



## ***Bacillus oryzicola* sp. nov., an Endophytic Bacterium Isolated from the Roots of Rice with Antimicrobial, Plant Growth Promoting, and Systemic Resistance Inducing Activities in Rice**

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Biological control of major rice diseases has been attempted in several rice-growing countries in Asia during the last few decades and its application using antagonistic bacteria has proved to be somewhat successful for controlling various fungal diseases in field trials. Two novel endophytic *Bacillus* species, designated strains YC7007 and YC7010<sup>T</sup>, with antimicrobial, plant growth-promoting, and systemic resistance-inducing activities were isolated from the roots of rice in paddy fields at Jinju, Korea, and their multifunctional activities were analyzed. Strain YC7007 inhibited mycelial growth of major rice fungal pathogens strongly *in vitro*. Bacterial blight and panicle blight caused by *Xanthomonas oryzae* pv. *oryzae* (KACC 10208) and *Burkholderia glumae* (KACC 44022), respectively, were also suppressed effectively by drenching a bacterial suspension (10<sup>7</sup> cfu/ml) of strain YC7007 on the rhizosphere of rice. Additionally, strain YC7007 promoted the growth of rice seedlings with higher germination rates and more tillers than the untreated control. The taxonomic position of the strains was also investigated. Phylogenetic analyses based on 16S rRNA gene sequences indicated that both strains belong to the genus *Bacillus*, with high similarity to the closely related strains, *Bacillus siamensis* KACC 15859<sup>T</sup> (99.67%), *Bacillus methylotrophicus* KACC 13105<sup>T</sup> (99.65%), *Bacillus amyloliquefaciens* subsp. *plantarum* KACC 17177<sup>T</sup> (99.60%), and *Bacillus tequilensis* KACC 15944<sup>T</sup> (99.45%). The DNA-DNA relatedness value between strain YC7010<sup>T</sup> and the most closely related

strain, *B. siamensis* KACC 15859<sup>T</sup> was 50.4 ± 3.5%, but it was 91.5 ± 11.0% between two strains YC7007 and YC7010<sup>T</sup>, indicating the same species. The major fatty acids of two strains were anteiso-C<sub>15:0</sub> and iso C<sub>15:0</sub>. Both strains contained MK-7 as a major respiratory quinone system. The G+C contents of the genomic DNA of two strains were 50.5 mol% and 51.2 mol%, respectively. Based on these polyphasic studies, the two strains YC7007 and YC7010<sup>T</sup> represent novel species of the genus *Bacillus*, for which the name *Bacillus oryzicola* sp. nov. is proposed. The type strain is YC7010<sup>T</sup> (= KACC 18228<sup>T</sup>). Taken together, our findings suggest that novel endophytic *Bacillus* strains can be used for the biological control of rice diseases.

**Keywords** : bacterial blight, endophytes, induced systemic resistance, leaf rot, novel *Bacillus*, rice growth promotion

Rice is the staple food over half of the world population and its production was estimated approximately 745 million tonnes in 2013 worldwide. Asia ranks highest in rice production but 24–41% of losses occur annually due to the major limiting factor, diseases (Anon., 2014; Savary et al., 2000). More than 70 diseases caused by fungi, bacteria, viruses, and nematodes have been recorded in rice and especially rice blast, bacterial leaf blight, grain rot, and bakanae are the most serious seed-borne diseases reducing yields (Ou, 1985). The bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* enormously affects yield losses 10–50% of total rice production, depending on the situation (Mew, 1992). Other bacterial diseases including seedling blight and rot, leaf sheath rot, leaf browning, panicle blight, bacterial wilt, and grain rot caused by *Burkholderia glumae* reduced the yield by up to 75%

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in some cases (Kim et al., 2010; Ura et al., 2006). The yield losses due to bakanae caused by *Fusarium fujikuroi* were estimated to be approximately 10–50% in the Asian rice-growing areas (Bonman, 1992). For controlling the diseases, chemical fungicides have been widely used in most Asian countries during past decades, but the efficacy of fungicides has decreased recently due to the occurrence of resistance (Yang et al., 2012). Additionally, the misuse of chemical fungicides has adverse effects on the agricultural environment and farmers, so the development of alternative control measures, specifically, biological controls using antagonistic microorganisms, has been attempted in many Asian countries (Gnanamanickam, 2009).

Biological control using antagonistic bacteria would be environmentally sound and can be implemented as an integrated disease management tool. Various bacterial genera, such as *Bacillus*, *Burkholderia*, *Lysobacter*, *Pantoea*, *Pseudomonas*, and *Streptomyces* have been used as biocontrol agents for controlling diseases of many crops, but a few studies have been reported on the biological control of rice diseases (Bouizgarne, 2013; McSpadden Gardener, 2010). The multiple use of antagonistic bacteria *Streptomyces* and *Bacillus* species has been attempted for the control of rice sheath blight (Sung and Chung, 1997). Rice blast and sheath blight were controlled effectively by *Bacillus vallismortis* EXTN-1 and two antagonistic bacterial strains, *Pseudomonas fluorescens* mc75 and pc78 (Choi et al., 2006; Park et al., 2006). Bakanae and seedling rot of rice caused by *Fusarium moniliforme* and *F. fujikuroi* were also controlled by antagonistic *P. fluorescens* and *Bacillus cereus* (Kazempour and Elahinia, 2007; Rosales and Mew, 1997). Among the diverse antagonistic bacteria, several *Bacillus* species have been developed as commercial bio-pesticides, because *Bacillus* species can produce endospores and persist successfully in natural environments for a long period after treatment (Hu et al., 2011).

*Bacillus* species widely used for biological control of many plant diseases in different hosts include *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Bacillus pasteurii*, *B. cereus*, *Bacillus pumilus*, *Bacillus mycoides*, and *Bacillus sphaericus* (Kloepper et al., 2004; McSpadden Gardener, 2010). *B. subtilis* GB03 and *B. amyloliquefaciens* IN937 were demonstrated to control the bacterial pathogen, *Erwinia carotovora* subsp. *carotovora* in *Arabidopsis* (Ryu et al., 2004). *B. cereus* AR156 and *B. subtilis* were also demonstrated to control *Pseudomonas syringae* pv. *tomato* DC3000 successfully in *Arabidopsis* by inducing resistance (Niu et al., 2011). Some of these *Bacillus* species have been well characterized in terms of their anti-fungal,

anti-bacterial, plant growth-promoting, and resistance-inducing activities in host plants (Park et al., 2009; Ryu, 2013). Diverse species of *Bacillus* have been isolated from various terrestrial and halophytic plants and some of them have been shown to be endophytic (Bibi et al., 2011, 2012). The genus *Bacillus* currently contains 299 species and more than 30 species have been reported to be novel during the last 5 years, based on polyphasic studies, including 16S rRNA gene sequences, DNA-DNA hybridization analyses, fatty acid profiles, and physical and biochemical tests (Parte, 2014).

