Metallosphaera cuprina sp. nov., an acidothermophilic, metal-mobilizing archaeon

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A novel acidothermophilic archaeon, strain Ar-4^T, was isolated from a sulfuric hot spring in Tengchong, Yunnan, China. Cells of strain Ar-4^T were Gram-staining-negative, irregular cocci and motile by means of flagella. Strain Ar-4^T grew over a temperature range of 55–75 °C (optimum, 65 °C), a pH range of 2.5–5.5 (optimum, pH 3.5) and a NaCl concentration range of 0–1 % (w/v). The novel strain was aerobic and facultatively chemolithoautotrophic. The strain could extract metal ions from sulfidic ore. It was also able to oxidize reduced sulfur compounds. In addition, it was able to use heterogeneous organic materials for organotrophic growth. The main cellular lipids were calditoglycerocaldarchaeol (CGTE) and caldarchaeol (DGTE). The DNA G + C content of the strain was 40.2 mol%. Analysis of 16S rRNA gene sequences showed that strain Ar-4^T was phylogenetically related to members of the genus *Metallosphaera* and had sequence similarities of 97.7 %, 97.0 % and 96.8 % with *Metallosphaera hakonensis* DSM 7519^T, *Metallosphaera sedula* DSM 5348^T and *Metallosphaera prunae* DSM 10039^T, respectively. Strain Ar-4^T showed DNA–DNA relatedness values of 47.5 %, 30.8 % and 29.1 % with *M. hakonensis* DSM 7519^T, *M. sedula* DSM 5348^T and *M. prunae* DSM 10039^T, respectively. The differences in cell motility, the temperature and pH ranges for growth, the ability to utilize carbon sources, the DNA G + C content, and the low DNA–DNA relatedness values distinguished strain Ar-4^T from recognized species of the genus *Metallosphaera*. On the basis of these results, it was concluded that strain Ar-4^T represents a novel species of the genus *Metallosphaera*, for which the name *Metallosphaera cuprina* is proposed. The type strain is Ar-4^T (=JCM 15769^T=CGMCC 1.7082^T).

Extremely acidothermophilic *Archaea* are thought to be important micro-organisms in the evolutionary history of Earth. They have been found in acidic hot springs, water holes and mud holes that contain elemental sulfur and soils within continental solfataric fields (Brierley & Brierley, 1973; de Rosa *et al.*, 1975). Representatives of the genera *Sulfolobus* (Brock *et al.*, 1972; Zillig *et al.*, 1980), *Acidianus* (Seeger *et al.*, 1986) and *Metallosphaera* (Takayanagi *et al.*, 1996) have been isolated from a large number of these habitats (Huber & Prangishvili, 2006). The genus *Metallosphaera* was first proposed by Huber *et al.* (1989) based on the physiological and DNA–DNA relatedness of metal-mobilizing strains isolated from a solfataric field. The type species of the genus is *Metallosphaera sedula*. Currently, the genus *Metallosphaera* contains three species

with validly published names (Huber *et al.*, 1989; Fuchs *et al.*, 1995; Kurosawa *et al.*, 2003). Members of this genus grow aerobically at low pH values and high temperatures in the presence of elemental sulfur. In this paper, a novel species of the genus *Metallosphaera* is described that was isolated from the muddy water of a sulfuric hot spring in Yunnan province, China.

Muddy water samples were collected from the edge of the hot springs (temperature and pH ranges were 55–80 °C and 4.5–5.5, respectively) of Tengchong county (N 24° 38′–25° 52′, E 98° 05′–98° 46′), Yunnan province, China. Samples were transferred to the laboratory within 3 days without temperature control and were processed immediately. Enrichment of acidothermophilic sulfur-oxidizers was performed in 250 ml Erlenmeyer flasks with 100 ml basal salt medium (BSM) (Chen *et al.*, 2007), which was modified by the addition of 1 ml trace element solution (containing per litre distilled water: 1.1 g FeCl₃·6H₂O, 0.05 g CuSO₄·5H₂O, 0.2 g H₃BO₃, 0.2 g MnSO₄·H₂O, 0.08 g Na₂MoO₄·2H₂O, 0.06 g CoCl₂·6H₂O and 0.09 g ZnSO₄·7H₂O). The final pH was adjusted to 2.5 with 5 mol H₂SO₄ l⁻¹. Elemental sulfur

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Two supplementary figures are available with the online version of this paper.

