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Full Paper

A novel symbiotic nitrogen-fixing member of the *Ochrobactrum* clade isolated from root nodules of *Acacia mangium*

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Ten strains of root nodule bacteria were isolated from the nodules of *Acacia mangium* grown in the Philippines and Thailand. Partial sequences (approx. 300 bp) of the 16S rRNA gene of each isolate were analyzed. The nucleotide sequences of strain DASA 35030 indicated high homology (>99%) with members of the genus *Ochrobactrum* in *Brucellaceae*, although the sequences of other isolates were homologous to those of two distinct genera *Bradyrhizobium* and *Rhizobium*. The strain DASA 35030 was strongly suggested to be a strain of *Ochrobactrum* by full length sequences of the 16S rRNA gene, fatty acids composition, G+C contents of the DNA, and other physiological characteristics. Strain DASA 35030 induced root nodules on *A. mangium*, *A. albida* and *Paraserianthes falcataria*. The nodules formed by strain DASA 35030 fixed nitrogen and the morphology of the nodules is the same as those of nodules formed by the other isolates. This is the first report that the strain of *Ochrobactrum* possesses complete symbiotic ability with *Acacia*.

Key Words——*Acacia mangium*; *Bradyrhizobium*; determinate nodule; nitrogen fixation; nodule bacteria; *Ochrobactrum*; *Rhizobium*; 16S rRNA

Introduction

Soil bacteria belonging to *Rhizobiaceae* are important to global agriculture and forestry since they form nitrogen-fixing nodules on roots or stems of leguminous plants that are responsible for one-third to half of the terrestrial nitrogen fixation (Burns and Hardy, 1975). Root- and stem-nodule bacteria associated with legumes have been classified into 6 genera (*Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Allorhizobium*, *Rhizobium*, *Sinorhizobium*) of the α-2 subclass of *Proteobacteria* (Wang and Martinez-Romero, 2000). Recently, *Methylobacterium nodulans* (Sy et al., 2001) and *Blastobacter denitrificans* (van Berkum and Eardly, 2002), which are members of α-*Proteobacteria*, were reported as nitrogen-fixing symbionts of legumes. Furthermore, two members of the β-*Proteobacteria*, *Burkholderia* (Moulin et al., 2001) and *Ralstonia* (Chen et al., 2001), have also been reported as possible nitrogen-fixing symbionts of legumes. These findings suggest that the genes responsible for symbiosis with legumes are transmissible horizontally and function in a relatively wide range of bacterial taxa (Fuentes et al., 2002).

Acacia mangium is one of the important woody

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legumes. In tropical and subtropical developing countries, the increasing introduction of *A. mangium*, inoculated with symbiotic nitrogen-fixing bacteria, in industrial plantations is largely due to its good silvicultural potential and ability to grow on degraded soils (Galiana et al., 1998). This woody legume has many applications such as fuel wood, timber, wind protection and animal fodder. *A. mangium* is used as a pioneer plant for reforestation. All these benefits of this plant are attributed to the nitrogen fixation ability of symbiotic bacteria. Nuswantara et al. (1997) reported the isolation of *Bradyrhizobium elkanii* strains from root nodules of *Acacia* trees in Indonesia. However, little information is available on the symbiosis between *A. mangium* and its microsymbionts.

In this report, root nodule bacteria isolated from *A. mangium* in the Philippines and Thailand were examined for their phylogenic relationship by nucleotide sequence of the 16S rRNA gene together with studies on their chemotaxonomic and symbiotic properties.

Materials and Methods

Bacteria and culture conditions. The nodule bacteria isolated from *A. mangium* nodules and analyzed in this study are listed in Table 1. The strains isolated from *Acacia* nodules and other rhizobial reference strains were cultured in Yeast Mannitol (YM) medium (Keele et al., 1969), or in TY medium (Beringer, 1974) at 28°C. *Escherichia coli* cells for cloning were cultured in Luria-Bertani (LB) medium (Sambrook et al., 1989). The antibiotics, kanamycin (50 μg/ml), ampicillin (50 μg/ml) or tetracycline (15 μg/ml), were used for selection markers.

For chemotaxonomic analyses, three strains isolated from *Acacia* (strains DASA 35030, DASA 35082 and PDA-1) and type strains of *Ochrobactrum* were cultured aerobically at 30°C in a medium containing 10 g of polypepton (Wako Pure Chemical Industries, Osaka, Japan), 2 g of Bacto yeast extract (Difco Laboratories, Detroit, USA), and 1 g of $MgSO_4 \cdot 7H_2O$ (per liter, pH 7.0), and *Bradyrhizobium* and *Rhizobium* strains were cultured in the medium containing 1 g of Bacto yeast extract, 5 g of mannitol, 0.7 g of K_2HPO_4, 0.1 g of KH_2PO_4, and 1.0 g of $MgSO_4 \cdot 7H_2O$ (per liter, pH 7.0).

Physiological characterization of symbiotic bacteria isolated from Acacia nodules. Generation time and growth range of pH and temperature were automatically monitored by a Bio-Photorecorder (TN-1506, ADVANTEC, Tokyo, Japan). Salt tolerance of bacteria was determined on the solid medium supplemented with NaCl at various concentrations and incubated at 28°C. IAA production of the isolates was determined as described previously (Nuntagij et al., 1997).

