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The Aquatic Budding Bacterium *Blastobacter denitrificans* Is a Nitrogen-Fixing Symbiont of *Aeschynomene indica*

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***Blastobacter* spp. are freshwater bacteria that form rosette structures by cellular attachment to a common base. Comparative analyses of ribosomal 16S rRNA gene and internally transcribed spacer region sequences indicated that *B. denitrificans* is a member of the α-subdivision of *Proteobacteria*. Among the α-*Proteobacteria*, *B. denitrificans* was related to a cluster of genera, including *Rhodopseudomonas palustris*, *Afipia felis*, *Nitrobacter hamburgensis*, and *Bradyrhizobium* spp. Although the precise phylogenetic relationships among these genera could not be established with a high degree of confidence, the sequences of *B. denitrificans* and several bradyrhizobial isolates from nodules of *Aeschynomene indica* were almost identical. Bradyrhizobia are bacteria that form nitrogen-fixing symbioses with legumes, including soybeans (*Glycine max*) and members of the genus *Aeschynomene*. From symbiotic infectiveness tests we demonstrated that the type strain for *B. denitrificans*, IFAM 1005, was capable of forming an effective nitrogen-fixing symbiosis with *A. indica*. Not only do these results reveal a previously unknown ecological adaptation of a relatively obscure aquatic bacterium, but they also demonstrate how evidence gathered from molecular systematic analyses can sometimes provide clues for predicting ecological behavior.**

Comparative sequence analyses of the 16S rRNA genes are widely used to reconstruct phylogenetic relationships among bacteria (15). A remarkable degree of ecological diversity exists within subgroup 2b of the α-*Proteobacteria* (9) because members include nitrogen-fixing symbionts of leguminous plants (*Bradyrhizobium* spp.), mammalian pathogens (*Afipia* spp.), nitrifying soil bacteria (*Nitrobacter* spp.), phototrophic bacteria (*Rhodopseudomonas palustris*), and the aquatic bud- and cluster-forming freshwater bacterium *Blastobacter denitrificans* (25).

Bradyrhizobium japonicum and *Bradyrhizobium elkanii* are most frequently associated with the formation of a nitrogen-fixing symbiosis with soybean. Perhaps it is no surprise that the stem-nodulating symbiotic bacteria of the flood-tolerant legume *Aeschynomene indica* (2, 29) are closely related to *Bradyrhizobium japonicum*, as estimated from 16S rRNA gene sequence divergence (17, 33, 34). However, from these reconstructions it also has been shown that *Bradyrhizobium japonicum* appears to be more closely related to the other genera of subgroup 2b than it is to *Bradyrhizobium elkanii* (25).

Not only does this indicate that the ecological adaptations within this group are poor indicators of phylogenetic relatedness, but it also implies that the genus *Bradyrhizobium* is polyphyletic.

The central focus of this study initially was to confirm the phylogenetic placement of bradyrhizobial isolates of *A. indica* since they reportedly are more closely related to *Bradyrhizobium japonicum* and other nonbradyrhizobial members of subgroup 2b than they are to *Bradyrhizobium elkanii* (17). From our analysis, which included type strains representing the named genera in the α-subdivision subgroup 2b, we concluded that some isolates were closely related to *B. denitrificans*. Therefore, we examined the possibility that the type strain of *B. denitrificans* (IFAM 1005) would form a symbiotic relationship with *A. indica*.

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MATERIALS AND METHODS

Bacterial strains. The strains used in this study, obtained from the U.S. Department of Agriculture Agricultural Research Service National *Rhizobium* Germplasm Collection, were originally isolated by van Berkum et al. (29) and are described in Table 1. *B. denitrificans* type strain IFAM 1005 (LMG 8443) and *Afipia felis* type strain ATCC 49715 were kindly provided by the Belgian Culture Collection of Microorganisms and the American Type Culture Collection, respectively. The sources of *A. indica* strain BTAi1, *A. americana* strain USDA 3177, *Methylobacterium extorquens* ATCC 8457 and ATCC 14718, *M. organophylum* ATCC 27886, *M. rhodinum* ATCC 14821, *Rhodobacter sphaeroides* 2.4.1, and *Rhodopseudomonas palustris* GH were as described previously (29). Sequencing results with *Rhodobacter sphaeroides* served as an outgroup for reconstruction of phylogenies from 16S rRNA gene and internally transcribed spacer (ITS) region sequence divergence.

