Characterization of Ferroplasma Isolates and Ferroplasma acidarmanus sp. nov., Extreme Acidophiles from Acid Mine Drainage and Industrial Bioleaching Environments

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Three recently isolated extremely acidophilic archaeal strains have been shown to be phylogenetically similar to Ferroplasma acidiphilum Y11 by 16S rRNA gene sequencing. All four Ferroplasma isolates were capable of growing chemoorganotrophically on yeast extract or a range of sugars and chemomixotrophically on ferrous iron and yeast extract or sugars, and isolate “Ferroplasma acidarmanus” Fer1T required much higher levels of organic carbon. All four isolates were facultative anaerobes, coupling chemoorganotrophic growth on yeast extract to the reduction of ferric iron. The temperature optima for the four isolates were between 35 and 42°C and the pH optima were 1.0 to 1.7, and “F. acidarmanus” Fer1T was capable of growing at pH 0. The optimum yeast extract concentration for “F. acidarmanus” Fer1T was higher than that for the other three isolates. Phenotypic results suggested that isolate “F. acidarmanus” Fer1T is of a different species than the other three strains, and 16S rRNA sequence data, DNA-DNA similarity values, and two-dimensional polyacrylamide gel electrophoresis protein profiles clearly showed that strains DR1, MT17, and Y1 group as a single species. “F. acidarmanus” Fer1T groups separately, and we propose the new species “F. acidarmanus” Fer1T sp. nov.

Acidophilic ferrous iron-oxidizing microorganisms have been implicated in the production of acid mine drainage (AMD), whereby metal sulfides are solubilized by oxidative dissolution, releasing metals and acid (32; for a review, see reference 30). The discharge of AMD causes considerable environmental damage via the release of metal-rich acidic effluents into groundwater. It was initially thought that the most important microorganism implicated in AMD was Acidithio- bacillus ferrooxidans (21). However, recent advances in molecular phylogenetic techniques have shown that other species are numerically dominant at certain acid-generating sites. An example of this is Iron Mountain, Calif., where the pyrite concentration in the ore is 95%, resulting in the generation of extremely acidic solutions (20). The microorganism population at the Iron Mountain acid-generating site is dominated by an archaeon of the genus Ferroplasma. Organisms from this genus were shown to constitute 85% ± 7% of the microorganism population by fluorescent in situ hybridization. An isolate obtained from the acid-leaching biofilm has been provisionally named “Ferroplasma acidarmanus” Fer1 (10). The “F. acidarmanus” Fer1T genome has been sequenced (97% complete), and draft results are available at the U.S. Department of Energy web site (http://genome.jgi-psf.org/draft_microbes/ferac/ferac_home.html). Details of the chemical and microbial aspects of sulfide dissolution at Iron Mountain (9) as well as an evaluation of the use of fluorescent in situ hybridization at the site (2) have been published.

Acidophilic microorganisms have been extensively exploited in biooxidation and bioleaching operations to remove metals from ore flotation concentrates, and three other Ferroplasma strains have been isolated from these environments. The type strain for the genus, Ferroplasma acidiphilum strain Y, was isolated from a pilot plant bioreactor for biooxidation of a gold-bearing arsenopyrite-pyrite concentrate (11). F. acidiphilum Y11 is described as being obligatory aerobic and autotrophic, as it is only capable of growth on Fe(II) or Mn(II) with the addition of a small concentration of organic carbon as a growth factor. F. acidiphilum Y11 is reported to be incapable of growth on organic carbon alone and is not capable of aerobic growth on reduced inorganic sulfur compounds.

Another member of the genus Ferroplasma is strain MT17, isolated from a South African pilot scale bioleaching reactor oxidizing a polymetallic sulfide concentrate at 45°C (22). This isolate was described as unable to grow in the absence of organic carbon but capable of chemomixotrophic growth on yeast extract either with Fe(II) or the reduced inorganic sulfur compound tetraethionate. MT17 was also reported to grow on yeast extract alone and was described as heterotrophic. It was also capable of anaerobically oxidizing yeast extract coupled to the reduction of Fe(III), but it was not ascertained if this was coupled to growth.

The final isolate, strain DR1, was cultured from a separate pilot scale biooxidation plant in South Africa and was shown to be similar to the genus Ferroplasma (by 16S ribosomal DNA [rDNA] sequence analysis). No phenotypic characterization of this isolate has been published.

For this study, we aimed to elucidate the genotypic and phenotypic characteristics of the genus Ferroplasma. Sequence comparisons of 16S rRNA gene sequences suggest that the isolates studied are closely related. However, key questions and inconsistencies regarding this genus need to be verified.

