Methylobacterium gossipiicola sp. nov., a pink-pigmented, facultatively methylotrophic bacterium isolated from the cotton phyllosphere

Munusamy Madhaiyan,^{1,2} Selvaraj Poonguzhali,¹
Murugaiyan Senthilkumar,¹ Jung-Sook Lee³ and Keun-Chul Lee³

Correspondence

Munusamy Madhaiyan
mmadhaiyan@hotmail.com

¹Department of Agricultural Microbiology, Tamilnadu Agricultural University, Coimbatore 641 003, Tamilnadu, India

²Temasek LifeSciences Laboratory, 1 Research Link, NUS, Singapore 117604

³Korean Collection for Type Cultures, Biological Resource Center, Korea Research Institute of Bioscience and Biotechnology, 111 Gwahangno, Yusong-gu, Daejeon 305-806, Republic of Korea

A pink, aerobic, facultatively methylotrophic, motile, Gram-negative rod, designated Gh-105^T, was isolated from the phyllosphere of cotton from Coimbatore (Tamilnadu, India). 16S rRNA gene sequence analysis showed clearly that the isolate belonged to the *Methylobacterium* cluster. Strain Gh-105^T was most closely related to *Methylobacterium adhaesivum* AR27^T (99% 16S rRNA gene sequence similarity) and *Methylobacterium iners* 5317S-33^T (97.5%). The isolate grew with C₁ compounds such as methanol and dichloromethane, but not with formaldehyde, formate, methylamine, trimethylamine or methane, as sole carbon sources and carried *mxoF*, which encodes methanol dehydrogenase and supports methylotrophic metabolism. The major fatty acid was C_{18:1ω7c} and the G + C content of the genomic DNA was 64.2 mol%. Physiological and biochemical data and DNA–DNA relatedness with *M. adhaesivum* KACC 12195^T and *M. iners* KACC 11765^T revealed clear phenotypic and genotypic differences. For this reason, we propose that strain Gh-105^T (=CCM 7572^T =NRRL B-51692^T) represents the type strain of a novel species, with the name *Methylobacterium gossipiicola* sp. nov.

The genus *Methylobacterium* is a group of strictly aerobic, Gram-negative, rod-shaped, pink-pigmented, facultatively methylotrophic bacteria that can grow on C₁ compounds such as methanol and other reduced C₁ compounds via the serine pathway (Green, 1992). Molecular taxonomy has classified the genus *Methylobacterium* as representing a line of descent in the α2 subgroup of the *Proteobacteria*. At the time of writing, the genus consisted of 34 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 ubiquitous and are found in soil, dust, fresh water and lake sediment as well as on solid surfaces. They are commonly found in association with plants, and it has been hypothesized that they dominate the phyllosphere bacterial population (Corpe & Rheem, 1989). Their association with plants varies from

strong (symbiotic) to loose (epiphytic) (Pirttilä *et al.*, 2000; Sy *et al.*, 2001; Kutschera, 2007; Schauer & Kutschera, 2008), a range that also includes intermediate associations (Lacava *et al.*, 2004), and their presence has also been detected using cultivation-independent methods (Araújo *et al.*, 2002; Idris *et al.*, 2004; Jackson *et al.*, 2006). Movements of methylobacteria within the plant as well as inter- and intracellular colonization of plant tissues are well documented (Pirttilä *et al.*, 2000; Sy *et al.*, 2005; Poonguzhali *et al.*, 2008). As phytosymbionts, methylobacteria utilize the methanol released from plants that is a by-product of cell-wall metabolism and emitted through stomatal pores (Nemecsek-Marshall *et al.*, 1995; Hüve *et al.*, 2007). In turn, methylobacteria impart a range of beneficial effects on plant growth and development through various mechanisms (Basile *et al.*, 1985; Koenig *et al.*, 2002; Trotsenko *et al.*, 2001; Holland & Polacco, 1992; Madhaiyan *et al.*, 2006, 2007a, b; Idris *et al.*, 2004). Recent studies have described the ubiquitous presence of methylobacteria on plant surfaces, including *Methylobacterium phyllosphaerae*, *M. mesophilicum* and *M. platani* from leaf phyllospheres (Green & Bousfield, 1983; Kang *et al.*, 2007; Madhaiyan *et al.*, 2009).

