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Thiobacillus cuprinus sp. nov., a Novel Facultatively Organotrophic Metal-Mobilizing Bacterium

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Five strains of mesophilic, facultatively organotrophic, ore-leaching eubacteria were isolated from solfatara fields in Iceland and a uranium mine in the Federal Republic of Germany. The new organisms are aerobic gram-negative rods. They can use sulfidic ores or elemental sulfur as sole energy source, indicating that they belong to the genus *Thiobacillus*. Alternatively, they grow on organic substrates such as yeast extract, peptone, and pyruvate. In contrast to the other leaching bacteria known so far, the new isolates are unable to oxidize ferrous iron. They consist of extreme and moderate acidophiles growing optimally at pH 3 and 4, respectively. The extreme acidophiles showed leaching characteristics similar to those shown by *Thiobacillus ferrooxidans*, while the moderate acidophiles exhibited a pronounced preference for copper leaching on some chalcopyrite ores. The G+C content of the DNA is between 66 and 69 mol%, depending on the isolate. In DNA-DNA hybridization experiments, the new strains showed homologies among each other of >70%, indicating that they belong to the same species. No significant DNA homology to *Thiobacillus* reference strains was detectable. Therefore, the new isolates represent a new species of *Thiobacillus*, which we name *Thiobacillus cuprinus*. Isolate Hö5 is designated as the type strain (DSM 5495).

Growth by oxidation of sulfidic ores is known only for a few bacteria. For mesophiles, this property is restricted to *Thiobacillus ferrooxidans* (3), *Thiobacillus prosperus* (8), and *Leptospirillum ferrooxidans* (1, 17). These species are characterized by an obligate chemolithoautotrophic metabolism, and they grow optimally at pH values of around 2. Furthermore, they are able to oxidize ferrous iron. In *T. ferrooxidans*, a specific electron carrier, rusticyanin, is thought to be involved in this reaction (10). The *Thiobacillus* strains also grow on sulfur compounds such as H_2S and elemental sulfur, on which they produce sulfuric acid.

We describe the isolation and properties of a new group of metal-mobilizing bacteria which are facultative organotrophs lacking the ability to oxidize ferrous iron.

MATERIALS AND METHODS

Strains. The type strains of *T. ferrooxidans* (ATCC 23270), *T. thiooxidans* (ATCC 19377), *T. perometabolis* (ATCC 23370), *T. novellus* (DSM 506), *T. neapolitanus* (DSM 581), and *T. thioparus* (DSM 505) were obtained from the American Type Culture Collection (ATCC), Rockville, Md., and from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM), Braunschweig, Federal Republic of Germany (FRG). *T. prosperus* (DSM 5130) was isolated in our laboratory (8).

Culture conditions. For cultivation of the new isolates, mineral salts medium M1 (6), adjusted to pH 3.5 with sulfuric acid, was used. The media used for the *Thiobacillus* reference strains were described elsewhere (8). All organisms were grown with shaking (100 rpm) in 100-ml Erlenmeyer flasks containing 30 ml of medium.

If not mentioned otherwise, in all experiments the ore mixture G1 (33 g/liter) (8) was used as the energy source for the new isolates. G1 consisted of equal parts of the following ores: pyrite (Grube Bayerland, Oberpfalz), chalcopyrite (Bad Grund, Harz), sphalerite (Grube Lüderich, Nordrhein-Westfalen), and pitch blend (Grube Höhenstein, Oberpfalz). This composition resulted in the following final contents of the minerals in G1: pyrite, 26%; chalcopyrite, 21%; sphalerite, 22%; galena, 1%; pitch blend, 0.25%. Elemental sulfur (3.3 g/liter), $FeSO_4$ (4%, wt/vol), $Na_2S_2O_3$ (0.5%), $K_2S_4O_6$ (0.5%), synthetic sulfides (1.7%), and natural ores (1.7%) were tested as possible substrates for chemolithotrophic growth. The synthetic sulfides were as follows: Ag_2S (content 99%; Merck), CdS (99%; Riedel-de Haen), CuS (>97%; Fluka), FeS (>97%; Merck), MoS_2 (99.5%; Riedel-de Haen), Sb_2S_3 (97%; Fluka), SnS (>97%; Merck), and ZnS (>98%; Fluka). The particle size of these sulfides was <0.125 mm.