Table 1. Bacterial strains isolated from *Acacia mangium*.

Strain	Geographic origin	Genus group[a]
PDA-1	Davao/Philippines	*Rhizobium*
PDA-2	Davao/Philippines	*Rhizobium*
DASA 35013	Naratiwat/Thailand	*Bradyrhizobium*
DASA 35022	Prachinburi/Thailand	*Bradyrhizobium*
DASA 35027	Prachinburi/Thailand	*Bradyrhizobium*
DASA 35030	Prachinburi/Thailand	*Ochlobactrum*
DASA 35042	Yasothorn/Thailand	*Bradyrhizobium*
DASA 35052	Ubonratchathani/Thailand	*Bradyrhizobium*
DASA 35082	Chachoengsao/Thailand	*Bradyrhizobium*
DASA 35092	Lopburi/Thailand	*Bradyrhizobium*

[a] Genus group was based on 16S rRNA gene partial sequence (approx. 300 bp).

Plant materials and nodulation assays. A. mangium WILLD., *A. albida* DELILE, *A. mellifera* (VAHL) BENTH. and *A. tortilis* (FORSK) HAYNE. and 3 other tree leguminous species *Paraserianthes falcataria* (L.) FOSBERG, *Leucaena glauca* (L.) BENTH. and *Parkinsonia aculeata* L. were tested as inoculation hosts. The seeds were scarified and surface sterilized with 97% sulfuric acid for 30 to 60 min and rinsed off with sterilized distilled water. The seeds were laid on a moistened paper towel in a petri dish and incubated at 25°C in continuous dark. The germinated seedlings (approx. 13 mm in root length) were inoculated with a bacterial suspension (2×10^7 cells/seedling) and planted in small pots filled with vermiculite, which was moistened with nitrogen deficient Fåhraeus medium (Fåhraeus, 1957). The seedlings were grown 14 h-light (150 μEs)/10 h-dark days at 25°C. After 60 to 80 days, nodule numbers were counted and the acetylene reducing activity of the whole plant was measured as nitrogen fixation activity (Fuentes et al., 2002). The amount of ethylene, the product of the acetylene reducing activity, was quantified by gas chromatography (Shimadzu GC 3BF, Kyoto, Japan).

DNA manipulation, amplification and sequencing. Genomic DNA was extracted from exponentially grown bacteria by the method of Masterson et al. (1985) with some modifications. Preparation of total genomic DNA

for quantification of G+C contents was described elsewhere (Marmur, 1961; Saito and Miura, 1963). Total DNAs for plasmid detection were prepared according to Casse et al. (1979). Plasmid DNA from *E. coli* was extracted by alkaline lysis and finally purified with 20% polyethylene glycol (PEG)-2.5 M NaCl (Birnboim and Doly, 1979). A part of 16S rRNA gene (approximately 300 bp) (Young and Eardly, 1991) was amplified with GeneAmp PCR System (PE Applied Biosystems, Norwalk, USA) using the primer pair; 16S-2F (5′-TGGCTCAGAACGAACGCTGGCGGC-3′) and 16S-1R (5′-CCCACTGCTGCCTCCCGTAGGAGT-3′). A nearly full length DNA fragment of the 16S rRNA gene was also amplified using the following primers: 9F (5′-GAGTTTGATCCTGGCTCAG-3′), 515F (5′-GTGCCAGCAGCCGCGGT-3′), 785F (5′-GGATTAGATACCCTGGTAGTC-3′), 1099F (5′-GCAACGAGCGCAACCC-3′), 1541R (5′-AAGGAGGTGATCCAGCC-3′), 1510R (5′-GGCTACCTTGTTACGA-3′), 1115R (5′-AGGGTTGCGCTCGTTG-3′), 802R (5′-TACCAGGGTATCTAATCC-3′), and 536R (5′-GTATTACCGCGGCTGCTG-3′) (Nakagawa and Kawasaki, 2001). The amplified fragments were purified by agarose gel electrophoresis and then cloned into the p3T-TA vector (Mo Bi Tec GmbH, Göttingen, Germany). The DNA sequence of the insert was determined using a BigDye Terminator Cycle Sequencing FS Reaction Kit (PE Applied Biosystems) and the sequences were read automatically using ABI PRISM 310 (PE Applied Biosystems). Sequences obtained were managed by Gene Works program (IntelliGenetics, Inc., Campbell, USA).

Phylogenetic analysis of the 16S rRNA gene. Partial sequences of 16S rDNA were aligned by using the ClustalW ver. 1.8 program and the phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) and visualized by using Tree View ver. 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). To analyze the nearly full sequences of the 16S rRNA gene, raw sequence data were compiled by using the software MacClade, ver. 4.03 (Maddison and Maddison, 2001). Multiple sequence alignments were facilitated by using ClustalX ver.1.81 (Thompson et al., 1997), and corrected manually by using MacClade. Phylogenetic trees were obtained from the data using the distance method. The most appropriate evolution model was determined for a given data set using PAUP* 4.0b10 (Swofford, 2002) and Modeltest 3.06 (Posada and Crandall, 1998).

