Numerical Taxonomy of Photosynthetic Rhizobia Nodulating *Aeschynomene* Species

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Fifty-two strains of photosynthetic rhizobia isolated from stem and root nodules of *Aeschynomene afraspera*, *Aeschynomene denticulata*, *Aeschynomene evenia*, *Aeschynomene indica*, *Aeschynomene nilotica*, *Aeschynomene pratensis*, *Aeschynomene rudis*, *Aeschynomene schemperi*, and *Aeschynomene sensitiva* were compared with reference rhizobial strains representing *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* species by performing a multivariate statistical analysis of 150 phenotypic characteristics. Eight phena, some of which contained subphena, were identified. *Aeschynomene* rhizobia which synthesize bacteriochlorophyll were grouped in phenon 6, which contained three subphena, while nonphotosynthetic *Aeschynomene* rhizobia were grouped in phena 1 and 2. Our results indicate that the photosynthetic rhizobia nodulating *Aeschynomene* species belong to a phenon (phenon 6) that is separate from the genera *Bradyrhizobium* (phena 1 to 5), *Azorhizobium* (phenon 7), and *Rhizobium* (phenon 8).

The following four genera of stem- and root-nodulating rhizobia are listed in the *Index of the Bacterial and Yeast Nomenclatural Changes* (15): *Rhizobium*, which includes six species (*Rhizobium leguminosarum*, *Rhizobium meliloti*, *Rhizobium loti*, *Rhizobium galegae*, *Rhizobium tropici*, and *Rhizobium huakuii*); *Bradyrhizobium*, which includes one species (*Bradyrhizobium japonicum*); *Azorhizobium*, which includes one species (*Azorhizobium caulinodans*); and *Sinorhizobium*, which includes fast-growing rhizobia that nodulate soybeans and contains two species (*Sinorhizobium fredii* and *Sinorhizobium xinjiangensis*).

Recently, several rhizobial isolates which produce bacteriochlorophyll have been isolated from stem nodules of members of the genus *Aeschynomene* (4, 14). At least 21 *Aeschynomene* species are known to express the trait of nodule formation on aboveground plant parts (13). Flood tolerance combined with fast growth and high N₂-fixing activity make many *Aeschynomene* species suitable for use as green manure in lowland rice cultivation (13). An initial attempt to characterize rhizobial isolates obtained from *Aeschynomene* stem nodules led to the conclusion that it would be difficult to include these rhizobial isolates in one of the four previously described genera (1, 2, 4), especially since some of the isolates had the unique ability to synthesize bacteriochlorophyll inside as well as outside nodules. Therefore, a new genus and species, "*Photorhizobium thompsonianum*," was proposed for the bacteria which are stem symbionts of members of the genus *Aeschynomene* and produce bacteriochlorophyll (4).

Young et al. (28) recently proposed that photosynthetic strain BTAi 1, which was isolated from stem nodules on *Aeschynomene indica*, may belong to the *Bradyrhizobium* cluster after they examined the DNA sequence of a 260-bp 16S rRNA gene fragment. The results of these authors suggested that designation of the genus "*Photorhizobium*" may be premature or unwarranted. However, since the

International Committee on Systematic Bacteriology has recommended that taxonomic designations should be based on examination of multiple phenotypes and phylogenetic traits of a large number of independent isolates (6), we initiated a large-scale study to characterize rhizobial strains nodulating *Aeschynomene* species. In this study, 68 photosynthetic and nonphotosynthetic rhizobium strains belonging to several species that nodulate stems and roots of members of the genus *Aeschynomene* were characterized. About 150 cultural, morphological, and physiological traits were used to study the phenotypic relationships among different groups of our isolates and several reference strains belonging to the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*.

MATERIALS AND METHODS

Bacterial strains. The strains which we used and their sources are listed in Table 1. Rhizobia were isolated from nine species of the genus *Aeschynomene* grown at different locations in the Philippines by using the method described by Vincent (23). Nonphotosynthetic rhizobia were isolated from root and stem nodules of *Aeschynomene afraspera* collected in Ubon, Thailand, and from *Aeschynomene fluminensis* grown in the Philippines. Root-nodulating strains from *Crotalaria juncea* grown in a sugar cane field in India. Root- and stem-nodulating strains were collected from *Sesbania rostrata* grown at four different locations (Laguna, Nueva Ecija, Cotabato, and Quezon) having different soil characteristics in the Philippines.

Growth media. All strains of *Rhizobium* and *Bradyrhizobium* species were grown and maintained on mannitol-yeast extract agar. Photosynthetic rhizobia and *Azorhizobium caulinodans* cultures were grown and maintained on tryptone-glucose-yeast extract agar containing 5 g of tryptone per liter, 1 g of glucose per liter, and 2.5 g of yeast extract per liter. "*Protaminobacter ruber*" NCIB 2879 and *Pseudomonas radiosa* IAM 12089 were grown in medium containing (per liter) 200 ml of potato extract, 10 g of Polypeptone, 10 g of glucose, and 2.5 g of yeast extract. All strains were grown at 30°C and stored on slants or in lyophilized form.

Cultural characteristics. Rhizobia in the logarithmic growth phase were harvested, washed, and resuspended in

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TABLE 1. Origins and hosts of the bacterial strains used.

