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Sulfolobus metallicus, sp. nov., a Novel Strictly Chemolithoautotrophic Thermophilic Archaeal Species of Metal-Mobilizers

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Summary

Five new isolates of archaeal coccoid thermoacidophiles were obtained from Icelandic solfataric fields. They are strict chemolithoautotrophs gaining energy by oxidation of S° and sulfidic ores. The new strains grow between 50 and 75 °C and pH 1 and 4.5 and tolerate NaCl concentrations of up to 3.0%. The GC-content of their DNA is 38 mol%.

The new isolates resemble members of *Sulfolobus* in their morphology, their ability to oxidize reduced sulfur compounds and their GC-content. They are different in their strictly chemolithoautotrophic mode of life, their ore-leaching capacity, DNA/DNA hybridization and incomplete serological cross-reaction of RNA polymerase. Therefore, we describe here a new species, *Sulfolobus metallicus*. Type strain is *Sulfolobus metallicus* (Kra 23; DSM 6482).

Key words: *Sulfolobus metallicus* – *Sulfolobaceae* – Archaea – Leaching – Acidophilic – Chemolithotrophic – Thermophilic

Introduction

All thermoacidophiles of the archaeal crenarchaeota kingdom are members of the *Sulfolobaceae* (*Stetter*, 1989; *Woese* et al., 1990). Up to now the genera *Sulfolobus*, *Acidianus*, *Desulfurolobus* and *Metallosphaera* have been described (*Brock* et al., 1972; *Segerer* et al., 1986; *Zillig* et al., 1986; *Huber* et al., 1989). They share a regular to irregular coccoid shape, the thermoacidophilic mode of life and the ability to oxidize elemental sulfur (*Stetter*, 1989). In addition, the *Sulfolobaceae* possess glycoprotein subunit cell envelopes and caldariellaquinone (*Weiss*, 1974; *Zillig* et al., 1980; *Huber* et al., 1989; *de Rosa* and *Gambacorta*, 1988). The different genera within the *Sulfolobaceae* can be distinguished from each other by their metabolic and biochemical properties: Members of *Sulfolobus* exhibit a GC-content of around 37 mol% and are able to utilize sugars, amino acids, and complex organic substances as energy and carbon sources (*Brock* et al., 1972; *Zillig* et al., 1980). So far, they have not been found to be able to extract metals (*Huber* et al., 1989). *Acidianus* and *Desulfurolobus* are closely related to each other (*Huber* et al., 1987). They show a GC-content of about 31 mol% and a facultatively aerobic metabolism with elemental sulfur as electron donor or aceeptor (*Segerer* et al., 1986; *Zillig* et al., 1986). They are weak ore-leachers (*Huber* et al., 1989).

The first ore-leaching acidophile, which had been described as *Sulfolobus brierleyi*, has recently been placed to the genus *Acidianus* (*Brierley* and *Brierley*, 1973; *Zillig* et al., 1980; *Segerer* et al., 1986). *Metallosphaera* is characterized by a GC-content of 45 mol% and a strong ore-leaching capacity (*Huber* et al., 1989). The subdivision into the genera on the basis of the metabolic and biochemical properties is further supported by the lack of significant DNA/DNA hybridization and the incomplete serological cross-reaction of their DNA-dependent RNA polymerases between members of them (*Stetter*, 1989). Within the genus *Sulfolobus*, the three species *S. acidocaldarius*, *S. solfataricus* and *S. shibatae* can be distinguished from each other by DNA/DNA hybridization (*Segerer* et al., 1986; *Grogan* et al., 1990) and by 16S rRNA sequencing (*Woese* et al., 1984; *Olsen* et al., 1985; *Grogan* et al., 1990).

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Here we describe a new group of aerobic metal-mobilizing thermoacidophiles representing a new species within the genus *Sulfolobus*.