Growth of the bacteria and DNA isolation. The phototrophic bradyrhizobia were grown in 50 ml of modified arabinose gluconate (MAG) broth (24) for the large-scale isolation of DNA purified by CsCl density centrifugation (19). MAG or charcoal-yeast extract broth (10 ml) was used to grow *B. denitrificans* or *Afipia felis*, respectively, for small-scale DNA preparations by using a Tissue and Blood DNA Extraction kit (Qiagen, Inc., Chatsworth, Calif.). Purification of DNA from the other cultures used in this study was as described by van Berkum et al. (29). Concentrations of DNA in solution were measured spectrophotometrically at 260 nm by using a Gilford Response Spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio).

PCR amplification and sequencing analysis. Primers 16Sa and 16Sb (26) were used for amplification of the 16S rRNA gene locus because PCR with primers fD1 and rD1 (31) sometimes failed or produced multiple bands. We determined by computer analysis by using Oligo (National Biosciences, Inc., Plymouth, Minn.) that the unsatisfactory amplification of the 16S rRNA gene with primers fD1 and rD1 probably resulted from the poor internal stability of these primers that may lead to false priming. The 16S rRNA genes were amplified in 120-μl volumes as described before (28) except for the primers and the PCR buffer [60 mM Tris-HCl, 15 mM $(NH_4)_2SO_4$, and 3.5 mM $MgCl_2$ at pH 9.0]. Primers 450 and 1440 (26) were used to amplify the ITS region and are located in conserved

TABLE 1. Bradyrhizobial strains originating from *A. indica* used in this study

USDA strain	Origin	Bchl phenotype[a]
USDA 4363	New Delhi, India	2
USDA 4366	New Delhi, India	3
USDA 4371	Maryland, United States	2
USDA 4379	Amazonas, Brazil	3
USDA 4393	Babungo, Cameroon	1
USDA 4399	Maryland, United States	2
USDA 4406	France	1
USDA 4409	Ibadan, Nigeria	1
USDA 4410	Ibadan, Nigeria	3
USDA 4415	Lampung, Indonesia	1
USDA 4421	Tamil Nadu, India	1
USDA 4424	Louisiana, United States	2
USDA 4430	Ibadan, Nigeria	–
USDA 4435	Maryland, United States	1
USDA 4440	Marondera, Zimbabwe	1

[a] Phenotypes: 1, no pigment formation; 2, pigment formation during day-night cycle; 3, pigment formation in the dark (29). –, Unknown.

regions of the 3′ end of the 16S rRNA gene and the 5′ end of the 23S rRNA gene. The PCR products generated with this primer pair also contained the intervening region (7, 10, 13, 14, 16, 20, 21, 32) of the 23S rRNA gene. PCR conditions were those described for the 16S rRNA gene. The PCR products were purified by using QIAquick Spin columns (Qiagen). A Perkin-Elmer 377 DNA Sequencer in combination with a DyeDeoxy Terminator Cycle Sequencing Kit (Perkin-Elmer, Foster City, Calif.) was used for sequencing the purified PCR products as described previously (26, 28). The sequences for USDA 4415, USDA 4409, USDA 4435, USDA 4440, USDA 4393, USDA 4363, USDA 4366, USDA 4410, USDA 4421, USDA 4424, BTAi1 (USDA 4362), USDA 4430, USDA 4371, USDA 4399, USDA 4406, USDA 4379, USDA 3177, *B. denitrificans* (LMG 8443), *Afipia felis* (ATCC 49715), *M. rhodinum* (ATCC 14821), *M. extorquens* (ATCC 8457), *M. organophylum* (ATCC 27886), and *Rhodobacter sphaeroides* (2.4.1) have been deposited in the GenBank database under accession numbers AF338159 through AF338182, respectively.

