Sulfur-inhibited *Thermosphaera aggregans* sp. nov., a new genus of hyperthermophilic archaea isolated after its prediction from environmentally derived 16S rRNA sequences

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Recently, a new procedure was developed which allowed for the first time the isolation of a hyperthermophilic archaeum tracked by 16S rRNA analysis from a terrestrial hot solfataric spring ('Obsidian Pool', Yellowstone National Park, WY, USA). This novel isolate is characterized here. Cells are round cocci with a diameter of 0.2-0.8 μm, occurring singly, in pairs, short chains and in grape-like aggregates. The aggregates exhibit a weak bluish-green fluorescence under UV radiation at 420 nm. The new isolate is an anaerobic obligate heterotroph, using preferentially yeast extract for growth. The metabolic products include CO₂, H₂, acetate and isovalerate. Growth is observed between 65 and 90 °C (optimum: 85 °C), from pH 5.0 to 7.0 (optimum: 6.5) and up to 0.7% NaCl. The apparent activation energy for growth is about 149 kJ mol⁻¹. Elemental sulfur or hydrogen inhibits growth. The core lipids consist mainly of acyclic and cyclic glycerol diphytanyl tetraethers. The cell envelope contains a cytoplasmic membrane covered by an amorphous layer of unknown composition; there is no evidence for a regularly arrayed surface-layer protein. The G+C content is 46 mol%. On the basis of 16S rRNA sequence comparisons in combination with morphological, physiological and biochemical properties, the isolate represents a new genus within the *Desulfurococcaceae*, which has been named *Thermosphaera*. The type species is *Thermosphaera aggregans*, the type strain is isolate M11TL (= DSM 11486).

**Keywords:** *Thermosphaera aggregans* sp. nov., archaea, hyperthermophile, 16S rRNA sequences

**INTRODUCTION**

Members of the *Desulfurococcaceae* are hyperthermophilic, anaerobic, coccoïd to disk-shaped cells, belonging to the *Crenarchaeota* branch within the *Archaea* domain. They grow either chemolithoautotrophically by sulfur reduction or strictly heterotrophically by sulfur respiration or fermentation (12, 46, 52, 54, 55). The family *Desulfurococcaceae* includes the genera 'Thermodiscus', 'Igneococcus', *Desulfurococcus* and *Staphylothermus* (12). Along with the type species *Desulfurococcus mucosus* (55), the genus *Desulfurococcus* (12, 55) also includes *Desulfurococcus mobilis* (55), 'Desulfurococcus saccharovorans' (48), 'Desulfurococcus amylolyticus' (9) and two as-yet-unnamed isolates S and SY (29). Members of *Desulfurococcus* were found in continental solfataric fields (9, 49, 55) and hydrothermal vent sites (29). The genus *Staphylothermus* is currently only represented by the type species *Staphylothermus marinus* (15), which has been isolated from both shallow and abyssal marine hydrothermal systems (15).

Cells of *Desulfurococcus* are coccoid and occur singly and in pairs (55), whereas *Staphylothermus* grows mainly in grape-like aggregates (15). They thrive under anaerobic conditions preferentially on complex organic substrates such as yeast extract, meat extract, bacterial and archaeal cell extracts, peptone, casein, starch or glycogen (9, 15, 19, 55). As metabolic...
products, CO₂, H₂, acetate, isovalerate, isobutyrate and H₂S (in sulfur-containing media) were identified (15, 19, 55). Members of the genus *Desulfitooccus* are not strictly sulfur-dependent but growth is stimulated by sulfur (9, 55). On the other hand, *Staphylothermus marinus* is strictly sulfur-dependent when grown as pure culture (15). However, in the absence of sulfur, it can be grown in syntrophic culture together with H₂-consuming organisms (10). In contrast, inhibition of growth by sulfur was found recently in the hyperthermophiles *Pyrobaculum aerophilum* (51) and *Pyrolobus fumarii* (6), both growing either by microaerophilic or by anaerobic nitrate respiration.

A great phylogenetic variety of cultured hyperthermophiles is already known (47). However, *in situ* 16S rRNA gene sequence analyses of samples from ‘Obsidian Pool’ (Yellowstone National Park, Wy, USA) revealed many unknown archaeal sequences, indicating the existence of many as-yet-uncultivated species (3, 4). Recently, a new procedure was developed, which allowed for the first time the isolation of a hyperthermophilic archaeum (M11TL³) from this natural environment by the combination of *in situ* 16S rRNA analyses, specific cell hybridization within enrichment cultures and ‘selected cell isolation’ by the use of a laser microscope (‘optical tweezers’; 4, 21). In an independent approach, Kane et al. (30) enriched and isolated a sulfate-reducing bacterium from an anaerobic laboratory bioreactor, originally identified as a molecular isolate phylogenetically related to *Desulfovibrio vulgaris*.