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These include chemoaotrophic versus chemooorganotrophic growth, oxidation of reduced inorganic sulfur compounds, and anaerobic growth. This study includes the first use of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) protein profile similarities to estimate the phylogenetic relationships between microorganisms.

**Materials and Methods**

**Isolates.** The Ferrobacteria isolates used in this study were the type strain for the genus, *F. acidiphilum* Y (11), acquired from Deutsche Sammlung von Mikroorganismen (DSM12658)*F. acidarmanus* Fer1 (10), kindly provided by J. F. Banfield and K. J. Edwards; isolate MT17 (22), supplied by D. B. Johnson; and isolate DRI, generously provided by D. E. Rawlings.

**Growth conditions.** Unless otherwise stated, all growth experiments were carried out in mineral salts medium (MSM) containing trace elements (7), 20 g of FeSO4 ÷ 7H2O liter-1, and 0.022% (wt/vol) yeast extract. The MSM containing yeast extract was adjusted to pH 1.2 with H2SO4 and autoclaved before filter-sterilized trace elements and ferrous iron were added. To ensure that cells were grown under the appropriate conditions for phenotypic characterization and comparison, all growth conditions and substrate-stimulated growth experiments were carried out with steady-state cells from continuous culture vessels. The cultures were inoculated with a viable cell mass equivalent to 10 µg of cell protein (measured by using a Bio-Rad protein assay reagent, with bovine serum albumin as the standard) from continuous culture vessels grown with MSM, trace elements, ferrous iron, and 0.022% (wt/vol) yeast extract (6a). The batch cultures were incubated on a rotary shaker at 150 rpm and 37°C for 63 h (unless otherwise stated). Growth curves were monitored by direct cell counts with a hemocytometer (Hawksley) on an Olympus BX50 phase-contrast microscope or by measurements of the optical density at 600 nm in a Philips PU8730 spectrometer. The cells for determining growth rates on yeast extract alone were previously subcultured in MSM and yeast extract. Unless otherwise stated, all experiments were performed in triplicate, with results reported as means ± standard deviations (SD) (calculated as the square roots of the sums of the squares for the test and control).

**Cellular response to organic and inorganic substrates.** *F. acidarmanus* Fer1 was either grown chemotaxotrophically on ferrous iron and yeast extract or chemooorganotrophically on yeast extract alone. After growth, cells were harvested by centrifugation at 10,000 g for 10 min (Heraeus 400R Labofuge), washed, resuspended in MSM, pH 1.2, and then stored on ice for no more than 2 h before use. Oxygen consumption was measured by using a Clark-type oxygen electrode (Rank) at 37°C. Cells were equilibrated for 3 min prior to the addition of substrate. Each substrate was added in the presence and absence of cells to determine biological and chemical oxygen consumption. The substrate-stimulated consumption of oxygen was recorded as a plus or minus response in (at least) duplicate (n = 2 to 5). The following substrates were tested (0.1% [wt/vol] final concentration, unless specified otherwise) on Fe(II)- and yeast extract-grown cells: 70 mM ferrous iron, 0.02% yeast extract, sucrose, D-glucose, starch, L-tryptophan, L-tyrosine, and L-valine. The vitamin mix was prepared according to the method of Golyshina et al. (11). Ferrous iron and yeast extract were also added separately by using primer pair 21Fa (6) and 1492R. The PCR incubation sequence was 94°C for 5 min on a Touchgene thermocycler (Techne). The PCR fragments were cleaned by using Sigma Spin postreaction purification (4). The 16S rRNA gene was amplified separately by using primer pair 21Fa (6) and 1492R (18) and primer pair 151F (15) and 1492R. The PCR incubation sequence was 94°C for 5 min followed by 25 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s, and then finally 72°C for 5 min on a Touchgene thermocycler (Techne). The PCR fragments were cleaned by using Chroma Spin + TE-1000 columns (Clontech Laboratories), and sequencing reactions were done with a Big Dye kit (Perkin-Elmer Biosystems), with incubations of 94°C for 30 s followed by 30 cycles of 94°C for 10 s, 50°C for 5 s, and 60°C for 4 min. The cycling sequence for sequencing was 21Fa, 515F, 906F (18), 519R (18), 907R (18), and 1492R. PCR fragments for sequencing were cleaned by using SigmaSpin postreaction purification.
cation columns (Sigma) and were analyzed on an ABI Prism 377 DNA sequencer. Evolutionary analyses of sequence data were performed by distance methods, using ARB (29) and PAUP (31), and by parsimony and maximum likelihood algorithms in PAUP. Distances were calculated in ARB according to the substitution algorithm of Jukes and Cantor (17), and phylogenetic trees were assembled by neighbor joining. Maximum likelihood analysis was done by using the GTR model (25), and heuristic searching was performed for the parsimony and maximum likelihood analyses.