Chemotaxonomic and G+C contents analyses. Isoprenoid quinones were prepared as described previously (Nakagawa et al., 1996), and analyzed by high-performance liquid chromatography (HPLC) (model LC-5A; Shimadzu, Kyoto, Japan) with a Cosmosil $5C_{18}$-AR packed column (4.6×150 mm) (Nacalai Tesque, Kyoto, Japan). Cellular fatty acid methyl esters were prepared according to a previous report (Suzuki and Komagata, 1983). The fatty acid methyl ester composition was determined by gas chromatography-mass spectrometry (model QP-5000, Shimadzu) with a BPX70 capillary column (0.22 mm×50 m) (SGE Japan, Inc., Tokyo, Japan) and a SPB-1 capillary column (0.25 mm×30 m) (Supelco, Inc., Bellefonte, USA). The guanine-plus-cytosine (G+C) content of the DNA was determined by HPLC with a Cosmosil $5C_{18}$-AR packed column (4.6×150 mm) (Mesbah et al., 1989).

Random amplified polymorphic DNA (RAPD) analyses. RAPD analysis was performed according to previous reports (Abe et al., 1998; Nuntagij et al., 1997) using two oligonucleotide primers RAPD-1 (GGTGCGGGAA) and RAPD-2 (GTTTCGCTCC) (Pharmacia Biotech, Uppsala, Sweden).

Morphological observation of root nodules. The nodules were detached from roots and the specimens were prepared for microscopic observations, Light Microscope (LM), Scanning and Transmission Electron Microscope (SEM, TEM). The procedures for preparation of specimens were basically performed according to a method previously reported (Fuentes et al., 2002; Tani et al., 2003).

Results

Host specificity of root nodule bacteria of Acacia

All tested strains could nodulate *A. mangium* and were confirmed to fix nitrogen as measured by acetylene reducing activity (ARA) (Table 2, Fig. 1). Seven isolates (strains PDA-1, PDA-2, DASA 35030, DASA 35022, DASA 35027, DASA 35052, and DASA 35082) were tested for nodulation and nitrogen fixation ability on 4 *Acacia* species and 3 other leguminous genera (Table 3). All tested strains nodulated *A. mangium*, *A. albida* and *P. falcataria* but not *A. mellifera*, *A. tortilis*, *P. aculeata* or *L. glauca*. First nodulation could be observed at 3 to 4 weeks after inoculation. Acetylene reducing activity could be detected in all nodulated plants (Table 3).

Table 2. Nodulation and acetylene-reducing activity (ARA) of the isolates from nodules of *A. mangium*.

Strains	Plant fresh wt. (g/plant)	Nodule No. (per plant)	ARA (nmol/plant/h)	ARA (nmol/nodule/h)
PDA-1	0.15	11	5.01	0.45
PDA-2	0.15	9	5.59	0.62
DASA 35013	0.11	6	3.85	0.64
DASA 35022	0.21	6	3.12	0.52
DASA 35027	0.14	4	1.68	0.42
DASA 35030	0.11	5	2.40	0.48
DASA 35042	0.07	2	2.98	1.49
DASA 35052	0.08	3	3.33	1.11
DASA 35082	0.12	10	3.12	0.31
DASA 35092	0.07	2	3.85	1.92

Data were scored at 60 days after inoculation (d.a.i.) from plural *Acacia* plants. Nitrogen fixation was estimated by acetylene-reducing activity.

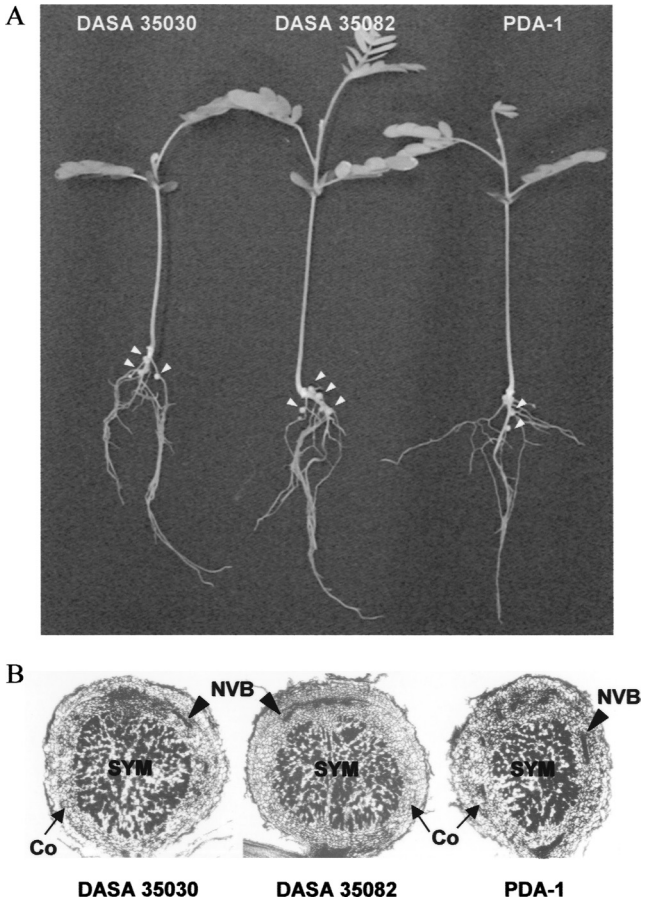

Fig. 1. Nodules of *Acacia mangium*.

