Methylobacterium hispanicum sp. nov. and *Methylobacterium aquaticum* sp. nov., isolated from drinking water

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Members of the genus *Methylobacterium* are ubiquitous in nature and can be isolated from almost any freshwater environment where dissolved oxygen exists. This genus is composed of a variety of pink-pigmented, facultatively methylotrophic (PPFM) bacteria. During a screening programme to monitor the bacterial population present in the drinking water of a municipal water supply in Seville (Spain) during the year 2003, five strains of PPFM bacteria were isolated and characterized. Analysis of their complete 16S rRNA gene sequences revealed that they constituted two separate phylogenetic groups (strains GP34^T and GR18, and strains GR16^T, GP22 and GP32, respectively) showing highest similarity to members of the genus *Methylobacterium*. The highest 16S rRNA sequence similarities of strain GP34^T were found with respect to the type strains of *Methylobacterium radiotolerans* (96·6 %) and *Methylobacterium fujisawaense* (96·4 %) and the highest 16S rRNA sequence similarities of strain GR16^T were to the type strains of *Methylobacterium extorquens* (96·0 %) and *Methylobacterium rhodesianum* (95·8 %). The G+C content of their DNA ranged from 66·5 to 67·8 mol%. DNA–DNA hybridization studies confirmed that they constituted two separate genospecies. On the basis of this phenotypic, phylogenetic and genotypic study, two novel species of the genus *Methylobacterium* are proposed: *Methylobacterium hispanicum* sp. nov., with type strain GP34^T (CECT 5997^T=CCM 7219^T=DSM 16372^T=CIP 108332^T), and *Methylobacterium aquaticum* sp. nov., with type strain GR16^T (CECT 5998^T=CCM 7218^T=DSM 16371^T=CIP 108333^T).

The genus *Methylobacterium* includes a group of pink-pigmented facultatively methylotrophic bacteria with the ability to grow on one-carbon compounds such as formate, formaldehyde and methanol as sole carbon and energy sources, as well as on a wide range of multi-carbon growth substrates (Green, 1992). This genus comprises 15 species with the type species *Methylobacterium organophilum*, which is not able to grow on methane. A recently described species isolated from poplar tissues, *Methylobacterium populi* (Van Aken *et al.*, 2004), is able to utilize methane as the sole source of carbon and energy. Methylotrophic bacteria that do not utilize methane are classified into two groups that use either the ribulose monophosphate or serine pathways for formaldehyde assimilation (Anthony, 1982). Members of the genus *Methylobacterium* that utilize methanol have the serine pathway for formaldehyde assimilation. 16S rRNA gene sequence analyses have shown that the genus *Methylobacterium* belongs to the α-subclass of the *Proteobacteria*, whereas ribulose monophosphate methylotrophs belong to the β-subclass (Bratina *et al.*, 1992; Brusseau *et al.*, 1994; Tsuji *et al.*, 1990).

The 15 species that belong to the genus *Methylobacterium* are *Methylobacterium aminovorans* (Urakami *et al.*, 1993), *Methylobacterium chloromethanicum* (McDonald *et al.*, 2001), *Methylobacterium dichloromethanicum* (Doronina *et al.*, 2000), *Methylobacterium extorquens* (Bousfield & Green, 1985), *Methylobacterium fujisawaense* (Green *et al.*, 1988), *Methylobacterium lusitanum* (Doronina *et al.*, 2002), *Methylobacterium mesophilicum* (Green & Bousfield, 1983), *M. organophilum* (Patt *et al.*, 1976), *M. populi* (Van Aken *et al.*, 2004), *Methylobacterium radiotolerans* (Green & Bousfield, 1983), *Methylobacterium rhodesianum* (Green *et al.*, 1988), *Methylobacterium rhodinum* (Green & Bousfield, 1983), *Methylobacterium suomiense* (Doronina *et al.*, 2002), *Methylobacterium thiocyanatum* (Wood *et al.*, 1998) and *Methylobacterium zatmanii* (Green *et al.*, 1988). In addition, the novel species *Methylobacterium nodulans* has recently been described (Jourand *et al.*, 2004). Members

