Methylobacterium salsuginis sp. nov., isolated from seawater

Xun Wang, Foday Sahr, Ting Xue and Baolin Sun

Correspondence

Baolin Sun
sunb@ustc.edu.cn

Hefei National Laboratory for Physical Sciences at Microscale and School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230027, People's Republic of China

Three pink-pigmented, facultatively methylotrophic strains, designated MP1, MP2 and MR^T, were isolated from seawater from southern China and characterized. Analysis of their complete 16S rRNA gene sequences revealed that they constituted three separate phylogenetic groups, showing the highest levels of similarity with respect to some members of the genus *Methylobacterium*. PCR amplification also showed the gene coding for the α -subunit of methanol dehydrogenase (*mxhF*) to be present in all strains, indicating a methylotrophic metabolism. All three strains utilized D-fructose, ethanol and nutrient agar as carbon sources, but did not utilize sucrose, citrate, acetate or formaldehyde. On the basis of the phenotypic, phylogenetic and genotypic analyses, strain MR^T represents a novel species, for which the name *Methylobacterium salsuginis* sp. nov. is proposed, with MR^T (=CGMCC 1.6474^T =NCCB 100140^T) as the type strain. Strains MP1 and MP2 respectively represent novel strains of the species *Methylobacterium oryzae* and *Methylobacterium lusitanum*.

Species of the genus *Methylobacterium* are strictly aerobic, facultatively methylotrophic, Gram-negative, rod-shaped bacteria that can grow on single-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, 2006).

The genus *Methylobacterium* was initially proposed by Patt *et al.* (1976) with the type species *Methylobacterium organophilum*. The genus *Methylobacterium* belongs to the α -2 subgroup of the *Proteobacteria* and consists of 23 species at the time of writing: *Methylobacterium amino- vorans* (Urakami *et al.*, 1993), *Mtb. aquaticum* (Gallego *et al.*, 2005a), *Mtb. chloromethanicum* (McDonald *et al.*, 2001), *Mtb. dichloromethanicum* (Doronina *et al.*, 2000), *Mtb. extorquens* (Bousfield & Green, 1985), *Mtb. fujisawaense* (Green *et al.*, 1988), *Mtb. hispanicum* (Gallego *et al.*, 2005a), *Mtb. lusitanum* (Doronina *et al.*, 2002), *Mtb. mesophilicum* (Green & Bousfield, 1983), *Mtb. nodulans* (Jourand *et al.*, 2004), *Mtb. organophilum* (Patt *et al.*, 1976), *Mtb. podarium* (Anesti *et al.*, 2004), *Mtb. populi* (Van Aken *et al.*, 2004), *Mtb. radiotolerans* (Green & Bousfield, 1983), *Mtb. rhodesianum* (Green *et al.*, 1988), *Mtb. rhodinum* (Green & Bousfield, 1983), *Mtb. suomiense* (Doronina *et al.*, 2002), *Mtb. thiocyanatum* (Wood *et al.*, 1998), *Mtb. zatmanii* (Green *et al.*, 1988), *Mtb. variabile* (Gallego *et al.*, 2005b), *Mtb. isbiliense* (Gallego *et al.*, 2005c), *Mtb.*

adhaesivum (Gallego *et al.*, 2006) and *Mtb. oryzae* (Madhaiyan *et al.*, 2007).

Several studies have reported the isolation of members of this genus from various natural environments (Green & Bousfield, 1981, 1983), including soil, freshwater, tap-water systems, lake sediments and as contaminants in various products and processes. Their existence in tap-water systems has been attributed to their acquired resistance to chlorination (Hiraishi *et al.*, 1995). More recently, strains of *Methylobacterium* have been associated with opportunistic urinary tract infection in immunocompromised persons (Lee *et al.*, 2004). Here we report the isolation and characterization of a novel species and two novel strains of *Methylobacterium* isolated from seawater off the coast of southern China.

The methods used for enrichment and isolation were as described by Bodrossy *et al.* (1995) but with slight modifications. One litre of seawater obtained off the coast near Xiamen (Fujian Province) was concentrated using a polysulfone-fibre membrane with pore size of 0.22 μ m. Aliquots (20 μ l) of the concentrated samples were added to 250 ml flasks containing 50 ml sterile mineral medium and were then incubated at 30 °C for 7 days. Serial dilutions of the active cultures were then spread onto 0.1 M methanol mineral medium agar plates and incubated at 30 °C for 5–7 days. Individual colonies were isolated and purified by streaking several times onto similar agar plates. Only those cultures with a single type of morphology (as observed under light microscopy) were considered to be pure.

The GenBank/EMBL/DDBJ accession numbers for the *mxhF* and 16S rRNA gene sequences of strain MP1 are EF030547 and EF015477, respectively, those for strain MP2 are EF030548 and EF015479, and those for strain MR^T are EF030550 and EF015478.

