Petrotoga halophila sp. nov., a thermophilic, moderately halophilic, fermentative bacterium isolated from an offshore oil well in Congo

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A novel thermophilic, moderately halophilic, rod-shaped bacterium, strain MET-B^T, with a sheath-like outer structure (toga) was isolated from an offshore oil-producing well in Congo, West Africa. Strain MET-B^T was a Gram-negative bacterium with the ability to reduce elemental sulfur, but not sulfate, thiosulfate or sulfite into sulfide. The optimum growth conditions were 60 °C, pH 6.7–7.2 and 4–6 % NaCl. The DNA G + C content was 34.6 mol%. Strain MET-B^T was phylogenetically related to members of the genus *Petrotoga*; *Petrotoga miotherma*, *Petrotoga olearia* and *Petrotoga mexicana* were the closest relatives, with type strains exhibiting more than 99 % identity in an analysis of small-subunit rRNA gene sequences. The values for DNA–DNA relatedness between the type strains of these three species and strain MET-B^T were less than 42 %. As MET-B^T was found to be genetically and physiologically different from other species of the genus *Petrotoga*, this strain is proposed as representing a novel species, for which the name *Petrotoga halophila* sp. nov. is proposed. The type strain is MET-B^T (= DSM 16923^T = CCUG 50214^T).

Members of the order *Thermotogales* (e.g. *Thermotoga*, *Geotoga* and *Petrogoga* species), considered as exclusively thermophilic micro-organisms, are known as common inhabitants of petroleum reservoirs (Magot *et al.*, 2000; Ollivier & Cayol, 2005). Their occurrence within the oilfield environment was established in the 1990s by Stetter *et al.* (1993) and Davey *et al.* (1993). However, in contrast to *Thermotoga* species, which have been isolated from various hot ecosystems throughout the world (e.g. thermal springs, hydrothermal vents), *Geotoga* and *Petrotoga* species have been found only in oil reservoirs (Ollivier & Cayol, 2005). In this respect, it has been suggested that members of the latter two genera might represent typical indigenous micro-organisms in this particular subterrestrial ecosystem (Ollivier & Cayol, 2005). Two *Geotoga* species and five *Petrotoga* species have been characterized so far. The genus *Petrotoga* comprises *Petrotoga miotherma*, *P. mobilis*, *P.*

olearia and *P. sibirica*, which originated from brines obtained from petroleum reservoirs located in Oklahoma and Texas (Davey *et al.*, 1993), a North Sea oil-production well (Lien *et al.*, 1998) and a continental petroleum reservoir in Western Siberia (*P. olearia* and *P. sibirica*; L'Haridon *et al.*, 2002), respectively. More recently, *Petrotoga mexicana* was recovered from an oil reservoir in the Gulf of Mexico (Miranda-Tello *et al.*, 2004).

Here we report on the isolation and characterization of a novel bacterial strain (MET-B^T), isolated from an offshore oil well in Africa. Although this strain is phylogenetically closely related to the five species of the genus *Petrotoga*, it exhibits significant phenotypic and genetic differences that are consistent with its placement within a novel species.

Strain MET-B^T was isolated from an oil/water mixture collected from well TBM111 of the Tchibouella oilfield (Congo). The oil produced was slightly biodegraded, with

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MET-B^T is AY800102.

selective and complete removal of the n-alkanes (Connan *et al.*, 1996), and the in-situ temperature and NaCl concentration were 45 °C and 98 g l⁻¹. The samples were collected in sterile glass bottles and kept at 4 °C until use. *P. miotherma* ATCC 51224^T was obtained from the American Type Culture Collection (Manassas, VA, USA). *P. olearia* DSM 13574^T was obtained from the Institut Universitaire Européen de la Mer (Plouzané, France) and *P. mexicana* DSM 14811^T was obtained from our culture collection in Marseille.

The enrichment culture was performed in 60 ml serum bottles inoculated with 2 ml sample. The medium contained the following (l⁻¹ distilled water): 0.2 g NH₄Cl, 0.3 g K₂HPO₄, 0.3 g KH₂PO₄, 0.1 g KCl, 0.1 g CaCl₂, 0.5 g MgCl₂·6H₂O, 90 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; 20 ml was dispensed into serum bottles under a stream of N₂/CO₂ (80:20, v/v) gas, and the sealed vessels were autoclaved for 45 min at 110 °C. Prior to inoculation, yeast extract, potassium nitrate and sodium acetate were injected from sterile stock solutions to final concentrations of 1 g l⁻¹, 10 mM and 20 mM, respectively. The bottles were incubated at 50 °C in a controlled-temperature incubator and three enrichment series were performed in the same medium before isolation. The strain was isolated by repeated use of the Hungate roll-tube technique (Hungate, 1969), using medium solidified with 0.8 % Phytagel (Difco). The process of serial dilution in roll tubes was repeated at least twice in order to purify the cultures.

