

# Leaching of Pyrite by Acidophilic Heterotrophic Iron-Oxidizing Bacteria in Pure and Mixed Cultures

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Seven strains of heterotrophic iron-oxidizing acidophilic bacteria were examined to determine their abilities to promote oxidative dissolution of pyrite ( $\text{FeS}_2$ ) when they were grown in pure cultures and in mixed cultures with sulfur-oxidizing *Thiobacillus* spp. Only one of the isolates (strain T-24) oxidized pyrite when it was grown in pyrite-basal salts medium. However, when pyrite-containing cultures were supplemented with 0.02% (wt/vol) yeast extract, most of the isolates oxidized pyrite, and one (strain T-24) promoted rates of mineral dissolution similar to the rates observed with the iron-oxidizing autotroph *Thiobacillus ferrooxidans*. Pyrite oxidation by another isolate (strain T-21) occurred in cultures containing between 0.005 and 0.05% (wt/vol) yeast extract but was completely inhibited in cultures containing 0.5% yeast extract. Ferrous iron was also needed for mineral dissolution by the iron-oxidizing heterotrophs, indicating that these organisms oxidize pyrite via the “indirect” mechanism. Mixed cultures of three isolates (strains T-21, T-23, and T-24) and the sulfur-oxidizing autotroph *Thiobacillus thiooxidans* promoted pyrite dissolution; since neither strains T-21 and T-23 nor *T. thiooxidans* could oxidize this mineral in yeast extract-free media, this was a novel example of bacterial synergism. Mixed cultures of strains T-21 and T-23 and the sulfur-oxidizing mixotroph *Thiobacillus acidophilus* also oxidized pyrite but to a lesser extent than did mixed cultures containing *T. thiooxidans*. Pyrite leaching by strain T-23 grown in an organic compound-rich medium and incubated either shaken or unshaken was also assessed. The potential environmental significance of iron-oxidizing heterotrophs in accelerating pyrite oxidation is discussed.

Microbiologically accelerated oxidation of pyrite ( $\text{FeS}_2$ ) and other sulfidic minerals is important in both environmental and applied microbiology. Sulfide mineral dissolution by acidophilic bacteria in active and derelict mines and mine spoils produces a noxious, metal-laden, often highly acidic effluent (acid mine drainage [AMD]), which is a serious and widespread form of stream and river pollution in many industrial and postindustrial areas. On the other hand, biological processing of sulfide-rich metal ores is an established area of biotechnology that is projected to increase both in scale and in the range of metals extracted (12, 23). Two species of mesophilic acidophilic bacteria, *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*, have been implicated as being the most significant microorganisms involved in sulfide mineral oxidation, although moderately thermophilic (or thermotolerant) bacteria and extremely thermophilic archaea are also known to be important in certain situations, such as self-heating coal spoils and bioleaching operations in which temperatures exceed 40°C. Both *T. ferrooxidans* and *L. ferrooxidans* are generally regarded as obligate chemolithotrophs and synthesize cell carbon via enzymic fixation of  $\text{CO}_2$ , although it has been shown that *T. ferrooxidans* has a limited capacity to utilize organic carbon (22).

Two mechanisms (“direct” and “indirect”) have been described as the mechanisms by which metal-mobilizing acidophilic bacteria degrade sulfide minerals, although electrochemical interactions which occur between minerals during bacterial leaching are also thought to be important in accelerating mineral dissolution (20). The direct mechanism is envisaged as

being mediated by microorganisms attached to mineral sulfides via enzymic oxidation of the ferrous iron or sulfide moieties of a mineral, whereas the indirect mechanism focuses on the role of ferric iron in abiotic chemical oxidation, in which the primary role of metal-mobilizing bacteria is regeneration of  $\text{Fe}^{3+}$ . However, Sand et al. (25) have presented evidence which indicates that the direct mechanism is also mediated by ferric iron oxidation of sulfide minerals. In the model of these authors, ferric iron bound to exopolymers produced by iron-oxidizing bacteria acts as an electron shuttle; it is reduced when it reacts with the sulfide and is reoxidized (in an energy-generating reaction) by the bacteria.

