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

Bernard Ollivier,[1] Marie-Laure Fardeau,[1] Jean-Luc Cayol,[1] Michel Magot,[2] Bharat K. C. Patel,[3] Gérard Prensier[4] and Jean-Louis Garcia[1]

Author for correspondence: Bernard Ollivier. Tel: + 33 4 91 82 85 76. Fax: + 33 4 91 82 85 70. e-mail: ollivier@esil.univ-mrs.fr

[1] Laboratoire ORSTOM de Microbiologie des Anaérobies, Université de Provence, 13288 Marseille Cedex 9, France

[2] Sanofi Recherche, Groupe Elf-Aquitaine, Unité de Microbiologie, 31676 Labège Cedex, France

[3] School of Biomolecular and Biomedical Sciences, Griffith University, Nathan 4111, Brisbane, Queensland, Australia

[4] Laboratoire de Microbiologie, Université Blaise Pascal, 63177 Aubière Cedex, France

Two irregular coccoid methanogens designated SEBR 4845^T and FR1T were isolated from an oilfield in Alsace, France. Strain SEBR 4845^T (T = type strain) is a hydrogenotrophic halotolerant methanogen, which grows optimally at 5% NaCl (w/v) and tolerates up to 12% NaCl. It does not use methylated compounds and therefore cannot be ascribed to any of the known genera of the halophilic methylotrophic methanogens. It differs from hydrogenotrophic members of the orders *Methanococcales* and *Methanomicrobiales* in the NaCl growth range (0–12% NaCl), which is the widest reported to date for any hydrogenotrophic methanogen. 16S rRNA gene sequence analysis indicated that strain SEBR 4845^T is a novel isolate for which a new genus is proposed, *Methanocalculus halotolerans* gen. nov., sp. nov. (= OCM 470^T) that might be indigenous to the oilfield ecosystem. Strain FR1T (= OCM 471) is a moderately halophilic methanogen which grows optimally at 10% NaCl and tolerates up to 20% NaCl. It grows on trimethylamine and methanol as carbon and energy sources. The G+C content of its DNA is 43 mol%. It is therefore phenotypically and genotypically related to members of the genus *Methanohalophilus*. This report provides evidence that methylotrophic and hydrogenotrophic, but not aceticlastic 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

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 surface 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 *Methanohalphilus* (Paterek & Smith, 1988). A similar micro-organism was also isolated from oilfield ecosystems (Davidova *et al.*, 1997; Obraztsova *et al.*, 1987a). Results are

The GenBank accession number for the 16S rRNA sequence of strain SEBR 4845^T 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 (l^{-1}) 1 g NH_4Cl, 0·3 g K_2HPO_4, 0·3 g KH_2PO_4, 14·2 g $MgCl_2.6H_2O$, 11·3 g $CaCl_2.2H_2O$, 0·17 g KCl, 87 g NaCl, 0·5 g CH_3COONa, 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 O_2-free N_2 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 N_2/CO_2 (80:20, v/v) at the atmospheric pressure. Vessels were autoclaved for 45 min at 110 °C. Prior to culture inoculations, $Na_2S.9H_2O$ and $NaHCO_3$ 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 H_2/CO_2 (80:20; 200 kPa) or trimethylamine (10 mM) as growth substrates. The pH of the medium under H_2/CO_2 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 4845^T and FR1T were isolated on H_2+CO_2 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) $NaHCO_3$ or Na_2CO_3 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 H_2/CO_2 (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 hydroxyapatite, and the G+C content was determined by using HPLC as described by Mesbah *et al.* (1989). Non-methylated lambda DNA (Sigma) was used as the standard.

16S rRNA sequence studies. A primer pair, designated FARCH-9 (5′-CTGGTTGATCCTGCCAG-3′) and Rd1 (5′-AAGGAGGTGATCCAGCC-3′) was used to amplify the 16S rRNA gene from genomic DNA of strain SEBR 4845^T. The amplified product was purified (Andrews & Patel, 1996; Love *et al.*, 1993; Redburn & 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-AG-3′), R2 (5′-GTATTACCGCGGCTGCTG-3′), R4 (5′-CCGTCAATTCCTTTGAGTTT-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 ae2 (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 dendrograms 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 H_2+CO_2 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 H_2+CO_2. Two cultures were obtained using this technique. The strain obtained on H_2+CO_2 was designated strain SEBR 4845^T (OCM 470^T) 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.

