microscopy (excitation, 488 nm; emission, 515 to 530 nm; Nikon Model Ni-E, Nikon Instech Co., Ltd., Tokyo, Japan).

Quantification of NO production by fluorescence spectrophotometry. A stock solution of DAF-FM was diluted 1,000-fold in water before use. Detached rice roots at 7 and 14 DAI were soaked in the DAF-FM solution for 1 min. The relative fluorescence units (RFU) of the DAF-FM solution were measured using a microplate reader fluorescence spectrophotometer (Varioskan; Thermo Scientific, USA). The wavelengths for excitation and emission were 470 and 515 nm, respectively (52). To examine the NO production from free-living cells of strains SUT-PR48 and SUTN9-2, the protocol described by Fukudome and coworkers (53) was modified. Bradyrhizobial strains SUT-PR48 and SUTN9-2 were grown in YM medium at 30°C for 5 days. Bacterial cells were collected by centrifugation at 10,000 × *g* for 5 min and were incubated with different N sources of 1 mM KNO_3, urea, and NH_4NO_3 in 1× phosphate-buffered saline (PBS) buffer (supplemented with 5 μM DAF-FM final concentration) for 16 h. The cell mixtures were centrifuged at 10,000 × *g* for 5 min. The supernatants were used to analyze NO production using a microplate reader fluorescence spectrophotometer (Varioskan; Thermo Scientific). The wavelengths for excitation and emission were 470 and 515 nm, respectively.

TABLE 6 Primers used in this study

Target gene by type	Primer name	Gene description	Primer sequence (5′→3′)	Description of design (reference or accession no.)
Housekeeping				
atpD	atpDF-SUT-PR48	ATP synthase subunit beta	TGTTGTCGACGAAGAACAGC	Designed from *atpD* of *Bradyrhizobium* sp. strain ORS285 (LT859959.1)
	atpDR-SUT-PR48		CACGAGTTCATCGAGTCCAA	
	atpDF-SUTN9-2		TGTTGTCGACGAAGAACAGC	Designed from *atpD* of *Bradyrhizobium* sp. strain SUTN9-2 (LAXE00000000)
	atpDR-SUTN9-2		CACGAGTTCATCGAGTCCAA	
Nitric oxide production				
norB	norB F-SUT-PR48	Nitric oxide reductase	AAGACCACGGTGACCAACAT	Designed from *norB* of *Bradyrhizobium* sp. strain ORS285 (LT859959.1)
	norB R-SUT-PR48		CGATCGATACCGTTGAGCTT	
	norBF-SUTN9-2		ACAGGAAGAAGATCGCAACG	Designed from *norB* of *Bradyrhizobium* sp. strain SUTN9-2 (LAXE00000000)
	norBR-SUTN9-2		GTGGCTGTGGTCGGTTATCT	
nirK	nirK F-SUT-PR48	Cu-containing nitrite reductase	TGCTGATCGTCCATTCTCAG	Designed from *nirK* of *Bradyrhizobium* sp. strain ORS285 (LT859959.1)
	nirK R-SUT-PR48		TGTGGGTGACGTAAGCGTAG	
	nirK F-SUTN9-2		TTGAAGTTGCCCTTCTCGTC	Designed from *nirK* of *Bradyrhizobium* sp. strain SUTN9-2 (LAXE00000000)
	nirK R-SUTN9-2		GGCGTGTTCGTGTATCACTG	
Nitrogen fixation				
nifH	nifH F	Dinitrogenase reductase	TACGGNAARGGSGGNATCGGCAA	Noisangiam et al. (62)
	nifH R		AGCATGTCYTCSAGYTCNTCCA	
nifH-GUS (transcriptional fusion)				
	nifHpm.SUTN9-2.F	*nifH* promoter region	ACCTATGTCGACGTGCTGAGCTGACTGAGTGG	Designed from *nifH* promoter region of *Bradyrhizobium* sp. strain SUTN9-2 (LAXE00000000)
	nifHpm.SUTN9-2.R		GCGTCGTCTAGAACTCAGCCCTCACTCAGTGT	
	pVO.Bis.r	pVO155 plasmid	GCACAGCAATTGCCCGGCTTTCTTG	Designed from pVO155 plasmid (to confirm *nifH*-GUS integrated into SUTN9-2 chromosome)
	CefoM15-Fin.f	Cefotaxime gene	GCTATGGCACCACCAACGATATC	Designed from CefoM15 gene (to confirm *nifH*-GUS integrated into SUTN9-2 chromosome)

Total RNA extraction and qRT-PCR analysis. The fresh rice roots were harvested at 7 days after inoculation with endophytic bradyrhizobial strains SUT-PR48 and SUTN9-2. Rice samples were sterilized with 70% ethanol for 30 s and 3% sodium hypochlorite, washed 5 to 6 times with sterilized water, immediately frozen in liquid nitrogen, and stored at −80°C for further total RNA extraction.