Chemolithotrophic iron-oxidizing acidophilic bacteria share mineral leaching environments with a range of other microorganisms, including fungi, algae, protozoans, and rotifers, as well as other bacteria (10, 15). Some data have indicated that the presence of heterotrophic acidophilic bacteria may enhance the rate of sulfide mineral oxidation by iron-oxidizing acidophiles (28); one way in which this may occur is by the heterotrophic bacteria metabolizing organic materials which inhibit the iron oxidizers and which accumulate in leachate liquors. Acidophilic bacteria which oxidize reduced sulfur compounds but not ferrous iron, such as the obligate chemolithotroph *Thiobacillus thiooxidans* and the mixotroph *Thiobacillus acidophilus*, may also aid leaching by producing sulfuric acid; this may be particularly beneficial when these bacteria are present in mixed cultures with *L. ferrooxidans*, which does not metabolize sulfur (21).

Recently, a novel group of mesophilic heterotrophic acidophiles has been described. These bacteria are able to oxidize ferrous iron to ferric iron, but, in contrast to chemolithotrophic acidophiles, they require organic carbon for growth. One isolate, which had a filamentous morphology similar to that of *Sphaerotilus* and *Leptothrix* spp., grew (and oxidized iron) in

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ferrous sulfate-yeast extract medium but could not oxidize pyrite in yeast extract-amended media (16). Unicellular iron-oxidizing heterotrophic acidophiles have been isolated from a diverse range of environments in both the United Kingdom and the United States, indicating that they may be widely distributed in acidic, metalliferous waters and soils (17). In this paper, we describe oxidation of pyrite by several isolates of these bacteria grown in pure cultures and in mixed cultures with *Thiobacillus* spp.

#### MATERIALS AND METHODS

**Bacteria.** Seven strains of iron-oxidizing heterotrophic bacteria were used in the leaching experiments. Three strains (strains T-21, T-23, and T-24) were isolated either directly or indirectly (via enrichment cultures) from AMD from inside an abandoned pyrite mine (Cae Coch) in the Conwy Valley, North Wales (19); strain T-25 was isolated from an AMD stream at the derelict Parys mine site in Anglesey, North Wales (26); strain CH13 was isolated from AMD at the Noranda Blackbird cobalt mine in Cobalt, Idaho (17); and strains SLC1 and SLC2 were isolated from regolith samples that were undergoing controlled accelerated sulfide mineral oxidation in humidity cell chambers at the former U.S. Bureau of Mines laboratories in Salt Lake City, Utah (27). Serial dilutions of AMD, cultures solutions, or suspensions of weathered regolith were plated onto ferrous iron-containing overlay media (11), and iron-oxidizing heterotrophic acidophiles were identified on the basis of their distinctive colony morphologies (13, 17). Isolates were purified by repeated isolation of single colonies on solid medium and were screened regularly for the presence of contaminant acidophilic bacteria. In addition, the following type strains of three acidophilic *Thiobacillus* spp. were used in the experiments: *T. ferrooxidans* ATCC 23270, *T. thiooxidans* ATCC 19377, and *T. acidophilus* ATCC 27807.

All heterotrophic iron-oxidizing bacteria were maintained in 10 mM ferrous sulfate–0.02% (wt/vol) yeast extract–basal salts liquid medium (13) adjusted initially to pH 2.0. *T. ferrooxidans* was grown in 20 mM ferrous sulfate–basal salts liquid medium (pH 2.0). Both *T. acidophilus* (which was grown exclusively as an autotroph) and *T. thiooxidans* were maintained in 1% (wt/vol) elemental sulfur–basal salts medium adjusted initially to pH 3.0. All of the bacteria were grown shaken at 30°C.

**Pyrite oxidation experiments.** Shake flask (250-ml) cultures containing 100 ml of basal salts and 1.0 g of pyrite were prepared. The pyrite used was obtained from the Cae Coch mine and was ground to particles that were <61 µm in diameter. An X-ray diffract analysis showed that the ore was ca. 80% FeS<sub>2</sub>; the remainder was mostly quartz and feldspars. No other sulfide minerals were detected in the ground ore. The pH of pyrite medium was adjusted with H<sub>2</sub>SO<sub>4</sub> to 2.0, and the medium was sterilized by autoclaving it at 120°C for 20 min. After the flasks were cooled to room temperature, they were inoculated (2%, vol/vol) with pure cultures of the seven heterotrophic iron-oxidizing bacteria, *T. ferrooxidans*, *T. thiooxidans*, and *T. acidophilus*. Pyrite oxidation by each of the seven heterotrophic iron-oxidizing isolates was also estimated in pyrite medium (see above) to which yeast extract (0.02%, wt/vol) was added. The effects of different yeast extract concentrations (0.005 to 0.5%, wt/vol) on pyrite oxidation by heterotrophic iron oxidizers were determined by using a single strain (strain T-21). The abilities of mixed cultures of acidophilic bacteria to accelerate pyrite oxidation were examined by inoculating unamended pyrite medium with all of the iron-oxidizing heterotrophic strains (as separate cultures) along with *T. thiooxidans*. Mixed cultures containing isolate T-21 or T-23 and the mixotroph *T. acidophilus* were prepared with yeast extract-amended and yeast extract-free pyrite media. The cultures were incubated at 30°C and shaken at 100 rpm for up to 50 days, and samples removed at weekly intervals for determinations of soluble iron concentrations and pH. All cultures were prepared in triplicate.