Material and Methods

Organisms. The type strains of *Sulfolobus acidocaldarius* (DSM 639), *Sulfolobus solfataricus* (DSM 1616) and *Acidianus brierleyi* (DSM 1651) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM, Braunschweig, FRG). *Sulfolobus shibatae* (B12; DSM 5389) was obtained from *W. Zillig,* Martinsried. *Acidianus infernus* (DSM 3191) and *Metallosphaera sedula* (DSM 5348) were obtained from the DSM and had been originally isolated by our laboratory (*Segerer* et al., 1986; *Huber* et al., 1989).

Growth conditions. The new isolates and the type strains were grown in modified ALLEN-medium (*Allen,* 1959; *Brock* et al., 1972) under shaking (150 rpm). *Sulfolobus acidocaldarius, Sulfolobus solfataricus, Sulfolobus shibatae* and *Acidianus infernus* were cultivated at 80 °C, *Acidianus brierleyi* and *Metallosphaera sedula* at 65 °C. The isolates were routinely grown at 65 °C on ore mixture "G6" (*Huber* et al., 1989). The cultures were usually transferred into fresh medium after one week of incubation (5% inoculum). Mobilization of metal ions was determined in 500 ml Erlenmeyer flasks containing 120 ml culture medium and 4 g ore mixture "G1N" equipped with an air condensor (*Huber* et al., 1989).

Growth measurement. Bacterial growth was followed by direct cell counting in a Thoma chamber (depth 0.02 mm) under a phase contrast light microscope (Zeiss Standard 16).

Electron microscopy. Pt-shadowed cells and thin sections were prepared and electron microscopy was carried out according to *König* and *Stetter* (1982).

Substrate utilization. In order to determine the substrates of the new isolates, the same organic and inorganic substances were assayed as described earlier (*Huber* et al., 1989). In addition, growth on the amino acids DL-alanine, DL-valine, L-methionine and L-glutamic acid (10 g/l) was examined.

Tolerance against heavy metal ions. Resistance against various heavy metal ions during growth on ore mixture "G6" was tested according to *Huber* et al. (1989).

Determination of metabolic products. Mobilization of metal ions from the ores and production of sulfate from elemental sulfur was measured in the supernatant of the cultures by "ICP" (Inductively Coupled Plasma, JY 70 Plus, Jobin Yvon) analyses.

Lipid analyses. Lipids were extracted according to the method of *de Rosa* et al. (1983). The total lipids were fractionated and identified as described previously (*Huber* et al., 1989).

Isolation of DNA. The DNA was prepared according to *Wildgruber* et al. (1982).

DNA base composition. The GC-content of DNA was determined by melting point analysis (*Marmur* and *Doty,* 1962) and by high performance liquid chromatography (HPLC) after digestion of the DNA with nuclease P1 (*Zillig* et al., 1980).

DNA similarity. DNA/DNA hybridization was performed as described elsewhere (*König,* 1984).

DNA-dependent RNA polymerase. The RNA polymerases of *S. acidocaldarius* and isolate Kra23 were purified according to *Zillig* et al. (1979). The activity was assayed for 20 min at 55 °C with poly[d(A–T)] as a template (*Zillig* et al., 1979). Exponential gradient SDS polyacrylamide gels (5–25%) were prepared according to *Laemmli* (1970) and *Mirault* and *Scherrer* (1971). The immunochemical cross-reaction of the RNA polymerase of isolate Kra23 was assayed as described by *Ouchterlony* (1962) employing rabbit antibodies (*Stetter,* 1977).

Results

Collection of samples and isolation of the new bacteria

Eleven aerobic samples were collected from water- and mudholes of terrestric Icelandic solfataric fields within the Krafla, Kerlingarfjöll, Krisuvik, Namarskarth and Hveragerthi areas. The original temperatures were between 55 and 100 °C and the pH between 1.5 and 5.0. All samples were carried to the laboratory without pH- and temperature control and were stored there at 4 °C. 1 ml of each of the original samples was transferred into 30 ml of mineral medium, pH 2.0 supplemented with pyrite, chalcopyrite or the ore mixture "G1N". After one week of incubation at 65 or 75 °C under shaking (150 rpm), irregular coccoid organisms, resembling *Sulfolobus* in shape, became visible within five (Kra23, Kra22, Ker2, NA4, Okri3) of the culture attempts from the samples of the Krafla, Kerlingarfjöll, Namarskarth and Krisuvik solfatares. Pure cultures were obtained by repeated serial dilutions in ore-containing (mixture "G1N") media.

