

Methanotherix soehngenii gen. nov. sp. nov., a New Acetotrophic Non-hydrogen-oxidizing Methane Bacterium

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Abstract. A new genus of methanogenic bacteria is described, which was isolated from a mesophilic sewage digester. It is most probably the filamentous bacterium, earlier referred to as *Methanobacterium soehngenii*, "fat rod" or "acetate organism". The single non-motile, non-sporeforming cells are rod-shaped ($0.8 \times 2 \mu\text{m}$) and are normally combined end to end in long filaments, surrounded by a sheath-like structure. The filaments form characteristic bundles. *Methanotherix soehngenii* decarboxylates acetate, yielding methane and carbon dioxide. Other methanogenic substrates ($\text{H}_2 - \text{CO}_2$, formate, methanol, methylamines) are not used for growth or methane formation. Formate is split into hydrogen and carbon dioxide. The temperature optimum for growth and methane formation is 37°C and the optimal pH range is 7.4–7.8. Sulfide and ammonia serve as sulfur and nitrogen source respectively. Oxygen completely inhibits growth and methane formation, but the bacteria do not lose their viability when exposed to high oxygen concentrations. 100 mg/l vancomycin showed no inhibition of growth and methanogenesis. No growth and methane formation was observed in the presence of: 2-bromoethanesulfonic acid, viologen dyes, chloroform, and KCN. The bacterium has a growth yield on acetate of 1.1–1.4 g biomass per mol acetate. The apparent " K_s " of the acetate conversion system to methane and carbon dioxide is 0.7 mmol/l. The DNA base composition is 51.9 mol% guanine plus cytosine. The name *Methanotherix* is proposed for this new genus of filamentous methane bacterium. The type species, *Methanotherix soehngenii* sp. nov., is named in honor of N. L. Söhngen.

Key words: Methane bacterium – Acetate decarboxylation – *Methanotherix soehngenii*

Acetate is one of the extracellular key intermediates in the anaerobic decomposition of organic material (Buswell and Hatfield 1936; Mah et al. 1976; Smith 1966) and serves as the principal substrate for methanogenesis. It is the precursor of 60–85% of the methane produced in anaerobic natural environments like sediments (Belyaev et al. 1975; Cappenberg and Prins 1974; Winfrey and Zeikus 1979), flooded soils (Takai 1970), paddy fields (Takai et al. 1963) and in man-made habitats like sewage digesters (Jeris and McCarty 1965;

Smith and Mah 1966; van den Berg et al. 1976) or biogas plants (Mackie and Bryant 1981; Mountfort and Asher 1978). Besides the quantitative, the qualitative importance of acetate was pointed out by Kaspar and Wuhrmann (1978) and van den Berg et al. (1976). Their experimental data indicated that acetate decarboxylation may be the rate-limiting step in the breakdown of dissolved organic matter.

Acetate was the first methanogenic substrate identified (Hoppe-Seyler 1876). However it took several decades until the first organism capable of converting acetate to methane (*Methanosarcina barkeri*) was isolated in pure culture (Schnellen 1947). In this report we describe a filamentous methane bacterium, *Methanotherix soehngenii* unable to oxidize hydrogen, using exclusively acetate for growth and methanogenesis. The isolation procedure and a preliminary characterization of this "acetate organism" has already been published earlier (Zehnder et al. 1980). In this paper we apprise the optimal growth conditions (medium composition, temperature, pH), kinetic data, the effect of some antibiotics and inhibitors on growth and methanogenesis, and the inability of several compounds to serve as a methanogenic substrate or cosubstrate (in presence of acetate). Further observations necessary for the characterization of the new species and its possible taxonomic position (G + C value, cell wall and lipid composition etc.) have been gathered.

Materials and Methods

Source of the Organism

Strains of *Methanotherix soehngenii* were isolated from samples of digested sludge from the sewage treatment plant in Opfikon near Zurich and from the Madison Metropolitan sewage treatment plant (Wisconsin, USA). In the following, only the Opfikon strain is considered.

Anaerobic Procedures

The strict anaerobic methods developed by Hungate (1950) and modified by Bryant (1972) and Balch and Wolfe (1976) were used throughout these investigations, including a freter-type anaerobic chamber (Coy, Lab. Products Inc., Ann Arbor, Michigan). Additions and withdrawals of all samples were made with sterile outgassed syringes and hypodermic needles. Gas samples from the headspace were removed with a Pressure Lok Syringe (Precisions sampling Co., Baton Rouge, LA, USA).

