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Phylogenetic Analysis of *Bradyrhizobium japonicum* and Photosynthetic Stem-Nodulating Bacteria from *Aeschynomene* Species Grown in Separated Geographical Regions

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Nearly complete and short partial 16S rRNA sequences were derived from PCR-amplified ribosomal DNAs of *Bradyrhizobium japonicum* USDA 136 and USDA 110 and five strains of bacteriochlorophyll-synthesizing bacteria isolated from stem nodules of *Aeschynomene indica* and other *Aeschynomene* species growing in different geographic regions, including India, The Philippines and North America. We confirmed that the five stem-nodulating strains examined synthesize bacteriochlorophyll *a*, and the absorption spectra of methanol-extracted cells contained a major absorbance peak at 770 nm. Strains isolated on different continents and from different *Aeschynomene* species were found to be phylogenetically homogeneous and exhibited levels of sequence similarity of more than 99%. The bacteriochlorophyll-synthesizing rhizobia, *Bradyrhizobium japonicum*, *Blastobacter denitrificans*, *Afipia felis*, and *Rhodopseudomonas palustris* exhibited levels of sequence similarity of 97% or greater and belong to a distinct line of descent within the alpha-2 subdivision of the *Proteobacteria*. Variable regions between positions 995 and 1045 provide potential target sites for design of a probe that is able to distinguish the photosynthetic rhizobia from closely related taxa.

Bacteria capable of inducing stem nodules in herbaceous legumes belonging to the genus *Aeschynomene* are of great significance because of their ability to produce the photosynthetic pigment bacteriochlorophyll *a* (Bchl *a*) (6, 9); this pigment is not produced by any other rhizobia that enter into plant symbioses. The first such bacterium described was strain BTAi 1, which was isolated from stem nodules of *Aeschynomene indica* (6); subsequently, similar bacteria have been isolated from stem nodules of *Aeschynomene afraspera*, *Aeschynomene denticulata*, *Aeschynomene evenia*, *Aeschynomene nilotica*, *Aeschynomene pratensis*, and *Aeschynomene sensitiva* (19) and from *Aeschynomene aspera* and *Aeschynomene rudis*. Bacteriochlorophyll (Bchl) is produced by these rhizobia under both symbiotic and free-living conditions. These organisms are representatives of the aerobic Bchl-synthesizing bacteria, also known as the aerobic photosynthetic bacteria, a physiological category distinct from the classical anoxygenic photosynthetic bacteria (31, 32).

These stem-nodulating endophytes are of special significance for improvements to symbiotic nitrogen fixation for crop production, since bacterial photophosphorylation decreases the demand for chloroplast-generated ATP by nitrogen fixation processes. The Bchl-containing stem nodules result in nitrogen fixation that exhibits greater energy self-sufficiency than a similar process that occurs in root nodules or non-Bchl-containing stem nodules, such as those produced by *Azorhizobium* spp. on *Sesbania* spp. (5, 6). The stem-nodulating symbiont BTAi 1 possesses the necessary Bchl and photosynthetic reaction centers, and illumination of *Aeschynomene indica*-BTAi 1 stem nodules with near-infrared radiation enhances the rate of chloroplast-independent acetylene reduction (9).

The presence of Bchl in stem-nodulating symbionts of *Aeschynomene* species raised the question of the phylogenetic relationship of these organisms to the anoxygenic photosynthetic bacterial genera *Rhodobacter* and *Rhodopseudomonas* on the one hand and the phytosymbiotic bacterial genera *Rhizobium* and *Bradyrhizobium* on the other. Progress was made in establishing the evolutionary position of the Bchl-synthesizing *Aeschynomene* stem-nodulating (BASN) rhizobia when partial 16S rRNA gene sequences of strain BTAi 1 and representative *Bradyrhizobium* strains became available (46). The results of a phylogenetic analysis based on a 260- to 264-bp segment of the 16S rRNA suggested that BTAi 1 is a very close relative of *Bradyrhizobium japonicum* and *Rhodopseudomonas palustris* (46).

