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Genetic and Phenetic Analyses of *Bradyrhizobium* Strains Nodulating Peanut (*Arachis hypogaea* L.) Roots

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Seventeen *Bradyrhizobium* sp. strains and one *Azorhizobium* strain were compared on the basis of five genetic and phenetic features: (i) partial sequence analyses of the 16S rRNA gene (rDNA), (ii) randomly amplified DNA polymorphisms (RAPD) using three oligonucleotide primers, (iii) total cellular protein profiles, (iv) utilization of 21 aliphatic and 22 aromatic substrates, and (v) intrinsic resistances to seven antibiotics. Partial 16S rDNA analysis revealed the presence of only two rDNA homology (i.e., identity) groups among the 17 *Bradyrhizobium* strains. The partial 16S rDNA sequences of *Bradyrhizobium* sp. strains form a tight similarity (>95%) cluster with *Rhodopseudomonas palustris*, *Nitrobacter* species, *Afipia* species, and *Blastobacter denitrificans* but were less similar to other members of the α-*Proteobacteria*, including other members of the *Rhizobiaceae* family. Clustering the *Bradyrhizobium* sp. strains for their RAPD profiles, protein profiles, and substrate utilization data revealed more diversity than rDNA analysis. Intrinsic antibiotic resistance yielded strain-specific patterns that could not be clustered. High rDNA similarity appeared to be a prerequisite, but it did not necessarily lead to high similarity values between RAPD profiles, protein profiles, and substrate utilization. The various relationship structures, coming forth from each of the studied features, had low compatibilities, casting doubt on the usefulness of a polyphasic approach in rhizobial taxonomy.

Bacterial classification can be based on phenotypic and/or genotypic features. Phenotyping can be based on morphological, physiological, or biochemical aspects and, in the case of the family *Rhizobiaceae*, also on symbiotic compatibility with legume host plants. Genotyping can be done by various methods such as DNA (rRNA) nucleotide sequence analysis, amino acid sequence analysis, DNA:DNA hybridization, DNA:rRNA hybridization, randomly amplified polymorphic DNA (RAPD) fingerprinting, restriction fragment length polymorphism (RFLP), fingerprinting for repetitive sequences in the genome, RNA oligonucleotide cataloguing, and mol% guanine plus cytosine (G+C) of total DNA.

The official classification of the genus *Bradyrhizobium*, as presented in *Bergey's Manual of Systematic Bacteriology* (22), considers only phenotypic features and mol% G+C. Later, genotypic features were also described (10). The subcommittee on the taxonomy of the *Rhizobiaceae* proposed that taxonomic amendments should adhere to minimal standards involving three genotypic features (DNA:DNA homology, rRNA homology, RFLP), three phenotypic features (symbiotic properties, cultural and morphological characteristics), and one mixed feature (multilocus enzyme electrophoresis) (13).

Determining genetic relationships between bacterial strains may well be done by comparative 16S rRNA sequence analysis. Relationships between members of the *Rhizobiaceae* have been phylogenetically delineated by using complete (51, 54, 55) and partial (56) 16S rRNA gene (rDNA) sequences. The presentation of 16S rDNA sequence dissimilarities as phylogenetic distances is, however, based on a number of inferences and assumptions that are not without controversy. We, therefore, present sequence dissimilarities in a purely Adansonian, numerical taxonomic fashion, treating each sequence alignment site as an operational taxonomic unit. This yields a dissimilarity dendrogram which is much less speculative than a phylogenetic tree yet presents the empirical data.

Genotypic characterization based on RAPD and on amplified repetitive sequences (REP/ERIC-PCR) is rapidly advancing and may replace and/or supplement RFLP. *Bradyrhizobium* strains have been included in such RAPD and REP/ERIC-PCR studies (7, 15, 23a).

Phenotypic characterization based on intrinsic antibiotic resistance has been used with a view to strain identification in ecological studies (23); serological techniques have, however, been used more often for such purposes (e.g., references 25 and 40a). Phenotyping based on substrate utilization tests is commonly used for numerical taxonomic purposes (e.g., references 8, 9, 11, and 28) and yields information with a strong ecological significance when the substrates used occur in the organism's habitat. Aromatic compounds may be important substrates for soilborne bacteria, as lignin yields aromatic degradation products.

Analysis of cellular protein fingerprints by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (9, 32) and multilocus enzyme electrophoresis are methods intermediary with respect to the phenetic-genetic dichotomy: the proteins analyzed represent gene products and metabolic functions.

Studies correlating genetic with phenetic data are essential for determining the taxonomic significance of results obtained with the various techniques. Stouthamer (45) found that relationships derived from 16S rRNA sequences correspond well with those derived from amino acid sequences of five proteins involved in several metabolic pathways.

This study aimed at developing methods to identify a *Bradyrhizobium* strains (*Arachis*). Some methods had to be tech-

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TABLE 1. Rhizobial strains with their MAR numbers and synonyms, original host plant, rDNA homology grouping, and GenBank accession numbers

MAR[a] no.	Synonym(s)[b]	Original host	rDNA[c]	GenBank no.
Bradyrhizobium sp. (*Arachis*)				
MAR 253	(Madison 625)	*Desmodium barbatum*	B	U 12888
MAR 411	IC 7011 (3G4b20)	*Arachis hypogaea*	A	U 12898
MAR 471		*Desmodium intortum*	A	U 13011
MAR 967		*Centrosema* sp.	A	U 12899
MAR 1445	TAL 1775; NC 92	*Arachis hypogaea*	B	U 12900
MAR 1505	TAL 169	*Vigna unguiculata*	A	U 12901
MAR 1510	TAL 309; CB 756	*Macrotyloma africanum*	A	U 12902
MAR 1555	TAL 11	*Vigna unguiculata*	B	U 12903
MAR 1574	TAL 1276	*Arachis hypogaea*	B	U 12904
MAR 1576	TAL 1380 (32H1)	*Crotalaria paulina*	A	U 12905
MAR 1586		*Arachis hypogaea*	B	U 12906
MAR 1587		*Arachis hypogaea*	B	U 12907
MAR 1589	NC 83.2, IC 7015	*Arachis hypogaea*	B	U 12908
MAR 1600	Z 55	*Vigna unguiculata*	B	U 12909
MAR 1605	Z 105	*Vigna unguiculata*	B	U 12910
Bradyrhizobium japonicum				
MAR 1491	USDA 110	*Glycine max*	A	U 12911
MAR 1526	USDA 123, LMG 6136	*Glycine max*	A	U 12912
Azorhizobium caulinodans				
MAR 1568	ORS 571, LMG 6465	*Sesbania rostrata*	Other	U 12913

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[b] CB, Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, Canberra, Australia; IC, International Center for Research In the Semi-Arid Tropics (ICRISAT), Patancheru, India; LMG, Laboratory of Microbiology, Gent University, Ghent Belgium; NC, Department of Microbiology, North Carolina State University, Raleigh; ORS, Collection de l'ORSTOM, Institut Français de Recherche Scientifique Pour le Développement en Coopération, Dakar, Senegal; TAL, Nitrogen Fixation in Tropical Agricultural Legumes (NifTAL), University of Hawaii, Paia; USDA, U.S. Department of Agriculture, Beltsville, Md.; Z, Department of Soil Science and Agricultural Engineering, University of Zimbabwe, Harare, Zimbabwe.
[c] rDNA homology group.

nically relatively simple, since they would be used in ecological studies concerning the competitive ability of these strains to nodulate peanut (*Arachis hypogaea* L. subsp. *fastigiata*) in controlled environment and field experiments performed in Zimbabwe. In this context, the screenings for intrinsic antibiotic resistance, substrate utilization, and protein fingerprinting were performed. Later, RAPD and rDNA nucleotide sequence analyses were performed in The Netherlands to supplement genetic data for classifying those *Bradyrhizobium* strains.

