Methylobacterium oryzae sp. nov., an aerobic, pink-pigmented, facultatively methylotrophic, 1-aminocyclopropane-1-carboxylate deaminase-producing bacterium isolated from rice

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A pink-pigmented, facultatively methylotrophic bacterium, strain CBMB20T, isolated from stem tissues of rice, was analysed by a polyphasic approach. Strain CBMB20T utilized 1-aminocyclopropane-1-carboxylate (ACC) as a nitrogen source and produced ACC deaminase. It was related phylogenetically to members of the genus Methylobacterium. 16S rRNA gene sequence analysis indicated that strain CBMB20T was most closely related to Methylobacterium fujisawaense, Methylobacterium radiotolerans and Methylobacterium mesophilicum; however, DNA–DNA hybridization values were less than 70 % with the type strains of these species. The DNA G+C content of strain CBMB20T was 70.6 mol%. The study presents a detailed phenotypic characterization of strain CBMB20T that allows its differentiation from other Methylobacterium species. In addition, strain CBMB20T is the only known member of the genus Methylobacterium to be described from the phyllosphere of rice. Based on the data presented, strain CBMB20T represents a novel species in the genus Methylobacterium, for which the name Methylobacterium oryzae sp. nov. is proposed, with strain CBMB20T (= DSM 18207T = LMG 23582T = KACC 11585T) as the type strain.

The genus Methylobacterium includes a group of strictly aerobic, Gram-negative, pink-pigmented, facultatively methylotrophic (PPFM) bacteria characterized by their ability to utilize single-carbon compounds like methanol and formaldehyde via the serine pathway, as well as a wide range of multicarbon growth substrates (Green, 1992). Methylobacterium is classified in the α2 subgroup of the Proteobacteria and presently consists of 22 species with validly published names (Heumann, 1962; Ito & Iizuka, 1971; Kouno & Ozaki, 1975; Patt et al., 1976; Rock et al., 1976; Austin & Goodfellow, 1979; Green & Bousfield, 1983; Urakami & Komagata, 1984; Bousfield & Green, 1985; Green et al., 1988; Urakami et al., 1993; Wood et al., 1998; Doronina et al., 2000, 2002; McDonald et al., 2001; Van Aken et al., 2004; Jourand et al., 2004; Anesti et al., 2004; Gallego et al., 2005a, b, c, 2006). Methylobacterium organophilum, the type species, remained the only PPFM bacterium reported with the ability to grow on methane until the description of Methylobacterium populi, a novel methane-utilizing species isolated from poplar trees. Members of the genus Methylobacterium are ubiquitous in nature, detected in soil, freshwater and lake sediments, as well as on other solid surfaces (Corpe & Rheem, 1989; Lidstrom & Chistoserdova, 2002), and are known particularly for their close association with plants (Holland & Polacco, 1994; Lidstrom & Chistoserdova, 2002; Sy et al., 2005). Associations of members of Methylobacterium with plants range from epiphytic to endophytic and symbiotic relations (Sy et al., 2001; Koenig et al., 2002; Pirtilă et al., 2006; Idris et al., 2006). Possible mechanisms of plant-growth promotion by Methylobacterium include production of phytohormones, such as indole-3-acetic acid (IAA), cytokinins or vitamins (Basile et al., 1985; Koenig et al., 2002).

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; PPFM, pink-pigmented, facultatively methylotrophic.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CBMB20T is AY683045.

Photomicrographs of strain CBMB20T, 16S rRNA gene sequence similarity data and biochemical reactions that differentiate the novel strains from related species are available as supplementary material in IJSEM Online.
Methylobacterium oryzae sp. nov.

