**Sulfurisphaera ohwakuensis gen. nov., sp. nov., a novel extremely thermophilic acidophile of the order Sulfolobales**

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Three spherical thermoacidophilic archaea (strains TA-1T, TA-13, TA-14) were obtained from acidic hot springs located in Ohwaku Valley, Hakone, Japan. All the isolates are facultatively anaerobic, and grew optimally at around 85 °C, pH 2.0. Isolate TA-1T was characterized further. The G+C content of DNA from TA-1T is 33 mol%. Although these properties resemble those of the genus Acidianus, the sequence of the 16S rRNA gene from strain TA-1T was more similar to that of species of Stygiolobus than of Acidianus. DNA-DNA hybridization experiments also indicated that strain TA-1T is clearly distinguished phylogenetically from the members of Acidianus, Sulfolobus and Metallosphaera. On the basis of the distinct physiological and molecular properties, we describe the new strains as members of the new genus Sulfurisphaera. The type species of the genus is Sulfurisphaera ohwakuensis, and the type strain of the species is TA-1T (=IFO 15161T).

Keywords: Sulfurisphaera, Sulfolobales, archaea, 16S rRNA, facultative anaerobe

INTRODUCTION

In the sulfur-dependent thermoacidophilic archaea, four genera have been assigned to the order Sulfolobales: (i) Sulfolobus, which is the type genus and includes the species Sulfolobus acidocaldarius (Brock et al., 1972), Sulfolobus solfataricus (Zillig et al., 1980), Sulfolobus shibatae (Grogan et al., 1990), Sulfolobus metallicus (Huber & Stetter, 1991), and Sulfolobus hakonensis (Takayanagi et al., 1996); (ii) Metallosphaera, which includes the species Metallosphaera sedula (Huber et al., 1989) and Metallosphaera prunae (Fuchs et al., 1995); (iii) Acidianus, which includes the species Acidianus brierleyi (Brierley & Brierley, 1973; Segerer et al., 1986; Zillig et al., 1980), Acidianus infernus (Segerer et al., 1986), and Acidianus ambivalens (Fuchs et al., 1996; Zillig et al., 1986); and (iv) Stygiolobus, with one species, Stygiolobus azoricus (Segerer et al., 1991). All members of the order Sulfolobales are acidophilic cocci. Sulfolobus and Metallosphaera are obligate aerobes. In contrast, Acidianus spp. are facultative anaerobes. Stygiolobus is an obligate anaerobe.

Two species from Japan, Sulfolobus shibatae and Sulfolobus kakovensis, have been formally described. During recent attempts to isolate additional thermoacidophilic archaean species from hot springs in Hakone, Japan, three spherical thermoacidophilic archaea (strains TA-1T, TA-13 and TA-14) were obtained. In this paper, we describe the isolation and characterization of these strains of thermoacidophilic archaea belonging to a novel genus of the order Sulfolobales.

METHODS

**Bacterial strains.** Strains TA-1T, TA-13 and TA-14 were obtained from muddy water at three locations in the acidic hot springs (70–80°C, pH 3) of Ohwaku Valley, Hakone, Japan. The samples were transported at ambient temperature, and cultured at 70 or 80 °C in TA medium, which we routinely use for enrichment. TA medium contains (g l−1 in parentheses): (NH₄)₂SO₄ (1.3), KH₂PO₄ (0.25), MgSO₄, 7H₂O (0.25), CaCl₂, 2H₂O (0.08), and yeast extract (1.0; Difco Laboratories). Prior to autoclaving, the pH was adjusted to 3.0 with 13% (w/v) H₂SO₄ at room temperature. Isolates were purified by isolation of single colonies on TA plates [TA medium solidified by 0.6% gellan gum (Merck)
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at 70 °C and pH 3.0. *Sulfobculus acidocaldarius* ATCC33909 was obtained from the American Type Culture Collection. *Sulfobculus solfataricus* DSM 1616, *Methanopyra sedula* DSM 5348, *Acidianus brierleyi* DSM 1651, *Acidianus infernus* DSM 3191, *Acidianus ambivalens* DSM 3772 and *Sulfolobus azoricus* DSM 6296 were obtained from the DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen.

