

# A New Species of *Devosia* That Forms a Unique Nitrogen-Fixing Root-Nodule Symbiosis with the Aquatic Legume *Neptunia natans* (L.f.) Druce

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**Rhizobia are the common bacterial symbionts that form nitrogen-fixing root nodules in legumes. However, recently other bacteria have been shown to nodulate and fix nitrogen symbiotically with these plants. *Neptunia natans* is an aquatic legume indigenous to tropical and subtropical regions and in African soils is nodulated by *Allorhizobium undicola*. This legume develops an unusual root-nodule symbiosis on floating stems in aquatic environments through a unique infection process. Here, we analyzed the low-molecular-weight RNA and 16S ribosomal DNA (rDNA) sequence of the same fast-growing isolates from India that were previously used to define the developmental morphology of the unique infection process in this symbiosis with *N. natans* and found that they are phylogenetically located in the genus *Devosia*, not *Allorhizobium* or *Rhizobium*. The 16S rDNA sequences of these two *Neptunia*-nodulating *Devosia* strains differ from the only species currently described in that genus, *Devosia riboflavina*. From the same isolated colonies, we also located their *nodD* and *nifH* genes involved in nodulation and nitrogen fixation on a plasmid of approximately 170 kb. Sequence analysis showed that their *nodD* and *nifH* genes are most closely related to *nodD* and *nifH* of *Rhizobium tropici*, suggesting that this newly described *Neptunia*-nodulating *Devosia* species may have acquired these symbiotic genes by horizontal transfer.**

*Neptunia natans* (L.f.) Druce is an aquatic legume native to several continents of the humid tropics and is used for both human consumption and as green manure for rice cultivation in Asiatic countries. This legume is unusual in that it normally develops buoyant floating stems that grow profusely on the surface of freshwater ponds, and in this aquatic environment it develops many stem-associated nitrogen-fixing nodules.

The developmental morphology of the infection process leading to formation of nitrogen-fixing nodules in *N. natans* has been examined under strict gnotobiotically controlled conditions, and several unique aspects of this specialized, aquatic plant-bacterium symbiosis distinguish it from other legume root-nodule symbioses (22). After colonizing the root and floating stem surfaces, the aquatic bacterium symbiont enters the primary root cortex and stem interior through natural wounds caused by splitting of the epidermis and emergence of young lateral and adventitious roots, respectively, and then stimulates early development of nodules in the cortex at the base of these roots primordia, but not in the stem itself (22). Following crack entry through the nodule periphery, the bacteria penetrate internal nodule host cells; induce formation of bona fide tubular infection threads that disseminate them fur-

ther intracellularly; and then release the bacteria into infection droplets, where they multiply. The endosymbiotic bacteria transform into nitrogen-fixing bacteroids within symbiosomes that eventually become filled with an unusual fibrillar matrix towards the end of their active nitrogen-fixing cycle in the aquatic environment. This symbiotic developmental process was the same for both bacterial isolates used in this previous study, named strains J1 and J2, isolated from surface-sterilized *N. natans* nodules collected in India. Those two strains exhibited a host preference for *N. natans* and were unable to nodulate several other legumes tested. Because these two isolates nodulated *Neptunia* roots, symbiotically fixed nitrogen, and were fast growing on standard *Rhizobium* culture media, they were provisionally called "*Rhizobium neptunii*."

The genera *Burkholderia* (13) and *Ralstonia* (3) are members of the  $\beta$ -subclass of the *Proteobacteria* ( $\beta$ -proteobacteria), recently reported to contain species that nodulate legumes, but most bacterial genera that form nitrogen-fixing nodules on legumes, including *Methylobacterium* (23), are in several families of  $\alpha$ -proteobacteria. Recently, several fast-growing *Neptunia*-nodulating bacteria isolated from Senegal (Africa) were assigned to a new rhizobial genus, *Allorhizobium* (10). This proposed bacterial genus included only one species, *Allorhizobium undicola*, belonging to the family *Rhizobiaceae*. We wondered whether all the bacteria that develop nitrogen-fixing root-nodule symbioses with *N. natans* are *Allorhizobium* species or whether they might include other taxa as well. Therefore, the primary objective of this work was to examine the

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phylogenetic relationship of the original isolates from India used in the previous study that documented the infection process during symbiotic root-nodule development in *N. natans* (22) and, by so doing, to determine if the taxon *A. undicola* as described by de Lajudie et al. (5) and Kuykendall and Dazzo (10) fully encompasses the unique group of diazotrophic nodule-inducing endosymbionts of this unusual aquatic legume.

#### MATERIALS AND METHODS

**Bacterial strains and culture media.** Strains J1 and J2 isolated from root nodules of *N. natans* in India (22) and the type strain of *Devosia riboflavina*, LMG 2277<sup>T</sup>, were cultivated in YMA medium (1) at 28°C.

