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Methanoplanus petrolearius sp. nov., a novel methanogenic bacterium from an oil-producing well

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Abstract

A disc-shaped methanogenic bacterium designated strain SEBR 4847^T (T = type strain) was isolated from a sample collected from an African offshore oil field. Strain SEBR 4847^T was non-motile, had a G+C content of 50 mol% and produced methane from H_2+CO_2, formate, and CO_2+propanol. Strain SEBR 4847^T grew optimally at 37°C; no growth was observed at 25°C or 45°C. It grew in the presence of up to 50 g/l NaCl; 10–30 g/l was required for optimal growth. The optimum pH for growth was 7.0. Doubling time was about 10 h under optimal conditions. Based on 16S rRNA sequence analysis, the isolate was identified as a new species of the genus *Methanoplanus* and designated *Methanoplanus petrolearius* sp. nov. The type strain is SEBR 4847^T (= OCM 486).

Keywords: Methanoplanaceae; *Methanoplanus*; Methanogen; Taxonomy; Oil well

1. Introduction

The study of the microbiology of oil reservoirs has shown the presence of fermentative bacteria [10,19], sulfate reducers [6,22,20,24], acetogens [9], and methanogens [3,8,15–18]. The methanogens that were isolated and characterized include (i) the hydrogenotrophic *Methanobacterium thermoautrophicum* [15], *M. bryantii* [8], *Methanococcus thermolithotrophicus* [16], and *Methanobacterium ivanovii* [3], (ii) phenotypic variants of *Methanobacterium thermoaggregans* [15], and *M. thermoalcaliphilum* [8], (iii) the methylotrophic *Methanococcoides euhalobius* [17], and (iv) the acetoclastic *Methanosarcina mazei* [18].

We have recently undertaken extensive microbial studies of oil fields. We report here on the enumeration, isolation and characterization of a new species of a dominant hydrogenotrophic methanogen present in an offshore oil field of the Gulf of Guinea, West Africa.

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2. Materials and methods

2.1. Sample collection and sample source

One liter sample was collected from the well-head of an offshore oil field of the Gulf of Guinea, West Africa, as previously described [4]. The in situ temperature was 33°C and the NaCl concentration was 32 g/l. The samples were air mailed to our laboratory and stored at 4°C until used.

2.2. Enumeration, enrichment, isolation and growth conditions

Enrichment, enumeration, and isolation of methanogenic cultures were achieved in a basal medium that contained (per liter) 1 g of NH_4Cl, 0.3 g of K_2HPO_4, 0.3 g of KH_2PO_4, 5 g of $MgCl_2$, 2 g of $CaCl_2 \cdot 2H_2O$, 0.2 g of KCl, 30 g of NaCl, 0.5 g of CH_3COONa, 0.5 g of cysteine-HCl, 1 g of yeast extract (Difco Laboratories, Detroit, MI), 1 g of bio-Trypticase (bioMérieux, France), 10 ml of the trace mineral element solution of Balch et al. [2], 1 mg of resazurin, and 1000 ml of 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, cooled to room temperature and 5 ml and 20 ml aliquots dispensed under a stream of N_2/CO_2 (80:20) gas mixture into Hungate tubes and serum bottles, respectively, and autoclaved for 45 min at 110°C. $Na_2S \cdot 9H_2O$ and Na_2CO_3 were injected from sterile stock solutions to a final concentration of 0.04% and 0.2% prior to culture inoculations.

Enumeration of methanogenic bacteria was performed using the Most Probable Number (MPN) technique. Tubes containing basal medium were amended with H_2/CO_2 (80:20, 2 bars) or a mixture of methanol (40 mM) and acetate (20 mM) as growth substrates and were inoculated in triplicate with serial dilutions prepared from the oil-field sample. Results were recorded after incubation at 37°C for 7 days by measuring methane production. Enrichment cultures were initiated by inoculating 1 ml of the last dilution of the positive hydrogenotrophic methanogen enumeration tubes, into serum bottles containing basal medium and H_2/CO_2 (80:20, 2 bars). 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 [12] using growth medium solidified with 1.5% Noble agar (Difco).

