Isolation and Characterization of New Metal-Mobilizing Bacteria

G. HUBER, H. HUBER, and K. O. STETTER
Lehrstuhl fur Mikrobiologie,
Universität Regensburg,
Universitätsstrasse 31,
8400 Regensburg, Fed. Rep. Germany

Summary

In a screening program, novel mesophilic, thermophilic and extremely thermophilic chemolithotrophic bacteria able to grow by mobilization of metals from natural ores were isolated from geothermal areas and from ore deposits. A first characterization revealed 3 groups of mesophiles differing in morphology, physiology, and biochemical properties from each other and from *Thiobacillus ferrooxidans*, although their taxonomic position is still unknown. Among the thermophiles, a *Sulfolobus*-shaped isolate is growing on ores at temperatures up to 85°C and is therefore the most extremely thermophilic leaching organism known to date.

INTRODUCTION

In spite of the increasing interest in microbial leaching, the variety of organisms known to be able to extract metals from ores is still very small. Within the mesophilic temperature range, mainly strains of *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* are known to be involved in hydrometallurgical processes [1,2], although the latter is only able to attack sulfidic ores in combination with *Th. ferrooxidans* [3]. In addition, *Leptospirillum ferrooxidans* and *L. ferrooxidans*-like organisms have been described which grow by oxidation of Fe^{2+} or pyrite at mesophilic temperatures [4,5]. The upper temperature for bacterial leaching has been reported to be around 75°C for *Sulfolobus brierleyi* [6] and related organisms [7]. Here, we present novel isolates from ore-containing biotopes growing chemolithotrophically on natural ores within the mesophilic, thermophilic, and extremely thermophilic temperature range.

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MATERIALS AND METHODS

Strains

The type strains of *Thiobacillus ferrooxidans* (ATCC 23270) and *Thiobacillus thiooxidans* (ATCC 19377) were obtained from the American Type Culture Collection. *Leptospirillum ferrooxidans* (DSM 2705) was supplied by the Deutsche Sammlung von Mikroorganismen.

Culture Conditions

The type strains and isolate SP5/1 were cultivated in 9K-medium [8]. For the isolates L7, LM1, LM3, MSB9, MSB11, MSB12, and Hö3, Hö5 we used a mineral salt medium (M1) containing in g per liter: KC1 0.33; $MgCl_2 \cdot 6\ H_2O$ 2.75; $MgSO_4 \cdot 7\ H_2O$ 3.45; NH_4Cl 1.25; $CaCl_2 \cdot 2\ H_2O$ 0.14; K_2HPO_4 0.14; KH_2PO_4 0.14; NaCl 0.5 and 10 ml trace mineral solution [9]. The pH was adjusted to 3.5 with H_2SO_4. The thermophilic isolates (TH2, SP3a, Kra23, NA4, VE2) were grown in Allen's medium [10]. To 100-ml Erlenmeyer flasks were added 30 ml of medium supplemented with 4 ml of a 20% ferrous sulfate solution or with 0.1 g S° or with 1 g ore mixture (G1) containing 0.25 g pyrite (Grube Bayerland, Oberpfalz), 0.25 g chalcopyrite (Bad Grund, Harz), 0.25 g sphalerite (Grube Lüderich,

