SULFATE REQUIREMENT FOR IRON OXIDATION BY *THIOBACILLUS FERROOXIDANS*

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ABSTRACT

LAZAROFF, NORMAN (British Columbia Research Council, Vancouver, B.C., Canada). Sulfate requirement for iron oxidation by *Thiobacillus ferrooxidans*. J. Bacteriol. **85**:78–83. 1963.—The growth of *Thiobacillus ferrooxidans* is initially inhibited in media containing ferrous chloride in place of ferrous sulfate. This inhibition of growth is due to the requirement of a high relative proportion of sulfate ions to chloride (or other anions) for iron oxidation. Adaptation takes place, producing strains which are able to oxidize iron in media containing an initially unfavorable anionic composition. Adaptation is possibly due to the selection of spontaneous mutants capable of oxidizing iron in high chloride, low sulfate media. Such cells are found at a frequency of 10^{-5} of the population of unadapted cultures.

The original isolation and studies of the iron-oxidizing bacteria, presently assigned to the genera *Thiobacillus* and *Ferrobacillus*, were carried out in acid media rich in sulfate ions (Colmer and Hinkle, 1947; Temple and Koehler, 1954; Leathen, Kinsel, and Braley, 1956). Although there appears to be some conflict as to whether all of these true autotrophic iron-oxidizing bacteria can produce sulfate as the result of biological oxidation of sulfide, thiosulfate, or sulfur, all isolates have been obtained from natural environments where sulfate is produced either directly or indirectly by biological oxidation (Leathen, McIntyre, and Braley, 1951; Leathen et al., 1956; Corrick and Sutton, 1961).

Unz and Lundgren (1960) described the growth of *T. ferrooxidans*, *F. ferrooxidans*, and *T. thiooxidans* on sulfur in media initially lacking sulfate ions. Their experiments were carried out in a medium modified from one used for growing *T. ferrooxidans* and *F. ferrooxidans* on iron. As formulated, it contained phosphate, chloride, and nitrate anions. However, their analyses for sulfate production indicated a considerable concentration of that anion in the growth flasks at the beginning of experiments, presumably due to carry-over with the heavy inoculum.

Unpublished experiments carried out at the British Columbia Research Council (Trussell et al., 1958–1961) indicated that the presence of various inorganic salts, particularly chlorides and nitrates, inhibited the oxidation of iron by suspensions of an iron-oxidizing bacterium isolated from an acidic mine-water. This organism was capable of oxidizing iron or elementary sulfur and was identified, on that basis, as *T. ferrooxidans*. Since several inorganic chlorides were found to inhibit iron oxidation, it was suspected that the phenomenon was caused by the anionic component. A study of the effects of differing relative concentrations of sulfate and chloride ions on iron oxidation was prompted by observations that the chloride inhibition of oxidation was diminished by the presence of increased levels of sulfate ions.

MATERIALS AND METHODS

The culture of *T. ferrooxidans* employed in this study was obtained from the copper leach waters of a mine at Britannia Beach, British Columbia. A pure culture was obtained by plating on the ferrous iron-silica gel medium of Leathen et al. (1951). The culture was recloned on this medium several times.

Cultures were maintained in the 9K medium of Silverman and Lundgren (1959). The solution contained the following constituents expressed as molar concentrations: $(NH_4)_2SO_4$, 2.3×10^{-2}; KCl, 1.3×10^{-3}; K_2HPO_4, 2.9×10^{-3}; $MgSO_4 \cdot 7H_2O$, 2.0×10^{-3}; $Ca(NO_3)_2$, 6.1×10^{-5}; $FeSO_4 \cdot 7H_2O$, 1.6×10^{-1}; H_2SO_4, 5.0×10^{-3}. For stock cultures, the basal medium without iron was sterilized by autoclaving at 15 lb of

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TABLE 1. *Inorganic ion requirements for suspensions of Thiobacillus ferrooxidans oxidizing Fe^{++}*

Medium	Rate of iron oxidation per ml†
Complete*	8.20
$(NH_4)_2SO_4$ omitted	7.95
$Ca(NO_3)_2$ omitted	7.50
KCl omitted	7.37
$MgSO_4 \cdot 7H_2O$ omitted	6.85
K_2HPO_4 omitted	6.60

* Complete medium was the 9K medium of Silverman and Lundgren (1959), containing 4% $FeSO_4 \cdot 7H_2O$ and adjusted to pH 2.5 with sulfuric acid. Each vessel contained 10 ml of medium at pH 2.5 and received 2.0 ml of a purified bacterial suspension containing 10^8 cells/ml. The rate of oxidation was determined by using the thiocyanate method to measure the concentration of ferric ions at zero time and at intervals after the addition of cells.

