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Thiobacillus prosperus sp. nov., represents a new group of halotolerant metal-mobilizing bacteria isolated from a marine geothermal field

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Abstract. From the shallow geothermally heated seafloor at the beach of Porto di Levante (Vulcano, Italy) 8 strains of long, tiny rods were isolated, which represent the first marine metal-mobilizing bacteria. Cells are Gram negative. They grow in a temperature range between 23 and 41°C with an optimum around 37°C at a salt concentration of up to 6.0% NaCl. The isolates are obligately chemolithotrophic, acidophilic aerobes which use sulfidic ores, elemental sulfur or ferrous iron as energy sources and procedure sulfuric acid. They show an upper pH-limit of growth at around 4.5. The G + C content of their DNA is around 64 mol%. Based on the results of the DNA-DNA hybridization they represent a new group within the genus *Thiobacillus*. Isolate LM3 is described as the type strain of the new species *Thiobacillus prosperus*.

Key words: *Thiobacillus* – Leaching – Chemolithotrophic – Acidophilic – Marine

The ability to grow chemolithoautotrophically on sulfidic ores is up to now restricted to *Thiobacillus ferrooxidans* (Colmer and Hinkle 1947), *Leptospirillum ferrooxidans* (Balashova et al. 1974) and to the archaebacterium *Acidianus brierleyi* (Brierley and Murr 1973; Segerer et al. 1986). During growth sulfuric acid is formed and heavy metals are solubilized („leaching").

The members of the genus *Thiobacillus* are Gram negative rod-shaped eubacteria, which are classified in three main groups based on the kind of energy metabolism (obligately or facultatively chemolithotrophic) and the possession of ubiquinone 10 or 8. They are further divided by the G + C content and the physiological properties of the different species (Katayama-Fujimura et al. 1982). All of them gain their energy by the oxidation of reduced sulfur compounds to sulfate (Vishniac 1974). *T. ferrooxidans* is able to grow also on water-insoluble metal sulfides and sulfidic ores like pyrite, chalcopyrite or sphalerite, performing the bacterial leaching process (Lundgren et al. 1986). *T. ferrooxidans* is further characterized by its ability to oxidize ferrous to ferric iron (Colmer and Hinkle 1947), enhancing the leaching process due to the strong oxidizing capacity of Fe^{3+} ("indirect leaching"). It was isolated from acid mine waters and from soils containing pyrite and marcasite (Vishniac 1974), but was not found in marine environments (Tuttle and Jannasch 1972). This corresponds to the inability of *T. ferrooxidans* to grow at salt concentrations above 1% (Razzell and Trussell 1963; Lazaroff 1963).

Here we describe a group of halotolerant marine metal-mobilizing rod-shaped thiobacilli which are different from *T. ferrooxidans*.

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Materials and methods

Strains

The type strains of *Thiobacillus ferrooxidans* (ATCC 23270), *Thiobacillus thiooxidans* (ATCC 19377) and *Thiobacillus perometabolis* (ATCC 23370) were obtained from the American Type Culture Collection, *Thiobacillus novellus* (DSM 506), *Thiobacillus neapolitanus* (DSM 581) and *Thiobacillus thioparus* (DSM 505) from the Deutsche Sammlung von Mikroorganismen, Braunschweig, FRG.

Culture conditions

T. ferrooxidans and *T. thiooxidans* were grown in "9K"-medium (Silverman and Lundgren 1959). *T. novellus* and *T. perometabolis* were cultivated in medium A (Katayama-Fujimura and Kuraishi 1980). For the cultivation of *T. thioparus* medium 2 (Starkey 1934) and for *T. neapolitanus* the *T. thioparus*-medium (Vishniac and Santer 1957) was used.

