Methylobacterium isbiliense sp. nov., isolated from the drinking water system of Sevilla, Spain

Virginia Gallego, Maria Teresa García and Antonio Ventosa

Correspondence
Antonio Ventosa
ventosa@us.es

Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Sevilla, 41012 Sevilla, Spain

Three pink-pigmented facultatively methylotrophic bacteria were isolated from drinking water. These strains (designated AR24^T, 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^T (=CECT 7068^T = CCM 7304^T).

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 C₁ 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-CT-3', and C1392R, 5'-CCACGGGCGGTGTGTAC-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^T,

Published online ahead of print on 13 October 2005 as DOI 10.1099/ij.s.0.63773-0.

The GenBank/EMBL/DBJ accession numbers for the 16S rRNA gene sequence of strains GR32, AR25 and AR24^T are AJ888241, AJ888240 and AJ888239, respectively.

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 *Methylobacterium* 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 (Gallego *et al.*, 2005b) by using Biolog Microplates.

Strains AR24^T, 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 *Methylobacterium* (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 mid-point value (*T*_m) 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 multiprimer 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

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence comparison showing the position of the three strains (AR24^T, AR25 and GR32) and other related species of the genus *Methylobacterium*. The tree was obtained using the maximum-parsimony method. GenBank accession numbers are included in parentheses. Bar, 2 % sequence divergence.

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 & Tijtgaat, 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 AR24^T 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 AR24^T 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 AR24^T 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 AR24^T, 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*.

Description of *Methylobacterium isbiliense* sp. nov.

Methylobacterium isbiliense (is.bil.i.en'se. L. neut. adj. *isbiliense* from Isbilía, 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*

Symbols: ND, Not determined; w, weak; v, variable; +, positive; –, negative. Bacteria are identified as: 1, *Methylobacterium isbiliense* AR24^T; 2, *Methylobacterium nodulans* LMG 21967^T (Jourand *et al.*, 2004); 3, *Methylobacterium variabile* GR3^T (Gallego *et al.*, 2005b); 4, *Methylobacterium hispanicum* CCM 7219^T (Gallego *et al.*, 2005a); 5, *Methylobacterium aquaticum* CCM 7218^T (Gallego *et al.*, 2005a); 6, *Methylobacterium aminovorans* JCM 8240^T (Urakami *et al.*, 1993); 7, *Methylobacterium suomiense* NCIMB 13778^T (Doronina *et al.*, 2002); 8, *Methylobacterium lusitanum* NCIMB 13779^T (Doronina *et al.*, 2002); 9, *Methylobacterium thiocyanatum* NCIMB 13651^T (Wood *et al.*, 1998); 10, *Methylobacterium chloromethanicum* NCIMB 13688^T (McDonald *et al.*, 2001); 11, *Methylobacterium populi* NCIMB 13946^T (Van Aken *et al.*, 2004).

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Cells occur:											
Singly	+	+	+	+	+	+	+	+	ND	ND	+
In pairs	Rarely	Rarely	+	Rarely	+	Rarely	Rarely	Rarely	ND	ND	+
Aggregates/rosettes	+	–	+	–	+	–	–	–	ND	ND	+
Cell length (µm)	2–5	1–1.5	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)	1–1.3	0.8–1.0	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	Not pigmented	Pink	Light pink	Pink to red	Pink or red	Pink	Pink	Pink	Pink	Pink to red
Diameter of colonies (mm)	1–2	0.5–1	2–7	1–2	1–2	1–3	0.5–1	1	1.5	ND	0.1–0.2
Oxidase	+(w)	+(w)	–	+(w)	–	+	+(w)	+(w)	v	ND	+
Growth on:											
Citrate	+	+	+	+	+	–	–	–	+	+	–
L-Glutamate	+	+	+	+	+	+	–	–	+	+	–
D-Glucose	–	–	+	–	+	–	+	–	+	–	–
D-Xylose	–	+	–	–	–	–	–	–	+	–	–
Fructose	–	–	+	+	+	+	+	+	+	–	+

