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Sulfolobus hakonensis sp. nov., a Novel Species of Acidothermophilic Archaeon

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We characterized a microbial strain that was isolated from a hot spring at a geothermal area in Hakone, Japan. This isolate, whose lobed-shaped cells were about 1.0 μm in diameter, was a facultative chemolithoautotroph that required aerobic conditions for growth. The optimum pH was 3.0 (pH range, 1.0 to 4.0), and the optimum temperature was 70°C (temperature range, 50 to 80°C). Lithotrophically, this strain grew on elemental sulfur and reduced sulfur compounds. The G+C content of the genomic DNA was 38.4 mol%. This organism contained calditoglycerocaldarchaeol, which is characteristic of members of the *Sulfolobaceae*. The levels of 16S rRNA sequence similarity between the new isolate and *Sulfolobus acidocaldarius*, *Sulfolobus solfataricus*, and *Sulfolobus shibatae* were less than 89.8%. Unlike *S. acidocaldarius*, *S. solfataricus*, and *S. shibatae*, the new isolate utilized sugars and amino acids poorly as sole carbon sources, and the levels of DNA-DNA hybridization between the new isolate and these *Sulfolobus* species were very low. Phenotypically, the new isolate was also distinct from the obligately lithotrophic organism *Sulfolobus metallicus*. We concluded that the new organism belongs to a new *Sulfolobus* species, for which we propose the name *Sulfolobus hakonensis*.

Extreme acidothermophiles are found in acidic hot springs, water holes, and mud holes that contain elemental sulfur (1, 2, 5). The genus *Sulfolobus*, which belongs to the family *Sulfolobaecae*, contains microorganisms that live in such habitats, and this genus was the first acidothermophilic genus of archaea (previously archaebacteria) described (2, 25, 29, 31). Members of this genus grow aerobically at low pH values and high temperatures in the presence of elemental sulfur (2, 9, 11, 31). Four *Sulfolobus* species have been identified and characterized previously (2, 9, 11, 31). The assignment of species to the genus *Sulfolobus* has been questioned recently (16, 30). *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus*, which are widely used in laboratory experiments, were isolated from hot springs at Yellowstone National Park in the United States and at Pisciarelli, Italy, respectively, and apparently are distributed worldwide (2, 31). As exact characterization of these taxa and taxonomic comparisons between *Sulfolobus* strains found in different places have not been performed, the possibility that the genus *Sulfolobus* includes many more species requires attention.

In this paper we describe a new species of the genus *Sulfolobus* that was isolated from an acidic hot spring at a geothermal area in Hakone, Japan. This species can be distinguished from the previously described *Sulfolobus* species by nutritional, DNA homology, and 16S rRNA sequences characteristics.

MATERIALS AND METHODS

Microbial strains. *S. acidocaldarius* DSM 639^T (T = type strain) and *S. solfataricus* DSM 1616^T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. *Escherichia coli* IAM 1264 was obtained from the IAM Culture Collection, Institute of Molecular and Cellular Biosciences, The University of Tokyo.

Microbial growth and growth media. Our new isolate and the type strains of *S. acidocaldarius* and *S. solfataricus* were cultivated in Allen's medium, as modified by Brock et al. (2) (modified Allen's medium) under aerobic conditions at 70°C. Modified Allen's medium contained basal salts, 0.1% (wt/vol) glucose, and 0.1% (wt/vol) yeast extract; the pH was adjusted to 3.0 at 70°C with 10% H_2SO_4 for most experiments and with HCl for the nutritional experiments. Microbial growth was monitored by measuring A_{520}, and cell numbers were determined by using a Thoma bacterial counting chamber. To examine chemolithoautotrophic growth, the new isolate and the type strains were cultivated in modified Allen's medium without glucose and yeast extract in the presence of 0.1% (wt/vol) elemental sulfur, potassium tetrathionate, or FeS as an energy source. When H_2S was used as an energy source, air containing 0.1% (vol/vol) H_2S was bubbled into modified Allen's medium without glucose and yeast extract at a constant flow rate (30 ml of H_2S per min per 10 ml of culture). To examine anaerobic and chemolithoautotrophic growth, the new isolate and the type strains were cultivated in modified Allen's medium without glucose and yeast extract in the presence of 0.1% (wt/vol) elemental sulfur, 0.001% (wt/vol) resazurin, and 0.1% (wt/vol) sodium sulfate under an H_2-CO_2 (4:1, vol/vol) gas phase. To examine the potential of the new isolate and the type strains to utilize sugars and amino acids as carbon sources, the organisms were cultivated in modified Allen's medium without glucose and yeast extract in the presence of each sugar or amino acid at a concentration of 0.1 or 1.0% (wt/vol). To determine whether 0.1% yeast extract was required, modified Allen's medium without glucose and yeast extract was used as the basal medium.

