

SELECTION AND CHARACTERIZATION OF MUNGBEAN ROOT NODULE BACTERIA BASED ON THEIR GROWTH AND SYMBIOTIC ABILITY IN ALKALINE CONDITIONS

Neelawan Pongsilp^{1*} and Achara Nuntagij²

Received: Apr 10, 2007; Revised: Jun 19, 2007; Accepted: Jun 25, 2007

Abstract

Ten strains of root nodule bacteria, isolated from mungbean (*Vigna radiata*) grown in five provinces of Thailand, were examined for their ability to grow in alkaline medium. Seven strains were able to grow at pH 8.0, whereas the growth of three strains was inhibited at pH 8.0. Therefore, the strains were grouped into 2 categories consisting of alkali-sensitive strains and alkali-tolerant strains. The growth of all strains tested was inhibited at pH 8.5, with a survival rate ranging between 0.03% to 2.13%. Of ten strains tested, the highest cell number was obtained from strain DASA02008 at pH 8.0. The cell number of strain DASA02008 reached $1.98 \times 10^8 \pm 5.90 \times 10^7$ CFU/ml, which differed significantly from other strains. At pH 8.5, the highest cell numbers were obtained from strains DASA02008, DASA02009 and DASA02010. The cell numbers obtained from strains DASA02008, DASA02009 and DASA02010 were $2.13 \times 10^3 \pm 9.97 \times 10^2$, $1.83 \times 10^3 \pm 4.80 \times 10^2$, and $1.97 \times 10^3 \pm 9.50 \times 10^2$ CFU/ml, respectively. In a nodulation test at pH 8.0, all strains could nodulate mungbean, and acetylene reduction activity (ARA) could be detected in all nodulated plants. However, there was no significant difference between alkali-tolerant strains and alkali-sensitive strains based on nodule numbers, nodule dry weight, plant shoot dry weight and acetylene reduction activity. Alkali-tolerant strains and alkali-sensitive strains were placed in the same cluster based on random amplified polymorphic DNA (RAPD) analysis. No small plasmids were found in any strains. The strain DASA02008, which had the highest cell number at both pH 8.0 and pH 8.5, was selected to analyze partial sequence (approximately 500 bp.) of the 16S rDNA. The nucleotide sequence indicated high homology (98%) with members of *Bradyrhizobium japonicum*, *Bradyrhizobium liaoningense* and *Bradyrhizobium* sp.

Keywords: Alkaline tolerance, root nodule bacteria, mungbean, RAPD, nodulation assay, *Bradyrhizobium*

Introduction

Mungbean (*Vigna radiata*) is one of the important legumes and a well-known economic crop in tropical and subtropical countries. It is often included in rice or corn-based crop rotation to replenish nitrogen, improve soil fertility and control pests and diseases.

¹ Department of Microbiology, Faculty of Science, Silpakorn University-SanamChandra Palace Campus, Nakorn Pathom, 73000. E-mail: neelawan@su.ac.th

² Soil Microbiology Research Group, Division of Soil Science, Department of Agriculture, Bangkok 10900

* Corresponding author

Mungbean is also used in several food products such as bean flour. As a leguminous plant, mungbean could be nodulated by rhizobia, causing the formation of a new organ (i.e. nodule) and establishing a nitrogen-fixing symbiosis (Loh and Stacey, 2003). Within the root nodules, these bacteria fix atmospheric nitrogen into ammonia, providing the nitrogen requirements of cultivated legumes (Hartmann and Amarger, 1991) and reducing the need for artificial fertilizer which can be expensive and cause environmental problems. Consequently, the symbiotic bacteria are of enormous agricultural and economic value (de Philip *et al.*, 1992). In biological nitrogen fixation, several environmental conditions are limiting factors to the growth and activity of the N₂-fixing plants. Typical environmental stresses of N₂-fixing systems include acidity, alkalinity, salinity, drought stress, nutrient deficiency, fertilizers, heavy metal, soil nitrate, temperature and biocides (Zahran, 1999). It is known that soil acidity, temperature, and salinity affect rhizobial persistence in the soil and the rhizosphere of plants, as well as the efficiency of nodulation (Bohloul *et al.*, 1992; Hungria *et al.*, 1993; Anyango *et al.*, 1995; Riccillo *et al.*, 2000). However, several strains, distributed among various species of rhizobia, are tolerant to stress effects (Zahran, 1999) and the responses of soil bacteria, such as rhizobia, to environmental stress factors have been investigated (Givskov *et al.*, 1994; Milcamps and de Bruijn, 1999; Riccillo *et al.*, 2000). In Thailand, soil alkalinity is one of the significant problems facing agricultural production in many areas and limits legume productivity. According to the survey of Land Development Department, soils in many agricultural fields are alkaline with an average pH above 7.0 to 8.5. Examples of alkaline soils in Thailand are some areas in Lopburi, Kamphangpet and Nakhon Pathom provinces (Land Development Department, 2007). A major problem in alkaline soils is reduced nutrient availability. Therefore, it makes good sense agriculturally to screen and develop strains that are tolerant of alkaline conditions as well as capable of nodulating mungbean and

