Growth Kinetics of *Thiobacillus ferrooxidans* Isolated from Arsenic Mine Drainage

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*Thiobacillus ferrooxidans* is found in many Alaskan and Canadian drainages contaminated by metals dissolved from placer and lode gold mines. We have examined the iron-limited growth and iron oxidation kinetics of a *T. ferrooxidans* isolate, AK1, by using batch and continuous cultures. Strain AK1 is an arsenic-tolerant isolate obtained from placer gold mine drainage containing large amounts of dissolved arsenic. The steady-state growth kinetics are described with equations modified for threshold ferrous iron concentrations. The maximal specific growth rate (μ<sub>max</sub>) for isolate AK1 at 22.5°C was 0.070 h<sup>-1</sup>, and the ferrous iron concentration at which the half-maximal growth rate occurred (K<sub>p</sub>) was 0.78 mM. Cell yields varied inversely with growth rate. The iron oxidation kinetics of this organism were dependent on biomass. We found no evidence of ferric inhibition of ferrous iron oxidation for ferrous iron concentrations between 9.0 and 23.3 mM. A supplement to the ferrous medium of 2.67 mM sodium arsenite did not result in an increased steady-state biomass, nor did it appear to affect the steady-state growth kinetics observed in continuous cultures.

The bacterium *Thiobacillus ferrooxidans* is responsible for metal leaching and acid production in water draining from sulfide deposits. *T. ferrooxidans* can catalyze the dissolution of metals either by directly attacking sulfide ores or by oxidizing reduced iron leading to indirect dissolution via the ferric ion (3). Sulfides and metals (such as arsenic) are commonly associated with lode gold deposits and are sometimes associated with placer deposits (46). Recently, *T. ferrooxidans* has been isolated from streams in Alaska and northern Canada that are affected by mining.

Brown et al. (6) found large numbers of *T. ferrooxidans* in drainage from 90% of the placer mines that they sampled. Concentrations of dissolved arsenic >10 parts per billion (10 μg/liter) were found in 30% of those same streams. *T. ferrooxidans* and arsenic were also present in streams affected by lode mining. In addition, *T. ferrooxidans* will leach substantial amounts of arsenic from placer gold mine material (H. V. Luong, J. F. Braddock, and E. J. Brown, Geomicrobiol. J., in press). These results suggest that *T. ferrooxidans* may be directly involved in the leaching of arsenic and other metals associated with active and abandoned mines to subarctic streams and groundwaters. However, the mechanisms by which these organisms are involved in the cycling of metals such as arsenic are not fully understood.

Many investigators have studied the microbiologically mediated dissolution kinetics of various sulfide minerals (10, 23, 26, 32, 42, 44). Although the growth kinetics of various thiothiobacilli when grown on reduced sulfur compounds have been described (2, 18, 25, 31), the iron-limited growth kinetics of *T. ferrooxidans* are not well understood. The partial kinetic descriptions that are found in the literature (21, 27–29, 33, 43) show inconsistent results. This may be due to the difficulties of growing this organism, particularly in continuous culture, and to taxonomic uncertainty about isolated strains of acidophilic iron oxidizers (13).

In this study, we used batch and continuous cultures to describe the iron-limited growth and iron oxidation kinetics of a *T. ferrooxidans* isolate (AK1) obtained from a stream near Fairbanks, Alaska. This stream is affected by placer mining and contains large amounts of dissolved arsenic. The growth and iron oxidation kinetics of isolate AK1 appear to deviate from traditional kinetic models. There was no apparent effect on the growth or iron oxidation kinetics of this isolate when the initial ferrous iron concentration was increased from 9.0 to 23.3 mM. Additionally, the presence of reduced arsenic in the feed ferrous iron did not affect the kinetics of this arsenic-tolerant isolate.

**MATERIALS AND METHODS**

*Microorganisms.* *T. ferrooxidans* AK1 was originally isolated from Eva Creek, located near Fairbanks, Alaska, and is the same isolate as strain TF133 described by Martin et al. (34). Eva Creek is heavily affected by placer gold mining and is known to contain large amounts of dissolved arsenic (6).

