Petrotoga mexicana sp. nov., a novel thermophilic, anaerobic and xylanolytic bacterium isolated from an oil-producing well in the Gulf of Mexico

Elizabeth Miranda-Tello,¹ Marie-Laure Fardeau,¹ Pierre Thomas,¹ Florina Ramirez,² Laurence Casalot,¹ Jean-Luc Cayol,¹ Jean-Louis Garcia¹ and Bernard Ollivier¹

Correspondence

Bernard Ollivier
ollivier@esil.univ-mrs.fr

¹IRD, UR 101 Extrémophiles, IFR-BAIM, Universités de Provence et de la Méditerranée, ESIL, Marseille, France

²Laboratorio de Tratamiento de Aguas Residuales, Universidad Autónoma Metropolitana, México, DF, Mexico

A novel anaerobic, thermophilic, xylanolytic, motile rod-shaped bacterium with a sheath-like outer structure (toga) was isolated from a Mexican oil well in the Gulf of Mexico. Strain MET12^T was a Gram-negative bacterium, reducing elemental sulfur, thiosulfate and sulfite to hydrogen sulfide. Its optimum growth conditions were 55 °C, pH 6.6, 3 % NaCl and 0.15 % MgCl₂·6H₂O. The DNA G + C content was 36.1 mol%. Phylogenetically, strain MET12^T was related to members of genus *Petrotoga*, with similarities to *Petrotoga mobilis*, *Petrotoga sibirica*, *Petrotoga miotherma* and *Petrotoga olearia* varying from 97.6 to 98.8 %. However DNA–DNA relatedness values between these species and strain MET12^T were lower than 70 %. As strain MET12^T (= DSM 14811^T = CIP 107371^T) was genomically and phenotypically different from existing *Petrotoga* species, it is proposed as the type strain of a novel species, *Petrotoga mexicana* sp. nov.

Anaerobes have always been considered as the dominant micro-organisms of petroleum reservoirs (Magot *et al.*, 2000). Among them, fermentative bacteria constitute an important microbial community of the oilfield environment. Thermophilic isolates are the most studied, probably because (i) most oil reservoirs occur at depths where the temperature is high and (ii) thermophiles possess thermostable enzymes of great interest in industrial processes. Stetter *et al.* (1993) provided evidence of the presence of *Thermotoga* strains, members of the order *Thermotogales*, in oilfields. The isolation of *Thermotoga elfii*, *Thermotoga subterranea* and *Thermotoga hypogea* from such ecosystems was reported shortly afterwards (Fardeau *et al.*, 1997a; Jeanthon *et al.*, 1995; Ravot *et al.*, 1995a). Microbial studies on various hot oil reservoirs throughout the world have provided evidence of the importance of micro-organisms morphologically and physiologically close to other members of *Thermotogales*, namely *Fervidobacterium*, *Thermosiphon*, *Geotoga* and *Petrotoga* (Grassia *et al.*, 1996; Magot *et al.*, 2000). However, no *Fervidobacterium* species originating from oilfield reservoirs has been characterized so far. Davey *et al.* (1993) first reported the presence of micro-organisms belonging to the genera *Geotoga* and *Petrotoga* in petroleum reservoirs located in Oklahoma and Texas.

Three novel species were fully characterized: *Geotoga petraea*, *Geotoga subterranea* and *Petrotoga miotherma*. Later, *Petrotoga mobilis* was isolated from a North Sea oil-production well (Lien *et al.*, 1998). *Petrotoga olearia* and *Petrotoga sibirica* were recently isolated from a continental petroleum reservoir in Western Siberia (L'Haridon *et al.*, 2002), from which L'Haridon *et al.* (2001) also isolated a novel species of the genus *Thermosiphon*. Members of the genus *Petrotoga* are thermophilic heterotrophs that grow in a broad range of salt concentrations (up to 10 % NaCl). During our microbial studies of oil waters originating from offshore reservoirs located in the Gulf of Mexico, we isolated a novel thermophilic strain phylogenetically related to the genus *Petrotoga*, which we propose to be assigned to a novel species, *Petrotoga mexicana* sp. nov.