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Media and Growth Conditions

Methanotrix soehngenii was routinely cultivated in a mineral salt (MS)-medium with acetate as sole energy source. The following stock solutions were used (grams per liter of distilled water): (A) KH_2PO_4 , 27.2; (B) $\text{Na}_2\text{HPO}_4 \times 2 \text{H}_2\text{O}$, 35.6; (C, mineral salt solution) NH_4Cl , 60; NaCl , 60; $\text{CaCl}_2 \times 2 \text{H}_2\text{O}$, 22; $\text{MgCl}_2 \times 6 \text{H}_2\text{O}$, 20; (D) NaHCO_3 , 80; (E, trace metal solution) according to Zehnder et al. (1980); (F) ethylenediaminetetracetate, 1; (G, vitamin solution) according to Wolin et al. (1963); (H) resazurin, 1. One liter of the MS-medium was prepared with 8 ml of solution A, 24 ml of solution B, 1 ml each of F and H, and made up with distilled water and 6.8 g sodium acetate (50 mmol/l) to 940 ml. This solution was boiled for 5 min in a waterbath and finally autoclaved (20 min, 121°C) under an atmosphere of 80% N_2 – 20% CO_2 . After cooling, 1 ml of trace metal solution E, 1 ml of vitamin solution G, 50 ml of solution D, 5 ml of mineral solution C and 100 mg vancomycin dissolved in 1 ml distilled H_2O were added aseptically to the autoclaved medium using a 0.2 μm disposable filter assembly (Gelman Sciences, Ann Arbor, MI, USA). Just before inoculation, 5 ml of a freshly prepared reducing solution, containing 0.24 g $\text{Na}_2\text{S} \times 9 \text{H}_2\text{O}$ and 0.03 g $\text{Na}_2\text{S}_2\text{O}_4$ were added in the same way. After inoculation, the medium was gassed again and the gas phase was brought to 152 kN/m². Unless stated otherwise, the gas mixture used was 80% N_2 – 20% CO_2 . The final pH value of the medium was 7.3–7.4. Any further supplements of the medium were added prior to the reduction solution.

Cells were cultivated in serum vials (35.5 or 118 ml) closed with black lip butyl rubber stoppers and sealed with an aluminium seal (Balch and Wolfe 1976), or in 1 liter serum bottles with serum caps (diameter 35 mm). Incubations were performed at 37°C in the dark without shaking. All individual experiments were done at least in duplicates, and the results reported were averaged.

Mass Cultivation

M. soehngenii was mass-cultured in 20 l carboys (inoculum 1%, vol/vol) on mineral salt medium with 100 mM acetate. At the end of the logarithmic growth phase (2–3 months) the cells were harvested with a continuously fed centrifuge (Christ, Zeta 20, rotor No. 8530) under anaerobic conditions (N_2 , 12,000 rpm, 100 ml/min). Cells were frozen in liquid nitrogen and were either lyophilized or stored at –20°C for further use. The biomass amounted to 25 g wet weight (2.2 g dry weight).

Purity Tests

Cultures were routinely checked for purity by examination of wet mounts using phase microscopy, fluorescence microscopy (with a 420 nm-filter), and by dilutions in the following complex media: 1. Thioglycollate-medium (Difco), 2. AC-medium (Difco), 3. CMC-medium (Holdemann and Moore 1972). The inoculated tubes were incubated for 2–3 months at 37°C. *M. soehngenii* showed no growth in these media. Contaminations were identified by visual and microscopic examination. Tubes inoculated with filter sterilized (0.2 μm) or heat treated (90°C, 10 min) cultures served as controls. The number of contaminants were calculated using the most probable number technique (MPN).

Substrate Utilization

Tests for the utilization of various compounds as substrates for growth and/or methane formation were performed in 35.5 ml serum vials containing either 20 ml MS-medium (acetate = 10 mmol/l) or 20 ml MS-medium without acetate. The compounds assayed were added to a final concentration of 10 mmol/l each. Duplicates were inoculated (50%, vol/vol), and the formation of methane determined after 10 weeks of incubation. Vials with inactive, heat treated cells (90°C, 10 min) served as controls.

Effect of Temperature and pH

Cultures in MS-medium (inoculum 0.1% vol/vol) were incubated at the appropriate temperatures (3, 10, 15, 22, 25, 30, 33, 37, 40, 50, 55, and 65°C) and assayed weekly for methane. A specific methane production rate constant (μ_{CH_4}) was calculated for the exponential growth phase. After 18 weeks, all of the bottles with only little or without growth and methane formation were placed for an additional 6 weeks at 37°C to determine whether the effect of these temperatures had actually been lethal to the organism. Cultures which did not return to active growth were considered dead.

To determine the optimal pH, experiments were performed in 35.5 ml serum vials with 20 ml of a bicarbonate-free MS-medium with 2 mM acetate. The desired pH was controlled with a 20 mM phosphate-buffer. The acetate concentration was kept low to reduce pH-changes due to bicarbonate formation from acetate. Triplicates were incubated for 4–8 weeks, and the methane production rate was determined.

Labeling Experiments

Experiments with [¹⁴C]-radiotracers were performed as described by Zehnder et al. (1980). ¹⁴CH₄ and ¹⁴CO₂ in the headspace were measured according to the method of Zehnder et al. (1979), using a liquid scintillation counter.

Studies on the metabolism of formate were done in 35.5 ml vials containing 20 ml MS-medium in which acetate was replaced by formate. [¹⁴C]-formate (specific activity 4.8 $\mu\text{Ci}/\text{mmol}$), methanol and hypophosphite were added from anaerobic stock solutions. For every experimental condition several replicates (4–6) were inoculated (10%, vol/vol) and sacrificed at different incubation times by acidifying them to pH 1 with HCl conc. Subsequently the headspace was assayed for CH₄, CO₂ and H₂ and the liquid for formate, methanol and acetate. Inactive, heat treated (90°C, 10 min) cultures served as controls.

