Thermoanaerobacter subterraneus sp. nov., a novel thermophile isolated from oilfield water

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A new thermophilic, anaerobic glucose-fermenting, Gram-positive, rod-shaped bacterium, designated strain SEBR 7858T, was isolated from an oilfield water sample. Under optimal conditions on a glucose-containing medium (3 % NaCl, 65 °C and pH 7.5), the generation time was 2.5 h. No growth occurred at 35 or 80 °C, nor at pH 5.5 or 9.0. Strain SEBR 7858T possessed lateral flagella. Spores were undetected but heat-resistant forms were present. Strain SEBR 7858T fermented a range of carbohydrates to acetate, L-alanine, lactate, H2 and CO2. The isolate reduced thiosulfate and elemental sulfur, but not sulfate or sulfite to sulfide. In the presence of thiosulfate, the ratio of acetate produced per mole of glucose consumed increased, suggesting a shift in the use of electron acceptors during carbohydrate metabolism. The DNA G+C content was 41 mol%. Based on 16S rRNA gene sequence analysis, the strain was almost equidistantly related to all members of the genus Thermoanaerobacter (mean similarity 92 %). Based on phenotypic, genomic and phylogenetic characteristics, strain SEBR 7858T was clearly different from all members of the genus Thermoanaerobacter and was therefore designated as a new species, Thermoanaerobacter subterraneus sp. nov. The type strain is SEBR 7858T ( = CNCM I-2383T, DSM 13054T).

Keywords: thiosulfate reduction, Thermoanaerobacter subterraneus sp. nov., thermophile, phylogeny, oil well

INTRODUCTION

In the last two decades, intensive research on anaerobic, thermophilic, carbohydrate-fermenting micro-organisms from marine and terrestrial volcanic hot springs has led to the isolation of several new genera and species in the domains Bacteria and Archaea. The major rationale for this research stems from the biotechnological potential and the basic evolutionary traits of these microbes. In Bacteria, particular attention has been paid to members of the order Thermotogales and the family Thermoanaerobiaceae. The latter includes the genera Thermoanaerobacter and Thermoanaerobacterium, whose members reduce thiosulfate or elemental sulfur, respectively, to sulfide (Lee et al., 1993; Wiegel & Ljungdahl, 1981). Thermoanaerobacter species are thermophilic, heterotrophic, saccharolytic anaerobes found in soil, faeces, sugar beet and sugar cane extraction juices, thermal spas, volcanic hot springs, non-volcanic geothermally heated subsurface aquifers, hydrothermal vents and oil-producing wells (Cayol et al., 1995; Cook et al., 1996; Jin et al., 1988; Kozianowski et al., 1997; Larsen et al., 1997; Lee et al., 1993; Leigh et al., 1981; Slobodkin et al., 1999; Wiegel, 1986; Wynter et al., 1996). Thermoanaerobacter species originating from oil-producing wells have only recently been studied and have been found to be similar to surface-inhabiting Thermoanaerobacter species (Cayol et al., 1995; Magot et al., 2000; Wynter et al., 1996). We report here on the characterization of a new member of this genus, Thermoanaerobacter subterraneus sp. nov., which was isolated from a French oilfield.

METHODS

Collection site. Strain SEBR 7858T was isolated from the ‘Lacq Supérieur’ oilfield located in south-west France. The in situ temperature of the geological formation, at a depth of 645 m, was 52 °C. A water sample was aseptically collected.
at the wellhead, as described previously (Bernard et al., 1992), transported to the laboratory and stored at 4 °C until required.

Medium, enrichment and isolation. Enrichment and routine growth were performed using a culture medium containing (l-): NH₄Cl, 1.0 g; K₂HPO₄, 0.3 g; KH₂PO₄, 0.3 g; MgCl₂.6H₂O, 1.0 g; CaCl₂.2H₂O, 0.1 g; NaCl, 10.0 g; KCl, 0.2 g; Na₂SO₄·3·16 g; glucose, 20 mM; sodium thiosulfate, 20 mM; cysteine/HCl, 0.5 g; yeast extract (Difco), 2.0 g; Bio-trypticase (BioMérieux), 2.0 g; trace mineral element solution (Balch et al., 1979), 10 ml; resazurin, 1.0 mg. The pH was adjusted to 7.0 with 10 M KOH and the medium was boiled under a stream of O₂-free N₂ gas and cooled to room temperature. Five or 20 ml aliquots were dispensed into Hungate tubes or serum bottles, respectively, under a stream of N₂/CO₂ (80:20, v/v) gas and the vessels were autoclaved for 45 min at 110 °C. Prior to inoculation, Na₂S, 9H₂O and NaHCO₃ were injected from sterile stock solutions to final collate coliforms and elemental sulfur were added to the culture medium at final concentrations of 20 mM, 2 mM and 2% (w/v), respectively. An aliquot of the water sample was inoculated into 20 ml medium and incubated at 70 °C without agitation to initiate an enrichment culture. Several pure cultures were obtained by picking well isolated colonies that developed in the culture medium solidified with 4% (w/v) Phytagel (Sigma; Deming & Baross, 1986) by the repeated use of the Hungate roll-tube method (Hungate, 1969). One of these was used for subsequent studies.