Strains CBMB20T and CBMB110 were respectively isolated from surface-disinfected stem and leaf tissues of rice (*Oryza sativa* L. ‘Nam-Pyeoung’). The strains were recovered on ammonium/mineral salts (AMS) medium (Whittenbury et al., 1970) supplemented with filter-sterilized cycloheximide (10 μg ml⁻¹) and methanol (0.5 % v/v) at 28 °C. The strains were maintained routinely on nutrient agar (NA; Difco) medium, supplemented with 1 % (v/v) methanol, or on selective AMS medium. Morphological properties were studied according to general protocols (Gerhardt et al., 1994). Scanning electron microscope (SEM) observations were performed on fixed material that was prepared for routine examinations as described by Bozolla & Russell (1998). Samples were critical-point-dried, mounted on stubs, sputter-coated with gold/palladium and visualized by using a Hitachi S-2500C SEM with GEMINI column equipped with a field-emission electron source.

Chromosomal DNA was extracted and purified from cells cultured on AMS medium as described by Sambrook et al. (1989). 16S rRNA genes were amplified using universal primers F1 and R2 (Weisberg et al., 1991) and 16S rRNA gene sequencing was performed by the Big-Dye primer method using an automated DNA sequencer (ABI Prism 310 Genetic Analyzer) as described previously (Brosius et al., 1978; Shima et al., 1994). Phylogenetic analyses were performed using MEGA version 3.1 (Kumar et al., 2004), after multiple alignment of the data by CLUSTAL W (Thompson et al., 1994). Distances were obtained using options according to the Kimura two-parameter model (Kimura, 1980) and clustering was performed using the neighbour-joining (Saitou & Nei, 1987) method. Bootstrap values from 1000 replications were used to obtain the confidence level of the branches (Felsenstein, 1985).

Nutritional features were determined using the Biolog Microstation (MicroLog-3, 4.01B). The analysis was carried out in Biolog GN2 microtitre plates according to the manufacturer’s instructions; the reactions were observed after incubating the plates at 28 °C for 7 days. Carbon-source utilization tests (excluding Biolog) were performed by using a standard protocol described by Green & Bousfield (1982). Other physiological and biochemical characteristics were tested using the API ZYM and API 20NE galleries (bioMérieux) following the manufacturer’s instructions. Cellular fatty acids were analysed in organisms grown on NA with 1 % methanol (v/v) for 48 h. The cellular fatty acids were derivatized to methyl esters (Sasser, 1990) and analysed by gas chromatography (Hewlett Packard 6890) using the Microbial Identification System (MIDI; Microbial ID) software package.
Hybridization temperatures were 60 and 65 °C using the DIG luminescent detection kit (Roche Diagnostics). The DIG-High Prime system (Roche Diagnostics) was used for filter hybridization as described by Seldin & Rock (1984). DNA hydrolysis and dephosphorylation (Mesbah et al., 1984; 8, Mtb. organophilum (Patt et al., 1976); 9, Mtb. rhodesi- num (Rock et al., 1976); 10, Mtb. zatmanii (Rock et al., 1976); 11, Mtb. rhodinum (Heumann, 1962); 12, Mtb. nodulans (Jourand et al., 2004); 13, Mtb. hispanicum (Gallego et al., 2005b); 14, Mtb. populi (Van Aken et al., 2004); 15, Mtb. adhaesivum (Gallego et al., 2006). +, Growth; −, no growth; v, variable; w, weak growth; NA, no data available.

### Table 1. Differential carbon-substrate utilization among *Methylobacterium* species

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The G+C content of genomic DNA was determined by HPLC analysis of individual nucleosides resulting from DNA hydrolysis and dephosphorylation (Mesbah et al., 1989) using a reversed-phase column (Supelcosil LC-18-S; Supelco). DNA–DNA hybridization was carried out following the filter hybridization method as described by Seldin & Dubnau (1985). Probe labelling was conducted by using the non-radioactive DIG-High Prime system (Roche Diagnostics) and hybridized DNA was visualized using the DIG luminescent detection kit (Roche Diagnostics). Hybridization temperatures were 60 and 65 °C and DNA–DNA relatedness was quantified by using a densitometer (Bio-Rad Laboratories).

