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REGULAR ARTICLE

Physiological and genetic characterization of rice nitrogen fixer PGPR isolated from rhizosphere soils of different crops

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Abstract
Aims We aimed to identify plant growth-promoting rhizobacteria that could be used to develop a biofertilizer for rice.
Methods To obtain plant growth-promoting rhizobacteria, rhizosphere soils from different crops (rice, wheat, oats, crabgrass, maize, ryegrass, and sweet potato) were inoculated to rice plants. In total, 166 different bacteria were isolated and their plant growth-promoting traits were evaluated in terms of colony morphology, indole-3-acetic acid production, acetylene reduction activity, and phosphate solubilization activity. Moreover, genetic analysis was carried out to evaluate their phylogenetic relationships based on 16S rRNA sequence data.
Results Strains of *Bacillus altitudinis*, *Pseudomonas monteilii*, and *Pseudomonas mandelii* formed associations with rice plants and fixed nitrogen. A strain of *Rhizobium daejeonense* showed nitrogen fixation activity in an in vitro assay and in vivo. Strains of *B. altitudinis* and *R. daejeonense* derived from rice rhizosphere soil, strains of *P. monteilii* and *Enterobacter cloacae* derived from wheat rhizosphere soil, and a strain of *Bacillus pumilus* derived from maize rhizosphere soil significantly promoted rice plant growth.
Conclusions These methods are effective to identify candidate species that could be developed as biofertilizers for target crops.

Keywords Rhizosphere · Plant growth promoting rhizobacteria · Rice · Nitrogen fixation · IAA · Phosphate solubilization · 16S rRNA · *Bacillus altitudinis* · *Pseudomonas monteilii* · *Pseudomonas mandelii* · *Rhizobium daejeonense*

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Abbreviations

PGPR	Plant growth promoting rhizobacteria
IAA	Indole-3-acetic acid
ARA	Acetylene reduction assay
DNA	Deoxyribonucleic acid
CTAB	Hexadecyltrimethylammonium bromide
PCR	Polymerase chain reaction
DDBJ	DNA data bank Japan
BLAST	Basic local alignment search tool
JAEA	Japan Atomic Energy Agency
CRD	Randomized block design

Introduction

The rhizosphere is defined as the volume of soil affected by plant roots (Hinsinger et al. 2009). The rhizosphere has important functions in plant nutrition, health, and yield. Different types of substances diffuse from plant roots into the rhizosphere, where they stimulate microbial activity. These substances include carbohydrates (sugars and oligosaccharides), organic acids, vitamins, nucleotides, flavonoids, enzymes, hormones, and volatile compounds (Prescott et al. 1990). The composition and amount of microorganisms in the rhizosphere vary among different plants because of variations in the quantity and quality of compounds exuded by the plants (Söderberg et al. 2002). Microbial communities in rhizospheres also vary among different plants, and are affected by the plant species (Grayston et al. 1998), soil type (Campbell et al. 1997), soil depth (Kuske et al. 2002), and cultivation practices (tillage/crop rotation) (Lupwayi et al. 1998). However, the plant species has the greatest effect on plant-associated habitats (Wieland et al. 2001).

Many microorganisms coexist in the rhizosphere, and bacteria are the most abundant among them. Rhizosphere bacteria that can inhabit plant roots are potentially useful for stimulating plant growth and increasing crop yields. These bacteria are collectively referred to as plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth 1978). They are distributed in different parts of the plants such as root tissues, leaves and stems. The PGPR can directly stimulate plant growth as a result of various bioactivities, including biological nitrogen fixation (Boddey and Dobereiner 1988), phytohormone production (Chabot et al. 1996; Bent et al. 2001), phosphate solubilization (Reyes et al. 2002), secretion of siderophores (Ahmad et al. 2008), and production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which reduces ethylene concentrations in plants (Duan et al. 2009). They can also indirectly affect plant growth by acting as biocontrol agents to suppress pathogenic organisms via production of diverse chemical compounds (Compant et al. 2005; Romero et al. 2007).

The greater part of plants species are associated with PGPR. Based on previous reports, bacterial strains with PGPR activities have been found in the following genera: *Acinetobacter*, *Agrobacterium*, *Agromonas*, *Alcaligenes*, *Arthrobacter*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Beijerinckia*, *Bradyrhizobium*, *Caulobacter*, *Chromobacterium*, *Derxia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Frankia*, *Herbaspirillum*, *Hyphomycrobium*, *Klebsiella*, *Micrococcous*, *Pseudomonas*, *Rhizobium*, *Serratia*, *Stenotrophomonas*, *Thiobacillus*, *Xanthomonas,* and *Zoogloea* (Tripathi et al. 2002; Hurek and Reinhold-Hurek 2003; Gray and Smith 2005; Choudhary and Johri 2009; Schmidt et al. 2012).

In many Asian countries, PGPR have been developed as biofertilizers for sustainable agriculture (Ohyama et al. 2006). However, few trials on PGPR as biofertilizers have been conducted in Japan. To our knowledge, there is only one report on a PGPR that promoted growth of rice in Japan. The *Azospirillum* sp. strain B510, which was isolated from a rice plant, was shown to enhance rice growth and yields (Isawa et al. 2010). Generally, it is considered that rhizospheres of different plants are colonized by different microbial communities of PGPR that differ genetically and physiologically. This may show differences in their ability to fix nitrogen, solubilize phosphate, and produce indole acetic acid (IAA).

Overall, the aim of our research is to isolate PGPR that could be developed as a biofertilizer for rice crops. In this study, we collected rhizosphere soils from seven different plants (rice, maize, wheat, oat, rye grass, crabgrass, and sweet potato) grown in soils with identical texture (Andosol) and basal fertilization management, and rhizosphere soil from rice grown in alluvial soil. We used the soils as inoculants to treat rice seedlings, and isolated 166 bacterial strains. We evaluated the physiological and genetic characteristics of these strains to

evaluate the diversity of PGPR in the rhizosphere soil from each plant.

Materials and methods

Soil sampling

We collected rhizosphere soil samples from seven different crops (rice, maize, wheat, oat, rye grass, crabgrass, and sweet potato) at a depth of 0–15 cm from two sites; Honmachi paddy (rice) and Saiwai-Cho upland fields (other crops), Tokyo University of Agriculture and Technology, Fuchu, Japan. Regarding to the topsoil depth collected, we considered that 0–15 cm depth is the active part for fibrous roots of the six gramineous plants selected with the exception of sweet potato. The samples were kept at 4 °C until use.

