Reprint from

SYSTEMATIC AND APPLIED MICROBIOLOGY

formerly Zentralblatt für Bakteriologie,
Mikrobiologie und Hygiene
I. Abt. Originale C

Gustav Fischer Verlag
Stuttgart · New York

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Professor Dr. *Karl O. Stetter*, Lehrstuhl für Mikrobiologie, Universität Regensburg, Universitätsstr. 31, D-8400 Regensburg

System. Appl. Microbiol. 12, 38–47 (1989)

Metallosphaera sedula gen. and sp. nov. Represents a New Genus of Aerobic, Metal-Mobilizing, Thermoacidophilic Archaebacteria

GERTRUD HUBER[1], CAROLA SPINNLER[1], AGATA GAMBACORTA[2], and KARL O. STETTER[1]

[1] Lehrstuhl für Mikrobiologie der Universität Regensburg, 8400 Regensburg, Federal Republic of Germany
[2] Istituto per la Chimica di Molecole di Interesse Biologico, Via Toiano 6, 80072 Arco Felice Naples, Italy

Received April 18, 1989

Summary

From a solfataric field in Italy three isolates of spherical thermoacidophilic metal-mobilizing archaebacteria were obtained. They are facultative autotrophs. From sulfidic ores they extract metal ions with very high efficiency. They are also vigorous S°-oxidizers. Alternatively, they are able to use heterogeneous organic material such as yeast extract. The isolates grow within a temperature range from 50 to 80 °C. The GC-content of their DNA is 45 mol %. No significant DNA homology is detectable between the isolates and the type strains of the members of the genera *Acidianus* and *Sulfolobus*. The DNA-dependent RNA polymerase of isolate TH2 shows incomplete serological cross-reaction with antibodies against the enzyme of *Sulfolobus acidocaldarius*. On the basis of the distinct physiological and molecular properties we describe the new strains as members of the new genus *Metallosphaera*. Type species and type strain is *Metallosphaera sedula* (TH2, DSM5348).

Key words: *Metallosphaera* – *Sulfolobaceae* – Archaebacteria – Leaching – Acidophilic – Chemolithotroph – Thermophilic

Introduction

Organisms able to extract metal ions from sulfidic ores ("bioleaching") are known within eu- and archaebacteria. The eubacterial metal-mobilizers are the mesophilic acidophilic species *Thiobacillus ferrooxidans* (*Colmer* and *Hinkle*, 1947), *Thiobacillus prosperus* (*Huber* and *Stetter*, 1989), and *Leptospirillum ferrooxidans* (*Balashova* et al., 1974). Within the archaebacteria (*Woese* et al., 1978), a thermophilic species capable of mobilizing metal ions is known (*Brierley* and *Murr*, 1973). It has been isolated from a thermal spring drainage in Yellowstone National Park (*Brierley* and *Brierley*, 1973). Brierley's isolate grows at temperatures up to 75 °C, within a pH range from 1 to 6 (*Segerer* et al., 1986), and is able to use elemental sulfur, ferrous iron, sulfidic ores, yeast extract, tryptone and casamino acids as energy sources (*Brierley* and *Brierley*, 1973; *Brierley* and *Murr*, 1973; *C. Brierley*, pers. comm.; *Zillig* et al., 1980; *Segerer* et al., 1986). Mainly on the basis of its similar morphology and its serologically cross-reacting RNA polymerase, Brierley's isolate was described as *Sulfolobus brierleyi* (*Zillig* et al., 1980). Further investigations demonstrated that *S. brierleyi* was a facultative anaerobe. Like *Acidianus infernus* it was capable of growing anaerobically by lithoautotrophic sulfur reduction. Since both organisms exhibited the same GC-content, it became evident that *S. brierleyi* belonged to the genus *Acidianus*. It has therefore been transferred to this genus and described as *Acidianus brierleyi* (*Segerer* et al., 1986). Two further coccoid isolates were reported to be able to extract metal ions from sulfidic ores at temperatures up to 75 °C (*Marsh* et al., 1983). However, their physiological features as well as their phylogenetic position remain unclear.

In this paper we describe the isolation and properties of three novel strains of metal-mobilizing archaebacteria belonging to an up to now unknown genus.

Materials and Methods

Strains. 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). *Acidianus infernus* (DSM 3191) was isolated in our laboratory (*Segerer* et al., 1986).

Culture conditions. For the cultivation of the new isolates and the type strains the medium of *Allen* (1959) modified by *Brock* et al. (1972) was used. The pH was adjusted to 2.0 with 10 N H_2SO_4. On sulfur or soluble substrates the organisms were grown in test-tubes (270 × 18 mm; 20 ml medium), on ores in 100 ml Erlenmeyer flasks (30 ml medium). The new isolates were routinely cultivated on the ore mixture "G6" (1 g/30 ml medium) consisting of equal parts of pyrite (Grube Bayerland, Oberpfalz), sphalerite (Grube Lüderich, Nordrhein-Westfalen) and pitch blend (Grube Höhenstein, Oberpfalz). Leaching experiments were carried out with ore mixture "G1N" which contained the three components of "G6" and, in addition, chalcopyrite from Norway (each 25% w/w).

Chemical composition of the ore mixture "G1N" (1 g dissolved in 30 ml aqua regia):

As	58 ppm;	Fe	6800 ppm;	Pb	250 ppm;	Y	2 ppm;
Cd	12 ppm;	Ge	30 ppm;	Th	4 ppm;	Yb	4 ppm;
Co	10 ppm;	Hg	13 ppm;	Ti	2 ppm;	Zn	4500 ppm;
Cu	800 ppm;	Mn	26 ppm;	U	95 ppm;	Zr	3 ppm;

Antimony, cerium, chromium, gold, molybdenum, nickel, silver, tin and vanadium were not found in detectable amounts.

