J. Gen. Appl. Microbiol., **43**, 183–187 (1997)

Short Communication

Characterization of *Bradyrhizobium* strains isolated from soybean cultivation in Thailand

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(Received February 26, 1997; Accepted June 20, 1997)

Key Words——acid tolerance; *Bradyrhizobium*; IAA production; RAPD; temperature tolerance; Thailand

Bradyrhizobium japonicum has been extensively used for soybean cultivation in Thailand. Due to the acidity of the soil, some strains of *B. japonicum* may not survive and nodulate well on soybean grown in this soil condition. Thus, the objective of this study was to develop strains that were compatible with soybean as well as tolerant to acidic soil conditions. Soybean is one of the most important economic crops in Thailand. It supplies fats, oils, and high proteins for people. Therefore, it requires a high amount of nitrogen for growth. There are many ways to improve the yield of soybean, but the effective use of symbiotic nitrogen fixation has been considered to be the most promising because the cost of utilizing nitrogen fixation for supplying nitrogen to the host plant is lower than that of the using chemical fertilizers. For N_2 fixation to be most beneficial, the effective strains must perform well under field conditions. It has been realized that soils in the tropics are usually acidic and have high Al contents. Some researchers (Richardson et al., 1988) reported that soil factors such as low pH and high Al affect induction of *nod* genes, but more intensive study is needed. Munns (1986) reported that the acid sensitivity of *Rhizobium meliloti* limits the productivity of *Medicago–R. meliloti* symbiosis in acidic soils and the strains have to be ameliorated to be more acid-tolerant in the field. However, little is known about why some strains are more tolerant than others. In this study, we report the characterization of *Bradyrhizobium* strains isolated in Thailand and refer to their ability of acid tolerance.

General characterization of rhizobia isolated from soybean nodule in Thailand. The root nodule bacteria used in this study were isolated from the mash of surface-sterilized nodules of soybean grown in the fields of Thailand. Five bacterial strains isolated from the nodules of soybean in Thailand and five reference *Bradyrhizobium* strains used in this study are listed in Table 1, summarizing the nodulation and nitrogen fixation ability, IAA production, and growth on acid plate. Nodulation and nitrogen fixation ability of the five isolates were decided by inoculating *Glycine max* cv. SJ5 as the host plant. After 30 days cultivation, nitrogen fixation ability was measured as acetylene reduction activity according to Somasegaran and Hoben (1994). All five isolates indicated nitrogen fixing ability. However, the nitrogen fixing ability of strain TAL211 was very low, so that the growth of the host plant inoculated with strain TAL211 was the same as the host plant inoculated with nitrogen fixation-deficient mutant strain SM5.

Fuhrmann (1993) described the diversity of *Bradyrhizobium* strains breaking them into two groups, *B. elkanii* and *B. japonicum*, according to IAA production. *Bradyrhizobium* that produces high IAA is classified as *B. elkanii*. IAA production of the Thailand isolates was detected as follows. The strains were grown at 28°C in the dark using Tris–TMRT broth medium containing 10 g of mannitol, 0.2 g of $CaCl_2 \cdot 2H_2O$, 0.25 g of $MgSO_4 \cdot 7H_2O$, 1.21 g of Tris base,

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Table 1. Characteristics of rhizobial strains.

Rhizobial strain	Nodulation	Nitrogen fixation	IAA production	Growth on acid plate (pH 3.0)	Source or reference
THA2	+	+	−	+	Thailand
THA5	+	+	−	−	Thailand
THA6	+	+	−	−	Thailand
THA7	+	+	−	+	Thailand
TAL211	+	+	−	+	Thailand
TAL102	+	+	+	−	NifTAL
USDA136b	+	+	−	−	USDA
SM5	+	−	−	−	Maier and Brill (1976)
USDA76	+	−	+	−	USDA
61A101C	+	+	−	+	Stripf and Werner (1978)

Table 2. Growth rate and growth-pH and -temperature of rhizobial strains from soybean.