(5 g l⁻¹), sterilized by steaming for 3 h on three consecutive days, was added for chemolithoautotrophic culture. Cultures were incubated at 65 °C for 6 days. After three enrichment steps, the culture was serially diluted (10-fold) in tubes containing 9 ml BSM. Samples of 0.2 ml were spread on BSM Gelrite (0.7%, Difco) plates with potassium tetrathionate as the energy source and the plates were incubated at 65 °C. Picked colonies were repeatedly streaked on plates for further purification. The method of Brock *et al.* (1972) was used to prepare the Gelrite plates. The purity of the clones was checked by 16S rRNA gene sequence analysis and morphological observations performed using a phase-contrast microscope (Axiostar plus, Zeiss).

The growth temperature of the novel strain was examined from 50 °C to 80 °C (with intervals of 5 °C) in BSM broth to which 2 g yeast extract l⁻¹ was added instead of sulfur, at pH 2.5. For determination of the optimum pH, strain Ar-4^T was cultured in the same medium at 65 °C. The pH of the media (from pH 0 to 9.0, at intervals of 0.5 pH units) was adjusted with 5 mol H₂SO₄ l⁻¹ or 5 mol NaOH l⁻¹. Cell growth was determined by measuring the increase of optical density at 520 nm (OD₅₂₀).

Cell morphology and flagella were examined by scanning electron microscopy (Quanta 200; FEI) and transmission electron microscopy (H600; Hitachi). Gram-staining was determined according to the method described by Gerhardt *et al.* (1994). Carbon source utilization and other biochemical characterization tests were performed with modified Allen medium (Allen, 1959; Brock *et al.*, 1972) as described previously (Huber *et al.*, 1989; Fuchs *et al.*, 1995; Takayanagi *et al.*, 1996; Dong & Cai, 2001). Growth of strain Ar-4^T under anaerobic conditions was tested by using H₂ as an electron donor and elemental sulfur as a terminal electron acceptor. To examine chemolithoautotrophic growth, the novel isolate was cultivated in Allen medium modified by the addition of (l⁻¹): 5 g elemental sulfur, 5 mmol K₂S₄O₆, 10 mmol Na₂S₂O₃, 13.9 g FeSO₄·7H₂O, 5 g pyrite or 5 g chalcopyrite. K₂S₄O₆, Na₂S₂O₃ or FeSO₄ were added as filter-sterilized solutions to the sterilized basal Allen medium (121 °C for 20 min). Growth on sulfur, K₂S₄O₆ or Na₂S₂O₃ was determined by examination with phase-contrast microscopy and by a decrease in the pH of the medium. Utilization of FeSO₄, pyrite or chalcopyrite at pH 2.0 was monitored by phase-contrast microscopy and by the detection of increased concentrations of Fe²⁺ and Cu²⁺ in the supernatant of centrifuged cultures, determined spectrophotometrically according to Tamura *et al.* (1974) and Bixian (2006).

