***Methanococcus infernus* sp. nov., a novel hyperthermophilic lithotrophic methanogen isolated from a deep-sea hydrothermal vent**

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An autotrophic, extremely thermophilic methanogen (ME^T) was isolated from a deep-sea hydrothermal chimney sample collected on the Mid-Atlantic Ridge at a depth of 3000 m. The heavily flagellated cells are motile and coccoid shaped. The new strain grows between 55 and 91 °C, with an optimum growth temperature at 85 °C. The optimum pH for growth is 6.5, and the optimum sea salt concentration for growth is around 25 g l⁻¹. The organism uses H₂ and CO₂ as the only substrate for growth and methane production. Tungsten, selenium and yeast extract stimulate growth significantly. In the presence of CO₂ and H₂, the organism reduces elemental sulphur to hydrogen sulphide. The G+C content of the genomic DNA is 33 mol%. As determined by 16S gene sequence analysis, this organism is closely related to *Methanococcus jannaschii* strain JAL-1^T. However, no significant homology was observed between them with DNA–DNA hybridization. It is proposed that this organism should be placed in a new species, *Methanococcus infernus*. The type strain is ME^T (= DSM 11812^T).

Keywords: deep-sea hydrothermal vents, thermophiles, *Archaea*, *Methanococcus*, *Methanococcus infernus*

INTRODUCTION

Marine hyperthermophilic methanogens that have been isolated from shallow water systems and deep-sea hydrothermal vents (Stetter, 1996a) belong to the genera *Methanococcus* and *Methanopyrus*. *Methanococcus thermolithotrophicus* strain SN-1^T isolated from coastal geothermally heated sediments close to Naples (Italy), was the first extremely thermophilic methanogen to be described (Huber *et al.*, 1982). A selenium-independent hyperthermophilic methanogen, *Methanococcus igneus* strain Kol 5^T, was isolated from a shallow submarine hydrothermal vent (Kolbeinsey ridge, Iceland) (Burggraf *et al.*, 1990). *Methanopyrus kandleri*, which represents one of the most extreme members of hyperthermophiles known so far, was isolated from the same shallow marine

system (Kurr *et al.*, 1991). This organism also thrives in hydrothermally heated deep-sea sediments in the Guaymas Basin (Kurr *et al.*, 1991; C. Jeanthon, unpublished results). Furthermore, *Methanococcus jannaschii* strain JAL-1^T and closely related isolates have been isolated exclusively from the Guaymas Basin (Jones *et al.*, 1983, 1989; Zhao *et al.*, 1988). In this paper we described the isolation and the characteristics of a novel extremely thermophilic *Methanococcus* isolated from a deep-sea hydrothermal vent chimney collected at 14° 45' N on the Mid-Atlantic Ridge.

METHODS

Reference strains. *M. jannaschii* strain JAL-1^T (DSM 2661^T), *M. igneus* strain Kol 5^T (DSM 5666^T) and *M. thermolithotrophicus* strain SN-1^T (DSM 2095^T) were obtained from the DSMZ–Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

Collection of chimney samples. During the Microsmoke cruise (November and December 1995), a chimney was

The GenBank/EMBL/DBJ accession number for the 16S rRNA sequence of ME^T is AF025822.

collected from the Logatchev hydrothermal field (14° 45'N, 44° 59'W) on the Mid-Atlantic Ridge at a depth of 3000 m (Krasnov *et al.*, 1995). The chimney was collected by the port manipulator of the manned submersible *Nautille* and stored in an insulated basket during retrieval to the surface. On board, subsampling across the sulphide structure was conducted as aseptically as possible. Chimney subsamples were transferred in 50 ml glass vials and immersed with a sterile solution of 3% (w/v) sea salts (Sigma). The vials were closed tightly with butyl rubber stoppers (Bellco), pressurized with N₂ (100 kPa), reduced with sodium sulphide if required, and stored at 4 °C until processed further.

