Existence of Hydrogen Sulfide in Iron-salts Grown *Thiobacillus ferrooxidans*

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Summary

It was found that iron-salts grown cells of *Thiobacillus ferrooxidans*, *Leptospirillum ferrooxidans*, and moderately thermophilic iron-oxidizing bacterial strain TH3 had 2.1 ~3.7 nmol hydrogen sulfide per mg of cell protein. The hydrogen sulfide in *T. ferrooxidans* AP19-3 cells was oxidized by hydrogen sulfide: ferric ion oxidoreductase in this bacterium with Fe^{3+} as an electron acceptor to produce Fe^{2+}. [^{14}C]-CO_2 was taken into the cells concomitantly with the oxidation of Fe^{2+} by iron oxidase. Since carbon dioxide fixation is absolutely dependent on energy supplied to the cells, the results suggest that hydrogen sulfide in *T. ferrooxidans* plays a role as an energy reserve.

Introduction

The iron-oxidizing bacterium *Thiobacillus ferrooxidans* is one of the most important bacteria for the leaching of sulfide ores.[3,15] This leaching ability is due to *T. ferrooxidans*' possession of unique enzyme systems that can oxidize both Fe^{2+} and reduced sulfur compounds. Thus, to breed valuable strains for industrial usage it is important to study the mechanism of both iron- and sulfur-oxidations in *T. ferrooxidans*. Recently, it was found that *T. ferrooxidans* has the ability not only to oxidize metal ions but also to reduce them[4,8,9,17-19]. Two unique enzymes, hydrogen sulfide: ferric ion oxidoreductase (SFORase) and sulfite: ferric ion oxidoreductase, which use Fe^{3+} as an electron acceptor for the oxidation of sulfide and sulfite ions, respectively, were purified from *T. ferrooxidans* AP19-3.[10,13,14] And a new route for sulfur oxidation other than that previously reported[5,6,21] has been proposed in *T. ferrooxidans* AP19-3[7,8,12]. SFORase was found to be present not only in *T. ferrooxidans* but also in *Leptospirillum ferrooxidans* and the moderately thermophilic iron-oxidizing bacteria[20], suggesting a wide distribution of this enzyme in iron-oxidizing bacteria. The actual substrate for SFORase in solid elemental sulfur oxidation by *T. ferrooxidans* AP19-3 was found to be hydrogen sulfide[12]. And a novel functional protein or hydrogen sulfide-binding protein (SBP), which reversibly binds hydrogen sulfide and supplies SFORase with hydrogen sulfide as a substrate, was recently isolated from the plasma membrane of *T. ferrooxidans* AP19-3[16]. The existence of SBP strongly suggests that hydrogen sulfide is synthesized in *T. ferrooxidans* cells. In this work, we show that iron-salts grown *T. ferrooxidans* cells synthesized hydrogen sulfide in the cells, and the hydrogen sulfide was oxidized by SFORase with Fe^{3+} as an electron acceptor to produce Fe^{2+}. Evidence that hydrogen sulfide in this bacterium was used as an energy source for carbon dioxide fixation is also presented. This is the first report that shows that hydrogen sulfide plays a role as an energy reserve in *T. ferrooxidans*.

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Materials and Methods

Microorganism and medium

The iron-oxidizing bacteria used in this study were: *Thiobacillus ferrooxidans* strains ATCC 33020, 13598, 19859, 21834, and 23270 from the American Type Culture Collection; *T. ferrooxidans* strain AP19-3[8]; *Leptospirillum ferrooxidans* P3A, and BKM-6-1339[20]; and the moderately thermophilic iron-oxidizing bacterial strain TH3.[20] Heterotrophic bacteria, such as *Escherichia coli* strains K-12, HB101 and JM109, *Acidiphilium facilis*, *Clostridium acetobutylicum* IFO 13948, and *Pseudomonas putida* ICRA 3460, were also used. The composition of the iron-salts medium used for cultivation of iron-oxidizing bacteria was described previously[11].

Determination of hydrogen sulfide content in the cells

Sulfide sulfur was measured by the methylene blue method.[1] N, N-dimethyl-p-phenylenediamine sulfate (400 mg) and ferric chloride (600 mg) were dissolved in 100 ml of 6 M HCl. Diamine-ferric chloride solution was prepared by mixing equal volumes of the two above-described solutions just before analysis. The diamine-ferric chloride solution (0.5 ml) was added to 2 ml of intact cell suspension (10 mg protein), incubated for 1 h at 30 °C, and centrifuged at 14,000×*g* for 5 min to separate cells. The blue color resulting from the production of methylene blue was measured by a Shimadzu UV-140 spectrophotometer at 670 nm. A linearity was obtained between hydrogen sulfide concentration (0—10 nmol hydrogen sulfide per ml) and absorbance at 670 nm.

