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Methylotrophic *Methylobacterium* Bacteria Nodulate and Fix Nitrogen in Symbiosis with Legumes

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Rhizobia described so far belong to three distinct phylogenetic branches within the α-2 subclass of *Proteobacteria*. Here we report the discovery of a fourth rhizobial branch involving bacteria of the *Methylobacterium* genus. Rhizobia isolated from *Crotalaria* legumes were assigned to a new species, "*Methylobacterium nodulans*," within the *Methylobacterium* genus on the basis of 16S ribosomal DNA analyses. We demonstrated that these rhizobia facultatively grow on methanol, which is a characteristic of *Methylobacterium* spp. but a unique feature among rhizobia. Genes encoding two key enzymes of methylotrophy and nodulation, the *mxaF* gene, encoding the α subunit of the methanol dehydrogenase, and the *nodA* gene, encoding an acyltransferase involved in Nod factor biosynthesis, were sequenced for the type strain, ORS2060. Plant tests and *nodA* amplification assays showed that "*M. nodulans*" is the only nodulating *Methylobacterium* sp. identified so far. Phylogenetic sequence analysis showed that "*M. nodulans*" NodA is closely related to *Bradyrhizobium* NodA, suggesting that this gene was acquired by horizontal gene transfer.

Symbioses between leguminous plants and soil bacteria commonly referred to as rhizobia are of considerable environmental and agricultural importance since they are responsible for most of the atmospheric nitrogen fixed on land. Rhizobia are able to elicit on most of the 18,000 species of the *Leguminosae* family the formation of specialized organs, called nodules, in which they reduce atmospheric nitrogen to ammonia to the benefit of the plant. Nodule formation is controlled by extracellular bacterial signal molecules, called Nod factors, which are recognized by the host plant (21, 34). The rhizobial species described so far are very diverse and do not form an evolutionary homogenous clade. They belong to three distinct branches within the α-2 subclass of *Proteobacteria* and are phylogenetically intertwined with non-symbiotic bacteria (40) (Fig. 1). A first large branch groups the genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, and *Allorhizobium* with *Agrobacterium*, a pathogenic bacterium of plants. A second branch contains the genus *Bradyrhizobium* together with photosynthetic free-living *Rhodopseudomonas*, whereas the third branch includes the genus *Azorhizobium* as well as the chemiautotroph *Xanthobacter.* Each rhizobial species has a defined host range, varying from very narrow, as in the case of *Azorhizobium caulinodans* (6), to very broad, as in the case of *Sinorhizobium* sp. strain NGR234 (30). Symbionts of legumes exhibiting ecological and agronomic potential should be characterized prior to their use in sustainable agriculture and environment management.

Crotalaria spp. are herbs and shrubs of the subfamily Papilionoideae; it is the largest plant genus in Africa. More than 500 species commonly occur in diverse climatological situations, from semidesert to rain forests and high mountains (1, 29). Some *Crotalaria* spp. are of great agronomic interest since they are used as green manure to improve soil fertility or control nematode populations in infested soils (4, 20). Characterization of a collection of rhizobia isolated from various *Crotalaria* species revealed two very distinct groups of symbiotic bacteria, a group of broad-host-range rhizobia related to *Bradyrhizobium* and a group of highly specific rhizobia of unknown taxonomic status (33).

We now report that the latter group of highly specific *Crotalaria* rhizobia belong to the *Methylobacterium* genus and assign them to a new species, for which we propose the name "*M. nodulans*." These *Methylobacterium* strains thus constitute a novel and fourth group of nitrogen-fixing legume-symbiotic bacteria. We demonstrated that "*M. nodulans*" is a facultative methylotroph, which is a unique property among rhizobia.

MATERIALS AND METHODS

Bacterial strains. Strains isolated from *Crotalaria* spp. are listed in Table 1. *Methylobacterium rhodesianum* LMG6086, *Methylobacterium organophilum* LMG6083, *Methylobacterium extorquens* LMG4250, *Methylobacterium rhodinum* LMG2275, *Methylobacterium zatmanii* LMG6087, *Methylobacterium mesophilicum* LMG5275, and *Methylobacterium* sp. strains LMG6378, LMG6085, and LMG6380 were from the collection of the Universiteit Gent (5). *Sinorhizobium meliloti* RCR2011, *Sinorhizobium medicae* A321, *Sinorhizobium fredii* USDA205, *Sinorhizobium terangae* ORS1009, *Rhizobium leguminosarum* bv. viciae 248, *Rhizobium etli* CFN42, *Rhizobium tropici* CIAT899, *Methylobacterium ciceri* UPMCa-7, *Methylobacterium loti* NZP2213, *Bradyrhizobium japonicum* USDA110, *Bradyrhizobium elkanii* USDA61, *Allorhizobium undicola* ORS995, and *Azorhizobium caulinodans* ORS571 were from our collection. The growth medium for *Methylobacterium* strains, including "*M. nodulans*" ORS2060 and ORS1917, was M72 (5) supplemented with 50 mM methanol. The complete medium for other strains was YM (37). Nodulating *Methylobacterium* strains were grown at 37°C; other strains were grown at 30°C.

