Journal of Agricultural Science; Vol. 6, No. 6; 2014
ISSN 1916-9752 E-ISSN 1916-9760
Published by Canadian Center of Science and Education

Screening of Nitrogen Fixing Endophytic Bacteria in *Oryza sativa* L.

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Received: March 3, 2014 Accepted: April 10, 2014 Online Published: May 15, 2014

doi:10.5539/jas.v6n6p66 URL: http://dx.doi.org/10.5539/jas.v6n6p66

Abstract

Nitrogen (N) is an essential element for the growth and yield of rice. Some endophytic bacteria can fix N_2 from the air and convert to nitrogen compounds that can be utilized by plants. In this study, endophytic bacteria were isolated from one-month-old seedlings of five rice (*Oryza sativa* L.) varieties (Muey Nong 24, Muey Nong 25, Pathum Thani 1, Suphan Buri 1 and Chai Nat 1) growing without nitrogen fertilizer in the farmers' field. One hundred and twenty-three isolates of endophytic bacteria were obtained from the roots, stems and leaves of these rice varieties. Nitrogenase activity of the bacteria in N-free culture medium was determined by acetylene reduction assay. Seven isolates of the bacteria with highest nitrogenase activity were identified by phylogenetic analysis of the 16S rRNA genes, and found to belong to *Burkholderia cepacia* (CS5), *Citrobacter* sp. (CR9), *Citrobacter* sp. (SS5), *Citrobacter* sp. (SS6), *Bacillus amyloliquefaciens* (25R14), *B. amyloliquefaciens* (SR1) and *B. thuringiensis* (25R2). Inoculation of *Bu. cepacia* (CS5) and *Citrobacter* sp. (CR9) to the seedlings of local rice variety (Muey Nong 24) significantly increased nitrogen concentration in the roots of rice.

Keywords: endophytic bacteria, nitrogen fixation, *Oryza sativa* L.

1. Introduction

Rice is the most important crop in Asia as the main staple food and to those who depend on rice farming for their livelihoods. Nitrogen is the most important nutrient in rice production. It is the primary input in modern rice farming (Craswell et al., 1981; Yoshida, 1981). Large amounts of nitrogen fertilizer, amounting to 112 million tons a year are used [globally in 2009 to 2011 (FAOSTAT, 2013)] in cultivation of rice and other economic crops. However, a notable exception is the commercial sugarcane production in Brazil which uses only limited input of N fertilizer and rely mainly on fixation of N_2 from the atmosphere by endophytic bacteria (Boddey et al., 2003). Subsistence rice production in Thailand is also rarely given any fertilizer. Economic crops, including rice, maize, wheat, sorghum and sugarcane have been reported to harbor endophytic bacteria that can convert N_2 from the atmosphere into combined N that can be utilized by plants while having no pathogenic effect on them (James et al., 1997; Bhattacharjee et al., 2008; Lima et al., 1987). Endophytic bacteria that can fix N_2 from the air are found in seeds, roots and stem of the rice plant include *Azoarcus* sp., *Herbaspirillum seropedicae*, *Bacillus megaterium*, *Azosprillium*, *Sphingomonas paucimobilis, Pantoea agglomerans*, *Idenella dechloratans* and *Burkholderia cepacia* (Stolzfus et al., 1997; Engelhard et al., 2000; Gyaneshwar et al., 2001; Jame et al., 2002). Inoculation of rice plants with N_2 fixing endophytic bacteria has resulted in increased growth and yield. Nitrogen fixing endophytic bacteria inoculation of rice seedlings has been shown to significantly increase biomass and grain yield of rice included *Gluconacetobacter diazotrophicus*, *Serratia marcescens*, *Burkholderia* sp. and *Azoarcus* sp. (Bhattacharjee et al., 2008; Choudhury & Kennedy, 2004). There is a possibility that endophytic N_2-fixing bacteria might play an important role in maintaining the yield of wetland rice in the mountains of northern Thailand without fertilizer input. This study was set out to (1) isolate endophytic N_2 fixing bacteria from rice plants grown without N fertilizer, (2) identify the bacteria selected on the basis of their N_2-fixing capacity and (3) evaluate the effect of selected bacteria on the growth of rice seedlings.