For analysis of lipid patterns, strain Ar-4^T, *M. sedula* DSM 5348^T and *Sulfolobus acidocaldarius* NBRC 15157^T [obtained from NITE Biological Research Center (NBRC)] were grown in modified Allen broth at 65 °C (strain Ar-4^T and *M. sedula*) or 70 °C (*S. acidocaldarius*). Total cellular lipids were extracted from stationary-phase cells (approximately 2 g wet weight) with 4.0 ml chloroform/methanol/water

(1:2:0.5, v/v) with sonication and centrifugation. The extraction procedure was repeated three times and supernatants were combined and dried using a rotary evaporator at 40 °C. Residual lipids were dissolved by adding 1 ml chloroform/methanol (2:1, v/v) and 0.1 ml water with thorough mixing. Methanol was then added to the mixture until the solution was clear. The mixture contained crude total lipid. Cyclic tetraether core lipids were prepared by acid methanolysis of total cellular lipids as described by Sugai *et al.* (1995), and were applied to TLC plates (Merck silica gel 60 HPTLC; Merck) that were activated at 110 °C for 2 h before use. The cyclic tetraether core lipids were separated by chromatography with two ascending runs as described by Itoh *et al.* (2001). The spots on the TLC plates were detected by spraying the plate with sulfuric acid/ethanol (1:1 v/v) followed by heating at 120 °C for 5–10 min.

The genomic DNA of strain Ar-4^T and other recognized species of the genus *Metallosphaera* was prepared as described previously (Marmur, 1961). The DNA G+C content was determined by the thermal denaturation method (Marmur & Doty, 1962) using DNA from *Escherichia coli* K-12 as a standard. DNA–DNA hybridization experiments were performed by the thermal denaturation and renaturation method as described by De Ley *et al.* (1970) and modified by Huß *et al.* (1983).

The nearly complete 16S rRNA gene of strain Ar-4^T was amplified with the primers 8aF (5'-TCYGGTTGATC-CTGCC-3') and 1512uR (5'-ACGGHTACCTTGTTACG-ACTT-3') and was sequenced. Alignments of 16S rRNA gene sequences were performed with the CLUSTAL_X program, version 1.64b (Thompson *et al.*, 1997), and positions with insertions or deletions were excluded during calculations. A neighbour-joining phylogenetic tree was constructed using MEGA 3.1 (Saitou & Nei, 1987; Kumar *et al.*, 2004) based on evolutionary distances that were calculated with the Kimura two-parameter model (Kimura, 1980).

Colonies of strain Ar-4^T on modified Allen plates supplemented with potassium tetrathionate were semi-transparent, smooth, rounded and convex with a diameter of 0.2–0.3 mm. Colonies on modified Allen plates supplemented with FeSO₄ and 0.2 g yeast extract l⁻¹ were brown, circular, flat and entire with a diameter of 0.7–0.8 mm. Cells of strain Ar-4^T were Gram-staining-negative, irregular cocci (lobed) with diameters of 0.9–1.0 µm (Fig. 1). Active motility was observed using a light microscope; long and curved flagella were observed with a transmission electron microscope (Fig. 1).

Strain Ar-4^T was strictly aerobic, grew at between 55 and 75 °C and over a pH range of pH 2.5 to 5.5. The optimal pH and temperature for growth were pH 3.5 and 65 °C. The initial pH range for growth on sulfur was between 1.0 and 6.0; the optimum cell density of approximately 8.0 × 10⁷ cells ml⁻¹ occurred at pH 3.5. The final pH of the medium decreased to approximately pH 0.98–2.15. Thus, the novel strain was apparently an extreme acidothermophile.

Fig. 1. Scanning (a) and transmission (b) electron micrographs showing the morphology of cells and flagella of strain Ar-4^T. Bars, 5 µm (a) and 1 µm (b).