Characterization studies. pH, temperature and NaCl ranges for growth were determined using the culture medium. The medium in Hungate tubes was adjusted to the desired pH, measured at ambient temperature, by injecting 10% (w/v) sterile anaerobic stock solutions of NaHCO₃ or Na₂CO₃. NaCl was weighed directly in the tubes prior to dispensing the culture medium. The strain was subcultured at least once under the same experimental conditions prior to determination of growth rates. Substrates were tested in culture medium lacking glucose at a final concentration of 20 mM. To test for electron acceptors, sodium thiosulfate, sodium sulfate, sodium sulfite and elemental sulfur were added to the culture medium at final concentrations of 20 mM, 20 mM, 2 mM and 2% (w/v), respectively.

Light and electron microscopy. Light microscopy was performed as described by Cayol et al. (1994). Electron microscopy was performed as described by Fardeau et al. (1997a).

Analytical techniques. Unless otherwise indicated, duplicate culture tubes were used throughout this study. Growth was measured by inserting tubes directly into a model UV-160A spectrophotometer (Shimadzu) and measuring changes in OD₅₇₅. Sulfide was determined photometrically as colloidal CuS by using the method of Cord-Ruwisch (1985). H₂S, CO₂, sugars, alcohols, volatile and non-volatile fatty acids were measured as described previously (Fardeau et al., 1996). l-Alanine was measured by HPLC as described by Moore et al. (1958).

Determination of DNA G+C content. The DNA G+C content was determined by the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). DNA was isolated and purified by chromatography on hydroxyapatite and its G+C content was determined by using HPLC as described by Mesbah et al. (1989). Non-methylated 16S DNA (Sigma) was used as the standard.

16S rDNA sequence analysis. The methods for the purification and extraction of DNA and the amplification and sequencing of the 16S rRNA gene have been described previously (Andrews & Patel, 1996). The 16S rRNA gene sequence was manually aligned with reference sequences of various members of Bacteria using the editor ae2 (Maidak et al., 1999). Reference sequences were obtained from the Ribosomal Database Project (Maidak et al., 1999), EMBL and GenBank databases. Positions of sequence and alignment uncertainty were omitted from the analysis. The pairwise evolutionary distances based on 1224 unambiguous nucleotides were computed by using the method of Jukes & Cantor (1969) and dendrograms were constructed from these distances by using the neighbour-joining method, both of which were from part of the PHYLIP suite of programs (Felsenstein, 1993). A maximum-likelihood approach with FastDNAml was used as an alternative method for tree construction (Olsen et al., 1994).

RESULTS

Enrichment and isolation

Enrichment cultures were positive after incubation at 70 °C for 3 d and H₂S was detected, presumably derived from thiosulfate reduction. Microscopic examination revealed the presence of rod-shaped bacteria. Single, well isolated colonies (3 mm diam.) that developed in Phytagel roll tubes after 3 d incubation at 70 °C were picked and serially diluted in Phytagel roll tubes at least twice before the culture was considered pure. Several axenic cultures were obtained and the culture designated strain SEBR 7858 was used for further studies.

Morphology

Strain SEBR 7858 was a rod-shaped bacterium (0.5–0.7 x 2–8 μm), which occurred singly (Fig. 1a). No motility was observed but the cells possessed laterally inserted peritrichous flagella (Fig. 1b). No spores were observed under microscopic examination (electron and light microscopy), but cultures exposed at 120 °C for 45 min could be subcultured, suggesting the presence of heat-resistant forms. Electron microscopy of ultrathin sections revealed a Gram-positive cell wall composed of a thin dense surface layer separated from a thicker inner layer (Fig. 1c).

Optimum growth conditions

Strain SEBR 7858 strictly anaerobic and did not grow in culture medium containing traces of oxygen, as indicated by the anaerobiosis indicator, resazurin. The strain grew optimally at 65 °C (temperature range between 40 and 75 °C, but not at 80 °C; Fig. 2a) and a pH of 7.5 (pH range between 6.9 and 8.5; Fig. 2b). The isolate grew in the presence of 0–3% NaCl with an optimum of 0% at pH 7-0 and 70 °C (Fig. 2c).