As phylogenetic data on the BASN rhizobia are limited, the extents of possible phylogenetic variation among the many BASN isolates and among strains isolated from the different host *Aeschynomene* species have not been determined. Given that these bacteria occupy a rather specialized ecological niche and share a unique combination of physiological characteristics, in this study we tried to determine whether they are in fact a phylogenetically coherent group. In addition, the short partial sequences available for analysis may not provide accurate separation of BTAi 1 from its close relatives. We determined nearly complete 16S rRNA sequences for BTAi 1 and representative *Bradyrhizobium japonicum* strains and also for additional BASN strains isolated from different *Aeschynomene*

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TABLE 1. *Bradyrhizobium* and photosynthetic stem-nodulating strains used in this study

Strain[a]	Source	Original host legume	Country of origin
BTAi 1[b]	D. E. Fleischman	*Aeschynomene indica*	United States
MKAa 2[c]	D. E. Fleischman	*Aeschynomene aspera*	India
MKAa 3[c]	D. E. Fleischman	*Aeschynomene aspera*	India
IRBG 230	J. K. Ladha	*Aeschynomene nilotica*	The Philippines
IRBG 2	J. K. Ladha	*Aeschynomene afraspera*	The Philippines
Bradyrhizobium japonicum USDA 136	R. A. Date	*Glycine max*	United States
Bradyrhizobium japonicum USDA 110	R. A. Date	*Glycine max*	United States

[a] IRBG, International Rice Bacterial Germplasm accession number, International Rice Research Institute, Manila, The Philippines; USDA, U. S. Department of Agriculture, Beltsville, Md.

[b] BTAi 1 is the recognized designation for the original Bchl-synthesizing rhizobial strain isolated from *Aeschynomene indica* by Eaglesham and coworkers at the Boyce Thompson Institute for Plant Research, Ithaca, N.Y. BTAi, Boyce Thompson *Aeschynomene indica* (34).

[c] MKAa 2 and MKAa 3 are the provisional designations for isolates collected from stem nodules on *Aeschynomene aspera* by Fleischman and coworkers at Madurai Kamaraj University, Madurai, India. MKAa, Madurai Kamaraj *Aeschynomene aspera*.

species. Emphasis was placed on including representative photosynthetic isolates obtained from *Aeschynomene* plants grown in widely separated geographic regions.

MATERIALS AND METHODS

Bacterial strains and culture methods. The rhizobial strains used and their origins are shown in Table 1. The organisms were grown routinely on mannitol-yeast extract agar (38) at 28°C for 4 to 7 days under aerobic conditions in the dark. *Bradyrhizobium japonicum* USDA 110 and USDA 136 were originally isolated from *Glycine max* in Florida and Beltsville, Md., respectively. Strains USDA 110 and USDA 136 are representatives of the core of *Bradyrhizobium* strains which infect soybeans (*G. max*).

Direct sequencing of PCR-amplified 16S rDNA. Plate-cultured cells were harvested with a sterile pick and suspended in 20 μl of high-performance liquid chromatography (HPLC) grade water. DNA was extracted by heat lysis of cells at 98°C for 10 min followed by pelleting of cell debris for 5 min with a microcentrifuge. The supernatant was used directly as a template for PCR amplification of the 16S ribosomal DNA (rDNA) (27). A fragment of the 16S rDNA up to 1,484 bp long was amplified by using a flanking pair of opposing universal primers. The use of forward primer 27f (5′-AGAGTTTGATC [C,A]TGGCTCAG-3′) and reverse primer 1492r (5′-TACG-G[C,T]TACCTTGTTACGACTT-3′) as described by Lane (20) allowed successful amplification of the strains studied. All numbering used for nucleotide positions corresponds to positions on the *Escherichia coli* 16S rRNA sequence as defined by Brosius et al. (3). The final reaction mixture (100 μl) consisted of 10 μl of 10× PCR buffer, 66 μl of HPLC grade water, 10 μl of a solution containing deoxynucleoside triphosphates at concentrations of 2 mM, 10 μl of template, and 2 μl of each primer solution (concentration, 0.5 μg μl^{-1}). The following program was used with a thermocycler (model 480; Perkin Elmer Cetus): initial denaturation at 98°C for 180 s, followed by addition of 2.5 U of *Taq* polymerase; and 30 cycles consisting of 93°C for 60 s, 48°C for 45 s, and 72°C for 90 s. An additional extension period of 72°C for 300 s was included after completion of the cycles.