Strain Ar-4^T grew chemolithoautotrophically on elemental sulfur, K₂S₄O₆ and FeSO₄. Pyrite and chalcopyrite were oxidized and supported the growth of the strain. On chalcopyrite, strain Ar-4^T mobilized around 10.6 % of the total copper within 12 days (maximum cell densities of approximately 3.0×10^7 cells ml⁻¹). Yeast extract stimulated the growth of strain Ar-4^T. Under anaerobic conditions and in the presence of hydrogen and elemental sulfur, neither growth nor H₂S production occurred. Organotrophic growth occurred on yeast extract, beef extract, peptone, tryptone, Casamino acids, L-tryptophan, L-arabinose, D-xylose and D-glucose. Strain Ar-4^T grew weakly in the presence of 1.0 % L-aspartic acid, L-glutamic acid, L-alanine, sucrose and raffinose; no growth occurred on D-fructose, D-galactose, D-mannose, D-rhamnose, lactose, maltose or other amino acids. Based on these phenotypic studies, strain Ar-4^T showed a range of characteristics that were different from those of recognized species of the genus *Metallosphaera* (Table 1). The optimal growth temperature of strain Ar-4^T was lower and the optimal pH for growth was higher than for other species of the genus *Metallosphaera*. Strain Ar-4^T was motile by means of flagella, but flagella have not been reported for *M. hakonensis* DSM 7519^T or *M. sedula* DSM 5348^T. Other

differences included the ability to assimilate various carbon sources and to oxidize inorganic ferrous iron or sulfuric compounds. In contrast to *M. hakonensis*, strain Ar-4^T grew well on elemental sulfur but weakly on pyrite (or FeS) and could not utilize H₂S as a sulfur source. In addition, strain Ar-4^T utilized more types of sugar (such as D-arabinose, D-xylose and D-glucose) and amino acids than the other three recognized species of the genus *Metallosphaera* (see Table 1), but used fewer kinds of organic carbon resources than most of the members of the genus *Sulfolobus*. The detailed physiological and biochemical characteristics of strain Ar-4^T and properties that distinguish it from its close relatives are given in the species description and shown in Table 1.

The analysis of the core lipids of strain Ar-4^T and type strains of *M. sedula* and *S. acidocaldarius* by TLC revealed that these strains produced identical core lipids (see Supplementary Fig. S1 in IJSEM Online) that were previously identified as calditoglycerocaldarchaeol (CGTE) (Nishihara *et al.*, 1987) and caldarchaeol (DGTE) (Sugai *et al.*, 1995). The DNA G+C content of strain Ar-4^T (40.2 mol%), was slightly lower than those of *M. hakonensis* DSM 7519^T, *M. sedula* DSM 5348^T and *M. prunae* DSM 10039^T, which were 40.5 mol%, 44.4 mol% and 44.4 mol%, respectively, as detected by the thermal denaturation method used in this study. The values were lower than those detected by the HPLC method as reported by Kurosawa *et al.* (2003) (shown in Table 1).

16S rRNA gene sequence analysis revealed that strain Ar-4^T was phylogenetically related to members of the genus *Metallosphaera* (sequence similarity range of 96.8–97.7 %), with the highest similarity (97.7 %) to *M. hakonensis*, and less than 90 % and 88 % similarities to other recognized species of the genera *Acidianus* and *Sulfolobus*, respectively. The neighbour-joining tree (Fig. 2) showed that strain Ar-4^T belonged to the family *Sulfolobaceae* and clustered with species of the genus *Metallosphaera*. This cluster of members of the genus *Metallosphaera* was also topologically supported by the minimal evolution and maximum-parsimony trees (see Supplementary Fig. S2).

The DNA–DNA hybridization values for strain Ar-4^T with *M. hakonensis* DSM 7519^T, *M. sedula* DSM 5348^T and *M. prunae* DSM 10039^T were 47.5 %, 30.8 % and 29.1 %, respectively, which were below the threshold value (70 %) recommended for the delineation of genomic species (Wayne *et al.*, 1987). This indicated that strain Ar-4^T represents a distinct genospecies of the genus *Metallosphaera*.

Based on these phenotypic and phylogenetic studies, it is concluded that strain Ar-4^T represents a novel species of the genus *Metallosphaera*, for which the name *Metallosphaera cuprina* sp. nov., is proposed.

