Methylobacterium variabile sp. nov., a methylotrophic bacterium isolated from an aquatic environment

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Strain GR3^T was isolated from drinking water during a screening programme to monitor the bacterial population present in the distribution system of Seville (Spain), and it was studied phenotypically, genotypically and phylogenetically. This pink-pigmented bacterium was identified as a *Methylobacterium* sp. Members of this genus are distributed in a wide variety of natural habitats, including soil, dust, air, freshwater and aquatic sediments. Phylogenetic analysis of the 16S rRNA gene sequence showed that strain GR3^T was closely related to *Methylobacterium aquaticum* (97.4 % sequence similarity), whereas sequence similarity values with respect to the rest of the species belonging to this genus were lower than 96 %. Furthermore, the DNA–DNA hybridization data and its phenotypic characteristics clearly indicate that the isolate represents a novel *Methylobacterium* species, for which the name *Methylobacterium variabile* sp. nov. is proposed. GR3^T (= DSM 16961^T = CCM 7281^T = CECT 7045^T) is the type strain; the DNA G + C content of this strain is 69.2 mol%.

The culturable and non-culturable bacterial populations from the tap water of four different parts of Seville (Spain) have been studied in order to monitor the microbiological water quality. Four sampling campaigns during a period of 1 year were completed, one each season. A group of slow-growing, pink-pigmented bacteria was isolated and identified as a *Methylobacterium* sp.

The genus *Methylobacterium* was created to include a group of strictly aerobic, Gram-negative, rod-shaped, pink-pigmented, facultatively methylotrophic (PPFM) bacteria that can grow on one-carbon compounds such as formate, formaldehyde and methanol as the sole source of carbon and energy, as well as on a wide range of multi-carbon growth substrates (Green, 1999). The genus *Methylobacterium* belongs to the α -Proteobacteria and has the serine pathway for formaldehyde assimilation. This genus now consists of 18 species: *Methylobacterium aminovorans* (Urakami *et al.*, 1993), *M. aquaticum* (Gallego *et al.*, 2005), *M. chloromethanicum* (McDonald *et al.*, 2001), *M. dichloromethanicum* (Doronina *et al.*, 2000), *M. extorquens* (Bousfield & Green, 1985), *M. fujisawaense* (Green *et al.*, 1988), *M. hispanicum* (Gallego *et al.*, 2005), *M. lusitanum*

(Doronina *et al.*, 2002), *M. mesophilicum* (Green & Bousfield, 1983), *M. nodulans* (Jourand *et al.*, 2004), *M. organophilum* (Patt *et al.*, 1976), *M. populi* (Van Aken *et al.*, 2004), *M. radiotolerans* (Green & Bousfield, 1983), *M. rhodesianum* (Green *et al.*, 1988), *M. rhodinum* (Green & Bousfield, 1983), *M. suomiense* (Doronina *et al.*, 2002), *M. thiocyanatum* (Wood *et al.*, 1998) and *M. zatmanii* (Green *et al.*, 1988).

The type species of the genus *Methylobacterium* is *M. organophilum*, which was the only PPFM bacterium reported to be capable of growth on methane until the description of a new methane-utilizing species, *M. populi*. Since *M. organophilum* has lost this ability, and neither the key enzyme of methanotrophic metabolism nor the genes encoding different forms of methane monooxygenase (MMO) have ever been detected in the PPFM bacteria, the methanotrophic ability of *M. populi* must be treated with considerable scepticism (Dedysh *et al.*, 2004).

Members of the genus *Methylobacterium* are ubiquitous in nature and are thus found in a variety of habitats (Green & Bousfield, 1981, 1983), including soil, dust, freshwater and lake sediments, leaf surfaces and root nodules, rice grains, air, hospital environments and as contaminants in various products and processes. Species of *Methylobacterium* have been reported to exhibit resistance to chlorination (Hiraishi *et al.*, 1995) and their presence in drinking water distribution systems is justified.

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Abbreviation: PPFM, pink-pigmented, facultatively methylotrophic.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain GR3^T is AJ851087.

Drinking water samples were concentrated by using a tangential flow filtration system (Filtron®) and plated on Plate Count Agar (Difco) and R2A agar (Difco). Plates were incubated at 28 °C for 7 days and different morphological colonies were plated in order to obtain pure cultures. We obtained 115 isolates, 32 of which were pink-pigmented. One of these pink-pigmented pure cultures was strain GR3^T, which was phenotypically characterized by using the methods described by Doronina *et al.* (1998). The nutritional features were determined as described by Gallego *et al.* (2005) by using Biolog Microplates (Biolog).

