

Sulfur Oxidation by the Iron Bacterium *Ferrobacillus ferrooxidans*

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ABSTRACT

MARGALITH, P. (Syracuse University, Syracuse, N.Y.), MARVIN SILVER, AND D. G. LUNDGREN. Sulfur oxidation by the iron bacterium *Ferrobacillus ferrooxidans*. J. Bacteriol. 92:1706-1709. 1966.—Sulfur and iron oxidation has been studied manometrically by use of *Ferrobacillus ferrooxidans* grown on either elemental sulfur or ferrous iron as the primary energy source. The iron-oxidizing enzyme was shown to be constitutive, since iron was oxidized as rapidly by sulfur-grown cells as by iron-grown cells. Sulfur-grown cells had a better capacity for oxidizing sulfur than did iron-grown cells; however, no lag in oxidation was seen in either case. The sulfur-oxidizing system was not inducible, and it is suggested that the different oxidative capacities are due to the heterogeneous mixture of cell types in the culture population.

The iron-oxidizing thiobacilli occupy a unique position among autotrophs in being able to use iron as a source of energy for the production of high-energy phosphate bonds and reducing power, for the fixation of carbon dioxide, and for the production of cell material (5). The microorganisms also use sulfur and other reduced inorganic sulfur compounds for the same purpose (1, 9). The comparative biochemistry and energetics of these oxidative reactions have been recently discussed (3). Current evolutionary concepts postulate that autotrophic microorganisms evolved as heterotrophic organisms became adapted to various inorganic environments. It therefore seemed desirable to study substrate oxidation of ferrous iron and elemental sulfur to better understand the nature of parallel pathways in chemolithotrophy.

An earlier report (1) showed that resting cells of *Thiobacillus ferrooxidans* can oxidize sulfur compounds such as elemental sulfur or thiosulfate after having been cultivated on iron as a source of energy. Cells grown on iron oxidized sulfur compounds in the presence of ferrous sulfate only after complete exhaustion of the bivalent iron. Furthermore, any oxidation of sulfur could take place only after a lag period of at least 40 min. Similar observations were made with cells grown in the presence of both substrates, that is, iron and sulfur.

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Unz and Lundgren (9) reported on the similarity of growth kinetics of *Ferrobacillus ferrooxidans* on both iron and sulfur substrates. However, cells grown in the presence of both minerals demonstrated a definite lag period of several days, as evidenced by cell counts and fall of pH, after the oxidation of all of the iron had taken place and before growth on the sulfur substrate commenced.

This report deals with some quantitative aspects of iron and sulfur oxidation by *F. ferrooxidans*.

MATERIALS AND METHODS

F. ferrooxidans, derived from the original culture of Leathen (4), was cultivated in two ways:

(i) Growth on ferrous iron. The cells were grown under conditions similar to those reported previously by Silverman and Lundgren (6). The organism was propagated in 16-liter glass carboys on the ferrous sulfate-9K medium (9,000 ppm of Fe⁺⁺, pH 3.3) under forced aeration and was harvested after 48 to 54 hr by use of a Sharples Centrifuge.

(ii) Growth on elemental sulfur. The cells were grown in 2-liter Fernbach flasks containing 500 ml of the 9K salts solution (pH 3.3), 1.0 ppm of FeSO₄, and 5 g of precipitated sulfur. These flasks were autoclaved for 5 min at 121 C prior to inoculation and were cooled rapidly to prevent sulfur from coalescing. Flasks were agitated on a reciprocating shaker for 5 to 7 days at 28 C and were harvested, after the pH had dropped below 2.0, with a Sorvall RC-2 refrigerated centrifuge. The sulfur in the flasks was not depleted during this time.

In both cases, inocula used were taken from pre-

viously harvested cells grown in the same manner. However, in the case of the sulfur-grown cells, the first inoculum had to be prepared by training iron-grown cells by repeated transfers on the sulfur medium. Attempts to grow cells on sulfur under forced aeration were not successful.

Harvested cells were washed twice with 0.01 M β -alanine buffer (pH 3.5) and stored at 4 C. Yields per 1,000 ml of medium were usually 60 to 70 mg (wet weight) of iron-grown cells, as compared to 150 to 180 mg of sulfur-grown cells. Oxidation experiments were carried out with 4-day-old cells by use of conventional manometric procedures (8).

The elemental sulfur suspension for oxidation experiments was prepared as described by Suzuki (7). Details of the Warburg vessel mixtures are given in the figure legend. Nitrogen was determined with Nessler's reagent (10) following cell digestion.