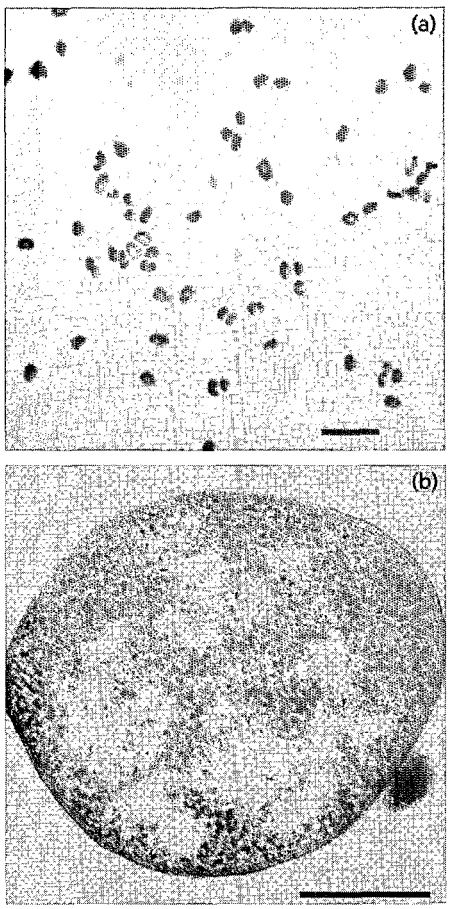

Fig. 1. (a) Phase-contrast micrograph of strain SEBR 4845^T showing irregular coccoid cells (bar, 5 μm); (b) electron micrograph of an ultrathin section of strain SEBR 4845^T showing the cell wall structure (bar, 0·2 μm).

Morphology

Strain SEBR 4845^T 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 4845^T 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).

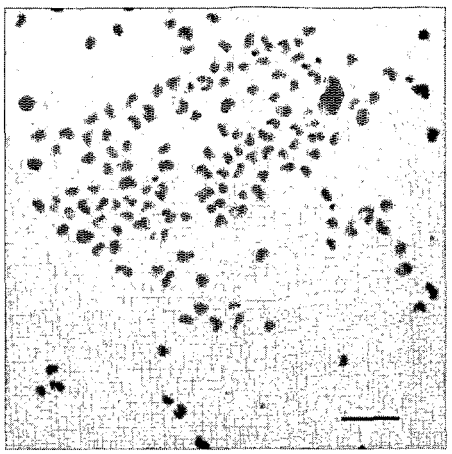

Fig. 2. Phase-contrast micrograph of strain FR1T showing irregular coccoid cells (bar, 5 μm).

Growth substrates

Strain SEBR 4845^T used H_2+CO_2 and formate to produce methane. Under a N_2/CO_2 atmosphere, it could not produce methane from acetate (20 mM), methanol (40 mM), trimethylamine (10 mM), lactate (10 mM), glucose (20 mM), CO_2 + 1-propanol (10 mM), CO_2 + 2-propanol (10 mM), CO_2 + 1-butanol (10 mM) or 2-butanol (10 mM) after 1 month incubation at 37 °C. Acetate (2 mM) was required for growth on H_2+CO_2, and yeast extract was stimulatory for growth.