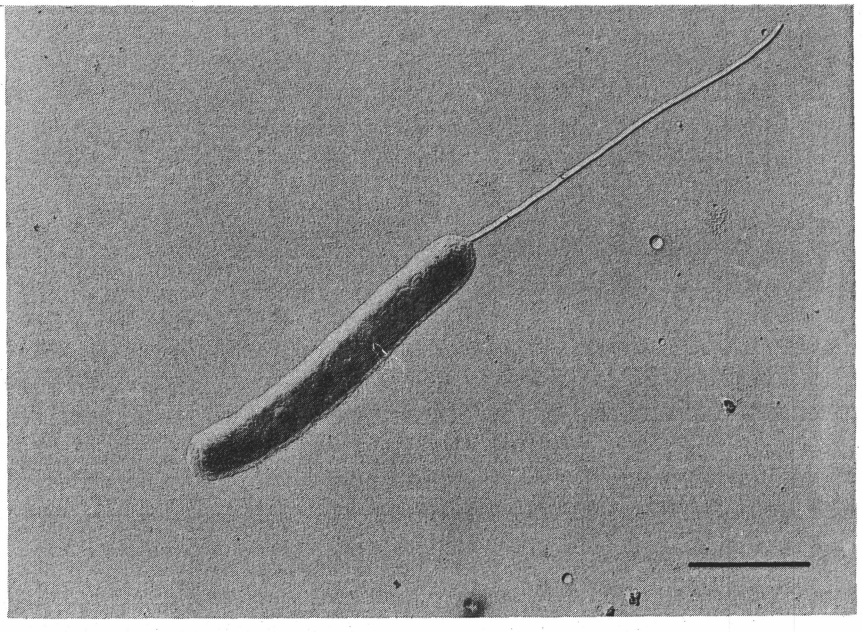

Fig. 1. Submarine isolate L7. EM-micrograph. Pt-shadowing. Bar, 1 μm.

Nordrhein-Westfalen) and 0.25 g pitch blend (Grube Höhenstein, Oberpfalz) as energy source. The particle size of this mixture was below 1 mm. The culture media were sterilized by autoclaving. Sulfur was sterilized by steaming for 30 min on three consecutive days at 100°C. The cultures were incubated in rotary shakers (New Brunswick) at 150 rpm.

Analysis of Cell Proteins

In order to obtain protein patterns, cells from 30-ml cultures (grown for 3 to 4 weeks) were harvested by centrifugation and washed twice with culture medium at pH 6.5 which did not contain ore. The washed cells were then sonicated after suspension in 50 μl of Laemmli's sample buffer [11]. The homogenates were heated for 15 min at 100°C, and 20 μg of protein were loaded on to an exponential polyacrylamide gel [11,12]. The electrophoresis was carried out at 100 V for 15 hours and then the gel was stained with Coomassie blue.

Metal Analyses

Metal ion concentrations within solutions were determined employing an ICP instrument (Lab Test).

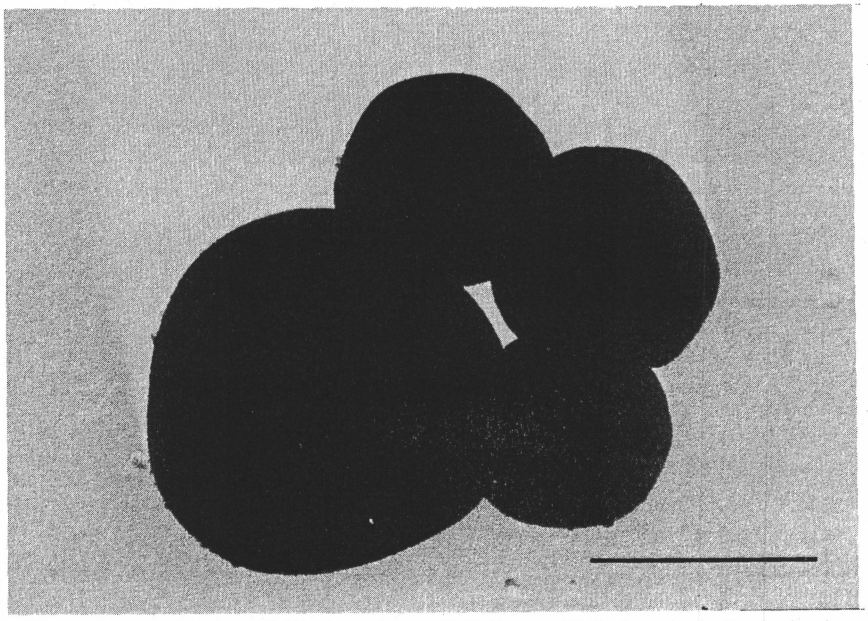

Fig. 2. Coccoid isolate SP5/1 from Pisciarelli Solfatara. EM-micrograph. Pt-shadowing. Bar, 1 μm.