Mineralogical composition of the natural ores (determined by microscopy of polished sections; D. Rose, personal communication) (level of precision, ±5%) was as follows.

arsenopyrite

St. Andreasberg (FRG): 95% arsenopyrite; traces of pyrite and galena

Veze (France): 30% arsenopyrite; traces of chalcopyrite, goethite, and pyrite

Reichenstein (Poland): 50% arsenopyrite; traces of ilmenite, rutile, and titanite

bornite

Butte (United States): 97% bornite; traces of pyrite and chalcocite

cinnabar

Almaden (Spain): 20% cinnabar; traces of pyrite

chalcopyrite

Bad Grund (FRG): 85% chalcopyrite; 10% pyrite; traces of galena

Cornwall (Great Britain): 90% chalcopyrite; 8% pyrite; traces of arsenopyrite

Kelchalpe (Austria): 80% chalcopyrite; 0.1% pyrite

Kopparberg (Sweden): 45% magnetite; 40% biotite; 6% chalcopyrite; 6% pyrrhotite; 3% sphalerite; traces of rutile

Murgul (Turkey): 30% chalcopyrite; 20% pyrite; traces of sphalerite

Norway: 38% pyrite; 32% chalcopyrite; 25% pyrrhotite; 3% sphalerite; traces of molybdenite and ilmenite

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TABLE 1. Origin of new isolates

Location	Sample taken from:	Original temp (°C)	Original pH	Strain designation
Hveragerdi (Iceland)	Large greenish mudhole	36	4.6	Hv10
Krisuvik (Iceland)	Black sediment form a small streamlet	30	5.0	Kris2
	Greyish sediment from a large water hole	30	3.0	Kris4
Poppenreuth (FRG)	Greyish mud and ore particles from a uranium-containing dump	10	5.5	Hö3
	Greyish mud from a small pond beneath the dump	10	6.0	Hö5

Tarn (France): 30% chalcopyrite; 20% pyrite; traces of covellite

chalcocite

Butte (United States): 90% chalcocite; traces of chalcopyrite and bornite

Eifel (FRG): 15% chalcocite; 15% bornite; traces of chalcopyrite, covellite, and hematite

St. Agnes (Great Britain): 70% chalcocite, 1% native bismuth

covellite

Butte (United States): 50% covellite; 20% chalcocite; 20% digenite; 5% bornite; 5% pyrite

galena

Clausthal (FRG): 80% galena; 15% sphalerite; traces of pyrite and marcasite

pitch blend

Schacht Höhenstein (FRG): 5% pyrite; 1% pitch blend; traces of anastase, zircon, and chalcopyrite

pyrite

Grube Bayerland (FRG): 90% pyrite; traces of chalcopyrite, rutile, and pyrrhotin

sphalerite

Grube Lüderich (FRG): 90% sphalerite; 5% galena; traces of pyrite and chalcopyrite

In all ores the matrix consisted of silicates. The particle size of all natural ores, including ore mixture G1, was <1 mm.

Usually yeast extract (0.05%; Difco Laboratories, Detroit, Mich.) served as the energy source for heterotrophic growth. In addition, peptone (0.05%), Casamino Acids (0.05%), meat extract (0.05%), sugars (arabinose, fructose, galactose, glucose, lactose, mannose, raffinose, ribose, sorbose, and sucrose; 0.1%), amino acids (DL-alanine, L-arginine, L-cysteine, L-glutamic acid, glycine, L-lysine, and DL-serine; 0.1%), and organic acids (acetate, lactate, succinate, citrate, malate, and pyruvate; 0.1%) were assayed as possible substrates.

Batch cultures (55 liters of medium) were grown in an enamel-protected fermentor (HTE; Bioengineering, Wald, Switzerland) with stirring (150 rpm) and aerating (2 liters/min).

Sensitivity to ampicillin was determined in liquid media containing 0.1, 1, 5, 10, 50, or 100 μg of the antibiotic per ml (incubation time, 4 weeks).

Determination of growth. Bacterial growth was determined by direct cell counting in a Thoma chamber (depth, 0.02 mm).

Fluorescence microscopy and electron microscopy. Cells attached to solid particles were visualized by a modified DAPI staining method (7). For electron microscopy, cells and thin sections were prepared and photographed as described previously (9).

Tolerance to heavy metals. Resistance to arsenic, cadmium, cobalt, copper, mercury, molybdenum, nickel, uranium, and zinc was determined. Stock solutions or salts (8) were added to the basal medium in the same concentrations as described before (8). The experiments were carried out at pH 4. The inhibitory concentration of each heavy metal was defined as the lowest concentration at which growth could not be obtained (incubation time, 4 weeks). The resistance to mercury could not be determined due to precipitation in the culture medium. The tested concentrations of all other metal ions remained constant during the incubation (checked by inductively coupled plasma analyses).

Quantitative determination of sulfate. Sulfate was precipitated by barium chloride and determined gravimetrically (23).

Isolation of DNA and determination of base composition. DNA was prepared as described earlier (22). The G+C content was determined by melting-point analyses in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (16) and by high-performance liquid chromatography of the nucleotides after digestion of the DNA with nuclease P1 (25).

DNA-DNA hybridization. DNA-DNA hybridizations were carried out (14) with radioactively labeled nick-translated DNA (12), using the filter technique (2, 4).

Metal analysis. Concentrations of dissolved metals were determined by inductively coupled plasma (Jobin Yvon, JY 70 Plus) analyses of the supernatant from centrifuged cultures or from aqua regia extractions of the ores. Three leaching experiments were carried out in parallel.