Rhizobial strain	Doubling time (min)	Growth pH range	Optimum pH range	Growth at				Cell conc. in YM-medi. (pH 4.0)[a]
				17	28	37	42 (°C)	
THA2	244	4–8	6–8	+	+	+	+	11.0
THA5	127	5–9	6–7	−	+	+	+	2.3
THA6	67	5–8	6–7	+	+	+	+	1.4
THA7	84	4–8	6–7	−	+	+	−	28.7
TAL211	314	3–8	6–7	−	+	+	+	50.0
TAL102	405	5–8	6–7	+	+	+	+	1.1
USDA136b	185	5–9	6–7	+	+	+	+	1.1
SM5	208	5–8	6–7	−	+	−	−	2.1
USDA76	252	5–8	6–7	+	+	−	−	3.7
61A101C	295	4–8	5–7	+	+	+	−	3.1

[a] Concentration of bacterial cells ($\times 10^5$/ml) was measured using YM agar plate (pH 6.8) after 7 days incubation in YM liquid medium (pH 4.0) at 28°C.

0.2 g of yeast extract, and 0.061 g of L-tryptophan in 1 *l* of distilled water (pH 6.8). IAA accumulated in the culture was determined by adding 2 ml of 0.01 M $FeCl_3$ in 35% $HClO_4$ to 1 ml of supernatant of the culture. The color reaction was detected after 30 min incubation in the dark at 30°C, and compared with negative and positive controls. Because all five isolates, THA2, THA5, THA6, THA7, and TAL211, produced no IAA or in a very small quantity, these strains were believed to be *B. japonicum*. The measurement of activity of uptake-hydrogenase and a sequence analysis of rRNA are necessary to classify the strains exactly as reported by Rumjanek et al. (1993). The colony-forming ability on acid plate was also investigated; yeast extract-mannitol (YM) agar plate (Keele et al., 1969) of which the pH was adjusted to 3.0 with HCl, was employed as the acid plate. Three of the isolates (strains THA2, THA7, and THA211) and *B. japonicum* 61A101C could form colonies on the acid plate. This preliminary result shows the possibility that these four strains are acid tolerant.

Effect of pH and temperature for bacterial growth. Bacteria were grown in YM liquid medium in 7 levels of pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0). Each pH medium was prepared by adding 1 ml of 1 M HEPES buffer, of which the pH was adjusted to the desired level, to 9.0 ml YM medium. Growth was measured after 65 h incubation by monitoring the optical density (O.D.) at 660 nm. For the temperature tolerance experiment, bacteria was grown in YM medium (pH 6.8) for 65 h at temperatures of levels 17, 28, 37, and 42°C, respectively. For both pH and temperature, the bacterial growth was judged to occur when the O.D. increased 0.01 unit after 65 h incubation. Doubling time was measured at 28°C using YM medium (pH 6.8). These results are summarized in Table 2. Strains THA2, THA5, and TAL211 indicated doubling times the same length as the *Bradyrhizobium* reference strains, while strains THA6 and THA7 indicated shorter doubling times than *Rhizobium* strains. The strains that could grow well at pH 4.0 were THA2, THA7, and TAL211 (Table 2). The concentration of rhizobial cells after culturing in media pH 4.0 for 7 days reached from 1.1×10^5 to 5.5×10^6 per milliliter but varied according to strain. Strains THA2, THA7, and TAL211 indicated relatively high concentrations after 7 days of incubation in acidic liquid medium. These data suggest that these three strains are acid tolerant. The tolerance to acidity depends on the ability to maintain an intracellular pH suitable for cell functions. Perez Gal-

Table 3. Enzymatic activities of rhizobial strains under different conditions of pH.