fixing nitrogen. Selecting root nodule bacteria that tolerate alkaline conditions will insure success in legume productivity and reduce the problems with nutrient deficiencies. Thus, in this study, mungbean-nodulating root nodule bacteria were examined for their ability to grow in alkaline medium, their ability to nodulate and fix nitrogen in alkaline condition, and for random amplified polymorphic DNA (RAPD) analysis and nucleotide sequence of partial 16S rRNA gene.

Materials and Methods

Iso lation of Bacteria and Culture Conditions

Root nodules were collected from mungbean (*Vigna radiata*) grown in different areas in Thailand. Bacteria were isolated from root nodules as described by Somasegaran and Hoben (1994). Undamaged nodules were immersed in 95% ethanol for 5 - 10 sec, transferred to 3% (v/v) solution of sodium hypochlorite for 2 - 4 min. and rinsed in five changes of sterile water. Surface-sterilized nodules were crushed and one loopful of the nodule suspension was streaked on yeast-mannitol agar (YMA) plates containing 25 µg/ml congo red. The strains used in this study are listed in Table 1. The purity of the strains was ensured by single-

Table 1. Bacterial strains isolated from mungbean grown in Thailand

Strain	Geographic origin
DASA02007	Saraburi/Thailand
DASA02008	Saraburi/Thailand
DASA02009	Chainat/Thailand
DASA02010	Chainat/Thailand
DASA02011	Chainat/Thailand
DASA02068	Sukhothai/Thailand
DASA02074	Pijitr/Thailand
DASA02077	Pijitr/Thailand
DASA02087	Petchaboon/Thailand
DASA02095	Petchaboon/Thailand

colony isolation, observing colony morphology on yeast-mannitol agar (YMA) containing 25 g/ml congo red, and by examining Gram-stain reaction and carbol fuchsin stain reaction (Somasegaran and Hoben, 1994). The strains were cultured in yeast-mannitol (YM) medium (Keele *et al.*, 1969) at 28°C. Pure cultures were maintained on yeast-mannitol agar (YMA) slants at 4°C and frozen in 50% glycerol at -80°C.

Selection of Alkali-Tolerant Strains

Bacteria were grown at 28°C at 200 rpm for 5 days and used as inoculum. The total cell counts of inoculum was examined by the standard plate count method and inoculated into yeast-mannitol (YM) liquid medium in 4 levels of initial pH (6.8, 7.5, 8.0, and 8.5). Each pH medium was controlled by adding 1 ml of 1M HEPES buffer, of which the pH was adjusted to the desired level, to 9 ml of yeast-mannitol (YM) medium (Nuntagij *et al.*, 1997). The initial cell number of each strain was 1×10^5 CFU/ml. Cultures were grown at 28°C at 200 rpm for 8 days and the cell numbers were measured by the standard plate count method.

Nodulation Assays

Mungbean seeds (variety Chainat 72) were scarified and surface sterilized with 3% sodium hypochlorite as described by Somasegaran and Hoben (1994). The seeds were laid on a moistened cotton plate and incubated at 25°C in the dark for 1 to 2 days. The germinated seeds were inoculated with a bacterial suspension and planted in hydroponics using Leonard's jars and foam sheets to maintain the stable pH. The pH of N-free nutrient solution (Somasegaran and Hoben, 1994) was adjusted to be 8.0 and 1M HEPES was added to control pH as described above. After 35 days cultivation, nodule numbers, nodule dry weight and plant shoot dry weight were determined. The nitrogen fixing ability was measured by using the acetylene reduction method (Somasegaran and Hoben, 1994). The initial cell number and the survival cell number of strains in N-free nutrient solution were estimated by the standard plate count method.

