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Evaluation of *Leptospirillum ferrooxidans* for Leaching

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The importance of *Leptospirillum ferrooxidans* for leach processes has been evaluated by studying the lithotrophic flora of three mine biotopes and a heap leaching operation, by percolation experiments with inoculated, sterilized ore, and by morphological, physiological, and genetic investigations of pure and mixed cultures of *L. ferrooxidans*, *Thiobacillus ferrooxidans*, and *Thiobacillus thiooxidans*. In biotopes of 20°C or above, *Leptospirillum*-like bacteria are as abundant as *T. ferrooxidans*. Leptospirilli represent at least one-half of the ferrous-iron-oxidizing population. Percolation experiments confirmed this result. Leptospirilli were as numerous as *T. ferrooxidans*. At reduced temperatures, the generation times of leptospirilli increase more so than those of *T. ferrooxidans*. At 14°C, *Leptospirillum* grows slowly and *T. ferrooxidans* dominates the population. Physiological investigations indicate that *L. ferrooxidans* is a strict chemolithoautotroph, metabolizing only ferrous iron and pyrite. Even an addition of 0.05% (wt/vol) yeast extract inhibited its growth. The maximum ferrous-iron-oxidizing activity of *L. ferrooxidans* amounts to about 40% of the activity of *T. ferrooxidans*. After growth on sulfidic ore, both species exhibit reduced iron-oxidizing activities, *L. ferrooxidans* exhibiting one-third and *T. ferrooxidans* exhibiting one-seventh of their maximum activities. Surprisingly, the absolute values are similar. For indirect leaching, *L. ferrooxidans* is as important as *T. ferrooxidans*. This was confirmed by the results of percolation experiments. *L. ferrooxidans* together with *T. thiooxidans* mobilized metals at least as well as *T. ferrooxidans* did. The best results were obtained with a mixed culture of all three species.

Various acidophilic iron- and/or sulfur-oxidizing bacteria are responsible for the oxidation of mineral sulfides in leach biotopes. At ambient temperatures, the most important bacteria are *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans*, and *Leptospirillum ferrooxidans* (26). The importance of thiobacilli is well documented (6, 7, 11, 13, 32). Yeasts, flagellates, molds, and chemoorganotrophic bacteria have been detected in such habitats. However, little information is available on the occurrence of leptospirilli in such environments. Since the first report of Markosyan (17) on the existence of a mesophilic, vibrioid-shaped, iron-oxidizing bacterium thriving in an environment which was believed to be occupied exclusively by lithotrophic bacteria of the *T. ferrooxidans* and *T. thiooxidans* type, a few reports of leaching involving *L. ferrooxidans* have been published. Until now, only limited information on the importance of *Leptospirillum* spp. in natural habitats has been available. Ecological studies are missing. Furthermore, the taxonomic status of the genus *Leptospirillum* is uncertain. In the latest edition of *Bergey's Manual*, *L. ferrooxidans* is mentioned but not described as an acknowledged genus or species (14). A valid description (by Markosyan) is still missing.

In the present study, several leach biotopes, namely, three mines and a dump, were examined for thiobacilli and leptospirilli. Pure cultures of selected samples were prepared. After characterization of the bacteria, leach studies were initiated to evaluate whether leptospirilli are of importance in natural leach biotopes.

MATERIALS AND METHODS

Microorganisms and growth conditions. *T. ferrooxidans* R1, R5, and R7 originated from samples from the Ilba mine in northwest Romania. *T. thiooxidans* R20 was also isolated from samples from the Ilba mine, and strains K6 and K16 originated from sewage pipelines in Hamburg, Germany (20). *L. ferrooxidans* R3 and R30 came from the Ilba mine in Romania, whereas strain P_3A was isolated from an enrichment culture obtained by D. Haisch (Lima, Peru).

For the ferrous-iron-oxidizing bacteria, a nutrient solution containing 50 g of $FeSO_4 \cdot 7H_2O$ per liter (about 10 g of ferrous ions per liter) at a pH of 1.8 was used (16). The sulfur-oxidizing bacteria were cultivated in S5 medium supplemented with 1% sulfur (12). Stock cultures were kept at 28°C in the dark for 2 months and then transferred into fresh medium (10% inoculum). Working cultures were either stirred and aerated or shaken vigorously on a shaker.

Enumeration of oxidizers of ferrous iron and sulfur. Samples were suspended in 100-ml bottles containing 50 ml of sterile, acidified tap water (pH 3) and incubated for 2 h on a rotary shaker at 250 rpm (Infors TR type) to detach cells from the substrates. To get reliable results for the *L. ferrooxidans* cell counts, 2 h was permitted for detachment to occur. Preliminary experiments had shown that this time period was necessary to achieve a high level of cell recovery, since these cells adhered more tightly to surfaces than did cells of *T. ferrooxidans*. The turbid suspension was diluted in 10-fold steps to 10^{-8}. Liquid samples (from the reservoir of the percolator or mine water) were diluted in the same way. For estimating the numbers of ferrous-iron- or sulfur-oxidizing bacteria, a three-tube most-probable-number (MPN) technique was applied. Since most cells grow attached to ore particles, only a few can be resuspended by known techniques and, thus, are available for counting by the MPN procedure. Hence, most MPN counts provide relative concentrations of microbes per unit of sample. For reliability, a carefully standardized procedure is necessary. For ferrous-iron oxidizers, 2.5 ml of medium containing 10 g of ferrous ions per liter at a pH of 1.8 (16) was used, whereas, for sulfur oxidizers, 2.5 ml of medium with a 10 g of sulfur per liter at a pH of 4.5 (12) was used. The inoculated tubes were shaken for 21 days in the dark at 28°C. Tests for *T. thiooxidans* were considered to be positive if the pH dropped below 2. For

