Isolation and characterization of methylotrophic methanogens from anoxic marine sediments in Skan Bay, Alaska: description of *Methanococcoides alaskense* sp. nov., and emended description of *Methanosarcina baltica*

Neha Singh, Melissa M. Kendall, Yitai Liu and David R. Boone

Biology Department, Portland State University, PO Box 751, Portland, OR 97207-0751, USA

Correspondence

Melissa M. Kendall
kendallm@pdx.edu

Three novel strains of methylotrophic methanogens were isolated from Skan Bay, Alaska, by using anaerobic cultivation techniques. The water was 65 m deep at the sampling site. Strains AK-4 (= OCM 774), AK-5^T (= OCM 775^T = DSM 17273^T) and AK-9 (= OCM 793) were isolated from the sulfate-reducing zone of the sediments. Each of the strains was a non-motile coccus and occurred singly. Cells grew with trimethylamine as a catabolic substrate and strain AK-4 could also catabolize methanol. Yeast extract and trypticase peptones were not required for growth, but their addition to the culture medium slightly stimulated growth. Each of the strains grew at temperatures of 5–28 °C; they were slight halophiles and grew fastest in the neutral pH range. Analysis of the 16S rRNA gene sequences indicated that strain AK-4 was most closely related to *Methanosarcina baltica*. DNA–DNA hybridization studies showed 88 % relatedness, suggesting that strain AK-4 represents a novel strain within this species. Strains AK-5^T and AK-9 had identical 16S rRNA gene sequences that were most closely related to the sequence of *Methanococcoides burtonii* (99.8 % sequence similarity). DNA–DNA hybridization studies showed that strains AK-5^T and AK-9 are members of the same species (88 % relatedness value), but strain AK-5^T had a DNA–DNA relatedness value of only 55 % to *Methanococcoides burtonii*. This indicates that strains AK-5^T and AK-9 should be considered as members of a novel species in the genus *Methanococcoides*. We propose the name *Methanococcoides alaskense* sp. nov., with strain AK-5^T (= OCM 775^T = DSM 17273^T) as the type strain.

In the marine environment, sulfate reduction is the dominant microbial process in the upper sediment layers. Generally, when sulfate is present, sulfate reduction is the major catabolic process and methanogenesis is limited. Because of their higher affinity for hydrogen and acetate, sulfate-reducing bacteria out-compete methanogens for these important substrates (King *et al.*, 1983; King, 1984; Oremland & Taylor, 1978). As a result, methanogenesis becomes a dominant process only in deeper sediments in which the sulfate ions have been exhausted. The limited

methane production that occurs together with sulfate reduction is due to the activity of methylotrophic methanogens (King, 1984; Oremland & Taylor, 1978; Oremland & Polcin, 1982).

Methanogens that belong to the family *Methanosarcinaceae* are characterized as having the broadest substrate range of methanogens; many can grow by reducing CO₂ with H₂ or by the splitting of acetate and all can grow by dismutating methyl compounds (Kendall & Boone, 2004). The family *Methanosarcinaceae* includes eight genera: *Methanosarcina*, *Methanolobus*, *Methanococcoides*, *Methanohalobium*, *Methanohalophilus*, *Methanosalsum*, *Methanomethylovorans* and *Methanimicrococcus*. The only described species of *Methanimicrococcus*, *Methanimicrococcus blatticola*, is unique among the *Methanosarcinaceae* because it reduces methylated compounds only in the presence of H₂ (Sprenger *et al.*, 2000).

The genus *Methanosarcina* currently comprises nine species: *Methanosarcina barkeri*, *Methanosarcina acetivorans*,

Published online ahead of print on 12 August 2005 as DOI 10.1099/ijs.0.63886-0.

Abbreviation: TMA, trimethylamine.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains AK-5^T, AK-9 and AK-4 are AY941801, AY941802 and AY663809, respectively.

Graphs showing the effect of environmental factors on the growth rates of strains AK-4, AK-5^T and AK-9 are available as supplementary material in IJSEM Online.

