APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1984, p. 461–467
0099-2240/84/090461-07$02.00/0

Vol. 48, No. 3

Role of Ferrous Ions in Synthetic Cobaltous Sulfide Leaching of *Thiobacillus ferrooxidans*

TSUYOSHI SUGIO,* CHITOSHI DOMATSU, TATSUO TANO, AND KAZUTAMI IMAI

Department of Agricultural Chemistry, Faculty of Agriculture, Okayama University, Okayama 700, Japan

Received 6 March 1984/Accepted 30 May 1984

Microbiological leaching of synthetic cobaltous sulfide (CoS) was investigated with a pure strain of *Thiobacillus ferrooxidans*. The strain could not grow on CoS-salts medium in the absence of ferrous ions (Fe^{2+}). However, in CoS-salts medium supplemented with 18 mM Fe^{2+}, the strain utilized both Fe^{2+} and the sulfur moiety in CoS for growth, resulting in an enhanced solubilization of Co^{2+}. Cell growth on sulfur-salts medium was strongly inhibited by Co^{2+}, and this inhibition was completely protected by Fe^{2+}. Cobalt-resistant cells, obtained by subculturing the strain in medium supplemented with both Fe^{2+} and Co^{2+}, brought a marked decrease in the amount of Fe^{2+} absolutely required for cell growth on CoS-salts medium. As one mechanism of protection by Fe^{2+}, it is proposed that the strain utilizes one part of Fe^{2+} externally added to CoS-salts medium to synthesize the cobalt-resistant system. Since a similar protective effect by Fe^{2+} was also observed for cell inhibition by stannous, nickel, zinc, silver, and mercuric ions, a new role of Fe^{2+} in bacterial leaching in *T. ferrooxidans* is proposed.

Thiobacillus ferrooxidans inhabits drainage in acid mines and is used for bacterial leaching. Attention to bacterial leaching has increased in recent years because of its application to low-grade ores. However, there is one weak point in *T. ferrooxidans* when used for bacterial leaching under severe acidic conditions in which many toxic heavy metals are present: the sulfur-metabolizing system of the organism is sensitive to heavy metals. When grown on Fe^{2+}-salts medium, *T. ferrooxidans* is remarkably resistant to high concentrations of metal ions except mercuric and silver ions (4–8, 13, 16, 17, 20). In contrast, when grown on sulfur-salts medium, the organism is sensitive to those heavy metals. Tuovinen et al. reported that cobalt was the most toxic cation on the growth of *T. ferrooxidans* on sulfur-salts medium (20). Razzell and Trussell also showed that a relatively low concentration of cobaltous, cupric, and silver ions inhibited cell growth on sulfur medium (9). The results suggest that *T. ferrooxidans* that has a metal-tolerant, sulfur-oxidizing system is more available for enhanced leaching of sulfide ores, especially under conditions in which the concentration of ferrous ions is very low and the contribution of the cell to indirect contact mechanisms by ferric ions may be low; such strains attack the sulfur moiety of the ores directly and more rapidly solubilize metal ions from the ores despite the presence of heavy metals. Thus, it seems important to obtain metal-tolerant strains and study the mechanism by which the organism protects its sulfur-metabolizing system from metals.

The role of ferric ions in bacterial leaching has been previously established (12). It is well known as an indirect contact mechanism in which ferric ions act as primary oxidants of metal sulfide, and the resulting ferrous ions are enzymatically oxidized by *T. ferrooxidans* to complete a cyclic process. However, the role of ferrous ions in bacterial leaching in *T. ferrooxidans* was only considered as an energy source of this organism. This work showed that ferrous ions or the oxidation of ferrous ions protects *T. ferrooxidans* AP19-3 from inhibition by cobalt and other heavy metals and permits the organism enhanced CoS leaching.

* Corresponding author.

MATERIALS AND METHODS

Microorganism. The iron-oxidizing bacterium *T. ferrooxidans* AP19-3 was used throughout this study. This strain is an obligate autotroph and was isolated from an iron-grown culture of *T. ferrooxidans* AP-19 (15).

Media and conditions of cultivation. The composition of the basal salts solution used throughout this study was the same as that used by Silverman et al. (14): $(NH_4)_2SO_4$, 3.0 g; KCl, 0.1 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $Ca(NO_3)_2$, 0.01 g; deionized water, 1,000 ml; and concentrated H_2SO_4, 2.5 ml (pH 2.5).

The medium used for isolation of *T. ferrooxidans* AP19-3 was as follows: Fe^{2+}, 18 mmol; proline, 0.4 g; extra-pure agar (Ishizu Pharmaceutical Co., Ltd.), 1 g; and basal salts solution, 100 ml. Tiny creamy brown colonies appeared on the plate. After being examined with a microscope, a completely isolated colony was taken from the plate and transferred into Fe^{2+} (0.108 M)-salts medium and shaken for 1 week at 30°C. The isolated strain (AP19-3) was preserved in Fe^{2+} (0.108 M)-salts medium consisting of Fe^{2+} (0.108 mol) and salts solution (1,000 ml).

The composition of sulfur-salts medium was as follows: elemental sulfur, 1 g; Fe^{3+}, 0.5 mM; and salts solution, 100 ml. Sulfur was sterilized separately by tyndalization. The ferric ion solution was sterilized by being passed through a Millex-HA filter (0.45-μm pore size; Millipore Corp.) and added asceptically into the autoclaved salts solution before inoculation.

The composition of cobaltous sulfide-salts medium (CoS-salts medium) was as follows: CoS (99.9% purity, below 250 mesh; Ishizu Pharmaceutical Co., Ltd.), 1 g; Fe^{3+}, 0.5 mM; and salts solution, 100 ml. One gram of CoS was added to 100 ml of salts solution, and the pH was readjusted to 2.5 with sulfuric acid before autoclaving (10 min at 1.0 kg/cm^2). All growth experiments were performed by shaking 100 ml of inoculated medium in a 500-ml shaking flask at 30°C.

