Methylobacterium phyllosphaerae sp. nov., a pink-pigmented, facultative methylotroph from the phyllosphere of rice

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A pink-pigmented, aerobic, facultatively methylotrophic bacterial strain, CBMB27T, isolated from leaf tissues of rice (Oryza sativa L. ‘Dong-Jin’), was analysed using a polyphasic taxonomic approach. Comparative 16S rRNA gene sequence-based phylogenetic analysis placed the strain in a clade with the species Methylobacterium oryzae, Methylobacterium fujisawaense and Methylobacterium mesophilicum; strain CBMB27T showed sequence similarities of 98.3, 98.5 and 97.3 %, respectively, to the type strains of these three species. DNA–DNA hybridization experiments revealed low levels (<38 %) of DNA–DNA relatedness between strain CBMB27T and its closest relatives. The sequence of the 1-aminocyclopropane-1-carboxylate deaminase gene (acds) in strain CBMB27T differed from those of close relatives. The major fatty acid of the isolate was C18:1ω7c and the G+C content of the genomic DNA was 66.8 mol%. Based on the results of 16S rRNA gene sequence analysis, DNA–DNA hybridization, and physiological and biochemical characterization, which enabled the isolate to be differentiated from all recognized species of the genus Methylobacterium, it was concluded that strain CBMB27T represents a novel species in the genus Methylobacterium for which the name Methylobacterium phyllosphaerae sp. nov. is proposed (type strain CBMB27T = LMG 24361T = KACC 11716T = DSM 19779T).

Bacteria of the genus Methylobacterium, class Alphaproteobacteria, consist mainly of a group of pink-pigmented facultatively methylotrophic bacteria with the ability to utilize C1 compounds such as methanol or formaldehyde and other, multicarbon compounds (Green, 1992). Cells are strictly aerobic, Gram-negative rods and, at the time of writing, the genus Methylobacterium comprised 28 species with validly published names (http://www.bacterio.cict.fr/m/methylobacterium.html), with Methylobacterium organophilum as the type species (Patt et al., 1976). Members of the genus Methylobacterium are versatile in nature and ubiquitous on plant surfaces, potentially dominating the phyllosphere population (Corpe & Rheem, 1989). Their association with plants extends from free-living to epiphytic, endophytic and symbiotic (Pirttilä et al., 2000; Sy et al., 2001; Kutschera, 2007; Schauer & Kutschera, 2008) and their presence has been detected by cultivation-independent methods (Araújo et al., 2002; Idris et al., 2004; Jackson et al., 2006). Inter- and intracellular colonization of plant tissues by Methylobacterium species and their mode of transmission from seed to aerial parts have been illustrated (Pirttilä et al., 2000; Sy et al., 2005; Poonguzhali et al., 2008).

Methylobacteria, as plant symbionts, utilize the methanol emitted from plants and, in turn, impart beneficial effects on plant growth through one or more mechanisms that include production of phytohormones like indoleacetic acid or cytokinins and vitamins (Basile et al., 1985; Koenig et al., 2002; Trotsenko et al., 2001), synthesis of enzymes such as urease or 1-aminocyclopropane-1-carboxylate deaminase (ACCD) that modulate plant growth (Holland & Polacco, 1992; Madhaiyan et al., 2006, 2007b) and production of siderophores (Idris et al., 2004). Furthermore, they tolerate heavy metals and are able to reduce their phytotoxicity and improve plant growth (Idris et al., 2004; Madhaiyan et al., 2007a). The data presented in...
this paper include the formal taxonomic description of a novel species of the genus *Methylobacterium*, which was isolated from rice leaf tissues and produces ACCD.

