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Anaerobic Growth of *Thiobacillus ferrooxidans*

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The obligately autotrophic acidophile *Thiobacillus ferrooxidans* was grown on elemental sulfur in anaerobic batch cultures, using ferric iron as an electron acceptor. During anaerobic growth, ferric iron present in the growth media was quantitatively reduced to ferrous iron. The doubling time in anaerobic cultures was approximately 24 h. Anaerobic growth did not occur in the absence of elemental sulfur or ferric iron. During growth, a linear relationship existed between the concentration of ferrous iron accumulated in the cultures and the cell density. The results suggest that ferric iron may be an important electron acceptor for the oxidation of sulfur compounds in acidic environments.

Thiobacillus ferrooxidans is an obligately autotrophic, acidophilic bacterium. Autotrophic growth can be supported by the oxidation of a variety of inorganic sulfur compounds, ferrous iron (6, 8), molecular hydrogen (4), or formic acid (13). *T. ferrooxidans* and physiologically related bacteria are of great economical importance because of their involvement in the biological leaching of copper and uranium ores and the biooxidation of gold ores (16). The organisms are also applicable to the biological desulfurization of coal (1). Furthermore, acidophilic sulfur-oxidizing bacteria are involved in the weathering of mineral metal sulfides, a process which may have important environmental implications (9).

T. ferrooxidans is generally assumed to be an obligately aerobic organism (8). However, under anaerobic conditions, ferric iron can replace oxygen as an electron acceptor for the oxidation of elemental sulfur (2, 3). At pH 2, the free energy change of the reaction $S + 6Fe^{3+} + 4H_2O \rightarrow H_2SO_4 + 6Fe^{2+} + 6H^+$ is negative ($\Delta G = -314$ kJ/mol [2]). However, whether anaerobic ferric iron respiration can contribute to the energy metabolism of *T. ferrooxidans* has long remained unclear. We have recently demonstrated that this is indeed the case: active uptake of amino acids could be energized by anaerobic oxidation of elemental sulfur or formic acid (12).

The aim of the present study was to investigate whether *T. ferrooxidans* is capable of anaerobic growth on elemental sulfur with ferric iron as an electron acceptor.

MATERIALS AND METHODS

Microorganism and maintenance. Pure cultures of the type strain of *T. ferrooxidans* (LMD 81.44; originally from the American Type Culture Collection as ATCC 23270^T) were obtained from the culture collection of the Laboratory of Microbiology, Delft, The Netherlands. The organism was routinely maintained in ferrous iron-grown shake flask cultures, as described previously (11).

Growth media. The mineral medium used for anaerobic growth studies contained the following, per liter of distilled water: $(NH_4)_2SO_4$, 132 mg; K_2HPO_4, 41 mg; $MgSO_4 \cdot 7H_2O$, 490 mg; $CaCl_2 \cdot 2H_2O$, 9 mg; KCl, 52 mg; $ZnSO_4 \cdot 7H_2O$, 1 mg; $CuSO_4 \cdot 5H_2O$, 2 mg; $MnSO_4 \cdot H_2O$, 1 mg; $NaMoO_4 \cdot 2H_2O$, 0.5 mg; $CoCl_2 \cdot 6H_2O$, 0.5 mg; $Na_2SeO_4 \cdot 10H_2O$, 1 mg; $NiCl_2 \cdot 6H_2O$; 1 mg. In some experiments, Difco yeast extract was added to give a final concentration of 5 mg · liter^{-1}. After addition of 50 mM $Fe_2(SO_4)_3$, the pH of the medium was adjusted to 1.9 by addition of H_2SO_4. Media were filter sterilized by using a MediaKaP 0.2-μm-pore-diameter filter capsule. Aliquots, 50 mg, of finely ground elemental sulfur were suspended in 1.0 ml of mineral medium without ferric sulfate and sterilized at 110°C. After the suspension cooled, 10 ml of filter-sterilized mineral medium was added.