The new isolates were usually cultivated in the mineral salt medium "M1" (Huber et al. 1986), adjusted to pH 2.5 with sulfuric acid and supplemented with ore mixture "G1" (1 g/30 ml medium). "G1" consisted of equal parts of pyrite (Grube Bayerland, Oberpfalz), chalcopyrite (Bad Grund, Harz), sphalerite (Grube Lüderich, Nordrhein-Westfalen) and pitch blend (Grube Höhenstein, Oberpfalz). The particle size was below 1 mm. The mineral composition of these ores was:

Pyrite: 90% pyrite; traces of chalcopyrite, rutile and pyrrhotin;

Chalcopyrite: 85% chalcopyrite; 10% pyrite; traces of galena;

Sphalerite: 90% sphalerite; 5% galena; traces of pyrite and chalcopyrite;

Pitch blend: 5% pyrite; 1% pitch blend; traces of anastase, zircon and chalcopyrite;

In all ores the matrix consisted of silicates. Chemical composition of the ore mixture "G1" (1 g dissolved in 30 ml

aqua regia): As 45 ppm; Cd 14 ppm; Co 5 ppm; Cu 2000 ppm; Fe 6000 ppm; Ge 65 ppm; Hg 8 ppm; Mn 28 ppm; Pb 250 ppm; Th 5 ppm; Ti 3 ppm; U 90 ppm; Y 2 ppm; Yb 4 ppm; Zn 4000 ppm; Zr 4 ppm.

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

Ferrous sulfate (4%, w/v), sodium thiosulfate (0.5%), potassium tetrathionate (0.5%), sugars (e.g. glucose, galactose, saccharose; each 0.1%), yeast extract (0.05%), peptone (0.05%), elemental sulfur (0.05%), synthetic sulfides (Ag_2S, CdS, CuS, FeS, MoS_2, Sb_2S_3, SnS, ZnS; each 1.7%) and natural ores (arsenopyrite (FeAsS), Reichenstein, Poland; galena (PbS), Clausthal, FRG; each 3.3%) were tested as further possible substrates.

The salt tolerance of the organisms was determined in the presence of the ore mixture "G1" by adding sterile NaCl up to the final concentrations.

All organisms were grown aerobically under shaking (100 rev/min) in 100 ml Erlenmeyer flasks containing 30 ml medium. Large scale cultures were grown in 85 l enamel-protected fermentors (HTE, Bioengineering, Wald, Switzerland) under gassing with air (2 l/min) and stirring (150 rev/min). In these cultures the cell yield of the new isolates was about 1 g (wet weight)/100 l medium.

Light microscopy

The cultures were routinely observed with a Zeiss Standard 16 phase contrast microscope using an oil immersion objective 100/1.3. For the visualization of cells attached to ores a modified DAPI procedure (Huber et al. 1985) including the fluorescence equipment IV FL with an excitation filter BP 365 and a selection filter LP 420 was used.

Electron microscopy

Cultures, fixed with glutaraldehyde (2%), were dropped onto collodium coated grids and platinum shadowed at an angel of 7°. Thin sections were prepared according to Huber et al. (1982). Electron micrographs were taken with a Jeol JEM 100 C electron microscope.

Determination of growth

Bacterial growth was determined by direct cell counting in a "Thoma"-counting chamber, depth 0.02 mm.

Tolerance against heavy metals

Stock solutions or salts of different heavy metal ions were added after separate sterilization to the culture media in the following final concentrations:

salt	final metal ion concentrations (mM)
$AgNO_3$	0.0009; 0.009; 0.09; 0.9;
$NaAsO_2$	0.013; 0.13; 1.3; 13;
$(CH_3COO)_2Cd \times 2H_2O$	0.009; 0.09; 0.9; 9;
$CoSO_4 \times 7H_2O$	1.7; 17; 85;170; 850;
$CuSO_4 \times 5H_2O$	1.6; 16; 79; 160; 790;
$HgSO_4$	0.0005; 0.005; 0.05; 0.5;
$Na_2MoO_4 \times 2H_2O$	0.01; 0.1; 1; 10;
$NiSO_4 \times 6H_2O$	1.7; 17; 170; 850; 1700;
$SbCl_3$	0.008; 0.08; 0.8; 8;
$(CH_3COO)_2UO_2 \times 2H_2O$	0.004; 0.04; 0.4; 4;
$ZnSO_4 \times 7H_2O$	1.5; 15; 150; 750; 1500; 3000

Quantitative determination of sulfate

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

Test for diaminopimelic acid

Diaminopimelic acid was determined chromatographically (Rhuland et al. 1955).