† Expressed as μg of Fe^{+++} produced per min.

pressure for 15 min, and then was combined with the ferrous iron solution which had been sterilized by passage through a sintered-glass or Seitz filter. Experimental cultures or mass cultures for producing concentrated cell suspensions were prepared without prior sterilization. In such cases, the pH of 2.5, a heavy inoculum, and the unique energy source could be relied upon to produce exclusive growth of *T. ferrooxidans* without the cumbersome filtration of large volumes of acidic iron solutions.

Media containing increased levels of chloride ions were prepared by replacing all or a part of the ferrous sulfate in the 9K medium with equimolar amounts of ferrous chloride. For growth on sulfur, the medium of Waksman (1922) was used.

Cells were harvested from iron or sulfur media after 3 days of growth from a 10% inoculum. Satisfactory cell yields were produced at room temperature (21 to 24 C) in aerated 6-liter bottles or shaken 250-ml flasks. Preparations of purified

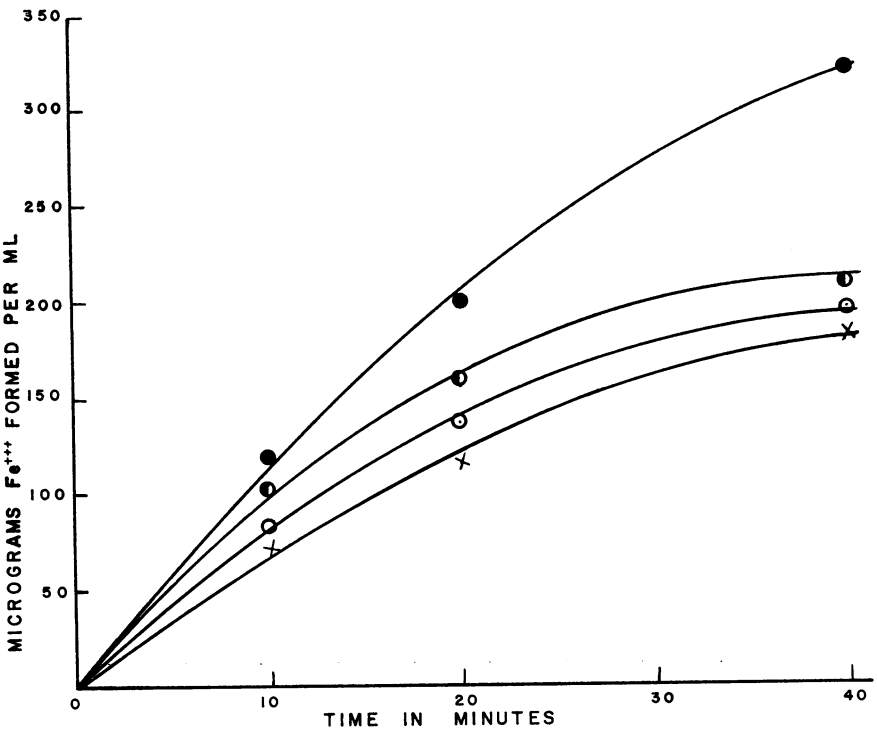

FIG. 1. *Effect of sodium chloride on the oxidation of ferrous ions by Thiobacillus ferrooxidans. Shaken reaction flasks at 30 C contained 0.16 M $FeSO_4 \cdot 7H_2O$ (pH 2.5). Washed bacterial suspension (2 ml), containing 10^9 cells/ml, was added to 20 ml of medium in each flask at zero time. ●, 0.017 M K_2HPO_4; ○, 0.017 M K_2HPO_4 + 0.17 M NaCl; ◐, 9K medium of Silverman and Lundgren (1959); ×, 9K medium + 0.17 M NaCl.*

cell suspensions were made by filtering the growth cultures through Whatman no. 1 filter paper to remove sulfur or precipitated iron. The filter paper was washed with a small quantity of dilute sulfuric acid (pH 2.5), and the washings were combined with the filtrate. The filtrate was then centrifuged at 10,000 × *g* for 20 min to sediment the bacterial cells. The cells were resuspended in dilute sulfuric acid (pH 2.5), and could be used directly in some experiments. Usually the cell suspension was diluted, refiltered, and recentrifuged to obtain a very clean preparation.