MATERIALS AND METHODS

Strains, culturing, and DNA extraction. Fifteen *Bradyrhizobium* sp. (*Arachis*) strains, two *B. japonicum* strains, and one *Azorhizobium caulinodans* strain were used. Strain designations, other synonyms, and the original legume hosts are presented in Table 1. All *Bradyrhizobium* sp. (*Arachis*) strains effectively nodulated peanut (48). *Bradyrhizobium* sp. strains were grown in yeast mannitol (YM) medium (48). *A. caulinodans* was grown in tryptone yeast (TY) medium (32). Genomic DNA was isolated as previously described (1) and dissolved in 10 mM Tris (pH 8.0)—1 mM EDTA (TE).

16S rDNA analysis. The initial PCR was performed with the forward primer 20F (5′-TGGCTCAGAACGAACGCTGGCGGC-3′) and the reverse primer 361R (5′-CCCACTGCTGCCTCCCGTAGGAGT-3′) (56). The PCR protocol was as follows (in a 50-μl total volume): 5 ng of genomic DNA, 500 nM 20F and 361R primers, 200 μM deoxynucleoside triphosphates, and 0.5 U of super-*Taq* polymerase in its reaction buffer (HT Biotechnology Ltd., Cambridge, United Kingdom). The temperature profile was run in a Perkin-Elmer thermal DNA cycler: 120 s at 94°C; 30 cycles of 45 s at 94°C, 45 s at 62°C, and 120 s at 72°C; and a final extension step of 300 s at 72°C. The second, nested PCRs employed the *20F and *361R primers, which had 18 extra nucleotides (5′-TGTAAAAC GACGGCCAGT-3′) of the M13-21 site added at the 5′ side (55). Two separate PCRs were performed with *20F-361R and 20F-*361R, using 1 μl of the initial PCR product per 100 μl. The two extended amplification products were, after electrophoresis, recovered from the agarose gel by using QIAGEN (Chatsworth), and dissolved in TE to yield 0.05 μg μl^{-1} of sequencing templates. Sequencing templates were pretreated by boiling for 2 min and snap cooling at −78°C. Sequencing reactions were performed according to the −21M13 Prism Ready Reaction Dye Primer Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif.) protocol. The DNA sequence was automatically analyzed by using an Applied Biosystems Model 373A sequencer. Both complementary strands were sequenced. Thirty 260- to 264-nucleotide-long fragments, from database-retrieved sequences, were aligned (GeneWorks, version 2.2, M. J. Glynias, Intelligenetics Inc.) with minor modifications (see notes, Table 2). PHYLIP software (version 3.5c, 1993, J. Felsenstein, University of Washington) was used for Jukes-Cantor distance (24) calculations and for dendrogram design. Pairwise dissimilarities were recalculated from Jukes-Cantor distances and presented in a neighbor-joining dendrogram (39). Alignment gaps were included as differences.

RAPD fingerprinting. Four primers were used: primer 1 (5′-GACGACGAC GACGAC-3′) (15), primer 2 (5′-TGCGCCGAATTATGCGG-3′), primer 3 (5′-ACGGAGTTGGAGGTC-3′) (primer 2 and 3 from the primer library of the Vrije Universiteit, selected on a mol% G+C of approximately 60%), and primer 4 (5′-GGTTATCGAAATCAGCCACAGCGC-3′) (15). The PCR protocol was as follows (in a 50-μl total volume): 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 0.001% (wt/vol) gelatin, 0.5 U of super-*Taq* polymerase, 0.1 ng of genomic DNA μl^{-1}, 200 μM deoxynucleoside triphosphates, 1 μM primer, and a drop of paraffin oil; the temperature profile was 5 min at 94°C, 40 cycles of 60 s at 94°C and 120 s at 36°C (primer 1 and 4) or 40°C (primer 2 and 3), 120 s at 72°C, and a final extension of 5 min at 72°C.

A 20-μl sample of the PCR reaction mix was electrophoretically separated on a 1.5% agarose gel. Marker VI (Boehringer Mannheim, Germany) was used as reference. The densitograms were analyzed using the GELCOMPAR software package (version 2.2, 1993 L. Vauterin and P. Vauterin) as described by Pot et al. (38). Computer manipulation of the densitograms involved standardization of each profile to the marker VI profile, background subtraction, equalization of total protein quantity per profile, and selection of the 200- to 2,300-bp segment for correlation analysis. The results for three primers were combined for each *Bradyrhizobium* strain. Pairwise Pearson product moment correlation coefficients (*r* values) of the densitograms were clustered by using the unweighted-pair-group-method-using-averages (UPGMA) algorithm (31).

Protein fingerprinting. Cells were precultured at 28°C on TY agar (32) slopes. Large surfaces of TY agar (in Roux flasks) were inoculated from these precultures and incubated at 28°C for 72 h. Cells were harvested, protein samples were prepared, and total cellular proteins were separated by SDS-PAGE (38). Protein profiles from different gels were normalized and standardized by using the *Psychrobacter immobilis* LMG 1125 profile as reference, of which several samples were run on each gel. The profiles between approximately 14 and 225 kDa were used for correlation analyses similarly as described for the RAPD profiles.