In this study, two *Bacillus* strains YC7007 and YC7010<sup>T</sup> isolated from the roots of rice were examined in developing a novel biocontrol agent with multifunctional activities and further characterized for identification by a polyphasic approach. The control efficacy of the novel strain YC7007 was also investigated through induction of resistance against bacterial blight and panicle blight as well as growth promotion in rice.

## Materials and Methods

**Isolation and cultivation of endophytic bacteria.** Endophytic bacterial strains were isolated from the rice roots collected at the paddy field of Gyeongsang National University Farm, Jinju, Korea. For the isolation, sample pieces were washed several times with running tap water and surface-sterilized with 70% ethanol for 5 min, 1.2% NaOCl solution for 10 min, and finally washed with sterile distilled water several times. To confirm the successful disinfection, washed segments were placed on one-tenth strength tryptic soy broth agar (1/10 TSA) at 28°C for 3 days and bacterial growth was observed. After confirmation of no bacterial colonies, the sample segments were again sterilized by 70% ethanol for a few seconds and ground in autoclaved distilled water with a sterile mortar and pestle (Bibi et al., 2012). Aliquots were 10-fold serially diluted in autoclaved distilled water and the diluted samples were kept in a hot water bath (80°C) for 10 min to select endospore-forming bacterial species. An aliquot (0.1 ml) was plated on 1/10 TSA media supplemented with cycloheximide (50 µg/ml) and the plates were incubated at 30°C for 3 days. The bacterial colonies grown on the medium were selected on the basis of distinct colony morphology. The bacterial strains isolated purely were subcultured on 1/10 TSA media and stored at –70°C for further use. For cultivation of bacterial strains, medium containing 10 g protease peptone, 10 g yeast extract, 4 g NH<sub>4</sub>Cl, 4 g MgSO<sub>4</sub>, 10 g glucose, and 15 g agar per liter distilled water was prepared.

**Antagonistic activity against fungal and bacterial pathogens.** The endophytic bacteria were tested against important plant fungal pathogens, such as *Alternaria panax* KACC 42461, *F. fujikuroi* KACC 44022, *F. oxysporum* KCTC 16909, *Sclerotinia sclerotiorum* GSCC 50501, *Pythium ultimum* GSCC 50651, *Bipolaris oryzae* KACC 40853, *Botrytis cinerea* KCTC 6973, *Magnaporthe grisea* KACC 40415, *Botryosphaeria dothidea* GSCC 50201, and *Rhizoctonia solani* KCTC 40101. The antagonistic activity of bacterial strains was determined by measuring the inhibition zone of mycelial growth of the fungal pathogens on potato dextrose agar (PDA) medium using *in vitro* confrontation bioassay (Bibi et al., 2012). For the antibacterial test, *B. glumae* KACC10359 and *X. oryzae* pv. *oryzae* KACC 10208 were grown on one-half strength R2A and YGC media (10 g glucose, 30 g CaCO<sub>3</sub>, 5 g yeast extract, and 15 g agar per liter distilled water), respectively and the inhibition activity was tested using a diffusion paper disc method. Culture filtrate of the bacterial strain YC7007 was prepared from the culture broth with different cultivation times by centrifugation (5,000 g, 10 min) and filtration through a Millipore filter (0.2 µm).

**Bioassay of induced resistance against bacterial pathogens.** Induction of resistance by strain YC7007 was tested against bacterial blight and panicle blight caused by *X. oryzae* pv. *oryzae* KACC 10208 and *B. glumae* KACC 10359, respectively, in pot tests. Seeds of rice (*Oryza sativa* L. cultivar Dongjin) were surface-sterilized with 1.2% NaOCl solution for 5 min and 70% ethanol for 5 min, then rinsed three times with sterile distilled water. The seeds were then kept at 30°C for 3 days in the dark for germination and the water was changed every day. The germinated seeds were sown in commercial nursery soil (Dasuran Sangto, Youngnong Sun Up, Korea) and placed in a greenhouse for cultivation. Then, 2-week-old seedlings were transplanted into plastic pots (9.5 × 8 × 7 cm<sup>3</sup>) containing about 150 g commercial nursery soils that had been autoclaved for 20 min at 121°C twice on two consecutive days. For the preparation of a bacterial suspension of YC7007, bacterial cells were cultivated for two days in the liquid mass culture medium on a rotary shaker (160 rpm, 28°C) and harvested by centrifugation (5,000 g, 10 min) and adjusted to different concentrations (5.6 × 10<sup>5</sup>, 3.6 × 10<sup>6</sup>, 2 × 10<sup>7</sup> cfu/ml) in buffer solution (10 mM MgSO<sub>4</sub>). The cell suspension (15 ml) of YC7007 was drenched into the plastic pot containing autoclaved soils (150 g) during transplanting 2-week-old rice seedlings at the same time. The optimal concentration of YC7007 (2 × 10<sup>7</sup> cfu/ml) was used for testing further resistance induction against

bacterial blight and panicle blight. The culture filtrate of strain YC7007 prepared from a 60 h culture broth that was diluted 10 times with the 10 mM MgSO<sub>4</sub> and sprayed to the leaves until to droplet for checking its control efficacy. Then, five days after treatment with YC7007, the bacterial pathogens were inoculated. The buffer solution was used as a control. All experiments were conducted using 10 plants with three replicates for each treatment.

**Inoculum preparation of bacterial pathogen.** The inocula of two bacterial pathogens, *B. glumae* KACC 10359 and *X. oryzae* pv. *oryzae* KACC 10208 were prepared using the cell suspension after cultivation in R2A and YGC media, respectively, at 28°C for 24 h on a rotary shaker (160 rpm). The culture broth of *B. glumae* was centrifuged (5,000 g, 10 min) and cell pellets were suspended in buffer solution (10 mM MgSO<sub>4</sub>) adjusted to 6 × 10<sup>7</sup> cfu/ml. A pin-prick bioassay was conducted for inoculation of the suspension of *B. glumae*. A bundle of 3–4 pins/needles was dipped into the suspension and then leaves were teased with the needles of bundle. Disease severity was evaluated 5 days after inoculation of the pathogen to the 5-week-old rice seedlings using a 0–3 disease rating scales: 0 = symptomless, 1 = slight infection, few small lesions, 2 = coalesced lesions to browning all over, and 3 = no description available (Cottyn et al., 1996). In case of bacterial blight, the suspension of *X. oryzae* pv. *oryzae* was prepared following the same procedure of *B. glumae* and concentration was adjusted to 1.2 × 10<sup>7</sup> cfu/ml. A clipping bioassay for bacterial blight was conducted using scissors that were dipped into the suspension and then used to cut the upper leaves. Disease severity was determined 7 days after inoculation according to a 1–9 scale (Misra et al., 1994). Disease reduction was calculated following the formula: [(disease severity of the control – disease severity of a treatment) / disease severity of the control] × 100%.