The amplified DNA was purified by two methods. The first method involved phenol-chloroform purification, after which the amplified sequencing template was selected by chromatographing the preparation on a 0.6% agarose gel, and a modification of the freeze-squeeze extraction method of Tautz and Renz (35) was then used. Excised gel slices containing the double-stranded product were placed in Ultrafree-MC tubes (pore size, 0.22 μm; Millipore Corp., Bedford, Mass.) and frozen at −70°C for 60 min. The frozen tubes were centrifuged at 4°C for 30 min, and the filtered product was precipitated. The second method was the Prep-A-Gene (Bio-Rad, Hercules, Calif.) purification method, as described by the manufacturer. Both methods gave comparable levels of template quality. To sequence the double-stranded template, we used a Sequenase version 2.0 kit (US Biochemical, Cleveland, Ohio) (4, 13) and universal primers complementary to conserved regions of the 16S rDNA (20, 40). The ^{32}P-labeled DNA was denatured at 98°C for 3 min before it was loaded onto the sequencing gel.

Analysis of 16S rRNA primary structure. The 16S rRNA sequences derived from direct gene sequencing were aligned with the 16S rRNA sequences of selected representatives of the alpha subgroup of the *Proteobacteria* obtained from the National Science Foundation Ribosomal RNA Database Project (25) or directly from the EMBL and GenBank data libraries. Variable positions due to sequence deletions or additions were noted, and alignments were appropriately adjusted. By using only unambiguously aligned sequence regions, a matrix of pairwise evolutionary distances was calculated by using the Jukes-Cantor algorithm (17) and the DNADIST program of the PHYLIP version 3.5c phylogenetic analysis software package (11). These distances were used to calculate similarity values and to generate an unrooted phylogenetic tree by the neighbor-joining method (26), using the NEIGHBOR program of the same PHYLIP package (10).

Spectrophotometric analysis of Bchl synthesis. Strains investigated for Bchl *a* production were grown under the following two illumination regimes: (i) cells on mannitol-yeast extract agar were incubated only in the dark; and (ii) cells on mannitol-yeast extract agar were subjected to a 16-h light–8-h dark cycle (the lighting cycle was that described by Evans et al. [9]). All cultures were incubated aerobically at 28°C.

All stem-nodulating *Aeschynomene* strains listed in Table 1 and *Bradyrhizobium japonicum* USDA 136 were examined for Bchl *a* production. Cells harvested from colonies grown on agar plates were suspended in 3 ml of methanol at 4°C and left in the dark for 2 h. Samples were centrifuged, and the supernatant was collected for analysis with a Hitachi model 150-20 spectrophotometer. Absorption spectra were generated by scanning over a wavelength range from 350 to 850 nm.

Nucleotide sequence accession numbers. All of the sequences determined for the strains listed in Table 1 were deposited in the GenBank data library under accession numbers L23330, L23331, and L23405 through L23409.

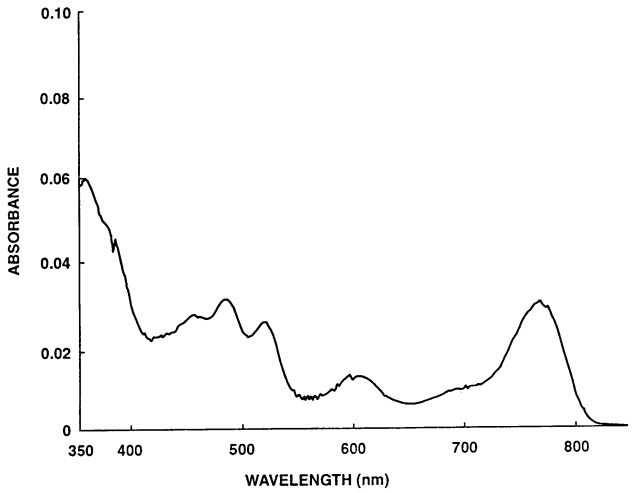

FIG. 1. In vitro absorption spectrum of methanol-extracted pigments from cells of stem-nodulating strain MKAa 2.

RESULTS

Bchl synthesis by stem-nodulating strains. To determine whether Bchl *a* synthesis was a characteristic shared by bacteria isolated from stem nodules of *Aeschynomene* species, spectra were obtained by using methanol extracts of dark-incubated cell biomass. All of the strains used in the 16S rDNA sequencing studies and originally isolated from stem nodules of *Aeschynomene* species (strains BTAi 1, MKAa 2, MKAa 3, IRBG 2, and IRBG 230) produced a major absorbance peak at 770 nm characteristic of Bchl *a* (24). The absorption spectra obtained were similar to the spectrum obtained for isolate MKAa 2 (Fig. 1). The presence of Bchl *a* confirms previous findings for strains BTAi 1, IRBG 2, and IRBG 230 (9, 19), but the results for strains MKAa 2 and MKAa 3 are new.