Morphology

In the light microscope, cells of the new isolates appeared as irregular, lobed cocci about 1.5 µm in diameter, sometimes reminiscent of pyramids or dishes (Fig. 1). The cells appeared immotile and occurred singly or in pairs (Fig. 2). They were surrounded by an envelope consisting most likely of protein subunits (Fig. 2).

Storage

Cultures frozen and stored over liquid nitrogen at −140 °C served as inocula for at least one year.

Optimal growth conditions

Growth on ores was obtained between 50 and 75 °C with an optimum around 65 °C (Kra23; doubling time 13 h) or 70 °C (Ker2; doubling time 8 h) (Fig. 3). The isolates grew in a pH range between 1.0 and 4.5 (not shown) and tolerated NaCl concentrations of up to 3.0%. Optimal growth (doubling time 8 h; final cell density about 2×10^8/ml) was observed at NaCl concentrations between 0

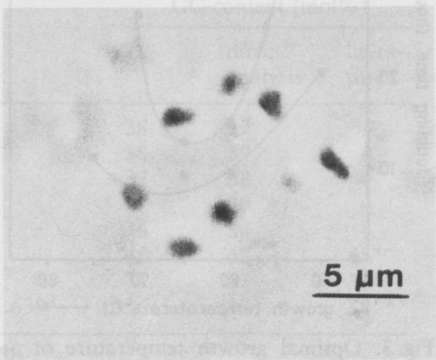

Fig. 1. Phase contrast micrograph of cells of *Sulfolobus metallicus* (isolate Kra23). Bar 5 µm.