**Measurement of growth promotion.** Growth promotion of rice by treatment with a bacterial suspension of strain YC7007 (2 × 10<sup>7</sup> cfu/ml) was examined during cultivation periods. Bacterial suspension (1 ml) of YC7007 was drenched on the rhizosphere of 5-day-old rice seedlings in test tubes (18 cm length) containing 10 ml 1/2 MS media with 0.8% agar. Growth data were recorded after 7 days of the bacterial treatment (12 days old). In case of the pot tests, 2-week-old seedlings were transplanted into plastic pots containing about 150 g autoclaved nursery soil and the bacterial suspension (15 ml) was drenched at 7 days from transplanting. Growth data were recorded



after 9 days of the bacterial treatment (30-day-old plants). Again, the bacterial suspension (15 ml) was drenched at the tillering stage (30 days old) and growth data were recorded at the booting stage after 40 days of bacterial treatment from the tillering stage (70 days old). Ten plants with three replicates for each treatment were conducted for growth promotion activities for the test tubes and pot tests. The buffer solution (10 mM MgSO<sub>4</sub>) was used for the control treatment.

#### **Phylogenetic analysis based on 16S rRNA gene sequences and DNA-DNA hybridization.**

The 16S rRNA gene was amplified from the genomic DNA extracted with a commercial extraction kit (Intron Biotech, Seoul, Korea) using bacterial universal primers 27F and 1492R and the purified PCR product was sequenced by GenoTech Inc. (Daejeon, Korea) (Lane, 1991). To ascertain the phylogenetic position of the novel endophytic bacteria and other closely related type strains, the 16S rRNA gene sequences of the strains were compared with sequences obtained from NCBI and the EzTaxon-e database server (Kim et al., 2012). Multiple alignments of the sequences were performed using the CLUSTAL\_X software (Thompson et al., 1997). Gaps were edited using the BioEdit program (Hall, 1999). The neighbor-joining method (Saitou and Nei, 1987), maximum-parsimony (Fitch, 1972), and maximum-likelihood algorithms in the MEGA 5.10 software (Tamura et al., 2011) were used to construct phylogenetic trees with bootstrap values based on 1,000 replications (Felsenstein, 1985). To determine the DNA-DNA relatedness values among strains, DNA-DNA hybridization was carried out using the DIG DNA labeling and detection kit (Roche Applied Science) according to the instructions of the manufacturer and the method described previously (Lee et al., 2011).

#### **Morphological, phenotypic and chemotaxonomical characteristics.**

Cell morphology was observed under a light microscope (Nikon) at 1000× magnification and the presence of a flagellum was investigated by transmission electron microscopy (Hitachi, model H-600) using a cell broth grown at 28°C for 24 h in R2A medium. Gram reaction was determined using the bioMérieux Gram stain kit according to the manufacturer's instruction. The hydrolysis tests of strains were performed by a standard protocol, using casein, esculin, gelatin, starch, L-tyrosine, urea, Tween 20, and Tween 80 (Reichenbach, 1992; Smibert and Krieg, 1994). Enzyme activities, acid production from different carbohydrates, assimilation of various substrates and growth on carbohydrates were determined using

commercial systems-API ZYM, API 20E, API 20NE, and API 50CH kits, respectively, at 28°C according to the manufacturer's instructions (BioMérieux). Growth at different temperatures and various pH values (pH 4.0–14.0 at intervals of 0.5 pH units) was investigated after 5 days incubation on R2A broth using appropriate buffers (Xu et al., 2005). Salt tolerance was tested in R2A broth supplemented with 1–14% (w/v, at 1% intervals) NaCl after 5 days incubation at 28°C. Duplicate antibiotic-sensitivity tests were performed using filter-paper disc diffusion assays with different antibiotics: 10 µg ampicillin, 30 µg chloramphenicol, 10 µg penicillin, 10 µg gentamycin, 30 µg kanamycin, 30 µg vancomycin, 30 µg streptomycin, and 30 µg tetracycline (Yasir et al., 2010). Preparation of cell walls and analyses of peptidoglycans were carried out using methods described by Schleifer (1985) with the modification that TLC was performed on cellulose sheets rather than by paper chromatography. For the analysis of cellular fatty acids, bacterial strains were cultivated in R2A broth at 28°C and the microbial cells were harvested in the mid-exponential growth phase (OD<sub>600</sub> = 0.4–0.5). The analysis of fatty acid methyl esters was performed according to the instruction of the microbial identification system (MIDI; Microbial ID, Inc.). Extracts were analyzed by GC (Agilent 6890) and identified by comparing the fatty acid profiles with the TSBA 40 database provided with the Sherlock software (ver. 4.0). Amino acids of whole-cell hydrolysates of bacterial strains were extracted and analyzed according to the method of Stanek and Roberts (1974). Isoprenoid quinones were extracted and analyzed using reverse-phase HPLC according to the method described by Komagata and Suzuki (1987). For the measurement of G+C content of the chromosomal DNA, the genomic DNA of strain YC7010<sup>T</sup> was extracted and purified as described (Ausubel et al., 1995). It was then enzymatically degraded into nucleosides and G+C content was determined with a reverse-phase C18 column (Mesbah et al., 1989). Polar lipids were extracted by the modified method of Minnikin et al. (1984) and separated by TLC on Merck Kieselgel 60-HPTLC. Aminolipids were detected by spraying the plate with 0.2% (w/v) solution of ninhydrin in butanol saturated with water followed by heating at 105°C for 10 min (Ross et al., 1985). Phospholipids were detected by spraying the plate with Zinzadze reagent of Dittmer and Lester (1964). Glycolipids were detected with 1-naphthol spray reagent by heating at 100°C for 3–5 min (Jacin and Mishkin, 1965). The presence of phosphatidylcholine was detected with Dragendorff reagent (Sigma-Aldrich; St. Louis, Mo). Total lipid profiles were detected by spraying with phosphomolybdic acid solution (Sigma-Aldrich; St. Louis, Mo) followed by heating

at 150°C for 10 min.

**Statistical analysis.** Data were analyzed by analysis of variance designed with a complete randomized single factor for *in vitro*, complete randomized block design for *in vivo*, and mean differences were compared using Duncan's multiple range test (DMRT). The SPSS software (ver. 17; SPSS Inc. in Chicago) was used for all analyses.