Isolation of plant growth-promoting rhizobacteria

The procedures used to isolate and characterize PGPR are shown in Fig. 1. To isolate PGPR, the seeds of two Japanese rice varieties (cv. Leaf star and cv. Nipponbare) were surface-sterilized in 70 % ethanol for 30 s, soaked in 3 % sodium hypochlorite for 3 min, and then the seeds were rinsed five times with sterilized distilled water. We took 20 g soil from each sample and used it to inoculate seeds in a pot containing sterilized vermiculite. Pots were kept in growth chamber under controlled conditions (16-h light/8-h dark photoperiod, at 25 °C/18 °C day/night temperatures). After 21 days, all plants were harvested from the pots, washed in running tap water to remove vermiculite, and then the roots and leaves were washed out five times with sterile distilled water in side of laminar flow cabinet (Sanyo Bio clean bench MCV-B91F, Tokyo). Leaves and roots were cut into 1–2 cm pieces, and the tissues were macerated using a sterilized mortar and pestle in the laminar flow cabinet. The turbid solution was diluted (10^{-1}–10^{-5}) with sterile distilled water. One loop from each of the diluted turbid solutions was inoculated into a screw-capped tube containing 15 ml Nitrogen-free NFb semi-solid medium (Döbereiner et al. 1976). The tubes were incubated at 28 °C for 48 h, and bacterial growth was observed as the formation of veil-like pellicles. Then, 10 μL from every layer (pellicle) was spread on N-free NFb agar medium plates. The plates were placed in anaerobic jars and microaerophilic conditions were established using a gas pack. The plates were incubated for 3 days at 28 °C. Bacterial colonies were re-streaked onto fresh NFb-Agar plates until typical bacterial colonies were obtained. Single colonies were picked and maintained on slants of NFb medium at 4 °C.

Morphological characteristics of bacterial isolates

To examine the morphological characteristics of isolates grown on NFb agar plates, one loopful of each bacterial colony was diluted in a microtube containing 1 ml sterilized distilled water and mixed well. Then, 5 μL was transferred onto an NFb agar plate and incubated for 3 days at 28 °C. The morphological characteristics of isolates including the colony form, elevation, margin, and color were observed and recorded under a stereo microscope (Olympus, Tokyo, Japan).

Indole-3-acetic acid (IAA) production

To detect and quantify IAA, each strain was inoculated into NFb broth containing 100 mg L^{-1} L-tryptophan. The cultures were incubated at 28 °C for 2 days in the dark. Then, the cell suspensions were centrifuged at 10,000 rpm for 15 min to remove cells, and the concentration of IAA in the supernatant was determined by the Salkovski colorimetric technique (Glickmann and Dessaux 1995) by measuring absorbance at 530 nm with a spectrophotometer (Ultrospec 3300 *pro*, Amersham Biosciences, Cambridge, United Kingdom). The cell density was determined by the plate dilution method.

Acetylene reduction assay

The acetylene reduction assay (ARA) provides an estimate of nitrogen-fixing activity. For the ARA, bacterial cultures were grown in vials containing N-free semi-solid NFb medium and incubated at 28 °C for 2 days. Then, 10 % acetylene (*v/v*) was injected into the culture vial and the cultures were further incubated for 1 h at 30 °C. Then, the concentration of ethylene in the vial was determined using a Shimadzu GC8A gas chromatograph (Shimadzu, Tokyo, Japan) equipped with a Poropak N column (Chrompack, Middelburg, The Netherlands). Un-inoculated tubes served as the control.

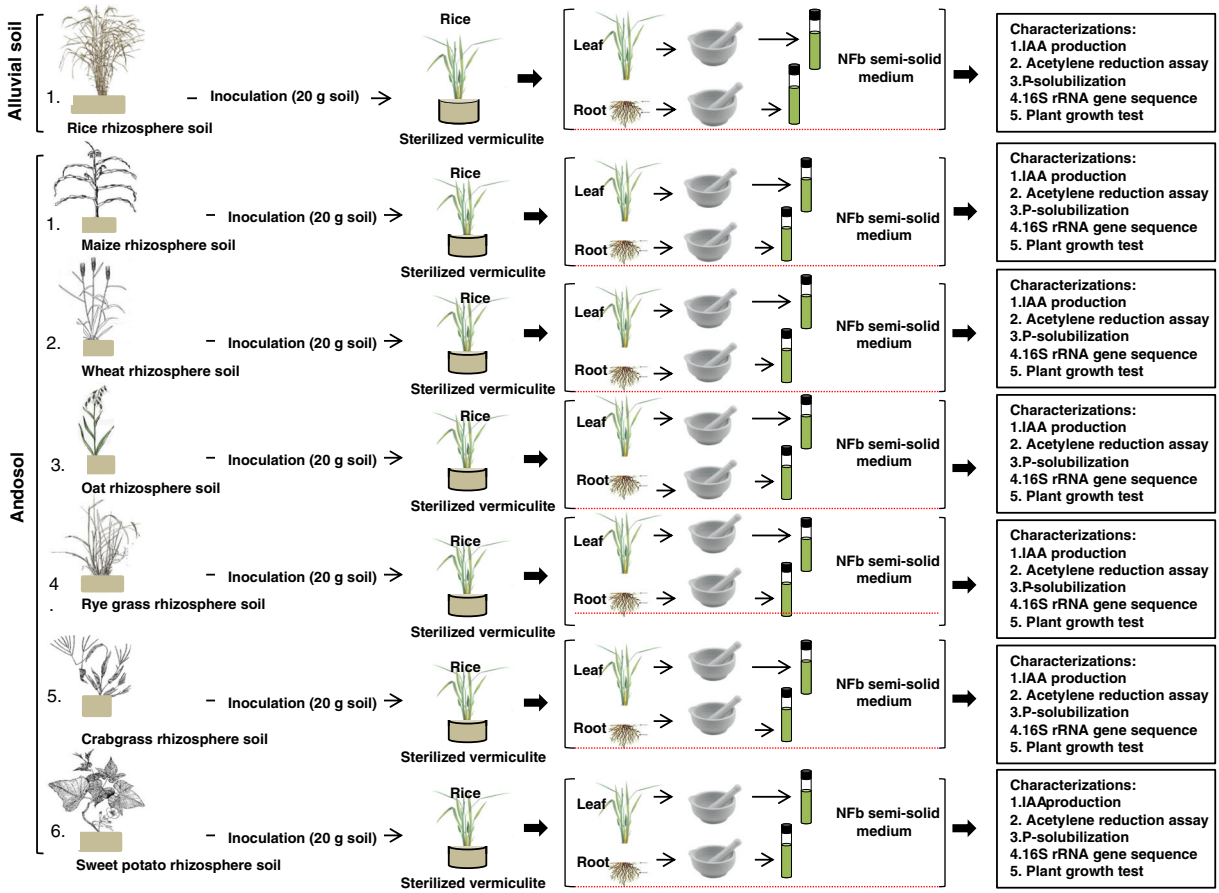

Fig. 1 Procedure to isolate and characterize PGPR derived from rhizosphere soils of seven different plants

The number of cells in each vial was determined by the plate dilution method.

Detection of inorganic phosphate solubilization activity

Bacterial isolates were grown in NFb broth medium at 28 °C for 48 h. Then, 5 μl of each culture was spotted onto Pikovskaia medium containing tricalcium phosphate (Pikovskaia 1948). The plates were incubated for 7 days at 28 °C. Formation of a clear zone around the bacterial colony indicated that the isolate was able to solubilize phosphate. The phosphate-solubilizing activity of each isolate was evaluated by measuring the size of the halozone.