Several strains were isolated from oil/water mixtures taken from production well heads (21-D) in Tabasco (Gulf of Mexico). Samples were collected in sterile plastic bottles and kept at room temperature until used. *P. mobilis* DSM 10674^T and *P. miotherma* DSM 10691^T were obtained from the DSMZ, whereas *P. olearia* DSM 13574^T and *P. sibirica* DSM 13575^T were obtained from the Institut Universitaire Européen de la Mer, Plouzané, France. Enrichment cultures were performed in 120 ml serum bottles containing 20 ml medium and inoculated with 2 ml water/oil sample. The medium contained (l⁻¹ distilled water): 1 g NH₄Cl, 0.3 g K₂HPO₄, 0.3 g KH₂PO₄, 0.1 g KCl, 0.1 g CaCl₂, 0.2 g

The GenBank accession number for the 16S rDNA sequence of strain MET12^T is AY125964.

MgSO₄·7H₂O, 0.5 g cysteine hydrochloride, 1 mg resazurin, 30 g NaCl and 10 ml trace mineral element solution (Balch *et al.*, 1979). The pH was adjusted to 7 with 10 M KOH. The medium was boiled under a stream of O₂-free N₂ gas and cooled to room temperature. Aliquots (20 ml) were dispensed into serum bottles under a stream of N₂/CO₂ (80:20, v/v) and the sealed vessels were autoclaved for 45 min at 110 °C. Prior to inoculation, Na₂S·9H₂O, NaHCO₃, peptone (Difco), yeast extract and sodium thiosulfate were injected from sterile stock solutions to respective final concentrations of 0.04, 0.2, 0.5 and 0.02 % (w/v) and 20 mM. H₂ pressure in the gas phase was 2 bars. The bottles were incubated at 60 °C and three enrichment series were performed in the same culture medium before isolation. Strains were isolated by repeated use of the Hungate roll-tube technique (Hungate, 1969), with medium solidified with 0.8 % phytagel (Difco). Serial dilution in roll tubes was repeated at least twice to purify the cultures. Growth experiments were performed in duplicate, using a medium differing from the enrichment medium by the absence of peptone and hydrogen in the gas phase. In addition to the basal medium, MgSO₄·7H₂O (0.2 g l⁻¹) was replaced by MgCl₂·6H₂O (1 g l⁻¹) and the concentration of yeast extract was increased to 0.1 %.

The pH, temperature and NaCl and MgCl₂ concentration ranges for growth were determined using basal medium supplemented with 20 mM glucose. The pH of the medium in Hungate tubes was adjusted by injecting 1 M HCl, NaHCO₃ or Na₂CO₃ from 10 % sterile anaerobic stock solutions. The characterized strain was subcultured twice under the same experimental conditions before growth rates were determined. All substrates were tested at a final concentration of 20 mM except for formate (80 mM), cellulose, starch and xylan (all 10 g l⁻¹). Elemental sulfur (1 %, w/v), sulfate (20 mM), thiosulfate (20 mM), sulfite (5 mM), nitrate (10 mM) and nitrite (5 mM) were tested as electron acceptors. H₂S production was determined photometrically as colloidal CuS by using the method of Cord-Ruwisch (1985). Sensitivity to ampicillin, chloramphenicol, kanamycin, rifampicin and vancomycin was tested at 10, 25, 50 and 100 µg ml⁻¹. Controls with ethanol and DMSO (solvents used for chloramphenicol and rifampicin) were performed. Growth was monitored by measuring OD₅₈₀ and by microscopic observations. Morphological characteristics were determined with an Optiphot (Nikon) phase-contrast microscope. For electron microscopic studies, preparations were negatively stained with sodium phosphotungstate as described by Koussémon *et al.* (2001). Growth was monitored by measuring OD₅₈₀ using a UV/visible spectrophotometer 50 Scan (Varian). Xylanolytic activity was measured as described previously (Fardeau *et al.*, 1997a); the assay was performed by adding dinitrosalicylic acid (Miller, 1959) and the xylose released from xylan degradation was determined from the A₅₄₀. The presence of L-alanine was determined enzymically at 340 nm as described previously (Fardeau *et al.*, 1997a). Fermentation products were determined by HPLC using a