It was found that autoclaving pyrite in acidic solutions solubilized significant quantities of iron, which was predominantly ferrous; concentrations up to 420 mg of total iron per liter were measured. To remove this soluble iron from autoclaved culture medium, the pyrite was separated by centrifugation (5,000 × g, 15 min) and resuspended in fresh, sterile basal salts (pH 2.0). This procedure was repeated, which produced a pyrite-basal salts medium in which the initial soluble iron concentration was less than 15 mg/liter. Culture flasks containing washed pyrite-basal salts medium were amended with either yeast extract (final concentration, 0.02% [wt/vol]) or yeast extract (0.02%) plus ferrous sulfate (1 mM) and then inoculated with either strain T-21 or strain T-23.

To examine the effect of culture aeration on pyrite oxidation by strain T-23, we compared the rates of mineral oxidation in cultures which were shaken (100 rpm) with the rates of mineral oxidation in cultures which were not shaken. The liquid medium used in this experiment contained 1% pyrite, 0.02% yeast extract, and 10 mM glycerol (which was metabolized by this iron-oxidizing heterotroph, as shown previously [1]). The cultures were incubated for up to 50 days at 30°C, and the concentrations of total iron and ferrous iron and culture pH values were recorded at regular intervals.

**Analytical methods.** Concentrations of total soluble iron in pyrite-containing cultures were measured by atomic absorption spectroscopy with a Pye Unicam model SP2900 instrument. Samples (ca. 1.0 ml) were removed aseptically from

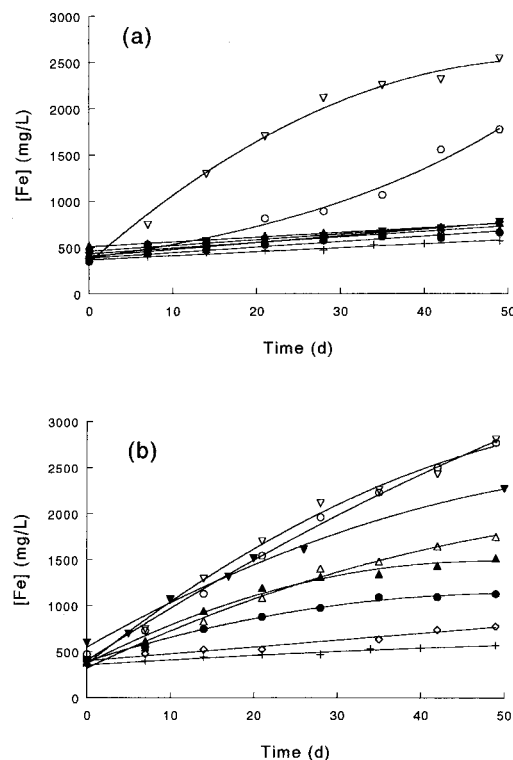


FIG. 1. Oxidation of pyrite by iron-oxidizing heterotrophs in medium containing 1% (wt/vol) pyrite (a) and medium containing 1% pyrite and 0.02% (wt/vol) yeast extract (b). Symbols: ▽, T-21; △, T-23; ○, T-24; ◇, T-25; ▲, CH13; ●, SLC1; +, uninoculated control; ▽, *T. ferrooxidans* ATCC 23270 control. d, days.

dispersed cultures and centrifuged at 13,000 × g for 3 min to remove the cells and particulate matter, and the supernatant was diluted 1:1 with 6 M HCl and stored at room temperature before analysis. Ferrous iron concentrations were determined by titration of cultures with a 1 mM potassium permanganate solution. A glass electrode was used to measure the culture pH.

#### RESULTS

**Trends and variability in pyrite bioleaching experiments.** Replicate cultures in each of the bioleaching experiments displayed very similar trends. The data shown below are the mean soluble iron concentrations determined with triplicate cultures at different times. Error bars are not shown because the variations observed were small and the error bars were often obscured by the symbols.