Enrichment cultures and purification. Enrichments were performed anaerobically in 50 or 100 ml vials containing 10 or 20 ml medium, respectively, according to Miller & Wolin (1974). The enrichment medium consisted of (l⁻¹ distilled water): 30 g sea salts, 1 g NH₄Cl, 0.35 g KH₂PO₄, 3.46 g PIPES, 1 g NaHCO₃, 2 g Difco yeast extract, 2 g Difco peptone, 1 g sodium acetate, 0.5 g cysteine·HCl, 1 ml trace element mixture (Widdel & Bak, 1992), 50 mg selenate, 30 mg tungstate, 1 ml vitamin mixture (Widdel & Bak, 1992), 1 ml thiamin solution (Widdel & Bak, 1992), 0.05 mg vitamin B₁₂, 1 ml growth-stimulating factors (Pfennig *et al.*, 1981) and 1 mg resazurin. The pH was adjusted to 6.5 using HCl 1M before autoclaving and the medium was reduced by adding appropriate amounts of sodium sulphide (Na₂S·9H₂O). H₂/CO₂ [80:20; 200 kPa (above atmospheric pressure)] was used as the gas phase. Unless indicated otherwise, cultures were incubated at 80 °C and the pH of the medium was readjusted after 1 h incubation. Positive enrichments were subcultured into the same medium without yeast extract, peptone and acetate (minimum medium, MM). Single colonies were obtained and purified by streaking onto MM that was reduced with a titanium(III) citrate solution (Zehnder & Wuhrman, 1976) and solidified with 0.7% (w/v) Phytagel (a gellan gum from Sigma). Plates were incubated in anaerobic jars at 75 °C for 3 d under a H₂/CO₂ atmosphere (80:20; 300 kPa). Stock cultures of the isolate ME were stored in culture medium at 4 °C. For long term storage, pure cultures were stored at -80 °C in the same medium containing 20% (w/v) glycerol.

Determination of growth. Growth was determined by measuring changes in turbidity at 600 nm with a Spectronic 20D spectrophotometer (Bioblock). Direct cell counts were determined using cells stained with acridine orange and counted by epifluorescence microscopy using an ocular grid (Hobbie *et al.*, 1977). All growth experiments were performed in duplicate.

Determination of growth parameters. The influence of the pH on growth was determined in the MM with various buffers at a concentration of 10 mM; acetate/acetic acid buffer from pH 4 to 5, MES at pH 5.5 and 6.0, PIPES buffer at pH 6.5 and 7.0, HEPES buffer at pH 7.5, Tris at pH 8 and 8.5. The pH of the medium was adjusted after one hour of incubation at the optimal temperature of growth. To determine the salt requirement, MM was prepared with different dilutions of sea salts. The effects of pH and salinity were determined at the optimal temperature for growth.

Determination of growth requirements. To investigate the ability to use substrates other than H₂, acetate (2 g l⁻¹), formate (5 g l⁻¹), methanol (0.5%; v/v), monomethylamine (2 g l⁻¹), and yeast extract (2 g l⁻¹) were added individually to the MM and with a N₂/CO₂ gas phase (80:20; 200 kPa). The same compounds added individually in the MM without bicarbonate at the same concentrations were tested as

possible carbon sources by using H₂ (100%; 200 kPa) as the gas phase. Selected nitrogenous compounds were tested for suitability as nitrogen sources, using ammonium-free mineral medium (Widdel & Bak, 1992). Nitrogenous compounds were added at 10 mM final concentrations. Electron acceptors were tested in the same medium without Na₂SO₄. To determine their potential stimulatory effects on the growth yield, the vitamin mixture, trace element solution, yeast extract, tungstate, and selenate were tested individually in the basal medium that consisted of (l⁻¹ distilled water): 30 g sea salts, 1 g NH₄Cl, 0.35 g KH₂PO₄, 3.46 g PIPES, 1 g NaHCO₃, 0.5 g cysteine·HCl, and 1 mg resazurin. The pH was adjusted as before and H₂/CO₂ (80:20; 200 kPa) was used as the gas phase.

