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Cytostructure, lipopolysaccharides, and cell proteins analysis from *Rhizobium fredii*

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Abstract. The symbiotic roles of rhizobia with legumes have been extensively investigated for agricultural applications. We isolated several strains of rhizobia from Taiwanese soils and analyzed their cellular proteins and lipopolysaccharide (LPS) profiles as well as their ultrastructure by electron microscopy. It was found that the flagella have different forms and locations on the surface of fast-growing and slow-growing rhizobia. In addition, the results of their ultrastructural visualization confirmed that poly-β-hydroxybutyrate (PHB) and polyphosphates are present in rhizobia. The ladder profiles of LPS and cellular proteins on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) indicated that different strains of rhizobia possess different profiles, but strains which belong to the same species have similar profiles. It was also shown by Western blot and immunoassay that strain specificity exists between rhizobia cellular proteins and the antisera of anti-rhizobia cells. These results confirm that indigenous *R. fredii* exhibits a significant difference from other species of Rhizobiaceae.

Keywords: Anti-cellular protein antibody; Anti-LPS antibody; Cell proteins; Cytostructure; Lipopolysaccharides; *Rhizobium fredii*; Soybean.

Introduction

The taxonomy of rhizobia, based on bacteria-plant cross inoculation groups, was at one time widely used (Elkan, 1992). However, it is now being replaced by numerical taxonomy, serology, metabolic components, and various molecular techniques (Hennecke et al., 1985; Javis et al., 1986; De Lajudie et al., 1994). All of these methods are able to recognize the fast-growing genus *Rhizobium* and the slow-growing genus *Bradyrhizobium* by their bacterial growth rate (Jordan, 1984; Elkan, 1992).

Based on our knowledge of *Rhizobium*-legume symbiosis, the rhizobial cell is involved in interactions with host plants during nodule development (Schmidt et al., 1989; Stacey et al., 1995). The cell surface components of *Rhizobium* have always been considered important factors in symbiotic effectiveness (Halverson and Stacey, 1986; Hitchcock et al., 1986; Dylan et al., 1990; Gray and Rolfe, 1990; Carlson et al., 1991; Yokota and Sakane, 1991; Niehaus et al., 1993; Ozga et al., 1994) in the specific interactions of rhizobia with legumes. Lipopolysaccharide (LPS) is one component of the bacterial outer membrane and consists of a polysaccharide and lipid A (Hitchcock et al., 1986). Various rhizobial strains differ in LPS composition and serological characters that can nodulate the legume host. Thus, LPS has taxonomic significance in Rhizobiaceae (Yokota and Sakane, 1991). In bacterial taxonomy, the diversity of cellular proteins in different species is also considered an important factor.

In the present study, we extracted LPS and cellular proteins from several strains of *Rhizobium* and *Bradyrhizobium*. These strains were isolated from indigenous soils and purchased from the American Type Culture and Collection (Rockville, MD). The LPS and cellular proteins were analyzed by SDS-polyacrylamide gel electrophoresis and by immunoassay after Western blotting. Based on the results, we can compare the SDS-PAGE profiles of *Rhizobium* and *Bradyrhizobium* as well as different species of *Rhizobium*. In addition, we analyzed the ultrastructural differences between *Rhizobium* and *Bradyrhizobium* by electron microscopy, because these results also provide some information for taxonomy, especially in Rhizobiaceae. Significant differences exist in the cellular structure between that of *Rhizobium* and *Bradyrhizobium*. From these results, we can obtain more information to confirm the taxonomic significance of indigenous *Rhizobium fredii*.

Materials and Methods

Strains of Rhizobia and Cultural Conditions

Nine strains of *R. fredii* and *R. loti* Lu7 were isolated from Taiwanese soils. The collected legumes (soybean) nodules (ca. 0.3 cm diameter) were washed with distilled water several times. This washing and cleaning procedure was done within 4 h of nodule collection. The nodules were then soaked in 4% H_2O_2 for 2 min and then washed

