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Microbial oxidation of pyrite: Experiments using microorganisms from an extreme acidic environment

KATRINA J. EDWARDS,[1,*] MATTHEW O. SCHRENK,[1] ROBERT HAMERS,[2] AND JILLIAN F. BANFIELD[1]

[1]Department of Geology and Geophysics, University of Wisconsin-Madison, Madison, Wisconsin 53706, U.S.A.
[2]Department of Chemistry, University of Wisconsin-Madison, Madison, Wisconsin 53706, U.S.A.

ABSTRACT

Surface colonization and microbial dissolution of pyrite were studied in the laboratory and by in situ surface colonization experiments conducted at Iron Mountain, California. Laboratory experiments involved organisms obtained from Iron Mountain and cultured in pH <1.0, 42 °C solutions designed to enrich for chemolithotrophic species present at acid-generating sites. Planktonic and sessile microorganisms grew in enrichment cultures containing small amounts of yeast extract. The maximum density of attached cells was approximately 8×10^6 cells/cm^2. Attachment was specific for pyrite and occurred nonrandomly; rod-shaped bacteria tended to orient parallel to {100} and {110} pyrite. Attachment resulted in formation of euhedral dissolution pits. Cultures grown without yeast extract contained only planktonic cells and euhedral dissolution pits were not developed on the pyrite surface. All cultured organisms were identified as bacteria by fluorescence in situ hybridization and domain-specific probes. *Leptospirillum ferrooxidans* comprised 10–40% of planktonic organisms in both enrichments. *Thiobacillus ferrooxidans* was not identified in either enrichment. Oxidation rates were approximately equivalent in both enrichments (4×10^{-7} μM Fe/cell·day) over a 28 day period.

Pyrite cubes were exposed to natural solutions at Iron Mountain for two months. A subset of samples was exposed only to solutions that had passed through 0.22 μm Teflon filters. Denser colonization (by distinctive elongate bacteria not observed in laboratory cultures) occurred on pyrite in filter-covered vessels. Attachment specificity, orientation, and resulting degradation morphology were similar to that observed in laboratory cultures. Results show that interaction between attached cells and pyrite surface is highly specific and the impact on surface morphology evolution is different from that associated with planktonic microorganisms, despite the similarity in effect (per cell) on total dissolution rates.

INTRODUCTION

Acid mine drainage (AMD) environments, and the microorganisms associated with them, have received considerable attention due to both the environmental problems associated with runoff and the industrial potential of bioleaching. The literature on this topic continues to be overwhelmed by studies involving only a few chemolithotrophic species (*Thiobacillus ferrooxidans* and, more recently, *Leptospirillum* spp.) and relatively moderate environmental conditions (pH = 2–3, 20–30 °C). Even for these fairly well-characterized conditions there exists considerable debate on the mechanisms and rates of microbial oxidation of sulfide minerals.

Many current models conclude that the predominant microbial impact on pyrite dissolution involves acceleration of the rate of oxidation of the pyrite surface by increased supply of Fe^{3+}(aq) resulting from microbial activity (Nordstrom and Southam 1997). Typically, this is referred to as an ''indirect'' mechanism because it involves non-enzymatic oxidation of Fe^{2+} to Fe^{3+} in solution. The term ''direct'' mechanism was introduced to refer to an enzymatic reaction taking place between an attached cell and the mineral surface (Silverman 1967).

The terms ''indirect'' and ''direct'' may not adequately describe the mechanisms involved in biological metal sulfide dissolution. The role of extracellular enzymes is uncertain, and other processes may operate at the cell-mineral surface. For example, Sand et al. (1995, 1997) and Schippers et al. (1996) proposed a model in which a surface-attaching species accumulates Fe^{3+} in its outer membranes. The Fe^{3+} ions can then be used by the attached cells for a non-enzymatic attack on sulfide surfaces. Extracellular enzymes have not been detected in acidophilic organisms. Evidence for extra cellular enzymatic involvement in Fe oxidation by *Leptothrix discophora* has been reported (Corstjens et al. 1992), and extracellular en-

* E-mail: katrina@geology.wisc.edu

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TABLE 1. Base media

Compound	mg/L
$(NH_4)_2SO_4$	800
KH_2PO_4	400
$MgSO_4 \cdot 7H_2O$	160
$MnCl_2 \cdot 4H_2O$	0.10
$ZnCl_2$	70
$CoCl_2 \cdot 6H_2O$	0.12
H_3BO_3	31
$Na_2MoO_4 \cdot 2H_2O$	85.2

zymes have been found to be important in catalyzing the initial breakdown of other insoluble substances (lignin, cellulose; e.g., Gosh et al. 1987). Extracellular enzymes also have been shown to participate in redox reactions involving Mn oxides (Tebo et al. 1997). Clearly, fundamental questions remain about the roles of surface-attaching species relative to their planktonic counterparts, the importance of attachment, and the enzymatic or non-enzymatic nature of the reaction.

The impact of attachment on oxidative dissolution rates is unresolved. In one study using an iron-oxidizing archaea, Larsson et al. (1993) showed that close contact between microorganisms and pyrite was necessary for optimum growth and oxidation rates. However, attachment to the sulfide surface did not appear to be necessary. In similar experiments using *T. ferrooxidans,* which do attach to sulfide surfaces, conflicting evidence exists for both the inhibition (Wakao et al. 1984) and acceleration (Arkesteyn 1979) of oxidation rates due to attachment.

In this study we report the rates and discuss mechanisms of oxidative dissolution as determined using mixed enrichment cultures obtained from an AMD environment. We provide new results relating to the specific attachment of bacteria to pyrite surfaces, both in the laboratory and field, and provide cell-normalized rates for attached vs. non-attached organisms in culture. Inclusion of field-based studies is important because enrichment culturing can introduce biases, particularly with respect to the microorganisms enriched for, which may falsely emphasize processes not relevant in the environment. In situ field experiments yield insights into the nature of the interactions between bacteria and pyrite surfaces that lead to acid mine drainage.

