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Thiobacillus plumbophilus spec. nov., a novel galena and hydrogen oxidizer

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Abstract. From an uranium mine three strains of rod-shaped, mesophilic, chemolithoautotrophic bacteria were isolated. They grow by oxidation of H_2S, galena (PbS) and H_2. Anglesite ($PbSO_4$) is formed from galena. No ferrous iron is oxidized by the isolates. They grow between pH 4 and 6.5 at temperatures of about 9 to 41 °C (optimum around 27 °C). The G+C content of the DNA is around 66 mol %. Based on their ability to oxidize sulfur compounds, the new organisms belong to the genus *Thiobacillus*. No significant homology with *Thiobacillus ferrooxidans* and *Thiobacillus cuprinus* was detected by DNA-DNA hybridization. Therefore the new isolates represent a new species within the genus *Thiobacillus*. Based on the unusual growth on galena, we name the new species *Thiobacillus plumbophilus* (type strain Gro7; DSM 6690).

Key words: *Thiobacillus* – Galena – Hydrogen oxidation – Chemolithotrophic

Members of the bacterial genus *Thiobacillus* are characterized by their ability to oxidize reduced sulfur compounds (Vishniac 1974). Different species exhibit further physiological properties like oxidation of ferrous iron (Colmer and Hinkle 1947), aerobic hydrogen oxidation (Drobner et al. 1990), nitrate reduction (Baalsrud and Baalsrud 1954) and growth on organic substrates (Guay and Silver 1985; Huber and Stetter 1990). *T. ferrooxidans* (Colmer and Hinkle 1947), *T. prosperus* (Huber and Stetter 1989) and *T. cuprinus* (Huber and Stetter 1990) are able to oxidize the sulfur moiety of sulfidic ores and, as a result of this property, are important organisms in bioleaching (Kelly 1988).

The leaching-efficiency of *T. ferrooxidans* parallels the solubility of the metal sulfide offered (Torma and Sakaguchi 1978). Therefore weakly soluble sulfides like cinnabar (HgS) and galena (PbS) are only slowly attacked by this organism. According to Kingma and Silver (1980) the oxidation of galena by *T. ferrooxidans* is enhanced in the presence of ferrous iron and elemental sulfur.

Here we describe the isolation and properties of a novel group of organisms which oxidize PbS, H_2S and H_2.

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Materials and methods

Strains

The type strain of *Thiobacillus ferrooxidans* (ATCC 23270) was obtained from the American Type Culture Collection (ATCC), Rockville, MD.. *T. cuprinus* (DSM 5495) was isolated in our own laboratory (Huber and Stetter 1990).

Culture conditions

T. ferrooxidans was grown in "9K"-medium (Silverman and Lundgren 1959), *T. cuprinus* in the mineral salts medium "M1" (Huber et al. 1986), adjusted to pH 3.5 with sulfuric acid. If not mentioned otherwise the new isolates were cultivated in "9K"-medium adjusted to pH 4.0 and supplemented with synthetic lead sulfide (PbS; 0.3 g/30 ml). All organisms were grown under shaking (100 rpm) in 100-ml Erlenmeyer flasks containing 30 ml medium.

Growth on H_2S was determined in a H_2S/oxygen gradient formed within soft agar. Five millilitres sterile "9K" agar-medium, pH 2, containing 0.025% Na_2S and 2% agar were filled into 28-ml rolltubes (Schott Mainz, FRG). After solidification, a soft agar overlay consisting of 10 ml "9K" medium, pH 4, containing 0.2% agar was prepared. After cooling down to 53 °C, 0.5 ml of a culture of isolate Gro3, Gro7 or Gro8 were mixed into the liquid soft agar (final concentration: 5×10^5 cells/ml) and an overlay above the H_2S containing layer was poured. The tubes were sealed under air by stoppers and were incubated at 28 °C.

Ferrous sulfate (4%, w/v), sodium thiosulfate (0.5%), potassium tetrathionate (0.5%), elemental sulfur (0.05%), synthetic metal sulfides (Ag_2S, CdS, CuS, CuS_2, FeS, HgS, MoS_2, Sb_2S_3, SnS, ZnS; each 1.7%), natural ores (galena, pyrite, chalcopyrite, sphalerite, pitch blend, each 1.7%; Huber and Stetter 1990), complex organic substrates (yeast extract, meat extract, peptone, casamino acids; each 0.05%), sugars (e.g. arabinose, glucose, galactose, lactose; each 0.1%), amino acids (DL-alanine, L-glutamic acid, DL-valine; each 0.1%) and organic acids (formiate, acetate, lactate, malate, propionate, pyruvate; each 0.1%) were tested as possible substrates. Aerobic oxidation of molecular hydrogen was assayed in mineral

salts medium „M1" adjusted to pH 6.0. The culture conditions were described elsewhere (Drobner et al. 1990).