Intrinsic antibiotic resistance. Antibiotic resistance was determined on YM agar plates with the antibiotic incorporated. Inoculation was done with a multipoint inoculator (49). Antibiotics (Sigma Chemical Co., St. Louis, Mo.) were used at the following concentrations (in milligrams $liter^{-1}$): carbenicillin (100, 250, 500, 750, 1,000), erythromycin (10, 25, 50, 100, 150, 200), kanamycin monosulfate (10, 25, 50, 100, 200, 400), nalidixic acid (25, 50, 100, 200, 250, 400),

TABLE 2. Pairwise nucleotide differences between 23 α-subdivision members of the *Proteobacteria* in their 16S rDNA fragments corresponding to *Escherichia coli* positions 44 to 337 (3)

Species no.	Species name	Accession no.	Reference	rDNA length	Pairwise nucleotide differences between strains																				
					1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	*Rhodobacter sphaeroides*	M 55498	56	262																					
2	*Azorhizobium caulinodans*	U 12913		260	25																				
3	*Beijerinckia indica*[a]	M 59060	53	262	34	29																			
4	*Caulobacter crescentus*[b]	M 83790	44	262	37	29	24																		
5	*Hyphomicrobium vulgare*[c]	X 53182	43	260	42	31	30	40																	
6	*Phyllobacterium rubiacearum*	D 12790	55	260	40	37	41	49	37																
7	*Rhizobium huakuii*	D 12797	55	260	39	37	40	45	34	10															
8	*Rhizobium meliloti* + others[d]	D 12783	55	260	39	32	33	38	39	15	17														
9	*Rhizobium leguminosarum*	D 12782	55	260	38	32	32	34	44	19	23	8													
10	*Agrobacterium tumefaciens*	D 12784	55	260	50	38	27	40	40	29	23	20	20												
11	*Paracoccus denitrificans*	X 69159	30	262	46	35	42	41	41	35	30	27	30	32											
12	*Bradyrhizobium* sp. (*Arachis*) A + others[d]	U 12898		264	36	35	24	34	39	38	37	30	27	36	45										
13	*Bradyrhizobium japonicum* RCR 3407 + other[d]	M 55488	56	264	35	35	24	34	40	38	38	30	27	36	46	1									
14	*Bradyrhizobium* sp. (*Lotus*) NZP 2257	M 55486	56	264	38	37	22	36	39	38	37	30	29	34	43	2	3								
15	*Rhodopseudomonas palustris*	M 55496	56	264	38	33	18	28	33	42	41	34	31	36	49	6	7	8							
16	*Bradyrhizobium* sp. (*Arachis*) B + other[d]	U 12888		264	36	31	20	30	35	40	39	32	29	38	47	4	5	6	2						
17	*Bradyrhizobium elkanii*	M 55490	56	264	36	33	23	29	36	43	39	35	32	38	49	10	11	12	8	6					
18	*Nitrobacter winogradskyi*	L 11661	35	261	35	31	18	29	32	43	42	36	33	37	48	10	9	12	4	6	10				
19	*Afipia felis*	M 65248	34	260	37	35	21	35	37	42	42	35	34	39	47	10	9	8	8	6	12	8			
20	*Bradyrhizobium* sp. (*Aeschynomene*) IRBG230[e]	L 23407	54	264	35	33	25	32	38	39	38	31	28	36	46	5	6	7	7	5	7	11	11		
21	*Rhodopseudomonas acidophila*[f]	M 34128	53	264	42	37	19	25	37	47	41	37	37	34	47	24	25	22	18	20	18	20	20	21	
22	*Rhodoplanes roseus*	D 25313	16	264	25	30	21	36	39	36	35	36	33	40	46	22	21	24	22	20	24	19	22	23	30
23	*Methylocystis minimus*	L 20844	4	263	49	40	29	39	39	42	41	43	39	40	54	30	30	32	24	26	28	24	28	31	30	35

[a] Four undetermined nucleotides (N) replaced with consensus nucleotides.
[b] One N replaced with consensus A.
[c] Nucleotides A and G added at beginning of sequence.
[d] See legend, Fig. 1.
[e] Consensus nucleotides A, G, G, C, T, and T added at beginning of sequence.
[f] Two N's replaced with consensus nucleotides.

rifampicin (2.5, 5, 10, 25, 50), spectinomycin hydrochloride (2.5, 5, 10, 25, 50), and streptomycin sulfate (2.5, 10, 20, 50, 150, 300). Each strain was inoculated in duplicate on the same plate. Plates were incubated at 28°C for 7 to 8 days and scored. The entire experiment was independently repeated once.

Substrate utilization. The auxanographic method used was analogous to the one described by Parke and Ornston (37). Strains were precultured to an optical density (660 nm) of 0.5 to 0.7 in Medium Stouthamer Mannitol (49) with a 1.5-fold greater phosphate concentration (MS1.5-Mannitol). Cells were rinsed twice by centrifugation and resuspension in MS1.5 medium to remove residual mannitol. Cells were seeded in MS1.5-agar (1.5%) plates. Then, 15 μmol (in 15 μl) of substrate was spotted on the edge of the plate. All aromatic stock solutions were prepared in dimethyl sulfoxide; other stock solutions were prepared in water. Plates were incubated at 30°C for 10 to 12 days and scored.

For the fixed substrate concentration experiments either MS medium or MS-Tris medium was used. In MSTris medium, phosphate was reduced to 2.0 mM KH_2PO_4 and 50 mM Tris buffer (pH 6.8) was added. Plate preparation and inoculation were performed as described under "Intrinsic antibiotic resistance," but substrate was incorporated in the plate. Substrate utilization was scored (+ or −) resulting in a matrix of 43 substrates versus 17 strains. Pairwise dissimilarity coefficients were calculated and presented as a UPGMA phenogram (PHYLIP software).

RESULTS AND DISCUSSION

16S rDNA analysis. 16S rDNA sequence, corresponding to *Escherichia coli* positions 44 to 337 (3), were determined for 17 *Bradyrhizobium* strains and 1 *Azorhizobium* strain. The rDNA fragment was 264 nucleotides long for all *Bradyrhizobium* strains and was 260 nucleotides long for *A. caulinodans* (Table 2).

All *Bradyrhizobium* strains, including two *B. japonicum* strains, gave highly homologous sequences, with differences at only four positions, yielding two different sequences, here designated as rDNA homology group A and B (Table 1). The sequences of rDNA homology group A and B strains are identical to the previously published sequences of *Bradyrhizobium* strain BTAi1 (56) and the *B. japonicum* type strain LMG 6138^T (50), respectively. The sequence of *A. caulinodans* was identical to that published by Willems and Collins (51). The four