MATERIALS AND METHODS

Enrichment cultures

Enrichment cultures were obtained using sediments and mine water from the Richmond 5-way at Iron Mountain mine in northern California. Temperature, pH, and dissolved oxygen conditions at the site of collection were 42 °C, 0.6, and 1.2 mg/L, respectively. Cultures were grown using 20 mL of sterile media (Table 1) added to 125 mL flasks (both autoclaved at 126 °C and 20 psi for 25 min), inoculated with 5 mL of a mixture of mine water and pyrite sediments (~3 g dry weight pyrite + <0.5 g quartz). Pyritic sediments generally range from 1.0 mm in diameter to <150 mm, however modal grain size distribution is highly heterogeneous within the mine. Surface area of representative sediments measured using a BET Surface Area Analyzer (Quantachrome Corp.) range from 0.1068 ± 0.0012 to 0.2373 ± 0.00012 m^2/g. An example of Richmond 5-way solution composition is listed in Table 2. Two media were used that varied in the presence or absence of yeast extract (0.02%). Both were adapted from standard 9K media (Silverman and Lundgren 1959). Each medium was adjusted to pH = 0.7– 0.8 with concentrated sulfuric acid and was not found to fluctuate significantly over the course of a two week incubation period (ΔpH ~ 0.2). Cultures were incubated at 42 °C in a gyratory shaker, and maintained through biweekly sub-culturing using 5 mL of previous enrichment for inoculum, 20 mL of media, and 2.0 g autoclaved pyritic sediment obtained from the Richmond 5-way.

TABLE 2. Composition of water from the Richmond 5-way (modified after Rodgers 1996, except where indicated)

Major anions	mg/L	Metals	mg/L
F	<1.5	Ca	248
Br	<5.5	Mg	1000
SO_4	3570	Na	233
DOC*	4–4.5	K	266
		SiO_2	127
		Al	1980
		Fe	28 600
		Mn	221
		B	9.75
		Cu	377
		Zn	2460
		Cd	19.7
		Co	1.9
		Cr	<10.4
		As	52.9
		Ti	8.17

* Robbins et al. 1998.

Partial analysis of the microbial populations in enrichments was performed with fluorescence in situ hybridization (FISH), using selected oligionucleotide probes. Organisms were analyzed at the Domain level for Bacteria, Eukarya, and Archaea, and at the species level for *Leptospirillum ferrooxidans* and *Thiobacillus ferrooxidans.* Methods for FISH can be found elsewhere (Schrenk et al. 1998).

Laboratory colonization experiments

Colonization and growth on pyrite surfaces were studied over six day periods. Six pyrite crystals (approximately $10 \times 10 \times 3.2$ mm slabs, 320 mm^2 surface area, and 1.65 g each), each displaying one natural face, were autoclaved in separate 125 mL flasks. Twenty milliliters of sterile media (pH ~ 0.7) with 0.02% yeast extract were added to each flask, and inoculated with 3 mL enrichment culture. Pyrite cubes were taken out of the medium every 24 h and rinsed with double-distilled sterile water, coated with gelatin (0.25%; Sigma), and fixed in paraformaldehyde (3%, pH = 7) overnight. Organisms adhering to

pyrite were stained with 4′, 6-diamidino-2-phenylindole (DAPI; Kapuscinski 1995) for observation with Ultraviolet (UV) epifluorescent microscopy using a Leica LEITZ DMRX microscope to quantify cell densities, distributions, and alignment and to assess specificity of attachment sites.

In situ colonization experiments

Pyrite cubes were characterized using reflected differential interference contrast imaging (rDIC; Leica LEITZ DMRX microscope) and then placed at seven localities within the Richmond mine 5-way for two months. One set of pyrite samples was placed directly in the environment so that the surfaces were surrounded by solution (pH $\sim$ 0.7, $T \sim 42$ °C) and sediments, and another set was placed in sterile chambers that were filled with filtered (0.22 μm) water from the environment and covered with 0.22 μm Teflon filters to allow for diffusive exchange.

Oxidation experiments

Pyrite dissolution and oxidation rates by mixed enrichment cultures were measured for four weeks. The two types of enrichment culture, varying in presence or absence of yeast extract, as well as corresponding abiotic controls, were used for batch experiments. Cells were grown for two weeks, as described above, then harvested by centrifugation and washed twice in media (Table 1). Pyrite slabs (1.65 g, 10 × 10 × 3.2 mm each) were used for all experiments. Pyrite was characterized by rDIC prior to inoculation. Pyrite slabs were placed in separate 125 mL flasks and autoclaved at 121 °C for 20 min. Twenty-one milliliters of media (with and without yeast extract) were added to flasks containing autoclaved pyrite and inoculated with 4 mL suspended cells. Twenty-five milliliters of sterile media with and without yeast extract (pH = 0.7) were added to flasks containing pyrite slabs for controls. Flasks were incubated at 42 °C on a gyratory shaker. Aliquots for Fe analysis were taken at the start of each experiment, and 3 h later. Thereafter, aliquots were taken daily. Cells and particulate matter were removed from liquid samples by centrifugation. Equal amounts of media were replaced after removal of each aliquot. Cell counts were made with a haemocytometer.

Total soluble Fe and total Fe^{2+} were measured using a Perkin-Elmer Lambda 3 UV/Vis spectrophotometer by the FerroZine method described by Dawson and Lyle (1990).

Results and discussion

Enrichment cultures

FISH analyses indicated that enrichment cultures were composed entirely of Bacteria. Enrichments with and without yeast extract contained *L. ferrooxidans* (10–40%) and both were devoid of *T. ferrooxidans*. The absence of *T. ferrooxidans* in enrichment cultures is not surprising, as FISH analyses of environmental samples from the Richmond 5-way also indicated the absence of *T. ferrooxidans* (Schrenk et al. 1998). Planktonic cells grown in the presence and absence of yeast extract are compared in Figure 1. The compositions of the two cultures are largely similar, with one notable exception. Only in the cultures containing yeast extract did we find organisms that attach irreversibly (not removed by rinsing) to pyrite surfaces. Cultures without yeast extract were found to contain only organisms that did not attach irreversibly to pyrite.

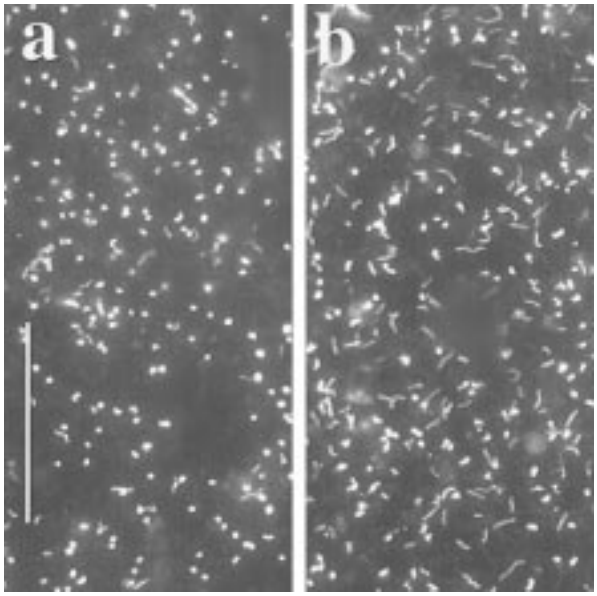

Figure 1. Example of sessile organisms in enrichment cultures grown on pyrite. (**a**) organisms from enrichments containing 0.02% yeast extract; (**b**) organisms grown in the absence of yeast extract. Cells are stained with DAPI and imaged in UV fluorescence. Scale bar is 25 μm, and applies to both images.

