Mahella australiensis gen. nov., sp. nov., a moderately thermophilic anaerobic bacterium isolated from an Australian oil well

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A novel Gram-positive, anaerobic and moderately thermophilic bacterium, strain 50-1 BON^T, was isolated from an Australian terrestrial oil reservoir. Cells were spore-forming straight rods, motile by peritrichous flagella. The optimum growth conditions were 50 °C, pH 7.5 and 0.1 % NaCl. Strain 50-1 BON^T fermented arabinose, cellobiose, fructose, galactose, glucose, mannose, sucrose, xylose and yeast extract. Glucose was fermented mainly into lactate, formate, hydrogen and CO₂. The major end product of pyruvate fermentation was acetate together with H₂ and CO₂. Thiosulfate, sulfate, elemental sulfur and nitrate were not used as terminal electron acceptors. The DNA G + C content was 55.5 mol%. The closest phylogenetic relative of strain 50-1 BON^T was *Thermoanaerobacterium thermosulfurigenes* (16S rRNA gene sequence similarity of 85.7 %). As strain 50-1 BON^T was physiologically and phylogenetically different from members of the order 'Thermoanaerobacteriales', it is proposed that strain 50-1 BON^T (= DSM 15567^T = CIP 107919^T) be classified as the type strain of a novel species of a new genus, *Mahella australiensis* gen. nov., sp. nov.

Mesophilic, thermophilic and hyperthermophilic fermentative bacteria constitute an important microbial community of the oilfield environment (Magot *et al.*, 2000). Cloning and sequencing of PCR-amplified 16S rRNA genes of oilfield microbial communities have suggested the presence of a limited number of anaerobes belonging to the class 'Clostridia', e.g. *Clostridium* spp. and *Eubacterium* spp. (Voordouw *et al.*, 1996). However, there is increasing evidence that mesophilic and thermophilic members of the Clostridiales are current inhabitants of oilfield ecosystems. They include: (i) sulfate-reducing bacteria of the genus *Desulfotomaculum*, family Peptococcaceae (Nazina *et al.*, 1988; Nilsen *et al.*, 1996), (ii) thermophilic fermentative micro-organisms of the genera *Thermoanaerobacter* and *Thermoanaerobacterium*, family 'Thermoanaerobacteriaceae' (Cayol *et al.*, 1995; Grassia *et al.*, 1996), and (iii) a mesophilic fermentative bacterium of the genus *Fusibacter*, family Clostridiaceae (Ravot *et al.*, 1999). We have recently performed investigations on the microbiology of anaerobes inhabiting oil reservoirs in Queensland, Australia. Here we describe a novel thermophilic anaerobic bacterium (strain 50-1 BON^T) isolated from a non-water-flooded terrestrial oil reservoir, which belongs to cluster VI of the order

Clostridiales. Strain 50-1 BON^T presented significant phenotypic and genotypic differences, and we propose that it be classified in a new genus and species, *Mahella australiensis* gen. nov., sp. nov.

The oil sample used in this study was collected from the Riverslea oilfield in the Bowen-Surat Basin of Queensland in eastern Australia. The sample has been designated OCA5 and was stored at 4 °C until used.

Enrichment was performed in a medium prepared anaerobically (Fardeau *et al.*, 2000) containing (l⁻¹ distilled water): 0.3 g K₂HPO₄, 0.3 g KH₂PO₄, 0.2 g MgCl₂·6H₂O, 0.1 g CaCl₂·2H₂O, 0.5 g cysteine hydrochloride, 1 mg resazurin, 0.1 g KCl, 1 g NaCl, 1 g NH₄Cl, 5 g yeast extract, 5 g bio-Trypticase (Difco) and 10 ml of the trace mineral solution of Balch *et al.* (1979). The pH was adjusted to 7.0 with 10 M KOH. Vessels were autoclaved for 45 min at 110 °C. Prior to inoculation, Na₂S·9H₂O and NaHCO₃ were added from sterile stock solutions. For enrichment, a 2 ml oil well water sample was inoculated into 20 ml medium and incubated at 50 °C. Three enrichment series were performed in the same medium before isolation. Strains were isolated by repeated use of the Hungate roll-tube technique (Hungate, 1969), with medium solidified with 2 % noble agar (Difco). The process of serial dilution in roll tubes was repeated at least twice to purify the cultures.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 50-1 BON^T is AY331143.