For large scale preparations the strains were grown in 85 l enamel-protected fermentors (HTE, Bioengineering, Wald, Switzerland) under stirring (150 rev/min) and slightly gassing with compressed air (25 ml/min). The cells were harvested in the exponential growth phase with a separator (Westfalia, West Germany).

Bacterial growth. Cell concentrations were determined with a Zeiss Standard 16 microscope by direct counting in a Thoma counting chamber (depth 0.02 mm). For the visualization of cells on solid particles they were stained by a modified "DAPI" (4'-6'-Diamidino-2-phenylindole) procedure (*Huber* et al., 1985) and observed with the fluorescence equipment IV FL with an excitation filter BP 365 and a selection filter LP 420.

Electron microscopy. Electron microscopy was performed as described previously (*Huber* and *Stetter*, 1989).

Substrate utilization. For heterotrophic growth one of the following organic substances was added to the mineral medium (10 g/l): yeast extract, tryptone, casamino acids (all: Difco); peptone, beef extract, L-(+)-arabinose, D-(−)-fructose, D-(+)-galactose, D-(+)-glucose, lactose, D-(+)-mannose, raffinose, D-(−)-ribose, sucrose, L-(−)-sorbose, D-(+)-xylose (all: Merck). In the presence of sulfur (10 g/l) or ores, the concentration of yeast extract was reduced to 0.2 g/l. In order to determine the inorganic energy sources the following compounds were used: elemental sulfur (Aldrich Chemical Company, Inc.; 99.999%; 10 g/l), synthetic sulfides (CdS, CuS, FeS, MoS_2, Sb_2S_3, SnS, ZnS; each 0.5 g/30 ml) and natural ores (each 0.5 g/30 ml):

- arsenopyrite: St. Andreasberg (Germany), Haute Loire (France), Reichenstein (Poland)
- bornite: Butte (Montana, USA), Hasborn (Germany)
- cinnabar: Almaden (Spain)
- chalcopyrite: Norway, Cornwall (GB), Kelchalpe (Austria), Kopparberg (Sweden), Tarn (France), Murgul (Turkey)
- chalcocite: Butte (Montana, USA), Eifel (Germany)
- covellite: Butte (Montana, USA)
- galena: Clausthal (Germany)
- pitch blend: Grube Höhenstein (Germany)
- pyrite: Grube Bayerland (Germany)
- sphalerite: Grube Lüderich (Germany)

Tolerance against heavy metals. For the examination of the resistance against heavy metal ions, media containing yeast extract or ore mixture "G6" as energy sources were supplemented by the following salts (final heavy metal concentration in mM):

$AgNO_3$ (0.009; 0.09; 0.9); $NaAsO_2$ (0.13; 1.3; 13); $(CH_3COO)_2Cd \times 2\ H_2O$ (0.09; 0.9; 9); $CoSO_4 \times 7\ H_2O$ (0.17; 0.85; 1.7); $CuSO_4 \times 5\ H_2O$ (0.16; 1.6; 16; 79); $HgSO_4$ (0.0005; 0.005; 0.05; 0.5); $Na_2MoO_4 \times 2\ H_2O$ (0.01; 0.1; 1; 10); $SbCl_3$ (0.08; 0.8; 8); $(CH_3COO)_2UO_2 \times 2\ H_2O$ (0.04; 0.4; 4); $ZnSO_4 \times 7\ H_2O$ (15; 150; 750).

Metal analysis. Mobilization of metal ions from the ores was determined by "ICP" (Inductively Coupled Plasma; JY 70 Plus, Jobin Yvon) analyses of the supernatant from centrifuged culture samples.

Quantitative determination of sulfate. Sulfate was determined gravimetrically as $BaSO_4$ according to *Williams* (1979). Simultaneously "soluble sulfur" was measured in the supernatant of the cultures by "ICP" analyses (JY 70 Plus, Jobin Yvon).

Lipid analyses. Lipids were extracted from dried cells according to *De Rosa* et al. (1983 a). The total lipid extract was treated with light petroleum (boiling point 40–70 °C). In order to analyze the quinone pattern, the soluble material was chromatographed on thin layer silica plates (TLC; solvent: light petroleum/diethyl ether 95:5, v/v). The insoluble light petroleum fraction contained the complex lipids. They were separated by TLC (solvent: chloroform/methanol/H_2O 65:25:4 by vol.) and hydrolyzed in 1 M methanolic HCl to cleave the polar head groups. After methanolysis, the different core lipids were separated by TLC with the following solvents (a) 2,3-di-O-phytanyl-*sn*-glycerol ($C_{20,20}$ diether) in chloroform/diethyl ether 95:5, v/v; (b) glycerol-dialkyl-glycerol tetraethers (GDGTs) in n-hexane/ethyl acetate 7:3, v/v (*Trincone* et al., 1988); (c) gycerol-dialkyl-nonitol tetraethers (GDNTs) in chloroform/methanol 9:1, v/v (*De Rosa* et al., 1983 a). Purified samples of caldariellaquinone, sulfolobusquinone, tricyclicquinone (*Lanzotti* et al., 1986), complex lipids, $C_{20,20}$ diether, GDGTs and GDNTs from *Sulfolobus solfataricus* ATCC 49155 were available for comparison by TLC (*Lanzotti* et al., 1986; *De Rosa* et al., 1983 a; b). All the compounds were detected by exposure to I_2 vapour, or by spraying with 0.1% $Ce(SO_4)_2$ in 2 N H_2SO_4 reagent, followed by heating at 150 °C for 5 min. The Dittmer and Lester reagent was used for phospholipids and α-naphtol/H_2SO_4 for glycolipids.

Preparation of DNA. The DNA was prepared as described previously (*Wildgruber* et al., 1982).