We expected that pigment production would be detected only in cells grown under the intermittent light provided by the light-dark cycle conditions (6, 9). However, we found that Bchl *a* synthesis could be detected and sustained in subcultured cells grown solely in the dark on solid mannitol-yeast extract medium. We noted that colonies grown in the dark appeared to be visibly paler than colonies grown under intermittent light and that the cells grown in the dark exhibited lower levels of absorbance for Bchl *a*-specific peaks. We found no evidence of Bchl synthesis by *Bradyrhizobium japonicum* USDA 136 even after growth under 16-h light–8-h dark conditions.

16S rDNA sequencing and phylogenetic analysis. Nearly complete 16S rDNA sequences were obtained for BASN strains BTAi 1, MKAa 2, and IRBG 230 and for *Bradyrhizobium japonicum* USDA 136 and USDA 110. The sequences were analyzed phylogenetically at a region covering a total of 1,124 nucleotide positions. The unrooted tree in Fig. 2 shows the phylogenetic relationships of the strains studied compared with other bacteria belonging to the alpha subclass of the class *Proteobacteria*.

The BASN isolates exhibited extremely high levels of sequence similarity to each other despite their differences in host origin and geographic origin. The levels of sequence similarity between the stem-nodulating rhizobia fell within the narrow range between 99.1 and 99.7% (Table 2). The phylogenetic analysis also revealed a close relationship between the BASN isolates and *Bradyrhizobium japonicum* USDA 136, USDA 110, and type strain LMG 6138 (Table 2). Our data for USDA 136 revealed a sequence identical to that of USDA 110. The BASN rhizobia occur together with the *Bradyrhizobium japonicum* strains, *Blastobacter denitrificans*, *Afipia felis* (42), and *Rhodopseudomonas palustris* (46) in a distinctly separate cluster within the alpha 2 subdivision of the *Proteobacteria* (43).

Partial sequences were obtained for strains MKAa 3 and IRBG 2, particularly for positions 70 to 400. An analysis of this region of 330 bases revealed that strains MKAa 2 and MKAa 3 had identical sequences and exhibited 98.2% sequence similarity to BTAi 1, while IRBG 230 and IRBG 2 exhibited levels of similarity to BTAi 1 of 97.9 and 97.3%, respectively. This 330-nucleotide segment allowed us to compare the sequences of the other BASN isolates and the BTAi 1 region from position 44 to position 337 sequenced by Young et al. (46). However, we believe that the data provided by longer sequences (Table 2) allow more accurate differentiation of close phylogenetic distances, where the added variables provide a higher level of analytical resolution.

DISCUSSION

Our analysis of several different strains of BASN bacteria may have revealed phylogenetic heterogeneity, as has been found with other symbiotic bacteria isolated from legumes, such as members of the root-nodulating genera *Rhizobium* and *Bradyrhizobium* and the stem-nodulating genus *Azorhizobium* (44). However, the BASN bacteria are monophyletic and cluster very closely together on the basis of the results of an analysis of nearly complete 16S rRNA sequences. We found that strains isolated from three *Aeschynomene* species and from widely separated geographic regions exhibit a very high degree of 16S rRNA sequence similarity.

It appears that in the case of the BASN bacteria, differences in host specificity for nodulation do not necessarily reflect phylogenetic distances, since strains falling into different groups based on host origin (1, 7) are in fact very closely related. The phylogenetic data are consistent with the suspicion that strains of *Aeschynomene* rhizobia may not be highly host specific, since such a phylogenetically coherent group would not be expected to exhibit phenotypic differentiation which might accompany host specificity. However, it has been noted that the sequence of the 16S rRNA molecule may be too highly conserved, even in hypervariable regions, to be used to reflect genetic differences responsible for host specificity.