*Growth medium.* The growth medium was modified from that described by Tuovinen and Kelly (45) and contained MgSO<sub>4</sub>·7H<sub>2</sub>O (0.4 g · liter<sup>-1</sup>), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (0.4 g · liter<sup>-1</sup>), KH<sub>2</sub>PO<sub>4</sub> (0.1 g · liter<sup>-1</sup>), and 10 N H<sub>2</sub>SO<sub>4</sub> to bring the medium to pH 1.85 to 1.95. Reduced iron as a sole energy source was supplied as FeSO<sub>4</sub>·7H<sub>2</sub>O, and concentrations varied in different experiments from 5 to 30 g · liter<sup>-1</sup> for batch cultures and 2.5 to 6.5 g · liter<sup>-1</sup> for continuous cultures. To eliminate iron precipitation formation, the components of the medium were autoclaved in two separate solutions and were aseptically mixed after the solutions cooled to room temperature. Solution A contained the FeSO<sub>4</sub> and about 2.5 ml of 10 N H<sub>2</sub>SO<sub>4</sub> per liter of final medium volume. Solution B contained the remaining salts diluted in water. The water used throughout these experiments was American Society for Testing Standards Type I quality.

In some continuous-culture experiments, 2.67 mM reduced arsenic (supplied as NaAsO<sub>2</sub>) was also incorporated into the culture medium. The arsenite was dissolved in water (pH 2) and was then aseptically filtered into the prepared medium.

*Batch culture.* For batch culture experiments, 5 to 10 ml of exponentially growing cells was inoculated into 500-ml Erlenmeyer flasks containing iron medium. The flasks were placed on a rotary shaker and were maintained at 22.5°C for the duration of each experiment. Microbiological and chemical parameters were measured periodically in small samples.
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measured for each flask. Compressed air to supply oxygen and carbon dioxide was added via the air port at a rate of 500 to 600 ml · min⁻¹. The sampling port was sealed with a silicone stopper through which a small piece of glass tubing was inserted. A small valve connected the glass tubing to a sterile syringe. We could rapidly and efficiently remove sterile samples with this system.

A magnetic stirring device continually agitated the contents of the reactor flask. The flasks were supported slightly above the stirrer to avoid any effect on the culture vessel from the heat generated by the motor of the stirrer. Silicone stoppers sealed the reactor vessel and feed carboy. Sterile cotton filters inserted in the feed carboy stopper and reactor vessel stopper maintained ambient pressure throughout the system. Glass tubing and a minimal amount of silicone tubing connected the feed carboy to the reactor vessel.

The continuous cultures were maintained at 22 to 23°C in a temperature-controlled room. At the start of an experiment, 5 to 10 ml of exponentially growing cells was added to 200 to 300 ml of fresh culture medium. The peristaltic pump was then started, the vessel was filled, and continuous cultivation was begun. Generally, a slow flow was used for several days until organism growth was observed, at which time the pump was adjusted to the desired flow rate. The flow rate for each culture was measured several times to assure continuous and constant delivery at a given flow rate by each pump. The dilution rate (D) was calculated as the flow rate divided by the volume of the culture vessel. The value of D was assumed to be equivalent to the specific growth rate (μ) of the organism at steady state. After at least three residence times (R, where R = D⁻¹ or μ⁻¹) had passed at a given flow rate, a steady state was assumed, and samples of the reactor vessel contents were removed for microbiological and chemical analysis.

Enumeration. Numbers of cells in laboratory cultures were determined by epifluorescent direct-count microscopy by a method adapted from that described by Hobbie et al. (22). Measured aliquots (0.1 to 0.5 ml) of flask or reactor vessel contents were placed in small test tubes containing filtered water. Two drops of filtered 0.1% (in water) acridine orange solution were then added to each tube to stain the cells. The stained-cell suspension in each tube was then filtered with a 3-ml syringe and a Swinnex filter apparatus (Millipore Corp.) onto an irgalan black-stained (22) filter (0.2-μm pore size, 25-mm diameter; Nuclepore Corp.). Filtered water (three 2-ml portions) was used to rinse each tube and the filter apparatus. The damp Nuclepore filter was then placed, with a drop of immersion oil, under a cover slip on a microscope slide and was held at 4°C in the dark for 30 min to 1 h, after which 15 to 20 microscope fields were counted per slide by using a Zeiss Standard microscope with an epifluorescence light source. Duplicate or triplicate filters were prepared and counted for each sample.