(A) Spherical nodules formed on young plants of *Acacia mangium* inoculated with the strains DASA 35030, DASA 35082 and PDA-1. Arrowheads indicate nodules. (B) Cross section of each nodule observed by light microscope. SYM, symbiotic zone; NVB, nodule vascular bundles; Co, cortex layer.

RAPD analyses of re-isolated strains from the nodules

To confirm that the bacteria in the nodules are identical to the original inoculated strains, the bacteria were re-isolated from the nodules and RAPD profiles were compared. The nodules formed by strains DASA 35030 and PDA-1 were excised from the host plant, *A. mangium* and symbiotic bacteria were isolated from the surface sterilized and squashed nodules. The RAPD profiles of the re-isolated bacteria were identical to those of the original inoculated strains (Fig. 2).

Phylogenetic analyses of 16S rRNA gene

Total DNA was extracted from 10 *Acacia* strains and partial sequences (300 bp) of 16S rRNA were analyzed. The strains were expected to be grouped into three different genera according to the homology obtained from a BLAST search. Seven strains were included in the genus *Bradyrhizobium*, and 2 strains grouped in the genus *Rhizobium*. Interestingly, one strain DASA 35030 indicated high homology with the genus *Ochrobactrum*, which is not a member of family *Rhizobiaceae* (data not shown). Three symbiotic strains, DASA 35030, DASA 35082 and PDA-1 were selected, one from each group, and the nearly full sequence of their 16S rRNA gene was determined. The strains DASA 35030, DASA 35082 and PDA-1 were aligned with the sequences of the type strains of the 70 known species belonging to the families *Rhizobiaceae*, *Phyllobacteriaceae*, *Bartonellaceae*, *Brucellaceae* and *Bradyrhizobiaceae*, and the strain PR17/sat of *Ochrobactrum* sp. isolated from a nematode (Babic et al., 2000), that were obtained from DNA

Table 3. Host specificity of the isolates from nodules of *A. mangium*.

Strains	*Acacia mangium*	*Acacia albida*	*Acacia mellifera*	*Acacia tortilis*	*Paraserianthes falcataria*	*Parkinsonia aculeata*	*Leucaena glauca*
PDA-1	+/+	+/+	−	−	+/+	−	−
PDA-2	+/+	+/+	−	−	+/+	−	−
DASA 35030	+/+	+/+	−	−	+/+	−	−
DASA 35022	+/+	+/+	−	−	+/+	−	−
DASA 35027	+/+	+/+	−	−	+/+	−	−
DASA 35052	+/+	+/+	−	−	+/+	−	−
DASA 35082	+/+	+/+	−	−	+/+	−	−

Nodulation was observed at 25 d.a.i (*A. mangium*), 30 d.a.i. (*A. albida*), 21 d.a.i. (*P. falcataria*), respectively. +/+, nodulation/nitrogen fixation; −, no nodulation.

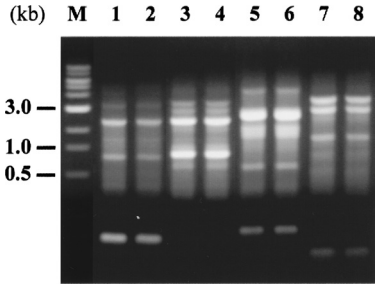

Fig. 2. RAPD profiles of original isolates and re-isolates from nodules.

Strains DASA 35030 and PDA-1 were inoculated to *A. mangium*, and then bacteria were re-isolated from the nodules, respectively. Lanes 1 to 4 are performed with primer 1 and lanes 5 to 8 are with primer 2. Lanes 1 and 5, original strain DASA 35030; lanes 2 and 6, re-isolates of DASA 35030. Lanes 3 and 7, original strain PDA-1; lanes 4 and 8, re-isolates of PDA-1. M, size marker.

databases. On the phylogenetic tree constructed on the basis of the aligned 16S rRNA sequences, the strain DASA 35030 was placed in *Ochrobactrum*/*Brucella* clade within the family *Brucellaceae*. Among the members of the *Ochrobactrum*/*Brucella* clade, *O. intermedium* and *Ochrobactrum* sp. PR17/sat are the most neighboring to the strain DASA 35030. The strain DASA 35082 is included in the *Bradyrhizobiaceae* clade, whereas the strain PDA-1 proved to belong to the *Rhizobiaceae* clade. *B. elkanii* and *R. tropici* are the most neighboring to the strains DASA 35030 and PDA-1, respectively (Fig. 3).