Batch cultures were grown in a 85 l enamel-protected fermentor (HTE, Bioengineering, Wald, Switzerland) either on PbS (30 g/55 l; gassing by 2 l of air per min) or on a mixture of H_2/CO_2/air (80 :20 : 3, 200 kPa).

Determination of growth

Growth was detemined by direct cell counting in a Thoma chamber (depth 0.02 mm).

Fluorescence microscopy

For the visualization of cells attached to solid particles a modified DAPI staining method was used (Huber et al. 1985).

Electron microscopy

For electron microscopy, cells were fixed with glutaraldehyde (2.5%) and formaldehyde (2%), applied to a carbon coated grid, washed with double distilled water and air dried. The grids were shadowed with platinum in an Edwards 306 shadowing unit.

For ultrathin sections, cells of an exponentially growing culture were fixed for 30 min by adding glutaraldehyde (0.5%) and formaldehyde (3%) to the culture medium. The harvested cells were resuspended in phosphate buffer (pH 6.1) supplemented with glutaraldehyde (0.5%) and formaldehyde (3%) and fixed again for 30 min. Cells were washed twice with Tris-buffer (pH 7.4), dehydrated successively with 50% ethanol, 70% ethanol, and a mixture of 95% ethanol plus LR Withe (1 + 2) at −20 °C and finally transferred into pure LR Withe (2 × 1 h at −20 °C, 1 × 12 h at 4 °C). The blocks were polymerized at 50 °C for 4 days. The ultrathin sections were stained with uranylacetate (2%) for 10 min and lead-citrate for 2 min. Electron micrographs were taken with a Philips CM12 electron microscope at an operating voltage of 100 kV.

Tolerance against heavy metals

Resistance to antimony, arsenic, cadmium, cobalt, copper, molybdenum, nickel, silver, uranium and zinc was determined in the presence of PbS. Stock solutions and tested concentrations were the same as described (Huber and Stetter 1989, 1990). Tolerance against iron was tested with and without PbS. $FeSO_4$-solution was added with the final Fe^{2+}-concentrations (mmol/l) 0.02, 0.2, 2, 4.

Quantitative determination of sulfate

Sulfate was determined gravimetrically after precipitation by $BaCl_2$ (Williams 1979).

Determination of hydrogenase activity

Cultures grown to stationary phase were resuspended in hydrogenase buffer. To destroy the cell wall, lysozyme solution was added (1000 U; 35 °C; 30 min). Hydrogenase activity was determined photometrically measuring the reduction of methylene blue (Schink and Schlegel 1979; Segerer et al., in preparation).

Hydrogen analysis

H_2 was analysed on a Hewlett Packard 5890 gas chromatograph (stainless steel column, packed with Molecular Sieve 5A, Supelco; oven temp. 140 °C, inject. temp. 190 °C, detector temp. 200 °C, detector TCD).

NMR analysis

To identify the product of aerobic hydrogen oxidation H_2 was substituted by D_2 and HDO was determined by NMR spectroscopy using a Bruker MSL300 NMR spectrograph.

Isolation of DNA

DNA was prepared as described earlier (Wildgruber et al. 1982).

DNA base composition

The G+C content of the DNAs was determined by melting point analysis in 0.1 × SSC (Marmur and Doty 1962) and by HPLC chromatography of the nucleotides after digestion of the DNAs with nuclease P1 (Zillig et al. 1980). Calf thymus DNA (G+C content 42 mol%) was used as reference.

DNA-DNA homology

DNA-DNA hybridizations were performed (König 1984) after radioactive in-vitro labelling of the DNA by nick translation (Kelly et al. 1970) using the filter technique (Gillespie and Gillespie 1971; Birnstiel et al. 1972).

Results

Enrichment and isolation

Aerobic samples of waters, sediments and ore particles with an original temperature of 11 °C and a pH-value of 6.5 were taken in an uranium mine near Großschloppen, FRG. In the laboratory sterile mineral medium supplemented with 0.5 g of the sulfidic ore galena (PbS) was inoculated with about 1 g of the material of different samples. After two weeks of incubation at 28 °C, rod-shaped organisms were visible in the enrichment attempts of the samples Gro3, Gro7 and Gro8. The enrichment cultures were purified by serial dilution carried out at least three times in the ore-containing medium. The isolates were designated the same as the samples.