Ferrous iron. Ferrous iron concentrations in solution (filtered through a Millipore type HA filter [0.22-μm pore size; 25-mm diameter]) and total ferrous iron concentrations (nonfiltered) were determined by the ortho-phenanthroline spectrophotometric method of the American Public Health Association (1). Ferrous iron concentrations were monitored in continuous-culture feed reservoir medium to assure chemical stability for the duration of an experiment. Ferrous iron was stable in the acid media for at least 6 months.

Arsenic. Arsenic concentrations were designated as arsenic in solution (filtered through a Millipore type HA filter [0.22-μm pore size; 25-mm diameter]) or as total arsenic (including arsenic in solution and associated with bacterial cells).

Total arsenic (micrograms per liter range) was analyzed by the graphite furnace atomic absorption method described by Wilson and Hawkins (46) as modified with a nickel matrix (19) or (milligrams per liter range) by the flame atomic absorption method with an Electrodeless Discharge Lamp at 193.7 nm as described for the Perkin-Elmer model 4000 atomic absorption spectrophotometer (Publication 0303-0152, 1982, The Perkin-Elmer Corp., Norwalk, Conn.).

Ionic species of arsenic were determined in culture filtrates by treating 2.0 ml of sample with 0.2 ml of a modified Murphy and Riley (36) molybdate reagent, containing 4 ml of ammonium molybdate (30 g · liter⁻¹), 5 ml of 10 N H₂SO₄, 2 ml of potassium antimony tartrate (1.36 g · liter⁻¹), and 0.108 g of ascorbic acid. The samples were incubated for ca. 6 h, at which time the arsenate fraction was extracted with

![Diagram](http://aem.asm.org/Downloaded from http://aem.asm.org on July 6, 2017 by guest)
isoamyl alcohol. The aqueous (arsenite-containing) phase was analyzed, as described above, by atomic absorption spectrophotometry (4). Arsenate ion concentrations were calculated as the differences between total arsenic and aqueous phase concentrations.

**Carbon.** Organic carbon was measured by the wet-chemical method described for the Technicon Auto Analyzer II (industrial method no. 451-76W/A, 1978, Technicon Instruments Corp., Inc., Tarrytown, N.Y.). This method was selected for its sensitivity (0.4 to 20.0 mg of C liter⁻¹) and small sample volume requirement (2 ml).

**Kinetic measurements.** A list of continuous-culture parameters is found in Table 1. The relationships among these parameters were used to describe the growth and iron oxidation kinetics of *T. ferrooxidans* in continuous culture. Growth rates (μᵢ) were plotted against residual ferrous iron concentrations with a modified Monod model (8). The modified Monod relationship can be expressed as

\[ \mu = \frac{\mu_{\text{max}}(S - S_i)}{K_\mu + (S - S_i)} \]

(1)

and can be linearized by the relationship

\[ (S - S_i)(\mu_{\text{max}})^{-1} = \frac{K_\mu}{\mu_{\text{max}}^{-1} + (S - S_i)(\mu_{\text{max}})^{-1}} \]

(2)

(see Table 1 for definition of symbols). This linearization was selected rather than the more commonly used double-reciprocal plot since it is less biased by deviations at low substrate concentrations (11) and, for these experiments, yields a more accurate estimate of the maximum growth rate (μᵢmax). Alternatively, μᵢmax was estimated with equations analogous to those from the internal stores model (17, 40).

The iron oxidation rate (v) was analyzed as a function of the residual ferrous iron concentration, specific growth rate, and carbon yield (Ycarbon). The relationship between v and the external ferrous iron concentration (S) was linearized as described for the Monod model. Regression analysis was used to estimate best-fit lines for all linear relationships. Curvilinear relationships were drawn by hand to indicate general trends in the data. The significance of the slope of linear relationships at the 95% confidence level was determined with F tests.

