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EXPERIMENTAL ARTICLES

Phenotypic Features of *Ferroplasma acidiphilum* Strains Y^T and Y-2

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Abstract—Earlier, we described a new family of mesophilic, strictly autotrophic Fe^{2+}-oxidizing archaebacteria, *Ferroplasmaceae*, which belongs to the order *Thermoplasmales* and includes the genus *Ferroplasma* and the species *F. acidiphilum* (strain Y^T) [1]. The present work is concerned with a comparative study of phenotypic characteristics of the type strain Y^T and a new strain, *F. acidiphilum* Y-2, isolated from dense pulps during oxidation of gold-containing arsenopyrite/pyrite concentrates from the Bakyrchikskoe (Kazakhstan) and Olimpiadinskoe (Krasnoyarsk krai) ore deposits, respectively. The G+C content of DNA from strains Y^T and Y-2 comprised 35.1 and 35.2 mol %, respectively; the level of DNA–DNA homology between the strains was 84%. Restriction profiles of chromosomal DNA from both strains exhibited a similarity coefficient of 0.87. Genotypic characteristics of these strains indicate their affiliation to the same species. The cells of both strains are polymorphic and lack cell walls. Strains of *F. acidiphilum* oxidized ferrous iron and pyrite as the sole source of energy and fixed carbon dioxide as the sole carbon source. The strains required yeast extract as a growth factor. Optimum pH for cell growth ranged from 1.7 to 1.8; the temperature optima for the growth of strains Y^T and Y-2 were 34–36 and 40–42°C, respectively. Comparative analysis of the total lipids revealed their close similarity in the strains; two glycophospholipids comprised 90% of the total lipids: lipid I, β-D-glucopyranosylcaldarchaetidylglycerol (about 55%), and lipid II, trihexosylcaldarchaetidylglycerol (26%), whose isopranyl chains contained no cyclopentane rings. The carbohydrate fraction of lipid I hydrolysate contained only D-glucose, whereas hydrolysate of lipid II contained both D-glucose and D-galactose in a molar ratio of 2 : 1. Thus, it was established that the intraspecies phylogenetic divergence within *F. acidiphilum* is manifested in the two strains by different temperature optima against a background of similarity in other phenotypic properties.

Key words: *Archaea, Ferroplasma*, acidophiles, chemolithoautotrophs, iron-oxidizing bacteria.

It is known that oxidation of sulfide minerals, Fe^{2+}, and elemental sulfur in nature and in biohydrometallurgical processes is realized by the mesophilic eubacteria *Acidithiobacillus ferrooxidans, Acidithiobacillus thiooxidans,* and *Leptospirillum ferrooxidans*; and moderately thermophilic bacteria of the genus *Sulfobacillus* and *Acidithiobacillus caldus*; and obligately thermophilic archaea *Acidianus brierleyi, Metallosphaera sedula, Metallosphaera prunae, Sulfolobus metallicus, Acidianus ambivalens,* etc.

Until recently, the order *Thermoplasmales* included only heterotrophic thermophilic archaea of the genera *Thermoplasma* [2–4] and *Picrophilus* [5].

Recently, the new family *Ferroplasmaceae* was described [1], which belongs to the order *Thermoplasmales* and includes the genus *Ferroplasma* and two species, *F. acidiphilum* [1] and *F. acidarmanus* [6]. These are mesophilic, acidophilic archaea oxidizing Fe^{2+} and FeS_2. *F. acidiphilum* is a strict autotroph, which requires, however, yeast extract for cell growth, whereas *F. acidarmanus* is capable of heterotrophic growth, in particular, on yeast extract.

This paper is concerned with a comparative study of strains *F. acidiphilum* Y^T and Y-2 isolated from dense pulps during industrial treatment of gold-containing arsenopyrite/pyrite concentrates from the Bakyrchikskoe (Kazakhstan) and Olimpiadinskoe (Krasnoyarsk krai) ore deposits, respectively.

MATERIALS AND METHODS

Isolation of pure cultures and cultivation conditions. Strains *F. acidiphilum* Y^T and Y-2 were isolated from dense pulps during leaching of gold-containing arsenopyrite/pyrite concentrates. The isolation was performed by the method of serial dilutions on medium

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containing (g/l) $MgSO_4$, 0.4; $(NH_4)_2SO_4$, 0.2; K_2HPO_4, 0.1; KCl, 0.1; $FeSO_4 \cdot 5H_2O$, 25, and 0.02% of yeast extract (Difco, United States). The pH was adjusted to 1.7 with 10 N H_2SO_4. The cultivation temperature was varied from 15 to 47°C; the pH was 1.8–2.0. The culture purity was judged from examinations under a phase-contrast microscope and from the results of inoculation of liquid and agar media containing glucose, yeast extract, and tryptone (1% each) at pH 1.8–2.0.

Pure cultures were grown in 250-ml Erlenmeyer flasks with 100 ml of the above-specified medium on a rotary shaker (150 rpm) at 42°C.

