Bacillus methylotrophicus sp. nov., a methanol-utilizing, plant-growth-promoting bacterium isolated from rice rhizosphere soil

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A Gram-positive bacterium, designated strain CBMB205^T, was isolated from the rhizosphere soil of traditionally cultivated, field-grown rice. Cells were strictly aerobic, motile, rod-shaped and formed endospores. The best growth was achieved at 30 °C and pH 7.0 in ammonium mineral salts (AMS) medium containing 600 mM methanol. A comparative 16S rRNA gene sequence-based phylogenetic analysis placed strain CBMB205^T in a clade with the species *Bacillus amyloliquefaciens*, *Bacillus vallismortis*, *Bacillus subtilis*, *Bacillus atrophaeus*, *Bacillus mojavensis* and *Bacillus licheniformis* and revealed pairwise similarities ranging from 98.2 to 99.2%. DNA–DNA hybridization experiments revealed a low level (<36%) of DNA–DNA relatedness between strain CBMB205^T and its closest relatives. The major components of the fatty acid profile were C_{15:0} anteiso, C_{15:0} iso, C_{16:0} iso and C_{17:0} anteiso. The diagnostic diamino acid of the cell wall was meso-diaminopimelic acid. The G+C content of the genomic DNA was 45.0 mol%. The lipids present in strain CBMB205^T were diphosphatidylglycerol, phosphatidylglycerol, a minor amount of phosphatidylcholine and two unknown phospholipids. The predominant respiratory quinone was MK-7. Studies of DNA–DNA relatedness, morphological, physiological and chemotaxonomic analyses and phylogenetic data based on 16S rRNA gene sequencing enabled strain CBMB205^T to be described as representing a novel species of the genus *Bacillus*, for which the name *Bacillus methylotrophicus* sp. nov. is proposed. The type strain is CBMB205^T (=KACC 13105^T=NCCB 100236^T).

Methylotrophy has been recognized as a property of specialized bacteria that are capable of growth on C1-compounds and members of numerous genera and species have been found to possess this ability (Whittenbury *et al.*, 1970; Arfman *et al.*, 1992; Kalyuzhnaya *et al.*, 2006; Lidstrom, 2006). It has been found that the occurrence of methylotrophy in prokaryotes does not correlate with traditional bacterial classification (Brautaset *et al.*, 2004), as facultative methylotrophy has been shown to be a property of diverse typically heterotrophic genera (Boden *et al.*, 2008). One of the earliest methylotrophic organisms to be

isolated was the Gram-positive bacterium, '*Bacillus methylicus*' (later renamed as '*Bacterium methylicum*', but no longer available in culture). This aerobic, non-spore-forming, facultative methylotroph produced red pigment when grown on formate or methanol and also grew on formaldehyde (Loew, 1892; Migula, 1900; Bergey *et al.*, 1939). Numerous specialized methylotrophs have since been described, including a great diversity of methanotrophs, some of which are obligate methane users, many that also use methanol and a few that are capable of growth on multicarbon compounds (Whittenbury *et al.*, 1970; Dedysh *et al.*, 2005). At the time of writing, the genus *Bacillus*, in the phylum *Firmicutes*, consisted of 225 species with validly published names (<http://www.bacterio.cict.fr/b/bacillus.html>) with *Bacillus subtilis* as the type species (Cohn, 1872; Skerman *et al.*, 1980). The genus includes the methanol-utilizing bacterium *Bacillus methanolicus*, which was isolated from soil samples (Arfman *et al.*, 1992).

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate deaminase; AMS, ammonium mineral salts; SEM, scanning electron microscope.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA sequence of strain CBMB205^T is EU194897.

A photomicrograph of cells of strain CBMB205^T is available with the online version of this paper.

Methylotrophic strains of the genus *Bacillus* display a strong resistance to high methanol concentrations and the molar growth yields on methanol at the optimum growth temperatures in methanol-limited chemostats are among the highest reported for any methylotrophic bacteria (Anthony, 1982; Dijkhuizen *et al.*, 1988). Currently Gram-negative bacteria remain the best-studied plant-interacting microbes, but representatives of high and low G+C Gram-positive bacteria also have excellent biocontrol, plant-growth-promoting and bioremediation activities. In addition, actinorhizal symbioses largely contribute to global biological nitrogen fixation and many Gram-positive bacteria promote other types of symbioses in tripartite interactions (Francis *et al.*, 2010). In this paper, a novel species of the genus *Bacillus* was isolated from the rice rhizoplane and was found to have the ability to produce 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase).