DNA technology. Genomic DNA was prepared by using the method of Chen and Kuo (7). Plasmid DNA was isolated with a Miniprep kit (Promega, Charbonières, France). PCR products were purified with a QIAquick gel extraction kit (Qiagen, Courtaboeuf, France). Restriction endonucleases and ligase were

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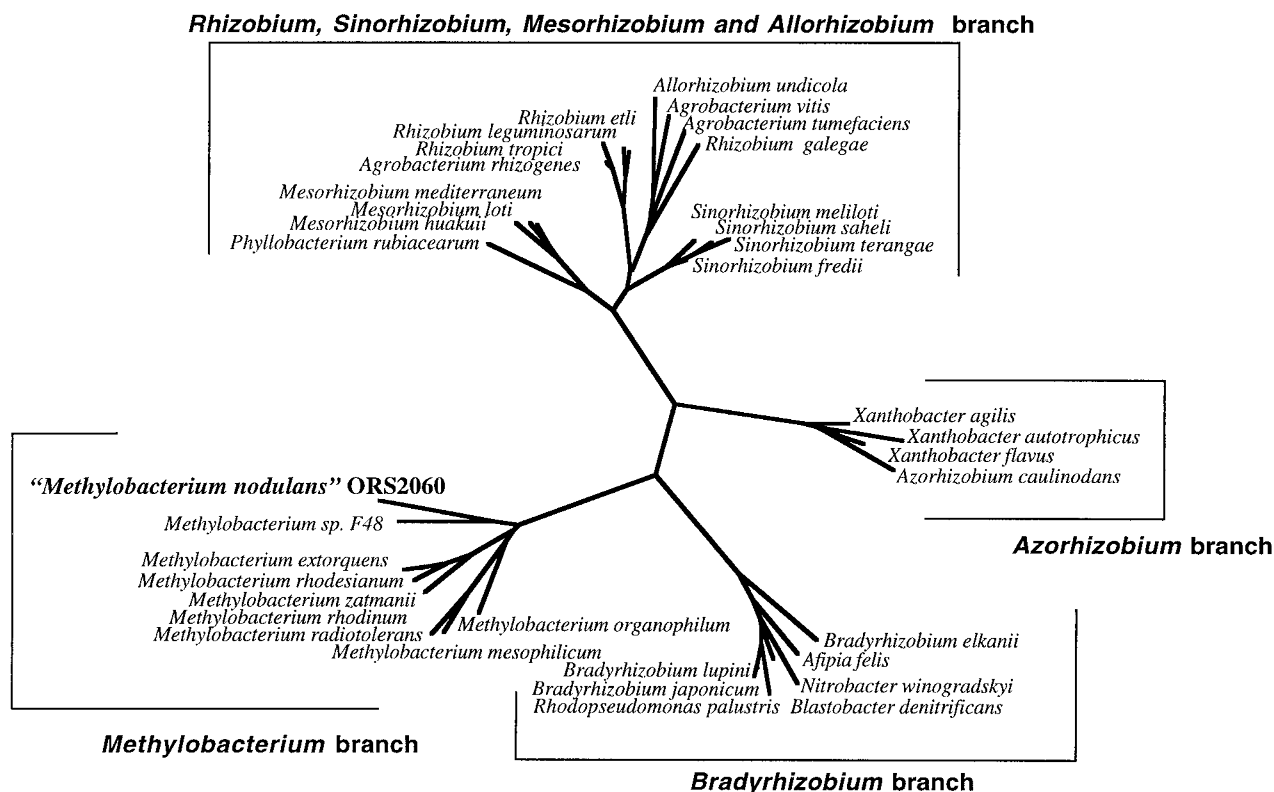

FIG. 1. Unrooted phylogenetic tree showing the different rhizobial branches, including the new "*M. nodulans*" in the α subdivision of *Proteobacteria*. The tree was constructed by using the neighbor-joining method from almost full-length 16S rDNA sequences. The GenBank/EMBL accession numbers are as follows (the first letters of the genus and species are given in parentheses): D 12790 (Pr), D12797 (Mh), X67229 (Ml), L38825 (Mmed), X67224 (Ar), X67234 (Rt), U29386 (Rl), U28916 (Re), Y17047 (Au), X67225 (Av), X67223 (At), X67226 (Rg), X67222 (Sm), X68390 (Ss), X68387 (St), X67231 (Sf), X94198 (Xag), X94201 (Xau), X94199 (Xf), D11342 (Ac), U35000 (Be), M65248 (Af), L11661 (Nw), S46917 (Bd), D25312 (Rp), D12781 (Bj), U69637 (Bl), D32226 (Mo), D32225 (Mmes), D32227 (Mrad), D32229 (Mrhodi), D32230 (Mz), D32228 (Mrhode), D32224 (Me), D32236 (Msp), and AF220763 (Mn).

used according to the manufacturer's specifications (Roche, Meylan, France, or Eurogentec, Seraing, Belgium). For Southern blot hybridization, restricted DNA was blotted to positively charged nylon membranes by the alkali transfer procedure and hybridized with digoxigenin (DIG)-dUTP using the DIG labeling kit supplied by Roche.