Determination of the GC-content. The GC-content of the DNA was determined by the T_M-method (*Marmur* and *Doty*, 1962) and by high performance liquid chromatography (HPLC) of the nucleotides (*Zillig* et al., 1980).

DNA-DNA hybridization. DNA-DNA hybridization was carried out according to *König* (1984).

Purification of DNA-dependent RNA polymerase. The RNA polymerases of *S. acidocaldarius* and of isolate TH2 were prepared by the procedure described by *Zillig* et al. (1979): (a) treatment with Polymin P, (b) chromatography on DEAE cellulose, (c) chromatography on heparin cellulose, (d) affinity chromatography on calf thymus DNA cellulose and (e) sucrose gradient ultracentrifugation. All steps were carried out in a buffer containing Tris-HCl (50 mM; pH 7.5), NH_4Cl (22 mM), EDTA (1 mM), 2-mercaptoethanol (10 mM) and 10% (v/v) glycerol. The activity of the enzymes was determined in a standard assay using poly[d(A-T)] as a template.

Polyacrylamide gel electrophoresis. Gel electrophoresis was carried out in a 5–25% exponential sodium dodecylsulfate polyacrylamide gel (*Laemmli*, 1970; *Mirault* and *Scherrer*, 1971).

Preparation of RNA polymerase antibodies. A rabbit was

immunized with a total of 180 µg of purified RNA polymerase from *S. acidocaldarius* using a micro method (*Stetter,* 1977).

Ouchterlony immunodiffusion test. The immunological cross-reaction of the antiserum against the RNA polymerase of *S. acidocaldarius* was assayed in the immunodiffusion test of *Ouchterlony* (1962).

Results

Isolation

Six aerobic samples of water, sediments and greyish mud were taken from an acidic drain from a hot water pond at Pisciarelli Solfatara (near Naples, Italy). The original temperatures were between 25 and 52 °C and the pH was around 2. The sediments consisted of whitish gravel covered with orange-, red-, or yellow-coloured layers at the surface. These coats contained mainly iron compounds like magnetite or hematite (*D. Rose,* pers. comm.). All samples were carried to the laboratory without pH- and temperature-control. The modified mineral medium of *Allen* (*Allen,* 1959; *Brock* et al., 1972) containing pyrite, chalcopyrite or the ore mixture "G1N" as sole energy source was inoculated with each 1 ml of these samples. The enrichment cultures were incubated at 65 °C under shaking. After one week, spherical cells became visible in two (TH2 and TH4) of the six culture attempts. The enrichment cultures were purified by repeated serial dilutions in the mineral medium containing pyrite (TH2) or "G1N" (TH4). For the isolates, the same designations as for the samples were used. Another isolate, strain SP3a, enriched and isolated from the same location on a mixture of elemental sulfur and yeast extract, was unable to grow on sulfidic ores when transferred directly from the isolation medium. However, it was capable of adapting during the following procedure (*C. Brierley,* pers. comm.): (a) incubation in a medium containing elemental sulfur (1%), ferrous sulfate (1%) and yeast extract (0.1%); (b) transfer and incubation in a medium containing ore mixture "G1N" (3.3%), ferrous sulfate (1%) and yeast extract (0.005%); (c) transfer and incubation in a medium containing "G1N" (3.3%) and ferrous sulfate (1%); (d) transfer and incubation in a medium containing only "G1N" (3.3%). The final cell densities were about 8×10^7/ml for steps (a) to (c) and 2×10^8/ml in the final step (ore mixture alone).

Morphology

In the phase contrast microscope cells of the three isolates appeared as round to slightly irregular cocci about 1 µm in width (Fig. 1). During growth on ores, up to 50% of the organisms were attached to the particles. In the fluorescence microscope a wiggling of the cells at the ore was observed after visualization by "DAPI" staining (*Huber* et al., 1985). In the electron microscope pilus-like structures protruding from the cells were detected (Fig. 2). They were about 20 nm wide and up to 3 µm long. These structures were not observed in cultures grown on yeast extract. The cells were Gram-negative. They were surrounded by a cell envelope consisting of protein subunits in regular arrangement (Fig. 3, 4).

Culture and storage

Cultures grown on ores were routinely transferred into fresh medium after one week (5% inoculum) of incubation. When stored over liquid nitrogen they served as inoculum for at least 18 months. It was not necessary to neutralize or concentrate the grown culture for successful storage.

Growth temperature and pH range

On ores, isolate TH2 grew between pH 1.0 and 4.5 (not shown). The optimal growth temperature was around 75 °C (Fig. 5; doubling time 315 min). No growth occurred at 45 °C and at 85 °C.

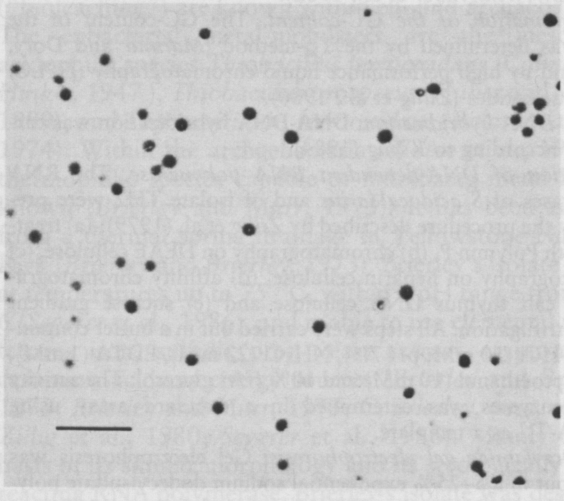

Fig. 1. Phase contrast micrograph of cells of *Metallosphaera sedula* (isolate TH2). Bar 5 µm.