For the kinetic experiments varying acetate concentrations (0.1, 0.2, 0.5, 1, 2, 5, 10 and 50 mmol/l; sodium [¹⁴C]-acetate with a specific activity of 3–70 $\mu\text{Ci}/\text{mmol}$) were inoculated (20%, vol/vol). During incubation the vials were assayed for methane and acetate and the corresponding metabolic rates (v_{CH_4} , methane production rate; v_{Ac} , acetate consumption rate) calculated.

Inhibitor Studies

Anaerobic solutions of the tested inhibitors were injected into the culture vessels 24 h prior to inoculation (20%, vol/vol). After incubation for 6 weeks the methane produced was measured. An untreated culture served as a control.

Antibiotics

The effect of several antibiotics on the growth of *M. soehngenii* was tested by inoculating (20%, vol/vol) MS-medium which contained the antibiotics in the desired final concentration. The increase in dry weight was determined after 4 weeks of incubation.

Growth Yield and Specific Metabolic Rates

Cell yields were determined at the end of growth in 1250 ml serum bottles containing 500 ml MS-medium (acetate = 100 mmol/l). Inocula of 0.5% (vol/vol) were used for all growth yield determinations. Following incubation, the cultures were sacrificed by adding HCl conc. to pH 3. Cell lysis was not observed at this pH (protein content of acidified cell suspensions not higher than controls at a neutral pH). The dry weight was determined by filtering triplicate aliquots (20 ml) with preweighed membrane filters (0.45 μm , Sartorius Nr. 11306, Göttingen) and drying them to constant weight for 24 h at 80°C. Cultures incubated with 2-bromoethanesulfonic acid (2-BES, 100 $\mu\text{mol/l}$), showing no growth and methane formation, served as controls. The specific methane production rate (k_{CH_4}) and the specific acetate consumption rate (k_{Ac}) are given as mmol methane (produced) $\times \text{g}^{-1}$ (dry weight) $\times \text{h}^{-1}$ and mmol acetate (consumed) $\times \text{g}^{-1}$ (dry weight) $\times \text{h}^{-1}$ respectively. They were determined in batch-cultures (500 ml MS-medium, temperature 37°C, pH 7.5–7.7).

DNA Base Composition

Cells were disrupted by passage through a French pressure cell at 140,000 kN/m² (treatment with lysozyme and/or sodium dodecyl sulfate did not cause cell lysis). Isolation and purification of the DNA proceeded as described by Stackebrandt and Kandler (1979).

The guanine plus cytosine (G + C) content of the deoxyribonucleic acid was determined from duplicate preparations by thermal denaturation (T_m -method), using an automatic recording spectrophotometer (Gilford Instrument Laboratories, Ind., Oberlin, OH, USA).

Analytic Procedures

Methane and acetate were detected by gaschromatography as described by Zehnder et al. (1980). Hydrogen was determined by a two-column gas chromatograph (Poropak Q) connected to a thermoconductivity detector (Gow Mac Instruments Co. Ltd., Shannon, Ireland).

Chemicals and Radioisotopes

All chemicals used were of the highest purity available (analytical grade). Coenzyme M as mercaptoethanesulfonic acid sodium salt was purchased from Pierce Chemical Co., Rockford, Ill. Resazurin, methylviologen and benzylviologen were obtained from BDH Chemicals Ltd., Poole. Vancomycin was a gift of the Swiss Serum and Vaccine Institute, Bern. The other antibiotics and inhibitors were supplied as follows: Novobiocin and valinomycin from Boehringer, Mannheim; 2-bromoethanesulfonic acid from Fluka AG, Buchs; penicillin-G from E. Lilly GmbH; D-cycloserine from

Hoffmann-La Roche; dihydrostreptomycin from Sigma Chemical Co.; ampicillin from Bayer AG, Leverkusen.

The following radiochemicals were obtained from New England Nuclear Corp., Boston, Mass.: Sodium [¹⁴C]-formate (2.8 mCi/mmol) and sodium [¹⁴C]-acetate (3.0 mCi/mmol).

Results

Morphology

The non-motile organism shows a Gram-negative reaction. No spores have been observed. *Methanotherix soehngenii* forms very long and flexible filaments which tend to aggregate in characteristic bundles (Fig. 1). Intensive shaking or stirring breaks the filaments into smaller pieces. The rod-shaped cells (0.8 \times 2 μm) have flat ends and are arranged end to end in the filaments.

Stoichiometry of the Conversion of Acetate to Methane

Acetate served as sole source of energy and organic carbon in a MS-medium. Hence, growth of *M. soehngenii* is chemoorganotrophic. A typical course of methane production and acetate consumption over an entire growth period of a batch culture in an atmosphere of 80% N₂ – 20% CO₂ is shown in Fig. 2. Acetate was stoichiometrically converted to methane (inset, Fig. 2) with a ratio of 0.97 mol of methane formed per mol of acetate consumed. Rates of methane production and acetate consumption were the same (maximum of 4.3 mmol l⁻¹ day⁻¹). Due to different lag phases in replicate vials methane production curves are inadequate to demonstrate inhibitory or stimulating effects on the conversion of acetate to methane. Methanogenic activity was defined, therefore, in terms of a specific methane production rate (μ_{CH_4}) which was calculated from the linear part of a half-logarithmic graph (CH₄ vs. time). The exponential methane production in Fig. 2 yielded for instance a μ_{CH_4} of 0.205 day⁻¹ or a "doubling time" $t_d(\text{CH}_4)$ of 3.4 days.

