**Methanocalculus halotolerans gen. nov., sp. nov., isolated from an oil-producing well**

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**INTRODUCTION**

Microbial studies of subsurface ecosystems, such as oilfields, have shown the presence of fermentative bacteria (Bernard et al., 1992; Bhupathiraju et al., 1991; Bhupathiraju & McInerney, 1993; Davydova-Charakhch’yan et al., 1993b; Fardeau et al., 1996; Holloway et al., 1980; Jeanthon et al., 1995; L’Haridon et al., 1995; Ravot et al., 1995; Stetter et al., 1993), sulfate reducers (Bhupathiraju & McInerney, 1993; Cord-Ruwisch et al., 1987; Nazina & Rozanova, 1978; Nilsen et al., 1996; Rees et al., 1995; Rozanova & Nazina, 1979; Rozanova & Galushko, 1990; Rozanova et al., 1989; Rueter et al., 1994; Stetter et al., 1993; Tardy-Jacquenod et al., 1996; Voordouw et al., 1996), and acetogens (Davydova-Charakhch’yan et al., 1993b). It has been established that methanogens constitute an important microbiological community inhabiting oilfields (Bhupathiraju & McInerney, 1993; Ivanov et al., 1983; Ng et al., 1989; Ni & Boone, 1991; Nilsen & Torsvik, 1996; Obraztsova et al., 1987a, b). However, very few studies have reported on methanogens from saline oilfields. Microbiological studies of surfritical hypersaline ecosystems have revealed the presence of methylotrophic methanogens growing at up to 30% (w/v) NaCl, whereas hydrogenotrophic halophilic methanogens grew at up to 9% NaCl (Ollivier et al., 1994). We describe a hydrogenotrophic methanogen isolated from a saline oilfield (8.7% NaCl). Phenotypic and phylogenetic characteristics of the isolate indicate that it is a novel halotolerant methanogen. In addition, we describe a methanogenic archaeon that is phenotypically and genotypically related to members of the genus *Methanohalophilus*. This report provides evidence that methylotrophic and hydrogenotrophic, but not acetotrophic methanogens are present in a saline subsurface oilfield environment, as already observed in surface saline to hypersaline environments.

Keywords: *Archaea, Methanocalculus halotolerans*, oilfield, halophily, taxonomy

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The GenBank accession number for the 16S rRNA sequence of strain SEBR 4845T is AF0033672.
discussed by comparing the ecology of methanogens from saline to hypersaline surface and subsurface ecosystems.

METHODS

Sample collection and sample source. A 1 l sample was collected from the well-head of the Scheibenhardt NDL 103 oilfield in Alsace (France) as previously described (Bernard et al., 1992). The in situ temperature was 38 °C and the NaCl concentration was 87 g l⁻¹.

Enrichment, isolation and growth conditions. Enrichment and isolation of methanogenic cultures were achieved in a basal medium that mimicked the mineral composition of the oilfield water and contained (1 l⁻¹) 1 g NH₄Cl, 0.3 g KH₂PO₄, 0.3 g K₂HPO₄, 14.2 g MgCl₂·6H₂O, 11.3 g CaCl₂·2H₂O, 0.17 g KCl, 87 g NaCl, 0.5 g CH₃COONa, 0.5 g cysteine. HCl, 0.5 g yeast extract (Difco), 0.5 g bio-Trypticase (bioMérieux), 10 ml of the trace element mineral solution of Balch et al. (1979), 1 mg resazurin and 1000 ml distilled water. The pH was adjusted to 7.0 with 10 M KOH. The medium was boiled under a stream of O₂-free N₂ gas and cooled to room temperature. Five millilitres of medium were dispensed into Hungate tubes and 20 ml in serum bottles under a stream of O₂-free N₂ gas and cooled to room temperature. Five millilitres of medium were dispensed into Hungate tubes and 20 ml in serum bottles under a stream of O₂-free N₂ gas and cooled to room temperature. Vessels were autoclaved for 45 min at 110 °C. Prior to culture inoculations, Na₅S·9H₂O and NaHCO₃ were injected from sterile stock solutions to a final concentration of 0.04% and 0.2% (w/v), respectively.