Analysis of the sequence data. The sequences were aligned by using the PILEUP program in the Wisconsin package of the Genetics Computer Group (Madison, Wis.). Aligned sequences were analyzed by using the Molecular Evolutionary Genetics Analysis (MEGA) package, version 1.01 (11), which was also used to generate bootstrap confidence values with 500 permutations of the data sets.

Southern hybridization analysis for detecting *nifHDK*. DNAs of *B. denitrificans* LMG 8443, the *A. indica* isolate USDA 4424, and *Bradyrhizobium japonicum* USDA 110 were restricted with *Eco*RI (Promega, Madison, Wis.) according to the manufacturer's protocol, and ca. 1 μg of digested DNA was separated according to molecular size in 0.7% agarose gels as described previously (29). Southern transfer and Southern hybridization with pDC4 (the *nifHDK* probe was kindly provided by Gary Ditta, University of California, San Diego) also were as described previously (29), except that the Gene Images random prime labeling module in conjunction with Gene Images CDP-Star detection module (Amersham Pharmacia Biotech, Piscataway, N.J.) was used according to the manufacturer's specifications to detect the *nifHDK* genes in the target DNAs.

Electron microscopy. Cultures of *B. denitrificans* recovered from nodules of *A. indica* were prepared for electron microscopy to examine cells for budding according to protocols used previously (30).

Plant tests. Seeds of *A. indica* were surface sterilized with concentrated H_2SO_4 for 3 min and then washed five times with sterile distilled water. The treated seeds were sown in sterile sand-filled Leonard jars (12), and 10 ml of MAG-grown late-log-phase 200-ml broth cultures was used to inoculate each jar. The cultures tested for symbiosis were BTAi1, USDA 4424, and *B. denitrificans* IFAM 1005 (LMG 8443). Each treatment was prepared in five replications, and five jars without inoculated bacteria served as controls. The plants were grown in a greenhouse without supplemental lighting for 34 days during July and August. The plants were uprooted, and the tops were cut off to determine nitrogenase activities as described by van Berkum et al. (29). Determinations for the concentration of ethylene in each chamber were as described by van Berkum and Sloger (27). The plant tops were dried at 60°C for 2 days to determine dry matter and total nitrogen contents as described previously (24). Nodules were used to isolate bacterial occupants in culture as described previously (29). Surface-sterilized seeds also were germinated on water agar plates at 30°C for 24 h to evaluate BTAi1 and *B. denitrificans* IFAM 1005 (LMG 8443) for nodulation of *A. indica* in growth pouches in triplicate on two separate occasions as described by van Berkum et al. (29).

RESULTS

Sequencing results. The molecular sizes of the PCR products obtained with amplification reactions with primers for the 16S rRNA gene were relatively uniform, varying in size only by ca. 100 bp. The products with *Afipia felis* strain ATCC 49715 and *B. denitrificans* strain IFAM 1005 (LMG 8443) were ca. 1,480 bp. Our 16S rRNA gene sequences for *B. denitrificans* and for *Afipia felis* were consistent with accessions S46917 and AF003937 for these two taxa in the GenBank database.

The molecular sizes of the PCR products obtained with amplification reactions with primers for the ITS region were variable. Among the 16 strains of *A. indica*, the products ranged from ca. 1,380 to 1,710 bp, and among the four methylotrophs, they ranged from ca. 1,085 to 1,165 bp. The products with *Bradyrhizobium* strain USDA 3177, *Rhodobacter sphaeroides* 2.4.1, *Afipia felis* strain ATCC 49715, *Rhodopseudomonas palustris* strain GH, and *B. denitrificans* strain IFAM 1005 (LMG 8443) were ca. 1,420, 1,165, 1,350, 1,400, and 1,670 bp, respectively.

Forty-four ITS region sequences were aligned and resulted in an alignment length of 1,650 bases. In a neighbor-joining tree generated from the aligned sequences, we placed the serotype strains representing *Bradyrhizobium elkanii* with strains representing *Bradyrhizobium japonicum* (Fig. 1). Placement of *N. hamburgensis*, *Afipia felis*, *Rhodopseudomonas palustris*, and *B. denitrificans* was proximal to the genus *Bradyrhizobium*. However, *B. denitrificans* IFAM 1005 (LMG 8443) and the *A. indica* isolates formed a distinct group separated from the other subgroup 2b genera.