Chemotaxonomic characteristics

Some chemotaxonomic characteristics of the strains are summarized in Table 4. All strains were characterized by ubiquinone 10 (Q-10), which supported that they belong to the α-*Proteobacteria*. The 16S rRNA gene sequencing analysis indicated that strain DASA 35030 was closely related to the genus *Ochrobactrum*. Chemotaxonomic analysis also suggested their phylogenetic resemblance, because strain DASA 35030 and *Ochrobactrum* strains showed a similar fatty acid composition and G+C content of the DNA. Their major fatty acids (more than 10% of the total fatty acids) were 16:0, 18:0, 18:1 and cyclo 19:0 and the G+C content of the DNA ranged from 56.0 to 58.5 mol%. Strain DASA 35082 and its phylogenetic neighbor, *Bradyrhizobium elkanii* IFO 14791^T, also shared similar chemotaxonomic characteristics. The closest organism to strain PDA-1, based on 16S rRNA sequence analysis, was *Rhizobium tropici*. However, the fatty acid composition and the G+C content of the DNA of strain PDA-1 were different from those of *R. tropici* IFO 15247^T. Strain PDA-1 contained 68% of 18:1 in the total fatty acids, although *R. tropici* IFO 15247^T contained only 3%. The G+C content of the DNA of strain PDA-1 and *R. tropici* IFO 15247^T were 65.1 mol% and 59.0 mol%, respectively.

Morphological observation of Acacia nodules

No significant differences were observed in the morphology among the nodules formed by strains, DASA 35030, DASA 35082 and PDA-1 (Fig. 1). The external appearance of *Acacia* nodules was judged as determinate-type morphology. The distinct apical meristem could not be observed by LM (Fig. 1B). The multiple vascular tissues were distributed in the outer cortex layer in a line. The symbiotic zone was observed at the central position of the nodule surrounded by a thick

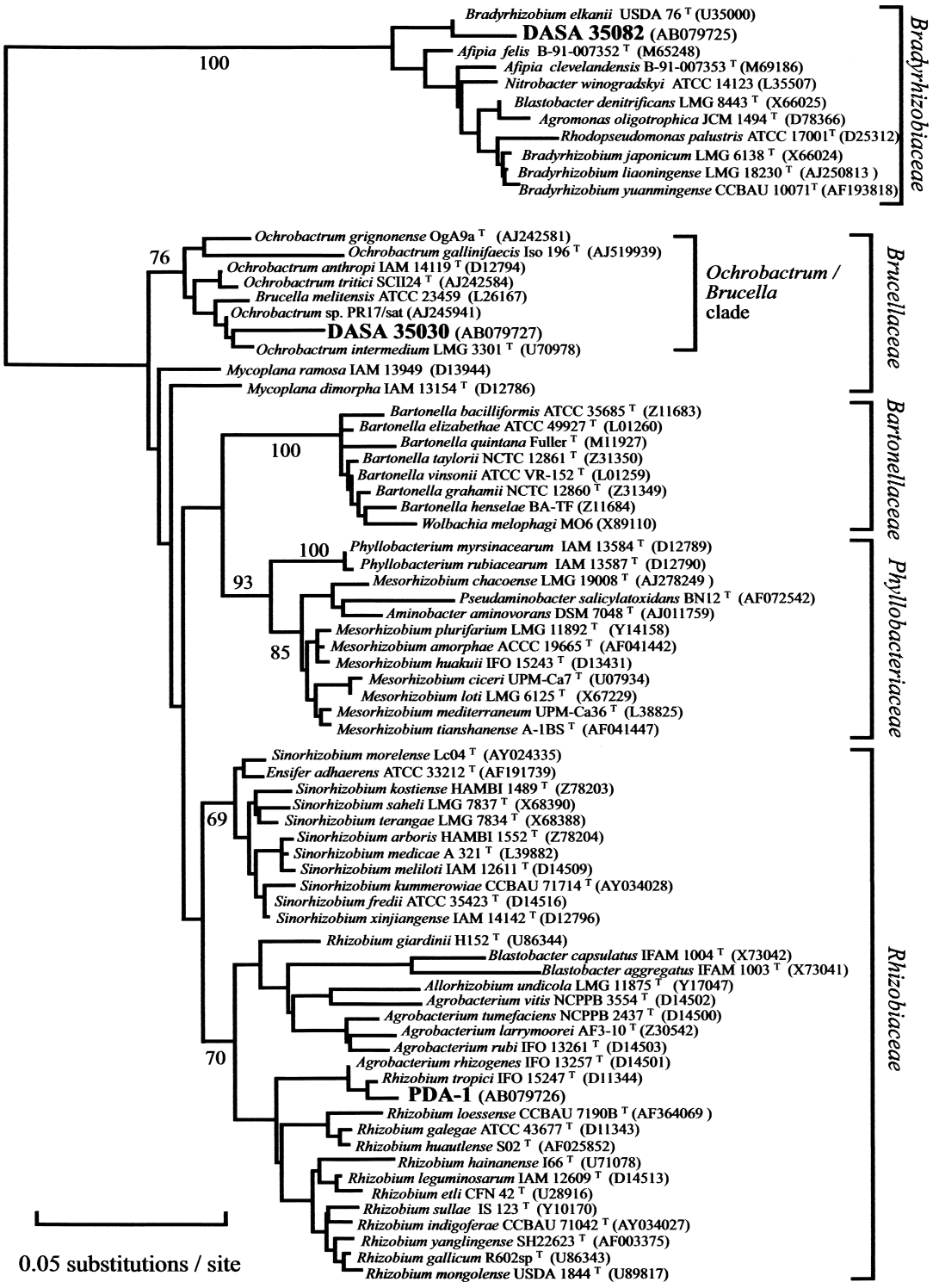

Fig. 3. A minimum-evolution (ME) tree for the strains DASA 35030, DASA 35082 and PDA-1.