Culture growth was monitored by determining the protein content of the culture liquid by the Lowry method. The concentration of ferric and ferrous iron in the medium was assayed by trilonometric titration. Elemental sulfur and sulfide minerals were sterilized with ethanol. To determine the antibiotic resistance of the strains, antibiotics were added to exponential-phase cultures.

Cell growth on organic substrates. Heterotrophic growth of strains *F. acidiphilum* Y-2 and Y^T was studied using the following substrates (0.1%): sugars (D-mannose, raffinose, L-glucose, D-xylose, L-rhamnose, D-cellobiose, L-arabinose, L-fructose, melibiose, and sorbitol), organic acids (acetate, formate, glucosaminic acid, succinate, and L-ketoglutarate), and amino acids (glutarate, histidine, glutamine, alanine, and asparagine). In addition, growth of the strains was studied on these substrates under mixotrophic conditions in the presence of Fe^{2+} (4 g/l) and FeS_2 (50 g/l).

Electron microscopic examinations. Whole-cell specimens were contrasted with 1% phosphotungstic acid (pH 2). To obtain ultrathin sections, cells were fixed with a 1% solution of OsO_4 in phosphate buffer (6.6 mM, pH 6.2) for 18 h at 5°C, dehydrated with ethanol, and embedded in Epon-812. Ultrathin sections of cells were prepared using an LKB-4800 ultramicrotome, contrasted with a 3% solution of uranyl acetate in ethanol for 20 min, stained additionally with lead citrate for 10 min, and examined under a JEM-100C electron microscope.

Fixation of $^{14}CO_2$. Nine-ml portions of an exponential-phase culture were placed into 20-ml bottles and sealed hermetically; then, 1 ml of a $NaH^{14}CO_3$ solution (pH 8.4) containing 0.232 μmol of $NaH^{14}CO_3$ with an activity of 0.005 μCi was introduced into each bottle with a syringe. The bottles were incubated on a shaker at 42°C for 2 h. Samples of the suspension (1.5 ml) were withdrawn and centrifuged at 16000 *g* for 6 min; after harvesting, the cells were washed three times with 0.01 N H_2SO_4 and transferred into bottles with 3 ml of ZhS-8 scintillation liquid. The radioactivity of preparations was measured in a Rackbeta liquid scintillation counter.

Determination of DNA base composition. DNA was extracted and purified by the Marmur method [7]. DNA base composition was determined from the melting profile of DNA [8]. The level of DNA–DNA homology was measured by the method of DNA renaturation according to De Ley *et al.* [9]. Spectroscopic characteristics were recorded on a Pye Unicam 1500 spectrometer.

Analysis of chromosomal DNA structure by pulsed-field gel electrophoresis. Obtaining of native DNA, its restriction, and fragment separation were carried out by the method of Schwartz and Kantor [10] with our modifications [11].

Analysis of lipid composition. Extraction of lipids from lyophilized cells, their purification from nonlipid admixtures, and fractionation on DEAE cellulose (Merck, Germany) were performed as described earlier [12]. The main lipid components were isolated from the fraction of strongly acidic lipids by chromatography on a column with Silica Gel L 100/160 (Lachema, Czech Republic) with elution by chloroform–methanol–water mixtures (60 : 10 : 1 and 60 : 20 : 2.5). Thin-layer chromatography was performed on plates with Silica Gel 60 (Merck, Germany). Monosaccharides were analyzed by gas-liquid chromatography, as described by Swain *et al.* [13]. Mass spectra were obtained by the MALDI method; positive ions were recorded on a Vision 2000 mass spectrometer (Thermo BioAnalysis Corp.). The ^{1}H-NMR (400 MHz) spectrum was recorded on a Unity Plus (Varian) spectrometer in a deuterochloroform–tetradeuteromethanol mixture (1 : 1).

Analysis of protein profile. Protein samples were obtained as described earlier [14]. Gel electrophoresis in polyacrylamide gel was carried out according to Gal'chenko and Nesterov [15] in a vertical chamber (Desaga, Germany) at 350 V and a current strength in the concentrating and separating gels of 86 and 60 mA, respectively.

Proteins were fixed with 50% trichloroacetic acid for 18 h and stained with a 0.01% solution of Coomassie Brilliant Blue R-250 for 5 h. Gels were washed with 7% acetic acid to colorlessness and scanned on a scanner for transparent films with a resolution of 600 dpi. Protein profiles were compared visually; similarity coefficients were calculated by the Dice formula [16].

RESULTS AND DISCUSSION

Cell morphology. The cell morphology of strain Y-2 was identical to that of strain Y^T [1]. At the beginning of the exponential growth phase, cells of both strains had an irregular round shape (Fig. 1a); in the middle of the exponential phase, electron-transparent zones were visible inside the cells (Fig. 1b), and some cells acquired vibrio-like shape (Fig. 1c). At the end of the exponential phase and in the stationary phase, most cells were pleomorphic (Fig. 1d). Cell reproduction proceeded by both binary division (Fig. 1a) and budding; buds formed either on the cell surface or on hyphae-like appendages (Fig. 1e).