Isolation of microbial strains. H_2S-containing water was obtained from a hot spring in a geothermal field at Owaku-dani in Hakone National Park (Hakone, Kanagawa, Japan). A 1-ml water sample was inoculated into 10 ml of modified Allen's medium (pH 2.5) and incubated at 70°C. After the culture became turbid (after about 1 week), a portion of the culture (1.0 ml) was transferred into 9.0 ml of fresh medium, and cultivation was continued. When the growth became exponential, portions of the culture were streaked onto 1.0% Gelrite (Difco Laboratories, Detroit, Mich.) plates containing modified Allen's medium, and the plates were incubated at 70°C. An isolated colony was transferred into broth, the culture was incubated, and portions were streaked onto plates as described above. This procedure was repeated once more. The isolate obtained was maintained by transferring it to fresh medium each week.

Electron microscopy. Cells were fixed with 4.0% glutaraldehyde for 8 h and postfixed with 1.0% osmic acid for 4 h. After dehydration in an ethanol series and substitution of ethanol with propylene oxide, the cells were embedded in Epon 812 resin (TAAB, Berkshire, England), and the resin was polymerized at 60°C for 12 h, as described by Kellenberger et al. (13). Thin sections prepared with a ultramicrotome were stained with 10% uranyl acetate and 0.02% lead citrate and observed with a model JEM 100B electron microscope (JEOL, Tokyo, Japan) operated at 80 kV, as described by Kellenberger et al. (13). To observe the cell surface by negative staining, cells were washed one to three times with 20 mM

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Tris-HCl (pH 7.5) and centrifuged at 10,000 × *g* for 1 min and then spread onto a water surface, as described previously (28). Intact or broken cells, cell membrane fragments, and cell ghosts floating on the surface were picked up with carbon-coated grids and stained with 1.0% uranyl acetate; they were then observed by electron microscopy directly or after rotary shadowing with Pt-Pd (4:1) at an angle of tan^1 (1/10), as described by Marmur (19).

Determination of the G+C content. Genomic DNA was isolated from microorganisms as described previously (19). The guanine-plus-cytosine (G+C) content of the DNA was determined by CsCl isopycnic centrifugation by using a type 70PRS rotor (Hitachi Koki Co., Ltd., Hitachi, Japan) at 40,000 rpm for 72 h, as described by De Ley (3). The distribution of DNA in the fraction was determined by the method of Kissane and Robins (15). *E. coli* IAM 1264 DNA (G+C content, 51.6 mol%) was used as the standard in the buoyant density analysis.

Lipid analysis. Total lipids were extracted from wet cells (packed volume, 1 ml) with 4.0 ml of chloroform-methanol-water (1:2:0.5, vol/vol/vol) with sonication. The extraction procedure was repeated three times. After degradation of the total lipids with acid methanolysis as described by Furuya et al. (8), the lipid cores were separated by thin-layer chromatography (TLC) on silica gel plates (type HPTLC; catalog no. 5641; Merck, Rahway, N.J.). The spots on the TLC plates were visualized by spraying the plates with 50% H_2SO_4 and then heating them at 140°C for 10 min. A quantitative analysis of the spots on the TLC plates was performed by a TLC-densitometry procedure by using a chromatoscanner (model CS-930; Shimadzu, Kyoto, Japan).

DNA-DNA hybridization. DNA was isolated from microorganisms as described by Marmur (19). The DNA was labeled with $[^3H]$dTMP by nick translation by using a nick translation kit (Takara Shuzo Co., Ltd., Kyoto, Japan), and 0.1 μg of the resulting labeled, sonicated DNA was the probe used for hybridization. After denaturation of the DNA, 10 μg of unlabeled DNA was immobilized on a nitrocellulose membrane (type HAWP; pore size, 0.45 μm; Millipore, Bedford, Mass.), and DNA-DNA hybridization experiments were performed by the membrane filter method, as described by Suzuki et al. (27).

