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Indigenous *Rhizobium* strains from Guam contain a mimosine degrading gene

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Abstract—Indigenous *Rhizobium* strains were isolated from nodules of *Leucaena leucocephala* (Lam.) de Wit on Guam. The bioassay of growing strains in *Rhizobium*-mimosine (RM) medium showed that the majority of isolates from the Barrigada site catabolized mimosine, a toxin which was produced by the plant, as a nutrient source, while only a few strains from Yigo site had this ability. The polymerase chain reaction (PCR) analysis was used to reveal the presence of a mimosine degrading gene (*mid*A). The PCR products of several *Rhizobium* strains isolated from Guam revealed that some isolates contained a mimosine degrading gene (*mid*A), the 1055-bp DNA fragment present in *Rhizobium* TAL1145, a strain from Hawaii.

Introduction

Leucaena leucocephala (Lam.) de Wit, commonly known as tangan-tangan or leucaena, is a leguminous shrub or tree grown in tropical and subtropical regions. All parts of the leucaena plants including roots and root nodules, contain a toxic compound called mimosine, [ß-N-(3-hydroxy-4-pyridone)-*a*-aminopropionic acid], which is known to have antimitotic activity (Jones 1979, Soedarjo & Borthakur 1996a). Mimosine is also found in leucaena rhizosphere (Soedarjo et al. 1994). Leucaena is nodulated by several types of *Rhizobium*, such as *Rhizobium* sp. strain TAL1145, *Rhizobium* sp. strain NGR234 and *Rhizobium tropici* strains such as CIAT899 (George et al. 1994). Among these rhizobia, only *Rhizobium* sp. strain TAL 1145 and some related strains can degrade mimosine and use it as a source of carbon and nitrogen, while the strains such as CIAT899 and NGR234 cannot degrade mimosine (Mid^- strains) (Soedarjo et al. 1994) It was also observed that when mimosine was added to tryptone-yeast extract medium, the growth of Mid^- strains was inhibited (Soedarjo & Borthakur 1996b). It

has been postulated that since mimosine is a bacteriostatic compound, it does not kill Mid^- strains which somehow withstand the inhibitory effect of mimosine in the nodule (Soedarjo et al. 1994). However, it was recently observed in Hawaii that the mid genes of Mid^+ strains are expressed in the leucaena nodules and the Mid^+ strains are found to be more competitive than the Mid^- mutants for the nodulation of leucaena (Soedarjo and Borthakur, unpublished data). The objective of this study is to investigate whether indigenous *Rhizobium* strains on Guam have the ability to degrade mimosine and to examine if these isolates contain the same mimosine degrading gene (*mid*A gene) which were isolated earlier from Hawaii strains.

Materials and Methods

Isolation of indigenous *Rhizobium* spp.

Soil samples were collected from fallow fields at three Guam Agricultural Experiment farms. The soil from the Yigo farm in northern Guam is characterized as Guam cobbly clay (clayey, gibbsitic, nonacid, isohyperthermic, Lithic Ustorthents), with a pH of 7.5. It is very shallow soil on limestone bedrock. The Barrigada farm soil is classified as Pulantat (clayey, montmorillonitic, isohyperthermic Lithic Ustorthents) with a pH of 7.0. The soil from the southern station farm, Ija, is Akina silty clay (very fine, kaolinitic, isohyperthermic Oxic Haplustalfs) with a pH of 4.5–5.5. Soil samples were collected from each site, by pooling 10 sub-samples. Ten grams of the soil sample from each site was mixed with 90 ml of sterile distilled water for 15 min using a wrist hand shaker.

Field-collected seeds of *L. leucocephala* were surface-sterilized with 1% NaOCI for 15 min. and rinsed three times with distilled water. Sixty seeds were scarified with concentrated sulfuric acid for 10 min and rinsed well with distilled water. Seeds were then soaked overnight in water for imbibition and were pre-germinated in sterile paper towels. Germinating seeds with about 4 mm long radicles were placed in a paper wick within a pouch (Mega International of Minneapolis, MN) which contained 40 ml of nitrogen-free nutrient solution with micro-nutrients (Somasegaran & Hoben 1985). One ml of soil sample solution was then applied to pre-germinating seeds. Ten seeds were treated with soil sample solution from each locality. Plants were grown in a screen house for 2-4 weeks. Ten to 20 well-developed nodules were harvested from plants inoculated with Yigo and Barrigada soils. *L. leucocephala* inoculated with Ija soil did not form nodules. Forty *Rhizobium* strains from Yigo and Barrigada sites were isolated in yeast extract-mannitol (YEM) medium with the standard procedure (Somasegaran & Hoben 1985).

