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Methanococcus Thermolithotrophicus, a Novel Thermophilic Lithotrophic Methanogen

Harald Huber[1], Michael Thomm[1], Helmut König[1], Gesa Thies[2], and Karl O. Stetter[1]

[1] Lehrstuhl für Mikrobiologie, Universität Regensburg, D-8400 Regensburg, Federal Republic of Germany
[2] Lehrstuhl für Zoologie, Universität Regensburg, D-8400 Regensburg, Federal Republic of Germany

Abstract. An autotrophic thermophilic motile coccoid methanogen was isolated from geothermally heated sea sediments close to Naples, Italy. Growth occurs on H_2/CO_2 and on formate between 30 and 70° C with an optimum at 65° C. The optimal doubling time is only 55 min. The NaCl-concentration ranges from 1.3% to 8.3% with an optimum around 4%. By its G + C-content of 31.3 mol%, its subunit envelope, and by DNA-RNA hybridization the new isolate is clearly defined to be a member of the genus *Methanococcus*. We name it *Methanococcus thermolithotrophicus*.

Key words: Methanogens – Archaebacteria – Autotrophic – Thermophilic

All thermophilic methanogens known so far are members of two orders, the Methanobacteriales and the Methanomicrobiales. The latter contains only one thermophilic strain, *Methanosarcina* TM-1 (Zinder and Mah 1979), which has been isolated from a sewage digestor and grows at a maximal temperature of 55° C. The Methanobacteriales contain the thermophilic *Methanobacterium thermoautotrophicum*, which occurs in thermal springs and sewage digestors (Zeikus and Wolfe 1972; Zeikus et al. 1980), and grows between 37° C (Stetter unpublished) and 75° C (Zeikus and Wolfe 1973; Zeikus et al. 1980), and the extreme thermophilic species *Methanothermus fervidus* (Stetter et al. 1981), which was recently isolated from a hot spring in Iceland, and grows between 65 and 97° C. In the order Methanococcales, 2 species, which can be cultivated only in the mesophilic temperature range, are known (Balch et al. 1979).

Here we report on the isolation and characterization of a new species of *Methanococcus* which grows at temperatures up to 70° C.

Materials and Methods

Strains

Methanococcus voltae, DSM 1937, was obtained from the Deutsche Sammlung von Mikroorganismen, Göttingen.

Culture Conditions

The organisms were cultivated by using the technique described by Balch and Wolfe (1976). *Methanococcus voltae* was grown in medium 3 of Balch et al. (1979), slightly modified (Wildgruber et al. 1982). For autotrophic growth of the new isolate, medium 3 of Balch et al. (1979), not containing sodium acetate, yeast extract, trypticase, and trace vitamines, pH 6.9 (adjusted with H_2SO_4), was used ("MGG medium"). Twenty milliliter cultures were grown in stoppered pressurized 100 ml serum bottles (Bormioli, Italy) made of "type III"-glass by incubation in shakers (New Brunswick) at 140 rpm, employing a glycerol bath.

Enrichment and isolation medium ("supplemented sea water"): 750 ml sea water was supplemented with the following ingredients: Na_2CO_3 1 g; NH_4HCO_3 2 g; sodium formate 1 g; sodium acetate 5 g; yeast extract (Difco) 1 g; tryptic digest of casein (Merck) 2 g; trace vitamines (Balch et al. 1979) 10 ml; trace minerals (Balch et al. 1979) 10 ml; 2 mercaptoethanesulfonic acid 0.1 mg; resazurin 1 mg; L-cysteine hydrochloride · H_2O 0.5 g; Na_2S · 9 H_2O 0.5 g. Then, the medium was completed to 1 l with H_2O dest. and the pH was adjusted to 7.5 (acetic acid). The media were sterilized for 20 min at 120° C.

Light Microscopy

The cells were observed and photographed with a Leitz Ortholux II microscope, equipped with a vario-orthomat camera system (Leitz). Fluorescence was observed in a Zeiss Standard fluorescence microscope with an excitation filter H436 and a selection filter LP470.

Electron Microscopy

For platinum shadowing, the cells were fixed on collodium coated grids and shadowed with platinum-iridium under an angle of 7°. To prepare thin sections, cells were centrifuged and then fixed in MGG-medium with 20 g glutaraldehyde/l for 2 h and postfixed with 10 g OsO_4/l for 1 h. Durcupan (Fluka) epoxy resin was used for embedding and thin sections were contrasted with lead citrate (5 min), uranylacetate (5 min) and again with lead citrate (3 min).

Electron micrographs were taken with a JEOL JEM 100 C electron microscope at 80 kV and with a 40 µm objective aperture.

Isolation of DNA

The DNA's of *Methanococcus voltae* and the new isolate were prepared as described elsewhere (Wildgruber et al. 1982).