Strain(s) ^a	Host	Origin ^b	Source ^c	Phenon	Subphenon
Photosynthetic rhizobia					
IRBG 2	<i>Aeschynomene afraspera</i>	Laguna	IRRI	6	A
ORS 322	<i>Aeschynomene afraspera</i>	Senegal	ORSTOM	6	B
IRBG 231	<i>Aeschynomene denticulata</i>	Laguna	IRRI	6	B
IRBG 232, IRBG 233, IRBG 234, IRBG 275	<i>Aeschynomene evenia</i>	Laguna	IRRI	6	B
IRBG 91, IRBG 92, IRBG 276, IRBG 277	<i>Aeschynomene indica</i>	Laguna	IRRI	6	B
BTai 1	<i>Aeschynomene indica</i>	United States	BTI	6	C
IRBG 210, IRBG 223, IRBG 224, IRBG 241	<i>Aeschynomene indica</i>	Iloilo	IRRI	6	B
IRBG 278, IRBG 279, IRBG 280, IRBG 281, IRBG 230, IRBG 282, IRBG 283	<i>Aeschynomene nilotica</i>	Laguna	IRRI	6	A
IRBG 126, IRBG 127, IRBG 284, IRBG 285, IRBG 286, IRBG 287	<i>Aeschynomene pratensis</i>	Laguna	IRRI	6	B
IRBG 123, IRBG 124, IRBG 125, IRBG 228	<i>Aeschynomene pratensis</i>	Iloilo	IRRI	6	B
IRBG 288, IRBG 289	<i>Aeschynomene rudis</i>	Laguna	IRRI	6	B
IRBG 249, IRBG 250, IRBG 251	<i>Aeschynomene schimperi</i>	Laguna	IRRI	6	B
IRBG 121, IRBG 127, IRBG 290, IRBG 291, IRBG 292, IRBG 293, IRBG 294, IRBG 296, IRBG 297	<i>Aeschynomene sensitiva</i>	Laguna	IRRI	6	C
IRBG 114, IRBG 115, IRBG 116, IRBG 117, IRBG 119, IRBG 118, IRBG 120	<i>Aeschynomene sensitiva</i>	Iloilo	IRRI	6	C
Azorhizobium spp.					
IRBG 31, IRBG 32	<i>Sesbania rostrata</i>	Cotabato	IRRI	7	
IRBG 8, IRBG 16	<i>Sesbania rostrata</i>	Laguna	IRRI	7	
IRBG 21	<i>Sesbania rostrata</i>	Nueva Ecija	IRRI	7	
ORS 571	<i>Sesbania rostrata</i>	Senegal	ORSTOM	7	
IRBG 43, IRBG 44, IRBG 45	<i>Sesbania rostrata</i>	Quezon	IRRI	7	
Bradyrhizobium spp.					
IRBG 251, IRBG 252, IRBG 253, IRBG 274, IRBG 298, IRBG 299, IRBG 300	<i>Aeschynomene afraspera</i>	Thailand	IRRI	1	
IRBG 270, IRBG 271, IRBG 272, IRBG 273, IRBG 301, IRBG 302, IRBG 303	<i>Aeschynomene fluminensis</i>	Maahas	IRRI	2	
IRBG 304, IRBG 305, IRBG 306, IRBG 307, IRBG 308, IRBG 309, IRBG 310, IRBG 311, IRBG 312	<i>Crotalaria juncea</i>	India	IRRI	2	
JIR 33, NIAES 3127	<i>Glycine max</i>	Japan	IB	3	
TAL 379, TAL 397	<i>Glycine max</i>	United States	NifTAL	3	
A1017	<i>Glycine max</i>	Japan	IB	4	
CB 1809	<i>Glycine max</i>	?		4	
TAL 102	<i>Glycine max</i>	United States	NifTAL	4	
TAL 1033	<i>Indigofera arrecta</i>	United States	NifTAL	3	
TAL 1037	<i>Indigofera brevicalyx</i>	United States	NifTAL	3	
TAL 289	<i>Indigofera endecaphylla</i>	United States	NifTAL	3	
TAL 760, TAL 761	<i>Indigofera hirsuta</i>	United States	NifTAL	5	
TAL 290, TAL 350	<i>Indigofera suffruticosa</i>	United States	NifTAL	4	
TAL 759, TAL 1038	<i>Indigofera</i> sp.	United States	NifTAL	4	
TAL 835	<i>Macrotyloma africanum</i>	United States	NifTAL	4	
325B	<i>Macrotyloma</i> sp.	United States	NifTAL	4	
TAL 420, TAL 441	<i>Vigna radiata</i>	United States	NifTAL	4	
TAL 169	<i>Vigna radiata</i>	United States	NifTAL	3	
32H1	<i>Vigna unguiculata</i>	?		4	
IRC 256	<i>Vigna unguiculata</i>	India	ICRISAT	5	
IRC 279	<i>Vigna unguiculata</i>	United States	BTI	4	
TAL 209	<i>Vigna unguiculata</i>	United States	NifTAL	3	
Rhizobium spp.					
TAL 1436R, TAL 1436S	<i>Acacia farnesiana</i>	United States	NifTAL	8	A
IC 3038, IC 3100, IC 3509	<i>Cajanus cajan</i>	India	ICRISAT	8	B
TAL 624	<i>Cajanus cajan</i>	United States	NifTAL	8	B
TAL 1768	<i>Gliciridia sepium</i>	United States	NifTAL	8	B
TAL 634	<i>Lathyrus hirsutus</i>	United States	NifTAL	8	B
TAL 638	<i>Lens culinaris</i>	United States	NifTAL	8	B
TAL 1145	<i>Leucaena leucocephala</i>	United States	NifTAL	8	B
TAL 1372, TAL 380	<i>Medicago sativa</i>	United States	NifTAL	8	A
TAL 1797, TAL 1383	<i>Phaseolus vulgaris</i>	United States	NifTAL	8	C
TAL 353	<i>Sesbania grandiflora</i>	United States	NifTAL	8	B
TAL 1123	<i>Sesbania macrocarpa</i>	United States	NifTAL	8	A
TAL 674	<i>Sesbania rostrata</i>	United States	NifTAL	8	A
IC 3071, IC 3073	<i>Sesbania</i> sp.	India	ICRISAT	8	B
TAL 1113, TAL 1115	<i>Sesbania</i> sp.	United States	NifTAL	8	B

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TABLE 1—Continued.

Strain(s) ^a	Host	Origin ^b	Source ^a	Phenon	Subphenon
TAL 382	<i>Trifolium semipilosum</i>	United States	NifTAL	8	B
45	<i>Trifolium</i> sp.	Philippines	BIOTECH	8	A
Photosynthetic nonrhizobia					
<i>Pseudomonas radiosa</i> IAM 12089		Japan	IAM		
<i>Protaminobacter ruber</i> NCIB 1933		Japan	SUT		

^a IRBG, The International Rice Research Institute Bacterial Germplasm, The International Rice Research Institute, Los Baños, Philippines; ATCC, American Type Culture Collection, Rockville, Md.; BIOTECH, National Institutes of Applied Microbiology and Biotechnology, UPLB, University of the Philippines at Los Baños, Los Baños, Philippines; BTI, Boyce Thompson Research Institute, New York, N.Y.; IAM, Institute of Applied Microbiology, The University of Tokyo, Tokyo, Japan; IB, Ibaraki University, Department of Agricultural Chemistry, Ibaraki-ken, Japan; ICRISAT, International Centre for Research in Semi-Arid Tropics, Patancheru, India; IRRI, The International Rice Research Institute, Los Baños, Philippines; NCIB, National Collection of Industrial Bacteria, Torrey Research Station, Aberdeen, Scotland; NifTAL, Nitrogen Fixation in Tropical Agricultural Legumes, University of Hawaii, Paia; ORSTOM, Institut Français de Recherche Scientifique Pour le Développement en Coopération, Paris, France; SUT, Department of Applied Science, Science University of Tokyo, Tokyo, Japan.

^b All locations are in the Philippines unless indicated otherwise.

0.1 M phosphate buffer. Cell suspensions (approximately 10^{15} cells per ml) were placed into individual wells of multiwell plates, and different test media were added by using a multipoint inoculator (Mast Laboratories, Ltd., Merseyside, United Kingdom). Loopfuls of cell suspensions were used to inoculate test media prepared in tubes. Cultures were incubated at $30 \pm 1^\circ\text{C}$ in the dark unless otherwise stated. Reactions were scored after 1, 2, and 7 days of incubation. Media devoid of test substrates were used as negative controls and were incubated along with the test media.

Morphological characteristics. Strains grown on mannitol-yeast extract agar for 7 days were examined for cell and colony morphology and for such characteristics as color, consistency, presence or absence of gummy substances, Gram reaction, presence of spores, and motility by using the methods described in the *Manual of Methods for General Microbiology* (18).