**LMW RNA analysis.** Low-molecular-weight (LMW) RNA extraction was accomplished following the phenol-chloroform method described by Höfle (8). The following commercial molecules from Boehringer Mannheim (Mannheim, Germany) and Sigma (St. Louis, Mo.) were used as reference: 5S rRNA from *Escherichia coli* MRE 600 (120 and 115 nucleotides) (2) and *E. coli* tRNAs specific for tyrosine (85 nucleotides) and valine (77 nucleotides) (21). Samples containing 5 µg of LMW RNA were added to 5 µl of loading solution (sucrose [300 mg/ml], urea [460 mg/ml], 20% sodium dodecyl sulfate [10 µl/ml], xylene cyanol [1 mg/ml]) and, after 10 min of heating at 70°C, applied to each well. LMW RNA profiles were obtained using staircase electrophoresis in gels (400 by 360 by 0.4 mm) using a vertical slab unit (Poker Face SE 1500 sequencer; Hoefer Scientific Instruments, San Francisco, Calif.). The separating gel contained 14% acrylamide-*N,N*-methylene bisacrylamide (29:1 [wt/wt]) and 7 M urea in TBE buffer (100 mM Tris, 83 mM boric acid, 1 mM EDTA [pH 8.5]). The system was stabilized at 50°C, and then a preelectrophoresis run of 30 min at 100 V was performed. The running buffer (1.2× TBE) was recycled at a flow rate of 300 ml/min with a peristaltic pump (MasterFlex; Cole Parmer Instruments, Chicago, Ill.). After electrophoresis, the gels were silver stained according to the method of Haas et al. (6).

**DNA extraction and 16S ribosomal DNA (rDNA) sequence analysis of *nodD* and *nifH* genes.** DNA was extracted as described previously (17) from a single colony of each strain. The PCR amplification of whole 16S rDNA of J1 and J2 strains was carried out using primers 8F (5'-AGAGTTTGATCTGGCTCAG-3') and 1522R (5'-AAGGAGGTGATCCANCCRCA-3') under the following conditions: preheating at 95°C for 9 min; 35 cycles of denaturing at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 2 min; and a final extension at 72°C for 7 min. The sequence reaction was performed on an ABI377 sequencer (Applied Biosystems Inc.) using a BigDye terminator (version 3.0) cycle sequencing kit as supplied by the manufacturer. The following primers were used: 5'-AACGCTGGCGCRKGCYTA-3', 5'-ACTCCTACGGGAGGCAGCAG-3', 5'-CTGCTGCCTCCCGTAGGAGT-3', 5'-CGTGCCAGCAGCCGCGGTAA-3', 5'-CAGGATTAGATACCTGGTAG-3', and 5'-GAGGAAGGTGGGATGACGTC-3', which correspond to *E. coli* small-subunit rDNA sequence positions 32 to 52, 336 to 356, 356 to 336, 512 to 532, 782 to 803, and 1173 to 1194, respectively. The PCR amplifications of *nodD* and *nifH* genes were carried out with the DNA extracted from the same isolated colonies using the primer pair 5'-CTCGTCGCTCGACGCATTGA-3' and 5'-TGCCCCATGGACATGTA-3' (*nodD*) and the primer pair 5'-GTCTCCTATGACGTGCTCGG-3' and 5'-GCTTCCATGGTATCGGGGT-3' (*nifH*) under the following conditions: preheating at 95°C for 9 min; 35 cycles of denaturing at 95°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 2 min; and a final extension at 72°C for 7 min. The sequence reaction was performed the same way as for the 16S rDNA gene. The same primers for amplification were used in the gene sequencing.

**16S rDNA analysis.** The sequences obtained were compared with those from the GenBank using the FASTA program (15). Sequences were aligned using the Clustal W software (24). The distances were calculated according to Kimura's two-parameter method (9). Phylogenetic trees were inferred using the neighbor-joining method (19). Bootstrap analysis was based on 1,000 resamplings. The PHYLIP package (Phylogenetic inference package, version 3.5c; University of Washington, Seattle) was used for all analyses. The phylogenetic trees were rooted using *Bradyrhizobium japonicum* as the outgroup.

**Plasmid profile analysis.** The isolated strains were subjected to plasmid profile analysis according to the method of Plazinski et al. (16) except that electrophoresis was done at 2 V cm<sup>-1</sup> for 90 min, followed by 3 V cm<sup>-1</sup> for 60 min and finally at 6 V cm<sup>-1</sup> for 3 h. The 175- and 205-kb plasmids of *Sinorhizobium meliloti* GR4 (25) were used as size markers. Plasmid DNA was capillary transferred to a nylon