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evaluation of *T. ferrooxidans* and *L. ferrooxidans*, those tubes were counted in which ferric iron formed and microscopy revealed the presence of bacteria. Differentiation between these two iron oxidizers was also determined by light microscopy. The highest positive dilution steps were analyzed for rods (indicating *T. ferrooxidans*) or curved and vibrioid-shaped cells (indicating *L. ferrooxidans*). Chemoorganotrophic microorganisms were counted by plating on DEV gelatin agar (for bacteria) or Sabouraud maltose agar (for fungi) of Merck (Darmstadt, Germany). Details have been described previously (28).

Isolation and characterization of ferrous-iron oxidizers. The highest positive dilution steps were used for preparation of pure cultures. From these samples, serial dilutions were made in 10-fold steps up to 10^{-9}. The dilution steps were repeated at least eight times. Selection for *T. ferrooxidans* was done by repeating the transfer rapidly, because microscopy had shown that the growth of *T. ferrooxidans* preceded the growth of *L. ferrooxidans*. The series of tests were performed at temperatures below 20°C. Accordingly, selection was done for *L. ferrooxidans* by using a late transfer. In addition, the incubation temperature was raised and the pH was decreased. The purity of the cultures obtained was checked by light microscopy during each stage of growth. Furthermore, cultures of *L. ferrooxidans* were checked for lack of sulfur-oxidizing capacity. Screening for heterotrophic contaminants was done by two methods, by using either the above-mentioned DEV gelatin agar and Sabouraud maltose agar (Merck) or the *Acidiphilium* medium (8). Thus, contaminants growing with high or low concentrations of nutrients could be detected. If these procedures resulted in pure cultures, characterization was done.

For transmission electron microscopy, cells were fixed in 5% glutaraldehyde in sodium cacodylate buffer (27) and then treated for 4 h with osmium tetroxide. Dehydration was done by increasing the ethanol concentration to 100%. The next step involved embedding the cells in Epon. Ultrathin sections of Epon-embedded cells were placed on copper grids and treated with uranium acetate and lead citrate. A Philips 201 electron microscope was used.

The guanine-plus-cytosine (G+C) content of the pure cultures was measured (18) with a Gilford 2600 spectrophotometer. References with known G+C contents were *Escherichia coli* K-12 and *T. ferrooxidans* ATCC 23270. Determinations were repeated at least three times.

Tests for substrate specificity were made with different organic and inorganic substrates in washing solution (16) supplemented with test substances. Volumes of solution (50 ml) in 100-ml flasks were placed on a rotary shaker in the dark at 28°C. Sulfur (S^0, 10 g/liter, pH 2.0), sulfite (SO_3^{2-}, 5 g/liter, pH 4.5), pyrite (FeS_2, 10 g/liter, pH 2.0), thiosulfate ($Na_2S_2O_3$, 5 g/liter, pH 4.5), and tetrathionate ($K_2S_4O_6$, 3.5 g/liter, pH 4.5) were tested for support of autotrophic growth.

These substances were also supplemented with yeast extract (0.05%) to test whether mixotrophic growth was possible. Acetate, citrate, malate, pyruvate, and glucose (10 mM, pH 4.5) were tested for support of chemoorganotrophic growth.

Growth was tested by microscopy and determinations of pH value, redox potential (25), sulfate (turbidimetry) (5), ferrous and ferric iron (phenanthroline) (2), protein (4, 31), total cell count by Helber chamber, and live cell count by the three-tube MPN method. Metal concentrations were analyzed by the atomic absorption spectrophotometry technique with a model 1100B Perkin-Elmer machine.

Evaluation of the importance of *L. ferrooxidans*. (i) Determinations of cell counts in leach biotopes. Three leach biotopes were selected for sampling. Site 1 is located in the Ilba mine (Venera vein) in northwest Romania near Baia Mare. It contains a sulfidic, complex copper-zinc ore with an average pH of 3.3 and has a temperature of 14°C. It contains pyrite, chalcopyrite, covellite, bornite, sphalerite, galena, and marcasite. Site 2 is located in the Baia Sprie mine in northwest Romania, also near Baia Mare. It contains a sulfidic copper ore of mainly chalcopyrite, pyrite, and covellite with an average pH of 3.0 and has a temperature of 28°C. Site 3 is located in west Romania near Rosia Poieni. It is a sulfidic, complex copper ore leach heap with an average pH of 2.5. The temperature at site 3 varies throughout the year. In winter, the surface of the dump is frozen and the plant is out of operation.

(ii) Determination of cell counts in percolators. Glass percolators with three sampling stubs were used. Each percolator was filled after sterilization with 4 kg of sterilized ore (8 h at 160°C, closed vessels) and inoculated with 10^8 cells of pure and mixed cultures of *T. ferrooxidans*, and/or *T. thiooxidans*, and/or *L. ferrooxidans*, per g of ore. The tests were run at 28°C in the dark. Washing solution (4.5 liters) was used (16). The pumping rate was 1.8 liters per h. The ore originated from the Ilba mine. It was ground into pieces smaller than 5 mm in diameter, and the pieces between 1 and 5 mm in diameter were used. The ore analysis yielded 4.7% Fe, 6.8% S, 0.3% Cu, 0.14% Zn, 0.23% Mn, 2.3% Mg, 0.002% Co, 0.05% Ni, and 0.1% Ca. The main minerals in the highly disseminated, partially oxidized ore were pyrite, chalcopyrite, galena, and sphalerite.