Antibiotic susceptibility. Sensitivity of strain ME^T to chloramphenicol (75 µg ml⁻¹), penicillin G (200 µg ml⁻¹), streptomycin (200 µg ml⁻¹), kanamycin (200 µg ml⁻¹), ampicillin (200 µg ml⁻¹) and rifampicin (50 µg ml⁻¹) (all from Sigma) was tested at 80 °C. Simultaneous experiments were performed with the thermophilic methanogens *M. jannaschii* strain JAL-1^T and *M. igneus* strain Kol 5^T at 80 °C and *M. thermolithotrophicus* strain SN-1^T at 65 °C.

Microscopy. An Olympus BH-2 microscope equipped with an Olympus OM-2 camera was used routinely to observe and count the cells. For negative staining, 20 µl bacterial suspension fixed with 2% (w/v) glutaraldehyde was dropped on Formvar/carbon-coated grids (400 mesh) and stained with 4% (w/v) uranyl acetate. Preparations of cells for freeze-fracturing and ultrathin sectioning was performed as described previously (Sleytr *et al.*, 1988). Electron micrographs were taken on a model CM100 electron microscope (Philips) with an acceleration voltage of 80 kV.

H₂S production. H₂S production was evaluated by adding 500 µl of a solution of CuSO₄ (5 mM) and HCl (50 mM) to 250 µl of a culture grown at 85 °C. The dark brown precipitate demonstrating its presence was compared to that of the uninoculated medium incubated in the same conditions.

Methane determination. Methane was measured with a Girdel model 3000 gas chromatograph equipped with a thermal conductivity detector. Samples (0.5 ml) were injected onto a Poropak Q (80-100 mesh) column and eluted with helium at a flow rate of 12 ml min⁻¹. Temperatures were as follows: injector, ambient temperature; column, 60 °C; detector, 150 °C. These conditions allowed us to follow methane production and hydrogen consumption simultaneously.

Isolation of DNA. Genomic DNA of strain ME^T was isolated by using the procedure described by Charbonnier & Forterre (1994). The DNA was purified on a caesium chloride gradient (Sambrook *et al.*, 1989) and purity was checked spectrophotometrically.

DNA base composition. The G+C content of the DNA was determined from the melting point according to Marmur & Doty (1962) using DNA (Sigma) from *Escherichia coli* (57 mol% G+C), *Clostridium perfringens* (26.5 mol% G+C) and *Micrococcus luteus* (77 mol% G+C) as standards.

Small-subunit rDNA sequencing after PCR amplification. The 16S rDNA (16S rDNA) was amplified by PCR as described previously (Reysenbach *et al.*, 1992). The double-stranded PCR products were sequenced with an ABI 373 automated sequencer. The 16S rDNA sequences were aligned manually with a representative set of 16S rRNA sequences obtained from the Ribosomal Database Project (Maidak *et al.*, 1996).

or from recent GenBank releases. The GenBank accession numbers for the 16S rRNA sequences reported in this paper are as follows: *Methanococcus jannaschii* JAL-1^T, M59126; *Methanococcus thermolithotrophicus* SN-1^T, M59128; *Methanococcus igneus* Kol 5^T, M59125; *Methanococcus voltae* PS^T, M59290; and '*Methanococcus aeolicus*' A, U39016. The secondary structure was used as a guide to ensure that only homologous regions were compared. A total of 1429 nucleotides were sequenced, and 1377 were used in the phylogenetic analysis. The absence of chimeric molecules was ensured by using the secondary sequence and computer analyses. The phylogenetic trees were constructed either with evolutionary distance matrices, using the program of De Soete (1983), or by maximum-likelihood analysis, using the program fastDNAm1 (Olsen *et al.*, 1994). Bootstrap values were obtained for maximum-likelihood analysis by using 100 replicate trees and random addition of sequence.

DNA homology. Genetic relatedness was investigated by slot-blot DNA–DNA hybridization by using a random-prime labelling and signal amplification system (Amersham Life Sciences) following the procedure described by Kristjánsson *et al.* (1994). Increasing amounts of target DNA (50–200 ng) denatured in 0.4 M NaOH were slotted onto a nylon hybridization membrane (Bio-Rad) and probed with 200 ng labelled tracer DNA. For each duplicate of DNA–DNA association (15 h in buffer 4× SSC with formamide, 0.5% blocking agent, 5% dextran sulphate, 100 µg denatured sheared salmon sperm DNA ml⁻¹), the temperature of hybridization chosen was in the optimal range in the hybridization buffer (Johnson, 1984; Ivanova *et al.*, 1988). Final high-stringency washes and signal amplification were performed according to the manufacturer's instructions. Hybridization signals were detected with a Storm fluorescent scanner (Molecular Dynamics) and analysed by using the IMAGE-QUANT program. Signal (maximum peak area) produced by self-hybridization of the probe with homologous target DNA was set as 100%.

