

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 °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 H₂/CO₂, 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. C₁ compounds, such as methanol and methylated nitrogen [methylamine, dimethylamine (DMA), trimethylamine (TMA)] or sulfur compounds (methanethiol 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 *Methermicoccaceae*. 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*, *Methanimicrococcus*, *Methanomethylivorans*, *Methanolobus*, *Methanococcoides*, *Methanohalobium*, *Methanohalophilus*, *Halomethanococcus* and *Methanosalsum* (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 cysteine 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 Nobel 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 SLH33^T 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 SLH33^T was 43.4 mol%.

DNA–DNA hybridization experiments were performed to further elucidate the level of DNA–DNA relatedness between strain SLH33^T and *Methanococcoides methylutens* TMA-10^T. 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-thermostated 6 × 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'-CGGTTGATCCTGCCGGA-3') and 1492R (5'-GGCTACCTTGTTACGACTT-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'-AGGGTTGCGCTCGTTG-3'; and 907R, 5'-GGTTACCTTGTTACGACTT-3').

The *mcrA* genes were amplified using primers ME1 (5'-GCMATGCARATHGGWATGTC-3') and ME2 (5'-TCA-TKGCRTAGTTDGGRTAGT-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 SLH33^T. Cells of strain SLH33^T 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 SLH33^T 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 SLH33^T 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 SLH33^T were *Methanococcoides* strains (97.7–99.6% similarity). The highest similarity was observed with *Methanococcoides* sp. strain NM1 (Watkins *et al.*, 2012) and *Methanococcoides methylutens* TMA-10^T, 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 SLH33^T and four other *Methanococcoides* strains is shown in Fig. 1. Strain SLH33^T fell into the phylogenetic cluster of the genus *Methanococcoides*. Clustering was supported by high bootstrap values. Phylogenetic analysis based on *mcrA* gene sequences confirmed that strain SLH33^T was a member of the genus *Methanococcoides*, in good agreement with the results of the 16S rRNA gene sequence analysis (Fig. 1). The *mcrA* gene sequence of strain SLH33^T fell within the genus *Methanococcoides* containing sequences from marine or similar habitats and was related most closely to *Methanococcoides* 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

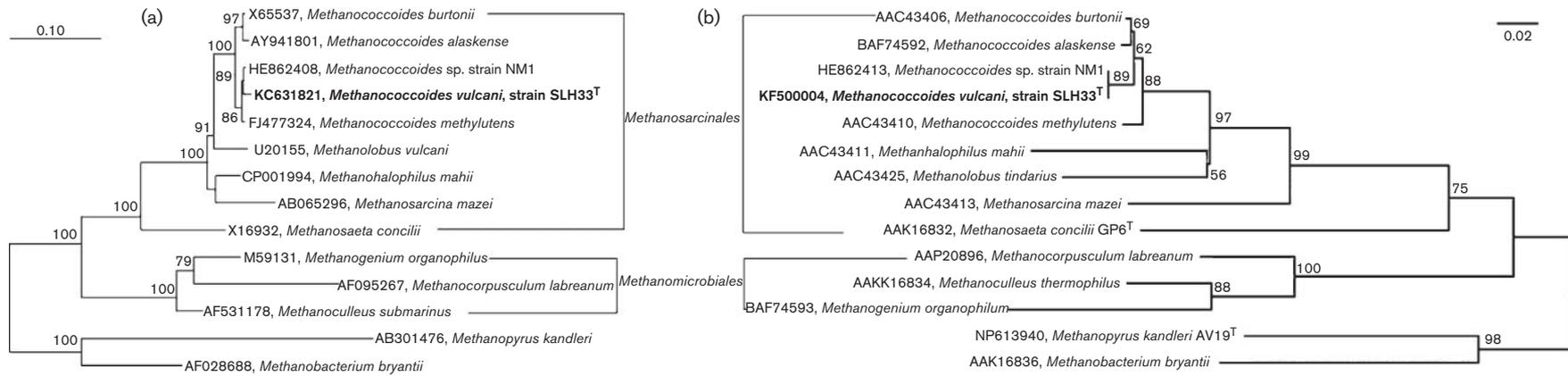


Fig. 1. (a) Phylogenetic tree showing the relationship between strain SLH33^T and methanogenic *Archaea* related to members of the genus *Methanococcoides*, based on a maximum-likelihood analysis of an alignment of archaeal 16S rRNA genes (1306 nt) in public databases; the sequence obtained in this study is marked in bold type. Bootstrap values (%) are based on 100 replicates and are indicated at nodes for branch bootstrap support values $\geq 50\%$. Bar, 0.1 substitutions per site. (b) Phylogenetic tree showing the relationship between strain SLH33^T and other orders of methanogenic *Archaea* and the genus *Methanococcoides*, 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 $\geq 50\%$. Bar, 0.02 substitutions per site.

Table 1. Characteristics differentiating strain SLH33^T from species of the genus *Methanococcoides*

Taxa: 1, strain SLH33^T (data from this study); 2, *Methanococcoides methylutens* TMA-10^T (Sowers & Ferry, 1983); 3, *Methanococcoides burtonii* DSM 6242^T (Franzmann *et al.*, 1992); 4, *Methanococcoides alaskense* AK-5^T (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.

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

*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 SLH33^T and *Methanococcoides methylutens* TMA-10^T (98.8% 16S rRNA gene sequence similarity) was low (<16%), far below the threshold value of 70% DNA-DNA relatedness generally accepted for the definition of a novel species (Wayne *et al.*, 1987).

Based on the data presented, strain SLH33^T is considered to represent a novel species of the genus *Methanococcoides*, for which the name *Methanococcoides vulcani* sp. nov. is proposed. Previously described species of the genus *Methanococcoides* have been isolated from coastal areas and Ace Lake. A description of the first organism belonging to this genus that has been isolated from a deep-sea environment is given here. The distinctive features between the novel isolate and recognized species of the genus *Methanococcoides* are given in Table 1. This distinctiveness was confirmed by random amplified polymorphic DNA fingerprinting between the novel isolate and validated species of the genus (Fig. S3).

Emended description of the genus *Methanococcoides*

The description is as given by Sowers (2000) with the following amendments. Cells are irregular cocci, occurring singly or in pairs; about 1 µm in diameter. The cell wall consists of a very thin protein monolayer approximately 10 nm thick. Susceptible to lysis by hypotonic or detergent shock. Mesophilic. Strictly anaerobic. Neutrophilic. Halophilic, growth between 0.08 and 1.1 M of NaCl. Cells can dismutate methylamines, methanol, betaine, choline and *N,N*-dimethylethanolamine for

growth but cannot catabolize acetate, DMS, H₂/CO₂ or formate.

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 Na⁺ and at pH 7.0. Doubling time under optimal conditions is 21 h.

The type strain, SLH33^T (=DSM 26966^T=JCM 19278^T), 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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