Methanocaldococcus villosus sp. nov., a heavily flagellated archaeon that adheres to surfaces and forms cell–cell contacts

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A novel chemolithoautotrophic, hyperthermophilic methanogen was isolated from a submarine hydrothermal system at the Kolbeinsey Ridge, north of Iceland. Based on its 16S rRNA gene sequence, the strain belongs to the order *Methanococcales* within the genus *Methanocaldococcus*, with approximately 95 % sequence similarity to *Methanocaldococcus jannaschii* as its closest relative. Cells of the novel organism stained Gram-negative and appeared as regular to irregular cocci possessing more than 50 polar flagella. These cell appendages mediated not only motility but also adherence to abiotic surfaces and the formation of cell–cell contacts. The new isolate grew at 55–90 °C, with optimum growth at 80 °C. The optimum NaCl concentration for growth was 2.5 % (w/v), and the optimal pH was 6.5. The cells gained their energy exclusively by reduction of CO₂ with H₂. Selenate, tungstate and yeast extract stimulated growth significantly. The genome size was determined to be in the range 1.8–2.0 kb, and the G+C content of the genomic DNA was 30 mol%. Despite being physiologically nearly identical to the other members of the genus *Methanocaldococcus*, analysis of whole-cell proteins revealed significant differences. Based on the results from phylogenetic, morphological and protein analyses, we conclude that the novel strain represents a novel species of the genus *Methanocaldococcus*, for which the name *Methanocaldococcus villosus* sp. nov. is proposed (type strain KIN24-T80^T = DSM 22612^T = JCM 16315^T).

At the time of writing, the order *Methanococcales* consists of two families, the mesophilic and slightly thermophilic *Methanococcaceae* and the hyperthermophilic *Methanocaldococcaceae*. The latter contains the genera *Methanocaldococcus* and *Methanotorris*, separated by more than 7 % 16S rRNA gene sequence divergence (Whitman & Jeanthon, 2006). All strains of the genus *Methanocaldococcus* described so far have been isolated from deep submarine habitats, e.g. hydrothermally heated material (Zhao *et al.*, 1988) or chimney samples (Jones *et al.*, 1983; Jeanthon *et al.*, 1998, 1999; L'Haridon *et al.*, 2003), whereas the genus *Methanotorris* was originally described from a shallow submarine hydrothermal vent (Burggraf *et al.*, 1990). In general, their

requirement for selenium and the high motility of cells allow differentiation of the genus *Methanocaldococcus* from *Methanotorris* (Whitman *et al.*, 2001).

Within the genus *Methanocaldococcus*, the 16S rRNA gene sequence similarity is higher than 95 %. The organisms share many metabolism and phenotypic characters (Whitman & Jeanthon, 2006), but reliable comparison of strains can be performed by SDS-PAGE of cellular proteins (Keswani *et al.*, 1996; L'Haridon *et al.*, 2003).

Samples were taken during the R/V *Poseidon* cruise in 1997 to the Kolbeinsey Ridge (Fricke *et al.*, 1989), north of Iceland (67° 05' 46" N 18° 42' 41" W), by the research submersible *Jago* at a depth of 105 m. The sample KIN24 originated from rocky ground next to effusing fluids of an active hydrothermal system. The material was pulverized, resuspended in seawater and stored at 4 °C for further cultivation analysis.

Abbreviations: SEM, scanning electron microscopy; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain KIN24-T80^T is FJ766848.

In order to enrich hyperthermophilic methanogens, MGG medium (modified from Huber *et al.*, 1982) was prepared according to the anaerobic technique described by Balch & Wolfe (1976). The medium consisted of (per litre distilled water) 18.0 g NaCl, 4.3 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 3.4 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5.5 g NaHCO_3 , 0.14 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.25 g NH_4Cl , 0.35 g KCl, 0.14 g $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 0.002 g $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ and 1 ml 10-fold trace mineral solution (Huber & Stetter, 2006). After reduction by adding 0.5 g $\text{Na}_2\text{S} \cdot 7\text{H}_2\text{O}$, the medium was adjusted to pH 6.5–7.0 with 1 M H_2SO_4 at room temperature. Medium was dispensed into 100 ml serum bottles (20 ml aliquots) with H_2/CO_2 as the gas phase (250 kPa; 80:20, v/v).