Enrichment cultures were initiated by inoculating 1 ml of the oilfield sample into serum bottles containing basal medium and H₂/CO₂ (80:20, 200 kPa) or trimethylamine (10 mM) as growth substrates. The pH of the medium under H₂/CO₂ was 7.0. The inoculated serum bottles were incubated at 37 °C without shaking. Pure cultures were obtained by the repeated use of the Hungate roll tube method (Hungate, 1969) using basal growth medium solidified with 1.5% (w/v) Noble agar (Difco). Two strains designated SEBR 4845T and FR1T were isolated on H₂/CO₂ and trimethylamine, respectively, as substrates and studied further.

Growth parameters. Growth at various pH values, temperatures and salt concentrations was tested in Hungate tubes in basal growth medium containing 40 mM sodium formate. The pH was adjusted to the desired value by injecting appropriate volumes of anaerobic sterile 10% (w/v) NaHCO₃ or Na₂CO₃ stock solutions. During growth, the pH of the medium increased by 0.1 units. Growth was tested at temperatures ranging from 20 to 50 °C in basal growth medium at the optimal pH. In this medium, the pH was not affected by temperature. To determine salt requirement for growth, sodium chloride was weighed directly into Hungate tubes and the medium subsequently dispensed as described above. The strain was subcultured at least once under the same experimental conditions prior to inoculation.

Substrate utilization. Substrates were added from sterile stock solutions to the basal medium at a final concentration of 10 mM (trimethylamine), 20 mM (acetate), or 40 mM (formate, methanol). Hydrogen oxidation was tested using H₂/CO₂ (80:20, 200 kPa) in the gas phase.

Analytical techniques. Unless otherwise indicated, duplicate culture tubes were used throughout the analytical studies. Light microscopy examinations were performed as previously described (Cayol et al., 1994). For electron microscopy examinations, exponentially grown cells were negatively stained and thin sections were prepared as already reported (Cayol et al., 1994). Growth was measured by inserting tubes directly into a Shimadzu model UV-160A spectrophotometer and measuring the optical density at 580 nm. Methane was quantified as described previously (Cord-Ruwisch et al., 1986).

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

16S rRNA sequence studies. A primer pair, designated FARCH-9 (5'-CTGTGTTGATCTTGCAGCAG-3') and Rd1 (5'-AAAGGGTAGATCCAGGCACCT-3') was used to amplify the 16S rRNA gene from genomic DNA of strain SEBR 4845T. The amplified product was purified (Andrews et al., 1986; Love et al., 1993; Reaburn & Patel, 1993) and the sequence determined with an ABI automated DNA sequencer by using a Prism dideoxy terminator cycle sequencing kit and the protocol recommended by the manufacturer (Applied Biosystems). The primers used for sequencing were F2 (5'-CAGGATTAGATACCCTGGT- TGC-3'), R2 (5'-GTATTACCCTGGTATCCAGCC-3'), F4 (5'-CGTCAATTCTCCTTTGAAGTTT-3') and the two amplification primers designated FARCH9 and Rd1 described above.

The 16S rRNA gene sequence was manually aligned with reference sequences of various members of the domain Archaea using the alignment editor aa2 (Maidak et al., 1996). Reference sequences were obtained from the Ribosomal Database Project (Maidak et al., 1996). Positions of sequence and alignment uncertainties were omitted from the analysis. Pairwise evolutionary distances based on 1224 unambiguous positions were computed by the method of Jukes & Cantor (1969), and dendograms were constructed from these distances by the neighbour-joining method. Both programs form part of the PHYLIP package (Felsenstein, 1993).

RESULTS

Enrichment and isolation

After 2 weeks incubation at 37 °C, positive enrichment cultures developed in the serum flasks containing H₂/CO₂ or trimethylamine, but not in those containing acetate as energy source. At 37 °C, circular colonies 1 mm in diameter developed in agar roll tubes after 1 month incubation on trimethylamine and after 1-5 months on H₂/CO₂. Two cultures were obtained using this technique. The strain obtained on H₂/CO₂ was designated strain SEBR 4845T (OCM 470T) and the strain obtained on trimethylamine was designated strain FR1T (OCM 471). Purity of the isolates was checked by microscopic examination of cultures inoculated in a complex rich medium containing yeast extract (1 g l⁻¹), bio-Trypticase (1 g l⁻¹) and glucose (20 mM) as the energy source; no growth was observed in such conditions.
Methanocalculus halotolerans gen. nov., sp. nov.

Fig. 1. (a) Phase-contrast micrograph of strain SEBR 4845T showing irregular coccoid cells (bar, 5 μm); (b) electron micrograph of an ultrathin section of strain SEBR 4845T showing the cell wall structure (bar, 0.2 μm).

