Sensitivity of Iron-Oxidizing Bacteria, *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*, to Bisulfite Ion

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When grown on iron-salt medium supplemented with the bisulfite ion, *Leptospirillum ferrooxidans* was much more sensitive to the ion than was *Thiobacillus ferrooxidans*. The causes of the sensitivity of *L. ferrooxidans* to the bisulfite ion were studied. The bisulfite ion completely inhibited the iron-oxidizing activities of *L. ferrooxidans* and *T. ferrooxidans* at 0.02 and 0.2 mM, respectively. A trapping reagent for the bisulfite ion, formaldehyde, completely reversed the inhibition. The treatment of intact cells with 1.0 mM bisulfite ion for 1 h and washing the bisulfite ion from the cells had no harmful effects on the iron-oxidizing activity of *T. ferrooxidans*. However, the treatment of *L. ferrooxidans* with 0.1 mM bisulfite ion for 1 h completely destroyed the iron-oxidizing activity. *T. ferrooxidans* had sulfite:ferric ion oxidoreductase activity. In contrast, a quite low level of sulfite:ferric ion oxidoreductase activity was found in *L. ferrooxidans*, suggesting that it is much more difficult for *L. ferrooxidans* to oxidize the bisulfite ion to the less harmful sulfate than it is for *T. ferrooxidans*. These results suggest that the sensitivity of *L. ferrooxidans* to the bisulfite ion is due to a lack of an active sulfite:ferric ion oxidoreductase and the sensitivity of its iron oxidase to bisulfite ion.

Thiobacillus ferrooxidans and *Leptospirillum ferrooxidans* are known as acidophilic chemolithotrophic iron-oxidizing bacteria. *T. ferrooxidans* has the ability to oxidize not only ferrous ion but also reduced sulfur compounds, and the ability to oxidize two different types of reduced inorganic compounds makes the bacterium a valuable microorganism for bacterial leaching. However, no strains of *L. ferrooxidans* have been reported to grow on sulfur-salt medium, although they grow on, and efficiently degrade, pyrite (4). Recently, we showed that not only *T. ferrooxidans* but also *L. ferrooxidans* has a hydrogen sulfide:ferric ion oxidoreductase (SFORase) (6, 10-13, 15, 16), which catalyzes the oxidation of elemental sulfur with Fe^{3+} as an electron acceptor to give Fe^{2+} and the bisulfite ion. To understand the basic physiology of *L. ferrooxidans* and to apply the bacterium to bacterial leaching, it is quite important to study the reason *L. ferrooxidans* strains failed to grow on sulfur-salt medium even though they had the enzymes involved in sulfur oxidation. We have shown that approximately 0.02 to 0.05 mM bisulfite ion, harmful to these iron-oxidizing bacteria, is produced as an intermediate during sulfur oxidation by SFORase not only in *T. ferrooxidans* but also in *L. ferrooxidans* (1, 8, 14, 16). Thus, to find a clue towards solving this complicated but interesting physiological problem in iron-oxidizing bacteria, we first compared the sensitivities of iron-oxidizing bacteria to the bisulfite ion. In this work, we show that *L. ferrooxidans* had a much lower level of sulfite:ferric ion oxidoreductase activity and an iron-oxidizing activity which was much more sensitive to the bisulfite ion than did *T. ferrooxidans*.

MATERIALS AND METHODS

Microorganisms, medium, and conditions of cultivation.

The iron-oxidizing bacteria used were as follows: *T. ferrooxidans* ATCC 13598, ATCC 13661, ATCC 14119, ATCC 19859, ATCC 21834, ATCC 23270, and ATCC 33020 from the American Type Culture Collection; *T. ferrooxidans* AP19-3 (7); *L. ferrooxidans* DSM 2391 from the Deutsche Sammlung von Mikroorganismen; *L. ferrooxidans* P3A and BKM-6-1339 from Wolfgang Sand, University of Hamburg, Hamburg, Germany. *T. ferrooxidans* and *L. ferrooxidans* were grown at 30°C on ferrous sulfate (3%)-salt medium at pH 2.5. The composition of the basal salt solution used throughout this study was described previously (16).