It is conceivable that the bacteria capable of stem nodulating *Aeschynomene* plants were introduced into different geographic regions along with the introduction of the plants themselves into those regions, which may explain the close phylogenetic relationships among geographically diverse strains. However, the existing evidence does not lend strong support to such a hypothesis. The exact habitats of origin of the BASN strains studied appear to be especially significant. Strain BTAi 1 was isolated from a submerged stem nodule on greenhouse-grown *Aeschynomene indica*; however, nodules occurred only on plants grown in nonsterilized sand and not on plants grown in sterilized sand. It was concluded that the rooting medium, quartz sand obtained from an open pit mine in West Virginia and subject to contamination from surface soil, was the source of the rhizobia (34). In this case, there seems to be little doubt that the strain is an indigenous North American strain and, furthermore, a strain that most probably originated from soil rather than a strain that was obligately associated with *Aeschynomene* plants.

However, there is a lack of correspondence in the origin of symbiosis between BTAi 1 and its host *Aeschynomene indica*, since the plant is not originally American but is native to the

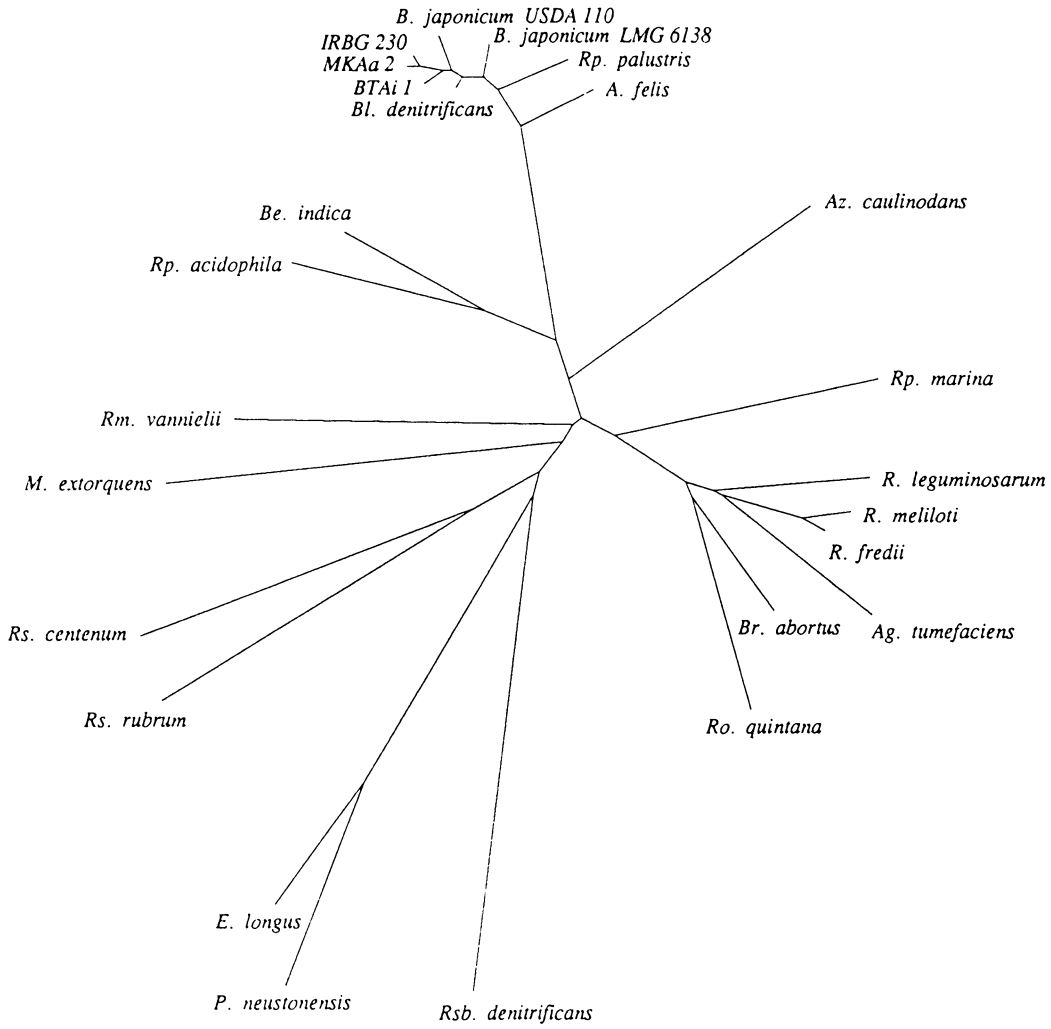

FIG. 2. Unrooted phylogenetic tree based on nearly complete 16S rDNA sequences, showing relationships of selected members of the alpha subclass of the *Proteobacteria* to Bchl-synthesizing stem-nodulating strains. Abbreviations: *A.*, *Afipia*; *Ag.*, *Agrobacterium*; *Az.*, *Azorhizobium*; *B.*, *Bradyrhizobium*; *Be.*, *Beijerinckia*; *Bl.*, *Blastobacter*, *Br.*, *Brucella*; *E.*, *Erythrobacter*; *M.*, *Methylobacterium*; *P.*, *Porphyrobacter*; *R.*, *Rhizobium*; *Rm.*, *Rhodomicrobium*; *Ro.*, *Rochalimaea*; *Rp.*, *Rhodopseudomonas*; *Rs.*, *Rhodospirillum*; *Rsb.*, *Roseobacter*.