RESULTS AND DISCUSSION

Collection of Samples

Within the area of Porto di Levante (Vulcano, Italy), 20-ml samples of pyrite-containing [13] geothermally heated sandy sediments of the beach and the shallow sea floor with original temperatures between 30 and 50°C were taken (samples L7, LM1, LM3, MSB9, MSB11, MSB12). Further samples were obtained from continental solfatara fields at Pisciarelli (Italy) with temperatures between 30 and 52°C (samples SP5/1, TH2, SP3a) and from Kraffla, Namaskarth and Hveravellir (all situated in Iceland) with original temperatures between 94 and 100°C (samples Kra23, NA4, VE2). The samples were brought to the laboratory without temperature or pH control. In addition, ore samples (Hö3, Hö5) were taken from a mud pond within the area of the uranium mine Höhenstein (Oberpfalz, FRG).

Enrichment and Isolation

For enrichment, 30 ml of ore-containing medium were inoculated with about 1 ml of samples and incubated at 30, 37 and 65°C, depending on the original temperatures of the samples. After one (thermophiles) to two weeks (mesophiles), bacteria became visible, partially attached to the ores which could be detected microscopically after fluorescence staining [14].

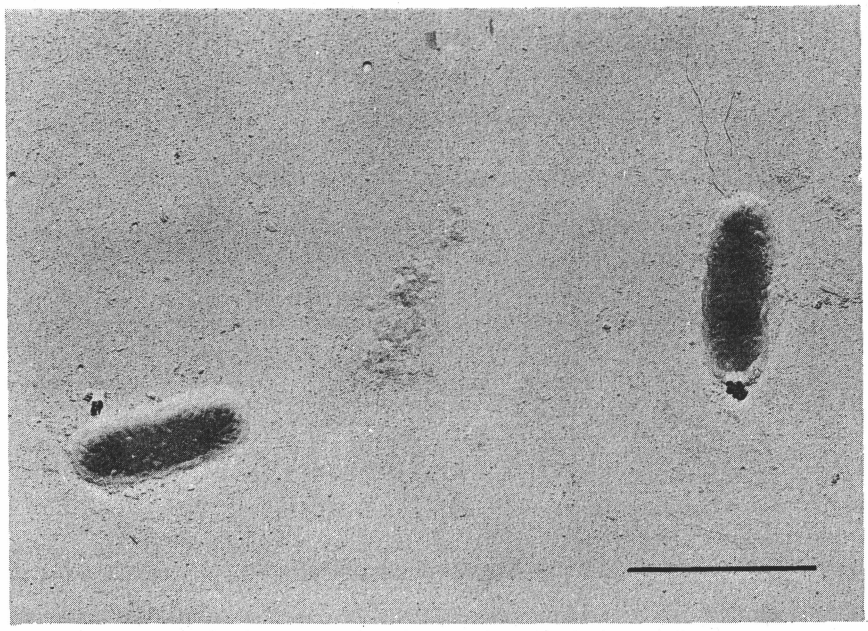

Fig. 3. Isolate Hö5 from an uranium mine. EM-micrograph. Pt-shadowing. Bar, 1 μm.