2. Method

2.1 Isolation of Endophytic Bacteria From Rice

Endophytic bacteria were isolated from 4 week-old seedlings of five rice varieties (local varieties Muey Nong 24 and Muey Nong 25, and modern varieties Pathum Thani 1, Suphan Buri 1 and Chai Nat 1) growing in farmers'

fields at the highland village of Thung Luang, in Mae Wang district of Chiang Mai (Lat. 18° 40′; Long. 98° 35′; Alt. 865 m) which had never received any fertilizer. The rice plant samples were washed with tap water and placed in 1.2% NaOCl for 5 min and immediately washed twice in sterile distilled water. The samples were then ground in a mortar. Serial ten fold dilutions of the ground samples were done with 0.85% NaCl solution. The suspension diluted to 10^{-1}-10^{-6} were spread on nitrogen free agar (NFA) plates, and incubated at 30°C for 3-7 days. The bacterial isolates on NFA were streaked and single colonies re-streaked on NFA for pure culture collection.

2.2 Determination of Nitrogenase Activity

Nitrogenase activity of the endophytic bacteria was examined by acetylene reduction assay (ARA) in 20 ml capped tube containing 9 ml of semisolid culture of NFA. The atmosphere in the capped tube was replaced with 10 % acetylene (v/v) and incubated at 30°C for 24 h. Ethylene formation was measured by gas chromatography. The nitrogenase activity was calculated in unit nmol C_2H_4/mg protein/h (Lee & Yoshida, 1997). The bacteria with highest nitrogenase activities were selected for identification.

2.3 Effect of Nitrogen Fixing Bacteria on Rice

The selected endophytic bacteria were tested for nitrogen fixation in rice variety Muey Nong 24. Seeds of the rice were surface sterilized in 1.2% NaOCl for 5 min, then washed three times with sterile distilled water. The seeds were placed on sterile wet tissue paper in Petri dish to germinate. The germinated seed was transferred to N-free nutrient solution (McDonald et al., 2001) to grow for 10 days. The rice seedlings were then inoculated by immersing the rice roots in 9×10^8 cells/ml bacterial suspension in 0.85 % NaCl for 60 min. For un-inoculated control treatment, the rice roots were immersed in 0.85 % NaCl for 60 min. The rice seedlings were transferred to pots containing 5 L of N-free nutrient solution. After growing for 49 days, the rice plants were added with 9×10^{10} cells/ml bacterial suspension and 1% yeast extract. The un-inoculated control plants were given 1 ml of 1% sterile yeast extract. Chlorophyll contents of the youngest emerged blade (YEB) were measured weekly with a chlorophyll meter (Minolta SPAD 502). The rice plants were harvested 84 days after the first inoculation. Nitrogen concentrations of the rice plants were determined by Kjeldahl method.

2.4 Bacterial Genomic DNA Extraction

The selected bacterial isolates were grown in nutrient agar and incubated at 30°C for 48 hr. Bacterial cells were harvested in 1.5 ml microcentrifuge tube. The bacteria were washed for 3 times with Tris EDTA buffer pH 8.0 and centrifuged at 6000 rpm for 3 min. Bacterial cells were lysed with 400 µl of 10% SDS under incubation at 37°C for 30 min. Protein was precipitated with chloroform: isoamyl alcohol (24:1) from the solution. Bacterial DNA was precipitated by adding cold isopropanol and centrifuged at 14,000 rpm, 4°C for 3 min. The DNA pellet was washed twice with 70% ethanol and suspended in 1X TE buffer at -20°C (Brunel et al., 1997).

2.5 PCR Amplification and Phylogenetic Analysis of 16S rRNA Gene

Universal primers pairs of 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) with 1525R (5′-AAGGAGGTGWTCC ARCC-3′) and 357F (5′-CTCCTACGAGGCAGG-3′) with 1392R (5′-ACGGGCGGTGTGTAC-3′) were used for amplification of 16S rRNA gene fragments. Total PCR reaction mixture contained 2x PCR Master mix (i-Taq) 10 µl, template DNA 2 µl, 10 pmol/µl of each primer 1 µl and distilled water 6 µl. For PCR cycle, an initial denaturation at 94°C for 2 min, each thermal cycling was followed by 30 cycles: denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s and final extension at 72°C for 7 min. PCR products were determined on 1% agarose gel (Brunel et al., 1997). The 16S rRNA genes were sequenced at Biobasic Inc. in Canada. The similarities of nucleotide were compared with those at the National Center for Biotechnology Information (NCBI) by BLAST program. Phylogenetic tree was analysed using BioEdit with MEGA 4 version 4.0.2 (Bootstrap=1000) (Tamura et al., 2007).