Strain CBMB205^T was isolated from the rhizoplane of rice (*Oryza sativa* L. cv O-dae) from samples collected by the Chungbuk Provincial Agricultural Research and Extension Services (Cheongwon, Republic of Korea). The strain was isolated on selective 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 for 5 days. The cells were maintained on AMS medium with 0.5 % (v/v) methanol or on nutrient agar (NA, Difco) with 1 % methanol (v/v). Morphological properties were studied according to general protocols (Gerhardt *et al.*, 1994). The nutritional features were determined using Biolog GP2 microplates (Madhaiyan *et al.*, 2007a; Poonguzhali *et al.*, 2006) and carbon-source utilization tests (excluding Biolog) were performed using standard protocols (Green & Bousfield, 1982). Gram staining was performed by using a Gram staining kit (Difco) and bacterial suspensions were examined by phase-contrast microscopy for cell morphology and motility. The pH range for growth was determined on R2A agar. The growth temperature range (4, 10, 20, 25, 30, 37, 45 °C) and NaCl tolerance [0, 1, 2, 3, 4, 5, 10 % (w/v)] were tested on R2A medium. All tests were incubated at 28 °C, unless otherwise indicated. Growth on nutrient agar, Luria-Bertani (LB), R2A, trypticase soy agar (Difco), King's B medium (KB), 2 % MH (see DSMZ list of media 620, http://www.dsmz.de/microorganisms/media_list.php), and Colby and Zathman medium was also evaluated at 30 °C. Catalase activity was determined by assessing bubble production in 3 % (v/v) H₂O₂ and oxidase activity was determined using 1 % (w/v) tetramethyl-*p*-phenylenediamine using Difco BBL catalase and oxidase reagent droppers according to the manufacturer's instructions (Difco). Tests for the hydrolysis of casein, glycerol tributyrates, gelatin, aesculin and pectin were performed and evaluated after 5 days (Atlas, 1993; Kouker & Jaeger, 1987; Ten *et al.*, 2004). Other physiological and biochemical characteristics were tested using the API ZYM, API 20NE and API 32GN galleries (bioMérieux) following the manufacturer's instructions. Preparation and examination

of cells under a scanning electron microscope (SEM) was carried out according to Bozzola & Russell (1998). Cells were visualized using a Hitachi S-2500C SEM with a GEMINI column equipped with a field-emission electron source. Cells of strain CBMB205^T were Gram-positive rods, aerobic, motile, endospore-forming, frequently branched and occurred singly or in pairs on solid AMS medium. Colonies were creamy white and convex with regular margins. Photomicrographs of cells of strain CBMB205^T grown on the solid surface of AMS medium supplemented with 0.5 % methanol are shown in Supplementary Fig. S1 (available with IJSEM Online). The novel strain grew on methanol and on diethanolamine and showed weak growth on trimethylamine and formaldehyde when they were supplemented as a carbon source in AMS medium (0.5 % v/v). The results of nutrient utilization tests and the results from the API 20NE, API 32GN galleries for strain CBMB205^T are compared with those of closely related species of the genus *Bacillus* in Table 1.

The 16S rRNA gene was amplified by PCR from extracted DNA using the universal primers 27F and 1492R (DeLong, 1992). The full gene sequence was determined by the fluorescent dye terminator method using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (version 3.1). The products were run on a ABI3730XL capillary DNA sequencer (ABI Prism 310 Genetic Analyzer). Sequence comparisons with close relatives available from GenBank were performed using BLAST (<http://www.ncbi.nlm.nih.gov.proxy.lib.siu.edu/blast/>) to determine an approximate phylogenetic affiliation and sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994). Phylogenetic trees were constructed with the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods, using the MEGA 4.1 program (Tamura *et al.*, 2007) and bootstrap values based on 1000 replications (Felsenstein, 1985). The results revealed that strain CBMB205^T was phylogenetically affiliated to the genus *Bacillus*, forming a phyletic lineage within the genus with a bootstrap value of 37 %. Strain CBMB205^T was closely related to *Bacillus vallismortis*, *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus mojavensis*, *Bacillus atrophaeus* and *Bacillus amyloliquefaciens* showing pairwise sequence similarities ranging from 98.2 to 99.2 % (Fig. 1).