Enzyme	Strain									
	THA2	THA5	THA6	THA7	TAL211	TAL102	USDA136b	SM5	USDA76	61A101C
Phosphatase, alkaline	○	○	–	–	–	–	–	–	○	–
Esterase (C4)	○	–	–	–	○	○	–	–	–	–
Esterase lipase (C8)	–	–	–	–	○	–	–	○	○	–
Lipase (14)	○	○	–	○	○	–	○	○	○	○
Leucine arylamidase	–	–	–	–	○	–	–	–	○	–
Valine arylamidases	–	○	○	–	○	○	○	○	○	○
Cystine arylamidase	–	○	–	–	○	–	○	–	○	○
Trypsin	–	–	–	–	×	○	–	–	○	○
Chymotrypsin	–	×	○	×	×	×	×	–	×	×
Phosphatase acid	–	○	+	○	○	○	–	–	○	–
Phosphoamidase	○	○	○	○	○	○	○	○	○	○
α-Galactosidase	○	×	–	×	○	×	×	×	○	○
β-Galactosidase	×	×	×	×	○	×	○	×	×	○
Glucuronidase	×	×	×	×	×	×	×	×	○	×
α-Glucosidase	○	×	×	×	×	×	×	○	×	×
β-Glucosidase	○	×	×	×	○	×	×	×	×	×
N-Acetyl-glucosaminidase	×	×	×	×	×	×	×	×	×	×
Mannosidase	×	×	○	×	×	×	×	×	×	×
Fucosidase	○	×	×	×	○	×	×	×	×	×

○, enzymatic activity did not change at either pH 4.5 or 6.8; +, enzymatic activity was increased at pH 4.5; –, enzymatic activity was decreased at pH 4.5; ×, enzymatic activity could not be detected at either pH 4.5 or 6.8.

dona and Hahn (1994) reported that acid-tolerant strains can regulate their internal pH by dissociating H^+ within the cell. Symbiotic N_2-fixing rhizobia are particularly sensitive to low pH, which is known to limit bacterial growth and the initiation of formation of nitrogen-fixing nodules on roots of host plants (O'Hara et al., 1989). The temperature also affected to the growth of rhizobia. All isolates grew well at 37°C as compared to the *Bradyrhizobium* reference strains isolated from the temperate zone. Heat tolerance is also a useful character of the Thailand isolates.

Activity of enzymes under different conditions of pH. The biochemical character of the strains was tested using an APIZYM-kit (API System, La Balme les Grottes, Montalieu–Vercieu, France) against 19 kinds of substrates. Bacterial cells were grown in YM media (pH 6.8 and 4.5) and the enzymatic activities under the different pH conditions were compared (Table 3). Preparation of the bacterial suspension for the kit, colorimetric detection of enzyme activity, and measurement of activity were performed according to the manufacturer's instructions. At pH 6.8, the enzymes that every strain could produce in high quantity were esterase, lipase, arylamidase, and phosphoamidase. Only two strains (SM5 and 61A101C) could produce a high amount of phosphatase. When the pH of the medium was dropped from pH 6.8 to 4.5, the activity of enzymes decreased generally, except for strain TAL211 (Table 3). Strain TAL211 maintained enzymatic activity at the same level as in the neutral medium even at a low pH, while the activity of many enzymes tested decreased in the other acid-tolerant strains, THA2 and THA7, at pH 4.5. This suggests that the mechanism of acid tolerance is different between strains THA2 and THA7, and strain TAL211. At pH 4.5, all strains could produce phosphoamidase in the same quantity as in the neutral medium.

Analysis of random amplified polymorphic DNA (RAPD). Cells grown in the middle log-phase (50 ml of TY medium; Beringer, 1974) were centrifuged at 6,000 rpm for 10 min at 4°C. The pellet was washed twice with 0.85% NaCl, suspended in 2 ml of SET buffer (20% sucrose, 50 mM Tris–HCl pH 7.6, 50 mM EDTA) and kept for overnight at −20°C. The cells were thawed at 65°C and added to 0.2 ml of lytic solution [5 mg/ml of lysozyme in TES buffer (pH 7.6) containing 10 mM Tris–HCl, 1 mM EDTA, and 10 mM NaCl] and 0.1 ml of RNase A [10 mg/ml in 0.1 M sodium acetate (pH 7.4) containing 0.3 mM EDTA]. The mixture was incubated at 37°C for 1 h. Then 0.05 ml of 25% SDS, was added and the mixture was shaken gently for 3–6 h, after which 0.3 ml of pronase (2 mg/ml in TES buffer) was added and shaking continued for 12 h. Sterile distilled water (1 ml) was added to the mixture, which was then shaken for 1 h. The solution was treated with phenol and a mixture of chloroform : iso-amyl alcohol (24 : 1), and the DNA was precipitated by adding 0.1 volume of 3 M sodium acetate (final concentration 0.3 M) and 2.5 volumes of 99% ethanol. Total cellular DNA was pelleted by centrifuga-

