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***Sulfolobus tengchongensis* sp. nov., a novel thermoacidophilic archaeon isolated from a hot spring in Tengchong, China**

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Abstract A novel thermoacidophilic strain, designated RT8-4, was isolated from an acidic hot spring in Tengchong, Yunnan, China, and characterized phenotypically and phylogenetically. Cells of strain RT8-4 are irregular cocci with peritrichous flagella. The strain grows aerobically in either a lithotrophic or a heterotrophic mode. No anaerobic growth is apparent. Growth on elemental sulfur occurs through the oxidation of sulfur. Strain RT8-4 is capable of utilizing tryptone, D-xylose, D-arabinose, D-galactose, maltose, sucrose, D-fructose, or L-glutamic acid as the sole source of carbon. D-Glucose and D-mannose are not utilized. RT8-4 grows optimally at 85 °C and pH 3.5. The G + C content of the genome of RT8-4 is 34.4 mol%. Phylogenetic analysis based on 16S rDNA sequence as well as DNA–DNA hybridization and phenotypic characterization identifies strain RT8-4 as a novel species in the genus *Sulfolobus*. It is proposed that strain RT8-4 be designated as *Sulfolobus tengchongensis* sp. nov. The type strain is RT8-4^T.

Keywords Archaea · Crenarchaeote · Phylogeny · *Sulfolobus tengchongensis* · Thermoacidophile

Introduction

Archaea of the genus *Sulfolobus*, a group of sulfur-oxidizing inhabitants of geothermal springs, are the most thermophilic aerobes currently known. Seven *Sulfolobus* species have been isolated so far from various geother-

mal sites around the world (Brock et al. 1972; Zillig et al. 1980; Grogan et al. 1990; Huber and Stetter 1991; Takayanagi et al. 1996; Jan et al. 1999; Suzuki et al. 2002). Since their initial description, these organisms have served as a preferred subject for the study of novel features in Archaea (Ciaramella et al. 2002). For instance, analysis of the transcription in *Sulfolobus* species provided the first evidence that Archaea are more closely related to Eukarya than to Bacteria in genetic mechanisms (Bell et al. 2001). The widespread presence of plasmids and viruses in *Sulfolobus* offers an apparent advantage in employing *Sulfolobus* as a model for genetic analysis (Zillig et al. 1998). In addition, these genetic elements are potentially useful in vector development. Research on *Sulfolobus* has recently gained a new momentum after the genomes of *Sulfolobus solfataricus* P2 and *Sulfolobus tokodaii* strain 7 were completely sequenced (Kawarabayashi et al. 2001; She et al. 2001). Efforts are being made to isolate and characterize new *Sulfolobus* species and their extrachromosomal genetic elements.

In this article we report the isolation and characterization of a novel *Sulfolobus* species. The strain was isolated from a sulfur-rich hot spring in Tengchong, which is located in the southwestern province of Yunnan, China, and denoted RT8-4. RT8-4 was characterized phenotypically and phylogenetically. Based on its phenotypic properties, 16S rDNA sequence, G + C content in genome, and DNA–DNA hybridization results, RT8-4 represents a new species in the genus *Sulfolobus*. We name the species *Sulfolobus tengchongensis*.

Materials and methods

Strains and growth conditions

Sulfolobus shibatae ATCC 51178^T (= DSM 5389^T) was obtained from the American Type Culture Collection. *Sulfolobus solfataricus* DSM 1616^T was a generous gift of K. Stedman (Portland, USA). Unless otherwise specified, type strains and isolated strains were grown aerobically in Brock's medium (Brock et al. 1972) supple-

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mented with 0.2% tryptone at 80 °C and initial pH of approximately 3.0–3.5 (at 22 °C).

Isolation of strains

Samples were collected from hot springs and mud holes with temperatures ranging from 61 to 94 °C and pH from 2.0 to 6.0 in a major solfataric field in Tengchong, Yunnan, China, and transported in sterile 50-ml centrifuge tubes at ambient temperature. Aliquots of the water sample (2–5 ml) or mud sample (1–2 g) were inoculated into Brock's medium (100 ml) in 250-ml serum bottles. After incubation at 80 °C with shaking (140 rpm) for 7–10 days, growth became apparent. Samples of the cultures were spread onto 0.8% gelrite plates containing Brock's medium (Zillig et al. 1994). Following incubation for 7 days at 80 °C, isolated colonies were picked, transferred to liquid Brock's medium, and purified by replating. The purity of the isolate was verified by colony and cell morphology examination.