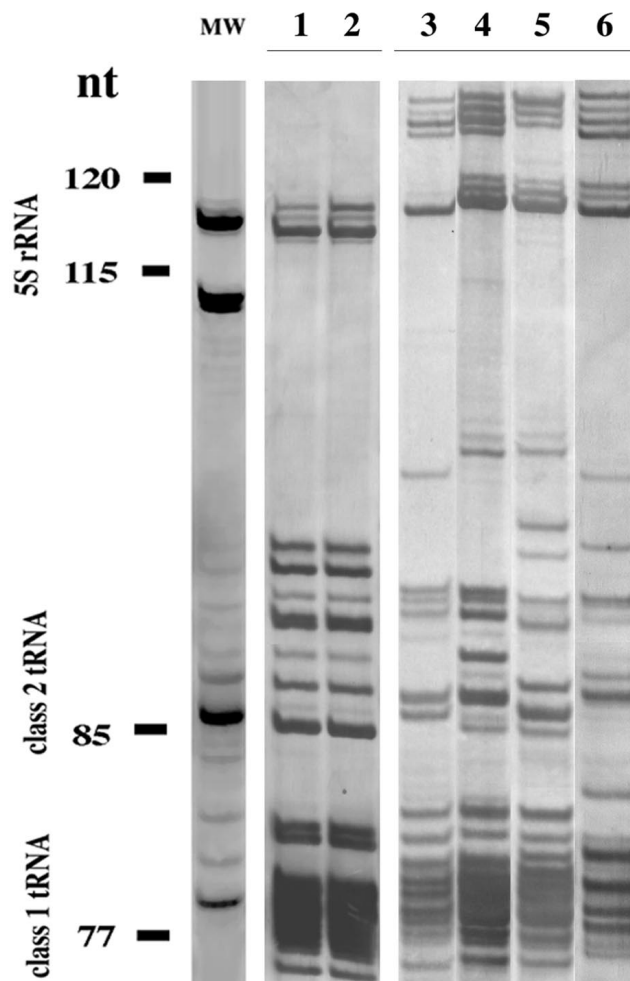


FIG. 1. LMW RNA profiles of type species of fast-growing genera of bacteria from the family *Rhizobiaceae* that nodulate various legumes and of strains that nodulate *N. natans*: strain J1 (lane 1), strain J2 (lane 2), *A. undicola* LMG 11875<sup>T</sup> (lane 3), *R. leguminosarum* ATCC 10004<sup>T</sup> (lane 4), *S. meliloti* ATCC 3390<sup>T</sup> (lane 5), and *M. loti* ATCC 3669<sup>T</sup> (lane 6).

membrane according to the method of Southern (20) and immobilized by baking at 80°C for 2 h.

**Oligonucleotide primers and preparation of DNA probe.** Oligonucleotide primers were designed to amplify a fragment of the *nodD* and *nifH* genes conserved among members of the family *Rhizobiaceae*. These primers were *nodD*1F (5'-CTCGTCGCTCGACGCATTGA-3') and *nodD*1R (5'-TGCCCCATGGACATGTA-3') (positions 31 to 53 and 585 to 568; *S. meliloti nodD* gene, AE007238) and primers *nifH*1F (5'-GTCTCCTATGACGTGCTCGG-3') and *nifH*1R (5'-GCTTCCATGGTATCGGGGT-3') (positions 367 to 389 and 794 to 774; *S. meliloti nifH* gene, AE007235).

The PCR-amplified fragments of *nifH* and *nodD* were digoxigenin labeled with the DIG DNA labeling kit (Roche Diagnostics Corp.) following manufacturer instructions and used as probes. Hybridization was detected with the DIG nucleic acid detection kit (Boehringer Mannheim, USA), using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium as substrates for alkaline phosphatase, according to the manufacturer instructions.

#### RESULTS AND DISCUSSION

**LMW RNA analysis.** Staircase electrophoresis typically separates LMW RNA molecules into three resolved zones: 5S rRNA and class 1 and class 2 tRNA (4). We have already