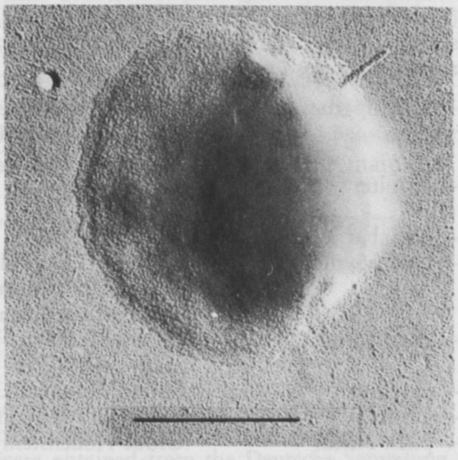

Fig. 2. Electron micrograph of *Metallosphaera sedula* (isolate TH2), platinum shadowed. Bar 0.5 µm.

Fig. 3. Electron micrograph of the cell surface of *Metallosphaera sedula* (isolate TH2). Bar 0.5 μm.

Metabolic properties

The new isolates grew aerobically on single sulfidic ores like pyrite, chalcopyrite and sphalerite and on combinations of them (ore mixtures "G6" and "G1N"). Growth was also obtained on the synthetic sulfides CdS, SnS and ZnS and on elemental sulfur. Arsenopyrite, bornite, cinnabar, chalcocite, covellite or galena and the synthetic sulfides CuS, FeS, MoS_2 and Sb_2S_3 did not serve as substrates. During growth on elemental sulfur, sulfuric acid was formed by the isolate TH2 (final concentration: 45 mM after 20 days; Fig. 6). The presence of yeast extract (0.005%) did not change the production rate of sulfate significantly (Fig. 6). The new isolates were able to grow on complex organic substrates such as beef extract, casamino acids, peptone, tryptone and yeast extract. No growth was obtained on sugars.

Under anaerobic conditions in the presence of hydrogen and elemental sulfur, neither growth nor H_2S production occurred (*A. Segerer*, pers. comm.).

Resistance against heavy metal ions

The resistance of isolate TH2 against different heavy metal ions was examined during growth on ore mixture "G6" and on yeast extract (Table 1). No differences between autotrophic and heterotrophic cultures were observed for arsenic, cadmium, cobalt, copper, antimony, uranium and zinc ions. Cells grown on ore tolerated higher concentrations of mercury (100 times) and molybdenum (10 times). As a reference *Thiobacillus ferrooxidans* ATCC 23270 was used. Isolate TH2 showed higher resistance against cadmium and molybdenum. The resistance against arsenic and uranium ions was similar for both

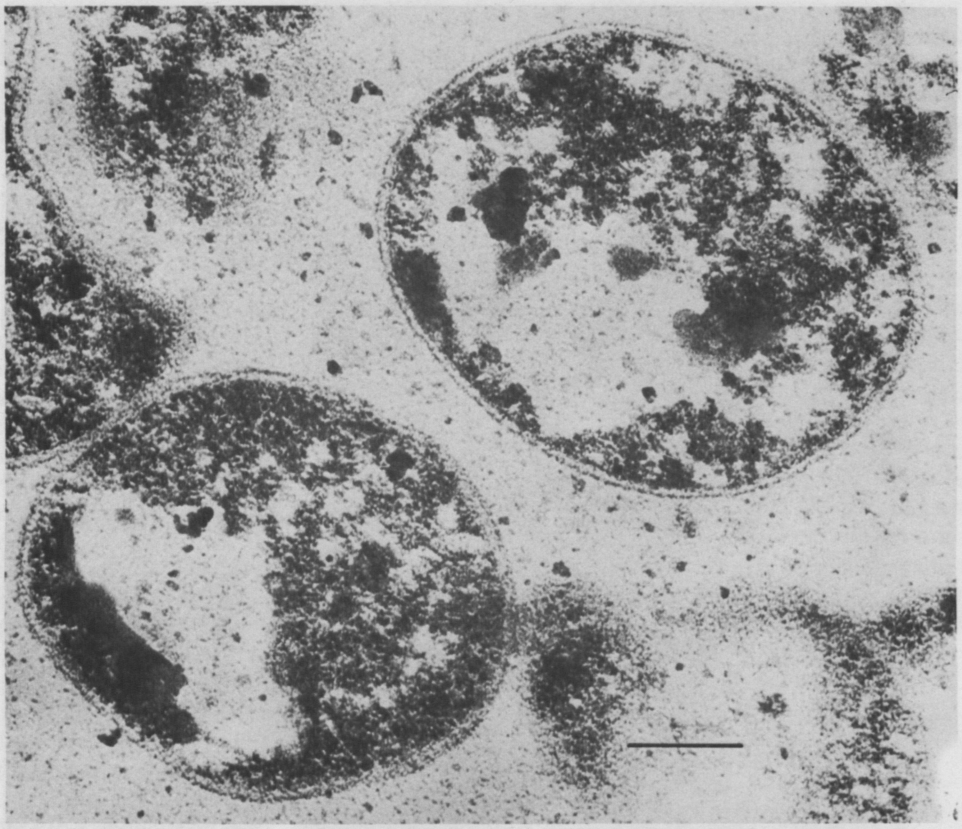

Fig. 4. Thin section of *Metallosphaera sedula* (isolate TH2) contrasted with lead citrate and uranyl acetate. Bar 0.2 μm.