Morphological characterization of the isolates included culture characterization on mineral salts media, Gram-staining and negative-staining for transmission electron microscopy. Cell growth at different pH values (5.0–10.0) and NaCl concentrations (0.1–10.0%) was determined by measuring the OD₆₀₀ using a spectrophotometer. Growth of the isolates at 4, 28, 30, 32 and 40 °C was also measured. Carbon-source utilization tests involving 12 carbon sources were performed by using a standard protocol described by Green & Bousfield (1982). Biochemical characterization of the isolates included catalase, urease, indole, methyl red and Voges–Proskauer tests. Hydrolysis of starch and gelatin, hydrogen sulfide production, the Simmons' citrate test and nitrate-reduction tests were also performed using standard protocols.

Genomic DNA from the three strains was extracted and purified according to the methods described by Möller *et al.* (1992), with slight modifications. Primers 5'-AGAGTTTG-ATCCTGGCTCAG-3' and 5'-AAGGAGGTGATCCAGCC-3', designed on the basis of previously published sequences of methanotrophic bacteria and synthesized by Invitrogen (Shanghai), were used as the forward and reverse primer, respectively, for 16S rRNA gene amplification. The *mxoF* gene, encoding the methanol dehydrogenase required for methanol utilization, was detected by PCRs in these three strains, using primers 1003f (5'-GCGGCACCAACTGGG-GCTGGT-3') and 1561r (5'-GGGCAGCATGAAGGGCT-CCC-3') as described by Heyer *et al.* (2002).

PCR products of the 16S rRNA and *mxoF* genes were cloned in the pCR2.1 vector (Invitrogen) and submitted to Invitrogen for sequencing. The 16S rRNA gene sequences determined for our isolates were aligned, using RDP II (<http://rdp.cme.msu.edu>), with 16S rRNA gene sequences available for known *Methylobacterium* species, and the *mxoF* gene sequences were aligned with reference sequences from GenBank by using BLAST (National Center for Biotechnology Information) and vector software. A phylogenetic tree was inferred by using the neighbour-joining (Saitou & Nei, 1987) method with MEGA (version 3.1) software.

On the basis of the criteria of Bodrossy *et al.* (1995), three pure colonies were isolated and labelled MP1, MP2 and MR^T. On mineral medium agar plates, the three isolates produced pink, circular colonies, 1–4 mm in diameter. Gram-staining established them as Gram-negative rods that occurred as single cells or in rosettes. Negative staining followed by transmission electron microscopy showed that all three isolates had a rod-shaped morphology (Fig. 1). Their cells were strictly aerobic, catalase-positive, motile, lacked flagella and were able to produce urease. Indole and H₂S were not produced and the methyl red and Voges–Proskauer tests gave negative results. Further biochemical analysis showed the strains to be capable of hydrolysing starch but not gelatin. The results of Simmons' citrate tests and nitrate-reduction tests were also positive. Growth occurred at 28, 30 and 32 °C, but not at 4 or 40 °C. For

Fig. 1. Transmission electron micrograph of cells of strain MR^T, isolated from seawater samples. Bar, 2.0 μm.

strains MP1 and MP2, optimal growth occurred at pH 7, whilst that for MR^T occurred at pH 6. For strains MP1 and MR^T, no growth occurred in the presence of ≥1.5% NaCl; for MP2, growth inhibition occurred at ≥2% NaCl.

Analysis of the 16S rRNA gene sequences identified our isolates as three distinct strains of the genus *Methylobacterium*. Strains MP1 and MP2 were identified as novel strains of two previously characterized species of the genus, *Mtb. oryzae* and *Mtb. lusitanum*, respectively, whereas strain MR^T appeared to represent a novel species of this genus (Fig. 2). Because of the chemotaxonomic homogeneity of the genus *Methylobacterium*, phylogenetic analyses constitute a critical tool in species identification (Green & Bousfield, 1982; Doronina *et al.*, 2002). Strain MP1 had 96.3% 16S rRNA gene sequence similarity with respect to a previously characterized strain of *Mtb. oryzae*, whereas strain MP2 shared 98% sequence similarity with respect to a previously characterized strain of *Mtb. lusitanum*. Strain MR^T was found to be most closely related to *Mtb. suomiense* F20^T; however, the level of sequence similarity was only 93.1%, suggesting that this strain represents a novel species of the genus *Methylobacterium*.