3. Results and Discussion

One hundred and twenty-three isolates of endophytic bacteria were collected on NFA. The number of endophytic bacteria in roots, stems and leaves of rice seedlings from farmers' field that had never received N fertilizer ranged from 3.0×10^2 to 2.9×10^8 cells/g fresh weight (Table 1). The population of endophytic bacteria was found to vary in density with rice variety and parts of the plant. The number of endophytic bacteria that could grow in N-free medium was highest in the leaves and roots of Chai Nat 1, a modern rice variety. Martin & Reinhold-Hurek (2002) reported that high density of N fixing bacteria were found in plants growing in cultivated field without chemical fertilizer. Acetylene reduction assay (ARA) confirmed that isolates of the endophytic bacteria from rice had nitrogenase activity (data not shown). Seven isolates of the bacteria with highest nitrogenase activity, in the range of 26.0-119.9 nmol C_2H_4/mg protein/h, were chosen for identification (Table 2). This range

of nitrogenase activity was comparable to 31.7 – 92.0 nmol C_2H_4/mg cell/h reported by Sugitha and Kumar (2009) for endophytic bacteria from the rhizosphere soil, rhizoplane, roots and stems of rice growing in Tamil Nadu State, India. In this experiment, the endophytic bacteria with the highest nitrogenase activity were from either stems or roots of modern high yield varieties Chai Nat 1 (CS5 from stems; CR9 from roots) and Suphan Buri 1 (SS5 and SS6 from stems; SR1 from roots) and from the roots of local variety Muey Nong 25 (25R14 and 25R2) isolated from the stems of Chai Nat 1.

Table 1. Number of endophytic bacteria in roots, stems and leaves from rice varieties

Rice varieties	Endophytic bacteria (cells/g fresh weight)		
	Roots	Stems	Leaves
Muey Nong 24	1.8×10^5c	1.7×10^6	2.3×10^5c
Muey Nong 25	3.1×10^3c	3.0×10^2	6.1×10^2c
Chai Nat 1	1.0×10^8b	8.4×10^3	2.9×10^8a
Suphan Buri 1	2.5×10^5c	2.9×10^5	1.5×10^5c
Pathum Thani 1	1.8×10^5c	2.3×10^5	2.4×10^3c
Effect by F-test	$P < 0.05$	NS	$P < 0.05$

Different letters designate significant difference within column by Duncan's Multiple Range Test at $P \leq 0.05$.

NS = Not significant at $P = 0.05$.

Table 2. Nitrogenase activity of selected endophytic bacterial isolates from rice

Bacterial isolates	Rice varieties	Plant parts	Nitrogenase activity (nmol C_2H_4/mg protein/h)
CS5	Chai Nat 1	Stem	119.9a
CR9	Chai Nat 1	Roots	44.6ab
25R2	Muey Nong 25	Roots	29.2b
25R14	Muey Nong 25	Roots	84.5ab
SR1	Suphan Buri 1	Roots	26.0b
SS5	Suphan Buri 1	Stem	36.5b
SS6	Suphan Buri 1	Stem	61.0ab
Effect by F-test			$P < 0.05$

Different letters designate significant difference within column by Duncan's Multiple Range Test at $P \leq 0.05$.

Amplification of 16S rRNA gene and phylogenetic analysis of bacterial isolate CS5 indicated that it was in the species of *Burkholderia cepacia* with bootstrap 99% (Figure 1). Singh et al. (2006) isolated *Bukholderia* from the rice roots. Procópio et al. (2009) isolated *Bu. cepacia* from stems of *Eucalyptus* spp. which was able to control *Magnaporthe grisea, Fusarium moniliforme* and *Rhizoctonia solani*. Govindarajan et al. (2007) determined the ability to reduce acetylene by endophytes from different sources and found that *Bu. vietnamiensis* (MGK3) had highest nitrogenase activity on glycerol, followed by mannitol and sucrose.