Methanosarcina siciliae, *Methanosarcina thermophila*, *Methanosarcina mazei* (= *Methanosarcina frisia*), *Methanosarcina vacuolata*, *Methanosarcina baltica*, *Methanosarcina lacustris* and *Methanosarcina semesiae*. The genus *Methanosarcina* includes slightly halotolerant and slightly halophilic species. Several species were originally isolated from anaerobic sewage sludge digesters (Barker, 1936; Bryant & Boone, 1987; Mah & Kuhn, 1984; Zhilina & Zavarzin, 1987; Zinder *et al.*, 1985) and other species of this genus have been isolated from various sediment environments, including marine and estuarine sediments (Elberson & Sowers, 1997; Lyimo *et al.*, 2000; Ni & Boone, 1991; Simankova *et al.*, 2001; Sowers *et al.*, 1984; von Klein *et al.*, 2002). Also, *Methanosarcina barkeri* strain CM1 has been isolated from sheep rumen (Jarvis *et al.*, 2000).

The genus *Methanococcoides* currently contains two species, both from environments with salinity near that of sea water. *Methanococcoides methylutens* was isolated from submarine canyon sediments that contained large deposits of organic material off the coast of southern California (Sowers & Ferry, 1983) and *Methanococcoides burtonii* was obtained from the anoxic hypolimnion of Ace Lake, Antarctica (Franzmann *et al.*, 1992).

These organisms can dismutate methylamines and methanol for growth but cannot catabolize acetate, dimethylsulfide, H_2/CO_2 or formate.

We describe three strains of methylotrophic methanogens that have been isolated from the sulfate-reducing zone in the sediments of Skan Bay, Alaska. Strain AK-4 was most closely related to *Methanosarcina baltica* and an emended description of *Methanosarcina baltica* is presented incorporating the phenotypic characteristics of this novel strain. Strains AK-5^T and AK-9 were most closely related to *Methanococcoides burtonii* and we propose a novel species, *Methanococcoides alaskense*, with strain AK-5^T (= OCM 775^T = DSM 17273^T) as the type strain.

Source of inoculum, enrichment and purification

All strains described here were isolated from sediment samples collected from Skan Bay, Alaska (57° N 167° W). Skan Bay lies on the north-west side of Unalaska Island in the Aleutian Island chain. Skan Bay was chosen as the study site because of its accessibility and its similarity (in temperature, pH and salinity) to deep-ocean sediments. Skan Bay is 65 m deep at the sampling site and the temperature of the sediment remains between 1 and 6 °C year-round. The sediments are permanently anoxic below the top centimetre and sulfate is depleted about 45 cm below the sediment-water interface (Reeburgh, 1980). Large amounts of organic matter are present, sedimented from diatoms and kelp. The sediment samples were collected with a multi-corer (Ocean Instruments) and samples from various depths (2–7 cm ranges) were immediately sealed in cans (while excluding air and gas) and stored at 4 °C during transport to the laboratory.

The anaerobic culture techniques of Hungate (1966), as modified by Sowers & Noll (1995), were used in this study. MSH enrichment medium (Ni & Boone, 1991) was used as the basal medium for characterization of the strains. The medium was prepared at pH 7.0 with a gas phase mixture of N_2 and CO_2 (7:3). The salinity of the medium was 0.2 M for growth of strain AK-9 and 0.3 M for growth of strains AK-5^T and AK-4.

A 1 g sample from the interval taken 32–39 cm deep in the sediment was inoculated into MSH enrichment medium (pH 7) containing 20 mM trimethylamine (TMA) as catabolic substrate. Another enrichment culture was inoculated with 1 g sediment collected from the interval 39–41 cm deep. Each of these suspensions was mixed, serially diluted and then the serial dilutions were inoculated into MSH enrichment medium with 20 mM TMA. All of the tubes were incubated at 15 °C and the cultures were screened periodically for methanogenesis by analysing a sample of the headspace gas (Maestrojuán & Boone, 1991). From each of the dilution series, the 10^{-3} culture [inoculated with the equivalent of 1 mg (wet weight) sediment] was the enrichment culture of highest dilution that produced methane. These cultures produced detectable methane after 4 weeks incubation and methanogenesis was complete after 16 weeks. No enrichments of higher dilution produced methane, even after 24 weeks of incubation.

Each culture isolated in this way was subjected to a second roll-tube isolation in order to ensure the purity of each strain. The purity of the cultures was checked microscopically and by ensuring that only a single colony type was seen in roll-tube media. Three pure cultures were obtained and named AK-4 (= OCM 774), AK-5^T (= OCM 775^T) and AK-9 (= OCM 793).