pump (Spectra Series P100; Thermo Separation Products), an automatic sampler (Spectra Series AS100), an Aminex HPX 87H (300 × 7.8 mm) column (Bio-Rad), a differential refractometer detector (Spectra System RI-150) and an integrator (Azur Microsoft). An aliquot of 20 µl cell-free supernatant was injected into the column, which was maintained at 37 °C. A 2.5 mM H₂SO₄ solution was used as solvent with a flow rate of 0.6 ml min⁻¹. The effect of O₂ on growth was determined in Hungate tubes containing anaerobic basal medium supplemented with 20 mM glucose. Tubes were inoculated and 20 ml sterile air was added to the gas phase. Cultures were incubated at 55 °C under agitation (150 r.p.m.). Growth was monitored by turbidity measurements (580 nm) and by microscopic observations. Experiments were conducted in duplicate and repeated at least twice. The presence of spores was determined by microscopic observation of cultures and pasteurization tests at 80 and 90 °C for 10 and 20 min.

The G + C content of DNA was determined at the DSMZ, using HPLC as described by Mesbah *et al.* (1989). DNA–DNA hybridization studies were carried out by the DSMZ as described by De Ley *et al.* (1970), with the modifications described by Huß *et al.* (1983) and Escara & Hutton (1980), using a model 2600 spectrophotometer equipped with a model 2527-R thermoprogrammer and plotter (Gilford Instruments). Renaturation rates were computed with the TRANSFER.BAS program (Jahnke, 1992). The 16S rDNA of strain MET12^T was amplified by adding 1 µl cell culture to a thermocycler microtube containing 5 µl 10 × Taq buffer, 0.5 µl 100 nM primers Fd1 and Rd1, 4 µl 25 mM MgCl₂·6H₂O, 0.5 µl 25 mM dNTPs, 0.5 µl Taq polymerase (5 U µl⁻¹) and 38 µl sterilized distilled water. The primers used (Fd1, 5'-AGAGTTTGATCCTGGCTC-AG-3'; Rd1, 5'-AAGGAGGTGATCCAGCC-3') were described by Winker & Woese (1991). The sample was placed in a hybrid thermal reactor thermocycler (GeneAmp PCR System 2400), denatured by heating for 3 min at 96 °C and subjected to 30 cycles of 20 s at 95 °C, 30 s at 52.9 °C and 2 min at 72 °C; this was followed by a final elongation step of 5 min at 72 °C. PCR products were cloned using the pGEM-T-easy cloning kit (Promega) according to the manufacturer's protocol. Clone libraries were screened by direct PCR amplification from a colony using the vector-specific primers SP6 (5'-ATTTAGGTGACACTATAGAA-3') and T7 (5'-TAATACGACTCACTATAGGG-3') and the following reaction conditions: 2 min at 96 °C, 40 cycles of 30 s at 94 °C, 1 min at 55 °C, 3 min at 72 °C, and a final extension of 10 min at 72 °C. Plasmids containing inserts of the expected length were isolated using the Wizard Plus SV Minipreps DNA purification system (Promega), according to the manufacturer's protocol. Purified plasmids were sent for sequencing to Genome Express (Grenoble, France). Sequence data were imported into the sequence editor BioEdit version 5.0.9 (Hall, 1999); base calling was examined and a contiguous consensus sequence was obtained. The full sequence was aligned using the RDP Sequence Aligner program (Maidak *et al.*, 2001). The

consensus sequence was then manually adjusted to conform to the 16S rDNA secondary structure model (Winker & Woese, 1991). A non-redundant BLASTN search (Altschul *et al.*, 1997) of the full sequence through GenBank (Benson *et al.*, 1999) identified its closest relative. Sequences used in the phylogenetic analysis were obtained from the RDP (Maidak *et al.*, 2001) and GenBank (Benson *et al.*, 1999). Positions of sequence and alignment ambiguity were omitted and pairwise evolutionary distances were calculated using the method of Jukes & Cantor (1969). A dendrogram was constructed using the neighbour-joining method (Saitou & Nei, 1987). Confidence in the tree topology was determined by using 100 bootstrapped trees (Felsenstein, 1985). rDNA accession numbers of reference organisms are included in Fig. 2. DNA–DNA hybridizations were performed at the DSMZ as described by Koussémon *et al.* (2001).