TABLE 2. *Experimental protocol for cultivation of Thiobacillus ferrooxidans in media of varied sulfate-chloride ratios*

9K medium containing 0.1 M $FeSO_4 \cdot 7H_2O$	9K medium containing 0.1 M $FeCl_2 \cdot 4H_2O$	$FeSO_4$	Iron supplied as $FeSO_4 \cdot 7H_2O$
ml	*ml*	*μmoles/ml*	%
90	0	100.0	100.0
75	15	83.4	83.4
60	30	66.6	66.6
45	45	50.0	50.0
30	60	33.3	33.3
15	75	16.6	16.6
0	90	0.0	0.0

Growth was measured in terms of bacterial cell counts determined with a Petroff-Hauser bacteria-counting chamber and a Tyoda phase-contrast microscope at 400×. In this system, precipitated iron particles were easily distinguished from bacterial cells.

Iron oxidation was determined spectrophotometrically by measuring the color of ferric thiocyanate complex in dilute hydrochloric acid (Kolthoff and Sandell, 1952). Prior to analysis, oxidation was stopped by diluting samples in 6 N HCl.

RESULTS

Preliminary experiments showed that cell suspensions of *T. ferrooxidans*, purified by means of filtration through paper, sedimentation, and washing in dilute sulfuric acid, were capable of oxidizing acidic ferrous sulfate solutions without the addition of any substances from the 9K medium of Silverman and Lundgren (1959). It was assumed that the cell preparations contained sufficient "carry-over" of any materials

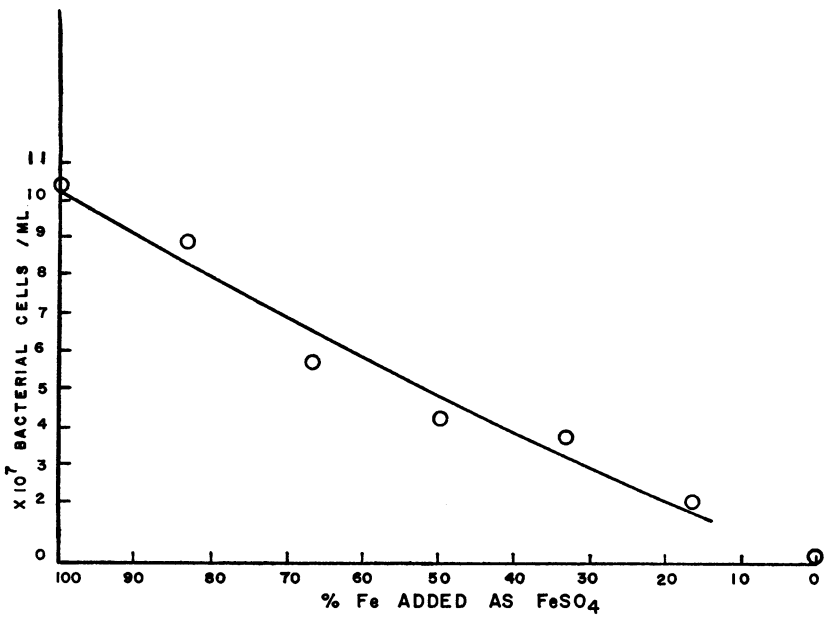

FIG. 2. *Growth of unadapted Thiobacillus ferrooxidans at ascending chloride to sulfate ratios. 9K medium of Silverman and Lundgren (1959), pH 2.5; iron added as various mixtures of 0.1 M $FeSO_4$ and 0.1 M $FeCl_2$ (see Table 2). Medium (90 ml) in 250-ml Erlenmeyer flasks was inoculated with 1.0 ml of a washed bacterial suspension containing 1.0 × 10^7 cells/ml. Cell counts were made after 9 days of incubation at room temperature on a rotary shake machine.*