DNA amplification, sequencing, and analysis. The primers used for DNA amplification and sequencing are described in Table 2. Nearly full-length 16S ribosomal DNA (rDNA) was amplified using the universal eubacterial 16S rDNA primers FGPS6 and FGPS1509 (28). To perform 16S rDNA PCR-restriction fragment length polymorphism analysis, 1,500-bp PCR products were digested with *Sau*96I, *Hin*fI, *Msp*I, and *Hae*III and restriction fragments were analyzed by horizontal agarose gel electrophoresis using Metaphor agarose (FMC Bioproducts, Hellerup, Denmark). The 1,500-bp fragments of ORS2060 and ORS1924 were sequenced by using the primers FGPS6, FGPS1509, 16S-370f, 16–1080r, 16S-870f, and 16S-1924r; 555-bp sequences homologous to the *mxaF* gene were amplified from *Crotalaria* rhizobia and sequenced by using the nondegenerate primers f1003 and r1561 (26). For rhizobial species, a fragment of about 440 bp homologous to *mxaF* was amplified and sequenced by using the degenerate primers mxaf916 and mxar1360. Two pairs of primers, nodAfbrad/nodArbrad and nodA1f/nodAb1r, were tested for *nodA* amplification of reference *Methylobacterium* strains (LMG6086, LMG6083, LMG4250, LMG2275, LMG6087, LMG5275, LMG6378, LMG6085, and LMG6380). *nodA* amplification and sequencing of ORS2060 were performed using three pairs of primers, nodAfbrad/nodArbrad, nodboxuniv2/nodArbrad, and nodAfbrad/NodB76r.

PCR amplification was performed with a Perkin-Elmer model 2400 thermocycler in a 25-μl (total volume) reaction mixture containing 50 ng of genomic DNA, each deoxynucleotide triphosphate (200 μM), primers (0.8 μM each), $MgCl_2$ (1.5 mM), 1.25 U of *Taq* DNA polymerase (Gibco BRL, Cergy Pontoise, France), and the buffer supplied with the enzyme. A touchdown PCR (12) was performed for primer pairs nodAfbrad/nodArbrad and nodA1f/nodAb1r (annealing temperature from 65 to 55°C in 20 cycles), primer pairs nodboxuniv2/nodArbrad and nodAfbrad/NodB76r (annealing temperature from 60 to 45°C in 30 cycles), and mxaf916/mxar1360 (annealing temperature from 60 to 50°C in 20 cycles). A standard PCR method was used for primer pairs FGPS6/FGPS1509 (60°C annealing temperature) and f1003/r1561 (55°C annealing temperature). Sequencing reactions were performed with the ABI Prism BigDye Terminator Cycle sequence kit (Applied Biosystems, Foster City, Calif.) and analyzed on an

TABLE 1. *Crotalaria* rhizobia used in this study

Strain(s)[a]	Host plant
"*M. nodulans*"	
ORS1917, ORS1991, ORS2060^T	*C. podocarpa*
ORS1924, ORS1928, ORS1937, ORS2030, ORS2092	*C. perrottetii*
ORS2026, ORS2045, ORS2076	*C. glaucoides*
Bradyrhizobium sp.	
ORS1810	*C. lathyroides*
ORS1816	*C. hyssopifolia*
ORS1813	*C. hyssopifolia*
ORS1929	*C. comosa*
ORS2077	*C. retusa*
ORS2088	*C. goreensis*

[a] All strains are described by Samba et al. (33).

TABLE 2. Primers used for DNA amplification and sequencing

Primer name	Primer sequence	Target gene	Reference
FGPS6	5′-GGA GAG TTA GAT CTT GGC TCA G-3′	16S rRNA	28
FGPS1509	5′-AAG GAG GGG ATC CAG CCG CA-3′	16S rRNA	28
16S-370f	5′-CCT GGG GAG TAC GGT CGC AAG-3′	16S rRNA	This study
16S1080r	5′-GGG ACT TAA CCC AAC ATC T-3′	16S rRNA	This study
16S-870f	5′-CCT GGG GAG TAC GGT CGC AAG-3′	16S rRNA	This study
16S-1924r	5′-GGC ACG AAG TTA GCC GGG GC-3′	16S rRNA	This study
f1003	5′-GCG GCA CCA ACT GGG GCT GGT-3	*mxaF*	26
r1561	5′-GGG CAG CAT GAA GGG CTC CC-3′	*mxaF*	26
mxaf916	5′-GGC GAC AAC AAG TGG WCS ATG-3′	*mxaF*/*xoxF*	This study
mxar1360	5′-ART CCA TRC ARA YGT GGT T-3′	*mxaF*/*xoxF*	This study
xoxFr	5′-CCG GAA CGG CTC GTA RTC CA-3′	*xoxF*	This study
nodAfbrad	5′-GTY GAG TGG AGS STK CGC TGG G-3′	*nodA*	This study
nodArbrad	5′-TCA CAR CTC KGG CCC GTT CGG-3′	*nodA*	This study
nodA1f	5′-TGC RGT GGA ARN TRB VYT GGG-3′	*nodA*	This study
nodAb1r	5′-GGN CCG TCR TCR AAS GTC ARG TA-3′	*nodA*	This study
nodboxuniv2	5′-ATC NAA ACA AWN RAT TTT AC-3′	*nod* box	This study
NodB76r	5′-GGR TKN GGN CCR TCR TCR AAN GT-3′	*nodB*	This study