Abbreviations. G + C: Guanine + Cytosine; SDS: Sodium dodecylsulfate (Sodium lauryl sulfate)

Offprint requests to: K. O. Stetter

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Analysis of the Cell Wall

The existence of a cell wall sacculus was checked for as described (Stetter et al. 1981).

Muramic acid and glycoprotein were analyzed as described (König and Stetter, in preparation).

Methane Detection

Methane was determined with a Hewlett Packard gas chromatograph, model 5880 A, using a 6 foot glass column filled with carbosieve S (Supelco) at 70° C isothermal.

Temperature Measurement

The temperature of the sea sediments was measured with an electronic thermometer (Metratherm 1200 d, BBC Metrawatt, Germany) equipped with a T 126 electrode.

Results

Collection of the Sample

The sample SN 1 was taken from the sandy geothermally heated sea floor of the beach at Stufe di Nerone close to Naples (Italy), about 3 m away from the shore in about 0.5 m depth. The original temperature of the sediment was 50° C, the pH 6.5 and the conductivity 7.5 mS, which is that of normal sea water. The sample was immediately filled into a sterile 100 ml storage bottle, which was sealed with a rubber stopper after the addition of 0.1 ml of resazurin (0.1 % w/v in water). The redox potential was then lowered as described (Wildgruber et al. 1982). The sample was carried to the laboratory at room temperature (around 20° C).

Enrichment and Isolation

In a freter type anaerobic chamber (Aranki and Freter 1972), a 100 ml serum bottle containing 20 ml "sea water supplemented" and 50 µg vancomycin/ml and 30 µg penicillin/ml was inoculated with 1 ml of sample SN 1. After sealing with a stopper, the bottle was pressurized (300 kPa H_2:CO_2 = 80:20; Balch and Wolfe 1976) and then incubated in a shaker (New Brunswick) at 50° C. After 4 days, the medium became turbid and methane could be detected in the gas phase. In the fluorescence microscope, masses of strongly green fluorescing cocci were visible.

One milliliter culture was transferred into the same medium, and incubated at 70° C. Next day the new organism, called SN 1, was grown without visible infection. This enrichment culture was streaked onto agar plates (2 % agar) prepared with "supplemented sea water". After 1 week, round convex smooth yellow colonies, about 0.5 – 1 mm in diameter became visible.

Culture and Storage

Cultures were transferred each day into fresh MGG-medium (2 % inoculation). For a long time preservation, the strain SN 1 was grown for 6 h and then, after renewing the gas atmosphere, it was stored at 4° C, from where it can serve at least 6 months as inoculum.

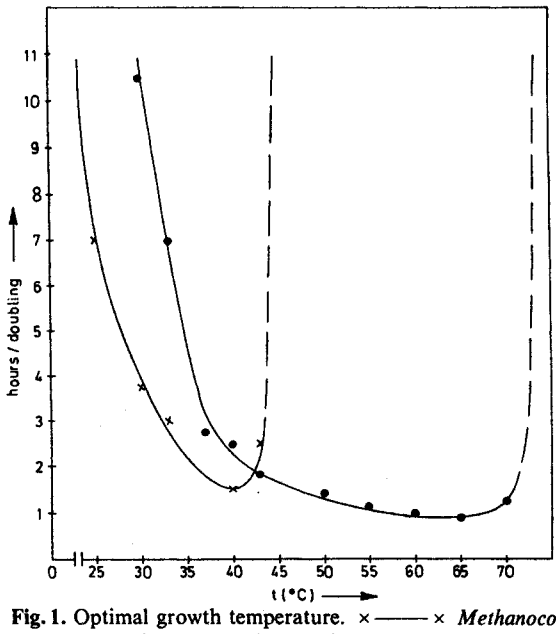

Fig. 1. Optimal growth temperature. × —— × *Methanococcus voltae*; ●——● *Methanococcus thermolithotrophicus*. Growth was determined several times during the exponential phase by O.D.$_{578}$-measurement. The hours/doubling were calculated from the slopes of the growth curves (not shown)

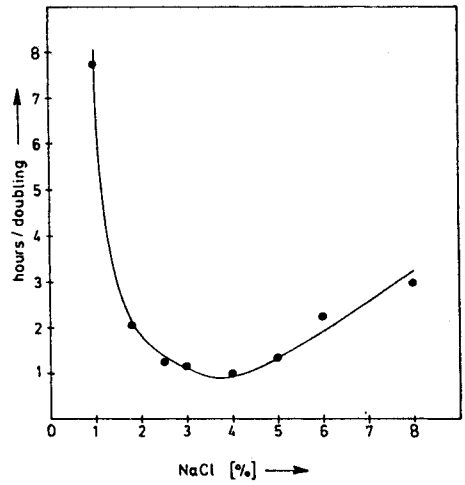

Fig. 2. Influence of NaCl-concentration on growth of *Methanococcus thermolithotrophicus*. The NaCl was added to a MGG-medium, not supplemented with NaCl. This basal medium already contains 0.33 % NaCl, which is not considered in the diagram