**Pyrite oxidation by pure cultures of heterotrophic iron-oxidizing acidophiles.** Only one of the seven isolates of heterotrophic iron-oxidizing acidophiles tested (strain T-24) catalyzed oxidative dissolution of pyrite in pyrite-basal salts medium (Fig. 1a). In contrast to *T. ferrooxidans*, with strain T-24 there was a lag period of about 20 days before bacterially enhanced pyrite dissolution was evident, although the subsequent rate of mineral oxidation by strain T-24 was similar to the rate of mineral oxidation by the autotroph. By the end of the 50-day leaching period, the total amount of pyrite oxidized by cultures of strain T-24 was ca. 35% less than the total amount of pyrite oxidized by cultures of *T. ferrooxidans*. We also found that the final recorded pH in T-24 cultures (mean pH, 1.50) was lower than the final recorded pH values in cultures of other heterotrophic isolates (pH range, 1.70 to 1.79) and of *T. ferrooxidans* (mean pH, 1.58).

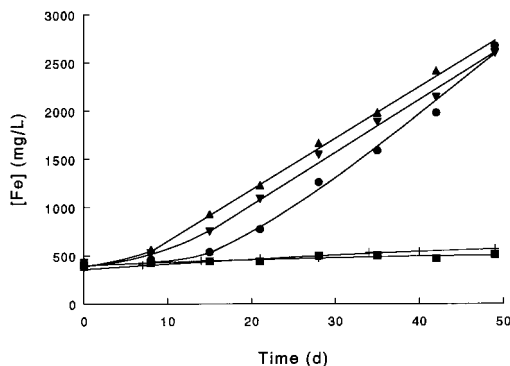


FIG. 2. Effects of different concentrations of yeast extract on the oxidation of pyrite by iron-oxidizing heterotrophic strain T-21. Cultures were amended with 0.005% (wt/vol) yeast extract, (▼), 0.01% (wt/vol) yeast extract, (▲), (●), 0.05% (wt/vol) yeast extract or 0.5% (wt/vol) yeast extract (■). +, uninoculated control. d, days.

In contrast to the results obtained in the pyrite-basal salts medium, all of the heterotrophic iron-oxidizing isolates with the possible exception of strain T-25 increased the rate of pyrite dissolution in pyrite-yeast extract medium (Fig. 1b). There were significant differences in the rates of pyrite solubilization between isolates. Strain T-24 was again found to be the most effective heterotrophic acidophile, oxidizing pyrite at a rate equivalent to that of the autotroph *T. ferrooxidans* (grown in pyrite-basal salts medium). The mean final pH for cultures of isolate T-24 (pH 1.39) was lower than the mean final pH values for cultures of other heterotrophic isolates (pH range, 1.60 to 1.73).

The effects of different concentrations of yeast extract on pyrite oxidation by strain T-21 are shown in Fig. 2. Bacterially enhanced mineral dissolution was observed in cultures containing between 0.005 and 0.05% yeast extract, although there was a more prolonged lag phase in cultures containing 0.05% yeast extract, but such dissolution was completely inhibited by a yeast extract concentration of 0.5%. The rates of pyrite oxidation were similar in these cultures, and there was no evidence that oxidation became carbon limited over the 50-day incubation period, even in cultures containing 0.005% yeast extract. Other work has confirmed that yeast extract acts as a carbon source for these bacteria rather than as a source of trace elements or an essential reduced sulfur compound (1).

In contrast to the results described above, mineral oxidation did not occur in pyrite-yeast extract cultures of isolates T-21 and T-23 in which the pyrite had been washed to remove the soluble iron. However, when 1 mM ferrous sulfate was added to washed pyrite-yeast extract cultures, both of these iron-oxidizing heterotrophic isolates oxidized the mineral (Fig. 3).

**Pyrite oxidation by mixed cultures of heterotrophic iron-oxidizing acidophiles.** When grown in mixed cultures with *T. thiooxidans* in pyrite-basal salts medium, three of the iron-oxidizing heterotrophic isolates (strains T-21, T-23, and T-24) accelerated mineral oxidation, while the other four isolates (strains T-25, CH13, SLC1, and SLC2) did not (Fig. 4). Isolate T-24 had previously been shown to oxidize pyrite in an "inorganic" medium (Fig. 1a) and therefore might have been anticipated to do so in a coculture with *T. thiooxidans*; we found, however, that the mean final concentration of soluble iron in T-24-*T. thiooxidans* mixed cultures was 12% greater than the mean final concentration of soluble iron in pure (yeast extract-free) cultures of T-24 (although it was about 28% less than the mean final concentration of soluble iron in cultures grown in

