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Synthesis of an Iron-Oxidizing System during Growth of *Thiobacillus ferrooxidans* on Sulfur-Basal Salts Medium

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It was found that the de novo synthesis of not only sulfur:ferric ion oxidoreductase (ferric ion-reducing system) but also iron oxidase was absolutely required when *Thiobacillus ferrooxidans* AP19-3 was grown on sulfur-salts medium. The results strongly suggest that iron oxidase is involved in sulfur oxidation. This bacterium could not grow on sulfur-salts medium under anaerobic conditions with Fe^{3+} as a terminal electron acceptor, suggesting that energy conservation by electron transfer between elemental sulfur and Fe^{3+} is not available for this bacterium.

Thiobacillus ferrooxidans inhabits drainage in acid mines and plays a crucial role in the bacterial leaching of sulfide ores. The value of *T. ferrooxidans* in bacterial leaching may be due to the ability of this bacterium to oxidize both ferrous ion (Fe^{2+}) and reduced-sulfur compounds.

A new route for sulfur oxidation other than that previously reported (1, 3–5, 12–15) has been proposed in *T. ferrooxidans* AP19-3, in which elemental sulfur is oxidized via two enzyme systems, namely, the sulfur:ferric ion oxidoreductase system (ferric ion-reducing system) and the iron-oxidizing system, and Fe^{3+} and Fe^{2+} play a role as mediators between the two systems (6). The first enzyme involved in sulfur oxidation, sulfur:ferric ion oxidoreductase, has been purified to an electrophoretically homogeneous state (8), and evidence that this enzyme is involved in aerobic sulfur oxidation in this strain has been accumulated (9–11).

Recently, Corbett and Ingledew also observed the reduction of Fe^{3+} by elemental sulfur in intact cells of iron-grown *T. ferrooxidans* NCIB 8455 (2). They showed that the reduction of Fe^{3+} by elemental sulfur was inhibited 50% by ca. 20 μM 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HOQNO), a specific inhibitor of electron transfer in the cytochrome bc_1 segment of respiratory chains, and suggested that site two energy conservation in electron transfer reactions between S^0 and either Fe^{3+} or O_2 occurs in *T. ferrooxidans* (2).

The apparent molecular weight of sulfur:ferric ion oxidoreductase is 46,000, with two identical 23,000-M_r subunits (8). Our results with a partially purified sulfur:ferric ion oxidoreductase strongly suggested that cytochrome bc_1 is not involved in the reduction of Fe^{3+} by elemental sulfur and this enzyme because the enzyme activity was not inhibited by 40 μM HOQNO but was slightly stimulated (data not shown). The activity of the ferric ion-reducing system determined with washed intact cells of *T. ferrooxidans* AP19-3 decreased to 81.3, 83.7, and 79.0% in the presence of 10, 20, and 40 μM HOQNO, respectively (data not shown).

According to the mechanism proposed by Corbett and Ingledew (2), there is no need for the de novo synthesis of cellular components involved in Fe^{2+} oxidation, such as cytochrome *c*, rusticyanin, and other periplasmic components, when *T. ferrooxidans* is grown on sulfur-salts medium because the cytochrome bc_1 complex and cytochrome oxidase contribute to the sulfur-oxidizing system under aerobic conditions. In contrast, according to our sulfur-oxidizing mechanism, the de novo synthesis of both the ferric ion-reducing system (sulfur:ferric ion oxidoreductase) and the iron-oxidizing system must occur in the cells during growth on sulfur-salts medium because these systems form part of the sulfur-oxidizing system of strain AP19-3. In this work we show that the de novo synthesis of both enzyme systems is absolutely required during the growth of *T. ferrooxidans* AP19-3 on sulfur-salts medium.