Old World tropics (2, 37). The cases of isolates IRBG 2 and IRBG 230 and their hosts (*Aeschynomene afraspera* and *Aeschynomene nilotica*, respectively) are similar. The host plants were originally imported from Senegal, West Africa, but were cultivated in The Philippines, and the rhizobial isolates most probably infected the legumes directly from Philippine soil. In these cases, it appears that the symbiont bacterium must have evolved as a soil saprophyte and must survive normally in soil as a chemoheterotrophic bacterium, possibly by using photosynthetic abilities under starvation conditions, as suggested for marine aerobic photosynthetic bacteria (30). If this is so, BASN rhizobia might be isolated from many different soils and geographic regions irrespective of the presence of the *Aeschynomene* hosts. Consistent with this hypothesis, 16S rRNA sequences closely corresponding to the BTAi 1 sequence have been found in the clone library generated from random samples of acidic forested Australian soil obtained from subtropical Queensland (21, 22, 33). In fact, a phylogenetic comparison has revealed that our BASN strains, bradyrhizobial strains, and *Rhodopseudomonas palustris* fall into a cluster with many of the derived 16S rDNA soil clones, as determined by using a ca. 300-nucleotide fragment of the gene for comparison (unpublished tree data from this study). One group of soil clone sequences has been previously reported to be closely related to and to differ by only one to four nucleotides from the sequences of members of the cluster containing BTAi 1, *Bradyrhizobium japonicum*, *Rhodopseudomonas palustris*, and *Afipia felis* (22).

The genus *Bradyrhizobium* has been found on the basis of DNA-DNA homology data to be unrelated to bacteria belonging to the genera *Rhizobium* and *Agrobacterium* (28), while 16S rRNA oligonucleotide catalogs and total DNA-rRNA homol-

TABLE 2. Sequence similarity values based on nearly complete 16S rDNA sequences of BASN rhizobia and selected members of the alpha subclass of the *Proteobacteria*

