APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1976, p. 567-571
Vol. 32, No. 4

Printed in U.S.A.

Ferric Iron Reduction by Sulfur- and Iron-Oxidizing Bacteria

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Received for publication 18 May 1976

Acidophilic bacteria of the genera *Thiobacillus* and *Sulfolobus* are able to reduce ferric iron when growing on elemental sulfur as an energy source. It has been previously thought that ferric iron serves as a nonbiological oxidant in the formation of acid mine drainage and in the leaching of ores, but these results suggest that bacterial catalysis may play a significant role in the reactivity of ferric iron.

Although the bacterial reduction of ferric iron has been extensively studied in environments of neutral pH (6–9), there has apparently been no report of ferric iron reduction by acidophilic bacteria. The bacterial reduction of ferric iron at acid pH would appear to be energetically and kinetically feasible, especially since it is only at acid pH values that ferric iron has any significant solubility in water (16). The role of ferric iron as an oxidant of pyrite and other sulfide minerals is well established (5, 14, 16, 17), but the evidence has suggested that this process is strictly nonbiological. For instance, Silverman (14) has shown that the nonbiological oxidation of pyrite by ferric iron proceeds rapidly in the absence of bacteria. Also, cinnabar, the mercury sulfide mineral, is oxidized by ferric iron, but at considerably slower rates (5).

During some work on the relationships between iron and sulfur oxidation states in acid geothermal habitats (4a; S. D. Zinder and T. D. Brock, submitted for publication), we discovered that ferric iron was relatively stable at low pH in the presence of elemental sulfur, although it reacted rapidly with sulfide. Since high concentrations of soluble ferric iron often develop in the highly acidic environments where iron- and sulfur-oxidizing bacterial occur, it seemed possible that these bacteria might use ferric iron as an oxidant when growing on elemental sulfur. Since elemental sulfur is thought to build up on the surface of sulfide minerals during their oxidation by O_2 (11), the ability of bacteria to oxidize elemental sulfur using ferric iron as an oxidant could be geochemically significant in the leaching of sulfide ores and in the development of acid mine drainage. For this reason, we initiated studies to determine whether these acidophilic bacteria might be able to reduce ferric iron during their oxidation of elemental sulfur. The bacteria chosen were *Thiobacillus thiooxidans* and *T. ferrooxidans*, two mesophilic sulfur-oxidizing bacteria, and *Sulfolobus acidocaldarius*, a thermophilic bacterium able to oxidize both sulfur (13) and ferrous iron (4a). All three organisms were found to be able to reduce ferric iron to the ferrous state.

MATERIALS AND METHODS

The cultures of *S. acidocaldarius* strains 98-3 and 79-13 were isolated from Yellowstone hot springs. Strain 98-3 is the type strain (4), and 79-13 was isolated on autotrophic sulfur-containing medium by J. L. Mosser in this laboratory. They were cultured routinely in screw-capped bottles at 70°C. The culture of *T. thiooxidans* was obtained from J. Shiveley of Clemson University. The *T. ferrooxidans* culture had been isolated on ferrous iron in this laboratory for some previous work (3) and was adapted for growth on elemental sulfur for the present study. Both *Thiobacillus* cultures were maintained at room temperature in Erlenmeyer flasks and were shaken gently with a Burrell wrist-action shaker.

The culture medium was that of Allen (1) adjusted to pH 2. For the ferric iron reduction experiments, the pH was lowered to 1.6 to maintain the solubility of ferric iron. Elemental sulfur was sterilized by Tyndallization and added at a level of 2 g/liter. Ferric chloride was added from a stock solution of $FeCl_3 \cdot 6H_2O$ to a final concentration of 400 μg of Fe^{3+} per ml.

Cell growth was quantified by microscopic counting using a Petroff-Hausser counting chamber. No attempt was made to count cells attached to elemental sulfur particles, so that the counts during the early stages of incubation were probably greatly underestimated. As shown with fluorescence microscopy for *S. acidocaldarius* (13), in the later stages of growth the majority of cells were unattached, so that Petroff-Hausser cell counts would be meaningful.

Ferric iron reduction was measured by assaying the formation of ferrous ions, using the phenanthroline reagent (2).

