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Methanosarcina baltica, sp. nov., a novel methanogen isolated from the Gotland Deep of the Baltic Sea

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Abstract A novel methanogen, *Methanosarcina baltica* GS1-A^T, DSM 14042, JCM 11281, was isolated from sediment at a depth of 241 m in the Gotland Deep of the Baltic Sea. Cells were irregular, monopolar monotrichous flagellated cocci 1.5–3 μm in diameter often occurring in pairs or tetrads. The catabolic substrates used included methanol, methylated amines, and acetate, but not formate or H_2/CO_2. Growth was observed in a temperature range between 4° and 27°C with an optimum at 25°C. The doubling time with methanol as substrate was 84 h at 25°C, 120 h at 9°C, and 167 h at 4°C. The doubling time with acetate as substrate was 252 h at 25°C and 425 h at 20°C. After the transfer of methanol-grown cultures, long lag phases were observed that lasted 15–20 days at 25°C and 25 days at 4°–9°C. The NaCl optimum for growth was 2%–4%, and the fastest growth occurred within a pH range of 6.5–7.5. Analysis of the 16S rDNA sequence revealed that the strain was phylogenetically related to *Methanosarcina*. The sequence similarity to described species of <95.7% and its physiological properties distinguished strain GS1-A^T from all described species of the genus *Methanosarcina*.

Key words Methanogens · *Methanosarcina* · Psychrotolerant · Marine sediments · Baltic Sea · Gotland Deep

Introduction

Methanogenesis in the world ocean occurs in the anaerobic part of the water column, in sediments, and also in microniches in oxic parts of the water column. Most of the deeper regions of the ocean have permanently low temperatures, and the microorganisms producing methane in these environments must be psychroactive or psychrophilic. Methanogens from cold environments, including from wetlands, are most likely the major source of atmospheric methane (Franzmann et al. 1997). Among the 83 validly described species of methanogens, so far only two psychrotolerant representatives have been identified (Franzmann et al. 1992, 1997; Garcia et al. 2000). This implies that the psychrotolerant methanogens contributing significantly to the global methane budget are poorly characterized and that their biodiversity is nearly unexplored.

In anaerobic parts of marine sediments and the ocean water column, sulfate reduction usually predominates over methanogenesis (Holmer and Kristensen 1994). However, methanogens can coexist with sulfate-reducers in these environments by using substrates such as methylamines and methanol. Many methanogens isolated from marine environments are obligately methylotrophic (Oremland and Boone 1994; Garcia et al. 2000).

The Baltic Sea is a large land-bounded brackish water body consisting of a series of basins with water depths ranging from 40 to 460 m. The Baltic Sea is supersaturated with methane compared with the open ocean (Bange et al. 1994), most likely because of its low sulfate content and because of the low extent of water exchange with the deeper, anaerobic water of its basins. Comparison of the upper 22 cm of sediments from different basins of the Baltic Sea revealed two types of methane-producing sediments. The high-methane-type sediment showed a methane concentration of up to 45 μmol dm^{-3} and was dominated by methane diffusion from deeper sediment layers. The low-methane-type sediment showed no distinct gradient below a sediment depth of 10 cm (Schmaljohann et al. 1998). The Gotland Deep is one of the major deep basins of the Baltic Sea. It is located east of the island of Gotland (Sweden) and has a maximum water depth of 241 m. The deep water of this basin is anaerobic most of the time because a permanent thermo/halocline at 60–80 m water depth largely inhibits water exchange between surface (salinity 7‰–8‰) and deeper

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waters (11‰–13‰). Only occasionally does intrusion of water masses from the North Sea cause a short interruption of the anoxic periods. Methane concentration increases in sediments of the central Gotland Deep from the surface to at least 80 cm, where values between 0.7 and 1.8 mM were found. The bulk of the methane was produced below the sulfate-containing surface sediment layer (0–20 cm depth), and the average methane production rate in deeper sediment layers (>80 cm) was calculated as 259 µmol m^{-2} day^{-1}. Carbon consumption in sediments of the Gotland Deep by methanogenesis is 0.1% of that consumed by dissimilative sulfate reduction in the upper, main sulfate-reduction layer (0–20 cm), and 4.6% when the layers deeper than 80 cm are included (Piker et al. 1998). Here, we report the characterization of a methanogen isolated from the deepest part of the Gotland Deep, which owing to its growth characteristics is able to contribute to methane formation under in situ conditions.

Materials and methods

Collection of the samples

During a cruise of the MS *Alkor* from April 25 to May 5, 1993, sediment samples were taken with a box corer on April 28 at station 303 of the Gotland Deep in the Baltic Sea (position, 57°17′65″ N and 20°06′05″ E) at a depth of 241 m. On board the ship, the sediment samples were placed in bottles (100 or 250 ml), which were closed with a rubber stopper, and reduced by the addition of 2.5% stock solution of sodium sulfide to a final concentration of 0.05%.

Two sediment fractions were collected: one containing the surface layer (GS1 = Gotland sediment 1) to a depth of 30 cm, and a second fraction containing sediment from depths > 30 cm (GS2). The redox potential of the sediment was –400 mV at the surface and increased linearly with depth to –475 mV at a depth of 9 cm. The samples were stored at 4°C.