Strains CBMB20T and CBMB110 were strictly aerobic, Gram-negative, non-spore-forming and formed pink- to red-pigmented colonies. Cells were rod-shaped, frequently branched and occurred singly or in rosettes on solid AMS and NA media. SEM photomicrographs of strain CBMB20T are available as Supplementary Figs S1 and S2 in IJSEM Online. The presence of an *mxaF* gene encoding methanol dehydrogenase for methanol utilization was examined by PCR using the non-degenerate primers f1003 and r1561 (McDonald & Murrell, 1997); a product of the expected size (560 bp) was detected, similar to the *mxaF* genes of other *Methylobacterium* species. The 16S rRNA gene sequence of strain CBMB20T obtained was a continuous stretch of 1416 bp. Sequence similarity calculations and phylogenetic analysis identified the strains as members of the genus *Methylobacterium* as a separate group and indicated the type strains of *Methylobacterium fujisawaense* (99.1% similarity), *Methylobacterium radiotolerans* (98.0%) and *Methylobacterium mesophilicum* (97.4%) as the closest relatives (Fig. 1). The 16S rRNA gene sequence similarity matrix is available in Supplementary Table S1.

Phenotypic differences between *Methylobacterium* species are limited and often rely on utilization of carbon and energy sources (Green & Bousfield, 1982; Green, 1992). Like other members of the genus, strain CBMB20T grew on C1 substrates, such as methanol and formate, but not methanolamine, trimethylamine or formaldehyde. Strain CBMB20T differed from its closest relatives in utilization of several carbon sources (summarized in Table 1). Carbon-source utilization, enzyme activities and other biochemical reactions that differentiate the strains from other related species are available as Supplementary Tables S2–S4. The fatty acid profile of strains CBMB20T and CBMB110 consisted mainly of cis-vaccenic acid (C40:0) and octadecanoic acid (stearic acid, C18:0) (Table 2).

Strains CBMB20T and CBMB110 utilize ACC as a nitrogen source, and the presence of ACC deaminase activity in cell-free extracts and the lowering of ethylene levels and promotion of root elongation in canola seedlings under gnotobiotic conditions have been reported elsewhere (Madhaiyan et al., 2006). This particular ability has not been tested among other members of the genus. The type strains of the closely related *Mtb. fujisawaense*, *Mtb. radiotolerans* and *Mtb. organophilum* were tested by preliminary plate assays on AMS medium spread with 30 µmol ACC as a nitrogen source, and the results revealed that all three strains are positive for ACC deaminase activity. The presence of ACC deaminase in bacteria plays an important role in plant growth promotion; the bacterial cells act as a sink for ACC, the immediate biosynthetic precursor of ethylene, thereby lowering plant ethylene levels and decreasing the negative effects of various environmental stresses (Stearns et al., 2005). The presence of ACC deaminase in various bacterial genera has been reported previously (Penrose & Glick, 2001; Belimov et al., 2001).

The DNA G+C contents of strains CBMB20T and CBMB110 are respectively 70.6 and 69.2 mol%. DNA–DNA reassociation performed to confirm the species status of the novel isolates in relation to their closest phylogenetic neighbours showed 88.63% relatedness, confirming the close relationship between the two strains. However, the type strains of closely related *Methylobacterium* species...
showed 42.09–63.09 % relatedness with CBMB20T, indicating that strain CBMB20T can be separated from other members of the genus *Methylobacterium* (Table 3). These results indicate that strain CBMB20T does not belong to any of these species when the recommendation of a threshold value of 70 % DNA–DNA relatedness for species definition is considered (Wayne et al., 1987).

The 16S rRNA gene sequence similarity data, DNA–DNA hybridization values and phenotypic characteristics allowed strains CBMB20T and CBMB110 to be separated from other members of the genus *Methylobacterium*. Strain CBMB20T is proposed as the type strain of a novel *Methylobacterium* species, for which the name *Methylobacterium oryzae* sp. nov. is proposed.