The basal medium used for characterization of pH, temperature and NaCl ranges for growth of the isolates was similar to the enrichment medium supplemented with 20 mM glucose. The culture medium was adjusted to different pH values by injecting NaHCO_3 from 10 % (w/v) sterile anaerobic stock solutions. Water baths were used to obtain incubation temperatures up to 100 °C. For studies of NaCl requirements, NaCl was weighed directly in the tubes prior to dispensing the medium. The characterized strain was subcultured at least once under the same experimental conditions prior to determination of growth rates. Substrates were tested in anaerobiosis in basal medium at a final concentration of 20 mM. To test for electron acceptors, sodium thiosulfate (20 mM), sodium sulfate (20 mM), sodium sulfite (2 mM), elemental sulfur (2 %, w/v), potassium nitrate (10 mM) and potassium nitrite (2 mM) were added to the medium. The use of electron acceptors was evaluated by measuring OD_{580} and sulfide, ammonium or nitrite production. The presence of spores was tested by phase-contrast microscopic observations of young and old cultures and after pasteurization tests performed at 80, 90 and 100 °C for 10 and 20 min. Antibiotics were added at 20, 25, 50, 100, 150 and 200 $\mu\text{g ml}^{-1}$ and the resulting growth was compared with a control with no antibiotic added.

Unless otherwise indicated, duplicate culture tubes were used throughout these studies. Growth was determined by measurement of OD at 580 nm using a UV-visible spectrophotometer 50 Scan (Varian). Sulfide was determined photometrically as colloidal CuS by the method of Cord-Ruwisch (1985). Nitrate and nitrite were estimated using the Quantofix test (Macherey-Nagel). Organic compounds were determined as described by Fardeau *et al.* (1997). Morphological characteristics of isolates were observed with an Optiphot phase microscope (Nikon). The electron microscopy studies were performed as described by Koussémon *et al.* (2001).

The G + C content of DNA was determined at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) using HPLC as described by Mesbah *et al.* (1989). Non-methylated λ DNA (Sigma) was used as the standard. The 16S rRNA gene of the isolate was amplified as described previously (Miranda-Tello *et al.*, 2003). PCR products were cloned using the pGEM-T Easy cloning kit (Promega), according to the manufacturer's protocol. The 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 and 3 min at 72 °C and a final extension of 10 min at 72 °C. Plasmids containing an insert of the correct length were isolated using the Wizard Plus SV Minipreps DNA Purification System kit (Promega), according to the manufacturer's protocol. Purified plasmids were sent to Genome Express (Grenoble, France) for sequencing.

Sequence data were aligned with a full-length consensus 16S rRNA gene sequence, assembled and checked for accuracy manually using the alignment editor BioEdit v5.0.9 (Hall, 1999). These were compared with other sequences in GenBank (Benson *et al.*, 1999) and RDP (Maidak *et al.*, 2001) using BLAST (Altschul *et al.*, 1997) to identify the closest relatives. Positions of sequence and alignment ambiguity were omitted and pairwise evolutionary distances based on 1373 unambiguous nucleotides were computed 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, 1993). The 16S rRNA gene sequence accession numbers of reference organisms are included in Fig. 2.

Enrichment cultures were positive after incubation at 50 °C for 3–4 days. Microscopic examination revealed the presence of motile, rod-shaped bacteria. White, round, smooth colonies (1–2 mm in diameter) developed on roll tubes after 7 days of incubation. Single colonies were picked and streaking was repeated three times before cultures were considered to be pure. Two strains similar in morphology were isolated: strain 50-1 BON^T and strain 50-2 BON. As these strains shared 100 % similarity in their 16S rRNA gene sequences, strain 50-1 BON^T was used for further characterization. Cells of strain 50-1 BON^T were straight rods, 3–20 μm in length and 0.5 μm in diameter (Fig. 1), motile by peritrichous flagella (mean of four flagella per cell). Terminal spores (mean 1.2 μm in diameter) swelling the cells were observed (Fig. 1). Ultra-thin sections of the cells showed a Gram-positive-type cell wall (data not shown). Cells divided by septation of the cell wall. Strain 50-1 BON^T grew at temperatures ranging from 30 to 60 °C, and growth was optimum at 50 °C (data not shown). Growth occurred at initial pH values between 5.5 and 8.8 at 50 °C, with the optimum at pH 7.5 (data not shown). The isolate grew at NaCl concentrations ranging from 0 to 4 %, with the

Fig. 1. Phase-contrast micrograph of strain 50-1 BON^T grown under optimal conditions showing sporulating cells. Bar, 10 μm .