Strain FR1T grew with methanol and trimethylamine as substrates for methanogenesis with N_2/CO_2 as the gas phase. It could not produce methane from H_2+CO_2 (80:20; 200 kPa), formate (40 mM), acetate (20 mM), lactate (10 mM), glucose (20 mM), CO_2 + 1-propanol (10 mM), CO_2 + 2-propanol (10 mM), CO_2 + 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 4845^T 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,

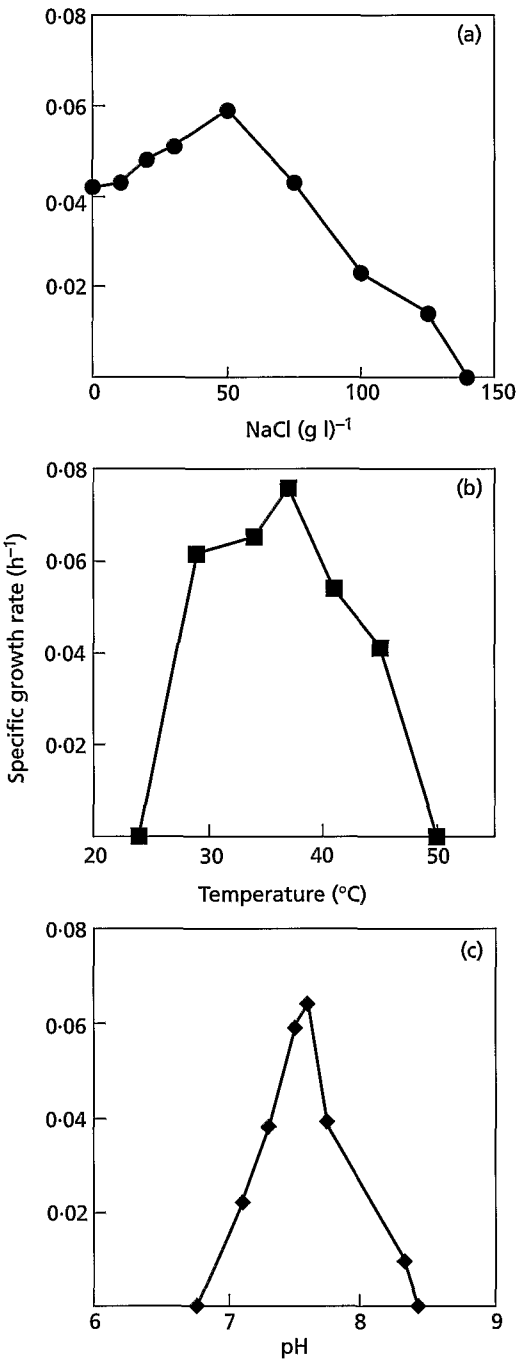

Fig. 3. Effects of (a) NaCl (temperature 37 °C; pH 7·0), (b) temperature (pH 7·0; 90 g NaCl l^{-1}) and (c) pH (temperature 37 °C; 90 g NaCl l^{-1}) on the growth of strain SEBR 4845^{T} cultivated in basal medium containing 1 g yeast extract l^{-1} and 1 g l^{-1} bio-Trypticase and sodium formate (40 mM).

phylogenetic study was only performed on strain SEBR 4845^{T}. Using five primers, we determined an almost complete sequence consisting of 1444 bases of the 16S rRNA gene of strain SEBR 4845^{T}. Several phylogenetic trees were constructed using a selection of different methanogen sequences obtained from the

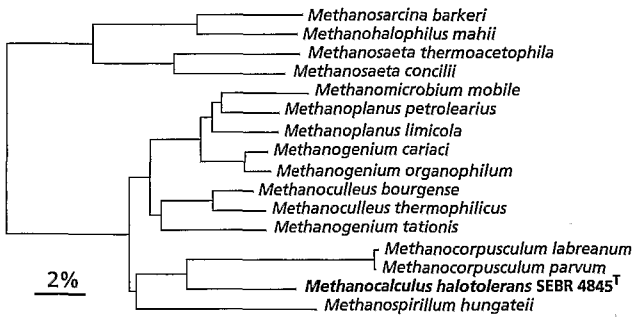

Fig. 4. Dendrogram showing the position of strain SEBR 4845^{T} amongst the order *Methanomicrobiales* of the methanogenic bacteria. The dendrogram was constructed using rRNA reference sequences from the Ribosomal Database Project (Maidak *et al.*, 1996). A pairwise Jukes–Cantor evolutionary distance matrix (Jukes & Cantor, 1969) was computed using 1224 unambiguous nucleotides and the dendrogram constructed from these distances by the neighbour-joining method. Both programs are part of the PHYLIP suite of programs (Felsenstein, 1993). Bar indicates evolutionary distance.