Applied Biosystems model 310 DNA sequencer. Sequences were aligned by using the PILEUP program (11). Phylogenetic trees were constructed by the neighbor-joining method (32), and a bootstrap confidence analysis was performed on 1,000 replicates to determine the reliability of the tree topology obtained (13).

Rhizobial *mxaF*-homologous partial sequences are available upon request.

Construction and screening of a genomic library of ORS2060. To obtain a genomic library of the ORS2060 strain, total DNA was subjected to partial digestion with *Sau*3AI and dephosphorylated with an alkaline phosphatase treatment. Fragments were then ligated to SuperCos I *Xba*I/*Bam*HI arms (Stratagene, La Jolla, Calif.) as instructed by the manufacturer. Ligated DNA was packaged by using the Gigapack III Gold packaging extract (Stratagene). Standard methods were used for titrating and cosmid propagation using *Escherichia coli* XL1-MR as the host. Approximately 2,000 white colonies were picked individually into 96-well microtiter plates containing Luria-Bertani medium plus kanamycin (50 μg/ml), grown overnight at 37°C, and stored with 30% glycerol at −80°C. Screening of *mxaF*-containing cosmids was performed by DNA amplification using the primer pair f1003/r1561. A selected clone, pSTM217, was confirmed by hybridization with an *mxaF* probe constructed by DIG labeling the ORS2060 555-bp *mxaF* internal fragment.

Methanol utilization tests. Cells were grown for 48 h to mid-log phase in minimum mineral medium M72 (5) supplemented with pyruvate (10 mM) and yeast extract (0.5g/liter). Bacterial suspensions were diluted in M72 medium to an optical density of 0.05, and one of the following compounds was added: methanol (MeOH) (10, 50, 100, or 500 mM), pyruvate (10 mM), or succinate (10 mM). Growth was monitored by measuring optical density at 620 nm. MeOH dosage in culture supernatants was performed as described previously (39) except that $KMnO_4$ was replaced by alcohol oxidase (EC 1.1.3.13) at 0.1 U/ml (Sigma, L'Isle d'Abeau, France).

Plant tests. Plant cultivation and nodulation tests were carried out as described previously (23), with the following modifications: seeds were superficially sterilized with concentrated sulfuric acid for 35 min (*Crotalaria podocarpa*), 30 min (*Crotalaria perrottetii*), 15 min (*Crotalaria comosa*), 40 min (*C. goreensis*), or 25 min (*Crotalaria ochroleuca*). Effectiveness was estimated by visual observation of plant vigor and foliage color of 30-day-old plants.

Fresh nodules were observed under an Olympus SHZ 10 stereomicroscope. Sections of 80-μm thickness were made using a Leica VT1000S Vibratome. Microscopic preparations were examined without further staining with an Olympus Provis microscope.

Bacteriochlorophyll detection. The presence of bacteriochlorophyll *a* in "*M. nodulans*" ORS2060 was checked as described previously (22).

Nucleotide sequence accession numbers. Accession numbers for 16S rRNA and the *mxaF* and *nodA* genes are as follows: AF220762 (ORS1924 16S rRNA gene), AF220763 (ORS2060 16S rRNA gene), AF220764 (ORS2060 *mxaF* gene), and AF266748 (ORS2060 *nodA* gene). Accession numbers for rhizobial partial *mxaF*-homologous sequences are AF304307 to AF304313.

RESULTS

Bacteria that specifically nodulate *Crotalaria* belong to the *Methylobacterium* genus. Rhizobia isolated from *Crotalaria glaucoides*, *C. perrottetii*, and *C. podocarpa* were previously shown to be highly specific, since they effectively nodulated only these three species (33). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis grouped almost all the strains into three related electrophoretic clusters separated from other rhizobial species (33).