Enrichment cultures were incubated at 60 °C for 1 week. Growth was regarded as positive based on optical density. Microscopic examination of the enrichment culture revealed the presence of rods with an outer, sheath-like structure (toga). Circular, transparent colonies, 2.5 mm in diameter, appeared after a 6 day incubation at 60 °C in solidified roll tubes containing phytagel. Three strains of rod-shaped bacteria were isolated that were morphologically and phylogenetically identical. Strain MET12^T was characterized further. Individual cells were 0.5–0.7 µm wide and 1.4–5.0 µm long and occurred singly, in pairs or in chains up to 30 µm long (Fig. 1a). Cells were surrounded by a sheath-like structure (Fig. 1b). They were motile and no spore formation was detected. Electron microscopy of cell sections exhibited a Gram-negative-type cell wall (Fig. 1c). Strain MET12^T was thermophilic and grew at temperatures ranging from 25 to 65 °C, with an optimum at 55 °C. The isolate was slightly halophilic and grew in the presence of 10–200 g NaCl l⁻¹, with an optimum at 30 g l⁻¹, and 0–200 g MgCl₂·6H₂O l⁻¹ (the basal culture medium contained 30 g NaCl l⁻¹) with an optimum at 1.5 g l⁻¹. The optimum pH for growth was 6.6 and growth occurred between pH 5.6 and 8.5. The isolate did not grow under aerobic conditions. Strain MET12^T fermented D-arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, raffinose, rhamnose, ribose, starch, sucrose, xylose, xylan and pyruvate. Casamino acids and peptone were only used in the presence of thiosulfate. Glucose was fermented into acetate as the major end product, together with lactate, H₂ and CO₂. L-Alanine was produced in trace amounts. Substrates that were not used were: acetate, butyrate, formate, fumarate, lactate, malate, butanol, methanol, propanol, methylamine, trimethylamine and H₂. The xylanase activity was shown to be endocellular. The isolate used elemental sulfur, thiosulfate and sulfite but not sulfate, nitrate or nitrite as electron acceptors. Strain MET12^T could grow in the presence of 10 µg chloramphenicol, kanamycin and vancomycin ml⁻¹ (no growth was obtained at 25 µg ml⁻¹) and in the presence of 25 µg ampicillin and rifampicin ml⁻¹ (no growth was obtained at

Fig. 1. (a) Phase-contrast micrograph of cells of strain MET12^T; bar, 5 µm. (b) Transmission electron micrograph of cells showing toga; bar, 2 µm. (c) Electron micrograph of an ultrathin section showing the Gram-negative cell-wall structure; bar, 0.25 µm.

50 µg ml⁻¹). The G+C content of strain MET12^T was 36.1 mol% (HPLC).

16S rDNA sequence analysis performed on 1195 unambiguous nucleotides revealed that strain MET12^T was a

Fig. 2. Phylogenetic dendrogram based on 16S rDNA sequences indicating the position of strain MET12^T amongst all known species of the family *Thermotogaceae*. Accession numbers of 16S rDNA sequences of reference organisms are included. Bootstrap values, expressed as percentages of 100 replications, are shown at branching points. Only values above 80% are shown. Bar, 2 nucleotide substitutions per 100 nucleotides.

member of the genus *Petrotoga* (family *Thermotogaceae*, order *Thermotogales*), with *P. miotherma* Davey *et al.* 1993 (98.9% similarity) and *P. olearia* L'Haridon *et al.* 2002 (98.6% similarity) being its closest phylogenetic relatives. Similarities to the two other species of the genus *Petrotoga* were 98.1% (*P. sibirica*) and 97.6% (*P. mobilis*) (Fig. 2). DNA–DNA hybridization studies indicated that strain MET12^T showed DNA–DNA relatedness of 57.1% to *P. mobilis*, 48.9% to *P. olearia*, 53.4% to *P. sibirica* and $65.4 \pm 3.2\%$ to *P. miotherma*.