Enrichment and isolation

The strains GS1-A^T, GS1-B, GS1-C, and GS1-PL4 were isolated from the surface sediment layer. Enrichment cultures of GS1-A^T were incubated at 20°C in MG medium (Wildgruber et al. 1982) containing 0.05% methanol (v/v); the other strains were enriched at 20°C in MG medium under a H_2/CO_2 atmosphere. Growth was observed after an incubation time of 3–4 weeks. Isolates were obtained by plating diluted cultures in an anaerobic chamber on MG medium solidified with 2% agar. Methanol was added to the medium after sterilization and before pouring the plates at 50°C. Small brown colonies (2–3 mm in diameter) were visible after 3–4 weeks. These were plated again and then transferred into liquid media.

Electron and light microscopy

Cells of GS1-A^T were fixed by injection into serum bottles of glutaraldehyde (final concentration, 3%; w/v) and a solution of ruthenium red (final concentration, 0.015%; w/v). Fixation time was 12 h. These prefixed cells were collected by centrifugation. The pellet was mixed with Noble agar at 50°C and cut into pieces. After washing with cacodylate buffer (0.15 m; pH 7.2), postfixation was done for 3 h in a mixture of 2 ml OsO_4 (4%) + 2 ml ruthenium red (0.15%) + 2 ml cacodylate buffer (0.15 m; pH 7.2) at 5°C. Dehydration was carried out in a graded series of ethanol. Embedding was done in ERL medium (Spurr, Fluka, Buchs, Germany). Sections were cut with a Reichert Ultracut E ultramicrotome (Leica, Benzheim, Germany), mounted on grids, and poststained with uranyl acetate and lead citrate.

Shadow-casting of air-dried cells with Pt/C was done with a Balzers BAF 300 freeze etching system with an angle of 35°. Electron micrographs were taken with a Philips EM 300 transmission electron microscope on Kodak (SO163, Rochester, NY, USA) electron microscope film no. 4489. Light microscope micrographs of GS1-A^T were made with a Zeiss (Jena, Germany) Photomicroscope II fitted with Nomarski interference contrast optics on Kodak Plus-X pan film.

Media and culture techniques

The isolated species was cultured in 120-ml serum bottles filled with 20 ml MG medium (Balch et al. 1979; Wildgruber et al. 1982) containing a trace elements solution modified according to Huber et al. (1992) and supplemented with 0.05% methanol. The salt composition of the MG medium was (g l^{-1}). KCl, 0.34; $MgCl_2$, 2.75; $MgSO_4$, 3.45; NH_4Cl, 0.25; $CaCl_2$, 0.14; K_2HPO_4, 0.14; and NaCl, 18. In addition, it contained 2 mg $Fe(NH4)_2(SO_4)_2$, a trace mineral and vitamin solution as described by Balch et al. (1979), Na-acetate, 1.0 g l^{-1}; yeast extract, 2 g l^{-1}; peptone, 2 g l^{-1}; $NaHCO_3$, 5 g l^{-1}; L-cysteine, 0.5 g l^{-1}; and $Na_2S{\cdot}9H_2O$, 0.5 g l^{-1}. The MGG medium had the same salt composition, but the organic compounds Na-acetate, peptone, and yeast extract were omitted. The headspace was N_2/CO_2 (150 kPa). The gas ratios used for N_2/CO_2 or H_2/CO_2 were 80 : 20 (v/v) at 150 kPa and 300 kPa, respectively. Cultures of strain GS1-A^T grew best without shaking.

Growth experiments

For substrate utilization experiments, anaerobic sterile solutions were added to the MGG medium (without organic substrates; Huber et al. 1982) prior to the inoculum. Each substrate, with the exception of dimethylsulfide, was tested at two different temperatures, indicated in parenthesis. The substrates tested were sodium acetate, 25 mM (25°C), 10 mM (20°C); sodium formate, 30 mM (25°C), 20 mM (20°C); methylamine, 40 mM (25°C), 20 mM (20°C); dimethylamine, 40 mM (25°C), 20 mM (20°C); trimethylamine,

20 mM (25°C), 10 mM (20°C); methanol, 40 mM (25°C), 20 mM (20°C); and dimethylsulfide, 1 mM (20°C) and 5 mM (20°C).

For the analysis of the optimal pH for growth of GS1-A^T, different buffers (20 mM) were used: glycyl/glycine for the pH range 4–4.5; 2-morpholinoethane sulfonic acid (MES), pH 5.0–6.5; and 1,4-piperazine-diethane sulfonic acid (PIPES), pH 7–7.5.

Growth measurements

Bacterial growth was determined by direct cell counting in a Thoma chamber (0.02 mm) under a phase contrast microscope (Zeiss Standard). The growth of parallel cultures was followed to determine optimal temperature, pH, and salt concentration.

Phylogenetic analyses

To characterize the gene encoding 16S rRNA, it was amplified by using PCR with the chromosomal DNA of GS1-A^T as template by using the following primer pairs: Arch21F (5′-TTCCGGTTGATCCYGCGGA-3′, Arch958R (5′-YCC GGCGTTGAMTCCAATT-3′); Universal 1100F (5′-AAC GAGCGMRACCC-3′), Universal 1400R (5′-GACGGGC GGTGTGTRC-3′) DeLong (1992); and EAF545 (5′-TTG AGCTCAAGCTTCCGCGGTAAYACCGRCRGYYC-3′, EAR952 (5′- TTTTGGATCCCCGGCGTTGARTCCAAT TRAACCG-3′) (M = A/C, R = A/G, Y = C/T).