Anaerobic growth studies. Cultures (11 ml) in 50-ml Erlenmeyer flasks were inoculated with 50 μl of a preculture which had been grown aerobically on ferrous sulfate (180 mM). The cultures were loosely stoppered with nylon wool and incubated in gas-tight jars. A strictly anaerobic, CO_2-enriched atmosphere was generated by using a commercially available H_2- and CO_2-generating system and a palladium catalyst (GasPak Atmospheric Systems Components; Becton Dickinson). Anaerobic conditions were achieved within 2 to 3 h, as checked by methylene blue indicator strips placed inside the jars. Cultures without ferric iron were included as controls to exclude the possibility that growth was due to leakage of oxygen into the jars.

Control of culture purity. Inocula and anaerobic cultures were checked for purity by immunofluorescence microscopy. Specific antisera against *T. ferrooxidans* were obtained as described previously (11).

Cell counts. To monitor growth as an increase of cell numbers, cultures were sacrificed at different time intervals. Tween 20 was added to cultures immediately before counting to give a final concentration of 0.03% (vol/vol). The cultures were then briefly shaken to release cells attached to elemental sulfur particles. After large sulfur particles had been allowed to sediment, cells were counted microscopically at ×400 magnification, using a Bürker-Türk counting chamber for bacteria. At least 300 cells were counted for each sample. All cell counts were performed in duplicate.

Analytical procedures. Samples for ferrous iron determination were prepared by filtration of culture samples through a 0.2-μm-pore-diameter Durapore membrane (Millipore Corp., Bedford, Mass.). Ferrous iron was determined by titration with potassium permanganate (17). The pH of cultures was measured with a combined pH glass electrode (Metrohm AG, Herisau, Switzerland).

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RESULTS

Quantification of growth. To demonstrate anaerobic growth of *T. ferrooxidans* on sulfur with ferric iron as an electron acceptor, some experimental problems needed to be solved. At low pH values, the solubility of ferric iron is much higher than at neutral pH. However, even at pH 2, the solubility of ferric iron is likely to limit the maximum biomass densities that can be attained in cultures grown anaerobically with ferric iron as the electron acceptor. The biomass concentrations that can be expected in anaerobic cultures of *T. ferrooxidans* can be estimated by assuming that the anaerobic growth yield on sulfur and ferric iron will be approximately equal to the aerobic growth yield on ferrous iron (0.23 g [dry weight]/mol of electrons [5]). If this assumption is correct, then the biomass concentration in an anaerobic culture containing an initial ferric iron concentration of 100 mM will not exceed 23 mg (dry weight) · $liter^{-1}$. In addition to the very low expected biomass concentrations, direct determination of biomass dry weight, organic carbon, or protein is complicated by high concentrations of soluble iron and by the presence of insoluble sulfur particles.

In view of the problems mentioned above, we resorted to cell counts as a method to measure anaerobic growth of *T. ferrooxidans* on elemental sulfur. A problem in this respect is that the organism attaches to the substrate during aerobic growth on elemental sulfur (10). This phenomenon was also observed during anaerobic growth (data not shown). To avoid inaccurate cell counts caused by attachment, cultures were shaken with 0.03% Tween 20 immediately before counting. Microscopic analysis revealed that this treatment resulted in an almost quantitative release of the bacteria from the sulfur particles. Control experiments showed that the Tween 20 treatment did not significantly affect cell counts of (homogeneous) aerobic ferrous iron-grown cultures (data not shown).

Anaerobic growth of *T. ferrooxidans*. Under anaerobic conditions, ferrous iron-grown cells of *T. ferrooxidans* exhibit significant rates of ferric iron-dependent sulfur oxidation (15). Therefore, cells from aerobic, ferrous iron-grown shake flask cultures were chosen as inocula for anaerobic growth experiments.