Total RNAs were directly isolated from plant samples using the RNeasy plant minikit (Qiagen, USA), according to the manufacturer's protocol. RNAs were treated with the DNase I to prevent contamination of genomic DNA and then converted to cDNA using iScript cDNA synthesis (Bio-Rad). The transcription levels were determined by real-time quantitative reverse transcription-PCR (qRT-PCR). The primers used for amplification (*atpD*, *nirK*, *norB*, and *nifH*) are listed in Table 6. qRT-PCR amplification was performed using QuantStudio 3 real-time PCR system mix (Applied Biosystems) with the following cycling conditions: an initial denaturation step at 95°C for 2 min; 35 cycles of 2 min at 95°C; 30 s at annealing temperatures of 53°C (*atpD*), 53°C (*nirK*), 54°C (*norB*), and 48°C (*nifH*), followed by a final 5-min extension at 72°C (Table 6). The relative gene expression was analyzed by comparative threshold cycle (C_T) method ($-\Delta\Delta C_T$) that was normalized to the endogenous housekeeping gene (*atpD*). Three biological replicates were pooled and analyzed. At least three PCR amplifications were performed for each sample.

Rice endophytic bradyrhizobia *nifH-GUS* assay and nitrogen fixation assay. To obtain *nifH-GUS*-labeled strain, the plasmid pVO155nifHpm9-2gus was constructed by amplification of the *nifH* promoter region from the *Bradyrhizobium* sp. SUTN9-2 genome (54). This plasmid, which is nonreplicative in *Bradyrhizobium* strains, is a derivative of plasmid pVO155 and carries the promoterless *gusA* gene (55). The resulting plasmid was introduced into *E. coli* S17-1 by electroporation (15 kV/cm, 100 Ω, and 25 μF) and was transferred into SUTN9-2 by triparental mating (56). Transconjugants were selected on YEM plates supplemented with 25 μg/ml nalidixic acid, 20 μg/ml cefotaxime, and 100 μg/ml kanamycin.

The rice plants inoculated with the SUTN9-2 *nifH-GUS*-labeled strain were grown in the test tube containing N-free nutrient and a 1 mM urea solution. The fresh rice roots and leaf sheaths were harvested at 2, 4, 10, and 12 weeks after inoculation. Rice samples were surface-sterilized and then macerated separately in a sterilized mortar and pestle with sterilized water. The SUTN9-2 *nifH-GUS*-labeled strain was separated from rice tissues by filtration using a 3-layer Miracloth and 8.0-μm membrane filter, respectively. Glucuronidase was assayed in a buffer consisting of 50 mM sodium phosphate (pH 7.0), 10 mM 2 mercaptoethanol, 0.1% Triton X-100, and 1 mM *p*-nitrophenyl β-D-glucuronide. Reactions occurred in a 1-ml volume mixture at 37°C and were terminated by the addition of 0.4 ml of 2.5 M 2-amino-2-methylpropanediol. *p*-Nitrophenol absorbance was measured at 415 nm (57).

The symbiotic abilities of bradyrhizobial strains were determined in Leonard's jars containing sterilized vermiculite, and 1 ml of bacterial strain SUTN9-2 equivalent to 10^7 cells was inoculated onto germinated *O. sativa* cv. PT1 seeds. Plants were harvested after 2, 4, 8, and 12 weeks, and five plants were

used to analyze nitrogenase activity using the acetylene reduction assay (ARA) (3). The reactions were carried out in an 80-ml test tube. Five percent (vol/vol) of gas phase in the headspace was replaced with acetylene and further incubated at 28°C for 24 h. Ethylene production was measured by using a gas chromatograph (GC) with a flame ionization detector and equipped with a PE-alumina column (50 m by 0.32 mm by 0.25 μm; PerkinElmer, USA).

Production of IAA and ACC deaminase by *Bradyrhizobium* sp. SUTN9-2 in rice tissues. The rice roots and leaf sheaths were harvested at 1 month after inoculation with endophytic bradyrhizobial strain SUTN9-2 and with an uninoculated control. Whole-rice samples were sterilized with 70% ethanol for 30 s and 3% sodium hypochlorite for 3 min, washed 5 to 6 times with sterilized water, and then macerated separately in liquid nitrogen with a sterilized mortar and pestle. Then, the supernatant was collected by centrifugation at 10,000 $\times$ *g* for 5 min. IAA and ACC deaminase production was determined as previously described (references 45 and 47, respectively).