Cell size varied from 0.4–0.9 μm in the exponential phase to 1–3 μm in length and 0.3–1.0 μm in width in

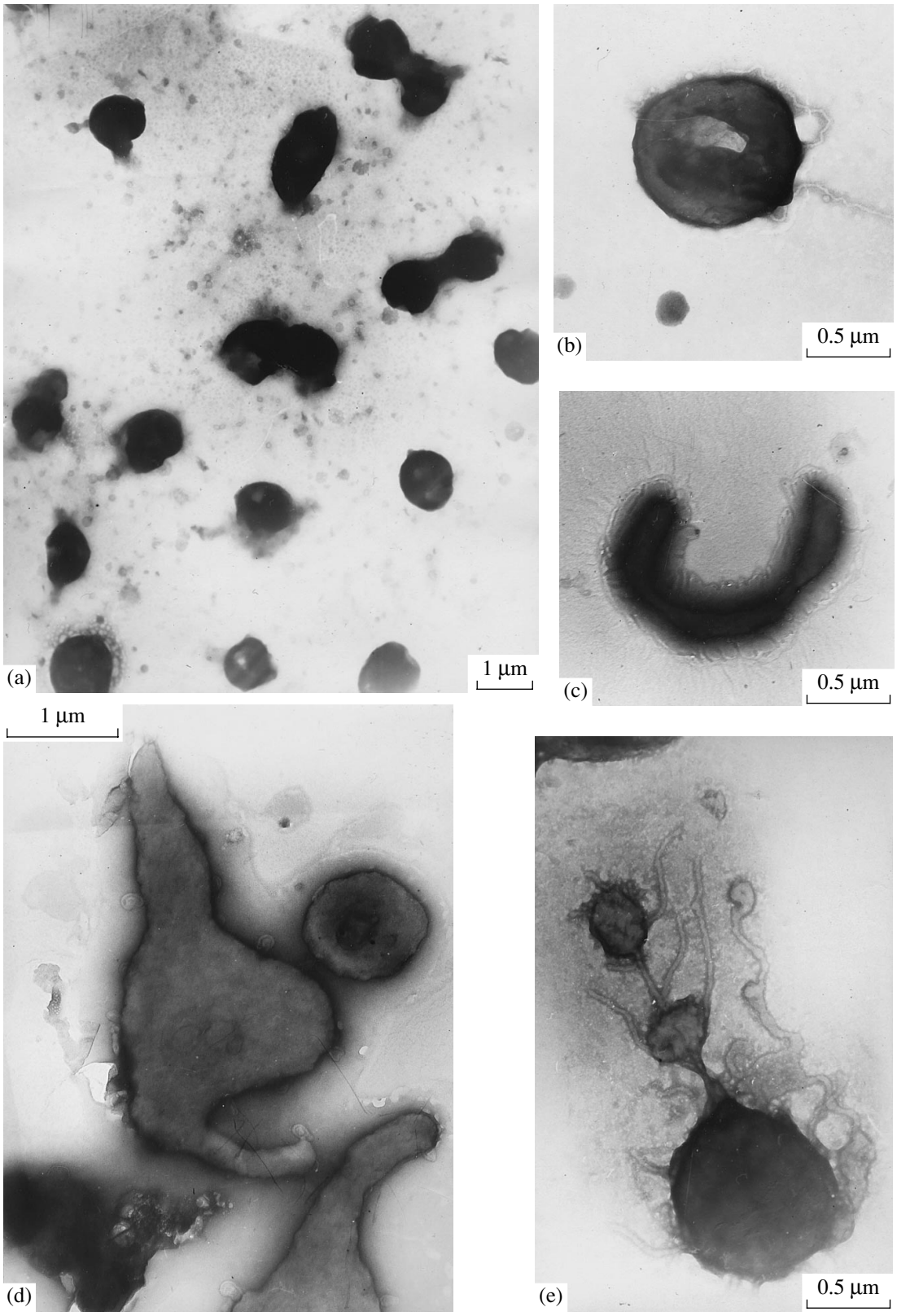


Fig. 1. Cell morphology of isolate Y-2: (a) beginning of the exponential growth phase; cells have irregular round shape; (b, c) middle of the exponential growth phase; electron-transparent zones are visible; some cells have vibrio-like shape; (d) stationary phase; cells are pleomorphic; (e) buds are formed on hyphae-like branches.

the stationary phase. In the sections of exponential-phase cells, membrane-bounded electron-transparent zones (Fig. 2a–2d) could be observed. Cell walls were absent (Figs. 2a, 2b).

Growth temperature and pH. Strains Y^T and Y-2 differed in temperature optima for their growth. Strain Y-2 grew in a temperature range from 15 to 47°C with the growth optimum at 40–42°C (Fig. 3a), whereas strain Y^T grew at 15–45°C with the growth optimum at 35–36°C. Strain Y-2 grew at higher temperature than strain Y^T possibly because it was isolated from the Olimpiadinskoe ore deposit, which contains pyrrhotite and is rich in sulfur, and this results in heating of the ore body in the quarry. Strain Y-2 grew in a pH range from 1.3 to 2.2, with the growth optimum at pH 1.7 (Fig. 3b), very much like strain Y^T [1].