Optimal Growth Temperatures

The isolate SN 1 shows a broad temperature range of growth between 30° C and 70° C (Fig. 1). At 75° C (data not shown) the O.D.$_{578}$ of the culture doubles within 5 h, followed by a period of cell lysis within the next 6 h. The shortest doubling time (Fig. 1) is only 55 min at 65° C. For comparison, *Methanococcus voltae* shows (Fig. 1) a temperature optimum around 40° C, under the same conditions.

Substrates and Organic Components of the Medium

H_2 and formate serve as substrates for methane formation. No organic components are required nor do they stimulate growth significantly (data not shown).

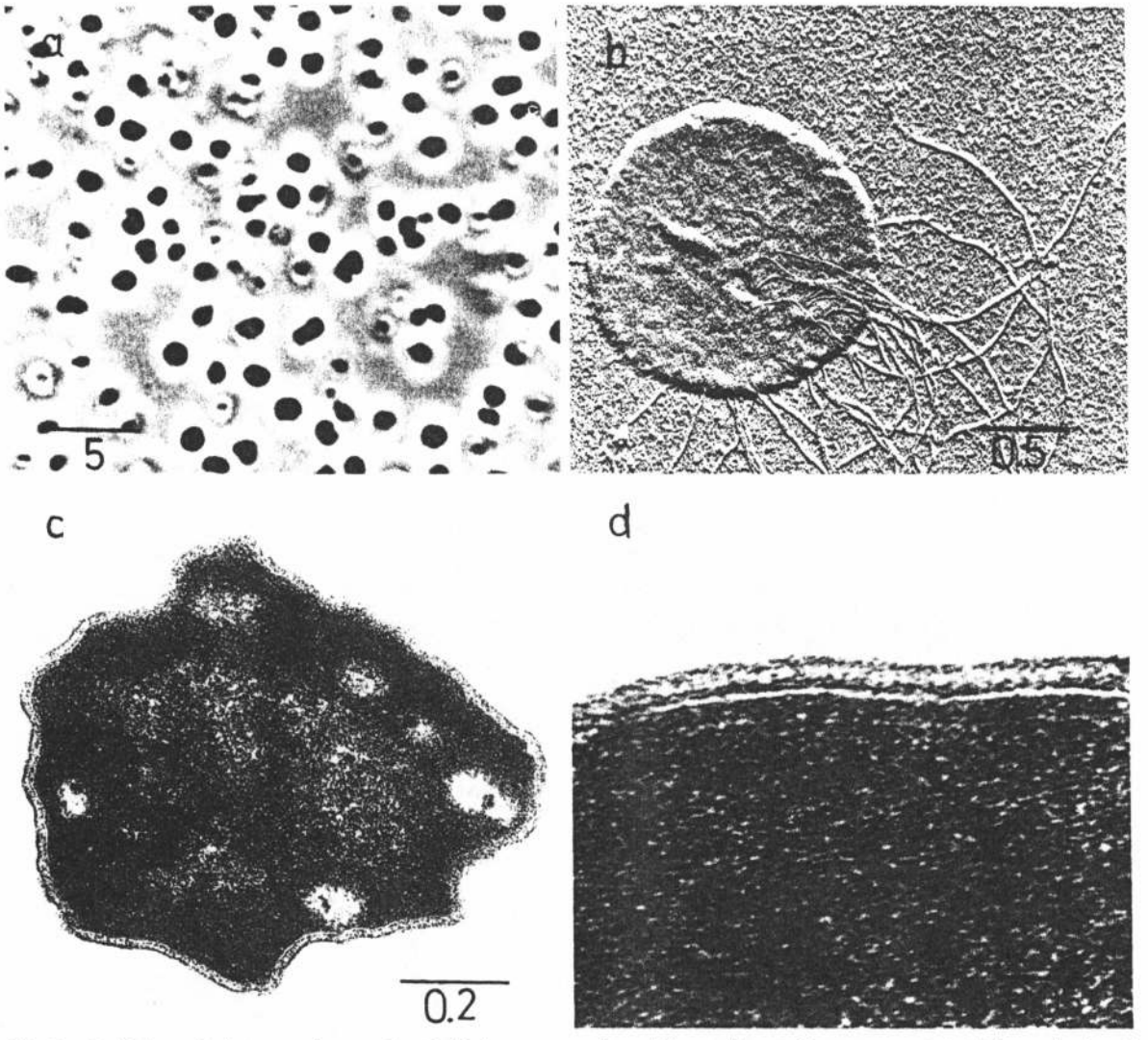

Fig. 3a–d. Light and electron micrographs of *Methanococcus thermolithotrophicus*. **a** Phase contrast; **b** platinum shadowed; **c, d** thin sections. Statements of size in μm

Optimal Salt Concentration

The isolate SN 1 grows in the presence of 1.3–8.3% NaCl (Fig. 2). The optimal concentration is around 4% NaCl.