The amplified PCR products were sequenced with an ABI Prism Dye Terminator Kit (Applied Biosystems, Foster City, CA, USA) and an ABI sequencer (373A, Applied Biosystems). The 16S rDNA sequence was compared to sequences in the European Molecular Biology Laboratory (EMBL) databases with the help of the Husar-Heidelberg server. Phylogenetic analyses were performed using the PHYLIP 3.5c package (Felsenstein 1989). The analyzed sequence of GS1-A^T rDNA consisted of 1,319 bp. Bootstrap analyses were based on 100 resamplings of the data set using the SEQBOOT program.

Results

Enrichment and isolation

One milliliter of the surface sediment fraction (see Materials and methods) collected at a depth of 241 m in the Gotland Deep was added to 20 ml modified MG medium (Materials and methods) containing 0.5% (v/v) methanol under an N_2/CO_2 atmosphere (80 : 20; 150 kPa) or to MG medium under a H_2/CO_2 atmosphere (80 : 20; 300 kPa). After an incubation time of 3–4 weeks at temperatures between 5° and 25°C, the growth of methanogens was detected microscopically in several enrichment cultures by analysis of fluorescence, and methane formation was shown by gas chromatography. The amount of methane formed increased with cell density (data not shown). Enrichment cultures were transferred into media containing in addition penicillin in concentrations ranging from 50 to 200 μg/ml. The cultures were further purified by repeated serial dilution, and isolates were obtained by repeated plating of cultures on media solidified with agar. Four isolates were obtained: GS1-A^T was cultivated in MG medium in the presence of methanol under a N_2/CO_2 atmosphere, and the isolates GS1-B, GS1-C, and GS1-PL4 grew both under a H_2/CO_2 atmosphere and under a N_2/CO_2 atmosphere in the presence of methanol. All isolates grew very slowly at temperatures between 5° and 25°C. It took up to 1 month before the final cell densities were obtained, which were 3×10^7 for GS1-A^T and 1×10^8 for GS1-B, GS1-C, and GS1-PL4. Sequence analysis of the gene encoding the 16S rRNA revealed that isolates GS1-B, GS1-C, and GS1-Pl4 were closely related to each other (sequence similarity between 99.7% and 99.9%) and to *Methanogenium cariaci* (sequence similarity, 99%). GS1-A^T was not closely related to any hitherto described methanogen. Therefore, only this strain was characterized in more detail.

Sensitivity against inhibitors and antibiotics

Growth of strain GS1-A^T in MGG medium in the presence of various antibiotics and inhibitors known to inhibit anaerobic bacteria but not methanogens (Garcia-Rodriguez et al. 1995; Böck and Kandler 1985) was tested. This strain did not grow in the presence of the methanogenesis inhibitor bromoethane sulfonate (10 mM). Growth was not affected by the antibiotics clindamycin (10 and 100 μg/ml), chloramphenicol (50 μg/ml), cycloserine (50 μg/ml), metronidazole (100 μg/ml), penicillin G (300 μg/ml), or piperamycin (50 μg/ml). Growth was not inhibited by *p*-chloromercuribenzoate (100 μg/ml).

Morphology

Cells of GS1-A^T were irregular cocci with a diameter of 1.5–3 μm (Fig. 1). The cells often formed pairs or tetrads; larger aggregates were never observed. Analysis of Pt/C-shaded cells by electron microscopy revealed a monopolar monotrichous flagellation (Fig. 2A). An S-layer was not detected. In thin sections, cells were observed to be connected by cytoplasmic bridges (Fig. 2B), suggesting that the cells often remain linked after cell division, resulting in the formation of pairs or tetrads of cells (see also Fig. 1).

Optimal growth conditions

Growth was obtained between 4°C and 27°C with optimum growth at 25°C. The doubling time at 25°C was 84 h (Fig. 3). GS1-A^T was also able to grow at low temperatures. At 4°C, the doubling time was 167 h and at 9°C, 120 h. No growth could be detected at temperatures >30°C. The maximum cell densities observed at 4°, 9°, 20°, and 25°C were 0.27×10^7, 0.4×10^7, 1.3×10^7, and 1.5×10^7, respectively. These find-

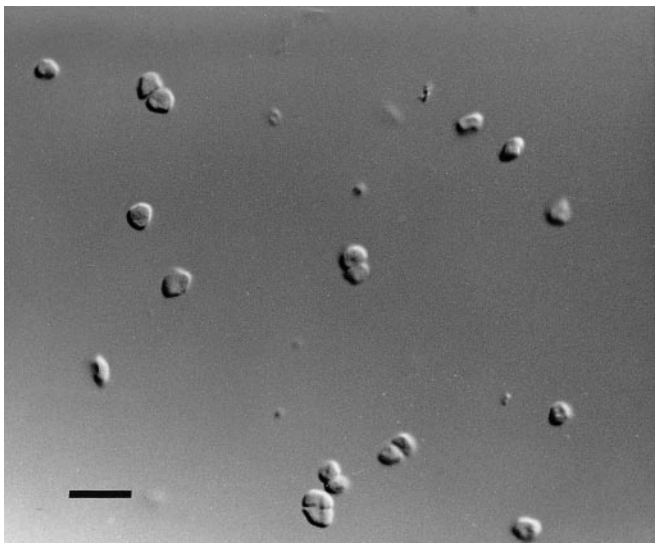

Fig. 1. Cells of *Methanosarcina baltica* mounted on agar slides and photographed using Nomarski interference optics. *Bar* 10 µm