RDP database. No variation in the placement of strain SEBR 4845^{T} was observed. The sequences listed in Fig. 4 were used to construct a representative tree. Phylogenetic analysis indicated that strain SEBR 4845^{T} 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 4845^{T} and *Methanocorpusculum* sp. or *Methanospirillum hungateii*. Despite a clear relationship between strain SEBR 4845^{T} 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 thermoautrophicum* (Ivanov *et al.*, 1983), *Methanobacterium bryantii* (Davydova-Charakhch'yan *et al.*, 1993a), *Methanococcus 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 thermoalcaliphilum* (Davydova-Charakhch'yan *et al.*, 1993a); (ii) methylotrophs, '*Methanohalophilus euhalobius*' (Davidova *et al.*, 1997; Obraztsova *et al.*, 1987a), *Methanosarcina siciliae* (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 4845^T from representative species of coccoid methanogens belonging to the order *Methanomicrobiales*

Species	Strain SEBR 4845^T*	*Methanoplanus limicola*†	*Methanocorpusculum parvum*‡	*Methanogenium organophilum*§	*Methanoculleus marisnigri*‖
Collection no.	OCM 470^T	DSM 2279^T	DSM 3823^T	DSM 3596^T	DSM 1498^T
Source	African oil well	Swamp	Waste water	Marine mud	Marine sediment
Temp. range (°C)	25–45	17–41	25–44	ND–39	15–40
Optimum temp. (°C)	38	40	37	30–35	20–25
pH range	7·0–8·4	ND	ND	ND	5·8–7·6
Optimum pH	7·6	6·5–7·5	6·8–7·5	6·4–7·3	6·6
NaCl concn range (%)	0–12·5	0·4–5·4	ND	ND	0–4·8
Optimum NaCl concn (%)	5	1	0–4·7	2	1
Generation time (h)	12	7	8	6	ND
G+C content (mol%)	55	48	49	47	61
Substrates used	H_2+CO_2 Formate	H_2+CO_2 Formate	H_2+CO_2 Formate CO_2+2-propanol CO_2+2-butanol	H_2+CO_2 Formate CO_2+2-propanol CO_2+2-butanol CO_2+1-propanol CO_2+ethanol	H_2+CO_2 Formate CO_2+2-propanol CO_2+2-butanol

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 4845^T. 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 archaea is 8·3% reported for *Methanococcus thermolithotrophicus* (Huber *et al.*, 1982). Strain SEBR 4845^T 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 4845^T which therefore cannot be placed as a member of the family *Methanosarcinaceae*. *Methanoplanus*, the only described genus in the family *Methanoplanaceae*, comprises three species, namely *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 4845^T morphologically. The genus *Methanocorpusculum*, which is the only described genus in the family *Methanocorpusculaceae*, comprises five species that grow optimally at much lower NaCl concentrations than that of SEBR 4845^T (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*, 4·8% (Zellner *et al.*, 1990); *Methanogenium cariaci*, 3·2% (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 4845^T 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 4845^T 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 *Methanolobus* (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 (Ollivier *et al.*, 1994). Phenotypic (Table 1) and phylogenetic studies of strain SEBR 4845^T 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. *methanum* 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 H_2+CO_2 and formate. The type species is *Methanocalculus halotolerans*.

Description of *Methanocalculus halotolerans* sp. nov

Methanocalculus halotolerans (ha.lo.to.le'rans. 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 H_2+CO_2 and formate. Requires acetate for growth; yeast extract is stimulatory. Cannot catabolize acetate, methanol, trimethylamine, lactate, glucose, CO_2+1-propanol, CO_2+2-propanol, CO_2+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 4845^T deposited in Oregon Collection of Methanogens (= OCM 470^T).

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