Optimal pH

The new organism grows in a pH range from 6 to 8 with an optimum around 7 (data not shown).

Morphology

In the light microscope, regular to irregular highly motile cocci about 1.5 μm in diameter, occurring singly and in pairs, can be seen (Fig. 3a). The cells are Gram-negative. In the presence of 2% SDS (w/v) the cells are immediately lysed. As seen in the electron microscope, a tuft of flagella is present, which is inserted in a distinct area on the cell surface (Fig. 3b). Each of the about 20 flagella of a cell is up to 3 μm long and 20 nm thick. In thin sections a cell envelope can be seen (Fig. 3c), which consists of subunits, most likely of protein (Fig. 3d), similar to other *Methanococci* (Jones et al. 1977). No muramic acid and no glycoproteins could be detected in preparations of the cell envelope.

DNA Base Composition

The DNA contains 31.3 mol% G + C as determined by the melting point in 1 × SSC (Marmur and Doty 1962) using DNA of *Methanococcus voltae* as a reference (30.7 mol% G + C).

A second analysis of the DNA base composition performed by HPLC chromatography of a nuclease P1 hydrolysate (Zillig et al. 1980) yielded at G + C-content of 31.6 mol% for the isolate SN 1.

Discussion

The isolate SN 1 is the first thermophilic *Methanococcus*. Its growth temperature optimum at 65°C is similar to that of *Methanobacterium thermoautotrophicum* (Zeikus et al. 1980). In contrast to the other coccoid methanogens described (Balch et al. 1979), yeast extract does not stimulate growth. With a doubling time of only 55 min, the new isolate is the fastest growing archaebacterium known to date, even faster than *Methanobacterium thermoautotrophicum*, strain Marburg, which has an optimal doubling time of 1.6 h (Brandis et al. 1981). This rapid growth is similar to that of

fast growing eubacterial anaerobians, e.g. *Lactobacilli* (K. O. Stetter, unpublished). Due to its vigorous growth and its autotrophy, the new isolate may be well suited for biochemical studies.

The new organism SN 1 is clearly determined to be a *Methanococcus* (Balch et al. 1979) by its coccoid shape, its substrate spectrum, its subunit cell envelope, and its DNA with a G + C-content of 31.3 mol%. In DNA-RNA hybridization experiments (Tu et al. 1982), very stable hybrids (fractional stability: 0.94) between the DNA of the isolate SN 1 and the labelled RNA of *Methanococcus voltae* were obtained, indicating a relatively close relationship with this species and confirming the affiliation to the Methanococcales.

Judging its place of isolation, its thermophily, its lithotrophic growth and its salt optimum, the isolate SN 1 seems to be distributed in volcanic areas on the sea floor. It is named *Methanococcus thermolithotrophicus.*

Description and Classification of the Methanococcus thermolithotrophicus

Order Methanococcales (Balch and Wolfe 1979)
Family Methanococcaceae (Balch and Wolfe 1979)
Genus *Methanococcus* (Barker 1936)
Methanococcus thermolithotrophicus; Huber, Thomm, and Stetter (sp. nov.)
ther.mo.li.tho.tro'phi.cus. Gr. fem. n. *therme* heat; Gr. masc. n. lithos stone; Gr. masc. n. trophos one who feeds; M. L. masc. adj. thermolithotrophicus thermophilic and lithotrophic.

Regular to irregular cocci, about 1.5 µm in diameter, occurring singly or in pairs. On agar, round, convex, smooth, yellowish colonies, around 1 mm in diameter are formed. About 20 flagella are inserted at a distinct area on the cell surface. The cells are Gram-negative, and are lysed by 2% SDS; the cell envelope consists of subunits. Optimal growth is at 65° C with a doubling time of 55 min. Growth takes place between 30 and 70° C. The optimal pH is 6.5 – 7.5. Culture is possible in the presence of 1.3 – 8.3% NaCl with an optimal growth at 4%. Methane is only formed from H_2/CO_2 and from formate. Organic material does not stimulate growth. The DNA base composition is 31.3 mol% G + C. In DNA-RNA hybridization, the DNA of the isolate forms hybrids with RNA of *Methanococcus voltae* with a fractional stability of 0.94.

The type strain is DSM 2095 Göttingen, FRG.

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