Growth rate. Cells were separated from iron particles, elemental sulfur, or a solid state of CoS by filtering cultures with Toyo paper filter no. 5C. Almost all of the cells present in culture fluid, namely, the cells not adhered to these

particles, could pass through the paper filter, and they were counted with a hemacytometer (Kayagaki Irika Kogyo Co., Ltd.). When the number of cells in the filtrates was too large to be counted directly, these filtrates were diluted with 0.1 N sulfuric acid.

Iron determination. Ferrous ions were determined colorimetrically by a modification of the *o*-phenanthroline method (11). Ferric ions were also determined by the *o*-phenanthroline method after being reduced with sodium sulfite.

Cobalt determination. Cultures were filtered through Toyo paper filter no. 5C and diluted 500 times with 0.1 N sulfuric acid. After centrifugation at 10,000 × *g* for 10 min, the amount of Co^{2+} in the supernatant was determined by atomic absorption spectroscopy with a Shimadzu AA-625-01 spectrophotometer, using an air-acetylene flame. The spectral line chosen was at 240.7 nm. The standard solution of Co^{2+} was diluted in preparation for atomic absorption spectroscopy (Ishizu Pharmaceutical Co., Ltd.).

RESULTS

Requirement of ferrous ions on synthetic cobaltous sulfide leaching. A fresh culture of Fe^{2+} (0.108 M)-salts-grown *T. ferrooxidans* AP19-3 was inoculated into 100 ml of Fe^{2+} (11, 18, and 36 mM)-salts media or CoS-salts media supplemented with or lacking Fe^{2+}. In Fe^{2+} (11, 18, and 36 mM)-salts media lacking CoS, the strain oxidized ferrous ions and grew to a maximum cell growth of ca. 0.25×10^8, 0.4×10^8, and 0.8×10^8 cells per ml of medium, respectively (Fig. 1A). In CoS-salts medium lacking Fe^{2+} or CoS-salts medium supplemented with Fe^{2+} (3.6 mM), no cell growth was observed. In CoS-Fe^{2+} (18 mM)-salts medium with killed cells or without cells, no cell growth was observed. The concentrations of cobaltous ions being leached in these four media slowly increased with cultivation time (Fig. 1B), but they were low. In contrast, in CoS-salts medium supplemented with Fe^{2+} (11, 18, and 36 mM), the strain grew vigorously after oxidizing ferrous ions completely or to a certain level, resulting in maximum cell growth of ca. 3.8×10^8, 6.1×10^8, and 6.5×10^8 cells per ml of medium, respectively. These values were much larger than those obtained when the strain grew on Fe^{2+}-salts media lacking CoS, suggesting that the strain utilizes not only Fe^{2+} but also sulfur moieties in CoS for growth. The amount of Co^{2+} being leached in these media also markedly increased after oxidizing ferrous ions completely or to a certain level. Leaching of Co^{2+} continued after the cells had virtually ceased growing, but after further cultivation it decreased to the level of chemical leaching. In this way, sulfur utilization and enhanced Co^{2+} solubilization by this strain were dependent on the amount of Fe^{2+} supplemented with CoS-salts medium. A higher concentration of Fe^{2+} (above 0.108 M) inhibited bacterial utilization of both Fe^{2+} and the sulfur moiety in CoS.

The effect of ferric ions (Fe^{3+}) on CoS leaching was also examined with sulfur-salts-grown AP19-3. However, it was impossible to test the true effect of Fe^{3+} on CoS leaching because some parts of Fe^{3+} added to the medium were spontaneously reduced by CoS (Fig. 2B). In CoS-Fe^{3+} (18 mM)-salts medium with active cells, the oxidation of Fe^{2+} (10.0 mM) produced by the chemical reduction of Fe^{3+} was accompanied by a rapid cell growth and Co^{2+} solubilization (Fig. 2A). The maximum cell growth of ca. 4.0×10^8 cells per ml of medium suggests that the strain utilized both Fe^{2+} and CoS for growth. In contrast, in CoS-Fe^{3+} (18 mM)-salts medium with killed cells or without cells, Fe^{2+} (10 or 10.7 mM) formed by the chemical reduction of Fe^{3+} remained throughout the cultivation. In CoS-salts medium lacking Fe^{3+} or supplemented with Fe^{3+} (2.5 mM), no cell growth was observed, and the amounts of Co^{2+} solubilized in these