It is not certain if attachment in enrichment cultures containing yeast extract is due to differences in bacterial species that may have resulted from the variation in media or due to differences in the surface properties of pyrite in the presence of the added organic material. The bacteria that attached to pyrite surfaces were neither *L. ferrooxidans* nor *T. ferrooxidans. L. ferrooxidans* occurred in the planktonic phase in both enrichments. In environmental samples from the Richmond 5-way, *L. ferrooxidans* also has been found to occur predominately as a planktonic phase (Schrenk et al. 1998). This is contrary to other studies, which suggest that *L. ferrooxidans* not only attaches firmly to pyrite, but is in fact required to do so in order to obtain energy (Sand et al. 1995). However, studies on microbial diversity in AMD environments show that *L. ferrooxidans* are a phylogenetically diverse group (Goebel and Stackebrandt 1995). Whether or not substrate-attachment affinity within this group is strain-specific is unknown. Alternatively, geochemical conditions such as pH or oxidation state of iron may play a role in attachment for leptospirilla. At some acid mine drainage sites, particularly where Fe^{3+}/Fe^{2+} ratios are

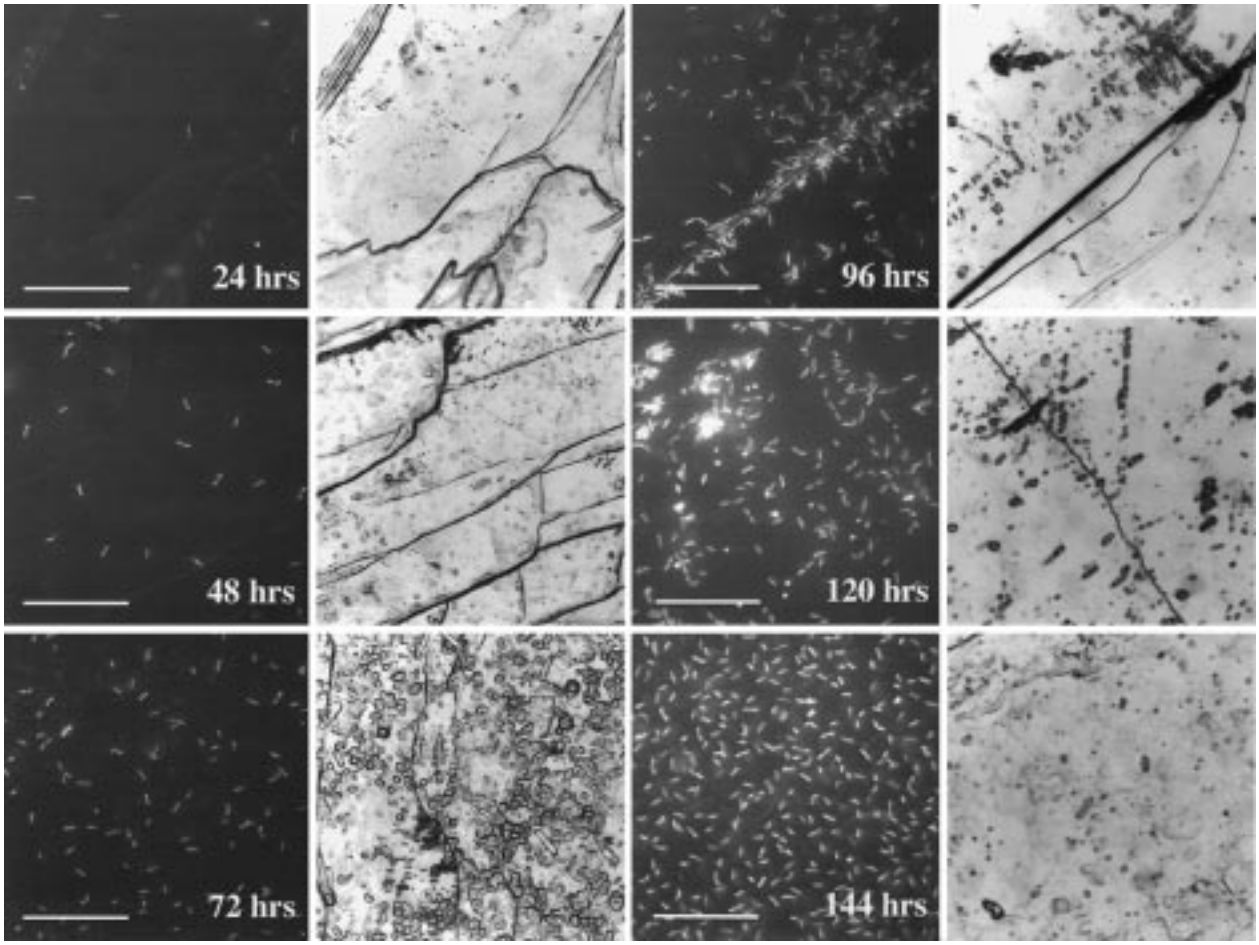

FIGURE 2. Incubation of cubic pyrite crystals with enrichment culture grown in base media (Table 1) with 0.02% yeast extract supplement. Dual images are shown for each 24 hr interval over 6 days. Cells shown on left are stained with DAPI and imaged in reflected UV fluorescence. Images to the right of each fluorescent image correspond to the same site, in plane reflected light. Scale bars are 25 μm.

high, it has been found that *L. ferrooxidans* attaches to pyrite surfaces and thus has a competitive advantage over other chemolithotrophs (e.g., Sand et al. 1992). However, at the Richmond 5-way, iron is predominately reduced ($Fe^{3+}/Fe^{2+} \sim 9/1$; Rodgers 1996), eliminating the need for *L. ferrooxidans* to attach to pyrite for a source of reduced iron.

Colonization of pyrite surfaces in laboratory studies

Surface colonization kinetics. Organisms in enrichments containing yeast extract began colonizing pyrite surfaces within the first 24 h of incubation. Figure 2 contains images that exemplify the average cell coverage on pyrite each day over the 6 day period. In experiments (images not shown) that were conducted for longer time periods (up to one month) under similar conditions, it was found that the average total cell density did not greatly exceed what is shown here after 144 h ($\sim 8 \times 10^6$ cells/cm^2).