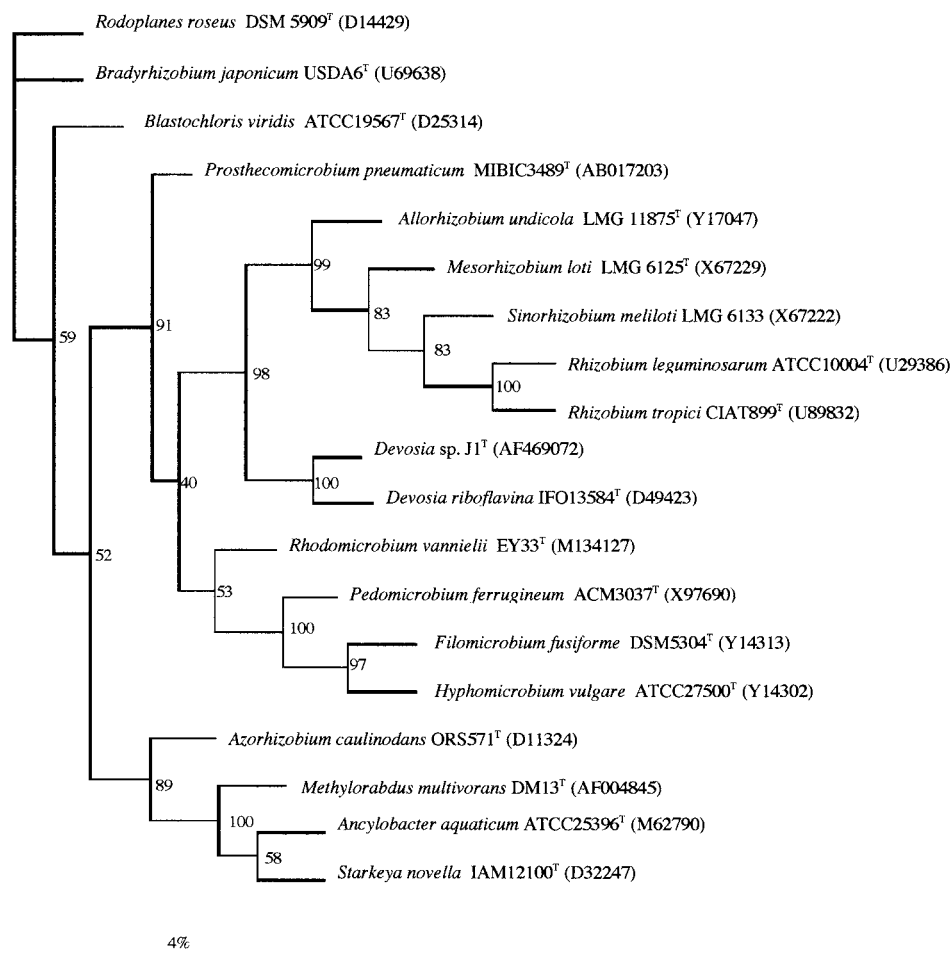


FIG. 2. Comparative sequence analysis of 16S rDNA from strain J1 and representative related strains from GenBank. The significance of each branch is indicated by a bootstrap value calculated for 1,000 subsets. Bar, 4 nt substitutions per 100 nt.

demonstrated that this powerful electrophoretic profiling technique can rapidly differentiate many bacterial genera on the basis of their 5S rRNA zone (26, 27, 28, 29) and many species (from the same or different genera) according to their tRNA profiles. Therefore, these profiles provide an excellent way to detect new microbial genera and species. For this reason we analyzed the LMW RNA profile of the *Neptunia*-nodulating isolates J1 and J2. Figure 1 (lanes 1 and 2) shows that the J1 and J2 strains are closely related in that they display identical LMW RNA profiles. However, the 5S rRNA zone of the profiles for both strains J1 and J2 is very different from the corresponding 5S rRNA zone of the type strain of *A. undicola* (Fig. 1, lane 3), which also nodulates *N. natans*. The 5S rRNA zone of strains J1 and J2 was also different from the corresponding zone in the LMW RNA profile of the other fast-growing rhizobia, *Rhizobium leguminosarum* (lane 4) and *S. meliloti* (lane 5), and from those of *Mesorhizobium loti* (lane 6). Therefore, staircase electrophoresis of LMW RNA profiling provided early, clear evidence that the *Neptunia*-nodulating strains J1 and J2 are unrelated to any of the type strains of fast-growing rhizobial genera already described. The profile of the 5S rRNA zones of strains J1 and J2 is different from those

of the genus *Allorhizobium*, which nodulates *Neptunia*, and the other genera of fast-growing rhizobia, *Rhizobium* and *Sinorhizobium*, indicating that these two *Neptunia*-nodulating isolates do not belong to these genera.

**16S rDNA sequencing and analysis.** As the accepted technique to identify bacteria at genus level is 16S rDNA sequencing, we have obtained the complete 16S rDNA sequences of strains J1 and J2. These sequences were compared with those from databanks using the FASTA program (15). Both sequences showed a 100% similarity to each other, and therefore only strain J1 (accession number AF469072) was included in developing the phylogenetic tree. Unexpectedly, the phylogenetic analysis of its 16S rDNA sequence (Fig. 2) showed that isolate J1 was most closely related to the genus *Devosia* in the family *Hyphomicrobiaceae* within the order *Rhizobiales* ( $\alpha$ -proteobacteria), which also contains the rhizobial species *Azorhizobium caulinodans*. The genus *Devosia* was proposed by Nakagawa et al. (14), and until now, this genus did not contain any nitrogen-fixing legume endosymbionts. *Devosia* currently includes only one known species, *Devosia riboflavina* (formerly *Pseudomonas riboflavina*) isolated from soil. This species is a gram-negative, aerobic, nonsporulated motile rod that con-