Morphology

Cells in the late-exponential growth phase were fixed directly in culture medium by adding an equal volume of 5% glutaraldehyde. Cells were fixed for 30 min at 4 °C and then collected by centrifugation. Cells were then fixed for 30 min in cacodylate buffer (pH 7.2) containing 1% osmium tetroxide. The cells were dehydrated in an ethanol series and embedded in low-viscosity epoxy resin for sectioning. Ultrathin sections were post-stained with uranyl acetate and lead citrate and viewed with a transmission electron microscope (EM-10CA; Zeiss).

Strain AK-4 formed colonies only after 60 days incubation at which time the surface colonies were about 1 mm in diameter, yellow, circular and concave. Cells were 2–3 µm in diameter, highly irregular coccoids that occurred singly and lacked flagella or pili (Fig. 1). The cells stained Gram-negative, were autofluorescent and lysed under alkaline conditions or after treatment with SDS, indicating a proteinaceous cell wall.

Surface colonies of strain AK-5^T were smaller than 1 mm after 45 days incubation, white, circular and concave with

Fig. 1. Electron micrograph of strain AK-4. This ultrathin section micrograph shows the irregular shape. Bar, 1 μm .

sharp edges. Typical cells were 1.5–2.0 μm in diameter, irregularly coccoid and occurred singly. The cells were autofluorescent and stained Gram-negative. The cells were non-motile and no flagella were seen by electron microscopy. Electron micrographs showed the presence of pili covering the cell surface (Fig. 2a). Some cells also contained

long rod-like structures with tubular subunits (Fig. 2c). Similar bodies have been reported close to the origin of the flagella in *Pyrococcus furiosus* (Fiala & Stetter, 1986); however, the exact nature of these structures is not known. Many cells also contained electron-dense, dark inclusions (Fig. 2b). The cells were lysed by SDS, indicating a proteinaceous cell wall.

Surface colonies of strain AK-9 were 1 mm after 45 days incubation. Colonies were circular, white to yellow and concave. Typical cells were 1–1.5 μm in diameter, irregularly coccoid and occurred singly. They were autofluorescent, non-motile and stained Gram-negative. The cells were susceptible to lysis by detergent. Although no motility was observed during microscopic examination of wet-mounts, the electron micrographs usually showed the presence of two appendages per cell that appeared to be flagella (Fig. 3b). Internal structures of electron-dense regions were seen in cells, but the nature of these cellular inclusions is unknown (Fig. 3a).

Growth characteristics

Growth was estimated from the accumulation of methane in the headspace gas, as measured by GC (Maestrojuán & Boone, 1991) and taking into account the methane produced

Fig. 2. Electron micrographs of strain AK-5^T. (a) Negative-stained micrograph shows numerous pili projecting from the surface. (b) Thin section transmission electron micrograph shows the irregular shape of the cells. Some cells contained protein inclusion bodies similar to those present in the cell on the left. (c) Transmission electron micrograph with an arrow pointing to a long rod-like cellular inclusion. Bars, 1 μm (a, b); 0.5 μm (c).

Fig. 3. Electron micrographs of strain AK-9. (a) Transmission electron micrograph shows the irregular coccoid morphology. Many cells contained inclusion bodies (dark spots). (b) Negative stain of AK-9. The cells bear flagella; however, no motility has been observed in microscopic examination. Bars, 1 μ m.

during the growth of the inoculum (Powell, 1983). The specific growth rate was calculated by fitting the Gompertz equation (Zwietering *et al.*, 1990) to these data. The optimal temperature for growth was determined from the specific growth rates of cultures incubated at various temperatures. We fitted the square-root equation (Powell, 1983; Ratkowsky *et al.*, 1983) to these data in order to obtain the maximum specific growth rate. All growth rate experiments were performed in triplicate.

Each strain grew between 5 and 25 °C, with fastest growth at 20–25 °C, but no growth at above 30.6 °C (see Supplementary Figs S1a and S2a in IJSEM Online). As indicated by the square-root equation (Ratkowsky *et al.*, 1983), strain AK-4 grew optimally at 21 °C, the minimum temperature was –2.3 °C and the maximum temperature was 28.4 °C. Strain AK-5^T grew fastest at 23.6 °C, the minimum growth temperature was –2.3 °C and the maximum temperature was 30.6 °C. Strain AK-9 grew optimally at 26.0 °C and the minimum and maximum temperatures for growth were –10.7 and 30.1 °C, respectively. The growth rate of AK-9 was faster than that of AK-5^T. Strain AK-4 showed the slowest growth of the three strains. Strains AK-5^T, AK-9 and AK-4 grew fastest at Na⁺ concentrations slightly below that of sea water (0.3 and 0.4 M). Cells grew in the range 0.1–0.7 M Na⁺ for strains AK-5^T and AK-4 and at 0.1–0.8 M Na⁺ for strain AK-9 (see Supplementary Figs S1b and S2b in IJSEM Online). Growth occurred in the pH range 6.3–7.5 for all three strains, but not at pH 6.0 or 8.0 (see Supplementary Figs S1c and S2c in IJSEM Online).