Organism	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1. *Rhodomicrobium vannielii*																									
2. *Methylobacterium extorquens*	88.6																								
3. *Rhodospirillum centenum*	85.8	86.9																							
4. *Rhodospirillum rubrum*	85.7	85.8	87.7																						
5. *Erythrobacter longus*	85.4	86.7	86.5	85.3																					
6. *Porphyrobacter neustonensis*	84.9	86.1	85.1	83.3	94.7																				
7. *Roseobacter denitrificans*	85.5	85.9	84.0	84.8	85.7	83.9																			
8. *Rhodopseudomonas marina*	89.6	88.2	85.6	87.6	87.0	85.4	86.5																		
9. *Rochalimaea quintana*	88.2	88.2	84.5	85.4	87.3	86.6	86.6	91.5																	
10. *Brucella abortus*	89.4	89.1	86.6	86.6	88.3	87.2	87.2	91.3	94.3																
11. *Agrobacterium tumefaciens*	88.4	88.2	84.8	85.5	87.8	86.4	86.9	91.3	92.6	93.5															
12. *Rhizobium leguminosarum*	89.7	88.7	86.2	86.0	87.4	86.7	87.8	90.6	92.9	94.2	94.2														
13. *Rhizobium fredii*	89.7	89.7	86.2	86.1	88.1	87.3	88.4	91.9	94.7	95.9	94.3	95.2													
14. *Rhizobium meliloti*	89.3	89.2	86.0	86.4	87.4	87.3	88.1	92.8	94.2	95.4	93.9	95.6	98.3												
15. *Rhodopseudomonas acidophila*	89.1	89.6	86.7	85.0	86.6	85.4	85.7	88.1	88.4	89.6	88.4	88.9	89.8	89.4											
16. *Beijerinckia indica*	89.7	91.0	86.6	85.3	86.7	86.6	86.5	89.3	90.1	90.7	90.1	90.4	91.0	91.3	94.7										
17. *Azorhizobium caulinodans*	89.6	88.4	86.0	86.0	86.0	85.0	86.1	90.2	88.8	90.3	89.7	90.4	90.4	90.5	91.2	91.6									
18. *Afipia felis*	89.2	89.8	85.5	85.0	86.6	85.3	85.0	88.0	87.7	89.6	88.0	88.8	89.8	89.2	92.3	92.1	90.7								
19. *Rhodopseudomonas palustris*	88.6	89.2	85.0	85.0	86.6	85.2	83.4	88.3	88.2	89.8	87.3	88.0	88.7	88.2	91.0	90.7	89.2	97.0							
20. *Bradyrhizobium japonicum* [a]	89.1	89.4	85.2	85.0	86.4	85.0	84.2	88.2	87.6	89.1	87.3	88.2	89.1	88.6	92.1	91.7	90.1	98.3	98.1						
21. *B. japonicum* USDA 110	88.8	89.6	85.2	84.9	86.4	85.0	84.3	88.3	87.2	89.0	87.3	88.3	89.1	88.7	91.7	91.2	89.6	97.8	97.7	99.4					
22. *Blastobacter denitrificans*	89.0	89.9	85.7	85.2	86.9	85.2	84.6	88.8	87.7	89.8	88.3	89.0	89.4	89.0	91.6	91.0	89.9	97.3	97.1	98.6	99.0				
23. BTAi 1	89.1	89.9	85.6	85.2	86.8	85.1	84.6	88.6	87.5	89.4	88.0	88.8	89.2	88.8	91.7	91.0	89.9	97.5	97.3	98.8	99.3	99.6			
24. MKAa 2	88.8	89.8	85.5	84.9	86.9	85.2	84.5	88.1	87.2	89.7	87.8	88.7	89.1	88.7	92.2	91.7	90.0	97.8	97.4	98.9	99.1	98.8	99.3		
25. IRBG 230	88.7	89.7	85.4	84.8	86.8	85.1	84.4	88.0	87.1	89.4	87.7	88.6	89.0	88.6	92.1	91.5	89.9	97.5	97.3	98.8	98.8	98.7	99.1	99.7	

[a] *Bradyrhizobium japonicum* type strain LMG 6138. LMG, Collection of the Laboratory of Microbiology, Rijksuniversiteit Gent, Ghent, Belgium.

ogy data have confirmed this separation and have also placed the genus *Bradyrhizobium* in a distinct branch along with the anaerobic phototroph *Rhodopseudomonas palustris* (14, 16). It appears that as a group, bacteria falling under the classification of *Bradyrhizobium japonicum* are not as phylogenetically coherent as the members of the BASN rhizobia (compare USDA 110 with LMG 6138^T for instance). Our sequence data and data obtained by Young et al. (46) suggest that *Bradyrhizobium japonicum* groups I and Ia (15) are phylogenetically closely related to the BASN rhizobia, while *Bradyrhizobium japonicum* group II strains (15) are recognizably distinct; the latter organisms have been reclassified as *Bradyrhizobium elkanii* (18). Our analysis confirmed that *Bradyrhizobium japonicum* and *Rhodopseudomonas palustris* share a phylogenetically distinct alpha 2 lineage with representatives of the BASN rhizobia (Fig. 2).

It is now apparent that the lineage occupied by the BASN rhizobia represents an independent phylogenetic cluster (Fig. 2) which appears to contain as diverse a collection of bacteria as the cluster occupied by the genus *Rhizobium* and its relatives (23, 41, 44). We predict that as the phylogenetic data base expands, additional bacterial types belonging to this line of descent will be discovered. For instance, data from 16S rRNA oligonucleotide catalogs have revealed high levels of homology between *Rhodopseudomonas palustris* and the soil and aquatic chemolithotrophic aerobes belonging to the genus *Nitrobacter* (29). Even more significant is the fact that the cluster containing the BASN rhizobia and other representatives is bound together by extremely high levels of sequence similarity despite nomenclatural and phenotypic heterogeneity. Members of this group exhibit levels of sequence similarity of 97% or greater, and the group appears to be much more homogeneous than the genus *Rhizobium* and its relatives, which Yanagi and Yamasato (44) have shown to have a collective integrity value of not less than 92%.