Strain CBMB27T was isolated from leaf tissues of rice (*Oryza sativa* L. ‘Dong-Jin’) collected from the National Honam Agricultural Experiment Station (Iksan, Republic of Korea) on selective ammonium mineral salts (AMS) medium (Whittenbury et al., 1970) supplemented with filter-sterilized cycloheximide (10 μg ml⁻¹) and 0.5 % (v/v) methanol at 28 °C by the leaf imprinting method (Chanprameet al., 1996). The strain was maintained on nutrient agar (NA; Difco) with 1 % (v/v) methanol or on AMS medium with 0.5 % (v/v) methanol. Morphological properties were studied according to standard protocols (Gerhardt et al., 1994). Nutritional characteristics were determined as described previously by using Biolog GN2 MicroPlates (Madhaiyan et al., 2007c) and utilization of carbon sources not included on the Biolog plates was determined as described by Green & Bousfield (1982). Other physiological and biochemical characteristics were tested using the API ZYM, API 20NE and API 32GN galleries (bioMérieux) following the manufacturer’s instructions. *Methylobacterium oryzae* CBMB20T was included as a positive control for these experiments. Scanning electron microscope observations were performed on fixed material that was prepared for routine examination 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 scanning electron microscope with a GEMINI column equipped with a field-emission electron source. The presence of the enzyme ACCD was determined by plate and quantitative assays as described previously (Madhaiyan et al., 2006). Cells of strain CBMB27T were Gram-negative, aerobic, non-endospore-forming rods that were frequently branched and occurred singly or in rosettes on solid AMS medium and formed pink- to red-pigmented colonies. A photomicrograph of strain CBMB27T grown on the solid surface of AMS medium supplemented with 0.5 % methanol is shown in Fig. 1. Strain CBMB27T grew on C₁ substrates such as methanol, methylamine and formate, but not on dimethylamine, trimethylamine or formaldehyde; differential utilization of several carbon sources by the isolate and its closest relatives is summarized in Table 1. A summary of enzyme reactions of strain CBMB27T compared with those of type strains of closely related *Methylobacterium* species is available as Supplementary Table S1 in IJSEM Online. Strain CBMB27T also utilized 1-amino-cyclopropane-1-carboxylate as a nitrogen source when tested with a plate assay, and considerable ACCD activity could be detected in cell-free extracts.

The 16S rRNA gene was PCR-amplified from the DNA extracts by using universal primers 27F (5’-AGAGTTTGATCTGGCTCAG-3’) and 1492R (5’-GTTACCTTGTGAGCTC-3’) and the full gene sequence was determined by the fluorescent dye terminator method using a sequencing kit (ABI PRISM BigDye Terminator cycle sequencing ready reaction kit v. 3.1) and products were run on a ABI3730XL capillary DNA sequencer (ABI PRISM 310 Genetic Analyzer). The resultant 16S rRNA gene sequence was compared with available sequences from GenBank using the program BLAST (http://www.ncbi.nlm.nih.gov.proxy.lib.siu.edu/blast/) to determine an approximate phylogenetic affiliation and gene sequences were aligned with those of closely related strains using the software CLUSTAL W (Thompson et al., 1994). Phylogenetic trees were constructed by the neighbour-joining method with the program package MEGA 3.1 (Kumar et al., 2004). Bootstrap confidence values were obtained using 1000 resamplings. The methanol dehydrogenase gene (mxaF), required for methanol utilization, and the acdS gene, which accounts for ACCD activity, were amplified from DNA extracts using primer pairs mxaF f1003 and mxaF r1561 (5’-GGCCGACAAACTGCGTGTT-3’ and 5’-GGGC-AGCATGAAGGGCTCCC-3’, respectively) for mxaF and F1936 and F1939 (5’-GHGAMGCACGTGGAAYWSYGGC-3’ and 5’-GARGCRCTCGAYVYCCRATC-3’, respectively) for acdS (McDonald & Murrell, 1997; Blaha et al., 2006). Products were sequenced directly and analysed as described by McDonald & Murrell (1997) and Blaha et al. (2006).