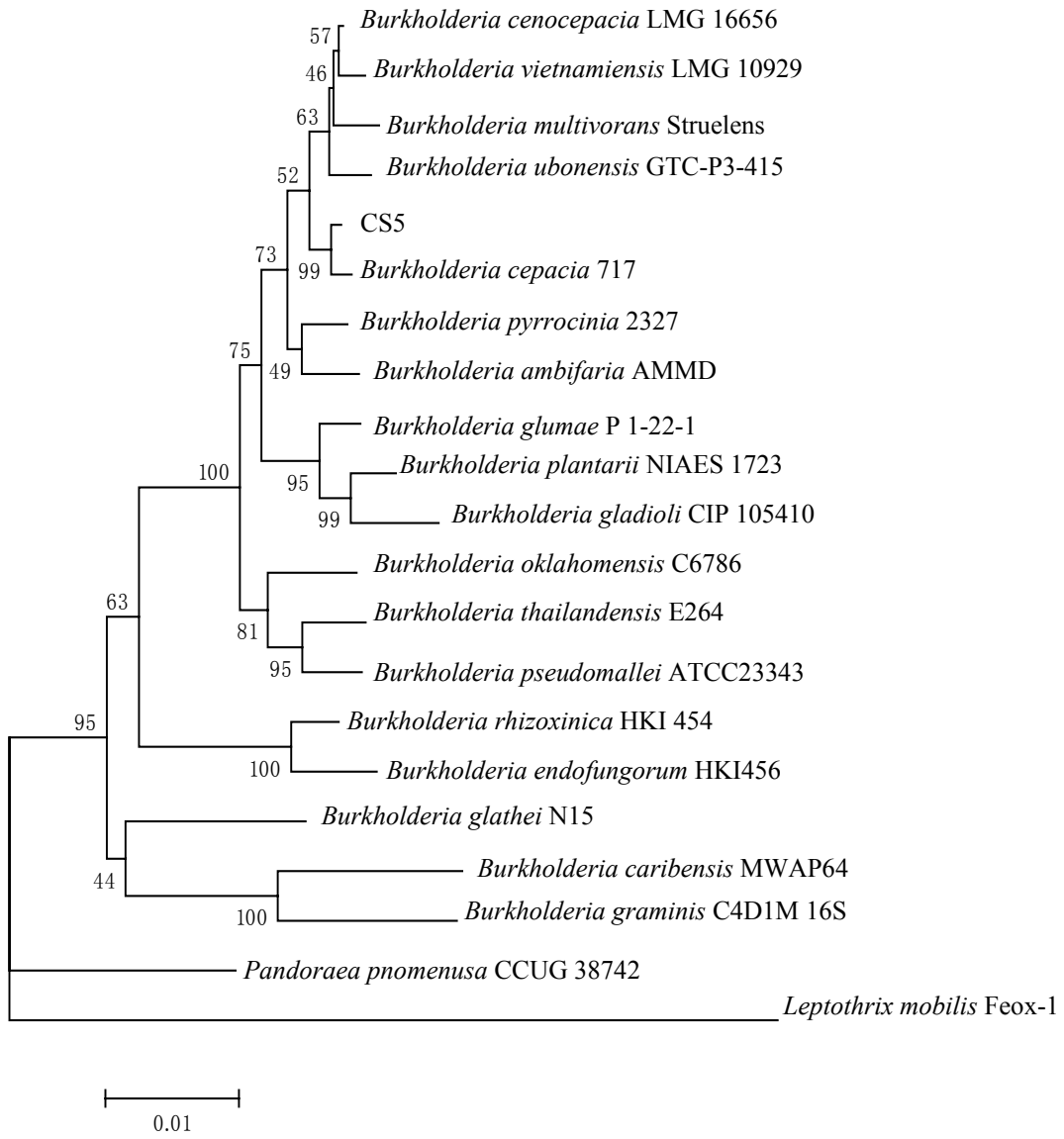

Figure 1. Phylogenetic tree based on 16S rRNA sequences of the endophytic bacterial isolate CS5

Bacterial isolates CR9, SS5 and SS6 showed similarity to the genus *Citrobacter*. Their close phylogenetic affinity suggested that these three isolates could be in the same species (Figure 2). However, the species of these isolates should be further identified. Tan et al. (2009) isolated bacteria closely related to *C. amalonaticus* from roots of *O. rufipogon* which showed nitrogenase activity in the range of 3.31- 36.89 µmol C_2H_4/ml/h. The isolates, 25R14 and SR1 were similar to *Bacillus amyloliquefaciens*, and 25R2 was similar to *B. thuringiensis* (Figure 3). Praça et al. (2012) reported that *B. thuringiensis* was found in soil, water, plants and dead insects. Cabbage seedlings inoculated with *B. thuringiensis* were detected inside roots and leaves of plants. It is a very important bacterium used as a biological control agent for insects. *B. amyloliquefaciens* is a bacterium used as a biological control agent of *Xanthomonas campestris* pv. *campestris* causing black root of cabbage and improve growth of the control plants (Wulff et al., 2002).