Sampling was done several times during the course of an experiment. Samples of about 5 g were taken from the ore body through the stubs. Cell counts were determined as described above.

RESULTS

Field studies for quantification of sulfur- and ferrous-iron-oxidizing bacteria. Eighty-five samples were taken from the Ilba mine during five sampling campaigns. The samples were characterized as either dry, wet, mud, or mine water. The results are presented in Table 1. It is evident that thiobacilli were present in each sample. The cell counts of the acidophiles were comparable (within the inherent inaccuracy of the MPN method). Even weakly acidophilic species such as *Thiobacillus intermedius* were detectable. *L. ferrooxidans* could be detected in 11 of 85 samples but not in mine water. Regularly, more cells of *T. ferrooxidans* than of *L. ferrooxidans* were found.

Chemoorganotrophic microorganisms were outnumbered by lithotrophs. Chemoorganotrophs represented less than 10% of the population. The nitrogen content ranged between 5 and 26 mg/kg of sample and, thus, was not limiting. For comparison, samples were taken from a mine biotope with a carbonaceous ore (Potcoava-Dubova in southwest Romania). The pH at this site was 8.5. Thiobacilli and leptospirilli were not detectable (results not shown). Thus, acidophilic lithotrophs were without importance in this alkaline biotope.

An additional sampling campaign was performed in the Baia Sprie mine. Twenty samples were taken and analyzed for lithotrophs. Samples were divided into humid and wet. In addition, a solid stone was taken as a sample. The results are presented in Table 2. In four samples, lithotrophs were not detectable; in the remaining 16 samples, oxidizers of ferrous iron were regularly demonstrated. The humid samples con-

TABLE 1. Quantification of acidophilic lithotrophs in the sulfidic Ilba mine (Romania)[a]

Sample type[b]	pH	Protein (μg/g of sample)	Nitrate (μg/g of sample)	Cell count of:					
				T. ferrooxidans	*T. thiooxidans*	Ti[c]	*L. ferrooxidans*	Chemoorganotrophs	Fungi
Dry	3.3	72	11	5×10^3	5×10^3	1×10^5	4×10^3	2×10^4	2×10^4
Wet	3.3	86	18	6×10^5	6×10^5	1×10^4	2×10^5	3×10^4	2×10^5
Mud	3.4	129	5	3×10^3	3×10^5	9×10^4	2×10^3	3×10^4	7×10^4
Water	3.2	71	8	2×10^6	8×10^5	1×10^5	+[d]	4×10^4	6×10^4

[a] Values are means for each sample type.
[b] Dry (water not visible), 40 samples; wet (water visible), 29 samples; mud (slurry), 8 samples; water, 8 samples.
[c] Ti, moderately acidophilic thiobacilli like *T. intermedius*, *T. novellus*, and *T. neapolitanus*, etc.
[d] +, detectable but not quantifiable.

tained 6×10^5 cells of ferrous-iron oxidizers and of sulfur oxidizers per g of ore. About 2×10^4 cells of weakly acidophilic thiobacilli per g of ore were also present. Microscopic evaluation of the highest dilution steps revealed that *T. ferrooxidans* dominated in 9 of 16 samples. In the other seven samples, *L. ferrooxidans* was the dominant organism.

The third sampling campaign was done on the heap leaching at Rosia Poieni. After 2 years of active leaching, the heap (20 by 20 m in area) had decreased from a height of 4 m to about 2 m because of biodegradation of mineral sulfides (resulting in smaller rock particles). Samples were taken from the surface, from a depth of 60 cm, from a puddle, and from the effluent at the bottom of the heap (results not shown). About 5×10^6 cells of sulfur- and ferrous-iron-oxidizing bacteria per g of ore were present. The cell count of the weak acidophiles amounted to about 4×10^3 cells per g of ore. In the effluent sample, the cell concentration was reduced to 1% or less of those of the ore samples. More than 99% of the bacterial population is living in the dump. Microscopic evaluation of the highest dilution steps demonstrated that *L. ferrooxidans* is as abundant in the heap as *T. ferrooxidans*. Every second sample contained only cells of *Leptospirillum*-like bacteria. On the surface of the heap, where the temperature at the sampling time was about 0°C, only *T. ferrooxidans* could be detected. The puddle and effluent samples contained only *T. ferrooxidans*. It is possible that *L. ferrooxidans* adheres more tightly to surfaces than does *T. ferrooxidans*. Similar evidence has been given previously (10, 23).

Laboratory studies for characterization of ferrous-iron-oxidizing pure cultures. (i) Morphology. From several samples of the Ilba mine, four pure cultures of ferrous-iron-oxidizing bacteria were prepared. An additional culture was made from an enrichment culture from Peru. Three cultures were obtained which resembled *T. ferrooxidans*, and two cultures were obtained which were similar to *L. ferrooxidans*. *T. ferrooxidans* R1, R5, and R7 were straight rods with a diameter of 0.3 to 0.6 μm and a length of 1 to 3.5 μm. The cells of two *L. ferrooxidans* strains, R3 and P_3A, had a diameter of 0.3 to 0.6 μm and a length of up to 3.5 μm. *L. ferrooxidans* showed strong pleomorphism. Young cultures (up to 4 days old) consisted mainly of vibriolike cells, whereas older cultures (1 to 2 weeks old) contained mostly spiral cells with up to four turns. Figure 1A to C demonstrates the appearance of the two strains. Ultrathin sections of *L. ferrooxidans* P_3A show that this strain contains a periplasmic space characteristic of lithotrophs. Carboxysomes, viruslike inclusion bodies with ribulose-bisphosphate carboxylase (30), were not detectable (Fig. 2), and intracytoplasmic membranes were not visible.