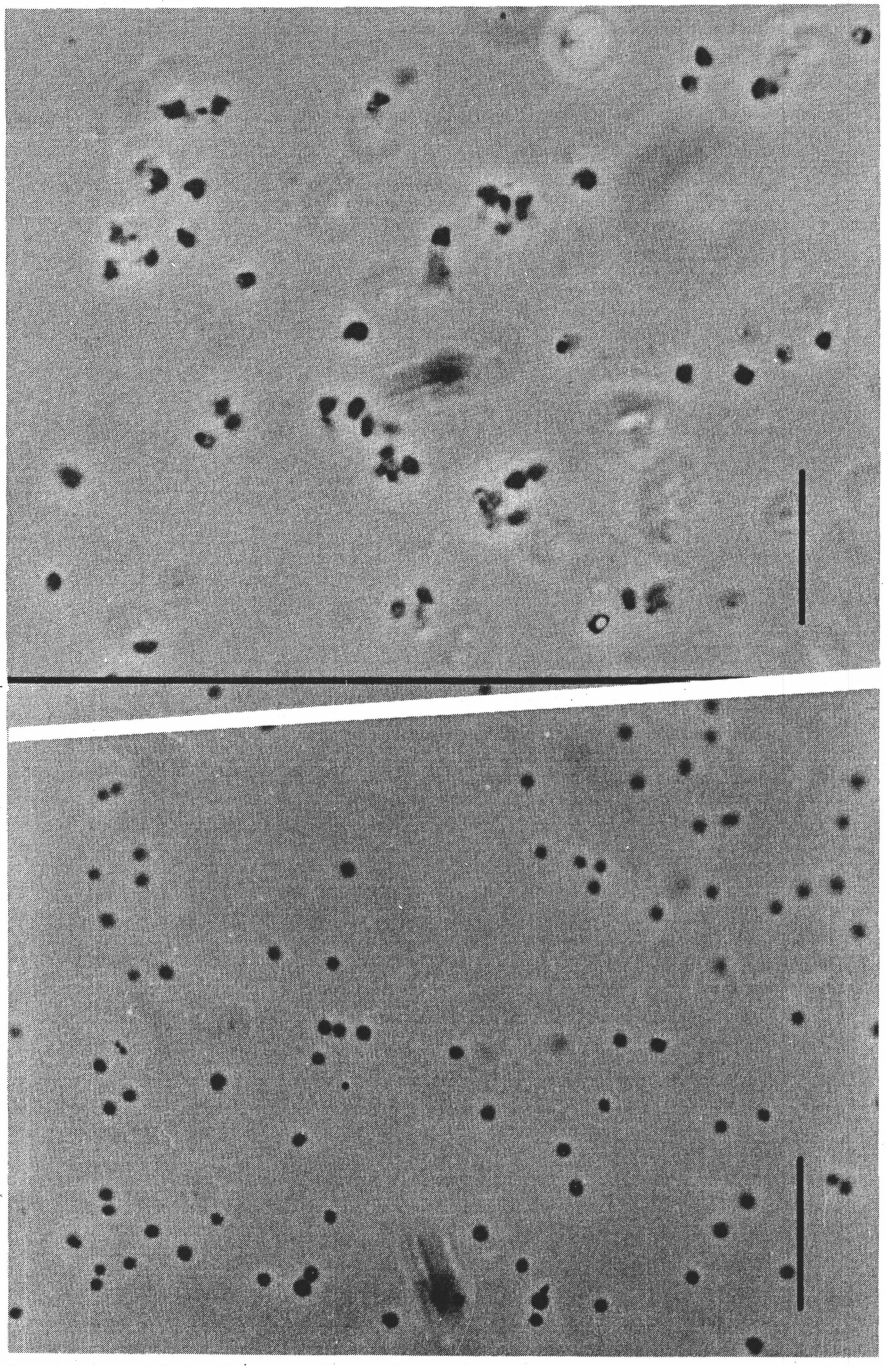

Fig. 4. Thermophilic isolates (a) TH2 from Pisciarelli Solfatara and (b) NA4 from Namaskarth. Phase-contrast micrographs. Bar, 10 μm.

TABLE I

Energy Sources of Mesophilic Isolates

Substrate	Strains			
	Th. ferrooxidans ATCC 23270	L7	Hö5	SP5/1
ore mixture G1 (3.3 %)	+	+	+	+
$FeSO_4$ (2.5 %)	+	±	−	+
S_o (0.3 %)	+	±	+	+
yeast extract (0.05 %)	−	−	+	−
sucrose (0.1 %)	−	−	+	n.d.

The organisms were purified by serial dilution repeated at least 3 times. For this purpose, cells attached to the ores were detached by strongly shaking (about 1 min.) with a whirlimix. The enrichment culture of sample SP5/1 were successfully plated on polysilicate plates [15] with $FeSO_4$ as an energy source.