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with sterile water for 3 min. Final nodule treatment included soaking in a 0.1% $HgCl_2$ solution for 2 min and then washing with distilled water. We visually selected the effective nodules based on their pink coloration, which indicated that they contained leghemoglobin. The nodule juice extract was streaked on YEM medium and incubated at 28°C for 4–5 days. After selecting resulting white colonies, the medium was restreaked several times to purify the culture growth. The cultural characteristics, biochemical identification, physiological characteristics and soybean-inoculation tests were compared. The control standard strains of *R. fredii*, *R. loti* ATCC 33669, *R. leguminosarum* bv. *viceae*, *R. leguminosarum* bv. *trifolii*, *R. meliloti* ATCC 9930, *B. japonicum* USDA 110, *B. japonicum* ATCC 103324 and *B. japonicum* ATCC 10927 were purchased from the American Type Culture Collection (Rockville, MD) and the United States Department of Agriculture (Beltsville, MD). All assays with seedlings were performed under microbiologically controlled conditions using nitrogen-free Fåhraeus medium (-NF) (Fåhraeus, 1957) as the plant growth medium.

Extraction of Lipopolysaccharides and Cellular Proteins

Both fast-growing and slow-growing rhizobia were grown on TY medium (tryptone 5 g, yeast extract 3 g, and $CaCl_2$ 1 g per 1 L distilled water) at 28°C for 3 days and 6 days, respectively (Beringer, 1974). They were then centrifuged, suspended in 0.1 M phosphate-buffered saline (0.9% NaCl, pH 7.0 PBS), stirred for 15 min, and recentrifuged at 8,000 rpm for 10 min. The cells were reacted with hot phenol water (0.1% $NaHCO_3$, 95% phenol) at 65°C for 15 min, cooled at 10°C for 15 min, centrifuged, and then the water layer was dialyzed against distilled water for 24 h. After being centrifuged, the distilled water layer was reacted with DNase I (10 μg/ml), RNase A (10 μg/ml), and proteinase K (600 μg/ml) for 1 h at 37°C, then incubated at 65°C for overnight. The solution was then dialyzed against distilled water and centrifuged at 9,000 rpm for 10 min. The supernatant was collected and lyophilized as partially purified LPS (Carlson et al., 1978; Cava et al., 1989). The DNase I and RNase A were purchased from Sigma Chemical Co., St. Louis, MO. The proteinase K was purchased from Merck, Germany.

To obtain cellular proteins, rhizobia were cultured and collected as above, then washed twice with TES buffer (10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.0). The OD_{600} of the cell suspension was adjusted to 0.6. Then the rhizobia were added to 1/4 volume of 5X sample buffer (0.3 M Tris, pH 6.8, 10% [w/v] SDS, 25% [v/v] 2-mercaptoethanol, 50% glycerol, 0.01% bromophenol blue) (Lipsanen and Lindström, 1989). The mixture was boiled for 5 min, centrifuged at 8,000 rpm for 10 min, and finally analyzed with electrophoresis (12.5% SDS-PAGE) and immunoassays (Western blots and agglutination tests) (Catty, 1989).

Preparation and Purification of Anti-Lipopolysaccharide and Anti-Bacterial Cell Antibody

The partially purified LPS (500 mg) or rhizobial cells (10^8 cells/ml) were suspended in 0.1 M PB, pH 7.0 and emulsified with an equal volume of complete Freund's adjuvant (Difco, Detroit, MI). Antiserum against the LPS or rhizobial cells was produced in two New Zealand white rabbits by administering four injections over 1–2 ml adjuvant treated cells over a 2 month period. The antiserum was further purified with ammonium sulphate (33%, w/v) and centrifuged at 7,800 *g* for 20 min. The pellets were resuspended in the same buffer and then dialyzed in 0.01 M PB for 24 h. Final purification used a DEAE-Sephacel (2.4 × 24 cm, Pharmacia) ion exchange column that was eluted with 0–0.5 M NaCl gradient in 5 mM Tris-HCl (pH 8.0) (Catty, 1989). Protein concentration was measured by Lowry's method and OD_{280} (Lowry et al., 1951).

SDS-PAGE, Immunoblotting and Agglutination Analyses of LPS and Cell Proteins

Twenty-five μg LPS from rhizobial cells were separated by SDS-PAGE and detected by silver stain after oxidation with periodic acid (Tsai and Frasch, 1982). The LBS was then electrotransferred to nitrocellulose paper (Transphor^r, 0.45 μm) and then immunoblotted with a 100 dilution of rabbit anti-LPS antisera, a 2,000 dilution of HRP [goat anti-rabbit Ig(A,G,M)]. Finally, AEC (3-amino-9-ethyl-carbazole, $C_{14}H_{14}N_2$, Pierce, Rockford, IL) was used for staining. Agglutination analysis was carried out by the addition of a 25 μl resuspension of cells to a serial dilution of rabbit anti-LPS or anti-cell antisera, which was then agglutinated overnight at 4°C. The titer of this reaction above the original antigen-antibody was determined, and we defined the reaction as positive (Catty, 1989).