Isolation of DNA

DNA of cells (0.5 g wet weight) was isolated according to Wildgruber et al. (1982).

DNA base composition

The G+C content of the DNAs was determined by the T_M-method in 0.1 × SSC (Marmur and Doty 1962) and by direct analysis of the nucleotides after digestion with nuclease P1 (Zillig et al. 1980) by HPLC chromatography.

DNA-DNA homology

DNA-DNA hybridization was 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).

Metal analysis

Concentrations of the elements, detected in the ore mixture "G1", were determined quantitatively in the aqua regia solutions or in the supernatant of the centrifugated cultures with an "ICP" (Inductively Coupled Plasma) instrument (Lab Test). The results of the bacterial leaching were the average from three parallel experiments. The variation of the extraction values was below 15%.

Results

Enrichment and isolation of the new organisms

Samples (20 ml) of sediments and waters were taken at different sites at the marine hydrothermal area at Porto di Levante, Vulcano, Italy (Table 1). In the laboratory, enrichment cultures were set up after the addition of about 1 g of samples into the sterile ore mixture "G"-containing mineral culture medium. After 2 weeks incubation at 37° C long thin rods became visible in the enrichment cultures from the samples L7, LM1, LM3, MSB9a, MSB11, MSB12, VC15 and VM17. The positive enrichments were purified by serial dilutions carried out at least three times in the ore-containing medium. The isolates were designated the same as the samples. Enrichment attempts from marine hydrothermal systems at Ischia, Naples, Iceland and Guaymas did not yield similar organisms (not shown).

Morphology

In the phase contrast microscope cells of the new isolates appeared as tiny rods, either motile in suspension or attached to the ore surface (about 50%). The organisms were up to 4 μm long and 0.2 – 0.4 μm wide (Fig. 1). The cells possessed

Table 1
Samples yielding positive enrichment cultures from the beach at Porto di Levante, Vulcano, Italy

Designation of sample	Sample taken from	Original temperature (°C)	Depth (m)
L7	Turbid water and small grey stones from a small spring	37	0.3
LM1	Light sand from a large spring	33	1
LM3	Coarse-grained sediment besides a large spring	35	1
MSB9a	Black sandy sediment from a small well	92	1
MSB11	Black sandy sediment	85	1
MSB12	Sulfur-covered lava stones in a spring	90	1
VC15	Grey sandy sediment	30	0.1
VC17	Strongly gassed grey and sandy sediment	37	0.5