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, γ -hydroxybutyric acid, α -ketoglutaric acid, L-lactic acid, L-malic acid, monomethyl succinate, propionic acid, pyruvic acid, succinamic acid, succinic acid and L-glutamic acid. The following compounds are not utilized as sole carbon and energy sources (Biolog): α -cyclodextrin, β -cyclodextrin, dextrin, glycogen, inulin, mannan, Tween 40, Tween 80, N-acetyl-D-glucosamine, N-acetyl-D-mannosamine, amygdalin, L-arabinose, D-arabitol, arbutin, cellobiose, D-fructose, L-fucose, D-galactose, D-galacturonic acid, gentibiose, D-gluconic acid, α -D-glucose, *m*-inositol, α -D-lactose, lactulose, maltose, maltotriose, D-mannitol, D-mannose, D-melezitose, D-melibiose, α -methyl-D-galactoside, β -methyl-D-galactoside, 3-methylglucose, α -methyl-D-glucoside, β -methyl-D-glucoside, α -methyl-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, α -hydroxybutyric acid, β -hydroxybutyric, *p*-hydroxyphenylacetic acid, α -ketovaleric acid, lactamide, D-lactic acid methyl ester, D-malic acid, methylpyruvate, N-acetyl-L-glutamic acid, alaninamide, D-alanine, L-alanine, L-alanyl-glycine, 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- α -glycerol phosphate. Isolated from drinking water. The DNA G + C content is 69.0–69.7 mol%. The type strain is strain AR24^T (=CECT 7068^T=CCM 7304^T). The DNA G + C content of the type strain is 69.0 mol%. The accession number for the 16S rRNA sequence of strain AR24^T is AJ888239.

Acknowledgements

V. Gallego was supported by a fellowship from the Spanish Ministerio de Educación y Ciencia. This work was supported by grants from the Quality of Life and Management of Living Resources Programme of the European Commission (QLK3-CT-2002-01972), Spanish Ministerio de Educación y Ciencia (BMC2003-01344) and from the Junta de Andalucía.

References

Bousfield, I. J. & Green, P. N. (1985). Reclassification of bacteria of the genus *Protomonas* Urakami and Komagata 1984 in the genus *Methylobacterium* (Patt, Cole, and Hanson) emend. Green and Bousfield 1983. *Int J Syst Bacteriol* **35**, 209.

Chen-Hsiang, L., Ya-Fen, T. & Jien-Wei, L. (2004). Underdiagnosis of urinary tract infection caused by *Methylobacterium* species with current standard processing of urine culture and its clinical implications. *J Med Microbiol* **53**, 755–759.

De Ley, J. & Tijtgaat, R. (1970). Evaluation of membrane filter methods for DNA–DNA hybridization. *Antonie van Leeuwenhoek* **36**, 461–474.

Doronina, N. V., Trotsenko, Y. A., Krausova, V. I., Boulygina, E. S. & Tourova, T. P. (1998). *Methylopila capsulata* gen. nov., sp. nov., a novel non-pigmented aerobic facultatively methylotrophic bacterium. *Int J Syst Bacteriol* **48**, 1313–1321.

Doronina, N. V., Trotsenko, Y. A., Tourova, T. P., Kuznetsov, B. B. & Leisinger, T. (2000). *Methylopila helvetica* sp. nov. and *Methylobacterium dichloromethanicum* sp. nov., novel aerobic facultatively methylotrophic bacteria utilizing dichloromethane. *Syst Appl Microbiol* **23**, 210–218.

Doronina, N. V., Trotsenko, Y. A., Kuznetsov, B. B., Tourova, T. P. & Salkinoja-Salonen, M. S. (2002). *Methylobacterium suomiense* sp. nov. and *Methylobacterium lusitanum* sp. nov., aerobic, pink-pigmented, facultatively methylotrophic bacteria. *Int J Syst Evol Microbiol* **52**, 773–776.

Gallego, V., Garcia, M. T. & Ventosa, A. (2005a). *Methylobacterium hispanicum* sp. nov. and *Methylobacterium aquaticum* sp. nov., isolated from drinking water. *Int J Syst Evol Microbiol* **55**, 281–287.

Gallego, V., Garcia, M. T. & Ventosa, A. (2005b). *Methylobacterium variabile* sp. nov., a methylotrophic bacterium isolated from an aquatic environment. *Int J Syst Evol Microbiol* **55**, 1429–1433.

Green, P. N. (2000). The genus *Methylobacterium*. In *the Prokaryotes*, Online Edn, <http://141.150.157.117:8080/prokPUB/index.htm>. Edited by M. Dworkin. New York: Springer.

Green, P. N. & Bousfield, I. J. (1981). The taxonomy of pink-pigmented facultatively methylotrophic bacteria. In *Microbial Growth on C1-Compounds*, pp. 285–293. Edited by H. Dalton. London: Heyden & Son Ltd.