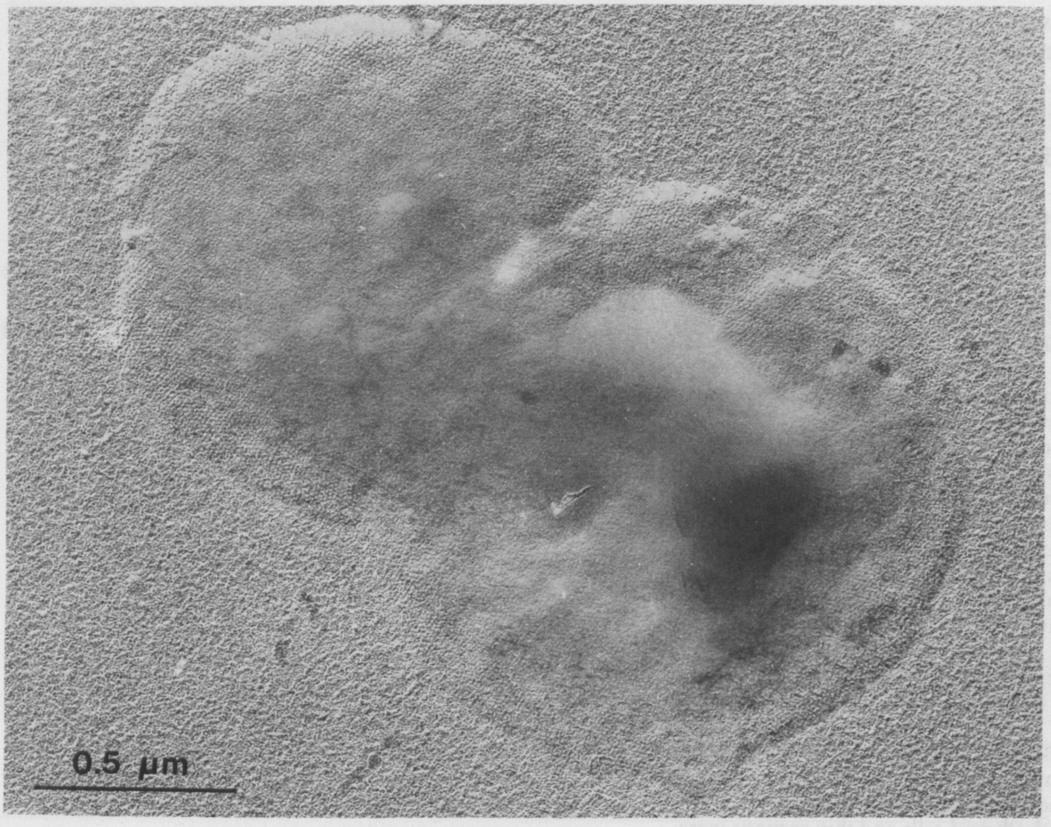

Fig. 2. Electron micrograph of *Sulfolobus metallicus* (isolate Kra23), platinum shadowed. Bar 0.5 µm.

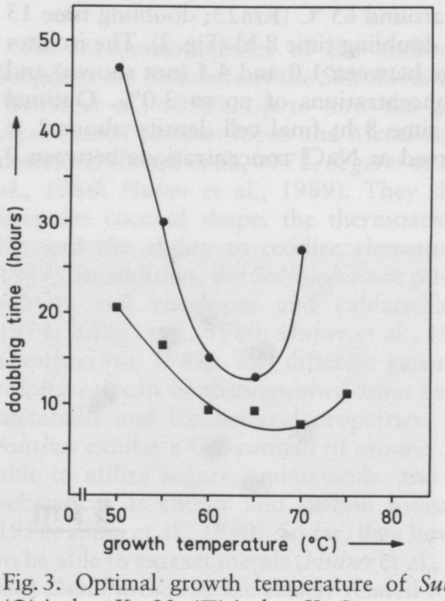

Fig. 3. Optimal growth temperature of *Sulfolobus metallicus*. (●) isolate Kra23; (■) isolate Ker2.
The doubling times were calculated from the slopes of the growth curves (not shown).

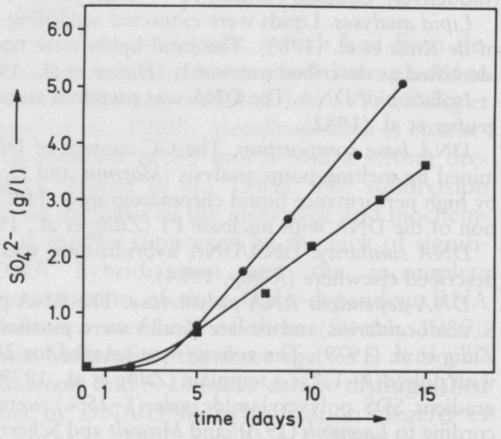

Fig. 4. Sulfate formation of *Sulfolobus metallicus* during growth on elemental sulfur.
(●) isolate Kra23; (■) isolate Ker2.

and 0.75%. *S. acidocaldarius* and *S. solfataricus* did not grow in the presence of 0.5 and 0.75% NaCl, respectively (not shown).

Metabolism

The new isolates grew chemolithoautotrophically by the oxidation of elemental sulfur, single sulfidic ores (pyrite, chalcopyrite, sphalerite) and on the ore mixtures "G6" and "GIN". They were even able to grow on the synthetic sulfides ZnS and CdS. On elemental sulfur within two weeks the isolates Kra23 and Ker2 formed 5100 mg/l and 3700 mg/l sulfate, respectively (Fig. 4). Sulfuric acid formation was not stimulated by the addition of 0.005% yeast extract (not shown). Arsenopyrite, bornite, cinnabar, chalcocite, covellite and the synthetic sulfides CuS, FeS, MoS_2, Sb_2S_3 and SnS did not serve as substrates. No growth was obtained on beef extract, casamino acids, peptone, tryptone and yeast extract, on arabinose, fructose, galactose, glucose, lactose, mannose, raffinose, ribose, sucrose, sorbose and xylose and on alanine, glutamic acid, methionine and valine. Under anaerobic conditions no growth and sulfur reduction by isolate Kra23 was observed (*A. Segerer*, pers. comm.).