RESULTS

Enrichment and isolation of the new organisms. Aerobic samples of waters, sediments, and ore particles with original temperatures of 10 to 36°C and pH values of 3.0 to 6.0 were taken from solfatara fields at Hveragerdi and Krisuvik, Iceland, and from a uranium mine near Poppenreuth, FRG. For enrichment, about 1 g of material from the various samples was transferred into 30 ml of mineral medium M1 supplemented with 1 g of ore mixture G1 and incubated at 37°C with shaking. After 2 weeks, thin rods were visible in the enrichment attempts of samples Hv10, Kris2, Kris4, Hö3, and Hö5 (Table 1). The enrichment cultures were purified by repeated serial dilutions in the ore-containing medium. The organisms turned out to be facultative heterotrophs (see below). They were cloned by plating five times in sequence on culture medium solidified with agar (1.5%) containing yeast extract (0.05%). Brownish round colonies about 1 mm in width were formed after an incubation time of 5 days at 37°C. The plated cultures were still able to grow on ore mixture G1. The isolates were designated as the samples.

Morphology. Cells of isolates Hö3, Hö5, and Hv10 appeared to be straight rods (length of isolates Hö3 and Hö5, about 1 μm [Fig. 1a]; length of isolate Hv10, about 2 to 4 μm [Fig. 1b]). In contrast, cells of strains Kris2 and Kris4 were curved rods (about 1.5 to 2.5 μm long [Fig. 1c]). Cells of all isolates were about 0.3 to 0.5 μm in width. They were motile by one polar flagellum (Fig. 1a to c). Cells stained gram

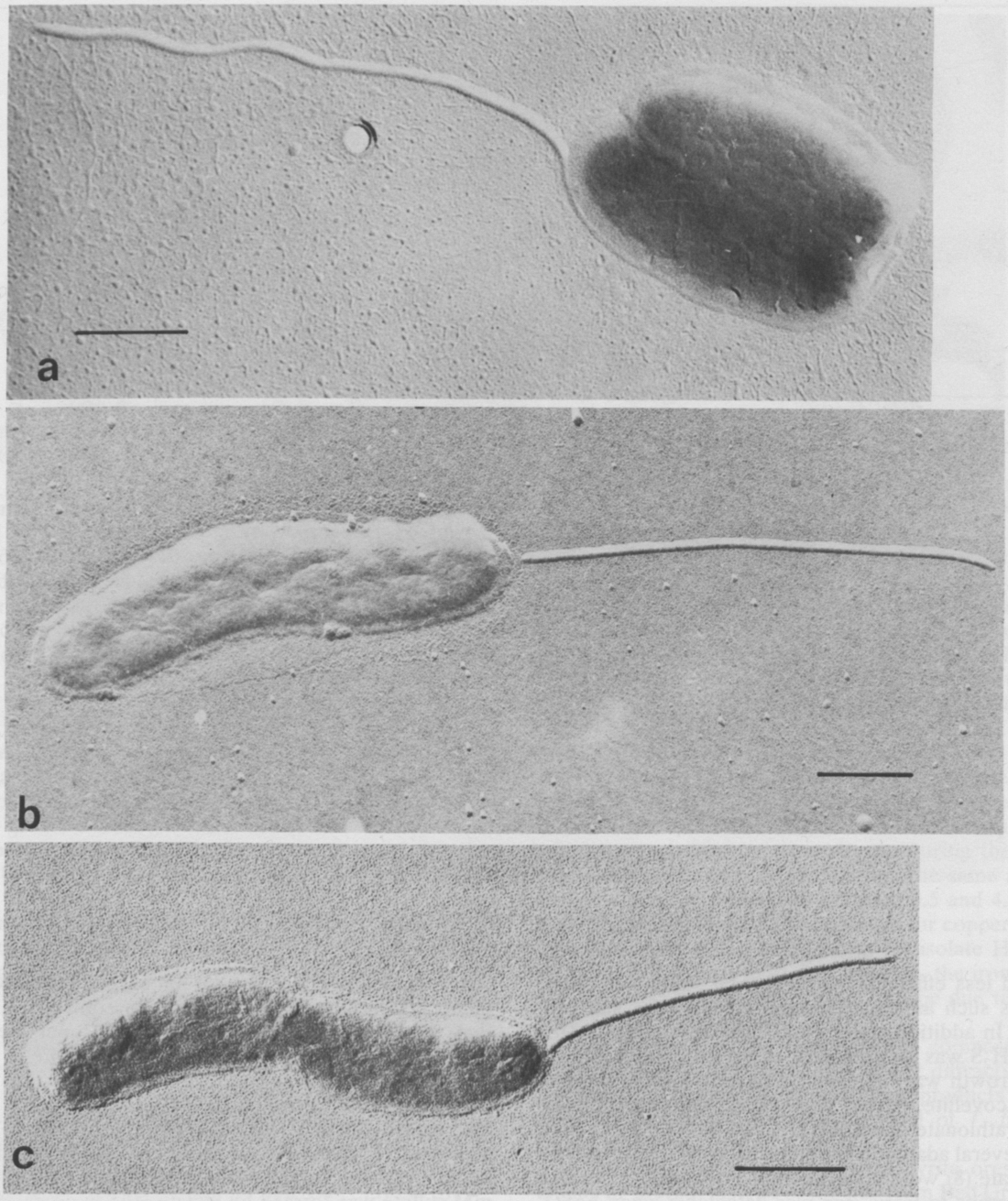

FIG. 1. Electron micrograph of *T. cuprinus*, platinum shadowed. Bar, 0.5 μm. (a) Isolate Hö5; (b) isolate Hv10; (c) isolate Kris2.

negative and showed a typical gram-negative cell wall (Fig. 2).