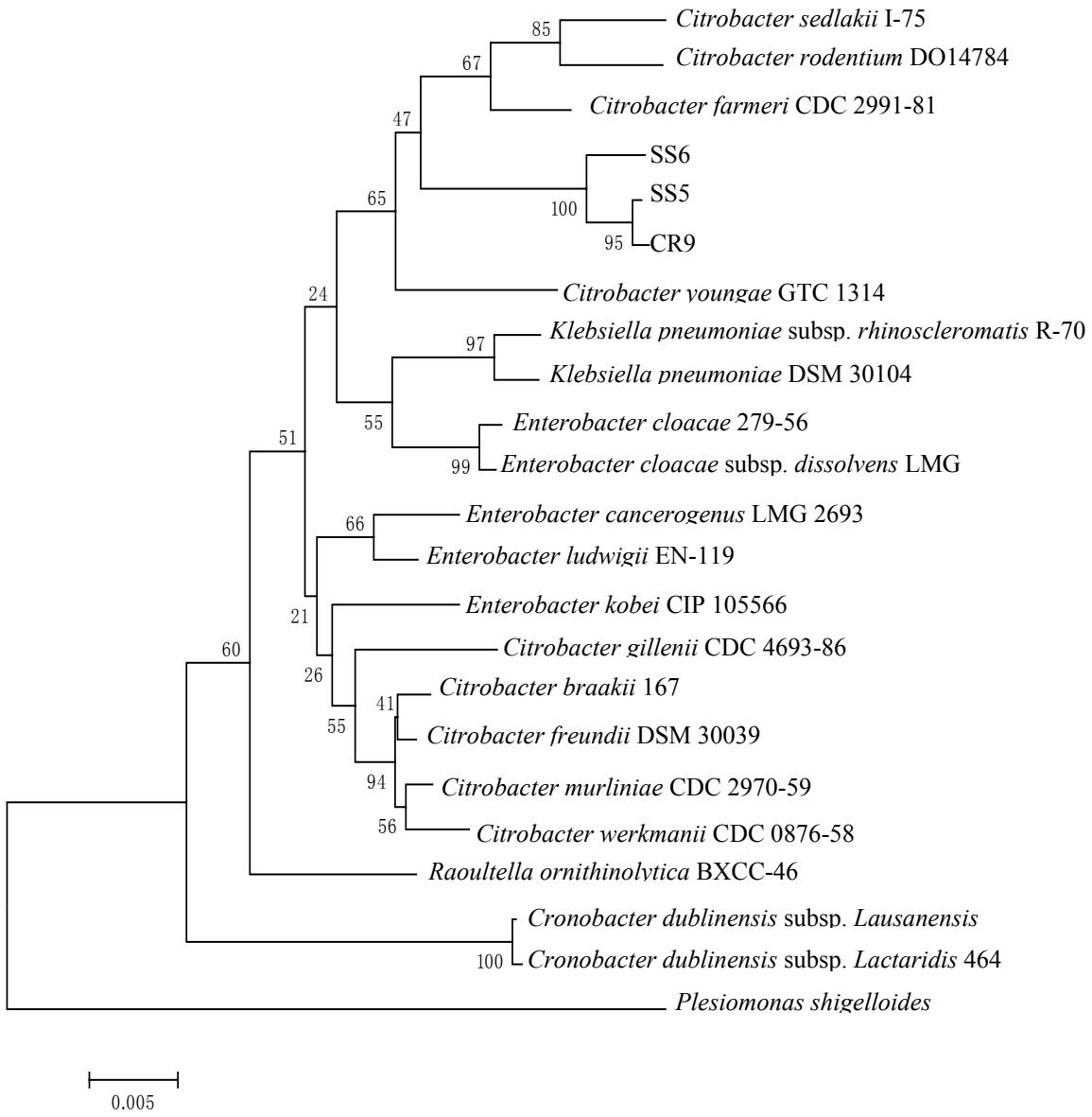

Figure 2. Phylogenetic tree based on 16S rRNA sequences of the endophytic bacterial isolates SS5, SS6 and CR9