Growth experiments were performed in duplicate, using Hungate tubes containing basal medium. The basal medium was the same as the enrichment medium but without potassium nitrate or sodium acetate and with 0.2 % yeast extract.

The pH, temperature and NaCl concentration ranges for growth were determined using basal medium supplemented with 20 mM glucose. The pH of the medium was adjusted by injecting aliquots of anaerobic stock solutions of 1 M HCl (acidic pH), 10 % NaHCO₃ or Na₂CO₃ (basic pH) into Hungate tubes. Water baths were used to obtain incubation temperatures ranging from 37 to 70 °C. To study NaCl requirements, NaCl was weighed directly in the tubes before the medium was dispensed. The strain was subcultured under the same experimental conditions before determination of the growth rate. Each substrate was tested in basal medium at a final concentration of 20 mM, except for starch and xylan (10 g l⁻¹). Elemental sulfur (1 %, w/v), sulfate (20 mM), thiosulfate (20 mM), sulfite (2 mM), nitrate (10 mM) and nitrite (2 mM) were tested as terminal electron acceptors. H₂S production was determined photometrically as colloidal CuS by using the method of Cord-Ruwisch (1985). Nitrate reduction was evaluated in the absence of ammonium chloride in the culture medium by measuring ammonium or nitrite production. The sensitivity of strain MET-B^T to chloramphenicol, kanamycin,

rifampicin and vancomycin was tested at 10, 25, 50 and 100 µg ml⁻¹. Controls containing ethanol and DMSO (solvents for chloramphenicol and rifampicin) were included. Growth was monitored by measuring OD₅₈₀ values and by making microscopic observations. Morphological characteristics and purity were observed with an Optiphot (Nikon) phase-contrast microscope. For transmission electron microscopy studies, cell preparations were negatively stained with sodium phosphotungstate, as described by Fardeau *et al.* (1997). Throughout the analytical studies, duplicate culture tubes were used. Analytical techniques were performed as described by Miranda-Tello *et al.* (2004). The effect of O₂ on growth was determined in Hungate tubes containing anaerobic basal medium supplemented with 20 mM glucose. Tubes were inoculated and various amounts of sterile air were added to the gas phase. The cultures were incubated at 55 °C with agitation at 150 r.p.m. Growth was monitored using turbidity measurements (580 nm) and microscopic observations. All of the experiments were conducted in duplicate and were repeated at least twice. The presence of spores was checked using microscopic observation of cultures and pasteurization tests performed at 80, 90 and 100 °C for 10 and 20 min.

The G+C content of the DNA was determined at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) (Braunschweig, Germany), using HPLC as described by Mesbah *et al.* (1989).

Genomic DNA of strain MET-B^T was extracted with the Wizard Genomic DNA purification kit (Promega), and the small-subunit rRNA gene (positions 8–1492, *Escherichia coli* numbering) was PCR-amplified (30 cycles) with the eubacteria-specific primers 8F (5'-AGAGTTTGATCCTGG-CTCAG-3') and 1492R (5'-GTCGTAACAAGGTAACCG-TA-3'), as described previously (Thabet *et al.*, 2004). The PCR product was purified (Nucleo Spin Extract kit; Macherey Nagel) and cloned using the pGEM-T-easy cloning kit (Promega). A plasmid containing an insert of the correct length was purified with the Wizard Plus SV Minipreps DNA purification system (Promega). The inserts of three plasmids obtained from independent cloning experiments were sequenced by Genome Express (Grenoble, France) using primers targeting the vector. The small-subunit rRNA gene sequence of strain MET-B^T and reference sequences available in GenBank (Benson *et al.*, 1999) were aligned using the sequence-aligner software from Ribosomal Database Project II (Maidak *et al.*, 2001) and the sequence alignment editor BioEdit (Hall, 1999). Phylogenetic analyses were conducted using programs implemented in the TREECON package (Van de Peer & De Wachter, 1994). Pairwise evolutionary distances of 1248 unambiguous nucleotides were computed using the method of Jukes & Cantor (1969). A phylogenetic tree was constructed using the neighbour-joining method (Saito & Nei, 1987). The robustness of the tree topology was tested by a bootstrap analysis involving 1000 resamplings (Felsenstein, 1985).

DNA–DNA hybridizations were performed at the DSMZ, as described previously (Miranda-Tello *et al.*, 2004).