**Description of *Methylobacterium oryzae* sp. nov.**

*Methylobacterium oryzae* (o.ry'zae. L. gen. n. oryzae of rice, from which the type strain was isolated).

Cells are strictly aerobic, Gram-negative, non-spor-forming, motile rods (0.60–0.80 × 2.10–2.75 μm), occurring singly, in pairs or in rosettes. Colonies are pink to red, convex and translucent with regular edges, slow-growing and 0.2–1.2 mm in diameter after 96 h at 28 °C on AMS. Growth occurs on NA and Luria–Bertani, R2A, PYG, succinate, glycerol-peptone and plate count agars. Growth occurs at 20–30 °C (optimal temperature 28 °C), but not at 4 or 40 °C, and at pH 5.0–10.0 (optimal pH 6.8). No growth in the presence of 2.0 % NaCl or higher. The pink pigment is water-insoluble and has absorption maxima at 233, 358, 505 and 534 nm in chloroform/methanol (1:1). Positive for catalase, oxidase, esterase and leucine arylamidase activities. Weak activity for esterase lipase, valine arylamidase, trypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase. No activity for pectinase, cellulase, protease or lipase. Indole, methyl red and Voges–Proskauer tests are negative. Gelatin, starch, lipid, casein and aesculin are not hydrolysed. Hydrogen sulfide is not produced. Simmons' citrate test is positive. Nitrate is reduced to nitrite. Methanol, ethanol, D-arabinose, D-xylene and fumarate are utilized as sole carbon sources. Does not use sucrose, 2-propanol, 1-butanol, dulcitol, L-lysine, betaine, oxalate, tartrate, salicylate, formaldehyde, methylamine, dimethylamine, trimethylamine, dichloromethane or methane. Ammonium sulfate,
potassium nitrate, sodium nitrate, ammonium chloride, L-alanine, L-glutamate, L-glutamine, urea, ACC and potassium thiocyanate are utilized as sole nitrogen sources. Does not utilize L-aspartate, glycine, L-tryptophan, methyamine or potassium cyanate. Carbon assimilation tests are positive for L-arabinose, potassium gluconate, adipic acid and malic acid. The intrinsic antibiotic-resistance pattern of the type strain shows high resistance to ampicillin, carbenicillin, nalidixic acid, chloramphenicol and streptomycin and sensitivity to kanamycin, gentamicin, spectinomycin and tetracycline. The following compounds are utilized as sole carbon and energy sources (Biolog): L-arabinose, D-galactose, pyruvic acid methyl ester, succinic acid monomethyl ester, formic acid, D-galactonic acid, lactone, D-gluconic acid, α-, β- and γ-hydroxybutyric acids, α-ketobutyric acid, α-ketoglutaric acid, α-koetovlalic acid, DL-lactic acid, malonic acid, propionic acid, D-saccaric acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, L-alaninamide, L-asparagine, L-aspartic acid, L-glutamic acid, L-prolylglutamic acid, glycerol and DL-β-glycolyl phosphate. Cellular fatty acids are hexadecanoate (palmitic acid; C16:0), 3.01 %; cis-7-ocadecenoate (cis-vaccenic acid; C18:1ω7c), 88.18 %; octadecenoate (stearic acid; C18:0), 4.61 %; and 3-hydroxyoctadecenoate (C18:3ω3-OH), 0.77 %.

The type strain, CBMB20T (=DSM 18207T=LMG 23582T=KACC 11585T), was isolated from stem tissues of rice (Oryza sativa ‘Nam-Pyeoung’) obtained from Chungbuk Provincial Agricultural Research and Extension Services, Cheongwon (36°58′ N 127°57′ E), Chungbuk, Republic of Korea. The DNA G+C content of strain CBMB20T is 70.6 mol%.

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References


