Methylobacterium nodulans sp. nov., for a group of aerobic, facultatively methylotrophic, legume root-nodule-forming and nitrogen-fixing bacteria

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Data on 72 non-pigmented bacterial strains that specifically induce nitrogen-fixing root nodules on the legume species *Crotalaria glaucoides*, *Crotalaria perrottetii* and *Crotalaria podocarpa* are reviewed. By SDS-PAGE analysis of total protein patterns and by 16S rRNA PCR-RFLP, these strains form a homogeneous group that is separate from other legume root-nodule-forming bacteria. The 16S rRNA gene-based phylogeny indicates that these bacteria belong to the genus *Methylobacterium*. They can grow on C_1 compounds such as methanol, formate and formaldehyde but not methylamine as sole carbon source, and carry an *mxaF* gene, encoding methanol dehydrogenase, which supports their methylotrophic metabolism. Presence of a *nodA* nodulation gene, and ability to nodulate plants of *Crotalaria* species and to fix nitrogen are features that separate the strains currently included in this group from other members of the genus *Methylobacterium*. The present study includes additional genotypic and phenotypic characterization of this novel *Methylobacterium* species, i.e. *nifH* gene sequence, morphology, physiology, enzymic and carbon source assimilation tests and antibiotic resistance. The name *Methylobacterium nodulans* sp. nov. (type strain, ORS 2060^T = CNCM I 2342^T = LMG 21967^T) is proposed for this group of root-nodule-forming bacteria.

The genus *Methylobacterium* includes a variety of pink-pigmented facultatively methylotrophic (PPFM) bacteria that are able to grow on C_1 compounds such as formate, formaldehyde and methanol as sole carbon and energy sources as well as on a wide range of multi-carbon growth substrates (Green, 1992). At the time of writing, the genus *Methylobacterium* consists of 14 PPFM species (Doronina *et al.*, 2002), with *Methylobacterium organophilum* as the type species (Patt *et al.*, 1976). *Methylobacterium* strains have been found on many plant tissues but no symbiotic association with plants has been reported (Holland, 1997). Recently and surprisingly, 16S rRNA gene-based phylogenetic analysis classified non-pigmented bacteria isolated from legume root nodules of three *Crotalaria* species (subfamily Papilionoideae, tribe Crotalarieae), i.e. *Crotalaria glaucoides*, *Crotalaria perrottetii* and *Crotalaria podocarpa*, in the genus *Methylobacterium* (Sy *et al.*, 2001a, b). More recently, other nitrogen-fixing isolates from root nodules of the legume *Lotononis bainesii* (Papilionoideae, Crotalarieae) were characterized as pigmented methylotrophic bacteria (Jaftha *et al.*, 2002). Here we review previous reports on *Methylobacterium* strains that nodulate *Crotalaria* species and add additional phenotypic and genotypic characterization data, such as morphology, physiology, enzymic and carbon sources assimilation tests, antibiotic resistance and *nifH* gene sequence, and conclude with the proposal of *Methylobacterium nodulans* sp. nov.

A group of 72 bacterial strains was isolated from root nodules samples from three *Crotalaria* species (*C. glaucoides*, *C. perrottetii* and *C. podocarpa*) sampled in five different geographical areas of Senegal (West Africa) (Samba *et al.*, 1999). SDS-PAGE protein pattern analysis clearly indicated that these strains constituted a homogeneous group that was separate from other known legume-nodule-forming bacteria (Samba *et al.*, 1999; Sy *et al.*, 2001a). Eleven strains, representative of the different SDS-PAGE subclusters and for plant and geographical origins, were chosen and studied further. The 16S rRNA PCR-RFLP profile analysis on these strains confirmed the homogeneity of this group

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and partial *nifH* gene sequences of strain ORS 2060^T are AF220763 and AJ512205, respectively.