Substrates for growth

Yeast extract or Bio-trypticase was required for growth on carbohydrates. Yeast extract could not be replaced by a vitamin mixture (Balch et al., 1979).
**Thermoanaerobacter subterraneus** sp. nov.

**Fig. 1.** (a) Phase-contrast micrograph of strain SEBR 7858\(^T\). Bar, 10 µm. (b) Electron micrograph of a negatively stained culture of strain SEBR 7858\(^T\) showing laterally inserted flagella. Bar, 2 µm. (c) Electron micrograph of an ultrathin section of strain SEBR 7858\(^T\) showing the cytoplasmic membrane (cm), the inner layer (il) and the outer layer (ol). Bar, 0.5 µm.

SEBR 7858\(^T\) grew on the following substrates (at a concentration of 20 mM unless otherwise indicated) in the presence of thiosulfate as the electron acceptor: D-fructose, D-galactose, D-glucose, DL-lactose, DL-maltose, D-mannose, D-xylene, D-ribose, mannitol, cellobiose, pyruvate, melibiose, starch and xylan, but not L-xylene, L-arabinose, L-rhamnose, sorbose or sucrose. Glycerol was used poorly. Acetate, L-alanine, lactate, H\(_2\) and CO\(_2\) were produced from glucose fermentation. From 0.6 to 0.8 mol L-alanine was produced per mole glucose consumed (Table 1).

**Effect of electron acceptors**

Strain SEBR 7858\(^T\) reduced thiosulfate, elemental sulfur and sulfite, but not sulfate to sulfide. The presence of thiosulfate altered the concentration of metabolites during glucose oxidation, indicating that thiosulfate modified the metabolic pathway of strain.
Table 1. End products from glucose metabolism by *Thermoanaerobacter subterraneus*, in the presence or absence of thiosulfate (20 mM)

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<th>Growth conditions</th>
<th>Amount of glucose consumed (mmol)</th>
<th>Amount of product (mmol)*</th>
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<tr>
<td></td>
<td></td>
<td>Acetate</td>
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<tr>
<td>Glucose</td>
<td>9.4</td>
<td>10.6</td>
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<tr>
<td>Glucose + thiosulfate</td>
<td>8.6</td>
<td>20.3</td>
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*Data were corrected for non-utilizable glucose present in the medium.

**DISCUSSION**

Strains of various physiological groups of thermophilic bacteria have been previously isolated from deep subsurface environments such as oilfields and aquifers. In addition to methanogens and sulfate-reducing bacteria, heterotrophic micro-organisms, including members of the order *Thermotogales* and the family *Thermoanaerobiaceae* (e.g. the genus *Thermoanaerobacter*), have been isolated and described (Andrews & Patel, 1996; Cayol et al., 1995; Fardeau et al., 1997a; Jeanthon et al., 1995; Magot et al., 2000; Ravot et al., 1995; Slobodkin et al., 1999; Stetter et al., 1993; 2144 International Journal of Systematic and Evolutionary Microbiology 50

**DNA G+C content**

The G+C content of isolate SEBR 7858T was 41 mol% (as determined by HPLC).
Wynter et al., 1996). Strain SEBR 7858^T, a Gram-positive thermophilic anaerobic bacterium, isolated from a French oilfield, is phylogenetically related to Thermoanaerobacter species. The presence of a Thermoanaerobacter species in oilfields was first reported in 1995. The isolate was found to be a phylogenetic relative of Thermoanaerobacter brockii and Thermoanaerobacter finnii (Cayol et al., 1995). It was recognized as a novel subspecies of the terrestrial microorganism Thermoanaerobacter brockii and named Thermoanaerobacter brockii subsp. falciiylicus. Because of the large number of new isolates belonging to Thermoanaerobacter, the taxonomy of this genus was revised on the basis of 16S rRNA sequence and DNA–DNA hybridization and now comprises 11 validated species, 3 subspecies and 3 non-validated species (Table 2).