Morphology

From all six samples from Vulcano, slim irregular rods, about 2.5 to 4 μm long and 0.3 μm in diameter with one polar flagellum, about 4 μm long and 17 nm in width were isolated (Fig. 1). From this group, isolate L7 was further characterized. A coccoid mesophilic isolate (SP5/1) varying in diameter between 0.8 to 2 μm (Fig. 2) was obtained from a source with an original temperature of 30°C and a pH of 1.5 from Pisciarelli Solfatara.

The samples of the Höhenstein mine yielded a third group of mesophilic isolates consisting of short small rods about 1 μm long and 0.3 μm in diameter from which isolate Hö5 was further investigated (Fig. 3). The thermophilic and extremely thermophilic isolates are regular (isolates TH2

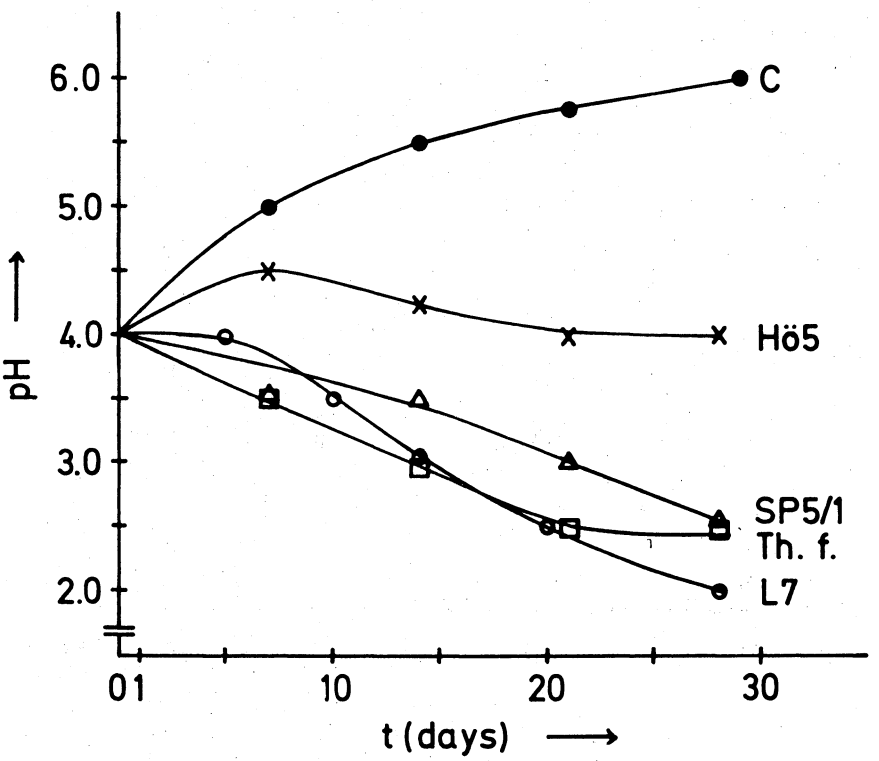

Fig. 5. pH profiles during growth of the new isolates on the sterile ore mixture G1.C = uninoculated control. Th. f. = *Thiobacillus ferrooxidans* ATCC 23270.

and SP3a; Fig. 4 a) or irregular (Kra23, NA4 and VE2; Fig. 4 b) spheres, about 0.7 to 1.5 μm in diameter. Isolate VE2 possesses a flagellum-like structure, about 30 nm in diameter and 12 μm long (not shown).