MATERIALS AND METHODS

The sulfur-salts medium used in this study consisted of elemental sulfur (1 g), Fe^{3+} (0.5 mM), and a basal salts solution (100 ml) (7). Elemental sulfur was sterilized by being heated for 30 min at 100°C and was incubated for 24 h at 30°C. The sterilization process was done three times, and elemental sulfur was added to the autoclaved salts solution before inoculation. *T. ferrooxidans* AP19-3 subcultured on sulfur-salts medium 53 times was inoculated asceptically into 100 ml of sulfur-salts medium. Growth experiments were performed by shaking 100 ml of inoculated medium in a 500-ml shake flask at 30°C. Sixty-six flasks were cultured to obtain sufficient cells for determining the activities of both the iron-oxidizing and ferric ion-reducing systems. The growth rate was determined by counting cells by a previously described method (7). After being shaken for various times (see Fig. 1), the cultures were passed through Toyo no. 5C filter paper to separate the cells from elemental sulfur, centrifuged at 10,000 × *g* for 20 min, and washed three times with 0.1 M β-alanine–SO_4^{2-} buffer (pH 3.0). The activities of both the iron-oxidizing and ferric ion-reducing systems were determined by previously described methods (6).

RESULTS AND DISCUSSION

The specific activity of the iron-oxidizing system markedly increased during the growth of *T. ferrooxidans* AP19-3 on sulfur-salts medium (Fig. 1). After 3 days of cultivation, the activity per l mg of cell protein was ca. eightfold higher than that in inoculated cells. The specific activity of the ferric ion-reducing system also increased during growth of this strain on sulfur-salts medium. After 3 days of cultivation, the activity per l mg of cell protein was ca. 2.5-fold higher than that in inoculated cells.

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After the maximum specific activity was reached, both of the activities rapidly decreased to the levels in inoculated cells. When washed cells were shaken in an acidic salts solution without elemental sulfur, both of the activities rapidly decreased, and the amount of decrease in both activities was greater, especially below pH 2.0 (data not shown). On the basis of these results, it is possible that the rapid decrease in both enzyme activities observed in Fig. 1 was due to the sulfuric acid produced by sulfur oxidation. The pH of the medium after 15 days of cultivation was 1.2.

The eightfold increase in the specific activity of iron oxidase during the growth of *T. ferrooxidans* AP19-3 on sulfur-salts medium strongly suggests that the de novo synthesis of not only the ferric ion-reducing system but also the iron-oxidizing system was absolutely required when the cells were grown on sulfur-salts medium. We previously showed that *T. ferrooxidans* AP19-3 subcultured 22 times on sulfur-salts medium had 62.5% of the iron-oxidizing activity of iron-grown cells and grew on iron-salts medium without a lag phase (9). These results also support the idea that iron oxidase is constitutive when *T. ferrooxidans* AP19-3 is grown on sulfur-salts medium. It is difficult to explain this de novo synthesis of iron oxidase during the growth of *T. ferrooxidans* AP19-3 on sulfur-salts medium by the mechanism of Corbett and Ingledew (2).

Under aerobic conditions in the presence of cyanide, which inhibits cytochrome oxidase and as a result inhibits the iron-oxidizing system of *T. ferrooxidans*, strain AP19-3 accumulated Fe^{2+} in the reaction mixture during the oxidation of elemental sulfur (6). When intact cells of this strain were incubated under aerobic conditions with elemental sulfur at a pH at which the iron oxidase of the cell scarcely operates (above pH 5.0), Fe^{2+} and sulfite accumulated in the reaction mixture (10). The production of Fe^{2+} during aerobic sulfur oxidation was observed when intact cells of *T. ferrooxidans* AP19-3 were incubated with elemental sulfur in the presence of *o*-phenanthroline, a chelator for Fe^{2+} (9). Growth on sulfur-salts medium and sulfur-oxidizing activity were completely inhibited by 5 and 50 mM *o*-phenanthroline, respectively (9). These results indicate that Fe^{2+} is produced as an intermediate during aerobic sulfur oxidation by this strain. If *T. ferrooxidans* has the ability to produce Fe^{2+} as an intermediate during aerobic sulfur oxidation, no matter how little, then iron oxidase is probably involved in sulfur oxidation.