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and *mxoF* sequences of strain Gh-105^T are EU912445 and HQ588892, respectively.

Two supplementary figures and two supplementary tables are available with the online version of this paper.

Strain Gh-105^T was isolated by the leaf imprinting method (Chanprame *et al.*, 1996) from the phylloplane of cotton (*Gossipium hirsutum* L. 'MCU12') collected from Tamilnadu Agriculture University, Cotton Research Station, Coimbatore, Tamilnadu, India, on selective ammonium mineral salts (AMS) agar (Whittenbury *et al.*, 1970) supplemented with filter-sterilized 10 µg cycloheximide ml⁻¹ and 0.5 % (v/v) methanol at 28 °C. The isolate was preserved at -80 °C in nutrient broth (Difco) with 1 % methanol (v/v), in AMS broth with 0.5 % methanol and 50 % glycerol (v/v) or by lyophilization. Morphological and phenotypic characteristics were studied according to the standard protocols described by Gerhardt *et al.* (1994) and Green & Bousfield (1982). The Gram-stain reaction was determined using a Gram-staining kit (Difco), according to the manufacturer's instructions. Cell morphology was observed at ×1000 magnification with a light microscope (Nikon) using cells grown for 72 h at 30 °C on AMS agar. Catalase activity was determined by assessing bubble production with 3 % (v/v) H₂O₂ and oxidase activity was determined using 1 % (w/v) tetramethyl-*p*-phenylenediamine using BBL catalase and oxidase reagent droppers (Difco), according to the manufacturer's instructions. Motility was tested in 1/10 R2A broth (Difco) supplemented with 0.2 % agar. Indole production was tested on MIL medium (Difco), according to the manufacturer's instructions. Anaerobic growth was tested by incubating cultures on R2A agar in a GasPak jar (BBL) for 14 days; aerobically incubated plates were used as controls. Growth at 5–45 °C and pH 4–10 (at intervals of 1 pH unit) was monitored in R2A broth. Growth with 0, 2, 3, 5 and 7 % (w/v) NaCl was tested in R2A broth. Other physiological and biochemical characteristics were tested using the API ZYM, API 20 NE and API 32 GN galleries (bioMérieux), according to the manufacturer's instructions. Nutritional characteristics were determined using GN2 MicroPlates (Biolog), as described previously (Madhaiyan *et al.*, 2007c). Utilization of other carbon sources not included in the MicroPlates was determined as described by Green & Bousfield (1982). Methanol, dichloromethane, methylamine, dimethylamine, trimethylamine and formaldehyde were added to minimal medium to concentrations of 0.2 and 0.5 % (v/v) and results were recorded after incubation for 14 days at 30 °C (Doronina *et al.*, 2000; Green & Bousfield, 1982). Scanning electron microscopy was performed on 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 using a Hitachi S-2500C microscope with a GEMINI column equipped with a field-emission electron source. 1-Aminocyclopropane-1-carboxylate deaminase activity was determined by plate and quantitative assays, as described previously (Madhaiyan *et al.*, 2006).

Cells of strain Gh-105^T were Gram-negative, aerobic, non-endospore-forming motile rods, were frequently branched and occurred singly or in rosettes after growth on AMS agar (Supplementary Fig. S1, available in IJSEM Online). Colonies were light pink. Strain Gh-105^T grew on NA,

R2A, PYG, succinate, glycerol peptone and plate-count agar media and did not grow on MacConkey agar. Strain Gh-105^T utilized C₁ substrates such as methanol and dichloromethane, but did not utilize methylamine, dimethylamine, trimethylamine or formaldehyde. Characteristics that differentiate strain Gh-105^T from its closest relatives are summarized in Table 1 and Supplementary Table S1.