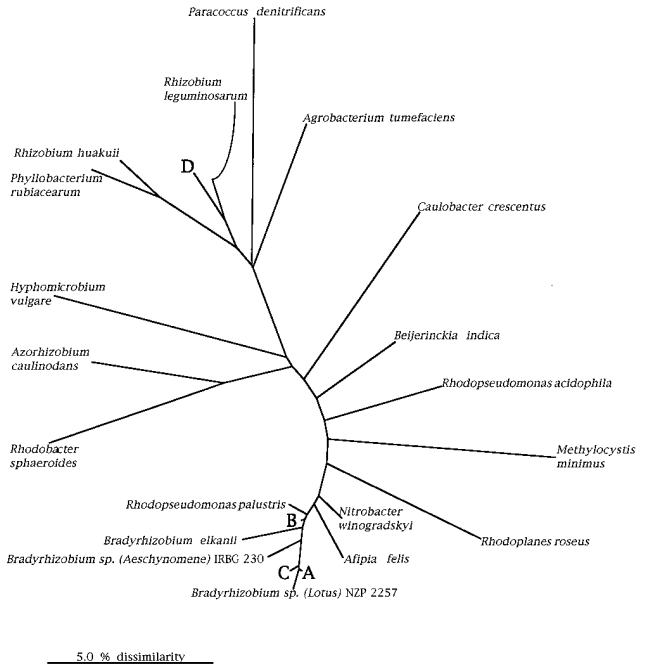

FIG. 1. Dendrogram showing relationships between 23 members of the α-Proteobacteria, comprising all strains from the *Bradyrhizobium-Rhodopseudomonas palustris* taxon (of which rDNA sequences were available) and other members of the family *Rhizobiaceae* and relatives. The tree gives a presentation of the dissimilarity matrix based on a 264-nucleotide alignment (derived from Table 2) using the neighbor-joining algorithm. (A) *Bradyrhizobium* sp. (*Arachis*) rDNA homology group A strains, *Bradyrhizobium* sp. (*Aeschynomene*) BTAi1 (56), *Blastobacter denitrificans* (50), *B. japonicum* MAR 1491, and MAR 1526; (B) *Bradyrhizobium* sp. (*Arachis*) rDNA homology group B strains, *B. japonicum* USDA 59 (56), *B. japonicum* LMG 6138^T (50), and *B. japonicum* IAM 12608^T (55); (C) *B. japonicum* RCR 3407 and *B. japonicum* USDA 110 (56); (D) *Rhizobium meliloti*, *R. fredii*, *Rhizobium* sp. NGR 234 (20), and *Sinorhizobium xinjiangensis* (55). References for the other sequences are given in Table 2.

positions that differ between the two *Bradyrhizobium* groups are located at positions [5′-184,185. . .195,196-3′]: rDNA homology group A has [5′-AG. . .CT-3′], while rDNA homology group B has [5′-GA. . .TC-3′]. These positions are structurally related as they are located opposite each other in stem-loop 7 of the secondary structure inferred for *Bradyrhizobium* sp. (*Aeschynomene*) strain BTAi1 (56). If such sequence dissimilarities are to be appropriately transformed in evolutionary distances, one would have to decide if the observed changes were actually 1, 2, or 4 changes, still leaving the problem concerning correction for multiple superimposed transitions and/or transversions. We refrained from such data transformations.

The rDNA segment analyzed is highly variable as evidenced from comparison of pairwise dissimilarities based on these 264 bp (Table 2) and those based on the almost complete 1.5-kb gene (51): variation in the former was 1.1 to 1.7 times higher than in the latter. The variation in the 264-bp segment was, however, still too low to differentiate between closely related strains. This problem could be resolved either by analyzing a larger segment of the 16S rDNA molecule or by invoking other, complementary methods (as done in this study; see other paragraphs). Increasing the alignment length to 1,124 bp enabled Wong et al. (54) to differentiate among three strains (*Blastobacter denitrificans* and *Bradyrhizobium* strains USDA 110 and BTAi1) that possess an identical 264-bp fragment (Fig. 1). The above comparison also shows that variation is site dependent and, if evolutionary distances are to be properly calculated, mutation rates should then be defined on a per-site basis.

DNA:DNA hybridization studies (18, 41) differentiated three strains belonging to rDNA homology group A: strains MAR 1526 (USDA 123), MAR 1510 (USDA 110) and MAR 1576 (32H1) belonged to DNA homology groups I, Ia, and III, respectively. Oyaizu et al. (36) found that DNA homology might only reach levels beyond 70% if the rDNA was fully identical between positions 1220 and 1377. A high rDNA homology appeared therefore to be a prerequisite for, but not necessarily leading to, a high DNA homology. It would be fruitful to further investigate the relationship between rDNA and DNA homology to show to which degree rDNA differences correspond with overall genomic differences.

The obtained sequences were compared with those of 20 other members of the α-subdivision of the class *Proteobacteria*. Pairwise sequence dissimilarities are expressed as nucleotide differences within the 264-nucleotide alignment (Table 2). The derived dissimilarities ranged from 0.38 to 20.7%. The pairwise dissimilarity matrix is depicted in an unrooted neighbor-joining dendrogram, or tree (Fig. 1). A neighbor-joining tree gave a more faithful representation of the dissimilarity matrix given in Table 2 than a Fitch tree. Naturally, no difference was observed between a phylogenetic tree constructed from the Jukes-Cantor distance matrix and a tree constructed from the dissimilarity matrix, as the transformation from dissimilarity to evolutionary distance involves only constants.

Three clusters within the family of the *Rhizobiaceae* became apparent: (i) a *Bradyrhizobium-Rhodopseudomonas palustris* cluster (dissimilarity, <5%), (ii) a *Rhizobium-Agrobacterium-Phyllobacterium* cluster, and (iii) *Azorhizobium*. The first cluster contains all *Bradyrhizobium* strains of which rDNA sequences are available, alongside *R. palustris*, *Blastobacter denitrificans*, *Nitrobacter* species, and *Afipia* species (Fig. 1). Recently, five more *Bradyrhizobium* sp. strains, together with a phototrophic *Bradyrhizobium* strain, were also located within this cluster (9, 12).

The tree topology of Fig. 1 is consistent with rRNA:DNA hybridization studies done with members of the rRNA superfamily IV (8, 21), and with trees based on larger rDNA fragments (51, 54, 55). This partial sequence thus provides enough taxonomic resolution to distinguish taxa in a highly reliable way, but a larger sequence may better resolve the finer branchings.

RAPD fingerprinting. Nine *Bradyrhizobium* sp. (*Arachis*) strains and two *B. japonicum* strains (Table 1) were fingerprinted by RAPD. All amplification products had lengths between 150 and 2,250 bp. The primers produced up to 10 products per strain, while the number of markers across all 11 strains were 9, 20, 13, and 0 for primers 1, 2, 3, and 4, respectively. Primer 2 produced the most RAPD markers, 20, making it the most successful oligonucleotide in generating polymorphic genomic-DNA patterns. Primer 4 gave no products but was successfully used with *Rhizobium* spp. (15). Since *Rhizobium* and *Bradyrhizobium* spp. have a similar mol% G+C, it is unlikely that this could account for the ineffectiveness of primer 4 with *Bradyrhizobium* strains. It is more likely that subtle experimental differences existed, as RAPD fingerprinting is known to be reproducible only under highly standardized conditions.