Nodulation tests. Plant tests were done to evaluate whether *B. denitrificans* was able to form a symbiosis with *A. indica* since, on the basis of rRNA sequence similarities, it is closely related to isolates originating from nodules of this legume host plant. All three (LMG 8443, USDA 4424, and BTAi1) nodulated *A. indica*, whereas uninoculated control plants formed no nodules. Nitrogen fixation in the inoculated plants was evident by their increased growth and by the presence of acetylene reduction activity in their roots (Table 2). The symbiotic response of *B. denitrificans* was similar to that of USDA 4424 and was superior to that of BTAi1. The ability of *B. denitrificans* to express nitrogenase activity probably was due to the presence of a DNA region with homology to *nifHDK* of *Sinorhizobium meliloti* cloned in pDC4 (Fig. 2). The *B. denitrificans* plant test was repeated two additional times. In each case *B. denitrificans* nodulated *A. indica*, whereas the uninoculated plants developed no nodules. Isolations were subsequently made from nodules of the plants inoculated with *B. denitrificans*, and the identity of the nodule occupants was confirmed because the ITS region sequences of *B. denitrificans* and the nodule isolates were identical (data not shown) and because these isolates had the ability to propagate by budding (Fig. 3).

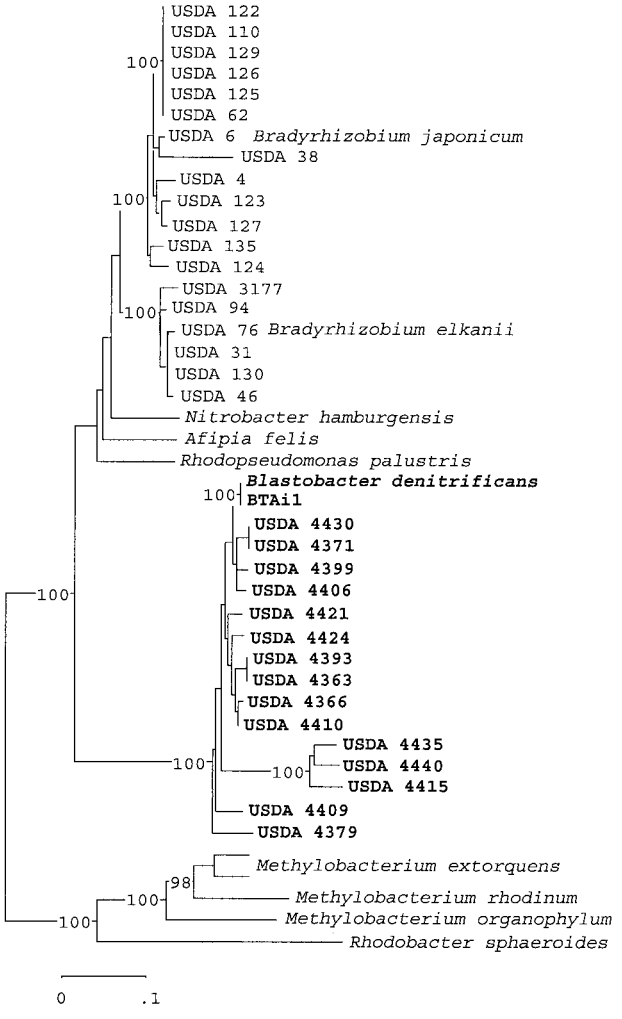

FIG. 1. Evolutionary relationship reconstructed from sequence divergence of the ITS region. Jukes-Cantor distances were derived from the aligned sequences to construct an unrooted tree by using the neighbor-joining method. The sequences were aligned by using the PILEUP program in the Wisconsin package of the Genetics Computer Group, and the aligned sequences were analyzed by using the MEGA package, version 1.01 (11). Five hundred permutations of the data set were generated in a bootstrap analysis to derive a majority rule consensus tree. The levels of support for the presence of nodes above a value of 90% are indicated in the neighbor-joining tree. The sequences for *Bradyrhizobium japonicum* USDA 122, USDA 123, USDA 124, USDA 125, USDA 126, USDA 127, USDA 129, USDA 135, USDA 38, USDA 4, USDA 62, and USDA 110; for *Bradyrhizobium elkanii* USDA 130, USDA 31, USDA 46, USDA 76, and USDA 94; and for *M. extorquens* ATCC 14718 were obtained from GenBank accession numbers AF208503, AF208504, AF208505, AF208506, AF208507, AF208508, AF208509, AF208511, AF208514, AF208515, AF208517, Z35330, AF208510, AF208512, AF208516, U35000, AF208518, and AF293375, respectively.