tion at 10,000 rpm for 10 min at 4°C, and the pellet was rinsed with 70% ethanol and dissolved in 0.5 ml of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.6). The concentration of DNA was measured, and 50 ng of total bacterial DNA was used as a template for PCR. The oligonucleotide primers were purchased from Pharmacia Biotech (Uppsala, Sweden). The sequences of the primers were as follows: Primer 1, GGTGCGGGA; Primer 2, GTTTCGCTCC; Primer 3, GTAGACCCGT; Primer 4, AAGAGCCCGT; Primer 5,

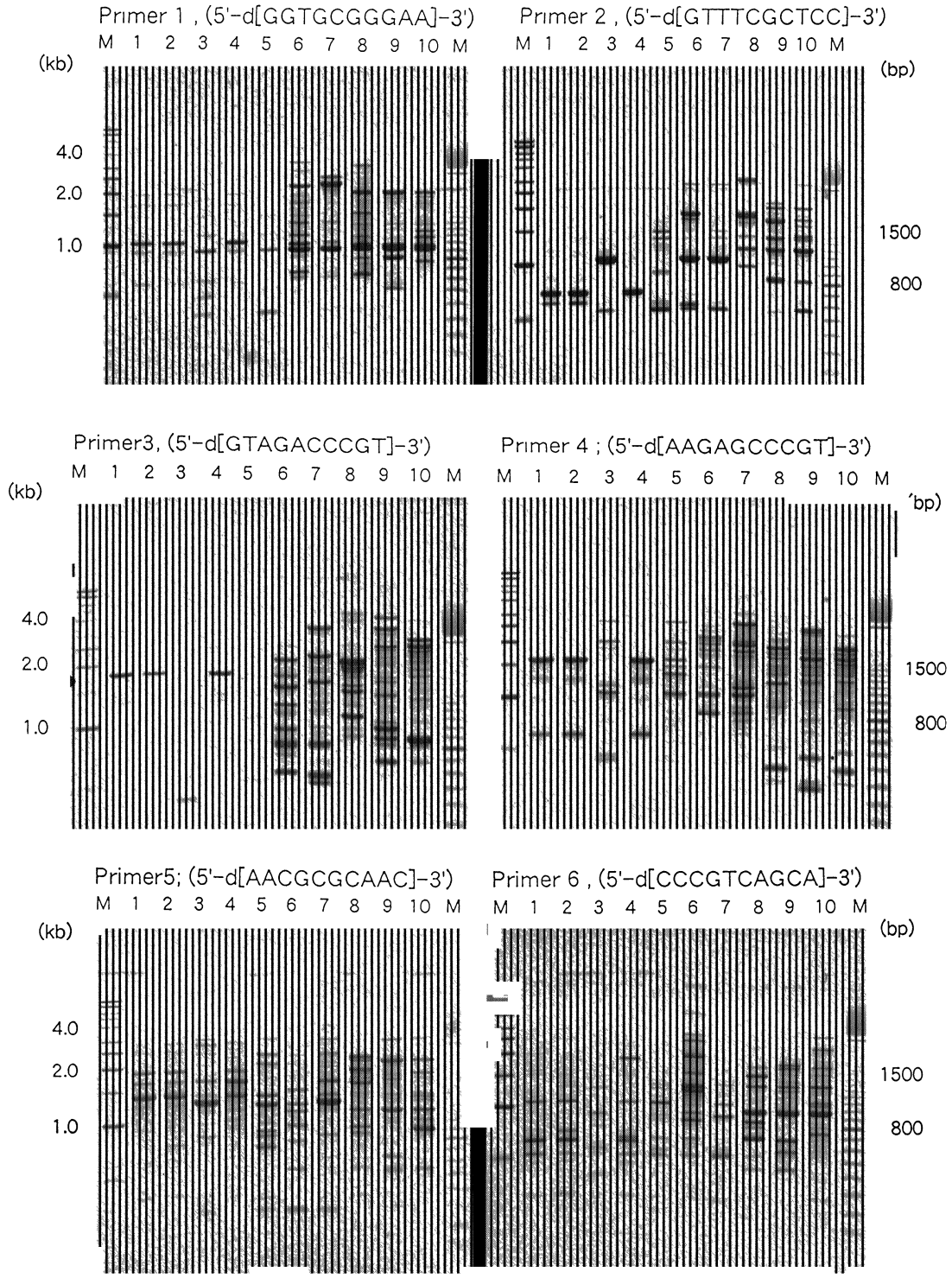

Fig. 1. RAPD analytical patterns of 10 rhizobial strains and 6 primers.
M, kb marker; lane 1, THA2; lane 2, THA5; lane 3, THA6; lane 4, THA7; lane 5, TAL102; lane 6, TAL211; lane 7, USDA136b; lane 8, SM5; lane 9, USDA76; lane 10, 61A101C.

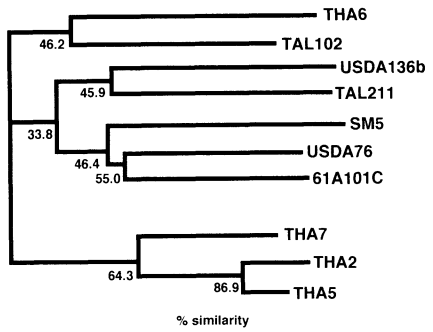

Fig. 2. Phenogram of 10 rhizobial strains by neighbour-joining method using Diversity One Software (PDI, NY, U.S.A.).

AACGCGCAAC; and Primer 6, CCCGTCAGCA. The reactions were carried out in 50 µl of reaction mixture containing 50 ng total bacterial DNA, 200 µM of each dNTPs, 50 pmol of one of the six primers, and 0.5 units of *Taq* polymerase (Takara Shuzo Co., Ltd., Kyoto, Japan) with the corresponding buffer (×10 buffer). The amplifications were performed with a Gene Amp PCR System 2400 (The Perkin–Elmer Corp., CA, U.S.A.). The products of reactions were separated electrophoretically using 1.5% agarose gel in TBE buffer (89 mM Tris base, 2 mM EDTA, 89 mM boric acid, pH 8.2). After the profiles of RAPD on agarose gel were incorporated into a Macintosh computer, the phenogram based on the similarity of RAPD profiles was obtained