Molecular characterization

We selected 30 of the 166 isolates, based on their ability to produce IAA and ethylene, and sequenced their 16S rRNA regions. The isolates were grown in NFb broth medium at 25 °C for 4 days. Prior to genomic isolation, the cells were harvested and washed twice with equal volumes of TNE buffer. The genomic DNA was extracted from isolates using the method of Yokoyama et al. (1996) with a slight modification, in that an additional 55 μL 10 % (*v/v*) cetyl trimethyl ammonium bromide (CTAB) was used. The DNA concentration and purity was checked using a NanoDrop 2000 UV–vis Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

DNA amplification and sequencing

The PCR amplifications and sequencing of the 16S rRNA gene were conducted as described previously (Yokoyama 2008). The bacterial universal primers 1F (5′-AGT TTG ATC CTG GCT C-3′) and 3R (5′-AAG GAG GTG ATC CAG CC-3′) were used for sequencing the 16S rRNA gene. These primers are located at positions 11–26 and 1,472–1,489 in the 16S rRNA gene of

Escherichia coli. Amplifications were performed using 50 μL reaction mixtures containing 2.5 μL primer set 1 F and 3R (10 μM each), 0.5 μL Taq DNA Polymerase (ExTaq polymerase 5 UmL-1, Takara Bio, Otsu, Japan), 5 μL 10× reaction buffer, 4 μL dNTP mixture, and 1 μL DNA template (200–250 ng DNA). The thermal cycling conditions were as follows: denaturation at 94 °C for 5 min. followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 3 min, and a final extension at 72 °C for 7 min. The PCR products were checked by electrophoresis on 1.5 (*w/v*) agarose gels. Amplified DNA bands corresponding to the 16S rRNA gene were purified using a QIAEX II agarose gel extraction kit (Qiagen, Valencia, CA, USA). The purified products were reacted using an ABI Prism BigDye Terminator v 3.1 cycle sequencing kit (Applied Biosystems, Foster City, USA) and 1F and 3R primers (3.2 pmol). Then, the sequencing reaction mixtures were analyzed using an ABI PRISM 3500 genetic analyzer (Applied Biosystems) according to the manufacturer's protocols to obtain DNA sequences of the 16S rRNA gene. The sequences obtained were compared with those deposited in the Genbank database using the online software BLAST. Sequence alignment and construction of the phylogenetic tree were performed using MEGA version 5.05.

Plant growth test

We cultured the 30 selected isolates in 20 ml NFb broth medium at 28 °C for 2 days. To eliminate the effects of indigenous soil microorganisms, Honmachi paddy soil was sterilized by gamma radiation at 50 kGy at the Takasaki institute of Japan Atomic Energy Agency (JAEA). Consequently, the soils were sterilized by a dose of 100 kGy to kill all microorganisms. Seeds of rice (cv. Leaf star) were surface-sterilized as described above. The two germinated seeds were transplanted into 300-ml plastic pots containing 200 g soil gamma-irradiated soils, and then 0.75 ml of each bacterial culture at a cell density of 10^9 colony forming unit /ml (CFU/ml) was applied to a seed in the pot. All pots were transferred to a greenhouse controlled at 28 °C±2 °C during the day (16 h) and 25 °C±2 °C during the night (8 h). Each pot was irrigated with sterilized distilled water. The experiment was performed in a completely randomized block design that consisted of three replicates for each treatment. Plants un-inoculated and inoculated with *Azosperillum brasilense* (Ts-13) were used as negative and positive controls, respectively (Meunchang et al. 2004). Plants were harvested after 3 weeks, and the roots were washed thoroughly in tap water to remove adhering soil. The fresh weight of roots and shoots was recorded, and then the roots and shoots were dried at 60 °C for 2 days before determining dry weight. The statistical significance of differences between treatments and controls was determined using Tukey's test ($P<0.05$) (Table 4).

Acetylene reduction assay of rhizosphere soils from inoculated rice seedlings

To measure the acetylene reduction activity of rhizosphere soil, 10 g soil from the rhizosphere of 21-day-old rice plants was placed in a sterilized vial, and the headspace was filled with 10 % acetylene gas (*v/v*). The vials containing 10 % acetylene were incubated at 28 °C for 24 h. Then, 1 ml air from the headspace of the vial was removed using a gas-syringe and injected to a GC8A gas chromatograph (Shimadzu) to analyze ethylene content. The rhizosphere soil from an un-inoculated plant was used as the control.

Results

Isolation of PGPR

We used rhizosphere soils from seven different plants as inoculants to isolate PGPR associated with rice plants, as shown in Fig. 1. We obtained a total of 166 isolates; 88 isolates were obtained from rice leaves and 78 were obtained from rice roots (Table 1).

Colony morphology of isolates

First, we evaluated the 166 isolates in terms of colony morphology and color, to determine whether there were morphological differences among isolates in rhizosphere soils from different plants. The results are shown in Table 2. The majority of the isolates (>80 %) from alluvial soil (rice rhizosphere soil) and Andosol (rhizosphere soils from the six other crops) formed circular colonies. Among the isolates from alluvial soil, 16.7 % formed filamentous or irregular colonies. The Andosol soils from the six different crops showed a wide variation in the proportion of isolates forming filamentous or irregular colonies, ranging from 0 % (sweet potato and

Table 1 Description of soil samples and number of isolates obtained from each sample

Soil group	Plant from which rhizosphere soil was obtained	Total number of isolates	Number of isolates obtained from rice root	Number of isolates obtained from rice leaf
Alluvial soil	Rice	24	18	6
Andosol soil	Maize	33	13	20
	Wheat	25	15	10
	Sweet potato	13	8	5
	Oat	30	11	19
	Crabgrass	17	8	9
	Rye grass	24	15	9

crabgrass rhizosphere soil) to 15.2 % (maize rhizosphere soil). In terms of colony color, 16.7 % of isolates from alluvial soil formed creamy colonies, 16.7 % formed transparent colonies, 16.7 % formed whitish colonies, and 50 % formed yellowish colonies. Among the isolates from Andosol soils, the most common colony colors were whitish (ranging from 12.0 % of isolates from wheat rhizosphere soil to 57.5 % of isolates from maize rhizosphere soil) and yellowish (ranging from 30.3 % of isolates from maize rhizosphere soil to 76.0 % of isolates from wheat rhizosphere soil). The majority of isolates, regardless of whether they were from alluvial or Andosol soils, formed raised colonies. Also, the majority of isolates from both soil types formed colonies with entire margins.

Physiological properties of isolates

Next, we evaluated the abilities of the isolates to produce IAA, to reduce acetylene, and to solubilize P. The ability to produce IAA is associated with plant growth-promoting activity, the ability to reduce acetylene indicates nitrogen-fixation activity, and the ability to dissolve calcium phosphate indicates that the isolate can provide P nutrition to its host plant. These tests were used to evaluate the potential of the isolates for use as biofertilizers. The frequencies of isolates in each soil type that were able to produce IAA are shown in Fig. 2a. Of the 166 isolates, 115 were able to produce IAA. The amount of IAA produced by individual isolates ranged from 0.04 to 231 μg L^{-1} (Table 3). Among isolates from sweet potato rhizosphere soil, 92.3 % were able to produce IAA. Among those from crabgrass and oat rhizosphere soils, at least 80 % were able to produce IAA. The JO32 isolate, which was derived from oat rhizosphere soil, produced the largest amount of IAA (231 μg L^{-1}; Table 3). Among the other rhizosphere soils, at least 50 % of isolates were able to produce IAA.