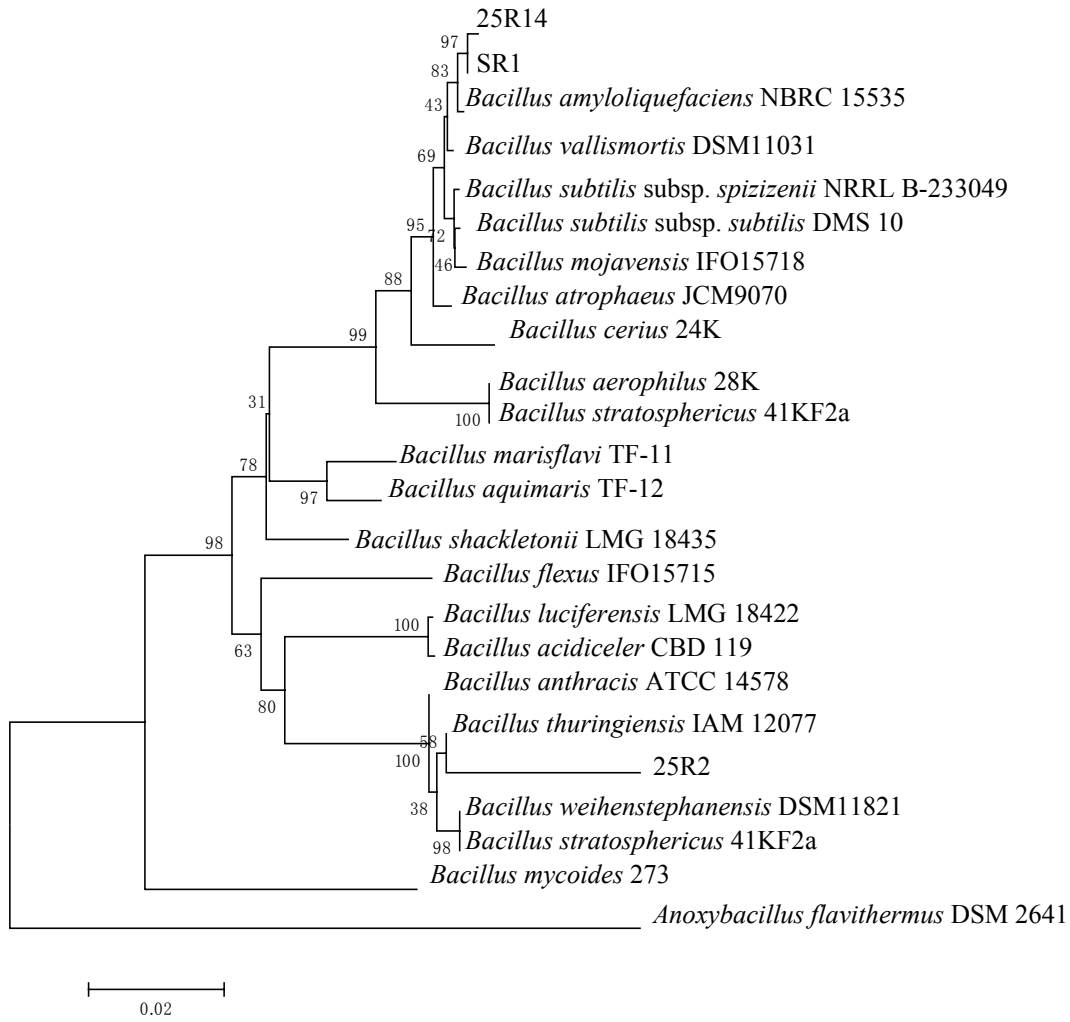

Figure 3. Phylogenetic tree based on 16S rRNA sequences of the endophytic bacteria 25R14, SR1 and 25R2