In cultures containing excess elemental sulfur and a limiting amount of ferric iron, ferric iron (100 mM) was quantitatively reduced to ferrous iron during a 20-day anaerobic incubation period (Table 1). Ferric iron reduction was not observed in noninoculated control cultures. This is in agreement with the conclusion of Brock and Gustafson (2) that ferric iron-dependent oxidation of elemental sulfur is a biological process. Reduction of ferric iron was accompanied by a decrease of the culture pH from 1.9 to 1.4. These observations are in accordance with the occurrence of anaerobic sulfur oxidation by the equation, $S + 6Fe^{3+} + 4H_2O \rightarrow H_2SO_4 + 6Fe^{2+} + 6H^+$.

Anaerobic oxidation of elemental sulfur supported growth of *T. ferrooxidans*, as evident from a 200-fold increase of the cell density (Table 1; Fig. 1). The final cell numbers reached in anaerobic cultures were not significantly affected by the addition of yeast extract to a final concentration of 5 mg · $liter^{-1}$ (Table 1). No growth was observed in cultures without elemental sulfur (Table 1). A slight increase of cell numbers was observed in cultures containing elemental sulfur which had not been supplemented with ferric iron (Table 1). This can be explained by the fact that the inoculum (0.5%, vol/vol) contained 180 mM ferric iron.

It has recently been reported that *T. ferrooxidans* can grow aerobically on molecular hydrogen (4). H_2 is one of the constituents of the anaerobic atmosphere used in the present study. Therefore, anaerobic growth on molecular hydrogen might have been expected. Drobner et al. (4) showed that aerobic growth on H_2 did not occur below a pH of 2.2, which is higher than the pH used in the present study. The low pH used in the present study may well have caused the absence of anaerobic growth on molecular hydrogen. Problems with the solubility of ferric sulfate prevented the use of a higher culture pH.

TABLE 1. Growth of *T. ferrooxidans* LMD 81.44 in anaerobic batch cultures[a]

Medium composition	Cell count (ml^{-1})	Ferrous iron (mM)	pH
Mineral medium	$<5 \times 10^6$	<0.5	1.9
Mineral medium + 0.5% elemental S	$(7 \pm 3) \times 10^6$	1.1 ± 0.2	1.9
Mineral medium + 50 mM $Fe_2(SO_4)_3$	$<5 \times 10^6$	<0.5	1.9
Mineral medium + 0.5% elemental S + 50 mM $Fe_2(SO_4)_3$	$(4 \pm 1) \times 10^8$	96 ± 3	1.4
Mineral medium + 0.5% elemental S + 50 mM $Fe_2(SO_4)_3$ + 5 mg of yeast extract · $liter^{-1}$	$(4 \pm 1) \times 10^8$	97 ± 1	1.4

[a] The mineral medium was supplemented with elemental sulfur, ferric iron, and/or yeast extract. Initial concentrations of ferric iron, elemental sulfur, and yeast extract were 100 mM, 0.5% (wt/vol), and 5 mg · $liter^{-1}$, respectively. The cultures (initial pH, 1.9) were inoculated with 50 μl of a ferrous iron-grown, aerobic batch culture to give an initial cell density of 1.5×10^6 ml^{-1}. Cultures were incubated at 30°C in gas-tight jars containing a nitrogen-carbon dioxide-hydrogen atmosphere. After 20 days, cell counts, ferrous iron assays, and pH measurements were performed as described in Materials and Methods. Data are presented as averages ± standard errors of at least three separate cultures.