Determination of ACC deaminase activity by plate and quantitative assays and tests for indole-3-acetic acid (IAA) production, sodium thiosulfate oxidation and siderophore production were carried out as described previously (Poonguzhali *et al.*, 2006; Madhaiyan *et al.*, 2006). A gnotobiotic growth pouch assay was performed to measure the degree of root elongation induced by ACC deaminase in tomato, canola and red pepper as these species are good representatives of ethylene sensitive plants (Glick *et al.*, 1994; Li *et al.*, 2000; Penrose & Glick, 2003; Ghosh *et al.*, 2003; Madhaiyan *et al.*, 2006, 2007b). Strain CBMB205^T possessed ACC deaminase activity, showed siderophore production and sulfur oxidation activity when tested by plate assays and produced negligible amounts of IAA (data

Table 1. Differential characteristics of strain CBMB205^T and some related species of the genus *Bacillus*

Strains: 1, CBMB205^T; 2, *B. amyloliquefaciens* KACC 12067^T; 3, *B. vallismortis* NRRL B-14890^T; 4, *B. subtilis* subsp. *subtilis* KACC 10854^T; 5, *B. subtilis* subsp. *spizizenii* NRRL B-23049^T; 6, *B. atrophaeus* NRRL NRS-213^T; 7, *B. mojavensis* NRRL B-14698^T; 8, *B. licheniformis* KACC 10476^T; 9, *B. methanolicus* DSM 16454^T. All the isolates were positive for motility and catalase; negative for nitrate reduction to N₂ and methylamine. In assimilation tests (API 20NE and API 32GN): all taxa were positive for aesculin hydrolysis, gelatin hydrolysis, D-glucose, D-mannose, D-mannitol, N-acetylglucosamine, maltose, L-alanine, glycogen, L-proline and potassium gluconate. All taxa were negative for indole production, glucose fermentation, itaconic acid, suberic acid, sodium malonate, potassium 5-ketogluconate, 3-hydroxybenzoic acid, L-serine, L-fucose, capric acid, potassium 2-ketogluconate, 3-hydroxybutyric acid, 4-hydroxybenzoic acid and phenylacetic acid. +, Growth; –, no growth; w, weak growth. All data were obtained in this study.

Characteristic	1	2	3	4	5	6	7	8	9
Pigmentation	Creamy white	Opaque	Creamy white	Opaque	Opaque	Dark brown	Creamy white	Opaque	Creamy white
Anaerobic growth	–	–	–	–	–	–	–	+	–
Oxidase	+	+	+	+	+	–	+	+	+
Growth in 10 % NaCl	–	+	+	+	+	+	+	+	–
Maximum growth temp (°C)	45	50	50	50	50	50	50	55	60
Minimum growth temp (°C)	15	10	10	10	10	10	10	15	35
Utilization of									
Diethanolamine	–	–	–	+	+	–	w	+	–
Ethanol	+	+	+	+	+	–	+	+	+
Methanol	+	+	+	+	+	–	+	+	+
Trimethylamine	w	+	–	w	w	–	w	+	–
API 20NE & API 32GN tests:									
Nitrate reduction to nitrite	+	–	+	+	+	+	+	+	–
Adipic acid	w	–	–	–	–	–	–	–	–
L-Arabinose	+	–	+	+	+	+	+	+	+
Arginine dihydrolase	–	+	w	+	+	+	+	+	+
β-Galactosidase	–	+	+	+	+	+	+	+	+
L-Histidine	+	+	–	+	+	w	+	–	+
Inositol	+	–	+	+	+	–	+	+	–
Lactic acid	+	+	+	+	+	–	+	w	–
Malic acid	+	+	+	+	+	w	+	+	+
Melibiose	+	+	+	+	+	–	+	+	+
Propionic acid	–	–	–	–	–	–	–	+	–
L-Rhamnose	+	–	–	–	–	–	+	+	–
D-Ribose	+	+	+	+	w	+	+	+	+
Salicin	+	+	–	+	+	w	+	+	–
Sodium acetate	–	–	–	–	–	–	–	w	–
D-Sorbitol	+	+	–	+	+	w	+	+	–
Sucrose	+	+	+	+	+	w	+	+	–
Trisodium citrate	+	–	+	+	+	–	+	+	+
Urease	–	+	–	+	+	+	+	–	–
Valeric acid	–	–	–	–	–	–	–	w	–
DNA G + C content (mol%)	45	43	43	43	43	42	43	46	48

not shown). The ACC deaminase activity in cell-free extracts of strain CBMB205^T was 8.25 nmol α-ketobutyrate per mg protein h^{–1}. The promotion of root elongation by strain CBMB205^T was checked with ethylene sensitive plants instead of with rice (the host crop), to verify the role of ACC deaminase in lowering the ethylene level. Inoculation of strain CBMB205^T increased the root lengths of tomato, red pepper and canola plants by 38.3, 4.2 and 22.4% over controls, respectively. Inoculation also increased the dry biomass of the plants (data not shown). The presence of the *acdS* gene (558 bp), encoding ACC

deaminase, was confirmed in the novel strain by detection with PCR amplification using the non-degenerate primers F1936 and F1939 (Blaha *et al.*, 2006). The presence of ACC deaminase in members of the genus *Methylobacterium* and various other bacterial genera has been reported previously (Madhaiyan *et al.*, 2006; Penrose & Glick, 2001; Belimov *et al.*, 2001). The novel strain did not show acetylene reduction activity, which was performed in order to indicate the presence of the nitrogenase enzyme. However, the presence of the *nifH* gene in the novel strain was confirmed by PCR amplification carried out with specific