Of the 166 isolates, 122 showed acetylene reduction activity (Fig. 2b). The ethylene production rates varied widely among individual isolates, ranging from 0.04 nmole C_2H_4 h^{-1} $(10^6$ cells$)^{-1}$ to 528.1 nmole C_2H_4 h^{-1} $(10^6$ cells$)^{-1}$ (Table 3). The majority of isolates (83.4 %) from rice rhizosphere soil showed nitrogen fixation activity (Fig. 2b). Similarly, the majority of isolates from Andosol rhizosphere soils showed nitrogen fixing activity, ranging from 63.7 % of isolates from maize rhizosphere soil to 79.2 % of isolates from rye grass rhizosphere soil. The JR5 strain associated with rice roots in rice rhizosphere soil showed the highest activity in the in vitro ARA [528.1 nmole C_2H_4 h^{-1} $(10^6$ cells$)^{-1}$; Table 3].

We evaluated the P-solubilization abilities of the 166 isolates, and found that only 36 isolates formed clear zones in the plate assay. Therefore, among all the isolates, 115 (69 % of the total) were able to produce IAA, 122 (73 % of the total) showed nitrogen fixing activity, but only 36 (21.6 %) were able to solubilize P. The highest frequency of P-solubilizing strains (40 % of isolates) was in maize rhizosphere soil (Andosol). The JR 37 strain associated with rice roots in rice rhizosphere soil showed the strongest P-solubilizing activity, which was observed as the largest clear zone in the plate assay (Table 3). Less than 10 % of the isolates from rhizosphere soils from oat and crabgrass, and none of those sweet potato rhizosphere soil, showed P-solubilization activity.

Genetic characterization of representative isolates based on 16S rRNA sequence

We selected 30 of the 166 isolates for 16S rRNA sequence analysis. The isolates were selected based on the type of rhizosphere soil they were derived from, their

Table 2 Summary of morphological characteristics of colonies of 166 bacterial isolates after incubation for 3 days using NFb medium

Morphological characteristic		Rhizosphere type						
		Saiwai-Cho upland soil (Andosol)						Honmachi paddy soil (Alluvial)
		Maize	Wheat	Sweet potato	Oat	Crabgrass	Rye grass	Rice
Form	Circular	28[a]	23	13	29	17	21	20
		84.8%[b]	92.0 %	100.0 %	96.6 %	100.0 %	87.5 %	83.3 %
	Filamentous	2	0	0	0	0	1	1
		6.1 %	0 %	0 %	0 %	0 %	4.2 %	4.2 %
	Irregular	3	2	0	1	0	2	3
		9.1 %	8.0 %	0 %	3.3 %	0 %	8.3 %	12.5 %
Color	Pink	1	0	0	0	0	0	0
		3.00 %	0 %	0 %	0 %	0 %	0 %	0 %
	Whitish	19	3	6	16	8	8	4
		57.5 %	12.0 %	46.1 %	53.3 %	47.1 %	33.3 %	16.7 %
	Yellowish	10	19	7	10	8	13	12
		30.3 %	76.0 %	53.8 %	33.3 %	47.1 %	54.2 %	50.0 %
	Creamy	2	2	0	1	0	2	4
		6.1 %	8.0 %	0 %	3.3 %	0 %	8.3 %	16.7 %
	Transparent	1	1	0	3	1	1	4
		3.0 %	4.0 %	0 %	10.0 %	5.8 %	4.2 %	16.7 %
Elevation	Convex	1	0	0	3	0	0	0
		3.03 %	0	0	10.0 %	0 %	0	0 %
	Flat	6	2	2	2	0	6	4
		18.2 %	8.0 %	15.4 %	6.7 %	0.0 %	25.0 %	16.7 %
	Raised	26	20	11	22	17	17	16
		78.8 %	80.0 %	45.8 %	73.3 %	100.0 %	70.8 %	66.7 %
	Umbonate	0	3	0	3	0	1	4
		0 %	12.0 %	0 %	10 %	0 %	4.20 %	16.7 %
Margin	Entire	28	23	13	28	13	21	20
		84.8 %	92.0 %	100.0 %	93.3 %	76.4 %	87.5 %	83.3 %
	Filiform	2	0	0	0	2	1	1
		6.1 %	0 %	0 %	0 %	11.8 %	4.2 %	4.2 %
	Undulate	3	2	0	2	2	2	3
		9.1 %	8.0 %	0 %	6.7 %	11.8 %	8.3 %	12.5 %

[a] Number of isolates
[b] Percentage of isolates

IAA production activity, and their acetylene reduction activity. We obtained almost complete sequences of the 16S rRNA gene for these isolates. The closest relative of each isolate, based on its 16S rRNA sequence, and its IAA production activity, acetylene reduction activity, and P-solubilizing activity, are shown in Table 3. The phylogenetic relatedness among the 30 representative isolates is shown in Fig. 3. There were clear differences in the distribution of bacterial genera between the two soil types (Andosol and alluvial soil). Among the Andosol soils, those from rhizospheres of five crops (all except ryegrass) contained isolates belonging to