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains GP34^T, GR18, GR16^T, GP22 and GP32 are AJ635304, AJ785570, AJ635303, AJ785571 and AJ785572, respectively.

of the genus *Methylobacterium* are distributed among a wide variety of natural habitats, including soil, dust, air, freshwater and aquatic sediments. These bacteria also occur in man-made environments, including potable water supplies, air-conditioning systems and masonry bathrooms and washstands, where they sometimes produce pink ropy masses of growth (Hiraishi *et al.*, 1995; Trotsenko *et al.*, 2001; Ultee *et al.*, 2004). Some species have been described as opportunistic human pathogens (Truant *et al.*, 1998; Hornei *et al.*, 1999). On the other hand, methylotrophic bacteria are frequently associated with terrestrial and aquatic plants, colonizing roots and leaf surfaces (Austin *et al.*, 1978; Yoshimura, 1982; Corpe & Rheem, 1989; Trotsenko *et al.*, 2001; Lidstrom & Chistoserdova, 2002). It is important to note that most of the *Methylobacterium* strains isolated from aquatic environments are highly resistant to chlorine (Hiraishi *et al.*, 1995). The chlorine tolerance of methylotrophic bacteria may explain why these organisms frequently occur in human-related environments.

Recent studies focusing on determination of the bacterial population in the drinking water of a municipal water supply in Seville (Spain) during 2003 permitted us to isolate a large number of bacteria. In the present paper, we have described the features of five novel isolates and shown that they constitute two novel species of the genus *Methylobacterium*, for which we propose the names *Methylobacterium hispanicum* sp. nov. and *Methylobacterium aquaticum* sp. nov.

The five strains, designated GR16^T and GR18 (isolated from culture medium R2A; Difco), and GP22, GP32 and GP34^T [isolated from plate count agar (PCA); Difco], were studied in detail. They were Gram-negative rods, strictly aerobic and motile (Table 1), occurring singly, in pairs or in rosettes (Fig. 1). Isolates were routinely maintained on PCA.

These bacteria, isolated from chlorinated water, were relatively slow growing and required between 5 and 7 days at 28 °C to form detectable colonies. The same growth characteristics were found in the chlorine-resistant strains isolated by Hiraishi *et al.* (1995). The methods used for phenotypic characterization have been described previously in detail (Doronina *et al.*, 1998). Because these bacteria are slow growing, identification by physiological testing is laborious. Moreover, the different species of this genus share a great number of phenotypic characteristics (Doronina *et al.*, 2002; McDonald *et al.*, 2001; Urakami *et al.*, 1993; Wood *et al.*, 1998; Van Aken *et al.*, 2004) and high chemotaxonomic homogeneity is also observed in the genus.

As with all other *Methylobacterium* species, the five isolates were strict aerobes, catalase positive and able to produce urease. Indole and H_2S were not produced, and methyl red and Voges–Proskauer tests were negative. The five isolates

Table 1. Differential phenotypic characteristics of *M. hispanicum* sp. nov., *M. aquaticum* sp. nov. and related species of the genus *Methylobacterium*