We observed attachment of cells to the surface and their subsequent division to form a monolayer, but we did not observe development of a multilayer biofilm. Monolayer biofilms are commonly observed in AMD environments (Southam et al. 1994). Monolayer rather than multilayer biofilm formation is favored because the dominant microbes are aerobes, and formation of a thick film would restrict diffusion of oxygen to the cell surfaces. The monolayer-type biofilm promotes the growth of chemolithotrophs that obtain energy from the metal sulfides to which they attach.

Direct microbial growth on pyrite surfaces has been observed previously. For example, Konishi et al. (1990) demonstrated cell division of *T. ferrooxidans* on pyrite surfaces. Average cell densities estimated on pyrite surfaces and in solution for our experiments are illustrated in Figure 3; note that the log-plot of surface cell density is nonlinear. Some increase in cell numbers must be due to initial attachment, yet the total rate of increase of cell numbers is less than exponential. The nonlinearity implies a suppression of cell growth, possibly due to the

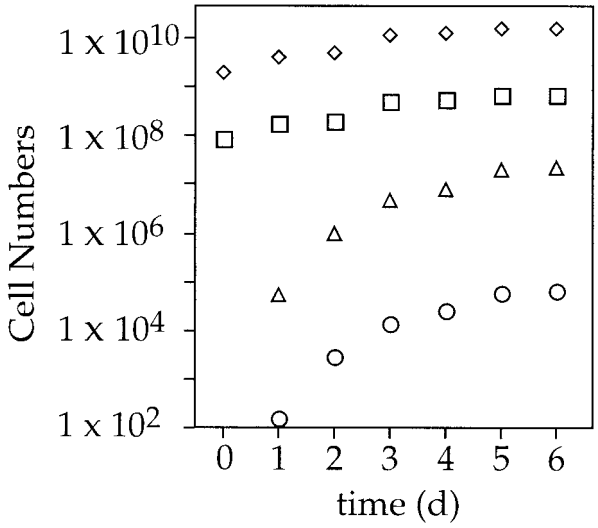

FIGURE 3. Cell numbers in solution and adhering to pyrite surface over six days. Symbols are as follows: diamond, total cells in solution; squares, cells/mL in solution; triangles, cells/mm^2 on surface; circles, total cells on surface. Total cells on surface based on 320 mm^2 available surface; total cells in solution based on 25 mL volume (see text).

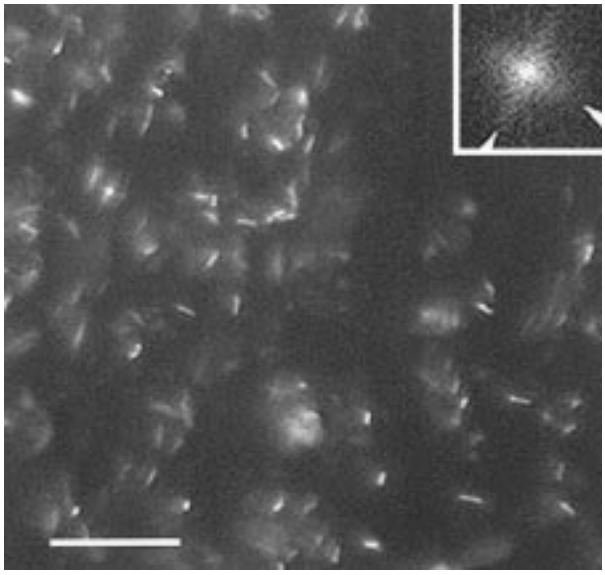

FIGURE 4. Organisms adhering to surface of a pyrite cube, stained with DAPI and imaged in UV fluorescence. Upper right: example Fourier transform image of organisms adhering to this surface. Scale bar is 20 μm.

limited surface area available in this experiment. This limitation on total cell numbers would occur much later at Iron Mountain, where the surface area of pyrite is orders of magnitude higher (0.1– 0.2 m^2/g pyrite). However, the maximum surface coverage observed in the laboratory experiments may be applicable in the natural environment under comparable geochemical conditions.

Cell surface distribution. Figure 4 shows that the orientation of attached cells on pyrite surfaces is non-random; alignment is commonly parallel to the <100> and <110> crystallographic directions. Preferential orientation is demonstrated by the presence of maxima at approximately 90° in the fast-Fourier transform (fft) shown in the upper right of this typical image of organisms attached to a pyrite surface. This has not been previously observed.

As can be seen in several of the images in Figure 2, localization of attached cells and the development of microcolonies occur at crystallographically controlled surface topographic features. Preferred sites include steps and noncrystallographic features such as scratches, microcracks, and grooves.

Attachment of bacterial cells occurs exclusively on the pyrite substrate. A pyrite crystal was cut and polished to expose silicate inclusions and then incubated for one week in an enrichment culture containing surface-colonizing bacteria (as in the colonization experiment described above). Figure 5 shows the resulting attachment specificity; a large silicate inclusion lacks attaching organisms, whereas the surrounding pyrite is colonized by bacteria and heavily pitted.

Dissolution by attaching organisms involves recognition of high surface energy sites, mineral specificity, and specificity with respect to crystallographic orientation. Whereas attachment and degradation of substrates involves an enzymatic system in certain cases (e.g., cellulosome; Bayaer et al. 1996), there is currently no evidence for such a system at low pH. Alternatively, attachment may be affected by diffuse effects that arise as the result of proximity between charged organic functional groups and the conductive pyrite surface (''charge-image charge'' interactions; Israelachvili 1991). These will be most important in proximity to surface steps, explaining the preferred orientation of cells at such sites.

Etching on pyrite surfaces due to attachment occurs in a crystallographically controlled manner, resulting in euhedral dissolution pits that are parallel to {100} and {110} (Fig. 6). Surface-etch patterns have been observed previously to result from surface attachment (e.g., Bennett and Tributsch 1978). This may be attributed to local chemical gradients developed in the attachment interface.

Localized etch pits are absent from pyrite incubated with cultures grown without any surface-attaching organisms and from controls [viewed at 10 (ocular) × 100 (lens) magnification by reflected light, differential interference contrast microscopy]. The absence of etch pits indicates that without attachment, when the primary pyrite dissolution mechanism involves generation of dissolved Fe^{3+}, surface dissolution is evenly distributed (at the sub-micrometer scale).