To determine the bacterial genus to which they belong, 16S rDNA analysis was performed on 11 representative strains belonging to the previously identified sodium dodecyl sulfate-polyacrylamide gel electrophoresis clusters (Table 1). All strains tested gave identical patterns by 16S rDNA PCR-restriction fragment length polymorphism analysis, showing that the strains form a very homogenous group. The 16S rRNA genes of two strains, ORS2060 isolated from *C. podocarpa* and ORS1924 isolated from *C. perrottetii*, were sequenced and shown to be identical (accession numbers AF220763 and AF220762, respectively). Phylogenetic 16S rDNA sequence analysis revealed that this group was distinct from the three main branches containing all known rhizobial species. Surprisingly, the specific *Crotalaria* symbionts belonged to the *Methylobacterium* lineage of the α-*Proteobacteria*, thus constituting a fourth phylogenetic rhizobium branch (Fig. 1). Sequence similarities with the different *Methylobacterium* species described so far ranged from 93.64% (*Methylobacterium radiotolerans*) to 94.52% (*M. extorquens*) and was 95.16% with its closest phylogenetic neighbor, *Methylobacterium* sp. strain F48. These ranges are comparable with those found between most *Methylobacterium* species, thus demonstrating that the specific *Crotalaria* rhizobia phylogenetically belong to the *Methylobacterium* genus.

Bacteria of the new species "*M. nodulans*" are facultative aerobic methylotrophs. *Methylobacterium* spp. oxidase methanol via a key periplasmic enzyme, methanol dehydrogenase, which belongs to the family of the pyrroloquinoline quinone (PQQ)-linked enzymes that are known as quinoproteins (2, 3). Methanol dehydrogenase is an $\alpha_2\beta_2$ tetramer noncovalently

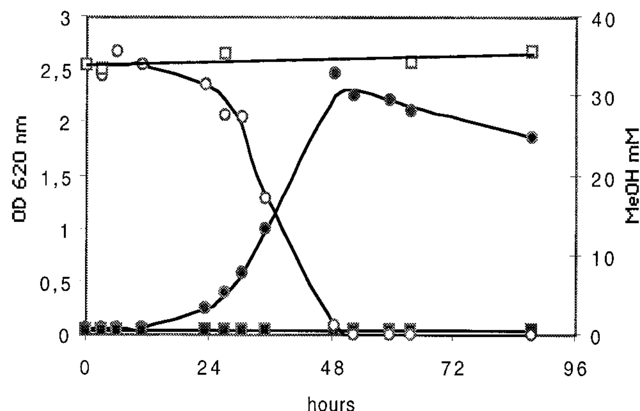

FIG. 2. Methanol utilization by "*M. nodulans*" ORS2060. *Bradyrhizobium* sp. strain ORS1810 (33) was used as a negative control. Shown is growth of ORS2060 (black circles) and ORS1810 (black squares) on minimum mineral medium 72 containing MeOH as the sole carbon source. MeOH concentrations in supernatants of cultures of ORS2060 (empty circles) and ORS1810 (empty squares) are also shown.

bound to PQQ (15). The structural gene for the α subunit of the methanol dehydrogenase encoded by *mxaF* is well conserved among gram-negative methylotrophic bacteria (24). Therefore, to evaluate the presence of methanol oxidation genes in *Crotalaria* rhizobia, we performed PCR amplifications using nondegenerate primers f1003 and r1561, defined from conserved parts of *mxaF* genes (26). These primers indeed produced an amplification product of the expected size for 9 of the 11 specific *Crotalaria* strains tested. The 555-bp PCR product obtained from the representative strain ORS2060 was homologous to *mxaF* genes. The full-length *mxaF* gene was obtained from a genomic library of ORS2060 probed with the 555-bp PCR product (see Materials and Methods for details). The corresponding nucleotide sequence contained a single extended 1,890-bp open reading frame (accession number AF220764) encoding a 629-amino-acid protein that exhibits 88% identity with the MxaF proteins of *M. extorquens* (accession number M31108) and *M. organophilum* (accession number M22629). A putative ribosome binding site was identified upstream from the proposed ATG start codon. The first 93 nucleotides of the structural gene encode a typical signal sequence for secretion (38). These results indicated that *Crotalaria*-nodulating *Methylobacterium* strains did contain, as all other *Methylobacterium* strains, the structural gene, *mxaF*, required for methanol oxidation.

In order to directly assess the methylotrophic properties of *Methylobacterium* species from *Crotalaria*, the strains were then tested for their ability to use methanol (50 mM) as the sole carbon source in liquid culture. Growth was compared to that of *M. extorquens* LMG4250 and *Bradyrhizobium* sp. isolated from *Crotalaria* species. No growth was observed for *Bradyrhizobium* strains over a 10-day period (Fig. 2). By contrast, all *Methylobacterium* strains from *Crotalaria* grew on this substrate, with generation times ranging from 9.5 to 40 h. The fastest growth rates were obtained with strains ORS2060 (Fig. 2) and ORS1917 (9.5-h doubling time) and were similar to the growth rate of *M. extorquens* LMG4250 on MeOH (50 mM) (7.5-h doubling time). Similar growth was noted on either pyruvate or succinate as the sole carbon source. Growth was obtained at an MeOH concentration up to 500 mM.

To confirm that the same bacterium isolated from *Crotalaria* exhibits both nodulation ability and methylotrophic properties, strain ORS2060 was grown on methanol, repurified from a single colony, and inoculated onto *C. podocarpa* and *C. perrottetii*. The bacteria formed nitrogen-fixing indeterminate nodules (Fig. 3). Single colonies reisolated from the nodules retained the ability to grow on methanol, clearly demonstrating that this new *Methylobacterium* strain is able both to grow on one-carbon (C_1) compounds and to effectively nodulate legumes.