Strains: 1, *M. hispanicum* sp. nov. GP34^T; 2, *M. aquaticum* sp. nov. GR16^T; 3, *M. aminovorans* JCM 8240^T (data from Urakami *et al.*, 1993); 4, *M. suomiense* NCIMB 13778^T (Doronina *et al.*, 2002); 5, *M. lusitanum* NCIMB 13779^T (Doronina *et al.*, 2002); 6, *M. thiocyanatum* NCIMB 13651^T (Wood *et al.*, 1998); 7, *M. chloromethanicum* NCIMB 13688^T (McDonald *et al.*, 2001); 8, *M. populi* NCIMB 13946^T (Van Aken *et al.*, 2004). +, Positive; −, negative; V, variable; W, weak; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8
Cells occur:								
Singly	+	+	+	+	+	ND	ND	+
In pairs	Rarely	+	Rarely	Rarely	Rarely	ND	ND	+
In rosettes	−	+	−	−	−	ND	ND	+
Cell length (μm)	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	1·5–1·7	0·8–1	0·8–1	1–1·2	0·8	0·8–1	0·8–1
Pigmentation	Light pink	Pink to red	Pink or red	Pink	Pink	Pink	Pink	Pink to red
Diameter of colonies (mm)	1–2	1–2	1–3	0·5–1	1	ND	ND	0·1–0·2
Oxidase	+ (W)	−	+	+ (W)	+	V	ND	+
Hydrolysis of Tween 80	−	+	ND	ND	ND	ND	ND	ND
Hydrolysis of starch	+ (W)	+	−	−	−	ND	ND	ND
Upper limit for growth on NaCl (%)	1	1	3	3	3	ND	ND	2
Growth on:								
Citrate	+	+	−	−	−	+	+	−
L-Glutamate	+	+	+	−	−	+	+	−
D-Glucose	−	+	−	+	−	+	−	−
D-Xylose	−	−	−	−	−	+	−	−
Fructose	+	+	+	+	+	+	−	+

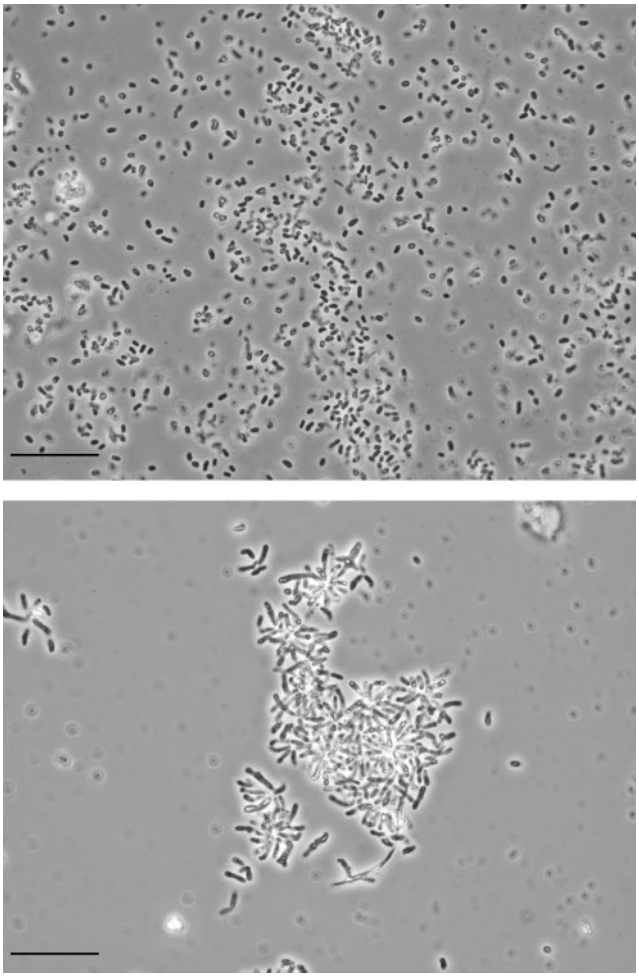

Fig. 1. Phase-contrast photomicrographs of *M. hispanicum* sp. nov. GP34^T (top) and *M. aquaticum* sp. nov. GR16^T (bottom). Bars, 10 μm.

possessed a number of phenotypic similarities. Colonies were pink to red and convex. All were capable of hydrolysing starch but not gelatin, aesculin, casein or DNA. Only strains GR16^T, GP22 and GP32 hydrolysed Tween 80. Nitrate was reduced to nitrite, Simmons' citrate test was positive and acid was oxidatively produced from D-arabinose and not from D-glucose, D-galactose, D-mannose or maltose. No growth occurred in the presence of 1·0 % NaCl.