Here we characterize the novel isolate M11TL³, the 16S rRNA sequence of which is identical to that of clone pSL91, known from ‘Obsidian Pool’ (4). It represents a new genus within the *Desulfitooccus* type species of which we name *Thermosphaera aggregans*.

**METHODS**

**Collection of samples, enrichment and isolation.** Sampling, enrichment and isolation of the new organism was described recently by Huber et al. (21).

**Strains and culture conditions.** For stock maintenance and experimental studies, the new isolate M11TL³ was cultivated in a medium containing the following components (1-l): 2.2 g MgCl₂.6 H₂O, 1.0 g NaHCO₃, 0.9 g NaCl, 17 mg KCl, 12.5 mg NH₄Cl, 7.0 mg CaCl₂, 2 H₂O, 7.0 mg K₂HPO₄, 3 H₂O, 0.05 mg Fe(III)Cl₃, 0.1 ml vitamin solution (2), 1.0 g Bacto yeast extract (Difco), 1.0 g Bacto peptone (Difco), 0.02 mg resazurine. The medium was adjusted to 6.5 with HCl. Strictly anaerobic culture media were prepared according to the anaerobic technique of Balch & Wolfe (1). Unless stated otherwise, the gas phase consisted of 300 kPa N₂/CO₂ (80:20). Isolate M11TL³ was routinely grown at 85 °C and approximately 60 r.p.m. in 28 ml serum tubes (borate silicate glass, Schott) containing 10 ml medium. For the determination of pH dependence of growth, cultures were grown in 100 ml flasks (Schott) in the presence of 200 kPa N₂. When organic compounds other than yeast extract or peptone were tested as substrates, the yeast extract and peptone concentration was reduced to 0.005% (w/v). To determine the optimal NaCl concentration for growth, only the medium NaCl was varied, while the concentrations of all other salts remained the same.

**Determination of growth.** Cell densities were determined by direct cell counting using a Thoma chamber (depth 0.02 mm).

**Effect of temperature on growth rate.** Influence of temperature on growth was assayed by the quantitative determination of the whole cell protein concentration as described (34, 42). For this experiment, M11TL³ was grown at different temperatures in 1 l culture bottles containing 250 ml medium. During growth, 20 ml samples were taken at different time intervals and the cells were collected by centrifugation (Beckman J2-21 centrifuge, 30 min, 4 °C; rotor: JA20). To solubilize the cells, the centrifuged cell sediment was resuspended in 100 μl SDS (5%) followed by incubation at 100 °C for 15 min. For protein determination, 100 μl solubilized cell material was incubated with 500 μl reagent A' and 4 ml reagent B of the Bio Rad DC Protein assay (Bio Rad). The protein concentration was determined spectrophotometrically (750 nm; Beckman). A BSA standard containing 0.03-1.5 mg protein ml⁻¹ was used. The protein concentration was used to determine the respective growth rate constant k. The logarithm of k was plotted against the reciprocal of the absolute temperature and the apparent activation energy for growth was calculated from an Arrhenius plot (18).

**Light and electron microscopy.** Cells were observed with a Zeiss standard phase-contrast microscope (Oberkochen) equipped with an oil-immersion objective (x100/1.3). Micrographs were taken on a Zeiss Axiophot 35 inverted phase-contrast microscope with a Olympus OM3 camera. To bring the cells into a horizontal position, the organisms were allowed to settle down onto the cover slide for approximately 30 min. Electron microscopy was carried out as described (24). For ultrathin sections, cells, fixed with 1% glutaraldehyde in the culture medium, were freeze-substituted in diethyl ether (containing molecular sieve, 0.4 mm).

**Detection of metabolic products.** Acetate was assayed by HPLC (25), H₂ and CO₂ by GC according to the methods of Huber et al. (26, 28). Methane was determined with a Hewlett-Packard 5890 gas chromatograph equipped with a Molecular Sieve 5A column (Supelco) and a flame ionization detector (oven temperature: 140 °C; detector and injector temperature: 170 °C). H₂S was detected qualitatively by addition of 20 μl saturated lead acetate solution to 0.5 ml samples. A dark brown precipitate demonstrated its presence.

Following growth on heat-treated xylan (121 °C, 20 min), the reducing sugars formed by M11TL³ were determined by the 3,5-dinitrosalicylic acid (DNSA) method, according to Miller (38). DNSA solution (750 μl) was added to a 500 μl sample, incubated for 20 min at 90 °C, and the colour reaction was measured spectrophotometrically at 595 nm.