**Growth conditions.** Isolates TA-1, TA-13 and TA-14 were grown in modified Brock’s basal salts mixture (Brock *et al.*, 1979) supplemented with 0.2 l-l, pH 2.5. *A. infernus* was cultured in modified Brock’s basal salts mixture supplemented with 0.2 g yeast extract l-l at 80 °C. The pH of the media was adjusted by the addition of 10 N HCl or 1 M NaOH and measured by a 

**Anaerobic media were prepared using the technique of**

- *Acidianus armbivalens* DSM 6296T were obtained from the American Type Culture Collection. *Acidianus brierleyi* was grown at 70 °C in DSM 150 medium (DSMZ catalogue of strains, 1993) (g l-1 in parentheses): (NH₄)₂SO₄ (30), KH₂PO₄ (0.5), MgSO₄, 7H₂O (0.05), NaMoO₄. 2H₂O (0.03), VO(OH)₂, 2H₂O (0.03), CoSO₄ (0.01), supplemented with 1.0 g yeast extract l-1, pH 2.5 at 80 °C. *Sulfobculus acidocaldarius, A. infernus* and *M. sedula* were grown in the same medium, at 80, 88 and 70 °C, respectively. *A. ambivalens* was grown at 80 °C in modified Brock’s basal salts mixture supplemented with 0.2 g yeast extract and about 0.5 g elemental sulfur (S) l-¹, pH 2.5.

**Growth was determined by direct cell counting using a Thoma counting chamber.** A good correlation was found between these two methods in exponential and stationary phases. The OD₆0₀ of 1 corresponds to a cell density of about 1x10⁶ cells ml⁻¹. The doubling times were calculated from the slopes of the growth curves. Anaerobic media were prepared using the technique of* Balch et al. (1979). Modified Brock’s basal salts mixture supplemented with S (1 g l-¹) was reduced by the addition of sodium sulfide (0.5 g l-¹) and adjusted to pH 2.5 with 10% H₂SO₄. Resazurin (1 mg l-¹) served as an O₂ indicator. Medium, 5 ml, was distributed into pressure-culture-tubes (18 mm diameter x 180 mm; SANSHIN) and pressurized with H₂ gas. A 1% inoculum of a dense standing culture of loosely capped glass tubes or long-necked conical flasks with stirring. Growth was determined by direct cell counting by using a Thoma counting chamber. A good correlation was found between these two methods in exponential and stationary phases. The OD₆₀₀ of 1 corresponds to a cell density of about 1x10⁶ cells ml⁻¹. The doubling times were calculated from the slopes of the growth curves. Anaerobic media were prepared using the technique of* Balch et al. (1979). Modified Brock’s basal salts mixture supplemented with S (1 g l-¹) was reduced by the addition of sodium sulfide (0.5 g l-¹) and adjusted to pH 2.5 with 10% H₂SO₄. Resazurin (1 mg l-¹) served as an O₂ indicator. Medium, 5 ml, was distributed into pressure-culture-tubes (18 mm diameter x 180 mm; SANSHIN) and pressurized with H₂ gas. A 1% inoculum of a dense culture of TA-1 (41 x 10⁶ cells ml⁻¹) was added by a syringe. The cultures were incubated at 80 °C without shaking. Growth was determined by direct cell counting using a Thoma counting chamber.

**Electrophoretic mobility.** The sections of TA-1 were prepared by rapid-freezing and substitution fixation as previously described (Dempsey & Bullivant, 1976) and examined using a JEOL 100S electron microscope at 80 kV. Strain TA-1, negatively stained with 2% phosphotungstic acid, was observed with a JEOL 2000EX electron microscope at 200 kV.

**Gram staining.** Gram-staining test of cells in the exponential phase was carried out using the modified Höcker’s method (Kruczak-Filipov & Shively, 1992). *Escherichia coli* LE392 and *Bacillus subtilis* 162 were used for negative and positive controls of the staining, respectively.

