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Characterization of Endophytic Diazotroph Bacteria Isolated from Rice

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Attempt to extend the biological nitrogen fixation to important crops such as rice has been conducted by isolating endophytic diazotrophs from rice rhizosphere and roots. In this study, three bacterial isolates of R2, R4, and E4 isolated from rice-legume rotation in the Nile Delta Egypt and four bacterial isolates of R38-O, R38-T, R53, and R58 isolated from wild rice in the Philippines were characterized using classical methods of bacterial identification and using biochemical test kits (API20E and API20NE). R2 and R4 isolates were identified as *Rhizobium* sp., E4 isolate was identified as *Rhizobium leguminosarum* bv. *trifolii*, and R38-T, R53, and R58 isolates were identified as *Sphingomonas*, *Azospirillum*, and *Agrobacterium*, respectively. Of all *Rhizobium* isolates, only E4 could form nodules on legumes other than their original host berseem clover (*Trifolium alexandrum* L.) as their original host.

Key words: endophytic bacteria, characterization, Nitrogen fixation, rice, nitrogen

INTRODUCTION

Biological nitrogen fixation (BNF) is a major contributor of nitrogen to the rhizosphere. Most of fixed nitrogen provided by BNF is from *Rhizobium*-legume symbiosis. Growing concern about the environment, energy, population growth, and agricultural sustainability has encouraged many research groups to explore whether rice could also take benefit from BNF (Ladha *et al*. 1997; Ladha & Reddy 2003).

Attempts to extend BNF to rice have been initiated through exploring nitrogen-fixing bacteria that reside naturally in rice tissues, known as endophytic diazotrophs. It is suggested that in a natural endophytic association, nature has selected stable endophytes that can competitively occupy niches within rice tissues without causing negative effects to the host plant (Ladha *et al*. 1997; Yanni *et al*. 1997). Natural endophytic diazotrophs has been found in sugarcane, which provide as high as 150 kg N/ha annually to the plant, mostly contributed by N_2-fixing bacteria living inside the plants (Boddey *et al*. 1995; James & Olivares 1998).

Screening of potential endophytic diazotroph-rice association has been conducted through isolation of endophytic diazotrophs from roots of various rice genotypes. Previous studies have shown that various rice genotypes including wild-type rice in nature harbour a large population of endophytic diazotrophs in their tissues (Barraquio *et al*. 1997; Yanni *et al*. 1997; Stoltzfus & de Bruijn 2000; reviewed in Ladha & Reddy 2003; Mano & Morisaki 2008). Barraquio *et al*. (1997) isolated endophytic diazotrophs from roots and stems of cultivated and wild-type rice, and from different rice cultures such as wetland, upland and deepwater rice fields. They found that the population of endophytic diazotrophs from surface-sterilized roots ranged between 10^3-10^7 MPN/g dry weight, or about 1-10% of the total bacterial population.

Another approach of finding potential endophytic diazotroph was through isolation of rhizobia from rice roots in rice-legume rotation. When the rotation practice has been run for a long period, *Rhizobium* is thought to evolve by forming an association with another host such as rice (Ladha *et al*. 1997). Several *Rhizobium leguminosarum* bv. *trifolii* isolates have been isolated from the Nile Delta region of Egypt (Yanni *et al*. 1997). In this region, rice has been cultivated in irrigated lowlands and rotated with berseem clover (*Trifolium alexandrum* L.) for more than seven centuries. Some isolates could promote rice growth both in the laboratory and field conditions (Yanni *et al*. 1997).

As part of the process of exploring the endophytic diazotroph-rice association, the bacteria obtained from isolation need to be identified to allow further studies and manipulations on potential isolates. In this study, some isolates from Barraquio *et al*. (1997) and Yanni *et al*. (1997) were characterized using morphological, physiological and biochemical methods. Some isolates were characterized as genus of *Rhizobium*, *Sphingomonas*, *Agrobacterium*, and *Azospirillum*.

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MATERIALS AND METHODS

Bacterial Isolates. R2, R4, and E4 isolates (Table 1) were obtained from nodules of a trap host berseem clover (Yanni *et al*. 1997). R38-O, R38-T, R53, and R58 isolates (Table 1) were obtained from surface-sterilized roots of wild rice grown in the Philippine (Barraquio *et al*. 1997).