Iron-oxidizing activity. Iron-oxidizing activity was determined by the following two methods. (i) It was measured by the decrease of Fe^{2+} in the reaction mixture by the *o*-phenanthroline method (5). The composition of the reaction mixture was as follows: 8 ml of 0.1 M β -alanine- SO_4^{2-} buffer (pH 3.0), 2 mg of protein from washed intact cells of *L. ferrooxidans* or *T. ferrooxidans*, and 160 μmol of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. The total volume was 10.0 ml. The reaction was carried out by shaking the reaction mixture at 30°C. (ii) Activity was measured by oxygen uptake, caused by the oxidation of Fe^{2+} , in a Warburg manometer. Each vessel contained 3.0 ml of reaction mixture plus 0.2 ml of 20% sodium hydroxide in the center well. The gas phase was air, and the temperature was 30°C. The composition of the reaction mixture was as follows (in 3.0 ml): 0.1 M β -alanine- SO_4^{2-} buffer (pH 3.0), 0.5 mg of protein from intact cells, and 100 μmol of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$.

Sulfite:ferric ion oxidoreductase activity. The composition of the reaction mixture used for determining sulfite:ferric ion oxidoreductase activity was as follows (11): 4 ml of 0.1 M sodium citrate buffer (pH 6.5), 5 mg of protein from washed intact cells of *L. ferrooxidans* or *T. ferrooxidans*, and 0.38 μmol of sodium sulfite. The total volume was 5 ml. When 2 mg of protein of cell extract was used as an enzyme source, 1 μmol of sodium sulfite was used. The reaction was carried out at 30°C

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TABLE 1. Differences in sensitivity to the bisulfite ion of various strains

Strain	Sulfite-oxidizing activity (nmol/mg/min)		Activity after bisulfite treatment							
			Iron-oxidizing activity ($\mu\text{mol/mg/min}$) ^a				SFORase activity (nmol/mg/min) ^b			
	Intact cells	Cell extract	0 ^c	0.1	0.5	1.0	0	0.1	0.5	1.0
<i>T. ferrooxidans</i>										
ATCC 13598	0.09	ND	1.2	0.9	0.7	0.7	9.5	3.4	2.4	1.9
ATCC 13661	0.04	ND	1.5	1.6	1.4	1.3	11.7	5.6	2.1	2.1
ATCC 19859	0.07	ND	0.7	0.6	0.6	0.6	9.1	1.5	0.4	0.4
ATCC 21834	0.12	ND	1.7	1.6	1.3	1.2	12.2	3.5	ND	1.8
ATCC 23270	0.10	ND	1.5	1.1	1.1	0.4	13.9	7.3	ND	3.5
ATCC 14119	ND	ND	1.5	1.5	1.3	0.9	8.1	4.2	2.2	1.1
ATCC 33020	0.04	ND	0.8	0.7	0.6	0.6	9.1	1.6	0.9	0.9
AP19-3	0.11	2.6	0.8	0.7	0.8	0.6	9.8	2.1	1.5	1.2
<i>L. ferrooxidans</i>										
DSM 2391	0.01	ND	ND	ND	ND	ND	4.8	4.8	4.9	4.8
P3A	0.01	0.7	0.9	0.0	0.0	0.0	6.3	6.5	6.7	6.6
BKM-6-1339	0.01	0.5	0.8	0.0	0.0	0.0	4.5	4.5	4.6	4.5

^a Iron-oxidizing activity was determined with intact cells by measuring the decrease of Fe^{2+} in the reaction mixture.

^b SFORase activity was determined with intact cells by measuring the increase of Fe^{2+} in the reaction mixture with elemental sulfur as an electron donor.