DNA-DNA hybridization and G+C content. G+C contents were calculated (in triplicate), with the type strain of *Methanobrevibacter ruminantium* serving as a reference. For establishment of the DNA-DNA reassociation values between *Ferroplasma* genomic DNAs, the spectrophotometric renaturation rate kinetic procedure adapted by Bowman et al. (5) from Huss et al. (16) was used. The optimal temperature for renaturation (25°C below melting temperature) was calculated, assuming a G+C content of 37 mol%, to be 64°C.

2D-PAGE. Cells for 2D-PAGE were harvested, washed, and prepared in urea-thiourea cell lysis buffer (13). The cell extract was focused in the first dimension by loading 200 μg of protein on pH 4 to 7 strips (Amersham) and then was separated in the second dimension in PAGE gels (6a). PAGE gels were stained with silver nitrate (1) and scanned by the Proteomic imaging system (Perkin-Elmer Life Sciences). Gels were analyzed with ProteomWeaver, version 1.3 (Definiens), and spot profiles were used to produce similarity values utilizing the Dice-Sorensen index (23), from which an unrooted neighbor-joining tree was constructed in PAUP (31).

Nucleotide sequence accession number. The DR1 16S rDNA sequence was elucidated and deposited into GenBank under accession number AY222042.

RESULTS

**Morphology.** The *Ferroplasma* isolates were either pleomorphic or irregular cocci, and the “*F. acidarmanus*” Fer1T diameter was found to be 0.66 ± 0.18 and 0.57 ± 0.20 μm (n = 52) for cells grown on Fe(II) and yeast extract (Fig. 1a) and for

FIG. 1. Transmission electron micrographs of “*F. acidarmanus*” Fer1T cells grown chemomixotrophically on ferrous iron and yeast extract (a) and chemoorganotrophically on yeast extract (b). Bars, 250 nm.

FIG. 2. Growth of *Ferroplasma* isolates in batch culture. “*F. acidarmanus*” Fer1T (■), MT17 (▲), and DR1 (▼) were grown chemomixotrophically on ferrous iron and yeast extract. The data points are means ± SD (n ≥ 2).
those grown on yeast extract alone (Fig. 1b), respectively. The size difference between the two sets of cells was analyzed by Student’s t test and was found to be significant, with a confidence of 98.4%.

**Growth rates and temperature and pH optima.** “F. acidarmanus” Fer1T was capable of growing chemomixotrophically on Fe(II) plus yeast extract (Fig. 2) and chemoorganotrophically on yeast extract alone (Fig. 3). The generation times for the chemomixotrophic and chemoorganotrophic cultures were 4.22 and 16.13 h, respectively. “F. acidarmanus” Fer1T grew to a higher peak cell density on Fe(II) plus yeast extract \((1.4 \times 10^9 \pm 2.9 \times 10^7 \text{ cells ml}^{-1}; n = 9)\) than on yeast extract alone \((8.9 \times 10^7 \pm 3.7 \times 10^7 \text{ cells ml}^{-1}; n = 12)\) (data not shown). Compared to “F. acidarmanus” Fer1T, strains MT17 and DR1 grew to higher cell densities, reaching \(2.7 \times 10^8 \pm 1.2 \times 10^7 \text{ cells ml}^{-1} (n = 2)\) and \(3.5 \times 10^8 \pm 1.7 \times 10^7 \text{ cells ml}^{-1} (n = 2)\), respectively (Fig. 2). The four Ferroplasma isolates were also grown chemoorganotrophically on yeast extract, and growth was measured by optical density readings (Fig. 3). Compared to the other isolates, chemoorganotrophically grown “F. acidarmanus” Fer1T had an extended lag phase and again achieved a lower cell density. With two further subcultures in MSM plus yeast extract, the generation time of the chemoorganotrophically grown culture decreased to 9.62 h and the lag phase decreased from 56 to 14 h (data not shown). We also tested whether the oxidation of manganese (72 mM) could support the growth of “F. acidarmanus” Fer1T and F. acidiphilum YT in the presence of 0.02% yeast extract. A statistically significant amount of growth was measured for both isolates, but it was less than their respective growth rates on yeast extract alone (data not shown).

“F. acidarmanus” Fer1T grew at pHs 0.2 to 2.5, with an optimum at 1.2 (Fig. 4a), and had a temperature optimum of 42°C (Fig. 4b). Although “F. acidarmanus” Fer1T did not appear to grow at pH 0.35 (Fig. 4a) after 63 h, it grew to \(0.27 \pm 0.15 \text{ and } 2.60 \pm 1.35 \mu g \text{ of protein ml}^{-1} (n = 3)\) at pHs 0.2 and 0.5, respectively. Within the time frame tested (63 h), isolates MT17 and DR1 grew at pHs 0.35 to 3 and 0.35 to 2.5, respectively (Fig. 4a), and at 37 to 51°C and 32 to 51°C, respectively (Fig. 4b).