The nutritional features of the isolates were determined using Biolog MicroPlates. Strains were grown on isolate medium (Biolog) at 28 °C for 72 h and suspended in sterile saline medium, within the density range specified by the manufacturer with a Biolog photometer model 21101. Immediately after suspending the cells in the saline solution, the suspensions were transferred into sterile multichannel pipetter reservoirs (Biolog) and the Biolog GN MicroPlates were inoculated with 125 μl of the cell suspension per well by means of an eight-channel repeating pipetter (Biolog). The inoculated plates were incubated at 28 °C for 7 days and the results were read with a MicroPlate Reader using Microlog 3.59 computer software to perform automated reading. The results of the nutritional tests are shown in the species descriptions and indicated a wide nutritional versatility of the five isolates.

Chromosomal DNA of the five strains 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 of the five isolates was amplified by PCR using two universal primers as described previously (Mellado *et al.*, 1995) and almost-complete nucleotide sequences (approx. 1400 bp) were determined. The ARB software package (Ludwig & Strunk, 1996) was used for 16S rRNA gene sequence analysis. Base-frequency filters were applied in the sequence comparison analysis and the effects on the results were evaluated.

16S rRNA gene phylogenetic analysis performed based on the neighbour-joining method (Saitou & Nei, 1987) clearly showed the position of this group of strains within the genus *Methylobacterium*. Maximum-parsimony- and maximum-likelihood-based trees using the full dataset or a selection of sequences were also obtained showing the same phylogenetic position of the group of isolates in the genus *Methylobacterium*, forming two clusters separated from the other species of this genus (Fig. 2).

Strains GP34^T and GR18 grouped together and their closest relatives were *M. organophilum* (95·6 % sequence similarity), *M. mesophilicum* (95·7 %), *M. fujisawaense* (96·4 %) and *M. radiotolerans* (96·6 %). Strains GR16^T, GP32 and GP22 also clustered together. Strain GR16^T was most closely related to *M. extorquens* (96·0 %), *M. rhodesianum* (95·8 %), *M. zatmanii* (95·5 %) and *M. thiocyanatum* (95·2 %). In addition, the 16S rRNA gene sequence similarity of strain GR16^T with respect to strains GP34^T and GR18 was 94·2 and 94·1 %, respectively. The 16S rRNA gene sequence similarity of strain GP34^T with respect to strains GR16^T, GP22 and GP32 was not greater than 94·5 %. According to these phylogenetic data, the isolates belonged to the genus *Methylobacterium*, but did not show similarity values higher than 96 % to the type strains of species of *Methylobacterium*, indicating that they constituted novel species of this genus.

The G+C content of genomic DNA was determined from the mid-point value (T_m) of the thermal denaturation profile (Marmur & Doty, 1962) using the equation of Owen & Hill (1979). The five isolates were shown to be very similar in their G+C content, ranging between 66·5 and 67·8 mol%. The G+C content of strains GP34^T and GR18 was 67·7 and 66·5 mol%, respectively, and of strains GR16^T, GP22 and GP32 was 67·5, 67·7 and 67·8 mol%, respectively.

Sequence similarity values obtained for these two groups of strains isolated from drinking water and all