Storage. When stored at room temperature without shaking, cultures grown on ores served as inoculum for at least 1 year. Cells cultivated on yeast extract or ores kept their viability for >2 years when they had been stored at −20°C or over liquid nitrogen (−140°C).

Growth temperatures. Isolates Hö5, Hv10, and Kris2 showed optimal growth at temperatures between 30 and 36°C (Table 2). Strain Hö5 grew at 20 to 45°C. No growth occurred at 15 and 50°C. Strains Hv10 and Kris2 grew at between 23 and 41°C. They did not grow at 20 and 45°C. The shortest doubling times for isolates Hö5, Hv10, and Kris2 on yeast extract were between 3 and 4 h.

pH dependence of growth. Isolates Hö3, Hö5, and Hv10 grew on yeast extract at between pH 3 and 7.2, with an optimum of around pH 4 (not shown). No growth was observed at pH 2.5 and 7.5. On sulfidic ores, they were able to start growing at between pH 3 and 5.5. No growth occurred at initial pH values of 2.5 and 6.0. During growth on sulfidic ores, the pH dropped due to the formation of sulfuric acid. Isolates Kris2 and Kris4 grew on yeast extract, as on sulfidic ores, at between pH 1.5 and 4.5. No growth occurred at pH 1 and 5. On yeast extract, optimal growth was obtained at pH 3.5. *T. ferrooxidans* showed a similar optimal pH of growth on ferrous iron (not shown).

Metabolism. All isolates were able to grow on ore mixture G1 and arsenopyrite (final cell densities, around 2×10^8 cells

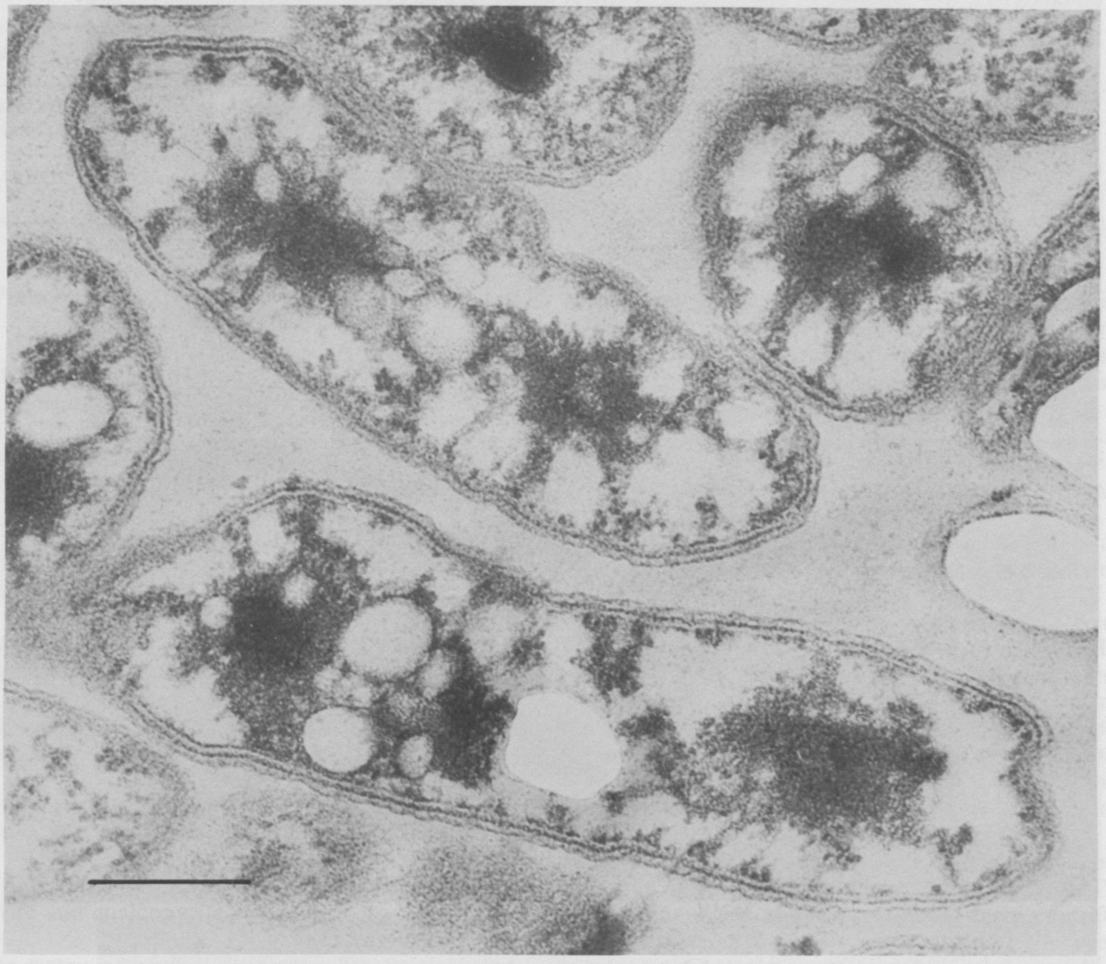

FIG. 2. Thin section of *T. cuprinus* (isolate Hö5). Bar, 0.2 μm.