Electron Microscopic Observation

Rhizobia were embedded in 2% agar at 40°C, cut into small pieces (1 mm^3), fixed with 2.5% glutaraldehyde in 0.1M PB (pH 7.0) for 2 h at 4°C, and then postfixed with 1% osmium tetroxide in the same buffer for 2–4 h at 4°C. The rhizobia were then dehydrated in an acetone (or ethanol) series and embedded Spurr resin (Yoshiharu and Nakamura, 1980). Ultrathin sections (80 nm, LKB Super Nova) were collected on Formvar-coated copper grids and stained with uranyl acetate, and lead citrate before examination with a JOEL 1200 EX II electron microscope at 80 kV. For negative contrast staining, rhizobia were centrifuged at low speed and washed. The cell suspensions were then dropped on Formvar-coated 300 mesh copper grids and stained with 1% phosphotungstic acid, and the type of flagella was determined with a transmission electron microscope at 80 kV.

Results and Discussion

Fast-growing rhizobia, *R. fredii*, have 2 to 6 peritrichous flagella (Figure 1A). Slow-growing rhizobia, *B.*

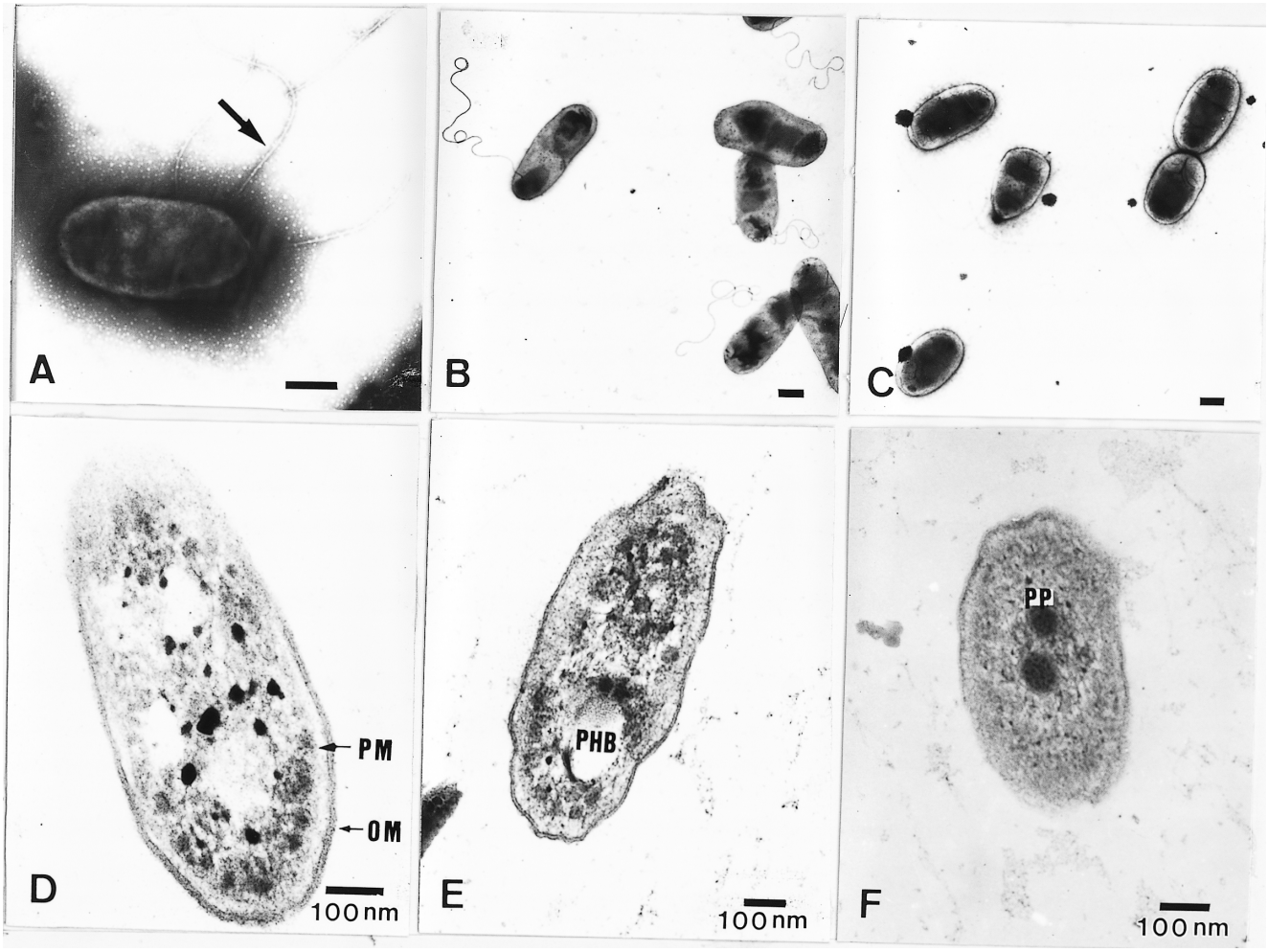

Figure 1. Surface structure and ultrasection of rhizobia. (A, D) *R. fredii* TU6, pH 6.8; (B, F) *B. japonicum* USDA 110; (C) *R. fredii* TU6, pH 5.0; (E) *R. loti* Lu7. OM: outer membrane, PM: plasma membrane, PHB: poly-β-hydroxybutyrate, PP: polyphosphate. Flagella (arrow). Bar present 0.5 μm.

japonicum, are sub-polar (Figure 1B). When the medium pH was decreased from 6.8 to 5.0, the number of *R. fredii* flagella fell (Figure 1C). This may be caused by the pI of flagellin near this pH value (Power et al., 1992). The rhizobia cells tend to be round because the low pH incubation will induce the secretion of extracellular polysaccharides (Song and Lin, unpublished data). Acidic conditions will also affect the activity of the bacterial colonies