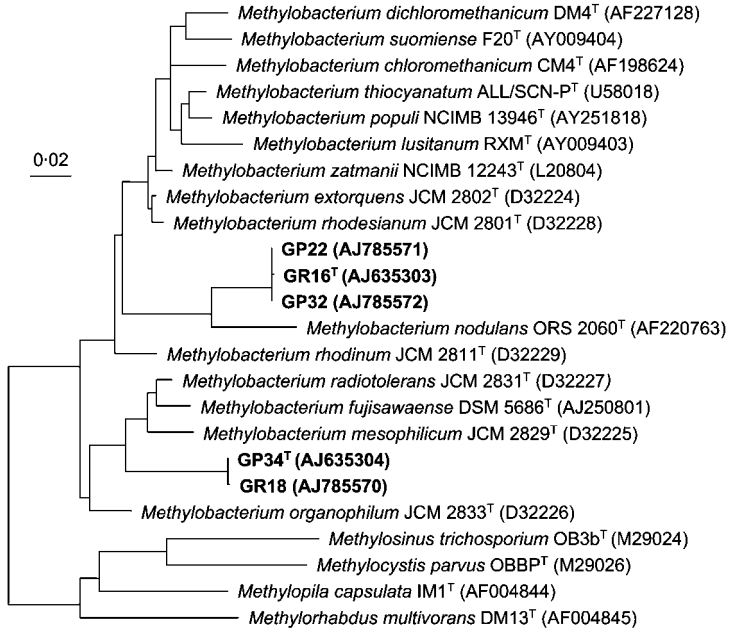

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequence comparisons showing the position of the two novel groups (strains GP34^T and GR18, and strains GR16^T, GP22 and GP32) compared with other related *Methylobacterium* species. The tree was obtained using the maximum-parsimony method. GenBank accession numbers are included in parentheses. Bar, 2 % sequence divergence.

Methylobacterium species with validly published names were low enough to propose their placement in two novel species within this genus. To confirm these results, DNA–DNA hybridization studies were performed following the competition procedure of Johnson (1994), described in detail in Mormile *et al.* (1999). The hybridization temperature was 60 °C, which was within the limit of validity for the filter method (De Ley & Tijtgat, 1970), and the percentage of hybridization was calculated according to Johnson (1994). DNA–DNA hybridization values between these five strains and the type strains of the *Methylobacterium* species that were more closely related phylogenetically are shown in Table 2. Our strains were found to have low levels of hybridization, showing relatedness values not higher than 45 % with the other *Methylobacterium* species studied. In contrast, a DNA–DNA hybridization value of 85 % was found between strain GP34^T and isolate GR18. In addition, strain GR16^T exhibited levels of DNA–DNA hybridization equal to or greater than 80 % with strains GP22 and GP32. These data indicated that the five isolates were genotypically distinct from the phylogenetically related type strains of *Methylobacterium* species. Furthermore, we have provided clear evidence that these novel isolates form two phylogenetic and genotypic groups, showing DNA–DNA hybridization values not higher than 45 % and 16S rRNA gene sequence similarities below 96 % with respect to previously described species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). On the basis of these results, two novel species within the genus *Methylobacterium* are proposed, with the names *Methylobacterium hispanicum* sp. nov. (with type strain GP34^T) and *Methylobacterium aquaticum* sp. nov. (with type strain GR16^T). The phenotypic characteristics that differentiate the two novel species from their phylogenetically closest relatives are summarized in Table 1.

Description of *Methylobacterium hispanicum* sp. nov.

Methylobacterium hispanicum (his.pa′ni.cum. L. neut. adj. *hispanicum* from Spain).

Gram-negative rods, 1·0–1·5 × 2·0–2·5 μm, occurring singly or in pairs. Cells are motile, non-spore-forming

Table 2. Levels of DNA–DNA hybridization between the five novel isolates and phylogenetically related species of the genus *Methylobacterium*

Source of unlabelled DNA	Relatedness (%) with ^{3}H-labelled DNA from:	
	GP34^T	GR16^T
M. hispanicum sp. nov.		
GP34^T	100	24
GR18	85	35
M. aquaticum sp. nov.		
GR16^T	45	100
GP22	33	80
GP32	32	89
M. mesophilicum NCIMB 11561^T	32	31
M. radiotolerans CCM 4464^T	42	25
M. fujisawaense NCIMB 12417^T	32	25
M. organophilum CCM 4460^T	37	19
M. rhodesianum NCIMB 12249^T	42	23
M. zatmanii CCM 4464^T	42	25
M. aminovorans CCM 4612^T	43	18
M. thiocyanatum NCIMB 13651^T	34	18
M. extorquens NCIMB 9399^T	45	18
M. suomiense NCIMB13778^T	45	33