Bacterial Characterization. Bacterial isolates were characterized using classical methods including morphological and physiological features. Morphological characterization was according to methods described by Lányi (1987) such as cell morphology, Gram staining, capsule formation, motility, and flagella arrangements. Flagella were stained with basic fuchsin after coating them with tannic acid. Physiological features of the bacteria were examined using API test kits (bioMérieux Pty Ltd, Australia) including API20E and API20NE. All API tests were conducted according to the manufacturer's instruction. The main characteristics of all isolates examined were compared to the standard clasification of Bergey's Manual and to the database of API systems. Some isolates were also compared to the reference isolates of Bally *et al*. (1983).

Bacterial Culture Medium. All isolates were grown on Bergersen's Modified Medium (BMM) plates (Rolfe *et al*. 1980) for three days or in BMM liquid medium overnight before examination. Nutrient composition of BMM medium (per 1000 ml) was as follow: $NaH_2PO_4 \cdot 2H_2O$ 179 mg, $MgSO_4 \cdot 7H_2O$ 80 mg, $FeCl_3$ 3 mg, $CaCl_2 \cdot 2H_2O$ 40 mg, thiamin 2 mg, biotin 0.2 mg, Na glutamate 0.5 g, yeast extract 0.5 g, mannitol 3 g, H_3BO_3 3 mg, $MnSO_4 \cdot 4H_2O$ 10 mg, $ZnSO_4 \cdot 7H_2O$ 3 mg, $CuSO_4 \cdot 5H_2O$ 0.25 mg, $Na_2MoO_4 \cdot 2H_2O$ 0.25 mg, and $CoCl_2 \cdot 6H_2O$ 0.25 mg. Semi-solid Nutrient Agar medium (0.5% agar in test tubes) containing 0.001% tetrazolium was used for motility test. To determine oxygen requirement of the isolates, BMM medium containing 0.2% sodium thioglycollate and 0.01% tetrazolium was used. Oxidative/Fermentative metabolism assay (OF) medium was also used for oxygen requirement test with the composition as follow: peptone 2 g, NaCl 5 g, K_2HPO_4 0.3 g, 0.2% bromthymol blue 15 ml, glucose 10 g, and yeast extract 1 g.

Nodulation Test. The ability of R2, R4, and E4 isolates to form nodules was tested on three clover hosts, berseem clover (*Trifolium alexandrum* L.), sub clover (*T. subterraneum* cv. Woogenellup), and white clover (*T. repens* cv. Haifa). Seeds of clovers were surface sterilized, germinated and inoculated according to Rolfe *et al*. (1980). The seedlings were grown on N-free Fahraeus agar plate. Three-day-old seedlings were inoculated with 20 µl bacterial suspension (OD_{600nm} = 0.01) from overnight culture. The plates containing inoculated plants were incubated vertically in a growth cabinet which was set at 21 °C (16 h day) and 19 °C (8 h night), and photon flux density of 140 mE/m²/s. The plants were grown for 4 weeks before nodules formed on roots were counted.

Table 1. Bacterial isolates used in this study

Isolate	Source	References
R2	Rhizosphere of rice-berseem clover rotation	Yanni *et al*. (1997)
R4	Rhizosphere of rice-berseem clover rotation	Yanni *et al*. (1997)
E4	Surface-sterilized roots of rice	Yanni *et al*. (1997)
R38-O	Surface-sterilized roots, *Oryza alta*	Barraquio *et al*. (1997)
R38-T	Surface-sterilized roots, *O. alta*	Barraquio *et al*. (1997)
R53	Surface-sterilized roots, *O. eichingeri*	Barraquio *et al*. (1997)
R58	Surface-sterilized roots, *O. longiglumis*	Barraquio *et al*. (1997)

RESULTS

All bacterial isolates were Gram-negative, rod-shape bacteria, except for R53 isolate which had a vibroid or helical shape (Table 2). Results of bacterial characterization indicated that isolates from the Nile Delta shared common features, and isolates from the wild rice in the Philippine had a diverse type of bacteria.