RESULTS

Chemical reaction between elemental sulfur and ferric iron. The nonbiological reaction between elemental sulfur and ferric iron was found to be virtually undetectable at room temperature, and quite slow even at 70°C. This was true even if colloidal sulfur was used (prepared by the method of Brierley [Ph.D. thesis, Montana State Univ., Bozeman, 1966]). Because colloidal sulfur quickly crystallizes in culture medium, there was no advantage to using colloidal sulfur in the experiments with bacteria. With sterile rolled sulfur, no reduction of ferric iron was observed in uninoculated controls even after 34 days of incubation at room temperature. At 70°C, significant nonbiological reduction occurred, but the extent of reduction was less than in inoculated flasks. Because of the slow reaction between elemental sulfur and ferric iron, it was possible to measure the bacterial reduction process, even during the relatively long incubation periods necessary during growth on elemental sulfur.

Reduction of ferric iron by *T. thiooxidans*. Figure 1 illustrates the results of an experiment in which ferrous iron formation and growth were measured simultaneously. As seen, growth and iron reduction were essentially parallel throughout the duration of the experiment. At the end of the experiment, virtually all of the ferric iron had been reduced to the ferrous form. It should be noted that this experiment was carried out aerobically, with continuous shaking of the cultures. At the low pH of the medium, the ferrous iron formed is stable to autooxidation, and, since *T. thiooxidans* is unable to oxidize ferrous iron, it accumulates.

Reduction of ferric iron by *T. ferrooxidans*. The *T. ferrooxidans* culture was adapted to growth on elemental sulfur after successive transfers over a number of months. The culture never grew as well on elemental sulfur as *T. thiooxidans*, but eventually it grew at a rate sufficient so that ferric iron reduction experiments could be initiated.

In initial experiments, no formation of ferrous iron could be detected during aerobic incubation. Since it seemed likely that any ferrous iron formed was being oxidized back to the ferric form by the bacteria, after 10 days of incubation, when good growth had been obtained, the cultures were rendered anaerobic by bubbling vigorously for 30 min with oxygen-free N_2. They were then returned to the shaker and incubation was continued. Within the next 24 h, significant ferrous iron could be detected in the inoculated flasks, but not in the uninoculated controls, and the amount of ferrous iron continued to increase during further incubation. Thus, *T. ferrooxidans* is able to reduce ferric iron, but the reduction is not seen aerobically because of the rapid bacterial reoxidation of the ferrous iron in the presence of oxygen.

Reduction of ferrous iron by *S. acidocaldarius*. Figure 2 shows the results of an experiment measuring the reduction of ferric iron by strain 79-13. As seen, there is significant nonbiological reduction, but the reduction in the inoculated flask is considerably greater. Note that this experiment was done with cultures that were unaerated but without exclusion of oxygen. Because of the low solubility of O_2 at 70°C, the system more closely resembles a microaerophilic one. Since natural populations of *S. acidocaldarius* do oxidize ferrous iron under

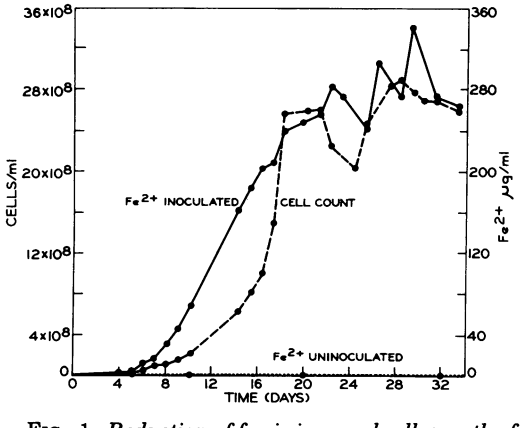

FIG. 1. *Reduction of ferric iron and cell growth of T. thiooxidans. Aerobic incubation at room temperature. Energy source, elemental sulfur.*