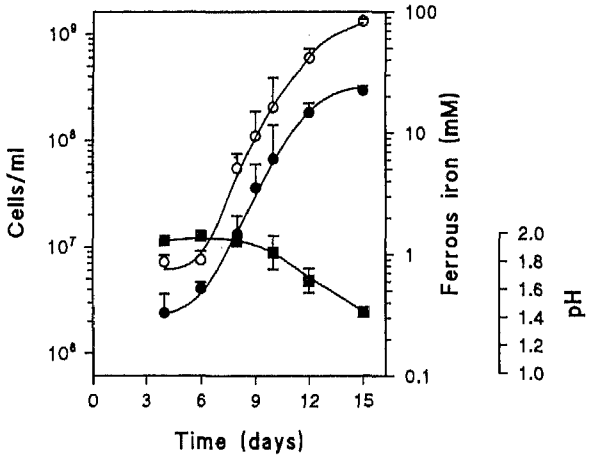

FIG. 1. Anaerobic growth of *T. ferrooxidans* LMD 81.44 on elemental sulfur (0.5%, wt/vol) and ferric sulfate (50 mM). Twenty-one cultures were inoculated with cells pregrown in aerobic, ferrous iron-grown shake flask cultures and divided among seven anaerobic jars (see Materials and Methods). The initial cell density was 1.2×10^6 cells · ml^{-1}. At the indicated times, a jar was opened and three cultures were analyzed for growth, ferrous iron concentration, and culture pH. Data are presented as averages ± standard errors. (Bars indicating standard error sometimes have been drawn upwards only to avoid overlap.) Symbols: ●, cell count; ○, ferrous iron concentration; ■, culture pH.

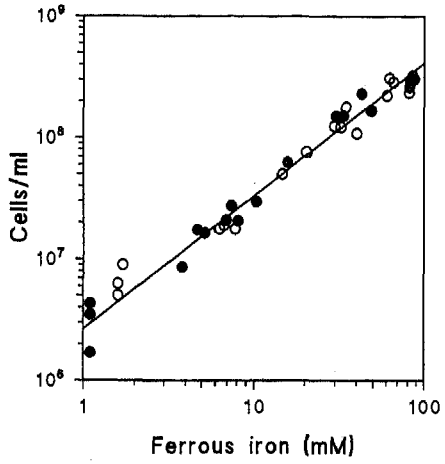

FIG. 2. Anaerobic growth of *T. ferrooxidans* LMD 81.44 on elemental sulfur (0.5%, wt/vol) and ferric sulfate (50 mM): relation between growth and ferric iron reduction. After various incubation times (see legend to Fig. 1), anaerobic cultures were analyzed for growth and ferrous iron concentration. Symbols: ●, cultures grown in defined medium; ○, cultures grown in defined medium supplemented with 5 mg of yeast extract · liter^{-1}. The correlation coefficient of the straight line (calculated by the least-squares method) was 0.97.

To estimate the specific growth rate of *T. ferrooxidans* under anaerobic conditions, an anaerobic growth curve was constructed. A series of replica cultures were incubated anaerobically for various times. This experimental approach resulted in a significant amount of scatter in the experimental data (Fig. 1). However, when the average cell counts of three independent cultures were calculated for each time point, a classic growth curve emerged (Fig. 1). After a lag phase, anaerobic growth proceeded exponentially with an approximate doubling time of 24 h (μ = 0.03 h^{-1}; Fig. 1). Growth was paralleled by an increase of the ferrous iron concentration and accompanied by a decrease of the culture pH (Fig. 1).

If ferric iron-dependent sulfur oxidation is the sole process responsible for the energization of autotrophic growth, a linear relationship is to be expected between the amount of ferric iron reduced and the increase of the cell number. Indeed, when ferrous iron concentration and cell counts of a number of independent cultures were plotted, the relationship between the parameters appeared to be linear (Fig. 2). The increase of the cell number per mole of ferrous iron formed amounted to approximately 3.6 × 10^9 cells · (mmol of Fe^{2+})$^{-1}$ (Fig. 2). The number of cells formed per mole of ferrous iron did not change when the medium was supplemented with 5 mg of yeast extract · liter^{-1} (Fig. 2).

The purity of all anaerobic cultures was confirmed by immunofluorescence microscopy. No significant morphological differences were observed between cells grown under aerobic and anaerobic conditions as judged by phase-contrast microscopy at ×1,000 magnification (data not shown).