Moderate to extreme thermophilic anaerobes belonging to the *Bacteria* and *Archaea* inhabit oil reservoirs originating from terrestrial or marine environments (Magot *et al.*, 2000). However, more attention has been paid so far to micro-organisms of the domain *Bacteria* belonging to the families *Thermoanaerobiaceae* and, particularly, *Thermotogaceae*, being well-known inhabitants of these terrestrial- and marine-subsurface ecosystems (Davey *et al.*, 1993; Fardeau *et al.*, 1997a; Grassia *et al.*, 1996; L'Haridon *et al.*, 2001, 2002; Lien *et al.*, 1998; Magot *et al.*, 2000; Ravot *et al.*, 1995a; Stetter *et al.*, 1993). The order *Thermotogales* and family *Thermotogaceae* comprise members of the genera *Thermotoga*, *Fervidobacterium*, *Thermosipho*, *Marinitoga*, *Petrotoga* and *Geotoga* (Alain *et al.*, 2002; Reysenbach, 2001; Wery *et al.*, 2001). Amongst these genera, strains belonging to *Petrotoga* and *Geotoga* have been isolated so far only from oil reservoirs (Davey *et al.*, 1993; L'Haridon *et al.*, 2002; Lien *et al.*, 1998). Based on phylogenetic studies, we provide evidence of the existence of a novel isolate (strain MET12^T) of the genus *Petrotoga* that inhabits an oil reservoir in the Gulf of Mexico. This confirms the close ecological relationship between micro-organisms of this genus and the oilfield ecosystem. Strain MET12^T has *P. miotherma* and *P. olearia* as its closest phylogenetic relatives, but DNA–DNA hybridization studies (values < 70%) with both these species and with *P. sibirica* and *P. olearia* allowed its assignment to a novel bacterial species (Wayne *et al.*, 1987). In addition, strain MET12^T possesses the highest G + C content within the genus *Petrotoga* (Table 1).

Besides phylogenetic and genomic differences between

strain MET12^T and other *Petrotoga* species, we also found numerous phenotypic differences. Strain MET12^T tolerates the highest NaCl concentration recorded in the genus (up to 20%, compared with a mean of 10% for the other species) (Table 1). Strain MET12^T differs from *P. olearia* and *P. sibirica* (L'Haridon *et al.*, 2002) by using thiosulfate as a terminal electron acceptor (Table 1). It also differs from *P. mobilis* (Lien *et al.*, 1998) by using raffinose, mannose and pyruvate. In contrast to *P. miotherma*, strain MET12^T is motile. Therefore, the combined phylogenetic, genomic and phenotypic characteristics of strain MET12^T allow us to assign it to the genus *Petrotoga* as the type strain of a novel species, *Petrotoga mexicana* sp. nov.

It is noteworthy that thiosulfate can be used as an electron acceptor and that L-alanine is a minor end product of glucose metabolism by *P. mexicana*. L-Alanine production together with thiosulfate reduction have been found as quite common metabolic features within members of the order *Thermotogales* (Ravot *et al.*, 1995b, 1996). Because of the position of these micro-organisms in the deepest branches of the phylogenetic tree, L-alanine production from glucose fermentation has been interpreted as a remnant of an ancestral metabolism (Ravot *et al.*, 1996). A thiosulfate-reducing activity shared by several members of this order might (i) help them when oxidizing hydrogen or amino acids such as leucine, isoleucine and valine (Fardeau *et al.*, 1993, 1997b) or (ii) have an undesirable influence on biocorrosion due to sulfide production (Magot *et al.*, 2000). The occurrence of *Petrotoga* species only in oil reservoirs so far raises questions about (i) their survival in this deep subsurface environment and (ii) their possible indigeneity to this peculiar ecosystem. Further microbial and ecological studies are needed to appreciate their impact in the oilfield environment.