Enumeration of endophytic bradyrhizobia. The plant most probable number (MPN) method was used to enumerate the bacterial endophytic population (19). Rice was grown under controlled environmental conditions of 28 $\pm$ 2°C on 16/8-h day/night cycle (full light, 639 $\mu E \cdot m^{-2} \cdot S^{-1}$) for 1, 2, 3, and 4 weeks. Then, the rice was surface-sterilized, and excised samples of roots and leaf sheaths were macerated with a sterilized mortar and pestle, diluted with saline solution (0.85% NaCl), and inoculated into plastic pouches using mung bean as a plant host for enumerating the population of endophytic bradyrhizobial nodulating mung bean.

Preparation of rice stubbles as bradyrhizobial inoculum for mung bean. The experiment was conducted under pot trial conditions. The experimental units consisted of pots (size 25.5 by 22.5 cm) sterilized with a 3% sodium hypochlorite solution overnight and then washed by adding boiled water into pots before filling them with 5 kg of low-organic-matter soil (pH 6.8; electrical conductivity [EC], 408 μS/cm; % organic matter [%O.M.], 0.63%; phosphorus [P], 49.9 ppm; potassium [K], 141 ppm; calcium [Ca], 689 ppm) and high-organic-matter soil (paddy soil) (pH 7.65; EC, 1,066 μS/cm; %O.M., 3.57%; P, 86.1 ppm; K, 932 ppm; Ca, 3,001 ppm). Soils were partially sterilized by adding 3 liters of boiled water into pots containing soil before seeding in order to eliminate native (indigenous) bradyrhizobia. Rice seeds were surface disinfected by washing them in 95% ethanol for 30 s, hydrogen peroxide (10% [vol/vol]) for 10 min, sodium hypochlorite solution (3% [vol/vol]) for 3 min, and then 5 to 6 times with sterilized water. Seeds were germinated in the dark at 30°C for 2 days on plates containing 0.85% agar YEM medium. Germinated seeds were soaked overnight with a culture of SUTN9-2 (10^8 CFU $\cdot$ ml^{-1}) containing the *GUS* reporter gene (SUTN9-2GUS) (Table 5). The experiment was conducted as five replicates of the following treatments: (i) control (without inoculation), (ii) low-organic-matter soil inoculated with SUTN9-2, and (iii) high-organic-matter soil collected from a paddy field inoculated with SUTN9-2. Rice plants were grown in the greenhouse until the seed maturation stage of rice. The number of plants per hill and the number of panicles per hill were evaluated.

Enumeration of rice endophytic bradyrhizobia from rice tissues. To enumerate the endophytic bradyrhizobia in rice tissues, 1- to 4-week-old rice tissues were surface-sterilized as previously described, and excised sample seeds, leaves, stems, roots, and stubbles were macerated separately with a sterilized mortar and pestle and then diluted in saline solution prior to spreading on YEM plates containing streptomycin (200 $\mu g \cdot ml^{-1}$) and 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) (10 mg $\cdot$ ml^{-1}). After 5 days of incubation at 28°C, the numbers of the blue colonies were counted to display the SUTN9-2 population densities in different rice tissues.

Enumeration of rice endophytic bradyrhizobia from soil. Plant most probable number (MPN) count was carried out for enumeration of the bacterial number in soil from rice cultivation where the rice was harvested and harvested 1 week later. The soils were mixed with sterilized water (1:1 [wt/wt]). All visible roots were removed from each suspension. The plant MPN using mung bean as the plant host was calculated from the dry weight of the soil. The number of bradyrhizobia was estimated by using tenfold dilutions (41). Three parallel dilution series based on a statistical treatment of the counting methods were used for enumeration analyses (58).

Investigation of mung bean nodulation from rice stubbles containing endophytic bradyrhizobia. After harvesting the rice inoculated with SUTN9-2, the remaining stubbles (~40g/pot) were immediately incorporated into the soil in each pot. After 1 week, 3 mung bean seeds were planted in each pot for 3 weeks under greenhouse conditions. The nodules from mung bean roots were collected and stained by a GUS assay (59).

Statistical analysis. Statistical analysis was performed with the SPSS 16.0 for Windows software (SPSS, Inc., Chicago, IL). The experimental data were statistically analyzed according to Steel and Torrie (60), and means were compared by Duncan's multiple range test (61).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01488-17.

SUPPLEMENTAL FILE 1, PDF file, 1.3 MB.

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