**RESULTS**

**Batch culture.** The results of a batch culture experiment in which ferrous iron in solution, organic carbon, and numbers of cells were monitored over time are shown in Fig. 2. The initial ferrous iron concentration in the culture medium was 108 mM in this experiment. These data can be used to predict an apparent maximum specific growth rate, a maximum iron oxidation rate (vᵢmax), and growth yields for this isolate.

The plot of log₁₀ numbers of cells over time can be used to estimate the apparent μᵢmax. The apparent μᵢmax was calculated to be 0.089 h⁻¹ for strain AK1 in this experiment and in a duplicate experiment (data not shown). Generation time (tᵢ), the time required for the population to double during exponential growth, was about 7.7 h. The relationship between ferrous iron in solution and time was used similarly to estimate an apparent maximum iron oxidation rate of 3.34 mmol liter⁻¹ h⁻¹ for this isolate.

The organic-carbon and cell number data were used to calculate growth yields, Ycarbon and Ycell, for strain AK1. The Ycarbon ranged from 0.12 to 0.20 mg of carbon per mmol of Fe²⁺ oxidized, whereas the Ycell demonstrated an increase of cells were monitored over time are shown in Fig. 2. The initial ferrous iron concentration in the culture medium was 108 mM in this experiment. These data can be used to predict an apparent maximum specific growth rate, a maximum iron oxidation rate (vᵢmax), and growth yields for this isolate.

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![Figure 2](http://aem.asm.org/)

**FIG. 2.** Ferrous iron in solution (Δ), log₁₀ number of cells (○), and carbon yield and cell yield (□) as functions of time for batch-grown cells.

![Figure 3](http://aem.asm.org/)

**FIG. 3.** Steady-state growth rate as a function of external ferrous iron concentration for *T. ferrooxidans*. Symbols represent influent ferrous iron concentrations (mM) as follows: ○, 9.0 to 9.5; •, 9.0 + 1.3 to 2.7 mM As³⁺; △, 17.9; ○, 22.4 to 23.3.

![Figure 4](http://aem.asm.org/)

**FIG. 4.** Linearization of the Monod growth curve (threshold corrected) for *T. ferrooxidans*. The equation for the line at the 95% confidence level is y = (14.30 ± 2.83)x + 11.12 ± 2.78 with r = 0.87. Symbols are the same as those in Fig. 3.
over time from 0.25 \cdot 10^9 \text{ to } 1.20 \cdot 10^9 \text{ cells per mmol of Fe}^{2+} \text{ oxidized. In a duplicate experiment (data not shown), a similar final } Y_{cell} \text{ value of } 1.3 \cdot 10^9 \text{ cells per mmol of Fe}^{2+} \text{ oxidized was calculated. These } Y_{carbon} \text{ and } Y_{cell} \text{ values suggest that the amount of carbon per cell decreased over time in batch-grown cells. At the stationary phase of growth (near the end of the experiment), there was ca. 0.12 pg of carbon per cell.}

**Continuous culture.** (i) **Growth kinetics.** The Monod relationship between growth rate and external ferrous iron concentration for isolate AK1 is shown in Fig. 3. This relationship describes the growth of this isolate at several different influent ferrous iron concentrations (S_R) and in the presence and absence of reduced arsenic. An apparent threshold ferrous iron concentration (S_t; x intercept) of about 0.25 mM was estimated from the results in Fig. 3. This extrapolated threshold value represents ferrous iron unavailable to the organism for growth (8).

The apparent threshold value was applied to a linearization of the Monod (35) growth curve (Fig. 4). The apparent \( \mu_{max} \) was 0.070 hr^{-1} and the threshold-corrected growth half-saturation constant (\( K_s \)) was 0.78 mM (Fig. 4).

\( Y_{carbon} \), expressed as carbon produced per iron oxidized as a function of growth rate, is shown in Fig. 5. The steady-state \( Y_{carbon} \) decreased with the increasing dilution rate for this isolate. This decrease is apparently related to a decrease in carbon per cell as a function of growth rate (Fig. 6) for this isolate. The carbon-to-cell ratio was not constant when strain AK1 was grown in batch cultures (Fig. 2).