DISCUSSION

In this study we estimated the phylogenetic relationships among *Bradyrhizobium* strains originating from either soybeans or *Aeschynomene* and compared their evolutionary relationships with several closely related nonrhizobial genera. Strains representing 17 different serogroups of the soybean bradyrhizobia were included (26). We also examined sequence divergence among the ITS regions in these strains because these loci appear to evolve more rapidly than the 16S genes in the same operons (3). The ITS region was previously used to demonstrate considerable genetic variation among a collection of bradyrhizobial isolates of soybean (26). The significance of our results is that the relative placements of members within subgroup 2b of the α-*Proteobacteria* differed between reconstructions derived from 16S and ITS region sequence divergence. Additional loci will need to be examined to provide a more informed decision about the phylogenetic placements of *B. denitrificans* and the bradyrhizobial isolates of *A. indica*.

TABLE 2. Nitrogen fixation by symbioses of *A. indica* and *B. denitrificans* (LMG 8443) compared to two other strains isolated from nodules of this host legume[a]

Strain	Plant dry wt (mg/plant)	Total plant nitrogen (mg/plant)	Acetylene reduction[b] (μmol of CH_2H_4/plant)
LMG 8443	302 A	11.7 A	11.5 A
USDA 4424	299 A	11.1 A	4.7 B
BTAi1	241 B	7.3 B	6.5 B
Control	44 C	0.4 C	0 C

[a] Values in the same column flanked by the same capital letter are not significantly different at the 5% level of probability as determined by Duncan's new multiple range test.
[b] That is, nitrogenase activity.

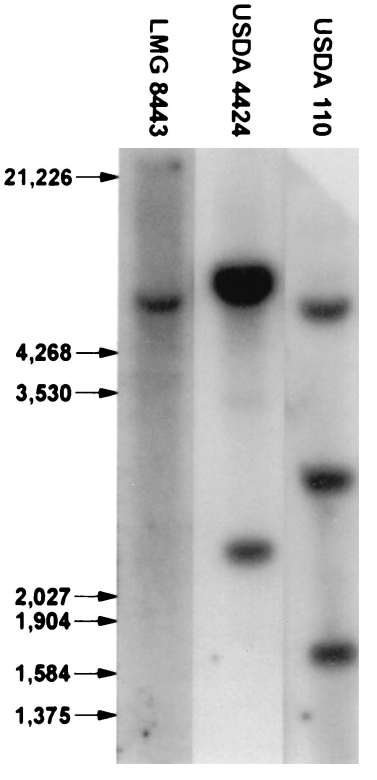

FIG. 2. Southern hybridization analysis with *nifHDK* genes of *S. meliloti* cloned in pDC4 as probe targeting *B. denitrificans* (LMG 8443), *A. indica* isolate USDA 4424, and *Bradyrhizobium japonicum* USDA 110 DNA. Molecular sizes in base pairs are indicated in the left margin.