Fig. 2. Phase-contrast micrograph of strain FR1T showing irregular coccoid cells (bar, 5 μm).

Morphology

Strain SEBR 4845T was an irregular coccus with a diameter of 0.8–1 μm, occurring singly or in pairs (Fig. 1a, b). It possessed two to three peritrichous flagella (data not shown). Strain FR1T was an irregular coccus with a diameter of 1 μm, also occurring singly or in pairs (Fig. 2). It possessed one flagellum (data not shown).

Optimum growth conditions

Strain SEBR 4845T did not grow in oxidized medium (oxidation was indicated by the pink colour of the resazurin). The isolate grew in the basal medium in the presence of 0–12.5% NaCl, with an optimum at 5% NaCl (Fig. 3a). It grew at an optimum temperature of 38 °C (Fig. 3b); it did not grow at 24 or 50 °C. Growth occurred between pH 7.0 and 8.4 with an optimum at pH 7.6 (Fig. 3c).

Strain FR1T grew at an optimum temperature of 35 °C; it did not grow at 24 °C or 50 °C (data not shown). The isolate grew in the presence of 1–17.5% NaCl, with an optimum between 5 and 10% NaCl (data not shown). Growth occurred between pH 6.6 and 8.0 with an optimum at pH 7.1 (data not shown).

Growth substrates

Strain SEBR 4845T used H₂+CO₂ and formate to produce methane. Under a N₂/CO₂ atmosphere, it could not produce methane from acetate (20 mM), methanol (40 mM), trimethylamine (10 mM), lactate (10 mM), glucose (20 mM), CO₂+1-propanol (10 mM), CO₂+2-propanol (10 mM), CO₂+1-butanol (10 mM) or 2-butanol (10 mM) after 1 month incubation at 37 °C. Acetate (2 mM) was required for growth on H₂+CO₂, and yeast extract was stimulatory for growth.

Strain FR1T grew with methanol and trimethylamine as substrates for methanogenesis with N₂/CO₂ as the gas phase. It could not produce methane from H₂+CO₂ (80:20; 200 kPa), formate (40 mM), acetate (20 mM), lactate (10 mM), glucose (20 mM), CO₂+1-propanol (10 mM), CO₂+2-propanol (10 mM), CO₂+1-butanol (10 mM) or 2-butanol (10 mM) after 1 month incubation at 37 °C. Acetate (2 mM) was not required for growth on trimethylamine.

G+C content of DNA

The G+C content of strain SEBR 4845T was 55 mol % and that of strain FR1T was 43 mol %.

16S rRNA gene sequencing and sequence analysis

Phenotypic and genomic characteristics of strain FR1T indicated similarities with members of the genus Methanohalophilus (Paterek & Smith, 1988). Since no marked phenotypic differences were observed between strain FR1T and species belonging to this genus,
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Fig. 3. Effects of (a) NaCl (temperature 37 °C; pH 7.0), (b) temperature (pH 7.0; 90 g NaCl l⁻¹) and (c) pH (temperature 37 °C; 90 g NaCl l⁻¹) on the growth of strain SEBR 4845T cultivated in basal medium containing 1 g yeast extract l⁻¹ and 1 g l⁻¹ bio-Trypticase and sodium formate (40 mM).

phylogenetic study was only performed on strain SEBR 4845T. Using five primers, we determined an almost complete sequence consisting of 1444 bases of the 16S rRNA gene of strain SEBR 4845T. Several phylogenetic trees were constructed using a selection of different methanogen sequences obtained from the RDP database. No variation in the placement of strain SEBR 4845T was observed. The sequences listed in Fig. 4 were used to construct a representative tree. Phylogenetic analysis indicated that strain SEBR 4845T could be a member of one of the families Methanomicrobiaceae or Methanocorpusculaceae, order Methanomicrobiales, since it was almost equidistant between members of the genus Methanospirillum or Methanocorpusculum (similarity of 86%) (Fig. 4). Bootstrap analysis indicated a robust relationship between strain SEBR 4845T and Methanocorpusculum sp. or Methanospirillum hungateii. Despite a clear relationship between strain SEBR 4845T and members of the families Methanomicrobiaceae and Methanocorpusculaceae, the characterization of more isolates is needed to affiliate unequivocally the isolate within one of these two families or in a new one.