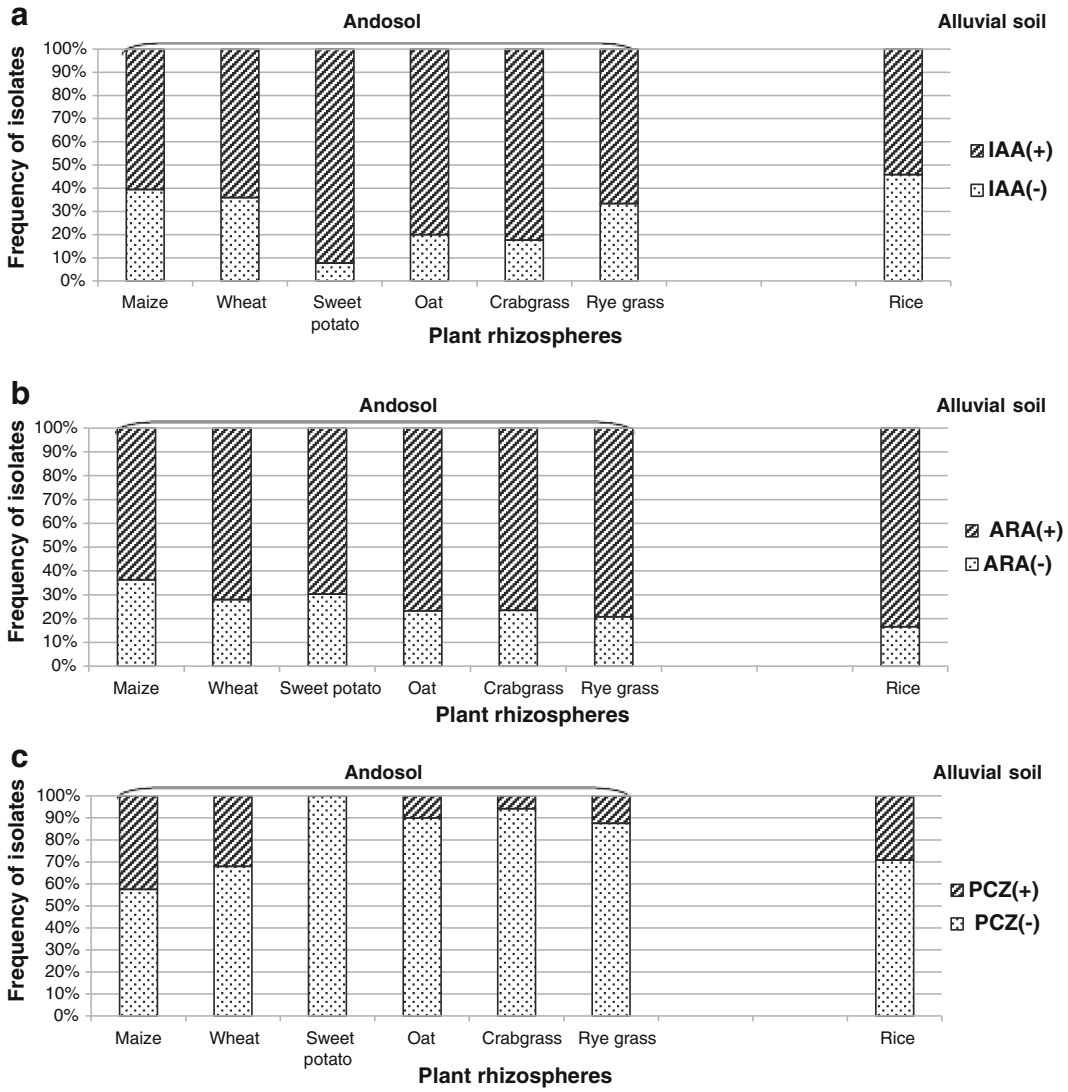

Fig. 2 Frequencies of isolates derived from various plant rhizospheres showing IAA production activity, acetylene reduction activity, and P-solubilization activity. **a** Indole-3-acetic acid production: **IAA (+)** Able to produce IAA. **IAA (−)** No IAA production. **b** Acetylene reduction assay: **ARA (+)** Acetylene reduction activity. **ARA (−)** No acetylene reduction activity. **c** Phosphorous solubilization: **PCZ (+)** Formation of clear zone formation representing phosphorous solubilizing activity. **PCZ (−)** No phosphorous solubilization activity

the genus *Enterobacter* and close relatives (Table 3, Fig. 3). Approximately 50 % of the isolates from these soils were in the genus *Enterobacter*. In contrast, the rice rhizosphere soil (alluvial soil) did not contain any *Enterobacter* isolates. Similarly, wheat and oat rhizosphere soils contained isolates in the genus *Klebsiella* and its close relatives, while rice rhizosphere soil did not contain any isolates in this genus. We obtained four *Pseudomonas* isolates; three were from maize and wheat rhizosphere soils, and one was from rice rhizosphere soil. The four *Pseudomonas* isolates represented different species; *Pseudomonas putida*, *Pseudomonas monteilii* (JW13), *Pseudomonas veronii* (JR37), and *Pseudomonas mandelii* (JRy205) (Table 3, Fig. 3). The latter three species have not been reported to be associated with rice plants previously. Both *P. monteilii* and *P. mandelii* showed acetylene reduction activity (Table 3), but neither have been reported to have nitrogen fixation activity in previous studies. We detected a strain of *Stenotrophomonas*, JR207, only in rice

Table 3 Details of isolate origin, closest relative and physiological characteristics

Soil group	Plant rhizosphere soil used as inoculant	Isolate name	Origin of isolates associated with rice plant	Closest relative based on 16S rRNA gene sequence	IAA production (μg/ml)[a]	ARA[b]	P-solubilizing activity[c]
Alluvial soil	Rice	JR207	Rice root	*Stenotrophomonas rhizophila*	25.6±1.5	0	0.8±0.2
		JR5	Rice root	*Rhizobium daejeonense*	65±3.9	528.1±37	0
		JR172	Rice root	*Agrobacterium tumefaciens*	10.6±0.3	0	0
		JR4	Rice root	*Bacillus altitudinis*	0	3.9±0.8	1.4±0.3
		JR37	Rice root	*Pseudomonas veronii*	0	7.8±2.3	4.5±1
		JR198	Rice root	*Bacillus altitudinis*	19±2	0	0.4±0.2
Andosol	Maize	JM160	Rice leaf	*Enterobacter sp*	4.1±1	1.2±0.7	0.8±0.3
		JM187	Rice leaf	*Enterobacter sp*	225.9±5	8.7±1.4	0.6±0.1
		JM185	Rice leaf	*Enterobacter ludwigii*	62.7±0.3	1.5±1	0
		JM51	Rice leaf	*Agrobacterium tumefaciens*	38.1±0.7	0	1±0.1
		JM158	Rice leaf	*Agrobacterium tumefaciens*	9.1±0.2	0	0
		JM195	Rice root	*Enterobacter sp*	30.6±0.9	0.9±0.6	1±0.3
		JM63	Rice root	*Enterobacter ludwigii*	38.6±0.14	1.1±0.5	0
		JM75	Rice root	*Pseudomonas putida*	0	0	1.5±0.2
		JM52	Rice root	*Bacillus pumilus*	20±0.9	5.9±2.7	0
	Wheat	JW2	Rice leaf	*Raoultella (Klebsiella) ornithinolytica*	4.2±0.1	3.7±1.6	0
		JW40	Rice leaf	*Raoultella (Klebsiella) ornithinolytica*	58±3	1.27±0.4	0
		JW191	Rice root	*Enterobacter ludwigii*	2.8±0.1	0.1 ±0.8	0
		JW69	Rice root	*Enterobacter asburiae*	28±2	0.8±0.4	0
		JW13	Rice root	*Pseudomonas monteilii*	8±0.2	6.12±2.1	1.7±0.4
	Sweet potato	JS183	Rice root	*Enterobacter asburiae*	85±5.6	3.84±0.6	0
	Oat	JO115	Rice leaf	*Enterobacter ludwigii*	25.6±0.2	1±0.5	0
		JO171	Rice leaf	*Rhizobium sp*	10.4±0.6	2.2 ±0.9	0
		JO143	Rice leaf	*Klebsiella oxytoca*	13.3±0.2	16.15±3.7	0
		JO32	Rice root	*Enterobacter sp*	231±10.5	0	0
		JO109	Rice root	*Enterobacter sp*	25.8±3.2	0.8±0.6	0
		JO126	Rice root	*Caulobacter henricii*	34±0.6	1.2±0.2	0
	Crabgrass	JC20	Rice leaf	*Enterobacter asburiae*	113±7.2	3.05±1.3	0
		JC100	Rice leaf	*Bacillus safensis*	5±0.2	1.4±0.8	0
	Rye grass	JRy205	Rice root	*Pseudomonas mandelii*	37±3	24.4±2.7	0

[a] Acetylene reduction assay (ARA). Values represent activity expressed as nmol C_2H_4 / h/ 10^6 cells
[b] Amount of IAA produced (μg IAA per ml per 10^6 cells)
[c] P-solubilizing activity. Units represent size of clear zone (in mm) caused by dissolution of calcium phosphate

rhizosphere alluvial soil. We detected four *Bacillus* isolates; two strains of *Bacillus altitudinis* were obtained from rice rhizosphere alluvial soil, one strain of *Bacillus pumilus* was obtained from maize rhizosphere soil (Andosol), and one strain of *Bacillus safensis* was obtained from crabgrass rhizosphere soil (Andosol) (Table 3, Fig. 3). One of the *B. altitudinis* strains, JR198, showed nitrogen-fixing activity (Table 3). This is the first report that *B. altitudinis* can be associated with rice plants and fix nitrogen. The *Caulobacter henricii* strain JO126 was unique to oat rhizosphere soil, and to our knowledge this is the first report of such species from rice. Interestingly, the acetylene reduction activity of the *Rhizobium daejeonense* strain JR5 was 240 times higher than that of another *Rhizobium* strain, JO171 (Table 3, Fig. 3).