Proposal of a new species was warranted by the low 16S rDNA sequence similarity values between *Methylobacterium* strains isolated from *Crotalaria* and other *Methylobacterium* strains. The unique symbiotic properties of these bacteria (see below) led us to propose the name "*Methylobacterium nodulans*." *Methylobacterium* spp., which are mostly isolated from water and leaf surface microflora, are known in the literature as pink-pigmented facultative methylotrophs (19). However, "*M. nodulans*" strains are not pigmented and the type strain,

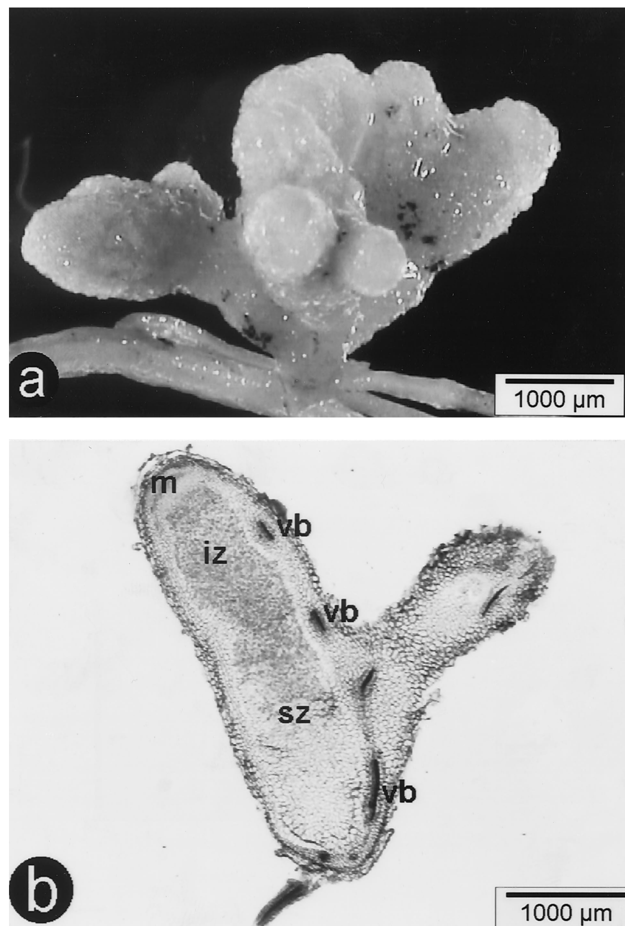

FIG. 3. Nodules of *C. perrottetii* inoculated with "*M. nodulans*" ORS2060. (a) Multilobed fresh nodule; (b) unstained longitudinal section displaying the classical structure of indeterminate nodules with an apical meristematic zone (m), an infection zone (iz), a senescent zone (sz), and peripheral vascular bundles (vb).

ORS2060, did not exhibit the characteristic bacteriochlorophyll absorption peak at 766 nm. This could account for their adaptation to the soil rather than to the phyllosphere or water.

"*M. nodulans*" is the only nodulating *Methylobacterium* species identified to date. The *Methylobacterium* genus has never been reported to contain nodulating bacteria. However, to be certain that nodulation is not a general but hitherto undetected *Methylobacterium* feature, we tested representative strains of several *Methylobacterium* species (*M. rhodesianum*, *M. organophilum*, *M. extorquens*, *M. rhodinum*, *M. mesophilicum*, *M. zatmanii*, *M. radiotolerans*, and "*M. nodulans*") and a few *Methylobacterium* sp. strains (LMG6378, LMG6085, and LMG6380) by inoculation on *Crotalaria* species nodulated by broad-host-range *Bradyrhizobium* (*C. comosa*, *C. goreensis*, and *C. ochroleuca*) or by "*M. nodulans*" (*C. perrottetii*). None of them nodulated, except "*M. nodulans*."

Specific rhizobial infection and nodulation of legumes is mainly controlled by a set of bacterial nodulation genes involved in the production of lipochitooligosaccharides (Nod factors) that act as morphogenic signal molecules on specific legume hosts (10, 36). Structural *nodABC* genes encoding key enzymes in Nod factor biosynthesis are present in all rhizobia. We thus looked for the presence of the *nodA* gene in the *Methylobacterium* representative strains listed above by PCR amplifications using two pairs of degenerate primers defined from conserved parts of NodA sequences. None of these strains responded positively, except "*M. nodulans*." The full-length *nodA* sequence of "*M. nodulans*" ORS2060 was determined after PCR amplification. The open reading frame, which probably corresponds to *nodA*, is 642 nucleotides long (accession number AF266748). Sequence similarity with the different complete rhizobial NodA protein sequences available in databases ranged from 53.06% (*A. caulinodans*) to 74.11% (*B. elkanii* USDA94). Phylogenetic analysis of available NodA proteins showed that "*M. nodulans*" is grouped with *Bradyrhizobium* spp. (Fig. 4).