Growth Conditions

Table 1 shows the effect of the cations K⁺, Na⁺ and Ca²⁺ which were added as acetate salts. Concentrations of 50 mmol/l induced almost the same methane production rate as found in the controls with only 10 mmol/l sodium acetate or glacial acetic acid. Clarified rumen fluid (20%, vol/vol) or digested sludge supernatant (20%, vol/vol) were of no significant effect on the rate of methane formation from acetate (Table 1). However, a reduced lag phase was observed (Huser 1981). Conversion of acetate to methane was not stimulated by yeast extract (6 g/l) (Table 1).

Temperature and pH-relationships in growing cultures of *M. soehngenii* are shown in Figs. 3 and 4. The optimal temperature was 37°C. At 50°C neither growth nor methane formation occurred and a subsequent incubation at 37°C could not restore the activity. However, at low temperatures (3°C) growth and methane production took place at a much reduced rate which increased when the cultures were brought back to 37°C. Between 10° and 37°C the temperature dependence of methane production from acetate followed the Arrhenius-relationship ($\theta^{10} = 2.05$; inset, Fig. 3).

Methane formation occurred within a range of pH 6.8–8.2. The optimum was at pH 7.4–7.8 (Fig. 4).

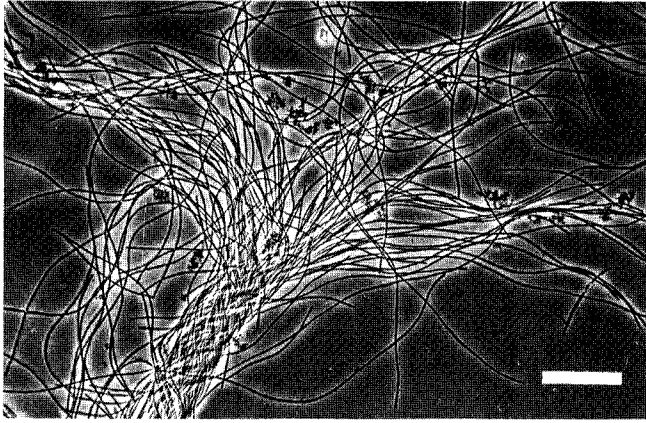


Fig. 1. Phase contrast photomicrograph of *Methanotherix soehngeni* showing the growth in long filaments which form characteristic bundles, bar indicates 50 μm

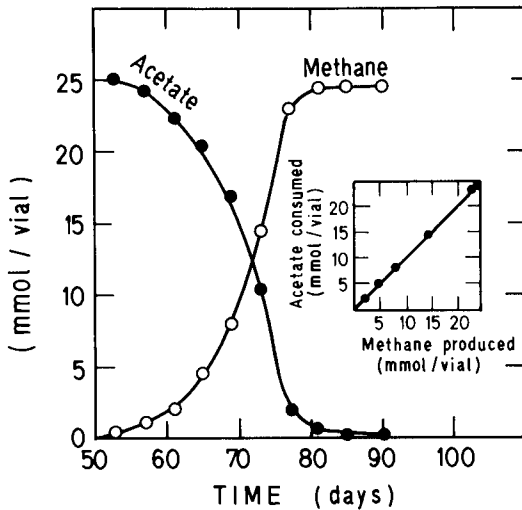


Fig. 2. Acetate conversion to methane during growth of *Methanotherix soehngeni* on acetate (50 mmol/l) in a batch culture with 500 ml MS-medium (Inoculum 0.5 ml)

Table 1. Specific methane production rate (μ_{CH_4}) for *Methanotherix soehngeni* under various growth conditions

Medium and growth condition	$\mu_{\text{CH}_4}^c$ (days $^{-1}$)
MS ^a + sodium acetate (10 mM)	0.157
MS + glacial acetic acid (10 mM)	0.164
MS + sodium acetate (50 mM)	0.168
MS + potassium acetate (50 mM)	0.158
MS + calcium acetate (50 mM)	0.154
MS ^b + clarified rumen fluid (20%, vol/vol)	0.181
MS ^b + digested sewage supernatant (20%, vol/vol)	0.191
MS ^b + yeast extract (6 g/l)	0.153
MS + sodium acetate (50 mM), shaking (100 rpm)	0.125

^a Experiments were performed in batch cultures with 500 ml MS-medium plus the indicated supplements