**Oxygen consumption and growth by Ferroplasma isolates.** An increase in the oxygen consumption rate above that of the cell-free controls was observed for all of the substrates listed in

![FIG. 3. Chemoorganotrophic growth of “F. acidarmanus” Fer1T (■), MT17 (▲), DR1 (▼), and F. acidiphilum YT (●) in batch cultures as measured by the optical density at 600 nm. The data points are means ± SD (n = 2).](http://aem.asm.org/)

![FIG. 4. Temperature and pH dependency of Ferroplasma strains. “F. acidarmanus” Fer1T (■), MT17 (●), and DR1 (▲) were grown on ferrous iron and yeast extract for 63 h at a range of pHs (a) and temperatures (b), and growth was measured as an increase in protein concentration. The results are averages of three experiments ± SD.](http://aem.asm.org/)
TABLE 1. Growth of Ferroplasma isolates on various substrates (1 mM final concentration, unless specified otherwise)

<table>
<thead>
<tr>
<th>Growth condition or substrate</th>
<th>Yeast extract (0.02%)</th>
<th>Casamino Acids (2%)</th>
<th>Sucrose</th>
<th>Sorbitol</th>
<th>Lactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fer1T</td>
<td>1.34 ± 0.38</td>
<td>1.56 ± 0.26</td>
<td>3.02 ± 0.36</td>
<td>1.98 ± 0.16</td>
<td>2.81 ± 0.41</td>
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<tr>
<td>MT17</td>
<td>4.09 ± 1.09</td>
<td>2.66 ± 0.37</td>
<td>2.56 ± 0.36</td>
<td>0.33 ± 0.24</td>
<td>2.90 ± 0.25</td>
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<tr>
<td>DR1</td>
<td>4.18 ± 0.68</td>
<td>3.12 ± 0.37</td>
<td>1.95 ± 0.29</td>
<td>2.61 ± 0.42</td>
<td>2.82 ± 0.34</td>
</tr>
<tr>
<td>YTN</td>
<td>1.81 ± 0.48</td>
<td>0.59 ± 0.48</td>
<td>2.19 ± 0.34</td>
<td>1.63 ± 0.36</td>
<td>1.24 ± 0.36</td>
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<table>
<thead>
<tr>
<th>Chemoorganotrophic [Fe(II) plus substrate]</th>
<th>Yeast extract (0.02%)</th>
<th>Saccharose</th>
<th>Glucose</th>
<th>Galactose</th>
<th>Fructose</th>
<th>Maltose</th>
<th>Xylose</th>
<th>Mannitol</th>
<th>Sorbitol</th>
<th>Lactose</th>
<th>Thiamine</th>
<th>Casamino Acids (2%)</th>
<th>Asparagine</th>
<th>Aspartic acid</th>
<th>Glutamic acid</th>
<th>Methionine</th>
<th>Phenylalanine</th>
<th>Serine</th>
<th>Tyrosine</th>
<th>Valine</th>
</tr>
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<tr>
<td>Fer1T</td>
<td>2.74 ± 0.18</td>
<td>1.86 ± 0.27</td>
<td>1.50 ± 0.45</td>
<td>2.22 ± 0.38</td>
<td>3.57 ± 0.20</td>
<td>3.23 ± 0.46</td>
<td>2.58 ± 0.23</td>
<td>3.50 ± 0.33</td>
<td>2.04 ± 0.34</td>
<td>2.28 ± 0.21</td>
<td>0.26 ± 0.14</td>
<td>2.78 ± 0.17</td>
<td>0.48 ± 0.15</td>
<td>0.37 ± 0.17</td>
<td>0.28 ± 0.18</td>
<td>0.25 ± 0.17</td>
<td>2.76 ± 0.41</td>
<td>0.44 ± 0.15</td>
<td>0.30 ± 0.18</td>
<td>0.26 ± 0.15</td>
</tr>
<tr>
<td>MT17</td>
<td>6.83 ± 0.20</td>
<td>2.86 ± 0.17</td>
<td>1.84 ± 0.18</td>
<td>1.26 ± 0.17</td>
<td>1.26 ± 0.17</td>
<td>1.86 ± 0.17</td>
<td>1.85 ± 0.34</td>
<td>1.10 ± 0.25</td>
<td>0.27 ± 0.23</td>
<td>2.52 ± 0.23</td>
<td>2.01 ± 0.18</td>
<td>4.40 ± 0.25</td>
<td>0.66 ± 0.23</td>
<td>0.60 ± 0.22</td>
<td>1.21 ± 0.21</td>
<td>0.08 ± 0.34</td>
<td>0.67 ± 0.20</td>
<td>0.00 ± 0.17</td>
<td>0.00 ± 0.18</td>
<td>0.13 ± 0.23</td>
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<tr>
<td>DR1</td>
<td>5.50 ± 0.47</td>
<td>1.30 ± 0.32</td>
<td>1.84 ± 0.28</td>
<td>1.46 ± 0.28</td>
<td>1.25 ± 0.18</td>
<td>1.60 ± 0.28</td>
<td>0.47 ± 0.28</td>
<td>0.49 ± 0.30</td>
<td>2.17 ± 0.30</td>
<td>3.38 ± 0.29</td>
<td>0.63 ± 0.35</td>
<td>4.90 ± 0.34</td>
<td>0.65 ± 0.32</td>
<td>0.72 ± 0.29</td>
<td>0.00 ± 0.28</td>
<td>1.37 ± 0.31</td>
<td>1.01 ± 0.38</td>
<td>0.00 ± 0.32</td>
<td>0.68 ± 0.31</td>
<td>0.68 ± 0.31</td>
</tr>
<tr>
<td>YTN</td>
<td>1.06 ± 0.64</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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| Absence of added organic carbon Fe(II)  | 0.00 ± 0.21          | 2.72 ± 0.25   | 3.08 ± 0.39   | 2.42 ± 0.32   |