The banding patterns of 11 *Bradyrhizobium* strains generated with the primers 1, 2, and 3 (with a total of 42 markers) were combined per strain in one synthetic (normalized) gel as shown in Fig. 2. The densitograms of those combined patterns were pairwise analyzed by correlation, resulting in correlation coefficients (r values) ranging from $r = 0.698$ downward to

TABLE 3. Pairwise correlation coefficients[a] between the densitograms corresponding to the RAPD patterns as shown in Fig. 2

Strain (group)[b]	Correlation coefficient											
	MAR 411	MAR 471	MAR 967	MAR 1445	MAR 1555	MAR 1574	MAR 1576	MAR 1586	MAR 1600	MAR 1491	MAR 1526	Marker VI-2nd
MAR 411 (A)												
MAR 471 (A)	53.5											
MAR 967 (A)	52.8	51.2										
MAR 1445 (B)	24.0	45.7	35.6									
MAR 1555 (B)	48.6	42.5	59.6	43.2								
MAR 1574 (B)	53.8	31.0	31.0	12.6	23.7							
MAR 1576 (A)	NS[c]	24.9	10.0	28.9	34.7	NS						
MAR 1586 (B)	43.6	22.9	20.7	26.1	NS	37.7	NS					
MAR 1600 (B)	30.6	24.1	29.0	18.4	NS	32.5	NS	24.9				
MAR 1491 (A)	50.6	61.1	69.8	34.4	41.2	10.5	10.5	32.4	39.6			
MAR 1526 (A)	61.8	55.8	46.2	13.7	37.6	13.2	13.2	21.0	19.5	54.9		
Marker VI-1st												94.8

[a] r values $\times$ 100.
[b] Denotes the rDNA homology group of the strain.
[c] NS means nonsignificant at $P = 0.001$ ($r < 0.078$).

nonsignificant levels below $r = 0.078$ (Table 3), evidencing considerable genetic diversity. Comparison of the two marker lanes ($r = 0.948$; Table 3), originating from two different gels, provides an estimate of the systematic error when using r values for pairwise comparisons. Generally, pairwise r values provide a more reliable tool to compare banding profiles than a comparison based on discrete data (14). Clustering analysis (Fig. 2) shows that there is only one reasonable tight cluster formed at $r > 0.50$, consisting of two *B. japonicum* strains (MAR 1491 and MAR 1526) and three *Bradyrhizobium* sp. (*Arachis*) strains (MAR 411, MAR 471, and MAR 967). All of these strains belong to rDNA homology group A; the sixth strain belonging to this rDNA homology group (MAR 1576) is, however, the most distantly related strain within this group of 11 strains. The strains belonging to rDNA homology group B are more polymorphic. They could only be clustered at $r < 0.50$ (Fig. 2). The genetic diversity as revealed by RAPD fingerprinting is clearly greater than that obtained with rDNA se-

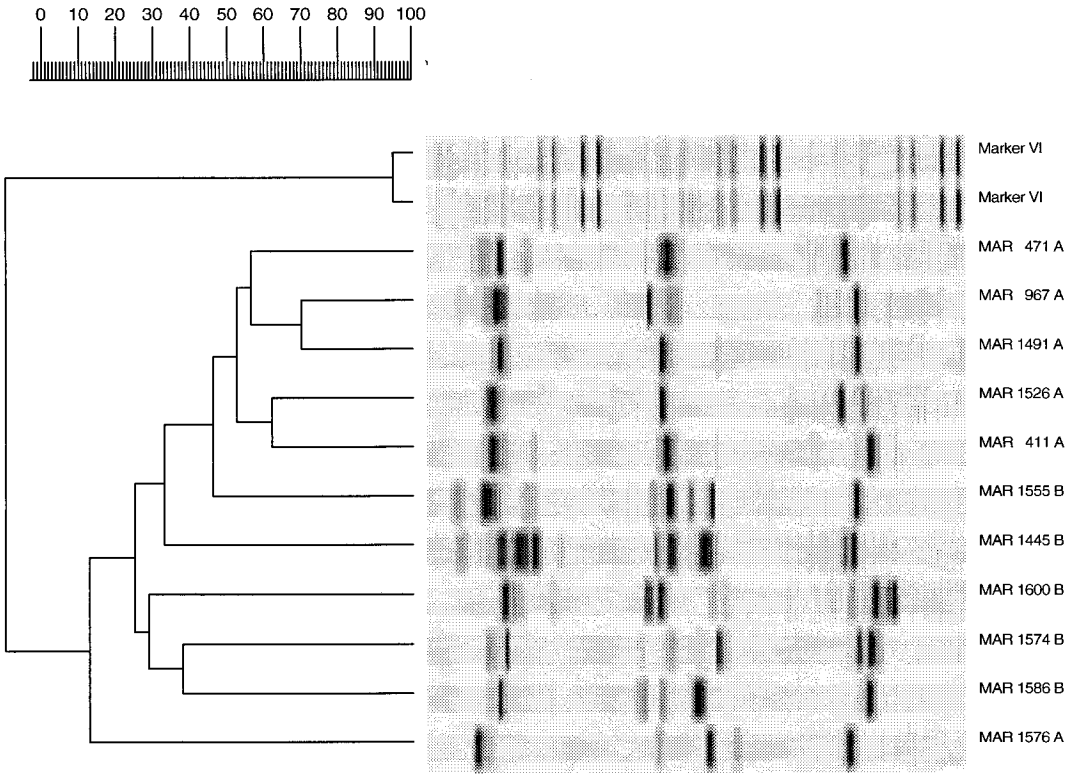

FIG. 2. RAPD patterns for nine *Bradyrhizobium* sp. (*Arachis*) strains and two *B. japonicum* strains produced with the primers 1, 2, and 3 as shown from right to left (the three gels are linked head to tail). The results of UPGMA clustering of strains based on pairwise correlation (r values) of the densitograms is shown. Letters A and B after the strain codes denote the rDNA homology groups. The marker bands are (from right to left) 2,176, 1,766, 1,230, 1,033, 653, 517, 453, 394, 298, 234, 220, and 154 bp long.

quence comparison. It appears again that rDNA homology is a prerequisite for RAPD profile similarity (as for the five clustered rDNA homology group A strains) but not a guarantee (as for the rDNA homology group B strains). RAPD profile similarity together with high rDNA sequence similarity are in our opinion good evidence for genetic relatedness of strains.

Polymorphism increases with increasing the numbers of primers used, because more genomic sites are covered. The use of more primers (yielding more markers) results in an (overall) reduced correlation between profiles: profile correlations based on primer 1 only resulted in *r* values between 0.80 and 1.00 (data not shown), while analyzing for the combined primers 1+2+3 resulted in *r* values below 0.70 (Table 3). A large number of RAPD markers (>150) together with a sufficient number of products per strain (>10) are necessary to draw reliable (taxonomic) conclusions (47). Such criteria were only partly met in this and in another study involving *Bradyrhizobium* strains (7).

Protein fingerprinting. Fifteen *Bradyrhizobium* sp. (*Arachis*) strains were fingerprinted for their total cellular protein profiles together with *A. caulinodans* and 37 representative strains of the nine different gel electrophoretic groups previously identified within the genus *Bradyrhizobium* by Dupuy et al. (9). The normalized protein patterns of these 53 strains, together with their clustering based on correlation coefficients (*r*) between their densitograms, are presented in Fig. 3.