Morphology

Tiny rods, either motile in suspension or attached to the metal sulfide (about 80%) were visible by phase contrast microscopy. The cells were up to 3 μm long and 0.15−0.25 μm in width. They possessed one polar flagellum (Fig 1). The cells showed a negative Gram-staining reaction. Ultrathin sections of cells grown on H_2 revealed a slime layer covering the cell wall (Fig. 2, arrow), possibly responsible for cell aggregation.

Storage

Cultures grown on PbS and stored at room temperature without shaking served as inocula for at least 1 year. Cells cultivated on PbS or H_2 kept their variability for at least 18 months when they had been stored at −20 °C or at −140 °C (liquid nitrogen; gas phase).

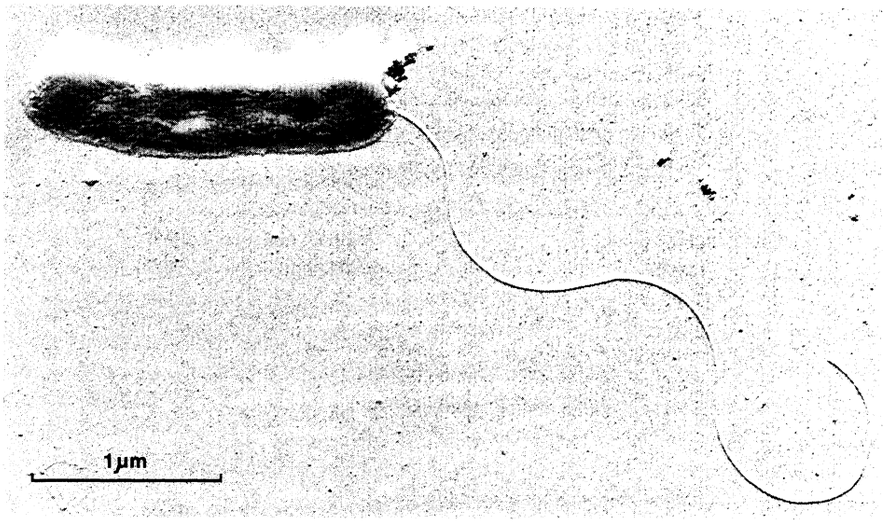

Fig. 1. Electron micrograph of *Thiobacillus plumbophilus* (isolate Gro7), shadowed unidirectionally with Pt

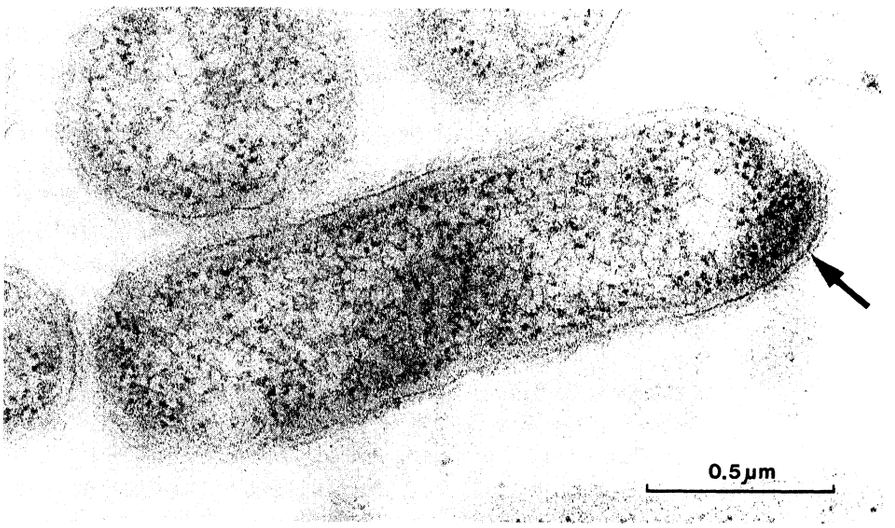

Fig. 2. Thin section of *Thiobacillus plumbophilus* (isolate Gro7), stained with uranylacetate and lead-citrate

Growth temperature and pH of growth

The three isolates Gro3, Gro7 and Gro8 grew up to 41 °C with a temperature optimum between 21 °C and 34 °C. The shortest doubling times were 24 h in the presence of PbS and 5 h in the presence of H_2. At 9 °C doubling time of cells grown on H_2 was 75 h. Growth below 9 °C was not determined. The new isolates grew between pH 4.0 and 6.5. On H_2S growth was only determined qualitatively.