Oxidation of inorganic substrates. Figure 4 shows the time course of the growth of strain *F. acidiphilum* Y-2 in Fe^{2+}-containing medium at 42°C and pH 1.8. Strain Y-2, like strain Y^T, can utilize Fe^{2+} and pyrite as the energy source; other minerals, such as antimonite, galenite, and chalcopyrite, as well as elemental sulfur, tetrathionate, and thiosulfate, were not utilized. Earlier, we reported that strain Y^T can grow on Mn^{2+}, although the growth is poorer than on Fe^{2+} [1]. In the present study, we revealed that poor growth of strains Y^T and Y-2 on $MnSO_4$-containing medium was possible only in the first culture passage and did not occur in subsequent passages. The archaeal strains Y-2 and Y^T resemble the eubacterium *Leptospirillum ferrooxidans* with respect to their response to inorganic substrates, although the latter organism does not require yeast extract.

Reaction to organic substrates. Strains Y^T and Y-2 did not grow on any of the organic substrates tested, with or without ferrous iron or pyrite. Yeast extract in the concentration of 0.02% was an essential vitamin source for the growth of both strains; in its absence, strains Y^T and Y-2 were able to oxidize Fe^{2+} and FeS_2 only in the first culture passage. At increased (to 0.2 or 0.4%) or decreased (0.002%) concentrations of yeast extract, growth of the strains did not occur (Fig. 5).

Fixation of $^{14}CO_2$. Earlier, it was revealed that cell suspensions of strain *F. acidiphilum* Y^T exhibited poor fixation of $^{14}CO_2$ [1]. In the present work, we performed a comparative study of $^{14}CO_2$ fixation by growing cells of strains *F. acidiphilum* Y^T and Y-2 and the strictly autotrophic bacterium *Acidithiobacillus ferrooxidans* under conditions of ferrous iron oxidation. As seen from the table, the incorporation of the radiolabel into the biomass of strains Y^T and Y-2 was approximately sixfold lower than the incorporation into *A. ferrooxidans* cells. However, these results, together with the data on the inability of strains Y^T and Y-2 to metabolize organic substrates, confirm the earlier inference that ferroplasmas are characterized by an autotrophic type of nutrition. The fact that strains Y^T and Y-2 exhibited a lower level of carbon dioxide fixation than *A. ferrooxidans* is due to their lower growth rate. The maximum specific growth rates (μ_{max}) of *F. acidiphilum* strains and *A. ferrooxidans* were 0.016 and 0.15 h^{-1}, respectively.

Fixation of $^{14}CO_2$ by strains Y-2 and Y^T of *F. acidiphilum* and by *A. ferrooxidans*

Microorganisms	Cell radioactivity, cpm/mg protein	$^{14}CO_2$ fixation, nmol $^{14}CO_2$/(min mg protein)
A. ferrooxidans	190592	0.125
Y-2	28258	0.01
Y^T	30783	0.01

Oxygen requirements. Strain Y-2, like strain Y^T, is a strict aerobe. No cell growth was observed under anaerobic conditions in the presence of sulfur, formate, or Fe^{3+}.

Antibiotic resistance. Strain Y-2, like strain Y^T, is resistant to ampicillin (50 μg/ml), chloramphenicol (10 μg/ml), and rifampicin (25 μg/ml) and sensitive to tetracycline (2 μg/ml) and gentiomycin (2 μg/ml). These data are indicative of the absence of cell walls in the archaea under study.

G+C content of DNA and DNA–DNA hybridization. The G+C content of DNA in strains Y-2 and Y^T was 35.2 and 35.1 mol %, respectively. According to the data reported by Golyshina *et al.* [1], the G+C content of DNA in strain Y^T as determined by the HPLC method was 36.5 mol %. The discrepancy between the results obtained by different methods lies within admissible limits. The level of DNA–DNA hybridization in these strains was as high as 84%.

Determination of DNA structure in strains Y^T and Y-2 by pulsed-field gel electrophoresis. The structures of chromosomal DNAs from strains Y-2 and Y^T determined by pulsed-field gel electrophoresis of native DNAs digested by the restriction endonuclease *Xho*I were different. The pattern of *Xho*I-digested chromosomal DNA from strain Y-2 lacked the fragment of 189 kb revealed in the chromosomal DNA of the type strain Y^T (Fig. 6a). The largest fragment of DNA from strain Y-2, of 117.2 ± 0.4 kb, resembled in size the second restriction fragment of the chromosomal DNA from strain Y^T (119.0 ± 1.3 kb) (Fig. 6b). The second fragment of DNA from strain Y-2, of 108.5 ± 0.4 kb, was close in size to the third fragment of DNA from strain Y^T (106.0 ± 0.5 kb). Chromosomal DNA from the type strain lacked the restriction fragment of 95 kb. This fragment in the chromosomal DNA from strain Y-2 possibly originated from the fragment of 189 kb due to the presence in the center of the corresponding nucleotide sequence of the CTCGAG site susceptible to *Xho*I. Smaller fragments of chromosomal DNA from the two strains were equal-sized [11]. The coefficient of similarity between the two cultures, calculated from the number of bands migrating at equal rates, was 0.87, which supports their affiliation to the same species.