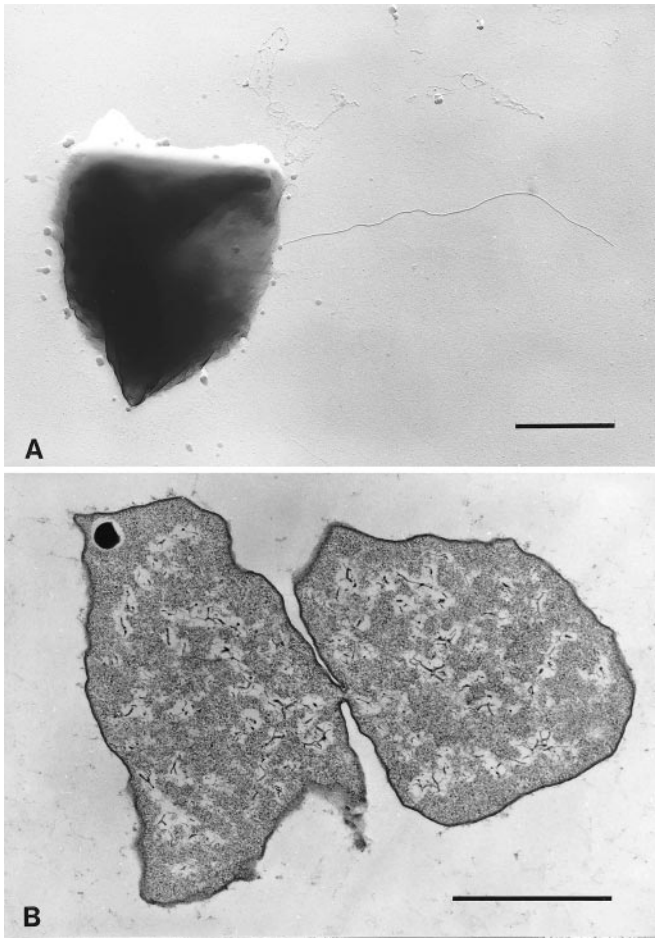

Fig. 2A,B. *Methanosarcina baltica*. **A** Single cell with flagellum. Shadow-casting with Pt/C. **B** Ultrathin section of two cells connected with a plasma bridge. *Bars* 1 µm

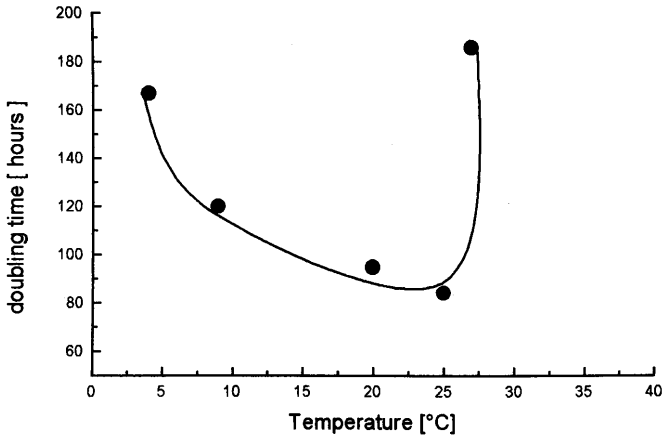

Fig. 3. Optimal growth temperature of *Methanosarcina baltica*. The doubling times were calculated from the slopes of the growth curves (three parallel cultures were analyzed, not shown). The NaCl concentration was 1.8%, the pH 6.9

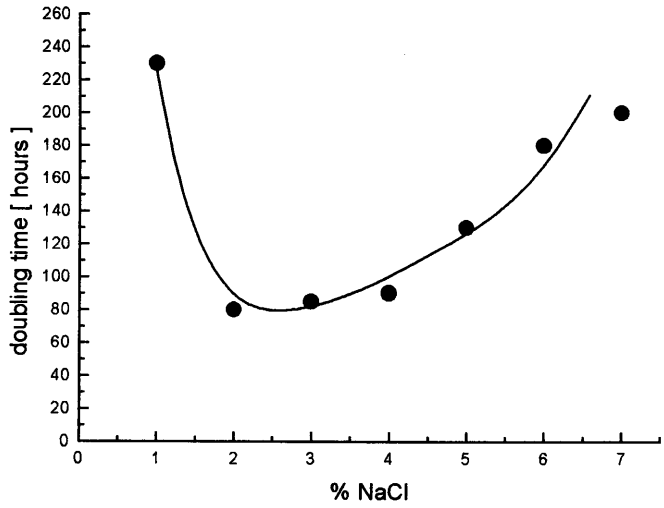

Fig. 4. Growth of *Methanosarcina baltica* at different concentrations of NaCl. The doubling times were calculated from growth curves (three parallel cultures were analyzed, not shown). The growth temperature was 25°C, and the pH 6.9

ings indicate that growth of GS1-A^T at low temperatures is poor. The optimal NaCl concentration in MG medium was between 1.8% (in normal MG medium) and 3%, with a doubling time of 80 h at 2% NaCl (Fig. 4). No growth was observed at 0% or 8% NaCl.

The isolate grew in a pH range between 4 and 8.5 (Fig. 5). At the optimal pH value of 6.5, the doubling time was 75 h. No growth occurred at pH 3, and the doubling time at pH 8.5 was 600 h.