Results

Existence of reduced sulfur compound in iron-salts grown T. ferrooxidans

The iron-oxidizing bacterium *T. ferrooxidans* has a unique enzyme, hydrogen sulfide: ferric ion oxidoreductase (SFORase) that oxidizes elemental sulfur (S^0) in the presence of glutathione (GSH) with Fe^{3+} as an electron acceptor to produce Fe^{2+} and sulfite ions[8,14]. When the cells of iron-salts grown *T. ferrooxidans* AP19-3 were incubated under aerobic conditions in the presence of S^0, Fe^{3+}, and sodium cyanide, Fe^{2+} was produced in the reaction mixture by the catalytic action of SFORase (Fig. 1)[8]. Interestingly, a large amount of Fe^{2+} was also produced when the cells were incubated with Fe^{3+} and sodium cyanide, but without S^0 (Fig. 1), suggesting that a reduced sulfur compound, which is used as an electron donor for SFORase, was present in the cells. The amount of Fe^{2+} produced in the presence of S^0 was larger than in its absence, suggesting that in the presence of S^0, SFORase used both S^0 and the reduced sulfur compound inside the cells as electron donor to produce Fe^{2+}. No Fe^{2+} was produced when Fe^{3+} was absent in the reaction mixture. Thus we tentatively named this Fe^{2+} production reaction observed in the absence of S^0, S^0-independent Fe^{2+} production. S^0-independent Fe^{2+} production was also observed under anaerobic conditions in the absence of sodium cyanide (Fig. 2).

The amount of hydrogen sulfide in T. ferrooxidans AP19-3 cells

Since hydrogen sulfide has been identified as an actual substrate of SFORase during the oxidation of S^0 [12], the unknown reduced sulfur compound found in *T. ferrooxidans*

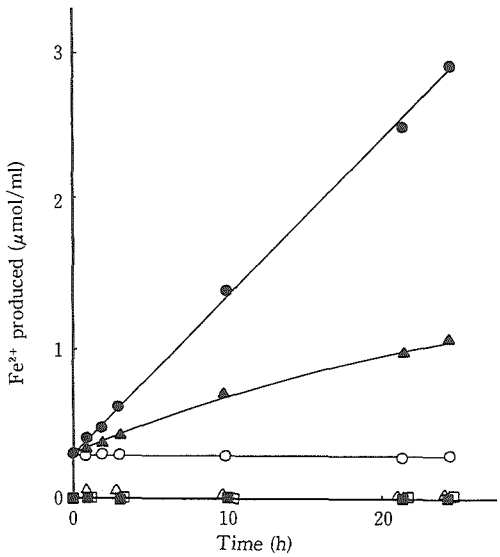

Fig. 1 Production of ferrous ion by *T. ferrooxidans* AP19-3 cells under aerobic conditions.

The reaction mixture was as follows: 9 ml of 0.1 M β-alanine-SO_4^{2-} buffer (pH 3.0); elemental sulfur, 200 mg; Fe^{3+}, 100 μmol; and washed intact cells, 5 mg of protein; sodium cyanide, 50 μmol. Total volume was 10.0 ml. Symbols: Fe^{2+} production in the reaction mixture containing cells, elemental sulfer, and Fe^{3+} (●), cells and Fe^{3+} (▲), cells (■), 10 min-boiled cells, elemental sulfur, and Fe^{3+} (○), 10 min-boiled cells and Fe^{3+} (△), 10 min-boiled cells (□).

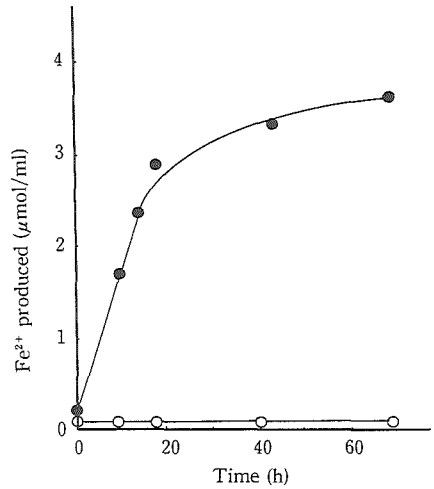

Fig. 2 Production of ferrous ion by *T. ferrooxidans* AP19-3 under anaerobic conditions.