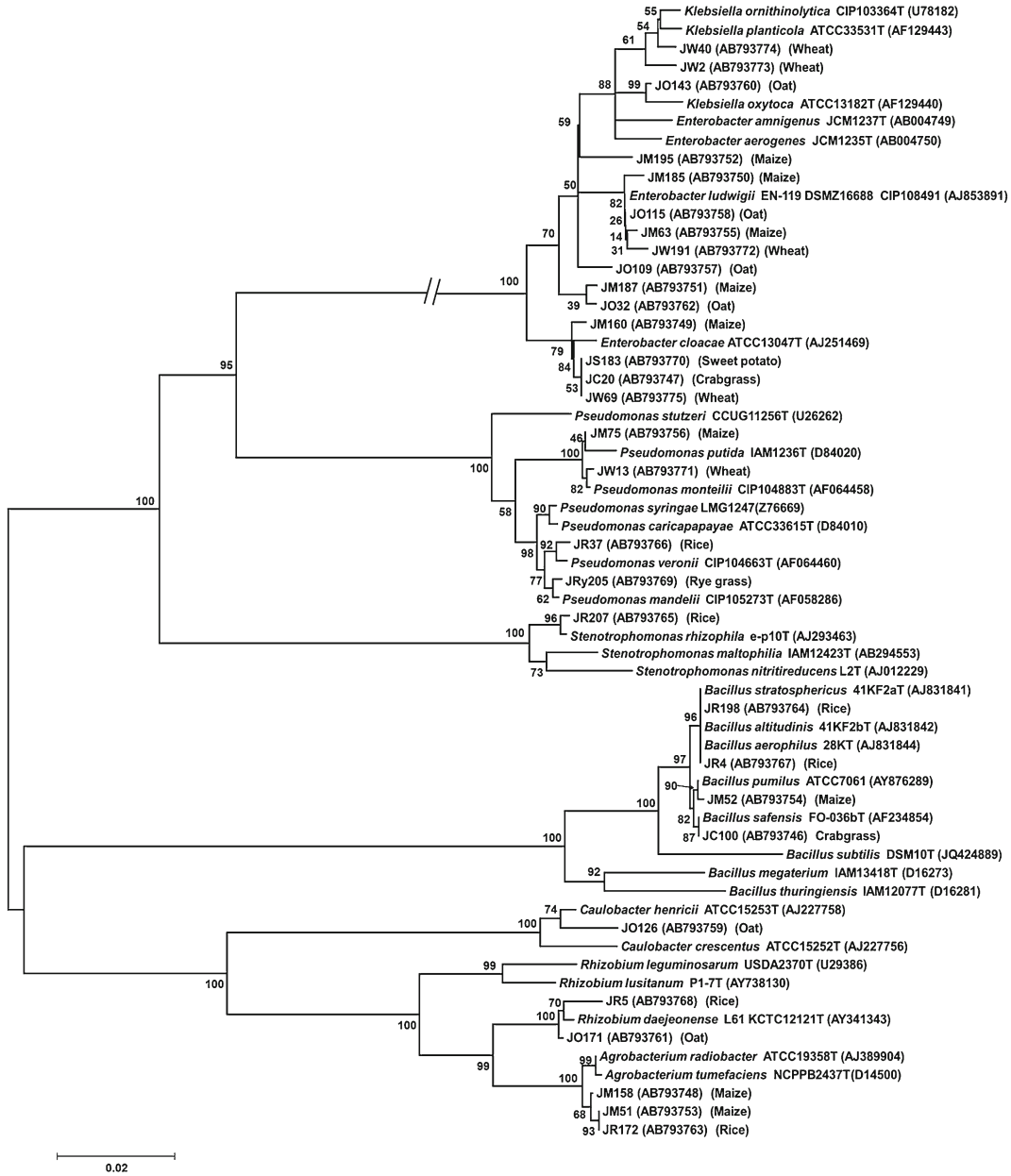

Fig. 3 Phylogenetic tree based on16S rRNA sequences showing positions of 30 isolates and type strains of species in different genera. Accession numbers of the 16S rRNA genes in DDBJ database are shown in parentheses. Numbers at the nodes indicate level of bootstrap support, based on neighbor-joining analysis of 1,000 re-sampled datasets. Scale bar indicates 0.02 changes/site

Effects of bacterial inoculation on rice plant growth

To ensure the effects of inoculation on plant growth and nitrogen fixing activity to rice plants, the 30 representative isolates were inoculated at the gamma-sterilized paddy soil. We also included a positive control in these experiments, the Ts-13 strain of *Azospirillum brasilense*, which is used as a biofertilizer for rice crops in Thailand (Meunchang et al. 2004).

All of the 30 tested isolates positively affected rice shoot length, compared with that of the control. The shoot lengths of inoculated plants compared with those of un-inoculated controls, are shown in Table 4. The shoot length of rice plants inoculated with JRy205,

Table 4 Summary of effects of inoculating isolates onto rice plants after 3 weeks of growth

Soil group	Plant rhizosphere	Isolate name	Bacterial species	Origin of isolate associated with rice plant	Shoot length (cm)	Root length (cm)	Shoot dry weight (mg)	Root dry weight (mg)	ARA[c]
		Control			41±2[a] 100[b]	3.2±0.3 100	42±2 100	13±2.5 100	0
Alluvial	Rice	JR207	*Stenotrophomonas rhizophila*	Rice root	46±3 124	5.5±1.5 171	68±22 161	28±14.9 215	0
		JR5	*Rhizobium daejeonense*	Rice root	50±5 135	5.6±0.4 175	96±18* 228	27±5.2 208	49±20
		JR172	*Agrobacterium tumefaciens*	Rice root	49±4 132	6.1±0.3 190	80±14 190	26±5.7 200	0
		JR4	*Bacillus altitudinis*	Rice root	47±5 127	5±0.1 156	78±21 185	19±5 146	61±3
		JR37	*Pseudomonas veronii*	Rice root	44±8 120	5.6±0.4 175	76±7ab 180	21±7.3 162	123±47
		JR198	*Bacillus altitudinis*	Rice root	51±5 138	5.8±1.3 181	75±10 178	40±5.6* 308	0
Andosol	Maize	JM160	*Enterobacter sp*	Rice leaf	45±6 122	4.3±0.8 134	60±13 142	20±1.3 154	21±15
		JM187	*Enterobacter sp*	Rice leaf	45±2 122	4.4±0.5 138	67±20 159	19±1.5 146	79±7
		JM185	*Enterobacter ludwigii*	Rice leaf	45±7 122	4.3±0.8 134	62±10 147	19±1.1 146	24±12
		JM51	*Agrobacterium tumefaciens*	Rice leaf	46±3 124	4.5±0.5 140	60±5 142	21±1.5 162	0
		JM158	*Agrobacterium tumefaciens*	Rice leaf	46.5±3 124	4.1±0.3 128	59±10 140	18±0.5 138	0
		JM195	*Enterobacter sp*	Rice root	45±4 122	4.8±0.8 150	68±2.5 161	21±3.2 162	19±1
		JM63	*Enterobacter ludwigii*	Rice root	49±1 132	4.5±0.5 140	69±8 164	20±2.2 154	25±13
		JM75	*Pseudomonas putida*	Rice root	46±5 124	5.6±1.3 175	65±13 154	24±3.7 185	40±20
		JM52	*Bacillus pumilus*	Rice root	50±6 135	7±1* 218	91±22* 216	29±13 223	45±19
		JW2	*Raoultella (Klebsiella) ornithinolytica*	Rice leaf	51±2 138	7.3±1.2* 228	82±21 195	28±7.2 215	34±27
		JW40	*Raoultella (Klebsiella) ornithinolytica*	Rice leaf	46±4 124	6.3±0.6 196	68±1.2 161	21±4.1 162	98±21
	Wheat	JW191	*Enterobacter ludwigii*	Rice root	44±3 119	5.4±0.7 168	65±10 154	18±5.8 138	24±1
		JW69	*Enterobacter asburiae*	Rice root	50±1 135	6.1±0.3 190	105±11* 250	27±0.5 208	49±21
		JW13	*Pseudomonas monteilii*	Rice root	55±1 149	8±0.9* 250	90±11* 214	38±9* 292	33±1
	Sweet potato	JS183	*Enterobacter asburiae*	Rice root	50±6 135	5.3±0.6 165	72±13 171	23±3.6 177	56±34
	Oat	JO115	*Enterobacter ludwigii*	Rice leaf	47±1 127	4.3±0.6 134	63±5 150	19±4.4 146	40±17
		JO171	*Rhizobium sp*	Rice leaf	46±5 124	6.6±0.6 206	43±6 102	31±13 238	17±11