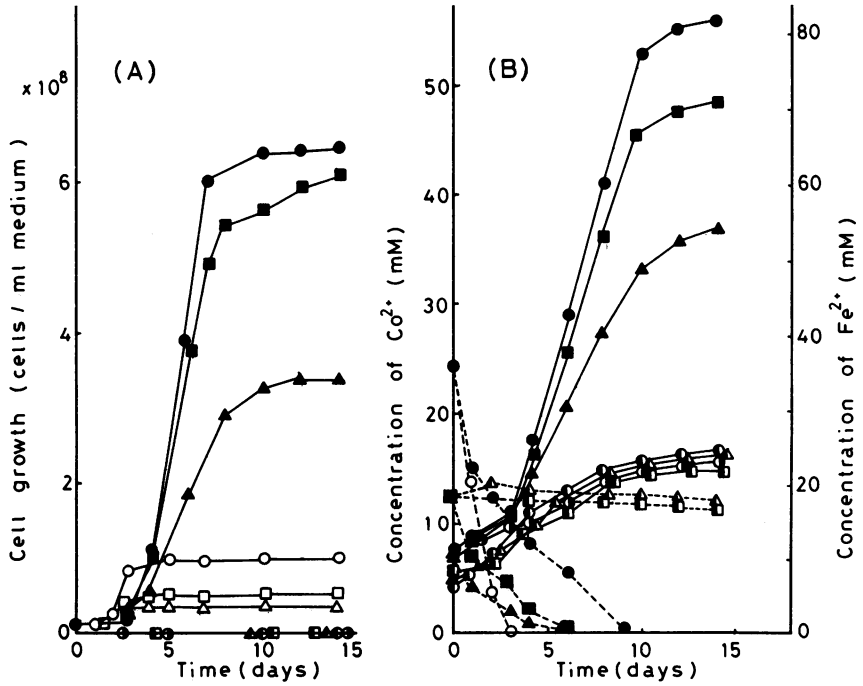

FIG. 1. Effect of ferrous ions on CoS leaching. The composition of salts solution and the method for analysis are described in the text. (A) Cell growth; (B) solid line, concentration of cobaltous ions; dotted line, concentration of ferrous ions. Symbols: Fe^{2+} (11 mM)-salts medium (△), Fe^{2+} (18 mM)-salts medium (□), and Fe^{2+} (36 mM)-salts medium supplemented with active cells (○); CoS-salts medium supplemented with active cells and Fe^{2+} at 3.6 mM (◑), 11 mM (▲), 18 mM (■), and 36 mM (●); CoS-salts medium supplemented with 18 mM Fe^{2+} and killed cells (◧); CoS-salts medium without cells (▲) or without Fe^{2+} (◐).

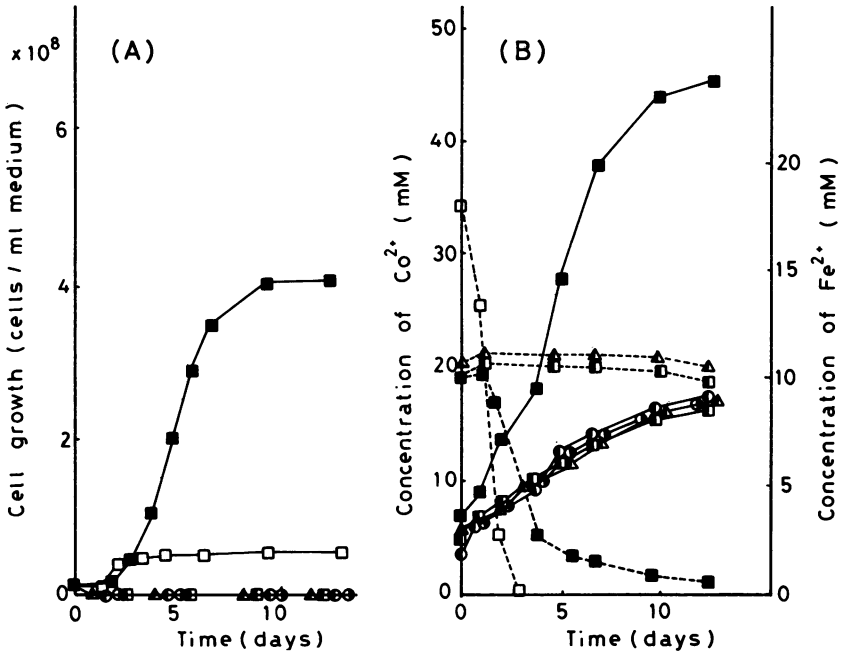

FIG. 2. Effect of ferric ions on CoS leaching. The composition of salts solution and the method for analysis are described in the text. (A) Cell growth; (B) solid line, concentration of cobaltous ions; dotted line, concentration of ferrous ions. Symbols: Fe^{2+} (18 mM)-salts medium supplemented with active cells (□); CoS-salts medium supplemented with active cells and Fe^{3+} at 2.5 mM (◑) and 18 mM (■); CoS-salts medium supplemented with 18 mM Fe^{3+} and killed cells (◧); CoS-salts medium without cells (▲) or without Fe^{3+} (◐).

media were similar to those of chemical leaching (no inoculum and killed control). Similar results were also obtained with iron-grown cells.

Effect of cobaltous ions on the cell growth on sulfur-salts medium. Strain AP19-3 grew on Fe^{2+} (0.108 M)-salts medium supplemented with 0.1 M Co^{2+} without any inhibition (data not shown). In contrast, cell growth on sulfur-salts medium was strongly inhibited by Co^{2+} at 0.1 mM (Fig. 3). This inhibition by Co^{2+} was completely protected by the addition of 18 mM Fe^{2+} (Fig. 4). The same concentration of Fe^{3+} did not have a similar protective effect. The results are in conflict with the data shown in Fig. 2, in which 18 mM Fe^{3+} was effective for rapid cell growth and enhanced solubilization of Co^{2+}. The reason why contradictory data were obtained can be explained by the difference of Fe^{2+} concentration in these two media. In CoS-salts medium supplemented with 18 mM Fe^{3+}, 10 mM Fe^{2+} was detected at the start of cultivation. In contrast, in sulfur-Co^{2+}-salts medium supplemented with 18 mM Fe^{3+}, Fe^{2+} was not detected throughout the cultivation. Since chemical or enzymatical reduction of Fe^{3+} with elemental sulfur is negligible in the case of sulfur salts medium, it can be said that the true effect of Fe^{3+} on the sulfur-metabolizing system of this strain in the presence of Co^{2+} can be decided by using sulfur-salts but not CoS-salts medium. Thus, it was concluded that Fe^{2+} but not Fe^{3+} was absolutely required to protect the strain from inhibition by Co^{2+}.

