Three pink-pigmented facultatively methylotrophic bacteria were isolated from drinking water. These strains (designated AR24<sup>T</sup>, AR25 and GR32) have been characterized on the basis of phenotypic traits, 16S rRNA gene sequence analysis and DNA–DNA hybridization. According to the results of these analyses, the three strains belong to the genus *Methylobacterium*. Analysis of 16S rRNA gene sequences revealed that the three isolates constituted a single phylogenetic group. The level of 16S rRNA gene sequence similarities with respect to the type strains of the genus *Methylobacterium* were less than 96·5%, except for the type strain of *Methylobacterium nodulans* (98·1%). The G+C content of their DNA ranged from 69·0 to 69·7 mol%. DNA–DNA hybridization values confirmed that they constitute a novel species for which we propose the name *Methylobacterium isbiliense* sp. nov. The type strain is AR24<sup>T</sup> (≡CCCT 7068<sup>T</sup> = CCM 7304<sup>T</sup>).

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, tap water systems, 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). Moreover, some *Methylobacterium* species are clinically opportunistic bacteria and have been found to cause urinary tract infections (Chen-Hsiang et al., 2004).

Species of this genus belong to the α-2 subclass of the *Proteobacteria* and are strictly aerobic, Gram-negative, rod-shaped, pink-pigmented, facultatively methylotrophic (PPFM) bacteria, which can grow on C<sub>1</sub> compounds such as formate, formaldehyde and methanol as sole source of carbon and energy, as well as on a wide range of multi-carbon growth substrates (Green, 2000). At the time of writing the genus *Methylobacterium* consists of 19 species (Patt et al., 1976; Green & Bousfield, 1983; Bousfield & Green, 1985; Green et al., 1988; Urakami et al., 1993; Wood et al., 1998; Doronina et al., 2000; McDonald et al., 2001; Doronina et al., 2002; Van Aken et al., 2004; Jourand et al., 2004; Gallego et al., 2005a, b), the type species being *Methylobacterium organophilum* (Patt et al., 1976).

Our previous studies focused on the determination of bacterial diversity found in drinking water, leading to the description of three new species of the genus *Methylobacterium*: *Methylobacterium aquaticum*, *Methylobacterium hispanicum* (Gallego et al., 2005a) and *Methylobacterium variabile* (Gallego et al., 2005b). The massive presence of these pink-pigmented bacteria in our first sampling campaign led us to carry out a second campaign for a more complete study of the incidence of these micro-organisms in potable tap water. In this paper we describe the features of three new pink-pigmented isolates that constitute a new species of the genus *Methylobacterium* for which we propose the name *Methylobacterium isbiliense* sp. nov.

A total of 25 litres of drinking water samples was collected from four different points in the water distribution system of Sevilla (Spain). Samples were concentrated by using a tangential flow filtration system (Filtron) and plated on Plate Count Agar (PCA-Difco) and R2A agar medium (Difco). Plates were incubated at 28°C for 7 days and pink-pigmented colonies were plated to obtain pure cultures. Chromosomal DNA of these isolates was extracted and the 16S rRNA gene of all isolates was amplified by using two universal primers (Eubak5, 5'-AGAGTTTGATC(AC)TGG-3'; and C1392R, 5'-CCACGGGCCGTTGTGAC-3'). Nearly full-length 16S rRNA gene sequences were obtained and phylogenetic analyses were performed by using the ARB software package (Ludwig & Strunk, 1996). Sequencing was performed by NBT-Newbiotechnic (Sevilla, Spain) using an automated DNA sequencer model 3100 (Applied Biosystems). Phylogenetic trees were inferred by using three tree-making algorithms: maximum-parsimony, neighbour-joining and maximum-likelihood methods. Strains AR24<sup>T</sup>,
AR25 and GR32 grouped together, forming a separate phylogenetic branch in the three phylogenetic trees, sharing 100% sequence similarity (Fig. 1). Their closest relative was *M. nodulans*, showing 98.1% sequence similarity, whereas the similarity values with respect to other type species belonging to the genus *Methyllobacterium* were lower than 96.5%. Although the 16S rRNA gene sequence similarity with respect to *M. nodulans* was higher than 97%, the colonies of this species are not pink-pigmented when they grow on MMS agar medium (Green, 2000), and this made us to continue with the characterization of these three strains.

The three pink-pigmented cultures were isolated from two different points in the drinking water distribution system, but all three were isolated from R2A agar isolation medium. They were phenotypically characterized by using the methods described by Doronina et al. (1998). Cell morphology, Gram staining, motility, and growth at different pH values, temperatures and concentrations of NaCl (0–4%) were determined on liquid and solid R2A medium. Nutritional features were determined as described previously (Gallo et al., 2005b) by using Biolog Microplates.