On the basis of partial 16S rRNA sequence analysis data and DNA-DNA and DNA-rRNA cistron data, the need for a taxonomic revision of the genera *Rhodopseudomonas* and *Bradyrhizobium* has been suggested, and the possibility that such a revision might lead to *Bradyrhizobium* soybean symbionts being reassigned to the genus *Rhodopseudomonas* has been raised (15, 16, 46). In view of the general lack of consensus concerning phylogenetic quantification with regard to formal taxonomy and the extremely high phylogenetic similarity values exhibited by these bacteria, we suggest that such major taxonomic changes (at the genus level) should be made cautiously. A reassignment relying on existing taxonomic priorities and based on phylogenetic relationships to *Rhodopseudomonas palustris* is made even less attractive by the apparent phylogenetic heterogeneity of the genus *Rhodopseudomonas* (45), as indicated in our analysis by the wide separation of *Rhodopseudomonas palustris* from *Rhodopseudomonas acidophila* and *Rhodopseudomonas marina* (Fig. 2).

The final resolution of the taxonomic questions concerning these organisms can only be resolved by using both molecular sequence data and more detailed phenotypic data than are presently available for these strains. A potentially useful chemotaxomic property for comparisons between these bacteria and other groups is the characteristic lipopolysaccharide sugar 2,3-diamino-2,3-deoxy-D-glucose found in *Rhodopseudomonas palustris* but not in the phylogenetically distantly related organism *Rhodopseudomonas acidophila* (36). This marker may also be found in the BASN bacteria and the other closely related taxa in the phylogenetic cluster containing *Rhodopseudomonas palustris*. The polyphasic approach to reconciliation of phenotype and phylogeny (39) should be applied to these organisms. In addition, genus designations must be of practical use at the bench. Incorporating such phenotypically diverse groups of bacteria as the human pathogen *Afipia felis*, the photosynthetic anaerobe *Rhodopseudomonas palustris*, the budding chemoheterotroph *Blastobacter denitrificans*, and the plant-symbiotic root- and stem-nodulating strains into one genus will lead to confusion for field agricultural scientists rather than order. A starting point for future polyphasic

TABLE 3. 16S rRNA positions that vary among BASN rhizobia and related bacteria and are potentially useful for oligonucleotide probe design

Taxon	Nucleotides at positions:		
	1000-1002	1019-1021	1038-1040
BASN rhizobia	UUG	CGU	CGA
Blastobacter denitrificans	UUG	CGU	CGA
Bradyrhizobium japonicum	CCA	UGU	UGG
Rhodopseudomonas palustris	CCA	CGC	UGG
Afipia felis	CCA	UGU	UGG

classification is the set of criteria and proposed minimal standards specifically recommended to aid in the taxonomy of root- and stem-nodulating bacteria (8, 12).

A segment of the 16S rRNA of particular interest in work on the BASN rhizobia is an area corresponding to positions 995 through 1045. This region forms a distinctive forked loop in the secondary structure and contains three notable areas of nucleotide variability in the bacteria closely related to the BASN rhizobia (Table 3). These nucleotide sequences may have the potential to be used to distinguish the BASN bacteria from the other taxa. The combination of the region from positions 1019 to 1021 and the region from positions 1038 to 1040 may be suitable for use in the construction of an approximately 25-mer specific probe with up to two to three nucleotide mismatches compared with the homologous sequence regions of non-BASN bacteria. Although *Blastobacter denitrificans* appears to have signature sequences identical to those of the BASN bacteria, it possesses two additional nucleotides, C and U, around positions 1025 and 1034, respectively, in the segment between positions 1021 and 1038, thus providing sufficient differentiation to allow specificity. If such a fragment is confirmed to be specific to the BASN rhizobia, it may enable rapid diagnostic detection of these efficient nitrogen-fixing bacteria, providing a useful addition to soil-testing procedures for tropical agriculture.

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