and the 16S rRNA gene sequence analysis of two of them, ORS 2060^T and ORS 1924 presenting 100 % identity, showed their close phylogenetic relationship with members of the genus *Methylobacterium* (Sy *et al.*, 2001a, b). The methylotrophic metabolism of the same 11 strains was confirmed by growth on MMS medium (Green, 1992) with C_1 compounds as sole carbon source: methanol, formate and formaldehyde but not methylamine (Dreyfus *et al.*, 1999; Sy *et al.*, 2001b). In addition, an *mxaF* gene, encoding the methanol dehydrogenase required for methanol utilization, was detected by PCR in the 11 strains and the *mxaF* gene sequence of ORS 2060^T showed 88 % identity to that of *M. organophilum* (Sy *et al.*, 2001b). Taken together, these data confirmed that the bacterial strains isolated from root nodules of *C. glaucoides*, *C. perrottetii* and *C. podocarpa* constituted a homogeneous bacterial group that belonged to the genus *Methylobacterium*.

Cross-inoculation and nitrogen fixation tests on legume plants revealed both nodulation specificity and nitrogen-fixing efficiency within the genus *Crotalaria* (Sy *et al.*, 2001a). Representative strains for the main *Methylobacterium* species, i.e. *Methylobacterium extorquens*, *Methylobacterium organophilum*, *Methylobacterium radiotolerans*, *Methylobacterium rhodinum*, *Methylobacterium mesophilicum*, *Methylobacterium rhodesianum* and *Methylobacterium zatmanii*, and two *Methylobacterium* spp. were tested for plant nodulation but none of them was able to induce any legume root nodule (Sy *et al.*, 2001b). In addition, the *nodA* gene, present in all legume-nodule-forming bacteria and encoding a key enzyme in Nod factor biosynthesis that induces legume nodulation (Martinez Romero, 1994; van Rhijn & Vanderleyden, 1995), was detected by PCR in the strains of the novel *Methylobacterium* species isolated from *C. glaucoides*, *C. perrottetii* and *C. podocarpa*. In contrast, *nodA* was not detected in any of the main representative strains of the genus *Methylobacterium* mentioned above (Sy *et al.*, 2001b). A comparative analysis of the NodA protein deduced from the *nodA* gene sequence showed a range of 53·1 % similarity with the NodA protein sequence from *Azorhizobium caulinodans* to 74·1 % similarity with that of *Bradyrhizobium elkanii* (Sy *et al.*, 2001b).

As a consequence of the above-mentioned results, Sy *et al.* (2001b) concluded that the group of strains made up of facultatively methylotrophic, root-nodule-forming and nitrogen-fixing bacteria may be regarded as a novel *Methylobacterium* species. In this report, we formally propose the name *Methylobacterium nodulans* sp. nov. to include these strains, with ORS 2060^T as the type strain.

Since the report of Sy *et al.* (2001b), novel *Methylobacterium* species have been described (Doronina *et al.*, 2002). Fig. 1 shows a 16S rRNA gene-based phylogenetic tree that includes *M. nodulans* ORS 2060^T, representative strains of *Methylobacterium* sp. isolated from *L. bainesii* (Jaftha *et al.*, 2002), 13 of the 14 validly published *Methylobacterium* species (the sequence of *Methylobacterium aminovorans* is not available) and their nearest phylogenetic neighbours. All *Methylobacterium* species and strains form a separate branch, consisting of three sub-branches. One sub-branch consists of *M. nodulans* ORS 2060^T and *Methylobacterium* sp. (isolated from *L. bainesii*) strains xct10, xct14 and xct17. *M. nodulans* ORS 2060^T shows sequence identity values of 95·8–97·6 % with *Methylobacterium* sp. strains isolated from *L. bainesii* (Jaftha *et al.*, 2002), and less than 95·2 % sequence identity with the other *Methylobacterium* species. These similarity values confirm that *M. nodulans* constitutes a separate species in the genus *Methylobacterium* and that it is distinct from *Methylobacterium* sp. strains isolated from *L. bainesii*.