Comparative 16S rRNA gene sequence analysis indicated that strain CBMB27T was affiliated phylogenetically to the genus *Methylobacterium*, forming a phyletic lineage within it with *M. oryzae* CBMB20T (bootstrap value of 54 %). Sequence similarity calculations and phylogenetic analysis revealed that strain CBMB27T was closely related to *M. oryzae* CBMB20T, *M. fujisawaense* DSM 5686T, *M. mesophilicum* DSM 1708T and *M. radiotolerans* JCM 2831T, with sequence similarities of 98.3, 98.5, 97.3 and 96.8 %, respectively (Fig. 2). Sequencing of the mxaF gene from CBMB27T resulted in a continuous stretch of 556 bp and, when compared with representative *Methylobacterium* species, revealed 99.3 % gene sequence similarity with sequences from the type strains of *M. oryzae* and *M. fujisawaense*. The acdS gene sequence of CBMB27T was closely related to those of the type strains of *M. oryzae* (98.1 % similarity) and *M. fujisawaense* (92.5 %). The
The presence of ACCD in *Methylobacterium* and various bacterial genera has been reported (Madhaiyan et al., 2006; Penrose & Glick, 2001; Belimov et al., 2001). For cellular fatty acid analysis, the strain was cultured in NA with 1% (v/v) methanol at 28°C for 48 h. Fatty acids were extracted, derivatized to methyl esters and analysed by...
GC (Hewlett Packard 6890) using the Microbial Identification System (MIDI; Microbial ID) software package, according to the standard protocol (Sasser, 1990). The G+C content of genomic DNA was determined by HPLC analysis of individual nucleosides using a reversed-phase column (Supelcosil LC-18-S; Supelco) as described by Mesbah et al. (1989). DNA–DNA hybridization was carried out to determine the relatedness between strain CBMB27T and its closest relatives according to the filter hybridization method (Seldin & Dubnau, 1985). Probe labelling was conducted by using the non-radioactive DIG-High Prime system (Roche Diagnostics). Hybridization temperatures were 60 and 65 °C and DNA–DNA relatedness was quantified by using a densitometer (Bio-Rad). The fatty acid profile of strain CBMB27T consisted mainly of cis-vaccenic acid (C18:1v7c), iso-C17:0 3-OH and octadecanoic (stearic) acid (C18:0) (summarized in Table 2). The DNA G+C content of strain CBMB27T was 66.8 mol%, which falls within the range described for the genus Methylobacterium (Green, 1992). Strain CBMB27T showed low levels of DNA–DNA relatedness with its closest relatives, which falls within the range described for the genus Methylobacterium (Green, 1992). Strain CBMB27T does not belong to any of the above 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 sequence similarity data, DNA–DNA hybridization values and other phenotypic characteristics allow strain CBMB27T to be distinguished from other members of the genus Methylobacterium. On the basis of these results, strain CBMB27T is considered to represent a novel species of Methylobacterium, for which the name Methylobacterium phyllosphaerae sp. nov. is proposed.

**Description of Methylobacterium phyllosphaerae sp. nov.**

Methylobacterium phyllosphaerae (phyllo.sphae’rae. N.L. n. phyllosphaera phyllosphere; N.L. gen. n. phyllosphaerae of the phyllosphere).

Gram-negative, non-endospore-forming, strictly aerobic, motile rods (0.63–0.64 × 1.8–2.7 µm) occurring singly, in pairs or in rosettes. Colonies are pink to red, convex, translucent with regular edges, slow-growing and 0.2–0.8 mm in diameter after 96 h at 28 °C on AMS. Grows on NA, R2A, peptone-yeast extract-glucose, sucrose, glycerol-peptone and plate count agar media. Does not grow in the presence of ≥ 2.0% NaCl. Growth occurs at 20–30 °C (optimal temperature 28 °C) and pH 5.0–9.0 (optimal pH 6.8). The pink pigment is water-insoluble and has absorption maxima at 233, 359, 505 and 545 nm in chloroform/methanol (1:1). Catalase, oxidase, arginine dihydrolase and urease tests are positive. Pectinase, cellulase, protease and β-galactosidase are absent. Tests for nitrate reduction, glucose fermentation, indole production and methyl red and Voges–Proskauer tests are negative. Gelatin, starch, glycerol tributyrante, casein and ascinulin are not hydrolysed. Hydrogen sulfide is not