Field-based attachment studies

In our field-colonization experiments, we found that many of the pyrite samples incubated at the Richmond 5-

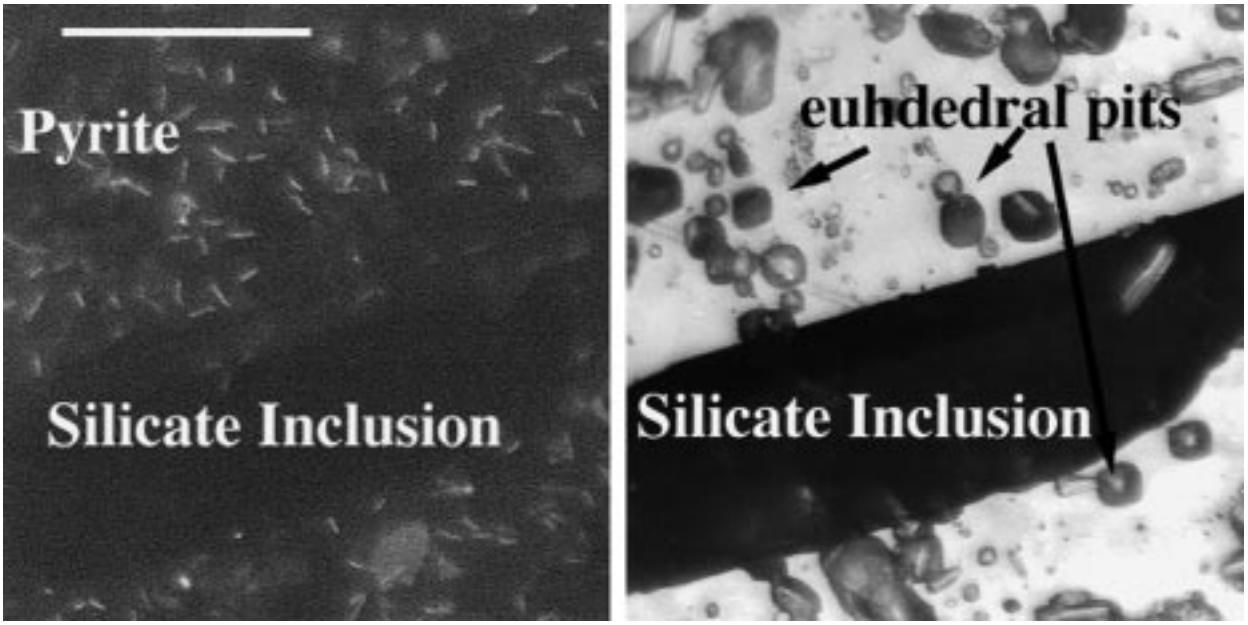

FIGURE 5. Left: organisms imagined in UV fluorescence (stained with DAPI) adhering to a polished cubic pyrite crystal, following one week of incubation with enrichment culture containing organisms that attach to pyrite surfaces. Right: reflected plane light image corresponding to image on left. Organisms did not adhere to silicate inclusions exposed by polishing. Scale bar is 25 μm, and applies to both images.

way were heavily colonized and pitted, as observed in laboratory experiments. The degree of colonization was heterogeneous among the different locations within the mine. As expected, physical degradation of the pyrite was more extensive in samples with a higher degree of colonization. Maximum colonization densities were approximately 8×10^7 cells/cm^2 for the filter-covered sample, and 6×10^6 cells/cm^2 for the uncovered sample. These cell densities are significantly higher than the value of 2×10^5 cells/cm^2 inferred by Nordstrom and Alpers (1998) from the field data of Southam and Beveridge (1992) for thiobacilli on pyrite surfaces.

Images from one location are shown in Figure 7. Image 7a is a sample that was incubated in a filter-covered chamber, whereas 7b shows a sample that was ''open'' to the environment. Organisms adhering to the sample shown in 7b are morphologically similar to the organisms found to adhere to pyrite in laboratory enrichment cultures. Note the preferred orientation of the elongate cells in Figure 7a. Organisms found adhering to the pyrite surface in the filter-covered chamber (Fig. 7a) are morphologically distinct from those in 7b and from any enrichment cultures. Organisms adhering to field-incubated cubes were all identified as bacteria by FISH. These results indicate that there are more than one surface-attaching bacterial species at Iron Mountain and that not all surface-attaching organisms are maintained in our enrichment cultures.

Another important result from the field colonization experiments is that organisms were able to penetrate the 0.22 μm filter membrane. The minimum cell width observed on this surface (measured from DAPI-stained cells) image was <0.3 μm (approximate, due to uncertainties in cell diameter measurement from the fluorescence signal) indicating the possibility that metabolically active cells entered the chambers. Alternatively, spore-

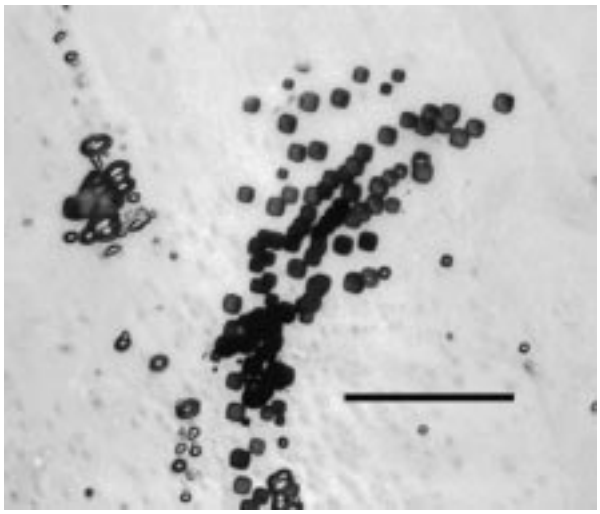

FIGURE 6. Reflected light image of a cubic pyrite crystal after incubation with enrichment culture containing pyrite-attaching organisms. Euhedral dissolution pits parallel to {100} and {110} develop within one week of incubation (conditions described in text). These features are absent in controls and after incubation with enrichment cultures without attaching organisms. Scale bar is 25 μm.

