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* (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.

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<sup>T</sup> and GR18, and strains GR16<sup>T</sup>, GP22 and GP32, respectively) showing highest similarity to members of the genus *Methylobacterium*. The highest 16S rRNA sequence similarities of strain GP34<sup>T</sup> 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<sup>T</sup> 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<sup>T</sup> (CECT 5997<sup>T</sup> = DSM 16372<sup>T</sup> = CIP 108332<sup>T</sup>), and *Methylobacterium aquaticum* sp. nov., with type strain GR16<sup>T</sup> (CECT 5998<sup>T</sup> = CCM 7218<sup>T</sup> = DSM 16371<sup>T</sup> = CIP 108333<sup>T</sup>).

The highest 16S rRNA sequence similarities of strain GP34<sup>T</sup> 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<sup>T</sup> 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<sup>T</sup> (CECT 5997<sup>T</sup> = DSM 16372<sup>T</sup> = CIP 108332<sup>T</sup>), and *Methylobacterium aquaticum* sp. nov., with type strain GR16<sup>T</sup> (CECT 5998<sup>T</sup> = CCM 7218<sup>T</sup> = DSM 16371<sup>T</sup> = CIP 108333<sup>T</sup>).
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 in man-made environments, including potable 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<sup>T</sup> and GR18 (isolated from culture medium R2A; Difco), and GP22, GP32 and GP34<sup>T</sup> [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<sub>2</sub>S 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*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells occur:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Singly</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>In pairs</td>
<td>Rarely</td>
<td>Rarely</td>
<td>Rarely</td>
<td>Rarely</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>In rosettes</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Cell length (μm)</td>
<td>2–2.5</td>
<td>4.5–9</td>
<td>1.5–4</td>
<td>1.2–2.5</td>
<td>1.7–2</td>
<td>2</td>
<td>2.5–3.5</td>
<td>1–10</td>
</tr>
<tr>
<td>Cell width (μm)</td>
<td>1–1.5</td>
<td>1.5–2.5</td>
<td>0.8–1</td>
<td>0.8–1</td>
<td>1–1.2</td>
<td>0.8</td>
<td>0.8–1</td>
<td>0.8–1</td>
</tr>
<tr>
<td>Pigmentation</td>
<td>Light pink</td>
<td>Pink to red</td>
<td>Pink or red</td>
<td>Pink</td>
<td>Pink</td>
<td>Pink</td>
<td>Pink</td>
<td>Pink to red</td>
</tr>
<tr>
<td>Diameter of colonies (mm)</td>
<td>1–2</td>
<td>1–2</td>
<td>1–3</td>
<td>0.5–1</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
<td>0.1–0.2</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+ (W)</td>
<td>-</td>
<td>+</td>
<td>+ (W)</td>
<td>+</td>
<td>V</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Hydrolysis of Tween 80</td>
<td>-</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Hydrolysis of starch</td>
<td>+ (W)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Upper limit for growth on NaCl (%)</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Growth on:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fructose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
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 GR16T, 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 GP34T 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 GR16T, GP32 and GP22 also clustered together. Strain GR16T 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 GR16T with respect to strains GP34T and GR18 was 94-2 and 94-1%, respectively. The 16S rRNA gene sequence similarity of strain GP34T with respect to strains GR16T, 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 GP34T and GR18 was 67-7 and 66-5 mol%, respectively, and of strains GR16T, 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

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**Fig. 1.** Phase-contrast photomicrographs of *M. hispanicum* sp. nov. GP34T (top) and *M. aquaticum* sp. nov. GR16T (bottom). Bars, 10 μm.
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 GP34T and isolate GR18. In addition, strain GR16T 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 GP34T) and Methylobacterium aquaticum sp. nov. (with type strain GR16T). The phenotypic characteristics that differentiate the two novel species from their phylogenetically closest relatives are summarized in Table 1.