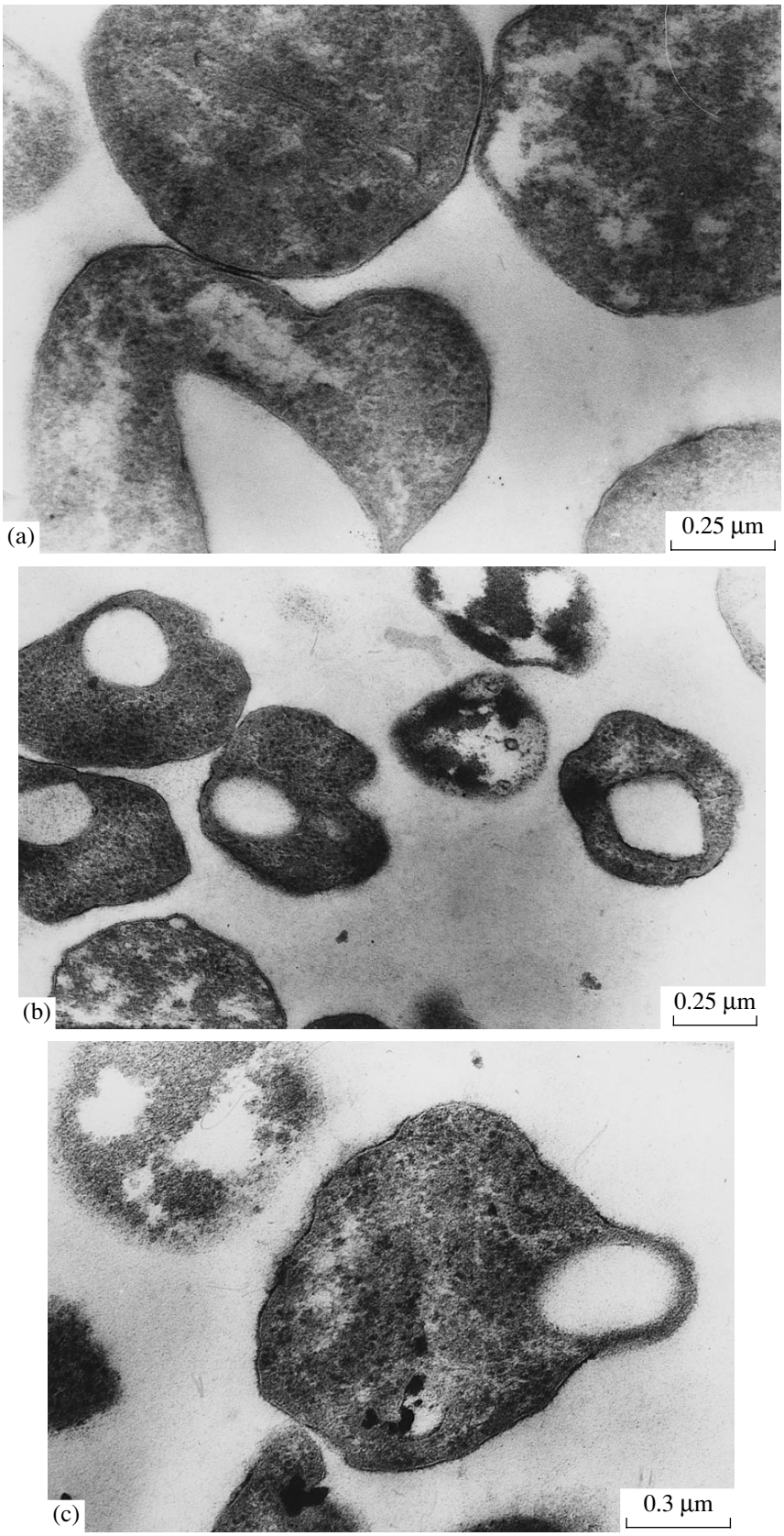


Fig. 2. Fine structure of strain Y-2 cells in the middle of the exponential growth phase: (a–c) electron-transparent zones bounded with membrane.

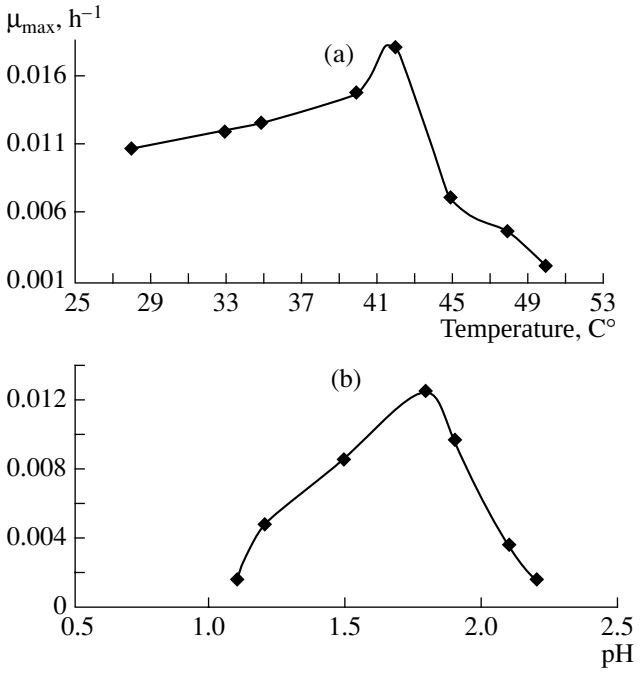


Fig. 3. Maximum specific growth rate of strain Y-2 as a function of (a) temperature and (b) pH.