Enrichment cultures were incubated at 50 °C for 2 weeks. Microscopic examination of the enrichment culture revealed the presence of rods with an outer sheath-like structure (toga). Circular, white colonies that were 2.0 mm in diameter appeared after 1 week incubation in roll tubes at 50 °C. Several non-motile strains similar in morphology, unable to reduce thiosulfate and producing the same end-products of glucose metabolism were isolated, but only strain MET-B^T was characterized further. The cells were 0.5–0.7 µm in width and 2–45 µm in length and occurred singly or in sheaths containing up to five cells (data not shown). The cells were not motile and no spore formation was detected. Electron microscopy of thin sections of strain MET-B^T revealed a typical toga cell-wall ultrastructure with a spongy periplasmic layer (data not shown). The cells stained Gram-negative. Strain MET-B^T was thermophilic and grew at temperatures ranging from 45 to 65 °C, with an optimum at 60 °C. The isolate was moderately halophilic and grew in the presence of NaCl concentrations ranging from 5 to 90 g l⁻¹, with an optimum between 40 and 60 g l⁻¹. The optimum pH range for growth was 6.7–7.2 and growth occurred between pH 5.6 and 7.8. Strain MET-B^T fermented D-arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, L-rhamnose, D-ribose, starch, sucrose, D-xylose, xylan and pyruvate. Glucose was fermented into acetate, lactate, L-alanine, H₂ and CO₂. No L-alanine was detected when strain MET-B^T was cultivated in the presence of elemental sulfur as the terminal electron acceptor. The following substrates were not used: acetate, fumarate, succinate, mannose, raffinose, methanol, Casamino acids, peptone and H₂ + CO₂. Sulfate, thiosulfate, sulfite, nitrate and nitrite were not used as terminal electron acceptors. Strain MET-B^T could grow with chloramphenicol, kanamycin or vancomycin (at 10 µg ml⁻¹ in each case) or 50 µg rifampicin ml⁻¹.

Members of the order *Thermotogales*, comprising anaerobic, rod-shaped micro-organisms whose cells are surrounded by sheath-like structures (like strain MET-B^T), are recognized as common inhabitants of deep, hot oil reservoirs located in marine or continental ecosystems (Magot *et al.*, 2000; Ollivier & Cayol, 2005). Of these, it is noteworthy that species belonging to the genera *Geotoga* or *Petrotoga* have been isolated only from petroleum environments, indicating that they might be indigenous to moderately deep and hot oil reservoirs (Ollivier & Cayol, 2005). The genus *Petrotoga* comprises five species, each of which is considered as being moderately thermophilic to thermophilic (i.e. with an optimum temperature for growth between 55 and 60 °C) heterotrophs growing in habitats covering a broad range of salt concentrations (0.5–20 %, depending on the species). In addition, they have DNA G + C contents ranging from 34 to 36.1 mol%. Culture enrichments that we performed to recover heterotrophic nitrate-reducing micro-organisms from an offshore oil well in Congo (West Africa) led to

the isolation of strain MET-B^T, which was able to use glucose as an energy source, but unable to use nitrate as a terminal electron acceptor. This strain showed phenotypic characteristics and genomic properties (e.g. a DNA G + C content of 34.6 mol%) that were consistent with its placement within the genus *Petrotoga* rather than the genus *Geotoga*. Moreover, small-subunit rRNA gene analysis based on 1248 unambiguous nucleotides confirmed that strain MET-B^T should be considered as a member of the genus *Petrotoga*, its closest phylogenetic relatives being the type strains of *P. miotherma* (Davey *et al.*, 1993), *P. olearia* (L'Haridon *et al.*, 2002), *P. mexicana* (Miranda-Tello *et al.*, 2004) and *P. sibirica* (L'Haridon *et al.*, 2002), which showed sequence similarities of 99.7, 98.8, 99.0 and 98.5 %, respectively (Fig. 1). *P. sibirica* clearly differs from our isolate and the *Petrotoga* species cited above as it does not grow at 60 °C and does not use the same range of substrates (Table 1). Moreover, despite the existence of close phylogenetic relationships between strain MET-B^T and *P. miotherma*, *P. olearia* and *P. mexicana*, a DNA–DNA hybridization analysis revealed relatedness values of only 41.9, 25.9 and 31.1 %, respectively, with respect to the type strains of these species. Therefore, according to the criteria of Wayne *et al.* (1987), strain MET-B^T is not related to these bacteria at the species level and should be considered as a novel species within the genus *Petrotoga*. In contrast to *P. miotherma*, strain MET-B^T fermented xylan and did not produce ethanol from sugar metabolism, but produced lactate (Table 1). Strain MET-B^T differed from *P. olearia* by the absence of motility, by not using peptone and by fermenting galactose (Table 1). Phenotypic differences were also observed between *P. mexicana* and strain MET-B^T, as the latter did not use thiosulfate as a terminal electron acceptor (Table 1). Finally, the optimum NaCl

Fig. 1. Phylogenetic dendrogram, based on small-subunit rRNA gene sequence data, indicating the position of strain MET-B^T with respect to all known species of the family *Thermotogaceae*. Bootstrap values above 50 % are shown. Bar, 2 nucleotide substitutions per 100 nucleotides.