Table 4 (continued)

Soil group	Plant rhizosphere	Isolate name	Bacterial species	Origin of isolate associated with rice plant	Shoot length (cm)	Root length (cm)	Shoot dry weight (mg)	Root dry weight (mg)	ARA[c]
		JO143	*Klebsiella oxytoca*	Rice leaf	45±2 122	5.3±0.3 165	79±15 188	15±9.2 115	27±14
		JO32	*Enterobacter sp*	Rice root	48.5±6 131	8±1.7* 250	62±2 147	21±8.7 162	35±5
		JO109	*Enterobacter sp*	Rice root	44.5±4 120	5±0.5 156	50±3 119	20±1.1 154	14±2
		JO126	*Caulobacter henricii*	Rice root	50±1 135	5.6±0.3 175	82±10 195	21±1 162	45±8
	Crabgrass	JC20	*Enterobacter asburiae*	Rice leaf	48±2 130	5±0.1 156	83±19 197	19±6 146	41±29
		JC100	*Bacillus safensis*	Rice leaf	45±6 122	5.6±0.8 175	65±21 154	14±4.2 108	46±22
	Rye grass	JRy205	*Pseudomonas mandelii*	Rice root	43±2 116	5.±0.5 156	86±19 204	23±3 177	66±2
	Reference strain	Ts-13	*Azospirillum brasilense*		53±5 143	6.8±0.8* 212	61±11 145	22±8.5 169	84±32

[a] Mean value (n=3 replicates)
[b] Percentage compared with control
[c] Rhizospheric-soil acetylene reduction activity (nmol C_2H_4 $tube^{-1}$ h^{-1}) in assay containing 10 g soil
*Value is significantly different from that of control, within each column (P<0.05)

derived from ryegrass rhizosphere soil, was 116 % that of the control, and that of rice plants inoculated with *Pseudomonas monteilii* (JW13), derived from wheat rhizosphere soil, was 149 % that of the control. However, none of these differences are statistically significant. All of the isolates also had a positive effect on root length. The statistically significant increases in root length (P<0.05), compared with that of the un-inoculated control, were in rice plants inoculated with JW2 [*Raoultella (Klebsiella) ornithinolytica*], JW13 (*P. monteilii*), JM52 (*B. pumilus*), and JO32 (*Enterobacter* sp.).

We determined the effects of the isolates on rice dry weight, and found that 29 of the 30 isolates showed positive effects. Only the *Rhizobium* strain JO171 isolated from oat rhizosphere soil did not affect rice dry weight (Table 4). Statistically significant increases in shoot dry weight (P<0.05), compared with that of the un-inoculated control, were observed in plants inoculated with JM52 (*B. pumilus*), JW69 (*Enterobacter asburiae*), JW13 (*P. monteilii*), and JR5 (*R. daejeonense*). All of the isolates positively affected root dry weight (Table 4). Out of the 30 isolates, 22 isolates (73 % of the total) resulted in root dry weights of 150 % or higher, compared with that of un-inoculated controls. The strains that resulted in statistically significant (P<0.05) increases in rice root dry weight, compared with that of the control, were JW13 (*P. monteilii*), which was derived from wheat rhizosphere soil, and JR198 (*B. altitudinis*), which was derived from rice rhizosphere soil. These strains resulted in root lengths approximately 300 % of that of the un-inoculated control.

We determined the acetylene reduction activities of rhizospheric soils of rice after inoculating each of the 30 isolates. Seven isolates showed no acetylene reduction activity in soil, even though they had previously shown acetylene reduction activity in the in vitro assay (Table 3). However, surprisingly, JO32 (*Enterobacter* sp.) showed rhizospheric acetylene reduction activity (Table 4). The *R. daejeonense* strain JR5, which was obtained from rice rhizosphere soil, showed the highest acetylene reduction activity in the in vitro assay (Table 3), but showed much lower activity in rhizosphere soil (49 nmol C_2H_4/h/10 g soil). The *P. veronii* strain JR37 showed the highest rhizospheric acetylene reduction activity among the 30 isolates tested.

We analyzed the relationship between plant growth promoting effects and rhizospheric nitrogen-fixation

ability. The *A. tumefaciens* strains JM51, JM158, and JR172, the *S. rhizophila* strain JR207, and the *B. altitudinis* strain JR198 showed no acetylene reduction activity in either test (Tables 3 and 4), but all of those isolates strongly promoted root and shoot growth of rice. As shown in Table 3, all of those isolates were able to produce IAA. The isolates that resulted in rice shoot and root growth of more than 150 %, compared with that of un-inoculated controls, were as follows: from maize rhizosphere soil, JM195 (*Enterobacter* sp.), JM63 (*E. ludwigii*), JM75 (*P. putida*), JM52 (*B. pumilus*); from wheat rhizosphere soil, JW2 and JW40 (*R. ornithinolytica*), JW191 (*E. ludwigii*), JW69 (*E. asburiae*), JW13 (*P. monteilii*); and from rice rhizosphere soil, JR207 (*S. rhizophila*), JR5 (*R. daejeonense*), JR172 (*A. tumefaciens*), JR37 (*P. veronii*), and JR198 (*B. altitudinis*).

Discussion

In this study, we isolated bacterial strains from rhizosphere of different plants, and evaluated their potential as PGPR for rice. In general, we expected that PGPR isolated from rice rhizosphere soil would perform better than those isolated from rhizosphere soils of different plants, because these bacteria would be adapted to the rice rhizosphere. However, we also tested the potential of bacteria obtained from other hosts as PGPR for rice, and found several PGPR that promoted rice growth.