per ml) and less efficiently on elemental sulfur and single sulfidic ores such as sphalerite and chalcopyrite and synthetic FeS. In addition, galena and CdS were used by Hö5 and Kris2. H_2S was an additional substrate (tested only for Hö5). No growth was obtained on ferrous sulfate, bornite, chalcocite, covellite, pyrite, pitch blend, cinnabar, thiosulfate, or tetrathionate or on Ag_2S, CuS, MoS_2, Sb_2S_3, SnS, and ZnS. Several adaptation attempts to grow the isolates on ferrous sulfate (8) were unsuccessful. Furthermore, no oxidation of ferrous to ferric iron was detectable when $FeSO_4$ was the sole energy source for the new organisms. In agreement with this result, during growth on FeS, the final concentration of ferric iron did not exceed the amount of the uninoculated control (not shown). In contrast, *T. ferrooxidans* produced 1,700 mg of soluble Fe^{3+} per liter on FeS within 1 week (sterile control, 1,000 mg of Fe^{3+} per liter). During growth on elemental sulfur or sulfidic ores, the new isolates formed sulfuric acid. From ore mixture G1 strain Hö5 formed about 5 mM sulfate within 28 days (Fig. 3). As a control, *T. ferrooxidans* produced about 12 mM sulfate under the same conditions. On elemental sulfur, about 4.5 mM sulfate was formed by isolate Hö5 in comparison to 30 mM produced by *T. ferrooxidans* (Fig. 4).

Under heterotrophic conditions, the new isolates grew on

TABLE 2. Temperature range of growth and shortest doubling times of new isolates Hö5, Hv10, and Kris2 and *T. ferrooxidans*[a]

Strain	Substrate	Growth at temp range (°C)	No growth at temp (°C) of:	Optimal growth at (°C):	Doubling time (h)
Hö5	Ore mixture G1	20–41	15, 45	ND	ND
	Yeast extract	20–45	15, 50	30	3.25
Hv10	Ore mixture G1	23–41	20, 45	ND	ND
	Yeast extract	23–41	20, 45	30	3
Kris2	Ore mixture G1	23–36	20, 41	ND	ND
	Yeast extract	23–41	20, 45	36	4
T. ferrooxidans ATCC 23270	Ore mixture G1	25–36	20, 41	ND	ND
	Ferrous sulfate	25–36	20, 41	36	4.5

[a] Doubling times were calculated from the slopes of the growth curves (not shown). ND, Not determined.

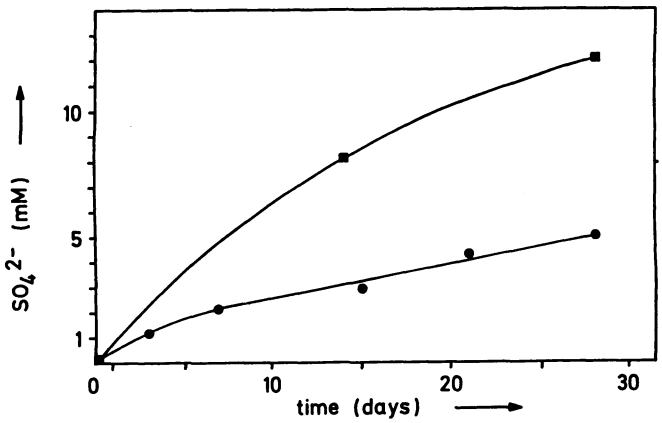

FIG. 3. Production of sulfate during growth on ore mixture G1; value of the sterile control was substracted. Symbols: (●) *T. cuprinus* (isolate Hö5); (■) *T. ferrooxidans*.

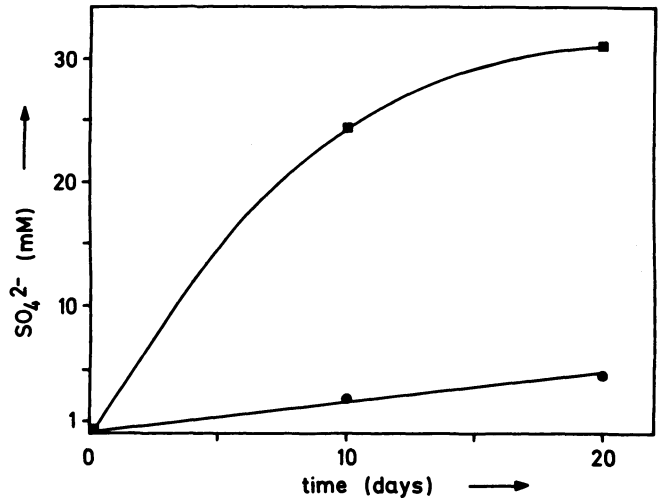

FIG. 4. Production of sulfate during growth on elemental sulfur; value of the sterile control was substracted. Symbols: (●) *T. cuprinus* (isolate Hö5); (■) *T. ferrooxidans*.