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence comparisons showing the position of strain CBMB205^T and related species of the genus *Bacillus*. Numbers at nodes indicate percentages of occurrence in 1000 bootstrapped trees; bootstrap values <70 % are not shown. Bar, 0.005 % substitutions per site.

primers (Ueda *et al.*, 1995) 19F (5'-GCIWTTYTAYGGI-AARGGIGG-3') and 407R (5'-AAICCRCCCAIACIACR-TC-3').

For the analysis of cellular fatty acids, the novel strain was cultured in trypticase soy agar at 28 °C for 48 h. The fatty acids were extracted, derivatized to methyl esters and analysed by GC (6890; Hewlett Packard) using the Microbial Identification System (MIDI; Microbial ID) software package, according to standard protocols (Sasser, 1990). Polar lipids and menaquinones were extracted and analysed according to Minnikin *et al.* (1984) and Kroppenstedt (1982). Preparation of cell walls from strain CBMB205^T and the analysis of peptidoglycan were carried out using methods described by Schleifer

(1985), with the modification that TLC was performed on cellulose sheets rather than by paper chromatography. The fatty acid profile of the strain CBMB205^T consisted mainly of C_{15:0} anteiso, C_{15:0} iso, C_{16:0} iso and C_{17:0} anteiso (summarized in Table 2).

DNA–DNA hybridization experiments were performed to determine the relatedness of strain CBMB205^T to its closest relatives. The filter hybridization method was used (Seldin & Dubnau, 1985). The hybridization temperature was 65 °C and DNA–DNA relatedness was quantified by using a densitometer (Bio-Rad Laboratories). Probe labelling was conducted by using the non-radioactive DIG-High prime system (Roche Diagnostics GmbH) and hybridized DNA was visualized using the DIG Luminescent Detection kit according to the manufacturer's instructions (Roche Diagnostics GmbH). The G + C content of the genomic DNA of the novel strain was determined by HPLC analysis using a reverse-phase column (Supelcosil LC-18 S, Supelco) of individual nucleosides as previously described (Mesbah *et al.*, 1989). Strain CBMB205^T showed a low level of DNA–DNA relatedness that ranged from 19.0 to 6.0 % with its closest relatives *B. amyloliquefaciens* (36.0 %), *B. vallismortis* (32 %), *B. subtilis* subsp. *subtilis* (31 %), *B. subtilis* subsp. *spizizenii* (30 %), *B. atrophaeus* (28 %), *B. licheniformis* (25 %) and *B. mojavensis* (19 %). These results indicated that strain CBMB205^T did not belong to the above species if the recommended threshold value of 70 % DNA–DNA similarity for the delineation of species is considered (Wayne *et al.*, 1987). The DNA G + C content of strain CBMB205^T was 45.0 mol%, which fell within the range previously described for the genus *Bacillus*.

The 16S rRNA gene sequence similarity data, DNA–DNA hybridization values and other phenotypic characteristics allowed strain CBMB205^T to be recognized as separate from other members of the genus *Bacillus*. On the basis of these results, strain CBMB205^T is considered to represent a

Table 2. Cellular fatty acid contents of strain CBMB205^T and type strains of related *Bacillus* species

Strains: 1, CBMB205^T; 2, *B. amyloliquefaciens* KACC 12067^T; 3, *B. vallismortis* NRRL B-14890^T; 4, *B. subtilis* subsp. *subtilis* KACC 10854^T; 5, *B. subtilis* subsp. *spizizenii* NRRL B-23049^T; 6, *B. atrophaeus* NRRL NRS-213^T; 7, *B. mojavensis* NRRL B-14698^T; 8, *B. licheniformis* KACC 10476^T; 9, *B. methanolicus* DSM 16454^T. Values are percentages of total fatty acids. Fatty acids representing <0.1 % in all strains were omitted. All data were obtained in this study.