and strictly aerobic. Colonies are pink, convex and translucent with regular edges, slow growing and 1–2 mm in diameter after 5 days at 28 °C on PCA. Cells do not grow in the presence of 1·0 % NaCl or higher. Growth occurs at 15–30 °C (optimal temperature 28 °C) and at pH 5·0–8·0 (optimal pH 6·5). Catalase- and urease-positive. Oxidase activity is weak. Indole, methyl red and Voges–Proskauer are negative. Starch is hydrolysed. Gelatin, Tween 80, casein, aesculin and DNA are not hydrolysed. Hydrogen sulfide is not produce. Simmons' citrate test is positive. Nitrate is reduced to nitrite. Produces acid 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): Tween 40, Tween 80, D-fructose, acetic acid, α-, β- and γ-hydroxybutyric acid, α-ketoglutaric acid, L-lactic acid, D- and L-malic acid, methyl pyruvate, monomethyl succinate, propionic acid, pyruvic acid, succinamic acid, succinic acid, *N*-acetyl-L-glutamic acid, L-asparagine, L-glutamic acid, glycyl-L-glutamic acid and glycerol. The following compounds are not utilized as sole carbon and energy sources (Biolog): α- and β-cyclodextrin, dextrin, glycogen, inulin, mannan, *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine, amygdalin, L-arabinose, D-arabitol, arbutin, cellobiose, L-fucose, D-galactose, D-galacturonic acid, gentiobiose, D-gluconic acid, α-D-glucose, *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, alaninamide, D- and L-alanine, L-alanyl-glycine, L-pyroglutamic acid, L-serine, putrescine, 2,3-butanediol, 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. Isolated from drinking water. The DNA G+C content is 67·5–68·0 mol% (T_m).

The type strain is GP34^T (=CECT 5997^T=DSM 16372^T=CCM 7219^T=CIP 108332^T). The DNA G+C content of strain GP34^T is 67·7 mol%.

Description of *Methylobacterium aquaticum* sp. nov.

Methylobacterium aquaticum (a.qua′ti.cum. L. neut. adj. *aquaticum* living in water).

Gram-negative rods, 1·5–1·7 × 4·5–8·0 μm, occurring singly, in pairs or in rosettes. Cells are motile, non-spore-forming and strictly aerobic. Colonies are pink to red, convex, not translucent, with regular edges, slow growing and 1–2 mm in diameter after 5 days at 28 °C on PCA. Cells do not grow in the presence of 1·0 % NaCl or higher. Growth occurs at 20–30 °C (optimal temperature 28 °C) and at pH 5·0–7·0 (optimal pH 6·0). Catalase- and urease-positive. Oxidase is negative. Indole, methyl red and Voges–Proskauer are negative. Starch and Tween 80 are hydrolysed. Gelatin, casein, aesculin and DNA are not hydrolysed. Does not form hydrogen sulfide. Simmons' citrate test is positive. Nitrate is reduced to nitrite. Produces acid 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): Tween 40, Tween 80, D-fructose, L-fucose, D-galactose, α-D-glucose, acetic acid, α-, β- and γ-hydroxybutyric acid, α-ketoglutaric acid, L-lactic acid, L-malic acid, monomethyl 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): α- and β-cyclodextrin, dextrin, glycogen, inulin, mannan, *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine, amygdalin, L-arabinose, D-arabitol, arbutin, cellobiose, D-galacturonic acid, gentiobiose, D-gluconic acid, *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, *N*-acetyl-L-glutamic acid, alaninamide, D- and L-alanine, L-alanyl-glycine, glycyl-L-glutamic acid, 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. Isolated from drinking water. The DNA G+C content is 67·3–67·9 mol% (T_m).

The type strain is strain GR16^T (=CECT 5998^T=CCM 7218^T=DSM 16371^T=CIP 108333^T). The DNA G+C content of strain GR16^T is 67·5 mol%.

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