^b MS incl. 10 mM sodium acetate

^c Mean value of duplicates

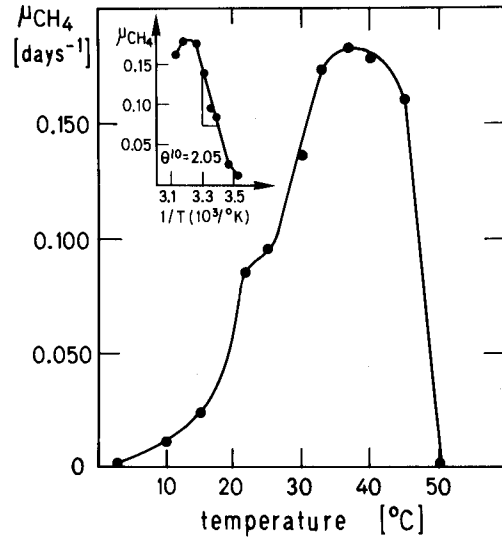


Fig. 3. Temperature-dependence of methane production from acetate by *Methanotherix soehngeni*. The data represent mean values with standard deviation from triplicates. Inset: Arrhenius-equation of temperature-dependence

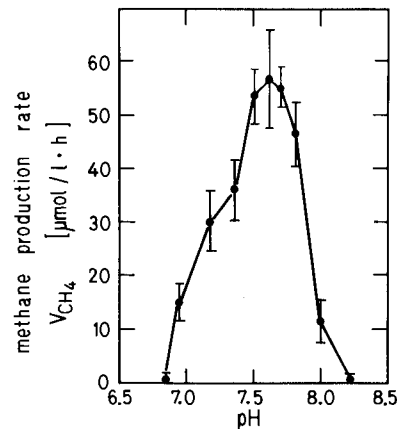


Fig. 4. Effect of pH on the methane production rate (v_{CH_4}) from acetate. The results are averages of a minimum of two replicates. Bars indicate standard deviation

Effect of Oxygen. *M. soehngeni* could be enriched not only from several sewage digesters but also from aerobic samples of pretreated raw sewage (Huser 1981). This suggested an oxygen tolerance of the organism. Replicate cultures were gassed with pure oxygen (0.2, 1, 6, 48 h). Aliquots (5 ml) were then centrifuged ($3000 \times g$, 15 min) and resuspended in 50 ml MS-medium. Untreated cell suspensions (5 ml) inoculated in 50 ml MS-medium served as controls. After variable lag phases (few hours to 3 days) the cultures, initially gassed with oxygen, and the un-gassed controls showed similar rates of methane production. Microscopic examination did not reveal any lysed cells.

Substrates for Growth and Methane Formation

Some details on the metabolism of *M. soehngeni* have already been published in our earlier paper (Zehnder et al. 1980). Besides acetate no other methanogenic substrate ($\text{H}_2 - \text{CO}_2$,

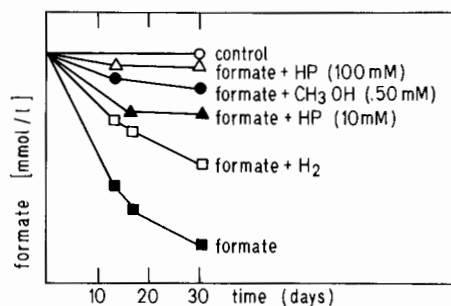


Fig. 5. The cleavage of formate under various experimental conditions. Heat treated cells (90°C , 10 min) served as controls. HP Hypophosphite

methanol, methylamines, formate) could serve for growth and methane formation. Formate was split into equal amounts of hydrogen and carbon dioxide which, however, were not utilized as substrates (results not shown). Adaptation to utilize hydrogen is unlikely, since cultures of *M. soehngenii* showed no consumption of hydrogen when incubated for over 6 months with a gas phase of H_2/CO_2 (80/20%) in the presence of acetate.

Methanol (50 mmol/l) and hydrogen (gasphase 80% H_2 - 20% CO_2) inhibited the formate cleaving system (Fig. 5). Hypophosphite, a selective inhibitor of formate dehydrogenase (Takamiya 1942), causes a concentration-dependent inhibition of the formate splitting activity. The presence of a formate dehydrogenase in *M. soehngenii* is strongly suggested by these observations. Interestingly enough hypophosphite (100 mmol/l) inhibited also the conversion of acetate to methane and carbon dioxide (results not shown). A variety of compounds (propionate, butyrate, pyruvate, citrate, glucose, glycerine; each at 10 mmol/l) were assayed in presence and without acetate for their ability to serve as sole or as cosubstrate for growth and/or methanogenesis. Methane formation and growth was exclusively observed in presence of acetate. The same was true in experiments with the following acetylated and/or methylated compounds (each at 10 mmol/l): dimethylsulfide, dimethylsulfoxide, acetoacetate, phenylacetate, N, N-dimethylformamide, N-methylacetamide, N, N-dimethylacetamide, N-acetylglucosamine, methanolsulfonic acid, 2-acetylbenzoic acid, 2-methoxyethanol, acetone, trimethylphosphate, acetaldehyde, formaldehyde. The last two substances caused a total inhibition of acetate conversion. Nitrate and sulfate were not utilized with acetate as an electron donor.