The isolation of strain SEBR 7858^T extends our knowledge on the diversity of microbes from subterranean ecosystems and confirms that Thermoanaerobacter species, similar to members of the order Thermotogales, may be important components of the heterotrophic microflora of oilfield reservoirs (Grassia et al., 1996; Magot et al., 2000). Studies on a methanogenic archaeon, Methanocalculus halotolerans isolated from an African oilfield (Ollivier et al., 1998), and the results reported by L’Haridon et al. (1995) suggest that indigenous micro-organisms might inhabit oilfield reservoirs. However, the presence of Thermoanaerobacter species in oilfield waters as a result of anthropogenic contamination rather than as an indigenous microflora cannot be ruled out entirely and further investigations are needed to understand their distribution. Nevertheless, Thermoanaerobacter species have a wide nutritional spectrum for growth, which includes carbohydrates and amino acids (Fardeau et al., 1997b; Faudon et al., 1995; Magot et al., 2000); oilfields may contain such nutrients, provided by other members of the bacterial community, that will possibly support their growth and/or viability.

Strain SEBR 7858^T oxidizes hydrogen in the presence of sulfur compounds. This may lead to corrosion of oilfield installations when these latter compounds are available in oilfield waters. The presence of a hydro- gen-oxidizing Thermoanaerobacter species was previously reported from oilfields (Fardeau et al., 1993a, b). In addition, thiosulfate reduction by Thermoanaerobacter species, including strain SEBR 7858^T, may be of significance in mineralization processes in thermal terrestrial or subterranean environments, since this reduction facilitates oxidation of organic matter as reported previously (Fardeau et al., 1996).

Strain SEBR 7858^T has many features typical of some members of the genus Thermoanaerobacter. It is Gram-positive, thermophilic (optimal temperature for growth is 65 °C), anaerobic and contains heat-resistant cells. The isolate reduces thiosulfate, sulfite and elemental sulfur, but not sulfate to sulfide. In addition, it ferments carbohydrates to l-alanine, acetate, lactate, H_2 and CO_2. This constitutes the first experimental evidence of l-alanine production by a Thermoanaerobacter species. We have detected the presence of l-alanine from glucose fermentation in Thermoanaerobacter brockii and Thermoanaerobacter ethanolicus, but it is a minor product compared to strain SEBR 7858^T (unpublished data). As for some members of Archaea (Kengen & Stams, 1994; Kobayashi et al., 1995), l-alanine production from glucose metabolism has been reported in hyperthermophilic and thermophilic members of Bacteria (e.g. Thermotoga, Thermotoga and Fervidobacterium, order Thermotogales) and has been suggested to be a remnant of an ancestral metabolism (Ravot et al., 1996). l-Alanine is also produced by a Gram-positive anaerobe belonging to the genus Clostridium (Örlygsson et al., 1995). It is possible that l-alanine production from carbohydrate fermentation is more widespread amongst members of Bacteria than previously believed.

Strain SEBR 7858^T differs from its closest phylogenetic relatives, such as Thermoanaerobacter brockii and Thermoanaerobacter ethanolicus, by fermenting meliobiose and by using sucrose and xylose poorly. In contrast to Thermoanaerobacter brockii (Zeikus et al., 1979), strain SEBR 7858^T has a much higher DNA G + C content (41 % for SEBR 7858^T compared to 30–31% for Thermoanaerobacter brockii). The isolate also differs from Thermoanaerobacter ethanolicus (Wiegel & Ljungdahl, 1981) as l-alanine is the major end product. Phylogenetic, genomic and phenotypic characteristics of strain SEBR 7858^T confirm that it represents a new species of the genus Thermoanaerobacter for which we propose the name Thermoanaerobacter subterraneus sp. nov., reflecting its subterranean origin.

**Description of Thermoanaerobacter subterraneus sp. nov.**

Thermoanaerobacter subterraneus (sub.ter. ra’ ne. us. L. pref. sub less than; L. n. terra earth; L. adj. subterraneus under the earth, describing its site of isolation).

Cells are rods (0.5–0.7 × 2–8 μm), occur singly or in pairs and possess laterally inserted flagella. Spores are not observed under microscopic examination but cultures exposed to 120 °C for 45 min could be subcultured, suggesting the presence of heat-resistant forms. Electron microscopic examinations reveal a Gram-positive cell wall. Round colonies (3 mm diam.) develop in Phytagel roll tubes after 3 d incubation at 70 °C. Chemo-organotrophic and obligately anaerobic member of the domain Bacteria. Optimum temperature for growth is 65 ºC at pH 7-5; temperature range for growth is 40–75 °C. Optimum pH is 7-5; growth occurs between pH 6-0 and 8-5. Halotolerant, growing in the presence of up to 3 % NaCl. Yeast extract or Bio-tryptique is required for growth on carbohydrates. Growth on sugars is highly enhanced by the presence of both yeast extract and Bio-tryptique. Yeast extract cannot be replaced by vitamins. Fer-
Table 2. Characteristics that differentiate members of the genus *Thermoanaerobacter*