The gene for the α-subunit of methanol dehydrogenase (*mxoF*) was amplified from strains MP1, MP2 and MR^T and then sequenced. A comparison of the *mxoF* gene sequences of the three isolates showed varying levels of similarity. Strain MP1 showed *mxoF* gene sequence similarities of 92.6 and 93.2% with respect to strain MP2 and strain MR^T, respectively, while strain MP2 showed 96.9% *mxoF* gene sequence similarity with respect to strain MR^T. When compared with representative *Methylobacterium* strains from the database, strain MP1 was most closely related to *Mtb. organophilum*, with 93% *mxoF* gene sequence similarity. Strains MP2 and MR^T were most closely related to *Mtb. podarium*, with 97 and 96% sequence similarity, respectively. Analysis of the *mxoF*

Fig. 2. Phylogenetic neighbour-joining tree, based on 16S rRNA gene sequences, showing the relationships between strains MP1, MP2 and MR^T and species belonging to the genus *Methylobacterium* and other related methylotrophic bacteria. Bar, 1 % sequence divergence.

genes of our three strains confirmed the 16S rRNA gene sequencing results, indicating that MP1, MP2 and MR^T belong to the genus *Methylobacterium*, exhibiting varying sequence similarity with respect to a number of previously characterized members of the genus (Table 1).

Three carbon sources, D-fructose, ethanol and nutrient agar, were utilized by all three strains. In comparisons between the carbon-source utilization profiles of previously characterized strains, strain MP1 and a previously characterized strain of *Mtb. oryzae* were both able to utilize D-glucose, D-xylose, L-arabinose, fructose, ethanol and nutrient agar, but MP1 differed from its relative by not utilizing citrate or acetate as carbon sources. Strain MP2 and a previously characterized strain of *Mtb. lusitanum* also showed the ability to utilize D-fructose and ethanol. Strain MP2 was also able to utilize betaine, but was unable to utilize acetate, unlike the previously characterized strain of *Mtb. lusitanum*, which utilized acetate but not betaine. Strain MR^T and its closest relative, *Mtb. suomiense*, were both able to utilize D-glucose, D-fructose, betaine and ethanol. Strain MR^T failed to utilize acetate, whilst its relative, *Mtb. suomiense*, was able to utilize acetate as an energy source.

The phylogenetic data and several phenotypic features indicate that strain MR^T represents a novel species and that strains MP1 and MP2 represent novel strains of *Methylobacterium oryzae* and *Methylobacterium lusitanum*, respectively. Strain MR^T represents a novel species of the genus *Methylobacterium*, for which we propose the name *Methylobacterium salsuginis* sp. nov.

Description of *Methylobacterium salsuginis* sp. nov.

Methylobacterium salsuginis (sal.su'gi.nis. L. gen. n. *salsuginis* of brine, seawater).

Cells are Gram-negative rods, 1.0–1.5 × 4.0–8.0 µm, occurring singly or in aggregates, and are strictly aerobic. Colonies are circular, regular in shape, pink to red, slow-growing and 1–2 mm in diameter after 3–4 days at 30 °C on methanol mineral medium agar plates. The pink pigment is water-insoluble. Catalase- and urease-positive, but negative for indole and in methyl red and Voges–Proskauer reactions. Simmons' citrate test is positive. Starch is hydrolysed, but gelatin is not. Hydrogen sulfide is

Table 1. Differential phenotypic characteristics of strain MR^T, strains MP1 and MP2 and type strains of related *Methylobacterium* species

Strains: 1, *Mtb. salsuginis* sp. nov. MR^T; 2, *Mtb. oryzae* MP1; 3, *Mtb. lusitanum* MP2; 4, *Mtb. aminovorans* JCM 8240^T (data from Urakami *et al.*, 1993); 5, *Mtb. suomiense* NCIMB 13778^T (Doronina *et al.*, 2002); 6, *Mtb. dichloromethanicum* DSM 6343^T (Doronina *et al.*, 2000); 7, *Mtb. thiocyanatum* NCIMB 13651^T (Wood *et al.*, 1998); 8, *Mtb. chloromethanicum* NCIMB 13688^T (McDonald *et al.*, 2001). +, Positive; –, negative; w, weak growth; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8
Cell arrangement								
Single	+	+	+	+	+	+	ND	ND
Pairs	+	Rarely	Rarely	Rarely	Rarely	Rarely	ND	ND
Aggregates/rosettes	+	+	+	–	–	–	ND	ND
Cell size								
Length (µm)	4.0–8.0	1.0–4.0	2.0–3.0	1.5–4.0	1.2–2.5	1.0–2.0	2.0	2.5–3.5
Width (µm)	1.0–1.5	1.0–1.5	0.8–1.0	0.8–1.0	0.8–1.0	0.8–1.0	0.8	0.8–1.0
Pigmentation	Pink to red	Pink	Pink	Pink to red	Pink	Pink	Pink	Pink
Colony diameter (mm)	1–2	2–4	1–2	1–3	0.5–1	1–3	1.5	ND
Growth on:								
D-Glucose	+	+	–	–	+	–	+	–
D-Xylose	–	+	–	–	–	–	ND	–
L-Arabinose	–	+	–	ND	–	ND	w	ND
D-Fructose	+	+	+	+	+	+	+	–
Sucrose	–	–	–	ND	ND	ND	ND	ND
Citrate	–	–	–	–	–	+	+	+
Acetate	–	–	–	+	+	+	+	+
Betaine	+	–	+	+	+	+	ND	+
Ethanol	+	+	+	ND	+	ND	ND	ND
Formaldehyde	–	–	–	ND	ND	ND	ND	ND
Formate	+	–	+	ND	ND	ND	ND	ND
Nutrient agar	+	+	+	ND	–	ND	+	ND