Description of *Petrotoga mexicana* sp. nov.

Petrotoga mexicana (me.xi.ca'na. N.L. fem. adj. *mexicana* Mexican, describing the site of sampling of the type strain).

Cells are motile rods, $0.5\text{--}0.7 \times 1.4\text{--}5.0\text{ }\mu\text{m}$, with an outer, sheath-like structure (toga), occurring singly, in pairs or in

Table 1. Differentiating characteristics between strain MET12^T and *Petrotoga* species

Species/strain: 1, *P. miotherma* (unless otherwise stated, data from Davey *et al.*, 1993); 2, *P. mobilis* (Lien *et al.*, 1998); 3, *P. olearia* (L'Haridon *et al.*, 2002); 4, *P. sibirica* (L'Haridon *et al.*, 2002); 5, *P. mexicana* sp. nov. (this study).

Characteristic	1	2	3	4	5
Type strain	ATCC 51224 ^T	DSM 10674 ^T	DSM 13574 ^T	DSM 13575 ^T	DSM 14811 ^T
Source	Petroleum reservoir	Hot oilfield water	Petroleum reservoir	Petroleum reservoir	Oilfield water
Cell size (µm)	0.6 × 2.0–7.5	0.5–1.5 × 1.0–50.0	0.3–0.6 × 0.9–2.5	0.3–0.6 × 0.9–2.5	0.5–0.7 × 1.4–30
No. of cells per sheath	1–5	1–20	1–2	1–2	1–10
Motility	–*	+	+	+	+
Spherical bodies	+	–	+	+	+
Temperature range (°C) [optimum]	35–65 [55]	40–65 [58–60]	37–60 [55]	37–55 [55]	30–65 [55]
pH range [optimum]	5.5–9.0 [6.5]	5.5–8.5 [6.5–7.0]	6.5–8.5 [7.5]	6.5–9.4 [8.0]	5.9–8.5 [6.6]
NaCl range (% w/v) [optimum]	0.5–10 [2]	0.5–9 [3–4]	0.5–8 [2]	0.5–7 [1]	1–20 [3]
Reduction of S ₂ O ₃ ^{2–}	+†	+	–	–	+
G + C content (mol%)‡	34.2 ^a /32 ^b †	34 ^a /31 ^b	35 ^a	33 ^a	36.1 ^a

*Data from this study.

†Data from Lien *et al.* (1998).

‡Determined by: *a*, HPLC method; *b*, thermal denaturation method.

chains up to 30 µm long. Electron microscopy of cell sections exhibits a Gram-negative-type cell wall. No spore formation is detected. Growth occurs from 25 to 65 °C (optimum 55 °C). Grows in the presence of 10–200 g NaCl l^{–1} (optimum 30 g l^{–1}) and 0–200 g MgCl₂·6H₂O l^{–1} (optimum 1.5 g l^{–1}). Growth occurs between pH 5.8 and 8.5 (optimum 6.6). Ferments D-arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, raffinose, rhamnose, ribose, starch, sucrose, xylose, xylan and pyruvate. The xylanase activity is endocellular. Glucose is fermented into acetate, lactate, H₂ and CO₂, with L-alanine as a minor end product. Casamino acids and peptone are used in the presence of thiosulfate. Acetate, butyrate, formate, fumarate, lactate, malate, butanol, methanol, propanol, methylamine, trimethylamine and H₂ are not used. Uses elemental sulfur, thiosulfate and sulfite but not sulfate, fumarate, nitrate or nitrite as electron acceptors. Grows in the presence of 10 µg chloramphenicol, kanamycin and vancomycin ml^{–1} and 25 µg ampicillin and rifampicin ml^{–1}. The G + C content is 36.1 mol% (HPLC).

The type strain, MET12^T (=DSM 14811^T=CIP 107371^T), was isolated from an oil well located in the Gulf of Mexico.