yeast extract, peptone, Casamino Acids, and meat extract. The final cell concentrations were about 5×10^8/ml for strains Hö3, Hö5, and Hv10 and about 5×10^7/ml for isolates Kris2 and Kris4. Isolates Hö3 and Hö5 showed excellent growth on pyruvate (0.2% in mineral medium M1, pH 5.5; final cell concentrations, about 1.5×10^{10}/ml) (B. Friedrich and A. Hofer, unpublished data). Isolates Hv10, Kris2, and Kris4 did not grow on pyruvate. All strains were unable to grow on arabinose, fructose, galactose, glucose, lactose, mannose, raffinose, ribose, sorbose, sucrose, L-arginine, L-cysteine, glycine, L-glutamic acid, L-lysine, DL-serine, acetate, lactate, succinate, citrate, and malate.

As expected, *T. ferrooxidans* and *T. prosperus* were unable to grow heterotrophically.

Resistance to antibiotics. Growth of all isolates was totally inhibited by 1 μg of ampicillin per ml. Partial inhibition was observed at 0.1 μg/ml.

Resistance to heavy metals. Isolate Hö5 grew in the presence of arsenic, copper, molybdenum, and zinc ions at 1.3, 7.9, 1, and 150 mM, respectively. These resistances were the same under heterotrophic and autotrophic conditions (Table 3). Cells grown on yeast extract were more sensitive to nickel (10 times), while they tolerated higher concentrations of uranium (100 times), cadmium (10 times), and cobalt (5 times). Isolates Hv10 and Kris2 exhibited the same tolerance to arsenic, cadmium, nickel, uranium, and zinc ions as Hö5 (grown on ores; Table 3). Isolates Hv10 and Kris2 grew in the presence of 0.79 and 1.6 mM Cu^{2+}, respectively. No growth was observed at 1.6 and 16 mM Cu^{2+} (Table 3).

As a control, *T. ferrooxidans* was more resistant than the new isolates to ions of copper, uranium, and zinc.

Extraction of metals. On ore mixture G1, the new isolates exhibited different metal extraction capacities. The extreme acidophilic strains Kris2 and Kris4 mobilized up to 160 mg of copper, 90 mg of uranium, and 1,450 mg of zinc per liter within 28 days (Table 4). Similar amounts of solubilized metal ions were obtained with *T. ferrooxidans*. In contrast, isolates Hö3, Hö5, and Hv10 extracted copper with relatively high efficiency (210 to 340 mg/liter), whereas the other metals were mobilized only in low amounts (uranium, 10 mg/liter; zinc, 200 to 250 mg/liter). During these leaching experiments, the pH of the cultures, the same as the uninoculated controls, remained between 3.5 and 4. The sterile controls did not show this preference for copper extraction. The concentration of soluble iron for isolate Hö5 was 110 mg/liter. In the case of *T. ferrooxidans*, the iron concentration was two times higher. This may be due to the higher solubility at the lower pH.

After incubation, the mineralogical composition of the residual ore was investigated by X-ray diffraction. Besides low amounts of jarosite, no additional products were found as a product of the new isolates and of *T. ferrooxidans* (Rose, personal communication).

In ore mixtures containing chalcopyrite ores from Kelchalpe or Murgul instead of the one from Bad Grund, isolate

TABLE 3. Tolerance of isolates Hö5, Hv10, and Kris2 and of *T. ferrooxidans* to heavy metal ions

Element	Concn (mM)				
	Hö5		Hv10 (ore)	Kris2 (ore)	*T. ferrooxidans* (ore)
	Yeast extract	Ore			
As	1.3 (13)[a]	1.3 (13)	1.3 (13)	1.3 (13)	1.3 (13)
Cd	0.9 (9)	0.09 (0.9)	0.09 (0.9)	0.09 (0.9)	0.09 (0.9)
Co	850 (1,700)	170 (850)	170 (850)	17 (85)	17 (85)
Cu	7.9 (16)	7.9 (16)	0.79 (1.6)	1.6 (16)	160 (790)
Mo	1 (10)	1 (10)	0.1 (1)	0.1 (1)	0.1 (1)
Ni	17 (85)	170 (850)	170 (850)	170 (850)	170 (850)
U	4 (ND)[b]	0.04 (0.4)	0.04 (0.4)	0.04 (0.4)	0.4 (4)
Zn	150 (750)	150 (750)	150 (750)	150 (750)	750 (1,500)

[a] Value in parentheses is concentration causing total inhibition.
[b] ND, Not determined; precipitations at higher concentrations.