in the rhizosphere. According to some reports (Smiley and Cook, 1983; Doherty et al., 1988; Streeter, 1988; Ozga et al., 1994) soybean nodulation and nitrogen-fixation activity are reduced in an acid environment. Such environments also influence root hair extension and curling (Lie, 1969), and/or expression of gene nodulation (Richardson et al., 1988).

The ultrastructures of *R. fredii* TU6, *R. loti* Lu7, and *B. japonicum* USDA 110 are shown in Figure 1D, E, F and present a double layer envelope. The dehydration step used for electron microscopy processing could make the capsule structure hard to retain because water-soluble gum components occupy 99% of exopolysaccharide (Gray and Rolfe, 1990). Large amounts of poly-β-hydroxybutyrate (PHB) are accumulated during the late-log or stationary phases (Figure 1E). Polyphosphates could be found in any growth phase (Figure 1F). Incubation of the ultrasection of *R. fredii* KR23 with anti-LPS-gold complex resulted in a deposition of gold particles in the outer layer and cell surface of the rhizobia.

Electrophoretic profiles of rhizobial LPS and cell proteins are shown in Figures 2 and 3. They profiles represent the ladder pattern, except for those of LPS of *R. fredii* TU6 and RO. Some reports (De Maagd et al., 1988; Carrion et al., 1990) have indicated that the O-antigens of these LPSs might commonly consist of oligosaccharides of specific lengths and presented in doublet form. The profiles of the cell proteins and LPS were to be strain-specific (Carrion et al., 1990; De Lajudie et al., 1994). Serological specificity of anti-LPS or anti-cell antisera was assessed by Western blots (Tables 1 and 2). The antisera prepared by injecting rabbits strongly hybridized with original antigen but, in general, failed to react with other rhizobia. The LPS of