Nutritional requirements

Cells were grown to the early stationary phase in Brock's medium supplemented with 0.2% tryptone and harvested by centrifugation (5,000 g, 15 min, 22 °C). The pelleted cells were washed with un-supplemented Brock's medium to avoid nutrient carryover. The washed cells were inoculated into 100 ml Brock's medium containing each of the tested sugars or amino acids at a concentration of 0.1% (wt/vol) in a 250-ml serum bottle. The cultures were incubated aerobically for 7 days at 80 °C. The cell densities of the cultures were measured at OD₆₀₀. To test the ability of the isolates to grow autotrophically on sulfur-containing inorganic compounds, cells were cultivated in Brock's medium supplemented with 0.1% (wt/vol) elemental sulfur in the presence of 5% CO₂ in head space.

G + C content

The genomic DNA was isolated from strain RT8-4^T by the method of Robb and Place (1995) and purified by CsCl density gradient centrifugation (Sambrook et al. 1989). The purified DNA was dissolved in 0.15 M NaCl and 0.015 M sodium citrate (pH 7.0). The T_m value of the sample was measured in a spectrophotometer (Beckman Coulter DU 800) (Marmur and Doty 1962). The G + C content was calculated using the following formula: T_m = 69.3 + 0.41 × (G + C).

DNA–DNA hybridization

The genomic DNA from RT8-4^T was fragmented by sonication. The DNA fragments (100 ng) were labeled with [α -³²P]dCTP by using a random primer DNA labeling kit (TaKaRa). Unincorporated nucleotides were removed by using a MicroSpin G-25 column (Amersham Pharmacia). Samples (5 µg) of DNA to be tested were briefly sonicated, denatured, and immobilized onto a Hybond-N+ membrane (Amersham Pharmacia). Hybridization was carried out at 68 °C for 20 h as described previously (Sambrook et al. 1989). After hybridization, the membrane was washed for 5 min at 22 °C in 2×SSC (Sambrook et al. 1989) and 0.5% SDS, 15 min at 22 °C in 2×SSC and 0.1% SDS, 60 min at 37 °C in 0.1×SSC and 0.5% SDS, and 60 min at 68 °C in 0.1×SSC and 0.5% SDS. Radioactivity retained on the membrane was determined by using a PhosphorImager (Molecular Dynamics).

16S rDNA sequencing and phylogenetic analysis

The 16S rDNA sequence of RT8-4^T was amplified from the genomic DNA by using the following pair of archaeal-specific

primers: 5'-CTCCGGTTGATCCTGCC (forward primer)/5'-GGCTACCTTGTTACGACTT (reverse primer) (Rice et al. 2001). The 1.4-kb PCR product was sequenced directly. The sequence has been deposited in GenBank (GenBank accession number: AY135637). To construct a phylogenetic tree, relevant 16S rDNA sequences were retrieved from GenBank and aligned using the program ClustalW. Phylogenetic analysis was performed using the program package Phylip 3.5c (Felsenstein 1989). The phylogenetic tree was constructed by the distance matrix method. Evolutionary distances, calculated by the Kimura's two-parameter model program, were used to construct the phylogenetic tree by the neighbor-joining method. Evaluation of the tree was performed by the bootstrap method with 1,000 replicates.

Electron microscopy

Cells in the early stationary phase were stained with 2% (wt/vol) uranyl acetate and observed under an electron microscope (Hitachi H-600A).

Results and discussion

Morphology

Cells of strain RT8-4^T were irregular cocci with a diameter of approximately 1.0–1.2 µm (Fig. 1). Colonies of the strain on Brock's medium solidified with 0.8% gelrite appeared following 5–7 days of incubation at 80 °C, and were smooth and flat in shape and dark yellow in color. Long and curved flagella (approximately 10 nm in diameter) were observed. Flagellar motility is a property shared by *Sulfolobus* species (Grogan 1989; Faguy et al. 1996; She et al. 2001).