The pH of all samples was around 6.5

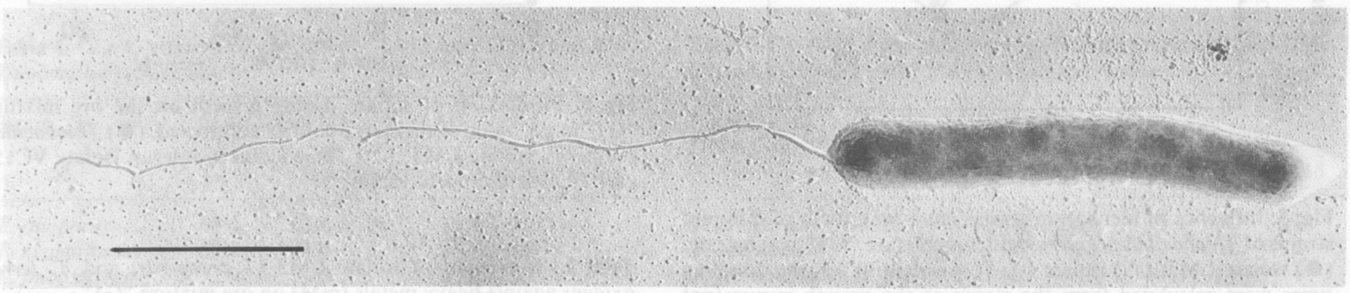

Fig. 1. Electron micrograph of *Thiobacillus prosperus* (isolate LM3) platinum shadowed, showing a monopolar monotrichous flagellation. Bar 1 μm

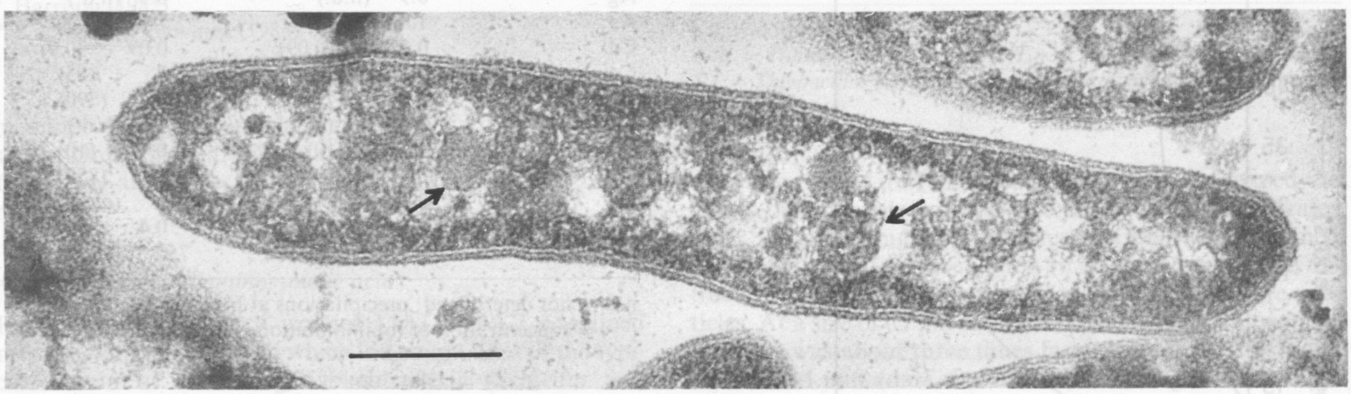

Fig. 2. Thin section of *Thiobacillus prosperus* (isolate LM3), contrasted with lead citrate and uranyl acetate. Bar 0.2 μm

one polar flagellum which was about 4 μm long (Fig. 1). They contained frequently granum-like bodies about 0.1–0.2 μm in width (Figs. 1, 2, arrows). They stained Gram-negative.

Storage

Cultures grown on ores and stored at room temperature served as inocula for at least 18 months. When stored in the cold room (4° C), the cells lost their viability within 2 weeks. Freezing at −20° C or −140° C (liquid nitrogen; gasphase) in the presence or absence of glycerol (20% w/v) or dimethylsulfoxid (5% w/v) led to complete inactivation of the cultures.

Growth temperatures

The isolates L7, LM3 and VC15 grew at temperatures above 20° C and below 45° C (strain VC15 below 41° C). For the isolates L7 and VC15 fastest growth (Fig. 3) was obtained at 37° C (doubling time 8, respectively 6 h). Isolate LM3 grew optimally at 33° C (doubling time 6 h).

pH of growth

All isolates grew between pH 1 and 4.5 with an optimum around 2. During growth on the ore mixture "G1" the pH dropped due to the production of sulfuric acid.

Although cells did not grow in media adjusted to pH 6.5, they survived and could successfully be transferred for at least 2 weeks into fresh media with pH 2.5 (storage temperature 37° C).