DISCUSSION

Besides sulfate-reducing and fermentative bacteria, oilfield facilities possess methanogens. The methanogens so far isolated and characterized include the (i) hydrogenotrophs: Methanobacterium thermoautotrophicum (Ivanov et al., 1983), Methanobacterium bryantii (Davydova-Charakhch'yan et al., 1993a), Methanosoccus thermolithotrophicus (Nilsen & Torsvik, 1996), Methanobacterium ivanovii (Belyaev et al., 1983), 'Methanoplanus petrolearius' (Ollivier et al., 1997) and phenotypic variants of Methanobacterium thermoaggregans (Ng et al., 1989) and Methanobacterium thermoaceticophilum (Davydova-Charakhch'yan et al., 1993a); (ii) methylotrophs, 'Methanohalophilus euhalobius' (Davidova et al., 1997; Obraztsova et al., 1987a), Methanosarcina sicilae (Ni & Boone, 1991; Ni et al., 1994); and (iii) an acetotroph, Methanosarcina mazei (Obraztsova et al., 1987b). Here we report on a new hydrogenotrophic methanogen inhabiting a saline
Table 1. Characteristics that differentiate strain SEBR 4845T from representative species of coccoid methanogens belonging to the order Methanomicrobiales

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain SEBR 4845T</th>
<th>Methanoplanus limicola†</th>
<th>Methanocorpusculus parum</th>
<th>Methanogenium organophilum</th>
<th>Methanoculleus marisnigri</th>
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<tr>
<td>Collection no.</td>
<td>OCM 470T</td>
<td>DSM 2279T</td>
<td>DSM 3823T</td>
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<td>DSM 1498T</td>
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<td>Waste water</td>
<td>Marine mud</td>
<td>Marine sediment</td>
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<td>Temp. range (°C)</td>
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<td>pH range</td>
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<td>Optimum pH</td>
<td>7-6</td>
<td>6.5-7.5</td>
<td>6-8-7.5</td>
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<td>NaCl concn range (%)</td>
<td>0-12.5</td>
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<td>1</td>
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<td>Optimum NaCl concn (%)</td>
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<td>1</td>
<td>8</td>
<td>6</td>
<td>ND</td>
</tr>
<tr>
<td>Generation time (h)</td>
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<td>7</td>
<td>49</td>
<td>47</td>
<td>61</td>
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<td>G+C content (mol%)</td>
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<td>48</td>
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<tr>
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<td>CO$_2$+ethanol</td>
<td>CO$_2$+ethanol</td>
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ND, Not determined.
* This study.
† Data from Wildgruber et al. (1982).
‡ Data from Zellner et al. (1987)
§ Data from Widdel et al. (1988).
∥ Data from Romesser et al. (1979).

(8.7%) oil-producing well and called strain SEBR 4845T. It is the first hydrogen-oxidizing methanogen growing between 0 and 12.5% NaCl, a range which is the widest reported to date for any hydrogenotrophic methanogen. To our knowledge, the upper NaCl limit for growth of hydrogenotrophic methanogenic archaebacteria is 8.3% reported for Methanococcus thermostholithothrophicus (Huber et al., 1982). Strain SEBR 4845T is an irregular coccus and has a G+C content of 55 mol%. Therefore, it cannot be placed as a member of the order Methanococcales which are irregular cocci with a G+C content ranging from 30 to 40 mol% (Garcia, 1990). The order Methanomicrobiales consists of the families Methanomicrobiaceae, Methanocorpusculaceae, Methanoplanaceae and Methanosarcinaceae. Acetate, methanol or methylamines are not used as substrates by strain SEBR 4845T which therefore cannot be placed as a member of the family Methanosarcinaceae. Methanoplanus, the only described genus in the family Methanoplanaceae, comprises three species, Methanoplanus endosymbiosus (Van Bruggen et al., 1986), Methanoplanus limicola (Wildgruber et al., 1982) and 'Methanoplanus petrolearius' (Ollivier et al., 1997). All are disc-shaped methanogens and therefore do not resemble strain SEBR 4845T morphologically. The genus Methanocorpusculum, which is the only described genus in the family Methanocorpusculaceae, comprises five species that grow optimally at much lower NaCl concentra-

tions than that of SEBR 4845T (Garcia, 1990) and have a G+C content < 55 mol%. The family Methanomicrobiaceae comprises five genera with only two genera having coccoid shapes, Methanogenium and Methanoculleus. Both genera contain five species with a G+C content ranging from 47 to 61 mol% (Garcia, 1990). All species grow at NaCl concentrations ranging from 2 to 7% (Garcia, 1990): Methanogenium liminatans, 48% (Zellner et al., 1990); Methanogenium cariaci, 32% (Romesser et al., 1979); and Methanogenium tationis, 7% NaCl (Zabel et al., 1984). Furthermore, the 16S rRNA sequence analysis indicated a distant relationship with the most halotolerant species of the genus Methanogenium, Methanogenium tationis (Fig. 4).