Some of the selected endophytic bacteria contributed N supply to the rice seedlings which was evident in leaf chlorophyll content (Table 3) and root N (Table 4). Chlorophyll meter has been effectively used to determineN status of rice, wheat (Singh et al., 2002) and other crops (Swiader & Moore, 2002; Fontes & de Araujo, 2006) in the youngest emerged blades (YEB) of rice plants (Muey Nong 24) the treatments were measured by chlorophyll meter (SPAD 502) every 7 days. It was shown that the inoculated treatments increased chlorophyll contents in YEB compared with the uninoculated control plant in 56 days (Table 3). For detection of nitrogen concentrations, the rice plants inoculated with *Bu. cepacia* (CS5) and *Citrobacter* sp. (CR9) had the highest nitrogen concentration in roots and higher than uninoculated control plants (Table 4). Potential of nitrogen fixing bacteria inoculated in rice grown in nitrogen free medium may be limited in carbon and nitrogen sources for initial growth. Inoculation with endophytic bacteria increased biomass of the rice plants (Table 5), but it was not significantly higher than the uninoculated rice plants. Chiarini et al. (1998) reported that sorghum inoculated with *Bu. cepacia* resulted in improved root biomass. Govindarajan et al. (2007) inoculated *Bu. vietnamiensis* (MGK3) to rice plants and showed that the bacterium could increase the grain yield of rice by 5.6-12.6%.

Application of nitrogen fixing endophytic bacteria may be potential biofertilizer integrated with green manure or compost fertilizer for rice production in organic farming. Therefore, further inoculum production of the nitrogen fixing endophytic bacteria and the optimum soil condition should be investigated.

Table 3. Chlorophyll content in young emerged blade (YEB) of rice variety Muey Nong 24

Bacterial isolates	Chlorophyll content in YEB (SPAD unit)			
	Days			
	7	28	56	84
Uninoculated control	18.2	17.79	23.8c	20.2c
Bu. cepacia (CS5)	18.85	17.45	25.4ab	20.4c
B. amyloliquefaciens (25R14)	18.2	17.3	24.0c	22.3ab
B. amyloliquefaciens (SR1)	18.5	16.7	24.4ab	22.1b
B. thuringiensis (25R2)	19.15	18.1	25.8a	22.9ab
Citrobacter (SS5)	18.43	17.0	24.7ab	23.9a
Citrobacter (SS6)	19.2	17.3	25.1ab	22.9ab
Citrobacter (CR9)	17.8	17.7	24.2ab	22.3ab
Effect by F-test	NS	NS	$P < 0.05$	$P < 0.05$

Different letters designate significant difference within column by Duncan's Multiple Range Test at $P \leq 0.05$.

NS = Not significant at $P = 0.05$.

Table 4. Nitrogen concentration in roots and shoot of rice (Muey Nong 24)

Bacrerial isolates	Nitrogen concentration (%)	
	Roots	Shoots
Uninoculated control	0.74b	0.70
Bu. cepacia (CS5)	0.93a	0.74
B. amyloliquefaciens (25R14)	0.80ab	0.75
B. amyloliquefaciens (SR1)	0.70b	0.72
B. thuringiensis (25R2)	0.80ab	0.74
Citrobacter (SS5)	0.82ab	0.71
Citrobacter (SS6)	0.75b	0.69
Citrobacter (CR9)	0.90a	0.68
Effect by F-test	$P < 0.05$	NS

Different letters designate significant difference within column by Duncan's Multiple Range Test at $P \leq 0.05$.

NS = Not significant at $P = 0.05$.

Table 5. Fresh and dry weight of rice plants variety Muey Nong 24 inoculated with different isolates of N fixing endophytic bacteria

Inoculation	Fresh weight (g/plant)		Dry weight (g/plant)	
	Roots	Shoots	Roots	Shoots
Uninoculated control	2.0d	3.3bc	0.28	0.63
Bu. cepacia (CS5)	2.5cd	3.9ab	0.30	0.67
B. amyloliquefaciens (25R14)	2.2d	3.9ab	0.28	0.63
B. amyloliquefaciens (SR1)	2.5cd	4.2a	0.32	0.67
B. thuringiensis (25R2)	2.4d	3.6ab	0.29	0.69
Citrobacter (SS5)	2.5cd	4.0ab	0.29	0.67
Citrobacter (SS6)	2.4d	4.2a	0.25	0.66
Citrobacter (CR9)	2.0d	4.0ab	0.25	0.66
Effects by F-test	$P < 0.05$	$P < 0.05$	NS	NS

Difference letters designate significant difference within column by Duncan's Multiple Range Test at $P \leq 0.05$.

NS = Not significant at $P = 0.05$.

Acknowledgements

The authors would like to acknowledge for financial support from the National Research Universities Program of Thailand Commission on Higher Education. We wish to thank the Department of Biology, Faculty of Science, Chiang Mai University and the Department of Plant Science and Natural Resources for the research facilities.

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