Acknowledgements

We thank Luis Fernández (Instituto Mexicano del Petróleo) for sampling. We also acknowledge financial support to E.M.-T. from Consejo Nacional de Ciencia y Tecnología, Secretaría de Educación Pública and Société Française d'Exportation des Ressources Éducatives. Many thanks to D. Alazard for valuable advice and P. Roger for improving the manuscript.

References

Alain, K., Marteinsson, V. T., Miroshnichenko, M. L., Bonch-Osmolovskaya, E. A., Prieur, D. & Birrien, J.-L. (2002). *Marinitoga*

piezophila sp. nov., a rod-shaped, thermo-piezophilic bacterium isolated under high hydrostatic pressure from a deep-sea hydrothermal vent. *Int J Syst Evol Microbiol* **52**, 1331–1339.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Balch, W. E., Fox, G. E., Magrum, R. J., Woese, C. R. & Wolfe, R. S. (1979). Methanogens: reevaluation of a unique biological group. *Microbiol Rev* **43**, 260–296.

Benson, D. A., Boguski, M. S., Lipman, D. J., Oullette, B. F. F., Rapp, B. A. & Wheeler, D. L. (1999). GenBank. *Nucleic Acids Res* **27**, 12–17.

Cord-Ruwisch, R. (1985). A quick method for the determination of dissolved and precipitated sulfides in cultures of sulfate-reducing bacteria. *J Microbiol Methods* **4**, 33–36.

Davey, M. E., Wood, W. A., Key, R., Nakamura, K. & Stahl, D. A. (1993). Isolation of three species of *Geotoga* and *Petrotoga*: two new genera, representing a new lineage in the bacterial line of descent distantly related to the “*Thermotogales*”. *Syst Appl Microbiol* **16**, 191–200.

De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.

Escara, J. F. & Hutton, J. R. (1980). Thermal stability and renaturation of DNA in dimethyl sulfoxide solutions; acceleration of the renaturation rate. *Biopolymers* **19**, 1315–1327.

Fardeau, M.-L., Cayol, J.-L., Magot, M. & Ollivier, B. (1993). H₂ oxidation in the presence of thiosulfate by a *Thermoanaerobacter* strain isolated from an oil-producing well. *FEMS Microbiol Lett* **113**, 327–332.

Fardeau, M.-L., Ollivier, B., Patel, B. K. C., Magot, M., Thomas, P., Rimbault, A., Rocchiccioli, F. & Garcia, J.-L. (1997a). *Thermotoga hypogea* sp. nov., a xylanolytic, thermophilic bacterium from an oil-producing well. *Int J Syst Bacteriol* **47**, 1013–1019.

Fardeau, M.-L., Patel, B. K. C., Magot, M. & Ollivier, B. (1997b). Utilization of serine, leucine, isoleucine, and valine by *Thermoanaerobacter brockii* in the presence of thiosulfate or *Methanobacterium* sp. as electron acceptors. *Anaerobe* **3**, 405–410.

- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Grassia, G. S., McLean, K. M., Glénat, P., Bauld, J. & Sheehy, A. J. (1996). A systematic survey for thermophilic fermentative bacteria and archaea in high temperature petroleum reservoirs. *FEMS Microbiol Ecol* **21**, 47–58.
- Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98 NT. *Nucleic Acids Symp Ser* **41**, 95–98.
- Hungate, R. E. (1969). A roll-tube method for the cultivation of strict anaerobes. *Methods Microbiol* **3B**, 117–132.
- HuB, V. A. R., Festl, H. & Schleifer, K. H. (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.
- Jahnke, K. D. (1992). Basic computer program for evaluation of spectroscopic DNA renaturation data from Gilford System 2600 spectrometer on a PC/XT/AT type personal computer. *J Microbiol Methods* **15**, 61–73.
- Jeanthon, C., Reysenbach, A. L., L'Haridon, S., Gambacorta, A., Pace, N. R., Glénat, P. & Prieur, D. (1995). *Thermotoga subterranea* sp. nov., a new thermophilic bacterium isolated from a continental oil reservoir. *Arch Microbiol* **164**, 91–97.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 211–232. Edited by H. N. Munro. New York: Academic Press.
- Koussémon, M., Combet-Blanc, Y., Patel, B. K. C., Cayol, J.-L., Thomas, P., Garcia, J.-L. & Ollivier, B. (2001). *Propionibacterium microaerophilum* sp. nov., a microaerophilic bacterium isolated from olive mill wastewater. *Int J Syst Evol Microbiol* **51**, 1373–1382.
- L'Haridon, S., Miroshnichenko, M. L., Hippe, H., Fardeau, M.-L., Bonch-Osmolovskaya, E., Stackebrandt, E. & Jeanthon, C. (2001). *Thermosipho geolei* sp. nov., a thermophilic bacterium isolated from a continental petroleum reservoir in Western Siberia. *Int J Syst Evol Microbiol* **51**, 1327–1334.
- L'Haridon, S., Miroshnichenko, M. L., Hippe, H., Fardeau, M.-L., Bonch-Osmolovskaya, E. A., Stackebrandt, E. & Jeanthon, C. (2002). *Petrotoga olearia* sp. nov. and *Petrotoga sibirica* sp. nov., two thermophilic bacteria isolated from a continental petroleum reservoir in Western Siberia. *Int J Syst Evol Microbiol* **52**, 1715–1722.
- Lien, T., Madsen, M., Rainey, F. A. & Birkeland, N.-K. (1998). *Petrotoga mobilis* sp. nov., from a North Sea oil-production well. *Int J Syst Bacteriol* **48**, 1007–1013.
- Magot, M., Ollivier, B. & Patel, B. K. C. (2000). Microbiology of petroleum reservoirs. *Antonie van Leeuwenhoek* **77**, 103–116.
- Maidak, B. L., Cole, J. R., Lilburn, T. G. & 7 other authors (2001). The RDP-II (Ribosomal Database Project). *Nucleic Acids Res* **29**, 173–174.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem* **31**, 426–428.
- Ravot, G., Magot, M., Fardeau, M.-L., Patel, B. K. C., Prensier, G., Egan, A., Garcia, J.-L. & Ollivier, B. (1995a). *Thermotoga elfi* sp. nov., a novel thermophilic bacterium from an African oil-producing well. *Int J Syst Bacteriol* **45**, 308–314.
- Ravot, G., Ollivier, B., Magot, M., Patel, B. K. C., Crolet, J.-L., Fardeau, M.-L. & Garcia, J.-L. (1995b). Thiosulfate reduction, an important physiological feature shared by members of the order *Thermotogales*. *Appl Environ Microbiol* **61**, 2053–2055.
- Ravot, G., Ollivier, B., Fardeau, M.-L., Patel, B. K. C., Andrews, K. T., Magot, M. & Garcia, J.-L. (1996). L-Alanine production from glucose fermentation by hyperthermophilic members of the domains *Bacteria* and *Archaea*: a remnant of an ancestral metabolism? *Appl Environ Microbiol* **62**, 2657–2659.
- Reysenbach, A.-L. (2001). Order I. *Thermotogales* ord. nov. Huber and Stetter 1992c, 3809. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 1, p. 369. Edited by D. R. Boone, R. W. Castenholz & G. M. Garrity. New York: Springer.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Stetter, K. O., Huber, R., Blöchl, E., Kurr, M., Eden, R. D., Fielder, M., Cash, H. & Vance, I. (1993). Hyperthermophilic archaea are thriving in deep North Sea and Alaskan oil reservoirs. *Nature* **365**, 743–745.
- 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.
- Wery, N., Lesongeur, F., Pignet, P., Derennes, V., Cambon-Bonavita, M.-A., Godfroy, A. & Barbier, G. (2001). *Marinitoga camini* gen. nov., sp. nov., a rod-shaped bacterium belonging to the order *Thermotogales*, isolated from a deep-sea hydrothermal vent. *Int J Syst Evol Microbiol* **51**, 495–504.
- Winker, S. & Woese, C. R. (1991). A definition of the domains *Archaea*, *Bacteria* and *Eucarya* in terms of small subunit ribosomal RNA characteristics. *Syst Appl Microbiol* **13**, 161–165.