Table 2. Morphological and physiological characteristics of the bacterial isolates

	Isolate						
	R2	R4	R38-O	R38-T	R53	R58	E4
Shape	rod	rod	rod	rod	vibroid	rod	rod
Gram reaction	-	-	-	-	-	-	-
Cell size (µm)	2 x 0.5	2.5 x 0.5	3 x 0.5	4 x 0.7	4 x 1	5 x 1	2 x 0.5
Colony morphology	mucoid, dome, entire	mucoid, dome, entire	mucoid, dome, entire	smooth, flat, irregular	smooth, raised, entire	smooth, entire, irregular	mucoid, dome, entire
Pigment production	-	-	-	-	pink	-	-
Motility	+	+	+	+	+	-	+
Flagella arrangement	monotrichous	monotrichous	peritrichous	monotrichous	monotrichous	-	monotrichous
Oxygen requirement	microaerophyl	microaerophyl	microaerophyl/ aerob	microaerophyl	microaerophyl	microaerophyl	microaerophyl
Pembentukan nodule	+	+	-	-	-	-	+
Oxidase	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
Hugh Leifson test	oxidizer	oxidizer	fermenter	fermenter	fermenter	fermenter	oxidizer

Bacterial Isolates from the Nile Delta. Morphological characteristics of R2 isolate were similar to that of R4 isolate (Table 2). Their colonies were dome shape, entire and mucoid when grown on BMM agar. The average cell diameter and cell length of both isolates were 0.5-0.8 and 2.0-2.5 µm, respectively. These rod bacteria had one flagellum at one end of the cell. Physiological tests showed that both isolates were microaerophilic bacteria either in BMM or in OF medium. These isolates were not able to acidify the medium in carbohydrate fermentation test of API20E (Table 3). Overall results of API20E or API20NE tests indicated that both isolates are identical (Table 3 & 4).

API20E test results showed that E4 isolate shared similar features to with R2 and R4 isolates (Table 3). E4 isolate was not able to assimilate all type of carbohydrate present in API20NE test kit, while R2 and R4 isolates were able to utilize certain types of carbohydrates including glucose, arabinose, mannose, mannitol, N-acetyl-glucosamine, and maltose (Table 4).

Bacterial Isolates from the Wild Rice. R38-O isolate was a motile rod with average diameter 0.5-0.7 µm and length 2-3 µm (Table 2), and possessed peritrichous flagella. On BMM plates, it formed mucoid, dome, entire, and opaque colonies. This isolate was an aerobic bacterium on both BMM and OF semi-solid medium API20E test showed that it hydrolized urease, esculin, gelatine, and β-galactosidase (Table 3). This isolate assimilated glucose, arabinose, mannose, mannitol, N-acetyl-glucosamine, maltose, gluconate, malate, and citrate (Table 3), and did

Table 4. API20NE test results of the isolates after incubation at 29 °C for 6 days

Test*	Isolate						
	R2	R4	E4	R38-O	R38-T	R53	R58
NO_3	-	-	-	-	-	+	-
TRP	-	-	-	-	-	-	-
GLU	-	-	-	-	-	-	-
ADH	-	-	-	-	-	-	-
URE	+	+	+	+	-	+	+
ESC	+	+	+	+	+	+	+
GEL	-	-	-	-	+	+	-
PNPG	+	+	+	+	+	+	+
Glu	+	+	-	+	-	+	+
Ara	+	+	-	+	-	+	+
Mne	+	+	-	+	+	-	+
Man	+	+	-	+	+	+	+
Nag	+	+	-	+	+	+	+
Mal	+	+	-	+	+	+	+
Gnt	-	-	-	+	+	+	+
Cap	-	-	-	-	-	+	-
Adi	-	-	-	-	-	+	-
Mlt	-	-	-	+	-	+	+
Cit	-	-	-	+	-	+	-
Pac	-	-	-	-	-	+	-
Ox	+	+	+	+	+	+	+

*Abbreviations: NO_3: nitrate reduction to nitrite to N_2; TRP: indole production from tryptophan; GLU: glucose acidification; ADH: arginine dihydrolase; URE: urease; ESC: esculin hydrolysis (β-glucosidase); GEL: gelatine liquefaction (protease); PNPG: p-nitro-phenyl βD-galacto-pyranoside (β-galactosidase); Glu: glucose assimilation; Ara: arabinose assimilation; Mne: mannose assimilation; Man: mannitol assimilation; Nag: N-acetyl-glucosamine assimilation; Mal: maltose assimilation; Gnt: gluconate assimilation; Cap: caprate assimilation; Adi: adipate assimilation; Mlt: malate assimilation; Cit: citrate assimilation; Pac: phenil acetate assimilation; Ox: cytochrome oxidase.