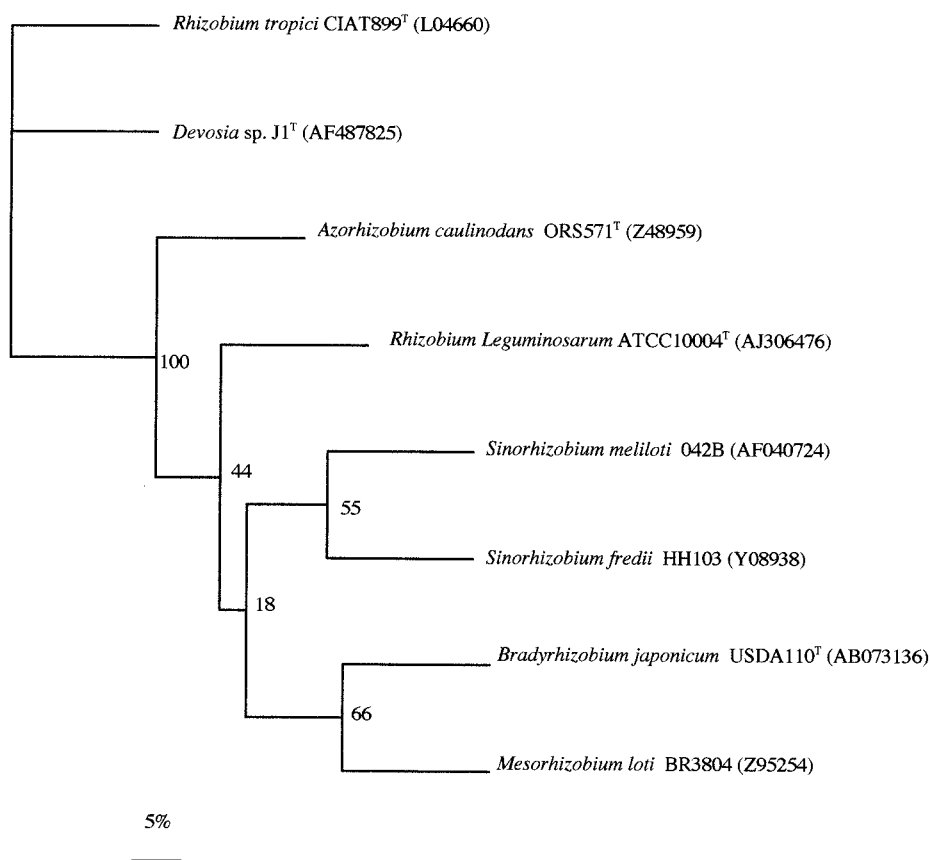


FIG. 3. Comparative sequence analysis of *nodD* gene sequences from strain J1 and representative related strains from GenBank. The significance of each branch is indicated by a bootstrap value calculated for 1,000 subsets. Bar, 5 nt substitutions per 100 nt.

tains long-chain 3-hydroxy fatty acids. Phylogenetic analysis of the 16S rDNA sequence of strain J1 indicates that its closest known species is *D. riboflavina* (95.9% similarity), but the difference of 30 nucleotide positions in the 16S rDNA sequences of both *Neptunia*-nodulating strains J1 and J2 strongly suggest that they are not *D. riboflavina*, but rather should belong to a new species of *Devosia*.

**Amplification and sequencing of *nodD* and *nifH* genes in strain J1.** The characteristics of legume nodulation and symbiotic nitrogen fixation in rhizobia are codified in *nod* and *nif* genes, respectively. Among these, the *nodD* and *nifH* genes are highly conserved among rhizobia. To design adequate primers for amplification of rhizobial *nodD* and *nifH* genes, we analyzed their sequences from several rhizobial and nonrhizobial species in GenBank. Based on highly homologous sequences, we designed the primers used in this work to amplify the *nodD* and *nifH* genes. Using total DNA from the same isolated colony of strain J1 that had also been used to analyze its 16S rDNA sequence, we have amplified a band corresponding to these *nodD* and *nifH* genes (accession numbers AF487825 and AF487824, respectively), and their sequences were obtained and compared with those of rhizobia (Fig. 3 and 4). As shown in Fig. 3 and 4, the sequences of *nodD* and *nifH* from strain J1 are closely related to the *nodD* and *nifH* genes of *Rhizobium tropici* CIAT 899<sup>T</sup>, respectively. CIAT 899 is a well-known, highly competitive and effective strain used in many studies of

the bean-rhizobium root-nodule symbiosis in Central and South America.

**Plasmid profile and location of *nodD* and *nifH* genes in strains J1 and J2.** The symbiotic genes that codify nodulation and nitrogen fixation are located in plasmids in fast-growing rhizobia and on chromosomes in slow-growing rhizobia. These symbiotic plasmids in fast-growing rhizobia can be transferred among species in the rhizosphere. We analyzed the plasmid profiles of J1 and J2 to test if their *nodD* and *nifH* genes are located in extrachromosomal DNA elements. This technique revealed two plasmids in both strains J1 and J2 (Fig. 5A, lanes 2 and 3) and a single plasmid in the type strain of *D. riboflavina* (Fig. 5A, lane 1). Specific probes detected both *nodD* and *nifH* in the same plasmid of ca. 170 kb in the plasmid profiles of both J1 and J2 isolates (Fig. 5B, lanes 5 and 6, and Fig. 5C, lanes 8 and 9, respectively), and neither of these two symbiotic genes was detected in the type strain of *D. riboflavina* (Fig. 5B and C, lanes 4 and 7, respectively).