**Table 2.** Cellular fatty acid compositions of strain CBMB27T and the type strains of related species of the genus Methylobacterium

| Strains: 1, CBMB27T; 2, M. oryzae CB20T; 3, M. fujisawaense KACC 10744T; 4, M. mesophilicum DSM 1708T; 5, M. radiotolerans DSM 1819T; 6, M. extorquens DSM 1337T; 7, M. adhaesivum KACC 12195T; 8, M. hispanicum DSM 16372T; 9, M. organophilum DSM 760T; 10, M. iners KACC 11765T; 11, M. aerolatum KACC 11766T; 12, M. platani KCTC 12901T. Data from this study (strains 1–9), Weon et al. (2008) and Kang et al. (2007). Values are percentages of total fatty acids; -, not detected. ECL, Equivalent chain-length. Fatty acids representing less than 0.3% in all strains were omitted.

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*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 2 contained iso-C16:1 I and/or C14:0 3-OH; summed feature 3 contained C16:1v7c and/or iso-C17:0 2-OH; summed feature 4 contained iso-C17:1 I and/or anteiso-C17:0 B.
produced. Simmon's citrate test is positive. D-Glucose, fructose, D-xylene, L-arabinose, fructose, L-glutamate, citrate, sebacate, acetate, ethanol, methylene and methanol are utilized as sole carbon sources. Ammonium sulfate, potassium nitrate, sodium nitrate, ammonium chloride, L-alanine, L-glutamate, L-glutamine, urea, methylene, trimethylamine, 1-aminocyclopropane-1-carboxylate and potassium thiocyanate are utilized as sole nitrogen sources. The type strain is highly resistant (up to 500 μg ml⁻¹) to ampicillin, carbenicillin, nalidixic acid and chloramphenicol, but sensitive (lowest concentration) to kanamycin (15 μg ml⁻¹), gentamicin (200 μg ml⁻¹), spectinomycycin (200 μg ml⁻¹) and tetracycline (100 μg ml⁻¹). The following compounds are utilized as sole carbon and energy sources (Biolog): Tween 80, L-arabinose, D-fructose, L-fucose, gentiobiose, pyruvic acid methyl ester, succinic acid monomethyl ester, cis-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-glucuronic acid, D-glucosaminic acid, ε-, β- and γ-hydroxybutyric acids, ε-ketobutyric acid, ε-ketogluconic acid, ε-ketovaleric acid, DL-lactic acid, propionic acid, D-saccharic acid, succinic acid, bromosuccinic acid, succinamic acid, N-α-galactosidase, N-α-mannosidase, and L-alaninamide, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, L-lysoglutamic acid, L-serine, 2-aminobenzaldehyde and glycerol. The following carbon sources are utilized in API 20NE and API 32GN tests: D-glucose, L-arabinose, potassium gluconate, adipic acid, malic acid, L-rhamnose, D-ribose, inositol, sucrose, itaconic acid, sodium malonate, lactic acid, L-alanine, potassium 5-ketoglucuronate, glycogen, 3-hydroxybenzoic acid, L-serine, salicin, melibiose, L-fucose, D-sorbitol, L-arabinose, potassium 2-ketogluconate, 3-hydroxybutyric acid, 4-hydroxybenzoic acid and L-proline. In API ZYM assays, esterase (C4), esterase lipase (C8), leucine arylamidase, trypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase are present, but alkaline phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, ε-chymotrypsin, ε-galactosidase, β-galactosidase, β-glucuronidase, ε-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are absent. Cellular fatty acids identified in the type strain are C₁₈:₁ω₇c, iso-C₁₇:₀ 3-0H, C₁₆:₀, C₁₈:₀ and C₁₈:₁ 3-OH.

The type strain is CBMB27T (=LMG 24361T =KACC 11716T =DSM 19779T), isolated from the leaf surface of rice (Oryza sativa L. ‘Dong-Jin’). The DNA G+C content of the type strain is 66.8 mol%.

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References