In the medium supplemented with both Co^{2+} (100 mM) and Fe^{2+} (18 mM), the strain oxidized Fe^{2+} and grew to a maximum cell growth of ca. 0.4×10^8 cells per ml of medium. The cell yield in this medium was similar to that obtained when the strain grew on Fe^{2+} (18 mM)-salts medium lacking sulfur, suggesting cell utilization of Fe^{2+} but not sulfur. With a higher concentration of Co^{2+}, 18 mM Fe^{2+} seems to be insufficient for protecting cell growth on sulfur from inhibition by Co^{2+}. When the Co^{2+} concentration added to sulfur-salts media was constant, the level of recovery from Co^{2+} inhibition was dependent upon the amount of Fe^{2+} added to the media (Fig. 5). Normal cell growth observed in sulfur-salts medium lacking Co^{2+} was obtained by the addition of 5.4 mM Fe^{2+}.

At the start of cultivation, CoS-salts medium contained ca. 5 mM Co^{2+}, which was enough to inhibit cell growth on sulfur-salts medium (Fig. 1B and 2B). Thus, it is supposed that in CoS-salts medium this chemically solubilized Co^{2+}

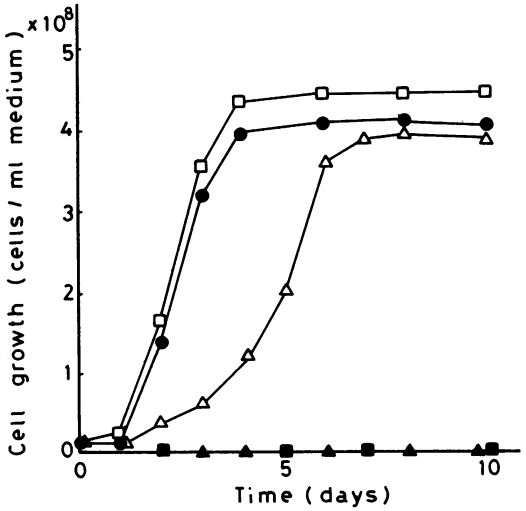

FIG. 3. Effect of cobaltous ions on cell growth on sulfur-salts medium. The composition of sulfur-salts medium and the method for analysis are described in the text. Symbols: sulfur-salts medium without Co^{2+} (●); sulfur-salts medium supplemented with Co^{2+} at 0.01 mM (△), 0.1 mM (■), and 1.0 mM (▲); sulfur-salts medium supplemented with 18 mM Fe^{2+} (□).

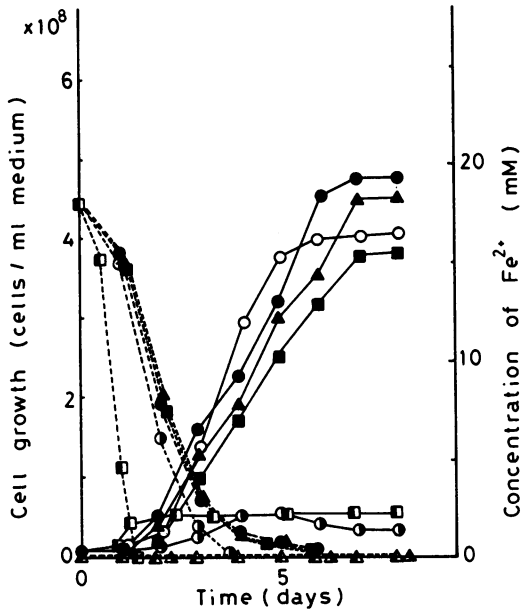

FIG. 4. Effect of ferrous or ferric ions on cell growth on sulfur-salts medium supplemented with cobaltous ions. The composition of salts solution and the method for analysis are described in the text. Solid line, cell growth; dotted line, concentration of ferrous ions. Symbols: Fe^{2+} (18 mM)-salts medium (◧); sulfur-salts medium (○); sulfur-salts medium with 18 mM Fe^{2+} but no added Co^{2+} (●); sulfur-salts medium supplemented with 18 mM Fe^{2+} and Co^{2+} at 1 mM (▲), 10 mM (■), and 100 mM (◑); sulfur-salts medium supplemented with 18 mM Fe^{3+} and 1 mM Co^{2+} (△); sulfur-salts medium supplemented with 1 mM Co^{2+} but no added Fe^{2+} (△).

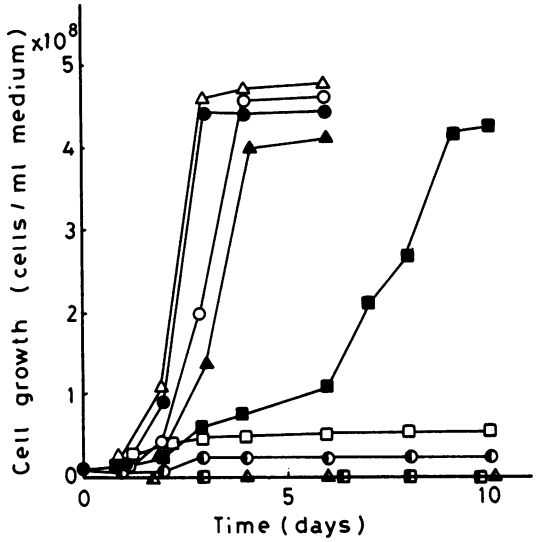

FIG. 5. Effect of concentration of ferrous ions on cell growth on sulfur-salts medium supplemented with cobaltous ions. The composition of salts solution and the method for analysis are described in the text. Symbols: Fe^{2+} (18 mM)-salts medium (□); sulfur-salts medium (○); sulfur-salts medium supplemented with 18 mM Fe^{2+} (△); sulfur-salts medium supplemented with 1 mM Co^{2+} and Fe^{2+} at 1.0 mM (▲), 1.8 mM (◐), 3.6 mM (■), 5.4 mM (▲), and 18 mM (●); sulfur-salts medium supplemented with 1 mM Co^{2+} but no added Fe^{2+} (◧).