2.3. pH, temperature, and NaCl ranges for growth

For pH studies we used Hungate tubes containing the growth medium, the pH of which was adjusted to the desired value by injecting appropriate volumes of sterile 10% $NaHCO_3$ or Na_2CO_3 anaerobic stock

Table 1
Characteristics that differentiate members of the genus *Methanoplanus*

Species	Strain SEBR 4847[T,a]	*Methanoplanus limicola*[b]	*Methanoplanus endosymbiosus*[c]
Type strain	OCM 486	DSM 2279	DSM 3599
Source	African oil well	swamp	marine ciliate
Temp range (°C)	28–43	17–41	16–36
Optimum Temp (°C)	37	40	32
pH range	5.3–8.2	ND	6.1–8.0
Optimum pH	7.0	6.5–7.5	6.8–7.3
NaCl concn. range (%)	0–5	0.4–5.4	0–4.5
Optimum NaCl concn. (%)	1–3	1	1.5
Generation time (h)	10	7	7
G+C content (mol %)	50	48	39
Substrates used	H_2+CO_2, formate CO_2+2-propanol	H_2+CO_2, formate	H_2+CO_2, formate

[a]This study.
[b]Data from Wildgruber et al. [25].
[c]Data from van Bruggen et al. [23].

solutions. Growth was tested at temperatures ranging from 25°C to 45°C. To determine salt requirement for growth, NaCl was weighed directly into Hungate tubes and the medium was subsequently dispensed as described above. The strain was subcultured at least once under the same experimental conditions prior to inoculation.

2.4. Substrate utilization

Substrates were added from sterile stock solutions to the basal medium at a final concentration of 10 mM (acetate, trimethylamine, lactate, glucose, 1-propanol, 2-propanol, 1-butanol, isobutanol) or 40 mM (formate, methanol). Hydrogen oxidation was tested using H_2/CO_2 (80:20, 2 bars) in the gas phase.

2.5. Analytical techniques

Unless otherwise indicated, all experiments were performed in duplicate. Phase contrast and fluorescence microscopy were performed as previously described [5]. Growth was quantified by inserting tubes directly into a model UV-160A spectrophotometer (Shimadzu Corp., Kyoto, Japan) and measuring the optical density at 580 nm. Methane was measured as described previously [7].

2.6. Determination of G+C content

The G+C content of DNA was determined at DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. The DNA was isolated and purified by chromatography on hydroxyapatite, and the G+C content was determined by using high-performance liquid chromatography (HPLC) as described by Mesbah et al. [14]. Nonmethylated lambda DNA (Sigma) was used as the standard.

2.7. 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 the methanogen. The amplified product was purified [1] 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 Inc.). The primers used for sequencing were F2 (5′-CAGGATTAGATACCCTGGTAG-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 which we determined was manually aligned with reference sequences of various members of the domain Archaea by using the alignment editor 'ae2' [13]. Reference sequences were obtained from the Ribosomal Database Project [13]. Positions of sequence and alignment uncertainty were omitted from the analysis. A pairwise evolutionary distances based on 1217 unambiguous nucleotides was computed by using the method of Jukes and Cantor and dendrograms were constructed from these distances by using the neighbour-joining method. Both programmes form part of the PHYLIP package [11].

2.8. Nucleotide sequence accession number

The 16S rRNA gene sequence of strain SEBR 4847^T has been deposited in the Genbank database under accession number U76631.

3. Results

3.1. Bacterial enumeration

MPN estimations indicated that 1.9×10^3 cells/ml hydrogenotrophic methanogens and 5×10^2 cells/ml methylotrophic non-acetoclatic methanogens were present in the oil-field sample. Total microflora, estimated by microscopy under epifluorescence, was 4.5×10^3 cells/ml.