Physiology

All isolates are able to grow chemolithoautotrophically on natural ores, e.g. mixture G1 (Table I). Isolate SP5/1 can also utilize $FeSO_4$ and S° as energy sources and therefore behaves like *Th. ferrooxidans*. The marine isolate L7 grows best on natural ores. On $FeSO_4$ and on S°, extremely slow and weak growth is obtained after a 2-months adaptation phase in the presence of both components together with ore mixture G1. In contrast, isolate Hö5 does not grow lithotrophically on $FeSO_4$ or S° but only on natural ores. However, this organism also grows on yeast extract and

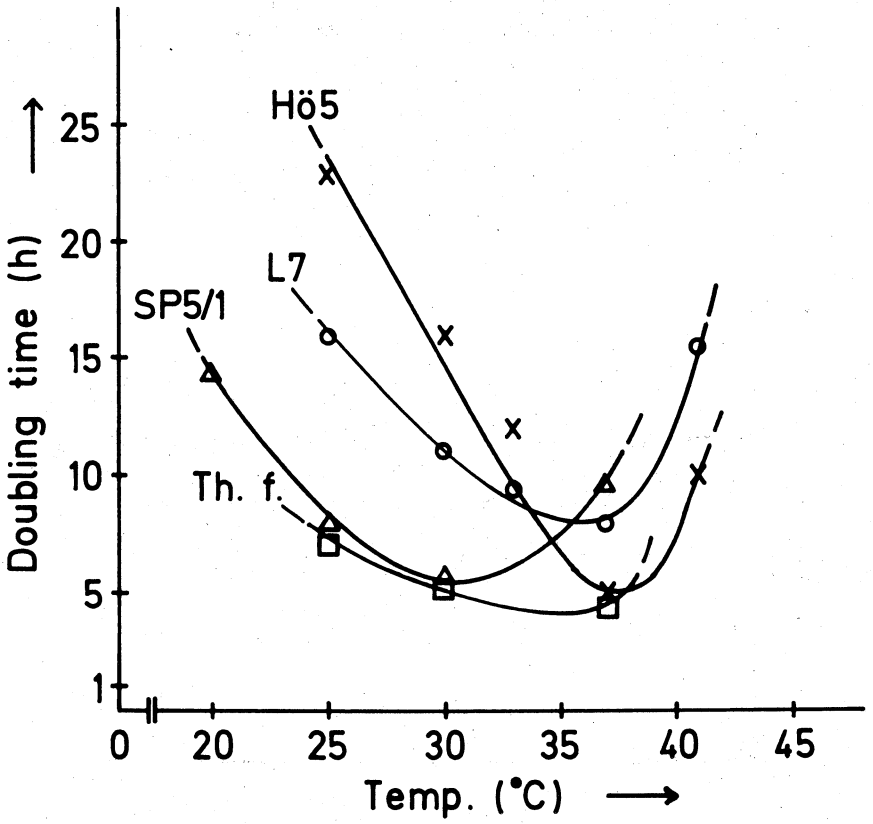

Fig. 6. Temperature optima of mesophilic strains. Th. f. = *Thiobacillus ferrooxidans* ATCC 23270. The strains were grown on ore mixture G1 as energy source in the basal media described under materials and methods. The doubling time was determined by direct counting in a Thoma counting chamber after vigorous shaking with a whirlimix.

even on sucrose as energy sources and is therefore a facultatively heterotrophic organism (Table I). The optimal pH for Hö5 is about 4.5. No growth occurs at pH 3 or below. During autotrophic growth on ores, the pH is only slightly lowered in contrast to all other isolates and *Th. ferrooxidans* (Fig. 5). The temperature optima of the three new mesophilic isolates and *Th. ferrooxidans* are between 30 and 37°C (Fig. 6).

The upper temperature limit of the thermophilic isolates is between 65 and 85°C, depending on the strains (Table II). The isolates SP3a and VE2 show the highest temperature maxima reported for ore-leaching bacteria. By their ability to grow lithoautotrophically on ores, the isolates are different from *S. brierleyi* (DSM 1651) (Table III). They can be distinguished from each other by their ability to utilize S° and yeast extract (Table III).