There were no clear differences in the trends in colony morphology of isolates among the seven different rhizosphere soils. However, the frequencies of colony colors differed slightly among the different rhizosphere soils. In case of the isolates obtained from the rhizosphere of sweet potato, 46.1 % of the isolates were whitish and the remaining isolates showed yellowish colonies, whereas in case of the isolates obtained from the rhizosphere of wheat, 12.0 % of the isolates had whitish colonies and 76.0 % yellowish. The reasons for the different frequencies of bacterial colony colors among rhizosphere soils are unknown.

We evaluated the ability of the 166 isolates to produce IAA. Most of the isolates from sweet potato rhizosphere soil (92.3 %) and from crabgrass rhizosphere soil (82 %) were able to produce IAA. Approximately 60 % of the isolates from the rhizosphere soils of maize, wheat, and rice were able to produce IAA (Fig. 2). Sweet potato and crabgrass can grow in soils with low fertility. This may be related to the ability of their rhizospheric bacteria to produce IAA. Yoshida and Ancajas (1971) was the first to discover nitrogen fixation in the rice rhizosphere. Later, Watanabe and Furusaka (1980) reported that many kinds of nitrogen fixing bacteria are distributed in the rice rhizosphere. In our experiments, 83.4 % of bacteria isolated from the rhizosphere soil of rice showed nitrogen-fixing abilities; this was the highest proportion of nitrogen-fixing bacteria among the seven rhizosphere soils. This finding implied that the rice rhizosphere is the most suitable environment for nitrogen fixation; however, approximately 60 %–80 % of isolates from rhizosphere soils of other plants also showed nitrogen-fixing abilities. Phosphorus is one of the most important plant nutrients, and there are limited natural resources suitable for producing phosphate fertilizers. Therefore, the P-solubilization ability of PGPR is one of the most important traits, since it means they can supply phosphorous nutrition to crops. Common genera of phosphate solubilizing bacteria include *Achromobacter, Agrobacterium, Bacillus, Enterobacter, Erwinia, Escherichia, Flavobacterium, Mycobacterium, Pseudomonas, Rhizobium, Burkholderia, Micrococcus, Aereobacter, Erwinia,* and *Serratia* (Sashidhar and Podile 2010). In our assay, only 21.6 % of isolates showed phosphate-solubilizing activity (Fig. 2), while 78.4 % of isolates showed IAA production and nitrogen fixation abilities. None of the isolates from rhizosphere soil of sweet potato, and few of those isolated from oat and crabgrass rhizosphere soil, were able to solubilize phosphate. The attributes of these rhizospheric bacteria may affect the nutrient uptake abilities of oat and crabgrass. However, further research is required to explore the relationships between the nutrient uptake patterns of these hosts (oat and crabgrass) and their rhizospheric bacteria. These results indicate that the best strategy to select isolates with all three plant-growth-promoting traits (IAA production, nitrogen fixing activity, and phosphate solubilizing activity), is to screen for phosphate solubilizing activity first.

We chose 30 isolates for genetic analysis. The isolates were selected based on differences in the rhizospheres they were derived from, their ability to produce IAA, and their ability to fix nitrogen. There was a clear difference in *Enterobacter* distribution between Saiwai-Cho upland fields (Andosol) and Honmachi paddy field soil (alluvial soil) (Table 3). Of the 24 isolates obtained from crops grown in the Saiwai-Cho upland fields, 12 isolates were *Enterobacter* strains,

whereas no *Enterobacter* isolates were obtained from the Honmachi paddy soil. *Enterobacter* species have been isolated from various crops such as cotton and sweet corn (Mcinroy and Kloepper 1995), rice (Mehnaz et al. 2001), sugarcane (Mirza et al. 2001), sunflower (Ambrosini et al. 2012), pine (Ribeiro and Cardoso 2012), and maize (Morales-Garcia et al. 2011). Therefore, the wide distribution of *Enterobacter* strains in rhizosphere soils in the Saiwai-Cho upland field may indicate that the members of this genus are dominant bacteria in the rhizospheres of diverse crops. The farmyard manure was applied at a rate of 10 tonnes per hectare per year to these two fields. In Saiwai-Cho upland field soil, this management practices may promote distribution of *Enterobacter* species. In paddy field soil, the management practices may result in an environment that is unsuitable for *Enterobacter* sp.

Bacillus species are common rhizospheric bacteria, and have been isolated from rhizospheres of different agricultural crops such as rice (Xie et al. 2003; Beneduzi et al. 2008b), wheat (Beneduzi et al. 2008a), and soybean (Park et al. 2005). There are several reports of *Bacillus* isolates that can fix nitrogen, produce plant hormones, and solubilize phosphate. We isolated four *Bacillus* strains, two of which were strains of *B. altitudinis*. This is the first report of this bacterium associating with rice roots and fixing nitrogen. Of the *B. altitudinis* isolates, JR4 showed nitrogen-fixing activity both in the in vitro assay and in vivo after inoculating rice plants. However, JR198 did not show nitrogen-fixation activity. The reason why nitrogen fixation activity is so variable among different strains of *B. altitudinis* is unknown.

There are contradictory reports about the ability of free-living *Rhizobia* to fix nitrogen. Alazard (1990) reported that a strain of *Rhizobium* was able to fix nitrogen under free-living conditions, whereas Chowdhury et al. (2007) reported that two free-living strains of *Rhizobium* isolated from *Lasiurus sindicus* could not fix nitrogen. In our assay, a *Rhizobium* strain isolated from the rice rhizosphere, JR5 (*R. daejeonense*) showed the highest acetylene reducing activity (528.1 nmol C_2H_4/h/10^6 cells) in the in vitro assay. Quan et al. (2005) reported that this species contained the *nif*H gene but formed ineffective nodules on roots of *Medicago sativa.* Therefore, this is the first report that the JR5 strain of *R. daejeonense* shows nitrogen fixing activity both in an in vitro assay and in vivo after inoculation onto rice roots (Tables 3 and 4). While JR5 showed the highest acetylene reducing activity in the in vitro assay, it was ranked 8th out of the 30 isolates in terms of in vivo acetylene reducing activity. Therefore, the acetylene reducing activity determined in vitro does not necessarily correlate with that in vivo. Further research is required to explore the reasons for this difference and to identify the factors that affect nitrogen-fixing ability in vivo. *Pseudomonas* species are common soil bacteria, and they have been well studied in terms of their plant growth-promoting activity (Vessey 2003), phosphate solubilizing activity (Naik et al. 2008), and nitrogen fixing activity (Hatayama et al. 2005). Three out of the four *Pseudomonas* isolated in this study (JW13, *P. monteilii*; JR37, *P. veronii*; JRy205, *P. mandelii*; Table 3 and 4) had not been reported to be associated with rice previously. Also, this is the first report of acetylene reducing activity in *P. monteilii* and *P. mandelii*.

In this study, we used rhizosphere soils from seven different crops to inoculate rice plants, and then isolated bacteria from the shoots and roots of the rice plants after 3 weeks of growth. Then, we conducted genetic and physiological analyses to characterize the isolates to evaluate their potential as PGPR for rice plants. We identified some isolates that showed potential as PGPR for rice: the *B. altitudinis* strain JR198 and the *R. daejeonense* strain JR5, which were derived from rice rhizosphere soil; the *P. monteilii* strain JW13 and the *E. cloacae* strain JW69, which were derived from wheat rhizosphere soil; and the *B. pumilus* strain JM52, which was derived from maize rhizosphere soil. Therefore, these methods are very effective for isolating and identifying candidates for development of biofertilizers for target crops.

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