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EXPERIMENTAL ARTICLES

Leptospirillum-Like Bacteria and Evaluation of Their Role in Pyrite Oxidation

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Abstract—Two strains of *Leptospirillum*-like bacteria isolated from dumps of Alaverdi and Akhtala sulfide ore deposits in Armenia were studied. The optimum and maximum temperatures for the growth of both strains were 37 and 40°C, respectively. The pH optimum was 2.0–2.3. Bacterial growth and ferrous iron oxidation were inhibited by yeast extract. The pyrite-leaching activity of the *Leptospirillum*-like bacteria under mesophilic conditions was close to that of *Acidithiobacillus ferrooxidans* and exceeded by 2.0–2.7 times the activity of these moderately thermophilic bacteria at 37°C. The leaching of pyrite by *Leptospirillum*-like bacteria increased in the presence of sulfur-oxidizing bacteria, particularly, in their association with a thermotolerant sulfur-oxidizing bacterium.

Key words: leptospirilla, sulfur-oxidizing bacteria, bacterial associations, pyrite leaching.

The chemolithotrophic bacterium *Leptospirillum ferrooxidans* capable of ferrous iron oxidation was isolated and described by Markosyan [1, 2]. Later, similar vibrioid microorganisms were revealed in uranium ores, acidic drainage of copper ores, and acidic hydrothermal vents [3–7]. At present, the genus *Leptospirillum* includes two species, the type species *L. ferrooxidans* L15(T) (=DSM 2705(T)), and the species *L. thermoferrooxidans* L-88(T) [8]. Leptospirilla are incapable of oxidizing elemental sulfur or its reduced compounds, such as thiosulfate and tetrathionate [1, 9]; however, *L. ferrooxidans* is able to oxidize pyrite [8, 10]. Moreover, pyrite was shown to promote the growth of leptospirilla, since some active strains of *Leptospirillum*-like bacteria were isolated from bioreactors during leaching of pyrite and cobaltiferrous pyrite [11]. It was shown that at a certain stage of continuous leaching of pyrite, *L. ferrooxidans* dominated and then completely displaced *Acidithiobacillus ferrooxidans* due to its greater tolerance to ferrous iron and low pH values [4, 11, 12]. Leptospirilla are considered to be promising for the removal of pyrite from coal [6]. Unlike mesophilic strains, the thermophilic bacterium *L. thermoferrooxidans* is unable to grow on pyrite [3].

Earlier, from sulfide ore deposits in Armenia, we isolated two strains of *Leptospirillum*-like bacteria that were capable of ferrous iron oxidation in a temperature range from 30 to 40°C.

The aim of this work was to study physiological properties of the isolated strains of leptospirilla and evaluate their role in pyrite leaching.

MATERIALS AND METHODS

The study was carried out with *Leptospirillum*-like bacteria (strains 64 and 72) isolated from dumps of Alaverdi and Akhtala sulfide ore deposits in Armenia. Enrichment cultures were incubated at 35°C; pure cultures were obtained by plating the enrichment culture on a Brierley medium [13] containing Fe^{2+} and 0.8% agarose and devoid of yeast extract. The purity of the isolates was tested by plating them onto the medium containing 0.02–0.05% of yeast extract with or without glucose. We also used the earlier isolated strains 69 and 86 of moderately thermophilic sulfur- and iron-oxidizing bacteria [14]; strains 33, 63, and 18 of *A. ferrooxidans*; and the thermotolerant sulfur-oxidizing bacterial strain 5 [15]. Strains of *A. ferrooxidans* were grown on a Leathen medium [16]; the other bacteria were cultivated on a Brierley medium. Ferrous iron or elemental sulfur were applied as the energy sources, depending on the bacteria used.

Physiological properties of *Leptospirillum*-like bacteria were studied under stationary conditions. In leaching experiments, pyrite samples were taken from the Alaverdi ore deposits and ground into 0.08-mm particles; batch cultivation of bacteria was performed in Erlenmeyer flasks on a shaker (180 rpm) at 30 or 37°C, depending on the strains used.

The titer of bacteria was determined by serial tenfold dilutions [16]; the biomass amount was determined from the protein content, analyzed by the Lowry method [17]. Concentrations of Fe^{2+} and Fe^{3+} ions were assayed by the method of complexometric titration with Trilon B [18].