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<td>Temperature range (°C)</td>
<td>Optimum temperature (°C)</td>
<td>pH range</td>
<td>Optimum pH</td>
<td>G + C content (mol%)</td>
<td>Reduction of Sº</td>
<td>Reduction of S-Oº₂</td>
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<td>40–73</td>
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Substrates used:  
- Arabinose: – + – – ND – – ND  
- Cellobiose: – + – – ND – – ND  
- Fructose: + + + + ND + + +  
- Galactose: + + + + ND + + +  
- Glucose: + + + + + + +  
- Inositol: ND ND ND ND – – –  
- Lactose: + + + + + + +  
- Maltose: + + + + + + +  
- Mannitol: + – ND ND + + – –  
- Mannose: + + + + + + +  
- Melitolose: ND ND ND ND ND – – –  
- Melibiose: + ND ND ND – – – –  
- Raffinose: ND + ND ND ND + – +  
- Rhamnose: – – – ND ND – – –  
- Ribose: + + ND ND + + – +  
- Starch: + + + + + + +  
- Sucrose: – + + + + + + +  
- Xylose: + + + + + + +  
- Xylan: + + ND ND + + – +  
- Formate: ND ND ND ND ND + ND ND  
- Lactate: ND – – – ND ND ND ND  
- Pyruvate: + + + + ND + + +  
- Beef extract: ND ND ND ND ND ND ND ND  
- Tryptone: ND ND ND + + ND ND ND ND  
- Yeast extract: ND – – + ND ND ND ND  
- Amygdalin: ND ND ND ND ND – – –  
- Cellulose: ND – ND – – – + –  
- Chitin: ND ND ND ND ND ND ND ND  
- Aesculin: ND ND ND ND ND ND ND ND  
- Glicosamine: ND ND ND ND ND ND ND ND  
- Glycerol: ND ND ND ND ND ND – –  
- Glycogen: ND ND ND ND ND ND – –  
- Hemichelulose: ND ND ND ND ND ND ND ND  
- Inulin: ND ND ND ND ND ND – –  
- Pectin: ND ND – – – – ND ND  
- Diagnostic fermentation products from glucose: Ethanol, **Ethanol, lactate, t-alanine, isobutyrate, isovalerate, butyrate, lactate**

*All species tested produced acetate, H₂ and CO₂ with the exception of *Thermoanaerobacter siderophilus* which did not produce acetate.
† Homooacetogetic species.
Table 2 (cont.)

Thermoanaerobacter kivui, Zeikus et al. (1979); 11, ‘Thermoanaerobacter lactoethylicus’, Kondratieva et al. (1989); 12, Thermoanaerobacter mathranii, Larsen et al. (1997); 13, Thermoanaerobacter siderophilus, Slobodkin et al. (1999); 14, Thermoanaerobacter sulfurophilus, Bonch-Osmolovskaya et al. (1997); 15, Thermoanaerobacter thermocopriae, Jin et al. (1988); 16, Thermoanaerobacter thermohydrosulfuricus, Wiegel et al. (1979); 17, Thermoanaerobacter wiegelii, Cook et al. (1996). ND, Not determined.

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<td>Ethanol, lactate, succinate</td>
<td>Ethanol, lactate, propionate, butyrate, isovalerate</td>
<td>Ethanol, lactate</td>
<td>Ethanol, lactate</td>
<td>Ethanol, butyrate, lactate</td>
<td>Ethanol, butyrate, formate, propionate, 2-propanol, butyrate, isovalerate, isocaproate</td>
<td>Ethanol, lactate, propionate</td>
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ments celllobiose, D-fructose, D-galactose, D-glucose, DL-lactose, DL-maltose, D-mannose, melibiose, D-ribose, starch, D-xylose, mannitol, pyruvate and xylan. Glycerol is poorly utilized and L-arabinose, L-rhamnose, sorbose, sucrose and L-xylose are not used. Acetate, L-alanine, lactate, H₂ and CO₂ are produced during glucose fermentation. Elemental sulfur, thiosulfate and sulfite, but not sulfate, are used as electron acceptor. The DNA G+C content is 41 mol% (as determined by HPLC). Isolated from oilfield water. Type strain is SEBR 7858™ (=CNCM I-2383®, DSM 13054™).

ACKNOWLEDGEMENTS

ELF Aquitaine is acknowledged for its financial support to M.M. and for authorizing the publication of this manuscript. The financial assistance in part to B.K.C.P. from the ELF Aquitaine is acknowledged for its financial support to M.M. and for authorizing the publication of this manuscript. We thank M. Camoin for technical assistance and P. A. Roger for improving the manuscript.

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