Based on these results, we conclude that natural isolates belonging to a new species in the genus *Devosia* are able to establish a nitrogen-fixing root-nodule symbiosis with the aquatic legume *N. natans* (L.f.) Druce. The aquatic growth habit and developmental morphology displayed in this beneficial plant-bacterium symbiosis has been thoroughly described and represents a unique ecology and infection process (22). These isolates of *Devosia* described here are phylogenetically unre-

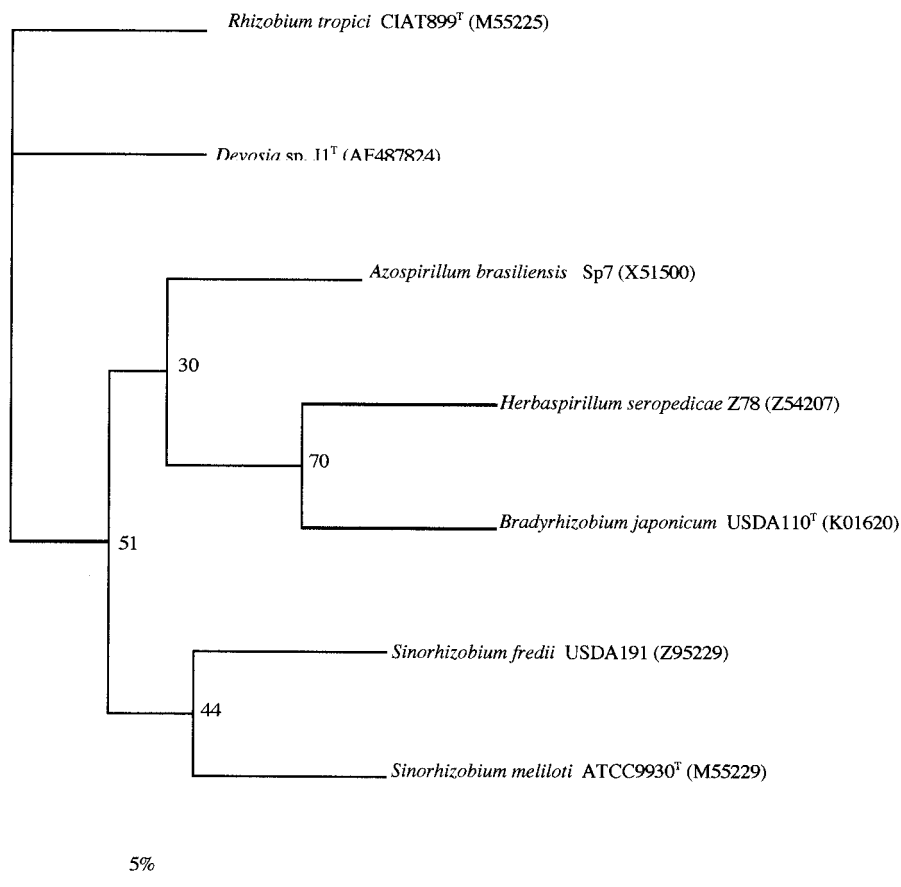


FIG. 4. Comparative sequence analysis of *nifH* gene sequences from strain J1 and representative related strains from GenBank. The significance of each branch is indicated by a bootstrap value calculated for 1,000 subsets. Bar, 5 nt substitutions per 100 nt.

lated to *R. tropici* (90% similarity in 16S rDNA sequences) and *A. undicola*, representing the only other bacterial species currently reported to form root nodules on the same aquatic legume host. These data add to the extension of legume-nodulating bacterial symbionts, e.g., the genera *Methylobacterium*, *Burkholderia*, and *Ralstonia* (3, 13, 23), that are away from the traditional rhizobia and bradyrhizobia, but because only about 50 of the 750 known legume genera have been studied, it is quite likely that more species of diazotrophic endosymbionts of legume root nodules unrelated to rhizobia will continue to be discovered by characterizing the bacteria nodulating unexplored legumes or geographical locations.

The *nod* and *nif* genes of fast-growing rhizobia required for nodulation and symbiotic nitrogen fixation in legumes commonly reside in plasmids, called pSym, that in some cases are autoconjugative. Consistent with this, we found that both *nodD* and *nifH* genes in strains J1 and J2 are located in a ca. 170-kb plasmid. The very similar sequences of the *nodD* and *nifH* of strains J1 and J2 with the corresponding genes of *R. tropici* suggest that these two *Neptunia*-nodulating *Devosia* isolates from India may have acquired these symbiotic genes by horizontal transference from rhizobia, possibly *R. tropici* that has a broad host range (7, 12) and carries Sym plasmids prone for horizontal transfer to different bacterial backgrounds (11, 18).