A partial *nifH* fragment (426 bp) was amplified from *M. nodulans* ORS 2060^T, as described by Jaftha *et al.* (2002), and sequenced (GenBank/EMBL/DDBJ accession no. AJ512205). Fig. 2 depicts a *nifH*-based phylogenetic tree showing the relationships of the *nifH* sequences of *Crotalaria*-nodulating strains with those of related nitrogen-fixing bacteria. A maximum-likelihood method was applied for the reconstruction of the phylogenetic tree based on inferred amino acid sequences, excluding third codon positions from the alignment (due to the saturation of this position). The phylogeny shows a close relationship among *M. nodulans* ORS 2060^T, *Methylobacterium* sp. isolated from *L. bainesii* and the *nifH* gene sequence from *Gluconacetobacter diazotrophicus*, a sugarcane endophyte of the α-*Proteobacteria*. Jaftha *et al.* (2002) suggested the closest relationship between their *Methylobacterium* sp. *nifH* sequence was with *Azospirillum brasilense*. However, they did not include the *G. diazotrophicus* sequence in their analysis and applied a phenetic method, including all nucleotide positions in their phylogenetic reconstruction, which is thus much more sensitive to homoplasy and false reconstruction than our maximum-likelihood approach that excluded saturated positions. To the best of our knowledge, nitrogen fixation and the presence of the *nifH* gene have never been reported in any other species belonging to the genus *Methylobacterium*.

Metabolic tests were performed on the 11 strains studied previously (Samba *et al.*, 1999; Sy *et al.*, 2001b): ORS 2026, ORS 2045 and ORS 2076 isolated from *C. glaucoides*; ORS 1917, ORS 1991 and ORS 2060^T isolated from *C. podocarpa*; and ORS 1924, ORS 1928, ORS 1937, ORS 2030 and ORS 2092 isolated from *C. perrottetii*. The following substrates were readily used as sole source of carbon in MMS medium at 30 °C (Green, 1992): succinate, citrate, pyruvate, glutamate and ethanol. In addition, bacterial enzymic activities were determined using the API 20NE galleries according to the manufacturer's protocol (bioMérieux); tests for nitrate reductase and urease were positive; tests for β-galactosidase, β-glucosidase, protease, indole production and glucose fermentation were negative. API Biotype 100 galleries (bioMérieux) were also used to check assimilation and growth on 100 carbon sources as according to Kersters *et al.* (1984). *M. nodulans* strains were able to grow on the following substrates as sole carbon sources: (+)-D-galactose,

