Methanococcoides vulcani sp. nov., a marine methylotrophic methanogen that uses betaine, choline and \(N,N\)-dimethylethanolamine for methanogenesis, isolated from a mud volcano, and emended description of the genus Methanococcoides

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A novel, strictly anaerobic, methylotrophic marine methanogen, strain SLH33\(^T\), was isolated from deep sediment samples covered by an orange microbial mat collected from the Napoli Mud Volcano. Cells of strain SLH33\(^T\) were Gram-stain-negative, motile, irregular cocci that occurred singly. Cells utilized trimethylamine, dimethylamine, monomethylamine, methanol, betaine, \(N,N\)-dimethylethanolamine and choline (\(N,N,N\)-trimethylethanolamine) as substrates for growth and methanogenesis. The optimal growth temperature was 30\(^\circ\)C; maximum growth rate was obtained at pH 7.0 in the presence of 0.5 M Na\(^+\). The DNA G+C content of strain SLH33\(^T\) was 43.4 mol%. Phylogenetic analyses based on 16S rRNA gene sequences placed strain SLH33\(^T\) within the genus Methanococcoides. The novel isolate was related most closely to Methanococcoides methylutens TMA-10\(^T\) (98.8 % 16S rRNA gene sequence similarity) but distantly related to Methanococcoides burtonii DSM 6242\(^T\) (97.6 %) and Methanococcoides alaskense AK-5\(^T\) (97.6 %). DNA–DNA hybridization studies indicated that strain SLH33\(^T\) represents a novel species, given that it shared less than 16 % DNA–DNA relatedness with Methanococcoides methylutens TMA-10\(^T\). The name Methanococcoides vulcani sp. nov. is proposed for this novel species, with strain SLH33\(^T\) (=DSM 26966\(^T\) =JCM 19278\(^T\)) as the type strain. An emended description of the genus Methanococcoides is also proposed.

Methanogens are divided into three metabolic groups based on substrates used: hydrogenotrophs use H\(_2\)/CO\(_2\), acetoclasts use acetate and methylotrophs use methylated compounds (Garcia et al., 2000). Generally, methanogenesis is limited in marine sediments when sulfate is present, because of the higher affinity of sulfate-reducing bacteria for hydrogen and acetate (Oremland & Taylor, 1978). However, methanogenic Archaea have been detected in upper sediment layers, where the concentration of the sulfate is relatively high, using cultivation, molecular methods or in situ measurement (Lazar et al., 2011a, b). The use of non-competitive substrates consumed exclusively by methanogens could explain the occurrence of methanogens in the presence of relatively high concentrations of sulphate. \(C_1\) compounds, such as methanol and methylated nitrogen [methylamine, dimethylamine (DMA), trimethylamine (TMA)] or sulfur compounds (methylthiol and dimethyl sulfide), could be utilized by some methanogens as substrate for growth and methanogenesis. The order Methanosarcinales is one of the seven orders of the methanogens, including a new

Abbreviations: DMA, dimethylamine; DMS, dimethylsulfide; MMA, monomethylamine; TMA, trimethylamine.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and mcrA gene sequences of strain SLH33\(^T\) are KC631821 and KF500004, respectively.

