A novel aerobic pink-pigmented facultative, 1-aminocyclopropane-1-carboxylate deaminase producing *Methylobacterium oryzae* sp. nov. isolated from rice

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Abstract
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.

Key Words
Methylobacterium, 1-aminocyclopropane-1-carboxylate, PPFM.

Introduction
The genus *Methylobacterium* includes a group of strictly aerobic, Gram-negative, pink-pigmented facultative methylotrophic (PPFM) bacteria characterized by their ability to utilize single carbon compounds like methanol, formaldehyde via the serine pathway (Green 1992). *Methylobacterium* is classified under the α2 subclass of *Proteobacteria* and presently consists of 22 species with validly published names. Possible mechanisms of plantgrowth promotion by *Methylobacterium* include production of phytohormones, such as indole-3-acetic acid (IAA), cytokinins or vitamins (Basile *et al.* 1985). Here we discuss the formal taxonomic description of a novel species of the genus *Methylobacterium*, Strain CBMB20, isolated from rice tissues with an ability to produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase.

Methods
Medium used for isolation
Strains CBMB20^T and CBMB110 were 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 mg/ml) 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.

Scanning electron microscope (SEM)
Scanning electron microscope (SEM) observations were performed on fixed material that was prepared for routine examinations as described by Bozzola and 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.

16S rRNA genes
16S rRNA genes were amplified using universal primers: fD1 and rP2 (Weisburg *et al.* 1991) and 16S rDNA sequencing was performed by big-dye primer method using an automated DNA sequencer (ABI Prism 310 Genetic Analyzer, Tokyo, Japan).

Nutritional features
Nutritional features were determined using the Biolog Microstation (MicroLog-3, 4.01B). The analysis was

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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
Carbon-source utilization tests (excluding biolog) were performed by using a standard protocol described by Green and Bousfield (1982).

Physiological and biochemical characteristics
Other physiological and biochemical characteristics were tested using the API ZYM and API 20NE galleries (bioMe´rieux) following the manufacturer's instructions. Cellular fatty acids were analysed in organisms grown on NA with 1% methanol (v/v) for 48 h.

Cellular fatty acids
The cellular fatty acids were derivatized to methylesters (Sasser 1990) and analyzed by a Gas Chromatograph (Hewlett Packard 6890) using Microbial Identification System (MIDI; Microbial ID) software package.

G+C content
The G+C content of genomic DNA was determined by HPLC analysis using a reverse-phase column (Supelcosil LC-18-S, Supelco) of individual nucleosides, resulting from DNA hydrolysis and dephosphorylation (Mesbah *et al*. 1989).

DNA-DNA hybridization
DNA-DNA hybridization was carried out following the filter hybridization method as described by Seldin and Dubnau (1985).

Results
Strains CBMB20^T and CBMB110 were strictly aerobic, Gram-negative, non-spore forming and forming pink to red-pigmented colonies. Cells were rod shaped, frequently branched and occurred singly or in rosettes on solid AMS and NA medium. Photomicrographs of strain CBMB20 ^{T} are shown in Figure 1.