not produced. Nitrate is reduced to nitrite. Carbon sources utilized include D-fructose, D-glucose, methanol, ethanol, formate, betaine and nutrient agar. Does not utilize D-xylose, L-arabinose, sucrose, citrate, acetate or formaldehyde. Nitrogen sources utilized include ammonium and nitrate. Growth occurs at 28, 30 and 32 °C (optimum, 30 °C) but not at 4 or 40 °C. Growth occurs at pH 5.0–8.0 (optimum, pH 7.0). No growth occurs in the presence of $\geq 1.5\%$ NaCl.

The type strain, MR^T (=CGMCC 1.6474^T =NCCB 100140^T), was isolated from seawater collected off the coast of southern China, near Xiamen (Fujian Province).

Acknowledgements

The authors wish to thank Liang Tao for collecting the seawater sample and Yonglong Zhuang for his assistance with the transmission electron microscopy. This research was supported by the One Hundred Talent Project of the Chinese Academy of Sciences.

References

Anesti, V., Vohra, J., Goonetilleka, S., McDonald, I. R., Sträubler, B., Stackebrandt, E., Kelly, D. P. & Wood, A. P. (2004). Molecular detection and isolation of facultatively methylotrophic bacteria,

including *Methylobacterium podarium* sp. nov., from the human foot microflora. *Environ Microbiol* **6**, 820–830.

Bodrossy, L., Murrell, J. C., Dalton, H., Kalman, M., Puskas, L. G. & Kovacs, K. L. (1995). Heat-tolerant methanotrophic bacteria from the hot water effluent of a natural gas field. *Appl Environ Microbiol* **61**, 3549–3555.

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.

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., García, 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., García, 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.

- Gallego, V., García, M. T. & Ventosa, A. (2005c).** *Methylobacterium isbiliense* sp. nov., isolated from the drinking water system of Sevilla, Spain. *Int J Syst Evol Microbiol* **55**, 2333–2337.
- Gallego, V., García, M. T. & Ventosa, A. (2006).** *Methylobacterium adhaesivum* sp. nov., a methylotrophic bacterium isolated from drinking water. *Int J Syst Evol Microbiol* **56**, 339–342.
- Green, P. N. (2006).** *Methylobacterium*. In *The Prokaryotes. A Handbook on the Biology of Bacteria*, 3rd edn, vol. 5, pp. 257–265. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer & E. Stackebrandt. 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.
- Green, P. N. & Bousfield, I. J. (1982).** A taxonomic study of some Gram-negative facultatively methylotrophic bacteria. *J Gen Microbiol* **128**, 623–638.
- 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. fujiisawaense* sp. nov. *Int J Syst Bacteriol* **38**, 124–127.
- Heyer, J., Galchenko, V. F. & Dunfield, P. F. (2002).** Molecular phylogeny of type II methane-oxidizing bacteria isolated from various environments. *Microbiology* **148**, 2831–2846.
- 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.
- Jourand, P., Giraud, E., Be'na, G., Sy, A., Willems, A., Gillis, M., Dreyfus, B. & de 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.
- Lee, C.-H., Tang, Y. F. & Lin, J. W. (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.
- Madhaiyan, M., Kim, B. Y., Poonguzhali, S., Kwon, S. W., Song, M. H., Ryu, J. H., Go, S. J., Koo, B. S. & Sa, T. M. (2007).** *Methylobacterium oryzae* sp. nov., an aerobic, pink-pigmented, facultatively methylotrophic, 1-aminocyclopropane-1-carboxylate deaminase-producing bacterium isolated from rice. *Int J Syst Evol Microbiol* **57**, 326–331.
- 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.
- Möller, E. M., Bahnweg, G., Sandermann, H. & Geiger, H. H. (1992).** A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. *Nucleic Acids Res* **20**, 6115–6116.
- 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.
- Saitou, N. & Nei, M. (1987).** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Urakami, T., Araki, H., Suzuki, K. & Komogata, 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., Doty, S. L., 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.
- 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.