Methylotrophy is not a common feature of rhizobia. We tested a collection of rhizobia belonging to various genera and species (*S. meliloti*, *S. medicae*, *S. fredii*, *S. terangae*, *R. leguminosarum* bv. viciae, *R. etli*, *R. tropici*, *M. ciceri*, *M. loti*, *B. japonicum*, *B. elkanii*, *A. undicola*, and *A. caulinodans*; see Materials and Methods) for their ability to grow on MeOH (50 mM) as the sole carbon source in solid and liquid culture. No growth of these strains was observed within 6 days. We also used PCR amplification to investigate the possible presence of a *mxaF*-homologous gene in the rhizobial strains. No amplification could be obtained using primers f1003 and r1561. When degenerate primers defined from conserved parts of both MxaF and MxaF homologs from α-*Proteobacteria* were used, all strains were found to contain a 440-bp sequence homologous to *mxaF*. These sequences however exhibited higher homology to two *mxaF* homologs, *mxaF′* from *M. extorquens* (72 to 80% amino acid identity) and *xoxF* from *Paracoccus denitrificans* (72 to 83% amino acid identity) than to *mxaF* from *M. extorquens* (50 to 53% amino acid identity). *xoxF* and *mxaF′* are thought to encode an alternative PQQ-dependent dehydrogenase (8, 18). Phylogenetic analysis further revealed that the rhizobial protein sequences are clustered with XoxF and MxaF′ and separated from the methanol dehydrogenase MxaF from α- and β-*Proteobacteria* (Fig. 5). We thus conclude that the sequences found in rhizobia probably do not correspond to a bona fide *mxaF* ortholog.

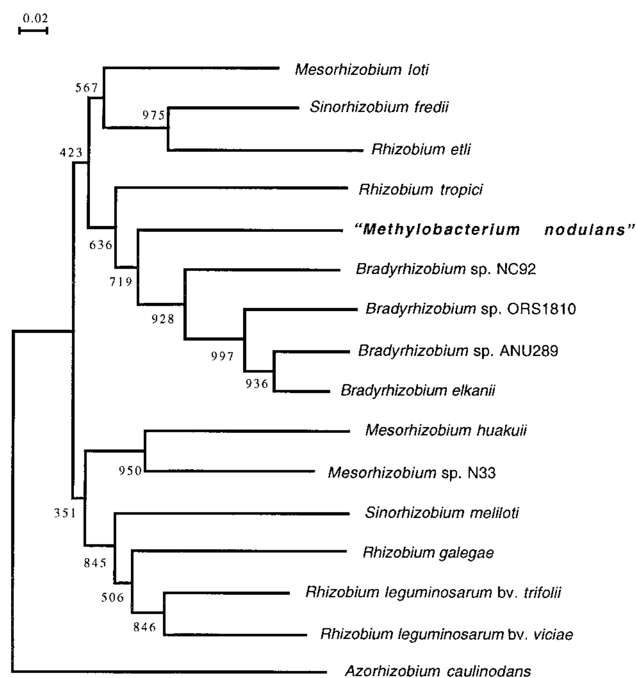

FIG. 4. Phylogenetic tree based on full-length NodA sequences construced by using the neighbor-joining method. Bootstrap values (from 1,000 replications) are indicated. The GenBank/EMBL accession numbers are as follows (the first letters of the genus and species are given in parentheses): L18897 (Ac), 106241 (Ml), M73699 (Sf), M58625 (Re), X98514 (Rt), AF266748 (Mn), U33192 (BspNC92), U04609 (BspANU289), U04609 (Be), AJ249353 (Mh), U53327 (MspN33), M11268 (Sm),X87578 (Rg), and X01650 (Rl). The NodA sequence of *Bradyrhizobium* sp. strain ORS1810 was kindly provided by T. Stepkowski.

DISCUSSION

The rhizobial species described so far belong to three distinct 16S rRNA branches within α-*Proteobacteria*, including nonsymbiotic bacteria such as the plant pathogen *Agrobacterium* and the human and animal pathogen *Afipia*. The discovery of a fourth rhizobial phylogenetic branch, constituted by bacteria of the *Methylobacterium* genus, thus confirms and extends the polyphyletic origin of rhizobia within the α subclass of *Proteobacteria* (40). It is noteworthy that although rhizobia have been studied for more than 100 years, symbionts of less than 50 of the 750 known legume genera have been fully characterized. Therefore, it is quite likely that much greater rhizobial diversity will be discovered by characterizing symbionts of unexplored legumes and by focusing on legumes of unexplored areas.