RESULTS

Enrichment and isolation

To obtain methanogenic thermophiles, 10 ml enrichment medium was inoculated with approximately 1 ml chimney suspensions. The enrichments were incubated at 80 °C in 50 ml vials with H₂/CO₂ as the gas phase (80:20; 200 kPa) without shaking. Within 2–3 d, turbidity caused by cell growth was observed. This growth consisted of motile and non-motile coccoid cells that fluoresced intense green at 420 nm. All positive enrichment cultures could be successfully transferred into MM. To obtain pure cultures, sub-cultures were streaked onto solidified medium and incubated in an anaerobic jar with the same gas phase at 75 °C. On solid medium, pale yellow round colonies (1 mm in diameter) were visible after incubation for up to 3 d. One colony was randomly picked and was designated as isolate ME.

Morphology

Cells of strain ME^T were irregular cocci, about 1–3 µm in diameter (Fig. 1a). Ultrathin sections of whole cells of strain ME^T possessed the typical archaeal cell

Fig. 1. Phase-contrast micrograph of strain ME^T (bar, 5 µm) (a) and electron micrographs of strain ME^T (b, c). Ultrathin sections (b) of whole cells showed the typical archaeal cell envelope profile (bar, 100 nm). Freeze-etched and shadowed preparations (c) showed the flagella and the S-layer (bar, 100 nm). S, S-layer; CM, cytoplasmic membrane.

envelope profile consisting of the cytoplasmic membrane and a single surface layer (S-layer) (Fig. 1b). They occurred singly or in pairs (Fig. 1a) and exhibited tumbling motility by means of at least three tufts of flagella, each containing a high number of flagella (Fig. 1c). Since the strain was heavily flagellated, parts of flagella were observed even on thin sections. The hexagonal S-layer lattice of strain ME^T with centre-to-centre spacings of approximately 12.2 nm was clearly visible (Fig. 1c).

Determination of growth parameters

Strain ME^T grew between 55 and 91 °C with optimum growth around 85 °C, while no growth was detected at 50 and 93 °C (Fig. 2a). Growth was observed between pH 5.25 and 7.0, with optimum growth around pH 6.5 (data not shown). Growth could be observed in sea salts concentrations ranging from 12.5 to 50 g l⁻¹ (Fig. 2b), with an optimum of approximately 25 g l⁻¹. No growth was observed at 6.25 and 56.25 g l⁻¹. Under optimal growth conditions (temperature, pH and NaCl), the doubling time of strain ME^T was approximately 35–40 min.

Determination of growth requirements

Strain ME^T is a strictly anaerobic autotrophic organism. Its growth is prevented in the presence of low levels of oxygen and H₂+CO₂ serves as the only substrate for growth. Growth was accompanied by exponential methane production which paralleled growth (data not shown). Large amounts of methane (up to 37 µmol ml⁻¹) were produced when cells entered the stationary phase. When sulphur was added to the medium in the presence of CO₂ and H₂, growth

Fig. 2. Growth rates of strain ME^T as a function of temperature (a) in the presence of sea salts (25 g l⁻¹) at pH 6.5 and (b) as a function of concentration of sea salts at 85 °C and at pH 6.5. Growth was followed (A_{600}) in MM under an atmosphere of H₂/CO₂ (80:20; 200 kPa).

occurred and H₂S was produced. Nitrate and ammonium were used as nitrogen sources (data not shown). When supplemented individually in the basal medium, the vitamin mixture, tungstate solution, selenate solution and yeast extract stimulated growth whereas trace element solution was found to be slightly inhibitory (data not shown). No growth was observed on acetate, formate, methanol, monomethylamine or yeast extract with a N₂/CO₂ (80:20; 200 kPa) or H₂ (100%; 200 kPa) headspace. As a control, *M. thermolithotrophicus* strain SN-1^T grew on formate at 65 °C in the presence of N₂/CO₂. No dissimilatory reduction of sulphate or thiosulphate was observed.