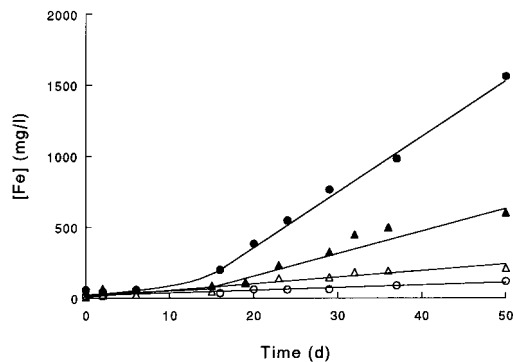


FIG. 3. Oxidation of washed pyrite by iron-oxidizing heterotrophic strains T-21 and T-23. Medium containing washed pyrite (1%, wt/vol) was amended with 0.02% (wt/vol) yeast extract (open symbols) or 1 mM Fe(II) plus 0.02% (wt/vol) yeast extract (solid symbols). Symbols:  $\Delta$  and  $\blacktriangle$ , T-21;  $\circ$  and  $\bullet$ , T-23. d, days.

pyrite-yeast extract medium). Strains T-21 and T-23 oxidized pyrite in cocultures with *T. thiooxidans*, an ability neither isolate (nor *T. thiooxidans*) displayed when it was grown as a pure culture in pyrite-basal salts medium. We also found that the order of "leaching efficiency" of these two heterotrophic acidophiles was reversed compared to order observed in pure cultures grown in pyrite-yeast extract medium (Fig. 4).

Mixed cultures of strain T-21 or T-23 and the mixotrophic sulfur oxidizer *T. acidophilus* also promoted mineral dissolution in inorganic pyrite medium (Fig. 5), although to a lesser extent than corresponding mixed cultures with *T. thiooxidans*. The rates of mineral oxidation by mixed cultures containing *T. acidophilus* were greatly enhanced in pyrite medium which was amended with yeast extract (Fig. 5). In mixed pyrite-yeast extract cultures containing strain T-23, the concentrations of soluble iron after 39 days of incubation were ca. 50% greater than the concentrations of soluble iron in the corresponding pure cultures of the iron oxidizer, while for mixed cultures containing strain T-21 they were ca. 10% less. Like *T. thiooxidans*, *T. acidophilus* was not able to oxidize pyrite in pure culture. The pH values of mixed cultures with both *Thiobacillus* spp. were invariably lower than the pH values of the corresponding pure cultures of iron-oxidizing heterotrophs (data not shown).

Cultures of strain T-23 grown in shaken or unshaken flasks containing pyrite-yeast extract-glycerol medium displayed dif-

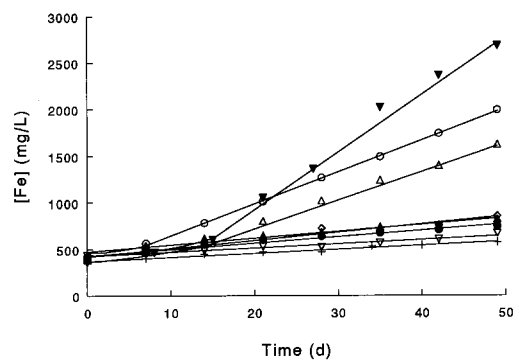


FIG. 4. Oxidation of pyrite by mixed cultures containing iron-oxidizing heterotrophic isolates and the sulfur-oxidizing autotroph *T. thiooxidans* in medium containing 1% (wt/vol) pyrite. Symbols: ▼, T-21;  $\Delta$ , T-23;  $\circ$ , T-24;  $\blacklozenge$ , T-25;  $\bullet$ , CH13;  $\bullet$ , SLC1; +, uninoculated control;  $\nabla$ , *T. thiooxidans* (pure culture) control. d, days.