## Results

**Antagonistic activity of isolated strains.** Among 250 bacterial strains isolated from the roots of rice plants, 15 endospore-forming bacteria showed inhibitory activity in the range of 2 to >20 mm to the mycelial growth of *F. fujikuroi*. These bacteria were found to be closely related to *Paenibacillus polymyxa*, *Bacillus siamensis*, *Paenibacillus jamilae*, *Bacillus methylotrophicus*, *Bacillus thuringiensis*, *B. cereus*, *Bacillus simplex*, and *Bacillus daliensis*, based on 16S rRNA gene sequences. These isolates were tentatively identified and their similarities were in the range of 99.27%–100% with the closely related genera. Of the isolates, two strains especially, YC7007 and YC7010<sup>T</sup>, with the highest similarity to *B. siamensis* showed strong antagonistic activity (>20 mm inhibition zone) against *F. fujikuroi* (Table 1). YC7007 also showed strong antagonistic activity against other rice pathogens,

*B. oryzae*, *M. grisea*, *F. fujikuroi*, and other major plant pathogens, with inhibition zones from 10 to 29 mm on PDA (Table 2). The culture filtrate of strain YC7007 prepared from different cultivation times also showed good growth inhibition against *F. fujikuroi* and two major seed-borne bacterial pathogens of rice, *B. glumae* and *X. oryzae* pv. *oryzae* (Fig. 1). The culture filtrate of 60 h-old broth had the strongest activity against three pathogens on the

**Table 2.** Antagonistic activity of endophytic strain YC7007 against important fungal and oomycete plant pathogens

Plant pathogens	Inhibition zone <sup>a</sup> (mm)
<i>Fusarium fujikuroi</i> KACC 44022	28.0 ± 0.6
<i>Magnaporthe grisea</i> KACC 40415	29.0 ± 0.6
<i>Bipolaris oryzae</i> KACC 40853	27.6 ± 0.3
<i>Rhizoctonia solani</i> KCTC 40101	23.6 ± 0.3
<i>Sclerotinia sclerotiorum</i> GSCC 50501	28.0 ± 0.6
<i>Botrytis cinerea</i> KCTC 6973	27.3 ± 0.3
<i>Fusarium oxysporum</i> KCTC 16909	25.0 ± 0.6
<i>Botryosphaeria dothidea</i> GSCC 50201	24.6 ± 0.3
<i>Pythium ultimum</i> GSCC 50651	28.3 ± 0.3
<i>Alternaria panax</i> KACC 42461	10.0 ± 0.6

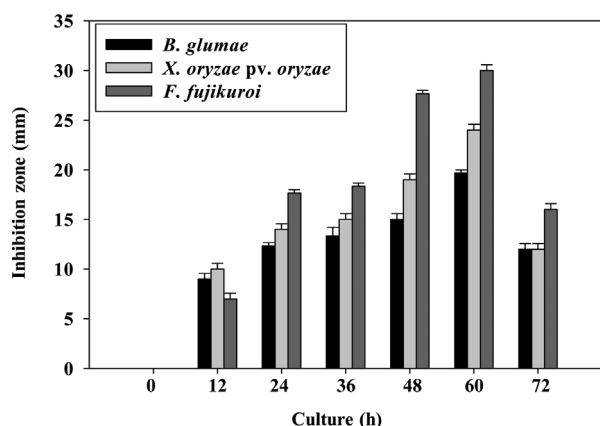
<sup>a</sup>The antagonistic activity was determined by an *in vitro* confrontation bioassay on PDA. The inhibition zone between mycelia of the pathogen and the border line of YC7007 was measured 7 days after incubation at 28°C and 20°C (*S. sclerotiorum* and *B. cinerea*). Data represent the average of three replicates.

**Table 1.** Identification of endophytic bacteria antagonistic to the bakanae pathogen of *Fusarium fujikuroi*

Origin	Closely related strain	% Identity <sup>a</sup>	Inhibition <sup>b</sup>
			<i>Fusarium fujikuroi</i>
YC7005	<i>Paenibacillus polymyxa</i> (AFOX01000032)	99.77	+
YC7006	<i>Paenibacillus polymyxa</i> (AFOX01000032)	99.77	+
YC7007	<i>Bacillus siamensis</i> (AJVF01000043)	99.67	++++
YC7008	<i>Paenibacillus polymyxa</i> (AFOX01000032)	99.27	+
YC7009	<i>Paenibacillus jamilae</i> (AJ271157)	100	+
YC7010 <sup>T</sup>	<i>Bacillus siamensis</i> (AJVF01000043)	99.67	+++
YC7012	<i>Bacillus siamensis</i> (AJVF01000043)	99.60	++
YC7013	<i>Bacillus methylotrophicus</i> (EU194897)	99.37	++
YC7014	<i>Bacillus methylotrophicus</i> (EU194897)	99.62	++
YC7015	<i>Bacillus methylotrophicus</i> (EU194897)	99.68	++
YC7016	<i>Bacillus methylotrophicus</i> (EU194897)	99.64	++
YC7022	<i>Bacillus thuringiensis</i> (ACNF010000156)	99.87	++
YC7023	<i>Bacillus cereus</i> (AE016877)	100	++
YC7025	<i>Bacillus simplex</i> (AB363738)	98.88	++
YC7027	<i>Bacillus daliensis</i> (ACNF010000156)	100	++

<sup>a</sup>Based on the partial 16S rRNA gene sequences analysis.

<sup>b</sup>The mycelial growth of a pathogen by antagonistic bacteria was determined as inhibition zone on PDA. +: 2–10, ++: 10–15, +++: 15–20, and ++++: > 20 mm.

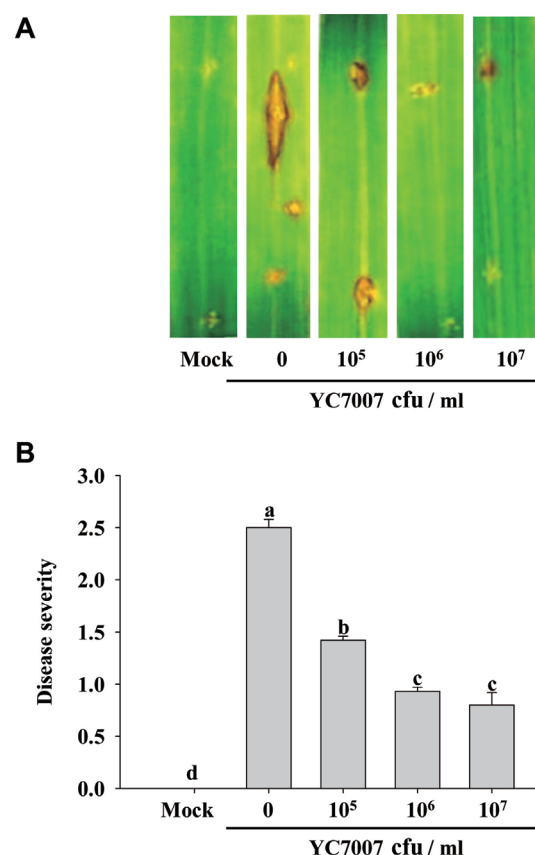


**Fig. 1.** Growth inhibition of major seed-borne rice pathogens (*Bulkholderia glumae*, *Xanthomonas oryzae* pv. *oryzae*, and *Fusarium fujikuroi*) by the culture filtrate of strain YC7007 at different culture times.

respective culture media with the inhibition zone of 30 mm, 24 mm and 19.7 mm for *F. fujikuroi*, *X. oryzae* pv. *oryzae* and *B. glumae*, respectively.