Bioassay of the mimosine degrading ability by indigenous *Rhizobium* Strains

Rhizobium strains were grown in liquid *Rhizobium*-mimosine (RM) medium, which was prepared according to the procedure described by Soedarjo et al.

(1994). A minor modification was the exclusion of biotin and DL-pantothenate. Each strain was cultured in 3 ml of RM media in a test tube for 5-6 days at 28°C. If a strain had the ability to utilize mimosine, the solution would change color from yellow to colorless. Cultures were examined for color changes.

Isolation of genomic DNA of indigenous *Rhizobium* strains

Rhizobium strains were grown in tryptone-yeasts extract (TY) medium (Beringer, 1974) for 4-6 days at 22-27°C. Genomic DNA from indigenous *Rhizobium* strains was isolated by using the modified procedure described by Pooyan et al. (1994). Ten mL of each cell culture was centrifuged. One mL of 1M NaCl was added and mixed well with a vortex shaker, re-centrifuged to spin down cells. The supernatant was discarded. The cells were re-suspended with 500 µL TE_{25} (10 mMTris, pH8.0 and 25 mM EDTA). After 0.5 mg lysozyme (10 µL 50mg/mL stock solution) was added, the cell cultures were incubated at room temperature for 10-15 min. Thirty two µL of 10% SDS (final SDS = 0.5%) was added and mixed gently. After adding 60 µL proteinase K (stock solution = 20mg/mL, final concentration = 200µg/mL), lysed cells were incubated at 50°C for 2 hours. Phenol extraction was repeated several times and the DNA containing upper aqueous phase was collected. After adding 100 µL 7.5 M ammonium acetate and 100% ethyl alcohol up to 1.5 mL, precipated DNA was collected and transferred to a new tube and stored in 500 µL 70% ethyl alcohol.

PCR amplification condition and resolution of MD+ gene

Genomic DNAs isolated from indigenous *Rhizobium* strains from Guam were examined for the presence of the mimosine degrading gene recognized *Rhizobium* strain TAL1145 at the Molecular Biology Lab of the University of Hawaii. The isolated DNA from *Rhizobium* strains was air-dried. The polymerase chain reaction (PCR) was done using the Gene Amp PCR system 2400 (Perkin Elmer Corp, Corwalk, CT). Modified PCR methods described by Jyothirmai & Borthakur (1996) were used with a final volume of 50 µL, which contained about 100 ng sample of DNA, 200 µM of each dATP, dCTP, dGTP, dTTP, 0.4 µM of both primer 1 (MAT-F) and primer 2 (MAT-R), 2.5 mM $MgCl_2$, and 2.0 U of *Taq* polymerase (Promega, Madison, WI). The initial template denaturation was programmed at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 seconds, primer annealing at 60°C for 45 seconds, and primer elongation at 72°C for 45 seconds. The final elongation period was 5 min at 72°C and the samples were cooled to 4°C. PCR products were resolved on 1.5% agarose gels and were visualized by staining with ethidium bromide. The results were photographed.

Results and Discussions

Eight out of eleven *Rhizobium* strains isolated from Barrigada site degraded mimosine in the bioassay. The brownish yellow color of the RM liquid medium turned colorless, indicating the mimosine was completely utilized by those strains

Table 1. Growth of indigenous *Rhizobium* strains in liquid rhizobium-mimosine (RM) medium after the incubation period of 6 days at 28°C, and detection of a mimosine degradation gene (*mid*A) in the PCR analysis[z].