16S rRNA sequencing. RNA was extracted from microorganisms, and complete sequences of the 16S rRNA were determined by the reverse transcriptase method (17). The following nucleotide primers that were complementary to universally conserved regions were used: TCCGGTTGATCCTGCCGGA (position 10, forward direction), GCGGACGGCTGAGTAACA (position 120, forward direction), CCTATAACGGGTAGGGGCCG (position 290, forward direction), CTACGGGGCGCACCAG (position 350, forward direction), CAGCCGCCGCGGTAATAC (position 520, forward direction), GGTACTCCCGGAGTAGGGGC (position 700, forward direction), GATTAGATACCCTGGTAG (position 800, forward direction), AAACTTAAAGGAATTGGC (position 920, forward direction), GTTAAGTCAGGCAACGAGCG (position 1110, forward direction), CACGCGGGTTACAATGGC (position 1240, forward direction), CCTTGCACACACCGCCCGTC (position 1240, forward direction), TGTTACTCAGCCGTCCGC (position 120, reverse direction), CGGCCCCTACCCGTTATAGG (position 290, reverse direction), CTGGTGCGGCCCCGTAG (position 350, reverse direction), GTATTACCGCGGCGGCTG(position 520, reverse direction), GCCCCTACTCCGGGAGTACC (position 700, reverse direction), CTACCAGGGTATCTAATC (position 800, reverse direction), GCCAATTCCTTTAAGTTT (position 920, reverse direction), CGCTCGTTGCCTGACTTAAC (position 1110, reverse direction), GCCATTGTAACCCGCGTG (position 1240, reverse direction), GACGGGCGGTGTGTGCAAGG (position 1400, reverse direction), and GAGGTGATCCAGCCGCAGG (position 1540, reverse direction) (*E. coli* 16S rRNA numbering). These primers were made with DNA synthesizer (model 380B; Applied Biosystems, Foster City, Calif.). Levels of homology and phylogenetic distances were determined by the method of Kimura (14). A phylogenetic tree was reconstructed by using the algorithm of the neighbor-joining method (22) and the phylogenetic distance values. The statistical significance of the positions of some groups on the tree was reexamined by using the bootstrap method of Felsenstein (7) and 1,000 replicates.

Nucleotide sequence accession numbers. The 16S rRNA nucleotide sequences of strain HO1-1 and *S. acidocaldarius* DSM 639^T have been deposited in the DDBJ, EMBL, and GenBank databases under accession numbers D14052 and D14053, respectively.

RESULTS AND DISCUSSION

Three isolates were obtained from hot spring water containing mud (pH 1.5; temperature, 91.5°C) which was obtained near a point from which H_2S-containing gas was actively gushing out in a lava field at Owaku-dani, Hakone, Japan. Strain HO1-1 was characterized in detail.

Strain HO1-1 did not grow in modified Allen's medium in the absence of glucose and yeast extract, but it did grow when the medium was supplemented with yeast extract under aerobic conditions at a low pH value and high temperature (Fig. 1 and Tables 1 and 2). This strain did not grow under anaerobic conditions with an H_2-CO_2 gas phase in medium supplemented with glucose and yeast extract, regardless of the presence of elemental sulfur (data not shown). It also grew when the elemental sulfur was replaced with other inorganic forms of sulfur (Table 2). Thus, strain HO1-1 is able to obtain energy by oxidizing sulfur.

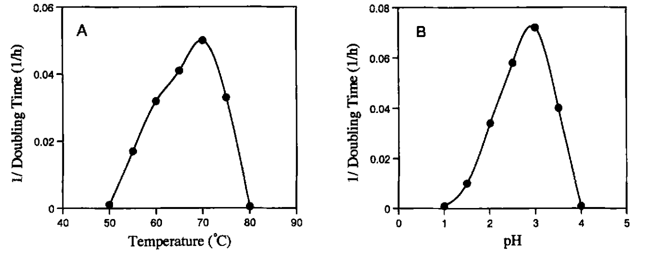

FIG. 1. Temperature (A) and pH (B) requirements for growth of strain HO1-1. Strain HO1-1 was cultivated under the aerobic conditions described in the text.

Strain HO1-1 grew at pH 1.0 to 4.0 and at 50 to 80°C (Fig. 1). The optimal pH and optimal temperature for growth were 3.0 and 70°C, respectively (Fig. 1). Thus, this strain is apparently an extreme thermophiles which is acidophilic.