The catabolic ranges of all strains were determined by adding (in separate tests) 160 mM formate, 40 mM acetate, 40 mM methanol, 25 mM TMA, 5 mM dimethylsulfide or 1 atm H₂

to the basal medium for each strain. When a given substrate did not support methanogenesis, possible inhibition was tested by inoculating the culture into culture medium containing that substrate plus 5 mM TMA (on which each strain could grow). The production of methane from cultures with TMA plus another substrate was taken to indicate that the other substrate was not inhibitory.

Each of the three strains grew with TMA as the catabolic substrate. Only strain AK-4 grew on methanol as the catabolic substrate. This is the first report of methyltrophic methanogens that can not grow on methanol, although *Methanohalobium vestigatum* can grow on low concentrations of methanol (Boone *et al.*, 1993). None of the strains could catabolize acetate, dimethylsulfide, formate or hydrogen.

All strains grew in MSH medium with TMA as the sole organic substrate. Although the strains did not require growth factors, specific growth rates increased when yeast extract and peptone (0.05–0.2 % final concentration) were added to the medium.

Phylogenetic analysis

The 16S rRNA gene sequences were determined after extraction of DNA from cell pellets of pure cultures of strains AK-4, AK-5^T and AK-9. DNA was extracted by using QIAamp DNA mini kit procedures (Qiagen). DNA for determination of base composition and for DNA–DNA hybridization experiments was extracted and purified by a modified method of Marmur (1961). Cells were lysed hypotonically and the lysate was mixed with chloroform/isoamyl alcohol to denature and remove proteins. DNA was extracted into the aqueous phase and then precipitated by

mixing 1:1 with cold 95 % (v/v) ethanol. The DNA was spooled onto a glass rod and dissolved into standard saline citrate solution (SSC; 0.15 M NaCl and 0.015 M trisodium citrate, pH 7.0). The 16S rRNA gene was amplified by PCR from genomic DNA by using the archaea-specific primer 4F (5'-TCCGGTTGATCCTGCCRG-3') and the universal primer 1492R (5'-GGTTACCTTGTTACGACTT-3'). Reactions contained 1.5 mM MgCl₂, 50 mM KCl, 30 mM Tris/HCl, 12.5 mM dATP, dGTP, dCTP and dTTP, 1 U *Taq* polymerase (USB Corporation), 20 pmol of each primer and approximately 100 ng DNA. The reactions were incubated under the following conditions: initial denaturation at 94 °C for 5 min, 35 cycles of primer annealing at 50 °C for 30 s, extension at 72 °C for 90 s and denaturing at 94 °C for 30 s; the final extension was at 72 °C for 10 min. PCR products were purified by using an UltraClean PCR Cleanup DNA purification kit (MoBio Laboratories). The purified products from strains AK-4, AK-5^T and AK-9 were used as the templates for sequencing. Sequencing reactions were performed with the ABI PRISM Big Dye Terminator Cycle Sequencing kit and an ABI 310 Genetic Analyzer (Applied Biosystems). The almost-complete sequences of both strands were obtained by using the primers 4F (described above), 515F (5'-GTGCCAGCMGCCGCGGTAA-3'), 765F (5'-TAGATACCCSSGTAGTCC-3' with S=C or G), 906F (5'-GAAACTTAAAKGAATTG-3' with K=G or T), 1100F (5'-GGCAACGAGCGMGACCC-3' with M=A or C), 1492R (described above), 1391R (5'-GACGGGCGGTGTGT-RCA-3' with R=A or G), 1098RA (5'-GGGTCTCGCTCGT-TSCC-3'), 907R (5'-GTGCCAGCMGCCGCGGTAA-3' with M=A or C) and 609R (5'-TCTACGCATTCACC-3') to produce an overlapping set of sequences that were assembled into one contiguous sequence by using the AutoAssembler Program (Applied Biosystems). The sequences were manually aligned to related sequences of described methanogens obtained from GenBank and to strain FRX-1, a methylo-trophic methanogen that we isolated from Lake Fryxell, Antarctica (unpublished data). Only homologous nucleotides were included in the phylogenetic analysis. Secondary structures were used as a guide to ensure correct alignment and that homologous regions were compared. Filters were used to exclude highly variable positions. The phylogenetic relationships were determined by using maximum-likelihood analysis using the HKY-85 model of evolution in PAUP* (Swofford, 2002). A bootstrap analysis was performed by using 1000 trial replications to provide confidence estimates for tree topologies.