(ii) **Iron oxidation kinetics.** The Michaelis-Menten relationship between the gross (total) iron oxidation rate and the external ferrous iron concentration at three different influent ferrous iron concentrations is shown in Fig. 7. The apparent threshold ferrous iron concentration for gross iron oxidation at three \( S_R \) values was ca. 0.25 mM, the same value

![Graph of iron oxidation rate vs. external ferrous iron concentration](image)

**Fig. 7.** Steady-state gross ferrous iron oxidation rate as a function of external ferrous iron concentration at the three different influent ferrous iron concentrations (A, B, C) as defined by the symbols in Fig. 3.

![Graph of growth rate vs. yield](image)

**Fig. 5.** The steady-state yield of cellular carbon as a function of growth rate for _T. ferrooxidans_. Symbols are the same as those in Fig. 3.

![Graph of steady-state carbon/cell ratio](image)

**Fig. 6.** The steady-state carbon/cell ratio as a function of growth rate. Symbols are the same as those in Fig. 3.

![Graph of threshold-corrected linearization](image)

**Fig. 8.** Threshold-corrected linearization of Fig. 7. The equations for the lines at the 95% confidence level are as follows: A, \( y = (0.45 \pm 0.39)x + 0.91 \pm 0.48 \) with \( r = 0.85 \); B, \( y = (0.57 \pm 0.78)x + 1.06 \pm 1.00 \) with \( r = 0.47 \); and C, \( y = (2.44 \pm 0.56)x + 0.87 \pm 0.39 \) with \( r = 0.91 \). Symbols are the same as those in Fig. 3.
estimated for growth kinetics. This indicates that iron oxidation and growth kinetics are tightly coupled. Threshold-modified linearization of the gross iron oxidation rate versus the external ferrous iron concentration is shown in Fig. 8. This figure can be used to predict a $v_{\text{max}}$ and a half-saturation constant ($K_{\text{Fe}}$) for different values of $S_R$. The apparent $v_{\text{max}}$ predicted from the reciprocal slope of the best-fit lines when $S_R = 9.0$ mM was 0.41 mmol · liter$^{-1}$ h$^{-1}$. The $v_{\text{max}}$ for $S_R = 22.4$ to 23.3 mM was 2.22 mmol · liter$^{-1}$ h$^{-1}$. The $K_{\text{Fe}}$ is estimated from $v_{\text{max}}$ times the $y$ intercept. Thus, $K_{\text{Fe}}$ was 0.36 and 2.02 mM for $S_R = 9.0$ and 22.4 to 23.3, respectively. Insufficient data were available at low dilution rates to yield a significant linear relationship when $S_R = 17.9$ mM, so no attempt was made to estimate $v_{\text{max}}$ or $K_{\text{Fe}}$ for this value of $S_R$.

The Michaelis-Menten relationship between net iron oxidation rate ($v_{\text{carbon}}$) and external ferrous iron concentration is shown in Fig. 9. Since saturation was not apparent, we assumed that the relationship between $v_{\text{carbon}}$ and $S$ was linear (first order). A theoretical maximum net iron oxidation rate must exist; therefore, the rates we observed for AK1 must be significantly below the maximum. The $x$ intercept (threshold) concentration of the linear relationship (Fig. 9) is 0.24 mM. The slope of the line (Fig. 9) is mathematically analogous to steady-state affinity ($a_I$) as defined by Button (9). The $a_I$ for isolate AK1 is ca. 0.22 liters · mg of $C^{-1}$ h$^{-1}$ and is a good parameter to use for comparative measures of specific (subsaturating) iron oxidation rate at low iron concentrations.

(iii) Arsenic. Reduced arsenic (1.33 or 2.67 mM) was added to the ferrous iron medium in several continuous-culture experiments. The results indicated that reduced arsenic was not oxidized in the presence of isolate AK1 growing at four different growth rates ranging from 0.007 to 0.034 h$^{-1}$. Virtually none of the reduced arsenic added was recovered in the oxidized form.