* The results are means of the protein concentration after 63 h minus the concentration of no-substrate controls ± SD. Growth results for F. acidiphilum Y† were taken from the work of Golyshina et al. (11). SDs were calculated as the square roots of the sums of the squares of the SDs for the test and controls (n = 3 for both the test and controls).

ND, not determined.

An aerobic growth of all isolates in the absence of Fe(III) was very low (Fig. 5) and was similar to that of the no-substrate (absence of Fe(III) and yeast extract) controls.

After 3 weeks of incubation at 37°C, small, white, round colonies appeared on plates containing MSM plus trace elements and yeast extract, but no growth was evident on plates grown with Fe(II) and yeast extract or ferrous iron plus Casamino Acids. The plating efficiencies for BRL Ultrapure and Sigma Type I agarose were 10.3% ± 2.1% and 7.7% ± 1.4% (n = 3), respectively. Single colonies were reisolated from both agarose types into shake flasks with Fe(II) plus yeast extract or yeast extract alone. After 8 days, all of the cultures had grown (data not shown).

Analysis of autotrophy. F. acidiphilum Y†, DR1, and MT17 grew in MSM plus Fe(II) in shake flasks (Table 1). This suggested that strains Y†, MT17, and DR1 could fix CO2 for autotrophic growth, as had previously been reported for F. acidiphilum Y† (11). Attempts to stimulate chemolithoautotrophic growth of F. acidarmanus Fer1T by the use of increased CO2 (2% [vol/vol] in air) in shake flasks and after a slow adaptation to reduced levels of yeast extract and increased CO2 (2% [vol/vol]) in the continuous culture vessel were unsuccessful.

As a further test, the Ferroplasma isolates were maintained in continuous culture vessels in the absence of organic carbon.
Neither DR1 nor “F. acidarmanus” Fer1T maintained a steady state after 3 volume changes in the absence of organic carbon (Fig. 6), although all isolates except “F. acidarmanus” Fer1T maintained a statistically valid protein concentration and could be revived in MSM plus Fe(II) and yeast extract (data not shown). The protein and yeast extract theoretical washout rates were also plotted, and the DR1 and “F. acidarmanus” Fer1T protein concentrations were maintained above the washout rate until the theoretical yeast extract concentration reached 0.0045 or 0.0003% (wt/vol), respectively. MT17 and F. acidiphilum YT also failed to maintain a steady state above the washout curve (data not shown).

We attempted to measure CO2 fixation on five separate occasions with various conditions and sampling times, but at no stage was a statistically significant concentration of 14CO2 incorporated into “F. acidarmanus” Fer1T compared to no-cell controls or no-growth controls (data not shown). We also tested whether F. acidiphilum YT, DR1, and MT17 incorporated more 14CO2 than the no-growth controls. No increase in 14CO2 incorporation was detected (data not shown).

Antibiotic sensitivity and heavy metal tolerance. With the exception of gentamicin, “F. acidarmanus” Fer1T was at least partly susceptible to all of the antibiotics at the concentrations tested (Table 2). “F. acidarmanus” Fer1T was only slightly inhibited by ampicillin, chloramphenicol, and kanamycin and was strongly inhibited by rifampin and tetracycline (Table 2). In general, “F. acidarmanus” Fer1T was less sensitive to inhibition by antibiotics than F. acidiphilum YT (11).