The 16S rRNA gene sequence was determined as described by Madhaiyan *et al.* (2009). Multiple sequence alignment and data analysis were performed using MEGA version 4 (Tamura *et al.*, 2007). Genetic distance calculations used the distance options according to the Kimura-2 model. Clustering was performed using the neighbour-joining (Fig. 1) and maximum-parsimony (not shown) methods and bootstrap values were calculated on the basis of 1000 replications. Pairwise comparisons were performed using the EzTaxon program (Chun *et al.*, 2007). The 16S rRNA gene sequence of strain Gh-105^T obtained in this study was a continuous stretch of 1351 bp. Strain Gh-105^T showed 99 % 16S rRNA gene sequence similarity with *Methylobacterium adhaesivum* AR27^T and 97.5 % with *Methylobacterium iners* 5317S-33^T. Sequence similarities with all other members of the genus *Methylobacterium* were <97.0 %. In the neighbour-joining phylogenetic tree (Fig. 1), strain Gh-105^T clustered with *M. adhaesivum* AR27^T and *M. iners* 5317S-33^T but formed a separate line of descent within this cluster.

The methanol dehydrogenase gene (*mxhF*), which is required for methanol utilization, was amplified from extracted DNA using the primer pair *mxhF* f1003 (5'-GCGGCACCAACT-GGGGCTGGT-3') and *mxhF* r1561 (5'-GGGCAGCATG-AAGGGCTCCC-3') (McDonald & Murrell, 1997). The *mxhF* sequence from strain Gh-105^T obtained in this study was a continuous stretch of 541 bp. Strain Gh-105^T showed 98 % *mxhF* sequence similarity with *M. adhaesivum* AR27^T and *M. iners* KACC 11765^T. In the neighbour-joining phylogenetic tree based on *mxhF* sequences (Supplementary Fig. S2), strain Gh-105^T formed a separate line in the cluster containing *M. adhaesivum* AR27^T.

Cellular fatty acids of cells grown on NA with 1 % methanol (v/v) at 28 °C for 48 h were analysed according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990) with the TSBA6 database and Sherlock version 6.0 (Microbial ID). The G+C content of genomic DNA was determined by reversed-phase HPLC (Supelcosil LC-18 S; Supelco) of individual nucleosides, as described by Mesbah *et al.* (1989). DNA-DNA hybridization was carried out as described by Seldin & Dubnau (1985). Probe labelling was conducted using the non-radioactive DIG High Prime DNA Labelling and Detection Starter Kit II (Roche Diagnostics). Hybridized DNA was visualized using the DIG Luminescent Detection Kit (Roche Molecular Biochemicals) and DNA-DNA relatedness was quantified using a densitometer (Bio-Rad Laboratories). Cell biomass for quinone analysis was obtained from cultivation in R2A agar at 28 °C for 10 days. The isoprenoid quinone was

Table 1. Differential characteristics of strain Gh-105^T and some members of the genus *Methylobacterium*