RESULTS AND DISCUSSION

Table 1 shows the effect of the energy source utilized during the cultivation of the chemolithotroph on the oxidation of ferrous iron and elemental sulfur by resting cells. Cells grown on iron will oxidize the ferrous iron at a rate [$Q_{O_2}(N)/hr$] similar to that of cells that had been grown on sulfur (Fig. 1 and Table 1, ratio of 1.01). The development of activity in cells grown in the absence of a substrate concentration of iron in the sulfur medium, as well as the lack of a significant change in the rate of iron oxidation by cells transferred on sulfur for over a period of 5 months, clearly indicates that the iron oxidation system of *F. ferrooxidans* is of a constitutive nature. This cannot be said with certainty about the sulfur-oxidizing mechanisms. In all the experiments conducted, the rate of sulfur oxidation of iron-grown cells was about half [$Q_{O_2}(N)/hr$] that of the sulfur-grown cells (Table 1, ratio of 0.40). These results are believed to be significant, because they have been substantiated by experimental observations of iron-grown and sulfur-grown cells taken from four separate cell batches and by using the same batch of cells in repeat experiments. This would rule out variation in oxidation because of insolubility of the substrate.

Beck (1) observed somewhat increased $Q_{O_2}(N)$ values for the iron-oxidizing organism grown in the combined presence of iron and sulfur, but dismissed this as insignificant in comparison with values obtained for the iron oxidation of different cell batches. Unz and Lundgren (9), working both with *F. ferrooxidans* and *T. ferrooxidans*, noted an increased count in the number of iron-oxidizing organisms when cultivated in the presence of iron and sulfur. In all cases, the increased count was shown to occur after 3 to 4 days of a lag period which followed the depletion of ferrous iron. The increased count preceded sulfur oxidation. Evi-

TABLE 1. Comparison of iron and sulfur oxidation of iron-grown and sulfur-grown *Ferrobacillus ferrooxidans*^a

Cells	$Q_{O_2}(N)-Fe^{++b}$	$Q_{O_2}(N)-S^0$
Iron-grown.....	3367.9 \pm 230	312.8 \pm 13
Sulfur-grown....	3333.5 \pm 203	782.5 \pm 23

^a Ratio of iron-grown to sulfur-grown cells was 1.01, for iron oxidation; and 0.40, for sulfur oxidation.

^b $Q_{O_2}(N)-Fe^{++}$ was calculated by dividing the milligrams of cellular nitrogen into twice the number of microliters of O_2 taken up during the first 30 min. $Q_{O_2}(N)-S^0$ was calculated by dividing the milligrams of cellular nitrogen into the number of microliters of O_2 taken up during the second 60 min. These calculations were determined this way because of convenience, and the values are used for comparative purposes. In 50 min, the 125 μ moles of $FeSO_4$ are completely oxidized; however, when 250 μ moles are used, similar $Q_{O_2}(N)$ values are found under these experimental conditions. Sulfur-grown cells consistently show a slight delay before the oxidation rate is linear. We can not explain this; thus $Q_{O_2}(N)$ values were determined from the linear portion of the curve.

dently, inocula for all the aforementioned experiments were iron-grown cells. To obtain a culture growing chemolithotrophically on sulfur as the sole energy source, *F. ferrooxidans* had to be trained for a period of about 3 to 4 weeks.

Our results, however, are not in agreement with the above observations. Neither iron-grown nor sulfur-grown cells show any significant lag period when the respectively grown resting cells carried out the oxidation of both substrates, although the sulfur-grown cells showed significantly higher values for the sulfur oxidation (Fig. 1).

The training of *F. ferrooxidans* to grow chemolithotrophically on sulfur may lead to a significant change in the quantitative or perhaps even qualitative nature of its oxidative machinery. However, with regard to the sulfur-oxidizing system, this change is not of a dramatic nature. A twofold increase in the $Q_{O_2}(N)$, however significant, does not indicate the presence of a true inductive system. To explain these findings, the following hypothesis is suggested.

Ferrobacillus preferentially oxidized ferrous iron, not because of its free energy ($-\Delta F$ 11.3 vs. 118 kcal/g of atom for sulfur) (5), but because of its solubility. Thus ferrous iron could reach the active site of the iron-oxidizing system much more rapidly than the slightly soluble sulfur. However, with our limited knowledge about the active site of the sulfur-oxidizing system, it cannot be excluded that, in the presence of ferrous iron, sulfur