To test the ability of previously described *Methanosarcina* species to grow slowly at low temperatures, *M. mazei* and *M. acetivorans* were incubated at 9°C in MG medium and medium 1 of Balch et al. (1979) containing 20 mM methanol. After 8 days, *M. acetivorans* grew to a cell density of 2.5×10^7. The same organism grew after 3 days of incubation at 37°C to cell densities of 4×10^8. At 37°C, *M. mazei* grew in large aggregates, but no growth at 9°C could

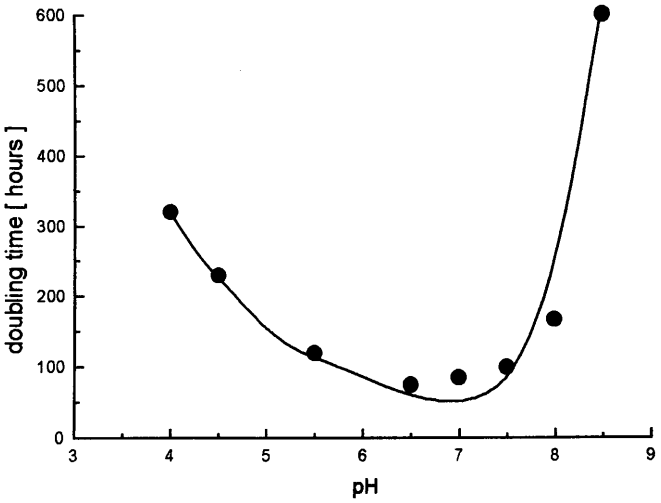

Fig. 5. Influence of pH on growth of *Methanosarcina baltica*. The doubling times were calculated from growth curves (three parallel cultures were analyzed, not shown). The growth temperature was 25°C, and the NaCl concentration, 1.8%

Table 1. Metabolic substrates utilized by *Methanosarcina baltica*

Substrate	T (°C)	Doubling time (h)	Final cell density $\times 10^7$ ml^{-1}
25 mM acetate	25	252	0.22
10 mM acetate	20	425	0.28
40 mM trimethylamine	20	124	1.8
20 mM trimethylamine	25	104	1.8
20 mM dimethylamine	20	285	0.4
40 mM dimethylamine	25	210	0.3
20 mM methylamine	20	48	1.2
40 mM methylamine	25	78	1.6
20 mM methanol	20	95	1.4
20 mM methanol	25	84	1.6

Growth on the various substrates was studied in MGG medium, not containing additional organic compounds. In MG medium, containing in addition Na-acetate, yeast extract, and peptone, final cell densities of up to 4×10^7 cells/ml were observed during growth on methanol as substrate

be detected. Growth of these *Methanosarcina* strains was inspected after 8, 15, 23, 29, and 36 days. These findings indicate that *M. acetivorans* is able to grow slowly with low cell yield at 9°C.

Catabolic substrates

MGG medium not containing additional organic substrates, except methanol, was used for the enrichment and isolation of GS1-A^T. No growth occurred when methanol was replaced by formate (20 mM at 20°C or 30 mM at 25°C), dimethylsulfide (1 mM and 5 mM at 20°C), or H_2/CO_2 (80:20; 300 kPa). Growth on various additional methanogenic substrates such as methylamine, dimethylamine, trimethylamine, and Na-acetate was studied. The strain was able to utilize all these compounds (Table 1). Best growth occurred on methylamine and trimethylamine. Growth on dimethylamine and acetate was poor (Table 1). Growth in the presence of 40 mM methanol was tested at 25°C, but these higher methanol concentrations seemed to be toxic for the cells, as very poor growth and subsequent lysis of the cells were observed (data not shown).

GS1-A^T cells incubated at 20°C in less-concentrated medium (20 mM di- or trimethylamine) had a longer lag phase compared with cultures incubated at 25°C in highly concentrated medium (40 mM di-or trimethylamine). But the exponential growth phase started earlier when cells were grown on 20 mM methylamine at 20°C compared with cells grown on 40 M methylamine at 25°C. In this regard, growth on methylamine was an exception.

Phylogeny

To identify the phylogenetic position of the new isolate, the 16S rDNA gene was amplified and sequenced. The DNA sequences were aligned with those of Archaea and then compared in a phylogenetic tree (Fig. 6) constructed by using distance matrix methods (Olsen 1987). The resulting tree was verified by using bootstrap analyses (Felsenstein 1989). Strain GSA-1^T was related to the genus *Methanosarcina*. It showed a sequence similarity of 93.3% with *Methanosarcina mazei* and of 95.6% with its closest relative, *Methanosarcina semesiae*.

Discussion

We describe here the isolation of a methanogen from an environment that is perennially cold, with temperatures typically between 3° and 7°C (Matthäus 1996). The strain GS1-A^T originates from the upper layer of the sediment of the Gotland Deep, where sulfate reduction predominates, but where the production of small amounts of methane has also been detected (Piker et al. 1998). An organism from such a habitat is predicted to grow via methylotrophy, and, as expected, the strain GS1-A^T was able to utilize methylamines and methanol and could not grow by CO_2 reduction. But, in contrast to the many methanogenic species that are obligate methylotrophs isolated from marine environments (Garcia et al. 2000), it was also able to utilize acetate.

Like the two methanogens *Methanococcoides burtonii* and *Methanogenium frigidum*, which were isolated from an Antarctic lake with a salinity level similar to that of seawater (Franzmann et al. 1992, 1997), GS1-A^T was able to grow at temperatures between 4° and 10°C.