Inhibitors and Antibiotics

The effect of some known inhibitors of methanogenesis on *M. soehngenii* was studied in the MS-medium inoculated with 20% (vol/vol) of an actively growing culture. Complete inhibition was caused by methylviologen (5 $\mu\text{mol/l}$), benzylviologen (5 $\mu\text{mol/l}$), chloroform (20 $\mu\text{mol/l}$), sodium arsenate (100 $\mu\text{mol/l}$) and potassium cyanide (100 $\mu\text{mol/l}$). The effect of fluoroacetate, nitrate and iodopropane is shown in Fig. 6. Iodopropane (0.1 mmol/l) completely suppressed methanogenesis from acetate. Fluoroacetate resulted in a concentration-dependent inhibition of methane formation. Trifluoroacetate (in a concentration range of 0.1 - 2 mmol/l) had no effect (results not shown). Methanogenesis from H_2 - CO_2 is inhibited by 2-bromoethanesulfonic acid (2-BES), an analogue of coenzyme M (2-mercaptoethanesulfonic acid;

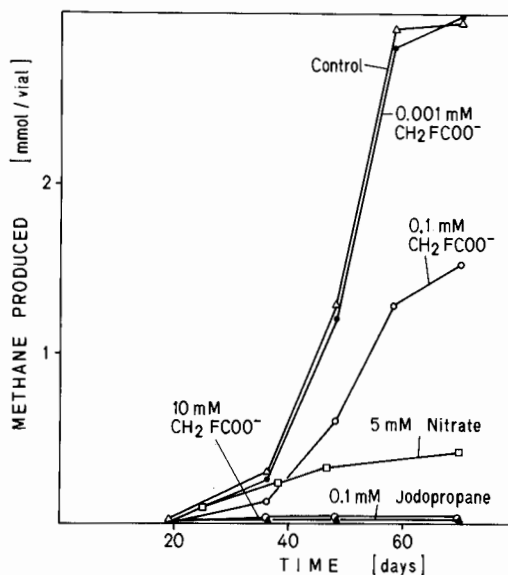


Fig. 6. Effect of inhibitors on methane formation from acetate. Vials containing 50 ml MS-medium were inoculated (2%, vol/vol). Values of two replicate cultures were averaged (standard deviation < 5%). Vials without any inhibitors served as controls

Balch and Wolfe 1979; Gunsalus 1977). It was shown to inhibit acetate conversion to methane in cultures of *Methanosarcina* strain 227 (Smith and Mah 1978) and *M. soehngenii* (Zehnder and Brock 1979) as well. The inhibition with 2-BES is relieved by addition of coenzyme M in *Methanosarcina* strain 227 (Smith and Mah 1978). This did not occur, however, with *M. soehngenii*, even with a 50-fold excess of coenzyme M.

Growing cultures of *M. soehngenii* were tested for their sensitivity to some known antibiotics in liquid media. It is known that methanogenic bacteria are insensitive to some antibiotics which act against eubacteria (Hammes et al. 1979; Hilpert et al. 1981). This fact has already been exploited for the selective enrichment and isolation of methanogens (Godsy 1980; Zinder and Mah 1979). The antibiotics examined in our work, together with their effects are listed in Table 2. Dihydrostreptomycin and novobiocin were chosen as a proof of the effectiveness of the experimental procedure. Since vancomycin (at 0.1 mg/ml) showed no negative effect on growth and methanogenesis it was routinely added to the MS-medium for subculturing and maintenance of *M. soehngenii*.

Kinetic Data on Methanogenesis from Acetate

Most of the kinetic data reported earlier for methanogenesis from acetate are of limited value because they were obtained from complex ecosystems (Kaspar and Wuhrmann 1978; Smith and Mah 1966) or from enrichment cultures (Lawrence and McCarty 1969). The only kinetic studies on acetate conversion to methane with pure cultures are those with *Methanosarcina* strain 227 (Smith and Mah 1978).

The methane production rate of *M. soehngenii* gets concentration-dependent at an acetate level of less than 10 mmol/l (Fig. 7). A " K_s "-value of 0.7 ± 0.05 mmol/l has been calculated for methanogenesis from acetate (concentration range 1 - 50 mmol/l). In the course of a batch-experiment the methane production rate was comparable to

Table 2. Effect of some antibiotics on the growth of *Methanotrix soehngenii*

Antibiotic	Concentrations (mg/ml)			
	0.01	0.1	1.0	10.0
Penicillin-G	15 ^a	80	88	100
Ampicillin	0	55	98	100
D-cycloserine	0	40	100	n.m. ^b
Vancomycin	n.m.	0	88	100
Novobiocin	67	75	97	100
Dihydrostreptomycin	97	100	100	n.m.

^a Percent inhibition of growth. Values of controls after 4 weeks incubation were taken as zero percent inhibition, based on dry weight determinations

^b n. m. not measured

the acetate consumption rate. In the initial phase (less than 5% acetate utilized), however, the acetate consumption rate was significantly greater than the corresponding methane production rate (Fig. 8, Fig. 2). This was true for all acetate concentrations assayed in the range of 0.1–50 mmol/l. Specific metabolic rates of $k_{Ac} = 1.62 \pm 0.1 \text{ mmol g}^{-1} \text{ h}^{-1}$ and $k_{CH_4} = 1.46 \pm 0.1 \text{ mmol g}^{-1} \text{ h}^{-1}$ were calculated from triplicate determinations.