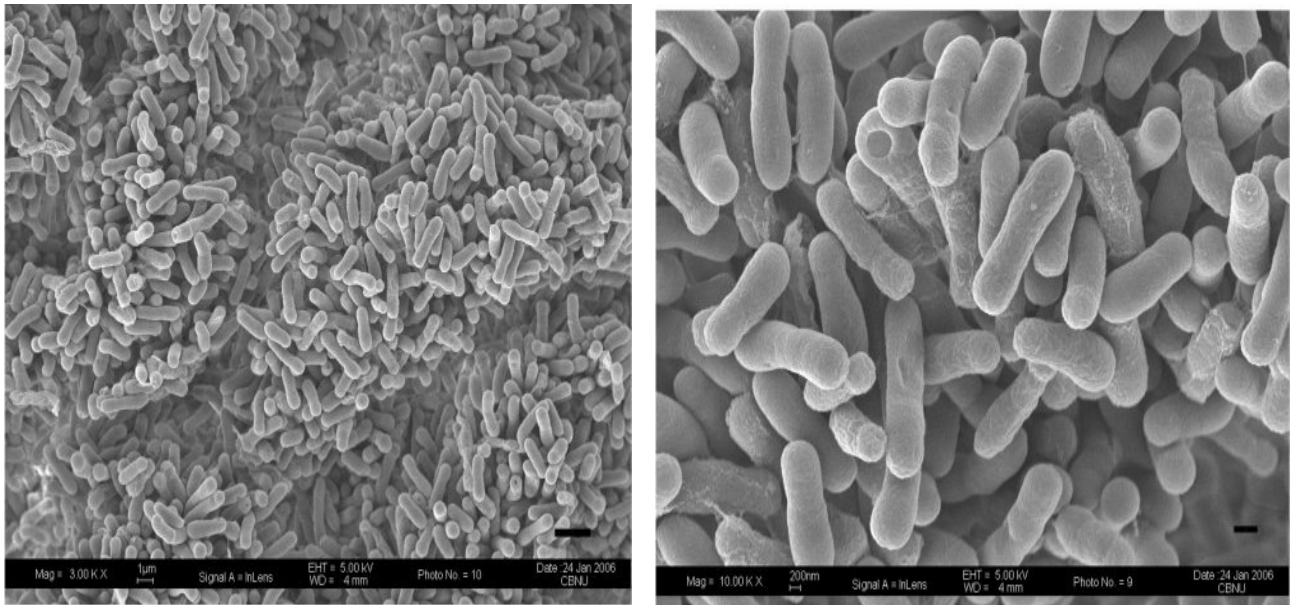

Figure 1. Scanning electron microscope (SEM) photomicrographs of *Methylobacterium oryzae* CBMB20 ^{T} on AMS medium supplemented with 0.5% methanol (v/v) (glutaraldehyde/osmium tetroxide fixation, gold/palladium coating: Hitachi S-2500C). Bar, 1µm and 200nm.

The strain CBMB20 utilized ACC as a nitrogen source and produced ACC deaminase and was phylogenetically related to members of the genus *Methylobacterium*.
The 16S rRNA gene sequence analysis indicated that the strain was most closely related to *Methylobacterium fujisawaense*, *Methylobacterium radiotolerans* and *Methylobacterium mesophilicum* (Figure 2).