Three supplementary figures are available with the online version of this paper.
lineage, *Methanomassiliicoccales* (Iino et al., 2013), whose name is not yet validly published. Members of the order *Methanosarcinales* have the widest substrate range of methanogens: many can grow by reducing CO₂ with H₂, by dismutating methyl compounds or by the splitting of acetate. The order *Methanosarcinales* comprises the families *Methanosarcinaceae*, *Methanosaetaceae* (an illegitimate name) and *Methylococcaceae*. The catabolism of methyl compounds to produce methane is accomplished by all known species of the family *Methanosarcinaceae*. This family comprises nine recognized genera: *Methanosarcina*, *Methanomicrococcus*, *Methanomethylovorans*, *Methanolobus*, *Methanococcoides*, *Methanohalobium*, *Methanohalophilus*, *Halomethanococcus* and *Methanosalts* (Kendall & Boone, 2006; Lomans et al., 1999; Sprenger et al., 2000; Yu & Kawamura, 1987). The genus *Methanococcoides* comprises, at the time of writing, three recognized species, namely *Methanococcoides methylutens*, *Methanococcoides burtonii* and *Methanococcoides alaskense*. *Methanococcoides methylutens* was isolated from submarine canyon sediments off the coast of southern California (Sowers & Ferry, 1983), *Methanococcoides burtonii* was obtained from the anoxic hypolimnion of Ace Lake in Antarctica (Franzmann et al., 1992) and *Methanococcoides alaskense* was isolated from permanently cold anoxic marine sediments at Skan Bay in Alaska (Singh et al., 2005). In this study, we describe a novel strain isolated from deep marine sediment, closely related to *Methanococcoides methylutens*.

Sediment samples were collected in the Napoli Mud Volcano in the eastern Mediterranean Sea during the Ifremer Medeco cruise with the research vessel *Pourquoi Pas?* in October and November 2007. Sediment push-core CT-21 was recovered during dive PL 331-10 by the remotely operated vehicle VICTOR 6000 (Ifremer) at 1938 m water depth (33° 43.4397′ N 24° 41.0385′ E). In the sampled area, sediments were recovered with dense orange microbial mats on top of the core. The sediment push-core sample contained bacterial orange filaments that penetrated the first 2–3 cm. Immediately after retrieval, the sediment core (10 cm long) was sectioned aseptically in 2 cm thick layers in the cooling room (4°C). Subsamples were transferred to 50 ml glass vials closed tightly with butyl rubber stoppers (Bellco), pressurized with N₂ (100 kPa), reduced with sodium sulfide and stored at 4°C until processed further.

Enrichment, isolation and cultivation of methylotrophic methanogens were performed in medium containing (per litre distilled water): 30 g NaCl, 4 g MgCl₂·6H₂O, 3.45 g MgSO₄·7H₂O, 0.25 g NH₄Cl, 0.33 g KCl, 0.14 g CaCl₂·2H₂O, 10 ml trace element mixture (medium 141; DSMZ), 0.2 g yeast extract, 1 g NaHCO₃, 0.14 g K₂HPO₄ and 1 mg resazurin. The pH of the medium was adjusted to pH 7 with HCl. The medium was boiled under nitrogen, cooled under nitrogen and 0.5 g cystein hydrochloride added. The medium was autoclaved, and then supplemented with 10 ml vitamin mixture (medium 141). Enrichments were performed anaerobically in 50 ml vials with 20 mM TMA. Enrichments were incubated at 15°C for up to 1 year. Positive enrichments, i.e. cocci that fluoresced blue–green when examined with a UV-fluorescence microscope, were subcultured and purified by streaking onto the same medium solidified with 15 g Noble agar l⁻¹. Plates were incubated in anaerobic jars at 20°C for 1 month. Purity of the isolate was confirmed by microscopic observations and subcultivation into rich media under aerobic and anaerobic conditions. Stock cultures were stored at −80°C with 5% (v/v) DMSO.

Unless otherwise stated, all phenotypic tests were performed at 20°C using the medium described above, at pH 7 with 30 g NaCl l⁻¹. Cell morphology and motility were examined by phase-contrast microscopy and transmission electron microscopy (JEM 100 CX II; JEOL) during the exponential growth phase. Transmission electron microscopy observations were performed after negative staining with uranyl acetate (2%, v/v).

The growth temperature range of the strain was examined between 5 and 40°C (5°C increments) by measuring the turbidity (600 nm) using a spectrophotometer (Genesys 20; ThermoScientific) of cultures incubated in Bellco tubes. The pH range for growth was examined at 20°C in the same medium with 20 mM MES, PIPES, HEPES or Tris over a range from pH 5.5 to 9.0. The effect of NaCl on growth was determined in the same medium with between 0 and 8% (w/v) NaCl (increments of 0.5%). Cell numbers were determined by direct cell counting using a flow cytometer (CyFlow Space; Partec). Growth rates were calculated using linear regression analysis from four to seven points along the logarithmic portion of the resulting growth curves.