Thus, analysis of the structure of chromosomal DNA in the strains isolated from different ores also testifies to their affiliation with the same species, *F. acidiphilum.*

Investigation of lipid composition. Comparative analysis of total lipids from strains Y-2 and Y^T by thin-layer chromatography using different solvent systems and specific detecting reagents revealed their close resemblance. Two components (I and II) comprised together about 90% of the total lipids, of which the less polar component (I) accounted for 55%. These components showed positive reactions with the molybdate reagent for phospholipids and with the α-naphthol reagent for carbohydrates; therefore, they were identified as glycophospholipids. Both lipids were purified by ion-exchange and adsorption chromatography.

The IR spectra of glycophospholipids I and II exhibited strong bands of phosphodiester group absorption at 1228, 1092, and 1035 cm^{-1}; absorption of the carbonyl group was not observed. Both lipids could be degraded to inorganic phosphate, glycerol, monosaccharides, and lipophilic products under stringent acid hydrolysis. The lipophilic product of lipid I degradation was identified as a caldarchaeol with one cyclopentane ring in each isopranyl chain based on its mass spectrum ($[M + H]^+$ with *m*/*z* 1296), the study of its degradation with hydroiodic acid, and the available data on the structure of polar lipids from thermoacidophiles [17, 18]. The lipophilic product formed upon hydrolysis of lipid II was assumed to be a cycle-free caldarchaeol, since the pseudomolecular ion $[M + H]^+$ in its mass spectrum was registered at *m*/*z* 1300. In the carbohydrate fraction of lipid I hydrolysate, only D-glucose was revealed,

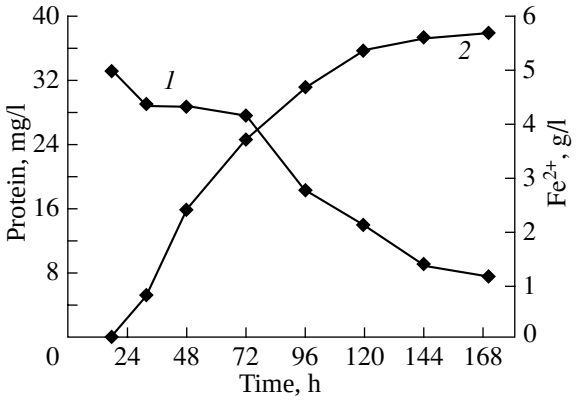


Fig. 4. Growth of strain Y-2 in mineral medium supplemented with 6 g/l of Fe^{2+} and 0.02% yeast extract (pH 1.8; 42°C): (*1*) Fe^{2+}; (*2*) protein.

whereas the hydrolysate of lipid II contained D-glucose and D-galactose in a molar ratio of 2 : 1.

Oxidation of lipids with periodate and subsequent acid hydrolysis resulted in the formation of the same lipophilic products, caldarchaeols, and a mixture of water-soluble compounds, among which glycerol was not revealed. This indicates that the glycerol residue in the lipids is phosphorylated at its primary hydroxyl group.

The ^{1}H-NMR spectrum of lipid I exhibited double signals of protons belonging to the ^{14}C-methyl groups in the region of 0.8–0.9 ppm: at 0.835 (12H), 0.840 (12H), 0.845 (6H), 0.875 (6H), and 0.880 (6H) ppm; the constant of spin–spin interaction (J) was 6.6 Hz. These data confirm our earlier inference on the structure of the lipophilic (core) moiety of this lipid molecule. In the weakest region, the double signal at 4. 265 ppm belongs to an anomeric proton; the found constant of spin–spin interaction of 7.92 Hz was typical of β-glucopyranose with the chair-like conformation C1 [19]. The results of mass-spectrometric analysis of intact lipid I ($[M + H]^+$ with *m*/*z* 1613 and $[M + Na]^+$ with *m*/*z 1*635) and lipid

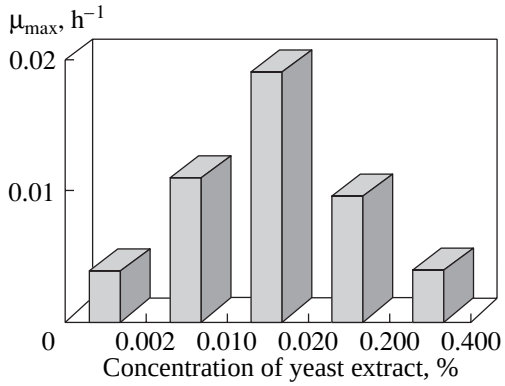


Fig. 5. Maximum specific growth rate of strain Y-2 as a function of yeast extract concentration.