We are currently performing a polyphasic analysis to thoroughly describe this new species of *Devosia* (tentatively named

“*Devosia neptuniae*”) and are studying several additional nodule isolates from *Neptunia* from Mesoamerica to compare the diversity of populations that nodulate this unique legume in several continents.

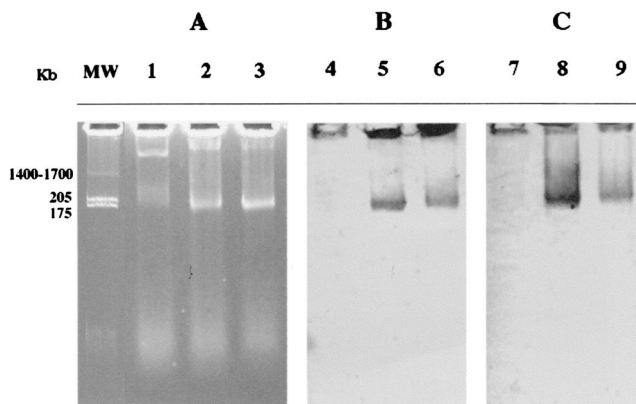


FIG. 5. (A) Plasmid profile in horizontal 0.7% agarose gel. Lanes: 1, *D. riboflavina* LMG2277<sup>T</sup>; 2, strain J1; 3, strain J2; MW, *S. meliloti* GR4. (B) Southern blot hybridization with the digoxigenin-labeled *nodD* probe. Lanes: 4, *D. riboflavina* LMG2277<sup>T</sup>; 5, strain J1; 6, strain J2. (C) Southern blot hybridization with the digoxigenin-labeled *nifH* probe. Lanes: 7, *D. riboflavina* LMG2277<sup>T</sup>; 8, strain J1; 9, strain J2.