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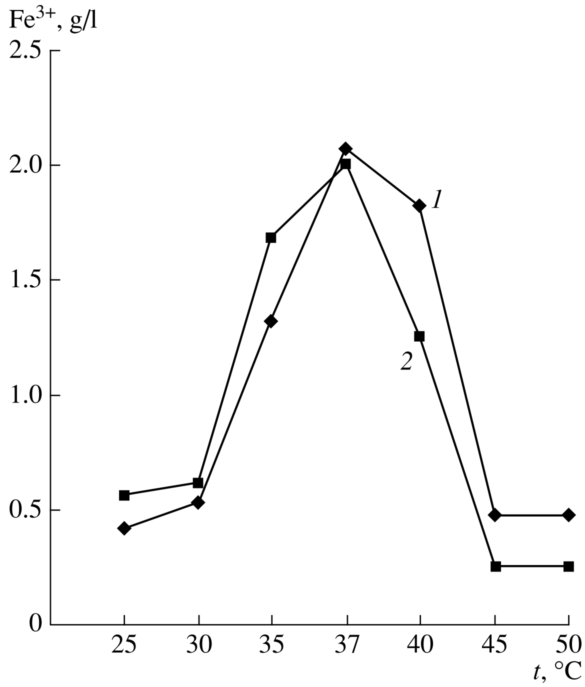

Fig. 1. Oxidation of Fe^{2+} by strains (*1*) 64 and (*2*) 72 of *Leptospirillum*-like bacteria grown for 3 days at different temperatures.

RESULTS AND DISCUSSION

The effect of temperature and pH. *Leptospirillum*-like bacteria were cultivated in media with ferrous iron as the energy source at temperatures from 25 to 50°C. Bacterial growth was monitored by measuring the amount of oxidized ferrous iron. Both strains grew in a temperature range from 30 to 40°C; the optimum temperature was 37°C. It is remarkable that iron-oxidizing activity at 40°C remained at a high level in strain 64 and sharply reduced in strain 72. Both strains did not oxidize Fe^{2+} at 45°C (Fig. 1).

When studying the effect of pH (in the range from 1.4 to 3.3) on the growth of isolates, iron-oxidizing activity was measured on the first day of cultivation, when no marked changes in the pH value had occurred. The pH optimum for the growth of strains 72 and 64 was 2.0 and 2.3, respectively; the pH value of 1.4 was the lower limit of bacterial growth (Fig. 2).

The effect of yeast extract. Iron-oxidizing activity of *Leptospirillum*-like bacteria was negatively affected by yeast extract concentrations as low as 0.01–0.02%, inhibited at 0.05 and 0.1%, and completely ceased at 0.2% (Fig. 3).

The effect of substrate concentration. An increase in Fe^{2+} concentration from 0.5 to 4.0 g/l promoted bacterial growth and enhanced iron oxidation; a further increase in ferrous iron concentration to 10.3 g/l caused retardation of growth and suppression of substrate oxidation. Both bacterial growth and iron oxidation were completely inhibited at a Fe^{2+} concentration of 15 g/l

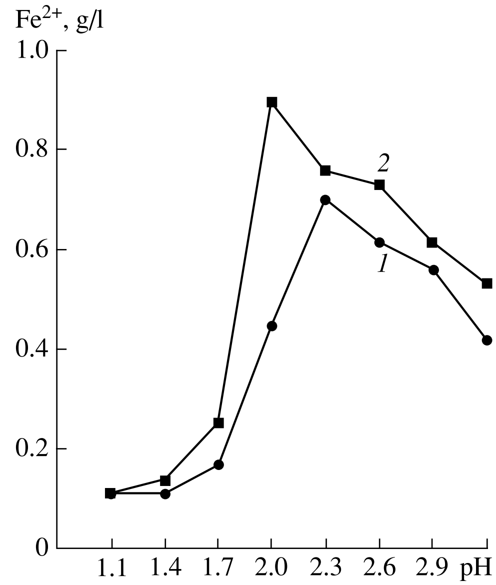

Fig. 2. Effect of initial pH values on the oxidation of Fe^{2+} by strains (*1*) 64 and (*2*) 72 of *Leptospirillum*-like bacteria.

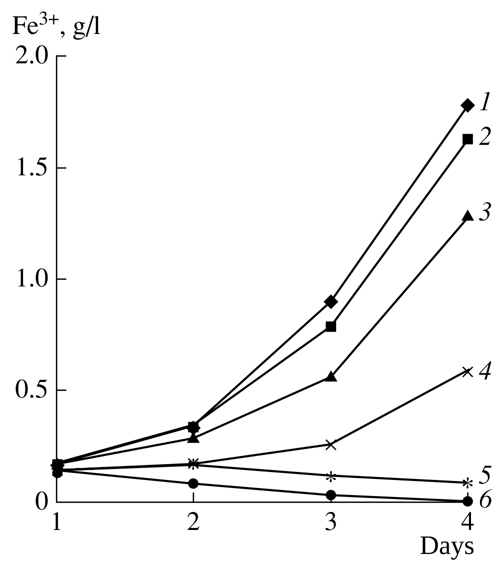

Fig. 3. Time course of Fe^{2+} oxidation by strain 64 in media (*1*) without yeast extract and in the presence of different yeast extract concentrations: (*2*) 0.01 and 0.02, (*3*) 0.05, (*4*) 0.1, (*5*) 0.2, and (*6*) 0.5%.