The catabolic growth range of the strain was determined in optimal conditions by adding (in separate tests) 80 mM formate, 40 mM acetate, 40 mM methanol, 20 mM TMA, 20 mM DMA, 20 mM monomethylamine (MMA), 5 mM dimethylsulfide (DMS), 6 mM betaine, 6 mM choline and 6 mM N,N-dimethylethanolamine. The ability to utilize methanol (40 mM) and hydrogen (100%, 100 kPa) was tested in standard culture conditions. Autotrophic growth was tested in medium buffered with NaHCO₃ and H₂/CO₂ (80:20; 200 kPa) was used as the gas phase. Stimulating compounds were tested under optimal growth conditions with TMA as the catabolic substrate.

The genomic DNA G+C content of strain SLH33T was determined by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) by HPLC analysis of DNAs as described by Mesbah et al. (1989). The G+C content of strain SLH33T was 43.4 mol%.

DNA–DNA hybridization experiments were performed to further elucidate the level of DNA–DNA relatedness between strain SLH33T and *Methanococcoides methylutens* TMA-10T. They were performed by the Identification Service of the DSMZ, as described by De Ley et al. (1970), with the modifications described by Huß et al. (1983),
using a Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier thermostatted 6 x 6 multicell changer and a temperature controller with in situ temperature probe (Varian).

Archaeal 16S rRNA genes were amplified by PCR from purified DNA extracts using the archaeal targeted primer pair 8F (5'-CGGTTGATATCCGGCGGA-3') and 1492R (5'-GCTGACCTTGTAGCTACGT-3') (Teske et al., 2002). The PCR products were visualized by agarose gel electrophoresis and ligated into pGEM T-Easy vector (Promega) and sequenced by Beckman Coulter Genomics using four primers (M13F, 5'-AGAGTTTGATCATGGCTCAG-3', M13R, 5'-CAGGAAACAGCTATGAC-3', 344F, 5'-AGGTTGTCGGTCGTGTTG-3', and 907R, 5'-GGTACCTTGTAGCTACGT-3').

The mcrA genes were amplified using primers ME1 (5'-GCMATGCARATHGGWATGTC-3') and ME2 (5'-TCA-TKGCRATGTTGDGGRTAGT-3') (Hales et al., 1996). The PCR conditions were as follows: 30 cycles of denaturation at 94 °C for 40 s, annealing at 50 °C for 90 s and extension at 72 °C for 3 min. The PCR products were visualized by agarose gel electrophoresis and ligated into pGEM T-Easy vector (Promega) and sequenced by Beckman Coulter Genomics using forward primer M13F and reverse primer M13R.

The 16S rRNA gene sequences obtained in this study were imported into version 111 of the Silva database (Pruesse et al., 2007; http://www.arb-silva.de) using the ARB software package (Ludwig et al., 2004). Sequences from other studies that were not included in Silva were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/). The sequences were automatically aligned against the entire database by using the ‘Integrated Aligner’ of ARB, and the alignments were refined manually. The final consensus sequence was assembled from three independent sequences from each primer used. Positions that had not been sequenced in one or more reference organisms were omitted. Phylogenetic trees of almost full-length sequences (1306 nt) were reconstructed using RaxML, a maximum-likelihood method (Stamatakis, 2006) with rate distribution model GTRMIX and a weighting mask based on positional variability of archaeal reference sequences calculated by parsimony. The outgroup-rooted (Methanopyrus kandleri) phylogenetic tree topology and node support (100 bootstraps) were tested.