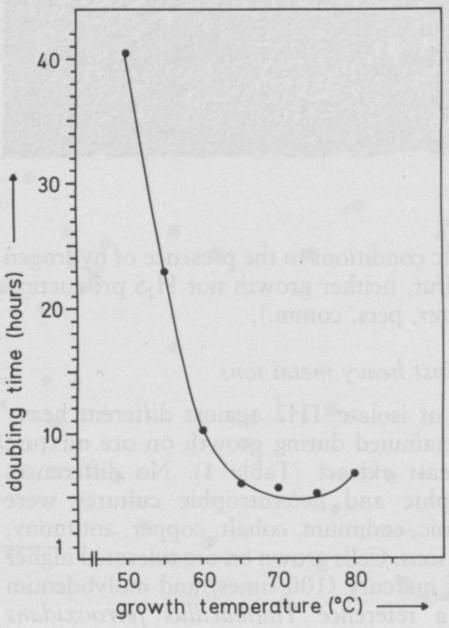

Fig. 5. Optimal growth temperature of *Metallosphaera sedula* (isolate TH2). The doubling times were calculated from the slopes of the growth curves (not shown).

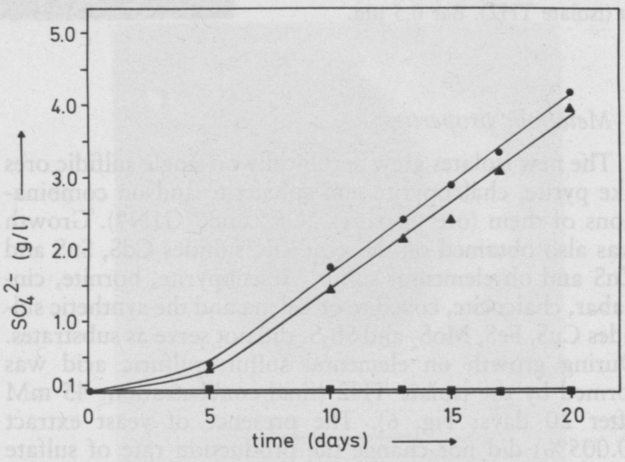

Fig. 6. Sulfate production of *Metallosphaera sedula* (isolate TH2) during growth on elemental sulfur.
(●–●) elemental sulfur; (▲–▲) elemental sulfur and yeast extract (0.005%); (■–■) medium without substrate.

Table 1. Tolerances of isolate TH2 and *Thiobacillus ferrooxidans* against heavy metal ions (mM)

Metal \ Strain	TH2 (YE)	TH2 (ore)	*T. ferrooxidans**
Ag	n.d.	0.09	0.9
	(n.d.)	(0.9)	(i.d.)
As	1.3	1.3	1.3
	(13)	(13)	(13)
Cd	0.9	0.9	0.09
	(9)	(9)	(0.9)
Co	0.85	0.85	17
	(1.7)	(1.7)	(85)
Cu	16	16	160
	(79)	(79)	(790)
Hg	0.0005	0.05	0.5
	(0.005)	(0.5)	(i.d.)
Mo	0.1	1	0.1
	(1.0)	(10)	(1)
Sb	0.8	0.8	8
	(8)	(8)	(i.d.)
U	0.4	0.4	0.4
	(4)	(4)	(4)
Zn	150	150	750
	(750)	(750)	(1500)

n.d. = not determined;
i.d. = impossible to determine due to precipitation;
( ) = concentration of total inhibition;
* *Huber* and *Stetter* (1989)

Table 2. Metal extraction by the new isolates and by the *Acidianus* and *Sulfolobus* type strains from the ore mixture "G1N" after 21 days of incubation (mg/l)

Strain \ Metal	Cu	Fe	U	Zn
TH2	750	40	90	4000
TH4	610	25	90	2400
SP3a	540	25	85	2000
A. brierleyi	140	20	80	1400
sterile control # 1	45	480	65	800
A. infernus	10	550	85	1200
sterile control # 2	4	300	75	300
S. acidocaldarius	1	110	20	170
S. solfataricus	1	100	30	270
sterile control # 3	1	120	20	260
total amount°	800	6800	95	4500

Sterile controls were carried out under the same pH and temperature conditions as the corresponding strains:
1: pH 2.5; T = 65 °C; # 2: pH 2.5; T = 80 °C; # 3: pH 5; T = 65 °C; ° = determined by chemical extraction with concentrated aqua regia. The type strains of *Acidianus* and *Sulfolobus* were cultivated in media containing yeast extract (0.02%) in addition to the ore mixture "G1N".

organisms. Against silver, cobalt, copper, mercury, antimony and zinc ions, the new isolate was more sensitive than *T. ferrooxidans*.

Ore leaching capacity

During growth on the ore mixture "G1N", isolate TH2 mobilized up to 100% of the total copper, uranium and zinc within 2 to 3 weeks (Table 2). For the *Sulfolobus* type strains, growth in the presence of ore was only obtained in combination with yeast extract. Within three weeks of incubation, the pH raised from 2.5 to 5.0. Concomitantly, no microbial metal extraction was evident when compared with the sterile control test (Table 2). As expected, *Acidianus brierleyi* grew autotrophically on ore mixtures (e.g. "G1N"). However, the final cell concentrations were rather low (about 1×10^7 cells/ml). They were raised about 10 times by the addition of low amounts of yeast extract (0.02%). Metal ion extraction continued in the presence of yeast extract (Table 2). *Acidianus infernus* was also able to grow autotrophically on ore mixtures (not shown), but metal mobilization with or without organic material was rather low (Table 2).

Lipid composition

The isolates TH2 and SP3a showed a lipid pattern (core and complex lipids) very close to that reported for *Sulfolobus solfataricus* (*De Rosa* et al., 1983 a, b). However, the relative proportions of glycolipids and minor complex lipids were different. Both strains contained essentially caldariellaquinone, which is a typical component of the membrane of *S. solfataricus* (*Lanzotti* et al., 1986). Sulfolobusquinone and tricyclicquinone were absent (*Lanzotti* et al., 1986; *Thurl* et al., 1986).