**Lipid analysis.** Total cellular lipids were extracted from stationary-phase cells as described previously (Sugai *et al.*, 1995) and analysed by TLC and a Shimadzu FTIR-8200 spectrophotometer. Cyclic tetraether core lipids were prepared by acid methanolysis of total cellular lipids as described in the same report (Sugai *et al.*, 1995). These core lipids were applied to TLC plates (silica gel 60 HPTLC; Merck) and were chromatographed by two ascending runs with the following solvent systems: 1st step, chloroform/methanol/water [7:5:2.5:2 by vol. (up. to 2 cm from the origin)]; 2nd step, hexane/diethyl ether/acetic acid (60: 40: 2 by vol.).

**DNA base composition.** The G+C contents of DNA were determined by reversed-phase HPLC of the DNA samples digested with nuclease P1 (Tamaoka & Komagata, 1984). The mean value was obtained from three independent experiments.

**DNA–DNA hybridization.** Levels of genomic DNA relatedness were determined by DNA–DNA slot-blot hybridization experiments. Four sets of aliquots containing 40 ng each genomic DNA were denatured by boiling for 10 min in 0.4 M NaOH and were transferred onto Hybond-N+ membrane (Amersham). Genomic DNAs were sheared by sonication to 500–2000 bp and used as probes. Labelling of probes, hybridization and detection of signals were performed using an ECL direct nuclease acid labelling and detection system (Amersham) according to the manufacturer’s instructions. The intensities of the signals were measured with a densitometer (CS-9000; Shimadzu).

**Cloning and sequencing of the 16S rRNA genes.** A 5.2 kbp EcoRI–XbaI fragment of TA-1* genome DNA, which contained the 16S rRNA gene, was cloned from a genomic DNA library constructed on plasmid Bluescript SK(−) (Stratagene) using standard methods (Sambrook *et al.*, 1989). The almost full-length 16S rRNA genes of *A. infernus, A. ambivalens* and *Sulfolobus azoricus* were amplified by PCR using the crenarchaeal universal primers 16S5 (5'-ATTCGGTTGATCCTG; positions 1–16; *Sulfolobus acidocaldarius* numbering) and 16S3A (5'-TGAGTTGA-TCCAGCC; complement of positions 1479–1493) with chromosomal DNAs as templates. These PCR primers were chosen by inspection of previously published sequences of the 16S rRNA gene from *Sulfolobus acidocaldarius* (Kurosawa & Itoh, 1993), *Sulfolobus solfataricus* (Kurosawa *et al.*, 1995), *A. brierleyi* (Kurosawa *et al.*, 1995), and *M. sedula* (Kurosawa *et al.*, 1995). PCR amplifications were performed in 100 μl reaction mixtures containing 10 mM Tris/HCl (pH 8-9), 80 mM KCl, 1.5 mM MgCl₂, 0.4% BSA, 0.1% sodium cholate, 0.1% Triton X-100, 200 μM each dNTP, 10 pmol each primer, 5 U Tth DNA polymerase (Toyobo), and template DNA. The following thermal cycle was used for 20 cycles: 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1.5 min.

To determine the almost full-length rDNA sequences (about 1490 bp), smaller fragments of PCR products were sub-
cloned into plasmid Bluescript SK(−) or KS(−) (Stratagene). DNA sequence analysis was performed on both strands and carried out by the dideoxynucleotide chain termination method with Texas-red-labelled primers using an automated DNA sequencer (SQ-5500; Hitachi). Bca-best DNA polymerase (Takara Shuzo) was used, and dGTP was replaced with dITP in all four sequencing mixtures to avoid "peak compression".