M. burtonii shared with GS1-A^T the ability to utilize methyl compounds, but, in contrast to this strain, GS1-A^T is also an acetoclastic methanogen. *M. frigidum* grew by carbonate reduction and on formate but was not methylotrophic. Sequence analysis of its 16S rRNA revealed that

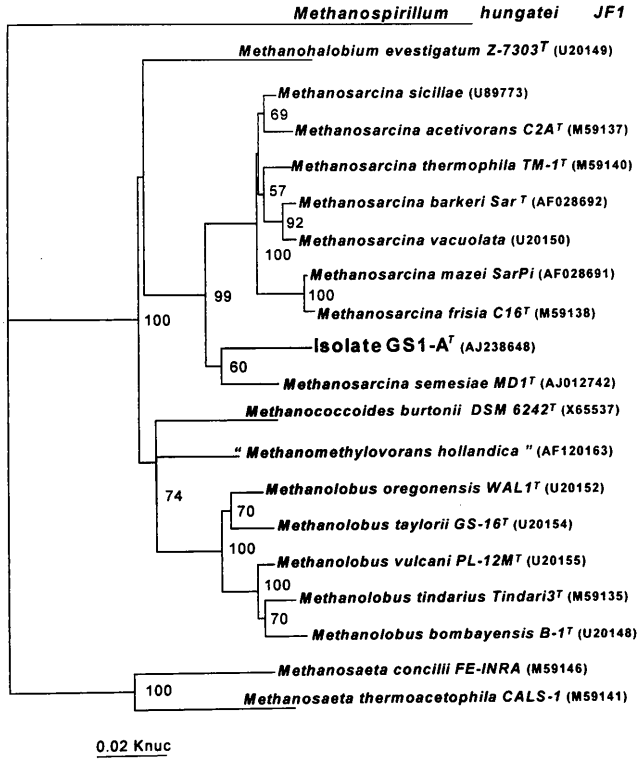

Fig. 6. Unrooted phylogenetic tree based on 16S rRNA sequences showing the relationship of GS1-A^T to members of the kingdom Euryarchaeota. The distance matrix was calculated using the Kimura method. The tree was constructed using the neighbor-joining method. *Methanospirillum hungatei* Jf1 was used as an outgroup. The *scale bar* indicates two estimated substitutions per 100 nucleotides. The *internal numbers* indicate absolute bootstrap values per 100 bootstraps. The accession numbers of the sequences are given *in parentheses*

GS1-A^T was not closely related to these psychrotolerant methanogens, but showed a common ancestry with members of the genus *Methanosarcina.*

Like the marine isolates *M. frisia*, *M. siciliae,* and *M. acetivorans* (Blotevogel and Fischer 1989; Ni et al. 1994; Sowers et al. 1984), strain GS1-A^T did not form large aggregates in its normal growth medium. The *Methanosarcina* strains *M. barkeri*, *M. thermophila*, *M. mazei*, *M. vacuolata*, and *M. acetivorans* can be adapted for growth at salt concentrations ranging from 0.05 to 1 M NaCl (Sowers et al. 1993). These *Methanosarcina* species, which grow at low salt concentrations as multicellular aggregates embedded in a methanochondroitin matrix, occur in the presence of marine concentrations of Na^+ and Mg^{2+} ions as single cells (Sowers et al. 1993). Considering this finding, it is not surprising that GSA-1^T grew only as single cells, pairs of cells, or tetrads, as this organism was always cultivated in a marine medium. Therefore, the possibility cannot be excluded that GSA-1^T also forms aggregates when adapted to low-salt conditions.

A methanogen growing optimally at 15°C was isolated from a freshwater pond at Abramtsevo near Moscow (Zhilina and Zavarzin 1991). Although the 16S rRNA sequence of this strain has not been determined, its morphological and physiological properties suggest that it belongs to the genus *Methanosarcina* (Zhilina and Zavarzin, personal communication): Like GS1-A^T, this isolate utilized both methyl compounds and acetate, and it was also unable to grow by CO_2 reduction. The lower temperature optimum of this strain (15°, compared with 25°C) and its origin from freshwater suggest that this not yet validly described *Methanosarcina* strain from Russia is a species different from GS1-A^T.

At first sight, GS1-A^T can be distinguished from all validly described *Methanosarcina* species by its ability to grow between 4° and 10°C (Garcia et al. 2000; Table 1). However, closer inspection of the growth of the two previously described mesophilic *Methanosarcina* species at 9°C revealed, after 8 days of incubation, significant growth of one of them, *M. acetivorans*, although the maximal cell density was lower than at the temperature optimum (40°C) by a factor of 16. In the original description of *M. acetivorans*, it was reported that growth below 10°C did not occur (Sowers et al. 1984). Our findings indicate that even an organism such as *M. acetivorans*, which has its optimal growth at 40°C, can show poor and slow growth at temperatures found in marine sediments. The growth rate and final cell yield of GS1-A^T cells were also much lower at temperatures below 10°C than at the optimal temperature of 25°C, indicating that an organism isolated from a perennially cold habitat with a low temperature optimum does not necessarily grow better a low temperatures than a mesophile. To our surprise, the growth rate of the mesophilic *M. acetivorans* at 9°C was faster by a factor of 3 than that of GS1-A^T, and the final cell densities reached by this methanogen at this temperature were similar to those of GS1-A^T. This finding indicates that a mesophilic strain can grow faster at low temperatures than a strain that – owing to its lower temperature optimum for growth – seems to be better adapted to growth at low temperatures. The determination of methane formation rates is required to address the question whether GS1-A^T shows higher metabolic activity at temperatures under 10°C than *M. acetivorans*. The inability of *M. mazei* to grow at 9°C showed that not all mesophilic *Methanosarcina* strains are able to thrive at low temperatures (Table 2).