Chromosomal DNA was isolated and purified according to the methods described by Wilson (1987) and Marmur (1961) and partially modified by Hood *et al.* (1987). The 16S rRNA gene was amplified by using the universal primers 16F27 and 16R1488 as described by Mellado *et al.* (1995). Sequencing was performed by NBT-Newbiotechnic using an automated DNA sequencer (model 3100; Applied Biosystems) and an almost-complete nucleotide sequence was determined. Alignment of the 16S rRNA gene sequence was carried out with the ARB software program (Ludwig & Strunk, 1996). Phylogenetic trees were inferred by using three tree-making algorithms – maximum-parsimony, neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood. The G + C content of genomic DNA was determined by the method of Marmur & Doty (1962) and by using the equation of Owen & Hill (1979). DNA–DNA

hybridization was carried out following the competition procedure of Johnson (1994), which is described in detail in Mormile *et al.* (1999). Hybridization temperatures were 60 and 61 °C, which are within the limit of validity for the filter method (De Ley & Tijtgaat, 1970), and the percentage of hybridization was calculated according to Johnson (1994). DNA relatedness values are the mean of three values.

Strain GR3^T is a Gram-negative, strictly aerobic rod that measures 1.0–1.5 µm in width by 2.0–6.0 µm in length when grown for 24 h at 28 °C. Cells are motile. Colonies of strain GR3^T are circular to slightly irregular in shape, pink in colour and 2–7 mm in diameter on R2A agar (after 7 days or more); sometimes colonies can have different pink pigmentation. Strain GR3^T is a slow-growing organism; no growth occurs in the presence of 1 % NaCl. Differential phenotypic characteristics of strain GR3^T are summarized in Table 1.

The almost-complete 16S rRNA gene sequence (approx. 1400 nt) of strain GR3^T was determined directly following PCR amplification. In addition, we determined the 16S rRNA gene sequence of *M. aminovorans* CCM 4612^T, the only species of the genus *Methylobacterium* for which the 16S rRNA gene sequence was not available, in order to include it in the phylogenetic analysis. Strain GR3^T exhibited 97.4 % 16S rRNA gene sequence similarity with

Table 1. Differential phenotypic characteristics of *M. variable* strain GR3^T and other related species of the genus *Methylobacterium*

Bacteria are identified as: 1, *M. variable* GR3^T; 2, *M. hispanicum* CCM 7219^T (Gallego *et al.*, 2005); 3, *M. aquaticum* CCM 7218^T (Gallego *et al.*, 2005); 4, *M. aminovorans* JCM 8240^T (Urakami *et al.*, 1993); 5, *M. suomiense* NCIMB 13778^T (Doronina *et al.*, 2002); 6, *M. lusitanum* NCIMB 13779^T (Doronina *et al.*, 2002); 7, *M. thiocyanatum* NCIMB 13651^T (Wood *et al.*, 1998); 8, *M. chloromethanicum* NCIMB 13688^T (McDonald *et al.*, 2001); 9, *M. populi* NCIMB 13946^T (Van Aken *et al.*, 2004). Symbols: ND, not determined; W, weak; V, variable; +, positive; –, negative.

Characteristics	1	2	3	4	5	6	7	8	9
Cells occur:									
Singly	+	+	+	+	+	+	ND	ND	+
In pairs	+	Rarely	+	Rarely	Rarely	Rarely	ND	ND	+
In rosettes	+	–	+	–	–	–	ND	ND	+
Cell length (µm)	2–6	2–2.5	4.5–8	1.5–4	1.2–2.5	1.7–2	2	2.5–3.5	1–10
Cell width (µm)	1–1.5	1–1.5	1.5–1.7	0.8–1	0.8–1	1–1.2	0.8	0.8–1	0.8–1
Pigmentation	Pink (V)	Light pink	Pink to red	Pink or red	Pink	Pink	Pink	Pink	Pink to red
Diameter of colonies (mm)	2–7	1–2	1–2	1–3	0.5–1	1	ND	ND	0.1–0.2
Oxidase	–	+(W)	–	+	+(W)	+	V	ND	+
Hydrolysis of starch	+(W)	+(W)	+	–	–	–	ND	ND	ND
Upper limit for growth on NaCl (%)	1	1	1	3	3	3	ND	ND	2
Growth on:									
Citrate	+	+	+	–	–	–	+	+	–
L-Glutamate	+	+	+	+	–	–	+	+	–
D-Glucose	+	–	+	–	+	–	+	–	–
D-Xylose	–	–	–	–	–	–	+	–	–
Fructose	+	+	+	+	+	+	+	–	+

Fig. 1. Phylogenetic tree showing the relationships of strain GR3^T, species belonging to the genus *Methylobacterium* and related methylotrophic bacteria. The tree was constructed by using the maximum-parsimony method and was based on a comparison of 16S rRNA gene sequences of approximately 1400 nt in length. Bar, 2 % sequence divergence.

respect to *M. aquaticum* and values lower than 95.6 % with respect the rest of the species of the genus *Methylobacterium* (Fig. 1). The DNA G+C content of strain GR3^T was 69.2 mol%, which is within the range described for the genus *Methylobacterium* (Green, 1999). This value is different to that of *M. aquaticum* (67.7 mol%) (Gallego *et al.*, 2005). Furthermore, to determine the genotypic relatedness between strain GR3^T and *M. aquaticum* CCM 7218^T, studies of DNA–DNA hybridization were performed. DNA–DNA hybridization values obtained were

30 and 37 %, indicating that the new isolate is genotypically different from the type strain of the *M. aquaticum*. Low DNA–DNA hybridization values (≤ 15 %) were also obtained between strain GR3^T and other related *Methylobacterium* species (Table 2).