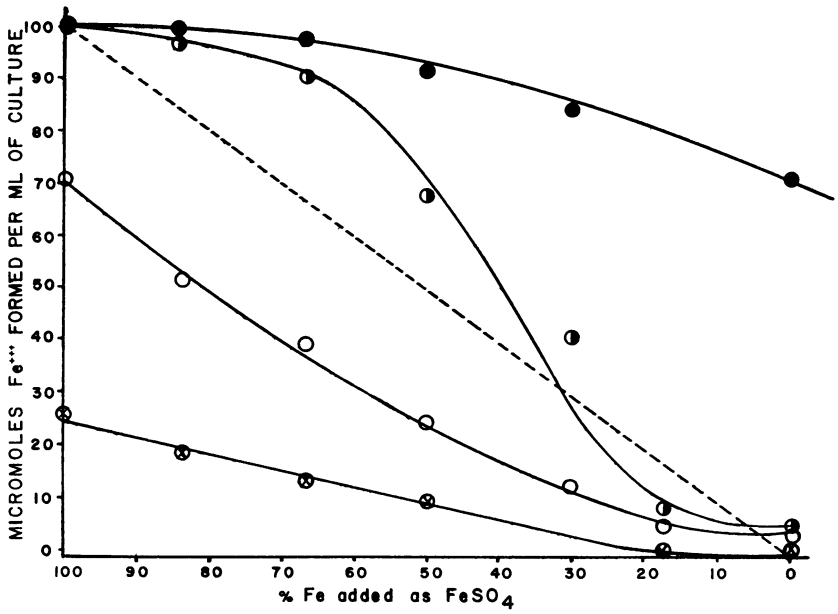

FIG. 3. *Oxidation of iron in cultures of Thiobacillus ferrooxidans adapting to ascending chloride-sulfate ratios. 9K medium of Silverman and Lundgren (1959), pH 2.5; iron added as various mixtures of 0.1 M $FeCl_2$ (see Table 2). Medium (90 ml) in 250-ml Erlenmeyer flasks was inoculated with 1.0 ml of washed suspension containing 1.0 × 10^7 cells/ml, grown on Waksman's (1922) medium. Incubation at room temperature on a rotary shake machine. ⊗, 5 days of incubation; ○, 7 days of incubation; ◑, 9 days of incubation; ●, 10 days of incubation. Dotted line, μmoles of Fe^{+++} which could be produced from $FeSO_4$ present.*

necessary for the oxidation of iron. Moreover, the omission of single constituents of 9K medium caused only slight decreases in the rate of iron oxidation by bacterial suspensions. The omission of potassium phosphate resulted in the least rapid oxidation of iron (Table 1).

It seemed likely from these results that oxidation of iron by cell suspensions might be studied in acid solutions made up solely with potassium phosphate and ferrous sulfate. Figure 1 shows that oxidation of ferrous ions by a bacterial suspension is considerably more rapid in a solution containing only 0.017 M potassium phosphate in addition to ferrous sulfate, than in 9K medium. The addition of 1% sodium chloride inhibited iron oxidation in 9K medium or in the potassium phosphate-ferrous sulfate solution. However, the inhibitory effect of the sodium chloride addition was relatively greater in the latter case.

An experiment to measure growth and iron oxidation at different sulfate concentrations was set up by varying the proportions of 0.1 M ferrous sulfate and 0.1 M ferrous chloride mixtures in 9K medium. The experimental mixtures with the amount of ferrous sulfate added in each case are shown in Table 2.

The number of cells per ml found after 9 days of incubation in shaken flasks, containing the experimental mixtures, is proportional to the amount of iron originally supplied as the sulfate (Fig. 2). As growth progresses, the oxidation of an increasing proportion of iron, supplied as chloride, occurs (Fig. 3). This may be attributed to an adaptation for oxidation of iron in an originally unfavorable environment.

In support of the information obtained from growing cultures, it was found that washed cell suspensions oxidize iron chloride solutions very slowly, but would oxidize such solutions more rapidly if a sulfate salt, such as potassium sulfate, were added. Figure 4 shows that the chloride ions in such cases still inhibited iron oxidation to an extent dependent on the relative concentrations of chloride and sulfate ions.