The tree was constructed based on nearly full length of 16S rDNA data together with the strain PR17/sat of *Ochrobactrum* sp. isolated from a nematode and 70 known species belonging to the families *Rhizobiaceae*, *Phyllobacteriaceae*, *Bartonellaceae*, *Brucellaceae* and *Bradyrhizobiaceae. Bradyrhizobiaceae* was used as the outgroup based on our recent study (Sawada et al., 2003). The tree was constructed from the distances estimated by the ML method with rate heterogeneous Tamura-Nei model (Tamura and Nei, 1993) (gamma shape parameter=0.5583; proportion of invariable sites=0.5871; number of rate categories=4). The horizontal length of each branch is proportional to the estimated number of nucleotide substitutions. Percent bootstrap support (1,000 replications) is indicated above or below nodes. Accession numbers of the nucleotide sequences are indicated in parentheses.

Table 4. Chemotaxonomic features of the isolates from nodules of *A. mangium*.

Strains	Normal acid									3-OH acid			G+C content of the DNA	Major quinone
	Saturated acid					Unsaturated acid								
	14 : 0	16 : 0	(i)17 : 0	17 : 0	18 : 0	16 : 1	18 : 1	(c)19 : 0	12 : 0	14 : 0	(i)15 : 0	16 : 0	18 : 0	
DASA 35030 (*Ochrobactrum* sp.)	t	14	0	1	18	1	34	17	0	1	0	3	7	58.5	Q-10
Ochrobactrum anthropi IFO15819^T	t	13	0	3	32	1	38	10	0	1	0	2	0	57.2[a]	Q-10
Ochrobactrum anthropi 1[a]	t	16	0	1	15	2	38	12	0	2	0	4	8	56.0	Q-10
Ochrobactrum intermedium IFO15820^T	t	14	0	2	23	t	32	19	0	2	0	2	3	57.8[a]	Q-10
Ochrobactrum tritici SCII24^T	t	11	0	2	15	t	54	10	0	1	0	2	3	56.3	Q-10
DASA 35082 (*Bradyrhizobium* sp.)	t	19	0	t	t	t	67	10	1	2	0	0	0	65.0	Q-10
Bradyrhizobium elkanii IFO14791^T	t	25	0	t	t	t	63	8	2	2	0	0	0	62.9	Q-10
PDA-1 (*Rhizobium* sp.)	0	20	0	t	t	4	68	4	1	2	0	0	0	65.1	Q-10
Rhizobium tropici IFO15247^T	t	36	3	0	t	t	3	25	0	7	8	9	8	59.0	Q-10

T, type strain; t=trace, less than 1%; (i), *iso-*; (c), *cyclo-*.
[a] Data from Holmes et al. (1988).

cortex layer (Fig. 1B). The mosaic-like distribution of the infected cells (IC) and uninfected cells (UIC) were observed by SEM (Fig. 4a, d, g). The infected cells were filled with a lot of bacteroid cells (Fig. 4b, e, h). The bacteroid existence in the infected cell was confirmed by TEM (Fig. 4c, f, i). The bacteroid cells were enwrapped by a peribacteroid membrane-formed symbiosome. The symbiosome in the IC by three strains (DASA 35082, DASA 35030 and PDA-1) contained plural bacteroid cells (Fig. 4c, f, i). The symbiosome of the strain DASA 35082 (*Bradyrhizobium*) contained high numbers (more than 10) of bacteroids in IC (Fig. 4f). Meanwhile, the symbiosome of the strain PDA-1 (*Rhizobium*) contained single or several bacteroid cells (Fig. 4i). Bacteroid existence in a symbiosome of the strain DASA 35030 showed up in the same manner as in the other two strains. The number of bacteroid cells in a symbiosome was variable for DASA 35030; both single and extremely numerous bacteroid cells could be observed in an IC of a nodule (Fig. 4c).

Physiological characterization of root nodule bacteria isolated from A. mangium

Relevant physiological characteristics of three strains, DASA 35030, DASA 35082 and PDA-1, are summarized in Table 5. The strain DASA35030 exhibited several typical features compared to other two rhizobial strains: especially short generation time, wide pH range for growth and salt tolerance (higher than 1 M NaCl).