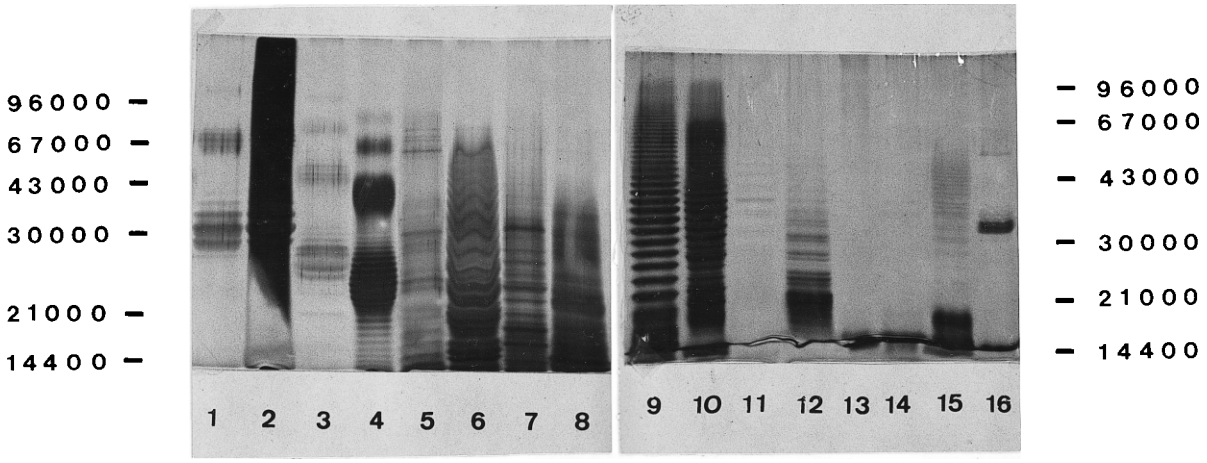

Figure 2. Silver-stained SDS-PAGE (12.5%) containing 25 μg of LPS. Lane 1–8: *R. fredii* TU6, *R. fredii* RO, *R. leguminosarum* bv. *viceae*, *R. loti* Lu7, *R. leguminosarum* bv. *trifolii*, *R. fredii* SB54, *R. fredii* KR23, *B. japonicum* USDA 110; lane 9–16: *R. fredii* KR25, *R. fredii* N23, *R. fredii* SB138, *B. japonicum* ATCC 11927, *R. fredii* NT, *R. meliloti* ATCC 9930, *R. lotii* ATCC 33669, *B. japonicum* ATCC 10324. Molecular markers are indicated.

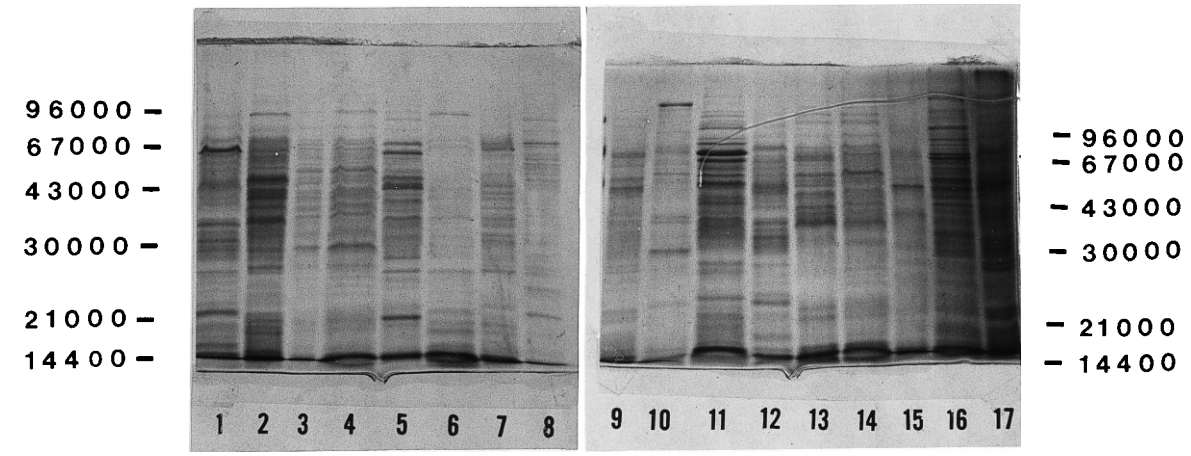

Figure 3. Electrophoretic banding pattern of total cellular proteins (12.5% SDS-PAGE). Lane 1–8: *R. fredii* TU6, *R. fredii* KR23, *R. fredii* KR25, *R. leguminosarum* bv. *viceae*, *R. meliloti* ATCC 9930, *R. lotii* ATCC 33669, *R. fredii* NT; lane 9–16: *R. fredii* RO, *R. fredii* SB138, *R. leguminosarum* bv. *trifolii*, *R. fredii* RO, *R. fredii* TuSO, *R. fredii* N23, *B. japonicum* USDA 110, *B. japonicum* ATCC 11927, *B. japonicum* ATCC 10324. Molecular markers are indicated.