Random Amplified Polymorphic DNA (RAPD) Analysis

Total DNA of the exponentially grown culture of each strain was extracted by using a Wizard Genomic DNA Purification kit (Promega, WI) according to the manufacture's instruction and used as a template for polymerase chain reaction (PCR). RAPD analysis were performed according to Nuntagij *et al.* (1997) using 3 oligonucleotide primers: RAPD-1 (5' GGTGCGGGAA 3'); RAPD-2 (5' GTTTCGCTCC 3'); and RAPD-3 (5' AAGAGCCCGT 3'). PCR conditions were as follows: 1 cycle at 94°C for 1 min, 45 cycles consisting of 94°C for 1 min., 36°C for 1 min, 72°C for 2 min, and 1 cycle at 72°C for 1 min. The products of the reactions were separated on a 1% agarose gel. The phenogram based on the similarity of RAPD profiles was obtained by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using an Image Master 1D Elite version 4.20 (Amersham, UK).

Plasmid Profile

Ten strains were examined for the presence of small plasmids. Plasmids were extracted by using a GF-1 plasmid extraction kit (Vivantis, Malaysia) according to the manufacture's instruction. Electrophoresis was run and the presence of plasmid bands was observed under UV illumination. The strain DASA64016, isolated from *Pueraria mirifica*, was used as a positive strain that bears plasmids.

Sequence Analysis of Partial 16S rDNA

The strain which had the highest cell number in liquid medium at both pH 8.0 and pH 8.5, was selected to analyze 16S rDNA. Partial 16S rDNA was amplified using universal primers UN16S 926f (5' AACTYAAAKGAA TTGACGG 3') and UN16S 1392r (5' ACGGGC GGTGTGTRC 3') (Lane, 1991). PCR reaction was done as described previously (Pongsilp *et al.*, 2002). PCR products were separated using 1% agarose gel in TBE buffer and purified using a QIA Quick Gel Extraction kit (Qiagen, Valencia, CA). The purified PCR product was sequenced by Macrogen, Korea. The nucleotide sequence

approximately 500 bp of the 16S rDNA of the selected strain was aligned using BLASTN (<http://www.ncbi.nlm.nih.gov>).

Statistical Analyses

Experimental data were compared by using the SPSS program (SPSS Inc., Chicago, IL).

Results and Discussion

Selection of Alkali-Tolerant Strains

In order to characterize the alkali-tolerant strains, their ability to grow in liquid medium at a different level of initial pH was assessed. Results in Figure 1 show the cell number of tested strains in liquid medium for 8 days at the level of pH 6.8, 7.5, 8.0, and 8.5. Ten strains showed a different level for alkaline tolerance based on the total cell counts obtained from the level of pH 6.8, 7.5, 8.0, and 8.5. While other strains could grow at pH 7.5, the strain DASA02074 was the most sensitive of alkaline because it was inhibited at pH 7.5. The survival rate of strain DASA02074 was about 7.67% in pH 7.5. According to the ability to grow at pH 8.0, ten strains were grouped into two categories consisting of alkali-tolerant strains and alkali-sensitive strains. Seven strains, DASA02007, DASA02008, DASA02009, DASA02010, DASA02011, DASA02077 and DASA02095, were able to grow in liquid medium pH 8.0 as the cell numbers were greater than the initial cell

number 1×10^5 CFU/ml. Therefore, they were considered as alkali-tolerant strains. The growth of three strains, DASA02068, DASA02074, and DASA02087, were inhibited at pH 8.0. Therefore, they were considered as alkali-sensitive strains. The alkali-tolerant strains showed different levels of tolerance. At pH 8.0, the highest cell number was obtained from strain DASA02008. The cell number of strain DASA02008 reached $1.98 \times 10^8 \pm 5.90 \times 10^7$ CFU/ml., which differed significantly from other strains ($\mu = 0.05$). This result revealed the existence of alkali-tolerant mungbean rhizobia in different areas in Thailand. The growth of all strains tested was inhibited at pH 8.5, with a survival rate ranging from 0.03% to 2.13%. At pH 8.5, the highest cell numbers were obtained from strains DASA02008, DASA02009 and DASA02010. The cell numbers obtained from strains DASA02008, DASA02009 and DASA02010 were $2.13 \times 10^3 \pm 9.97 \times 10^2$, $1.83 \times 10^3 \pm 4.80 \times 10^2$, and $1.97 \times 10^3 \pm 9.50 \times 10^2$ CFU/ml, respectively.