may inhibit cell utilization of sulfur moiety in CoS, and externally added Fe^{2+} may protect the cells from inhibition by Co^{2+}. These suppositions were ascertained as follows. CoS-salts medium was filtered before inoculation through Toyo paper filter no. 5C to remove solid CoS. One gram of elemental sulfur was added to the slightly pink filtrate obtained, in which 5.1 mM of Co^{2+} and required salts for sufficient cell growth were present. Sulfur-salts-grown cells were inoculated into this sulfur-filtrate medium. The strain oxidized Fe^{2+} and grew on sulfur-filtrate medium supplemented with 18 mM Fe^{2+} resulting in maximum cell growth of ca. 5.1×10^8 cells per ml of medium and suggesting cell utilization of both Fe^{2+} and sulfur for growth (Fig. 6). In contrast, on sulfur-filtrate medium supplemented with 18 mM Fe^{3+}, no cell growth and no Fe^{2+} were observed. Similar results were also obtained with iron-grown cells. From these results it was ascertained that cell utilization of the sulfur moiety in CoS was inhibited by Co^{2+} that was solubilized from CoS, and this inhibition was protected by externally added Fe^{2+}.

Growth of cobalt-resistant strain AP19-3 on CoS-salts medium. Exponential-phase cells from Fe^{2+} (0.108 M)-Co^{2+} (10 mM)-salts, Fe^{2+} (18 mM)-sulfur-Co^{2+} (10 mM)-salts, or Fe^{2+} (18 mM)-CoS-salts medium were harvested by passing the cultures through a membrane filter (0.45 μm) and were washed with salts solution. These washed exponential-phase cells were inoculated into sulfur-Co^{2+} (1 mM)-salts medium supplemented with or lacking Fe^{2+}. No cell growth was observed on sulfur-Co^{2+} (1 mM)-salts medium lacking Fe^{2+} (Fig. 7). However, normal cell growth was observed on sulfur-Co^{2+} (1 mM)-salts medium supplemented with Fe^{2+} (1.8 mM). Maximum cell growth of ca. 4.0×10^8 cells per ml of medium suggest that these cells utilized sulfur for growth. As the control, washed exponential-phase cells harvested from Fe^{2+} (0.108 M)-salts, Fe^{2+} (18 mM)-sulfur salts, or sulfur-salts medium were also inoculated into CoS-salts

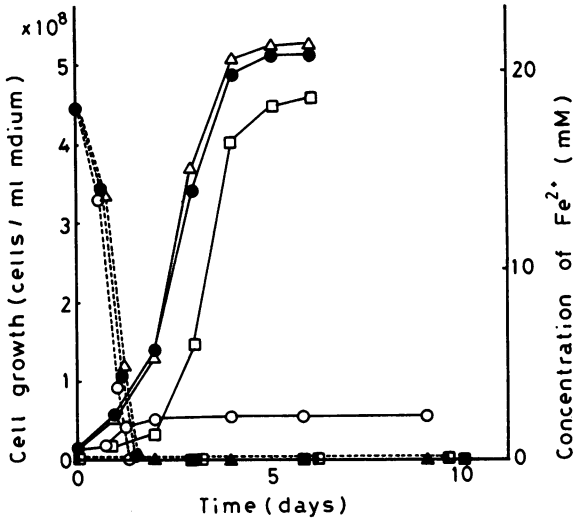

FIG. 6. Effect of cobaltous ions solubilized from CoS on cell growth on sulfur. CoS-salts medium was filtered before inoculation through a Toyo paper filter to remove solid CoS in which 5.1 mM Co^{2+} and salts required for sufficient cell growth were present. One gram of sulfur was added to this filtrate to make sulfur-filtrate medium. A solid line showed the cell growth, and a dotted line showed the concentration of ferrous ions. Symbols: Fe^{2+} (18 mM)-salts medium (○); sulfur-salts medium (□); sulfur-salts medium supplemented with 18 mM Fe^{2+} (△); sulfur-filtrate medium supplemented with Fe^{2+} at 1.8 mM (▲) and 18 mM (●); sulfur-filtrate medium supplemented with 18 mM Fe^{3+} (◧); sulfur-filtrate medium without Fe^{2+} or Fe^{3+} (■).