3.2. Enrichment and isolation

After 1 week of incubation at 37°C, positive enrichment cultures developed in tubes that had been inoculated from the last dilution of positive hydrogenotrophic methanogen enumeration tubes. Microscopic examination revealed the presence of disc-shaped bacteria. Circular colonies developed in

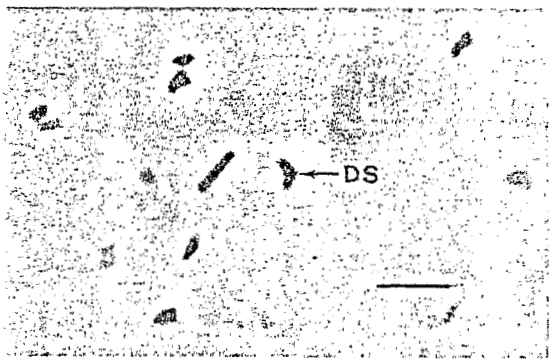

Fig. 1. Phase-contrast micrograph of strain SEBR 4847^T showing irregular disc shaped cells (DS). Bar = 5 μm.

agar roll tubes after 1 month incubation at 37°C. Two axenic cultures were obtained using this technique. They appeared similar in morphology and physiology; therefore only strain SEBR 4847^T (T = type strain) was further characterized and designated the type strain.

3.3. Morphology

Strain SEBR 4847^T was a strictly anaerobic, nonmotile, irregular disc-shaped bacterium with a diameter of 1–3 μm, occurring singly or in pairs (Fig. 1).

3.4. Optimum growth conditions

Strain SEBR 4847^T grew at an optimum temperature between 35 and 40°C. It did not grow at 25°C or at 45°C. The isolate grew in the presence of NaCl concentrations ranging from 0 to 5%, with an optimum between 1 and 3% NaCl. Growth occurred between pH 5.3 and pH 8.4 with an optimum at pH 7.0.

3.5. Substrate used for growth

Strain SEBR 4847^T used H_2–CO_2, formate, and CO_2+2-propanol to produce methane. Strain SEBR 4847^T could not utilize acetate, methanol, trimethylamine, lactate, glucose, CO_2+1-propanol, CO_2+1-butanol, and isobutanol. Acetate was required for growth, and the presence of yeast extract was stimulatory for growth.

3.6. G+C content of DNA

The G+C content of isolate SEBR 4847^T was 50 mol%.

3.7. 16S rRNA gene sequencing and sequence analysis

Using five primers, we determined an almost complete sequence consisting of 1429 bases of the 16S rRNA gene of strain SEBR 4847^T. Phylogenetic analysis revealed that strain SEBR 4847^T was a member of the family Methanoplanaceae, order Methanomicrobiales and the closest relatives was *Methanoplanus limicola* and *Methanoplanus endosymbiosus* (average similarity of 93.5%). A dendrogram generated by the Neighbour-Joining method showing

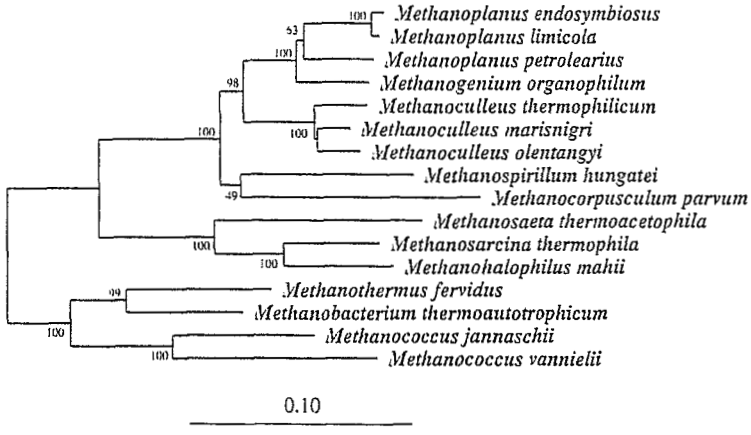

Fig. 2. Dendrogram showing the position of strain SEBR 4847^T amongst the methanogenic bacteria. Bar indicates evolutionary distance.

this relationship is shown in Fig. 2. Bootstrap analysis indicated that the relationship of strain SEBR 4847^T to *M. limicola* and *M. endosymbiosus* was not robust.