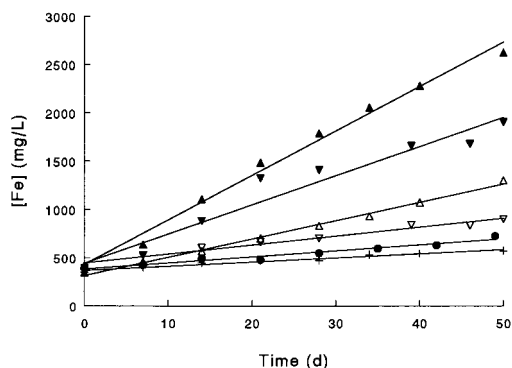


FIG. 5. Oxidation of pyrite by mixed cultures containing iron-oxidizing heterotrophs and the sulfur-oxidizing mixotroph *T. acidophilus* in medium containing 1% (wt/vol) pyrite (open symbols) or medium containing pyrite plus 0.02% (wt/vol) yeast extract (solid symbols). Symbols:  $\triangle$  and  $\blacktriangle$ , T-21;  $\nabla$  and  $\blacktriangledown$ , T-23; +, uninoculated control;  $\bullet$ , *T. acidophilus* (pure culture) control. d, days.

ferent trends both in the rate of pyrite dissolution and in the ratios of ferrous and ferric iron present (Fig. 6). These cultures were designed to be either aerobic (shaken flasks) or microaerobic (unshaken flasks), although dissolved oxygen concentrations were not measured during the experiment. In the shaken cultures, ferric iron was the dominant soluble species throughout the 50 days of incubation. In contrast, in the unshaken cultures, the concentrations of ferrous iron increased dramatically after 24 days of incubation, and by the end of the experiment all of the soluble iron was essentially ferrous (Fig. 6b). We found that pyrite oxidation, as assessed by measuring concentrations of total soluble iron over time, was greater in the unshaken cultures than in the shaken cultures (Fig. 6a) for

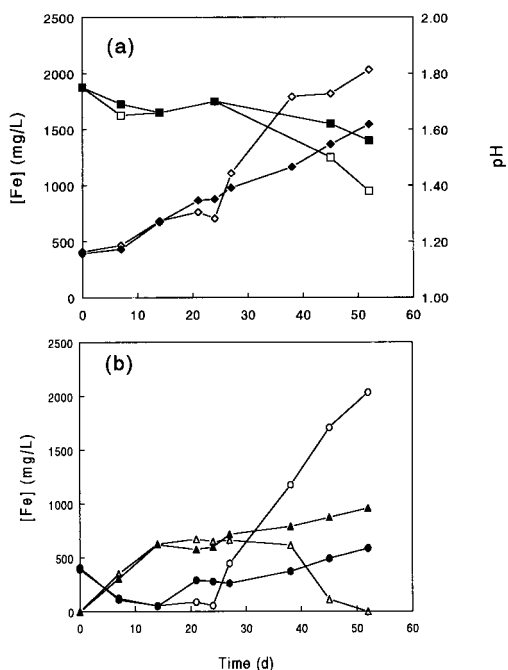


FIG. 6. Oxidation of pyrite by heterotrophic iron-oxidizing isolate T-23 in a medium containing 1% (wt/vol) pyrite, 0.02% (wt/vol) yeast extract, and 10 mM glycerol. Cultures were either shaken (solid symbols) or not shaken (open symbols). (a) Symbols:  $\diamond$  and  $\blacklozenge$ , total soluble iron;  $\square$  and  $\blacksquare$ , pH. (b) Symbols:  $\triangle$  and  $\blacktriangle$ , ferric iron;  $\circ$  and  $\bullet$ , ferrous iron. d, days.

much of the experiment; the mean culture pH values also tended to be lower in the unshaken flasks. In addition, pyrite leaching in the control cultures was greater (and the pH values were lower) in unshaken flasks than in shaken flasks, although the mineral dissolution in uninoculated media was always considerably less than the mineral dissolution in media containing bacteria (data not shown).

## DISCUSSION

All of the acidophilic bacteria investigated in the present study are able to oxidize ferrous iron to ferric iron, but they are not able to fix inorganic carbon (1); this clearly differentiates these organisms from the well-characterized autotrophic iron-oxidizing acidophiles. Phylogenetically, they are also distinct from other acidophilic bacteria. Five of the novel isolates used in this study (all of the isolates except SLC1 and SLC2) have been subjected to a 16S ribosomal DNA sequence analysis and have been shown to be members of a distinct cluster at the cusp between the gram-negative bacteria and the gram-positive bacteria (14), although they appear to belong to more than a single species (24). The name "*Ferromicrobium acidophilus*" has been proposed for strain T-23, although this name has not been officially confirmed yet. Database searches have revealed that the microorganism that is most closely related to these mesophilic heterotrophic acidophiles is the moderately thermophilic iron oxidizer *Acidimicrobium ferroxidans*, although the relationship is quite distant (overall level of sequence similarity, 67%) (14). Physiological differences among the strains were observed in the present study; the isolates often exhibited different propensities to oxidize pyrite in both pure and mixed cultures.