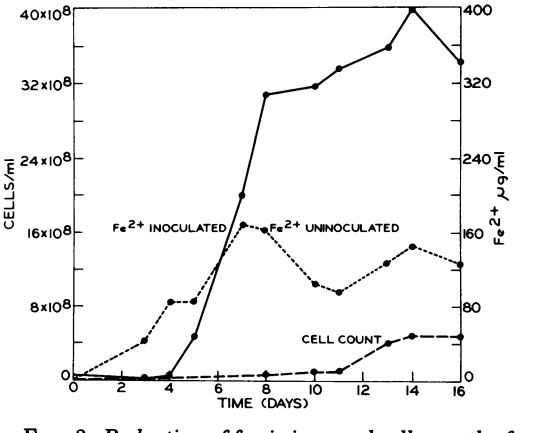

FIG. 2. *Reduction of ferric iron and cell growth of S. acidocaldarius. Aerobic incubation at 70°C. Energy source, elemental sulfur.*

these conditions (4a), it might have been thought that the reduction of ferric iron would be undetectable, yet almost quantitative reduction of ferric iron occurred during the 14- to 16-day incubation period. The pure culture used was isolated on sulfur and may be unable to oxidize ferrous iron.

Since *S. acidocaldarius* grows readily heterotrophically on yeast extract or a variety of simple organic carbon sources (4), experiments were set up to see whether ferric iron reduction would occur with heterotrophically grown cells. Strain 98-3 was adapted to growth using 0.5% glutamate as sole carbon and energy source, in a medium at pH 2. Under these conditions, instead of growth requiring weeks for completion, as in sulfur, stationary phase was reached after 3 to 4 days of incubation. Rapid growth and ferric iron reduction occurred during the 3- to 4-day incubation period under aerobic (microaerophilic, see above) conditions (Fig. 3). Experiments were also set up to see whether *S. acidocaldarius* could grow using ferric iron as electron acceptor anaerobically with glutamate as energy source, but these experiments were thwarted when it was discovered that under anaerobic conditions glutamate (and several other organic compounds used by *S. acidocaldarius* as energy sources) reduced ferric iron non-biologically. It is conceivable that the reduction of ferric iron seen with heterotrophic cells under aerobic conditions could be nonspecific, resulting from the utilization of dissolved O_2 by the bacteria, with the subsequent chemical reduction of ferric iron by residual glutamate. Because *S. acidocaldarius* does not grow well under forced aeration, it was impossible to increase the O_2 tension to test this point.

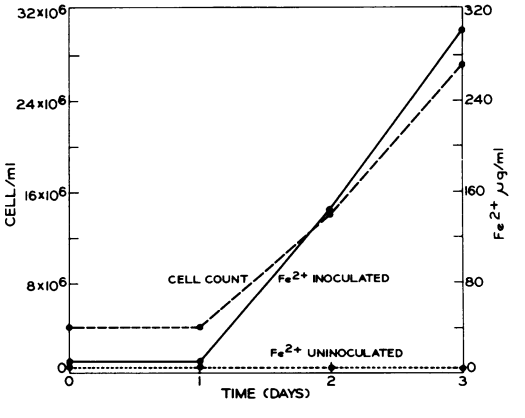

FIG. 3. *Ferric iron reduction and cell growth of S. acidocaldarius in glutamate-containing medium. Aerobic incubation at 70°C.*

DISCUSSION

These results show that three acidophilic, sulfur-oxidizing bacteria are able to reduce ferric iron using elemental sulfur as electron donor. In the case of *T. ferrooxidans*, the process can only be demonstrated under anaerobic conditions, since the ferrous iron formed by the reduction process is reoxidized to ferric iron when O_2 is present. *T. thiooxidans*, which does not oxidize ferrous iron, is the most favorable organism for study of the ferric iron reduction process, since the ferrous iron formed is chemically stable at the pH values used and accumulates quantitatively.

Still to be answered by further work is the question of whether these bacteria can grow anaerobically using ferric iron as an electron acceptor. Because the free energy of the reaction $S^0 + 6Fe^{3+} + 4H_2O = HSO_4^- + 6Fe^{2+} + 7H^+$ (assuming pH 2) is -75 kcal, and the free energy of the reaction $2S^0 + 3O_2 + 2H_2O = 2HSO_4^- + 2H^+$ (assuming pH 2) is -124 kcal/S atom (-247 kcal for the whole reaction), there is more energy avilable using O_2 than Fe^{3+} as electron acceptor. However, the oxidation of elemental sulfur using nitrate is known to serve for growth (of *Thiobacillus denitrificans*), and the free energy of the reaction $S^0 + H_2O + 3NO_3^- = 3NO_2^- + SO_4^{2-} + 2H^+$ (assuming pH 7) is -84 kcal, only slightly higher than the reaction using ferric iron (if complete denitrification to N_2 occurs, then the free energy is considerably higher using NO_3^-, -130 kcal/S atom). Thus, it seems reasonable that anaerobic growth of *Thiobacillus* and *Sulfolobus* using ferric iron could be possible. Since the concentrations of ferric iron in the environments where these organisms live is often quite high (over 100 µg/ml), ferric acid is not uncommon in Yellowstone acid springs (4a), it seems likely that these organisms have evolved the ability to use this electron acceptor when O_2 is absent.