Strains AR24, AR25 and GR32 were Gram-negative rods, motile and strictly aerobic, measuring 1.0–1.3 μm wide by 2.0–5.0 μm long in 24 h, R2A liquid medium cultures, incubated at 28 °C. Colonies of these strains were circular, regular in shape and pink-pigmented. The three strains could grow from pH 4 to pH 10, and from 20 to 37 °C; no growth occurred in the presence of 1% NaCl. Urease was produced, the methyl-red and Voges-Proskauer tests were negative and H2S was not produced, as for all species of the genus *Methyllobacterium* (Green, 2000).

Chromosomal DNA for DNA–DNA hybridization and G+C content determination was isolated and purified according to the methods described by Wilson (1987) and Marmur (1961), as partially modified by Hood et al. (1987). The DNA G+C content was determined from the midpoint value (Tm) of the thermal denaturation profile (Marmur & Doty, 1962) in 0.1 × SSC buffer and was obtained with a Perkin–Elmer Lambda 20 spectrophotometer at 260 nm programmed for temperature increases of 1.0 °C min⁻¹. The G+C content was calculated from this temperature by using the equation of Owen & Hill (1979).

DNA was labelled by the multiprime system with a commercial kit (Prime-a-Gene Labelling System; Promega). The labelled DNA was denatured prior to hybridization by heating at 100 °C for 5 min and then placed on ice. DNA–DNA hybridization studies were performed by the competition procedure of the membrane method described by Wilson (1987). Base 2% sequence divergence.

![Phylogenetic tree based on 16S rRNA gene sequence comparison showing the position of the three strains (AR24, AR25 and GR32) and other related species of the genus *Methyllobacterium*. The tree was obtained using the maximum-parsimony method. GenBank accession numbers are included in parentheses. Bar, 2% sequence divergence.](image-url)
Methylobacterium isbiliense sp. nov.

Methylobacterium isbiliense (is.bil.i.en’se. L. neut. adj. isbiliense from Isbilia, the old name for the city of Sevilla, Spain, from where the bacterium was isolated).

Gram-negative rods, 1–1.3 × 2–5 μm, occurring singly or forming aggregates. Cells are motile, non-spore-forming and strictly aerobic. Colonies are pink, circular, regular in shape and 1–2 mm in diameter after 7 days at 28 °C on R2A medium. Slow-growing (no growth is obtained before 3–4 days in R2A solid medium) and does not grow in the presence of 1-0 % NaCl or higher. Growth occurs at 20–37 °C (optimal temperature 28 °C) and at pH 4.0–10.0 (optimal pH between 6-0 and 7-0). Oxidase and catalase activities are weakly positive. Urease is positive. Indole production, methyl red and Voges–Proskauer are negative. Starch is hydrolysed. Gelatin, Tween 80, caseine and DNA are not hydrolysed. Hydrogen sulfide is not produced. Simmons’ citrate test is positive. Nitrate is not reduced. Acid is not produced from D-glucose, D-galactose, D-mannose,

Table 1. Differential phenotypic characteristics of Methylobacterium isbiliense and other 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>
<th>9</th>
<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells occur:</td>
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<tr>
<td>Singly</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>In pairs</td>
<td>Rarely</td>
<td>Rarely</td>
<td>+</td>
<td>Rarely</td>
<td>+</td>
<td>Rarely</td>
<td>+</td>
<td>Rarely</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Aggregates/rosettes</td>
<td>+</td>
<td>-</td>
<td>+</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>2–5</td>
<td>1–1.5</td>
<td>2–6</td>
<td>2–2.5</td>
<td>4–5–8</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>1–1.3</td>
<td>0.8–1.0</td>
<td>1–1.5</td>
<td>1.5–1.7</td>
<td>0.8–1</td>
<td>0.8–1</td>
<td>0.8–1</td>
<td>0.8–1</td>
<td>0.8–1</td>
<td>0.8–1</td>
<td>0.8–1</td>
</tr>
<tr>
<td>Pigmentation</td>
<td>Pink</td>
<td>Not pigmented</td>
<td>Pink</td>
<td>Light pink</td>
<td>Pink to red</td>
<td>Pink to red</td>
<td>Pink</td>
<td>Pink</td>
<td>Pink</td>
<td>Pink</td>
<td></td>
</tr>
<tr>
<td>Diameter of colonies (mm)</td>
<td>1–2</td>
<td>0.5–1</td>
<td>2–7</td>
<td>1–2</td>
<td>1–2</td>
<td>1–3</td>
<td>0.5–1</td>
<td>1</td>
<td>1.5</td>
<td>0.1–0.2</td>
<td></td>
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<tr>
<td>Oxidase</td>
<td>+ (w)</td>
<td>+ (w)</td>
<td>-</td>
<td>+ (w)</td>
<td>-</td>
<td>+</td>
<td>+ (w)</td>
<td>+ (w)</td>
<td>V</td>
<td>ND</td>
<td>+</td>
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<tr>
<td>Growth on:</td>
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<td></td>
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<tr>
<td>Citrate</td>
<td>+</td>
<td>+</td>
<td>+</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>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>D-Glucose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td>D-Xylose</td>
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<tr>
<td>Fructose</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Johnson (1994), as described in detail by Mormile et al. (1999). The hybridization temperature was 61 °C, which is within the limit of validity for the filter method (De Ley & Tijtgat, 1970) and the percentage hybridization was calculated according to Johnson (1994). The experiments were carried out in triplicate.