With one exception, all of the heterotrophic iron-oxidizing bacteria required a source of organic carbon (provided in the form of yeast extract) in order to catalyze the oxidative dissolution of pyrite when they were grown in pure culture. The reason why strain T-24 oxidized pyrite in an inorganic medium is unclear but may be related to the exceptional ability of this bacterium to scavenge for traces of organic materials (1), which are invariably present as contaminant and airborne materials in liquid media (6). Other work has indicated that the concentrations of dissolved organic carbon can increase from  $\sim 1$  mg/liter in freshly prepared inorganic acidophilic media to 10 to 20 mg/liter in uninoculated cultures stored in a laboratory (9). Strain T-24 has been shown to have no capacity to fix  $\text{CO}_2$  (1). In addition, strain T-24 has been found to be unique among the iron-oxidizing heterotrophic isolates in that it is able to oxidize elemental sulfur; this characteristic was reflected by the fact that the pH values recorded in cultures of this isolate were lower than the pH values recorded in cultures of the other isolates. The non-iron-oxidizing heterotrophic acidophile *Acidiphilium cryptum* has also been reported to promote limited oxidation of sulfur (8).

In pyrite-yeast extract medium, all of the iron-oxidizing heterotrophic isolates (with the possible exception of strain T-25) were able to accelerate oxidation of pyrite. Clear differences in the leaching efficiencies of the bacteria were apparent, with cultures of the most efficient isolate (strain T-24) displaying rates of pyrite oxidation similar to the rates of pyrite oxidation by the type strain of *T. ferroxidans* (grown in yeast extract-free medium). Interestingly, the rates of pyrite oxidation by strain T-21 grown in media containing low concentrations of yeast extract (as low as 0.005% [wt/vol]) were similar to the rates of pyrite oxidation observed in the presence of higher yeast extract concentrations, indicating that this bacterium may effectively oxidize pyrite in oligotrophic acidic environments.

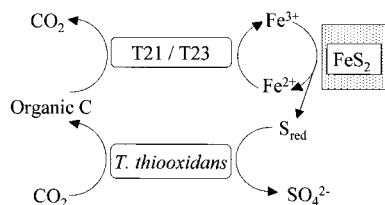


FIG. 7. Hypothetical scheme for the leaching of pyrite by mixed cultures containing the sulfur-oxidizing organism *T. thiooxidans* and heterotrophic iron-oxidizing bacteria.

Pyrite oxidation by the heterotrophic iron-oxidizing acidophiles appeared to operate via the indirect mechanism, which is consistent with the hypothesis proposed by Sand et al. (25). The requirement for ferrous iron (as well as prefixed carbon) suggests that these bacteria accelerate the oxidation of pyrite by producing ferric iron; this oxidizes pyrite in an abiotic reaction, in which ferric iron is reduced back to ferrous iron. Whether iron is oxidized by planktonic cells or by cells attached to pyrite crystals (or by both populations) is not clear, although it is known that strain T-23 is a highly hydrophobic bacterium and that its propensity to attach to pyrite is similar to that of *T. ferrooxidans* (2).

Mixed cultures of iron-oxidizing heterotrophic strain T-21 or T-23 and the sulfur-oxidizing autotroph *T. thiooxidans* were able to oxidize pyrite; pure cultures of these bacteria were not able to accelerate oxidative dissolution of the mineral in inorganic medium. Previous studies (14, 21, 28) which have indicated that mixed cultures of acidophiles may, in some situations, promote accelerated mineral leaching have all included at least one bacterium in the consortium (e.g., *T. ferrooxidans* or *L. ferrooxidans*) which could also oxidize sulfide minerals when it is grown in pure culture. The synergistic relationship between the bacteria described in this report is, therefore, distinct from other synergistic relationships which have been described for acidophiles. Like other autotrophic acidophiles, *T. thiooxidans* releases organic materials into culture media (3), and these materials may be used by heterotrophic acidophiles (10). It is interesting that the order of leaching efficiency for strains T-21, T-23, and T-24 was different when the organisms were grown in mixed cultures with *T. thiooxidans* than when they were grown in pyrite-yeast extract medium as pure cultures; this may to some extent reflect different abilities of the iron-oxidizing heterotrophs to utilize the organic materials in yeast extract and the organic materials originating from *T. thiooxidans*. A hypothetical scheme summarizing how mixed cultures of strain T-21 or T-23 and *T. thiooxidans* accelerate pyrite oxidation is shown in Fig. 7, which is based in part on the model for bacterial pyrite leaching proposed by Sand et al. (25). Iron oxidation by the heterotrophs produces ferric iron, which reacts with pyrite, producing thiosulfate. In acidic solutions, thiosulfate is hydrolyzed to elemental sulfur, a variety of polythionates, and sulfate. The various reduced forms of sulfur are substrates for *T. thiooxidans*, which fixes carbon dioxide and releases organic carbon into the culture medium; some or all of this carbon is utilized by the heterotrophs, which continue the recycling of iron. Oxidation of both ferrous iron and sulfur (as shown by acid production) indicated that strain T-21 or T-23 and *T. thiooxidans* were active in the mixed cultures. In other mixed-culture experiments, large numbers of both *T. thiooxidans* and heterotrophic iron-oxidizing bacteria have been recovered (by plating onto selective media) at the end of the oxidation period (9).