Antibiotic resistance tests. The agar diffusion disc method and a Sensi-Disc eight-place dispenser (BBL, Cockeysville, Md.) were used for antibiotic resistance tests. Zones of inhibition were measured for each disc, and the organisms were scored as resistant (inhibition zone, 0 to 2 mm) or susceptible (inhibition zone, >2 mm). The following antibiotics and amounts per disc were used: amikacin, 10 µg; ampicillin, 10 µg; bacitracin, 10 U; carbenicillin, 100 µg; cephalothin, 30 µg; cefoperazone, 75 µg; cefuroxime, 30 µg; chlortetracycline, 30 µg; ciprofloxacin, 5 µg; cloxacillin, 1 µg; doxycycline, 30 µg; gentamicin, 10 µg; imipenem, 10 µg; kanamycin, 30 µg; lincomycin, 2 µg; methicillin, 5 µg; minocycline, 30 µg; nafcillin, 1 µg; nalidixic acid, 30 µg; netilmicin, 30 µg; nitrofurantoin, 100 µg; novobiocin, 30 µg; oxacillin, 1 µg; penicillin, 100 µg; polymyxin B, 100 U; streptomycin, 10 µg; sulfisoxazole, 250 µg; tobramycin, 10 µg; and vancomycin, 30 µg.

Acetylene reduction assays. Acetylene reduction (nitrogen fixation) activity was determined on basal N_2 -free semisolid agar by using the method described by Watanabe et al. (24).

Bacteriochlorophyll determination. Rhizobia were grown on $0.1\times$ tryptone-glucose-yeast extract agar with a light-dark cycle for 7 days. The cells were harvested and suspended in 25% bovine serum albumin (BSA), and the cells were then used for in vivo absorption spectrum determinations. Fresh root and stem nodules were macerated with a homogenizer in acetone-methanol (7:2, vol/vol), and the suspension was centrifuged for 15 min at 5,000 rpm to separate the solvent extract from the plant debris. The absorption spectra of cell suspensions in 25% BSA and nodule extracts in the acetone-

methanol solution were determined by using a scanning spectrophotometer (Shimadzu Co., Inc., Kyoto, Japan) at wavelengths of 300 to 900 nm.

Utilization of sole carbon sources. Utilization of carbon compounds as sole carbon sources was tested by using a basal medium supplemented with different carbon sources at a final concentration of 0.2% (wt/vol). Bromothymol blue at a concentration of 0.0025% was used as the pH indicator. The pH of each medium was adjusted to 7.0 ± 1 by using sterile 1 M NaOH or 1 N HCl after the carbon source was added. The following carbon sources were tested: acetate, adonitol, L-arabinose, cellobiose, citrate, dextrin, dulcitol, D-fructose, fumarate, galactose, gluconate, glucose, glyc-erol, inulin, meso-inositol, lactose, trehalose, malonate, maltose, D-mannose, D-melibiose, pyruvate, L-rhamnose, D-ribose, saccharic acid, sorbose, sucrose, trehalose, xylitol, and xylose. All of the carbon compounds were filter sterilized before they were added to the basal medium. Basal medium supplemented with mannitol was used as the positive control, and basal medium without mannitol was used as the negative control. Changes in the pH values of the media were recorded after 7 days of incubation.

Utilization of amino acids as sole nitrogen sources. Utilization of amino acids as sole nitrogen sources was tested by using a basal medium in which NH_2SO_4 was replaced by the following amino acids: L-alanine, L-arginine, L-asparagine, L-glycine, L-lysine, L-methionine, L-ornithine, DL-phenylalanine, DL-threonine, DL-tryptophan, and L-tyrosine. Basal medium containing NH_2SO_4 was used as the positive control, and basal medium lacking a nitrogen source was used as the negative control. All amino acids were prepared as stock solutions and were filter sterilized before they were added to basal medium to obtain a final concentration of 10 mM.

Miscellaneous other tests. The oxidase test was performed with cytochrome oxidase test paper (Nissui Seiyaku, Tokyo, Japan). Catalase activity was determined by gas production after addition of a 3% hydrogen peroxide solution. Tests for tolerance for different NaCl concentrations (0.5, 1, 2, and 3%), different pH levels (4, 5, 6, 7, 8, and 9), and different temperatures (20, 30, and 45°C) were performed on mannitol-yeast extract medium. The reaction to litmus milk was examined by using Difco litmus milk medium. Tests for gelatinase activity, starch hydrolysis, nitrate reduction, denitrification, DNase activity, and penicillinase activity were carried out by using the methods described in the *Manual of Methods for General Microbiology* (18).

Nodulation tests. Nodulation by all of the rhizobium strains isolated in this study was verified by examining nodulation in

the homologous host plants, using the methods described by Vincent (23). Legumes were grown in test tubes containing Jensen N₂-free agar medium (23). Stem inoculation tests were performed with 4-week-old plants by applying gum arabic-based cell suspensions with sterile brushes. The plants were incubated at 30°C during the day and at 25°C during the night in a light- and humidity-controlled growth chamber. The presence or absence of nodules and the appearance of the plants were recorded after 2 weeks of incubation.

Numerical analyses. All tests were performed in duplicate, and the data were recorded in binary code. A cluster analysis of 150 phenotypic variables was performed by using single-linkage, average-linkage, and Ward minimum variance methods, using the Clustan software package (25) and an IBM mainframe computer. The unweighted pair group method with averages was chosen instead of the single-linkage, complete-linkage, or Ward method, because the best separation was obtained with this method (19).

RESULTS

A numerical taxonomic analysis of *Aeschynomene* rhizobia showed that all of the photosynthetic rhizobia could be included in one phenon (phenon 6) if a similarity level of 86% was used; three subphenons were formed at a similarity level of 89% (Fig. 1). Slowly growing rhizobia formed the first five phenons, and phenon 1 and 2 contained the nonphotosynthetic *Aeschynomene* rhizobia. Phenon 7 included all of the *Azorhizobium* strains, while phenon 8, which was composed of three subphenons, included all fast-growing rhizobia. The important characteristics of each phenon are described below.

Phenon 1 contained seven strains of rhizobia isolated from *Aeschynomene afraspera* grown in a rice field in Ubon, Thailand. Six strains formed a group at a similarity level of 88%, while strain IRBG 251 clustered with the other strains in the phenon at a similarity level of 83%. All of the strains formed effective root and stem nodules on *Aeschynomene afraspera* but not on other *Aeschynomene* species (data not shown) and were devoid of bacteriochlorophyll when they were grown under the conditions used for photosynthetic rhizobia. The characteristics shared by the members of phenon 1 are shown in Table 2. Strain IRBG 251 is phenotypically distinct from the other rhizobia in the phenon and could be differentiated from the other strains on the basis of utilization of pyruvate, arginine, tryptophan, and tyrosine.