Liquid medium was inoculated with 0.5 ml sample KIN24, which had been stored for 8 years at 4 °C. After 4 days of incubation with agitation (60 r.p.m.) at 85 °C, regular to irregular cocci of different cell sizes were detected by phase-contrast microscopy (Nikon Labophot-2). Fluorescence of the cells was determined as described previously (Burggraf *et al.*, 1990). The larger cocci showed an intense blue–green fluorescence, which was stable for at least 10 s. Pure cultures of the fluorescent cocci were obtained by applying the optical tweezer (Huber *et al.*, 1995), and the new isolate was designated KIN24-T80^T. Unless otherwise stated, routine incubation was done overnight, resulting in stationary cells. Stock cultures of the cells were stored in culture medium at 4 °C. For long-term storage, cells were centrifuged anaerobically (3000 g, 30 min) in rubber-stopped glass bottles, resuspended in fresh culture medium containing 5% (v/v) DMSO and dispensed into glass capillaries, which were heat-sealed and stored over liquid nitrogen at our in-house culture collection.

Cells of the new isolate were regular to irregular, highly motile cocci with a mean diameter of 1 µm, usually occurring singly or in pairs. For description of colony morphology, liquid cultures were plated on solid MGG medium [containing 0.7% (w/v) Phytigel; Sigma] followed by incubation in an anaerobic jar at 75 °C for at least 24 h. The cells formed round, regular-edged, pale-yellow colonies, 1–2 mm in diameter. Production of methane was determined according to Burggraf *et al.* (1990). Gram staining and susceptibility to lysis by hypotonic solution and detergents were performed as described previously (Boone & Whitman, 1988). The organism stained Gram-negative, and cells of the mid- to late-exponential growth phase lysed rapidly in distilled water, hypotonic solution or 0.01% (w/v) SDS, as described for *Methanocaldococcus* (Jeanthon *et al.*, 1999; L'Haridon *et al.*, 2003).

To evaluate the physiological properties of the novel strain, parameters including optima for temperature, pH and NaCl concentration were analysed in duplicate in the culture medium according to the minimum standards described by Boone & Whitman (1988). Growth rates were determined by direct cell counting using a Thoma counting chamber (depth 0.02 mm). Strain KIN24-T80^T grew between 55 and 90 °C, with an optimum at 80 °C; no

growth could be detected at 50 or 95 °C. Phase-contrast microscopy revealed that, in comparison with cells grown at the optimal temperature, cells were considerably larger at lower temperatures, with a mean diameter of 1.5 µm, with single cells up to 2 µm in diameter. Hence, all further experiments were performed at the optimal growth temperature of 80 °C. To ascertain the pH dependence of the organism, the pH of the medium was adjusted with diluted sulphuric acid or sodium hydroxide, as indicated above, without the usage of additional buffers. Growth was observed between pH 5.5 and 7.0, with an optimum at pH 6.5; no growth was detected at or below pH 5.0 or at and above pH 7.5. Different amounts of NaCl were added to the culture medium (MGG medium prepared without NaCl) to determine the optimum growth rate with regard to salt concentration. The minimal requirement for growth was 0.5% (w/v) NaCl, the upper limit was 5.5% (w/v) NaCl and the optimum was 2.5% (w/v) NaCl. The minimum doubling time for growth of strain KIN24-T80^T under optimal conditions was 45 min.