Chemotaxonomy

All mesophilic isolates show a negative gram staining reaction and contain meso-diaminopimelic acid and are sensitive to the antibiotics ampicillin, vancomycin and kanamycin, indicating that they belong to the gram-negative eubacteria. As a first indication, patterns of SDS-soluble cell proteins were compared with those of the type strains of *Th. ferroooxidans, Th. thiooxidans*, and *L. ferrooxidans* (Fig. 7). It is obvious, that the protein patterns of isolates L7 and Hö5 are very different from each

TABLE II
Growth Temperatures of Thermophilic Isolates

Strains	Temperatures			
	65°C	75°C	80°C	85°C
TH2	+	+	-	-
SP3a	+	+	+	n.d.
Kra23	+	-	-	-
NA4	+	-	-	-
VE2	+	+	+	+
S. brierleyi DSM 1651	+	+	-	-

TABLE III

Energy Sources of Thermophilic Isolates

Strains	Substrate		
	ore mixture G1 (3.3 %)	S_o (0.3 %)	yeast extract (0.1 %)
TH2	+	+	+
SP3a	+	−	+
Kra23	+	+	−
NA4	+	+	−
S. brierleyi DSM 1651	−	−	+

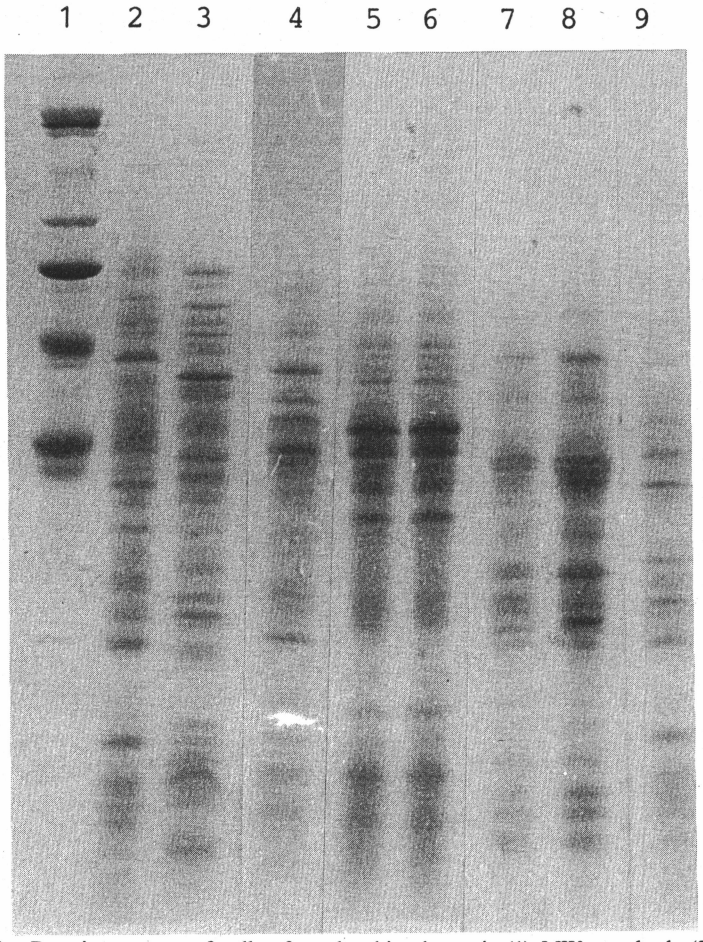

Fig. 7. Protein patterns of cells of ore-leaching bacteria (1) MW standards (200; 116; 92; 65; 45 kD) (2) *Th. ferrooxidans* ATCC 23270 (3) *L. ferrooxidans* DSM 2705 (4) *Th. thiooxidans* ATCC 19377 (5) L7 (6) LM3 (7) Hö3 (8) Hö5 (9) SP5/1.

other and from the type strains, while that of the coccoid isolate SP5/1 shows some similarity with *Thiobacillus ferrooxidans*. These findings are in agreement with the metabolic properties of these isolates and support the assumption that L7 and Hö5 may represent novel leaching organisms. The thermophiles possess protein subunit envelopes, suggesting that they belong to the sulfur-dependent archaebacteria [16].