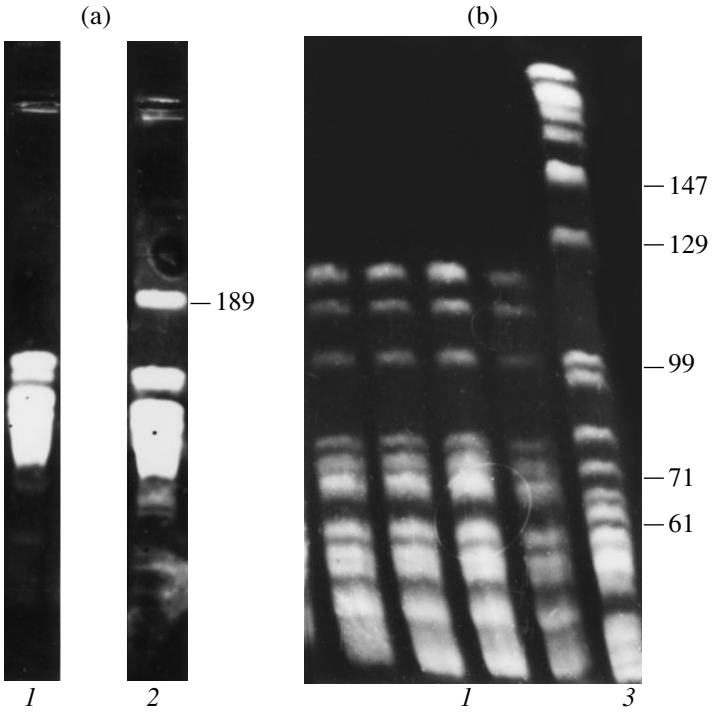
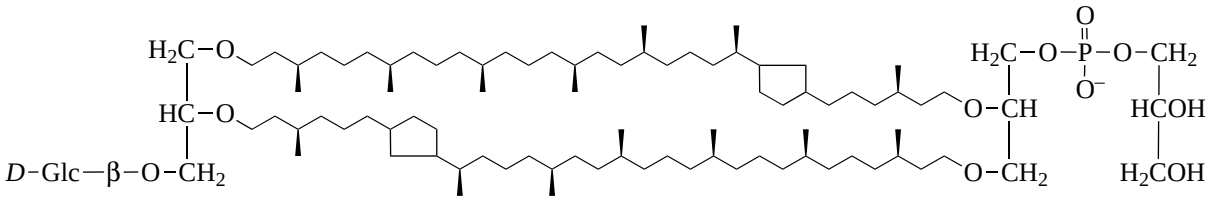
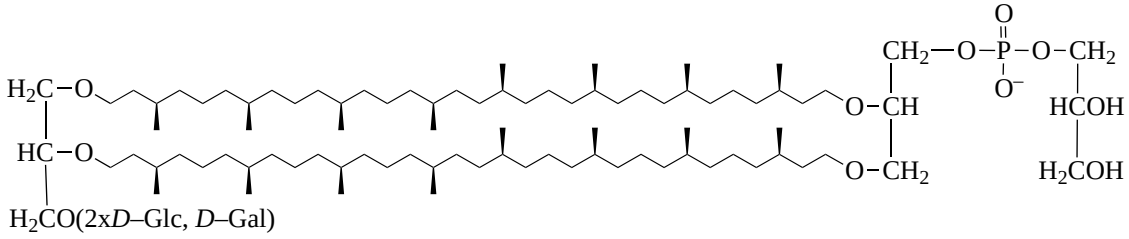


Fig. 6. Pulsed-field gel electrophoresis of chromosomal DNA from strains of *F. acidiphilum* (*1*) Y-2 and (*2*) Y^T and (*3*) *A. ferrooxidans* VKM B-458 digested with *XhoI* restriction endonuclease (40 units/30 μl). Conditions of pulsed-field gel electrophoresis: (a) 120 V; 14°C; pulse time, 25 s; duration, 44 h; (b) 130 V; 19°C; pulse time, 10 s; duration, 68 h. Molecular weights (kb) of DNA fragments from strain *A. ferrooxidans* VKM B-458 are shown on the right side of the electrophoregrams.



Glycophospholipid I



Glycophospholipid II

Fig. 7. Structure of the major lipids of strains *F. acidiphilum* Y^T and Y-2.

II ($[M + H + Na]^+$ with *m*/*z* 1964), together with the above-presented data on the lipid composition, make it possible to conclude that lipid dominant in both strains is β-D-glucopyranosylcaldarchaetidylglycerol (Fig. 7, glycophospholipid I). Such glycosides of caldarchaetidylglycerol have not been described earlier.