Growth

Strain RT8-4^T grew well in Brock's medium supplemented with 0.2% tryptone under aerobic conditions. To determine the optimal growth temperature for the



Fig. 1 Electron micrograph of an RT8-4^T cell. The sample was negatively stained with uranyl acetate and viewed under an electron microscope. Bar 1 µm

strain, we cultivated the culture (100 ml) in a 250-ml serum bottle with shaking (140 rpm) at various temperatures from 65 to 95 °C with the initial pH adjusted to 3.0, and determined the maximal growth rate of the strain at each temperature. RT8-4^T grew fastest at 85–90 °C and was capable of slow growth at 95 °C (Fig. 2A). Therefore, RT8-4^T has a higher optimal temperature than other *Sulfolobus* species described so far. We also determined the maximal growth rate of RT8-4^T at 80 °C at various pH values ranging from 1.7 to 6.5, and found that the strain grew optimally at pH 3.5 (Fig. 2B). Both temperature and pH optima for the growth of RT8-4^T are characteristic of *Sulfolobus*.

Strain RT8-4^T was able to grow aerobically in Brock's medium containing elemental sulfur, and the growth resulted in the acidification of the medium. To test if the RT8-4^T was able to grow anaerobically, we

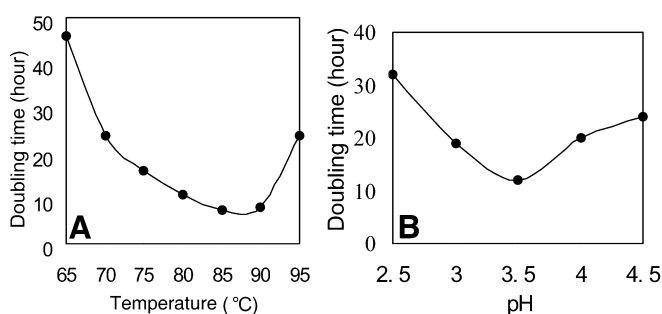


Fig. 2A, B Effects of temperature and pH on the growth rate of strain RT8-4^T. **A** Cells were grown aerobically in Brock's medium supplemented with 0.2% tryptone at the indicated temperatures. Growth was measured in a spectrophotometer. The doubling time in the exponential phase was calculated. **B** Cells were grown aerobically at 80 °C in Brock's medium with pH adjusted to the indicated values. The doubling time for the strain was determined as described above

incubated the strain in Brock's medium containing 0.2% tryptone or sulfur in an atmosphere of H₂/CO₂ (80:20). No growth was apparent under either conditions. Therefore, RT8-4^T is unable to grow anaerobically in either a heterotrophic or an autotrophic mode.

Nutrient utilization

Sixteen compounds were tested for suitability as sole sources of carbon and energy for strain RT8-4^T (Table 1). The results show multiple differences between RT8-4^T and the described *Sulfolobus* species in the utilization of carbon sources. RT8-4^T grew well on D-arabinose and D-xylose, but failed to grow on D-glucose and D-mannose. By comparison, both hexoses are readily utilized by *Sulfolobus shibatae* DSM 5389^T, *Sulfolobus solfataricus* DSM 1616^T, *Sulfolobus acidocaldarius* DSM 639^T, and *Sulfolobus yangmingensis* YM1^T. On the other hand, the two pentoses are poor carbon sources for *S. acidocaldarius* DSM 639^T. D-fructose was used by RT8-4^T, but D-rhamnose and D-sorbitol were not. Among the tested disaccharides, lactose could not serve as the sole carbon source for RT8-4^T, a property which distinguishes RT8-4^T from *S. shibatae* DSM 5389^T and *S. solfataricus* DSM 1616^T. RT8-4^T was also able to utilize L-glutamic acid but not L-aspartic acid as the only carbon source.

G + C content

Sulfolobus species are a group of archaea with a low G + C content in their genome. The melting temperature of the genomic DNA of strain RT8-4^T was determined to be 83.4 °C. This T_m value corresponds to a

Table 1 Utilization of compounds as sole carbon and energy sources. Strains were incubated aerobically at 80 °C for 7 days in Brock's medium supplemented with each of the tested compounds at a concentration of 0.1% (wt/vol). Cell densities were measured in a spectrophotometer at 600 nm. The amount of growth was expressed as the ratio of A₆₀₀ after 7 days of incubation to A₆₀₀ before incubation: –, a ratio of 1 or less; ±, a ratio between 1 and 2; +, a ratio greater than 2; NA not available. Data for strains other than RT8-4^T were taken from Takayanagi et al. (1996) (*S. shibatae*, *S. solfataricus*, and *S. acidocaldarius*) and Jan et al. (1999) (*S. yangmingensis*)