All three of the Ferroplasma isolates tested exhibited a degree of resistance to cadmium, copper, arsenate, and arsenite (Table 3), although “F. acidarmanus” Fer1T appeared to be less sensitive to heavy metal inhibition. Increased levels of resistance to some of the metals could be induced by a previous exposure to subtoxic concentrations (for example, cadmium inhibition of DR1) (Table 3). We also found that in the presence of low concentrations of arsenate, “F. acidarmanus” Fer1T grew to a higher cell density than in the absence of As(V) (Table 3).

16S sequence data, DNA hybridization, and 2D-PAGE protein profiles. The DR1 16S rDNA sequence was elucidated, and a similarity matrix was calculated for the four isolates (Table 4). A phylogenetic tree was constructed for the Ferroplasma isolates and related microorganisms (Fig. 7). It can be seen that although the 16S rDNA similarities are very close, “F. acidarmanus” Fer1T forms a separate group from the other three strains, and this relationship is supported by all phylogenetic estimations (Fig. 7). The DNA-DNA similarities between the four isolates are also listed in Table 4, and an unrooted tree was constructed (Fig. 8). The results clearly show that strains DR1 and YT are from the same species and that MT17 is more closely related to DR1 and YT than to “F. acidarmanus” Fer1T. 2D-PAGE protein profiles were obtained with whole-cell extracts from all four Ferroplasma isolates grown chemomixotrophically on Fe(II) and 0.02% yeast extract (6a). The unrooted neighbor-joining tree constructed from the protein
is that an autotroph presented in the literature. The genus
leaching environments.

An autotroph (34), whereas the second definition (used in this study) is that
perhaps for some vitamins that may be required for growth

Means

Values are means ± SD (n = 3).

profile data had the same topology as that produced from
DNA-DNA similarity data (Dopson et al., submitted), with
the longest branch separating “F. acidarmanus” FerI1T from
the other isolates.

DISCUSSION

This paper is a detailed study of the phenotypic and geno-
typic characteristics of four isolates from the genus Fer-
roplasma which were isolated from different acid bioleaching
and AMD environments. A summary of all of the phenotypic
and genotypic characteristics of the four isolates is given in Table 5. The
results from this study make possible an assessment of the
biogeochemical role of these Ferroplasma isolates in acid-
leaching environments.

The question of autotrophy is central to the classification of
the genus Ferroplasma, and at least two definitions are presented
in the literature. The first, used by Golyshina et al. (11),
is that an autotroph fixes CO2 as its carbon source “except
perhaps for some vitamins that may be required for growth”
(34), whereas the second definition (used in this study) is
that an autotroph fixes CO2 as its sole source of organic carbon
(19). Although F. acidiphilum YT has been described as strictly
chemoautotrophic, the culture medium used in that study in-
cluded 0.02% (wt/vol) yeast extract (11). It was argued that
the amount of yeast extract in the medium was insufficient to be a
carbon and energy source, and therefore by the definition
utilized by Golyshina et al., F. acidiphilum YT grew autotrophi-
cally. In this study, we have shown that the four Ferroplasma
isolates could grow chemoorganotrophically on the same con-
centration of yeast extract as that used in the F. acidiphilum YT
study. Therefore, under the conditions described, F. acidipi-
hum YT cannot be described as chemoautotrophic and must be
referred to as chemomixotrophic.

The protein concentration in continuous culture vessels in
the absence of organic carbon was initially maintained above
the theoretical protein washout rate. After 3 volume changes,
DR1 (Fig. 6b), F. acidiphilum YT, and MT17 (data not shown)
maintained a statistically valid level of protein above zero, but
“F. acidarmanus” FerI1T did not (Fig. 6a). This reflects
the relative requirement for organic carbon for the four isolates.
We also attempted to measure 14CO2 uptake with all four Ferro-
plasma isolates, but we were unsuccessful. As in a previous
study (11), low levels of 14CO2 uptake were detected. However,
these levels were not statistically higher than those
for no-cell controls or cells incubated in the presence of
14CO2 at 4°C. Therefore, our results clearly show that F. acidipi-
hum YT, DR1, and MT17 have a much lower requirement for or-
ganic carbon than “F. acidarmanus” FerI1T and that they
possibly fix organic carbon. This pattern of carbon requirement
among the isolates was supported by the revival of all isolates
except “F. acidarmanus” FerI1T after three culture volume
changes in continuous culture vessels in the absence of added
organic carbon. Also, protein production was detected in batch
cultures in the absence of organic carbon for all isolates except
“F. acidarmanus” FerI1T (Table 1). The annotation of the 97%
complete genome sequence of “F. acidarmanus” FerI1T does
not indicate the presence of genes for a complete CO2 fixation
pathway. The presence of genes for CO2 fixation cannot be
completely ruled out given the number of hypothetical proteins
with unknown functions and given that the genome sequence is
incomplete. However, in compliance with the organic carbon
requirements detected in this study, it is possible that the other
Ferroplasma isolates (not “F. acidarmanus” FerI1T) have genes
encoding autotrophy.