Nodulation Assays

The initial cell number and the survival cell number of strains in N-free nutrient solution are shown in Figure 2. After 35 days in N-free nutrient solution pH 8.0, the highest cell number was obtained from the strain DASA02008 at $4.40 \times 10^5 \pm 5.66 \times 10^4$ CFU/ml, which differed significantly from other strains ($\mu = 0.05$). This result is consistent with the results obtained

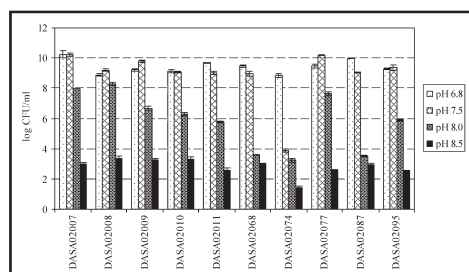


Figure 1. Total cell counts of tested strains grown in liquid medium at the various levels of pH. The values shown are the mean values of 3 replicates. Error bars indicate standard deviations

from the growth in alkaline liquid medium. In a nodulation test at pH 8.0, all strains could nodulate mungbean and acetylene reduction activity (ARA) could be detected in all nodulated plants. However there is no significant difference between alkali-tolerant strains and alkali-sensitive strains based on nodule numbers, nodule dry weight, plant shoot dry weight

weight and ARA. Nodule numbers, nodule dry weight, plant shoot dry weight and ARA obtained from each strain are shown in Figure 3. Figure 4 shows the comparison between mungbeans inoculated with the strains and the uninoculated control. Nodulation inhibition of alkalinity has also been reported in previous studies. Tang *et al.* (1998) reported that nodula-

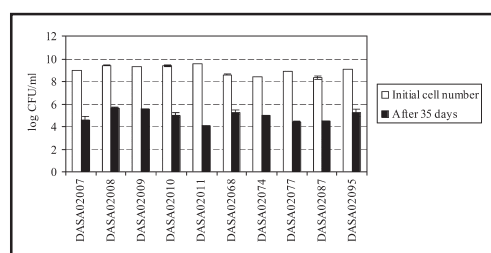
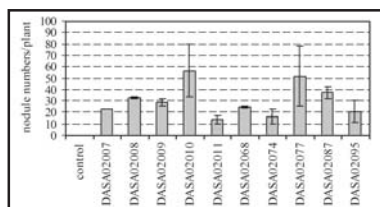
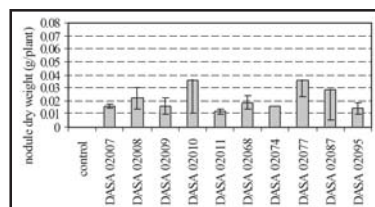


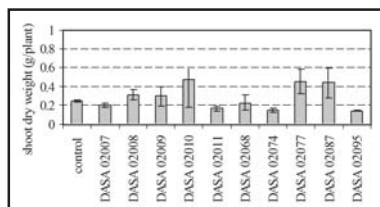
Figure 2. Total cell counts of tested strains remained in N-free nutrient solution pH 8.0. The values shown are the mean values of 2 replicates. Error bars indicate standard deviations



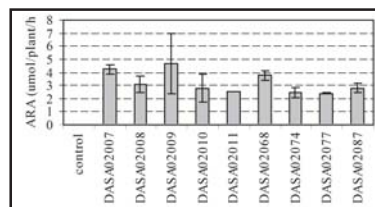
(a)



(b)



(c)



(d)

Figure 3. Average nodule numbers (a), nodule dry weight (b), plant shoot dry weight (c) and acetylene-reducing activity (ARA) (d) of mungbean inoculated with the strains. The values shown are the mean values of 2 replicates. Error bars indicate standard deviations

tion of groundnut (*Arachis hypogaea*) was inhibited when plants grow in a nutrient solution containing carbonate, and nodulation of *Lupinus angustifolius* was also inhibited when plants grow in a limed sand at a pH of > 7.0. Tang and Robson (1993) reported that high pH (>6.0 and up to 10.0) totally inhibited the nodulation of some lupins. Moreover, it has been hypothesized that rhizobia appear to be more tolerant to alkalinity than do their legume hosts (Zahran, 1999). This hypothesis can be an explanation of our results. Although the

strains were able to grow at pH 8.0, nodulation and nitrogen- fixing ability were inhibited because of the sensitivity of the plants to alkalinity.