Phenon 2 consisted of eight rhizobial strains isolated from *Aeschynomene fluminensis* and eight strains isolated from *Crotalaria juncea*. The rhizobia isolated from *Aeschynomene fluminensis* exhibited a very high degree of similarity (97%), while the strains isolated from *Crotalaria juncea* clustered at a similarity level of 94%. The overall level of similarity between the strains isolated from *Aeschynomene fluminensis* and the strains isolated from *Crotalaria juncea* was 84%. A total of 97 phenotypic characteristics (including negative characteristics) were shared by the rhizobia belonging to this phenon. The following key characteristics differentiated the strains isolated from *Aeschynomene fluminensis* and the strains isolated from *Crotalaria juncea*: utilization of acetate, fumarate, and pyruvate as energy sources; and resistance to gentamicin and polymyxin B but not to doxycycline, minocycline, netilmicin, and novobiocin.

Phenon 3 included rhizobial strains isolated from *Glycine max* (two strains), *Indigofera* spp. (three strains), and *Vigna radiata* (one strain). Variable carbon compound utilization

reactions were observed for the members of this phenon. All of the strains assimilated all of the amino acids tested except glycine and tryptophan. Litmus milk remained unchanged even after 7 days of incubation. The resistance to antibiotics varied, but all strains were susceptible to amikacin, imipenem, minocycline, and streptomycin.

Phenon 4 contained 15 rhizobial strains isolated from *Glycine max* (5 strains), *Vigna unguiculata* (2 strains), *V. radiata* (2 strains), *Indigofera* sp. (2 strains), *Indigofera suffruticosa* (2 strains), *Macrotyloma africanum* (1 strain), and *Macrotyloma* sp. (1 strain). Hup⁺ *B. japonicum* A1017 and NIAES 3127 and Hup⁻ *B. japonicum* JIR 33 were included in this phenon. Utilization of carbon compounds varied among the strains. Only a few of the strains did not utilize glycine and tryptophan. All strains were resistant to most of the antibiotics tested; the exceptions were amikacin, gentamicin, imipenem, kanamycin, netilmicin, minocycline, and streptomycin. None of the strains turned litmus milk alkaline, but 13% of the strains exhibited an acid reaction and a serum zone after 7 days of incubation. Nitrate was reduced to nitrite by 60% of the strains, but no strain could further reduce nitrite to gaseous nitrous oxide. Strain 32H1 and two strains isolated from *Indigofera* spp. fixed N₂ under free-living conditions.

Phenon 5 contained 2 rhizobial strains isolated from *Indigofera hirsuta* and 2 strains isolated from *V. unguiculata*; these strains clustered at a similarity level of 82%. The carbon utilization patterns of these strains varied widely, while all strains utilized 4 of the 11 amino acids tested (alanine, arginine, asparagine, and ornithine) as carbon and nitrogen sources. All of the strains exhibited multiple antibiotic resistance (Table 2).

Phenon 6 contained 54 photosynthetic rhizobial strains isolated from stem nodules on eight species belonging to the genus *Aeschynomene*; these strains formed a cluster at a similarity level of 84%. All of the strains except ORS 322 and BTAi 1 were isolated in this study. The 54 strains could be subdivided into subphenons 6A, 6B, and 6C. Strain BTAi 1, an isolate obtained from the United States, (22), clearly differed from Philippine isolates obtained from *Aeschynomene indica* and other strains belonging to subphenons 6A and 6B (Fig. 1). The most interesting characteristic of this group was the ability of the rhizobia to synthesize bacteriochlorophyll inside stem nodules under symbiotic and free-living conditions (Fig. 2). All strains belonging to this group had the ability to grow and fix N₂ in the absence of combined N. The symbiotic nitrogenase activity observed in the strains belonging to this group has been reported by other workers (1, 2, 4, 5).

Subphenon 6A contained seven rhizobial strains isolated from *Aeschynomene nilotica* and 1 strain isolated from *Aeschynomene afraspera*. The similarity level for all of the strains isolated from *Aeschynomene nilotica* was very high (96%), while the level of similarity between the strains isolated from *Aeschynomene nilotica* and the strains isolated from *Aeschynomene afraspera* was 91%. Key characteristics that differentiated the strains isolated from the two *Aeschynomene* species included utilization of inulin as a carbon source, acidification of glycerol, alkalization of lactose and xylose, and resistance to vancomycin (Table 3).

Subphenon 6B contained rhizobial strains isolated from several species belonging to the genus *Aeschynomene* (4 strains isolated from *Aeschynomene evenia*, 10 strains isolated from *Aeschynomene pratensis*, 8 strains isolated from three accessions of *Aeschynomene indica*, 2 strains isolated from *Aeschynomene schimperii*, 2 strains isolated from

FIG. 1. Dendrogram showing phenotypic relationships of rhizobia isolated from *Aeschynomene* species and other species of stem and root nodule bacteria. Phenon 1, 2, and 6 contained rhizobia isolated from *Aeschynomene* species, and phenon 6 contained photosynthetic rhizobia.