DISCUSSION

The ability of *T. ferrooxidans* to use ferric iron as an electron acceptor for the oxidation of elemental sulfur was first demonstrated in 1976 (2). However, so far it has remained unclear whether this process can support autotrophic growth under anaerobic conditions.

When studying anaerobic growth in batch cultures, it is of key importance to rule out the possibility that growth is due to leakage of oxygen into the cultures. Several results indicate that oxygen leakage was not responsible for the increase in cell numbers observed in the present study. (i) Growth in cultures which had not been supplemented with ferric iron was only marginal and could be attributed to the presence of ferric iron in the inocula. (ii) Growth proceeded exponentially (Fig. 1), which would not be expected in the case of a slow, growth-limiting rate of oxygen leakage into the cultures. (iii) A linear relation existed between the amount of ferrous iron produced and the cell numbers in the anaerobic cultures (Fig. 2).

The data presented in this paper demonstrate that *T. ferrooxidans* is capable of anaerobic growth on elemental sulfur, using ferric iron as an electron acceptor. The anaerobic growth rate of approximately 0.03 h^{-1} (Fig. 1) is of the same order of magnitude as the aerobic growth rate on a number of sulfur compounds. In this respect, it should be realized that the growth conditions used in the present study may not have been optimal. Anaerobic growth of *T. ferrooxidans* is therefore clearly not a marginal phenomenon, and the organism can be considered a real facultative anaerobe.

The biomass yield observed during anaerobic growth on elemental sulfur (3.6 × 10^9 cells · mmol of electrons^{-1}), as calculated from the amount of ferrous iron produced, is comparable to cell yields in aerobic, ferrous iron-grown cultures (approximately 3 × 10^9 cells · [mmol of Fe^{2+}]$^{-1}$ [14], corresponding to 0.23 mg [dry weight] · [mmol of Fe^{2+}]$^{-1}$ [5]). In contrast, aerobic growth yields of *T. ferrooxidans* on inorganic sulfur compounds are over twofold higher than the growth yield on ferrous iron (5). The cell yields observed in the present study are consistent with a model proposed previously (13) in which the ferric iron-reducing enzyme system accepts electrons at the redox level of the ferrous iron oxidoreductase. However, more information about cell size and biomass composition is required before definitive conclusions can be drawn on the energetic efficiency of anaerobic growth.

Johnson and McGinness (7) demonstrated that many heterotrophic acidophiles are capable of anaerobic ferric iron respiration and suggested that ferric iron may be an important electron acceptor under acidic conditions. Anaerobic, ferric iron-dependent autotrophic growth of *T. ferrooxidans*, and possibly other acidophiles, on sulfur compounds may play an important role in the iron and sulfur cycles in acidic environments. In addition to this, the ability of *T. ferrooxidans* to grow under anaerobic conditions may be relevant to its application in biohydrometallurgy. In particular during large-scale in situ leaching operations, *T. ferrooxidans* may often encounter environments with low dissolved oxygen concentrations which are rich in dissolved ferric iron. It is often assumed that the oxidation of sulfur compounds by ferric iron that occurs in leaching operations is a strictly chemical process and that the role of the bacteria is limited to the regeneration of the chemical oxidant (16). The results presented in this paper seem to support the suggestion of Brock and Gustafson (2) that ferric iron may be a major electron acceptor for the biological oxidation of metal ores in leaching operations. However, the key substrates in biological leaching are sulfidic minerals rather than elemental sulfur. Further research is required to investigate whether or not these compounds are also substrates for anaerobic growth of *T. ferrooxidans* and, if so, whether anaerobic biological leaching is quantitatively significant compared

with the strictly chemical oxidation of sulfidic minerals by ferric iron.

T. ferrooxidans and related bacteria have been implicated as the causative agents in the mobilization of heavy metals from metal-sulfur complexes (9). If the oxidation of these metal-sulfur compounds can also occur with ferric iron as the electron acceptor, the maintenance of anaerobic conditions as such (e.g., in landfills) is not a guarantee of their immobilization.

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