Sampling site and soil type	Strain No.	Ability to utilize mimosine as carbon and nitrogen source	Detection of the 1055-bp fragment in PCR analysis
Barrigada:	B1	+	-
Pulantat soil	B2	+	-
	B4	+	-
	B5	+	-
	B7	ND (contaminated)	+ (faint)
	B9	+	+
	B10	+	+ (faint)
	B12	+	+
	B19	-	-
	B24	-	+/- (very faint)
	B26	+	-
Yigo:	Y1	-	ND
Guam cobbly clay	Y2	-	ND
	Y3	-	ND
	Y4	+	ND
	Y5	-	+
	Y7	-	ND
	Y9	-	ND
Control:	*Rhizobium* TAL1145	+	+
	Bradyrhizobium sp.	-	ND

[z]The "+" sign means that a strain has the ability to utilize mimosine as a nutrient, and indicates the positive detection of 1055-bp fragment. The "-" sign symbolizes a lack of the ability to catabolize mimosine, and indicates no detection of the 1055-bp fragment. ND means that a strain was not included in either the bioassay or the PCR analysis.

(Table 1). Previously, Soedarjo et al. (1994) demonstrated that mimosine was not detected in the medium by high performance liquid chromatography (HPLC) when the medium became colorless with the presence of mimosine degrading rhizobia. Among the eight mimosine degrading strains from Barrigada, three strains generated the 1055-bp PCR fragment (Table 1; Fig. 1 & 2), suggesting that those strains contained the *mid*A genes found in strain TAL1145. A faint PCR band was observed in some strains. A diversity in the *mid*A gene sequence in Guam strains was found from the presence of such a faint band or the absence of the 1055-bp PCR fragment. The PCR primers used in this study were based on the *midA* gene sequence of strain TAL1145. It is likely that the PCR primers did not bind to the genomic DNA due to differences in the sequences in the variable regions. It would be necessary to test several sets of primers to select the most suitable set of primers which could reveal the most conserved regions of the DNA sequences within the *mid*A gene.

The majority of the strains collected from Guam cobbly clay soils at Yigo were mimosine non-degrading rhizobia (Mid^{-}). Among seven isolates, only one

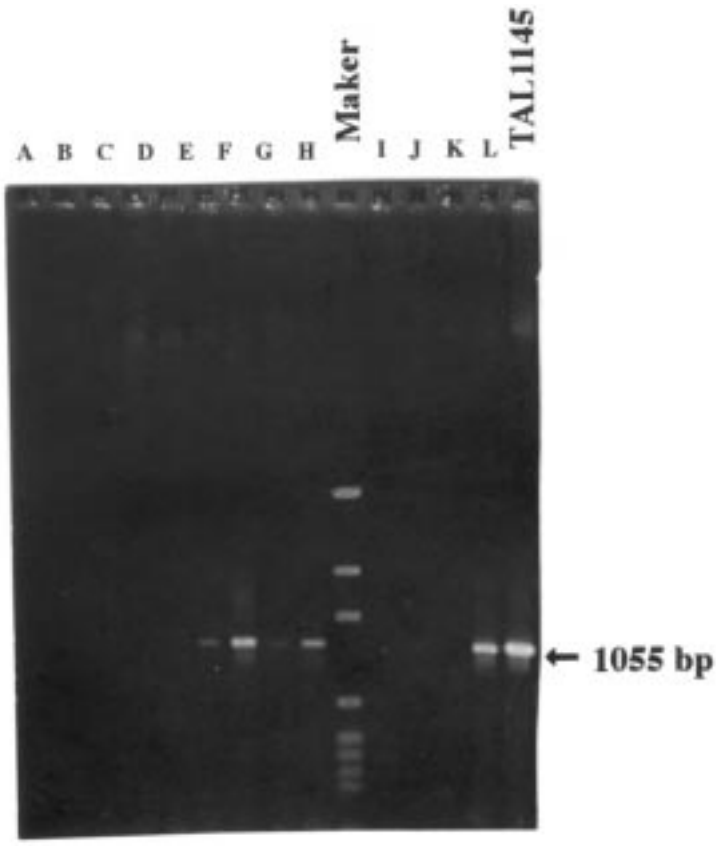

Figure 1. The PCR screening of indigenous *Rhizobium* genomic DNA using two primers (MAT-F and MAT-R). Two primers amplified a 1055-bp fragment of mimosine degrading gene (*mid*A) which was previously isolated from TAL1145 strain. The marker bands (M) from the top are 2,645-bp, 1,605-bp, 1,198-bp, 676-bp, 517-bp, 396-bp, and 350-bp. Indigenous *Rhizobium* strains, B1 (lane A), B2 (lane B), B4 (lane C), B5 (lane D), B7 (lane E), B9 (lane F), B10 (lane G), B12 (lane H), B19 (lane I), B24 (lane J), and B26 (lane K) were isolated from Pulantat soil at Barrigada while a strain Y5 (lane L) was isolated from Guam cobbly clay soil at Yigo.