Strain HO1-1 were spherical or irregular polyhedrons (lobed) with diameters of 0.9 to 1.1 μm and had no flagella; motility was not observed at any phase of growth (Fig. 2A and Table 1). The cells had a plasma membrane that was about 4 nm thick and was surrounded by a layer (cell wall) that was about 21 nm thick (Fig. 2A). The surface of the layer was composed of regularly arranged structures that had a wavy appearance (Fig. 2B). The layer was associated with a network-like structure throughout the surface, and the meshes of the network were equal in size and were located at an angle of 60° with respect to each other at constant intervals, as shown by the negatively stained preparations (Fig. 2C and D).

The characteristics of strain HO1-1, including its lobed-cells, its acidothermophilic mode of life, its aerobic metabolism, and its lithotrophic dependence on sulfur for growth, are consistent with the characteristics of the family *Sulfolobaceae* (25).

An analysis of the structure of the lipid core of strain HO1-1 by TLC revealed that this strain produced two major spots, which corresponded to caldarchaeol (26) and calditoglycerocaldiarchaeol (CGTE) (20) (Fig. 3). We estimated that CGTE accounted for about 80% of the core lipid content by densitometry of the TLC spots. Essentially the same results were obtained for *S. acidocaldarius* and *S. solfataricus* (Fig. 3). The spot corresponding to strain HO1-1 CGTE on the TLC in Fig. 3 was also analyzed by fast atom bombardment-mass spectrometry and positive-ion fast atom bombardment spectroscopy, and the presence of CGTE molecules with various degrees of cyclization was confirmed (detailed chemical structures will be reported elsewhere). CGTE has been found only in members of the *Sulfolobaceae* (4, 6, 18).

The family *Sulfolobaceae* comprises the genera *Sulfolobus*, *Acidianus*, and *Metallosphaera* (4, 10, 23). Recently, it has been suggested that members of the genus *Desulfurolobus* which previously was considered a genus that belongs to the family *Sulfolobaceae*, should be placed in the genus *Acidianus* (24, 32). Strain HO1-1 can be distinguished from the genus *Acidianus* because strain HO1-1 does not grow under anaerobic conditions, while *Acidianus* strains grow under both aerobic and anaerobic conditions (2, 9, 11, 23, 31, 32).

The G+C content of genomic DNA is a useful way to distinguish the genera *Sulfolobus*, *Acidianus*, and *Metallosphaera*, whose G+C contents have been reported to be 34 to 39, 30 to

TABLE 1. Morphological, physiological, and biophysical properties of strain HO1-1 and other *Sulfolobus* strains

Characteristic	Strain HO1-1	*S. acidocaldarius* DSM 639^{T}	*S. solfataricus* DSM 1616^{T}	*S. shibatae* DSM 5389^{T}[a]	*S. metallicus* DSM 6482^{T}[b]
Colony morphology					
Color	Dark yellow	Dark yellow	Grayish	Pale tan	
Shape	Smooth, flat	Smooth, flat	Smooth, flat		
Diam (μm)	0.5–1.0	0.5–1.0	2.0		
Cell shape	Lobed	Lobed	Lobed		Irregular, coccoid
Cell diam (μm)	0.9–1.1	0.8–1.0	0.8–2.0	0.7–1.5	1.5
Flagella	–	–	–		
Motility	–	–	–	Weak	
Cell wall	+	+	+	+	+
Temp range for growth (°C)	50–80	55–85	55–85		50–75
Optimum temp (°C)	70	70	70	81	65
pH range for growth	1.0–4.0	2.0–4.0	2.0–4.0		1.0–3.5
Optimum pH	3.0	2.5	4.5	3.0	
G+C content (mol%)	38.4 ± 1.5[c]	38.2 ± 1.5[c]	36[d]	34.6	37

[a] Data from reference 9.

[b] Data from reference 11.

[c] Mean ± standard deviation from three experiments.

[d] Data from reference 31.