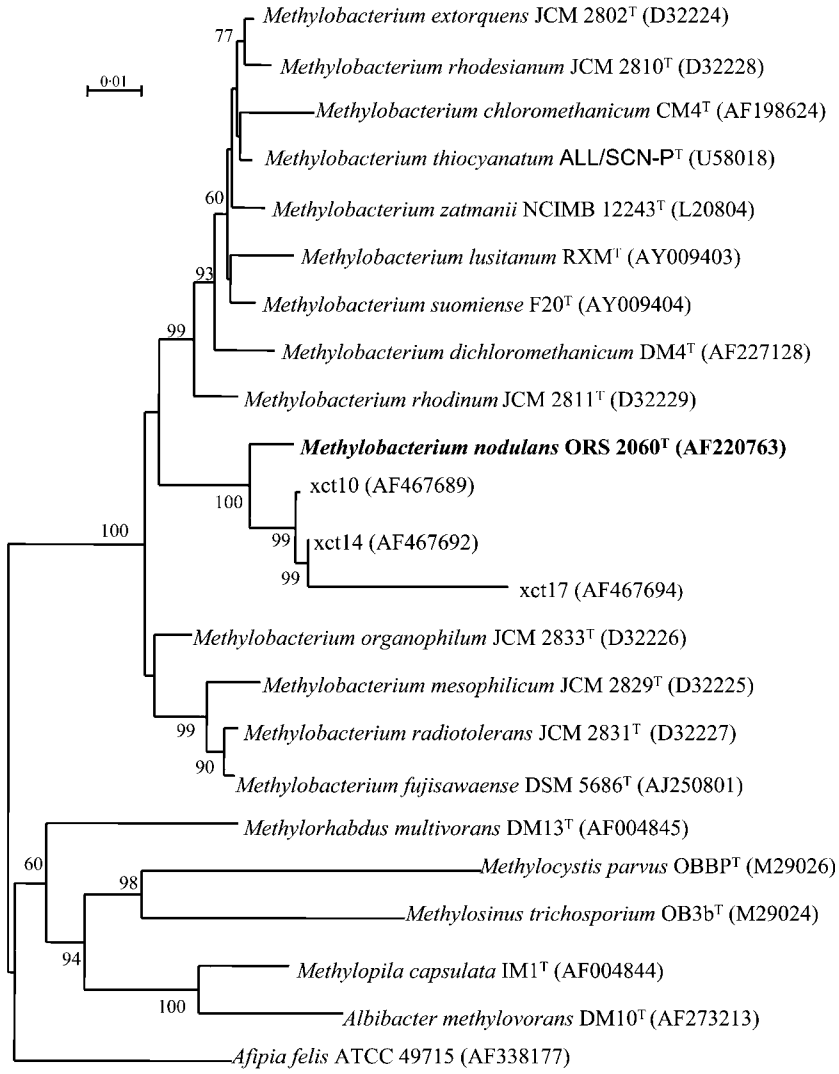

Fig. 1. Phylogenetic analysis of the 16S rRNA gene sequences of strains of *M. nodulans* sp. nov. and other representatives of the genus *Methylobacterium* and related genera. The 16S rRNA gene was PCR-amplified from pure DNA of strain ORS 2060^T and sequenced using primers as described by Sy *et al.* (2001b) and an ABI Prism BigDye terminator cycle sequence kit (Applied Biosystems). The sequence was analysed on an Applied Biosystems model 310 DNA sequencer and its software, Sequence Navigator. Other 16S rRNA gene sequences from representative *Methylobacterium* species and related genera were from the GenBank database (accession numbers are given in parentheses). All sequences were aligned using the Multalign software (Corpet, 1988). The tree was generated using the neighbour-joining method of Saitou & Nei (1987) and CLUSTAL_X software (Thompson *et al.*, 1997). Bootstraps values (100 replicates) are shown at branch points. Bar, 1 estimated substitution per 100 nucleotide positions.

(−)-D-ribose, (+)-L-arabinose, (+)-D-xylose, glycerol, D-lyxose, D-saccharate, mucate, (+)-L- and (−)-D-tartrate, (+)-D- and (−)-L-malate, *cis*- and *trans*-aconitate, tricarballylate, glucuronate, 2- and 5-keto-D-gluconate, D-gluconate, phenylacetate, *p*-hydroxybenzoate, quinate, benzoate, betaine, α-DL-amino-n-butyrate, DL-lactate, fumarate, glutarate, DL-glycerate, β-DL-hydroxybutyrate, L-aspartate, L-proline, L-alanine, L-serine and 2-oxoglutarate. Strains were unable to use α-(+)-D-glucose, β-(+)-D-fructose, (+)-D-trehalose, (+)-D-mannose, (+)-L-sorbose, α-(+)-D-melibiose, sucrose, α-lactose, (+)-D-raffinose, maltotriose, maltose, lactulose, (+)-D-cellobiose, β-gentobiose, aesculin, α-L-rhamnose, α-(−)-L-fucose, (+)-D-arabitol, xylitol, dulcitol, *myo*-inositol, D-mannitol, D-sorbitol, tryptophan, *N*-acetylglucosamine, coumarate, trigonelline, putrescine, histamine, L-histidine, ethanolamine, tryptamine, D-glucosamine, D-alanine, malonate, propionate and L-tyrosine. The intrinsic antibiotic resistance patterns of the strains show fairly high resistance to ampicillin, carbenicillin and nalidixic acid but sensitivity to kanamycin, gentamicin and tetracycline. Table 1 summarizes features useful for distinguishing *M. nodulans* sp. nov. from the 14 recognized species of the genus *Methylobacterium* (Green, 1992; Doronina *et al.*, 2002).