**DISCUSSION**

**Growth kinetics.** We have used batch and dilute (low biomass) continuous cultures to describe the growth kinetics of an arsenic-tolerant subarctic isolate of *T. ferrooxidans*. The growth and iron oxidation kinetics of this isolate deviate from traditional kinetic models.

The conventional Monod model which was originally derived from Michaelis-Menten enzyme kinetics is useful for describing the carbon-limited growth of heterotrophs when the growth-limiting substrate is incorporated as cellular material. When *T. ferrooxidans* is grown on reduced iron, the energy-limiting substrate is not incorporated as cellular material. The Monod model depends on a constant yield which links specific growth rate to substrate removal. The Monod model has also been modified to accommodate the possibility that yield may increase with increasing growth rate (i.e., maintenance energy; see reference 39). The model is less readily adaptable to the possibility that yields may decrease with increasing growth rate.

Decreasing yields with increasing growth rate have commonly been observed for a variety of microorganisms grown in chemostats under the limitation of inorganic ions such as phosphate, magnesium, silicate, ammonium, and nitrate and of vitamins such as B12 (5, 12, 30, 40). The internal-stores kinetic model originally proposed by Droop (17) and modified by Rhee (40) has been used to describe growth rates as a function of internal rather than external concentrations of limiting substrates. It is essential in this kinetic model that yields vary inversely with growth rate since concentrations of intracellular substrate increase with increasing growth rate (38, 40). The application of either the Monod or the internal-stores model to the observed growth of dilute, iron-limited cultures of isolate AK1 is limited. Despite these recognized limitations, either model can be used to mathematically describe some growth and iron oxidation functions for this isolate.

The Monod model was used to estimate iron-limited growth parameters for strain AK1 with respect to external ferrous iron concentrations. The values we found for $\mu_{\text{max}}$ and $K_s$ were somewhat lower than those reported for other isolates of *T. ferrooxidans*. Values reported for other isolates cultured in chemostats include the following: a $\mu_{\text{max}}$ of 0.14 h$^{-1}$ and a $K_s$ of 37 mM (43); a $\mu_{\text{max}}$ of 0.161 h$^{-1}$ and a $K_s$ of 3.8 mM (33); and a $\mu_{\text{max}}$ of 1.25 to 1.78 h$^{-1}$ and a $K_s$ of 0.7 to 2.4 mM (27, 28). These apparent discrepancies are probably a result of growing the organism under different physiological regimes (i.e., pH and temperature) and of variations in the microorganisms classified as *T. ferrooxidans*. Several investigators recently demonstrated that variations of taxonomic importance may occur among different isolates of *T. ferrooxidans*. Variations have been found in DNA base composition studies (20, 34), physiological studies (41), and morphological studies (13). Growth and iron-oxidation kinetics of these iron-oxidizing bacteria may be yet another way of describing a functional classification for these microorganisms.

Our results also show apparent threshold ferrous iron concentrations of about 0.25 mM for both iron-limited growth and iron oxidation. The threshold ferrous iron concentration observed here represents a concentration of substrate unavailable to strain AK1 for energy utilization but is not analogous to maintenance energy since the same threshold exists for both growth rate and iron oxidation rate (i.e., maintenance energy is observed as a positive threshold only for growth in conventional kinetic models). Thresholds have not been reported for other *T. ferrooxidans* strains growing on reduced iron. However, most other studies have used much higher iron concentrations, resulting in much more biomass in the continuous-culture vessels. Eccleston and
Kelly (18) did observe a maintenance energy coefficient for a _T. ferrooxidans_ strain grown in either thiommalate- or thiosulfate-limited chemostat cultures. However, the lowest dilution rate used in these studies was 0.02 h⁻¹. The apparent threshold of 0.25 mM for both growth and iron oxidation indicates that growth and iron oxidation are tightly coupled in isolate AK1. The threshold value may be an indication of the enzyme activity and rate of ferric iron to ferrous iron. However, increasing ferric iron/ferrous iron ratios do not change specific (biomass-adjusted) ferrous oxidation rates (7).

