Methanocaldococcus indicus sp. nov., a novel hyperthermophilic methanogen isolated from the Central Indian Ridge

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An autotrophic, hyperthermophilic methanogen, strain SL43^T, was isolated from a deep-sea hydrothermal chimney sample collected on the Central Indian Ridge at a depth of 2420 m. The coccoid, surface-layer-carrying, Gram-negative-staining cells were heavily flagellated and exhibited a slight tumbling motility. The temperature range for growth at pH 6.5 was 50–86 °C, with optimum growth at 85 °C. The optimum pH for growth was 6.6 and the optimum NaCl concentration for growth was 30 g l⁻¹. The novel isolate used H₂ and CO₂ as the only substrates for growth and produced methane. Selenium and yeast extract stimulated growth significantly. In the presence of CO₂ and H₂, the organism reduced elemental sulfur to hydrogen sulfide. Growth was inhibited by chloramphenicol and rifampicin, but not by ampicillin, kanamycin, penicillin or streptomycin. The G + C content of the genomic DNA was 30.7 mol%. On the basis of 16S rRNA gene sequence analysis, this organism was most closely related to *Methanocaldococcus infernus* ME^T (3.2 % distance). Its phylogenetic distinctiveness was confirmed by RFLP analysis of the 16S rDNA, a reliable tool for differentiating hyperthermophilic methanococci. On the basis of phylogenetic and physiological characteristics, it is proposed that strain SL43^T (= DSM 15027^T = JCM 11886^T) be designated as the type strain of a novel species, *Methanocaldococcus indicus* sp. nov.

On the basis of differences in 16S rRNA gene sequences and the diversity of temperature growth ranges, the strictly anaerobic, methane-producing members of the order *Methanococcales* have recently been separated into two families, the mesophilic and thermophilic members of the *Methanococcaceae* (optimal temperature ≤ 70 °C) and the hyperthermophilic members of the *Methanocaldococcaceae* (Whitman *et al.*, 2001). The family *Methanocaldococcaceae* includes two new genera, *Methanocaldococcus* and *Methanotorris* (Whitman & Jeanthon, 2002). Members of these genera possess less than 93 % 16S rRNA gene sequence similarity and have been exclusively isolated from deep-sea hydrothermal vents. Within the genus *Methanocaldococcus*, the 16S rRNA sequence similarity between species is greater than 95 %.

To date, *Methanocaldococcus* species have been isolated from actively venting sulfide deposits at deep-sea hydrothermal vents at the East Pacific Rise (13°N and 21°N) (Jones *et al.*, 1983; Jeanthon *et al.*, 1999a) and the Mid-Atlantic Ridge (14°45'N and 23°N) (Jeanthon *et al.*, 1998, 1999b) and from hydrothermally heated sediments from Guaymas Basin in the Gulf of California (Zhao *et al.*, 1988; Jones *et al.*, 1989; Jeanthon *et al.*, 1999b).

Because of the paucity of readily identifiable phenotypic characteristics, taxonomic distinction between *Methanocaldococcus* species on this basis is difficult. Reliable comparison of strains can be made by RFLP analysis of the 16S rDNA (Jeanthon *et al.*, 1999b). This method was used to reveal a novel species isolated from sulfide deposits of a deep-sea hydrothermal vent on the Central Indian Ridge. In this paper, we describe the isolation and characterization of this novel organism.

The novel strain was isolated from chimney samples

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The GenBank accession number for the 16S rDNA sequence of *Methanocaldococcus indicus* SL43^T is AF547621.

collected in April 2001 at the Kairei vent field on the Central Indian Ridge (25°19'S, 70°02'E) at a depth of 2420 m (Van Dover *et al.*, 2001). Using the hydraulic arm of the remotely operated vehicle *Jason*, the chimney samples were placed in insulated containers for the trip to the surface. Once on board, samples were ground anaerobically and stored in serum vials at 4 °C with N₂ in the headspace.