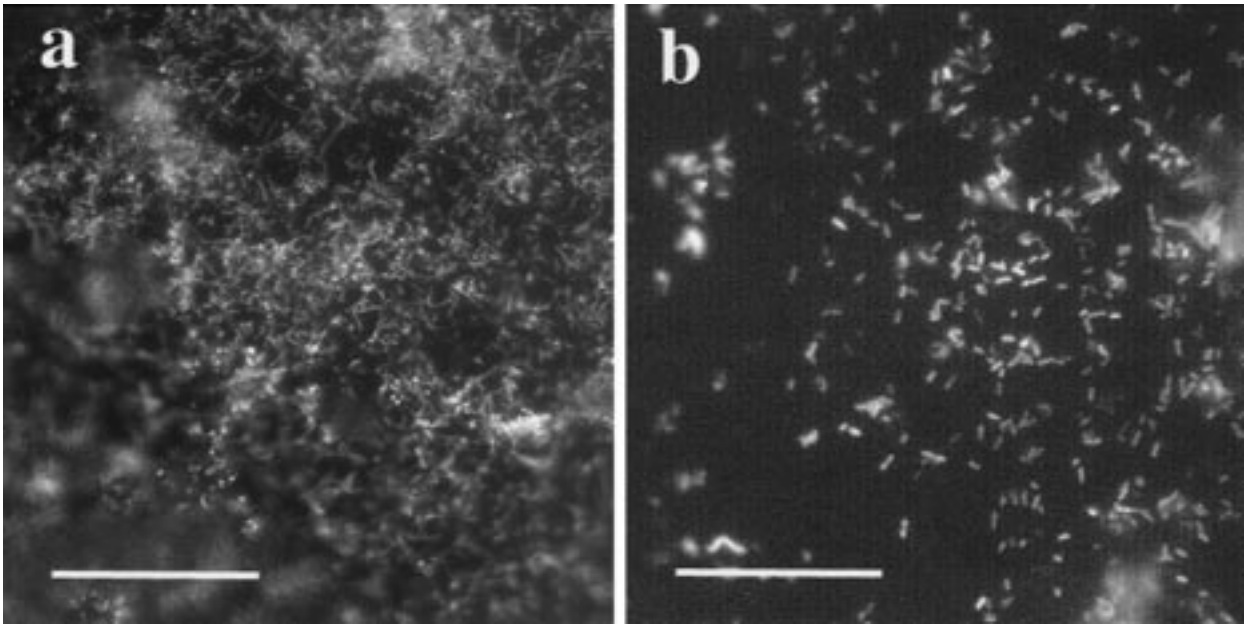

FIGURE 7. In situ colonization of pyrite: DAPI-stained reflected fluorescent image of organisms adhering to pyrite surface following a two-month incubation at the field site. (**a**) Cubic pyrite crystal incubated in a chamber filled with filtered water (0.22 μm filter) from site. (**b**) Cubic pyrite crystal incubated ''open'' to environment. Scale bars are 25 μm.

forming bacteria have been well documented in AMD environments (*Sufobacillus* spp.) (Dufrense et al. 1996). Spore formation occurs as the result of nutrient-limiting conditions or other environmental stresses. Conditions at Iron Mountain vary considerably as the result of the seasonal rainfall in the region, which is reflected by variations in dissolved oxygen content, conductivity, pH, temperature, and other variables. Thus, it is possible that spores could have entered the filter-covered chambers diffusionally during a period of environmental stress. However, phylogenetic analysis of solutions from the Richmond 5-way have not yet detected spore-forming bacteria at this site (Edwards et al. 1998). Additionally, the high cell densities observed on the pyrite incubated in the chamber relative to the pyrite incubated in the open indicates that the chamber environment may have been more favorable to colonization. This difference suggests that metabolically active organisms entered the chambers. Microorganisms may favor the chamber environment due to the absence of protozoa that inhabit this environment (Edwards, unpublished data).

Dissolution and oxidation of pyrite

Figure 8 shows the concentration of Fe in solution from pyrite dissolution over a four-week period. Total cell numbers in solution over the same time period are shown in Figure 9. Overall dissolution rates were significantly higher in the enrichment containing organisms that attach to pyrite surfaces than the one without (1.0×10^{-5} mol/m^{-2} s compared with 1.4×10^{-6} mol/m^2·s, respectively). However, bacterial growth rates were also higher in the former, resulting in approximately equal amounts of total Fe being released per organism, at $\sim 4 \times 10^{-7}$ μm Fe/cell·day. The enrichment culture containing organisms that attach to pyrite was dominated by Fe^{3+} (Fig. 10a) whereas the enrichment without attaching organisms was dominated by Fe^{2+} (Fig. 10b).

The rate of dissolution in the enrichment culture without surface-attaching organisms is approximately five times higher than the corresponding control. Therefore, the predominance of reduced Fe in this culture indicates that the rate of Fe oxidation was slow relative to the rate of consumption of Fe^{3+} at the pyrite surface in oxidative dissolution reactions. In the enrichment that contained surface-attaching organisms, the higher cell numbers supported a total oxidation rate that was higher than the rate at which Fe^{3+} could be consumed at the surface, resulting in the predominance of Fe^{3+} in solution.

It has been suggested in other reports that the rate of pyrite oxidation proceeds about as fast as the rate at which aqueous Fe^{3+} can be produced from Fe^{2+} through microbial catalysis (e.g., Nordstrom and Alpers 1998). If so, then our results suggest that the attaching cells and their polymers block access of Fe^{3+} to the surface, resulting in build up of Fe^{3+} in solution. Alternatively, the rate of Fe^{2+} oxidation in the culture that contains attaching cells may have exceeded the abiotic rate of pyrite oxidation by Fe^{3+}. Further work is needed to distinguish these possibilities. Both controls were dominated by Fe^{2+}, as would be expected at pH = 0.7 without an oxidation catalyst.

The microbial oxidation rates measured here are substantially faster than those measured in many other studies (Table 3). From an interlaboratory study comparing

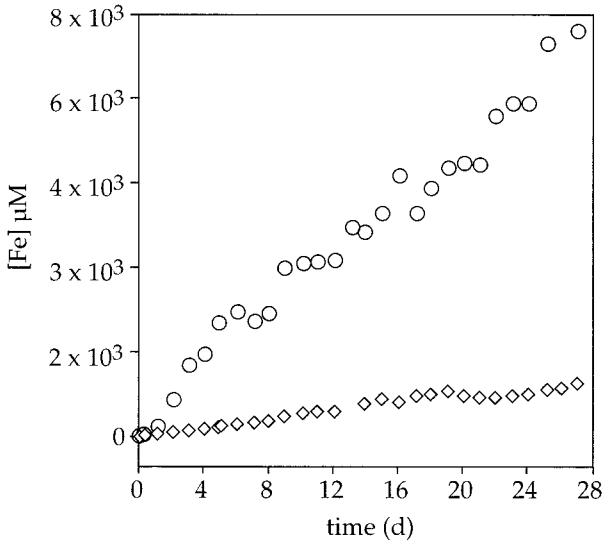

FIGURE 8. Total Fe released into solution over four weeks in enrichment cultures incubated with a single pyrite crystal (320 mm^2 surface area). Symbols are as follows: circles, Fe released in enrichment cultures that contained bacteria that attached to pyrite; diamonds, Fe released in enrichment cultures lacking attaching organisms.

the bioleaching rates of pyrite using *T. ferrooxidans* (Olson 1991), Nordstrom and Alpers (1998) calculated a microbial oxidation rate of 8.6×10^{-8} mol/m^2·s. However, this, and most other bioleaching experiments, were conducted under conditions for optimized growth of *T. ferrooxidans* (pH ~ 2, T ~ 25 °C). Our experiments were conducted under conditions that were selected based on field measurements at the source of inoculum used for cultures (pH ~ 0.7, T = 42 °C). The higher temperature