Our results provide some insights into the possible activities
of these organisms in their habitats. Mixotrophic growth ox-
idizing Fe(II) is a key phenotypic trait which is important for
growth and survival of these microorganisms in acid-leaching
environments. This trait would also be key to contributing to

| TABLE 3. Metal resistance in strains “F. acidarmanus” FerI1T, MT17, and DR1 in uninduced and induced cells |
|-----------------|---------|----------|----------|
| Metal           | Conc (g liter-1) | Growth of strain (µg of protein ml-1) |
|                 | FerI1T | DR1      | MT17     |
| No metal        | 0.12   | 0.12     | 0.12     |
| Cd              | 0.12   | 0.12     | 0.12     |
| Reinoculated Cd | 0.12   | 0.12     | 0.12     |
| Cu              | 0.12   | 0.12     | 0.12     |
| Reinoculated Cu | 0.12   | 0.12     | 0.12     |
| As(III)         | 0.12   | 0.12     | 0.12     |
| As(V)           | 0.12   | 0.12     | 0.12     |
| Reinoculated As(V) | 0.12 | 0.12 | 0.12 |

Growth was measured as the protein concentration after 63 h. Values are means ± SD (n = 3).

| TABLE 4. Degrees of 16S rDNA (upper sector) and DNA-DNA (lower-sector) similarity among the four Ferroplasma strains |
|-----------------|----------|----------|
| Strain (for DNA-DNA similarity) | FerI1T | DR1 | MT17 | YT |
| % 16S rDNA or DNA-DNA similarity with indicated strain |
| FerI1T       | 100.0   | 99.9   | 99.7   | 99.6   |
| DR1          | 99.9   | 100.0   | 99.7   | 99.6   |
| MT17         | 99.7   | 99.6   | 100.0   | 99.9   |
| YT           | 99.6   | 99.9   | 99.7   | 100.0   |

Similarity values are means of results of three or four replicates ± SDs. Values in the upper sector indicate similarities in 16S rDNAs, and values in the lower sector indicate similarities between DNAs.
The production of acid-leaching solutions affected by the biological generation of the strong oxidant Fe(III) and protons. The lack of chemolithoautotrophic growth by "F. acidarmanus" Fer1T was surprising considering its high level of biofilm growth in association with fungi in a low-organic-carbon-level environment (3). It is possible that "F. acidarmanus" Fer1T is autotrophic, but not under the conditions tested, or that it may require a growth factor that was not supplied in the laboratory but that may be provided in its natural environment, possibly by a fungus. Another alternative is that the natural population contains both chemolithoautotrophic and nonchemolithoautotrophic strains that are indistinguishable by the 16S rDNA probe used. "F. acidarmanus" Fer1T grows at both lower temperatures and over a wider temperature range (23 to 46°C) than the other three strains. This possibly reflects the fact that it was isolated from a natural mine site, where it would be expected to experience a wider range of temperatures than the controlled environment in a biooxidation vessel. Also, "F. acidarmanus" Fer1T grew at a lower and narrower pH range and has previously been reported to grow at pH 0 (10). It is likely that this reflects the extremely low pH of the Iron Mountain AMD site (20). One other phenotypic difference is that "F. acidarmanus" Fer1T grows to an approximately 10-fold-lower cell density (based on protein concentration [11]) than F. acidiphilum YT and has significantly lower cell counts than strains DR1 and MT17. This could be due to the culture conditions for "F. acidarmanus" Fer1T being below optimum. For example, "F. acidarmanus" Fer1T has a higher requirement for organic carbon than the other strains do.

Also, in contrast to a previous report (11), we found that manganese could not support the growth of "F. acidarmanus" Fer1T or F. acidiphilum YT, as the amount of chemomixotrophic growth was less than that of chemoorganotrophic growth on yeast extract alone. This suggests that the manganese added (72 mM) was not only unable to be used as an energy source but was also slightly inhibitory for both strains. Finally, in contrast to the F. acidiphilum YT study, all four Ferroplasma isolates have been demonstrated to grow anaerobically via the oxidation of yeast extract coupled to the reduction of Fe(III). In natural AMD and biolaelching environments, Ferroplasma species grow in the form of biofilms (4), which is an advantageous form of growth for avoiding washout. Anaerobic growth is an important phenotype for Ferroplasma, as the dissolved oxygen concentrations in biofilms vary greatly, and thus within the interior Fe(III) reduction may be important for activity and cell maintenance.