Initial enrichments were done on-board ship in MSH medium (Boone *et al.*, 1989; http://methanogens.pdx.edu/OCM_media.html). Seven chimney samples were ground under a stream of N₂. Chimney samples were hard and contained pyrite, pyrrhotite, some chalcopyrite, anhydrite and iron oxides. Each sulfide slurry (0.5 ml) was inoculated into 5 ml MSH medium and the serum tubes were incubated at 80 °C for 2–3 days or until turbidity was noted. When growth occurred, it consisted of motile coccoid cells that fluoresced intense green at 420 nm. Positive enrichments were transferred in a culture medium that contained the following (l⁻¹ distilled water): 30 g NaCl, 3 g MgCl₂·2H₂O, 4 g Na₂SO₄, 0.5 g KCl, 0.25 g NH₄Cl, 0.2 g KH₂PO₄, 3.46 g PIPES, 0.15 g CaCl₂·2H₂O, 0.5 g Difco yeast extract, 1 ml trace-element mixture (Widdel & Bak, 1992), 0.2 mg sodium tungstate, 50 µg sodium selenate, 1 ml vitamin mixture (Widdel & Bak, 1992), 1 ml thiamin solution (Widdel & Bak, 1992), 0.05 mg vitamin B₁₂, 1 ml growth-stimulating factors (Pfennig *et al.*, 1981) and 1 mg resazurin. Prior to autoclaving, the pH of the medium was adjusted to 6.5 using 5 M HCl. After autoclaving, the pH of the medium was readjusted to 6.5 at room temperature. H₂/CO₂ (80:20; 200 kPa) was used as the gas phase. Pure cultures were obtained and purified on the same medium solidified with 0.7% (w/v) Phytigel (Sigma) and reduced with a titanium(III) citrate solution (Zehnder & Wuhrmann, 1976).

Pure cultures were obtained by streaking subcultures onto solidified culture medium. Pale-yellow, round colonies (1 mm in diameter) were visible on plates after 3 days at 80 °C in anaerobic jars under a H₂/CO₂ atmosphere (80:20; 300 kPa). Seven colonies were randomly picked and subcultured. Analysis of the RFLP profiles of the 16S rRNA genes showed that all the patterns were identical and were distinct from those of the described species of the genera *Methanothermococcus*, *Methanocaldococcus* and *Methanoterris* (Jeanthon *et al.*, 1999b). One of the pure cultures, designated isolate SL43^T, was selected for further characterization. Stock cultures of isolate SL43^T were stored in culture medium at 4 °C. For long-term storage, pure cultures were stored at -80 °C in the same medium containing 10% (w/v) DMSO.

Cells of strain SL43^T were irregular cocci, about 1–3 µm in diameter depending on the growth phase (Fig. 1). The cells stained Gram-negative (Murray *et al.*, 1994), occurred singly or in pairs and exhibited tumbling motility. As reported for *Methanococcales* (Whitman & Jeanthon, 2002), cells from the mid-exponential to late-exponential growth phase lysed rapidly in SDS (0.01%) and in distilled water (Boone

Fig. 1. Electron micrograph of strain SL43^T. Negative staining of a whole cell, showing the flagellation. Bar, 500 nm.

& Whitman, 1988). Ultrathin sections of cells, prepared as described previously (Sleytr *et al.*, 1988), revealed a delicate, hexagonal S-layer with a lattice constant of approximately 13.1 nm. The S-layer could not be recognized clearly all over the cell surface, in particular, in areas of heavy flagellation. Negative staining revealed that flagellation was mainly restricted to one specific region of the cell body, where numerous flagella originated (Fig. 1).

Unless otherwise stated, growth experiments were performed in duplicate in the culture medium. Growth was monitored by measuring the increase in OD₆₀₀ with a Spectronic 401 spectrophotometer (Bioblock). The pH range for growth was determined by adding increasing volumes of sterile solutions of 10% (w/v) NaHCO₃ or 8% (w/v) Na₂CO₃ to media buffered with 20 mM PIPES (for pH ≥ 6.5) and 20 mM MES (for pH ≤ 6). Since they may vary with incubation temperature, the pH values of these media were checked at room temperature after overnight incubation of uninoculated tubes under H₂/CO₂ at 85 °C.