**Phylogenetic analysis.** The previously published sequences of the 16S rRNA genes (reference and DNA database accession no. in parentheses) from *Sulfolobus acidocaldarius* 98-3 (Kurosawa & Itoh, 1993; D14876), *Sulfolobus solfataricus* P1 (Kurosawa et al., 1995; D26490). *A. brierlei* DSM 1651 (Kurosawa et al., 1995; D26489), *M. sedula* TH2 (Kurosawa et al., 1995; D26491), and *Sulfolobus shibatae* B12 (Grogan et al., 1990; M32504) were included in the analysis. Sequences from TA-1T and eight type strains belonging to the order *Sulfolobales* were aligned with five outgroup species; *Pyrodictium occultum* (Stetter et al., 1983; M21087) of the order *Pyrodictiales*, *Desulfurococcus mobilis* (Zillig et al., 1982; M36474), *Thermophilum pendens* (Zillig et al., 1983a; X14835) and *Thermoproteus tenax* (Zillig et al., 1981; M35966) of the order *Thermoproteales*, and *Thermococcus celer* (Zillig et al., 1983b; M21529) of the order *Thermococcales*. All sites with gaps in any sequences and the regions of the PCR primers were removed from alignment. Pairwise distances between all sequences were estimated by the Kimura's two-parameter method (Kimura, 1980). The percentage similarities within the 16S rRNA gene sequences were estimated by using GENETYX-MAC software (Software Development). Distances and percentage similarities between known species of *Sulfolobales* and TA-13 or TA-14 were calculated from partial sequences (positions 19-669; *Sulfolobus acidocaldarius* numbering) of their 16S rRNA genes. Phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1987) and maximum-likelihood methods (Felsenstein, 1981) using 1409 positions. To test the stability of the phylogenetic trees, the sequence data were sampled 1000 times for bootstrap analysis. These analyses were performed by using the CLUSTAL V program developed by Dr. D. Higgins of the EMBL and the PHYLIP program (Felsenstein, 1993).

**RESULTS AND DISCUSSION**

Supernatants of muddy water (70–80 °C, pH 3) from three locations in the acidic hot springs in Ohwaku Valley, Hakone, were inoculated into 10 ml TA medium and incubated at 70 or 80 °C. After 3 d, all three samples produced turbid cultures at both temperatures. A portion (0-1 ml) of each turbid culture grown at 80 °C, was spread onto gellan gum plates. Colonies appeared after 8 d incubation and were grown for one more day. The colonies were replated to purify the organisms. Three stable cultures, which were derived from different samples, were named TA-1T, TA-13 and TA-14. Preliminary analysis of the partial sequences of the 16S rRNA genes showed that these three isolates possessed over 99% sequence similarity with one another and were closely related. However, they only possessed 87–94% sequence similarity with 16S rRNA genes from the type strains of the species belonging to the order *Sulfolobales*. We studied strain TA-1T in detail for characterization and taxonomic classification of these isolates.

Under the phase-contrast microscope, cells of isolate TA-1T appeared as round to slightly irregular cocci about 1 μm in width. The diameter of the cells was determined by electron microscopy, and varied from 1.2 to 1.5 μm when determined by negative staining and by thin sections, respectively (Fig. 1). The thin sections of TA-1T revealed an envelope 24 nm thick surrounding the cell membrane. This value was almost identical to the corresponding values for *A. infernus* (Segeret et al., 1986), *Stygiolobus azoricus* (Segeret et al., 1991), *M. sedula* (Huber et al., 1989) and *Sulfolobus acidocaldarius* (our observation). A coat was observed outside of the envelope, but pili- or flagella-like structures were not found. Colonies of TA-1T on the gellan gum plates were smooth, roundly convex, and slightly yellow. The cells stained Gram-negative.

The major core lipids of thermoacidophilic archaea of the order *Sulfolobales* are caldarchaeol (glycerol-dialkyl-glycerol tetraethers) and calditoglycerocaldar-
TA-1T grew between pH 1.0 and 5.0, and the optimum pH was 2.0. The doubling time of TA-1T was 5.9 h under optimal conditions (Fig. 2). TA-1T grew on pH values (b).

**Fig. 2.** Growth rates of TA-1T at various temperatures (a) and pH values (b).

The temperature range for growth of TA-1T was from 63–92 °C, and the temperature optimum was 84 °C. TA-1T grew between pH 1.0 and 5.0, and the optimum pH was 2.0. The doubling time of TA-1T was 5.9 h under optimal conditions (Fig. 2). TA-1T grew on proteinaceous, complex substrates such as yeast extract or tryptone (final cell density was $3.4 \times 10^8$ ml$^{-1}$).