To test the ability of the isolate to utilize energy sources other than H_2 , the medium was prepared with a gas phase of N_2/CO_2 (250 kPa, 80:20, v/v) and the following substrates were added separately to final concentrations of 0.1% (w/v): acetate, formate, methanol, pyruvate and yeast extract. No growth could be detected over a period of 3 days by phase-contrast microscopy. Therefore, the strain was considered to grow exclusively by reduction of CO_2 using H_2 as an electron donor, like all members of the genus *Methanocaldococcus* with validly published names (Jones *et al.*, 1983; Jeanthon *et al.*, 1998, 1999; L'Haridon *et al.*, 2003). As the aforementioned studies have reported a requirement for or stimulus by trace elements or organic compounds for growth of *Methanocaldococcus* species in the presence of H_2 and CO_2 , MGG medium was prepared without trace minerals and the following substances were added individually or in combination: yeast extract (0.1 g l^{-1}), selenate (0.05 g l^{-1}), tungstate (0.05 g l^{-1}), 1-fold trace mineral solution (Huber & Stetter, 2006; 10 ml l^{-1}) and 1-fold vitamin solution (Balch *et al.*, 1979; 10 ml l^{-1}). Experiments in MGG medium without trace mineral solution resulted in slower growth and two- to fourfold lower final cell densities compared with the original culture medium. Adding selenate to the medium compensated for the effects on growth rate and final cell densities caused by leaving out the trace mineral solution, whereas addition of tungstate and yeast extract had no influence on the doubling time.

Genomic DNA was prepared from the new isolate according to Jahn (2003). Cells from 60 ml culture were harvested (27 000 g, 30 min) and resuspended in 400 µl buffer A (500 mM Tris/HCl, pH 8.0, 100 mM NaCl, 1 mM sodium citrate). For cell lysis, one volume of buffer B [500 mM Tris/HCl, pH 8.0, 100 mM NaCl, 4% (w/v) SDS] and 50 µl proteinase K (20 mg ml^{-1}) were added and the mixture was incubated for 1 h at 65 °C. DNA was extracted from the resulting cell lysate as described by Muscholl *et al.* (1993).

The 16S rRNA gene was PCR-amplified using primers 8aF (5'-TCYGGTTGATCCTGCC; Eder *et al.*, 1999) and 1512uR (5'-ACGGHTACCTTGTTACGACTT; Lane, 1991). Bidirectional sequencing (GENEART, Regensburg) resulted in a total of 1490 bp; 1378 positions were aligned with a set of representative archaeal 16S rRNA gene sequences using the ARB software package (Ludwig *et al.*, 2004). Dendrograms computed with the neighbour-joining method revealed *Methanocaldococcus jannaschii* JAL-1^T to be the closest relative of strain KIN24-T80^T, with a phylogenetic distance of 5.0% (Fig. 1). All other members of the genus *Methanocaldococcus* grouped apart at a distance of 5.1–6.0%, whereas *Methanoterris igneus* Kol 5^T was even less closely related (7.4% distance). A maximum-likelihood tree confirmed the relatedness of the novel strain to the other *Methanocaldococcus* species, but also corroborated the deep branching within this genus (not shown).

For determination of the genome size of strain KIN24-T80^T, *Methanocaldococcus jannaschii* DSM 2661^T (Jones *et al.*, 1983) was chosen as a reference. Both strains were grown in modified MGG medium [MJ medium; modification for 1 l medium: 30.0 g NaCl, 1.0 g NaHCO₃, 0.01 g (NH₄)₂Fe(SO₄)₂·6H₂O, 0.5 mg NiCl₂·6H₂O and 1 ml 10-fold vitamin solution (Balch *et al.*, 1979)]. Cultures were harvested (27 000 g, 10 min) and PFGE was performed according to Murray *et al.* (1990) except that the lysis solution was prepared without lysozyme and the concentration of proteinase K in buffer ESP was raised to 1 mg ml⁻¹. Washing in TE was performed three times for at least 1 h and 200 µl of 7 mg PMSF ml⁻¹ (Serva) was added to the second wash step. A 1.0% (w/v) agarose gel [Agarose Broad Range (Roth) in 0.5 × TBE buffer] was loaded directly with the plugs; Lambda Ladder and *S. cerevisiae* chromosome (CHEF DNA size markers; Bio-Rad) were used as standards. DNA was separated on a CHEF-DRIII system (Bio-Rad); the run time was 24 h at 6 V cm⁻¹ with a switching time of 1.17 s at the beginning and 4.26 min in the end, at an included angle of 120°. The gel was afterwards subjected to staining with ethidium bromide (final concentration 1 µg µl⁻¹) and destained in distilled water. The genome size of *Methanocaldococcus jannaschii* DSM 2661^T was calculated to be 1.5 Mb, which was somewhat smaller than the reported size of 1.66 Mb (Bult *et al.*, 1996). In the experiment, two additional bands of less than 100 kb were detected, representing the two plasmids of *Methanocaldococcus*