Deoxyribonucleic Acid Base Composition (Mol Percent Guanine Plus Cytosine)

The mol percent G+C was calculated to be 51.9% for *M. soehngenii*.

Discussion

The physiological and morphological characteristics of the new methane bacterium resemble those of the filamentous organism first reported by Söhngen (1906). The same type of bacterium was later observed by various other investigators in their cultures with acetate as sole energy substrate (Barker 1936; Groenewege 1920; Mylroie and Hungate 1954; Smit 1930) or in enrichment cultures on various substrates with acetate as a free intermediate e. g. on propionate (Boone and Bryant 1980), on butyrate, (McInerney et al. 1979), on benzoate (Ferry and Wolfe 1976), on cellulose (Khan and Mes-Hartree 1981) or on lignin derivatives (Healy et al. 1980). A similar organism was recently described in a highly enriched culture (Colvin et al. 1979; van den Berg et al. 1976). However, its isolation was never successful. We have cultured *Methanotrix soehngenii* with more than 20 transfers in a mineral salt medium with acetate as the sole organic carbon compound. No requirement for a growth factor developed within this period. Growth on acetate exhibited exponential kinetics. Thus, the splitting of acetate into methane and carbon dioxide apparently generated sufficient energy for growth. Our finding that no other substrates (e. g. H₂, propionate, butyrate, pyruvate, citrate, glucose, glycerine) were cometabolized, excluded the need for supplementary energy sources for growth and/or methanogenesis from acetate contrary to reports from other authors (Toerien et al. 1971; Zeikus et al. 1975).

Methane Formation. The mechanism by which methane is formed from acetate and the concomitant energy generating

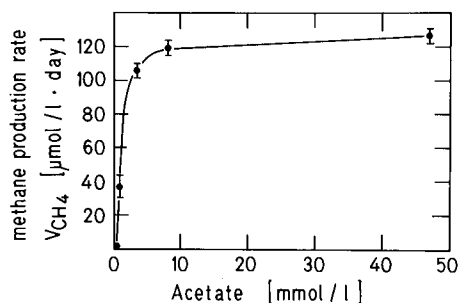


Fig. 7. Methane production rate (v_{CH_4}) at different acetate concentrations (0.1–50 mmol/l). Duplicate values were averaged and are given with the standard deviation

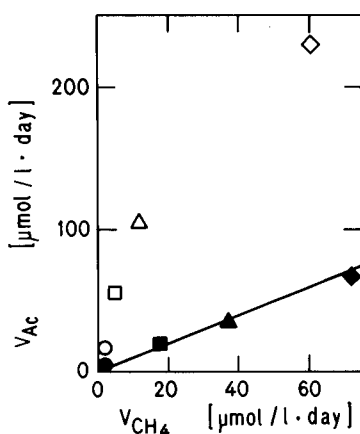


Fig. 8. Rates of acetate consumption (v_{Ac}) and methane production (v_{CH_4}) in batch experiments with the following initial acetate concentrations (mM): 0.2 (●, ○), 0.5 (■, □), 1.0 (▲, △), 2.0 (◆, ◇). Open signs represent data obtained at an early phase of the experiment (less than 5% of the added acetate consumed). Closed symbols show data obtained later (10–15% acetate consumed). The line indicates the theoretical value of $v_{Ac}/v_{CH_4} = 1$

system are not known. The inhibition of the acetate conversion to methane by known inhibitors of the hydrogenotrophic methanogenesis, e. g. 2-bromoethanesulfonic acid, viologen dyes, CHCl₃ (Balch and Wolfe 1979; Gunsalus 1977; McBride and Wolfe 1971; Robertson and Wolfe 1970) suggests a similarity of the mechanisms for methane production from acetate and from H₂–CO₂. The inhibitory effect of 2-bromoethanesulfonic acid is of particular interest in view of the fact that coenzyme M is involved in the methylreductase system in hydrogenotrophic (Gunsalus and Wolfe 1980) as well as in acetotrophic methanogenesis (Baresi and Wolfe 1981). Labeling experiments with cultures of *M. soehngenii* clearly demonstrated that the methyl group of acetate was converted to methane and the carboxyl group to carbon dioxide (Huser 1981; Zehnder et al. 1980), in analogy to the acetate conversion by *Methanosarcina* species (Mah et al. 1978; Smith et al. 1980; Zinder and Mah 1979).

The efficiency of energy generation estimated by the molar growth yields on acetate is similar in *M. soehngenii* and in *Methanosarcina barkeri*. Values (g biomass per mol acetate utilized) of 1.1 (Hutten et al. 1981), 1.8–2.7 (Smith and Mah 1978), 1.6–1.9 (Weimer and Zeikus 1978) and 2.1 (Zinder and Mah 1979) were reported for *Methanosarcina* species, compared to 1.1–1.4 for *M. soehngenii* and 1.1 for a similar

organism in an enrichment culture (van den Berg et al. 1976). This does not mean, however, that the mechanism of acetate conversion to methane is the same in both organisms. Growth of *Methanosarcina* is concentration-dependent at acetate concentrations of less than 50 mmol/l, reflecting the relatively high K_s -value (5 mmol/l; Smith and Mah 1978).

