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, CBMB27^T, 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 CBMB27^T 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 CBMB27^T and its closest relatives. The sequence of the 1-aminocyclopropane-1-carboxylate deaminase gene (*acdS*) in strain CBMB27^T differed from those of close relatives. The major fatty acid of the isolate was C_{18:1}ω7c 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 CBMB27^T represents a novel species in the genus *Methylobacterium* for which the name *Methylobacterium phyllosphaerae* sp. nov. is proposed (type strain CBMB27^T = LMG 24361^T = KACC 11716^T = DSM 19779^T).

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

Abbreviation: ACCD, 1-aminocyclopropane-1-carboxylate deaminase.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *mxrF* and *acdS* gene sequences of strain CBMB27^T are EF126746, EF562496 and EU430438, respectively.

API ZYM reactions of strain CBMB27^T and related strains are available as supplementary material with the online version of this paper.

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 CBMB27^T 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 (Chanprame *et 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* CBMB20^T 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 Bozzola & 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 CBMB27^T 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 CBMB27^T grown on the solid surface of AMS medium supplemented with 0.5 % methanol is shown in Fig. 1. Strain CBMB27^T 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 CBMB27^T compared with those of type strains of closely related *Methylobacterium* species is available as Supplementary Table S1 in IJSEM Online. Strain CBMB27^T also utilized 1-aminocyclopropane 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'-AGAGTT-TGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTT-GTTACGACTT-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 (*mxoF*), required for methanol utilization, and the *acdS* gene, which accounts for ACCD activity, were amplified from DNA extracts using primer pairs *mxoF* f1003 and *mxoF* r1561 (5'-GCGGCACCAACTGGGGCTGGT-3' and 5'-GGGC-AGCATGAAGGGCTCCC-3', respectively) for *mxoF* and F1936 and F1939 (5'-GHGAMGACTGCAAYWSYGGC-3' and 5'-GARGCRTCGAYVCCRATCAC-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 CBMB27^T was affiliated phylogenetically to the genus *Methylobacterium*, forming a phyletic lineage within it with *M. oryzae* CBMB20^T (bootstrap value of 54 %). Sequence similarity calculations and phylogenetic analysis revealed that strain CBMB27^T was closely related to *M. oryzae* CBMB20^T, *M. fujisawaense* DSM 5686^T, *M. mesophilicum* DSM 1708^T and *M. radiotolerans* JCM 2831^T, with sequence similarities of 98.3, 98.5, 97.3 and 96.8 %, respectively (Fig. 2). Sequencing of the *mxoF* gene from CBMB27^T 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 CBMB27^T was closely related to those of the type strains of *M. oryzae* (98.1 % similarity) and *M. fujisawaense* (92.5 %). The

Fig. 1. Photomicrograph of cells of strain CBMB27^T on AMS medium supplemented with 0.5 % (v/v) methanol. Bar, 2 µm.

Table 1. Differential carbon source utilization of strain CBMB27^T and related *Methylobacterium* species

Utilization of various compounds as sole sources of carbon and energy is shown. Adapted from Green (1992). Species/strains: 1, strain CBMB27^T (*M. phyllosphaerae* sp. nov.); 2, *M. oryzae* (Madhaiyan *et al.*, 2007c); 3, *M. fujisawaense* (Kouno & Ozaki, 1975); 4, *M. mesophilicum* (Austin & Goodfellow, 1979); 5, *M. radiotolerans* (Ito & Iizuka, 1971); 6, *M. extorquens* (Urakami & Komagata, 1984); 7, *M. adhaesivum* (Gallego *et al.*, 2006); 8, *M. suomiense* (Doronina *et al.*, 2002); 9, *M. organophilum* (Patt *et al.*, 1976); 10, *M. rhodesianum* (Rock *et al.*, 1976); 11, *M. zatmanii* (Rock *et al.*, 1976); 12, *M. rhodinum* (Heumann, 1962); 13, *M. nodulans* (Jourand *et al.*, 2004); 14, *M. hispanicum* (Gallego *et al.*, 2005); 15, *M. populi* (Van Aken *et al.*, 2004). +, Growth; –, no growth; v, variable; w, weak growth; NA, no data available. All species are positive for growth on methanol.

Carbon source	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
D-Glucose	w	–	+	+	+	–	–	+	–	–	–	+	–	–	–
Fucose	+	–	+	+	+	–	–	NA	–	–	–	–	–	–	–
D-Xylose	+	–	+	+	+	–	–	–	–	–	–	+	+	–	–
L-Arabinose	+	+	+	+	+	–	–	–	–	–	–	–	+	–	–
Fructose	+	–	–	–	–	–	+	+	+	+	+	+	–	–	+
L-Glutamate	+	+	+	+	+	+	+	–	+	–	–	+	+	+	–
Citrate	+	–	+	+	+	–	+	–	–	–	–	+	+	+	–
Sebacate	+	+	+	v	+	–	NA	NA	–	–	–	–	NA	NA	–
Acetate	w	–	+	–	+	+	+	+	+	+	+	+	+	+	+
Betaine	–	–	–	–	+	+	NA	+	–	+	–	+	+	NA	+
Tartrate	–	–	v	–	–	v	NA	–	–	–	v	–	+	NA	+
Ethanol	w	w	+	+	v	+	NA	+	+	+	+	+	+	NA	+
Methane	–	–	–	–	–	–	NA	–	v	–	–	–	NA	NA	+
Methylamine	w	–	–	–	–	+	NA	+	+	+	+	+	–	NA	+
Dimethylamine	–	–	–	–	–	–	NA	–	–	+	–	–	NA	NA	–
Trimethylamine	–	–	–	–	–	–	NA	–	+	–	–	–	NA	NA	–
Nutrient agar	+	+	+	–	+	+	NA	–	+	+	+	+	NA	NA	+

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

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequence comparison showing the position of strain CBMB27^T and related species of the genus *Methylobacterium*. Numbers at nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets (only values greater than 50 % are shown). Bar, 0.01 substitutions per site.