jannaschii DSM 2661^T. For strain KIN24-T80^T, the genome size was 1.8–2.0 Mb. As no additional bands could be identified, it was concluded that the new isolate did not possess any extra-chromosomal DNA.

For estimation of the G+C content of the novel strain, genomic DNA was purified initially by applying the Genomic-tip 100/G system (Qiagen) according to the manufacturer's instructions for bacteria. Shotgun sequencing of 96 clones and sequence analysis was performed by LGC Genomics (Berlin, Germany). The G+C content of the assembled contigs, making up 4.5% of the genome, was determined to be 30 mol%, which was very similar to the reported G+C content of *Methanocaldococcus jannaschii* JAL-1^T (31 mol%; Jones *et al.*, 1983; Bult *et al.*, 1996).

A detailed morphological analysis of strain KIN24-T80^T was done by transmission electron microscopy (TEM). Cells grown in liquid medium were fixed with 1% (final concentration; v/v) glutaraldehyde for 15 min at room temperature. In the case of suspensions, concentrated cells were placed on a carbon-coated 400-mesh copper grid (Plano). Samples were either negatively stained for 1 min with 2% (w/v) uranyl acetate or shadowed unidirectionally with Pt/C at 15° (CFE 50; Cressington). Freeze etching was performed as described previously (Rachel *et al.*, 2002). For ultrathin sections, cells were concentrated (13 000 g, 10 min) and high-pressure frozen (EM-PACT 2; Leica). After freeze substitution and embedding (EM AFS2; Leica) as described elsewhere (Burghardt *et al.*, 2007), samples were ultrathin sectioned with an ultramicrotome (UltracutE, Reichert-Jung; Leica) equipped with a diamond knife (knife angle 35°; Diatome). These sections were stained with 2% (w/v) uranyl acetate for 20 min and 0.5% (w/v) lead citrate for 1 min. TEM images were recorded using a CM 12 transmission electron microscope (FEI) operated at 120 keV equipped with a slow-scan charge-coupled device camera (TEM 1000; TVIPS).

TEM analysis revealed that cells of strain KIN24-T80^T possessed about 50 polarly inserted flagella (Fig. 2a). In some cases, the flagella formed a tuft that dissolved into single filaments at around 500 nm from the cell or, in about 5% of the cells, the tufts were seen to connect two cells to each other (Fig. 3). Cable-like structures made of flagella mediating the formation of cell–cell contacts were also described for *Pyrococcus furiosus* (Näther *et al.*, 2006).

Fig. 1. Phylogenetic position of strain KIN24-T80^T and members of the genus *Methanocaldococcus* based on neighbour-joining analysis of 16S rRNA gene sequences. GenBank accession numbers are provided in parentheses. Bootstrap percentages based on 1500 replications are given at branching points. Bar, 10 estimated substitutions per 100 nucleotide positions.

Fig. 2. Electron micrographs of cells of strain KIN24-T80^T. Transmission electron micrographs of swimming cells (a, b) and scanning electron micrographs of cells grown on glassy carbon (c, d). (a) Pt/C-shadowed cell showing flagellation. (b) Ultrathin section with arrows outlining the cytoplasmic membrane (CM) and S-layer (SL). (c) Cell surrounded by a network of flagella mediating contact to the surface material and revealing the extraordinary surface structure of the cell. (d) Cell-cell connection. Bars, 1 μ m.