Discussion

The nodules of *A. mangium* formed by strains DASA 35030, DASA 35082 and PDA-1 showed common morphological features (Figs. 1 and 4). In the infected cells, the symbiosome can be divided into two different types depending on the numbers of bacteroids. One type, called the singular type, is single bacteroid in a symbiosome. This type of symbiosome is generally observed when the symbiont is genus *Rhizobium*. The other one is the so-called plural type. This type of symbiosome containing plural bacteroids is generally observed when the symbiont is genus *Bradyrhizobium*. The symbiosome in *Acacia* nodules in this study was observed as a plural type (Fig. 4). In particular, the symbiosomes of strains DASA 35082 and DASA 35030 contained multiple bacteroids.

It is known that nodule bacteria symbiotic with legu-

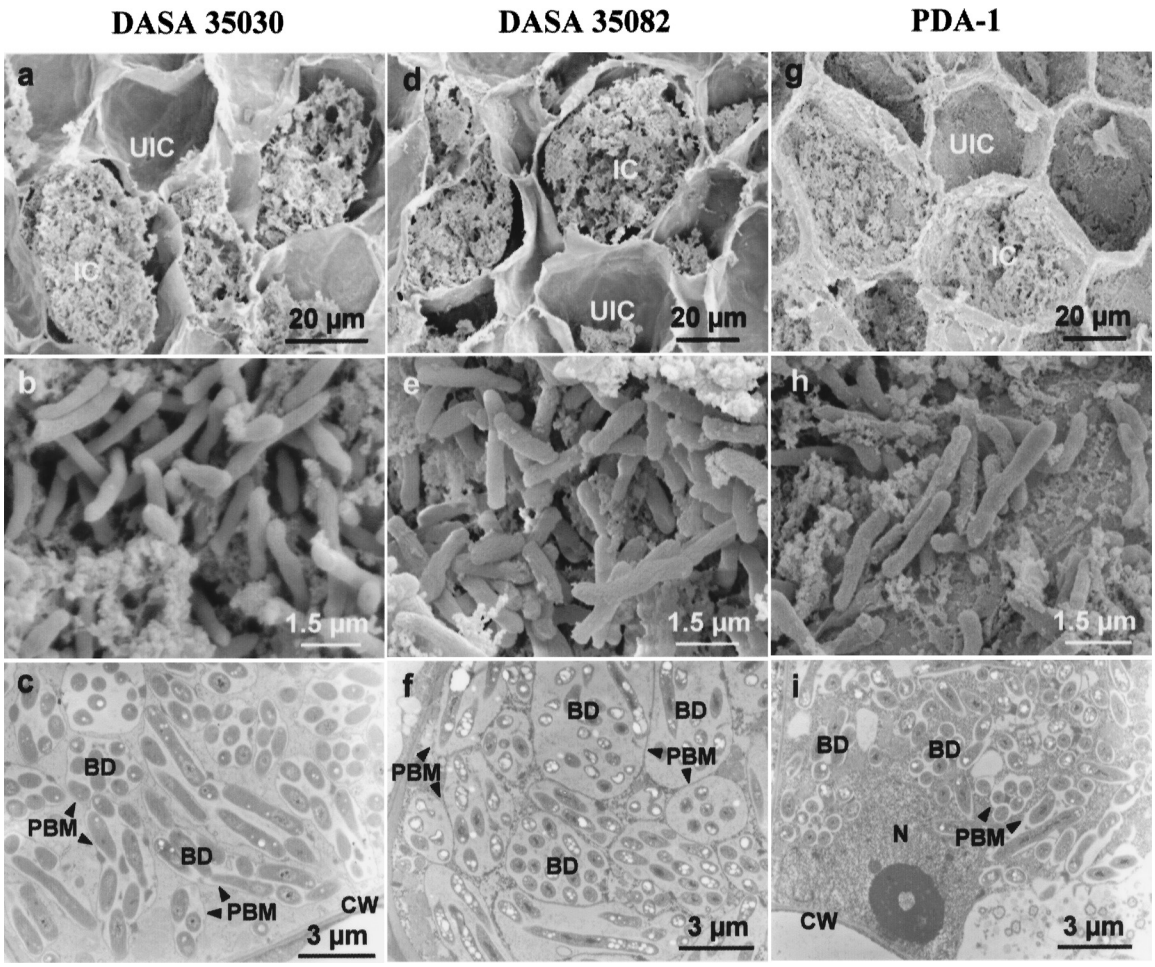

Fig. 4. SEM and TEM observations of *Acacia* nodules formed by DASA 35030, DASA 35082 and PDA-1.

Nodules harvested at 6 weeks after inoculation were observed by SEM (a, b, d, e, g, h) and TEM (c, f, i). Many bacteroids surrounded by peribacteroid membrane were observed. IC, infected cell; UIC, uninfected cell; BD, bacteroid; PBM, peribacteroid membrane; CW, cell wall; N, nucleus.

Table 5. Summary of characteristics of the isolates from nodules of *A. mangium*.