TABLE 2. Characteristics of rhizobia belonging to different phena^a

Characteristic	% of strains positive											
	Phenon 1 (n = 7) ^b	Phenon 2 (n = 16)	Phenon 3 (n = 6)	Phenon 4 (n = 15)	Phenon 5 (n = 4)	Phenon 6			Phenon 7 (n = 9)	Phenon 8		
						Sub- phenon A (n = 8)	Sub- phenon B (n = 29)	Sub- phenon C (n = 17)		Sub- phenon A (n = 7)	Sub- phenon B (n = 14)	Sub- phenon C (n = 2)
Gummy soft	100	50	83	73	100	0	0	0	0	86	100	100
Gummy hard	0	0	17	13	0	0	0	0	0	0	0	0
Not gummy	0	50	0	13	0	88	100	100	100	14	0	0
Penicillinase	100	100	100	73	75	100	100	100	100	86	71	50
Litmus milk reaction												
Alkaline	57	56	0	0	75	0	14	6	89	14	36	0
Acid	0	0	0	13	25	0	0	0	11	0	21	0
Neutral	43	44	100	73	0	100	86	94	0	86	21	50
Serum formed	0	0	17	13	50	0	0	0	67	29	50	0
Gelatinase	0	63	50	67	50	0	0	0	0	29	36	100
DNase	29	0	0	0	0	0	0	0	0	0	0	50
Starch hydrolysis	0	0	0	0	0	0	0	0	0	0	0	0
Nitrogen fixation	0	0	0	44	0	100	100	100	100	0	0	0
Bacteriochlorophyll	0	0	0	0	0	100	100	100	0	0	0	0
Hayne's medium	100	0	33	33	25	75	59	47	100	43	21	0
Alpha-ketolactose	0	0	17	7	0	0	0	0	0	0	14	0
Denitrification	14	0	0	0	0	0	38	18	0	0	0	0
Nitrate reduction	86	50	33	60	50	88	100	82	0	43	43	0
Growth at:												
pH 4	100	100	17	20	50	13	0	6	100	14	64	0
pH 5	100	100	67	93	100	100	93	100	100	57	93	50
pH 8	100	100	50	53	75	100	93	65	100	71	79	50
pH 9	100	100	33	20	25	13	3	6	100	43	71	0
Growth in the presence of:												
0.5% NaCl	100	100	67	73	75	100	100	100	100	100	86	100
3% NaCl	0	0	33	13	0	0	0	0	11	29	14	50
Utilization (as a carbon source) of:												
Acetate	0	44	0	33	50	88	100	94	100	100	71	50
Adonitol	100	94	100	60	50	88	100	100	100	100	100	0
Arabinose	71	94	100	73	75	100	83	100	0	100	100	0
Cellobiose	71	94	67	20	50	100	0	100	0	100	100	50
Citrate	0	0	17	33	50	100	79	82	44	43	57	50
Dextrin	43	94	83	13	50	13	0	94	100	86	93	0
Dulcitol	57	44	83	20	25	13	10	100	0	86	93	50
Fructose	57	50	83	73	100	100	93	100	100	86	100	50
Fumarate	14	44	33	40	25	0	100	100	100	100	86	0
Galactose	86	94	100	53	50	100	100	100	0	100	100	50
Gluconate	100	94	83	53	100	100	100	100	100	29	100	100
Glucose	71	94	100	73	75	100	100	100	100	100	100	100
Glycerol	100	94	100	73	75	100	100	100	100	100	100	0
Inulin	0	94	83	0	25	88	0	0	0	0	7	0
Inositol	100	94	50	7	25	0	0	100	0	100	100	100
Lactose	43	94	100	20	75	0	14	94	100	100	100	100
Malate	43	0	17	60	50	100	100	100	100	100	79	100
Malonate	100	0	17	7	0	0	52	94	100	86	86	50
Maltose	71	94	83	27	25	100	0	100	0	100	100	50
Mannose	43	94	100	60	75	100	100	100	0	100	100	50
Melibiose	71	13	33	40	50	0	0	6	100	100	100	50
Pyruvate	14	44	33	47	50	0	100	6	100	100	93	100
Rhamnose	57	94	50	40	50	88	14	6	100	100	100	0
Ribose	100	94	83	93	100	88	90	100	100	100	100	0
Saccharic acid	57	50	33	67	100	88	100	100	100	14	36	0
Sorbitose	57	94	17	40	50	0	0	100	100	57	79	0
Sucrose	71	6	0	13	25	0	21	94	0	100	100	0
Trehalose	57	94	33	33	25	88	24	100	100	100	100	50
Xylitol	57	94	17	13	25	0	0	100	0	100	100	50
Xylose	13	94	83	80	100	100	97	100	100	100	100	0
Alkalinization on:												
Arabinose	0	0	33	20	25	0	0	24	100	0	0	0
Fructose	14	50	33	20	25	0	0	0	100	0	7	0
Galactose	71	44	83	33	25	0	0	0	0	0	7	0

Continued on following page

Characteristic	% of strains positive											
	Phenon 1 (n = 7) ^b	Phenon 2 (n = 16)	Phenon 3 (n = 6)	Phenon 4 (n = 15)	Phenon 5 (n = 4)	Phenon 6			Phenon 7 (n = 9)	Phenon 8		
						Sub- phenon A	Sub- phenon B	Sub- phenon C		Sub- phenon A	Sub- phenon B	Sub- phenon C
						(n = 8)	(n = 29)	(n = 17)		(n = 7)	(n = 14)	(n = 2)
Gluconate	100	50	50	13	25	100	0	100	0	0	7	0
Glucose	86	0	83	33	75	0	3	94	100	0	0	0
Glycerol	86	0	33	33	25	0	0	94	0	0	0	0
Lactose	14	44	83	20	75	88	0	0	100	14	7	0
Maltose	71	0	17	33	25	100	97	100	0	57	14	0
Rhamnose	57	50	50	33	50	0	0	6	100	0	0	0
Sucrose	14	6	0	7	0	0	0	0	0	0	0	0
Xylose	0	94	33	27	50	88	0	0	0	0	0	0
Acidification on:												
Arabinose	0	44	17	7	0	0	3	41	0	43	86	0
Fructose	0	0	17	0	0	0	0	0	0	29	21	0
Galactose	0	0	0	7	25	88	0	0	0	57	79	0
Glucose	0	50	0	0	0	100	0	6	0	57	86	0
Glycerol	14	94	50	7	0	88	0	6	0	29	71	0
Lactose	0	0	0	0	0	0	0	0	0	29	29	0
Rhamnose	0	44	0	0	0	0	0	0	0	14	43	0
Sucrose	0	0	0	0	0	0	0	0	0	29	57	0
Xylose	0	0	33	0	0	13	14	100	0	29	57	0
Utilization (as a carbon and nitrogen source) of:												
Alanine	100	100	100	100	100	100	100	100	100	71	93	100
Arginine	14	100	100	87	100	100	100	100	100	57	93	100
Asparagine	100	100	100	93	100	100	100	100	100	100	100	100
Glycine	86	0	17	7	0	0	0	0	0	43	43	100
Lysine	100	56	100	80	75	100	100	100	100	71	71	100
Methionine	100	100	100	87	75	100	66	24	100	57	86	100
Ornithine	100	100	100	93	100	100	100	94	0	86	100	100
Phenylalanine	100	100	100	100	75	100	66	24	0	57	64	100
Threonine	100	100	100	100	75	100	66	24	100	43	64	100
Tryptophan	14	100	50	27	25	100	66	24	100	43	50	100
Tyrosine	14	100	100	80	50	100	66	24	0	86	57	100
Resistance to:												
Amikacin (10 µg/disc)	100	0	0	0	0	0	3	0	0	0	0	0
Ampicillin (10 µg/disc)	100	100	67	80	75	100	90	100	100	57	21	0
Bacitracin (10 U/disc)	100	100	83	80	75	100	90	94	100	57	43	100
Carbenicillin (100 µg/disc)	100	100	17	73	25	100	66	88	0	14	0	0
Cephalothin (30 µg/disc)	100	100	50	93	75	25	7	6	0	29	0	0
Cefoperazone (75 µg/disc)	100	100	100	100	100	100	66	59	100	71	36	0
Cefuroxime (30 µg/disc)	100	100	17	40	75	63	21	0	100	57	29	0
Chlortetracycline (30 µg/disc)	100	0	50	73	100	0	0	0	0	0	36	0
Ciprofloxacin (5 µg/disc)	100	100	83	100	100	38	48	53	100	14	7	0
Cloxacillin (1 µg/disc)	100	100	83	100	100	100	97	94	100	100	79	100
Doxycycline (30 µg/disc)	100	56	100	100	100	0	0	0	0	0	7	0
Gentamicin (10 µg/disc)	100	44	33	13	50	0	69	41	0	0	0	0
Imipenem (10 µg/disc)	0	0	0	7	0	0	0	0	100	0	0	0
Kanamycin (30 µg/disc)	0	0	17	13	50	0	3	0	0	0	0	0
Lincomycin (2 µg/disc)	100	100	100	100	100	100	97	100	100	100	100	100
Methicillin (5 µg/disc)	100	100	100	100	100	100	100	100	100	100	86	100
Minocycline (30 µg/disc)	0	56	0	20	25	0	0	0	0	0	0	0
Nafcillin (1 µg/disc)	100	100	100	100	100	100	97	100	100	100	100	100
Nalidixic acid (30 µg/disc)	100	100	100	93	100	13	14	29	100	100	64	0
Netilmicin (30 µg/disc)	0	56	17	7	50	0	7	35	0	0	0	0
Nitrofurantoin (100 µg/disc)	100	100	100	100	100	100	100	100	0	100	71	0
Novobiocin (30 µg/disc)	100	56	33	67	100	0	17	0	100	71	50	0
Oxacillin (1 µg/disc)	100	100	100	100	100	100	100	100	100	100	100	100
Penicillin (100 U/disc)	100	100	83	87	100	100	93	100	100	71	36	100
Polymyxin B (100 U/disc)	100	44	100	100	100	100	97	100	0	14	43	0
Streptomycin (10 µg/disc)	100	0	0	20	25	0	3	24	0	14	36	0
Sulfisoxazole (250 µg/disc)	100	100	83	100	100	100	100	100	100	100	100	100
Tobramycin (10 µg/disc)	100	100	50	87	100	0	0	0	0	0	7	0
Vancomycin (30 µg/disc)	100	100	100	100	100	88	76	59	100	0	7	0