Random Amplified Polymorphic DNA (RAPD) Analysis

RAPD profiles and the phenogram generated by primers RAPD-1, RAPD-2, and RAPD-3 are shown in Figures 5, 6, and 7, respectively. Alkali-tolerant strains and alkali-sensitive strains were placed in the same



Figure 4. Comparison between mungbeans inoculated with the strains and uninoculated control. Plant shoot dry weight obtained from the alkali-tolerant strains, DASA02010, DASA02011 and DASA02077 is not significantly different from that of the alkali-sensitive strain DASA02087

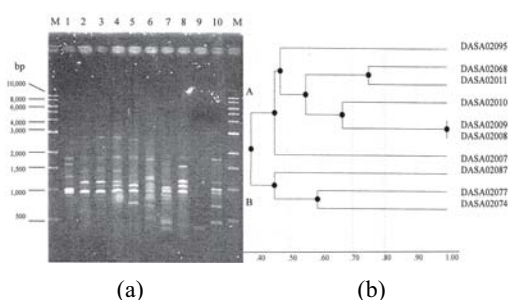


Figure 5. RAPD profile (a) and phenogram (b) of tested strains generated by primer RAPD-1. Lane M, 1 kb ladder; 1, DASA02007; 2, DASA02008; 3, DASA02009; 4, DASA02010; 5, DASA02011; 6, DASA02068; 7, DASA02074; 8, DASA02077; 9, DASA02087; 10, DASA02095

cluster. The phenogram generated by primer RAPD1 was consisted of 2 main clusters. Cluster A was comprised of the 6 alkali-tolerant strains and 1 alkaline-sensitive strains. Cluster B contained 1 alkali-tolerant strain and 2 alkaline-sensitive strains. The phenogram generated by primer RAPD2 contained 2 main clusters. Cluster A was comprised of the 2 alkali-tolerant strains and the 2 alkaline-sensitive strains. Cluster B was comprised of the 2 alkali-tolerant strains and 1 alkaline-sensitive strain. Similar result was obtained from the phenogram

generated by primer RAPD3. Cluster A was consisted of the 7 alkali-tolerant strains and 1 alkaline-sensitive strain. Cluster B contained 1 alkali-tolerant strain and 1 alkaline-sensitive strain. These results suggest that there was no significant correlation between RAPD profile and the tolerance of alkalinity. RAPD analysis has been used mostly for intraspecies discrimination, but the primer binds and PCR fragment are amplified randomly. However, each primer used in this study showed a different level of specificity for tested strains. Primer RAPD-1

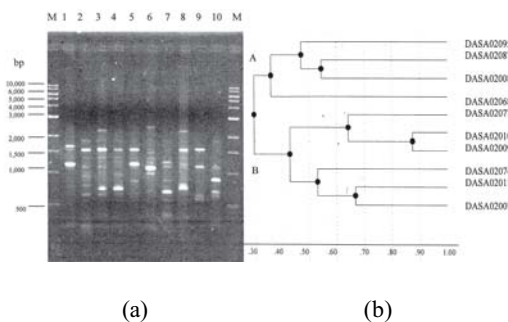


Figure 6. RAPD profile (a) and phenogram (b) of tested strains generated by primer RAPD-2.
Lane M, 1 kb ladder; 1, DASA02007; 2, DASA02008; 3, DASA02009; 4, DASA02010; 5, DASA02011; 6, DASA02068; 7, DASA02074; 8, DASA02077; 9, DASA02087; 10, DASA02095

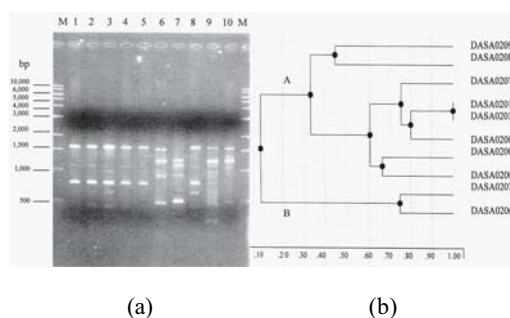


Figure 7. RAPD profile (a) and phenogram (b) of tested strains generated by primer RAPD-3.
Lane M, 1 kb ladder; 1, DASA02007; 2, DASA02008; 3, DASA02009; 4, DASA02010; 5, DASA02011; 6, DASA02068; 7, DASA02074; 8, DASA02077; 9, DASA02087; 10, DASA02095