The 16S rDNA sequence of GS1-A^T shows a sequence similarity of 95% with *M. barkeri*, *M. frisia*, *M. siciliae*, and *M. mazei. M. frisia* (Blotevogel and Fischer 1989) has been proposed as a synonym of *M. mazei* (Maestrojuan et al. 1992), but, because the two strains differ in their 16S rRNA sequence (Fig. 6) and physiological properties, *M. frisia* is listed as a separate species in Table 2.

In contrast to GS1-A^T, which grows on methyl compounds and is acetoclastic but unable to utilize H_2/CO_2, most of the described *Methanosarcina* species can grow via CO_2 reduction as well (Table 2). *M. barkeri* and *M. vacuolata* show lower NaCl optima than GS1-A^T. The closest relative with respect to the 16S rRNA sequence, *M. semesiae*, can be clearly distinguished from GS1-A^T by its higher temperature optimum and its inability to utilize acetate (Lyimo et al. 2000; Table 2). GS1-A^T can be further physiologically distinguished from *M. semesiae* by its inability to utilize dimethylsulfide (Table 2).

The 16S rRNA sequence of strain GS1-A^T differed by 4.3%–5% from all described species of the genus

Table 2. Characteristic properties of isolate GS1-A^T and related strains of the genus *Methanosarcina*

	GS1-A^T	*M. semesiae*	*M. frisia**	*M. barkeri*	*M. siciliae*	*M. vacuolata*	*M. mazei*	*M. acetivorans*
Morphology	Cocci; singly, in pairs and tetrads	Cocci; singly	Cocci; singly or in pairs	Large and small aggregates	Cocci; aggregates	Cocci; small aggregates	Cocci; singly or in aggregates; cysts	Cocci; cysts
Temperature optima	25	30–35	36	30–40	37	40	35–42	35–40
Growth at 4°–10°C	+	–	–	–	–	–	–	+
NaCl optima (M)	0.3–0.4	0.2–0.6	0.3–0.4	<0.2	0.5	0.1	0.2–0.4	0.1–0.4
pH optima	6.5	6.5–7.5	6.5–7.2	6.5–7.5	6.4–6.8	6	6–7	6.5
Substrate								
Acetate	+	–	+	+	+/–	+	+/–	+
Methylamines	+	+	+	+	+	+	+	+
Methanol	+	+	+	+	+	+	+	+
H_2/CO_2	–	–	+	+	–	+/–	+/–	–
Dimethylsulfide	–	+	n.d.	n.d.	+	n.d.	–	+

Data were taken from this work, Mah and Kuhn (1984), Zhilina and Zavarzin (1987), Sowers et al. (1984), Boone et al. (1993, 2001), and Lyimo et al. (2000). Growth of *M. acetivorans* and *M. mazei* at 9°C was analyzed in this study for a period of 36 days. After an incubation period of 8 days, significant growth of *M. acetivorans* was found, although in the original description no growth below 10°C was reported (Sowers et al. 1984). It is possible that more *Methanosarcina* strains are able to grow below 10°C when incubated for a longer period of time. In this study, only for *M. mazei* and *M. acetivorans* was growth at low temperature inspected for a period of 36 days
n.d., not determined

Methanosarcina. In view of this deep phylogenetic separation of this strain from other strains of *Methanosarcina* and owing to its low temperature growth optimum, we propose GS1-A^T as a new species of the genus *Methanosarcina*, which we have named *Methanosarcina baltica.*

Description of *Methanosarcina baltica* sp. nov.

Methanosarcina baltica (bal' ti. ca. L. n. mare balticum, the Baltic Sea, N.L. fem. adj. baltica pertaining to the Baltic Sea, from which the organism was isolated).

Cells are irregular cocci with diameters of 1.5–3 µm, and they occur often in pairs and tetrads. They exhibit monopolar, monotrichous flagellation. Cells utilize methanol, methylamines, and acetate as substrates, but display no growth on H_2/CO_2, formate, or dimethylsulfide. They grow between 4° and 27°C, with an optimum at 25°C. The doubling time is 84 h at 25°C, 120 h at 9°C, and 167 h at 4°C. After transfer of the cultures, long lag phases lasting 15–20 days at 25°C and 25 days at 4°–9°C were observed. Fastest growth was observed at NaCl concentrations between 2% and 4% and at pH values between 6.5 and 7.5. Sequence comparisons of 16S rDNA sequences placed strain GS1-A^T in the family *Methanosarcinaceae*. The type strain was isolated from the anoxic surface layer of the sediment at a depth of 241 m in the Gotland Deep of the Baltic Sea. The type strain is GS1-A1^T (= DSM 14042^T; JCM 11281).

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References

Balch WE, Fox GE, Magrum LJ, Woese CR, Wolfe RS (1979) Methanogens: reevaluation of a unique biological group. Microbiol Rev 43:260–296

Bange HW, Bartell UH, Rapsomanikis S, Andrae MO (1994) Methane in the Baltic and North Sea and a reassessment of the marine emissions of methane. Global Biogeochem Cycles 8:465–480

Blotevogel K-H, Fischer U (1989) Transfer of *Methanococcus frisius* to the genus *Methanosarcina* as *Methanosarcina frisia* comb. nov. Int J Syst Bacteriol 39:91–92

Böck A., Kandler O (1985) Antibiotic sensitivity of archaebacteria. In: Woese CR, Wolfe RS (eds) Archaebacteria. Academic, New York, pp 525–544