Description of *Methylobacterium nodulans* sp. nov.

Methylobacterium nodulans (no′du.lans. N.L. v. *nodulare* to nodulate; N.L. part. adj. *nodulans* nodulating, expressing the original feature of strains to induce nitrogen-fixing nodules on roots of legume plants).

Short asporogenous Gram-negative rods (0·8–1·0 × 1·0–1·5 μm) that occur singly or occasionally in pairs; some are motile with one or more polar flagella. Colonies on MMS medium + agar (Green, 1992) with methanol as sole carbon source are shiny, smooth, raised, entire and 0·5–1 mm in diameter after 3 days at 30 °C and are not pigmented. Optimal growth occurs at pH 6·8–7·5 and at 30–37 °C. Strictly aerobic, catalase-positive and weakly oxidase-positive; urease-positive and able to reduce nitrate into nitrite. Table 1 shows other phenotypic traits of the

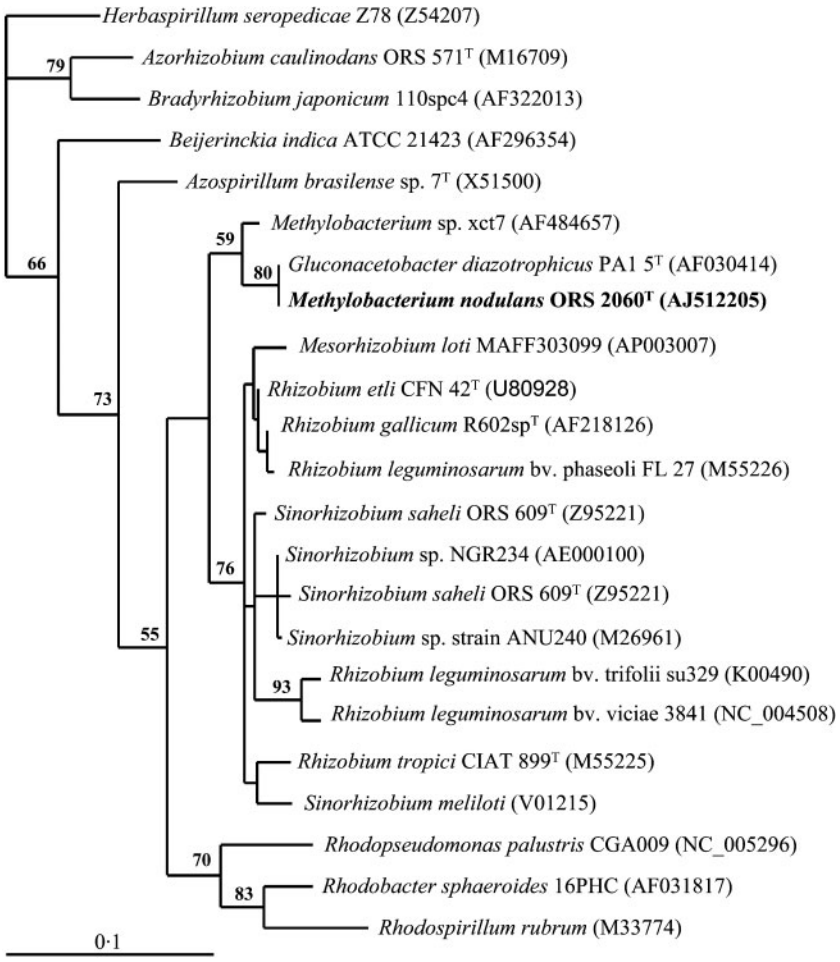

Fig. 2. Phylogenetic tree based on a 426 bp fragment of the *nifH* gene, showing relationships of *M. nodulans* ORS 2060^T with other nitrogen-fixing bacteria of the α- and β-*Proteobacteria*. *nifH* was PCR-amplified using primers as described by Jaftha *et al.* (2002) and sequenced as described in Fig. 1. *nifH* sequences from representative species of nitrogen-fixing bacteria were from the GenBank database. Sequences were multi-aligned using the CLUSTAL_X software (Thompson *et al.*, 1997), and the phylogenetic tree was generated using PAUP* version 4.0b10 (Swofford, 1998) applying a maximum-likelihood method, estimating base frequencies, the six-parameters instantaneous rate matrix substitution, the shape of the gamma distribution used to accommodate among-site rate variation (three categories) and, finally, estimating the proportion of sites unable to accept substitutions (GTR+Γ_3+I model). The reconstruction is based on the first and second codon positions only. Bootstrap values are indicated in bold and were estimated from 100 replicates. GenBank/EMBL/DDBJ accession numbers are in parentheses.

species. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *mxaF*, partial *nodA* and partial *nifH* gene sequences of strain ORS 2060^T are AF220763, AF220764, AF266748 and AJ512205, respectively. Can form nitrogen-fixing root nodules in symbiosis with *Crotalaria glaucoides*, *Crotalaria perrottetii* and *Crotalaria podocarpa*.

Table 1. Characteristics useful for distinguishing *M. nodulans* sp. nov. from other species of the genus *Methylobacterium*

Species: 1, *Methylobacterium nodulans* ORS 2060^T; 2, *M. organophilum*; 3, *M. extorquens*; 4, *M. rhodinum*; 5, *M. zatmanii*; 6, *M. mesophilicum*; 7, *M. rhodesianum*; 8, *M. fujisawaense*; 9, *M. radiotolerans*; 10, *M. aminovorans*; 11, *M. thiocyanatum*; 12, *M. chloromethanicum*; 13, *M. dichloromethanicum*; 14, *M. suomiense*; 15, *M. lusitanum*. Tests are based on the assimilation of various compounds as sole source of carbon. Abbreviations: +, growth; −, no growth; W, weak growth; V, variable; ND, not determined.

Compound	1*	2†‡	3†‡	4†‡	5†‡	6†‡	7†‡	8†‡	9†‡	10‡	11‡	12‡	13‡	14‡	15‡
Methylamine	−	+	+	+	+	−	+	−	−	+	+	+	+	+	+
Acetate	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+
Citrate	+	−	−	+	−	+	−	+	+	−	+	+	+	−	−
L-Glutamate	+	−†, +‡	V†, +‡	+	−	+	V†, −‡	+	+	+	+	+	+	−	−
D-Glucose	−	+†, −‡	−	W†, +‡	−	+	−	+	+	−	+	−	−	+	−
D-Xylose	+	−	−	−†, +‡	−	+	−	+	+	−	+	−	−	−	−
Fructose	−	+	−	+	+	−	+	V†, −‡	−	+	+	−	+	+	+
Betaine	+	−	+	+	−	−	+	−	+	+	ND	+	+	+	−

*Results of the 11 strains studied including ORS 2060^T.
†Data from Green (1992).
‡Data from Doronina *et al.* (2002).

The type strain is ORS 2060^T (=CNCM I 2342^T=LMG 21967^T), isolated from *C. podocarpa* from the Bel-Air area, Dakar, Senegal. Most of the molecular and physiological studies were conducted on this strain.

Note added in proof

While this article was being reviewed, *Methylobacterium populi* was validly described (Van Aken *et al.*, 2004).

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