Table 1. Western blots between *Rhizobium* LPS and rabbit anti-*Rhizobium* LPS antisera.

Strain	Antisera			
	R. fredii TU6	*R. fredii* KR23	*R. loti* Lu7	*B. japonicum* USDA 110
R. fredii TU6	+	–	–	–
KR23	–	+	–	–
KR25	–	–	–	+
SB54	–	–	–	–
RO	+	–	–	–
N23	–	+	–	–
NT	–	–	–	–
TuSO	–	–	–	–
SB138	–	–	–	–
R. loti Lu7	–	+	+	+
ATCC 33669	–	–	–	–
R. leguminosarum bv. *viceae*	–	–	–	–
R. leguminosarum bv. *trifolii*	–	–	–	–
R. meliloti ATCC 9930	–	–	–	–
B. japonicum USDA 110	–	–	–	+
ATCC 10324	–	–	–	–
ATCC 11927	–	–	–	–

Table 2. Western blots between *Rhizobium* cell proteins and rabbit anti-*Rhizobium* whole cell antisera.

Strain	Antisera			
	R. fredii TU6	*R. fredii* KR23	*R. loti* Lu7	*B. japonicum* USDA 110
R. fredii TU6	+	–	–	–
KR23	–	+	–	–
KR25	–	–	–	–
SB54	–	–	–	–
RO	–	–	–	–
N23	–	+	–	–
NT	–	–	–	–
TuSO	–	–	–	–
SB138	–	–	–	–
R. loti Lu7	–	–	+	–
ATCC 33669	–	–	–	–
R. leguminosarum bv. *viceae*	–	–	–	–
R. leguminosarum bv. *trifolii*	–	–	–	–
R. meliloti ATCC 9930	+	–	–	–
B. japonicum USDA 110	–	–	–	+
ATCC 10324	–	–	–	–
ATCC 11927	–	–	–	–

R. loti Lu7 did also react with the antisera of *R. fredii* KR23 and *B. japonicum* USDA 110. Antisera of *R. fredii* KR23 reacted with *R. fredii* N23 and *R. loti* Lu7. As to anti-rhizobial cell antisera, those of *R. fredii* KR23 reacted with *R. fredii* KR23 and N23. Antisera of *R. loti* Lu7 and *B. japonicum* USDA 110 only interacted with the original antigen. Antisera of *R. fredii* TU6 cross-reacted with *R. meliloti* ATCC 9930. These results show that the *R. fredii* KR23 and N23 have the same epitopes on their surfaces, and *R. loti* Lu7 might have the same LPS structure, but not in cellular proteins.

The results of electrophoresis and immunoassays indicated that LPS of *R. fredii* was strain-specific, but not species-specific. Thus, the rhizobial LPS of our studies might not behave as a host-recognition determinant because specific relationships exist between the rhizobial species and the leguminous host. We have previously shown that LPS participates in the attachment step of rhizobia to the soybean root hairs (Yang and Lin, 1993). In *Rhizobium*-clover symbiosis, the preferential adsorption to clover root hairs is through a 2-deoxy-glucose sensitive receptor site (Dazzo et al., 1976). Various rhizobial strains differing in LPS composition, serological character, and gel patterns were indicated in previous reports (Carlson et al., 1978; Lipsanen and Lindström, 1989; Yokota and Sakane, 1991). Generally, *R. leguminosarum* strains were found to be formed by a heterogeneous group of biovar subspecies; in which individual strains could be identified by their LPS profiles (Carrion et al., 1990). Given the differences in LPS profiles among strains, separate analysis of them may be very useful for distinguishing between the individual strains of *R. fredii*.

Recently, taxonomic classifications based on a combination of nucleic acid hybridization, protein analysis, cross-inoculation groups, and serology have offered rhizobia as two genus, *Rhizobium* and *Bradyrhizobium*. From the results herein, it is clear that rhizobial cell ultrastructure and some cell components obtained by electron microscopy can be used to distinguish between *Rhizobium* and *Bradyrhizobium*. The profiles of LPS and cellular proteins on SDS-PAGE can reveal the differences that exist between species and strains. Also, we are able to compare the differences of surface epitopes among different species and the relation of phylogeny through serological identification. We can therefore confirm, through the combination of the results in this and our other studies, that *R. fredii* isolated from Taiwanese soil exhibits significantly more taxonomic diversity than other species of Rhizobiaceae.

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