Bacterial nodulation (*nod)* genes have been shown to play a central role in the molecular dialog between the plant and the bacterium, leading to plant recognition, infection, and nodulation (10, 34, 36). The presence of structural *nodABC* genes in all rhizobia indicates the unique origin of these genes, which could have been disseminated among *Proteobacteria* via self-transmissible plasmids (25) or other mechanisms allowing

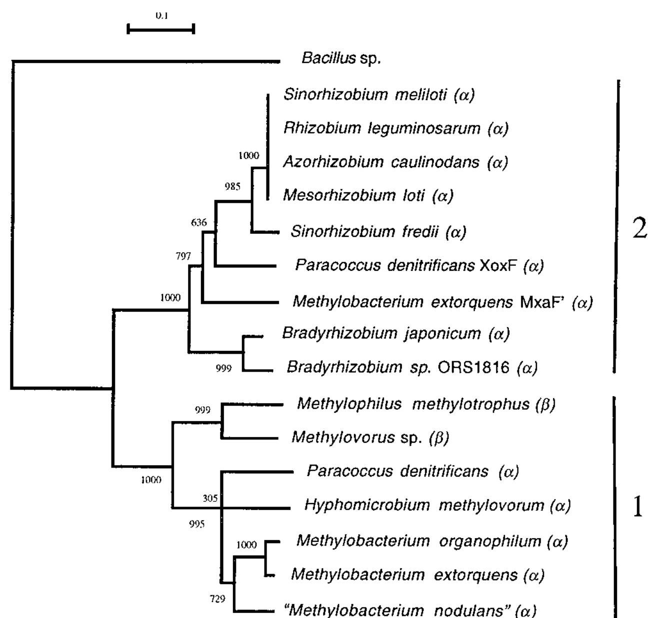

FIG. 5. Phylogenetic tree based on about 140-amino-acid MxaF-homologous sequences constructed by using the neighbor-joining method. Bootstrap values (from 1,000 replications) are indicated. Cluster 1 groups only MxaF sequences with the following GenBank/EMBL accession numbers (the first letters of the genus and species are given in parentheses): M17339 (Pd), AB004097 (Hm), M22629 (Mo), M31108 (Me), AF220764 (Mn), U41040 (Mm), and AF184915 (Msp). Cluster 2 groups the rhizobial sequences (accession numbers given in Materials and Methods) together with XoxF from *P. denitrificans* (U34346) and MxaF′ from *M. extorquens* (U72662). MxaF from *Bacillus* sp. (M65004) was used as the root. The topology of the tree constructed with the full-length MxaF-homologous sequences (thus excluding the rhizobial sequences) is similar. (α), member of α-*Proteobacteria*; (β), member of β-*Proteobacteria*.

transfer of "symbiotic islands" (35). The presence of the *nodA* gene in "*M. nodulans*" is consistent with nodulation data and suggests that this bacterium is able to establish symbiosis by the same molecular mechanisms as other rhizobia. The NodA phylogenetic analysis confirms the monophyletic origin of this structural common *nod* gene (Fig. 4). The deduced absence of *nodA* in nonsymbiotic *Methylobacterium* spp. together with the close phylogenetic relationship between "*M. nodulans*" and *Bradyrhizobium* NodA proteins suggests that "*M. nodulans*" has acquired nodulation properties by lateral gene transfer.

Bacteria of the *Methylobacterium* genus are facultative methylotrophs capable of growing on one-carbon compounds such as formate, formaldehyde, and methanol as the sole source of carbon and energy, as well as on a wide range of multicarbon substrates (16, 17). They constitute one of the major methylotrophic branches in the α-2 subclass of *Proteobacteria*. It should be noted that other branches, including the three rhizobial branches described to date (Fig. 1), also contain methylotrophs. Indeed, *Xanthobacter* species are all autotrophic methylotrophs (27), most *Rhodopseudomonas* species grow photosynthetically with methanol (31), and a methyl bromide-utilizing methylotroph closely related to *Rhizobium* species was recently identified (9). *Methylobacterium* species are known to occur in man-made environments such as drinking water supplies, swimming pools, and hospital washbasins, where they often become highly resistant to chlorine. They are also widespread in natural environments, including soil, dust, air, and fresh water, wherever one-carbon compounds are abundant. Because of their ability to metabolize various plant decomposition compounds such as methylated compounds, methylotrophic bacteria play an important ecological role in the environmental carbon cycle. Although *Methylobacterium* strains have been frequently found on plant tissues (19), there is no previous evidence of symbiotic association of such microorganisms with plants. We demonstrated here that a group of *Methylobacterium* strains, identified as the new species "*M. nodulans*," are able to form nitrogen-fixing nodules on the roots of leguminous plants. However, this case may not be unique since we recently learned that CB376, a nodulating photosynthetic strain from *L. bainesii* (14), could be classified in the *Methylobacterium* genus on the basis of its 16S rRNA sequence (W. Heumann, personal communication).

A polyphyletic origin of rhizobia versus a monophyletic origin of common nodulation genes suggests that rhizobia have evolved through acquisition of nodulation functions in different bacterial branches susceptible to adaptation to different legume environments. "*M. nodulans*" is a facultative methylotroph, a unique property among rhizobia, raising the question of the role of methylotrophy in *Crotalaria*-"*M. nodulans*" symbiosis. We are currently starting a genetic analysis to understand why *Methylobacterium* strains are specifically associated with some particular legumes.

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