Strains: 1, *Methylobacterium gossipiicola* sp. nov. Gh-105^T; 2, *M. adhaesivum* KACC 12195^T; 3, *M. mesophilicum* DSM 1708^T; 4, *M. phyllosphaerae* CBMB27^T; 5, *M. oryzae* CBMB20^T; 6, *M. fujiisawaense* KACC 10744^T; 7, *M. organophilum* DSM 760^T; 8, *M. radiotolerans* DSM 1819^T; 9, *M. extorquens* DSM 1337^T; 10, *M. hispanicum* DSM 16372^T; 11, *M. iners* KACC 11765^T (data from Weon *et al.*, 2008); 12, *M. aerolatum* KACC 11766^T (Weon *et al.*, 2008); 13, *M. platani* KCTC 12901^T (Kang *et al.*, 2007). Data were obtained in this study unless indicated. All strains grow on methanol and have singly occurring cells. +, Positive; w, weakly positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
Colony diameter (mm)	0.4–1.3	0.5–0.8	0.4–1.4	0.2–0.8	0.8–1.7	0.8–1.2	1.0–1.6	0.7–1.5	0.6–1.7	1.0–2.0	0.4–0.7	0.5–0.8	0.5–1.8
Colony pigmentation*	LP	P	WP	P to R	P to R	P	LP	P to R	P	LP	P	P	P
Cell characteristics													
Pairs	+	Rare	+	+	+	Rare	Rare	Rare	+	+	Rare	Rare	+
Rosettes	+	+	+	+	+	+	–	+	–	+	–	–	+
Length (µm)	1.4–2.2	1.5–5	2.3–4.4	1.8–2.7	2.1–2.8	ND	ND	1.4–2.5	2–2.5	2–2.5	1.5–2.5	1.5–3	2.0–6.5
Width (µm)	0.4–0.5	1.0–1.2	1–2.2	0.6	0.6–0.8	ND	ND	0.6–0.8	1–1.5	1–1.5	1.0	1.0	1.4–1.5
Conditions for growth													
Maximum NaCl (%)	1.5	1	1	1	1.5	1	1.5	<2	<2	<1	<2	<2	1
Optimum temperature (°C)	28	28	30	28	28	28	25–30	20–32	30	28	25–28	28	25–30
pH range	5–8	5–9	6–8	5–9	5–10	6–8	5–8	6–8	5–9	5–8	6–8	5–9	6–8
Nitrate reduction	–	–	–	–	+	–	–	+	–	–	–	–	+
Oxidase	+	+ / w	+ / w	+	+	–	+ / w	+	+	+	+	+	+
Urease	+	+	–	+	–	–	–	–	+	+	–	+	+
Gelatin hydrolysis	–	–	–	–	–	–	–	–	–	–	+	–	–
Utilization of:													
L-Arabinose	+	–	+	+	+	+	–	+	–	–	–	w	+
L-Glutamate	+	+	+	+	+	+	+	+	+	+	+	–	–
Citrate	+	+	+	+	–	+	–	+	–	+	–	–	–
D-Fucose	–	–	+	+	–	+	–	+	–	–	–	–	–
D-Fructose	w	+	–	+	–	–	+	–	–	–	–	–	+
D-Glucose	+	–	+	w	–	+	–	+	–	–	–	w	–
Acetate	+	+	–	w	–	+	+	+	+	+	+	–	+
L-Aspartate	–	–	+	+	–	+	+	–	–	+	+	–	+
DNA G + C content (mol%)†	64.2	63.6	69.5	66.8	70.6	69.9	70.5	71.2	68.2	67.7	68.0	73.2	68.5

*LP, Light pink; P, pink; R, red; WP, whitish pink.

†Data for reference strains from Madhaiyan *et al.* (2007c, 2009), Gallego *et al.* (2006) and Urakami *et al.* (1993).

investigated using reversed-phase HPLC, as described by Komagata & Suzuki (1987). The fatty acids of strain Gh-105^T consisted mainly of C_{18:1ω7c}, summed feature 3 (comprising C_{16:1ω7c} and/or C_{16:1ω6c}) and C_{16:0} (Supplementary Table S2). The DNA G + C content of strain Gh-105^T was 64.2 mol%, which falls within the range described for the genus *Methylobacterium* (Green, 1992) (Table 1). Strain Gh-105^T showed low DNA–DNA relatedness with *M. adhaesivum* KACC 12195^T (52.1 ± 3.1 %) and *M. iners* KACC 11765^T (15.1 ± 1.9 %). When the recommendation of a threshold value of 70 % DNA–DNA relatedness for the definition of species is considered (Wayne *et al.*, 1987), this result indicates that strain Gh-105^T does not belong to *M. adhaesivum* or *M. iners*.