Analysis of the 16S rRNA gene sequences revealed that the sequence of strain AK-4 had a similarity of 99.5 % to that of *Methanosarcina baltica*. No other 16S rRNA gene sequences of established species showed more than 97 % similarity to that of strain AK-4. The 16S rRNA gene sequences of strains AK-5^T and AK-9 were identical and were 99.8 % similar to the sequence of the type strain of *Methanococcoides burtonii*. An evolutionary tree based on the maximum-likelihood-generated comparison of the 16S rRNA gene sequences is shown in Fig. 4.

DNA base composition

The genomic DNA G+C content was calculated by using the thermal denaturation method developed by Marmur & Doty (1962). The DNA melting temperature (T_m) for each of the isolates was determined by thermal denaturation in 1 × SSC buffer with a UV spectrophotometer (Varian Cary). *Escherichia coli* ATCC 1174, with a T_m of 51.7 °C (Starr & Mandel, 1968), was used as the standard during each determination. The T_m value was then substituted in the Sly equation (Sly *et al.*, 1986) to calculate the DNA G+C content.

The T_m values for strains AK-4, AK-5^T and AK-9 were 89.1, 91 and 90 °C, respectively, which correspond to DNA G+C contents of 37.3, 41.9 and 39.5 mol%, respectively.

DNA–DNA hybridization

DNA–DNA hybridization values were determined quantitatively with the method described by De Ley *et al.* (1970). The DNA to be tested was diluted to a final concentration of 50 µg ml⁻¹ (Huß *et al.*, 1983) in 1 × SSC buffer. DNA was fragmented to 700 bp by sonication (Microson XL 2000; Misonix) for 2 min. The fragment size was verified by electrophoresis and by comparison with a 1 kb ladder (Invitrogen).

DNA–DNA relatedness between strain AK-4 and *Methanosarcina baltica* GS1-A^T was 88 % and thus strain AK-4 should be classified as a reference strain of *Methanosarcina baltica*.

DNA–DNA hybridization tests indicated a DNA–DNA relatedness value of 55 % between strain AK-5^T and its closest relative, *Methanococcoides burtonii* DSM 6242^T. This suggests that strain AK-5^T should not be classified as a member of this species if it can be distinguished by phenotypic traits (Wayne *et al.*, 1987). DNA–DNA relatedness values of 88 % between strain AK-5^T and strain AK-9 suggest that these strains belong together as members of the same species.

Comparisons with related species

On the basis of DNA–DNA relatedness and 16S rRNA gene sequence analysis, strain AK-4 was assigned as a member of the psychrophilic, methanogenic species *Methanosarcina baltica*. However, strain AK-4 possesses unique phenotypic properties compared with the *Methanosarcina baltica* type strain, GS1-A^T (Table 1). *Methanosarcina baltica* GS1-A^T can utilize TMA, methanol and acetate as substrate, but strain AK-4 can only use TMA and methanol. Morphologically, the strains are both highly irregular cocci, but *Methanosarcina baltica* GS1-A^T has flagella and occurs mainly singly or as diploids; strain AK-4 has no flagella and occurs both singly and as pseudosarcinae. The optimum pH for *Methanosarcina baltica* GS1-A^T is 6.5, whereas that for strain AK-4 is 7.2. In addition, strain AK-4 grows over a broader Na⁺ range, from 0.1 to 0.7 M Na⁺.

Fig. 4. Maximum-likelihood phylogenetic tree showing the positions of strains AK-4, AK-5^T and AK-9. The numbers at the branch nodes are bootstrap values based on 1000 replications. The sequences of '*Methanococcus aeolicus*' and *Methanogenium cariaci* were used as outgroup references. Bar, 0.01 substitutions per site.