Salt tolerance

Isolates L7, LM1, LM3, MSB9a, MSB11, MSB12 and VC15 grew at NaCl concentrations of up to 3.5% (Fig. 4; not shown), added to the basal medium. Strain VM17 grew

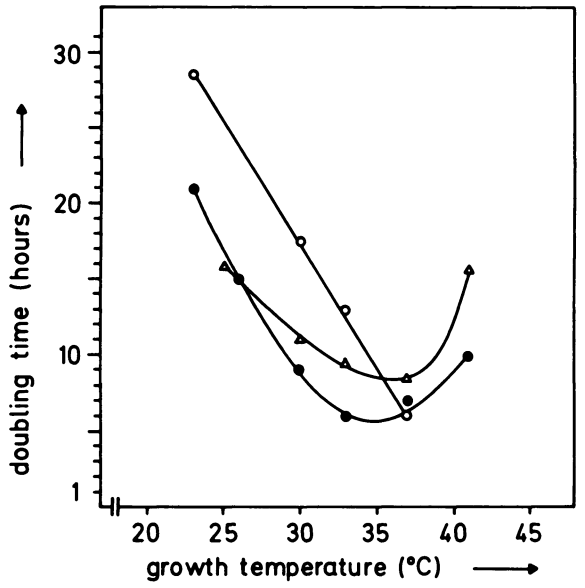

Fig. 3. Influence of incubation temperature on growth of different strains of *Thiobacillus prosperus* with ore mixture "G1" as substrate. (●) isolate LM3; (△) isolate L7; (○) isolate VC15; the doubling times were calculated from the slopes of the growth curves (not shown)

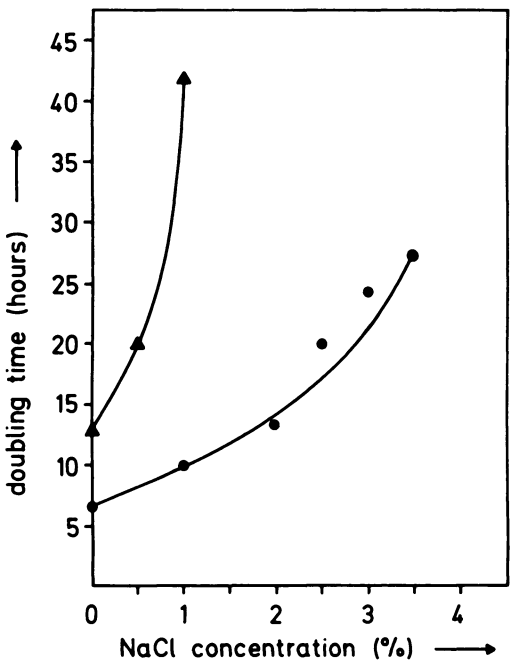

Fig. 4. Effect of NaCl concentrations added to the basal medium with "G1" as substrate. (●) *Thiobacillus prosperus*, isolate LM3; (▲) *Thiobacillus ferrooxidans*; the doubling times were calculated from the growth curves (not shown)

even up to concentrations of 6% NaCl (not shown). Fastest growth (Fig. 4) and highest final cell concentrations were obtained without additional salt. As expected *T. ferrooxidans* did not grow at NaCl concentrations higher than 1.0% (Fig. 4).

All new isolates were able to grow in the presence of seawater.