**Induction of systemic resistance against panicle blight and bacterial blight.** Strain YC7007 was investigated for the activity of inducing systemic resistance to the panicle blight and bacterial blight of rice. Drenching of three different concentrations of YC7007 to soils at  $5.6 \times 10^5$ ,  $3.6 \times 10^6$ , and  $2 \times 10^7$  cfu/ml revealed significantly lower disease severity index of the leaf rot ( $p < 0.05$ ) by 1.4, 0.9, and 0.8, respectively, than the control, 2.5 (Fig. 2). However, there was no significant difference in the disease severity between bacterial concentrations of  $10^6$  and  $10^7$  cfu/ml. The concentration ( $2 \times 10^7$ , cfu/ml) of strain YC7007 drenched to the rhizosphere soils showed good control efficacy against panicle blight and bacterial blight, by 65.2% and 61.2%, respectively. Its culture filtrate sprayed to the leaves also showed significantly lower disease severity than the control, with disease reduction by 70.8% and 70.5% for panicle blight and bacterial blight, respectively (Table 3). The strain YC7007 showed significantly lower disease severity ( $p < 0.01$ ) against panicle blight and bacterial blight than the control.

**Growth promotion by YC7007.** Strain YC7007 had growth promoting activity with significantly ( $p < 0.05$ ) better growth than the control at all stages tested such as the seedling, tillering, and booting stages of rice. Treatment with the bacterial suspension ( $2 \times 10^7$  cfu/ml) to the rhizosphere of the rice increased shoot length from 11.67 cm to 19.33 cm in the test tube at the seedling stage, 36.45



**Fig. 2.** Effect of YC7007 drenching in the rice rhizosphere on disease severity of panicle blight caused by *Bulkholderia glumae*. (A) Necrotic lesions developed at 5 days after inoculation of pathogen at 30°C. (B) Cell suspension of YC7007 (15 ml) at different concentrations was drenched to the pots containing autoclaved soil (150 g) 5 days before inoculation of the bacterial pathogen. The pathogen was challenged by pin-prick inoculation in each treatment (20 leaves) with three replicates. Different letters indicate values that are significantly different ( $P < 0.05$ ) by Duncan's multiple range test. 'Mock' means no inoculation of the pathogen.

cm to 46.33 cm, and 55.33 cm to 61.0 cm in the tillering and booting stages, respectively. The length of primary roots increased from 2.67 cm to 7.67 cm at the seedling stage and the numbers of tillers were also increased, from 1.9 to 2.9 at the tillering stage and 5.3 to 7.0 at the booting stage (Table 4).

**Identification of two antagonistic bacteria YC7007 and YC7010<sup>T</sup>.** Two endophytic strains YC7007 and YC7010<sup>T</sup> with the strongest antagonistic activity out of 15 antagonistic bacteria were identified using a polyphasic approach. The 16S rRNA gene sequence of the two strains was a continuous stretch of 1513 base pairs, which aligned

**Table 3.** Suppression of panicle blight and bacterial leaf blight of rice by YC7007

Treatment <sup>a</sup> Pathogen <sup>b</sup>	Disease severity		% Disease reduction	
	<i>B. glumae</i>	<i>X. oryzae</i> pv. <i>oryzae</i>	<i>B. glumae</i>	<i>X. oryzae</i> pv. <i>oryzae</i>
Control	2.50 ± 0.08 a	5.67 ± 0.13 a	–	–
YC7007 (suspension)	0.87 ± 0.12 b	2.20 ± 0.23 b	65.2	61.2
YC7007 (culture filtrate)	0.73 ± 0.07 b	1.67 ± 0.13 bc	70.8	70.5

<sup>a</sup>The cell suspension 15 ml ( $2 \times 10^7$  cfu/ml) was drenched to the pots 5 days before inoculation of the bacterial pathogen. Culture filtrate prepared with 60 h old culture broth was diluted 10 times with 10 mM MgSO<sub>4</sub> and sprayed to the leaves as droplets before 5 days of pathogen inoculation. Control plants were sprayed with only the buffer solution (10 mM MgSO<sub>4</sub>).

<sup>b</sup>The inoculation techniques for panicle blight and bacterial blight was conducted by pinprick and clipping assay, respectively, described details in the Materials and Methods. Different letters indicate values that are significantly different ( $P < 0.01$ ) by Duncan's multiple range tests. Mean ± SE; standard error was calculated using 10 plants with three replicates for each treatment.

**Table 4.** Effect of YC7007 on the growth promotion of rice at different growing stages

Treatment	Growth promoting activity					
	Seedling stage <sup>a</sup>		Tillering stage <sup>b</sup>		Booting stage <sup>c</sup>	
	Shoot length (cm)	Root length (cm)	Shoot length (cm)	Tiller no.	Shoot length (cm)	Tiller no.
Control	11.67 ± 0.33 b	2.67 ± 0.33 b	36.45 ± 1.18 b	1.89 ± 0.22 b	55.33 ± 0.33 b	5.33 ± 0.67 b
YC7007	19.33 ± 0.33 a	7.67 ± 0.33 a	46.33 ± 2.0 a	2.89 ± 0.11 a	61.0 ± 2.00 a	7.00 ± 0.58 a

<sup>a</sup>Bacterial suspension (1 ml) of YC7007 ( $2 \times 10^7$  cfu/ml) was inoculated with 5-day-old seedlings in the test tube.

<sup>b</sup>5 ml of YC7007 ( $2 \times 10^7$  cfu/ml) was drenched to 21-day-old seedlings in pots and data were recorded after 9 days of bacterial treatment.