Metabolism

The new isolates grew aerobically on natural galena (e.g. Clausthal, FRG) and on synthetic PbS. The only metabolic product was anglesite ($PbSO_4$). Elemental sulfur was not detectable (D. Rose, personal communication). Due to the low solubility of lead sulfate, no rise of sulfate concentration in the liquid phase and, therefore, no drop of pH occurred during growth. Growth was neither stimulated nor inhibited by the addition of organic substrates. Elemental sulfur, thiosulfate, tetrathionate, the synthetic metal sulfides Ag_2S, CdS, CuS, CuS_2, FeS, HgS, MoS_2, Sb_2S_3, SnS and ZnS, purified UO_2 and the natural ores pyrite, chalcopyrite, sphalerite, and pitch blend did not serve as substrates. No oxidation of Fe^{2+} to Fe^{3+} was detectable neither when $FeSO_4$ was the sole energy source nor in combination with PbS.

PbS could be replaced by H_2S, offered as a gradient within a soft agar column. The cells grew as a turbid ring, about 3 mm in height.

Alternatively the new isolates gain energy by aerobic hydrogen oxidation (Fig. 3). When H_2 was replaced by D_2, HDO was detectable by NMR spectroscopy (Fig. 4).

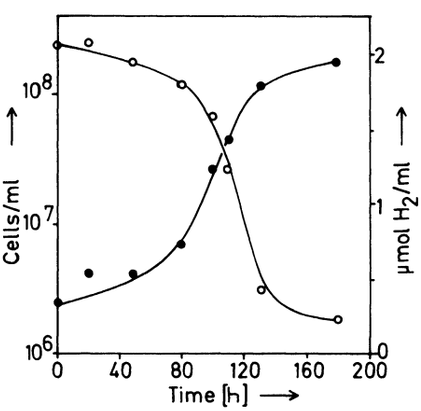

Fig. 3. Growth of *Thiobacillus plumbophilus* (isolate Gro7) by hydrogen oxidation. *Symbols:* ●, cell concentration; ○, H_2 concentration

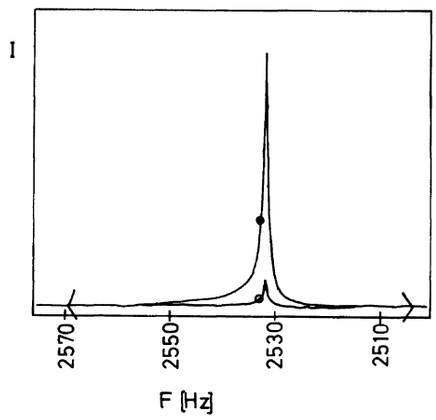

Fig. 4. NMR spectrograph of the liquid phases of cultures of *Thiobacillus plumbophilus* (isolate Gro7; ●; numerical value = 45.5) and uninocculated medium (○; numerical value = 6.1); gas phase D_2/CO_2/air. I = intensity; F = frequency; ⟨⟩ = integral

Resistance to antibiotics

Growth of the three isolates was totally inhibited by 50 µg/ml ampicillin and 100 µg/ml rifampicin.

Resistance to heavy metals

Isolate Gro7 grew in the presence of antimony, arsenic, cadmium, cobalt, copper, molybdenum, nickel, silver, uranium and zinc ions at 0.8, 1.3, 0.9, 1.7, 1.6, 1, 0.17, 0.4 and 1.5 mmol/l respectively (Table 1). In comparison to *Thiobacillus ferrooxidans*, isolate Gro7 was more resistant against ions of cadmium and molybdenum, while it was more sensitive against ions of antimony, cobalt, copper, nickel and zinc. Furthermore, the isolates did not tolerate Fe^{2+} concentrations higher 2 mmol/l.

Content of quinones

The new isolates contained ubiquinone 8 (CoQ_8) as their main quinone (96.5%). Ubiquinone 10 was not detectable (D. Collins, personal communication).