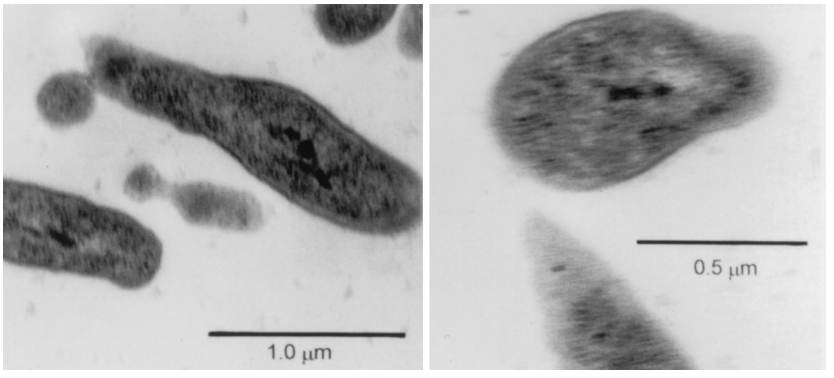

FIG. 3. Electron micrographs of *B. denitrificans* LMG 8443 after isolation in culture from nodules of *A. indica*.

We predicted from our results that *B. denitrificans* could well be a symbiotic bacterium of *A. indica*, since it was placed very close to several isolates of *A. indica* in our estimates of phylogenies based on 16S rRNA gene and ITS region sequence divergence. We confirmed this hypothesis through several plant tests in which the type strain of *B. denitrificans*, IFAM 1005 (LMG 8443), nodulated and formed nitrogen-fixing symbioses with *A. indica*. Since this strain of *B. denitrificans* was originally isolated from lake water in Germany (8) and not from a plant nodule, there was no reason (besides the rRNA data) to suspect that it might also be a nitrogen-fixing symbiont of legume plants. These results demonstrate how evidence gathered from the study of molecular systematics can sometimes provide clues for predicting ecological characteristics of poorly understood species. Even though our prediction was accurate in this case, it is important to recognize the need for caution when associating a metabolic function with an organism from molecular systematic data alone (1).

From our result the natural tendency might be to view the bradyrhizobia of *A. indica* as *Blastobacter* species or to consider changing the genus *Blastobacter* to *Bradyrhizobium*. However, before such changes in nomenclature can be proposed it is important to examine both the taxonomic status of *Blastobacter* and the bradyrhizobia that include the phototrophs.

Zavarzin (35) originally proposed the genus *Blastobacter* and described the type species, *B. henricii*, from cells observed in lake water without isolating it in pure culture. Four additional isolates were examined and each was proposed as a separate species, *B. aggregatus*, *B. capsulatus*, *B. denitrificans*, and *B. natatorius* (23), each listed on the *Approved Lists of Bacterial Names* (18). However, the genus *Blastobacter* is highly heterogeneous since these described species fall into at least three distinct branches of the α-subdivision of the *Proteobacteria* (9). Therefore, Hugenholtz et al. (9) suggested that, with the exception of *B. aggregatus* and *B. capsulatus*, the different species should not be incorporated into the same genus. As a solution to the problems in the taxonomy in *Blastobacter*, Sly and Cahill (22) proposed that new genera be created for the validated species and transferred the type strain for *B. natatorius* to the new genus *Blastomonas* as the type strain *Blastomonas natatoria*. Therefore, from our results it would seem justified that the type strain for *B. denitrificans* (IFAM 1005, LMG 8443) either be transferred to an existing genus or become the type strain for a new genus.

The symbiotic bacteria of *A. indica* are unique because they nodulate the plant stems, branches, and roots; some produce photosynthetic pigments (2, 4, 5, 6). Based on these and other phenotypic characters, Eaglesham et al. (5) proposed that these photosynthetic symbiotic bacteria be classified as *Photorhizobium thompsonianum* with BTAi1 (USDA 4362) as the type strain. However, this proposal was not validated and, on the basis of 16S rRNA gene sequence comparisons, it was concluded that these bacteria would be more appropriately classified as *Bradyrhizobium* (18, 33, 34).

Except for our ITS sequence data, it would seem warranted to suggest that *B. denitrificans* be reclassified as a *Bradyrhizobium*, perhaps as a separate species from *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii*, and *Bradyrhizobium liaoningense*. However, we propose that the taxonomy of *B. denitrificans* and the symbionts of the *A. indica* cross-inoculation group (2) be left unchanged until their phylogenetic placement in relationship with the genus *Bradyrhizobium* can be confirmed by using sequence divergence of other gene loci in addition to the 16S rRNA gene and the ITS region.

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