Boone DR, Whitman WB, Rouvière P (1993) Diversity and taxonomy of methanogens. In: Ferry JG (ed) Methanogenesis. Chapman and Hall, pp 35–80

Boone DR, Whitman WB, Koga Y (2001) Order II *Methanosarcinales*. In: Boone DR, Castenholz GM (ed) Bergey's manual of systematic bacteriology, Vol I, Springer, New York, pp 268–275

DeLong E (1992) Archaea in coastal marine environments. Proc Natl Acad Sci U S A 89:5685–5689

Felsenstein J (1989) PHYLIP: phylogeny inference package (version 3.2). Cladistics 5:164–166

Franzmann PD, Springer N, Ludwig W, Conway de Macario E, Rohde M (1992) A methanogenic archaeon from Ace Lake, Antarctica: *Methanococcoides burtonii* sp. nov. Syst Appl Microbiol 15:573–581

Franzmann PD, Liu Y, Balkwill DL, Aldrich HC, Conway de Macario E, Boone DR (1997) *Methanogenium frigidum* sp. nov., a psychrophilic H_2-using methanogen from Ace Lake, Antarctica. Int J Syst Bacteriol 47:1068–1072

Garcia JL, Patel BK, Ollivier B (2000) Taxonomy, phylogenetic and ecological diversity of methanogenic archaea. Anaerobe 6:1205–1226

Garcia-Rodriguez, JA, Garcia-Sanchez JE, Munoz-Bellido JL (1995) Antimicrobial resistance in anaerobic bacteria: current situation. Anaerobe 1:69–80

Holmer M, Kristensen E (1994) Coexistence of sulfate reduction and methane production in an organic-rich sediment. Mar Ecol Prog Ser 107:177–184

Huber H, Thomm M, König H, Thies G, Stetter KO (1982) *Methanococcus thermolithotrophicus*, a novel thermophilic lithotrophic methanogen. Arch Microbiol 132:47–50

Huber R, William T, Huber D, Trincone A, Burggraf S, König H, Rachel R, Rockinger J, Fricke H, Stetter KO (1992) *Aquifex pyrophilus* gen nov. sp nov, represents a novel group of marine hyperthermophilic hydrogen-oxidizing bacteria. Syst Appl Microbiol 15:340–351

Lyimo TJ, Pol A, Op den Camp HJM, Harhangi HR, Vogels GD (2000) *Methanosarcina semesiae* sp nov., a dimethylsulfide-utilizing methanogen from mangrove sediment. Int J Syst Evol Microbiol 50:171–180

Maestrojuan GA, Boone JN, Mah RA, Menaia JAGF, Sachs MS, Boone DR (1992) Taxonomy and halotolerance of mesophilic *Methanosarcina* strains: assignment of strains to species, and synonymy of *Methanosarcina mazei* and *Methanosarcina frisia*. Int J Syst Bacteriol 42:561–567

Mah RA, Kuhn DA (1984) Transfer of the type species of the genus *Methanococcus* to the genus *Methanosarcina*, naming it *Methanosarcina mazei* (Barker 1936) comb. nov. et emend., and conservation of the genus *Methanococcus* with *Methanococcus vannielii* as the type species. Int J Syst Bacteriol 34:263–265

Matthäus W (1996) Temperatur, Salzgehalt, Dichte. In: G. Rheinheimer (ed) Meereskunde der Ostsee. Springer, New York, pp 75–81

Ni S, Woese CR, Aldrich HC, Boone DR (1994) Transfer of *Methanolobus siciliae* to the genus *Methanosarcina*, naming it *Methanosarcina siciliae*, and emendation of the genus *Methanosarcina*. Int J Syst Bacteriol 44:357–359

Olsen GJ (1987) The earliest phylogenetic branchings: comparing rDNA-based evolutionary trees inferred with various techniques, Cold Spring Harbor Symp Quant Biol 52:825–838

Oremland RS, Boone DR (1994) *Methanolobus taylorii* sp nov., a new methylotrophic, estuarine methanogen. Int J Syst Bacteriol 44:573–575

Piker L, Schmaljohann R, Imhoff J-F (1998) Dissimilatory sulfate reduction and methane production in Gotland Deep sediments (Baltic Sea) during a transition period from oxic to anoxic bottom water (1993–1996). Aquat Microb Ecol 14:183–193

Schmaljohann, R., Piker L, Imhoff J-F (1998) The distribution of methane and hydrogen sulfide in basin sediments of the central and southern Baltic Sea. Meyniana 50:191–211

Sowers KR, Baron SF, Ferry JG (1984) *Methanosarcina acetivorans* sp nov., an acetotrophic methane-producing bacterium isolated from marine sediments. Appl Environ Microbiol 47:971–978

Sowers, K, Boone JE, Gunsalus RP (1993) Disaggregation of *Methanosarcina* spp and growth as single cells at elevated osmolarity. Appl Environ Microbiol 59:3832–3839

Wildgruber G, Thomm M, König H, Ober K, Ricchiuto T, Stetter KO (1982) *Methanoplanus limicola*, a plate-shaped methanogen representing a novel family, the *Methanoplanaceae*. Arch Microbiol 132:31–36

Zhilina TN, Zavarzin GA (1987) *Methanosarcina vacuolata* sp. nov., a vacuolated *Methanosarcina*. Int J Syst Bacteriol 37:281–283

Zhilina TN, Zavarzin GA (1991) Low temperature methane production by a pure culture of *Methanosarcina* sp. Dokl. Akad. Nauk SSSR 317:1242–1245