Sensitivity to antibiotics

M. jannaschii strain JAL-1^T, *M. igneus* strain Kol 5^T, strain ME^T, and *M. thermolithotrophicus* strain SN-1^T were resistant to ampicillin and kanamycin (200 µg ml⁻¹) and were sensitive to chloramphenicol (75 µg ml⁻¹). Among these strains, only *M. thermolithotrophicus* strain SN-1^T was inhibited by penicillin and streptomycin (200 µg ml⁻¹). Finally, *M. jannaschii* strain JAL-1^T and strain ME^T were sensitive to

Fig. 3. Phylogenetic relationships of strain ME^T and other *Methanococcales* produced by maximum-likelihood analysis. The sequence of the ME^T small subunit was aligned with other rRNA from the Ribosomal Database Project (17). The scale bar represents the expected number of changes per sequence position.

rifampicin (50 µg ml⁻¹) whereas *M. igneus* strain Kol 5^T and *M. thermolithotrophicus* strain SN-1^T were resistant to this compound.

DNA base composition

The G+C content of the DNA of strain ME^T determined by the thermal denaturation (T_m) method was 33 mol%. As a control, the base composition of *Fervidobacterium nodosum* was determined to be 35 mol% [34 mol% by the T_m method (Patel *et al.*, 1985) and by direct base analysis reported by (Huber *et al.*, 1990)].

16S rDNA sequence analysis

The 16S rDNA sequence analysis placed strain ME^T as a close relative of *M. jannaschii* strain JAL-1^T (Fig. 3). Based on their 16S rDNA sequences, a similarity matrix generated using the correction of Jukes & Cantor (1969) as modified by Olsen *et al.* (1994) revealed that ME^T was 96.5% similar to *M. jannaschii* strain JAL-1^T. Phylogenetic trees generated using maximum-likelihood analyses and distance matrices were similar. Bootstrap values in both cases place ME^T unequivocally with *M. jannaschii* strain JAL-1^T (in 100% of the samplings of 100 bootstrap resamplings).

DNA-DNA homology

No significant homology (< 10%) was obtained between bulk cellular DNA of the isolate ME^T and *M. jannaschii* strain JAL-1^T.

DISCUSSION

The novel marine extremely thermophilic strain ME^T belongs to the archaeal domain on the basis of the cell envelope composition, its resistance to antibiotics, and

Table 1. Characteristics of *Methanococcus* species and strain ME^T

Organism	Motility	Temperature (°C)		Substrate	Stimulation by:			Reference
		Range	Optimum		Selenium	Tungsten	Yeast extract	
Strain ME ^T	+	55–91	85	H ₂	+	+	+	This work
<i>M. jannaschii</i> strain JAL-1 ^T	+	50–86*	85	H ₂	+	ND†	–	Jones <i>et al.</i> (1983)
<i>M. igneus</i> strain Kol 5 ^T	–	45–91	88	H ₂	–	ND	+	Burggraf <i>et al.</i> (1990)
<i>M. thermolithotrophicus</i> strain SN-1 ^T	+	30–70	65	H ₂ , formate	ND	–	ND	Huber <i>et al.</i> (1982)

ND, Not determined.

* In our laboratory, the maximum growth temperature measured was 91 °C. No growth was obtained at 93 °C.

† In our laboratory, tungstate (30 mg l⁻¹) was found to stimulate growth.

the 16S rDNA sequence (Hilpert *et al.*, 1981; Woese *et al.*, 1990; Sleytr *et al.*, 1996). The morphology, metabolism, G + C content and 16S rDNA sequence indicate that strain ME^T belongs to genus *Methanococcus*.