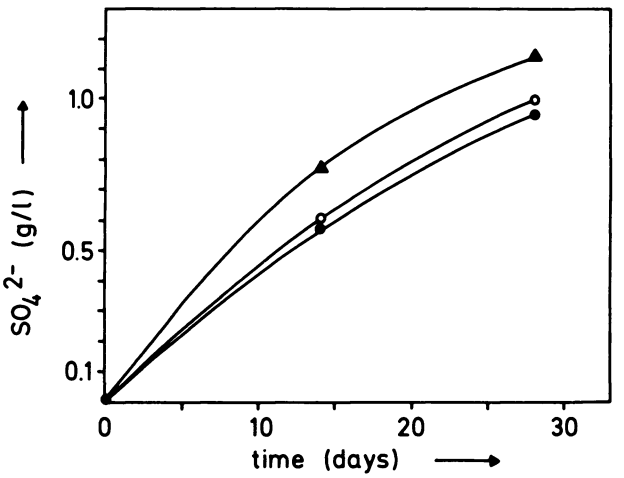

Fig. 5. Production of sulfate during growth on the ore mixture "G1" in g/l, value of the sterile control subtracted. (●) *Thiobacillus prosperus*, isolate LM3; (○) *Thiobacillus prosperus*, isolate VC15; (▲) *Thiobacillus ferrooxidans*

Table 2. Tolerances of isolate LM3 (*T. prosperus*) and *T. ferrooxidans* against heavy metals (mM) on ore mixture "G1"

Element	Isolate LM3	*T. ferrooxidans*
Ag	0.9 (n.d.)	0.9 (n.d.)
As	1.3 (13)	1.3 (13)
Cd	0.009 (0.09)	0.09 (0.9)
Co	170 (850)	17 (85)
Cu	16 (79)	160 (790)
Hg	0.05 (0.5)	0.5 (n.d.)
Mo	1 (10)	0.1 (10)
Ni	850 (1700)	170 (850)
Sb	8 (n.d.)	8 (n.d.)
U	0.04 (0.4)	0.4 (4)
Zn	1500 (3060)	750 (1530)

n.d. = not determined; precipitations at higher concentrations
( ) = concentration of total inhibition

Metabolism

The new isolates grew aerobically on single sulfidic ores like pyrite, sphalerite, chalcopyrite, arsenopyrite and galena as energy source. During growth on the ore mixture "G1" the new isolates and *T. ferrooxidans* formed about 10 mM sulfate within 28 days (Fig. 5; final cell concentration about 2×10^8/ml). Growth of all isolates was neither stimulated nor inhibited by the addition of yeast extract (0.02%). The sulfidic ores could be substituted by H_2S as substrate. Thiosulfate, tetrathionate and the synthetic sulfides Ag_2S, CuS, FeS, MoS_2, Sb_2S_3, SnS and ZnS did usually not serve as substrate. As an exception, isolate VC15 was able to grow by oxidation of synthetic FeS. All isolates were unable to grow on organic substrates like yeast extract, saccharose and glucose.

Elemental sulfur and ferrous sulfate were used as substrates by the strains LM1, MSB9a and VM17. Isolates L7, LM3, VC15, MSB11 and MSB12 appeared to be unable to use one or both of these substrates when they were precultured on sulfidic ores. However, they could be adapted to these substrates after about 5 transfers in "G1"-containing

Table 3. Metal extraction by the new isolates and by *Thiobacillus ferrooxidans* from the ore mixture "G1" in 28 days (g/l)

Element	Strain								Sterile control[a]	Total amount[b]
	L7	LM1	LM3	MSB9	MSB11	MSB12	VC15	T.f.		
Cu	0.15	0.09	0.16	0.13	0.12	0.13	0.12	0.16	0.04	2.00
Fe	0.13	0.25	0.19	0.22	0.23	0.25	0.18	0.24	0.22	6.00
U	0.08	0.05	0.09	0.05	0.05	0.05	0.06	0.06	0.01	0.09
Zn	1.43	1.45	1.65	1.41	1.24	1.27	1.55	1.85	0.50	4.00

T.f. = *Thiobacillus ferrooxidans*
[a] Chemical extraction by sulfuric acid from the medium
[b] Determined by chemical extraction with concentrated aqua regia

Table 4. G + C content of the isolates LM3 and VC15 and some *Thiobacillus* type strains