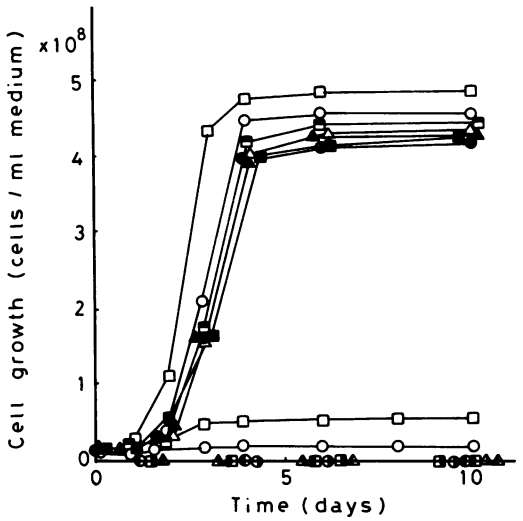

FIG. 7. Growth of cobalt-resistant strain AP19-3 on sulfur-salts medium supplemented with Co^{2+}. The composition of salts solution and the method for preparation of cobalt-resistant strain AP19-3 are described in the text. Symbols: washed exponential-phase cells harvested from Fe^{2+} (0.108 M)-Co^{2+} (10 mM)-salts (●), Fe^{2+} (18 mM)-sulfur-Co^{2+} (10 mM)-salts (▲), or Fe^{2+} (18 mM)-CoS-salts (■) medium inoculated into sulfur-Co^{2+} (1 mM)-salts medium supplemented with 1.8 mM Fe^{2+}; washed exponential-phase cells harvested from Fe^{2+} (0.108 M)-salts (◐), Fe^{2+} (18 mM)-sulfur-salts (◭), or sulfur-salts (◧) medium inoculated into sulfur-Co^{2+} (1 mM)-salts medium supplemented with 1.8 mM Fe^{2+}; washed exponential-phase cells harvested from Fe^{2+} (0.108 M)-Co^{2+} (10 mM)-salts (◑), Fe^{2+} (18 mM)-sulfur-Co^{2+} (10 mM)-salts (◭), or Fe^{2+} (18 mM)-CoS-salts (◨) medium inoculated into sulfur-salts medium lacking Fe^{2+}; washed exponential-phase cells harvested from a culture of cobalt-resistant cells inoculated into sulfur-Co^{2+} (1 mM)-salts (⬒) or sulfur-Co^{2+} (10 mM)-salts (△) medium lacking Fe^{2+}; washed exponential-phase cells harvested from Fe^{2+} (0.108 mM)-salts medium inoculated into sulfur-salts medium lacking Fe^{2+} (○) or Fe^{2+} (18 mM)-sulfur-salts medium (□).

medium supplemented with or lacking Fe^{2+} in which these control cells could not grow. Thus, when subcultured in media supplemented with both Fe^{2+} and Co^{2+}, the strain was able to decrease the amount of Fe^{2+} absolutely required for growth on sulfur-Co^{2+} (1 mM)-salts medium. When these sulfur-Co^{2+} (1 mM)-Fe^{2+} (1.8 mM)-salts-grown cells were subcultured in the same sulfur-Co^{2+} (1 mM)-Fe^{2+} (1.8 mM)-salts medium two or three times, cobalt-resistant strains that can grow on sulfur-Co^{2+} (1 mM or 10 mM)-salts medium lacking Fe^{2+} were obtained (Fig. 7). However, the absence of Fe^{2+}, Co^{2+}, or both in subculturing media never produced cobalt-resistant cells, suggesting that oxidation of Fe^{2+} in the presence of Co^{2+} is absolutely required for the strain to become cobalt resistant.

Washed exponential-phase cells harvested from Fe^{2+} (0.108 M)-Co^{2+} (10 mM)-salts, Fe^{2+} (18 mM)-sulfur-Co^{2+} (10 mM)-salts, or Fe^{2+} (18 mM)-CoS-salts medium and washed exponential-phase cells harvested from the culture of cobalt-resistant cells that grew on sulfur-Co^{2+} (10 mM)-salts medium lacking Fe^{2+} were inoculated into CoS-salts media supplemented with or lacking Fe^{2+}. They could not grow on CoS-salts medium lacking Fe^{2+} but grew on CoS-salts medium supplemented with 1.8 mM of Fe^{2+} (Fig. 8). Maximum cell growth of ca. 3.0×10^8 cells per ml of medium suggests that these cells utilized sulfur moiety of CoS for growth. Solubilization of Co^{2+} was accompanied by cell growth. As the control, washed exponential-phase cells harvested from Fe^{2+} (0.108 M)-salts, Fe^{2+} (18 mM)-sulfur-salts, or sulfur-salts medium were also inoculated into CoS-salts medium supplemented with or lacking Fe^{2+} in which these control cells could not grow, and the amount of Co^{2+} solubilized was comparable to that of chemical leaching. Cobalt-resistant cells and the cells that were subcultured in the medium supplemented with both Fe^{2+} and Co^{2+} brought a marked decrease in the amount of Fe^{2+} absolutely required for growth on CoS-salts medium, suggesting that one part of Fe^{2+} externally added to CoS-salts medium was utilized for the strain to synthesize the cobalt-resistant system. The results that cobalt-resistant strains could not grow on CoS-salts medium lacking Fe^{2+} and the amount of cell growth on CoS-Fe^{2+} (1.8 mM)-salts medium was always lower than that of CoS-salts medium supplemented with 18 mM Fe^{2+} suggest that besides being used for synthesizing the cobalt-resistant system, Fe^{2+} must be utilized for another purpose which is now unknown but is important for the strain to grow on CoS-salts medium. All of the attempts at growing strain AP19-3 on CoS-salts medium lacking Fe^{2+} have never done well.

Effect of ferrous ions on cell growth on sulfur-salts medium

TABLE 1. Cell growth on iron or sulfur in the presence of heavy metal[a]

Addition	Concn (mM)	Cell growth with the following energy source:		
		Iron[b]	Sulfur[c]	Iron + sulfur[d]
None		+	+++	+++
$HgCl_2$	0.001	+	−	+++
	0.010	−	−	−
$AgNO_3$	0.001	+	+++	+++
	0.010	+	−	+++
$SnCL_2$	0.1	+	+++	+++
	1.0	+	−	+++
	10.0	−	−	−
$UO_2SO_4 \cdot 3H_2O$	0.1	+	+++	+++
	1.0	+	−	+++
	10.0	−	−	−
$ZnSO_4$	1.0	+	+++	+++
	5.0	+	−	+++
$NiSO_4$	1.0	+	+++	+++
	5.0	+	−	+++
$Pb(NO_3)_2$	5.0	+	+++	+++
	10.0	+	−	+++
$CdCl_2$	5.0	+	+++	+++
	10.0	+	−	+++
$CuSO_4$	10.0	+	+++	+++
	100.0	+	+++	+

[a] The composition of salts solution was described in the text. Sulfur-salts-grown cells were inoculated into iron, sulfur, or iron-plus-sulfur medium in which each of the heavy metals listed above was added. Symbols show the amount of cell growth: −, no cell growth; +, 0.4×10^8 cells per ml of medium which showed the maximum cell growth on iron medium; +++, 4.5×10^8 to 5.5×10^8 cells per ml of medium which showed the maximum cell growth on sulfur medium or iron-sulfur medium.

[b] Fe^{2+} (18 mM)-salts medium.

[c] Sulfur (1%)-salts medium supplemented with 0.5 mM Fe^{3+}.