Strain ME^T differs from *M. thermolithotrophicus* strain SN-1^T in its inability to use formate and in its temperature range and optimum for growth (Huber *et al.*, 1982; Jones *et al.*, 1989). It differs from *M. igneus* strain Kol 5^T in its temperature range and optimum for growth, its ability to grow in the presence of sulphur, and its positive response to the effect of yeast extract and selenium (Burggraf *et al.*, 1990). The newly described strain ME^T is most similar to *M. jannaschii* strain JAL-1^T with respect to motility, temperature optimum and range for growth (Table 1), and with respect to susceptibility to antibiotics. However, phenotypically, it differs from the type strain JAL-1^T in several aspects. In contrast with strain JAL-1^T, strain ME^T did not grow at 50 °C. Moreover, the growth of strain ME^T in basal medium was enhanced by the presence of yeast extract. This compound has no stimulatory effect on growth of strain JAL-1^T [Jones *et al.*, 1983; this study (data not shown)].

The results of phylogenetic analyses of 16S rDNA gene sequences indicated that strain ME^T is related to *M. jannaschii* strain JAL-1^T. The level of 16S rDNA sequence similarity between strain ME^T and this organism (96.5%) was less than the limit (97%) used to define distinct species at the DNA level without the requirement for DNA–DNA reassociation tests (Stackebrandt & Goebel, 1994). The low level of genetic relationship between strains ME^T and JAL-1^T was confirmed by DNA–DNA hybridization studies, indicating that these organisms cannot be assigned to the same species (Johnson, 1984). From the above results, we concluded that strain ME^T represents a new *Methanococcus* species. We propose to name it *Methanococcus infernus* reflecting its high temperature of growth and the nature of its extreme habitat, the deep-sea hydrothermal vent chimneys.

At the Mid-Atlantic 'Snake Pit' vent, high numbers of cells of *Methanopyrus* spp. [10⁸ (g chimney material)⁻¹]

have been enumerated (Stetter, 1996b). However from an ecological point of view, thermophilic *Methanococcus* spp. were thought to represent the dominant methanogenic archaea occurring at deep-sea hydrothermal vents (Jones *et al.*, 1989). Our recent survey of thermophilic subpopulations of methanogens at the 23°N site on the Mid-Atlantic Ridge seems to confirm this postulate (Harmsen *et al.*, 1997). Furthermore, their widespread occurrence and their diversity in the deep-sea hydrothermal vent sites from Guaymas Basin, East Pacific Rise (13°N) and Mid-Atlantic Ridge (14.5°N and 23°N) suggests that they may play a significant role in this unique habitat (C. Jeanthon, S. L'Haridon, N. Pradel & D. Prieur, unpublished results).

Description of *Methanococcus infernus* sp. nov.

Methanococcus infernus (in.fer'nus. L. masc. adj. *infernus* referring to the place of isolation, deep-sea hydrothermal vents).

Cells exhibit a tumbling motility by means of tufts of flagella. They are cocci (diameter, 1–3 µm) and occur singly and in pairs. Pale yellow colonies about 1 mm in diameter formed on Phytigel plates. Growth occurs between 55 and 91 °C, with an optimum around 85 °C (doubling time 35–40 min). Growth occurs between pH 5.25 and 7 with an optimum of approximately pH 6.5, and with sea salt concentrations between 12.5 and 56.25 g l⁻¹ with an optimum of approximately 25 g l⁻¹. Obligately anaerobic. Chemolithotrophic. Uses H₂ and CO₂ as energy and carbon sources to produce methane. Growth is stimulated by selenate, tungstate and yeast extract. Sulphur is reduced to hydrogen sulphide in the presence of CO₂ and H₂. Growth is inhibited by chloramphenicol (75 µg ml⁻¹) and rifampicin (50 µg ml⁻¹) but not by streptomycin, penicillin G, kanamycin and ampicillin (all at 200 µg ml⁻¹). DNA base composition of the type strain is 33 mol% G + C (as determined by the thermal denaturation method). The type strain is *Methanococcus infernus* ME^T, which was obtained from a deep-sea hydrothermal vent chimney at Mid-Atlantic Ridge (14° 45'N). Strain ME^T has been deposited in the DSMZ–Deutsche Sammlung von Mikroorganismen

und Zellkulturen under accession number DSM 11812^T.

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