(Figs. 4a, 4b). The value of the saturation constant (K_s) for Fe^{2+} was 6.7 mg/l, which was comparable with the value found in experiments with *L. thermoferrooxidans* [3].

Pyrite leaching. In the first series of experiments, the activity of pyrite oxidation by *Leptospirillum*-like bacteria (strain 64) was compared with that of *A. ferrooxidans* (strains 33, 63, and 18) by measuring the amount of iron that was leached from pyrite in the

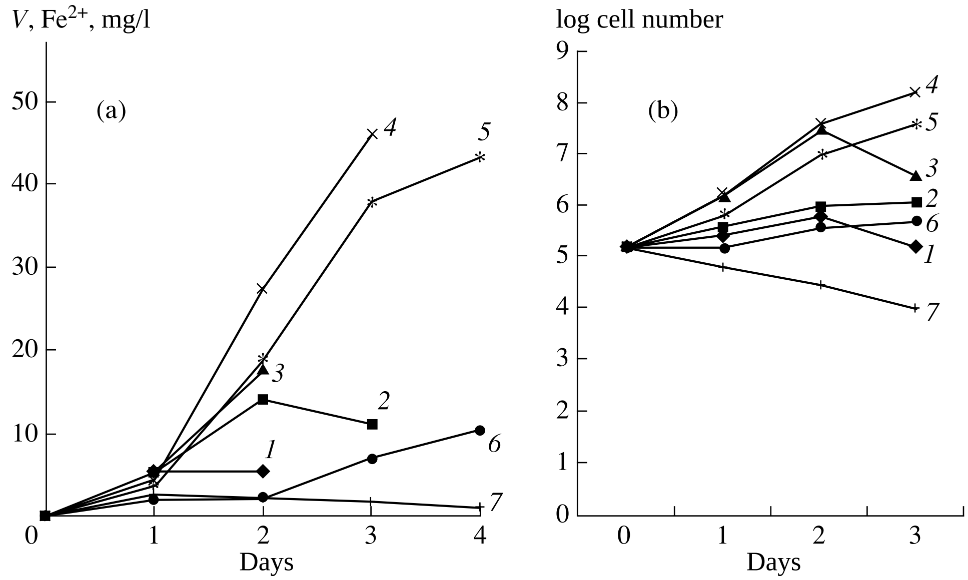

Fig. 4. (a) Iron oxidation and (b) growth of strain 64 at different Fe^{2+} concentrations in the medium: (*1*) 0.5, (*2*) 1.12, (*3*) 2.0, (*4*) 4.0, (*5*) 5.2, (*6*) 10.4, and (*7*) 15.4 and 20 g/l.

course of bacterial growth at 30°C for 14 days. As seen from Fig. 5, *Leptospirillum*-like bacteria exhibited a higher pyrite-leaching activity than strains 33 and 63 of *A. ferrooxidans* but were inferior to strain 18.

In the second series of experiments, pyrite leaching by *Leptospirillum*-like bacteria and earlier isolated moderately thermophilic sulfur- and iron-oxidizing bacteria (strains 69 and 86) [14, 19] was compared.

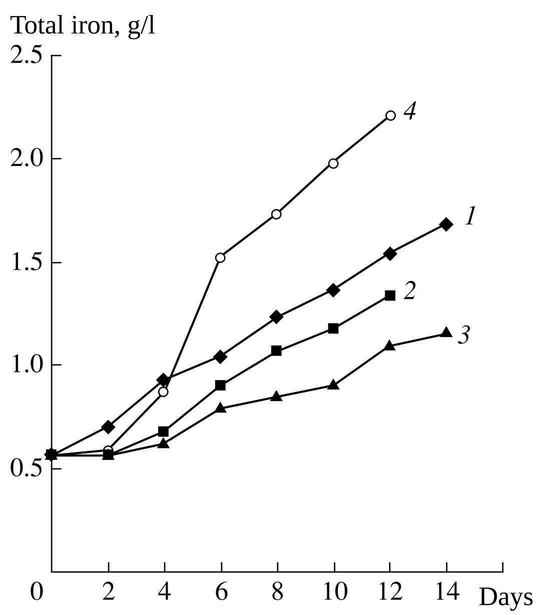

Fig. 5. Pyrite oxidation at 30°C by (*1*) strain 64 of *Leptospirillum*-like bacteria and strains (*2*) 33, (*3*) 63, and (*4*) 18 of *A. ferrooxidans.*