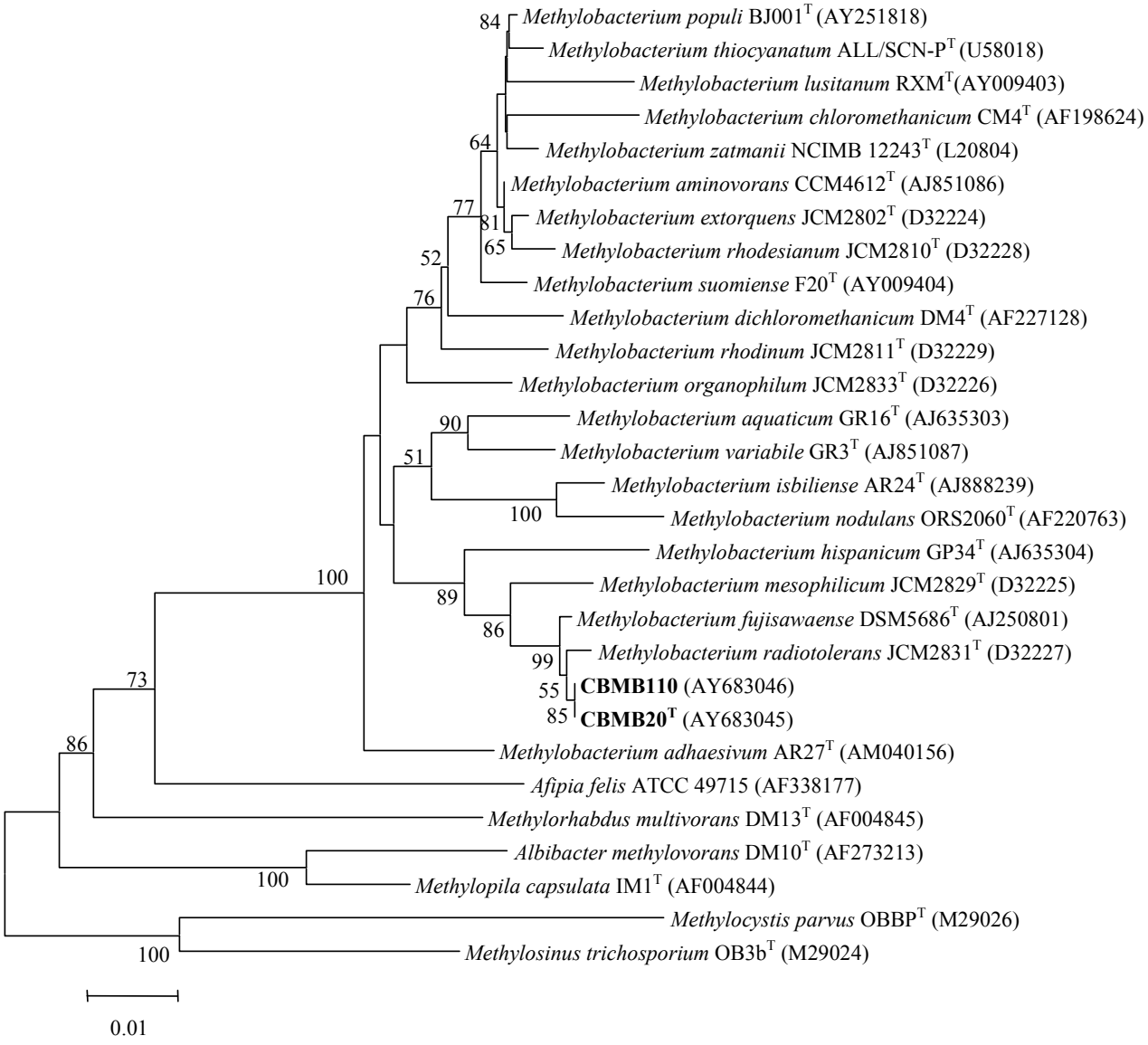

Figure 2. Phylogenetic tree based on 16S rRNA gene sequence comparison showing the position of the two strains (CBMB20T and CBMB110) and other related species of the genus *Methylobacterium*. The numbers at the nodes indicate the levels of the bootstrap support based on a neighbor-joining analysis of 1,000 resampled data sets. The bootstrap values below 50% were not indicated. Bar, 0.01 substitution per site.

However the DNA-DNA hybridization values were less than 70% with the type strains of these species (Table 1).

Table 1. DNA-DNA hybridization values between *Methylobacterium oryzae* CBMB20 T and its closest relatives.

Species	DNA-DNA hybridization (%)
CBMB20T	100.00
CBMB110	88.63
Methylobacterium fujisawaense KACC 10744T	42.09
Methylobacterium mesophilicum DSM 1708T	54.51
Methylobacterium radiotolerans DSM 1819T	63.09

The fatty acid profile of the strains CBMB20T and CBMB110 consisted mainly cis vaccenic acid (C18:1 w7c) and octadecanoate (stearic acid, C18 : 0) (Table 2).

Table 2. Cellular fatty acid compositions (as percentages of the total) of strains CBMB20^T and CBMB110 and related species of the genus *Methylobacterium*.
Summed feature 2 contained one or more of iso-6 $C_{16:1}$ I and/or $C_{14:0}$ 3-OH; Summed feature 3 contained one or more of $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH. 1, CBMB20^T; 2, CBMB110; 3, *M. fujisawaense* KACC10744^T; 4, *M. hispanicum* DSM 16372^T; 5, *M. mesophilicum* DSM 1708^T; 6, *M. organophilum* DSM 760^T; 7, *M. radiotolerans* DSM 1819^T; 8, *M. populi*; 9, *M. suomiense*; 10, *M. lusitanum*; 11, *M. goesingense*. 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 acids	1	2	3	4	5	6	7	8	9	10	11
$C_{9:0}$	ND	ND	ND	1.2	ND	ND	ND	ND	ND	ND	ND
$C_{12:0}$	ND	ND	ND	4.53	ND	ND	ND	ND	ND	ND	ND
$C_{14:0}$	ND	0.3	ND	2.35	ND	ND	ND	ND	ND	ND	ND
$C_{16:0}$	3.01	3.5	1.96	4.57	3.18	2.98	3.01	6.40	ND	ND	3.3
$C_{18:0}$	4.61	3.66	5.42	4.57	4.1	6.27	5.29	11.9	ND	ND	2.4
$C_{18:0}$ 3-OH	0.77	0.72	0.69	ND	1.52	0.49	0.64	ND	ND	ND	ND
$C_{18:1}$ ω7c	88.2	86.8	88.7	68.5	88.6	88.1	88.8	86.1	84.7	83.1	82.0
Summed Feature 2*	0.77	1.69	0.80	7.97	1.06	0.79	0.81	ND	ND	ND	ND
Summed Feature 3*	1.79	2.1	1.74	6.27	0.84	0.69	0.81	ND	ND	ND	ND
Summed Feature 4*	ND	0.81	ND	ND	ND	ND	ND	ND	ND	ND	ND
Unknown fatty acid 14.959 (ECL)	0.36	0.46	0.47	ND	0.45	0.46	0.50	ND	ND	ND	ND

*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-6 $C_{16:1}$ I and/or $C_{14:0}$ 3-OHSummed 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**.**

Conclusion

The 16S rRNA sequence similarity data, DNA-DNA hybridization values, and other phenotypic characteristics allowed the strains CBMB20^T and CBMB110 to separate from other members of the genus *Methylobacterium.* Strain CBMB20^T is proposed as the type strain of novel *Methylobacterium* species, for which the name *Methylobacterium oryzae* sp. nov is proposed.

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