The mcrA gene sequences were imported into a seed alignment. The mcrA gene sequences were then translated into amino acid sequences and aligned using CLUSTAL X (Larkin et al., 2007). Trees were calculated at the amino acid level (153 aa) using a neighbour-joining method (Saitou & Nei, 1987) with a Poisson correction implanted in the MEGA4.0.2 program (Tamura et al., 2007). The outgroup-rooted (Methanopyrus kandleri) phylogenetic tree topology and node support (1000 bootstraps) were tested.

After the third streaking on a plate, a single yellow colony of 1–2 mm in diameter was picked and transferred back into liquid medium. This was designated strain SLH33T. Cells of strain SLH33T were irregular cocci, occurred singly, were autofluorescent and exhibited a slight tumbling motility with one to four polar flagella (Fig. S1, available in the online Supplementary Material). Cells of strain SLH33T were irregular cocci with a mean diameter about 0.6–1.7 μm. Cells stained Gram-negative. The cells were lysed by SDS, indicating a proteinaceous cell wall.

The strain grew in media with an Na+ concentration of 0.25–1 M with no growth at 0.17 or 1.2 M. Optimum growth was at 0.5 M Na+. The pH range for growth was pH 6.0–7.8. No growth was detected at pH 5.5 or 8.0, and optimum pH was about 7.0. The optimum temperature for growth was 30 °C, and no growth was observed at 37 °C. Growth was observed at 4 °C, but the minimal temperature for growth was not determined (Fig. S2).

Growth and methanogenesis were supported by the methylotrophic substrates TMA, DMA, MMA, methanol, betaine, choline, and N,N-dimethylethanolamine. No growth was observed on DMS, formate, acetate or H2/CO2. Hydrogen was not used when reducing methanol. When supplemented individually in the basal medium vitamin mixture (medium 141), yeast extract (0.02 %), peptone, tryptone and Casamino acids stimulated the growth yield. Under optimal growth conditions, the doubling time of strain SLH33T was around 21 h.

To test the phylogenetic position of strain SLH33, we added the 16S rRNA and mcrA gene sequences to a comprehensive set of sequences from public databases. According to 16S rRNA gene sequence comparisons, the closest neighbours of strain SLH33T were Methanococoides strains (97.7–99.6 % similarity). The highest similarity was observed with Methanococoides sp. strain NM1 (Watkins et al., 2012) and Methanococoides methylutens TMA-10T, the latter being closest reference strain (98.8 % similarity) representing a species with a validly published name. A phylogenetic tree based on the maximum-likelihood method for strain SLH33T and four other Methanococoides strains is shown in Fig. 1. Strain SLH33T fell into the phylogenetic cluster of the genus Methanococoides. Clustering was supported by high bootstrap values. Phylogenetic analysis based on mcrA gene sequences confirmed that strain SLH33T was a member of the genus Methanococoides, in good agreement with the results of the 16S rRNA gene sequence analysis (Fig. 1). The mcrA gene sequence of strain SLH33T fell within the genus Methanococoides containing sequences from marine or similar habitats and was related most closely to Methanococoides sp. strain NM1 isolated from Napoli Mud Volcano sediments (Watkins et al., 2012). The topologies of the 16S rRNA and mcrA gene trees resembled each other strongly (Fig. 1). The topologies of the trees were also checked with alternative methods (parsimony) and appear to be the same (data not shown).