The relevance of ferric iron reduction for the generation of acid mine drainage and for microbial leaching of ores is of considerable interest. The mechanism of bacterial pyrite oxidation has been clarified by the work of Silverman (14) and Singer and Stumm (15). The early work of Sato (11) is also relevant. Two mechanisms for bacterial pyrite oxidation have been recognized, a direct mechanism in which the bacteria attach to the pyrite crystals and oxidize the mineral and an indirect mechanism in which the bacteria oxidize soluble ferrous iron to the ferric state, and the latter reacts with pyrite chemically to oxidize the mineral. The indirect mechanism is considered to be the most likely reaction under most conditions. This mecha-

nism requires the presence of molecular O_2, since this is the only electron acceptor with an electrode potential more positive than that of the ferrous/ferric couple. As long as O_2 is present, oxidation of pyrite can continue to completion, since ferrous iron formed during the reaction can continue to be returned to the ferric state by the bacteria. However, Sato (11) has shown that during the oxidation of sulfide minerals, the metal atoms move into the surrounding solution to become aqueous cations, thus enriching the remaining solid phase with sulfur atoms. When solid sulfur is finally left over, it becomes oxidized to sulfate. With pyrite and marcasite, Sato (11) concluded that the sulfur released from the crystal structure was converted to unstable S_2 molecules, which would be instantly oxidized in the presence of oxidizing agents. In the case of the other metal sulfides studied (copper, lead, silver, and zinc), Sato (11) concluded that stable solid sulfur would be formed, which would eventually be oxidized to sulfate. Such solid sulfur, probably at the oxidation state of S^0, reacts slowly with ferric iron in the absence of bacteria, so that the bacterial utilization of ferric iron as an electron acceptor for the oxidation of elemental sulfur should greatly speed up the reaction. Thus, in the case of the oxidation of metal sulfides other than those of iron, the ability of sulfur-oxidizing bacteria to utilize ferric iron as an electron acceptor would appear to be of considerable importance, when O_2 is absent, and possibly even when O_2 is present (10).

What is the availability of O_2 in environments where sulfide minerals are undergoing oxidation? In leaching dumps, it seems likely that O_2 would often be limiting within the depths of the piles. Most of the copper leach dumps in operation in the United States are quite large in size (see Table 1 of Sheffer and Evans [12]), and O_2 penetration to the center must be markedly restricted. Since there is a considerable O_2 demand during the oxidation of sulfide minerals, anaerobic or microaerophilic conditions must often develop. All of the influent leach liquors used in the leaching of copper sulfide minerals have high concentrations of ferric iron, derived from the scrap metal used in the copper recovery process. Ferric iron is probably the prime oxidant in the leaching process, since all of the ferric iron in the influent leach solution is found in the ferrous form in the effluent (12). It has generally been assumed that the oxidation of sulfide minerals by ferric ions is strictly a nonbiological process (15). However, since solid sulfur builds up on the mineral particles as the oxidation proceeds, and elemental sulfur does not react rapidly with Fe^{3+}, it seems reasonable that the ability of *T. ferrooxidans* to utilize ferric iron as an electron acceptor would provide an important mechanism for speeding up the rate of oxidation of the sulfide minerals. Unfortunately, most of the work on microbial leaching (see 17) has been done under laboratory conditions where good aeration has been maintained. It would be of considerable importance to measure O_2 concentrations within leach piles and to measure the rate of bacterial oxidation of sulfide minerals anaerobically in the presence of ferric iron. If the proposed mechanism is correct, it suggests that more rapid or effective leaching with ferric iron would be obtained if care were taken to develop and maintain a large active population of bacteria within the leach dump.

ACKNOWLEDGMENTS

This work was supported by a research grant from the National Science Foundation (GB-35046), a contract from the Energy Research and Development Agency (100-2161-31), and the College of Agricultural and Life Sciences.

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