[d] Fe^{2+} (18 mM)-sulfur (1%)-salts medium.

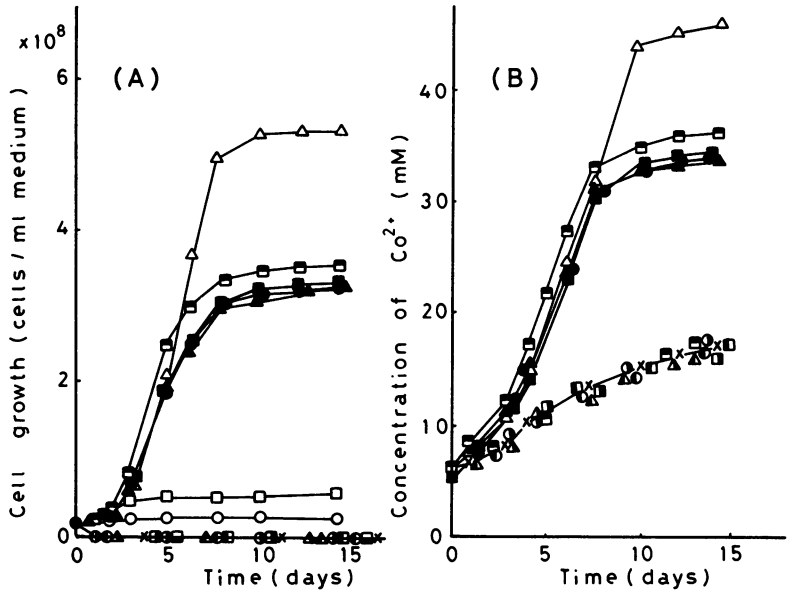

FIG. 8. Growth of cobalt-resistant strain AP19-3 on CoS-salts medium. The composition of salts solution and the method for preparation of cobalt resistant-strain AP19-3 are described in the text. (A) Cell growth; (B) concentration of Co^{2+}. Symbols: washed exponential-phase cells harvested from Fe^{2+} (0.108 M)-Co^{2+} (10 mM)-salts (●), Fe^{2+} (18 mM)-sulfur-Co^{2+} (10 mM)-salts (▲), or Fe^{2+} (18 mM)-CoS-salts (■) medium and washed exponential-phase cells harvested from a culture of cobalt-resistant cells that grew on sulfur-Co^{2+} (10 mM)-salts medium lacking Fe^{2+} (⬒) inoculated into CoS-salts medium supplemented with 1.8 mM Fe^{2+}; washed exponential-phase cells harvested from Fe^{2+} (0.108 M)-salts (◐), Fe^{2+} (18 mM)-sulfur-salts (◭), or sulfur-salts (◧) medium inoculated into CoS-salts medium supplemented with 1.8 mM Fe^{2+}; washed exponential-phase cells harvested from Fe^{2+} (0.108 M)-Co^{2+} (10 mM)-salts (◑), Fe^{2+} (18 mM)-sulfur-Co^{2+} (10 mM)-salts (◭), or Fe^{2+} (18 mM)-CoS-salts (◨) medium and washed exponential-phase cells harvested from a culture of cobalt-resistant cells that grew on sulfur-Co^{2+} (10 mM)-salts medium lacking Fe^{2+} (⬓) inoculated into CoS-salts medium lacking Fe^{2+}; washed exponential-phase cells harvested from Fe^{2+} (0.108 M)-salts medium inoculated into Fe^{2+} (1.8 mM)-salts (○), Fe^{2+} (18 mM)-salts (□), or Fe^{2+} (18 mM)-CoS-salts (△) medium; cell growth and Co^{2+} solubilization on CoS-salts medium lacking both Fe^{2+} and cells (×).

supplemented with heavy metals. Cell growth on Fe^{2+}-salts medium was not inhibited by the concentrations of heavy metals listed in Table 1. In contrast, cell growth on sulfur-salts medium was inhibited by these heavy metals, such as mercuric, silver, and stannous ions at 1 μM, 10 μM, and 1 mM, respectively. Nickel, cadmium, and zinc ions also inhibited cell growth at much higher concentrations. However, when 18 mM Fe^{2+} was added to these sulfur-heavy metal-salts media, the strain could utilize not only Fe^{2+} but also sulfur for growth, resulting in maximum cell growth of ca. 5×10^8 cells per ml of medium. The results suggest that similar protection by Fe^{2+} also occurred for these heavy metals. A higher concentration of cupric ions (100 mM) did not inhibit growth on sulfur-salts medium lacking Fe^{2+}. Curiously, Fe^{2+} added to this sulfur-Cu^{2+} (100 mM)-salts medium inhibited cell utilization of sulfur, suggesting that the mechanism of action for Cu^{2+} on the sulfur-metabolizing system of the strain is different from that of other heavy metals.

DISCUSSION

What makes *T. ferrooxidans* one of the most valuable microorganisms for bacterial leaching of sulfide ores? It may be due to an ability to utilize both Fe^{2+} and the sulfur moiety in sulfide ores for growth. By using this ability, *T. ferrooxidans* directly and indirectly attacks sulfide ores to carry out rapid solubilization of metals.

Microbiological leachings of ZnS (9, 18, 19), CoS (2), and NiS (3) have been studied with *T. ferrooxidans*; CoS leaching seems to be interesting because cell growth on sulfur is strongly inhibited by a very low concentration of Co^{2+} (0.1 mM). Groudev showed that the leaching rate of Co^{2+} from CoS in *T. ferrooxidans* was slightly increased by the addition of Fe^{2+} or Fe^{3+} but that the leaching rate of *T. thiooxidans* was not accelerated or slightly retarded by the addition of Fe^{2+} (2). In CoS leaching in *T. ferrooxidans* strain AP19-3, both the amount of Co^{2+} solubilized from CoS and cell utilization of the sulfur moiety in CoS were primarily dependent on the presence of Fe^{2+} or oxidation of Fe^{2+}. Ferrous ions added to CoS-salts medium or sulfur-Co^{2+}-salts medium protected the cells from inhibition by Co^{2+}.