The reproducibility of profiles ranged from $r = 0.96$ for the same sample on the same gel to $r = 0.93$ for the same sample on different gels and to $r = 0.91$ for different samples of the same strain. Differences between protein profiles of strains are due to either a different genetic constitution or a different physiological state. Standardized procedures generally reduce differences but may also increase them: slowly growing *Bradyrhizobium* strains sampled at late lag phase or early log phase may exhibit differences that relate to their physiological state at harvest. *Bradyrhizobium* strains, under standard assay conditions, generally, gave protein profiles that were less sharp than those obtained for *Rhizobium* strains.

Twelve of the fifteen *Bradyrhizobium* sp. (*Arachis*) strains belong to cluster 1 sensu Dupuy (9), while two strains (MAR 967 and MAR 1589) belong to cluster 3 sensu Dupuy and one strain occupies a separate position (Fig. 3). Seven of the cluster 1 strains (MAR 411, 1445, 1555, 1574, 1587, 1600, and 1605) are close relatives of the type strain of *B. japonicum* LMG 6138^T; the other five strains in cluster 1 (MAR 471, 1505, 1510, 1576, and 1586) are further removed from the *B. japonicum* type strain. The two strains in cluster 3 (MAR 967 and 1589) group together with *B. japonicum* strains LMG 8321 (USDA 135) and LMG 6136 (USDA 123; MAR 1526). All of the 15 *Bradyrhizobium* sp. (*Arachis*) strains, inclusive of the least associated strain MAR 253, are thus shown to be authentic *Bradyrhizobium* strains. The photosynthetic *Bradyrhizobium* sp. (*Aeschynomene*) strains (including BTAi1) were also located in the vicinity of the *B. japonicum* type strain (29, 32). Two strains of *B. elkanii*, including the type strain LMG 6134^T, are members of cluster 6 sensu Dupuy.

At or above $r = 0.93$ a separation between the two rDNA homology groups A and B is observed (Fig. 3). The six strains clustering most closely with the *B. japonicum* type strain, itself a B strain (Fig. 1), are all B strains; the three closely associated strains further removed from the *B. japonicum* type strain (MAR 1505, 1510, and 1576) are all A strains.

The close affiliation of a number of *Bradyrhizobium* sp. (*Arachis*) strains to the *B. japonicum* type strain, for both protein profile and rDNA sequence similarity, suggest that these strains belong to this latter species; more genotypic data (e.g., DNA:DNA homology) would, however, be necessary to justify such taxonomic conclusions.

Intrinsic antibiotic resistance. Seventeen *Bradyrhizobium* sp. strains and one *A. caulinodans* strain were tested for resistance to seven antibiotics. The highest concentrations, of the five or six test concentrations, at which strains grew are given in Table 4.

Each strain had a specific pattern across the seven tested antibiotics. Attempts to group the *Bradyrhizobium* sp. strains according to particular antibiotic profiles failed, since the diversity is too wide. Remarkable is the high kanamycin resistance (>400 $\mu g\ \mu l^{-1}$) of the strains MAR 1587, MAR 1600, and MAR 1605, all recent Zimbabwean isolates. rDNA homology grouping was not reflected in antibiotic resistance profiles (Table 4). Kuykendall et al. (27) found that *B. japonicum* strains from DNA homology group II (*B. elkanii* strains) were more resistant to a wide array of antibiotics than group I strains. This showed that genetic and phenetic traits may be correlated, but a correlation between rDNA data and antibiotic resistance was not observed with the *Bradyrhizobium* strains used in this study.

Resistance of *Bradyrhizobium* sp. (*Arachis*) strains to antibiotics may be used for strain identification in ecological studies, because strain-specific resistance patterns are obtained when several (three or more selected) antibiotics are used in combination.

Substrate utilization. The utilization for growth of 43 compounds by up to 17 *Bradyrhizobium* sp. strains is summarized in Table 5.

Of the 21 aliphatic compounds tested, all strains used pentoses and hexoses for growth. It is well documented that monosaccharides are mainly metabolized by the Entner-Doudoroff pathway in *Bradyrhizobium* spp. (46). Of the polyols (sugar alcohols) tested, mannitol was utilized by all strains and glycerol was utilized by almost all, while sorbitol and inositol were utilized by fewer strains. The tricarboxylic acid (TCA) cycle intermediates aconitate, succinate, and malate were utilized by most strains (88 to 100%) indicating that the TCA cycle is also active in these *Bradyrhizobium* strains, as it is in other rhizobia (46). Glutamate was used for growth by all strains. Di- and polysaccharides were utilized by no more than 67% of the tested strains. This contrasts with the purported view that *Bradyrhizobium* strains are unable to utilize polysaccharides (10, 46). However, Dreyfus et al. (8) already reported that some *Bradyrhizobium* strains could utilize disaccharides.

The 22 aromatic compounds were divided into compounds that are degraded by the protocatechuate and catechol pathways (40). Protocatechuate was utilized as a sole carbon and energy source by all tested strains. The compounds immediately prior to protocatechuate in the degradation route, 4-hydroxybenzoate, vanillate, and caffeate, were also utilized by all strains. The compounds further "upstream" in the degradation route could not be utilized by all strains, except coniferyl alcohol and coumarate. All strains could thus utilize the key compounds in lignin degradation, namely vanillate and coniferyl alcohol (40), indicating metabolic adaptation to the natural habitat. 4-Methyl benzoate and tyrosine could by utilized by the least number of strains. Catechol could be utilized by 12 out of 17 strains. The other compounds further upstream in the catechol pathway could be utilized by fewer strains, except benzoate. Benzoate can, however, also be degraded by the protocatechuate pathway, which explains why more strains were able to grow on benzoate than on catechol. Salicylate is a product of polycyclic aromatic compound degradation (40). Strains able to grow on salicylate may be involved in degradation of polycyclic aromatic compounds. The degradation prod-