Fig. 2. Dendrogram based on 16S rRNA gene sequences generated by the neighbour-joining method (Saitou & Nei, 1987) indicating the phylogenetic position of strain 50-1 BONT. GenBank accession numbers are shown. Bootstrap values from 100 replications are shown at branching points. Only values above 80 were considered significant and are reported. Bar, 5 substitutions per 100 nucleotides.

optimum at 0.1% NaCl (data not shown). The doubling time under optimal conditions was 11 h.

Yeast extract was not required for growth but increased growth. In the presence of yeast extract, the OD was four times higher than in its absence. Strain 50-1 BONT grew under anaerobiosis with the following substrates as carbon and energy sources: arabinose, cellobiose, fructose, galactose, glucose, mannose, sucrose, xylose and yeast extract. Glucose was degraded mainly into lactate [1.3 mol (mol glucose consumed)⁻¹], formate [1.5 mol (mol glucose consumed)⁻¹], hydrogen and CO₂, together with acetate [0.2 mol (mol glucose consumed)⁻¹] and ethanol [0.5 mol (mol glucose consumed)⁻¹]. Acetate together with H₂ and CO₂ were the major end products of pyruvate fermentation. The following compounds were not fermented: ethanol, methanol, 1-propanol, glycerol, 1,2-propanediol, olive oil, starch, benzoate, formate, succinate, lactate, fumarate, acetate, propionate, peptone and Casamino acids. Electron acceptors tested were not used.

Penicillin and ampicillin did not inhibit growth at concentrations up to 200 µg ml⁻¹, but growth was inhibited by chloramphenicol at a concentration of 50 µg ml⁻¹.

The first isolations and partial characterizations of thermophilic fermentative micro-organisms originating from high-temperature petroleum reservoirs were performed in the 1980s (Levi *et al.*, 1985; Shenman & Vance, 1987). Further studies provided evidence that thermophilic anaerobes are an important microbial community of the oilfield ecosystems (Cayol *et al.*, 1995; Grassia *et al.*, 1996; L'Haridon *et al.*, 1995; Magot *et al.*, 2000; Stetter *et al.*, 1993). Strain 50-1 BONT was isolated from a non-water-flooded Australian oilfield in Queensland. It was found to be a moderately thermophilic, spore-forming anaerobe that utilized a wide range of carbohydrates, lactate being a major end product of glucose metabolism. Spores were round, terminal, distending the cells and were brightly refractile under phase-contrast microscopy (Fig. 1). However, similarly to *Thermoanaerobacter brockii* (formerly *Thermoanaerobium*

brockii), non-refractile structures could be observed in the same position within the cells and were assumed to be pre-spores (Cook *et al.*, 1991). 16S rRNA gene sequence analysis revealed that strain 50-1 BONT was a member of cluster VI of the order *Clostridiales* and its closest phylogenetic relatives were members of the genus *Thermoanaerobacterium*, family 'Thermoanaerobacteriaceae', class 'Clostridia' (Fig. 2). This family also includes members of the genus *Thermoanaerobacter*, which, similarly to members of the genus *Thermoanaerobacterium*, are known to use thiosulfate as a terminal electron acceptor, reducing it to sulfide or elemental sulfur (Lee *et al.*, 1993; Wiegel & Ljungdahl, 1981). This is the case for *Thermoanaerobacterium thermosulfurigenes*, isolated from a thermal, volcanic, algal-bacterial community (Schink & Zeikus, 1983), and *Thermoanaerobacterium aotearoense*, isolated from geothermally heated water and sediments collected in New Zealand (Liu *et al.*, 1996), the closest phylogenetic relatives of strain 50-1 BONT (similarities of 85.7 and 85.5% with *Thermoanaerobacterium thermosulfurigenes* and *Thermoanaerobacterium aotearoense*, respectively). In contrast to *Thermoanaerobacterium thermosulfurigenes* and *Thermoanaerobacterium aotearoense*, strain 50-1 BONT was unable to reduce thiosulfate and did not grow at temperatures above 60 °C. In addition, strain 50-1 BONT had a different DNA G+C content (55.5 mol% for strain 50-1 BONT compared with 32.6 mol% for *Thermoanaerobacterium thermosulfurigenes* and 34.5–35 mol% for *Thermoanaerobacterium aotearoense*). Strain 50-1 BONT also differed from both *Thermoanaerobacterium* species by the range of optima and growth conditions, and by the range of substrates used (Table 1). Within the genus *Clostridium*, strain 50-1 BONT has *Clostridium thermocellum* as its closest phylogenetic relative (similarity of 84.8%). However, the latter uses cellulose and cellulose relatives but not sugars (Ng *et al.*, 1977). In addition, it grows at temperatures above 60 °C and has a lower DNA G+C content (38.1–39.5 mol% for *C. thermocellum*). In this respect, strain 50-1 BONT clearly differed phylogenetically and phenotypically from anaerobic spore-forming bacteria known so far. This strain