33, and 45 to 47 mol%, respectively (9, 10, 11, 23, 32). The G+C content of strain HO1-1 was determined by the buoyant density method. As *Sulfolobus* DNA preparations apparently contain materials which affect the DNA G+C content determinations (13; unpublished data), we determined DNA G+C contents directly with centrifuged fractions by using the fluorometric method. The G+C content of strain HO1-1 was estimated to be 38.4 mol%, a value that was very similar to the value obtained for *S. acidocaldarius* DSM 639^{T} (38.2 mol%) (Table 1). Thus, strain HO1-1 seems to belong to the genus *Sulfolobus* and not to the genus *Acidianus* or the genus *Metallosphaera.*

Four *Sulfolobus* species, *S. acidocaldarius*, *S. solfataricus*, *Sulfolobus shibatae*, and *Sulfolobus metallicus*, have been de-

TABLE 2. Utilization of sulfur compounds, sugars, amino acids, and yeast extract for growth[a]

Characteristic	Strain HO1-1	*S. acidocaldarius* DSM 639^{T}	*S. solfataricus* DSM 1616^{T}	*S. shibatae* DSM 5389^{T}[b]	*S. metallicus* DSM 6482^{T}[c]
Utilization of sulfur sources					
Elemental S	+	+	+	+	+
FeS	++	++	+		–
Tetrathionate	+	+	+		
H_2S	+	+	+		
Utilization of sugars[d]					
D-Arabinose	–	±	±	+	–
D-Ribose	–	–	±		–
D-Xylose	–	–	±		–
D-Fructose	–	±	±		–
D-Galactose	–	–	++	+	–
D-Glucose	–	+	++	+	–
D-Mannose	–	++	++	+	–
L-Rhamnose	–	–	–		
Lactose	–	–	++	+	–
Maltose	(±)	+	++	+	
Sucrose	–	++	+	+	–
Raffinose	–	++	+	+	–
Utilization of amino acids[e]					
L-Aspartic acid	–	±	±		
L-Glutamic acid	(±)	+	±		–
L-Tryptophan	(±)	+	–		
Utilization of yeast extract	++	++	++	++	–

[a] The microbial strains were incubated at 70°C for 1 week in the presence of various substances under the conditions described in the text. We tested each sugar and amino acid at concentrations of 0.1 and 1.0%; the same results were obtained with both concentrations, except as indicated below. The amount of growth was expressed as the ratio (R) of A_{520} after 1 week of incubation to A_{520} before incubation (which was 0.05), as follows: –, $R = 1$; ±, $1 < R \leq 2$ ($R \geq 2$ after 2 weeks of incubation); +, $2 < R \leq 10$; ++, $R > 10$; (±), $R = 1$ and $1 < R \leq 2$ in the presence of 0.1 and 1.0% test substrate, respectively.

[b] Data from reference 9.

[c] Data from reference 11.

[d] The sugars that were not utilized were inositol, D-mannitol, and D-sorbitol.

[e] The amino acids that were not utilized were L-alanine, L-arginine, L-asparagine, L-cysteine, L-glutamine, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-ornithine, L-phenylalanine, L-proline, L-serine, L-threonine, and L-tyrosine.