GC-content of the DNA

The three isolates TH2, TH4 and SP3a exhibited GC-contents of 45, 45 and 44 mol % (Table 3). The DNA base composition of the *Acidianus* and *Sulfolobus* type strains served as references (Table 3).

Sensitivity to diphtheria toxin

After addition of diphtheria toxin to the crude extract of isolate TH2, a protein, most likely elongation factor II, was ADP-ribosylated (*F. Klink*, pers. comm.; *Kessel* and *Klink*, 1980).

Table 3. GC-content of the new isolates and of the *Acidianus* and *Sulfolobus* type strains

Strain	T_M	GC-content (mol %) direct analysis	literature
TH2	44.6	46.7	–
TH4	44.1	46.1	–
SP3a	43.7	n.d.	–
A. brierleyi	30.5	30.0	31*
A. infernus	30.5	33.0	31**
S. acidocaldarius	35.6	36.7	38*
S. solfataricus	34.0	33.7	35*

* *Zillig* et al. (1980); ** *Segerer* et al. (1986); n.d. = not determined.

DNA homology

DNA – DNA cross hybridization experiments between the new strains revealed homologies of 94% and above indicating that they belong to the same species (Table 4). As was expected cultures of isolate TH2, grown on ores and on yeast extract, were 100% homologous (within the accuracy of the method; Table 4). No specific relationship of the new organisms could be detected with the *Sulfolobus* and *Acidianus* type strains as indicated by insignificant hybridization rates of 12% and below (*Steigerwalt* et al., 1976; *Schleifer* and *Stackebrandt*, 1983; Table 4).

Table 4. DNA-DNA homologies between the new isolates and the *Acidianus* and *Sulfolobus* type strains (in %)

Filter-bound DNA from	^{32}P-labelled DNA from					
	A. brier.	*S. acido.*	*S. solf.*	TH2 ore	TH2 YE	TH4 YE
A. brier.	100	5	9	7	10	n.d.
A. infernus	6	3	5	6	n.d.	n.d.
S. acido.	4	100	12	12	n.d.	n.d.
S. solf.	7	9	100	8	n.d.	n.d.
TH2 ore	12	8	10	100	100	n.d.
TH2 YE	10	n.d.	n.d.	98	100	94
TH4 YE	n.d.	n.d.	n.d.	n.d.	97	100

n.d. = not determined; YE = yeast extract;
A. brier. = *A. brierleyi* DSM 1651; *S. acido.* = *S. acidocaldarius* DSM 639; *S. solf.* = *S. solfataricus* DSM 1616.

Fig. 7. Subunit patterns of the RNA polymerases after sodium dodecylsulfate polyacrylamide gel electrophoresis.
(1) *S. acidocaldarius* DSM 639; (2) *Metallosphaera sedual* (isolate TH2).

DNA-dependent RNA polymerase

In the sodium dodecylsulfate polyacrylamide gel the DNA-dependent RNA polymerase of isolate TH2 exhibited the "BAC" pattern characteristic for sulfur-metabolizing archaebacteria (Fig. 7; *Schnabel* et al., 1983). The RNA polymerase of *Sulfolobus acidocaldarius* was used as molecular weight standard (*Prangishvilli* et al., 1982). The molecular weights of the subunits of the RNA polymerase of isolate TH2 were calculated after coelectrophoresis with the *S. acidocaldarius* enzyme (Table 5). It consisted of two heavy subunits (130 and 104 kD) and 9 light polypetide chains with molecular weights between 42 and 11.3 kD. In the Ouchterlony immunodiffusion test (Fig. 8), antibodies prepared against the purified RNA polymerase of *S. acidocaldarius* yielded an incomplete cross-reaction with the enzyme of isolate TH2 and with the enriched RNA polymerases of *S. solfataricus* and *A. brierleyi*. As was expected, no serological cross-reaction with a member of the *Thermoproteales, Pyrobaculum islandicum*, was obtained (not shown).

Table 5. Molecular weights (in kilodaltons) of components of the DNA-dependent RNA polymerases of *Sulfolobus acidocaldarius* and *Metallosphaera sedula* (isolate TH2)

Subunit* \ Strain	*Sulfolobus acidocaldarius*°	*Metallosphaera sedula*
B	122	130
A	101	104
C	44	42
D_1	33	33.5
D_2	32	32
E	26	24.5
F	17.5	15.8
G	13.8	14.5
H	11.8	13
I	11.2	11.8
J	10.8	11.3

° Molecular weights according to *Prangishvilli* et al. (1982)
* Designations in analogy to *S. acidocaldarius* following the molecular sizes.

Discussion

The three new isolates TH2, TH4 and SP3a are spherical to slightly irregular thermophilic organisms able to extract metal ions from sulfidic ores. They belong to the archaebacterial kingdom (*Woese* et al., 1978) as demonstrated by the occurrence of isopranyl ether lipids (*De Rosa* et al., 1977; *Langworthy* et al., 1982; *De Rosa* and *Gambacorta*, 1988), the presence of an ADP-ribosylable elongation factor II-like protein (*Kessel* and *Klink*, 1982), and the complex structure of the DNA-dependent RNA polymerase (*Zillig* et al., 1980; *Zillig* et al., 1982). By their acidophilic mode of life and the presence of benzothiophenquinone and glycerol-dialkyl-nonitol tetra-