As seen from Table 1, the *Leptospirillum*-like bacterial strain 64 showed a greater intensity of pyrite leaching (by 4.5 and 2.7 times, respectively) than strain 86 grown under autotrophic or mixotrophic (in the presence of 0.02% yeast extract) conditions. Similarly, the *Leptospirillum*-like bacterial strain 72 oxidized pyrite more efficiently than the moderately thermophilic bacterial strain 69.

It is important that pyrite leaching by *Leptospirillum*-like bacteria was accompanied by the accumulation of ferric iron exclusively, whereas, in the case of thermophilic bacteria grown either autotrophically or mixotrophically, a considerable amount of ferrous iron (0.3–0.5 g/l) was revealed in the medium in addition to ferric iron.

It should be noted that the amount of iron leached from pyrite by different bacterial strains correlated with the biomass accumulation and sulfuric acid formation (acidification of the medium) (Table 1).

As mentioned by some researchers [6, 11], pyrite oxidation by *Leptospirillum*-like bacteria is accompanied by the accumulation of sulfur both in the medium and on the surface of the mineral, which prevents further pyrite leaching. One way to solve this problem may be the application of *Leptospirillum*-like bacteria in associations with sulfur-oxidizing bacteria. Therefore, we tested the associations of *Leptospirillum*-like bacteria with *A. thiooxidans* VKM B-460, *A. acidophilum* 13 (*T. organoparus* 13), and the thermotolerant sulfur-oxidizing strain 5.

As seen from Table 2, the efficiency of pyrite oxidation was maximal in the association of *Leptospirillum*-like bacteria with the thermotolerant strain 5 and

Table 1. Pyrite oxidation by *Leptospirillum*-like bacteria and moderately thermophilic bacteria*

Strains	Addition of yeast extract	Leached iron, g/l		Protein, mg/ml	Final pH
		Fe^{3+}	Fe^{2+}		
L. ferrooxidans, strain 64	–	4.760	0.028	0.076	1.25
strain 86	–	1.064	0.476	0.02	1.4
strain 86	+	1.764	0.308	0.042	1.3
L. ferrooxidans, strain 72	–	2.604	0.056	0.040	1.12
strain 69	–	1.372	0.532	0.02	1.3
strain 69	+	2.196	0.168	0.025	1.2

* Conditions of the experiments: FeS_2, 2%; pH 2.0; 37°C; cultivation time, 10 days.

Table 2. Pyrite oxidation by associations of *Leptospirillum*-like bacteria and sulfur-oxidizing bacteria*

Strains	Leached iron, mg/l		SO_4^{2-}, mg/l	Final pH	Titer of bacteria, cells/ml
	Fe^{3+}	Fe^{2+}			
Control (without bacteria)	56	448	600	1.7	–
L. ferrooxidans, strain 64	9324	84	1700	1.1	10^5
A. thiooxidans VKM B-460	56	448	650	1.6	0
L. ferrooxidans, strain 64 + + *A. thiooxidans* VKM B-460	12180	28	2200	1.0	10^5 10^8
A. acidophilum, strain 13	56	504	600	1.65	0
L. ferrooxidans, strain 64 + + *A. acidophilum*, strain 13	9968	28	1950	1.15	10^5 10^5
strain 5	84	476	620	1.7	10
L. ferrooxidans, strain 64 + + strain 5	13024	56	230	0.7	10^5 10^6

* Conditions of the experiments: FeS_2, 2%; pH 1.7; 35°C; cultivation time, 14 days.

slightly lower in the association with *A. thiooxidans* VKM B-460.

Thus, the isolated thermotolerant strains of *Leptospirillum*-like bacteria showed pyrite-leaching activity similar to that of *A. ferrooxidans.* When grown at the optimal temperature, these bacteria showed activity that was twice as great as that in moderately thermophilic sulfur- and iron-oxidizing bacteria grown under mixotrophic conditions. The pyrite-leaching activity of *Leptospirillum*-like bacteria increased in the presence of sulfur-oxidizing bacteria, particularly, in the association with the thermotolerant strain 5.

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