**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 acceptor (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).
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 1631) 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 "GIN" 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/1) 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 Mirautil and Scherrer (1971). The immununodinoclonal 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 terrestrical Icelandic solfataric fields within the Krafá, Kerlingarfjóll, 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 “GIN”. After one week of incubation at 65 or 75 °C under shaking (150 rpm), irregular coccolid organisms, resembling *Sulfolobus* in shape, became visible within five (Kra23, Kra22, Ker2, NA4, Okri3) of the culture attempts from the samples of the Krafá, Kerlingarfjóll, Namarskarth and Krisuvik solfatares. Pure cultures were obtained by repeated serial dilutions in ore-containing (mixture “GIN”) 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 × 10^9/ml) was observed at NaCl concentrations between 0
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.
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 CuS, FeS, MoS₂, SnS and SnS. 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, MoS₂, Sb₂S₃ 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 urnol/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 small 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)

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

* Data from Huber et al. (1989).

### Table 2. Metal extraction by the new isolates from the ore mixture “GIN” within 28 days (mg/l)

<table>
<thead>
<tr>
<th>Metal</th>
<th>Extraction by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kra23</td>
</tr>
<tr>
<td>Cu</td>
<td>700</td>
</tr>
<tr>
<td>U</td>
<td>94</td>
</tr>
<tr>
<td>Zn</td>
<td>8125</td>
</tr>
</tbody>
</table>

* Sterile control: pH 2.5, 65 °C.

** Determined by chemical extraction of ore mixture GIN with concentrated aqua regia.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>GC-content (mol%)</th>
<th>T_M</th>
<th>direct analysis</th>
<th>literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kra23</td>
<td>38</td>
<td>37</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Kra22</td>
<td>38</td>
<td>39</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ker2</td>
<td>37</td>
<td>38</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Okri3</td>
<td>36</td>
<td>38</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>M. sedula DSM 5348T</td>
<td>45</td>
<td>47</td>
<td>45*</td>
<td></td>
</tr>
<tr>
<td>A. infernus DSM 3191T</td>
<td>31</td>
<td>33</td>
<td>31**</td>
<td></td>
</tr>
<tr>
<td>S. acidocaldarius DSM 639T</td>
<td>36</td>
<td>37</td>
<td>38***</td>
<td></td>
</tr>
</tbody>
</table>

* Huber et al. (1989).
** Segerer et al. (1986).
*** Zillig et al. (1980).
Filter-bound DNA from

32P-labelled DNA from

| A. brierleyi | 100 | 5 | 9 | 7 |
| A. infernus | 6 | 3 | 5 | 6 |
| S. acidocaldarius | 4 | 100 | 12 | 12 |
| S. solfataricus | 7 | 9 | 100 | 8 |
| M. sedula | 12 | 8 | 10 | 100 |
| Kra23 | 5 | 3 | 6 | 6 |

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 immunological identity with that of strain Kra23 (Fig. 5).

Discussion

The five new isolates belong to the archaeal domain in that they contain isoprenyl ether lipids (de Rosa et al.,
Sulfolobus metallicus, sp. nov.

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 cocoid shape, their thermocodiophilic 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 caldariella-quinone 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 Acidiums infernus (31 mol%) and 7% lower than that of Metallosphaera sedula (45 mol%). The phylogenetic distance of S. metallicus to the genera Acidiumus 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 terrestrial 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.

Sulfolobus metallicus, sp. nov. is a 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 chloropyrite and on elemental sulfur. Formation of sulfuric acid. No sulfur reduction anaerobically. Cell envelope consists of a layer. Isopropyl ether lipids and caldariella-quinone 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 Acidiums 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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