Table 3. API20NE test results of the isolates after incubation at 29 °C for 6 days

Test*	Isolate							Reference strains**		
	R2	R4	E4	R38-O	R38-T	R53	R58	Br10	Sp7	C58
ONPG	+	+	+	-	+	+	+	+	+	+
ADH	-	-	-	-	-	-	-	-	-	-
LDC	-	-	-	-	-	-	-	-	-	-
ODC	-	-	-	-	-	-	-	-	-	-
CIT	-	-	-	-	-	+	-	+	-	-
H_2S	-	-	-	-	-	-	-	-	-	-
URE	+	+	+	+	-	+	+	+	+	+
TDA	-	-	-	-	-	-	-	-	-	-
IND	-	-	-	-	-	-	-	-	-	-
VP	+	+	+	+	+	+	+	+	+	+
Gel	-	-	-	-	+	-	-	-	-	-
Glu	-	-	-	+	-	+	+	+	+	+
Man	-	-	-	+	+	-	-	-	-	-
Ino	-	-	-	-	-	-	-	-	-	-
Sor	-	-	-	-	-	-	-	-	-	-
Rha	-	-	-	+	-	-	+	-	+	+
Sac	-	-	-	-	+	-	-	-	-	-
Mel	-	-	-	+	-	-	+	-	+	+
Amy	-	-	-	-	+	-	+	+	+	-
Ara	-	-	-	+	-	+	+	+	+	+
Ox	+	+	+	+	+	+	+	+	+	+

*Abbreviations: ONPG: ortho-nitro-phenyl-galactoside (β-galactosidase); ADH: arginine dihydrolase; LDC: lysine decarboxylase; ODC: ornithine decarboxylase; CIT: citrate utilization; H_2S: hydrogen sulfide production; URE: urease; TDA: tryptophan desaminase; IND: indole production; VP: Voges-Proskauer (acetoin production); Gel: Kohn's gelatinase (gelatine liquefaction); Glu: glucose acidification; Man: mannitol acidification; Ino: inositol acidification; Sor: sorbitol acidification; Rha: rhamnose acidification; Sac: sucrose acidification; Mel: melibiose acidification; Amy: amygdalin acidification; Ara: arabinose acidification; Ox: cytochrome oxidase. **Bally *et al*. (1983). Br10: *Azospirillum lipoferum*; Sp7: *Azospirillum brasilense*; C58: *Agrobacterium tumefaciens.*

not acidified inositol, sorbitol, sucrose, and amygdalin (Table 4). Identification of R38-O isolate based on API20E test was unacceptable in the manufacturer's database. Likewise, API20NE test showed low discrimination (56%).

R38-T isolate was a motile rod, averaging 0.7-1.0 µm in diameter and 3-4 µm in length (Table 2). It formed smooth, irregular, and transluscent colonies on BMM agar plate. R38-T isolate was a fastidious bacterium, it required low level of yeast extract for growth in mineral media. This isolate possessed two or three flagella at one end. It was not able to grow in BMM medium with thioglycollate and tetrazolium, but was able to grow in OF medium containing bromthymol blue. In OF medium it was microaerophyl (Table 2). It could acidify mannitol, sucrose and amygdalin (Table 3), and assimilate mannose, mannitol, N-acetyl-glucosamine, maltose, and gluconate (Table 4). Based upon API20NE tests and the manufacturer's database, this isolate was close to *Sphingomonas paucimobilis* with 84.8% similarity.

