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 CBMB20^T, isolated from stem tissues of rice, was analysed by a polyphasic approach. Strain CBMB20^T 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 CBMB20^T 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 CBMB20^T was 70.6 mol%. The study presents a detailed phenotypic characterization of strain CBMB20^T that allows its differentiation from other *Methylobacterium* species. In addition, strain CBMB20^T is the only known member of the genus *Methylobacterium* to be described from the phyllosphere of rice. Based on the data presented, strain CBMB20^T represents a novel species in the genus *Methylobacterium*, for which the name *Methylobacterium oryzae* sp. nov. is proposed, with strain CBMB20^T (=DSM 18207^T=LMG 23582^T=KACC 11585^T) 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 $\alpha 2$ 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; Pirttilä *et al.*, 2000; 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.*,

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 CBMB20^T is AY683045.

Photomicrographs of strain CBMB20^T, 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.

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence comparison showing the position of strains CBMB20^T and CBMB110 and related species of the genus *Methylobacterium*. Numbers at nodes indicate percentages of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets. Values below 50% are not indicated. Bar, 0.01 substitutions per site.

2002; Trotsenko *et al.*, 2001). In addition, members of *Methylobacterium* are associated with nitrogen metabolism of plants by means of bacterial urease (Holland & Polacco, 1992). *Methylobacterium* strains are able to establish efficient nitrogen-fixing symbioses by nodulating legume roots (Sy *et al.*, 2001) and have been identified as endosymbionts within the buds of Scots pine (Pirttilä *et al.*, 2000). Furthermore, we have reported the enhancement of plant growth in canola by 1-aminocyclopropane-1-carboxylate (ACC) deaminase-producing *Methylobacterium* strains (Madhaiyan *et al.*, 2006). The data presented in this paper include the formal taxonomic description of a novel species of the genus *Methylobacterium* for two strains isolated from rice tissues with the ability to produce ACC deaminase.

Strains CBMB20^T and CBMB110 were respectively isolated from surface-disinfected stem and leaf tissues of rice (*Oryza sativa* L. 'Nam-Pyeong'). 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 Bozzola & 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 fd1 and rp2 (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.

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

Utilization of various compounds as sole sources of carbon and energy is shown. Adapted from Green (1992). Species/strains: 1, CBMB20^T; 2, CBMB110; 3, *Mtb. radiotolerans* (data from Ito & Iizuka, 1971); 4, *Mtb. mesophilicum* (Austin & Goodfellow, 1979); 5, *Mtb. fujisawaense* (Kouno & Ozaki, 1975); 6, *Mtb. suomiense* (Doronina *et al.*, 2002); 7, *Mtb. extorquens* (Urakami & Komagata, 1984); 8, *Mtb. organophilum* (Patt *et al.*, 1976); 9, *Mtb. rhodesianum* (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.

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

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 CBMB20^T 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 CBMB20^T are available as Supplementary Figs S1 and S2 in IJSEM Online. The presence of an *mxhF* gene encoding methanol

dehydrogenase required 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 *mxhF* genes of other *Methylobacterium* species. The 16S rRNA gene sequence of strain CBMB20^T 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 CBMB20^T grew on C₁ substrates, such as methanol and formate, but not methylamine, trimethylamine or formaldehyde. Strain CBMB20^T 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 CBMB20^T and CBMB110 consisted mainly of *cis*-vaccenic acid (C_{18:1}ω7c) and octadecanoic acid (stearic acid, C_{18:0}) (Table 2).