Strain	G + C (mol%)		
	T_M	Direct analysis	Reference value
T. thioparus	64.4	62.4	62–66[a]
T. neapolitanus	55.1	54.4	55–57[a]
T. thiooxidans	52.0	54.4	52[b]
T. ferrooxidans	59.2	58.7	58[b]
LM3	64.4	64.4	
VC15	63.1	62.1	

[a] Harrison (1982)
[b] Kuenen and Tuovinen (1984)

Table 5. DNA-DNA homologies (%) between the new isolates LM3, L7 and VC15 and some *Thiobacillus* type strains

Filter-bound DNA from	^{32}P-labelled DNA from						
	T.tp.	*T.n.*	*T.f.*	*T.t.*	LM3	L7	VC15
T. thioparus	100	18	n.d.	n.d.	15	n.d.	10
T. neapolitanus	9	100	1	n.d.	19	n.d.	1
T. ferrooxidans	3	8	100	12	14	13	6
T. thiooxidans	n.d.	n.d.	3	100	15	n.d.	1
LM3	6	11	4	12	100	98	15
L7	n.d.	n.d.	9	12	91	100	n.d.
VC15	12	n.d.	7	n.d.	36	n.d.	100

n.d. = not determined
T.tp. = *Thiobacillus thioparus*; *T.n.* = *Thiobacillus neapolitanus*; *T.f.* = *Thiobacillus ferrooxidans*; *T.t.* = *Thiobacillus thiooxidans*

medium which were supplemented with Fe^{2+} or elemental sulfur. On Fe^{2+} and elemental sulfur the final cell concentrations of all isolates were rather poor (about 4×10^7 cells/ml).

Analysis of meso-diaminopimelic acid

Hydrolysates of cells of isolates LM3 and L7 contained meso-diaminopimelic acid which is a component of the typical murein of Gram-negative eubacteria (Schleifer and Kandler 1972).

Resistance against heavy metals

The resistances of isolate LM3 and of *Thiobacillus ferrooxidans* against heavy metals were examined in the presence of various concentrations of different heavy metal ions (Table 2). In comparison with *T. ferrooxidans* strain LM3 was about one order of magnitude more sensitive against ions of cadmium, mercury and uranium, while it was more resistant against cobalt, molybdenum, nickel and zinc. Isolate LM3 was even able to grow in the presence of 850 mM of nickel and of 1500 mM of zinc ions.

Ore leaching capacity

On the ore mixture "G1" the new isolates and *Thiobacillus ferrooxidans* showed very similar patterns and final concentrations of solubilized metal ions (Table 3). The new strains extracted up to 100% uranium, 40% zinc and up to 8% copper within 28 days. Solubilized iron was precipitated as jarosite (D. Rose, pers. communication). Therefore, the concentration of iron in the supernatant did not exceed the values of the sterile control. The maximal extraction rates of 5, 120 and 7 mg/l per day, respectively, were obtained about 3 weeks after inoculation (data not shown). The extraction rates depended strongly on the size of the ore particles. At a size of 63 µm and below, the extraction of copper and zinc was about three times faster than that of particles of below 1 mm (data not shown). The higher extraction velocity may be due to the larger surface available to the organisms. In media containing 3.5% NaCl in addition no significant differences in the leaching capacity were detected for all isolates (data not shown).

Content of quinones

The new isolates contained ubiquinone 8 (CoQ_8) as their main quinone (96.5%). No ubiquinone 10 was detected (D. Collins, pers. communication).

DNA composition

The G + C content of the DNA was determined for the isolates LM3 and VC15 and for some *Thiobacillus* reference strains (Table 4). The isolates LM3 and VC15 exhibited G + C contents of around 64 and 63 mol% respectively.

DNA-DNA hybridization

DNA-DNA hybridization between the isolates L7, LM3, VC15 and *Thiobacillus* reference strains was carried out.

Isolates L7 and LM3 were chosen since they were the first strains obtained in pure culture. Strain VC15 was different from the other isolates by its ability to grow on FeS. Isolates LM3 and L7 showed more than 90% homology between each other (Table 5). There was only a low DNA homology between isolates VC15 and LM3 (25% average; Table 5). No significant homology could be detected between isolates L7, LM3, VC15 and the *Thiobacillus* reference strains.