Extrapolation to lower acetate concentrations (< 3 mM) resulted in specific growth rates comparable to those of *M. soehngenii* at these acetate concentrations. The low K_s -value for the conversion of acetate to methane by *M. soehngenii* (0.7 mmol/l), which is in the concentration range of the optimum of the functioning of sewage digesters (Kaspar and Wuhmann 1978; Capenberg 1976) may be one reason for the successful competition of *M. soehngenii* in various ecosystems. *Methanosarcina* is usually not present in great amounts in digested sludge (Mah et al. 1978; Pretorius 1972; van den Berg et al. 1976; Wolfe 1971; own observations) or in freshwater sediments (Winfrey and Zeikus 1979; own observations). The presence, however, of *Methanotrix* in all of nine municipal sewage digesters observed in Switzerland and in the USA, in mechanically pretreated raw sewage, in a sanitary landfill and in various Indian biogas plants fed on cattle manure or nightsoil (Huser 1981; unpublished observations), indicate its wide distribution in nature as possibly the major acetate-utilizing methanogen (McInerney et al. 1981).

Taxonomy. The lack of murein in the cell envelope of *M. soehngenii* and the presence of ether-linked polyisoprenoid alcohols clearly characterize the newly isolated organism as an archaebacterium (Woese 1981). Methane formation, and the presence of the unique coenzyme CoM classify the bacterium as a methanogen. Morphological and physiological features are similar to organisms first described by Söhngen (1906) and later named *Methanobacterium soehngenii* in an impure culture by Barker (1936). However, the morphology and ultrastructure (Huser 1981; Zehnder et al. 1980), the absence of a pseudomurein-type cell wall (Kandler and König, personal communication), the lack of C_{40} -tetraethers as polar lipids (Langworthy, personal communication) and, most conclusive, the comparative cataloging of the 16S rRNA (Stackebrandt et al. 1982), clearly show that it does not belong to the order Methanobacteriales. A new genus *Methanotrix* within the order Methanomicrobiales (Balch et al. 1979) was therefore proposed (Stackebrandt et al. 1982; Zehnder et al. 1980).

Description

Methanotrix soehngenii gen. nov. et sp. nov. Me.tha'no.thrix. M.L.n. *methanum* methane; Gr.n. *thrix* hair; M.L. fem.m. *Methanotrix* the methane (-producing) hair, soehn'gen.i.i., M.L.gen.n. *soehngenii* of Söhngen; named in honor of N.L. Söhngen who in 1906 first described a similar organism converting acetate to methane.

A combined generic and specific description follows.

Morphology

The non-sporeforming cells are rodshaped with flat ends occurring as long filaments in unshaken cultures. The Gram reaction is negative. The outer layer of the cell wall is composed of proteins (O. Kandler and H. König, personal

communication). It has a sheath-like structure which shows a regular striation. Cells are non-motile.

Cell Wall

The unstable cell wall is devoid of muramic acid (O. Kandler, personal communication).

Lipids

Di-o-phytanyl glycerol ether (C_{20}) was found as the only polar lipid. Tetraether (C_{40}) and phosphoglycerolipids are absent (T. Langworthy, personal communication).

Nutrition

Anaerobic. Grows chemoorganotrophically in a defined mineral salt medium that contains vitamins, trace metals and acetate as the only organic component. Other organic supplements are not required. Methane is exclusively formed from the methyl group of acetate. $H_2 - CO_2$, formate, methanol and methylamines cannot be used for growth or methane formation. Formate is split into hydrogen and carbon dioxide. Acetate and carbon dioxide are assimilated.

Growth Characteristics

The organism grows at temperatures between 3° and 45° C with an optimum at 37° C, and at pH values between 6.8 and 8.2, with optimum growth between pH 7.4–7.8.

Oxygen Tolerance

Exposure to oxygen has no lethal effect. Growth and methane formation occurs, however, only under strictly anaerobic and highly reducing conditions.

Biochemical Features

Coenzyme M (0.35 nmol/mg dry cells) and F_{420} (0.55 µg/mg protein) were determined in cell extracts. Two different types of membrane-bound cytochromes were found (W. Kühn, personal communication).

DNA Base Composition

The G+C content of the DNA is 51.9 moles %.

Phylogenetic Relationship

Comparative analysis of the 16S rRNA revealed only a distant relationship to other methanogens. The highest degree of relatedness was found to *Methanosarcina barkeri* ($S_{AB} = 0.44$).

Habitat

Methanotrix soehngenii was isolated from digested sewage sludge. Enrichments were obtained from all of nine examined sewage digesters for domestic wastes, from biogas fermenters for animal wastes, from a sanitary landfill and from mechanically pretreated raw sewage. It was not found in enrichments from anoxic freshwater sediments.

Type Strain

The type strain of *Methanotherix soehngenii* is strain Opfikon. The description of the type strain is identical to that of the species. This strain has been deposited in the German Collection of Microorganisms (DSM) under the number 2139.

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