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 CBMB27^T 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) 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). The fatty acid profile of strain CBMB27^T consisted mainly of *cis*-vaccenic acid (C_{18:1}ω7c), iso-C_{17:0} 3-OH and octadecanoic (stearic) acid (C_{18:0}) (summarized in Table 2). The DNA G + C content of strain CBMB27^T was 66.8 mol%, which falls within the range described for the genus *Methylobacterium* (Green, 1992). Strain CBMB27^T showed low levels of DNA–DNA relatedness with its closest relatives, *M. fujisawaense* KACC 10744^T (37.6%), *M. mesophilicum* DSM 1708^T (31.5%), *M. radiotolerans* DSM 1819^T (31.8%), *M. adhaesivum* KACC 12195^T (23.2%), *M. extorquens* KACC 11434^T (13.8%) and *M. oryzae* KACC 11585^T (8.06%). These results indicate that strain CBMB27^T 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 CBMB27^T to be distinguished from other members of the genus *Methylobacterium*. On the basis of these results, strain CBMB27^T 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 (phyl.lo.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, succinate, 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 tributyrates, casein and aesculin are not hydrolysed. Hydrogen sulfide is not

Table 2. Cellular fatty acid compositions of strain CBMB27^T and the type strains of related species of the genus *Methylobacterium*

Strains: 1, CBMB27^T; 2, *M. oryzae* CBMB20^T; 3, *M. fujisawaense* KACC 10744^T; 4, *M. mesophilicum* DSM 1708^T; 5, *M. radiotolerans* DSM 1819^T; 6, *M. extorquens* DSM 1337^T; 7, *M. adhaesivum* KACC 12195^T; 8, *M. hispanicum* DSM 16372^T; 9, *M. organophilum* DSM 760^T; 10, *M. iners* KACC 11765^T; 11, *M. aerolatum* KACC 11766^T; 12, *M. platani* KCTC 12901^T. 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.

Fatty acid	1	2	3	4	5	6	7	8	9	10	11	12
C _{9:0}	–	–	–	–	–	–	–	1.2	–	–	–	–
C _{12:0}	–	–	–	–	–	–	–	4.5	–	0.8	–	–
C _{14:0}	–	–	–	–	–	–	–	2.4	–	0.8	–	0.4
C _{16:0}	4.1	3.0	2.0	3.2	3.0	2.6	4.5	4.6	3.0	3.3	3.9	7.6
iso-C _{17:0} 3-OH	11.5	–	–	–	–	9.1	–	–	–	–	–	–
C _{18:0}	4.4	4.6	5.4	4.1	5.3	2.3	–	4.6	6.3	2.9	2.4	1.7
C _{18:0} 3-OH	0.9	0.8	0.7	1.5	0.6	–	–	–	0.5	–	1.7	3.5
C _{18:1} ω7c	75.1	88.2	88.7	88.6	88.8	64.8	79.3	68.5	88.1	82.3	82.6	81.0
Summed feature 2*	2.7	0.8	0.8	1.06	0.8	6.3	2.2	8.0	0.8	2.2	3.50	2.0
Summed feature 3*	1.1	1.8	1.7	0.8	0.8	12.6	8.5	6.3	0.7	6.5	6.0	2.2
Summed feature 4*	–	–	–	–	–	1.31	5.1	–	–	–	–	–
Unknown ECL 14.959	–	0.4	0.5	0.5	0.5	–	–	–	0.5	–	–	–

*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-C_{16:1} I and/or C_{14:0} 3-OH; summed feature 3 contained C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; summed feature 4 contained iso-C_{17:1} I and/or anteiso-C_{17:0} B.

produced. Simmons' citrate test is positive. D-Glucose, fucose, D-xylose, L-arabinose, fructose, L-glutamate, citrate, sebacate, acetate, ethanol, methylamine and methanol are utilized as sole carbon sources. Ammonium sulfate, potassium nitrate, sodium nitrate, ammonium chloride, L-alanine, L-glutamate, L-glutamine, urea, methylamine, 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), spectinomycin (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-gluconic acid, D-glucosaminic acid, α -, β - and γ -hydroxybutyric acids, α -ketobutyric acid, α -ketoglutaric acid, α -ketovaleric acid, DL-lactic acid, propionic acid, D-saccharic acid, succinic acid, bromosuccinic acid, succinamic acid, L-alaninamide, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, L-pyroglutamic acid, L-serine, 2-aminoethanol 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-ketogluconate, 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, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase are absent. Cellular fatty acids identified in the type strain are C_{18:1}ω7c, iso-C_{17:0} 3-OH, C_{16:0}, C_{18:0} and C_{18:0} 3-OH.

The type strain is CBMB27^T (=LMG 24361^T =KACC 11716^T =DSM 19779^T), 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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