generated the same profile from strains DASA 02008 and DASA02009, with 100% similarity in the phenogram; as well, primer RAPD-3 generated the same profile from strains DASA02010 and DASA02011, while primer RAPD-2 could generate the distinguishable profiles from these strains. This is in agreement with De Bruijn (1992) that PCR with a suitable primer could be applied for molecular genetic characterization of rhizobia. The usefulness of RAPD analysis in the characterization and classification of *Bradyrhizobium* strains has also been reported (Lunge *et al.*, 1994; Nishi *et al.*, 1996; Nuntagij *et al.*, 1997). The development of RAPD analysis provided a new tool for investigating genetic polymorphisms in many different organisms and recently has been used for *Rhizobium* identification and *Bradyrhizobium* genetic analyses (Kosier *et al.*, 1993; Kay *et al.*, 1994). Lunge *et al.* (1994); Nishi *et al.* (1996); Nuntagij *et al.* (1997) reported the usefulness of RAPD analyses in the characterization of *Bradyrhizobium* strains. Paffetti *et al.* (1996); de Oliveira *et al.* (2000) investigated the genetic diversity of *Rhizobium* populations by RAPD. Paffetti *et al.* (1996) demonstrated the considerable level of genetic diversity among *Rhizobium meliloti* strains which were phenotypically indistinguishable. de Oliveira *et al.*

(2000) showed the great genetic heterogeneity among *Rhizobium tropici* and *Rhizobium leguminosarum* bv. *phaseoli* strains. In this work, RAPD profiles generated by primer RAPD-2 were characteristic specific for each strain and can be used to discriminate the strains isolated from the root nodules of mungbean.

Plasmid Profile

The profiles from small plasmid extraction are shown in Figure 8. No small plasmids were found in any strains.

Sequence Analysis of Partial 16S rDNA

The partial nucleotide sequence of the 16S rRNA gene of strain DASA02008 was sequenced. Analysis of the sequence by BLASTN indicated the strain was a member of the genus *Bradyrhizobium* which shared 98% similarity with *Bradyrhizobium japonicum* USDA110 (GenBank accession number AF363150.1), *B. japonicum* USDA123 (GenBank accession number AF363151.1), *B. japonicum* USDA62 (GenBank accession number AF208517.1), *B. japonicum* USDA4 (GenBank accession number AF208515.1), *B. japonicum* USDA38 (GenBank accession number AF208514.1), *B. japonicum* USDA127 (GenBank accession number AF208508.1), *B. japonicum*

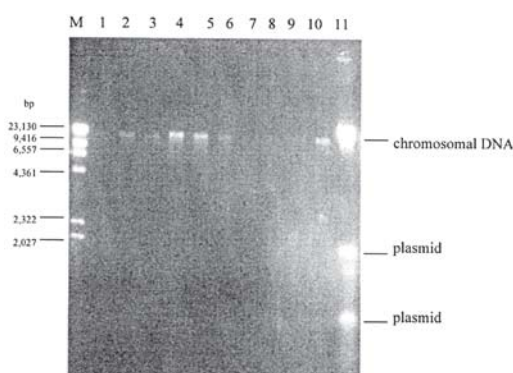


Figure 8. The profiles from small plasmid extraction of tested strains. Lane M, λ /HindIII DNA ladder; 1, DASA02007; 2, DASA02008; 3, DASA02009; 4, DASA02010; 5, DASA02011; 6, DASA02068; 7, DASA02074; 8, DASA02077; 9, DASA02087; 10, DASA02095; 11, DASA64016

USDA126 (GenBank accession number AF208507.1), *Bradyrhizobium* sp. CCBAU 23013 (GenBank accession number DQ836144.1), *Bradyrhizobium liaoningense* DdE3 (GenBank accession number DQ786798.1) The partial nucleotide sequence of gene encoding for 16S rRNA of strain DASA02008 has been deposited in GenBank under accession number EF513150.

Conclusion

In this work, we revealed the existence of alkali-tolerant mungbean-nodulating bacteria from different geographical origins in Thailand. The selected strains could nodulate mungbean at pH 8.0 and nitrogen fixation ability could be detected in all nodulated plants. RAPD profiles generated with primer RAPD-2 (5' GTTTCG CTCC 3') were found to be characteristic specific for each strain. The 16S rDNA sequence data showed that the representative strain DASA02008 was closely related to members of *Bradyrhizobium japonicum*, *Bradyrhizobium liaoningense* and *Bradyrhizobium* sp.

Acknowledgments

This work was supported in part by the Faculty of Science, Silpakorn University. We thank the staff of the Soil Microbiology Research Group, Department of Agriculture for their assistance on nodulation assays.

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