Description of *Metallosphaera cuprina* sp. nov.

Metallosphaera cuprina (cu'pri.na. L. n. *cuprum* copper; L. suff. *-inus -a -um* suffix used with the sense of belonging to;

Table 1. Morphological, physiological and biophysical properties of strain Ar-4^T and some related strains of the family *Sulfolobaceae*

Strains: 1, *M. cuprina* Ar-4^T; 2, *M. hakonensis* DSM 7519^T (data from Takayanagi *et al.*, 1996); 3, *M. sedula* DSM 5348^T (Huber *et al.*, 1989); 4, *M. prunae* DSM 10039^T (Fuchs *et al.*, 1995); 5, *S. acidocaldarius* ATCC 33909^T (Takayanagi *et al.*, 1996; Brock *et al.*, 1972; Zillig *et al.* 1980); 6, *S. solfataricus* DSM 1616^T (Takayanagi *et al.*, 1996; Brock *et al.*, 1972; Zillig *et al.*, 1980); 7, *A. infernus* DSM 3191^T (Seegerer *et al.*, 1986; He *et al.*, 2004; Yoshida *et al.*, 2006); 8, *A. brierleyi* DSM 165^T (Seegerer *et al.*, 1986; He *et al.*, 2004; Yoshida *et al.*, 2006). +, Positive; −, negative; NA, not available; w, weakly positive. Data for taxa 1–3 for the sugar, amino acid and organic substrate experiments were obtained as described by Takayanagi *et al.* (1996). The amount of growth was expressed as the ratio (R) of A₅₂₀ after 6 days of incubation to A₅₂₀ before incubation, as follows: −, R=1; ±, 1 ≤ R ≤ 2; +, 2 ≤ R ≤ 10; ++, R ≥ 10. The tests on the other three species of the genus *Metallosphaera* were run in parallel with strain Ar-4^T. When the results from this study are different from previously reported data or when no other data are available, the results from this study are given in parentheses. Strain Ar-4^T did not utilize L-cysteine, L-glutamine, L-lysine, L-methionine, L-tyrosine, L-valine or L-serine. Other amino acids that were utilized weakly included L-threonine, L-arginine, L-asparagine, L-glycine, L-isoleucine, L-leucine, L-phenylalanine and L-proline. All species were Gram-staining-negative and utilized elemental sulfur.

Characteristic	1	2	3	4	5	6	7	8
Cell shape*	IC	L	IC	L	L	L	IC	IC
Cell diameter (µm)	0.9–1.0	0.9–1.1	1.0	1.0	0.8–1.0	0.8–2.0	0.5–1.5	1–1.5
Flagella	+	−	NA	+	−	−	NA	NA
Motility	+	−	+	+	−	−	−	−
Growth temperature range (°C)	55–75	50–80	50–80	55–80	55–85	55–85	65–96	45–75
Optimum growth temperature	65	70	75	75	70	70	90	70
Growth pH range	2.5–5.5	1.0–4.0	1.0–4.5	1.0–4.5	2.0–4.0	3.5–5.0	1.0–5.5	1.0–6.0
Optimum pH	3.5	3.0	2.5	NA	2.5	4.5	2.0	1.5–2.0
DNA G + C content (mol%)	40.2 ± 0.5†	38.4 ± 1.5†	46.2‡	45	46.0	38.2 ± 1.5†	36	31
Utilization of sulfur sources								
Tetrathionate	+	+	+	−	+	+	NA	NA
H ₂ S	−	+	NA	NA	+	+	NA	NA
Pyrite (or FeS)	+	++	+	+	++	+	NA	NA
Anaerobic with H ₂ and S	−	−	−	−	−	−	+	+
Utilization of sugars								
L-Arabinose	+	−	−	−	±	±	−	−
D-Ribose	−	−	−	−	−	±	−	−
D-Xylose	+	−	−	−	−	±	−	−
D-Fructose	−	−	−	−	±	±	−	−
D-Galactose	−	−	−	−	−	++	−	−
D-Glucose	+	−	−	−	+	++	−	−
D-Mannose	−	−	− (±)	− (±)	++	++	−	−
Lactose	−	−	−	−	−	++	−	−
Maltose	−	± (+)	−	−	+	++	−	−
Sucrose	±	−	−	−	++	+	−	−
Raffinose	±	−	−	−	++	+	−	−
Utilization of amino acids								
L-Aspartic acid	±	−	(±)	(+)	±	±	−	−
L-Glutamic acid	±	± (+)	(±)	(+)	+	w	−	−
L-Tryptophan	+	±	(+)	(±)	+	−	−	−
L-Alanine	±	−	(+)	(+)	NA	NA	−	−
Utilization of organic substrate								
Yeast extract	+	++	+	+	++	++	−	+
Beef extract	+	(+)	+	+	NA	NA	−	+
Peptone	++	(+)	+	+	+	NA	−	+
Tryptone	+	(±)	++ (+)	(+)	+	NA	−	+
Casamino acids	++	(+)	++ (+)	− (+)	+	NA	−	+