Strains CBMB20^T 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 CBMB20^T 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

Table 2. Cellular fatty acid compositions of strains CBMB20^T and CBMB110 and related species of the genus *Methylobacterium*

Species/strains: 1, CBMB20^T; 2, CBMB110; 3, *Mtb. fujisawaense* KACC 10744^T; 4, *Mtb. hispanicum* DSM 16372^T; 5, *Mtb. mesophilicum* DSM 1708^T; 6, *Mtb. organophilum* DSM 760^T; 7, *Mtb. radiotolerans* DSM 1819^T (data in columns 1–7 from this study); 8, *Mtb. populi* (data from Van Aken *et al.*, 2004); 9, *Mtb. suomiense* (Doronina *et al.*, 2002); 10, *Mtb. lusitanum* (Doronina *et al.*, 2002); 11, '*Mtb. goesingense*' (Idris *et al.*, 2006). Values are percentages of total fatty acids. Fatty acids representing less than 0.3 % in all strains were omitted. ND, Not detected; NR, not reported; ECL, equivalent chain length.

Fatty acid	1	2	3	4	5	6	7	8	9	10	11
C _{9:0}	ND	ND	ND	1.2	ND	ND	ND	NR	NR	NR	NR
C _{12:0}	ND	ND	ND	4.53	ND	ND	ND	NR	NR	NR	NR
C _{14:0}	ND	0.3	ND	2.35	ND	ND	ND	NR	NR	NR	NR
C _{16:0}	3.01	3.5	1.96	4.57	3.18	2.98	3.01	6.40	NR	NR	3.3
C _{18:0}	4.61	3.66	5.42	4.57	4.1	6.27	5.29	11.9	NR	NR	2.4
C _{18:0} 3-OH	0.77	0.72	0.69	ND	1.52	0.49	0.64	NR	NR	NR	NR
C _{18:1} ω7c	88.18	86.77	88.65	68.53	88.63	88.10	88.76	86.11	84.7	83.1	82.0
Summed feature 2*	0.77	1.69	0.80	7.97	1.06	0.79	0.81	NR	NR	NR	NR
Summed feature 3*	1.79	2.1	1.74	6.27	0.84	0.69	0.81	NR	NR	NR	NR
Summed feature 4*	ND	0.81	ND	ND	ND	ND	ND	NR	NR	NR	NR
Unknown ECL 14.959	0.36	0.46	0.47	ND	0.45	0.46	0.50	NR	NR	NR	NR

*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.

showed 42.09–63.09 % relatedness with CBMB20^T, indicating that strain CBMB20^T can be separated from other members of the genus *Methylobacterium* (Table 3). These results indicate that strain CBMB20^T 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 CBMB20^T and CBMB110 to be separated from other members of the genus *Methylobacterium*. Strain CBMB20^T is proposed as the type strain of a novel *Methylobacterium* species, for which the name *Methylobacterium oryzae* sp. nov. is proposed.

Table 3. DNA–DNA hybridization between strain CBMB20^T and its closest relatives

Strain	DNA–DNA hybridization with strain CBMB20 ^T (%)
CBMB20 ^T	100.00
CBMB110	88.63
<i>Mtb. fujisawaense</i> KACC 10744 ^T	42.09
<i>Mtb. mesophilicum</i> DSM 1708 ^T	54.51
<i>Mtb. radiotolerans</i> DSM 1819 ^T	63.09

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-spore-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-xylose 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, methylamine 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 mono-methyl ester, formic acid, D-galactonic acid, lactone, D-gluconic acid, α -, β - and γ -hydroxybutyric acids, α -ketobutyric acid, α -ketoglutaric acid, α -ketovaleric acid, DL-lactic acid, malonic acid, propionic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, L-alaninamide, L-asparagine, L-aspartic acid, L-glutamic acid, L-pyroglutamic acid, glycerol and DL- α -glycerol phosphate. Cellular fatty acids are hexadecanoate (palmitic acid; C_{16:0}), 3.01 %; *cis*-7-octadecenoate (*cis*-vaccenic acid; C_{18:1 ω 7c}), 88.18 %; octadecanoate (stearic acid; C_{18:0}), 4.61 %; and 3-hydroxyoctadecanoate (C_{18:0} 3-OH), 0.77 %.

The type strain, CBMB20^T (=DSM 18207^T=LMG 23582^T=KACC 11585^T), was isolated from stem tissues of rice (*Oryza sativa* L. 'Nam-Pyeong') 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 CBMB20^T is 70.6 mol%.

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