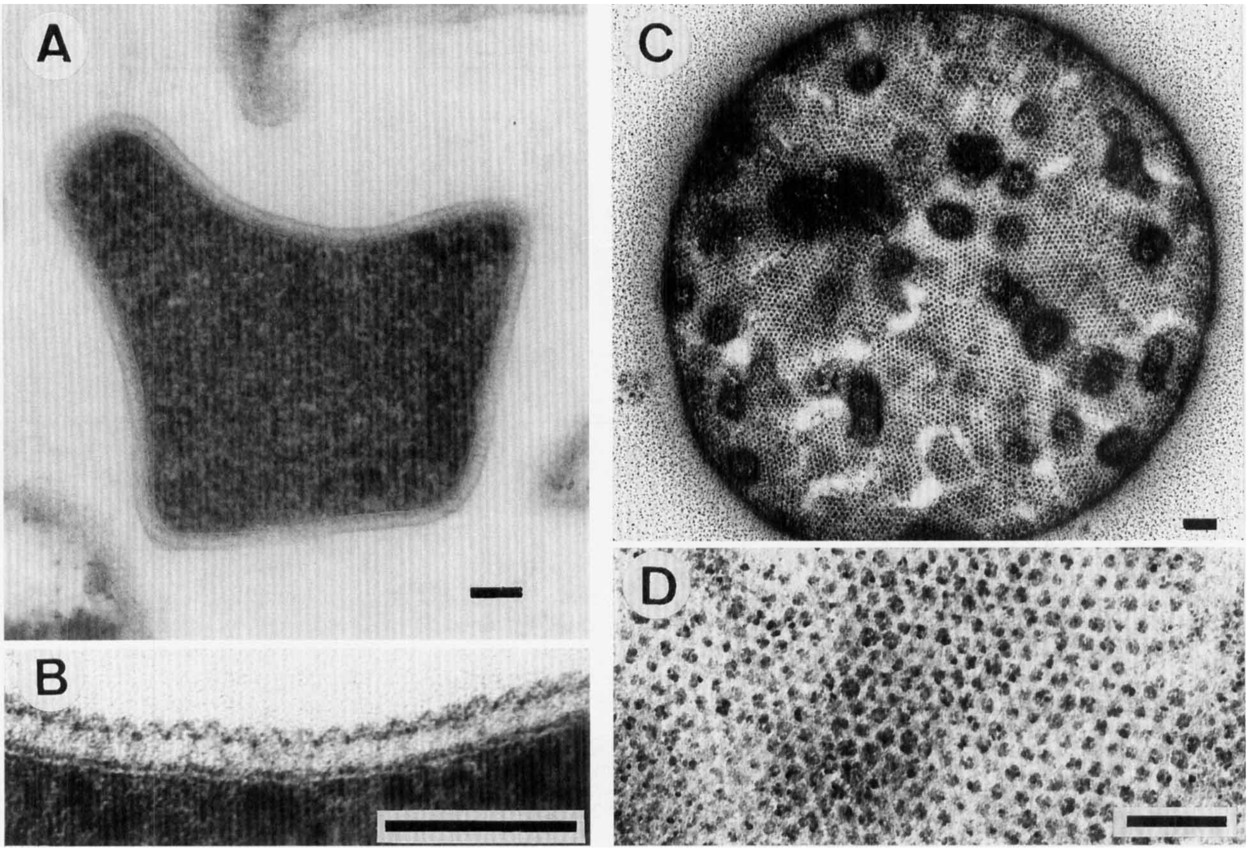

FIG. 2. Electron micrographs of microbial cells. (A and B) Thin sections of a strain HO1-1 cell. Panel B shows the peripheral region of the cell. (C and D) Negatively stained cell envelope. A ghost of a strain HO1-1 cell was stained negatively, shadowed, and observed by electron microscopy, as described in the text. Panel D is a higher magnification of the cell shown in panel C. Bars = 0.1 μm.

scribed previously (2, 9, 11, 31, 32). Although these four species are similar morphologically, are all acidothermophiles, and have similar genomic DNA G+C contents (Table 1) (2, 9, 11, 31, 32), they can be distinguished from each other by their nutritional requirements. Three of these species, *S. acidocaldarius*, *S. solfataricus*, and *S. shibatae*, are facultative chemolithoautotrophs, whereas *S. metallicus* is an obligate autotroph (2, 9, 11, 31, 32). Strain HO1-1, *S. acidocaldarius*, *S. solfataricus*, and *S. shibatae* utilize organic nutrients in yeast extract and some sugars and amino acids, but *S. metallicus* cannot utilize these compounds (Table 2); this characteristic distinguishes strain HO1-1 from *S. metallicus*. We compared the abilities of strain HO1-1 and the three facultative chemolithoautotrophs to utilize carbon compounds (Table 2). A total of 15 sugars were tested, and strain HO1-1 grew slightly in the presence of 1.0% maltose, while *S. acidocaldarius* and *S. solfataricus* grew in the presence of 0.1 and 1.0% maltose and several other sugars (Table 2). *S. acidocaldarius* could be distinguished from *S. solfataricus* by its ability to utilize galactose and lactose (Table 2). We found that *S. shibatae* has a sugar utilization profile that is similar to that of *S. solfataricus* and quite different from that of strain HO1-1 (Table 2). When amino acids were tested, strain HO1-1 grew weakly in the presence of 1.0% glutamic acid and 1.0% tryptophan, while *S. acidocaldarius* grew weakly in the presence of 1.0% glutamic acid and 1.0% tryptophan, while *S. acidocaldarius* grew weakly in the presence of 0.1% glutamic acid and 0.1% tryptophan and *S. solfataricus* grew weakly in the presence of 0.1 and 1.0% aspartic acid and 0.1 and 1.0% glutamic acid (Table 2). Thus, strain HO1-1 could be distinguished from *S. acidocaldarius*, *S. solfataricus*, and *S. shibatae* on the basis of utilization of carbon compounds, just as *S. acidocaldarius* can be distinguished from *S. solfataricus* and *S. shibatae* on the basis of this characteristic.