The composition of the reaction mixture was as follows: 9 ml of 0.1 M β-alanine-SO_4^{2-} buffer (pH 3.0); Fe^{3+}, 100 μmol; and washed intact cells, 20 mg of protein. The total volume was 10.0 ml. The reaction mixture was bubbled with nitrogen gas through a narrow glass tube in an ice bath for 10 min. After the glass tube was sealed, reaction was started. Symbols: Fe^{2+} production in the reaction mixture containing cells and Fe^{3+} (●), and 10 min-boiled cells and Fe^{3+} (○).

AP19-3 cells seems to be hydrogen sulfide. Thus we measured the amount of hydrogen sulfide in the cells of *T. ferrooxidans*, *L. ferrooxidans*, and some heterotrophs by the methylene blue method (Table 1). Iron-salts grown cells of *T. ferrooxidans* AP19-3 had 2.4 nmol hydrogen sulfide per mg of cell protein. Interestingly, all of the iron-oxidizing bacteria tested had about 4～18 times more hydrogen sulfide than the heterotrophs, such as *E. coli*, *A. facilis*, *C. acetobutylicum*, and *P. putida*.

When *T. ferrooxidans* AP19-3 cells were incubated under aerobic conditions with Fe^{3+} and sodium cyanide, the hydrogen sulfide in the cells decreased concomitantly with the production of Fe^{2+} (Fig. 3). When the cells were treated with potassium permanganate for 1.5 h, the hydrogen sulfide decreased in approximately one fifth of the control cells (Fig. 3), suggesting that hydrogen sulfide was chemically oxidized by permanganate. The activity of S^0-independent Fe^{2+} production reaction in permanganate-treated cells markedly decreased. From the results we concluded that the reduced sulfur compound found in *T. ferrooxidans* AP19-3 cells was hydrogen sulfide.

Table 1 The amount of hydrogen sulfide in the cells

Bacteria	H_2S[a] (nmol/mg protein)	Bacteria	H_2S[a] (nmol/mg protein)
T. ferrooxidans AP19-3	2.4	*Leptospirillum ferrooxidans* BKM-6-1339	3.7
T. ferrooxidans ATCC 33020	2.3	Moderately thermophilic iron-oxidizing bacterium TH3	2.3
T. ferrooxidans ATCC 13598	2.2	*Escherichia coli* K12	0.5
T. ferrooxidans ATCC 19859	2.1	*Escherichia coli* HB101	0.5
T. ferrooxidans ATCC 21834	2.0	*Escherichia coli* JM109	0.5
T. ferrooxidans ATCC 23270	2.1	*Acidiphilium facilis*	0.2
Leptospirillum ferrooxidans P3A	3.1	*Clostridium acetobutylicum* IFO 13948	0.6
		Pseudomonas putida ICRA 3460	0.2

a) The method for analysis of hydrogen sulfide in washed intact cells was described in Materials and Methods. Values shown are the means of duplicate experiments.

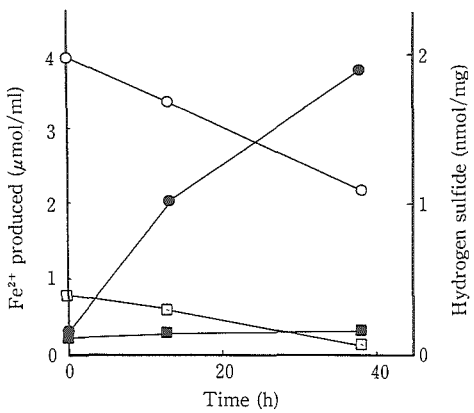

Fig. 3 Production of ferrous ion by permanganate-treated cells of *T. ferrooxidans* AP19-3.
T. ferrooxidans AP19-3 cells (15 mg of protein) were treated with 30 ml of 0.1 mM potassium permanganate for 1 h, washed with 0.1 M β-alanine-SO_4^{2-} to remove the reagent. Production of Fe^{2+} was determined under aerobic conditions. The composition of reaction mixture was as follows: 9 ml of 0.1 M β-alanine-SO_4^{2-} buffer (pH 3.0); permanganate-treated cells or control cells without treatment, 10 mg of protein; Fe^{3+}, 100 μmol; sodium cyanide, 50 μmol. Total volume was 10.0 ml. Symbols: Fe^{2+} production by permanganate-treated cells (■); Fe^{2+} production by control cells wihtout treatment (●); H_2S content of permanganate-treated cells (□); H_2S content of control cells without treatment (○).