16S rRNA gene sequence similarity, DNA–DNA relatedness and phenotypic characteristics allowed the separation of strain Gh-105^T from other members of the genus

Methylobacterium. Therefore, strain Gh-105^T represents a novel species of *Methylobacterium*, for which the name *Methylobacterium gossipiicola* sp. nov. is proposed.

Description of *Methylobacterium gossipiicola* sp. nov.

Methylobacterium gossipiicola (gos.si.pi.i'co.la. N.L. n. *Gossipium* a scientific botanical genus name; L. suff. *-cola* inhabitant, dweller; N.L. n. *gossipiicola* *Gossipium* dweller, referring to the isolation of the type strain from the cotton phyllosphere).

Gram-negative, non-endospore-forming, strictly aerobic, motile rods (0.4–0.5 × 1.4–2.2 µm), occurring singly, in pairs or in rosettes. On AMS agar at 28 °C, colonies are light pink, convex, translucent with regular edges and slow growing (0.4–1.3 mm in diameter after 96 h). Grows on NA, R2A, PYG, succinate, glycerol peptone and plate-count

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain Gh-105^T in the genus *Methylobacterium*. Bootstrap values (>70%) based on 1000 replications are shown at branch nodes. Bar, 0.01 % nucleotide sequence divergence.

agar media. Does not grow in the presence of $\geq 2\%$ NaCl. Grows at 20–30 °C (optimum 28 °C) and at pH 5.0–9.0 (optimum pH 6.8). Catalase, oxidase, arginine dihydrolase and urease are present, but pectinase, cellulase, protease and β -galactosidase are absent. 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 produced. Simmons' citrate test is positive. As sole nitrogen sources, ammonium sulfate, potassium nitrate, sodium nitrate, ammonium chloride, L-alanine, L-tryptophan, urea, L-aminocyclopropane 1-carboxylate and potassium thiocyanate are utilized, but methylamine, trimethylamine, glycine, potassium cyanate, diphenylamine and diethylamine are not utilized. As sole carbon and energy sources (MicroPlate), dextrin, glycogen, L-arabinose, cellobiose, D-fructose, D-galactose, α -D-glucose, lactulose, D-mannitol, D-mannose, D-psicose, D-sorbitol, sucrose, trehalose, turanose, pyruvic acid methyl ester, succinic acid monomethyl ester, acetic acid, *cis*-aconitic acid, citric acid, formic acid, D-gluconic acid, D-glucuronic acid, α -, β - and γ -hydroxybutyric acids, α -ketobutyric acid, α -ketoglutaric acid, α -ketovaleric acid, DL-lactic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, L-alanine, L-alanyl glycine, L-glutamic acid, glycyl L-aspartic acid, L-leucine, L-serine,

L-threonine, DL-carnitine, γ -aminobutyric acid, glycerol, DL- α -glycerol phosphate, α -D-glucose 1-phosphate and D-glucose 6-phosphate are utilized. With API ZYM, esterase (C4), esterase lipase (C8), leucine arylamidase, trypsin, alkaline phosphatase and naphthol-AS-BI-phosphohydrolase are present, but acid phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, α -chymotrypsin, α - and β -galactosidases, β -glucuronidase, α - and β -glucosidases, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase are absent. The major isoprenoid quinone is Q-10. The major fatty acids are C_{18:1} ω 7c and summed feature 3 (comprising C_{16:1} ω 7c and/or C_{16:1} ω 6c). The DNA G+C content of the type strain is 64.2 mol%.

The type strain, Gh-105^T (=CCM 7572^T =NRRL B-51692^T), was isolated from the leaf surface of cotton (*Gossypium hirsutum* L. 'MCU12') collected from Tamilnadu Agriculture University, Cotton Research Station (Coimbatore, Tamilnadu, India).

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