The G + C content of the DNAs of the three strains ranged from 69-0 to 69-7 mol%; the G + C of strain AR24T was 69 mol%. These values are within the range of G + C content described for the genus Methylobacterium (Hood et al., 1987; Urakami et al., 1993). The percentage DNA–DNA hybridization between the type strain AR24T and strain GR32 was 99%, confirming the close relationship between these strains (Fig. 1). On the other hand, the percentage hybridization between closely related Methylobacterium species and strain AR24T ranged between 14 and 37% for Methylobacterium aquaticum DSM 16371 T and Methylobacterium nodulans LMG 21967 T, respectively. On the basis of these results, and taking into consideration the criteria recommended by Wayne et al. (1987), these strains constitute a new taxonomic unit.

The DNA–DNA hybridization results, the phylogenetic data and several phenotypic features allowed strains AR24T, AR25 and GR32 to be differentiated from other members of the genus Methylobacterium. A novel species, Methylobacterium isbiliense sp. nov., is therefore proposed to accommodate the three strains. Table 1 shows some phenotypic features that differentiate this species from other related species of the genus Methylobacterium.
maltose, D-arabinose and fructose. Methanol, formate and formaldehyde are utilized as sole carbon sources. Ammonium sulfate, aspartate and glutamate are utilized as sole nitrogen sources. The following compounds are utilized as sole carbon and energy sources (Biolog): acetic acid, \( \gamma \)-hydroxybutyric acid, \( \alpha \)-ketoglutaric acid, L-lactic acid, L-malic acid, monomethyl succinate, propionic acid, pyruvic acid, succinic acid, succinic acid and L-glutamic acid. The following compounds are not utilized as sole carbon and energy sources (Biolog): \( \alpha \)-cyclodextrin, \( \beta \)-cyclodextrin, dextrin, glycogen, inulin, mannann, Tween 40, Tween 80, N-acetyl-D-glucosamine, N-acetyl-D-mannosamine, amygdalin, L-aranobinose, D-arabitol, arbutin, cellobiose, D-fructose, L-fucose, D-galactose, D-galacturonic acid, gentibiose, D-gluconic acid, \( \alpha \)-D-glucose, \( m \)-inositol, \( \alpha \)-D-lactose, lactulose, maltose, maltotriose, D-mannitol, D-mannose, D-melezitose, D-melobiose, \( \alpha \)-methyl-D-galactoside, \( \beta \)-methyl-D-galactoside, 3-methylglucoside, \( \alpha \)-methyl-D-glucoside, \( \beta \)-methyl-D-glucoside, \( \alpha \)-methyl-D-mannoside, palatinose, D-psicose, D-raffinose, L-rhamnose, D-ribose, salicin, sedoheptulanos, D-sorbitol, stachyose, sucrose, D-tagatose, D-trehalose, turanose, xylitol, D-xylose, \( \alpha \)-hydroxybutyric acid, \( \beta \)-hydroxybutyric acid, \( p \)-hydroxyphenylacetate acid, \( \alpha \)-ketolactic acid, lactamide, D-lactic acid methyl ester, D-malic acid, meptivryurate, N-acetyl-L-glutamic acid, alaninamide, D-alanine, L-alanine, L-alanylglycine, L-asparagine, 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-\( \alpha \)-glycerol phosphate. Isolated from drinking water. The C content of the type strain is 69–75 mol%. The type strain is CECT 7068T = CCM 7304T. The DNA G+C content of the type strain is 69–70 mol%. The accession number for the 16S rRNA sequence of strain AR24T is AJ888239.

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