^c Bisulfite ion concentration (millimolar).

on a reciprocal shaker. A sample of the reaction mixture (0.5 ml) was withdrawn and centrifuged at $10,000 \times g$ for 2 min, and the concentration of the bisulfite ion in the supernatant solution thus obtained (0.2 ml) was determined spectrophotometrically by the pararosaniline method (17). The amount of chemically oxidized bisulfite ion was always verified with a reaction mixture containing intact cells or cell extract boiled for 10 min instead of the native one.

SFORase activity. The SFORase activity was determined with intact cells by measuring the amount of Fe^{2+} produced in the reaction mixture. The composition of the reaction mixture used for aerobic Fe^{3+} reduction by elemental sulfur was as follows: 9 ml of 0.1 M β -alanine- SO_4^{2-} buffer (pH 3.0), 200 mg of elemental sulfur, 100 μmol of Fe^{3+} , 5 mg of protein from intact cells, and 50 μmol of sodium cyanide. The total volume was 10.0 ml. A sample of the reaction mixture was withdrawn and centrifuged at $12,000 \times g$ for 2 min to remove elemental sulfur and cells, and the concentration of ferrous ion in the supernatant solution was measured colorimetrically by the *o*-phenanthroline method (5).

Protein content. Protein content was determined by the biuret method (2) or the method of Lowry et al. (3) with crystalline bovine serum albumin as the reference protein.

RESULTS

Effect of bisulfite ion on the growth of *L. ferrooxidans* and *T. ferrooxidans*. *T. ferrooxidans* ATCC 13598, ATCC 13661, ATCC 14119, ATCC 19859, ATCC 21834, ATCC 23270, and ATCC 33020 and *T. ferrooxidans* AP19-3 grew without a lag phase on Fe^{2+} (3%)-salt medium supplemented with 2 mM sodium sulfite at 30°C (data not shown). Cell growth of *T. ferrooxidans* AP19-3 on Fe^{2+} (3%)-salt medium was not affected by 5 mM sodium sulfite (8). In contrast, cell growth of *L. ferrooxidans* DSM 2391, *L. ferrooxidans* BKM-6-1339, and *L. ferrooxidans* P3A was not observed in the medium supplemented with 2 mM sodium sulfite, suggesting that *L. ferrooxidans* was much more sensitive to the bisulfite ion than was *T. ferrooxidans*.

Species differ in sensitivity to the bisulfite ion. Approximately 0.02 to 0.05 mM of bisulfite ion was produced during oxidation of elemental sulfur by *T. ferrooxidans* AP19-3. Sulfite:ferrous ion oxidoreductase, which catalyzes oxidation of the

bisulfite ion with Fe^{3+} as an electron acceptor to give sulfate and ferrous ions, has been purified to an electrophoretically homogeneous state from the plasma membrane of *T. ferrooxidans* AP19-3. Two physiological roles of this enzyme were proposed (9, 11). One of them is detoxification of a harmful bisulfite ion to a less harmful sulfate. To study the sensitivity of iron-oxidizing bacteria to the bisulfite ion, sulfite:ferrous ion oxidoreductase activity was studied with intact cells and cell extracts. Intact cells of *T. ferrooxidans* ATCC 13598, ATCC 19859, ATCC 21834, and ATCC 23270 had sulfite:ferrous ion oxidoreductase activity comparable to that of *T. ferrooxidans* AP19-3 (Table 1). *T. ferrooxidans* ATCC 13661 and ATCC 33020 had 36% of the sulfite:ferrous ion oxidoreductase activity found in *T. ferrooxidans* AP19-3. Interestingly, all of the *L. ferrooxidans* strains tested showed only 9% of the sulfite:ferrous ion oxidoreductase activity found in *T. ferrooxidans* AP19-3. The cell extracts from *L. ferrooxidans* P3A and BKM-6-1339 had 27 and 19%, respectively, of the sulfite:ferrous ion oxidoreductase activities found in cell extracts of *T. ferrooxidans* AP19-3. These results suggest that the rate of decomposition of the bisulfite ion is much slower in *L. ferrooxidans* than in *T. ferrooxidans*.