Discussion

The new isolates are mesophilic rod-shaped Gram-negative eubacteria able to oxidize sulfur compounds and even sulfidic ores to sulfate and are therefore members of the genus *Thiobacillus* (Vishniac 1974). Due to their obligately chemolithotrophic acidophilic mode of life and their possession of ubiquinone Q-8 they can be attached to the chemotaxonomic group III-3 within the genus *Thiobacillus* (Katayama-Fujimura et al. 1982). *Thiobacillus ferrooxidans* belongs to the same group. The eight new isolates can be easily distinguished from *T. ferrooxidans* by their tiny slender shape, their growth in the presence of at least 3.5% NaCl and their very poor growth on elemental sulfur and on ferrous iron. The isolates LM3, L7 and VC15 had been inspected more closely. They exhibit a G + C content of their DNA 5% higher than *T. ferrooxidans* and show no significant DNA hybridization with *T. ferrooxidans* and other *Thiobacillus* group III members. Between each other, isolates LM3 and L7 exhibit over 90% DNA hybridization and are therefore strains of the same new species. Isolate VC15 shows only a rather low DNA hybridization with isolates LM3 and L7 and is therefore different. Up to now the only taxonomic feature further distinguishing isolate VC15 from the other isolates is its ability to grow on synthetic ferrous sulfide. Therefore, the description of a separate taxon seems to us not to be justified at the moment. On the basis of the morphological, physiological and molecular differences mentioned above, which separate the new isolates clearly from the known members of the genus *Thiobacillus*, we describe one new species which we name *Thiobacillus prosperus* sp. nov. due to its ability to extract pretious metal ions from ores. The type strain is *Thiobacillus prosperus* LM3 (DSM 5130).

The new species *Thiobacillus prosperus* represents the first halotolerant metal-mobilizing bacterium isolated. Similar to *T. ferrooxidans* it shows vigorous extraction of metal ions from sulfidic ores. *T. prosperus* shows an extremely high resistance against cobalt, nickel and zinc ions. Due to its high tolerance of salt (even 6% in the case of isolate VM17), *T. prosperus* may be suitable for industrial leaching in salt-containing environments where *T. ferrooxidans* is unable to grow. Within its natural environment at the marine hydrothermal system at Vulcano, *T. prosperus* may grow mainly by oxidation of pyrite deposits, which are currently formed (Wauschkuhn and Gröpper 1975). Although *T. prosperus* cannot grow at the high pH of regular sea water, there may be most probably acidic microenvironments formed by SO_2-containing submarine fumaroles suitable for it to grow.

Description of a new species

Thiobacillus prosperus Huber and Stetter, sp. nov. pros'pe.rus L. masc. adj. *prosperus* prosperous, referring to its ability to gain precious metals by ore "leaching".

Cells are Gram-negative rods, about 3 to 4 μm long and 0.3 μm in width and are motile by one polar flagellum. Optimal growth around 37° C and up to 41° C. Growth from 0% to 3.5% NaCl, and between pH 1.0 and 4.5. Strictly chemolithoautotrophic and aerobic. Growth on sulfidic ores like pyrite, sphalerite, chalcopyrite, arsenopyrite, galena and on H_2S. Poor growth on elemental sulfur and ferrous iron. Produces sulfuric acid from reduced sulfur compounds. Sensitive to ampicillin and vancomycin, possess m-DAP and ubiquinone Q-8. G + C content of the DNA 64 mol%. Insignificant DNA hybridization to *T. ferrooxidans*, *T. thiooxidans*, *T. neapolitanus* and *T. thioparus*. Lives in marine sediments in hydrothermal areas.

Type strain is *Thiobacilllus prosperus*, LM3, DSM 5130, Braunschweig, FRG.

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