^a Phenon 1 and 2 contained nonphototrophic rhizobia isolated from *Aeschynomene* species, phenon 6 contained all of the phototrophic rhizobial strains isolated from *Aeschynomene* species, phenon 7 contained all of the *Azorhizobium* strains, and phenon 8 contained all of the fast-growing *Rhizobium* strains. All of the strains examined were gram negative, motile, aerobic, and positive for cytochrome oxidase and catalase, did not produce clots in litmus milk, did not hydrolyze starch, did not tolerate 5 to 10% NaCl, did not grow at 39 to 45°C, and grew at 20 to 30°C at pH 6 to 7.

^b n is the number of strains examined.

FIG. 2. Absorption spectra for photosynthetic rhizobia (dotted line) in 25% BSA and *Aeschynomene afraspera* stem nodule extracts in acetone-methanol (7:2, vol/vol) (solid line), showing the positions of chlorophyll and bacteriochlorophyll *a* at wavelengths of 660 and 770 nm, respectively.

Aeschynomene rudis, 1 strain isolated from *Aeschynomene denticulata*, and 1 strain isolated from *Aeschynomene afraspera* [ORS 322]). Most of the strains utilized the majority of the carbon compounds tested. Only the strains isolated from *Aeschynomene pratensis* utilized trehalose. None of the rhizobial strains isolated from *Aeschynomene evenia* utilized most of the amino acids as nitrogen sources. Of the 29 antibiotics tested, only 5 were found to be inhibitory. Nitrate was reduced, but denitrification was observed only in strains isolated from *Aeschynomene evenia* and in a few strains isolated from *Aeschynomene pratensis*.

Subphenon 6C contained 16 rhizobial strains isolated from *Aeschynomene sensitiva* and 1 strain isolated from *Aeschynomene indica* (strain BTAi 1). The rhizobia isolated from *Aeschynomene sensitiva* were homogenous (similarity level, 90%). A total of 93 phenotypic characteristics (including negative characteristics) were shared by all of the strains in the subphenon. Strains isolated from *Aeschynomene sensitiva* differed from BTAi 1 in the following characteristics: utilization of acetate, lactose, and malonate; and alkalization of glucose and glycerol. Arginine was the only amino acid utilized by strains isolated from *Aeschynomene sensitiva* strains but not by BTAi 1. All strains isolated from *Aeschynomene sensitiva* were resistant to bacitracin and carbenicillin, in contrast to BTAi 1.

The phenotypic groups of stem-nodulating rhizobia isolated from *Aeschynomene* species are somewhat similar to the cross-inoculation groups. The strains belonging to cross-inoculation groups 2 and 3 of Alazard et al. (1) were similar to the strains belonging to phenotypic subphenon 6A and 6B (Fig. 1). However, strains isolated from *Aeschynomene indica* and *Aeschynomene sensitiva*, which formed a separate phenotypic group (subphenon 6C), also belonged to cross-inoculation group 2 (1).

Phenon 7 was a cluster that contained closely related

organisms and formed at a similarity level of 88%. All of the phenon 7 strains except strain ORS 571 were isolated from stem and root nodules of *Sesbania rostrata*. Strain ORS 571, the type strain of *Azorhizobium caulinodans*, was obtained from Institut Francais de Recherche Scientifique Pour le Developpement en Cooperation. This group was homogeneous, and its members shared 129 phenotypic characteristics (including negative characteristics). All of the strains fixed N_2 ex planta, but they did not produce bacteriochlorophyll inside or outside the nodules.

Phenon 8 contained 23 rhizobial strains and was divided into three subphenon, subphenon 8A, 8B, and 8C. The strains grew rapidly on mannitol-yeast extract agar, were motile, did not produce alkali in sugar media, and utilized a wide range of carbon compounds. These characteristics are similar to those generally found in members of the genus *Rhizobium* (11).

Subphenon 8A contained six rhizobial strains (two variant strains isolated from *Acacia farnesiana*, two strains isolated from *Medicago sativa*, 1 strain isolated from *S. rostrata*, and 1 strain isolated from *Sesbania macrocarpa*. Strain TAL 674, which supposedly was isolated from *S. rostrata*, appeared to be either mislabeled or a contaminant. These strains were obtained from the Nitrogen Fixation in Tropical Agricultural Legumes culture collection. The strains belonging to this subphenon utilized a wide range of carbon compounds and acidified most of them. Aspartate was the only amino acid used as a nitrogen source. All of the strains were resistant to cloxacillin, lincomycin, methicillin, nafcillin, nalidixic, nitrofurantoin, oxacillin, and sulfisoxazole; 43% of the strains reduced nitrate, but none could denitrify.

Subphenon 8B was large and diverse; it contained strains isolated from *Cajanus cajan* (four strains), *Sesbania* sp. (five strains), *Gliciridia sepium* (one strain), *Leucaena leucocephala* (one strain), *Lens culinaris* (one strain), *Lathyrus hirsutus* (one strain), and *Trifolium semipilosum* (one strain). These strains used a wide range of carbon compounds as energy sources and were resistant to lincomycin, nafcillin, oxacillin, and sulfisoxazole (Table 2).

Subphenon 8C contained two rhizobial strains isolated from *Phaseolus vulgaris*, and these strains exhibited a similarity level of 90%. Strains belonging to this subphenon could be differentiated from other strains on the basis of their ability to utilize various carbon sources.