The second main lipid II, accounting for 35% of the total lipids, is a triglycoside of caldarchaetidylglycerol (Fig. 7, glycophospholipid II). The isopranyl chains in its molecule do not contain cycles; the trisaccharide fragment consisted of a D-galactose residue and two residues of D-glucose. It is probably also a new lipid, but its structure needs clarification.

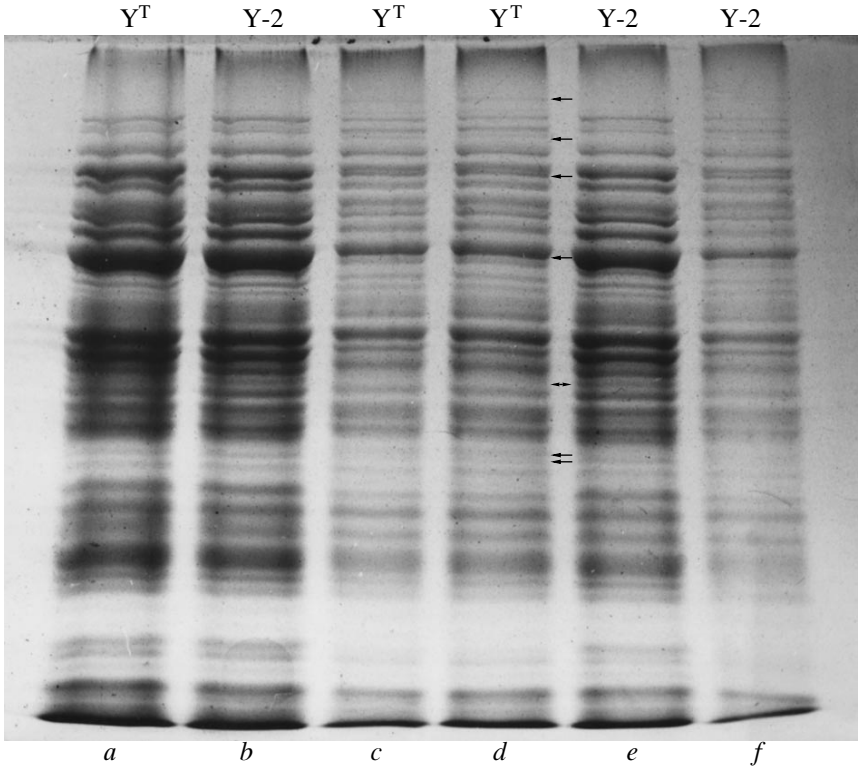


Fig. 8. Protein profiles of strains *F. acidiphilum* Y-2 and Y^T. Sample volume (μl): (*a*) 15; (*b*) 15; (*c*) 5; (*d*) 10; (*e*) 10; (*f*) 5.

The study of protein profiles. The protein profiles of ferroplasmas have not been investigated earlier. In this study, three electrophoregrams of protein profiles were obtained for each strain. As can be seen from Fig. 8, the protein profiles of strains Y-2 and Y^T appeared to be closely allied and contained 33–36 proteins. The coefficient of similarity between isolates Y-2 and Y^T was 90%, which was in agreement with their affiliation with the same species.

Thus, the results obtained in this work point to the fact that the strains *F. acidiphilum* Y-2 and Y^T are fairly similar in their main properties (pH optimum for cell growth, requirements for oxygen and organic substrates, chemolithoautotrophic metabolism, resistance to antibiotics, and cell morphology). The strains are characterized by similar protein and lipid profiles, close values of the G+C content of DNA, and a high level of DNA–DNA homology. However, the strains somewhat differed in the optimum temperature for cell growth and in the structure of chromosomal DNA as determined by pulsed-field gel electrophoresis. The higher temperature optimum for the growth of strain Y-2 can be explained by its isolation from the Olimpiadinskoe ore deposit, which is characterized by elevated temperature due to a high content (about 34%) of easily oxidizable pyrrhotite.

The data obtained on the structure of the chromosomal DNA are indicative of genetic divergence of the strains under different environmental conditions and may be applied for their monitoring. Earlier, from *F. acidiphilum* cells, we isolated a phospholipid fraction of low polarity and showed, by mass spectrometry, that it contained archaetidylglycerol and archaetidic acid, which were minor components of the cell lipids [1]. The analysis of two major lipids from *F. acidiphilum* strains performed in the present work showed that the dominant lipid is a monoglycoside of caldarchaetidylglycerol. In this respect, *F. acidiphilum* is similar to the species *Thermoplasma acidophilum* [13]. However, in the latter species, the dominant lipid, unlike glycophospholipid I, contains L-gulose [13], and a glucoside of caldarchaetidylglycerol is a minor component and its monosaccharide residue has an anomeric α-configuration [20].

In a previous work [1], the ability of *F. acidiphilum* to oxidize Mn^{2+} was noted. However, more thorough studies did not support this fact: strains Y-2 and Y^T were found to oxidize Mn^{2+} only in the first culture passage; the reason for this phenomenon is not yet understood.

The results obtained also point to wide distribution of archaea of the genus *Ferroplasma* in ore deposits of remote regions (Kazakhstan, Siberia, and the United States).

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