On the basis of the data presented above, strain GR3^T represents a novel species of the genus *Methylobacterium*, for which we propose the name *Methylobacterium variabile* sp. nov.

Table 2. Levels of DNA–DNA hybridization between *M. variabile* strain GR3^T and *M. aquaticum* CCM 7218^T and other related *Methylobacterium* species

Source of unlabelled DNA	Relatedness (%) with ³ H-labelled DNA from strain:	
	GR3 ^T	CCM 7218 ^T
<i>M. variabile</i> GR3 ^T	100	37
<i>M. aquaticum</i> CCM 7218 ^T	30	100
<i>M. aminovorans</i> CCM 4612 ^T	4	18
<i>M. extorquens</i> NCIMB 9399 ^T	15	18
<i>M. radiotolerans</i> CCM 4464 ^T	5	25
<i>M. nodulans</i> LMG 21967 ^T	13	ND

ND, Not determined.

Description of *Methylobacterium variabile* sp. nov.

Methylobacterium variabile (L. neut. adj. *variabile* variable).

Gram-negative rods, 1.0–1.5 µm × 2.0–6.0 µm, occurring singly in pairs or in rosettes. Cells are motile, non-spore-forming and strictly aerobic. Colonies are pink, circular to slightly irregular and 2–7 mm in diameter after 7 days at 28 °C on R2A agar; sometimes colonies have different pink pigmentations. Slow-growing; does not grow in the presence of 1.0% NaCl or higher. Growth occurs at 20–30 °C (optimal temperature 28 °C) and at pH 5.0–8.0 (optimal pH 6.0). Catalase- and urease-positive. Oxidase-negative. Indole, methyl red and Voges–Proskauer are negative. Starch is weakly hydrolysed. Tween 80 is hydrolysed. Gelatin, casein, aesculin and DNA are not hydrolysed. Hydrogen sulfide is not produced. Simmons' citrate test is positive. Nitrate is reduced to nitrite. Acid is produced oxidatively from D-arabinose, but not from D-glucose, D-galactose, D-mannose or maltose. Methanol, formate and formaldehyde are utilized as sole carbon sources. Ammonium sulfate, nitrate, aspartate and glutamate are utilized as sole nitrogen sources. The following compounds are utilized as sole carbon and energy sources (Biolog): D-fructose, L-fucose, D-galactose, D-gluconic acid, α-D-glucose, acetic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, α-ketoglutaric acid, L-lactic acid, L-malic acid, mono-methyl succinate, propionic acid, pyruvic acid, succinamic acid, succinic acid, L-asparagine and L-glutamic acid. The following compounds are not utilized as sole carbon and energy sources (Biolog): Tween 40, Tween 80, α-cyclodextrin, β-cyclodextrin, dextrin, glycogen, inulin, mannan, N-acetyl-D-glucosamine, N-acetyl-D-mannosamine, amygdalin, L-arabinose, D-arabitol, arbutin, cellobiose, D-galacturonic acid, gentibiose, m-inositol, α-D-lactose, lactulose, maltose, maltotriose, D-mannitol, D-mannose, D-melezitose, D-melibiose, methyl α-D-galactoside, 3-methyl glucose, methyl α-D-glucoside, methyl β-D-glucoside, methyl α-D-mannoside, palatinose, D-psicose, D-raffinose, L-rhamnose, D-ribose, salicin, sedoheptulosan, D-sorbitol, stachyose, sucrose, D-tagatose, D-trehalose, turanose, xylitol, D-xylose, p-hydroxyphenyl acetic acid, α-ketovaleric acid, lactamide, D-lactic acid methyl ester, D-malic acid, methyl pyruvate, alaninamide, N-acetyl-L-glutamic acid, D-alanine, L-alanine, glycyl-L-glutamic acid, L-alanyl-glycine, L-pyroglutamic acid, L-serine, putrescine, 2,3-butanediol, glycerol, adenosine, 2'-deoxyadenosine, inosine, thymidine, uridine, adenosine 5'-monophosphate, thymidine 5'-monophosphate, uridine 5'-monophosphate, fructose 6-phosphate, glucose 1-phosphate, glucose 6-phosphate and DL-α-glycerol phosphate.

The type strain is GR3^T (=DSM 16961^T=CCM 7281^T=CECT 7045^T), which was isolated from drinking water. The DNA G+C content of the type strain is 69.2 mol%.

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