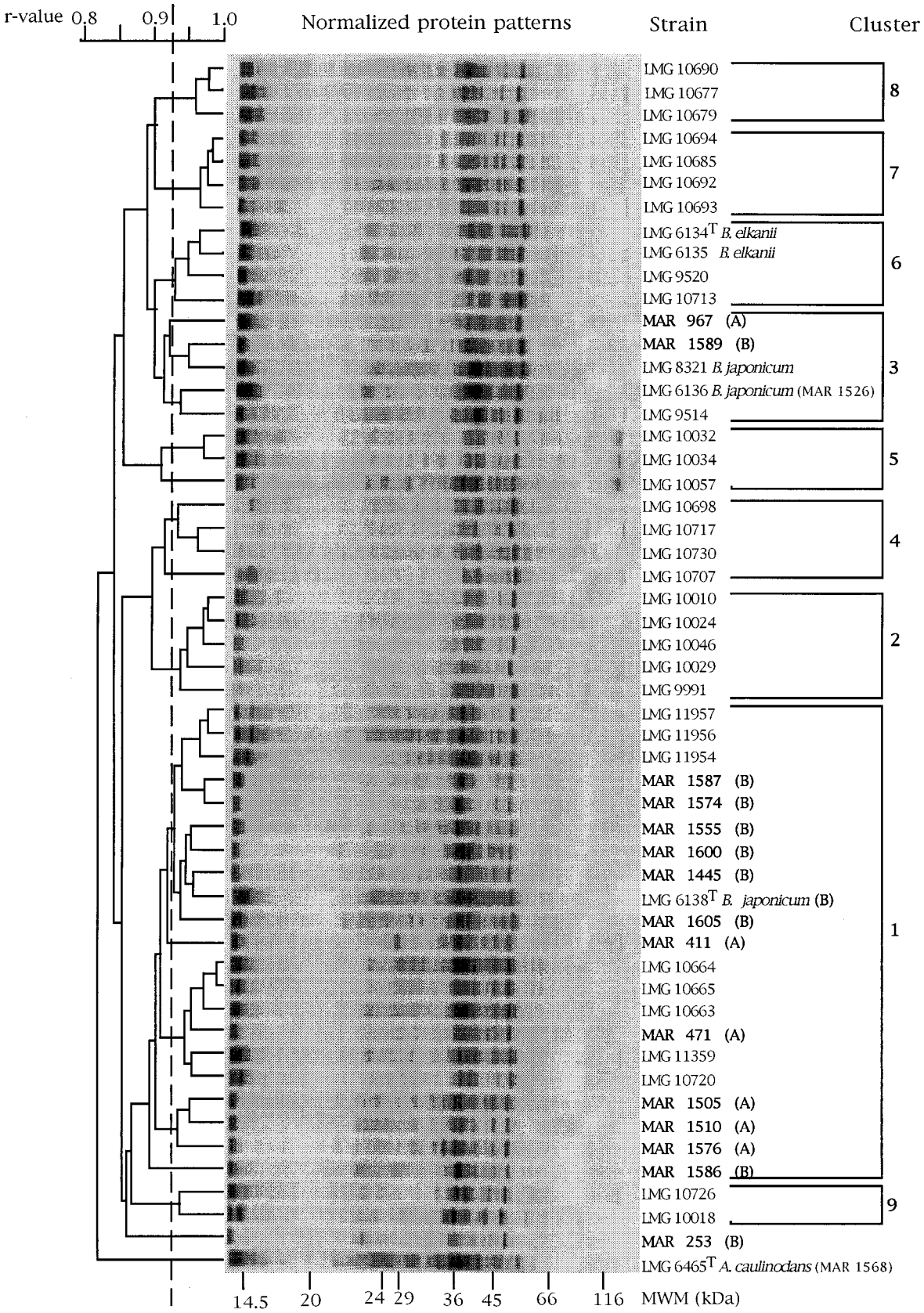

FIG. 3. UPGMA dendrogram showing the relationships between the normalized electrophoretic protein profiles of 15 *Bradyrhizobium* sp. (*Arachis*) strains (MAR codes), 37 *Bradyrhizobium* strains representing the nine electrophoretic clusters found by Dupuy et al. (9), and 1 *A. caulinodans* strain (LMG codes; T means type strain). The molecular weight markers (MWM) are indicated at the bottom. Letters between brackets (A or B) denote rDNA homology group; the dotted vertical line (r = 0.93) separates A from B strains.

TABLE 4. Intrinsic antibiotic resistance of 18 rhizobial strains to seven antibiotics

Strain no.	Group[a]	Antibiotic resistance (mg liter^{-1})[b]						
		Ery	Kan	Str	Spe	Rif	Nal	Car
Bradyrhizobium sp. (*Arachis*)								
MAR 967	A	0	0	0	10	0	25	100
MAR 1555	B	10	0	0	50	25	400	0
MAR 1574	B	10	0	0	25	10	200	100
MAR 1586	B	10	0	0	50	5	400	0
MAR 471	A	10	10	10	25	50	100	100
MAR 1589	B	25	10	10	50	10	400	250
MAR 411	A	50	50	10	50	50	100	750
MAR 1510	A	50	50	10	50	50	250	500
MAR 253	B	100	10	50	50	50	0	750
MAR 1505	A	100	25	10	50	50	250	500
MAR 1576	A	100	50	10	50	50	400	500
MAR 1445	B	100	50	20	50	50	400	1,000
MAR 1587	B	100	400	2.5	50	50	100	250
MAR 1600	B	150	400	10	50	10	25	250
MAR 1605	B	150	400	10	50	50	50	250
Bradyrhizobium japonicum								
MAR 1491	A	100	10	10	50	50	100	500
MAR 1526	A	50	10	0	50	25	0	250
Azorhizobium caulinodans								
MAR 1568		100	0	0	25	10	25	1,000

[a] rDNA homology group designation.
[b] Maximum resistance within the range of test concentrations (see Materials and Methods).

ucts of flavonoids and tannins, phloroglucinol and protocatechuic acid, can be utilized by almost all strains. However, no auxanographic growth of five *Bradyrhizobium* strains was observed with the flavonoids apigenin, biochanin A, chrysin, daidzein, naringenin, phloretin, or quercetin (data not shown). All strains could utilize adipate, an analog of β-ketoadipate, indicating the presence of an active β-ketoadipate pathway in which aromatic rings are cleaved by dioxygenases (37).

A numerical taxonomic analysis was performed with the substrate utilization data. Pairwise similarities were calculated, ranging from 94 to 63% (data not shown), and used for a clustering analysis (Fig. 4). At or above the 84% similarity level the 17 strains can be divided into five clusters and two separate strains. Considering *Bradyrhizobium* sp. (*Arachis*) strains only, i.e., excluding *B. japonicum*, it is observed that within a cluster all strains belong to the same rDNA homology group; only MAR 1576 violates this observation. The two *B. japonicum* strains have a pairwise similarity of 83%. The cluster MAR 411/MAR 1510 (93% similar) is notable, since both strains are superior peanut microsymbionts (48). The cluster MAR 1586/MAR 1587 (88% similar) is interesting, as both strains were recently isolated from the same site in Zimbabwe.

Five recent numerical taxonomic studies, all heavily relying on substrate utilization, had *Bradyrhizobium* strains included (5, 6, 8, 11, 28). All found a clear separation of the genus *Bradyrhizobium* from other genera in the family *Rhizobiaceae*. *B. japonicum* strains always clustered together with other *Bradyrhizobium* sp. strains, except in the analysis of Dreyfus et al. (8). All reports, as well as this one (Fig. 4), grouped all nonphototrophic *Bradyrhizobium* strains at or above the 70% similarity level; the phototrophic *Bradyrhizobium* strains grouped with the others at 67% (28). Among the nonphototrophic *Bradyrhizobium* strains, three to five subphena have been observed above 80% similarity (11, 28): these subphena could constitute subspecies from *B. japonicum* according to the common finding that within species 80% or higher similarity occurs (42).