16S rRNA gene sequences provide limited resolution when closely related micro-organisms are analysed. Stackebrandt & Ebers (2006) recommend DNA–DNA reassociation
Fig. 1. (a) Phylogenetic tree showing the relationship between strain SLH33\(^T\) and methanogenic *Archaea* related to members of the genus *Methanococcoides*, based on a maximum-likelihood analysis of an alignment of archaeal 16S rRNA genes (1306 nt) in public databases; the sequence obtained in this study is marked in bold type. Bootstrap values (%) are based on 100 replicates and are indicated at nodes for branch bootstrap support values ≥ 50%. Bar, 0.1 substitutions per site. (b) Phylogenetic tree showing the relationship between strain SLH33\(^T\) and other orders of methanogenic *Archaea* based on a neighbour-joining analysis of an alignment of *mcrA* genes (153 aa) in public databases; the sequence obtained in this study is marked in bold type. Bootstrap values (%) are based on 1000 replicates and are indicated at nodes for branch bootstrap support values ≥ 50%. Bar, 0.02 substitutions per site.
Table 1. Characteristics differentiating strain SLH33T from species of the genus *Methanococcoides*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin</td>
<td>Napoli Mud Volcano, eastern Mediterranean Sea</td>
<td>Submarine canyon sediment, California</td>
<td>Hypolimnion of Ace Lake, Antarctica</td>
<td>Anoxic sediment, Skan Bay, Alaska</td>
</tr>
<tr>
<td>Extracellular structure</td>
<td>Flagella</td>
<td>-</td>
<td>Flagella</td>
<td>-</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Temperature range for growth (optimum) (°)</td>
<td>ND–35 (30)</td>
<td>15–ND (30–35)</td>
<td>1.7–29.5 (23.4)</td>
<td>−2.3 to 30.6 (23.6)</td>
</tr>
<tr>
<td>pH range for growth</td>
<td>6–7.8</td>
<td>6–8.0</td>
<td>5.5–8.0</td>
<td>6.0–8.0</td>
</tr>
<tr>
<td>NaCl concentration for growth (M)</td>
<td>0.08–1.02</td>
<td>0.15–1.1</td>
<td>0.2–0.5</td>
<td>0.1–0.7</td>
</tr>
<tr>
<td>Catabolic substrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>N,N-Dimethylethanolamine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Choline</td>
<td>+</td>
<td>−*</td>
<td>−*</td>
<td>−*</td>
</tr>
<tr>
<td>Betaine</td>
<td>+</td>
<td>−*</td>
<td>−*</td>
<td>−*</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>43.4 (HPLC)</td>
<td>42 (Tm)</td>
<td>39.6 (Tm)</td>
<td>39.5 (Tm)</td>
</tr>
</tbody>
</table>

*Data from this study.

Experiments to test the genomic uniqueness of a novel isolate at a 16S rRNA gene sequence similarity range of 98.7–99%. Thus, the level of DNA–DNA relatedness between strain SLH33T and *Methanococcoides methylutens* TMA-10T (Wayne *et al.*, 1983; 3, *Methanococcoides burtonii* DSM 6242T (Franzmann *et al.*, 1992); 4, *Methanococcoides alaskense* AK-5T (Singh *et al.*, 2005). +, Positive; −, negative; ND, not determined. All are positive for growth with TMA, DMA and MMA but negative for growth with DMS.

**Description of *Methanococcoides vulcani* sp. nov.**

*Methanococcoides vulcani* (vul.ca’ni. L. gen. n. vulcani of Vulcanus, the Roman god of fire; relating to the place of isolation from Napoli Mud Volcano).

Cells exhibit a slight tumbling motility by means of one to four polar flagella. They are irregular cocci (diameter 0.6–1.7 μm) and occur singly. Colonies are yellow, circular and convex. Cells exhibit a specific F420 blue autofluorescence under UV illumination. TMA, DMA, MMA betaine, choline, N,N-dimethylethanolamine and methanol serve as catabolite substrates with methane as the product. DMS, formate, acetate and hydrogen are not used as catabolic substrates. Hydrogen is not used with methanol. Growth is stimulated by vitamins, yeast extract, peptone, tryptone and Casamino acids. Fastest growth occurs at 30 °C, at a salinity of 0.5 M NaCl and at pH 7.0. Doubling time under optimal conditions is 21 h.

The type strain, SLH33T (=DSM 26966T=JCM 19278T), was isolated from sediment in Napoli Mud Volcano covered by a dense orange microbial mat at 1938 m water depth. The DNA G+C content of the type strain is 43.4 (HPLC).

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REFERENCES