The heavy inoculation of a series of flasks (similar to those shown in Table 2) with cells

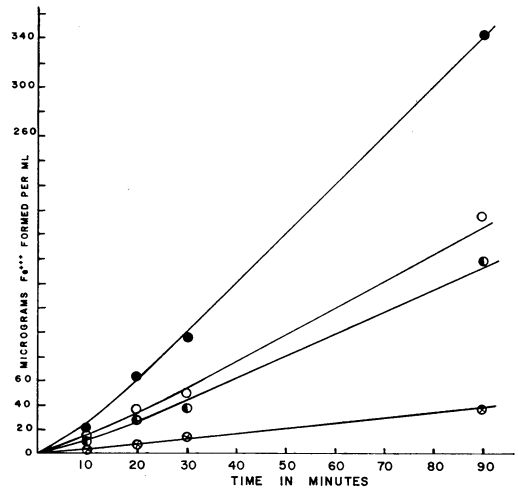

FIG. 4. *Oxidation of Fe^{++} by suspensions of Thiobacillus ferrooxidans in solutions containing different levels of sulfate and chloride ions. Shaken reaction flasks at 30 C contained 10 ml of 9K basal medium of Silverman and Lundgren (1959); pH 2.5, plus the following additions:* ●, *0.05 M $FeSO_4$;* ○, *0.05 M $FeCl_2$ + 0.1 M K_2SO_4;* ◐, *0.05 M $FeCl_2$ + 0.05 M K_2SO_4;* ⊗, *0.05 M $FeCl_2$. Washed bacterial suspension (0.5 ml) containing 4.0 × 10^9 cells/ml was added to each flask at zero time.*

which had adapted to high chloride, low sulfate levels produced good growth and iron oxidation within a few days. Growth and iron oxidation was still somewhat slower in the high chloride treatments than in flasks containing high sulfate levels.

By successive dilution of chloride-grown cell suspensions in media with high concentrations of sulfate (9K), it was found that approximately all adapted cells were capable of growing in high sulfate media. However, only one cell out of 10^5 ordinary sulfate-grown cells was capable of initiating growth in chloride media.

Since heavy inocula of adapted cells were required to initiate growth in chloride media, further experimentation will be required before the mode of adaptation to low sulfate tolerance can be explained.

DISCUSSION

The phenomenon of chloride inhibition of iron oxidation may be viewed equally well as a sulfate requirement for iron oxidation by *T. ferrooxidans*. The presence of chloride ions increases the requirement for sulfate in the system studied. Although it was possible to adapt *T. ferrooxidans* to growth on iron in media containing all iron supplied as the chloride, it was not possible to obtain adaptation to media in which sulfate was completely replaced by chloride.

Previous work suggests that many other anions beside chloride are capable of interacting similarly with the sulfate requirement for iron oxidation. In view of the high concentration of sulfate involved as well as the adaptation to initially unfavorable levels, it is difficult to entertain the possibility that sulfate serves as co-factor for the iron oxidase system. In consideration of the interaction with other anions, it seems possible that the sulfate balance controls the entrance of ferrous ions into the cell. Another possibility is that sulfate is a substrate required for synthesis of a sulfur compound involved in energy transfer from the iron oxidase system.

Although adaptation of *T. ferrooxidans* to oxidation of iron in media containing a low level of sulfate ions occurs readily, it is difficult to maintain sustained growth of adapted cells in a medium where most of the sulfate is replaced with chloride.

It has not yet been unequivocally determined whether the mechanism for adaptation to lower sulfate to chloride ratios involves the selection of mutants which arise spontaneously in the prototrophic stock. However, this mechanism seems likely to play some part, since approximately one cell of 10^5 in the unadapted stock can initiate growth in tubes of medium with high concentrations of chloride and low concentrations of sulfate.

It is interesting to note that the ecological niche which these microorganisms inhabit in nature not only is one of high acidity, but is also rich in sulfate ions (Temple and Koehler, 1954; Leathen et al., 1951). If the provision of high levels of sulfate is prerequisite for efficient bacterial oxidation of reduced iron, then the ability of *T. ferrooxidans* to simultaneously oxidize iron and sulfur or sulfides uniquely fits it for the oxidation of reduced mineralized iron in nature. Although sulfides can be oxidized by ferric ions in acid solution, oxidized iron is rather insoluble in dilute sulfuric acid and is removed from the reacting system. This is evident in nature by the frequent accumulation of oxidized iron precipitates (ochers) in the acid run-off

from leaching sulfide minerals. This limitation of the chemical oxidation of iron sulfide minerals due to the precipitation of the chemical oxidant is compensated for in nature by the ability of an organism like *T. ferrooxidans* to oxidize directly the sulfur atoms of sulfide molecules, thereby making the mineral substrate soluble and providing sulfate and hydrogen ions required for iron oxidation.

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