	Strain RT8-4 ^T	<i>S. shibatae</i> DSM 5389 ^T	<i>S. solfataricus</i> DSM 1616 ^T	<i>S. acidocaldarius</i> DSM 639 ^T	<i>S. yangmingensis</i> YM1 ^T
Sulfur compounds					
Elemental S	+	+	+	+	+
FeS	NA	NA	+	+	+
Carbohydrates					
D-Arabinose	+	+	+	±	+
D-Xylose	+	NA	±	–	+
D-Galactose	+	+	+	–	+
D-Glucose	–	+	+	+	+
D-Mannose	–	+	+	+	+
D-Rhamnose	–	+	–	–	+
Lactose	–	+	+	–	+
Maltose	+	+	+	+	+
Sucrose	+	+	+	+	+
D-sorbitol	–	–	NA	NA	NA
D-fructose	+	+	NA	NA	NA
Raffinose	NA	+	+	+	+
Amino acids					
L-Aspartic acid	–	–	±	±	+
L-Glutamic acid	+	+	+	+	+
Tryptone	+	+	+	+	+
Yeast extract	+	+	+	+	+

34.4 mol% G+C content in the DNA. The G+C content of the RT8-4^T DNA is close to that reported for *S. shibatae* DSM 5389^T (34.6 mol%) and is within the range of G+C contents for the genus *Sulfolobus* (34–42 mol%) (Grogan et al. 1990; Huber and Stetter 2001).

Phylogenetic analysis

Sequence analysis showed that the 16S rDNA of strain RT8-4^T was most closely related to those of *S. shibatae* DSM 5389^T (94.72% identity) and *S. solfataricus* DSM 1616^T (94.65% identity), and more distantly related to those of other characterized *Sulfolobus* species (<90% identity). This result is supported by the phylogenetic analysis on the basis of 16S rDNA sequences. As revealed by the phylogenetic tree (Fig. 3), constructed from the evolutionary distances by using the neighbor-joining method, the order *Sulfolobales* represents a monophyletic group within the Crenarchaeota kingdom of the Archaea domain, and is composed of two clusters

with a high bootstrap value (999 out of 1,000 replicates), one containing *Acidianus* and *Metallosphaera* and the other consisting of *Sulfolobus* and *Stygiolobus* (Suzuki et al. 2002). The topology of the neighbor-joining tree agrees well with the phylogenetic tree constructed by the parsimony method (data not shown). *Sulfolobus* species are found in several branches within *Sulfolobales*, suggesting that they do not belong phylogenetically to a single genus (Fuchs et al. 1996). In the phylogenetic tree, strain RT8-4^T forms a branch with *S. shibatae* and *S. solfataricus* with a bootstrap value of 997 in 1,000 replicates.

DNA–DNA hybridization

DNA–DNA hybridization experiments suggested low relatedness of strain RT8-4^T with *S. shibatae* DSM 5389^T and *S. solfataricus* DSM 1616^T (Table 2). The relatedness between RT8-4^T and each of the two described *Sulfolobus* species is lower than that between the

Fig. 3 A phylogenetic tree for the *Sulfolobales*. The tree was constructed on the basis of the alignments of the 16S rDNA sequences of selected archaea by the neighbor-joining method. The sequences from the euryarchaeotes are used as the outgroups in the phylogenetic analysis. Numbers denote the bootstrap values obtained with 1,000 replicates