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

<table>
<thead>
<tr>
<th>Source of unlabelled DNA</th>
<th>Relatedness (%) with 3H-labelled DNA from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GP34T</td>
</tr>
<tr>
<td>M. hispanicum sp. nov.</td>
<td></td>
</tr>
<tr>
<td>GP34T</td>
<td>100</td>
</tr>
<tr>
<td>GR18</td>
<td>85</td>
</tr>
<tr>
<td>M. aquaticum sp. nov.</td>
<td></td>
</tr>
<tr>
<td>GR16T</td>
<td>45</td>
</tr>
<tr>
<td>GP22</td>
<td>33</td>
</tr>
<tr>
<td>GP32</td>
<td>32</td>
</tr>
<tr>
<td>M. mesophilicum NCIMB 11561T</td>
<td>32</td>
</tr>
<tr>
<td>M. radiotolerans CCM 4464T</td>
<td>42</td>
</tr>
<tr>
<td>M. fujisawaense NCIMB 12417T</td>
<td>32</td>
</tr>
<tr>
<td>M. organophilum CCM 4460T</td>
<td>37</td>
</tr>
<tr>
<td>M. rhodesianum NCIMB 12249T</td>
<td>42</td>
</tr>
<tr>
<td>M. zatmanii CCM 4464T</td>
<td>42</td>
</tr>
<tr>
<td>M. aminovorans CCM 4612T</td>
<td>43</td>
</tr>
<tr>
<td>M. thiocyanatum NCIMB 13651T</td>
<td>34</td>
</tr>
<tr>
<td>M. extorquens NCIMB 9399T</td>
<td>45</td>
</tr>
<tr>
<td>M. suomiense NCIMB13778T</td>
<td>45</td>
</tr>
</tbody>
</table>

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 µm x 2·0–2·5 µm, occurring singly or in pairs. Cells are motile, non-spore-forming
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 produced. 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, succinic acid, succinamic acid, N-acetyl-L-glutamic acid, L-asparagine, L-glutamic acid, glycy1-L-glutamic acid and glycerol. The following compounds are not utilized as sole carbon and energy sources (Biolog): α- and β-cyclodextrin, dextrin, glycochen, glycinogen, inulin, mannannan, N-acetyl-D-glucosamine, N-acetyl-D-mannosamine, amygdalin, L-arabinose, D-arabitol, arbutin, cellobiose, L-fucose, D-galactose, D-galacturonic acid, gentiobiose, D-glucosamine, m-inositol, α-D-maltose, lactulose, maltose, maltotriose, D-mannitol, D-mannose, D-melezitose, D-melibiose, methyl α-D-galactoside, 3-methyl glucose, methyl α-D-glucose, methyl β-D-glucoside, methyl α-D-mannoside, palatinose, D-psicose, D-rhamnose, D-ribose, salicin, sedoheptuloban, D-sorbitol, stachyose, sucrose, D-tagatose, D-trehalose, turanose, xylitol, D-xylene, p-hydroxyphenyl acetic acid, α-ketovaleric acid, lactamide, D-lactic acid methyl ester, alaninamide, D- and L-alanine, L-alanyl-glycine, L-proplyglutamic acid, L-serine, putrescine, 2,3-butanediol, adenosine, 2′-deoxadenosine, 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% (Tm).

The type strain is GP34T (= CECT 5997T = DSM 16372T = CIP 108332T). The DNA G+C content of strain GP34T 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-sporforming 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, acetan 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, succinic acid, succinamic acid, N-acetyl-L-glutamic acid, L-asparagine, L-glutamic acid, glycy1-L-glutamic acid and glycerol. The following compounds are not utilized as sole carbon and energy sources (Biolog): α- and β-cyclodextrin, dextrin, glycochen, glycinogen, inulin, mannannan, N-acetyl-D-glucosamine, N-acetyl-D-mannosamine, amygdalin, L-arabinose, D-arabitol, arbutin, cellobiose, D-galacturonic acid, gentiobiose, D-glucosamine, m-inositol, α-D-maltose, lactulose, maltose, maltotriose, D-mannitol, D-mannose, D-melezitose, D-melibiose, methyl α-D-galactoside, 3-methyl glucose, methyl α-D-glucose, methyl β-D-glucoside, methyl α-D-mannoside, palatinose, D-psicose, D-rhamnose, D-ribose, salicin, sedoheptuloban, D-sorbitol, stachyose, sucrose, D-tagatose, D-trehalose, turanose, xylitol, D-xylene, p-hydroxyphenyl acetic acid, α-ketovaleric acid, lactamide, D-lactic acid methyl ester, D-malic acid, methyl pyruvate, N-acetyl-D-glutamic acid, alaninamide, D- and L-alanine, L-alanyl-glycine, L-prolylgutamic 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% (Tm).

The type strain is strain GR16T (= CECT 5998T = DSM 7218T = CIP 108333T). The DNA G+C content of strain GR16T is 67·5 mol%.

Acknowledgements

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References