The effect of bisulfite treatment on iron-oxidizing activity was studied with intact cells of *L. ferrooxidans* and *T. ferrooxidans*. The cells were incubated in 0.1 M β -alanine- SO_4^{2-} buffer (pH 3.0) containing 0.1, 0.5, and 1.0 mM sodium sulfite for 1 h at 30°C. After 1 h of incubation with the bisulfite ion, these cells were washed three times with 0.1 M β -alanine- SO_4^{2-} buffer (pH 3.0) to remove the bisulfite ion, and then the activity of iron oxidase was measured. Most of the iron-oxidizing activity remained after 0.1 mM bisulfite treatment in all of the *T. ferrooxidans* strains tested (Table 1). In contrast, iron-oxidizing activities of *L. ferrooxidans* P3A and BKM-6-1339 were completely lost by this treatment (Table 1). Even after 1.0 mM bisulfite treatment, 58, 87, 86, 71, 27, 60, 75, and 75% of iron-oxidizing activity remained in *T. ferrooxidans* ATCC 13598, ATCC 13661, ATCC 19859, ATCC 21834, ATCC 23270, ATCC 14119, ATCC 33020, and AP19-3, respectively. These results indicate that iron oxidase in *L. ferrooxidans* was completely destroyed by treating the cells with a low level of the bisulfite ion.

SFORase plays a crucial role in the oxidation of reduced sulfur compounds (6, 10–13, 15, 16). The effect of the bisulfite

FIG. 1. Effect of the bisulfite ion on iron-oxidizing activities of *T. ferrooxidans* (A) and *L. ferrooxidans* (B). Iron-oxidizing activity was determined with washed intact cells by measuring oxygen uptake caused by the oxidation of Fe^{2+} in a Warburg manometer. Symbols: ▲, 0.2 mM sodium sulfite; ●, 0.1 mM sodium sulfite; ◻, 0.05 mM sodium sulfite; ●, 0.02 mM sodium sulfite; △, 0.01 mM sodium sulfite; ◻, 0.05 mM sodium sulfite and 1.0 mM formaldehyde; and ○, without sodium sulfite.

ion on the activity of SFORase was also studied. Washed intact cells of *L. ferrooxidans* and *T. ferrooxidans* were incubated in 0.1 M β -alanine- SO_4^{2-} buffer (pH 3.0) containing 0.1, 0.5, and 1.0 mM sodium sulfite for 1 h at 30°C. After 1 h of incubation with the bisulfite ion, the cells were washed three times with 0.1 M β -alanine- SO_4^{2-} buffer (pH 3.0) to remove the bisulfite ion, and then SFORase activity was measured with the reaction mixture containing 5 mM sodium cyanide, which completely inhibits iron oxidase of *T. ferrooxidans* and *L. ferrooxidans* but not SFORase (6). The SFORase activity was markedly lost by 0.1 mM bisulfite treatment in *T. ferrooxidans* but not in *L. ferrooxidans* (Table 1). After 1.0 mM bisulfite treatment, *T. ferrooxidans* ATCC 13598, ATCC 13661, ATCC 19859, ATCC 21834, ATCC 23270, ATCC 14119, ATCC 33020, and AP19-3 had 20, 18, 4, 15, 25, 14, 10, and 12%, respectively, of the SFORase activity found in the cells without bisulfite treatment. These results suggest that, in *T. ferrooxidans* strains, SFORase was easily damaged by bisulfite ion. In contrast, SFORase activities of *L. ferrooxidans* DSM 2391, P3A, and BKM-6-1339 were not affected even after 1.0 mM bisulfite treatment, suggesting that SFORase of *T. ferrooxidans* is quite unstable to the bisulfite ion but those of *L. ferrooxidans* are comparatively stable to the bisulfite ion.