(Y4) was confirmed to be Mid^+. Another isolate (Y5), that appeared to be Mid^-, was found to generate the 1055 bp *mid*A-specific PCR fragment indicating that this strain contained a mutant or non-functional copy of the genes. It also suggested the possible existence of gene interactions to suppress the ability of mimosine degradation in Y5 strain.

No nodules were formed from the plant infection experiment using Ija soil as the inoculum. In a previous rhizobia population survey, it was found that Akina soil in Ija site contained more *Bradyrhizobium* spp. than *Rhizobium* spp., and that generally the population of *Rhizobium* spp. was low in this site (Marutani & Manalastas, unpublished data).

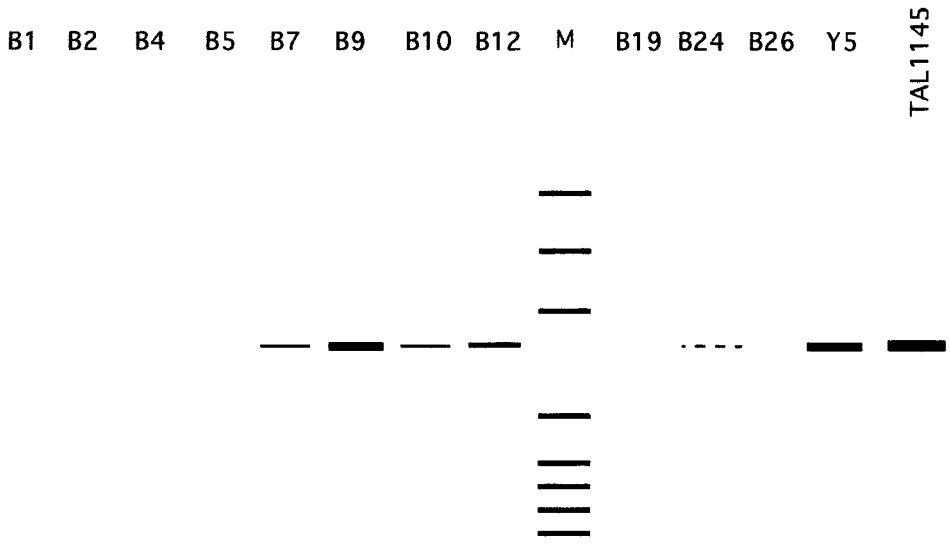

Figure 2. The schematic diagram of the PCR screening indigenous *Rhizobium* genomic DNA using two primers (MAT-F and MAT-R) shown in Fig. 1. The marker is shown in lane M and TAL1145 is the isolate with the 1055-bp fragment of mimosine degrading gene (*mid*A) originated from Hawaii.

This is the first report to demonstrate that *Rhizobium* strains isolated from Guam contain the mimosine degrading gene. The study suggests that there is genetic diversity among *Rhizobium* strains nodulating *L. leucocephala* in Guam. The occurrence of genetic variants of *Rhizobium* is also influenced by the soil type or locality. The strains isolated in the experiments can be classified into four groups: (i) Mid^+ strain similar to TAL1145; (ii) Mid^+ strain different from TAL1145; (iii) Mid^- strain containing mutant or deleted form of the *mid* genes; and (iv) Mid^- strains without sequence homology with *mid*A genes. The first two groups were found more in Pulantat soils of Barrigada.

Further study is needed to include a larger number of *Rhizobium* strains to reveal the detailed relationship of the soil environment and strains with the possession of mimosine catabolizing ability. It is also of interest to isolate nitrogen fixing bacteria from nodules of other leguminous species such as indigenous leucaena, *L. insularum* var. *guamensis* Fosberg & Stone, to investigate the mimosine degrading ability of rhizobia and the general relationship between the indigenous plants and *Rhizobium* spp.

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