R53 isolate was a motile bacterium, cell diameter 1.0-1.5 µm and length 3-5 µm, and had a single polar flagellum. When cultured for 3 days on BMM plate, this isolate showed various shapes from slightly curved to mostly-S-shape or helical cells with pointed ends. Many cells were filled with highly refractile granules. It produced pink and often wrinkled colonies on agar medium after 3 days of growing. When growing in BMM or OF semi-solid media, it initially formed a fine white pellicle (a thin layer of bacterial growth) just below the surface of the media within 48 h. Later it became microaerophylic at 3 days after inoculation (Table 2). R53 isolate could utilize most sugars in API20NE test, except for mannose (Table 4). This isolate reduced nitrate to nitrite within 48 h as shown by red colour formation in the NO_3 tube of API 20NE test kit. R53 isolate had a close similarity to *Azospirillum lipoferum* Br10 based on API20E test, except for amygdalin acidification (Table 3).

R58 isolate was a non-motile rod bacterium with average diameter 1.0-1.5 µm and length 2-4 µm. The cells become ovoid and pleomorphic when cultured on BMM medium for more than a week. On BMM agar medium it formed smooth, irregular and entire colonies. Capsule formation was observed on cells of this strain. The isolate was microaerophyl either in BMM or OF semi-solid medium. API20E test results indicated that R58 isolate was closely related to the reference strain *A. tumefaciens* C58 (Table 3), while API20NE test showed that R58 isolate was related to *A. radiobacter* with 97.9% similarity. Overall results suggest that R58 isolate belongs to the genus *Agrobacterium*.

Nodulation Test. R2, R4, and E4 isolates were obtained from nodules of trap host berseem clover. Nodulation tests were used to verify the ability of those isolates to nodulate clover hosts as an important feature of the genus *Rhizobium*, and to determine the host range of each strain. After 4 weeks, E4 isolate was able to nodulate all berseem clover plants (100%), and most of white (74%), and subterranean clover plants (75%). The average nodule number per plant on berseem, white and subterranean clover following inoculation with E4 isolate was 5.7, 4.2, and 3.9, respectively (Table 5). R2 and R4 isolates formed nodules on berseem clover in a lower percentage (21 and 26%, respectively), with the average nodule numbers of 3.2 and 2.3, respectively. R2 and R4 isolates were not able to form nodules on white and subterranean clovers. Visual observation showed that E4 isolate induced the formation of mature nitrogen-fixing nodules on three clover hosts as indicated by pink colour of nodule interior. In contrast, R2 and R4 isolates induced the formation of immature nodules on berseem clover.

DISCUSSION

Rhizobium and several unclassified soil bacteria have been isolated and shown to form potential beneficial association with rice either in laboratory, glass house or field experiments (Yanni *et al*. 1997; Barraquio *et al*. 1997). Using standard methods of bacterial characterization combined with API20E and API20NE kits, we identified three isolates from Nile Delta Egypt (Yanni *et al*. 1997) and three isolates from the Philippine (Barraquio *et al*. 1997) as *Rhizobium*, *Sphingomonas*, *Agrobacterium*, and *Azospirillum*. The use of API test kits is sufficient to characterize isolates commonly found in soil such as *Agrobacterium*, *Azospirillum*, and *Sphingomonas*. Poor identification of R38-O isolate suggest that this isolate may not widespread in soil. Alternatively, low discrimination found on API test could be a result of the limited bacterial library in the manufacturer's database.

R53 isolate, which was assigned as an *Azospirillum*, harbour about 1% of the total population of endophytes in rice tissue (Barraquio *et al*. 1997). It has been known that bacteria of the genus *Azospirillum* are widely distributed in soil and are associated with the roots of

Table 5. Nodule formation of bacterial isolates on legume hosts using agar plate methods

Strain	Host					
	Berseem clover		Sub clover		White clover	
	% nodulated plants*	No. nodule/plant**	% nodulated plants	No. nodule/plant	% nodulated plants	No. nodule/plant
Control	0 (0/42)	0	0 (0/42)	0	0 (0/22)	0
R2	21 (13/63)	3.2 ± 2.7	0 (0/51)	0	0 (0/23)	0
R4	26 (19/72)	2.3 ± 2.0	0 (0/45)	0	0 (0/29)	0
E4	100 (51/51)	5.7 ± 4.6	74 (34/46)	4.2 ± 3.1	75 (18/24)	3.9 ± 2.8