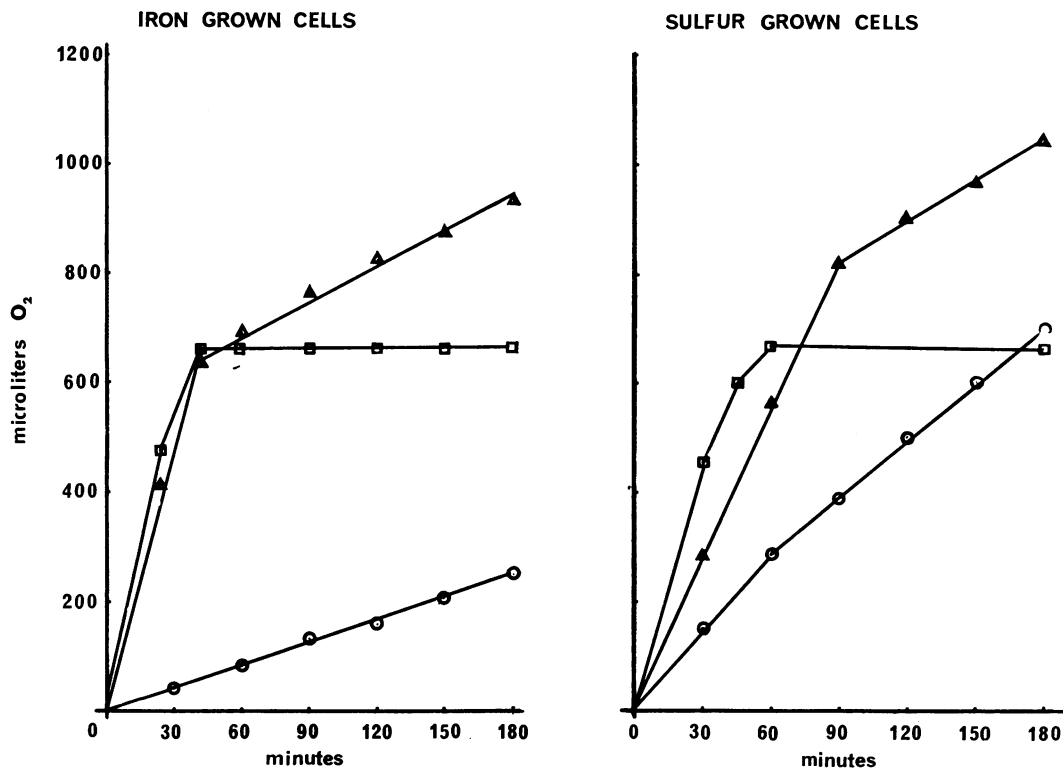


FIG. 1. Oxidation of ferrous iron and elemental sulfur by iron-grown and sulfur-grown *Ferrobacillus ferrooxidans*. Each Warburg vessel contained β -alanine- SO_4 buffer (pH 3.5) and a cell suspension (0.15 ml) containing 270.2 μg of N for iron cells and 271.5 μg of N for sulfur cells. Symbols: \square = 125 μmoles of FeSO_4 ; \circ = 48 mg of elemental sulfur; \triangle = 125 μmoles of FeSO_4 and 48 mg of elemental sulfur. The center well contained 0.2 ml of 20% KOH. Gas phase, air; temperature, 30 C; shaking rate, 148 oscillations/min with a 1.5-cm stroke. Equilibration time was 15 min before the cells were tipped into the main compartment from one of the side arms.

is also oxidized, as suggested by Beck's (1) experiments and our observations.

In the presence of both inorganic substrates ($\text{Fe}^{++} - \text{S}^0$), the iron-oxidation rate was decreased for both iron- and sulfur-grown cells; the effect was more noticeable with sulfur cells (Fig. 1). The observation was consistently shown with different cell batches. The reason for the inhibition is not known, but a speculation is that electrons from Fe^{++} and S^0 are competing for the same cytochrome pathway, assuming that sulfur is oxidized via sulfite through the sulfite-oxidase system of Charles and Suzuki (2).

The increased activity of the sulfur-oxidizing system of sulfur-grown cells may be due to the fact that the population of iron bacteria is heterogenous. The strain employed in these experiments was isolated from acid mine water several years ago by Leathen and associates (4) and has been maintained by repeated transfers in liquid mineral solutions containing a high concentration of ferrous iron. It is very unlikely

that, under these conditions of low pH (usually between 2.0 and 3.0), any organisms but chemolithotrophs could thrive. In fact, plating of these cultures on various nutrient media never revealed any contaminant. Also sulfur-oxidizing thio-bacilli are unlikely to develop under these conditions because of the absence of substrate amounts of reduced sulfur compounds in the 9K medium employed. However, even under these drastic environmental conditions, a heterogenous culture of iron-oxidizing bacteria may be involved. Assuming a nonidentity of sulfur- and iron-oxidizing sites (at least in the beginning of the electron transport chain), one might conceive of a cell population consisting of some cells, endowed with the potential of ferrous iron oxidation only, and other cells having, in addition, the sulfur-oxidizing system. In the absence of appreciable amounts of elemental sulfur and in an environment of ferrous compounds, there would be no advantage to either of the iron-oxidizing types. Under conditions of cultivation on elemental

sulfur, those cells able to oxidize sulfur would predominate, leading to an increased sulfur-oxidizing potential of the population without appreciably affecting its iron-oxidizing capacity. If this interpretation is correct, the observations on the necessity of "adapting" iron cells to sulfur substrates, or the so-called "lag period" following growth on iron in a medium containing mixed substrates, should be explained simply by the low oxidation rate of sulfur by a population that, only in part, is capable of iron and sulfur oxidation.

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