Final OD₆₀₀ values of cultures after 6–8 h incubation were used to determine the growth ranges and optima for temperature, pH and NaCl concentration for strain SL43^T. The effects of pH and NaCl concentration were determined at the optimal temperature for growth. Under these conditions, strain SL43^T grew between 50 and 86 °C, with optimum growth at around 85 °C; no growth was detected at 45 or 88 °C. Growth was observed between pH 5.5 and 6.7, with optimum growth at around pH 6.5. No growth was observed at pH 5.2 or 7. Growth occurred in NaCl concentrations ranging from 15 to 50 g l⁻¹, with optimum growth at 30 g l⁻¹. No growth was observed at 10 or 60 g l⁻¹. Under the optimal conditions for growth, the doubling time of the novel organism was 25–30 min.

Strain SL43^T is a strictly anaerobic, autotrophic organism. Growth was prevented in the presence of low levels of oxygen, and H₂ and CO₂ served as the only substrates for

growth. No growth was observed on acetate (2 g l⁻¹), formate (5 g l⁻¹), methanol (0.5 %, v/v), monomethylamine (2 g l⁻¹) or yeast extract (2 g l⁻¹) with a N₂/CO₂ (80:20; 200 kPa) or H₂ (100 %; 200 kPa) headspace. In the presence of H₂ and CO₂, methane production paralleled growth (Jeanthon *et al.*, 1998). Ammonium (10 mM) was the preferred nitrogen source, but significant growth also occurred in the presence of yeast extract, tryptone, urea, glutamate (all at 2 g l⁻¹) and nitrate (10 mM). When supplemented individually, selenate, tungstate and yeast extract stimulated the growth rate. When sulfur (5 g l⁻¹) was added to the sulfate-free medium in the presence of CO₂ and H₂, growth occurred and H₂S was produced. No dissimilatory reduction of cystine (5 g l⁻¹), sulfate or thiosulfate (at 20 mM) was observed.

Sensitivity to antibiotics supplemented at 25, 50, 75, 100 and 200 µg ml⁻¹ was tested in the culture medium at 80 °C. Strain SL43^T was resistant to ampicillin, penicillin, streptomycin and kanamycin (200 µg ml⁻¹) and was sensitive to chloramphenicol (75 µg ml⁻¹) and rifampicin (25 µg ml⁻¹).

DNA was isolated after disruption of cells using a French pressure cell (Thermo Spectronic) and purified by hydroxyapatite chromatography (Cashion *et al.*, 1977). The DNA was hydrolysed with P1 nuclease and the nucleotides were dephosphorylated with bovine alkaline phosphatase (Mesbah *et al.*, 1989). The G+C content of the DNA of strain SL43^T was 30.7 mol%, as determined by the HPLC method (Tamaoka & Komagata, 1994), and is in the range reported for other members of the genus *Methanocaldococcus* (Whitman *et al.*, 2001).

A total of 1409 nucleotides of the 16S rRNA gene were sequenced as described previously (Götz *et al.*, 2002). Distance and maximum-likelihood trees (De Soete, 1983; Olsen *et al.*, 1994) (1354 nucleotides were used) placed strain SL43^T as a relative of *Methanocaldococcus infernus* ME^T (3.2 % distance), *Methanocaldococcus jannaschii* JAL-1^T (4.1 % distance), *Methanocaldococcus vulcanius* M7^T and *Methanocaldococcus fervens* AG86^T (5.3 % distance)

Fig. 2. Phylogenetic relationship of strain SL43^T and other members of the *Methanocaldococcaceae* revealed by maximum-likelihood analysis. The sequence of the SL43^T small subunit was aligned with other 16S rRNA gene sequences from the Ribosomal Database Project (Maidak *et al.*, 2001) and GenBank (Benson *et al.*, 2002). The scale bar represents the expected number of changes per sequence position. Numbers at branch nodes are bootstrap values based on 100 bootstrap resamplings.

(Fig. 2). However, the level of 16S rDNA sequence similarity between strain SL43^T and these organisms is lower than the limit (97 %) used to define distinct species at the DNA level without the requirement for DNA–DNA reassociation tests (Stackebrandt & Goebel, 1994). This significant phylogenetic distinctiveness was confirmed by RFLP analysis of the 16S rDNA (Jeanthon *et al.*, 1999b), a reliable and useful method for distinguishing species of *Methanocaldococcus* from each other (Whitman *et al.*, 2001).