**Lipid analysis.** Lipids were extracted from cell mass as described by Huber et al. (25). The core lipids were identified by TLC (50). As reference substances, extracted core lipids of *Thermococcus stetteri* (39) and *Pyrobaculum organotrophi* (23) were used.

**DNA isolation and base composition.** DNA was isolated as described by Lauerer et al. (33). The G+C content was
Thermosphaera aggregans sp. nov.

Results

Morphology

Cells of the isolate M11TL\textsuperscript{T} were regular cocci. During the exponential growth phase, they grew predominantly in grape-like aggregates consisting of five to several hundred individuals. Under the UV microscope at 420 nm, these aggregates exhibited a weak bluish-green fluorescence which faded rapidly under UV radiation. The aggregates with diameters up to 10 \( \mu \)m could not be disintegrated into single cells by supersonic treatment, mechanical stirring, enzyme treatment (trypsin, proteinase K, cellulase) or periodic acid (0.7 \%, w/v). In the late-logarithmic and stationary growth phases, smaller aggregates, single cells, duplex forms and short chains with up to four cocci were visible (Figs 1, 2a). The diameter of the cells varied between 0.2 and 0.8 \( \mu \)m. Morphology was influenced by culture conditions: at the upper temperature limit of growth (90 \(^\circ\)C) or in the presence of sulfur (e.g. 0.05 \%, final concen), single cells up to 3.5 \( \mu \)m in diameter containing one or two dark granules became visible.

Grape-like cell aggregates possessed several flagella (Fig. 2a), single cells possessed up to eight flagella. Ultrathin sections of freeze-substituted cells revealed a cytoplasmic membrane (4 nm), plus an amorphous layer, approximately 10–15 nm thick (Fig. 2b). In a micrograph of a freeze-etched cell aggregate, no regular lattice, indicative of a surface-layer protein, was detected (Fig. 2c). The fracture face revealed a fine granular cytoplasm (Fig. 2c).

Physiological characterization

M11TL\textsuperscript{T} grew from 67 to 90 \(^\circ\)C with an optimal doubling time of 110 min at 85 \(^\circ\)C (Fig. 3). No growth occurred at 65 or 92 \(^\circ\)C. The Arrhenius plot yielded a straight line between growth temperatures of 75 and 85 \(^\circ\)C. The apparent activation energy for growth calculated from the mid-zone was about 149 kJ mol\(^{-1}\).

Growth of isolate M11TL\textsuperscript{T} occurred between pH 5.0 and 7.0 with an optimum of 6.5. No growth was observed at pH 4.5 or 7.5. M11TL\textsuperscript{T} grew when between 0 and 0.7 \% NaCl was added to the medium. To adapt cultures to NaCl concentrations above 0.4 \%, the NaCl concentration was increased stepwise by 0.1 \% during each culture transfer. Growth was optimal in the absence of exogenous NaCl.

Metabolism

In closed serum tubes, isolate M11TL\textsuperscript{T} grew strictly heterotrophically with yeast extract (final concentration 0.1 \%, w/v) and peptone (0.1 \%) under anaerobic culture conditions up to cell titres of about 2 \times 10^8 cells ml\(^{-1}\). With yeast extract as single substrate, titres of about 1 \times 10^8 cells ml\(^{-1}\) were obtained. Maximal cell densities of M11TL\textsuperscript{T} with up to 4 \times 10^8 cells ml\(^{-1}\) were obtained by flushing exponentially growing cultures periodically with N\(_2\)/CO\(_2\) (80:20) to remove hydrogen, a product of fermentation (see below) that is a potent growth inhibitor for this species. No growth occurred, when cultures were pressurized with hydrogen-containing gas (200 kPa N\(_2\)/CO\(_2\) = 80:20 + 100 kPa H\(_2\)/CO\(_2\) = 80:20). This inhibition was reversible, for growth resumed after gas exchange (300 kPa N\(_2\)/CO\(_2\) = 80:20).