Carbon dioxide fixation by using hydrogen sulfide as an energy source

Whether the hydrogen sulfide in *T. ferrooxidans* AP19-3 cells can be used as a biological energy source for this bacterium was studied by measuring the activity of [^{14}C]-CO_2 uptake into the cells, because CO_2 fixation is absolutely dependent on energy supplied to the cells.[2] Ferrous iron was produced anaerobically through a S^0-independent Fe^{2+} production reaction. The time course of Fe^{2+} production is shown in Fig. 2. Part of the reaction mixture (5 ml) was removed periodically into 30 ml of a flask. After adding [^{14}C] Na_2CO_2 (1 μCi) and cold Na_2CO_3 (10 nmol), the flask was sealed with a silicon stopper and incubated under aerobic conditions at 30 °C for 30 min. After the reaction was stopped by adding mercuric chloride, the reaction mixture was centrifuged at 14,000×g for 10 min. The concentration of Fe^{2+} in the reaction mixture removed after 0, 15, and 70 h of incubation was 0.0, 0.0 and 0.14 μmol/ml, respectively, indicating that Fe^{2+} produced by S^0-independent Fe^{2+} production reaction was oxidized by iron oxidase

under aerobic conditions. The cells harvested by centrifugation were washed, and their radioactivity was measured by using an Aloka-LSC-liquid scintillation system.[2] The amounts of carbon dioxide fixed into the cells were 0.000, 0.012, and 0.017 μmol/mg protein·min for the cells removed after 0, 15, and 70 h incubation, respectively. The stoichiometry between the amounts of Fe^{2+} oxidized and CO_2 fixed by the cells corresponds well with the results obtained previously in *T. ferrooxidans* AP19-3, in which ferrous sulfate was used as an energy source for CO_2 fixation.[2] These results indicate that hydrogen sulfide observed in *T. ferrooxidans* AP19-3 cells was used as an energy source for CO_2 fixation.

Discussion

It was found that iron-oxidizing bacteria, *T. ferrooxidans*, *L. ferrooxidans*, and moderately thermophilic iron-oxidizing bacterial strain TH3, had about 4~18 times more hydrogen sulfide in the cells than the heterotrophs tested. The results suggest the special role of hydrogen sulfide in the metabolism of iron-oxidizing bacteria. In the presence of Fe^{3+}, the hydrogen sulfide in *T. ferrooxidans* AP19-3 cells was oxidized by SFORase to produce Fe^{2+}. Under aerobic conditions, the Fe^{2+} thus produced was oxidized by iron oxidase and $[^{14}C]$-CO_2 was fixed into the cells concomitantly with the Fe^{2+} oxidation. Since CO_2 fixation depends absolutely on energy supplied to the cells, the results strongly suggest that hydrogen sulfide found in *T. ferrooxidans* AP19-3 plays a role as an energy reserve. In this text we propose the existence of a new type of energy reserve compound, hydrogen sulfide, for *T. ferrooxidans*.

Recently, we showed that various kinds of iron-oxidizing bacteria have SFORase in the cells[20]. Since the discovery of SFORase in *T. ferrooxidans* AP19-3 cells[8], we have been attracted to an interesting problem: why SFORase or the enzyme involved in elemental sulfur oxidation is constitutively synthesized in not only sulfur-salts grown but also iron-salts grown cells ? The finding that hydrogen sulfide is present in iron-salts grown *T. ferrooxidans* AP19-3 cells supplies an answer to this question. That is, iron-oxidizing bacteria seem to have SFORase to oxidize hydrogen sulfide in the cells to produce energy for growth and/or cell maintenance. The mechanism of how *T. ferrooxidans* AP19-3 cells synthesize hydrogen sulfide is now under investigation.

Acknowledgements

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鉄酸化細菌 *T. ferrooxidans* におけるサルファイドの存在

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二価鉄—無機塩培地で生育した鉄酸化細菌 *Thiobacillus ferrooxidans*, *Leptospirillum ferrooxidans*, 中等度好熱性鉄酸化細菌細胞中に, 細胞タンパク質1mg当たり2.1～3.7nmolのサルファイドが存在していることを明らかにした. *T. ferrooxidans* AP19-3株中のサルファイドは, 本菌の硫黄酸化に関与する酵素 hydrogen sulfide: ferric ion oxidoreductase によって, 三価鉄（Fe^{3+}）を電子受容体にして酸化され二価鉄（Fe^{2+}）を生成した. このようにして生成された Fe^{2+} は, 鉄酸化酵素によって酸化され, これに伴って $[^{14}C]$-CO_2 が細胞内に取り込まれた. 炭酸ガスの固定反応はエネルギーの供給に絶対的に依存しているので, 今回得られた結果は, *T. ferrooxidans* 中に合成されたサルフィイドが貯蔵物質としての役割を持っていることを示唆している.