As "F. acidarmanus" Fer1T does not possess a cell wall, it was unsurprising that ampicillin was ineffective. Similar results were found for F. acidiphilum YT (11) and for members of another acidophilic archaeal genus, Picrophilus (27). A difference between the antibiotic sensitivities of "F. acidarmanus" Fer1T and Picrophilus was that "F. acidarmanus" Fer1T was resistant to >50 μg of chloramphenicol ml⁻¹ (a concentration expected to be inhibitory), while Picrophilus was susceptible. "F. acidarmanus" Fer1T, DR1, and MT17 all exhibited a degree of resistance to cadmium, copper, arsenite, and arsenate. This is not surprising given the metal-rich environments from which they were isolated. In some instances, either induction of the Ferroplasma isolates to metal resistance or adaptation to higher levels of metals occurred. It was also observed that small quantities of arsenate stimulated the growth of "F. acidarmanus" Fer1T. An increase in the oxidation rate of reduced inorganic sulfur compounds in the presence of low levels of arsenite has also been observed for Acidithiobacillus caldus (12), possibly due to the increased energy demand for efflux via...

FIG. 7. Phylogenetic relationship of 16S rRNA gene sequences with representative sequences from databases. The dendrogram was generated by distance matrix and neighbor-joining methods and was rooted thereafter at the branch with Archaeoglobus fulgidus as the outgroup. Accession numbers for sequences are given in parentheses. The scale bar represents 0.05 substitutions per site. Branch points supported by distance, maximum likelihood, and parsimony estimations are indicated by open circles.

FIG. 8. Unrooted neighbor-joining tree of DNA-DNA similarity. The scale bar represents 10% changes.
the ArsB protein (8). Also, in its natural environment “F. acidarmanus” Fer1T grows in very high metal concentrations, including arsenopyrite at the Iron Mountain site (20), and it is possible that it may require small quantities of metals for optimal growth.

The four Ferroplasma isolates have 16S rRNA gene similarities between 98.9 and 99.7%, although phylogenetic trees constructed using distance, maximum likelihood, and parsimony estimations all supported branch points that separated “F. acidarmanus” Fer1T from the other three Ferroplasma strains (Fig. 7). DNA-DNA similarity data are currently viewed as the most reliable method of defining taxonomic relationships, and a similarity of 70% or higher has been defined as the species border (33). Even though 16S rDNA sequence similarities of >97% are accepted as a single species, it has been found that DNA-DNA similarity values for the Ferroplasma isolates and when other phylogenetic methods are inconclusive.

The relationship of “F. acidarmanus” Fer1T to the other Ferroplasma isolates is summarized in Table 5. Based on 16S rDNA sequence data and 2D-PAGE profiles, “F. acidarmanus” Fer1T groups as a separate phylotype, and it is phenotypically and genotypically different from the other Ferroplasma strains. While DNA-DNA similarity values are borderline, we propose that it is a separate species and retain it as “F. acidarmanus” Fer1T.

Updated description of Ferroplasma genus based on the work of Golyshina et al. (11). Grows chemooorganotrophically, chemomixotrophically, and possibly chemoautotrophically. Facultative anaerobes. Colonies formed on yeast extract solid medium are small, white, and round. G+C content is 36.5 to 37 mol%. The type strain is Ferroplasma acidiphilum YT and there are three other isolates, namely DR1, MT17, and “Ferroplasma acidarmanus” Fer1T.

Description of Ferroplasma acidarmanus sp. nov. Dopson, Baker-Austin, Hind, Bowman, and Bond 2004. F. acidarmanus (a.c.id’.a.r’man.us. L. n. acidus, acid; L. adj. arman.us, pertaining to Arman, the owner of the mine from which the species was isolated; N.L. adj. acidarmanus, an acidophilic archaeon isolated from a mine belonging to Arman).

Isolated from an AMD site in California. Morphology as described for the genus. G+C content of 36.8 mol%. Growth occurs between 23 and 46°C (mesophilic) and pHs 0 and 1.5 (acidophilic). Grows chemooorganotrophically on yeast extract, Casamino Acids, and sugars and chemomixotrophically on Fe(II) and the above organic carbon sources. Facultative anaerobe, coupling oxidation of yeast extract to Fe(III) reduction. “F. acidarmanus” Fer1T has been deposited in the American Type Culture Collection in the patent pending collection.

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