Table 1. Heavy metal ion tolerances of isolate Gro7 *(Thiobacillus plumbophilus)* and *T. ferrooxidans* (mmol/l)

Element	Isolate Gro7*		*T. ferrooxidans***	
	growth	no growth	growth	no growth
Ag	0.9	n.d.	0.9	n.d.
As	1.3	13	1.3	13
Cd	0.9	9	0.09	0.9
Co	1.7	17	17	85
Cu	1.6	16	160	790
Fe	2.0	4	250	n.d.
Mo	1	10	0.1	1
Ni	0.17	1.7	170	850
Sb	0.8	8	8	n.d.
U	0.4	4	0.4	4
Zn	1.5	15	750	1530

n.d. = not determined; precipitations
* grown on PbS
** grown on ore mixture „G1" (Huber and Stetter 1989)

Table 2. DNA-DNA homologies (%) between the new isolates Gro7 and Gro8 and *Thiobacillus* reference strains *T. ferrooxidans* and *T. cuprinus*

Filter-bound DNA from	^{32}P-labelled DNA from	
	Gro7	Gro8
Gro7	(100)	100
Gro8	100	(100)
T.f.	0	3
T.c.	7	0

T.f. = *Thiobacillus ferrooxidans*
T.c. = *Thiobacillus cuprinus*

DNA base composition

The G+C content of the DNA was determined for the isolates Gro7 and Gro8. The organisms exhibited a G + C content of around 66 mol %.

DNA-DNA hybridization

DNA-DNA hybridizations between isolates Gro7 and Gro8 exhibited a DNA homology of 100%. No significant homology could be detected between the new isolates and *Thiobacillus* reference strains (Table 2).

Discussion

The new isolates are mesophilic, aerobic, Gram-negative tiny rods gaining energy by the oxidation of inorganic sulfur compounds. Therefore they are members of the genus *Thiobacillus* (Vishniac 1974). By 16S rRNA sequencing, isolate Gro7 belongs to the beta subdivision of the purple bacteria which contains also other members of *Thiobacillus* (Burggraf and Woese, personal communication; Huber and Stetter 1990). Since the new organisms are obligate chemolithotrophs and possess UQ-8, they are members of the group III of *Thiobacillus* (Katayama-Fujimura et al. 1982). The metal-mobilizers *T. ferrooxidans* and *T. prosperus* belong to the same group. Similar to *T. ferrooxidans* the new isolates are facultative hydrogen oxidizers (Drobner et al. 1990). However, they are different by (a) their slender tiny rod-shape of the cells, (b) an 8% higher G+C content (66 mol %) of their DNA, (c) their moderate acidophily, (d) their unability to oxidize ferrous iron, and (e) the lack of significant DNA-DNA homology. Therefore, the new isolates represent a new species within the genus *Thiobacillus*. Due to its utilization of galena as sole sulfidic ore we name it *Thiobacillus plumbophilus*, the leadloving *Thiobacillus*. Compared to *T. ferrooxidans*, the apparent lead specifity of *T. plumbophilus* could be explained by its much higher sensitivity against various heavy metal ions. Heavy metal ions become usually soluble during growth of ore-leaching bacteria on sulfidic ores. Anglesite ($PbSO_4$), however, the product of galena oxidation, is only very weakly soluble (135 µmol/l at 20 °C) and, therefore does not affect growth of *T. plumbophilus*.

The natural biotope of *T. plumbophilus* is so far unknown. It could not be enriched from solfataric fields or sulfidic ore mines. Within the uranium mine close to Großschloppen, *T. plumbophilus* may grow on expense of lead compounds like galena which may have formed as decay products from uranium.

Description of a new species

Thiobacillus plumbophilus Drobner, Huber, Rachel, and Stetter, sp. nov. plum.bo'philus L. neut. n. plumbum, lead; Gr. verb. philein, to love; M. L. adj. plumbophilus loving lead, referring to its ability to grow with PbS as sole energy source.

Cells are rod-shaped, gram-negative, about 3 μm long and 0.25 μm in width, and are motile by one polar flagellum. Optimal growth between 21 °C and 34 °C and up to 41 °C. Growth between pH 4.0 and 6.5. Strictly chemolithoautotrophic and aerobic. Oxidation of galena (PbS), H_2S and H_2. Sensitive to ampicillin and rifampicin, possess 96.5% ubiquinone Q-8. G+C content of the DNA 66 mol %. 16S rRNA sequence data show *T. plumbophilus* to be a member of the beta subdivision of the purple bacteria. Insignificant DNA hybridization to *T. ferrooxidans* and *T. cuprinus*. Isolated from an uranium mine in Germany.

Type strain is *Thiobacillus plumbophilus*, Gro7, DSM 6690, Braunschweig, FRG.

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