TABLE 4. Metal extraction by the new isolates and by *T. ferrooxidans* from ore mixture G1 in 28 days

Element	Extraction (g/liter)								
	Hö3	Hö5	Hv10	Sterile control (pH 3.5)[a]	Kris2	Kris4	*T. ferrooxidans*	Sterile control (pH 2.5)[a]	Total amt[b]
Cu	0.23	0.36	0.30	0.02	0.18	0.09	0.16	0.04	2.00
Fe	0.11	0.10	0.10	0.04	0.18	0.23	0.24	0.22	6.00
U	0.02	0.02	0.01	0.01	0.10	0.03	0.06	0.01	0.10
Zn	0.62	0.60	0.65	0.40	1.95	1.89	1.85	0.50	4.00

[a] Chemical extraction from the medium by sulfuric acid.
[b] Determined by chemical extraction with concentrated aqua regia.

Hö5 also showed a preferential extraction of copper: 450 to 575 mg of copper per liter was solubilized, but only 26 to 29 mg of uranium per liter and not more than 140 mg of zinc per liter (Table 5). Although final cell densities in the ore mixtures were nearly the same when the chalcopyrite ore of Bad Grund was replaced by chalcopyrite ores derived from Cornwall, Kopparberg, Tarn, or Norway, no preferential extraction of copper was obtained (Table 5).

Analysis of *meso*-diaminopimelic acid. In cell hydrolysates of isolate Hö5, *meso*-diaminopimelic acid, a component of the murein present in many gram-negative eubacteria (18), was found.

Quinone composition. Like all members of *Thiobacillus* sp. groups II and III (11), cells of isolates Hö3 and Hö5 (grown on yeast extract) contained ubiquinone-8 (CoQ_8) as the main quinone (97 and 98%, respectively). Ubiquinone-10 was not detectable (D. Collins, personal communication).

Resistance to diphtheria toxin. Cell homogenates of isolate Hö5 did not show ADP-ribosylation of elongation factor G by diphtheria toxin (F. Klink, personal communication), indicating that the isolate belongs to the eubacteria (13).

DNA base composition. Isolates Hö3, Hö5, Hv10, and Kris2 exhibited G+C contents of DNA of between 66 and 69 mol% (Table 6), determined by thermal denaturation (16) and direct analysis of the mononucleotides (25). As expected, the G+C content of the cells was independent of the mode of cultivation (ore or yeast extract).

DNA-DNA hybridization. DNA-DNA hybridizations of isolates Hö5 and Hv10 between cultures grown on ore and on yeast extract revealed homologies of 81 to 100% (Table 7). Within the accuracy of the method, this high homology again demonstrated identity of the heterotrophic and chemolithoautotrophic culture lines. Isolates Hö3, Hö5, Hv10, and Kris2 exhibited very high DNA homologies among themselves (71 to 100%; Table 8). No specific relationship to the *Thiobacillus* reference strains was detected, as indicated by insignificant hybridization rates of 17% and below (Table 8) (19, 20).

TABLE 5. Metal extraction by isolate Hö5 within 28 days[a]

Ore mixture	Extraction (mg/liter)			
	Cu	Fe	U	Zn
G6 + Bad Grund	340	60	10	200
G6 + Murgul	450	80	26	140
G6 + Kelchalpe	575	0	29	0
G6 + Cornwall	200	0	0	110
G6 + Kopparberg	6	35	10	100
G6 + Tarn	20	30	10	140
G6 + Norway	50	50	3	40

[a] Values of the sterile controls were subtracted. The ore mixtures contained equal parts of pyrite, sphalerite, and pitch blend (= G6) and 1 part of one of the given chalcopyrites (total amount in each test was 1 g of ore in 30 ml of medium M1).

TABLE 6. DNA base composition of isolates Hö3, Hö5, Hv10, and Kris2 and some *Thiobacillus* type strains

Strain or isolate	G+C content (mol%)		
	T_m[a]	Direct	Reference value
T. thioparus	64.4	62.4	62–66[b]
T. thiooxidans	52.0	54.4	52[c]
T. ferrooxidans	59.2	58.7	58[c]
T. prosperus	64.4	64.4	64[d]
Hö3 (yeast extract)	68.1	65.5	
Hö3 (ore)	68.1	n.d.	
Hö5 (yeast extract)	66.4	65.7	
Hö5 (ore)	66.6	65.2	
Hv10 (yeast extract)	69.7	68.0	
Hv10 (ore)	69.3	66.3	
Kris2 (ore)	66.8	65.2	

[a] T_m, Thermal denaturation.
[b] From reference 5.
[c] From reference 15.
[d] From reference 8.