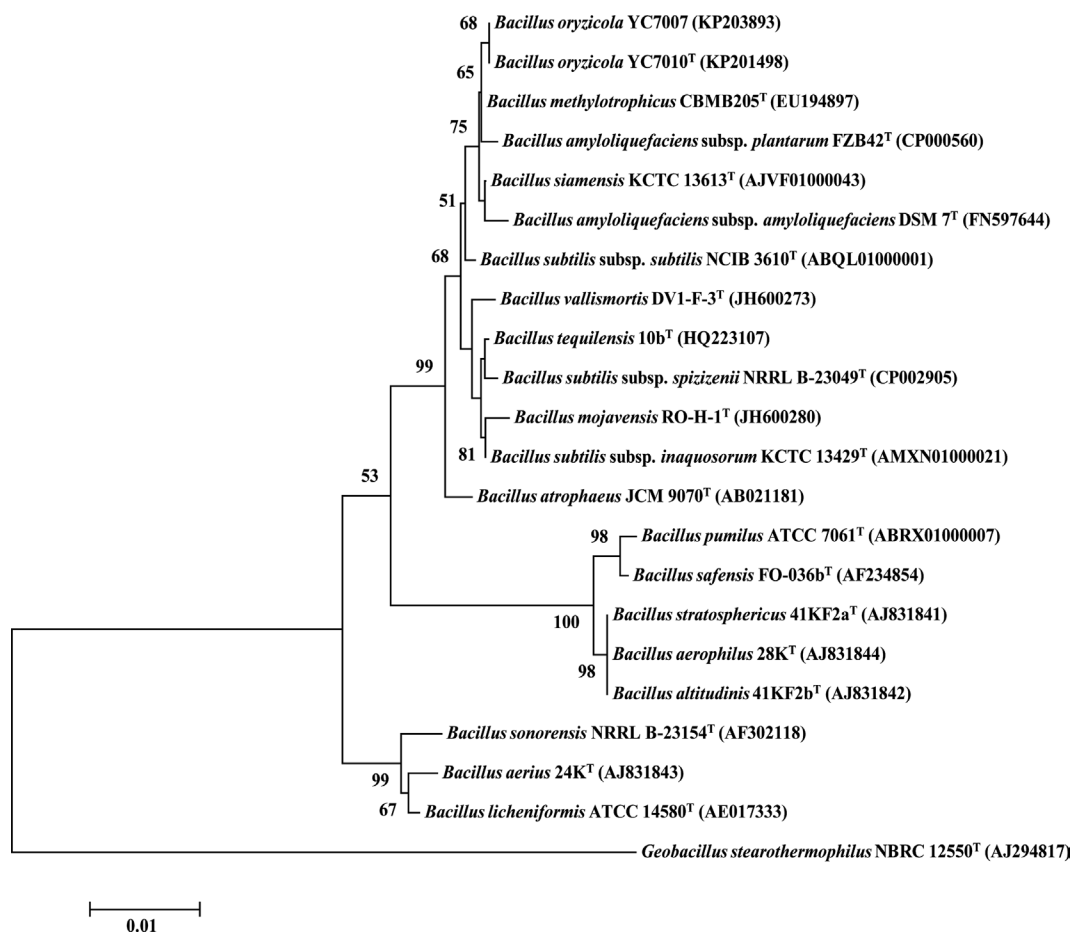
<sup>c</sup>5 ml of YC7007 ( $2 \times 10^7$  cfu/ml) was drenched once again to 30-day-old seedlings in pots. Data were recorded after 40 days of bacterial treatment. Control plants of all stages were treated with the buffer solution (10 mM MgSO<sub>4</sub>) as the same amount of the corresponding treatments by the YC7007. Different letters indicate statistically significant differences between treatments by Student's *t*-test. Mean ± SE; standard error was calculated using 10 plants with three replicates for each treatment.

identically except only one nucleotide, with the database accession numbers KP203893 (YC7007) and KP201498 (YC7010<sup>T</sup>) in GenBank/EMBL/DDBJ. Based on the comparative 16S rRNA gene sequence analysis, the two strains are in the same clade with the same branch length of the phylogenetic tree, showing the highest similarity to *B. siamensis* KACC 15859<sup>T</sup> (99.67%), *B. methylotrophicus* KACC 13105<sup>T</sup> (99.65%), *B. amyloliquefaciens* subsp. *plantarum* KACC 17177<sup>T</sup> (99.60%), and *B. tequilensis* KACC 15944<sup>T</sup> (99.45%) (Table 1, Fig. 3). The DNA-DNA relatedness value between strain YC7010<sup>T</sup> and its most closely related strain *B. siamensis* KACC 15859<sup>T</sup> was 50.4 ± 3.5, but it was 91.5 ± 11.0% between YC7007 and YC7010<sup>T</sup> (Table 5). Both strains were Gram-positive, motile, rod-shaped, and grew well at 13–60°C (optimum at 28–30°C) and pH (4–12) (optimum at pH 7). Strains were able to grow at 0.1 TSA media with 1–13% NaCl (w/v), but NaCl was not required for growth. Strains were resistant to chloramphenicol by 30 µg/ml. Strains contained meso-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan and MK-7 as a major respiratory quinone system. Other physiological and biochemical characteristics of two strains YC7007 and YC7010<sup>T</sup>

are summarized in Table 6. The major fatty acids of the strains YC7007 and YC7010<sup>T</sup> were the anteiso-C<sub>15:0</sub> (38.4 and 32.0%) and iso C<sub>15:0</sub> (28.1 and 27.7%), respectively. The other cellular fatty acids profiles of YC7010<sup>T</sup> consisted of C<sub>16:0</sub> (7.7%), iso C<sub>17:0</sub> (6.4%), anteiso-C<sub>17:0</sub> (5.3%), iso C<sub>16:0</sub> (5.2%), C<sub>18:0</sub> (5.1%), C<sub>16:1</sub> ω7c alcohol (3.4%), iso C<sub>14:0</sub> (2.9%), iso-C<sub>17:1</sub> ω10c (1.7%), C<sub>16:1</sub> ω11c (1.4%), C<sub>14:0</sub> (1.1%), and C<sub>20:1</sub> ω7c (0.2%) (Table 7). The G+C contents of the genomic DNA of two strains YC7007 and YC7010<sup>T</sup> were 50.5 mol% and 51.2 mol%, respectively. Strains exhibited a polar lipid profile consisting of phosphatidylethanolamine (PE), phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), unknown glycolipid (GL) and unknown aminolipids (AL1-2) (Fig. S1). Based on 16S rRNA gene sequence analysis, DNA-DNA relatedness value, fatty acid composition and biochemical and physiological characteristics, strains YC7007 and YC7010<sup>T</sup> represent a novel species of the genus *Bacillus*, for which the name *Bacillus oryzicola* sp. nov. is proposed.

**Description of *Bacillus oryzicola* YC7010<sup>T</sup> sp. nov.** *Bacillus oryzicola* (o.ry.zi'co.la L. fem. n. oryz, rice; L. v. colere, to inhabit; L. adj. oryzicola, living in rice). Cells are Gram-





**Fig. 3.** Phylogenetic tree constructed from the comparative analysis of 16S rRNA gene sequences showing the relationship of strains YC7007 and YC7010<sup>T</sup> with other related taxa. The phylogenetic tree was constructed using the neighbor-joining method and the Jukes & Cantor evolutionary distance matrix data obtained from aligned nucleotides. Bootstrap values (expressed as percentage of 1,000 replications) greater than 50% are shown at the branch points.

positive, rod-shaped (0.8–0.9 × 2.0–3.0 μm). Colonies grown on R2A agar at 28°C for 2 days are white-cream, circular, and flat elevation with an entire margin. Cells are motile with a single polar flagellum. The cell wall contains *meso*-diaminopimelic acid as the diagnostic diamino acid. Cells usually occur singly or in pairs that are catalase-positive and oxidase-negative. Cells grow at 13–60°C and at pH 4–12.0. Hydrolysis of casein and gelatin is positive

while negative for starch, Tween 20, Tween 80, tyrosine, and carboxymethyl cellulose. It uses D-glucose, D-fructose, D-mannose, D-mannitol, methyl- $\alpha$ -D-glucopyranoside, N-acetyl-glucosamine, amygdalin, arbutin, esculin ferric citrate, salicin, D-celiobiose, D-maltose, D-lactose, D-saccharose, D-trehalose, D-raffinose, starch, glycogen, gentiobiose, trisodium citrate, and Kohn's gelatin. With the API ZYM kit, it showed enzymatic activities of esterase (C4),