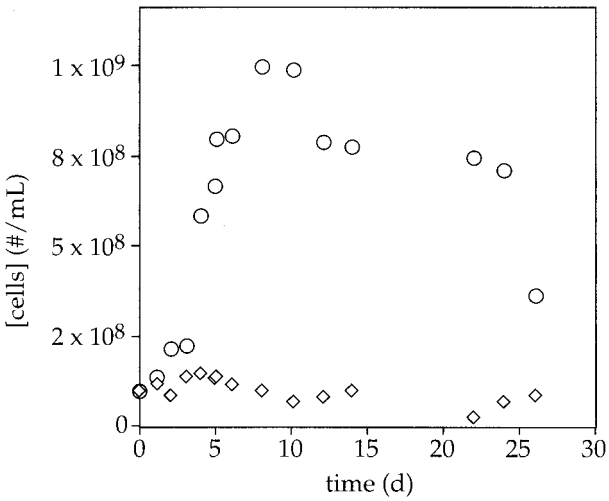

FIGURE 9. Total cells in solution during four week dissolution of a single pyrite crystal. Symbols are the same as in Figure 8. Cell counts were estimated with haemocytometer and normalized to 25 mL total volume.

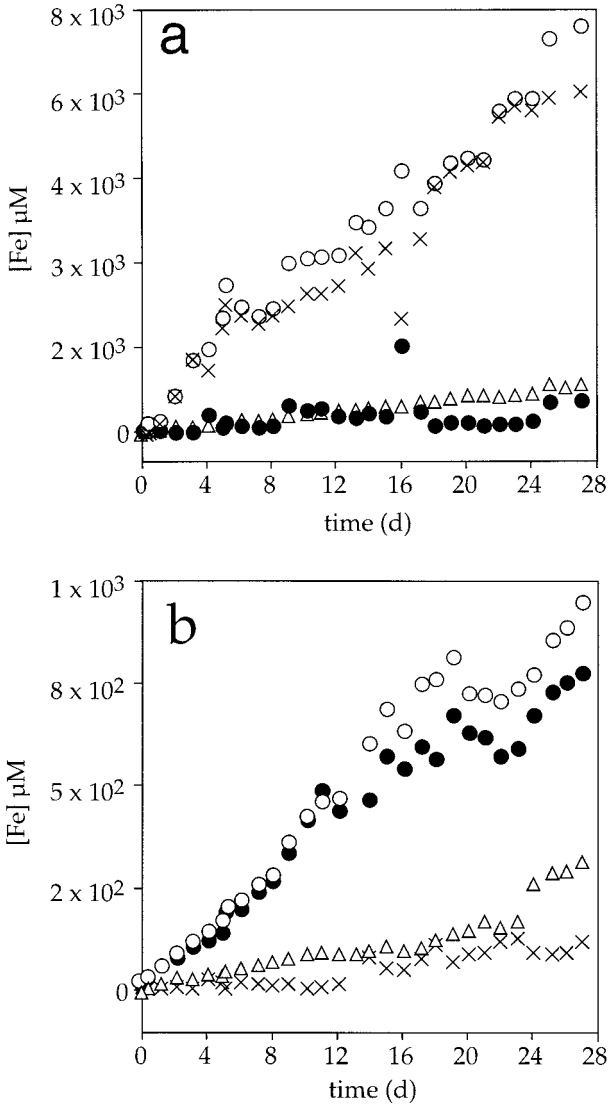

FIGURE 10. Iron speciation data for enrichment cultures containing organisms that attach to pyrite (**a**), and cultures that lack attaching phases (**b**). Symbols are as follows: open circles = total Fe released in enrichment cultures; triangles = total Fe released in corresponding control experiments; crosses and filled circles = Fe^{3+} and Fe^{2+}. Speciation is not shown for controls; in both cases, it was predominantly Fe^{2+}.

used for our experiments almost certainly accounts in part for the higher overall rates measured. However, these conditions may not have optimized growth and oxidation rates for each species present. The microbial rate of Olson (1991) at 25 °C, while lower than the rate measured in our experiment, was 34 times faster than their abiotic controls (2.6×10^{-9} mol/m^2·s, calculated using 1 g pyrite in 50 mL solution), whereas in our experiments, the microbial rate is only 5–8 times faster than abiotic controls. This difference in rates may be attributed to differences in culture composition, as the experiments of Olson (1991) used pure cultures of *T. ferrooxidans*, while ours

TABLE 3. Experimentally determined oxidation rates

	Abiotic (mol/m²·s)	Biotic (mol/m²·s)
This study (T = 42 °C, pH = 0.7)	1.3×10^{-6}–2.8×10^{-7}	1.0×10^{-5}–1.4×10^{-6}
Olson (1991) (T = 25 °C, pH = 2–3)	2.5×10^{-9}	8.6×10^{-8}
McKibben and Barnes (1986) (T = 30 °C, pH = 2–4)	3.0×10^{-6}–1.7×10^{-8}	

used natural mixed populations that lacked *T. ferrooxidans.* Other factors that may explain this result include differences in media composition, differences in microbe numbers in biotic experiments (our growth rates were apparently surface area limited), and errors in surface area estimation (discussed below).

McKibben and Barnes (1986) reported oxidation rates by oxygen that are comparable to the abiotic rates reported here, and faster than the abiotic rate reported by Olson (1991). These differences in rates may be due to inferred surface area in the experiments of Olson (1991). Pyrite surface area was not measured but was calculated using the weight and grain size distribution of the sample (Nordstrom and Alpers 1998).

Despite the suggestion of Wakao et al. (1984) that adsorption of cells onto the pyrite surface inhibits dissolution, we find that the rate of pyrite dissolution per attached cell is closely comparable to that per planktonic cell. The prevailing view is that aqueous catalysis by Fe- and S-oxidizing bacteria (sessile or planktonic) explains the link between the chemistry and biology of sulfide mineral oxidation. However, we conclude that the contribution of attached cells is important because the surface of pyrite and associated solutions evolve differently when attached cells are present. Our results show that to understand acid generation at very low pH, the impact of attached as well as planktonic chemolithotrophs must be considered.

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REFERENCES CITED

Arkesteyn, G.J. (1979) Pyrite oxidation by *Thiobacillus ferrooxidans* with special reference to the sulfur moiety of the mineral. Antonie van Leeuwenhoek Journal of Microbiology, 45, 423–436.

Bayaer, E.A., Morag, E., Tormo, J., and Lamed, R. (1996) The cellulosome: A cell surface organelle for the adhesion to and degradation of cellulose. In M. Fletcher, Ed., Bacterial Adhesion: Molecular and Ecological Diversity, p. 155–182. Wiley, New York.