Optimum temperature for growth of strain SEBR 4845T corresponded to that of the oil well (38 °C). In addition, the highest NaCl concentration supporting hydrogen oxidation via methanogenesis in terrestrial ecosystems was reported in a lake containing 9% NaCl (Ollivier et al., 1994; Oremland & King, 1989). Therefore we can suggest that the ability of strain SEBR 4845T to grow in a wide NaCl range and optimally at NaCl concentration and temperature close to that of the oil well from which it was isolated might be indicative of its indigenous origin as recently hypothesized (L'Haridon et al., 1995; Nilsen et al., 1996).
The second isolate, strain FR1T, is a moderately halophilic methanogen, that grows optimally at 10% NaCl and tolerates up to 20% NaCl. It grows on trimethylamine as energy sources and has a G+C content of 43 mol%. It is therefore phenotypically and genetically more related to the members of the genus *Methanohalophilus* (Paterek & Smith, 1988) than to the members of the genus *Methanoblobus* (Stetter, 1989). The latter grow optimally at a lower NaCl concentration and have a narrower range of NaCl concentration for growth than that of *Methanohalophilus* species (Oremland & Boone, 1994; Stetter, 1989). A member of the genus *Methanohalophilus*, ‘*Methanohalophilus euhalobius*’ was also isolated from oilfield ecosystems (Davidova et al., 1997; Obraztsova et al., 1987a).

Here we also report that enrichments to show the presence of aceticlastic methanogens in the saline oil sample were unsuccessful. These results finally suggest that methylotrophic, but not aceticlastic methanogenic archaea are also representative of a subterrestrial saline ecosystem as already described for terrestrial saline to hypersaline environments, hydrogenotrophs being adapted to lower NaCl concentrations than methylotrophs (Olivier et al., 1994). Phenotypic (Table 1) and phylogenetic studies of strain SEBR 4845T allow its proposal as the type species of a new genus, *Methanocalculus halotolerans* gen. nov., sp. nov.

**Description of Methanocalculus gen. nov.**

*Methanocalculus* (Me.tha.no.cal’cu.lus. M.L. n. meth-anum methane; M.L. n. calculus pebble, gravel; M.L. masc. n. Methanocalculus a methane-producing pebble-shaped bacterium).

Cells are irregular cocci and possess peritrichous flagella. Methanogenic and obligately anaerobic member of the domain *Archaea*. Mesophilic and neutrophilic; growth occurring at NaCl concentrations ranging from 0 to 12-5% with optimum at 5% NaCl. Produces methane from H₂+CO₂ and formate. The type species is *Methanocalculus halotolerans*.

**Description of Methanocalculus halotolerans sp. nov**

*Methanocalculus halotolerans* (hal.o.to.ler’ans. Gr. n. hals, halos salt; tolerans L. pres. part. of tolero tolerate; M.L. masc. adj. halotolerans salt-tolerating).

Round colonies (diameter, 1 mm) are present after 10 weeks incubation at 37°C. Cells are irregular cocci with a diameter of 0.8–1 μm. The cells occur singly or in pairs and possess two to three peritrichous flagella. The optimum temperature for growth is 38°C with no growth occurring at 24 and 50°C. The optimum pH is 7.6; growth occurs from pH 7.0 to 8.4. The optimum NaCl concentration for growth is 5% with growth occurring in 0–12.5% NaCl. Doubling time is about 12 h under optimal conditions. Produces methane from H₂+CO₂ and formate. Requires acetate for growth; yeast extract is stimulatory. Cannot catabolize acetate, methanol, trimethylamine, lactate, glucose, CO₂+1-propanol, CO₂+2-propanol, CO₂+1-butanol, or 2-butanol as substrates for methanogenesis. The G+C content of the DNA is 55 mol% (as determined by HPLC). Isolated from an oil-producing well. The type strain is SEBR 4845T deposited in Oregon Collection of Methanogens (= OCM 470T).

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