While mixed cultures of the heterotrophic iron oxidizer

strain T-21 or T-23 and the sulfur oxidizer *T. acidophilus* also accelerated pyrite oxidation, the effect was relatively marginal in inorganic pyrite medium. *T. acidophilus* differs from *T. thiooxidans* in that it is mixotrophic and readily changes from using inorganic carbon sources to using organic carbon sources (18). We anticipate that in contrast to *T. thiooxidans*, *T. acidophilus* may compete with heterotrophic iron-oxidizing bacteria for dissolved organic carbon, thereby limiting the growth of and iron oxidation by the heterotrophic iron-oxidizing bacteria in mixed cultures. Addition of yeast extract increased the rate of pyrite oxidation by mixed cultures containing both iron oxidizers and *T. acidophilus*, although only in the case of strain T-23 was the mixed culture more effective than corresponding pure cultures of the iron-oxidizing heterotroph. Like *T. thiooxidans* cultures, the lower pH values in mixed cultures containing *T. acidophilus* than in pure cultures of the iron-oxidizing heterotrophs indicate that the mixotroph actively oxidized reduced sulfur in the former cultures.

Maintenance of high ferric iron/ferrous iron ratios in pyrite cultures should favor mineral oxidation. However, Evangelou (5) has pointed out that low concentrations of ferric iron can result in very effective oxidation of pyrite, and other workers (4, 7) have shown that the autotroph *T. ferrooxidans* is able to bring about extensive leaching of mineral sulfides when it is grown under anaerobic conditions. A comparison of pyrite leaching by strain T-23 incubated under aerobic conditions and pyrite leaching by strain T-23 incubated under microaerobic conditions indicated that pyrite oxidation was more extensive under the latter conditions from the time when the ferrous iron/ferric iron ratios increased (day 24 onward), although there were significant amounts (~100 to 650 mg/liter) of ferric iron present in these cultures during most of this period. The reduction of ferric iron during days 24 to 52 could have occurred by the following two mechanisms (alone or in tandem): (i) an abiotic mechanism involving reaction with pyrite and reduced sulfur species; and (ii) a biological mechanism involving strain T-23, which has been shown to use ferric iron as an electron acceptor when it is grown under low oxygen tensions (1). Reduction of ferric iron caused the pH in unshaken cultures to be lower than the pH in aerobic cultures, and it is possible that enhanced pyrite solubilization could have been due to more rapid abiotic oxidation (by ferric iron) at the lower pH. It is also conceivable that some of the additional solution phase iron found in unshaken cultures originated from reductive dissolution of solid phase ferric iron compounds (hydroxide, jarosites, etc.), which may form as secondary products when sulfide minerals are leached, although there was no visual evidence of ferric iron precipitates in any of the cultures. The possibility that pyrite oxidation by at least some species of acidophilic mineral-mobilizing bacteria is accentuated by a low redox potential (high ferrous iron/ferric iron ratios) warrants more extensive investigation.

Surveys of environments associated with the oxidation of sulfide minerals have shown that iron-oxidizing heterotrophic bacteria are widespread at such sites (13, 17). In light of the data presented in the current work, we surmised that these bacteria are important net contributors to mineral oxidation in such situations. The requirement of these organisms for organic carbon is minimal and might well be satisfied by the organic carbon originating from indigenous autotrophic acidophiles, as well as from extraneous sources (soil leachates, etc.). Future experimental work in which mixed cultures containing these bacteria and iron-oxidizing autotrophs (*T. ferrooxidans*, *L. ferrooxidans*) will be assessed for bioleaching of a range of sulfide ores should provide insight into the potential for using these organisms in commercial mineral-processing operations.

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