The 16S rRNA gene sequences of strains AK-5^T and AK-9 were identical to each other and the DNA–DNA relatedness value, determined by DNA–DNA hybridization, was 88 %. Strains AK-5^T and AK-9 were phenotypically similar and it is clear that the strains represent a single species. 16S rRNA gene sequence analysis indicated that the strains belong within the genus *Methanococcoides* and their closest relative is *Methanococcoides burtonii*. The DNA–DNA relatedness value between strain AK-5^T and the type strain of *Methanococcoides burtonii* was only 56 %, indicating that strains AK-5^T and AK-9 represent a separate species. As strains AK-5^T and AK-9 were phenotypically distinct from *Methanococcoides burtonii* (Table 1), we propose that they represent a novel species, *Methanococcoides alaskense* sp. nov., with strain AK-5^T as the type strain.

Emended description of *Methanosarcina baltica* von Klein et al. 2002

Methanosarcina baltica (bal'ti.ca. L. n. *mare balticum* the Baltic Sea; N.L. fem. adj. *baltica* pertaining to the Baltic Sea).

Cells are irregular cocci with diameters of 1.5–3 µm and occur singly, in pairs or in tetrads. Cells may be non-motile and lack flagella or cells may exhibit monopolar, monotrichous flagellation. Methanol and methylamines serve as catabolic substrate, with methane as the end product. Some strains may use acetate as catabolic substrate. Dimethylsulfide, formate and H₂/CO₂ are not used as energy sources. Growth occurs between –22.3 and 28 °C, with an optimum at 25 °C for the type strain. The doubling time is 84 h at 25 °C, 120 h at 9 °C and 167 h at 4 °C. After

Table 1. Comparison of physiological characteristics of isolates AK-4, AK-5^T, AK-9 and the related species *Methanosarcina baltica* and *Methanococcoides burtonii*

Taxa: 1, *Methanosarcina baltica* GS1-A^T; 2, *Methanococcoides burtonii* DSM 6242^T; 3, AK-4; 4, AK-5^T; 5, AK-9. Data were taken from this study, von Klein *et al.* (2002) and Franzmann *et al.* (1992). All strains were able to use TMA as a catabolic substrate. NR, Not reported.

Characteristic	1*	2	3	4	5
DNA G+C content (mol%)	NR	39.6	37.3	41.9	39.5
Extracellular structures	Flagella	Flagella	None	Pili	Flagella
Motility	NR	+	—	—	—
Range for growth:					
Temperature (°C)	−22.3–27	1.7–29.5	−2.3–28.4	−2.3–30.6	−10.7–30.1
Optimum temperature (°C)	25	23.4	21	23.6	26
Na ⁺ (M)	0.1–0.5	0.2–0.5	0.1–0.7	0.1–0.7	0.1–0.8
pH	4.9–8.5	6.8–8.2	6.3–7.5	6.3–7.5	6.3–7.6
Catabolic substrates:					
Methanol	+	+	+	—	—
Acetate	+	—	—	—	—

*The square-root equation (Ratkowsky *et al.*, 1983) was applied to the data of von Klein *et al.* (2002) to determine the minimum, optimum and maximum temperatures for growth for *Methanosarcina baltica*.

transfer of cultures, long lag phases lasting 15–20 days at 25 °C and 25 days at 4–9 °C are observed. Growth occurs at 0.1–0.7 M Na⁺, with fastest growth occurring at 0.3–0.4 M Na⁺ and at pH values between 6.5 and 7.5.

The type strain, GS1-A^T (=DSM 14042^T=JCM 11281^T), was isolated from the anoxic surface layer of the sediment at a depth of 241 m in the Gotland Deep of the Baltic Sea. Strain AK-4 (=OCM 774), isolated from marine sediments at Skan Bay, Alaska, is a reference strain.

Description of *Methanococcoides alaskense* sp. nov.

Methanococcoides alaskense (a.las'ken.se. N.L. neut. adj. *alaskense* referring to Skan Bay, Alaska, from where the type strain was isolated).

Irregular cocci 1.5–2.0 µm in diameter, occurring singly. Non-motile. Pili are present. TMA serves as catabolic substrate with methane as the product. Methanol, acetate, dimethylsulfide, formate and hydrogen are not used as catabolic substrate. TMA serves as sole organic substrate. Fastest growth occurs at 23.6 °C, with salinity between 0.3 and 0.4 M and a pH of 6.3–7.5.