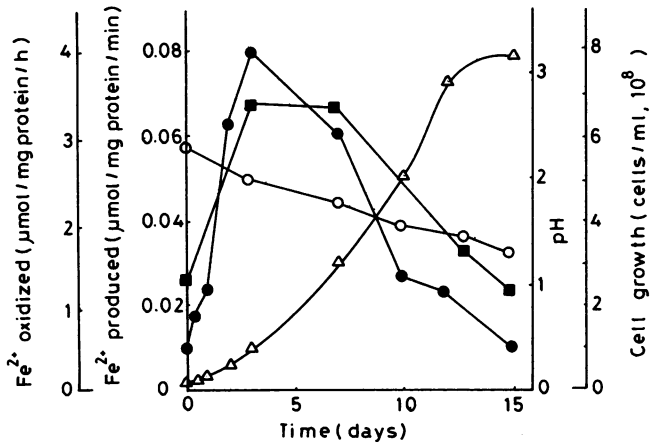

FIG. 1. Synthesis of iron-oxidizing and ferric ion-reducing systems during growth of *T. ferrooxidans* AP19-3 on sulfur-salts medium. The composition of the sulfur-salts medium and the methods used for analysis are described in the text. Symbols: △, cell growth; ○, pH of the medium; ●, specific activity of the iron-oxidizing system; ■, specific activity of the ferric ion-reducing system (sulfur:ferric ion oxidoreductase).

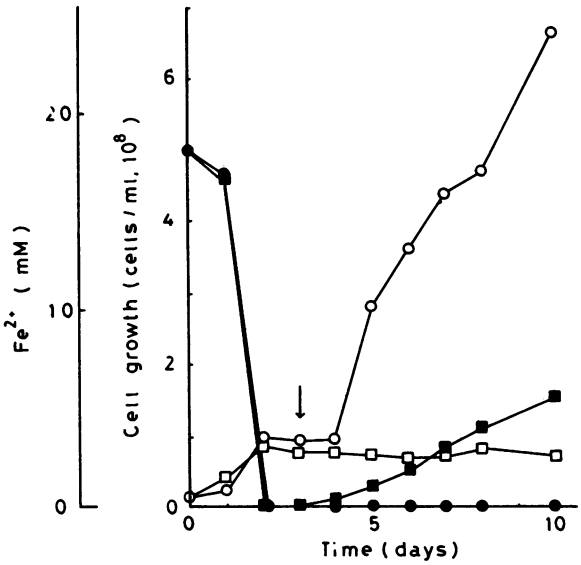

Fig. 2. Attempted anaerobic growth of *T. ferrooxidans* AP19-3 with a ferric ion as the terminal electron acceptor. *T. ferrooxidans* AP19-3 was grown on Fe^{2+} (18 mM)-salts medium at 30°C. At the point indicated by the arrow, at which Fe^{2+} in the medium was completely oxidized to Fe^{3+}, elemental sulfur (1 g) and $NaHCO_3$ (0.2 g) were added. The headspace of one of the cultures was purged with nitrogen gas and incubated anaerobically with Fe^{3+} as the terminal electron acceptor at 30°C; the other was incubated aerobically at 30°C. The amount of Fe^{2+} produced in the medium and the growth rate were determined by a previously described method (7). Symbols: ○, number of cells in the medium incubated under aerobic conditions; □, number of cells in the medium incubated under anaerobic conditions; ●, Fe^{2+} content in the medium incubated under aerobic conditions; ■, Fe^{2+} content in the medium incubated under anaerobic conditions.

The possibility of anaerobic growth of *T. ferrooxidans* AP19-3 with Fe^{3+} as a terminal electron acceptor was studied. Under anaerobic conditions in the presence of elemental sulfur and Fe^{3+}, Fe^{2+} was produced during incubation with the cells, but cell growth was not observed (Fig. 2). In contrast, under aerobic conditions in the presence of elemental sulfur and Fe^{3+}, normal cell growth on sulfur-salts medium was observed, but Fe^{2+} production was not. These results indicate that *T. ferrooxidans* AP19-3 cannot grow on sulfur-salts medium with Fe^{3+} as a terminal electron acceptor, namely, site two energy conservation in electron transfer reactions between S^0 and Fe^{3+} is not available for this bacterium.

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