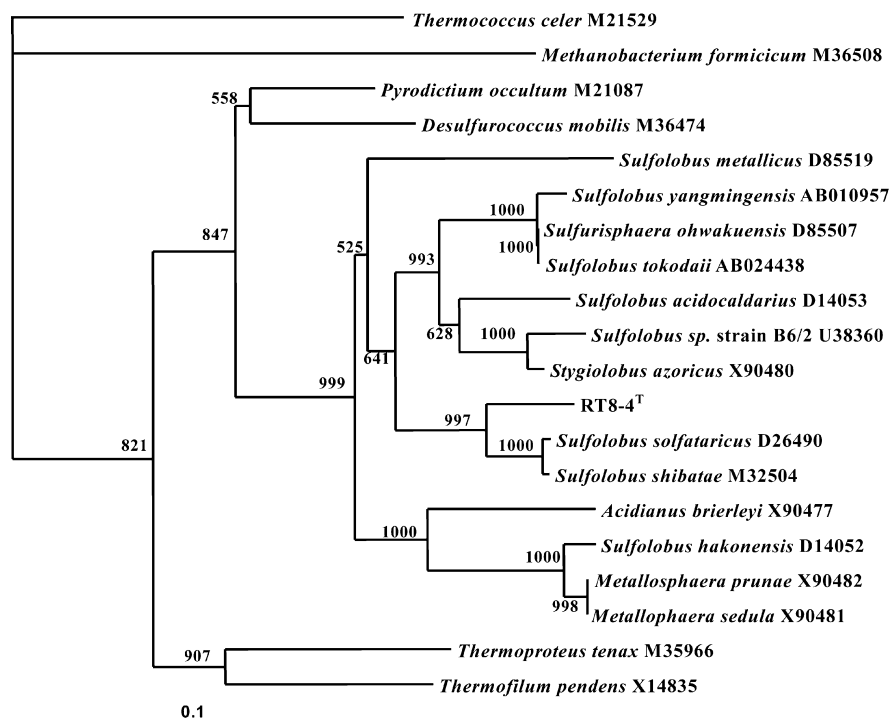


Table 2 DNA–DNA relatedness between strain RT8-4^T, *S. solfataricus* DSM 1616^T and *S. shibatae* DSM 5389^T. Retention of a labeled DNA by a filter bound by the DNA of the same source is designated as 100%. Each value is an average of three independent measurements and expressed as a mean ± SD

Source of filter-bound DNA	Percent retention of the labeled DNA of the following source by an indicated DNA-bound filter		
	Strain RT8-4 ^T	<i>S. shibatae</i> DSM 5389 ^T	<i>S. solfataricus</i> DSM 1616 ^T
Strain RT8-4 ^T	100	< 1	< 1
<i>S. shibatae</i> DSM 5389 ^T	1 ± 1	100	6.7 ± 3
<i>S. solfataricus</i> DSM 1616 ^T	1 ± 1	4.7 ± 2	100

latter two *Sulfolobus* species. These data indicate that RT8-4^T does not belong to either *S. shibatae* or *S. solfataricus*. Crosshybridization between the *S. shibatae* DSM 5389^T DNA and the *S. solfataricus* DSM 1616^T DNA was approximately 5–7% of each set of self hybridization. This number is lower than that reported previously (26%; Grogan et al. 1990). The discrepancy probably arose from the use of different hybridization methods in the two studies.

In conclusion, strain RT8-4^T possesses many phenotypic properties characteristic of the genus *Sulfolobus*. These include acidothermophily, highly irregular cell shape, and ability to grow lithotrophically via oxidation of elemental sulfur. However, the strain differs from other known *Sulfolobus* species in nutrient utilization and DNA relatedness. Phylogenetic analysis based on 16S rDNA sequences groups the strain with and distinguishes it from *S. shibatae* DSM 5389^T and *S. solfataricus* DSM 1616^T in the order *Sulfolobales*. Therefore, we conclude that RT8-4^T represents a new species of *Sulfolobus* and propose the name *Sulfolobus tengchongensis* for this species. The type strain is RT8-4^T.

Description of *Sulfolobus tengchongensis* sp. nov.

Sulfolobus tengchongensis (teng.chong.en'sis, M. L. adj. *tengchongensis*, pertaining to Tengchong, Yunnan, China, where the organism was isolated). Cells are irregular cocci with a diameter of 1.0–1.2 µm. Peritrichous flagella (approximately 10 nm in diameter) are long and curved. Colonies are dark yellow, smooth and flat.

Cells grow optimally on Brock's medium supplemented with tryptone. D-Arabinose, D-xylose, D-galactose, D-fructose, sucrose, and L-glutamic acid can be utilized as the sole source of carbon and energy. D-Glucose, D-mannose, rhamnose, D-sorbitol, lactose, and L-aspartic acid are not utilized. Growth also occurs on Brock's medium supplemented with elemental sulfur under aerobic conditions. No anaerobic growth is apparent under either heterotrophic or autotrophic growth conditions. Under aerobic and chemoheterotrophic growth conditions, cells grow at temperatures from 65 to 95 °C, and optimal growth occurs at 85 °C. Cells grow at pH 1.7–6.5, and optimal growth occurs at pH 3.5.

The G + C content of the genome of *S. tengchongensis* strain RT8-4^T is 34.4 mol%, as calculated from a T_m value of 83.4 °C for the DNA of the strain. The 16S rDNA of the strain shares 94.72% and 94.65% sequence identity with those of *S. shibatae* DSM 5389^T and *S. solfataricus* DSM 1616^T, respectively.

The type strain is *Sulfolobus tengchongensis* RT8-4^T, which has been deposited with China General Microbiological Culture Collection Center as strain AS1.3345.

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