The yield of organic carbon for strain AK1 was relatively constant in batch cultures, ranging from 0.12 to 0.20 mg of carbon per mmol of iron oxidized. However, steady-state (continuous-culture) data indicate a decreasing curvilinear dependence of _Y_ carbon on the specific growth rate, particularly below the point where _μ_ = 0.03 h⁻¹ (Fig. 5). If _Y_ carbon data below this point had not been available, the yield might have been assumed to be constant with increasing dilution rate. _Y_ cell (number of cells / mmol of iron oxidized⁻¹) also decreased as a function of _μ_ (data not shown). The exponential function (Fig. 5) can be approximated by the linear function in _Y_ = -0.019μ + 0.89 (r = -0.89) which describes the observed relationship between _Y_ carbon and _μ_. The steady-state carbon/cell ratio also appears to decrease with increasing dilution rate for isolate AK1 (Fig. 6). We observed, when counting AK1 cells by epifluorescence microscopy, that cell size markedly increased at low dilution rates. Other investigators have reported similar observations (D. W. Tempest and O. M. Neijssel. Abstr. Third Int. Symp. Microbial Ecol., 1983. II2, p. 8). Our data indicate that at low growth rates strain AK1 is most efficient at utilizing reduced iron. The highest _Y_ carbon we observed was 0.50 mg of C per mmol of iron oxidized at _μ_ = 0.007 h⁻¹. This yield is close to the theoretical maximum growth yield of 0.53 mg of C per mmol of iron for _T. ferrooxidans_ growth at pH 2 (24).

The steady-state growth kinetics of strain AK1 apparently do not depend on the initial ferrous iron concentration (Fig. 3) over the range of _S₀_ values used in these experiments. Additionally, the growth kinetics of this isolate are not affected by the addition of reduced arsenic to the ferrous iron-containing feed.

**Iron oxidation kinetics.** The gross ferrous iron oxidation rate at steady state depends on the influent ferrous iron concentration (see Fig. 7 and 8 for strain AK1). The _v_ max and the _Kᵦₑᵦᵦ_ both appear to increase with increasing _Sᵦ_. This increase in maximum iron oxidation rates with increasing _Sᵦ_ was also observed by Guay et al. (21). Guay et al. examined the steady-state iron oxidation kinetics of a _T. ferrooxidans_ strain at influent ferrous iron concentrations of 89 to 161 mM. Their strain did not reach its maximum capacity for iron oxidation even at an _Sᵦ_ of 161 mM.

The observed dependence of _v_ max on influent substrate concentration is predicted from Michaelis-Menten kinetics. The Michaelis-Menten parameter, _v_ max, is a relative rather than a specific value. The apparent maximum rate of the enzyme is attained when all of the enzyme sites are saturated with substrate. Therefore, if more enzyme is present at a given substrate concentration, the apparent _v_ max is greater. When the influent ferrous iron concentration is increased, a larger concentration of organic carbon (as a function of ambient substrate concentration or of growth rate) is produced by strain AK1. Assuming that cellular carbon represents about 50% of the dry weight of the AK1 cell under iron-limited, steady-state conditions, an increase in cell dry weight (biomass) should result in an increased concentration of ferro-oxidase per unit volume. Therefore, the total activity of the enzyme is greater at increasing influent ferrous iron concentrations as long as the cells remain iron limited.

The gross iron oxidation rate can be converted to a specific rate by defining iron oxidation per unit of carbon (v_ carbon) or per number of cells (v_ cell). These specific iron oxidation rates are analogous to the specific growth rate constant described in the Monod growth model. The _v_ carbon as a function of the _S-S₀_ is independent of the _S₀_ for strain AK1 (Fig. 9). Therefore, enzyme concentration, not ferric inhibition of ferrous-iron oxidation, appears to be responsible for the results observed in Fig. 7 and 8, which may also be used as evidence that ferrous iron remained the limiting factor in these experiments. Additionally, reduced arsenic added to the feed does not affect the net iron oxidation rate of this organism.

Expressed mathematically, _v_ carbon = _μ_ _Y_ carbon. When _μ_ _Y_ carbon⁻¹ is plotted against _Y_ carbon⁻¹ by analogy to the internal-stores growth model, the relationship described by the equation _μ_ = 0.070 - 0.179 _Y_ (r = 0.933) predicts a _μ_ max of 0.070 h⁻¹, the same value predicted by the Monod equation.