No growth was observed on native xylan (final concentration 0.2 \%, w/v) or xylose (0.1 \%). However, growth took place on heat-treated xylan (0.2 \%) with cell densities of about 7 \times 10^7 cells ml\(^{-1}\). After growth, an increase of reducing sugars in the culture medium was detected. Furthermore, growth was observed on gelatin (0.2 \%), on a defined mixture of amino acids (0.04 \%) and on glucose (0.1 \%), yielding cell densities of only 2–5 \times 10^7 cells ml\(^{-1}\). No growth occurred on meat extract, bovine heart infusion, peptone, amylose, glycogen, cellulose, cellobiose, maltose, raffinose, pyruvate and acetate (final concentration 0.1 \%, w/v).
Elemental sulfur was a potent inhibitor of growth. Even at the very low concentration of 0.05% (w/v), only about 10% of the final cell titres were determined compared to cultures without sulfur. When 0.05% sulfur was added to exponentially growing cultures, cell growth stopped. During further incubation, H$_2$S
was formed in large amounts and cells began to lyse. Similar observations have been reported for methanogens and hyperthermophilic sulfate-reducers (13, 56). Sulfide did not inhibit growth of M11TL\textsuperscript{T}, when added to the medium in the same concentration as sulfur.

When M11TL\textsuperscript{T} was cultivated on yeast extract and peptone, isovalerate, acetate, CO\textsubscript{2} and H\textsubscript{2} were identified as fermentation products.

Lipid composition

Thin-layer chromatograms of the core lipids of M11TL\textsuperscript{T} showed mainly acyclic (R\textsubscript{f} value 0.43) and cyclic glycerol diphytanyl tetraethers with one to four pentacyclic rings (R\textsubscript{f} values 0.4, 0.39, 0.37, 0.35, respectively). Traces of glycerol diphytanyl diethers (R\textsubscript{f} 0.75) were detected in addition.

DNA base composition, DNA–DNA hybridization and 16S rRNA phylogeny

The G+C content of isolate M11TL\textsuperscript{T} was 46 mol%, as determined by melting point analysis (36).

In DNA–DNA hybridization studies, DNA of isolate M11TL\textsuperscript{T} showed no hybridization signal with the isolated DNA of Staphylothermus marinus and Desulfurococcus mobilis.

By 16S rRNA sequence comparisons, isolate M11TL\textsuperscript{T} was identified as a member of the Crenarchaeota within the domain Archaea. The closest relatives of M11TL\textsuperscript{T} are members of the genera Desulfurococcus and Staphylothermus, as indicated by an estimated exchange of 3.5 and 3.9 nucleotides per 100 positions, respectively (12). Fig. 4 shows the phylogenetic relationship of M11TL\textsuperscript{T} with other members of the Crenarchaeota.

DISCUSSION

Based on 16S rRNA sequence comparisons, the novel isolate M11TL\textsuperscript{T} was identified as a member of the Crenarchaeota. Within this branch, M11TL\textsuperscript{T} was clearly affiliated to the Desulfurococcaceae with closest relationship to Staphylothermus and Desulfurococcus (12, 15, 55). However, M11TL\textsuperscript{T} differs in several important phenotypical properties from Staphylothermus and Desulfurococcus. A considerable difference to these genera is obvious with respect to the utilization of sulfur. In contrast to the sulfur-respiring Desulfurococcus and Staphylothermus, growth of M11TL\textsuperscript{T} is prevented in the presence of elemental sulfur. This sulfur inhibition was unexpected, because all other genera within the Desulfurococcaceae respire sulfur (12). However, within other main phylogenetic branches of the Crenarchaeota, the type of metabolism also varies. Within the otherwise strictly anaerobic,
sulfur-respiring Thermoproteales and Pyrodictiaceae (12), Pyrobaculum aerophilum (51) and Pyrolobus fumarii (6) utilize aerobic or anaerobic respiration with nitrate. Furthermore, Stygilobus azoricus (45), a strictly anaerobic member of the Sulfolobales, is the closest relative of the strict aerobe Sulfolobus acidocaldarius (8). In view of these unexpected properties, it is not always justified to infer the metabolic type based upon the metabolic properties of the phylogenetic relatives.

In contrast to Desulfurococcus, M11TL grows in large, grape-like cell aggregates similar to Staphylothermus. However, cell aggregates of M11TL exhibit a bluish-green fluorescence at 420 nm. The source of this fluorescence is unknown. A unique feature of M11TL is evident in its cell architecture, with no surface-layer protein being detectable: there is no evidence for a regular surface lattice like that in Desulfurococcus or any other members of the Crenarchaeota (12), nor for a structure like the tetrapyrrhon of Staphylothermus (Fig. 2c; 41, 53).

M11TL is also unique in the composition and structural features of its core lipids. In contrast to Staphylothermus and Desulfurococcus, where glycerol diphtyanyl diether- and acyclic glycerol diphtyanyl tetraether lipids have been identified, acyclic tetraethers and cyclic tetraethers with one to four pentacyclic rings are the predominant lipids in M11TL (14, 15). The occurrence of cyclic tetraether lipids in members of the Desulfurococcaceae was so far unknown and emphasizes again the uniqueness of M11TL within this family.