(ii) Physiology. Growth curves (not shown) of these five strains proved that all were able to grow lithoautotrophically with ferrous iron as the source of energy and carbon dioxide as the source of cell carbon. Table 3 gives a summary of the results. It is evident that the properties of *T. ferrooxidans* and *L. ferrooxidans* are similar. One exception is the generation time of 4 h for *T. ferrooxidans* R1, which is considerably lower than the generation times of the other strains. It is also obvious that the two strains of *L. ferrooxidans* grow at considerably lower pH values than *T. ferrooxidans*. There is also some evidence that *T. ferrooxidans* grows best with ferrous iron concentrations that are higher than those for *L. ferrooxidans*. Thiobacilli as well as leptospirilli are mesophiles. The optimum growth temperatures vary from 22 to 35°C. However, considerable differences were noted for growth at reduced temperatures. The generation times of the three strains of *T. ferrooxidans* increased at 15°C to 44, 33, and 32 h (for R1, R5, and R7, respectively). The generation times of the two *Leptospirillum* strains increased even further, to 63 h (R3) and 93 h (P_3A). Even strain R1 of *T. ferrooxidans*, which has a growth temperature optimum of 35°C which is well above those of leptospirilli, exhibits at 15°C a shorter generation time than the two *L. ferrooxidans* strains.

Additional tests were performed to assay substrate specificity. Five substrates were used to test for lithotrophic growth. For a second test series, these substrates were supplemented with 0.05% yeast extract to test for mixotrophic growth. The third test series was run with organic compounds to test for chemoorganotrophic growth. The results are given in Table 4. It is evident that *L. ferrooxidans* is able to grow on pyrite. None of the other substrates supported autotrophic or mixotrophic growth. *T. ferrooxi-*

TABLE 2. Quantification of acidophilic lithotrophs in the sulfidic Baia Sprie mine (Romania)[a]

Sample type[b]	pH	Cell count of:			No. of samples with prevalence of:	
		T. ferrooxidans or *L. ferrooxidans*	*T. thiooxidans*	Ti[c]	*T. ferrooxidans*	*L. ferrooxidans*
Stone	4.6	ND[d]	ND	ND		
Humid	3.4	+(3)[e]	ND	ND	3	
Wet	3.0	6×10^5	6×10^5	2×10^4	6	7

[a] Values are means for each sample type.
[b] Stone (solid block), 1 sample; humid (water not visible), 6 samples; wet (water visible), 13 samples.
[c] Ti, moderately acidophilic thiobacilli like *T. intermedius*, *T. novellus*, and *T. neapolitanus*, etc.
[d] ND, not detectable.
[e] +, detectable but not quantifiable; (3), three of six samples were evaluated.