DISCUSSION

The phenotypic character-based numerical taxonomy method used in this study clearly showed that all photosynthetic rhizobial strains can be grouped together at a similarity level of 86% and cluster with other well-defined groups of rhizobia only at low levels of similarity. This suggests that the photosynthetic rhizobia are members of a new genus, but this conclusion is contradicted by the results of Young et al. (28), which were based on a 16S rRNA gene sequence analysis and suggested that one of the photosynthetic isolates obtained from *Aeschynomene indica* clusters with the *B. japonicum* group. Moreover, the results of our 16S rRNA gene sequence and fatty acid profile analyses of a number of photosynthetic isolates obtained from *Aeschynomene* species also suggest that these organisms should be included in the *Bradyrhizobium* cluster (20), although clearly not in the three previously described *B. japonicum* DNA homology groups distinguished by Hollis et al. (9). The apparent discrepancy between these results and the observations of

TABLE 3. Characteristics that differentiate the nonphotosynthetic and photosynthetic rhizobia isolated from *Aeschynomene* species

Characteristic	Reaction of:											Nonphotosynthetic	
	Photosynthetic rhizobia isolated from:												
	<i>A. afraspera</i> (n = 2) ^a	<i>A. denticulata</i> (n = 1)	<i>A. evenia</i> (n = 4)	<i>A. indica</i> (n = 8)	<i>A. indica</i> (n = 1) ^b	<i>A. nilotica</i> (n = 7)	<i>A. pratensis</i> (n = 10)	<i>A. rudis</i> (n = 2)	<i>A. schemperi</i> (n = 3)	<i>A. sensitiva</i> (n = 16)	<i>A. afraspera</i> (n = 5)	<i>A. fluminensis</i> (n = 7)	
Nitrogen fixation	+	+	+	+	+	+	+	+	+	+	—	—	
Bacteriochlorophyll <i>a</i>	+	+	+	+	+	+	+	+	+	+	—	—	
Litmus milk reaction													
Alkaline	D	—	—	D	+	—	—	—	—	—	D	+	
Neutral	D	+	+	D	—	+	+	+	+	+	D	—	
Growth at:													
pH 4	D	—	—	—	+	—	—	—	—	—	+	+	
pH 8		+	+	+	—	+	+	+	+	D	+	+	
pH 9	D	—	D	—	—	—	—	—	—	—	+	+	
Denitrification	—	+	+	—	—	—	D	—	—	—	—	—	
Utilization of carbon sources													
Acetate	D	+	+	+	—	+	+	+	+	+	—	+	
Adonitol	D	+	+	+	+	+	+	+	+	+	+	+	
Cellobiose	D	—	—	—	+	+	—	—	—	+	D	+	
Citrate	+	+	+	+	+	+	D	+	+	+	—	—	
Dextrin	D	—	—	—	—	—	—	—	—	+	D	+	
Dulcitol	D	—	—	—	+	—	D	—	—	+	D	+	
Fumarate	D	+	+	+	+	—	+	+	+	+	—	+	
Inositol	—	—	—	—	+	—	—	—	—	+	—	+	
Inulin	—	—	—	—	—	+	—	—	—	—	+	+	
Lactose	—	—	—	—	—	—	D	—	—	+	D	+	
Malate	+	+	+	+	+	+	+	+	+	+	D	—	
Malonate	—	+	D	D	—	—	—	+	+	+	+	—	
Maltose	D	—	—	—	+	+	—	—	—	+	D	+	
Pyruvate	D	+	+	+	+	—	+	+	+	—	—	+	
Rhamnose	D	—	—	D	+	+	—	—	—	—	D	+	
Ribose	D	+	+	D	+	+	+	+	+	+	+	+	
Sorbitol	—	—	—	—	+	—	—	—	—	+	D	+	
Trehalose	D	—	—	—	+	+	+	—	—	+	D	+	
Acidification of carbon sources													
Galactose	—	—	—	—	—	+	—	—	—	—	—	—	
Glucose	D	—	—	—	+	+	—	—	—	—	—	—	
Glycerol	—	—	—	—	+	+	—	—	—	—	—	+	
Rhamnose	—	—	—	—	—	—	—	—	—	—	—	+	
Xylose	D	—	—	—	+	—	D	—	—	+	—	—	
Alkalinization of carbon sources													
Gluconate	D	—	—	—	+	+	—	—	—	+	+	—	
Glucose	—	—	—	—	—	—	—	—	D	+	+	—	
Glycerol	—	—	—	—	—	—	—	—	—	+	+	—	
Lactose	—	—	—	—	—	+	—	—	—	—	D	+	
Maltose	D	+	+	+	+	+	+	+	+	+	D	—	
Xylose	—	—	—	—	—	+	—	—	—	—	—	+	
Utilization of amino acids as C and N sources													
Arginine	+	+	+	+	—	+	+	+	+	+	—	+	
Glycine	—	—	—	—	—	—	—	—	—	—	+	—	
Methionine	+	+	—	+	+	+	D	+	+	—	+	+	
Phenylalanine	+	+	—	+	+	+	D	+	+	—	+	+	
Threonine	+	+	—	+	+	+	D	+	+	—	+	+	
Tryptophan	+	+	—	+	+	+	D	+	+	—	—	+	
Tyrosine	+	+	—	+	+	+	D	+	+	—	—	+	
Resistance to antibiotics													
Amikacin (10 µg)	—	—	—	—	—	—	—	—	—	—	+	—	
Ampicillin (10 µg)	D	—	D	+	+	+	+	+	+	+	+	+	
Bacitracin (10 U)	+	+	+	+	—	+	+	+	—	+	+	+	
Carbenicillin (100 µg)	D	—	D	D	—	+	+	D	—	+	+	+	
Cefoperazone (75 µg)	+	—	—	D	+	+	+	—	+	D	+	+	
Cefuroxime (30 µg)	—	—	D	D	—	D	—	—	+	—	+	+	
Cephalothin (30 µg)	—	—	—	D	+	D	—	—	—	—	+	+	
Chlortetracycline (30 µg)	—	—	—	—	—	—	—	—	—	—	+	—	
Ciprofloxacin (5 µg)	—	+	+	D	+	D	D	D	—	D	+	+	
Doxycycline (30 µg)	—	—	—	—	—	—	—	—	—	—	+	—	
Gentamicin (10 µg)	—	+	D	D	—	—	+	D	+	D	+	+	
Nalidixic acid (30 µg)	—	—	D	D	+	—	+	—	—	D	+	+	
Netilmicin (30 µg)	—	—	D	—	—	—	—	—	—	D	—	—	
Novobiocin (30 µg)	—	—	D	—	—	—	—	—	+	—	+	—	
Streptomycin (10 µg)	—	—	D	—	—	—	—	—	—	D	+	—	
Tobramycin (10 µg)	—	—	—	—	—	—	—	—	—	—	+	+	
Vancomycin (30 µg)	—	—	+	D	+	+	+	D	+	D	+	+	

^a *n* is the number of strains examined.^b Strain BTAi 1.^c +, 80% or more of the strains are positive; —, 80% or more of the strains are negative; D, 79% or fewer of the strains are negative.