Heavy metal resistance

During growth on the ore mixture "G6", the isolate Kra23 tolerated concentrations of 1.7 mmol/l cobalt (*M. sedula* 0.85 mmol/l), 300 mmol/l zinc (*M. sedula* 150 mmol/l) and 17 mmol/l nickel ions (*M. sedula* 1.7 mmol/l) (Table 1). For *S. acidocaldarius*, a tolerance against arsenate of 10 mmol/l was reported (*Lindström* and *Sehlin* 1989), which is in the range of isolate Kra23. Non metal-mobilizing bacteria, e.g. *E. coli*, are inhibited by a 30,000 fold lower copper ion concentration (0.5 µmol/l) than isolate Kra23 (*Domek* et al., 1984).

Ore leaching capacity

All isolates mobilized 90 to 100% of the total copper within four weeks (Table 2). The final zinc ion concentrations in the supernatant varied between 70 and 100% depending on the strain (Table 2). 100% of the uranium were mobilized by all isolates within 2 weeks with the exception of strain Kra22.

Lipid composition

The isolates Kra23 and Kra22 contained glycerol-dialkyl-nonitol tetraethers and caldariellaquinone in the membrane. Sulfolobusquinone and tricyclicquinone were not found. Between the two new strains only little differences in the proportions of minor complex lipids were detectable (*M. de Rosa* and *A. Gambacorta*, pers. comm.).

GC-content of the DNA

The GC-content of all isolates was around 38 mol% (Table 3). The type species of the different genera within the *Sulfolobaceae* served as references (Table 3).

Table 1. Growth of *S. metallicus* (isolate Kra23) and *Metallosphaera sedula* on ore mixture "G6" in the presence of different heavy metal ion concentrations (in mmol/l)

Metal ion	Strain			
	Kra23		*M. sedula**	
	growth	no growth	growth	no growth
Ag	0.09	0.9	0.09	0.9
As	1.3	13	1.3	13
Cd	0.9	9	0.9	9
Co	1.7	17	0.85	1.7
Cu	16	160	16	79
Hg	0.05	0.5	0.05	0.5
Mo	1	10	1	10
Ni	17	170	1.7	17
Sb	0.8	8	0.8	8
U	0.4	4	0.4	4
Zn	300	750	150	750

* Data from *Huber* et al. (1989).

Table 2. Metal extraction by the new isolates from the ore mixture "G1N" within 28 days (mg/l)

Extraction by	Metal		
	Cu	U	Zn
Kra23	700	94	4125
Kra22	700	60	3000
NA4	770	90	3900
Ker2	760	87	4025
Okri3	700	90	4500
sterile control*	90	65	1300
"G1N" metal ion content**	800	95	4500

* Sterile control: pH 2.5, 65 °C.
** Determined by chemical extraction of ore mixture G1N with concentrated *aqua regia*.

Table 3. DNA base composition of the isolates and of representatives of the *Sulfolobaceae*

Strain	GC-content (mol%)		
	T_M	direct analysis	literature
Kra23	38	37	–
Kra22	38	39	–
NA4	37	38	–
Ker2	37	39	–
Okri3	36	38	–
M. sedula DSM 5348^T	45	47	45*
A. infernus DSM 3191^T	31	33	31**
S. acidocaldarius DSM 639^T	36	37	38***

* *Huber* et al. (1989).
** *Segerer* et al. (1986).
*** *Zillig* et al. (1980).

Table 4. DNA/DNA similarity between *Sulfolobus metallicus* (isolate Kra23) and the type strains of the *Sulfolobaceae* (in %)

Filter-bound DNA from	^{32}P-labelled DNA from				
	A. brierleyi DSM 1651^T	*S. acidocaldarius* DSM 639^T	*S. solfataricus* DSM 1616^T	*M. sedula* DSM 5348^T	Kra23 DSM 6482^T
A. brierleyi	100	5	9	7	7
A. infernus	6	3	5	6	8
S. acidocaldarius	4	100	12	12	9
S. solfataricus	7	9	100	8	7
M. sedula	12	8	10	100	5
Kra23	5	3	6	6	100

Sensitivity to diphtheria toxin

In the crude extracts of isolates Kra23 and NA4, an elongation factor II-like protein was ADP-ribosylated by diphtheria toxin (*F. Klink*, pers. comm.; *Kessel* and *Klink*, 1980).