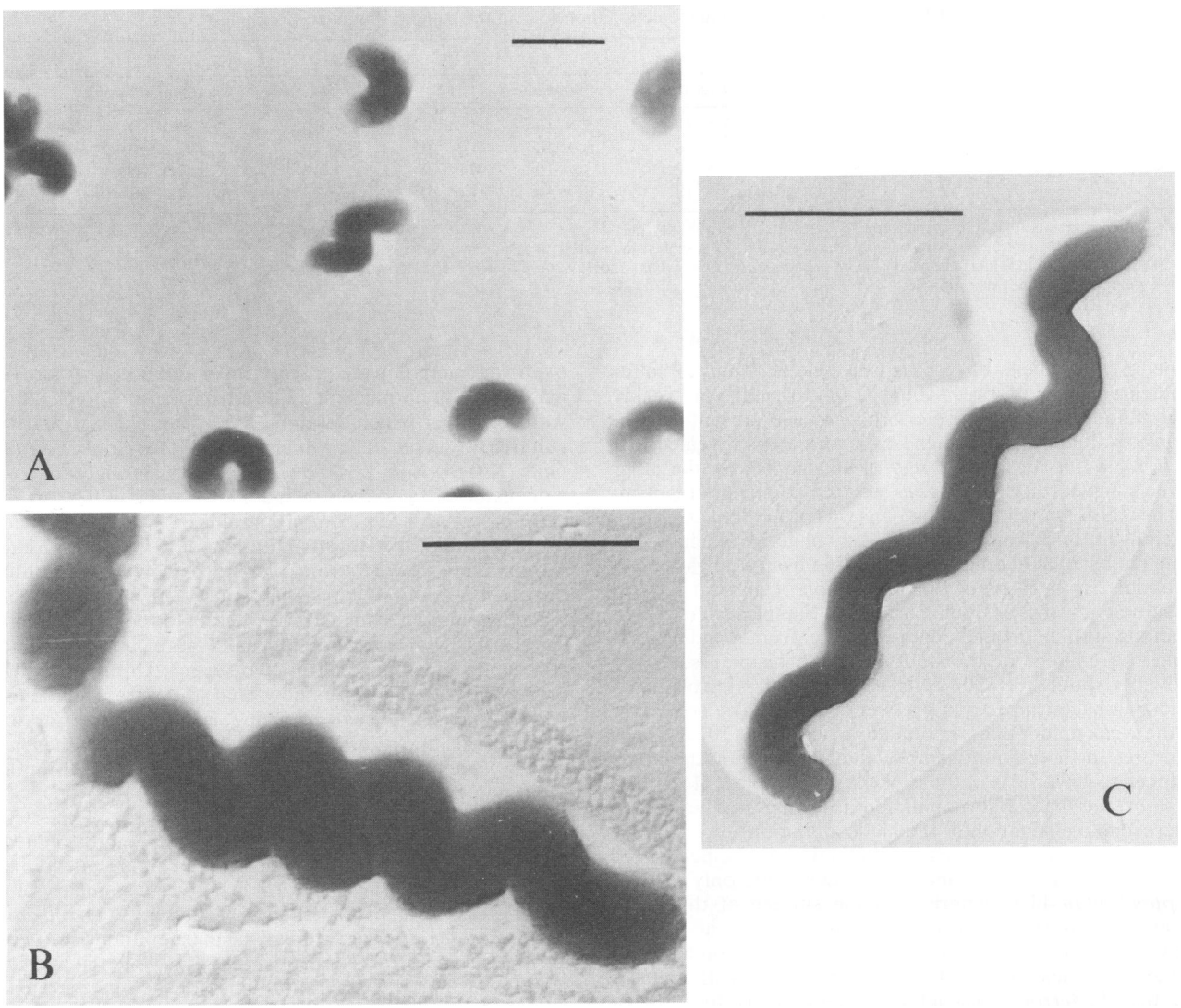

FIG. 1. Transmission electron micrographs of cells of *L. ferrooxidans* R3 after 4 days of growth (A), *L. ferrooxidans* R3 after 10 days of growth (B), and *L. ferrooxidans* P_3A after 10 days of growth (C). Bars, 1 μm.

dans was able to grow autotrophically with the five substrates, but mixotrophic growth was not detectable. The organic compounds did not support any growth of either species.

Furthermore, G+C analyses of the five strains were prepared. For reference, *E. coli* K-12 and *T. ferrooxidans* ATCC 23270 were used. The determinations of the melting points yielded G+C contents for the *T. ferrooxidans* strains 51.9% (R5), 58.8% (R1), and 63.1% (R7). ATCC 23270 had a G+C content of 58.1%. The G+C contents for the *L. ferrooxidans* strains were 53.9% (P_3A) and 56.4% (R3). The standard deviation was about ±0.4% for at least six determinations each. The values for *T. ferrooxidans* R1 and R7 are in accordance with other data (9) for the G+C content spectrum of *T. ferrooxidans*. The value for strain R5 is below this range, but, because of its growth characteristic, the strain belongs to the species *T. ferrooxidans*. The data for *L. ferrooxidans* fall within the range.

Laboratory studies for evaluation of leaching capacities. In addition to the above-mentioned five strains, *L. ferrooxidans* R30 and *T. thiooxidans* K6 and K16 were used. For leach experiments in glass percolators with crushed sulfidic ore from the Ilba mine (Romania), 4 kg of sterilized ore (pieces 1 to 5 mm in diameter) was needed. The ore was inoculated with different combinations of pure and mixed cultures. *L. ferrooxidans* was tested in combination with *T. thiooxidans*, *T. ferrooxidans*, or both. The cell count was 10^8 cells per g of ore for sulfur or ferrous-iron oxidizers. The high cell count was chosen to avoid any lag phase before the start of leaching. Figure 3 summarizes the results of a typical experiment. The ore had been inoculated with *T. ferroxidans*, *T. thiooxidans*, and *L. ferrooxidans*. It is obvious that the ferrous-iron-oxidizing bacteria grew on the ore. The cell counts of *T. ferrooxidans* and of *L. ferrooxidans* increased slightly to 5×10^8 cells per g of ore (with some deviations) throughout the experiment. *L. ferrooxidans* grew as well as *T. ferrooxidans*. In contrast, the ability of *T. thiooxidans* to grow on the ore was limited. To evaluate the influence of temperature on the growth of the mixed culture, an experiment at a temperature of 14°C (instead of 28°C) was in-