Characters	DASA 35030	PDA-1	DASA 35082
Deduced genus	*Ochrobactrum*	*Rhizobium*	*Bradyrhizobium*
No. of plasmid[a]	2	2	0
Nodulation (*Acacia mangium*)	+	+	+
ARA[b] (nmol/nodule)	0.48	0.45	0.31
Generation time (min)	36	85	301
IAA production	±	+	+++
Opt. pH for growth (pH range)	6–7 (4.5–11)	6 (5–9.5)	6–7 (5–9)
Opt. temperature for growth (°C)	37	28	28
NaCl tolerance (M)	1.02	0.34	0.34
Cell size (µm) (length×width)			
Free-living	1.2×0.2	1.8×0.4	1.7×0.5
Bacteroid	2.2×0.4	2.1×0.4	2.3×0.4

[a] Total DNA was extracted following the technique reported by Casse et al. (1979). Plasmid profile was determined by agarose gel electrophoresis.

[b] ARA was defined in Table 2.

minous plant growing in tropical and sub-tropical areas are very diverse; namely, both genera *Rhizobium* and *Bradyrhizobium* can nodulate the same host plant (Fuentes et al., 2002; Gault et al., 1994; Haukka et al., 1998; Keyser et al., 1982). The strains isolated from *Acacia* trees in this work were classified into three different genera, *Rhizobium* and *Bradyrhizobium* belonging to the family *Rhizobiaceae*, and *Ochrobactrum*, a member of *Brucellaceae*. Strain DASA 35082 was expected to be a member of *Bradyrhizobium* judging from the analyses of the 16S rRNA gene and other taxonomic characters. Another isolate, strain PDA-1, was closely related with *R. tropici* and a few species in genus *Agrobacterium* in 16S rRNA gene analysis (Fig. 3). The genera *Rhizobium* and *Agrobacterium* are closely related to each other phenotypically and genotypically, and members of these two genera are randomly dispersed and intermingled with one another within one clade of the 16S rRNA gene-based phylogenetic tree (Fig. 3) (Sawada et al., 1993, 2003; Willems and Collins, 1993; Yanagi and Yamasato, 1993; Young et al., 2001). Moreover, chemotaxonomic profiles of these genera are also similar to each other (Kuykendall et al., 2004; Tighe et al., 2000; Yokota et al., 1993; Young et al., 2004). Based on these results, it was proposed that these two genera should be amalgamated into one genus named *Rhizobium* (Young et al., 2001). The 16S rRNA sequence analysis suggested that strain PDA-1 belonged to the genus *Rhizobium* and its phylogenetic neighbor is *R. tropici*. However, chemotaxonomic characteristics of strain PDA-1 are different from those of *R. tropici*. The amount of 18:1 in the total fatty acids of strain PDA-1 and *R. tropici* IFO 15247^T were 68% and 3%, respectively. 3-OH *iso* 15:0, 3-OH 16:0 and 3-OH 18:0 were present in *R. tropici* IFO 15247^T but not in strain PDA-1. Furthermore, 16S rRNA sequence similarity between strain PDA-1 and *R. tropici* IFO 15247^T is 98.1%. These results suggested that strain PDA-1 constitutes an independent species in the genus *Rhizobium*, although further taxonomic studies are required to establish a new taxon for the strain PDA-1.

Strain DASA 35030, which exhibits complete symbiotic ability on the original host *Acacia* tree, was identified as a member of *Ochrobactrum*/*Brucella*. The genus *Ochrobactrum* was first described by Holmes et al. (1988) as an isolate from human clinical specimens, and later this genus was found in soil samples and in wheat rhizosphere (Velasco et al., 1998). The close relation between the genus *Ochrobactrum* and *Brucella* is also reported (Lebuhn et al., 2000; Velasco et al., 1998). The strain DASA 35030 was placed in *Ochrobactrum*/*Brucella* clade according to the phylogenetic analysis of 16S rRNA gene (Fig. 3). The strain DASA 35030 was also confirmed as a member of genus *Ochrobactrum* by the fatty acid composition, G+C content (Table 4) and enzymatic activity detected by bacterial identification system for *Enterobacteriaceae* and other gram negative rods (API 20E, bioMerieux sa, l'Etoile, France) (data not shown).

There was no significant difference in the morphology of nodules formed by DASA 35030 compared with those of strains DASA 35082 and PDA-1. These results show that the strain DASA 35030 is an *Ochrobactrum* strain possessing symbiotic nitrogen-fixing ability with leguminous plants. Recent studies based on 16S rRNA sequence analyses revealed that *Burkholderia* (Moulin et al., 2001), *Ralstonia* (Chen et al., 2001), *Methylobacterium* (Sy et al., 2001) and a new species of *Devosia* sp. (Rivas et al., 2002) have the ability to establish symbiosis with leguminous plants. The strain DASA 35030 in this report is the first evidence that a member of *Ochrobactrum*/*Brucella* has the ability to establish symbiotic nitrogen-fixing root nodules with host leguminous trees. How could root nodule bacteria that do not belong to *Rhizobiaceae* acquire the symbiotic ability of leguminous plants? What kind of genetic background is required to become endo-symbiont of leguminous plants? The infection- and nodulation-process of DASA 35030 should be compared with the typical rhizobial strains and the other root nodule bacteria that do not belong to *Rhizobiaceae*. The DNA sequence and structural arrangement of symbiotic genes such as *nod* and *nif* genes will give us the clue to answer the questions.

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