Ultrathin sections showed that the cell envelope was not completely regular and smooth (Fig. 2b). The different methods used for preparation showed that the cells had an S-layer with a lattice constant of 13.1 nm, as measured in images of negatively stained cell envelopes. Subunits were arranged in a sixfold or pseudo-sixfold (e.g. threefold) symmetry. These findings were consistent with those published for *Methanocaldococcus indicus* (L'Haridon *et al.*, 2003).

Adherence of strain KIN24-T80^T to surfaces was tested as described previously (Rieger, 1998; Näther *et al.*, 2006). Gold grids for TEM (400-mesh, with/without carbon coat) were placed in Teflon holders and medium was prepared as described above. Grown cultures were fixed with 1% (v/v) glutaraldehyde for 10 min at room temperature. Cells adhering to the grids were either analysed by DAPI (4',6'-diamidino-2-phenylindole) staining and subsequent epifluorescence microscopy or negatively stained for TEM.

For testing growth on glassy carbon (20 × 10 × 1 mm, in Teflon holders), cells were grown to exponential or stationary growth phase in liquid medium and were prepared for scanning electron microscopy (SEM) according to Schopf *et al.* (2008). Specimens were examined with a Hitachi S-4100 field emission scanning electron microscope. Scanning electron micrographs were taken with Digiscan hardware and were processed with the digital micrograph 3.4.4 software (Gatan Digital Micrograph).

Analysis of the gold grids revealed that cells of strain KIN24-T80^T covered the whole surface and formed biofilm-like structures. TEM analysis of carbon-coated grids indicated that all cells were embedded in a dense network of flagella and up to half of the cocci were connected to each other via flagella bundles. In contrast, only about 50% of freely swimming cells prepared for TEM showed this heavy flagellation, and formation of flagella bundles was rarely observed. These striking

Fig. 3. (a) Transmission electron micrograph of freeze-etched cells of strain KIN24-T80^T showing a cell–cell connection. (b, c) Enlargements of (a) demonstrating that the contact is formed by a bundle of flagella. Bar, 1 μ m.

observations were confirmed by SEM analysis of cells of strain KIN24-T80^T grown on glassy carbon. More than 80 % of the cells exhibited a striated pattern on their surface, the orientation of which changed on an individual cell, as exemplified in Fig. 2(c). These surface structures were clearly observed during exponential growth, but were decreasingly detected afterwards. In comparative studies, we did not see such a pattern for either *Methanocaldococcus jannaschii* DSM 2661^T or *Methanocaldococcus indicus* DSM 15027^T. Since this pattern is also more irregular than that observed on many crenarchaeota with ordered S-layers (Rachel, 2009), the conclusion was drawn that it is unique to the new isolate. Fig. 2(d) gives another example of the unusual surface pattern of the new isolate, indicating its irregular striation, and, in addition, a cell–cell connection is visible, as described for *Pyrococcus furiosus* (Näther *et al.*, 2006).

Because species of the genus *Methanocaldococcus* can barely be distinguished by phenotypic and nutrient characteristics (Whitman & Jeanthon, 2006), the following type strains

were included in comparative analyses. *Methanocaldococcus jannaschii* DSM 2661^T (Jones *et al.*, 1983), *Methanoterris igneus* DSM 5666^T (Burggraf *et al.*, 1990) and *Methanothermococcus thermolithotrophicus* DSM 2095^T (Huber *et al.*, 1982) were available from our in-house culture collection. *Methanocaldococcus fervens* DSM 4213^T (Jeanthon *et al.*, 1999), *Methanocaldococcus indicus* DSM 15027^T (L'Haridon *et al.*, 2003), *Methanocaldococcus infernus* DSM 11812^T (Jeanthon *et al.*, 1998) and *Methanocaldococcus vulcanius* DSM 12094^T (Jeanthon *et al.*, 1999) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). For comparative studies, all strains were grown in MJ medium.