DNA/DNA similarity

All new isolates exhibited DNA similarity between 73 and 100% among each other (not shown). No significant hybridization between strain Kra23 as a representative of this group and the type species of the different genera within the *Sulfolobaceae* was observed (Table 4) indicating phylogenetic distance.

DNA-dependent RNA polymerase

The molecular masses of the polypeptides (between 130 and 11 kD) of the Kra23 RNA polymerase were determined by coelectrophoresis with the *S. acidocaldarius* enzyme (Table 5). In order to investigate the immunological relationships of the RNA polymerase of isolate Kra23 to other thermoacidophilic sulfur-metabolizers, antibodies against the enzyme of strains Kra23 were prepared (*Stetter*, 1977). In the Ouchterlony immunodiffusion assay, the enzymes of *S. acidocaldarius*, *S. solfataricus* and *A. brierleyi* spurred against that of isolate Kra23 (Fig. 5). As expected from the DNA homology, the RNA polymerase of strain Okri3 showed immunochemical identity with that of strain Kra23 (Fig. 5).

Table 5. Molecular masses (in kilo daltons) of the subunits of the DNA-dependent RNA polymerases of *Sulfolobus metallicus* (isolate Kra23) and *Sulfolobus acidocaldarius*

Subunit*	Strain	
	*Sulfolobus acidocaldarius***	Kra23
B	122	130
A	101	103
C	44	45
D_1	33	34.5
D_2	32	33.5
E	26	27
F	17.5	20.5
G	13.8	14.5
H	11.8	13
I	11.2	11
J	10.8	–

* Designations in analogy to *S. acidocaldarius* following the molecular sizes.

** Molecular weights according to *Prangishvilli* et al. (1982).

Discussion

The five new isolates belong to the archaeal domain in that they contain isopranyl ether lipids (*de Rosa* et al.,

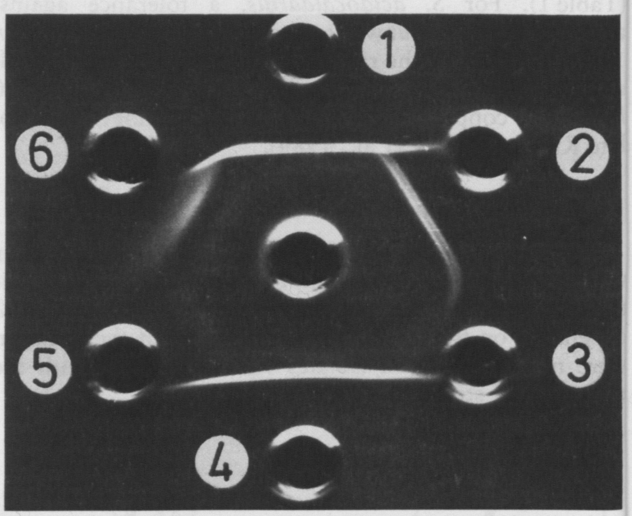

Fig. 5. Ouchterlony immunodiffusion test of *Sulfolobus metallicus* (isolate Kra23) RNA polymerase antibodies against the DNA-dependent RNA polymerases of members of the *Sulfolobaceae*.
(center) antibodies against the DNA-dependent RNA polymerase of *Sulfolobus metallicus* (isolate Kra23)
Purified or enriched RNA polymerases of:
(1, 4) *Sulfolobus metallicus* (isolate Kra23), purified
(2) *Sulfolobus acidocaldarius*, purified
(3) *Metallosphaera sedula*, purified
(5) *Acidianus brierleyi*, enriched
(6) *Sulfolobus metallicus* (isolate Okri 3), enriched