Fatty acid	1	2	3	4	5	6	7	8	9
C _{14:0} iso	—	2.9	1.2	1.3	1.5	1.9	1.1	1.1	0.9
C _{15:0} iso	20.8	21.5	17.5	28.6	29.2	14.5	25.2	33.6	27.5
C _{15:0} anteiso	52.7	44.2	49.5	44.2	42.5	50.2	46.5	43.6	32.5
C _{16:0}	—	8.5	5.6	4.5	4.4	5.2	5.5	4.6	5.7
C _{16:0} iso	13.9	—	—	2.2	2.1	—	—	—	11.2
C _{17:0} iso	—	9.7	8.6	8.9	8.5	9.5	9.1	6.5	6.1
C _{17:0} anteiso	12.6	12.5	17.5	9.6	11.2	17.9	11.5	10.5	15.8

novel species of the genus *Bacillus*, for which the name *Bacillus methylotrophicus* sp. nov. is proposed.

Description of *Bacillus methylotrophicus* sp. nov.

Bacillus methylotrophicus [me.thy.lo.tro.phi.cus. N.L. suff. *methylo* pertaining to the methyl radical; N.L. adj. *trophicus* (from Gr. adj. *trophikos*) nursing, tending or feeding; N.L. masc. adj. *methylotrophicus* feeding on methyl radical, methyl radical-consuming].

Gram-positive, endospore-forming, strictly aerobic, motile rods (0.63–0.64 × 1.8–2.7 µm) occurring singly or in pairs. Colonies are creamy white, 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, LB, R2A, TSA, KB, 2 % MH and Colby and Zathman medium. Does not grow in the presence of >4.0 % (w/v) NaCl. Growth occurs at 20–45 °C (optimal temperature 28 °C) and at pH 2.0–10.0 (optimal pH 7.0). Catalase, oxidase, pectinase and protease activities are positive. Cellulase, arginine dihydrolase, urease and β-galactosidase activities are absent. Nitrate reduction and hydrolysis of gelatin, glycerol tributyrates and aesculin are positive. Tests for glucose fermentation and indole production are negative. Methanol, trimethylamine and ethanol are utilized as sole carbon sources. Ammonium sulfate, potassium nitrate, sodium nitrate, ammonium chloride, L-alanine, L-glutamine, L-tryptophan, glycine, trimethylamine, 1-aminocyclopropane-1-carboxylate, potassium cyanate and potassium thiocyanate are utilized as sole nitrogen sources, but urea, methylamine, L-glutamate, diphenylamine and L-aspartic acid are not utilized as nitrogen sources. None of the compounds in Biolog tests are utilized as sole carbon and energy sources. The following carbon and nitrogen sources are assimilated in the API 20NE test: D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetylglucosamine, maltose, potassium gluconate, adipic acid, malic acid and trisodium citrate. Negative for capric acid and phenylacetic acid. The following carbon sources are assimilated in the API 32GN test: N-acetylglucosamine, D-ribose, inositol, sucrose, maltose, lactic acid, L-alanine, glycogen, D-mannitol, D-glucose, salicin, melibiose, L-rhamnose, D-sorbitol, L-arabinose, trisodium citrate, L-histidine and L-proline. Negative for itaconic acid, suberic acid, sodium malonate, sodium acetate, potassium 5-ketogluconate, 3-hydroxybenzoic acid, L-serine, L-fucose, propionic acid, capric acid, valeric acid, potassium 2-ketogluconate, 3-hydroxybutyric acid and 4-hydroxybenzoic acid. In API ZYM assays, alkaline phosphatase, esterase (C4), esterase lipase (C8), naphthol-AS-BI-phosphohydrolase and α-glucosidase are present, but lipase (C14), leucine arylamidase, trypsin, acid phosphatase, valine arylamidase, cystine arylamidase, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are absent. The cell wall contains meso-diaminopimelic acid as the diagnostic diamino acid. Predominant polar lipids are diphosphatidylglycerol, phosphatidylglycerol, a minor amount of phosphatidylcholine

and two unknown phospholipids. The major respiratory quinone is MK-7. Major cellular fatty acids are C_{15:0} anteiso, C_{15:0} iso, C_{16:0} iso and C_{17:0} anteiso.

The type strain, CBMB205^T (=KACC 13105^T=NCCB 100236), was isolated from the rhizoplane of rice (*Oryza sativa* L. cv O-dae). The sample was collected at reproductive stage (R9) and was obtained from the Chungbuk Provincial Agricultural Research and Extension Services, Cheongwon (36° 58' 0" N, 127° 57' 0" E), Chungbuk, Republic of Korea. The DNA G+C content of the type strain is 45.0 mol%.

Acknowledgements

M.M. is indebted to the BrainKorea21, Republic of Korea, for a position as an invited scientist. The author also acknowledges support from the Rural Development Administration, Republic of Korea.

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