Metal Mobilization

During dissolution of the ore mixture G1, isolates L7 and SP5/1 and *Th. ferrooxidans* 23270 are similar in their ability to solubilize various metal ions, e.g. Cu^{2+} (Fig. 8 a), Zn^{2+} (Fig. 8 b) and U^{6+} (not shown). In

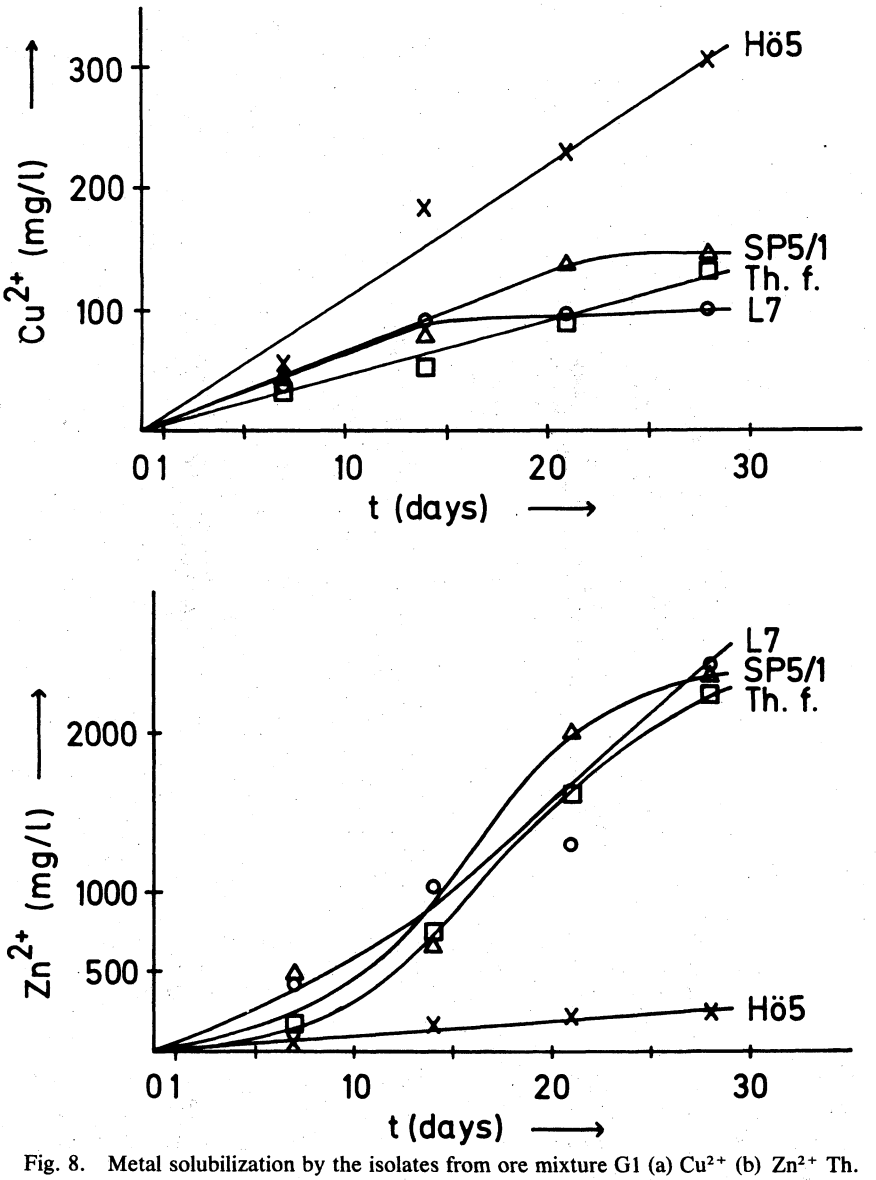

Fig. 8. Metal solubilization by the isolates from ore mixture G1 (a) Cu^{2+} (b) Zn^{2+} Th. f. = *Thiobacillus ferrooxidans* ATCC 23270.

contrast, isolate Hö5 shows practically no solubilization of Zn^{2+} (Fig. 8 b) and uranium, but a pronounced preference for copper (Fig. 8 a), indicating a metal specificity unknown up to now.

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