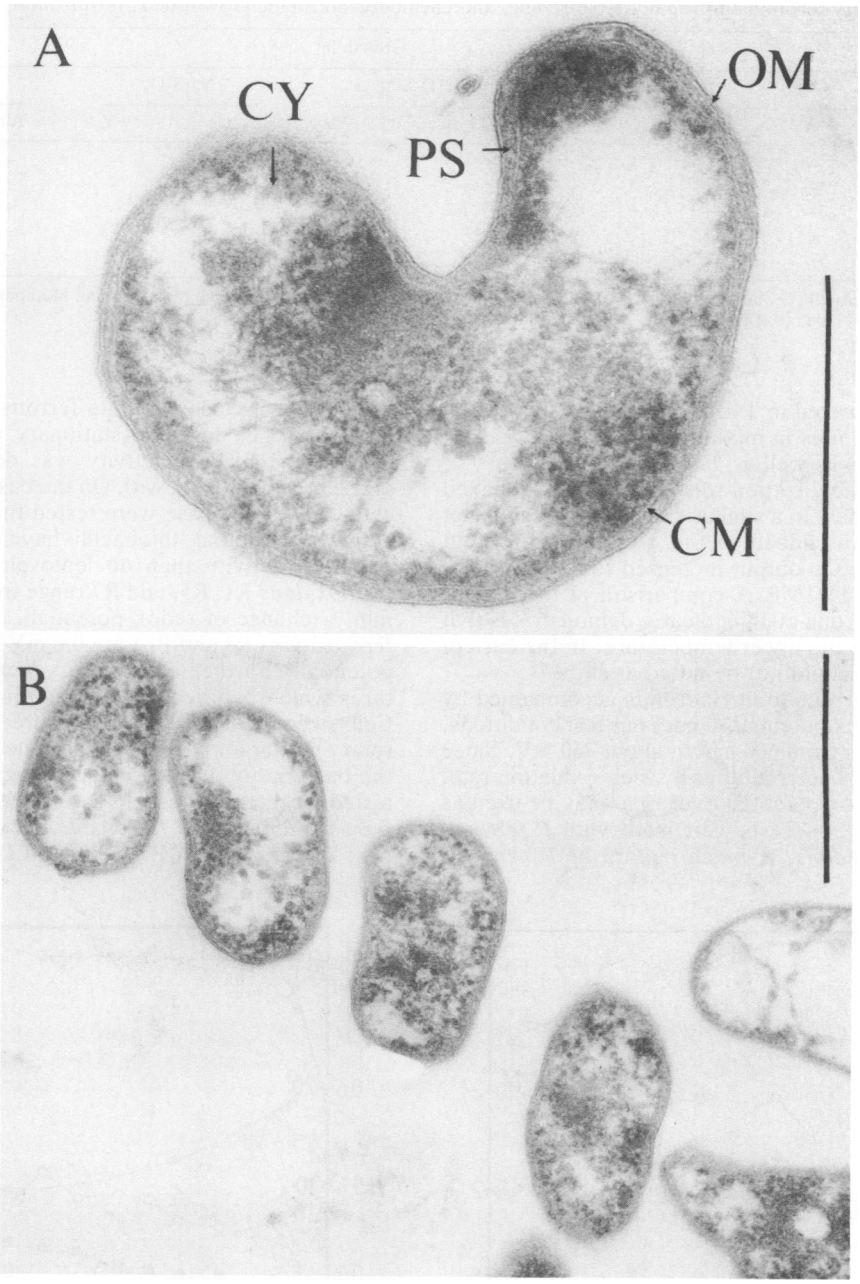

FIG. 2. Transmission electron micrographs from ultrathin sections of cells of *L. ferrooxidans* P_3A. (A) Vibriolike cell (after 4 days of growth). CY, cytoplasma; CM, cytoplasmic membrane; OM, outer membrane; PS, periplasmic space. (B) Section along the longitudinal axis of a spiral cell (after 10 days of growth). Several parts of the spiral cell are visible. Bars, 0.5 μm.

cluded. This temperature is comparable to the temperature in the Ilba mine. The results are presented in Fig. 4. It is evident that leaching proceeded at a lower rate compared to the values shown in Fig. 3. There was also a striking difference in the cell counts: the cell counts of *T. ferrooxidans* increased under these conditions, the cell counts of *T. thiooxidans* remained at the inoculation level, and the cell counts of *L. ferrooxidans* decreased throughout the experiment (from 10^6 to 10^4 cells per g of ore). Such a reduction was never noted at 28°C.

The metal output was analyzed for all experiments, and

TABLE 3. Physiological characterization of the three strains R1, R5, and R7 of *T. ferrooxidans* and the two strains R3 and P_3A of *L. ferrooxidans*

Strain	Generation time (h)	Cell count/ml	Protein (mg/liter)	pH optimum	Temp optimum (°C)	Fe^{2+} optimum (g/liter)
R1	4	1×10^8	2.4	2.2	35	≥10
R5	12	8×10^7	1.7	2.3	22	8
R7	13	8×10^7	1.6	2.0	22	≥10
R3	12	1×10^8	1.4	1.7	28	8
P_3A	10	8×10^7	1.3	1.5	30	6

TABLE 4. Capacity for lithoautotrophic, mixotrophic, and chemoorganotrophic growth of *T. ferrooxidans* and *L. ferrooxidans*

Strain	Growth in[a]:										
	S^0		FeS_2		Na_2SO_3		$Na_2S_2O_3$		$K_2S_4O_6$		OC[b]
	Alone	+Y	Alone	+Y	Alone	+Y	Alone	+Y	Alone	+Y	
R1	++	−	++	−	+	−	+	−	++	−	−
R5	++	−	++	−	+	−	++	−	++	−	−
R7	+	−	+	−	+	−	+	−	++	−	−
R3	−	−	+	−	−	−	−	−	−	−	−
P_3A	−	−	+	−	−	−	−	−	−	−	−

[a] ++, good growth; +, growth; −, no growth; +Y, 0.05% yeast extract added. For further details, see Materials and Methods.
[b] OC, organic compounds (acetate, citrate, malate, pyruvate, glucose).

the results are summarized in Table 5. They clearly demonstrate that *L. ferrooxidans* in mixed culture with *T. thiooxidans* mobilizes metals as well as *T. ferrooxidans*.

The highest metal mobilization was obtained with a mixed culture of all acidophiles. In a similar experiment (results not shown), 1% pyrite was added to the ore. The Zn output increased to 63%, the Co output increased to 32%, and the Mn output increased to 16%. A comparison of the experiment at 28°C with the one at 14°C clearly demonstrates that leaching is reduced at the lower temperature. In the case of Co and Mn, leaching could not be noted at all.