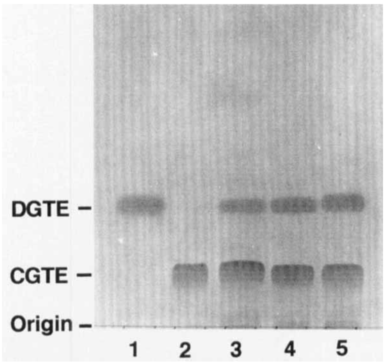

FIG. 3. Analysis of the lipids of strain HO1-1 and related organisms. The core lipids of the microorganisms were analyzed by TLC as described in the text. The plate was first developed with chloroform-methanol (4:1, vol/vol) up to 3 cm and then dried and redeveloped in the same direction with hexane-ether-acetic acid (30:20:1, vol/vol/vol) up to the top. Lanes 1 and 2 contained authentic caldarchaeol (DGTE) and CGTE, respectively, as controls. Lanes 3, 4 and 5 contained the core lipids of strain HO1-1, *S. acidocaldarius*, and *S. solfataricus*, respectively.

TABLE 3. Levels of DNA-DNA hybridization between the *Sulfolobus* strains

Source of filter-bound DNA	% of [^{3}H]-labeled probe DNA associated with filter-bound DNA[a]		
	Strain HO1-1	*S. acidocaldarius* DSM 639^T	*S. solfataricus* DSM 1616^T
Strain HO1-1	100	2.8 ± 0.5	6.1 ± 2.8
S. acidocaldarius	3.6 ± 3.4	100	2.8 ± 1.8
S. solfataricus	3.1 ± 2.6	6.3 ± 2.6	100
E. coli IAM 1264	<1	<1	<1
Calf thymus	<1	<1	<1

[a] Mean ± standard deviation from three experiments.

Similarity of genomic DNAs as determined by DNA-DNA hybridization has been used to distinguish species (12). The lack of genetic relatedness of strain HO1-1 to previously described species was revealed by the levels of hybridization of genomic DNAs (Table 3). The levels of hybridization between strain HO1-1 and *S. acidocaldarius* DNAs and between strain HO1-1 and *S. solfataricus* DNAs were estimated to be 3.6 and 3.1%, respectively, and the level of hybridization between *S. acidocaldarius* and *S. solfataricus* DNAs was 6.1% (Table 3). These results indicate that strain HO1-1 is not a member of *S. acidocaldarius* or *S. solfataricus.*

To examine the taxonomic position of strain HO1-1 at the generic level, we determined the base sequences of the 16S rRNAs of strain HO1-1 (1,451 nucleotides) and *S. acidocaldarius* DSM 639^T, the type strain of the type species of the genus *Sulfolobus*. Sequence data for *S. solfataricus* IFO 15331^T (derived from DSM 1616^T) were provided by Y. H. Itoh (11a). Sequence data for *S. solfataricus* "P1" (not strain P1^T deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen as strain DSM 1616^T were reported by Olsen et al. (21), and the level of sequence similarity between this strain and *S. acidocaldarius* DSM 639^T was 99.9% (unpublished data). Recently, strain "P1" proved to be erroneously labeled and was assigned to *S. acidocaldarius* (30). The levels of similarity between strain HO1-1 16S rRNA and *S. acidocaldarius, S. solfataricus*, and *S. shibatae* 16S rRNAs were 89.8, 89.3, and 89.4%, respectively, when 1,346 nucleotides were compared. The levels of similarity between strain HO1-1 and members of other genera were less than 87.5%. The levels of similarity for the three *Sulfolobus* species and strain HO1-1 were 89.3 to 92.1%, except when *S. solfataricus* and *S. shibatae* were compared (level of similarity, 99.6%) (Table 4). Figure 4 shows a reconstructed phylogenetic tree. As Table 4 and Fig. 4 show, strain HO1-1 seems to be closely related to the genus *Sulfolobus*, but different from previously described *Sulfolobus* species.

On the basis of phenotypic characteristics and genetic distinctness, we concluded that strain HO1-1 represents a new *Sulfolobus* species, and we propose the name *Sulfolobus hakonensis* for this species.