Table 1. Discriminating characteristics between strain 50-1 BONT and its closest relatives *Thermoanaerobacterium thermosulfurogenes* and *Thermoanaerobacterium aotearoense*

Data for reference species were taken from Schink & Zeikus (1983) (*T. thermosulfurogenes*) and Liu *et al.* (1996) (*T. aotearoense*). ND, Not determined.

Characteristic	Strain 50-1 BONT	<i>T. thermosulfurogenes</i>	<i>T. aotearoense</i>
Temperature range (°C)	30–60	35–75	35–66
Optimum temperature (°C)	50	60	60–63
pH range	5.5–8.8	4.0–7.6	3.8–6.8
Optimum pH	7.5	5.5–6.5	5.2
DNA G + C content (mol%)	55.5	32.6	34.5–35.0
Reduction of thiosulfate	–	+	+
Substrates used:			
Arabinose	+	+	–
Starch	–	+	+
Pyruvate	+	–	–
Doubling time on glucose (h)	11.0	1.9	ND
End products of glucose metabolism	Lactate, formate, ethanol, acetate, H ₂ , CO ₂	Ethanol, acetate, lactate, H ₂ , CO ₂	Ethanol, acetate, lactate, H ₂ , CO ₂

was isolated from a non-water-flooded reservoir, which is the best model for studying indigenous bacteria. Whether this strain is of indigenous or exogenous origin is difficult to conclude, since contamination could occur in a number of ways during working of the oilfields (e.g. drilling, well equipment operations and damaged tubing). However, the hypothesis that micro-organisms might be indigenous to petroleum reservoirs has been presented by several authors (Grassia *et al.*, 1996; L'Haridon *et al.*, 1995; Ollivier *et al.*, 1998) and deserves further consideration. Indeed, the improvement of our knowledge of the metabolic diversity of oil reservoir microbial inhabitants will be helpful in developing microbial processes to enhance oil recovery.

Because of its phenotypic, genotypic and phylogenetic characteristics, strain 50-1 BONT represents a novel species of a new genus within the family *Thermoanaerobacteriaceae* that we propose to name *Mahella australiensis* gen. nov., sp. nov.

Description of *Mahella* gen. nov.

Mahella (Mah.el'la. L. dim. ending *-ella*; N.L. fem. n. *Mahella* named in honour of the American microbiologist Professor R. A. Mah, for his important contribution to the taxonomy of anaerobes).

Cells are straight rods. Gram reaction is positive. Spores are formed. Growth is anaerobic. Moderately thermophilic member of class 'Clostridia', family 'Thermoanaerobacteriaceae'. Sugars serve as main substrates with lactate and formate being major end products of sugar metabolism. The type species is *Mahella australiensis*.

Description of *Mahella australiensis* sp. nov.

Mahella australiensis (aus.tra.li.en'sis. N.L. fem. adj. *australiensis* related to Australia).

Displays the following properties in addition to those given in the genus description. Cells (3–20 × 0.5 µm) occur singly or in pairs and possess peritrichous flagella. Electron microscopy shows a Gram-positive-type cell wall. Round colonies (1–2 mm diameter) develop in roll tubes after 7 days of incubation at 50 °C. Chemo-organotrophic and obligately anaerobic. Ferments arabinose, cellobiose, fructose, galactose, glucose, sucrose, D-xylose and pyruvate. The optimum temperature for growth is 50 °C at pH 7.5; temperature range between 30 and 60 °C. The optimum pH is 7.5; growth occurs between pH 5.5 and 8.8. Halotolerant, growing in the presence of up to 4 % NaCl with an optimum at 0.1 %. Yeast extract is not required for growth. Elemental sulfur, sulfate, thiosulfate, sulfite, nitrate or nitrite is not used as an electron acceptor. The G + C content of the DNA is 55.5 mol% (HPLC).

The type strain, 50-1 BONT (=DSM 15567^T=CIP 107919^T), was isolated from an Australian oil well in Queensland.

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