Growth was not observed on the following single sugars or amino acids: D-glucose, D-galactose, D-fructose, D-xylene, lactose, maltose, sucrose, alanine, glutamate, glycine or histidine. Aerobic cultures stored at $-80$ °C served as inocula for at least 10 months.

Within the order *Sulfolobales*, *Acidianus* species are facultatively anaerobic organisms that grow by either oxidation or reduction of elemental sulfur, forming $H_2SO_4$ and $H_2S$, respectively. To test the ability of strain TA-1T to grow anaerobically, it was inoculated into the reduced mineral medium (initial cell density $4.1 \times 10^6$ ml$^{-1}$) and cultivated under anaerobic conditions. *Sulfolobus acidocaldarius* failed to grow under these conditions (details in Methods). After 4 d, turbidity was visible in cultures of TA-1T. After 6 d, the cell density was $7.8 \times 10^6$ ml$^{-1}$. Anaerobic growth was not observed in the absence of $S^0$. Addition of yeast extract (0.2 g l$^{-1}$) to anaerobic media yielded better growth (final cell density $2.6 \times 10^7$ ml$^{-1}$) than in the absence of yeast extract. This result suggested that TA-1T is also able to grow organotrophically under anaerobic conditions.

The G+C content of TA-1T was $32.9 \pm 0.8$ mol%. This value is almost identical to corresponding values of *A. infernus* (Huber *et al.*, 1989) and *A. ambivalens* (Zillig *et al.*, 1986) and slightly higher than that of *A. brierleyi* (30%) (Huber *et al.*, 1989). The facultative anaerobic growth properties and mol% of G+C content of TA-1T were characteristic of *Acidianus* species.

To analyse the phylogenetic position of TA-1T, the 16S rRNA gene sequences from the isolate TA-1T, *A. infernus*, *A. ambivalens* and *Stygiolobus azoricus* were determined. The 16S rRNA gene of TA-1T showed 87.1, 90.4 and 90.2% sequence similarity with the genes from *A. brierleyi*, *A. infernus* and *A. ambivalens*, respectively. These similarity values are lower than those between TA-1T and *Sulfolobus acidocaldarius* or *Stygiolobus azoricus* (about 94%). No significant similarity was observed with other type strains analysed. We constructed phylogenetic trees by the neighbour-joining method (Fig. 3) and maximum-likelihood method (not shown). On both phylogenetic trees, TA-1T, TA-13 and TA-14 formed a new cluster which was clearly independent of the previously described species of the order *Sulfolobales* with 100% bootstrap probabilities.

DNA–DNA hybridization experiments also demonstrated that TA-1T had 1% or lower hybridization values with *A. brierleyi* and *A. infernus* (data not shown). These molecular phylogenetic data indicate that the isolate TA-1T is clearly distinguished from previously described species of the genus *Acidianus*. Facultative anaerobic isolates of the order *Sulfolobales* which exhibit 30–33 mol% G+C content have been classified into the genus *Acidianus* until now. However, TA-1T should be classified into a new genus based on...
**Description of Sulfurisphaera ohwakuensis gen. nov.**

*Sulfurisphaera ohwakuensis* (oh.wa.ku.en'sis. M.L. adj. *ohwakuensis* from the Ohwaku Valley, referring to the place of isolation).

Exponentially growing cells are 1.2–1.5 μm in diameter. Thin sections of cells reveal an envelope about 24 nm wide covering the cell membrane. Facultatively anaerobic. TA-1 is able to grow organotrophically on yeast extract or peptone. Cells grow at 63–92 °C, with optimal growth occurring at 84 °C. The pH range for growth is 1.0–5.0, with optimal growth around pH 2.0. The doubling time was 5.9 h under optimal conditions. The G+C content of DNA of the type strain is 32.9 mol%. Isolate obtained from muddy water of an acidic hot spring of Ohwaku Valley, Hakone, Japan. The type strain is isolate TA-1T (=IFO 15161T).

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