**Table 5.** DNA-DNA hybridization values of YC7010<sup>T</sup> with closely related strains

Strains	% DNA-DNA hybridization
YC7007	91.5 ± 11.0
<i>B. siamensis</i> KACC 15859 <sup>T</sup>	50.4 ± 3.5
<i>B. methylotrophicus</i> KACC 13105 <sup>T</sup>	48.7 ± 9.4
<i>B. subtilis</i> subsp. <i>inaquosorum</i> KACC 17047 <sup>T</sup>	47.4 ± 4.0
<i>B. amyloliquefaciens</i> subsp. <i>plantarum</i> KACC 17177 <sup>T</sup>	41.9 ± 7.9
<i>B. tequilensis</i> KACC 15944 <sup>T</sup>	37.9 ± 5.7



**Table 6.** Differential phenotypic characteristics between strains YC7007 and YC7010<sup>T</sup> and closely related species of the genus *Bacillus*

Characteristic	<sup>a</sup> 1	2	3	4	5	6	7
Growth at pH 13	–	+	–	–	–	+	+
Growth at 14% NaCl	–	–	–	–	–	–	+
Hydrolysis of							
Gelatin	+	–	+	+	+	+	+
Carboxymethyl cellulose	–	–	–	–	+	–	–
API kits							
Arbutin	+	w	–	w	+	w	+
D-Lactose	+	w	w	w	–	w	–
Starch	+	w	–	w	w	–	+
Glycogen	+	w	–	w	w	w	+
Gentiobiose	+	w	–	–	–	–	+
D-Turanose	–	–	w	–	–	–	–
Potassium-5-ketogluconate	–	–	w	–	–	–	–
Alkaline phosphatase	w	+	+	+	–	+	+
Acid phosphatase	w	w	–	–	–	+	–
Naphtol-AS-BI-phosphohydrolase	+	–	–	+	+	+	+
$\alpha$ -Glucosidase	–	–	+	–	–	–	–
N-acetyl- $\beta$ -glucosaminidase	+	+	+	–	–	–	–
$\alpha$ -Mannosidase	–	–	w	–	–	–	–
G+C content (mol%)	50.5	51.2	41.4 <sup>b</sup>	45 <sup>c</sup>	ND	ND	ND

<sup>a</sup>Taxa: 1, strain YC7007; 2, strain YC7010<sup>T</sup>; 3, *B. siamensis* KACC 15859<sup>T</sup>; 4, *B. methylotrophicus* KACC 13105<sup>T</sup>; 5, *B. subtilis* subsp. *inaquosorum* KACC 17047<sup>T</sup>; 6, *B. amyloliquefaciens* subsp. *plantarum* KACC 17177<sup>T</sup>; 7, *B. tequilensis* KACC 15944<sup>T</sup>. +: positive; –: negative; w: weakly positive; ND: not determined. Data for the related type strains are from this study unless indicated.

<sup>b</sup>Sumpavapol et al., 2010.

<sup>c</sup>Madhaiyan et al., 2010.

esterase lipase (C8), naphthol-AS-BI-phosphohydrolase, and N-acetyl- $\beta$ -glucosaminidase, but not lipase (C-14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin,  $\alpha$ -chymotrypsin,  $\alpha$ -galactosidase,  $\beta$ -galactosidase,  $\beta$ -glucosidase,  $\alpha$ -mannosidase, and  $\alpha$ -fucosidase. It was resistant to 30  $\mu$ g of chloramphenicol and streptomycin, but susceptible to 10  $\mu$ g of ampicillin, penicillin, gentamycin, and 30  $\mu$ g of kanamycin, vancomycin, and tetracycline. Growth was observed in R2A broth containing 13% (w/v) NaCl but not 14% (w/v). The major quinone is MK-7. The polar lipids are phosphatidylethanolamine (PE), phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), unknown glycolipid and unknown aminolipids. The DNA G+C content of the type strain is 51.2 mol%. The type strain, YC7010<sup>T</sup> (= KACC18228<sup>T</sup>), was isolated from the root of rice in Jinju, Korea.

## Discussion

Among many endophytic bacterial strains isolated from the rice roots, 6% of the *Bacillus* species were antagonistic and inhibited the mycelial growth of phytopathogens in

this study. About 9% of endophytic bacteria including several *Bacillus* species and other Gram-negative bacteria isolated from tidal flat plants were antagonistic against oomycetous phytopathogens in a previous study (Bibi et al., 2012). Our strains, belonging to the genus *Bacillus* based on the 16S rRNA gene sequence, were examined for their antagonistic activity against the major rice fungal and bacterial pathogens *in vitro* and *in vivo* tests. Of these isolates, two strains YC7007 and YC7010<sup>T</sup> with the highest similarity to *B. siamensis* showed strong antagonistic activity in suppressing mycelial growth of the bakanae pathogen, *F. fujikuroi*, one of the most important seed-borne pathogens of rice. The culture filtrate of YC7007 also had strong inhibiting activity against bakanae, bacterial blight, and grain rot, suggesting that the strain may produce antifungal compounds. It has been reported that some *Bacillus* species produce small peptides and lipopeptides, such as fengycin, iturin, and surfactin, which have good suppression of phytopathogens (Bais et al., 2004; Crane et al., 2013; Dimkic et al., 2013). Drenching with a cell suspension of strain YC7007 suppressed the severity of panicle blight and bacterial blight of rice in pot tests, which

**Table 7.** Cellular fatty acid composition (%) of strains YC7007, YC7010<sup>T</sup>, and other related taxa

Fatty acids	<sup>a</sup> 1	2	3	4	5
C <sub>12:0</sub>	1.7	–	–	–	–
C <sub>14:0</sub>	–	1.1	0.7	–	1.1
C <sub>15:0</sub>	–	–	8.2	–	–
C <sub>16:0</sub>	6.1	7.7	3.3	5.1	4.1
C <sub>17:0</sub>	–	–	0.9	–	–
C <sub>18:0</sub>	–	5.1	–	–	1.1
iso-C <sub>14:0</sub>	4.6	2.9	2.7	6.1	2.6
iso-C <sub>15:0</sub>	28.1	27.7	30.7	28.8	31.9
iso-C <sub>16:0</sub>	6.8	5.2	2.5	5.9	3.9
iso-C <sub>17:0</sub>	6.4	6.4	5.6	6.9	7.6
iso-C <sub>17:1</sub> ω10c	2.0	1.7	1.6	–	2.6
anteiso-C <sub>15:0</sub>	38.4	32.0	37.2	39.8	35.6
anteiso-C <sub>17:0</sub>	5.9	5.3	4.7	7.6	5.8
C <sub>16:1</sub> ω7c alcohol	–	3.4	–	–	1.2
C <sub>16:1</sub> ω11c	–	1.4	2.0	–	1.5
C <sub>20:1</sub> ω7c	–	0.2	–	–	–
Summed feature 4*	–	–	–	–	1.1

<sup>a</sup>Taxa: 1, strain YC7007; 2, strain YC7010<sup>T</sup>; 3, *B. siamensis* KACC 15859<sup>T</sup>; 4, *B. methylotrophicus* KACC 13105<sup>T</sup>; 5, *B. amyloliquefaciens* subsp. *plantarum* KACC 17177<sup>T</sup>. –: not detected.\*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 4 contained C<sub>17:1</sub> ISO I/ANTEL.