1977; *Langworthy* et al., 1982; *de Rosa* and *Gambacorta*, 1988), possess an elongation factor II-like protein sensitive against diphtheria toxin (*Kessel* and *Klink*, 1982) and exhibit a complex subunit structure of their DNA-dependent RNA polymerase (*Zillig* et al., 1980; *Zillig* et al., 1982). On the basis of their irregular coccoid shape, their thermoacidophilic mode of life, their aerobic metabolism and their ability to oxidize elemental sulfur, they are members of the *Sulfolobaceae* (*Stetter*, 1989). Furthermore they resemble the members of this family in their possession of glycerol-dialkyl-nonitol tetraethers and of caldariellaquinone in their membrane (*de Rosa* and *Gambacorta*, 1988). In their GC-content of around 38 mol%, the new isolates are similar to *S. acidocaldarius*, *S. solfataricus* and *S. shibatae*. However they can be distinguished from these species by their obligately chemolithoautotrophic mode of nutrition and their leaching capacity. Further differences are their salt tolerance, lack of DNA/DNA similarity and the incomplete cross-reaction of antibodies against the DNA-dependent RNA polymerase of isolate Kra23 with the enzymes of *S. acidocaldarius* and *S. solfataricus*. Therefore we consider the new isolates as representatives of a new species of the genus *Sulfolobus*, which we name *Sulfolobus metallicus*, because of their ability, to mobilize metal ions from sulfidic ores. Due to their equal metabolic properties, the identical GC-content and a DNA homology of more than 73% between each other (*Schleifer* and *Stackebrandt*, 1983), all five isolates are members of the new species *S. metallicus*. In their ability to mobilize metals from sulfidic ores, *S. metallicus* resembles the genera *Acidianus* and *Metallosphaera*. In contrast to the *Acidianus* species however, *S. metallicus* is unable to reduce elemental sulfur under anaerobic conditions. In addition, the GC-content of the DNA of *S. metallicus* is around 7% higher than that of the type strains *Acidianus infernus* (31 mol%) and 7% lower than that of *Metallosphaera sedula* (45 mol%). The phylogenetic distance of *S. metallicus* to the genera *Acidianus* and *Metallosphaera* is further evident by the lack of significant DNA homology (*Schleifer* and *Stackebrandt*, 1983) and the incomplete immunological cross-reaction of antiserum against the native RNA polymerase of isolate Kra23 (*Stetter* et al., 1981). So far, members of *S. metallicus* were only isolated from samples of terrestric Icelandic solfataric fields. Because of their salt tolerance of up to 3% NaCl, however, geothermally heated acidic marine environments could possibly be another biotope.

Description of *Sulfolobus metallicus*, sp. nov.

me.tal'li.cus. L. masc. n. *metallicus*, the miner.

Gram-negative irregular cocci about 1.5 μm in width. Growth between 50 and 75 °C, at pH 1.0 to 4.5 and at 0 to 3% NaCl. Aerobic. Obligately chemolithoautotrophic growth on sulfidic ores like pyrite, sphalerite and chalcopyrite and on elemental sulfur. Formation of sulfuric acid. No sulfur reduction anaerobically. Cell envelope consists of a S-layer. Isopranyl ether lipids and caldariellaquinone present. An elongation factor II-like protein is sensitive to diphtheria toxin. DNA base composition 38 mol% GC. DNA similarity below 9% to the type strains of the genera *Acidianus*, *Metallosphaera* and *Sulfolobus*. "BAC" type DNA-dependent RNA polymerase with molecular masses of the polypeptides of 130, 103, 45, 34.5, 33.5, 27, 20.5, 14.5, 13 and 11 kD (determined by SDS polyacrylamide gel electrophoresis). Incomplete cross-reaction of antibodies against the native RNA polymerase of isolate Kra23 with RNA polymerases of the type strains of *Acidianus infernus Metallosphaera sedula* and *Sulfolobus acidocaldarius*.

Isolated from continental solfataric fields in Iceland.

Type strain: *Sulfolobus metallicus*, Kra 23, DSM 6482, Braunschweig, FRG.

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