*numbers in parentheses are the number of nodulated plants per total plant tested; **values are mean and standard deviation of nodules per plant.

forage grasses, cereals, and non-gramineous plants (Bashan *et al*. 2004). Azospirilla are known as free living nitrogen-fixing bacteria, but they can penetrate the root system and grow endophytically in intercellular spaces (Mano & Morisaki 2008). Among plant-associated bacteria, *Azospirillum* has been found to be a promising bacterium for promoting plant growth. Besides producing plant hormones and fixing nitrogen, species of this genus display versatile C- and N-metabolisms (Table 4; Steenhoud & Vanderleyen 2000), which makes them well adapted to establish in the competitive environment of the rhizosphere. Five species of the genus *Azospirillum*, namely *A. brasilense*, *A. lipoferum*, *A. irekense*, *A. halopraeferens*, and *A. amazonense* have been recognized. Except for *A. halopraeferens*, most *Azopirillum* species mentioned above have been isolated from wild rice and cultivated rice (reviewed in Mano & Morisaki 2008). Isolation of *A. lipoferum* has often been reported from maize, while *A. brasilense* has been commonly obtained from wheat and rice (Bashan *et al*. 2004).

R58 isolate, which was present at about 6% of the resident bacterial population in rice tissues (Barraquio *et al*. 1997) is assigned into the genus *Agrobacterium*. *Agrobacterium*, which is closely related to *Rhizobium*, is a classic obligate wound-infecting pathogen on plants. It has been suggested that legume nodules may have arisen from a wound tumor, with rhizobia evolving from an ancestor of *Agrobacterium* (Sprent & Raven 1985). *Agrobacterium* has been reported previously as a diazotroph (Kanvinde & Sastry 1990). *A. tumefaciens* strain C58 and B6 have been shown to reduce acetylene to ethylene and to incorporate ^{15}N supplied as $^{15}N_2$ (Kanvinde & Sastry 1990). These isolates were reported capable of fixing N_2 in the free-living state and growing on N-free medium, despite that DNA from those isolates did not hybridize with *nif* genes from *Klebsiella pneumoniae*, *R. meliloti*, or *Azorhizobium caulinodans* (Ruvkun & Ausulbel 1980; Kanvinde & Sastry 1990). *A. tumefaciens* has been used worldwide to mediate rice transformation by T-DNA transfer (Nishimura *et al*. 2007).

R38-T isolate, which was identified in this study as *S. paucimobilis*, was isolated from roots of *O. alta* in the Philippines (Table 1). *S. paucimobilis* has been isolated from roots of *O. officinalis* and *O. sativa* by using nitrogen free medium (Engelhard *et al*. 2000). The occurrence of this bacterium in the rice rhizosphere has also been shown by Bally *et al*. (1983) using the 'spermosphere' model.

R2 and R4 isolates shared similar features with E4 isolate in both morphological and physiological tests using API20E kits, but different features were obtained using API20NE kits, particularly carbohydrate assimilation test. These results suggest that R2 and R4 isolates have a close relationship to E4 isolate and can be assigned as species of *Rhizobium*. Recent study using gfp-tagged bacteria have shown that R4 isolate were observed only at the peripheral of nodule structure in berseem clover (Perrine-Walker *et al*. 2007), indicating that the bacteria were not fixing nitrogen. The inability of R2 and R4 isolates to form mature nodule in berseem clover suggests that these isolates could have lost some genes necessary for complete nitrogen-fixing nodule formation. Perrine-Walker *et al.* (2007) have speculated that R4 isolate have evolved to become a rice-*Rhizobium*-specific isolate during rice-clover rotation practice for more than 700 years in the Nile Delta of Egypt. Further characterization of these isolates using molecular methods will be useful in determining the relationship of these isolates with *R. leguminosarum* bv. *trifolii*.

All isolates examined were microaerophylic on either BMM or OF semisolid medium (Table 2). This microaerophylic nature may indicate that the bacteria were adapted to live in niche inside the plant, which is low in O_2 and relatively high in carbon. This environment is conducive to N_2 fixation and allows the efficient transfer of fix N products to the host. It is therefore necessary to determine the ability of these isolates in promoting rice growth and to examine their possible mode of action in endophyte-rice association.

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