indicated the induction of systemic resistance. Disease severity of panicle blight was reduced by treatment with the strain YC7007 at 10<sup>5</sup> (cfu/ml) and higher concentrations between 10<sup>6</sup> to 10<sup>7</sup> cfu/ml suppressed disease, over 62%. The culture filtrate of YC7007 also decreased panicle blight and bacterial blight significantly over 70% compared with the control. It was demonstrated that treatment with the cell suspension of *B. subtilis* (2.5 × 10<sup>8</sup> cfu/ml) and *B. cereus* AR156 (5 × 10<sup>8</sup> cfu/ml) controlled root and leaf speck diseases of *Arabidopsis*, respectively (Bais et al., 2004; Niu et al., 2011). The bacterial concentrations of these two isolates *B. subtilis* and *B. cereus* AR156 (10<sup>8</sup> cfu/ml) was over 100 times higher than that of strain YC7007 (10<sup>6</sup> cfu/ml), which may not be feasible for practical use at commercial farms. For developing antagonistic bacteria as biological agents, the optimal concentration of bacteria should be equal or lower than 10<sup>7</sup> cfu/ml, considering the dilution factor in the final use by farmers (Chen et al., 2009). As the bacterial suspension drenched to the rhizosphere of rice and culture filtrate sprayed on the leaves, had an antagonistic activity against the pathogens, strain YC7007 probably has indirect and/or direct interactions between them. Diseases of panicle blight and bacterial blight were controlled by the

treatment – both drenching and spraying – suggesting that the strain YC7007 may react through induced systemic resistance (ISR) or systemic acquired resistance (SAR) for the suppression of the diseases (Ahn et al., 2007; Niu et al., 2011). The reaction mechanism needs to be determined in terms of the hormonal signaling via the salicylic, jasmonic, or ethylene pathways for SAR or ISR.

Several species of *Bacillus* have been commercialized as biological agents due to their long persistence in adverse environments over a long period. Some of these were functional in many plants, by inducing systemic resistance (Hu et al., 2011; Kloepper et al., 2004). *B. vallismortis* EXTN-1 and *B. cereus* have been used to control rice blast, sheath blight, and bakanae (Kazempour and Elahinia, 2007; Park et al., 2006). To our knowledge, this is the first report that *Bacillus* strain YC7007 has long-lasting activity in resistance induction in rice against major bacterial diseases of panicle blight and bacterial blight. Furthermore, strain YC7007 showed good growth promotion of rice by a one-time application of a bacterial suspension at the seedling stage, just after germination. YC7007 increased shoot and root length by 1.1–2.9 fold at the seedling, tillering, and booting stages, and increased tiller numbers by over 1.3-fold at the tillering and booting stages compared with the control. This demonstrates that resistance-inducing and growth-promoting activity by the probiotic bacterium YC7007 could be long lasting, from the seedling to the booting stage (Picard et al., 2008). It has been reported that the induction of host resistance negatively affects host plants, in suppressing growth associated with the plant hormones, gibberellic acid (GA) and auxin. The immune system is mediated by brassinosteroids and salicylic acid, repressing GA and auxin responses, at least in part via negative cross talk with the hormone, respectively, in rice and *Arabidopsis* (De Vleeschauwer et al., 2012; Wang et al., 2007). A chemical derivative of salicylic acid (SA), benzothiadiazole (BTH), induced systemic resistance against biotrophic pathogens in rice, cucumber, *Arabidopsis*, and pepper plants via salicylic acid signaling networks, but it retarded the plant growth by inhibiting the auxin response. In contrast, some rhizobacteria, including *Bacillus* species, enhanced resistance induction with no negative effect on plant growth (Ahn et al., 2005; Ryu et al., 2004; Yang et al., 2009). In this respect, strain YC7007 may be a good candidate for developing a biocontrol agent with multiple functions of resistance induction and growth promotion in rice without negative effect. Additionally, strain YC7007 has anti-fungal and anti-bacterial activity, probably via production of antibiotics.

Our two endophytic strains, designated YC7007 and

YC7010<sup>T</sup>, were further characterized based on a polyphasic study including 16S rRNA gene sequences, DNA-DNA hybridization, fatty acid analyses, and other physical and biochemical tests. The two strains were identified as novel species of the genus *Bacillus*, in the same clade with the same branch length in the phylogenetic tree. The overall percentage sequence divergences of the two strains were the same, but differed from the other closely related species in the clade of the phylogenetic tree. Strains YC7007 and YC7010<sup>T</sup> showed 100% similarity and very high DNA-DNA relatedness values of 91.5% between them, indicating that the two strains are the same *Bacillus* species. The DNA-DNA hybridization values of other related strains *B. siamensis*, *B. methylotrophicus*, *B. subtilis* subsp. *inaquosorum*, *B. amyloliquefaciens* subsp. *plantarum*, and *B. tequilensis* in comparison to the type strain YC7010<sup>T</sup> were below 70%, which indicated that YC7010<sup>T</sup> was a novel species (Goris et al., 2007; Stackebrandt and Goebel, 1994). Additionally, the two strains showed almost the same reactions in physical and biochemical tests using API kits, but showed different responses to other reference strains, indicating that the two strains are distinct from other related *Bacillus* strains. In tolerance to NaCl, the two strains can survive up to 13% NaCl, but *B. methylotrophicus* did not grow at 10% NaCl (Madhaiyan et al., 2010). Major cellular fatty acids of YC7007 and YC7010<sup>T</sup> were anteiso-C<sub>15:0</sub> and iso C<sub>15:0</sub>, which are same as other related species of the genus *Bacillus*. Other minor fatty acids of the two strains differed from those of closely related *Bacillus* strains. Each bacterium has a specific fatty acid profile and fatty acid analysis is used as an effective tool in bacterial taxonomy (Kämpfer, 1994). The major isoprenoid quinones of strains YC7007 and YC7010<sup>T</sup> were menaquinone-7 (MK-7) as those of the type strains of the genus *Bacillus* species (Kang et al., 2013). The DNA G+C contents of both strains were 50.5–51.2 mol%, which are similar to but slightly higher than those of other *Bacillus* species (Madhaiyan et al., 2010; Sumpavapol et al., 2010). Major polar lipids of both strains were PE, PG, and DPG, which are in accordance with those of *B. siamensis* and *B. songklensis* (Kang et al., 2013; Sumpavapol et al., 2010).

Based on all the data from the polyphasic studies, the two strains YC7007 and YC7010<sup>T</sup>, belonged to the genus *Bacillus*; we therefore, proposed strain YC7010<sup>T</sup> and YC7007 as a novel species and suggested the name *Bacillus oryzicola* YC7010<sup>T</sup> sp. nov. with the type strain. In conclusion, the endophytic strain *B. oryzicola* YC7007 has multifunctional activities in rice: direct inhibition of fungal and bacterial pathogens, systemic resistance induction, and plant growth promotion, which could possibly be used practically as a

microbial inoculant on commercial farms.

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