by the neighbour-joining method using Diversity One Software (PDI, New York, U.S.A). The RAPD profiles of the 10 strains generated by each primer are presented in Fig. 1. The profiles were found to be characteristic specific for each strain. Ten rhizobial strains divided into three clusters. All *Bradyrhizobium* strains isolated in the temperate zone (USDA136b, SM5, USDA76, and 61A101C) belonged to the same cluster. Acid-tolerant strains THA2 and THA7 constructed independent clusters with strain THA5, while another acid-tolerant strain, TAL211, belonged to the cluster which consists of *Bradyrhizobium* strains in the temperate zone. Strains THA2, THA5, and THA7 are believed to be tropical zone-specific strains.

The results of RAPD analysis support the findings of De Bruijn (1992), that PCR with a suitable primer could be applied for molecular genetic characterization of rhizobia. Nishi et al. (1996) and Lunge et al. (1994) reported the usefulness of RAPD analyses in the characterization of *Bradyrhizobium* strains. Thus, this RAPD technique could be successfully used for characterization and classification of *Bradyrhizobium* in Southeast Asia.

We are grateful to Mr. Preecha Wadisirisak, Mr. Somsak Kotepong, and Miss Panan Rerngsamran at the Soil Microbiology Research Group, Division of Soil Science, Department of Agriculture, Thailand, for their kind assistance in this work. This work was supported by a Grant-in-Aid for Scientific Research (Project No. 06044159) and Creative Basic Research (Project No. 08NP0901) from the Ministry of Education, Science, Sports and Culture, Japan.

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