The type strain, AK-5^T (=OCM 775^T=DSM 17273^T), was isolated from permanently cold, anoxic marine sediments at Skan Bay, Alaska. Strain AK-9 (=OCM 793) is a reference strain.

Acknowledgements

We thank David Valentine, Bill Reeburgh and the crew of the R/V *Alpha Helix* for help in sample collection and Jill Mikucki for providing

a sediment sample from Antarctica. We also thank Karl Rusterholtz, Jane Boone, Martin Sobieraj, Susan Masta and Amy Banta for help in the analysis of the 16S rRNA gene sequences. We are grateful to Henry Aldrich (University of Florida) for preparing the electron micrographs. This work was supported by a grant from the National Science Foundation's LExEn program [prime contract OCE-0085607 via a subcontract from the University of California at Irvine (L00OCE0085607)] and by a NSF IGERT Subsurface Biosphere fellowship to M.M.K.

References

- Barker, H. A. (1936). Studies upon the methane-producing bacteria. *Arch Microbiol* 7, 420–438.
- Boone, D. R., Whitman, W. B. & Rouvière, P. E. (1993). Diversity and taxonomy of methanogens. In *Methanogenesis: Ecology, Physiology, Biochemistry and Genetics*, pp. 35–80. Edited by J. G. Ferry. New York: Chapman & Hall.
- Bryant, M. P. & Boone, D. R. (1987). Emended description of strain MS^T (DSM 800^T), the type strain of *Methanosarcina barkeri*. *Int J Syst Bacteriol* 37, 169–170.
- De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* 12, 133–142.
- Elbersson, M. A. & Sowers, K. R. (1997). Isolation of an acetoclastic strain of *Methanosarcina siciliae* from marine canyon sediments and emendation of the species description for *Methanosarcina siciliae*. *Int J Syst Bacteriol* 47, 1258–1261.
- Fiala, G. & Stetter, K. O. (1986). *Pyrococcus furiosus* sp. nov. represents a novel genus of marine heterotrophic archaeobacteria growing optimally at 100 °C. *Arch Microbiol* 145, 56–61.
- Franzmann, P. D., Springer, N., Ludwig, W., Conway de Macario, E. C. & Rohde, M. (1992). A methanogenic Archaeon from Ace Lake, Antarctica: *Methanococcoides burtonii* sp. nov. *Syst Appl Microbiol* 15, 573–581.
- Hungate, R. E. (1966). *The Rumen and its Microbes*. New York: Academic Press.