Bennett, J.C. and Tributsch, H. (1978) Bacterial leaching patterns on pyrite crystal surface. Journal of Bacteriology, 134, 310–326.

Corstjens, P.L., de Vrind, J.P., Westbroek, P., and de Vrind-de Jong, E.W. (1992) Enzymatic iron oxidation by Leptothrix discophora: Identification of an iron-oxidizing protein. Applied and Environmental Microbiology, 58, 450–454.

Dawson, M.V. and Lyle, S.J. (1990) Spectrophotometric determination of iron and cobalt with ferrozine and dithizone. Talanta, 37, 1189–1191.

Dufresne, S., Bousquet, J., Boissinot, M., and Guat, R. (1996) Sulfobacillus disulfidooxidans sp. nov., a new acidophilic, disulfide-oxidizing, gram-positive, spore-forming bacterium. International Journal of Systematic Bacteriology, 46, 1056–1064.

Edwards, K.J., Goebel, B.M., Rodgers, T.M., Schrenk, M.O., Gihring, T.M., Cardona, M.M., Hu, B., McGuire, M.M., Hamers, R.J., Pace, N.R., and Banfield, J.F. (1998) Geomicrobiology of pyrite (FeS_2) dissolution: A case study at Iron Mountain, California. Geomicrobiology Journal, in press.

Goebel, B.M. and Stackebrandt, E. (1995) Molecular analysis of the microbial biodiversity in a natural acidic environment. In C.A. Jerez, T. Vargas, H. Toledo, and J.V. Wiertz, Eds., Proceedings of the International Biohydrometallurgy Symposium IBS-95, vol. II, p. 43–52. University of Chile, Santiago.

Gosh, B.K., Gosh, A., and Salnar, A (1987) Cellulase secretion from a hypercellulolytic mutant. In J. Chaloupka and V. Krumphanzl, Eds., Extracellular Enzymes of Microorganisms, p. 157–172. Plenum Press, New York.

Israelachvili, J.N. (1991) Intermolecular and surface forces, 450 p. Academic Press London, U.K.

Kapuscinski, J. (1995) DAPI: A DNA-specific fluorescent probe. Biotechnic & Histochemistry, 70, 220–233.

Konishi, Y., Asai, S., and Sakai, H.K. (1990) Bacterial dissolution of pyrite by *Thiobacillus ferrooxidans.* Bioprocess Engineering, 5, 5–17.

Larsson, L., Gunnel, O., Holst, O., and Karlsson, H.T. (1993) Oxidation of pyrite by *Acidianus brierleyi*: Importance of close contact between the pyrite and the microorganisms. Biotechnology Letters, 15, 99–104.

McKibben, M.A. and Barnes, H.L. (1986) Oxidation of pyrite in low temperature acidic solutions: Rate laws and surface textures. Geochimica et Cosmochimica Acta, 50, 1509–1520.

Nordstrom, D.K. and Alpers, C.N. (1998) Geochemistry of acid mine waters. In G. Plumlee and M. Logsdon, Eds., Reviews in Economic Geology, in press.

Nordstrom, D.K. and Southam, G. (1997) Geomicrobiology of sulfide mineral oxidation. In Mineralogical Society of America Reviews in Mineralology, 35, 361–382.

Olson, G.J. (1991) Rate of pyrite bioleaching by *Thiobacillus ferrooxidans*: Results of an interlaboratory comparison. Applied and Environmental Microbiology, 57, 642–644.

Robbins, E.I., Rodgers, T.M., Nordstrom, D.K., and Alpers, C.N. (1998) Subsurface geochemistry and ecology of the food chain at pH 0–4 in Iron Mt., Calif. USA. In Proceedings of the Chemistry and Ecology of Highly Acidic Environments Conference, p. 10–12. University of Durham, England.

Rodgers, T.M. (1996) Bacterial diversity in acid mine drainage from iron mountain, Shasta County, California: A 16S ribosomal RNA approach, 660 p. M.S. thesis, University of Wisconsin, Madison.

Sand, W., Gerke, T., Jozso, P., and Shippers, A. (1997) Novel mechanism for bioleaching of metal sulfides. In Proceedings of the International Biohydrometallurgy Symposium IBS97-Biomine 97, p. QP2.1–QP2.10. Austrailian Mineral Foundation, South Australia.

Sand, W., Gerke, T., Hallmann, R., and Schippers, A. (1995) Sulfur chemistry, biofilm, and the (in)direct attach mechanism-a critical evaluation of bacterial leaching. Applied Microbiology and Biotechnology, 43, 961–966.

Sand, W., Rohde, K., Sobotke, B., and Zenneck, C. (1992) Evaluation of *Leptospirillum ferrooxidans* for leaching. Applied and Environmental Microbiology, 58, 85–92.

Schippers, A., Jozsa, P.G., and Sand, W. (1996) Sulfur chemistry in bacterial leaching of pyrite. Applied and Environmental Microbiology, 62, 3424–3431.

Schrenk, M.O., Edwards, K.J., Goodman, R.M., Hamers, R.J., and Banfield, J.F. (1998) Distribution of *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*: Implications for generations of acid mine drainage. Science, 279, 1519– 1522.

Silverman, M.P. (1967) Mechanism of bacterial pyrite oxidation. Journal of Bacteriology, 94, 1046–1091.

Silverman, M.P. and Lundren, D.G. (1959) Studies on the growth of

Thiobacillus ferrooxidans. I. An improved medium and harvesting procedure for securing high cell yields. Journal of Bacteriology, 77, 642–647.

Southam, G. and Beveridge, T.J. (1992) Enumeration of thiobacilli with pH-neutral and acidic mine tailings and their role in the development of secondary mineral soil. Applied and Environmental Microbiology, 58, 1904–1912.

Southam, G., Ferris, F.G., and Beveridge, T.J. (1994) The role of bacterial biofilms in mineral leaching of sulfide tailings and in downstream metal recovery. In W.J. Costerton and H.M. Lappin-Scott, Eds., Bacterial Biofilms, p. 148–170. Cambridge University Press, Cambridge, U.K.

Tebo, B.M., Ghiorse, W.C., van Waasbergen, L.G., Seiring, P.L., and Caspi, R. (1997) Bacterially-mediated mineral formation: Insights into Manganese (II) oxidation from molecular genetic and biochemical studies. In Mineralogical Society of America Reviews in Mineralology, 35, 225–260.

Wakao, N. Mishina, M., Sakurai, Y., and Shiota, H. (1984) Bacterial pyrite oxidation: 3. Adsorption of *Thiobacillus ferrooxidans* cells on solid surfaces and its effect on iron release from pyrite. Journal of General and Applied Microbiology, 30, 63–77.

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