Although the novel isolate shared a number of phenotypic features with its relatives, obvious differences exist (Table 1). Strain SL43^T is distinct from other *Methanocaldococcus* species, and particularly from *Methanocaldococcus infernus*, its closest phylogenetic relative, in its maximum pH and temperature for growth and in the numbers of regions of insertion of flagella into the cell body. This distinctiveness was confirmed by comparing whole-cell protein patterns of strain SL43^T with those of type strains of described

Table 1. Differentiating characteristics of *Methanocaldococcus* species

Taxa: 1, strain SL43^T; 2, *Methanocaldococcus infernus*; 3, *Methanocaldococcus jannaschii*; 4, *Methanocaldococcus vulcanius*; 5, *Methanocaldococcus fervens*. Data were obtained from Jones *et al.* (1983), Zhao *et al.* (1988) and Jeanthon *et al.* (1998, 1999a). ND, Not determined.

Characteristic	1	2	3	4	5
Temperature for growth (°C):					
Range	50–86	55–91	50–91	49–89	48–92
Optimum	85	85	85	80	85
pH range for growth	5.5–6.7	5.25–7.0	5.2–7.0	5.25–7.0	5.5–7.6
Flagellation apparatus	One extended insertion site	Three tufts of flagella	Two bundles of flagella	Three tufts of flagella	ND
Resistance to rifampicin*	–	–	–	–	+

*Concentration tested was 50 µg ml⁻¹; +, resistant; –, sensitive.

Fig. 3. Electrophoretic analysis of whole-cell proteins of strain SL43^T (lane 2), *Methanocaldococcus infernus* ME^T (3), *Methanocaldococcus jannaschii* JAL-1^T (4), *Methanocaldococcus fervens* AG86^T (5), *Methanocaldococcus vulcanius* M7^T (6) and *Methanocaldococcus igneus* Kol5^T (7). Precision protein standards (Bio-Rad) were used as size markers (lane 1). Electrophoretic analysis of whole-cell proteins was performed as described by Keswani *et al.* (1996).

Methanocaldococcus and *Methanoterris* species (Fig. 3). We propose that strain SL43^T represents a novel *Methanocaldococcus* species, named *Methanocaldococcus indicus* sp. nov.

Description of *Methanocaldococcus indicus* sp. nov.

Methanocaldococcus indicus (in'di.cus. L. masc. adj. *indicus* of India, referring to the Indian Ocean, where the type strain was isolated).

Cells exhibit a tumbling motility by means of numerous flagella, predominantly inserted at one specific region of the cell body. They are cocci (1–3 µm in diameter), covered with a hexagonal S-layer lattice, and occur singly and in pairs. Cells lyse rapidly in SDS (0.01 %) and in distilled water. Pale-yellow, round colonies about 1 mm in diameter form on Phytagel plates. Growth occurs at between 50 and 86 °C, with an optimum at 85 °C, between pH 5.5 and 6.7, with the optimum at around pH 6.5, and between 15 and 50 g NaCl l⁻¹, with an optimum of 30 g l⁻¹. Obligately anaerobic. Chemolithotrophic. Uses H₂ and CO₂ as energy and carbon sources to produce methane. Growth is stimulated by selenate, tungstate and yeast extract. Sulfur is reduced to H₂S in the presence of CO₂ and H₂. Growth is inhibited by chloramphenicol (75 µg ml⁻¹) and rifampicin (25 µg ml⁻¹) but not by streptomycin, penicillin G, kanamycin or ampicillin (200 µg ml⁻¹). The DNA G+C content of the type strain is 30.7 mol% (as determined by HPLC). Distinguished from other *Methanocaldococcus* strains by comparison of whole-cell protein patterns.

The type strain, strain SL43^T (= DSM 15027^T = JCM 11886^T), was obtained from a deep-sea hydrothermal vent chimney in the Kairei vent field on the Central Indian Ridge.

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