Green, P. N. & Bousfield, I. J. (1983). Emendation of *Methylobacterium* Patt, Cole, and Hanson 1976; *Methylobacterium rhodinum* (Heumann 1962) comb. nov. corrig.; *Methylobacterium radiotolerans* (Ito & Iizuka 1971) comb. nov., corrig.; and *Methylobacterium mesophilicum* (Austin & Goodfellow 1979) comb. nov. *Int J Syst Bacteriol* **33**, 875–877.

Green, P. N., Bousfield, I. J. & Hood, D. (1988). Three new *Methylobacterium* species: *M. rhodesianum* sp. nov., *M. zatmanii* sp. nov., and *M. fujisawaense* sp. nov. *Int J Syst Bacteriol* **38**, 124–127.

Hiraishi, A., Furuhashi, K., Matsumoto, A., Koike, K. A., Fukuyama, M. & Tabuchi, K. (1995). Phenotypic and genetic diversity of chlorine-resistant *Methylobacterium* strains isolated from various environments. *Appl Environ Microbiol* **61**, 2099–2107.

Hood, D. W., Dow, C. S. & Green, P. N. (1987). DNA:DNA hybridization studies on the pink-pigmented facultative methylotrophs. *J Gen Microbiol* **38**, 709–720.

Johnson, J. L. (1994). Similarity analysis of DNAs. In *Methods for General and Molecular Bacteriology*, pp. 655–681. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.

Jourand, P., Giraud, E., Bena, G., Sy, A., Willens, A., Gillis, M., Dreyfus, B. & Lajudie, P. (2004). *Methylobacterium nodulans* sp. nov., for a group of aerobic, facultatively methylotrophic, legume root-nodule-forming and nitrogen-fixing bacteria. *Int J Syst Evol Microbiol* **54**, 2269–2273.

Ludwig, W. & Strunk, O. (1996). ARB – a software environment for sequence data (<http://www.mikro.biologie.tu-muenchen.de>).

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J Mol Biol* **3**, 208–218.

Marmur, J. & Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* **5**, 109–118.

- McDonald, I. R., Doronina, N. V., Trotsenko, Y. A., McAnulla, C. & Murrell, J. C. (2001).** *Hyphomicrobium chloromethanicum* sp. nov. and *Methylobacterium chloromethanicum* sp. nov., chloromethane-utilizing bacteria isolated from a polluted environment. *Int J Syst Evol Microbiol* **51**, 119–122.
- Mormile, M. R., Romine, M. F., Garcia, M. T., Ventosa, A., Bailey, T. J. & Peyton, B. M. (1999).** *Halomonas campisalis* sp. nov., a denitrifying, moderately haloalkaliphilic bacterium. *Syst Appl Microbiol* **22**, 551–558.
- Owen, R. J. & Hill, L. R. (1979).** The estimation of base compositions, base pairing and genome size of bacterial deoxyribonucleic acids. In *Identification Methods for Microbiologists*, 2nd edn, pp. 217–296. Edited by F. A. Skinner & D. W. Lovelock. London: Academic Press.
- Patt, T. E., Cole, G. C. & Hanson, R. S. (1976).** *Methylobacterium*, a new genus of facultatively methylotrophic bacteria. *Int J Syst Bacteriol* **26**, 226–229.
- Urakami, T., Araki, H., Suzuki, K.-I. & Komagata, K. (1993).** Further studies of the genus *Methylobacterium* and description of *Methylobacterium aminovorans* sp. nov. *Int J Syst Bacteriol* **43**, 504–513.
- Van Aken, B., Peres, C. M., Lafferty-Doty, S., Yoon, J. M. & Schnoor, J. L. (2004).** *Methylobacterium populi* sp. nov., a novel aerobic, pink-pigmented, facultatively methylotrophic, methane-utilizing bacterium isolated from poplar trees (*Populus deltoides* × *nigra* DN34). *Int J Syst Evol Microbiol* **54**, 1191–1196.
- Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987).** International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.
- Wilson, K. (1987).** Preparation of genomic DNA from bacteria. In *Current Protocols in Molecular Biology*, pp. 2.4.1–2.4.2. Edited by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith & K. Struhl. New York: Wiley.
- Wood, A. P., Kelly, D. P., McDonald, I. R., Jordan, S. L., Morgan, T. D., Khan, S., Murrell, J. C. & Borodina, E. (1998).** A novel pink-pigmented facultative methylotroph, *Methylobacterium thiocyanatum* sp. nov., capable of growth on thiocyanate or cyanate as sole nitrogen sources. *Arch Microbiol* **169**, 148–158.