The polyphasic approach in rhizobial classification. The relatedness between 17 *Bradyrhizobium* strains was studied using two genetic features, two phenetic features, and one mixed feature. 16S rDNA sequences are generally considered to be good indicators for (genotypic) relatedness (52), but few studies have correlated rDNA data with other taxonomic features. This study intended to link the various results, coming forth from five separate studies, in one coherent taxonomy, i.e., a polyphasic study in a nutshell.

The rDNA-nucleotide dissimilarity tree, deduced from a 264-nucleotide alignment, is in good agreement with phylogenetic trees that are based on larger rDNA alignments, inferred by others (see rDNA section). These rDNA-based relationships were compared with relationships coming forth from the other four studies. In several, but not all, cases the rDNA clustering corresponded with clusterings based on RAPD, PAGE, or substrate utilization: it appeared that high rDNA homology (identity) is a prerequisite, but not a guarantee, for high similarity values obtained with other techniques. A similar observation was previously made when rDNA similarity was correlated with DNA homology (36). The rDNA homology groups A and B, as described in this study, were generally separately clustered in the RAPD, PAGE, and substrate utilization analyses; i.e., A and B strains, generally, did not occur in one cluster. A clustering of strains based on intrinsic antibiotic resistance did not occur: all strains had highly specific resistance profiles. The rDNA homology group A strains generally clustered more tightly than the B strains; the B strains always had some single members that were clustered with other B strains at low similarity values. The 2 *B. japonicum* strains included in this study clustered together with the 15 *Bradyrhizobium* sp. (*Arachis*) strains, indicating a close association between these strains.

In this study the difficulties of a polyphasic study clearly surfaced: strains that are clustered together by one method may be scattered over various separate clusters when other methods are employed. Some methods allow for very detailed characterization (e.g., RAPD and intrinsic antibiotic resistance patterns), useful for the separation of strains within a single species, while other methods may only elucidate interspecies or

TABLE 5. Utilization of 43 substrates by *Bradyrhizobium* sp. strains (see Table 1)

Compound type and substrate	%[a]	n[b]	Method[c]
Aliphatic			
Polysaccharide (dextrin)	29	17	A
Disaccharides			
Maltose	58	12	F(50)
Sucrose	67	12	F(50)
Lactose	67	12	F(50)
Trehalose	12	17	A
Hexoses			
Glucose	100	12	F(50)
Galactose	100	12	F(50)
Pentoses			
Arabinose	100	12	F(50)
Xylose	100	17	A
Polyols			
Glycerol	91	11	F(10)
Mannitol	100	17	F(50)
Sorbitol	59	17	A
Inositol	18	17	A
TCA cycle intermediates			
Aconitate	100	4	A
Succinate	88	17	A
Malate	94	17	A
Others			
Acetate	0	16	F(1)
Adipate	100	16	F(1) and A
Muconate	75	16	F(1)
Glutamate	100	17	A
Nicotinate	76	17	A
Aromatic			
Protocatechuate pathway			
Protocatechuate	100	17	A
4-Hydroxybenzoate	100	17	A
Vanillate	100	17	A
Caffeate	100	17	A
Ferulate	94	17	A
Veratrate	29	17	A
Quinate	88	16	F(1)
Anisate	76	17	A
4-Methyl benzoate	0	17	A
Coniferyl alcohol	100	17	A
Coumarate	100	17	A
Tyrosine	12	17	A
Catechol pathway			
Catechol	71	17	A
Benzoate	100	17	A
Salicylate	18	17	A
Guaiacol	12	17	A
Phenylalanine	50	16	A
Others			
Gallate	100	17	A
Syringate	53	17	A
Syringic aldehyde	47	17	A
Phloroglucinol	82	17	A
Gentisate	100	17	A

[a] Fraction (%) of the tested strains growing.
[b] Number of strains tested.
[c] Method used to determine growth on a substrate was by auxanography (A) and/or by growth at a fixed concentration (F) with the micromolar concentration given in brackets.

intergeneric differences (e.g., rDNA). In general, we found unacceptable inconsistencies across the various methods, preventing us from drawing taxonomic conclusions from the combination of the five studies.

The rDNA nucleotide similarities clearly show that *Bradyrhizobium* strains, including the phototrophic strains, cluster together with *Rhodopseudomonas palustris*, *Nitrobacter* species, *Afipia* species, and *Blastobacter denitrificans* (Fig. 1). It has been suggested by others that members of this cluster may be regrouped into one redefined genus (35, 50, 56). However, comparative phenotypic data are scarce. A number of arguments in favor of such a redefinition are here presented. (i) Phototrophy is present both in *R. palustris* and in *Bradyrhizobium* sp. (*Aeschynomene*) strains, both utilize bacteriochlorophyll A as pigment, and the former are obligate anaerobic while the latter are aerobic phototrophs (54). (ii) Dinitrogen fixation occurs with *R. palustris* (anaerobically) and with *Bradyrhizobium* strains (microaerobically). (iii) Anaerobic dissimilatory nitrate reduction occurs with some *R. palustris* strains (26), *Nitrobacter* strains (2), and *Bradyrhizobium* strains (33). (iv) *Nitrobacter* strains possess an intracytoplasmic membranous structure reminiscent of that of *R. palustris* (2, 35). (v) *R. palustris* and *Blastobacter* strains multiply by budding (17, 19). (vi) *Nitrobacter* species and *R. palustris* both have an uncommon deoxy-sugar (2,3-diamino-2,3-deoxy-D-glucose) instead of glucosamine in their lipopolysaccharide (19). (vii) Among all *Rhodopseudomonas* strains only *R. palustris* can utilize benzoate as substrate (19), while all *Bradyrhizobium* strains are able to utilize this substrate (Table 5).

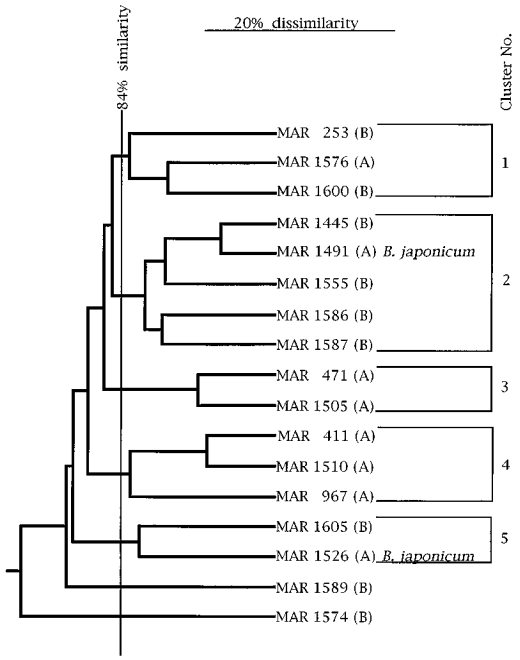

FIG. 4. Phenogram showing the relationships among 17 *Bradyrhizobium* sp. strains concerning the utilization of 43 substrates, based on UPGMA cluster analysis of pairwise dissimilarities. Letters A or B between brackets denote the rDNA homology group.

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