The oxidation of ferrous to ferric iron is accompanied by an increase in the redox potential. Under our test conditions, the potential increases from 600 mV to about 750 mV. Since ferrous-iron-oxidizing bacteria differ by their oxidation rate, the change of the redox potential over time may be used as a measure of activity (25). Tests were made with *T. ferrooxidans* and *L. ferrooxidans*. A batch culture of *T. ferrooxidans* R5 was tested for its ferrous-iron-oxidizing activity during the lag, log, and stationary growth phases. As expected, the highest activity was detected during the log phase (results not shown). On the basis of these results, only cells of the log phase were tested further. The experiments demonstrated that thiobacilli have a higher ferrous-iron-oxidizing activity than do leptospirilli. The values for *T. ferrooxidans* R1, R5, and R7 range from 1.2 to 1.3 to 1.5 mV min^{-1} (change of redox potential). The values for both *L. ferrooxidans* R3 and P_3A are 0.6 mV min^{-1} (change of redox potential). Further experiments were done with pure cultures which had been growing with Ilba ore for 10 weeks. Cells were detached from the ore by being shaken on a rotary shaker for at least 60 min. The ore was separated from the bacteria by differential centrifugation. These cells were tested for their ferrous-iron-oxidizing activity. As a result, *T. ferrooxidans* and *L. ferrooxidans* exhibited reduced activities: *T. ferrooxidans* R5 possessed 14% of its original activ-

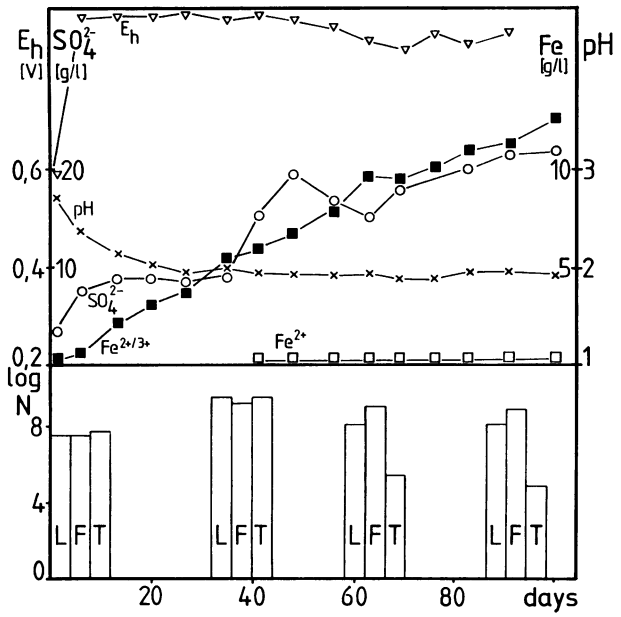

FIG. 3. Growth of *L. ferrooxidans*, *T. ferrooxidans*, and *T. thiooxidans* on sulfidic ore at 28°C in percolation experiments. Ore used was from the Ilba mine (Romania), 1 to 5 mm in diameter. E_h, redox potential; SO_4^{2-}, sulfate content; $Fe^{2+/3+}$, ferrous iron plus ferric iron content; Fe^{2+}, ferrous iron content; log N, logarithmically derived cell counts per gram of ore; L, *L. ferrooxidans* R3; F, *T. ferrooxidans* R7; T, *T. thiooxidans* R20.

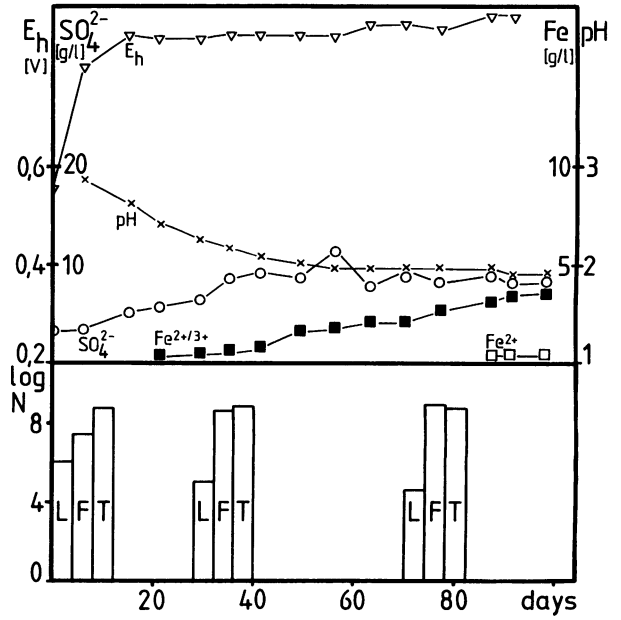

FIG. 4. Growth of *L. ferrooxidans*, *T. ferrooxidans*, and *T. thiooxidans* on sulfidic ore at 14°C in percolation experiments. Ore used was from the Ilba mine (Romania), 1 to 5 mm in diameter. E_h, redox potential; SO_4^{2-}, sulfate content; $Fe^{2+/3+}$, ferrous iron plus ferric iron content; Fe^{2+}, ferrous iron content; log N, logarithmically derived cell counts per gram of ore; L, *L. ferrooxidans* R3; F, *T. ferrooxidans* R7; T, *T. thiooxidans* R20.

TABLE 5. Metal output in leach tests of a Romanian ore inoculated with pure and mixed cultures of *T. ferrooxidans* (A), *T. thiooxidans* (B), and *L. ferrooxidans* (C)

Metal	Metal output (%) from cultures of:					
	Sterile	A (28°C)	C (28°C)	B+C (28°C)	A+B+C	
					28°C	14°C[a]
Cu	6	19	18	25	40	16
Zn	16	55	28	44	56	40
Co	14	30	17	28	26	14
Mn	11	14	10	13	13	10

[a] To this culture was added 0.5% sulfur.

ity, and *L. ferrooxidans* R3 possessed about 33%. The absolute value in both cases was 0.2 mV min^{-1}. Thus, the two species had similar ferrous-iron-oxidizing activities.