The evolutionary distances between isolate M11TL and its nearest relatives Staphylothermus marinus and Desulfurococcus mobilis are 3-9 and 3-5%, respectively (12). This divergence is similar to that between the genera Staphylothermus and Desulfurococcus or Pyrobaculum and Thermoproteus (12), which indicates that isolate M11TL represents a new genus. The significance of these differences is also evident in the branching of the 16S rRNA phylogenetic tree, which strengthens this criterion (Fig. 4) and is also reflected in hybridization studies by the lack of significant DNA homology.

Therefore, on the basis of 16S rRNA sequence comparisons as the main criterion, in combination with unique morphological, physiological and biochemical properties, we propose that M11TL represents a new genus, which we describe here as Thermosphaera ('the hot sphere'). The type species is Thermosphaera aggregans, type strain Thermosphaera aggregans, isolate M11TL (DSM 11486).

Growth of Thermosphaera aggregans occurs within a temperature range of 25°C similar to many hyperthermophilic archaea (5). From 75 to 85°C, Thermosphaera aggregans grows within the Arrhenius relation with an activation energy of 149 kJ mol⁻¹, which is significantly higher than that described for mesophilic and psychrophilic bacteria (17, 27).

Recently, we were able to isolate additional hyperthermophiles from the ‘Obsidian Pool’ (22); their presence was predicted from extraction and sequencing of DNA from this site (3, 4). One of the isolates is S10TFL (sequence identical to clone sequence pJP6; 3, 12), a strict anaerobe, which grows preferentially on archael cell extracts (22). S10TFL and Thermosphaera aggregans use different biopolymers for growth, and therefore together they act as degraders of organic matter in their biotope. Thermosphaera aggregans produces isovalerate, acetate, hydrogen and CO₂, and can provide growth substrates for other hyperthermophiles present in the ‘Obsidian Pool’. One of these consumers is a new isolate S10L (sequence identical to clone sequence pSL23; 4), which represents a novel, hyperthermophilic sulfate-reducer (22).

**Description of Thermosphaera gen. nov.**

Thermosphaera (Thermosp.ahe'ra. Gr. fem. n. therme, heat; L. fem. n. sphæra, sphere; L. fem.n. Thermospha'era, the hot sphere).

Cells are cocci, occurring singly, in pairs, in short chains and in aggregates. No spores formed. Cell envelope consists of a cytoplasmic membrane with an amorphous layer on top. Membrane contains acyclic and cyclic glycerol diphtyanyl glycerol tetraethers. Growth up to 90°C and up to 0.7% NaCl. Anaerobic. Strictly heterotrophic. Growth by fermentation. Sulfur or hydrogen inhibits growth. DNA base composition 46 mol% G+C. No significant DNA homology with Staphylothermus marinus and Desulfurococcus mobilis.

By 16S rRNA analysis, Thermosphaera represents a new genus belonging to the Desulfurococcaceae. Habitat: solfataric hot spring.

The type species is Thermosphaera aggregans.

**Description of Thermosphaera aggregans sp. nov.**

Thermosphaera aggregans (ag'gre.gans. L. v. aggregare to aggregate, referring to the ability of the cells to form grape-like aggregates).

Cells are cocci from 0.2 to 0.8 µm in diameter, occurring singly, in pairs, in short chains and in grape-like aggregates. During growth, aggregates up to 10 µm with several hundred individuals visible. Cell aggregates exhibit a bluish-green fluorescence at 420 nm. Spores are not formed. Flagella present. Cell envelope composed of a cytoplasmic membrane (width: 4 nm), covered by an amorphous layer. 16-15 nm thick. No evidence for a regular arrayed surface-layer protein. Core lipids consist mainly of acyclic and cyclic glycerol diphtyanyl glycerol tetraethers with one to four pentacyclic rings. Growth between 67 and 90°C (optimum: 85°C), pH 5.0-7.0 (optimum: 6.5) and 0-0.7% NaCl (optimum: 0%). Optimal doubling time 110 min. Anaerobic. Heterotrophic growth with yeast extract. Peptone stimulates growth. CO₂, H₂, acetate and isovalerate formed as metabolic products. Growth inhibition by sulfur or hydrogen. G+C content 46 mol%. 39 and 35%
evolutionary distance to *Staphylococcus marinus* and *Desulfurococcus mobilis*, respectively.

The type strain is isolate M11TL\(^T\), DSM 11486, Braunschweig, Germany (isolated from ‘Obsidian Pool’ in the Mud Volcano area of Yellowstone National Park, WY, USA).

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