DISCUSSION

The novel isolates represent the first facultatively organotrophic ore-leaching eubacteria. On the basis of their rod shape, their negative gram-staining reaction, and their ability to oxidize sulfur, they can be affiliated with the genus *Thiobacillus* (21). This classification is confirmed by sequence comparison of the 16S and 23S rRNAs of isolate Hö5 which have been assigned to the "β_1 purple bacteria" (24; M. Bachleitner, Ph.D. thesis, Technische Universität, Munich, FRG, 1989). This phylogenetic group already contains other species of the genus *Thiobacillus*. In their ability to grow chemolithoautotrophically on sulfidic ores, the new isolates resemble *T. ferrooxidans* and *T. prosperus* (3, 8). They are different by (i) their growth with organic compounds as carbon and energy source; (ii) their inability to oxidize ferrous iron, which is in line with the absence of rusticyanin (A. Hofer, H. Huber, and K. O. Stetter, unpublished data); and (iii) an insignificant DNA homology. Furthermore, the new isolates exhibit an 8-mol%-higher G+C content than *T. ferrooxidans*. In contrast to *T. prosperus*, they are unable to grow in seawater. The very high DNA homology among the isolates demonstrates a close relationship at the species level. They are all acidophiles. However, two groups with different pH ranges of growth are evident: (i) isolates Kris2 and Kris4, which grow at between pH 1.5 and 4.5 (optimum, around 2.5) and are therefore extreme

TABLE 7. DNA-DNA homologies between the heterotrophic and autotrophic culture lines of isolates Hö5 and Hv10[a]

Filter-bound DNA from:	Homology (%) with ^{32}P-labeled DNA from:			
	Hö5 (YE)	Hö5 (ore)	Hv10 (YE)	Hv10 (ore)
Hö5 (YE)	**100**	88	ND	98
Hö5 (ore)	100	**100**	ND	ND
Hv10 (YE)	88	ND	**100**	100
Hv10 (ore)	ND	ND	81	**100**

[a] YE, Yeast extract; ND, not determined.

TABLE 8. DNA-DNA homologies among the new isolates and some *Thiobacillus* type strains

Filter-bound DNA from:	Homology (%) with ^{32}P-labeled DNA from:							
	T. thioparus	*T. neapolitanus*	*T. ferrooxidans*	*T. thiooxidans*	Hö3	Hö5	Hv10	Kris2
T. thioparus	**100**	18	ND	ND	5	7	8	15
T. neapolitanus	9	**100**	1	ND	6	9	10	12
T. ferrooxidans	3	8	**100**	12	1	3	3	6
T. thiooxidans	ND	ND	3	**100**	ND	14	ND	ND
T. prosperus	6	11	4	12	9	17	5	9
Hö3	7	14	2	ND	**100**	79	86	94
Hö5	9	14	5	7	86	**100**	98	80
Hv10	9	17	3	ND	100	88	**100**	89
Kris2	7	14	4	ND	71	80	80	**100**
Calf thymus	2	6	3	9	1	1	1	3

[a] ND, Not determined.

acidophiles similar to *T. ferrooxidans* and *T. prosperus*; and (ii) strains Hö3, Hö5, and Hv10, which grow at between pH 2.5 and 7.2 (optimum, around pH 4) and are moderate acidophiles. The two groups also differ in their ore-leaching characteristics. On ore mixture G1, the extremely acidophilic isolates show a pattern of metal ion extraction similar to those of *T. ferrooxidans* and *T. prosperus*. The moderate acidophiles, however, exhibit a less efficient mobilization of uranium and zinc compared with the extreme acidophiles, while copper extraction is significantly enhanced. The preferential leaching of copper is evident only on several chalcopyrite ores used in the mixtures. It therefore depends on the source and composition of the chalcopyrite. The basis of this different behavior is still unknown.

Due to the features mentioned above, the new isolates represent a new species which we name *Thiobacillus cuprinus*. The type strain is isolate Hö5 (DSM 5495). *T. cuprinus* can be isolated from ore- and sulfur-containing environments of the mesophilic temperature range such as mines and solfataric areas.

Description of a new species. *Thiobacillus cuprinus* Huber and Stetter, sp. nov.; cu.pri'nus L. masc. adj. *cuprinus*, copper, describing its ability to extract copper ions from ores.

Cells are gram-negative rods, about 1 to 4 μm long and 0.3 to 0.5 μm wide. They are motile due to one polar flagellum. Colonies on agar plates (heterotrophic growth conditions) have a brownish color. Temperature optimum between 30 and 36°C, no growth at 50 or 15°C. pH optimum between 3 and 4; no growth at pH 1 and 7.5. Facultatively chemolithoautotrophic; aerobic. Organotrophic growth on yeast extract, peptone, Casamino Acids, and meat extract. Some (Hö3 and Hö5) grow on pyruvate.

Lithoautotrophic growth on chalcopyrite, sphalerite, arsenopyrite, galena, H_2S, and elemental sulfur. Sulfuric acid formed during lithotrophic growth. No oxidation of ferrous iron. Sensitive to ampicillin. *meso*-Diaminopimelic acid and ubiquinone-8 present, but no rusticyanin. G+C content of DNA between 66 (Hö5) and 69 (Hv10) mol%. Insignificant DNA-DNA hybridization to *T. ferrooxidans*, *T. neapolitanus*, *T. prosperus*, *T. thioparus*, and *T. thiooxidans*. Lives in continental solfataric fields and mines.

Type strain is *Thiobacillus cuprinus* Hö5 DSM 5495, Braunschweig, FRG.

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