## ACKNOWLEDGMENTS

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## REFERENCES

- Bergersen, F. J. 1961. The growth of *Rhizobium* in synthetic media. *Austr. J. Biol.* **14**:349–360.
- Bidle, K. D., and M. Fletcher. 1995. Comparison of free-living and particle-associated bacterial communities in the Chesapeake Bay by stable low-molecular-weight RNA analysis. *Appl. Environ. Microbiol.* **61**:944–952.
- Chen, W. M., S. Laevens, T. M. Lee, T. Coenye, P. de Vos, M. Mergeay, and P. Vandamme. 2001. *Ralstonia taiwanensis* sp. nov., isolated from root nodules of *Mimosa* species and sputum of a cystic fibrosis patient. *Int. J. Syst. Evol. Microbiol.* **51**:1729–1735.
- Cruz-Sánchez, J. M., E. Velázquez, P. Mateos, and E. Martínez-Molina. 1997. Enhancement of resolution of low molecular weight RNA profiles by staircase electrophoresis. *Electrophoresis* **18**:1909–1911.
- de Lajudie, P., E. Laurent-Fullele, A. Willems, U. Torck, R. Coopman, M. D. Collins, K. Kersters, B. Dreyfus, and M. Gillis. 1998. *Allorhizobium undicola* gen. nov., sp. nov., nitrogen-fixing bacteria that efficiently nodulate *Neptunia natans* in Senegal. *Int. J. Syst. Bacteriol.* **48**:1277–1290.
- Haas, H., B. Budowle, and G. Weiler. 1994. Horizontal polyacrylamide gel electrophoresis for the separation of DNA fragments. *Electrophoresis* **15**:153–158.
- Hernández-Lucas, I., L. Segovia, E. Martínez-Romero, and S. G. Pueppke. 1995. Phylogenetic relationships and host range of *Rhizobium* spp. that nodulate *Phaseolus vulgaris* L. *Appl. Environ. Microbiol.* **61**:2775–2779.
- Höfle, M. G. 1988. Identification of bacteria by low molecular weight RNA profiles: a new chemotaxonomic approach. *J. Microbiol. Methods* **8**:235–248.
- Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**:111–120.
- Kuykendall, L. D., and F. B. Dazzo. *Allorhizobium* De Lajudie, Laurent-Fullele, Willems, Torck, Coopman, Collins, Kersters, Dreyfus & Gillis, 1998. *Allorhizobium*. In D. Brenner, N. Krieg, J. Staley, and G. Garrity (ed.), *The Proteobacteria*. Bergey's manual of systematic bacteriology, 2nd ed., vol. 2, in press. Springer-Verlag, New York, N.Y.
- Martínez, E., R. Palacios, and F. Sánchez. 1987. Nitrogen-fixing nodules induced by *Agrobacterium tumefaciens* harboring *Rhizobium phaseoli* plasmids. *J. Bacteriol.* **169**:2828–2834.
- Martínez-Romero, E., L. Segovia, F. M. Mercante, A. A. Franco, P. Graham, and M. A. Pardo. 1991. *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. *Int. J. Syst. Bacteriol.* **41**:417–426.
- Moulin, L., A. Munive, B. Dreyfus, and C. Boivin-Masson. 2001. Nodulation of legumes by members of  $\beta$ -subclass of Proteobacteria. *Nature* **411**:948–950.
- Nakagawa, Y., T. Sakani, and A. Yokota. 1996. Transfer of "Pseudomonas riboflavina" (Foster 1944), a gram-negative, motile rod with long-chain 3-hydroxy fatty acids, to *Devosia riboflavina* gen. nov., sp. nov., nom. rev. *Int. J. Syst. Bacteriol.* **46**:16–22.
- Pearson, W. R., and D. J. Lipman. 1988. Search for DNA homologies was performed with the FASTA program. *Proc. Natl. Acad. Sci. USA* **85**:2444–2448.
- Plazinski, J., Y. H. Chen, and B. G. Rolfe. 1985. General method for the identification of plasmid species in fast-growing soil microorganisms. *Appl. Environ. Microbiol.* **48**:1001–1003.
- Rivas, R., E. Velázquez, A. Valverde, P. F., Mateos, and E. Martínez-Molina. 2001. A two primers random amplified polymorphic DNA procedure to obtain polymerase chain reaction fingerprints of bacterial species. *Electrophoresis* **22**:1086–1089.
- Rogel, M. A., I. Hernández-Lucas, L. D. Kuykendall, D. L. Balkwill, and E. Martínez-Romero. 2001. Nitrogen-fixing nodules with *Ensifer adhaerens* harboring *Rhizobium tropici* symbiotic plasmids. *Appl. Environ. Microbiol.* **67**:3264–3268.
- Saitou, N., and M. Nei. 1987. A neighbour-joining method: a new method for reconstructing phylogenetics trees. *Mol. Biol. Evol.* **44**:406–425.
- Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503–517.
- Sprinzi, M., J. Moll, F. Meissner, and T. Hartmann. 1985. Compilation of tRNA sequences. *Nucleic Acids Res.* **13**:1–49.
- Subba-Rao, N. S., P. F. Mateos, D. Baker, H. Pankraz, J. Palma, J., F. B. Dazzo, and J. I. Sprent. 1995. The unique root-nodule symbiosis between *Rhizobium* and the aquatic legume *Neptunia natans* (L.f.) Druce. *Planta* **196**:311–320.
- Sy, A., E. Giraud, P. Jourand, N. García, A. Willems, A., P. de Lajudie, Y. Prin, M. Neyra, M. Gillis, C. Boivin-Masson, and B. Dreyfus. 2001. Methylo-trophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. *J. Bacteriol.* **183**:214–220.
- Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The clustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **24**:4876–4882.
- Toro, N., and J. Olivares. 1986. Characterization of a large plasmid of *Rhizobium meliloti* involved in enhancing nodulation. *Mol. Gen. Genet.* **202**:331–335.
- Velázquez, E., E. Cervantes, J. M. Igual, A. Peix, P. F. Mateos, S. Benamar, A. Moiroud, C. T. Wheeler, J. Dawson, D. Labeda, C. Rodríguez-Barrueco, and E. Martínez-Molina. 1998. Analysis of LMW RNA profiles of *Frankia* strains by staircase electrophoresis. *Syst. Appl. Microbiol.* **21**:539–545.
- Velázquez, E., J. M. Igual, A. Willems, M. P. Fernández, E. Muñoz, P. F. Mateos, A. Abril, N. Toro, P. Normand, E. Cervantes, M. Gillis, and E. Martínez-Molina. 2001. Description of *Mesorhizobium chacoense* sp. nov. that nodulates *Prosopis alba* in the Chaco Arido region (Argentina). *Int. J. Syst. Evol. Microbiol.* **51**:1011–1021.
- Velázquez, E., E. Martínez-Romero, D. N. Rodríguez-Navarro, M. E. Trujillo, A. Daza, P. F. Mateos, E. Martínez-Molina, and P. Van Berkum. 2001. Characterization of rhizobial isolates of *Phaseolus vulgaris* by staircase electrophoresis of low-molecular-weight RNA. *Appl. Environ. Microbiol.* **67**:1008–1010.
- Velázquez, E., M. E. Trujillo, A. Peix, J. L. Palomo, P. García-Benavides, P. F. Mateos, A. Ventosa, and E. Martínez-Molina. 2001. Stable low molecular weight RNA analyzed by staircase electrophoresis, a molecular signature for both prokaryotic and eukaryotic microorganisms. *Syst. Appl. Microbiol.* **24**:490–499.