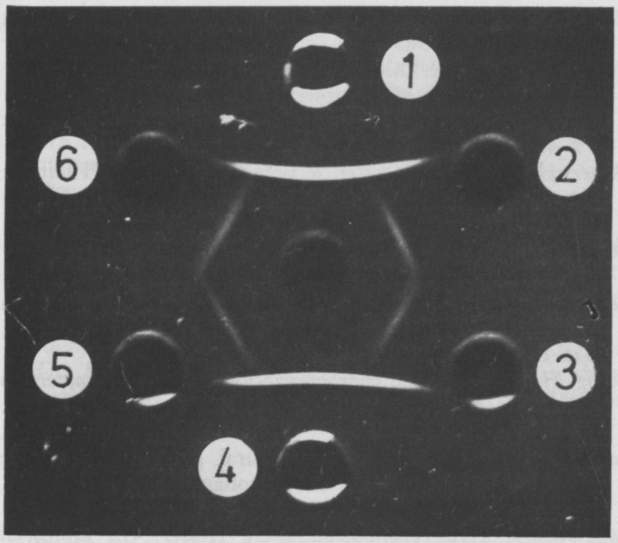

Fig. 8. Ouchterlony immunodiffusion test of antibodies against the DNA-dependent RNA polymerase of *Sulfolobus acidocaldarius* DSM 639. Purified or enriched RNA polymerases of (1, 4) *S. acidocaldarius*; (2) *Sulfolobus*-like isolate Kra23; (3) *Metallosphaera sedula* (isolate TH2); (5) *A. brierleyi*; (6) *S. solfataricus*.

ethers they resemble members of the genera *Sulfolobus* and *Acidianus* (*De Rosa* and *Gambacorta,* 1988). Due to their ore-leaching capacity and their ability to grow by oxidation of elemental sulfur and organic material, they are reminiscent of *Acidianus brierleyi* (*Brierley* and *Brierley,* 1973; *Segerer* et al., 1986). In contrast to *A. brierleyi,* the new isolates show a nearly round shape, grow at 80 °C and tolerate pH values up to 5. A further difference to all members of *Acidianus* is the inability of the isolates to grow anaerobically (*Segerer* et al., 1986). The new strains mobilize metal ions from sulfidic ores like chalcopyrite about 10 times faster than *A. brierleyi* and are therefore highly efficient ore leachers. In column leaching experiments on pyrite at 65 °C, their metal extraction rate was around 1 g/l × d which is more than 50 times higher than values of mesophilic metal-mobilizers e. g. *Thiobacillus ferrooxidans* ATCC 23270 at 30 °C (*G. Huber, H. Huber* and *K. O. Stetter,* unpublished). Therefore, the new strains may be suitable for reactor leaching and for in situ leaching of geothermally heated ore deposits. Isolates TH2, TH4 and SP3a can be further distinguished from members of the genus *Acidianus* by a 14 mol% higher GC-content of their DNA. Organisms with differences in the GC-content of more than 10% are usually believed to belong to different genera (*Suoka,* 1961; *Bradley,* 1971). By their strictly aerobic mode of life, the isolates resemble the two *Sulfolobus* species, *S. acidocaldarius* and *S. solfataricus*. Members of the genus *Sulfolobus* are characterized by their ability to oxidize elemental sulfur to sulfuric acid (*Brock* et al., 1972; *Zillig* et al., 1980). In contrast to the primary description, the type species *S. acidocaldarius,* represented by the type strain DSM 639, is unable to oxidize elemental sulfur and to form sulfuric acid (*Huber* and *Stetter,* unpublished). A similar result has been reported by *Marsh* et al. (1983) suggesting that the *S. acidocaldarius* type strain may have lost this property. In contrast to our new isolates, *S. acidocaldarius* and *S. solfataricus* are not able to grow on sulfidic ores and to mobilize metal ions. Strains TH2, TH4 and SP3a can be further distinguished from the *Sulfolobus* species by their inability to grow on sugars and their DNA not hybridizing with *Sulfolobus*. The incomplete cross-reaction of antibodies against the RNA polymerase of *S. acidocaldarius* with the enzyme of isolate TH2 indicates that the new strains are no members of the genus *Sulfolobus* (*Stetter* et al., 1981). Thermophilic organisms, growing on sulfur and yeast extract, with a GC-content similar to the new isolates have been reported by *Furuya* et al. (1977) and *Golovacheva* et al. (1987). These strains were not available to us and nothing is known about their taxonomic position and their ability to mobilize metal ions. Due to their morphology, physiology and biochemical characteristics, strains TH2, TH4 and SP3a represent a new genus, *Metallosphaera,* the "metal-mobilizing sphere". The three isolates share the same physiological properties and the DNA homologies between each other exceed 94%. Therefore they are strains of the same species. In consequence of their strong ore-leaching activity, we name the new species *Metallosphaera sedula*. The type species is *Metallosphaera sedula* DSM5348. Like *A. brierleyi* (*Brierley* and *Brierley,* 1973), *Metallosphaera sedula* was isolated from a continental solfataric field. In their natural habitat, H_2S is present due to the volcanic activity (*Hohl,* 1985). With heavy metal ions, metal sulfides may be formed, which then serve as natural substrates for ore-leaching bacteria. In accordance with this suggestion, numerous pyrite crystals (up to about 5 mm in length) were observed in the bluish-grey mud (80 °C; pH 4) of a solfataric field close to Cape Reykjanes, Iceland (*K. O. Stetter,* unpublished).

Description of a novel genus and one new species

Metallosphaera gen. nov. *Me.tal.lo.sphae'ra.* L. neut. n. metallum, ore; L. fem. n. sphaera, sphere; M.L. fem. n. *Metallosphaera,* the metal-mobilizing sphere.