- HuB, V. A. R., Festl, H. & Schleifer, K. H. (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.
- Jarvis, G. N., Strompl, C., Burgess, D. M., Skillman, L. C., Moore, E. R. B. & Joblin, K. N. (2000). Isolation and identification of ruminal methanogens from grazing cattle. *Curr Microbiol* **40**, 327–332.
- Kendall, M. M. & Boone, D. R. (2004). The Order *Methanosarcinales*. In *The Prokaryotes: an Evolving Electronic Resource for the Microbiological Community*. Release 3.17. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K. -H. Schleifer & E. Stackebrandt. New York: Springer. <http://link.springer-ny.com/link/service/books/10125/>
- King, G. M. (1984). Utilization of hydrogen, acetate, and “non-competitive” substrates by methanogenic bacteria in marine sediments. *Geomicrobiol J* **3**, 275–306.
- King, G. M., Klug, M. J. & Lovley, D. R. (1983). Metabolism of acetate, methanol, and methylated amines in intertidal sediments of Lowes Cove, Maine. *Appl Environ Microbiol* **45**, 1848–1853.
- Lyimo, T. J., Pol, A., Op den Camp, H. J. M., Harhangi, H. R. & Vogels, G. D. (2000). *Methanosarcina semesiae* sp. nov., a dimethylsulfide-utilizing methanogen from mangrove sediment. *Int J Syst Evol Microbiol* **50**, 171–178.
- Maestrojuán, G. M. & Boone, D. R. (1991). Characterization of *Methanosarcina barkeri* MS^T and 227, *Methanosarcina mazei* S-6^T, and *Methanosarcina vacuolata* Z-761^T. *Int J Syst Bacteriol* **41**, 267–274.
- Mah, R. A. & Kuhn, D. A. (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* (Approved Lists 1980) with *Methanococcus vannielii* (Approved Lists 1980) as the type species. *Int J Syst Bacteriol* **34**, 263–265.
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.
- Marmur, J. & Doty, P. A. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* **5**, 109–118.
- Ni, S. & Boone, D. R. (1991). Isolation and characterization of a dimethyl sulfide-degrading methanogen, *Methanolobus siciliae* HI350, from an oil well, characterization of *M. siciliae* T4/M^T, and emendation of *M. siciliae*. *Int J Syst Bacteriol* **41**, 410–416.
- Oremland, R. S. & Polcin, S. (1982). Methanogenesis and sulfate reduction: competitive and noncompetitive substrates in estuarine sediments. *Appl Environ Microbiol* **44**, 1270–1276.
- Oremland, R. S. & Taylor, B. F. (1978). Sulfate reduction and methanogenesis in marine sediments. *Geochim Cosmochim Acta* **42**, 209–214.
- Powell, G. E. (1983). Interpreting gas kinetics of batch culture. *Biotechnol Lett* **5**, 437–440.
- Ratkowsky, D. A., Lowry, R. K., McMeekin, T. A., Stokes, A. N. & Chandler, R. E. (1983). Model for bacterial culture growth rate throughout the entire biokinetic temperature range. *J Bacteriol* **154**, 1222–1226.
- Reeburgh, W. S. (1980). Anaerobic methane oxidation: rate depth distributions in Skan Bay sediments. *Earth Planet Sci Lett* **47**, 345–352.
- Simankova, M. V., Parshina, S. N., Tourova, T. P., Kolganova, T. V., Zehnder, A. J. B. & Nozhevnikova, A. N. (2001). *Methanosarcina lacustris* sp. nov., a new psychrotolerant methanogenic archaeon from anoxic lake sediments. *Syst Appl Microbiol* **24**, 362–367.
- Sly, L. I., Blackall, L. L., Kraat, P. C., Tian-Shen, T. & Sangkhobol, V. (1986). The use of second derivative plots for the determination of mol% guanine plus cytosine of DNA by the thermal denaturation method. *J Microbiol Methods* **5**, 139–156.
- Sowers, K. R. & Ferry, J. G. (1983). Isolation and characterization of a methylotrophic marine methanogen, *Methanococcoides methylutens*, gen. nov., sp. nov. *Appl Environ Microbiol* **45**, 684–690.
- Sowers, K. R. & Noll, K. M. (1995). Techniques for anaerobic growth. In *Archaea: a Laboratory Manual*, pp. 15–47. Edited by K. R. Sowers & H. J. Schreier. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sowers, K. R., Baron, S. F. & Ferry, J. G. (1984). *Methanosarcina acetivorans* sp. nov., an acetotrophic methane-producing bacterium isolated from marine sediments. *Appl Environ Microbiol* **47**, 971–978.
- Sprenger, W. W., van Belzen, M. C., Rosenberg, J., Hackstein, J. H. P. & Keltjens, J. T. (2000). *Methanomicrococcus blatticola* gen. nov., sp. nov., a methanol- and methylamine-reducing methanogen from the hindgut of the cockroach *Periplaneta americana*. *Int J Syst Evol Microbiol* **50**, 1989–1999.
- Starr, M. P. & Mandel, M. (1969). DNA base composition and taxonomy of phytopathogenic and other enterobacteria. *J Gen Microbiol* **56**, 113–123.
- Swofford, D. L. (2002). PAUP*: Phylogenetic analysis using parsimony (*and other methods). Sunderland, MA: Sinauer Associates.
- von Klein, D., Arab, H., Volker, H. & Thomm, M. (2002). *Methanosarcina baltica*, sp. nov., a novel methanogen isolated from the Gotland Deep of the Baltic Sea. *Extremophiles* **6**, 103–110.
- Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.
- Zhilina, T. N. & Zavarzin, G. A. (1987). *Methanosarcina vacuolata* sp. nov., a vacuolated methanosarcina. *Int J Syst Bacteriol* **37**, 281–283.
- Zinder, S. H., Sowers, K. R. & Ferry, J. G. (1985). *Methanosarcina thermophila* sp. nov., a thermophilic, acetotrophic, methane-producing bacterium. *Int J Syst Bacteriol* **35**, 522–523.
- Zwietering, M. H., Jongenburger, I., Rombouts, F. M. & van't Riet, K. (1990). Modeling of the bacterial growth curve. *Appl Environ Microbiol* **56**, 1875–1881.