Analysis of whole-cell proteins was performed as follows. Concentrated cells from the exponential phase (13 000 g, 10 min) were resuspended in aerobic medium (without resazurin) and incubated with 1 % (w/v) SDS for 10 min. The remaining cell debris was removed by centrifugation (6000 g, 10 min). Protein samples were resolved by SDS-PAGE either in 12.5 % (w/v) acrylamide gels according to

Fig. 4. Electrophoretic analysis of Coomassie-stained whole-cell proteins of strain KIN24-T80^T and related strains. Lanes: 1, strain KIN24-T80^T; 2, *Methanocaldococcus fervens* DSM 4213^T; 3, *Methanocaldococcus indicus* DSM 15027^T; 4, *Methanocaldococcus infernus* DSM 11812^T; 5, *Methanocaldococcus jannaschii* DSM 2661^T; 6, *Methanocaldococcus vulcanius* DSM 12094^T; 7, *Methanotorris igneus* DSM 5666^T; 8, *Methanothermococcus thermolithotrophicus* DSM 2095^T. NEB Broad Range protein markers were used as size markers (lanes M). (a) Gradient gel; (b) detail of a 12.5% gel.

Laemmli (1970) or in 8–20% (w/v) acrylamide gradient gels as described previously (Schägger & von Jagow, 1987; Burghardt *et al.*, 2007). Proteins were visualized by Coomassie blue G250 staining. The whole-cell protein pattern of strain KIN24-T80^T differed from those of the type strains of described *Methanocaldococcus* species, especially in the range between 10 000 and 30 000 Da (Fig. 4a). As can be seen in Fig. 4(b), the similarity of the protein bands between 50 000 and 60 000 Da emphasized the relatedness of the novel strain to the genus *Methanocaldococcus*, whereas *Methanotorris igneus* DSM 5666^T and *Methanothermococcus thermolithotrophicus* DSM 2095^T were distinct from these species.

The results of phenotypic characterization confirm the affiliation of KIN24-T80^T to the genus *Methanocaldococcus*. Nevertheless, 16S rRNA gene sequence analysis in combination with the unique whole-cell protein SDS-PAGE pattern proved its distinctiveness from any previously described species. Based on the data presented herein, strain KIN24-T80^T represents a novel species, for which the name *Methanocaldococcus villosus* sp. nov. is proposed. Herewith, we describe the first hyperthermophilic *Methanocaldococcus* species isolated from a shallow submarine hydrothermal system.

Description of *Methanocaldococcus villosus* sp. nov.

Methanocaldococcus villosus (vil.lo'sus. L. masc. adj. *villosus* hairy, rough, shaggy, villous, referring to the numerous flagella that form a dense network or bundle and the extraordinary surface structure in SEM preparations).

Cells are regular to irregular cocci, 1–2 µm in diameter, occurring singly or in pairs. They possess an S-layer with (pseudo-) sixfold symmetry and have an irregular, striated pattern on the cell surface. Highly motile by means of over 50 flagella which can also serve as adherence structures and for formation of cell–cell contacts. Chemolithoautotrophic. Uses solely CO₂ and H₂ as carbon and energy sources for production of methane. Anaerobic. Growth occurs at 55–90 °C (optimum 80 °C), 0.5–5.5% (w/v) NaCl [optimum 2.5% (w/v)] and pH 5.5–7.0 (optimum pH 6.5). Optimal doubling time is 45 min. Growth is stimulated by selenate, tungstate and yeast extract. Cells stain Gram-negative. Colonies are round and pale yellow, about 1–2 mm in diameter, when grown on Phytagel-solidified plates. Cells lyse rapidly in water, hypotonic solution and 0.01% (w/v) SDS. Distinguished from other members of the genus *Methanocaldococcus* by 16S rRNA gene sequence analysis and comparison of whole-cell protein SDS-PAGE patterns.

The type strain is KIN24-T80^T (=DSM 22612^T =JCM 16315^T), isolated from a shallow submarine hydrothermal system at Kolbeinsey Ridge, north of Iceland.

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