Description of *Sulfolobus hakonensis* sp. nov. *Sulfolobus hakonensis* (ha. ko. nen'sis. M.L. masc. adj. *hakonensis*, pertaining to Hakone National Park, Japan, the location of the hot spring from which the organism was isolated). The cells are lobed, gram-negative cocci (diameter, 0.9 to 1.1 μm) and are surrounded by cell wall which consists of regularly arranged structures. Growth occurs at temperatures between 50 and 80°C (optimum temperature, 70°C) and at pH 1.0 to 4.0 (optimum pH, 3.0). Aerobic and facultatively chemolithoautotrophic. Lithotrophic growth occurs on elemental sulfur, ferrous sulfide, sodium tetrathionate, and hydrogen sulfide. Weak growth occurs in the presence of 1.0% maltose, glutamic acid, or tryptophan as a sole carbon and energy source. The G+C content of the genomic DNA is 38.4 mol%. The main cellular lipids are CGTE and caldarchaeol. The levels of similarity between the 16S rRNA of *S. hakonensis* and the 16S rRNAs of *S. acidocaldarius* DSM 639^T, *S. solfataricus* IFO 15331^T, and *S. shibatae* DSM 5389^T are 89.8, 89.3, and 89.4%, respectively. The type strain is strain HO1-1 (= IAM 14250 = JCM 8857 = DSM 7519 = ATCC 51241).

TABLE 4. Levels of similarity and phylogenetic distances (K_{nuc} values) for 1,346-nucleotide regions of the 16S rRNA genes of strain HO1-1 and thermophilic microorganisms

Organism	Nucleotide sequence accession no.	% Similarity or K_{nuc} value[a]									
		Strain HO1-1	*Sulfolobus acidocaldarius* DSM 639^T	*Sulfolobus solfataricus* IFO 15331^T	*Sulfolobus shibatae* B12^T	*Desulfurococcus mobilis*	*Pyrodictium occultum*	*Thermoproteus tenax*	*Thermofilum pendens*	*Thermococcus celer*	*Methanobacterium formicicum*
Strain HO1-1			89.8	89.3	89.4	87.4	87.5	83.3	83.7	79.0	75.5
Sulfolobus acidocaldarius DSM 639^T		9.6		92.1	92.1	89.3	89.8	85.5	86.0	79.9	76.5
Sulfolobus solfataricus IFO 15331^T	D26490	10.1	7.4		99.6	88.6	89.1	85.1	86.8	79.6	76.8
Sulfolobus shibatae B12^T	M32504	10.1	7.3	0.3		88.6	89.2	85.2	86.9	79.6	77.0
Desulfurococcus mobilis	M36474	12.1	10.1	10.8	10.8		94.1	88.0	88.6	84.1	78.7
Pyrodictium occultum	M21087	12.0	9.5	10.3	10.2	5.3		89.4	90.5	85.1	79.3
Thermoproteus tenax	M35966	16.5	14.0	14.4	14.3	11.4	10.0		89.2	82.7	76.5
Thermofilum pendens	X14835	16.1	13.5	12.7	12.5	10.8	8.9	10.2		84.1	78.9
Thermococcus celer	M21529	21.5	20.3	20.8	20.8	15.6	14.5	17.1	15.6		82.0
Methanobacterium formicicum	M36508	26.1	24.7	24.3	24.1	22.0	21.3	24.9	21.8	8.2	

[a] The values on the upper right are levels of similarity (expressed as percentages), and the values on the lower left are K_{nuc} values.

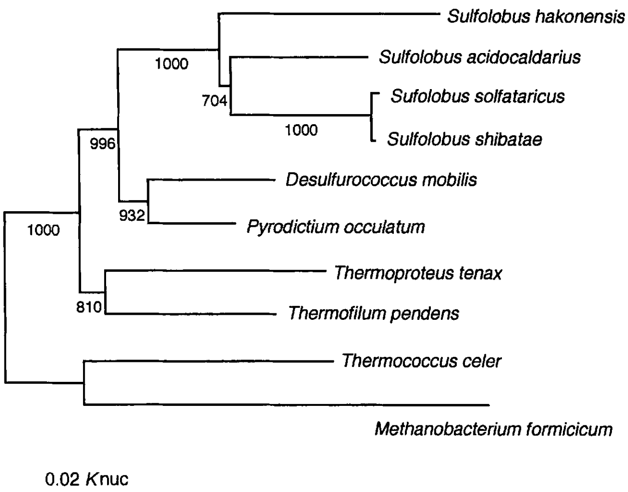

FIG. 4. Phylogenetic relationships among strain HO1-1 (*S. hakonensis*) and related microorganisms. The phylogenetic tree was reconstructed by the neighbor-joining method on the basis of 16S rRNA sequences data. The numbers are the bootstrap values obtained with 1,000 replicates. See Table 4 for the strains used in this analysis.

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