DISCUSSION

In summarizing the results, it became obvious that the bacterium *L. ferrooxidans* is an important member of the biocoenosis in leach habitats. Under appropriate conditions, one of every two oxidizers of ferrous iron belongs to this genus (and species). This result is of importance for studies on leaching with lithotrophic acidophiles. Most studies are done with *T. ferrooxidans*, sometimes combined with *T. thiooxidans* (if pure cultures are used), or with unspecified enrichment cultures. In the former case, the results are incomplete because of the absence of the effect of *L. ferrooxidans* whereas in the latter case *L. ferrooxidans* may have inadvertently contributed to the leaching. Muyzer et al. (21) reported such a finding when an immunofluorescence-DNA-fluorescence staining technique for monitoring a leaching experiment on desulfurization of coal was used. At 16 days after the inoculation of nonsterile coal samples, the autochthonous flora had overgrown the inoculated *T. ferrooxidans* and effectively removed pyrite. Since their antiserum was shown not to react with *L. ferrooxidans*, these bacteria may well have been the dominant leaching organisms. An important factor favoring the presence of *T. ferrooxidans* and/or *L. ferrooxidans* seems to be the temperature of the habitat. At 20°C or above, leptospirilli have generation times which are comparable to those of thiobacilli. However, at reduced temperatures, the generation times of the former increase more so than those of the latter. *T. ferrooxidans* can overgrow the leptospirilli in this habitat, as demonstrated by the percolation experiments. It is known that *T. ferrooxidans*-type bacteria show substantial growth even at temperatures as low as 4°C (1). Our field studies also revealed that temperature is of importance. At sites with temperatures of 20°C or higher (28°C, Baia Sprie), leptospirilli were as abundant as *T. ferrooxidans*, sometimes even outnumbering the latter, whereas at sites with temperatures below 20°C, leptospirilli were rarely detectable (Ilba mine). The percolation experiments with pure and mixed cultures confirm the results of the field study. At 14°C, a temperature which corresponds to the average temperature in the Ilba mine, *L. ferrooxidans* did not grow well. The cell counts of *L. ferrooxidans* declined, whereas those of *T. ferrooxidans* increased.

Much work on the use of *Leptospirillum* spp. in leaching processes has already been done (10, 19, 22, 24). In the opinion of the authors of those earlier works, *Leptospirillum*-like bacteria may be used as successfully as *T. ferrooxidans*. In some cases, the use of *Leptospirillum* spp. seems to be preferable because of their biochemical properties. The inhibiting concentration of ferric iron (23) for leptospirilli is more than 10 times higher than that for *T. ferrooxidans* (42.8 versus 3.1 mM Fe^{3+}). In addition, the high acid tolerance of the *Leptospirillum*-like bacteria may favor their growth in acidic biotopes (22). Our comparison of the optimum pH values confirms that leptospirilli have a lower pH optimum than *T. ferrooxidans*, although it has been described that *T. ferrooxidans* strains are still metabolically active at a pH of 0.8 (29). Another remarkable property of *L. ferrooxidans* is the substrate optimum. Again these data are in accordance with those of Norris et al. (23). They described a K_m value for ferrous iron of *Leptospirillum* spp. which is well below that of *T. ferrooxidans*. Obviously the ferrous-iron-oxidizing system needs less substrate for saturation than the system of *T. ferrooxidans*. The ability of *Leptospirillum* spp. to leach ore is reflected by its substrate specificity. Ferrous iron and pyrite serve as substrates for growth. Other sulfur compounds as well as yeast extract-supplemented substrates (mixotrophy) failed to support growth. Chemoorganotrophic growth also was not detectable. The data are in accordance with those of the first work on *L. ferrooxidans* (17).

Considerable evidence for the importance of *L. ferrooxidans* can likewise be derived from the fact that it has the same ferrous-iron-oxidizing activity as *T. ferrooxidans* when growing on pyrite or complex sulfidic ore. Measurements of the activities of both (25) yielded the same values. Leptospirilli are as important as thiobacilli at least in the mechanism of indirect leaching via ferric iron.

In addition, it seems that leptospirilli adhere to surfaces more tightly than do *T. ferrooxidans* cells. As an result of this finding, the impact of *L. ferrooxidans* will usually be underestimated or perhaps not be detected at all because of low cell counts.

The G+C contents of the original strain and of some other isolates were analyzed (10); the values ranged between 50 and 57%. Our two strains fit well in this range. The wide range of values indicates the existence of a phenospecies with several genospecies. This has been demonstrated for the phenospecies *Nitrosomonas* (15) and will also apply to *L. ferrooxidans* and *T. ferrooxidans*. In the case of *T. ferrooxidans*, a G+C content range of 57 to 65% has been described (9) and, accordingly, a subdivision of the species was proposed. Additional data on DNA-DNA homologies indicate that our two strains of *L. ferrooxidans* belong to two genospecies (15a). Further work will be necessary to establish the evolutionary tree of the acidophilic ferrous-iron-oxidizing bacteria (9, 10, 33). Because of their acid tolerance and possession of unique enzyme systems for oxidation of ferrous iron (3), these bacteria are able to grow in an ecological niche which is poisonous for most other microorganisms. These unique properties make them useful for microbial metal leaching.

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