4. Discussion

Strain SEBR 4847^T produced methane from H_2 and CO_2 and a green fluorescence under UV light microscope, thus indicating that the isolate was a methanogen. Strain SEBR 4847^{T}is a disc-shaped irregular bacterium and has a G+C content of 50 mol% and therefore cannot be placed as member of the order Methanococcales which are irregular cocci and have G+C content ranging from 30 to 40 mol%. The order Methanomicrobiales contains families Methanomicrobiaceae, Methanocorpusculaceae, Methanoplanaceae, and Methanosarcineae. Acetate, methanol or methylamines cannot be used as substrates; therefore strain SEBR 4847^T cannot be placed as member of the family Methanosarcinaceae. Members of the families Methanomicrobiaceae and Methanocorpusculaceae are irregular cocci or rods; they are therefore morphologically distinct from strain SEBR 4847^T. *Methanoplanus* which is the only described genus in the family Methanoplanaceae, is comprised of two species, namely *Methanoplanus endosymbiosus* and *M. limicola*. Both are described as disc-shaped hydrogenotrophic methanogens [23,25] and therefore resemble strain SEBR 4847^T morphologically and phenotypically. The analysis of the 16S rRNA sequence of strain SEBR 4847^T confirmed its affiliation to *M. limicola* and *M. endosymbiosus* (average similarity 93.5%). Strain SEBR 4847^T differed from *M. endosymbiosus* [23] in the temperature growth range and a lower G+C content in the DNA (Table 1). In contrast to *M. limicola* [25], strain SEBR 4847^T grew at 42°C and had an optimum NaCl concentration ranging from 1% up to 3%. Phylogenetically, *M. limicola* and strain SEBR 4847^T were also distinct (similarity of 95%), a feature which alone warrants to place SEBR 4847^T as a new species of the genus *Methanoplanus* [21].

Strain SEBR 4847^T was isolated from a subsurface ecosystem. In contrast, *M. limicola* was isolated from a small Italian swamp containing drilling waste and was assumed to inhabit swamps of freshwater and seawater. However, on the basis of our results we can hypothesize that methanogens similar to *M. limicola* and strain SEBR 4847^T could originate from subsurface ecosystems since phylogenetically similar strains were also isolated from another offshore oil field off the Gulf of Guinea (data not shown). Furthermore strain SEBR 4847^T exhibited optimum growth over a wide range of NaCl concentration (1–3%) indicating that it could grow optimally in the saline conditions of the oil field (3%). Based on phylogenetic and phenotypic characteristics, we propose that strain SEBR 4847^T be designated as a new species of the genus *Methanoplanus*, *M. petrolearius* sp. nov.

4.1. Description of *Methanoplanus petrolearius* sp. nov.

Methanoplanus petrolearius (pe.tro.le.a'riusL. fem. n. *petra* rock; L. adj. *olearius* related to vegetal oil: *petrolearius* L. masc. adj. related to mineral oil). Round colonies (diameter: 1–2 mm) are present after 3 weeks of incubation at 37°C. Cells are irregular disc-shaped with a diameter of 1–3 μm. The cells occur singly or in pairs and are non-motile under microscope. Methanogenic and obligately anaerobic member of the domain Archaea. The optimum temperature for growth is 37°C with no growth occurring at 25°C and 45°C. The optimum pH is 7.0; growth occurs from pH 5.3 to 8.4. The optimum NaCl concentration for growth is between 1 and 3% NaCl with growth occurring at NaCl concentration ranging from 0 and 5%. Doubling time is about 10 h under optimal conditions. Produces methane from H_2–CO_2, formate, and CO_2+2-propanol. The strain requires acetate for growth and yeast extract is stimulatory. The strain cannot use acetate, methanol, trimethylamine, lactate, glucose, CO_2+1-propanol, CO_2+1-butanol, and isobutanol. The G+C content of the DNA is 50% (as determined by HPLC). Isolated from oil-producing well. The type strain is SEBR 4847^T(=OCM 486).

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