The steady-state _v_ carbon as a function of _S_ (Fig. 9) for strain AK1 appears to be a linear relationship. Since a theoretical maximum net iron oxidation rate must exist, the function must asymptotically approach a maximal value. This indicates that the observed rates are significantly lower than the maximum rate obtainable by strain AK1. The maximum rate would presumably occur when much higher ferrous iron concentrations are added to iron-starved batch cultures (30).

**Arsenic.** _T. ferrooxidans_ oxidizes many reduced metals including cuprous copper (37), elemental selenium (44), and uranous sulfate (14-16). Furthermore, _T. ferrooxidans_ may be able to obtain energy from some of these inorganic oxidations (15, 16).

Strain AK1 was isolated from placer gold mine drainage containing dissolved arsenic. Speciation of the dissolved arsenic in this stream indicated that arsenate species and other species (including arsenite) were present in about equal amounts (6). Arsenate was not the only species present despite the fact that the stream was saturated with oxygen. In laboratory cultures, strain AK1 was tolerant to at least 900 mg of arsenite or arsenate · liter⁻¹ when the organism was grown in a medium containing ferrous iron (J. M. Forshaug, M.S. thesis, University of Alaska, Fairbanks, 1983).

For these reasons, we hypothesized that _T. ferrooxidans_ isolate AK1 can oxidize reduced arsenic and derive energy from such an oxidation. However, the results of steady-state experiments to test this hypothesis were inconclusive. When arsenite was added to the continuous-culture medium containing ferrous iron, we observed no oxidation of the arsenite ion. The presence of either 1.33 or 2.67 mM arsenate in the medium did not yield an increase in biomass, nor did it have any observable effect on growth-kinetic or iron oxidation kinetic parameters being measured. If arsenite can be used for energy production by isolate AK1, an observable increase in biomass would be expected.

**Summary.** The importance of the iron-oxidizing thiobacilli in catalyzing biogeochemical cycles has only recently been recognized. With the depletion of high-grade ores, methods of bioleaching for the recovery of valuable metals are becoming increasingly important. At some time in the future, genetic manipulation may be used to produce highly efficient leaching microorganisms (3). The iron-limited growth kine-
ics have not been well described for *T. ferrooxidans*. Published kinetics are inconsistent. These inconsistencies probably result from apparent strain-to-strain differences and from the difficulties in culturing this organism, especially in iron-limited continuous cultures. A complete kinetic model must describe both cell growth and substrate utilization with some function that relates the specific growth rate to the substrate utilization rate. The yield constant links the equations of the Monod model. Alternatively, the Monod model can be modified to accommodate an increasing yield with increasing growth rate when a maintenance coefficient is observed. Other kinetic models have been developed (17, 38, 40) to describe the growth of microorganisms as a function of internal rather than external concentrations of a limiting nutrient.

We have described the growth kinetics, ferrous iron oxidation kinetics, and arsenite oxidation potential of an arsenic-tolerant *T. ferrooxidans* isolate, AK1. The growth of this organism is described in both transient (batch) culture and steady-state (continuous) culture. We have observed that the growth and iron oxidation kinetics of this autotrophic isolate deviate from Monod kinetics. The kinetics of this isolate show some similarities to kinetic models derived to describe substrate-limited growth from internal rather than external substrate concentrations. These observations indicate that additional studies are needed, particularly at low dilution rates, to accurately model the growth and substrate utilization of *T. ferrooxidans* and possibly other autotrophic microorganisms.

We have also described a method for the successful continuous cultivation of *T. ferrooxidans* in dilute, iron-limited continuous cultures. This continuous-culture method can be used to predict the growth and iron oxidation kinetics of other strains of *T. ferrooxidans* and to investigate the ability of *T. ferrooxidans* to oxidize and produce energy from reduced metals other than iron. Such studies are important for understanding the biogeochemical cycling of many metals and for applying these microorganisms to biohydrometallurgy.

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**LITERATURE CITED**