*IC, irregular cocci; L, lobed.

†Values given are means with standard deviations from three replications.

‡The value of 38.4 mol% was determined from the CsCl isopycnic centrifugation method (Takayanagi *et al.*, 1996), the value of 46.2 mol% was determined by HPLC (Kurosawa *et al.*, 2003).

Fig. 2. Phylogenetic tree constructed with the neighbour-joining method according to 16S rRNA gene sequence evolutionary distances among strain Ar-4^T and other members of the family Sulfolobaceae. *Pyrodictium occultum* PL-19^T (M21087) was used as the outgroup. GenBank accession numbers are given in parentheses. Numbers represent confidence levels (values >50 % are shown) from 1000 replicate bootstrap samplings. Bar, 0.01 substitutions per nucleotide position.

N.L. fem. adj. *cuprina* belonging to copper, describing its ability to extract copper ions from ores).

Cells are irregular cocci (0.9–1.0 µm), Gram-staining-negative and motile by means of flagella. Clones on modified Allen plates supplemented with potassium tetrathionate are semi-transparent, smooth, rounded and convex with a diameter of 0.2–0.3 mm. Clones on modified Allen plates supplemented with FeSO₄ are brown, circular and flat. Growth occurs in extremely acidothermophilic conditions with growth temperatures from 55 to 75 °C (optimum 65 °C) and at pH 2.5 to 5.5 (optimum pH 3.5). Cells grow well in modified Allen medium containing 0–1 % (w/v) NaCl and growth is inhibited by 2 % (w/v) NaCl. Aerobic, facultatively chemolithoautotrophic growth on elemental sulfur, K₂S₄O₆, FeSO₄, pyrite (or FeS) and chalcopyrite. Chemolithoautotrophic growth is stimulated by yeast extract. Hydrogen sulfide is not produced when grown on elemental sulfur and hydrogen under anaerobic conditions. Organotrophic growth on yeast extract, beef extract, peptone, Casamino acids, L-tryptophan, D-arabinose, D-xylose and D-glucose; weak growth on sucrose, raffinose, L-aspartic acid, L-glutamic acid and L-alanine. The main cellular lipids are calditoglycerocaldarchaeol (CGTE) and caldarchaeol (DGTE). 16S rRNA gene sequence similarity between *M. cuprina* and *M. hakonensis* DSM 7519^T, *M. sedula* DSM 5348^T and *M. prunae* DSM 10039^T is 97.7 %, 97.0 % and 96.8 %, respectively.

The type strain, Ar-4^T (=JCM 15769^T=CGMCC 1.7082^T), was isolated from muddy water samples from a sulfuric hot spring of Tengchong county, Yunnan province, China. The DNA G+C content of the type strain is 40.2 mol%.

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