Table 1. Characteristics useful for differentiating between strain MET-B^T and phylogenetically related *Petrotoga* species

Strains: 1, *P. miortherma* ATCC 51224^T (Davey *et al.*, 1993); 2, *P. mobilis* DSM 10674^T (Lien *et al.*, 1998); 3, *P. olearia* DSM 13574^T (L'Haridon *et al.*, 2002); 4, *P. sibirica* DSM 13575^T (L'Haridon *et al.*, 2002); 5, *P. mexicana* DSM 14811^T (Miranda-Tello *et al.*, 2004); 6, strain MET-B^T (this study). ND, No data available.

Characteristic	1	2	3	4	5	6
Source	Petroleum reservoir	Hot oilfield water	Petroleum reservoir	Petroleum reservoir	Oil well water	Oil well 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	0.5–0.7 × 2–45
Cells per sheath (<i>n</i>)	1–5	1–20	1–2	1–2	1–10	1–5
Motility	–	+	+	+	+	–
Spherical bodies	+	–	+	+	+	+
Range [optimum] for growth						
Temperature (°C)	35–65 [55]	40–65 [58–60]	37–60 [55]	37–55 [55]	30–65 [55]	45–65 [60]
pH	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]	5.6–7.8 [6.7–7.2]
NaCl (%)	0.5–10 [2]	0.5–9 [3–4]	0.5–8 [2]	0.5–7 [1]	1–20 [3]	0.5–9 [4–6]
Reduction of thiosulfate	+	+	–	–	+	–
DNA G + C content (mol%)†	34.2 ^a /32 ^b	34 ^a /31 ^b	35 ^b	33 ^b	36.1 ^a	34.6 ^a
Substrate utilization						
Arabinose	+	+	+	–	+	+
Cellobiose	ND	+	+	–	+	+
Galactose	+	+	–	+	+	+
Xylan	–	+	+	+	+	+
Xylose	+	+	+	–	+	+

*Data from Lien *et al.* (1998).

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

concentration for growth strain of MET-B^T was higher (4–6 %) than that of any *Petrotoga* species, so the novel isolate should be considered as a moderate halophile (Table 1). Therefore, on the basis of the phenotypic and phylogenetic data in this study, strain MET-B^T represents a novel species within the genus *Petrotoga*, for which the name *Petrotoga halophila* sp. nov. is proposed.

Description of *Petrotoga halophila* sp. nov.

Petrotoga halophila (ha.lo'phi.la. Gr. n. *halos* salt; Gr. adj. *philos* loving; N.L. fem. adj. *halophila* salt-loving).

Cells are rods, 0.5–0.7 × 2–45 µm in size, with an outer sheath-like structure (toga), occurring singly or in sheaths. Electron microscopy of sections of cells exhibits a Gram-negative-type cell wall. Flagella are not observed. No spore formation is detected. Grows at temperatures in the range 45–65 °C, with an optimum at 60 °C. Grows in the presence of NaCl at concentrations ranging from 5 to 90 g l⁻¹, with an optimum at 40–60 g l⁻¹. The optimum pH for growth is 6.7–7.2, but growth occurs between pH 5.6 and 7.8. Ferments D-arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, rhamnose, ribose, starch, sucrose, xylose, xylan and pyruvate. Under anaerobic conditions, glucose is fermented into acetate, lactate, L-alanine, H₂ and CO₂. No L-alanine is produced in the presence of elemental sulfur as the terminal electron acceptor. Acetate, fumarate,

succinate, mannose, raffinose, methanol, Casamino acids, peptone and H₂ are not used. Elemental sulfur is used as a terminal electron acceptor, but sulfate, sulfite, thiosulfate, fumarate, nitrate and nitrite are not. Grows with chloramphenicol, kanamycin and vancomycin (each at 10 µg ml⁻¹) and with 50 µg rifampicin ml⁻¹. The DNA G + C content is 34.6 mol% (HPLC).

The type strain, MET-B^T (= DSM 16923^T = CCUG 50214^T), was isolated from an oil well in Congo, West Africa.

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