TABLE 4. Differential characteristics of aerobic and anaerobic photosynthetic bacteria^a

Characteristic	Aerobes			Facultative anaerobes	
	Rhizobia	<i>Protaminobacter ruber</i> NCIB 2879	<i>Pseudomonas radiora</i> IAM 12089	<i>Rhodopseudomonas palustris</i>	<i>Rhodopseudomonas gelatinosa</i>
Colony color	Pink ^b	Pink	Pink	Red-brown	Yellow-brown
Flagellation	Subpolar to polar	Polar	Polar	Subpolar	Subpolar
Denitrification	D	—	—		
Nitrate reduction	+	—	+		
Penicillinase	+	+	—	+	+
Anaerobiosis	—	—	—	+	+
Gelatinase	—	—	—	+	— ^c
Bacteriochlorophyll production					
Aerobic conditions	+	+	+	+	+
Anaerobic conditions	—	—	—	+	+
Continuous light	—	—	—	+	+
Intermittent light	+	+	+	+	+
Nitrogen fixation (free living)	+	—	—	+	+
Growth at pH 5	+	+ ^d	+	+	
Utilization (as a carbon source) of:					
Arabinose	+	—	+		
Citrate	D	—	+	—	+
Fructose	+	—	—	—	—
Glucose	+	—	+	—	+
Glycerol	+	+ ^e	+ ^e	+ ^c	— ^c
Malonate	D	—	—	+ ^c	+ ^c
Mannose	+	— ^e	— ^e	— ^c	+ ^c
Pyruvate	D	—	—	+	+
Succinate	+	—	—	—	D
Trehalose	D	—	—		
Utilization (as a nitrogen source) of:					
Alanine	+			— ^c	+ ^c
Asparagine	+			— ^c	+ ^c
Resistance to:					
Amikacin (10 µg)	—	+	—		
Carbenicillin (100 µg)	D	—	—		
Cefoperazone (75 µg)	D	—	—		
Cefuroxime (30 µg)	—	+	—		
Ciprofloxacin (30 µg)	D	+	+		
Cloxacillin (1 µg)	+	+	+		
Gentamicin (10 µg)	D	+	—		
Nalidixic acid (30 µg)	—	+	+		
Netilmicin (30 µg)	—	+	—		
Nitrofurantoin (100 µg)	+	+	+		
Streptomycin (10 µg)	—	+	—		
Sulfisoxazole (250 µg)	—	+	+		
Tobramycin (10 µg)	—	+	—		
Vancomycin (30 µg)	D	+	+		
Nodulation on <i>Aeschynomene</i> spp. ^f	E	N	N	N	N

^a +, 80% or more of the strains are positive; —, 80% or more of the strains are negative; D, 79% or fewer of the strains are negative. All data were obtained in this study unless indicated otherwise.

^b Color of colonies when the organisms are grown under intermittent light-dark illumination conditions.

^c Data from reference 22b.

^d Weak reaction.

^e Data from reference 22a.

^f E, nodulates the host legume; N, no nodules are formed on either the stems or the roots of *Aeschynomene* species.

Young et al. (28) may not be significant per se, but rather may be a reflection of the currently very preliminary and inconclusive taxonomy of the highly diverse genus *Bradyrhizobium* (9, 10, 17, 21). At this time, there are valid arguments for and against including photosynthetic rhizobia in the *Bradyrhizobium* cluster.

Previous studies of the phenotypic characteristics of strain BTA11 isolated from stem nodules of *Aeschynomene indica* (22) and strain BICC 608 isolated from stem nodules of *Aeschynomene aspera* (3) led to the conclusion that these strains are examples of an intermediate type, having char-

acteristics of members of both the fast-growing *Rhizobium* species and the slowly growing *Bradyrhizobium* species. The relatively fast growth of these strains, their ability to utilize disaccharides and tricarboxylic acid cycle intermediates (Table 2), the presence of a significant level of NADP-linked 6-phosphogluconate dehydrogenase activity, and the pentose phosphate pathway (3) are characteristics of *Rhizobium* strains. Characteristics such as subpolar flagellation, alkalization of various compounds, reaction to litmus milk, and resistance to carbenicillin (Table 2) are characteristics of *Bradyrhizobium* strains. In addition, Chakrabarti et al. (3)

found that strain BICC 608 exhibited DNA homology with representative *Rhizobium* and *Bradyrhizobium* strains.

On the basis of 16S rRNA oligonucleotide catalogs and the results of DNA-rRNA hybridization studies, it has been suggested that there is a close relationship between *Bradyrhizobium* strains and the facultatively anaerobic photosynthetic organism *Rhodopseudomonas palustris* (8, 10). Young et al. (28) have confirmed this observation and have also reported that the 16S rRNA gene sequences of strain BTAi 1 and *Rhodopseudomonas palustris* resemble *Bradyrhizobium* sequences. Jarvis et al. (10) have suggested that phylogenetic classifications should group the genera *Bradyrhizobium* and *Rhodopseudomonas* together. However, the aerobic photosynthetic bacteria, including the rhizobia, differ from the anaerobic photosynthetic bacteria in that the former synthesize bacteriochlorophyll only under aerobic conditions, whereas the latter require anaerobic conditions (7). The photosynthetic rhizobia produce the bacteriochlorophyll pigment during incubation under intermittent light-dark conditions but not during incubation under continuous illumination or continuous darkness (data not shown); however, this was not the case for marine bacteria (7) and other aerobic photosynthetic bacteria (16). The photosynthetic rhizobia also differ from other aerobic photosynthetic bacteria in that the former use a wide range of carbon compounds and are susceptible to many antibiotics (Table 4). In addition, the photosynthetic rhizobia are able to nodulate and form symbiotic associations with legumes (homologous plants), characteristics which are not found in nonrhizobial photosynthetic bacteria (Table 4). Phylogenetically, the aerobic photosynthetic bacteria, including the photosynthetic rhizobia, and the anaerobic anoxygenic phototrophs are closely related since they belong to the alpha purple groups (26, 27), but physiologically there are distinct differences between these groups.

In our dendrogram analysis we used a total of 150 characters, and these were weighted equally in accordance with the Adansonian principle. Besides the presence of bacteriochlorophyll, the following characteristics distinguish strains of photosynthetic rhizobia from strains of bradyrhizobia: gum production; dinitrogen-dependent growth and N₂ fixation; utilization of acetate, citrate, and malate; alkalization on maltose; susceptibility to cephalothin, doxycycline, and tobramycin; and resistance to nalidixic acid. The genus *Bradyrhizobium* is highly diverse (9–11, 17), and some of the diagnostic characteristics listed above are not completely unknown in this genus. The five phenotypes representing *Bradyrhizobium* strains clustered at a much lower similarity level (73%) than the three subphenotypes in the group of photosynthetic rhizobia do.

Although until recently the genus *Bradyrhizobium* was thought to have only one species (*B. japonicum*), the genus has been reported to be composed of at least three distinct subgroups (9, 17, 21). Recently, on the basis of DNA homology data another species, *Bradyrhizobium elkanii* sp. nov., has been described (12). On the basis of phenotype characteristics, in this study we distinguished the photosynthetic rhizobia from members of the genera *Bradyrhizobium*, *Rhizobium*, and *Azorhizobium* at similarity levels of 73, 74, and 79%, respectively. Determining whether the photosynthetic rhizobia should be assigned to a new genus or to the genus *Bradyrhizobium* as a new species will require further work.

ACKNOWLEDGMENTS

We thank Pete Malabuyoc for technical assistance and F. de Bruijn (Michigan State University, East Lansing) and Peter Young

(University of York, United Kingdom) for providing valuable suggestions.

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