Effect of the bisulfite ion on iron-oxidizing activity. Effects of sodium sulfite on iron-oxidizing activity were studied with washed intact cells of *L. ferrooxidans* BKM-6-1339 and *T. ferrooxidans* AP19-3. Iron-oxidizing activity was nearly completely inhibited by 0.02 mM sodium sulfite in *L. ferrooxidans* and by 0.2 mM sodium sulfite in *T. ferrooxidans* AP19-3 (Fig. 1), indicating that iron oxidase of *L. ferrooxidans* was 10 times more sensitive to the bisulfite ion than that of *T. ferrooxidans*. The inhibition of iron-oxidizing activity by 0.05 or 0.07 mM sodium sulfite was completely reversed by the addition of 1.0 mM formaldehyde, a trapping reagent for the bisulfite ion, in the reaction mixture (Fig. 2), strongly suggesting that the bisulfite ion added to the reaction mixture as sodium sulfite really inhibited iron-oxidizing activity. Iron-oxidizing activity of *L. ferrooxidans* BKM-6-1339 was inhibited by 0.5, 1.0, and 5.0 mM of formaldehyde at 17, 29, and 48%, respectively (data not shown).

FIG. 2. Effects of formaldehyde and the bisulfite ion on iron-oxidizing activities of *L. ferrooxidans*. Iron-oxidizing activity was determined with washed intact cells by measuring oxygen uptake caused by the oxidation of Fe^{2+} in a Warburg manometer. Symbols: △, 0.07 mM sodium sulfite; ▲, 0.07 mM sodium sulfite and 1.0 mM formaldehyde; ◻, 0.5 mM sodium sulfite; ◻, 0.05 mM sodium sulfite and 1.0 mM formaldehyde; ○, without sodium sulfite or formaldehyde.

DISCUSSION

It was found that *L. ferrooxidans* was much more sensitive to the bisulfite ion than was *T. ferrooxidans*, and the causes of sensitivity of *L. ferrooxidans* to the bisulfite ion were studied. In striking contrast to the case of *T. ferrooxidans*, iron oxidase of *L. ferrooxidans* was sensitive to quite a low level of the bisulfite ion (0.02 mM) and, moreover, was easily destroyed by treating cells with a low level of the bisulfite ion (0.1 mM) for 1 h. Since iron oxidase is the most important enzyme for iron-oxidizing bacteria, a sensitivity of iron oxidase to the bisulfite ion seems to be a main reason for the inability of *L. ferrooxidans* to grow on Fe^{2+} -salt medium supplemented with the bisulfite ion. The absence or low level of sulfite:ferric ion oxidoreductase in *L. ferrooxidans* seems to be another reason that the growth of *L. ferrooxidans* on Fe^{2+} -salt medium was strongly inhibited by the bisulfite ion.

It is quite strange that *L. ferrooxidans* cannot grow on elemental sulfur-salt medium even though it has an enzyme involved in sulfur oxidation or SFORase. The results obtained in this study seem to provide a clue towards solving this physiologically interesting problem in iron-oxidizing bacteria. We have shown that approximately 0.02 to 0.05 mM bisulfite ion was produced in the reaction mixture when not only *T. ferrooxidans* but also *L. ferrooxidans* was incubated with elemental sulfur (14, 16). The bisulfite ion produced by these iron-oxidizing bacteria may be sufficient for the iron oxidase of *L. ferrooxidans* but not of *T. ferrooxidans* to be completely inhibited and/or damaged. In contrast, *T. ferrooxidans* seems to decompose the bisulfite ion more rapidly because the bacterium has an active bisulfite oxidase and, moreover, has an iron oxidase comparatively insensitive to this harmful ion.

It is interesting that the effects of the bisulfite ion on SFORases of two iron-oxidizing bacteria were quite different. The SFORase of *T. ferrooxidans* was easily damaged by a low level of bisulfite ion (0.1 mM), suggesting that SFORase in *T. ferrooxidans* may not operate when a high concentration of the bisulfite ion is present, and this stops further production of the harmful bisulfite ion by SFORase reaction. In contrast, bisul-

fite production in *L. ferrooxidans* may not be stopped in the presence of a high level of bisulfite ion because SFORase of *L. ferrooxidans* was not affected by treating the cells with 1.0 mM bisulfite ion for 1 h.

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