Cells are regular to slightly irregular Gram-negative cocci, about 0.8 to 1.2 µm in width. Growth between 50 °C and 80 °C (optimum around 75 °C) and pH 1.0 to 4.5. Aerobic, facultatively chemolithoautotrophic. Lithotrophic growth on sulfidic ores like pyrite, sphalerite and chalcopyrite and on elemental sulfur. Production of sulfuric acid. No sulfur reduction by molecular hydrogen. Organotrophic growth on beef extract, casamino acids, peptone, tryptone and yeast extract, no utilization of sugars. Cell envelope composed of protein subunits, isopranyl ether lipids and caldariellaquinone in the cell membrane. ADP-ribosylation of an elongation factor II-like protein by diphtheria toxin. 45 mol % GC-content of the DNA. DNA homology below 12% to the type strains of *Acidianus infernus* DSM 3191 and *Sulfolobus acidocaldarius* DSM 639. RNA polymerase exhibits the "BAC" type like the thermophilic sulfur metabolizing archaebac-

teria and shows incomplete cross-reaction with antibodies against the enzyme of *S. acidocaldarius*. The molecular weights of the subunits are (in kilodaltons) 130, 104, 42, 33.5, 32, 24.5, 15.8, 14.5, 13, 11.8, 11.3 as determined by sodium dodecylsulfate polyacrylamide gel electrophoresis.

Metallosphaera sedula sp. nov., *se'du.la.* L. adj. *sedulus*, busy, describing the efficient metal mobilization. Description as for the genus.

Isolated from a continental solfataric field.

Type species is *Metallosphaera sedula*, TH2, DSM5348, Braunschweig, FRG

Acknowledgement. The excellent technical assistance of *Lucia Schwarzfischer* and *Silvia Sur* is greatly appreciated. We wish to thank *F. Klink* for providing unpublished data.

This work was supported by grants of the Bundesministerium für Forschung und Technologie (BMFT, Projektleitung Rohstoffforschung, Förderungskennzeichen 03 C 142 0 and 03 R 085 A 2) and by the Fonds der Chemischen Industrie.

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Prof. Dr. *K. O. Stetter,* Lehrstuhl für Mikrobiologie der Universität Regensburg, Universitätsstr. 31, D-8400 Regensburg, Federal Republic of Germany

System. Appl. Microbiol. 12, 48–55 (1989)

Nucleic Acid Hybridization Studies on *Leuconostoc* and Heterofermentative Lactobacilli and Description of *Leuconostoc amelibiosum* sp. nov.

ULRICH SCHILLINGER[1], WILHELM HOLZAPFEL[2], and OTTO KANDLER[3]

[1] Bundesanstalt für Fleischforschung, E. C. Baumann-Str. 20, 8650 Kulmbach, Federal Republic of Germany
[2] Bundesforschungsanstalt für Ernährung, Engesserstraße 20, 7500 Karlsruhe 1, Federal Republic of Germany
[3] Botanisches Institut der Universität München, Menzingerstraße 67, 8000 München 19, Federal Republic of Germany

Received January 20, 1989

Summary

DNA-DNA and DNA-rRNA hybridizations were carried out with the type strains of all known *Leuconostoc* species and the taxonomically exceptional species of the heterofermentative lactobacilli which possess some phenetic characteristics unusual in lactobacilli but common in leuconostocs, e.g. particular chemotypes of peptidoglycan and slime formation. The results confirm the genomical relationship of the exceptional species of the heterofermentative lactobacilli with particular species of the genus *Leuconostoc* as well as the isolated position of the acidophilic species *Leuconostoc oenos* within the genus *Leuconostoc*.

Strains which have been tentatively named *Leuconostoc mesenteroides* subsp. *"amelibiosum"* were found to be only distantly related with any of the known *Leuconostoc* species and are described as *Leuconostoc amelibiosum* sp. nov.

Key words: *Leuconostoc* – *Lactobacillus* – *Leuconostoc amelibiosum* – Lactic acid bacteria – Nucleic acid hybridization – Relationship – Taxonomy – Peptidoglycan types

Introduction

Apart from its typically irregular coccoid morphology, the genus *Leuconostoc* is distinguished from the gas-forming heterofermentative lactobacilli mainly by two phenetic criteria: its inability to produce ammonia from arginine and the formation of only D(-)-lactate from glucose (*Garvie*, 1984; 1986). However, some taxonomically exceptional species of the heterofermentative lactobacilli, such as *Lactobacillus viridescens* and *Lactobacillus fructosus*, do not hydrolyze arginine and form predominantly D(-)-lactate. Moreover, the cell wall of such lactobacilli contains a peptidoglycan type similar to that of the leuconostocs (*Kandler*, 1970; *Kandler* and *Weiss*, 1986). In contrast with most heterofermentative lactobacilli, where the crosslinkage of the peptidoglycan subunits is mediated by an asparagine residue (L-Lys-D-Asp type), the amino acids alanine and serine are found to be typical constituents of the interpeptide bridge in the exceptional lactobacilli and in leuconostocs (Table 1). For instance, the peptidoglycan type of *Leuconostoc mesenteroides* (Lys-Ser-Ala_2) was also found in *Lactobacillus minor* (*Kandler* et al., 1983) while *Lb. viridescens* and *Lactobacillus halotolerans* contain the same type of peptidoglycan as *Leuconostoc oenos* (Lys-Ala-Ser) (*Kandler* et al., 1967). Some of the exceptional lactobacilli may share additional characteristics with leuconostocs, e.g. formation of very short coccoid rods, production of slime from sucrose, the same electrophoretic mobility or an immunological relatedness of various enzymes, such as pyruvate reductase (*Sharpe* et al., 1972), D(-)lactate dehydrogenase (*Gasser* and *Gasser*, 1971; *Garvie*, 1975), glucose-6-phosphate dehydrogenase (*Gasser* and *Hontebeyrie*, 1977). These findings indicate a specific relationship between the leuconostocs and the taxonomically exceptional heterofermentative lactobacilli.

This paper reports on DNA-DNA and DNA-rRNA hybridization studies among strains of the various species of *Leuconostoc* and some taxonomically exceptional heterofermentative lactobacilli.