It has been very difficult to determine precisely whether iron oxidation or merely the presence of Fe^{2+} causes protection from inhibition by Co^{2+}. The best way to decide this problem seems to be with a selective inhibitor that can block iron oxidation without inhibiting sulfur oxidation. However, there have been no such good selective inhibitors, because sulfur oxidation is usually more sensitive to inhibition than iron oxidation. Tuttle and Dugan showed that some organic acids strongly inhibited iron oxidation, sulfur oxidation, and growth on iron (21). In *T. ferrooxidans* strain AP19-3, cell growth on both iron and sulfur were completely inhibited by pyruvate, succinate, acetate, lactate, and hydroxybutyrate at 1, 5, 10, 10, and 10 mM, respectively. Though direct evidence has not been obtained, the results described below seem to suggest that iron oxidation is important in protecting cells against inhibition by Co^{2+}. (i) When the strain grew on CoS-Fe^{2+}-salts medium or sulfur-salts medium supplemented with both Fe^{2+} and Co^{2+}, growth on sulfur was always observed after Fe^{2+} was oxidized completely or to a certain level. (ii) If the oxidation of Fe^{2+} present in sulfur-Fe^{2+}-Co^{2+}-salts medium was markedly inhibited by inhibitors such as the organic acids described above or much higher concentrations of heavy metals listed in Table 1, growth on

sulfur was never observed. (iii) Cobalt-resistant cells were obtained only when the strain oxidized Fe^{2+} in the presence of Co^{2+}.

Though the mechanism for this protective effect by Fe^{2+} seems to be rather complex, it can be said that one part of Fe^{2+} added to CoS-salts medium was utilized to make strain AP19-3 cobalt-resistant. The other part of Fe^{2+} may be consumed for another unknown purpose for cells to grow on CoS-salts medium. This unknown mechanism of action is now under investigation.

The results obtained in this experiment suggest that the protective effect by Fe^{2+} cannot be definitely observed in iron-containing sulfides, such as pyrite (FeS_2), chalcopyrite ($CuFeS_2$), bornite (Cu_5FeS_4), jamesonite ($Pb_5FeSb_6S_{14}$), etc., because iron present in these ores may be utilized for protection. Duncan and Walden showed that the addition of Fe^{2+} had no influence on the rate or extent of copper extraction from chalcopyrite (1).

LITERATURE CITED

1. **Duncan, D. W., and C. C. Walden.** 1972. Microbiological leaching in the presence of ferric iron. Dev. Ind. Microbiol. **13**:66–75.
2. **Groudev, S. N.** 1982. Leaching of cobalt from synthetic cobalt sulphide by *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans*. Dokl. Bolg. Akad. Nauk. **34**:217–220.
3. **Groudev, S. N.** 1982. Leaching of nickel from sulphide minerals by pure and mixed cultures of chemolithotrophic bacteria. Dokl. Bolg. Akad. Nauk. **35**:1113–1116.
4. **Hoffman, L. E., and J. L. Hendrix.** 1976. Inhibition of *Thiobacillus ferrooxidans* by soluble silver. Biotechnol. Bioeng. **18**:1161–1165.
5. **Imai, K., T. Sugio, T. Tsuchida, and T. Tano.** 1975. Effect of heavy metal ions on the growth and iron-oxidizing activity of *Thiobacillus ferrooxidans*. Agric. Biol. Chem. **39**:1349–1345.
6. **Malouf, E. E., and D. Prater.** 1961. Role of bacteria in the alteration of sulfide minerals. J. Met. **May**:353–356.
7. **Olson, G. J., W. P. Iverson, and F. E. Brinckman.** 1981. Volatilization of mercury by *Thiobacillus ferrooxidans*. Curr. Microbiol. **5**:115–118.
8. **Olson, G. J., F. D. Porter, J. Rubinstein, and S. Silver.** 1982. Mercuric reductase enzyme from a mercury-volatilizing strain of *Thiobacillus ferrooxidans*. J. Bacteriol. **151**:1230–1236.
9. **Razzell, W. E., and P. C. Trussel.** 1963. Isolation and properties of an iron-oxidizing *Thiobacillus*. J. Bacteriol. **85**:595–603.
10. **Sadler, W. R., and P. A. Trudinger.** 1967. The inhibition of microorganisms by heavy metals. Miner. Deposita **2**:158–168.
11. **Sandell, E. B.** 1950. Colorimetric determination of trace metals, 2nd ed. Interscience Publishers, Inc., New York.
12. **Silverman, M. P.** 1967. Mechanism of bacterial pyrite oxidation. J. Bacteriol. **94**:1046–1051.
13. **Silverman, M. P., and H. L. Ehrlich.** 1964. Microbial formation and degradation of minerals. Adv. Appl. Microbiol. **6**:153–206.
14. **Silverman, M. P., and D. G. Lundgren.** 1959. Studies on the chemoautotrophic iron bacterium *Ferrobacillus ferrooxidans*. I. An improved medium and a harvesting procedure for securing high cell yields. J. Bacteriol. **77**:642–647.
15. **Sugio, T., Y. Anzai, T. Tano, and K. Imai.** 1981. Isolation and some properties of an obligate and a facultative iron-oxidizing bacteria. Agric. Biol. Chem. **45**:1141–1151.
16. **Sugio, T., T. Kougami, T. Tano, and K. Imai.** 1982. Glutathione transport system in *Thiobacillus ferrooxidans* strain AP-44. Agric. Biol. Chem. **46**:2919–2924.
17. **Sugio, T., T. Tano, and K. Imai.** 1981. Isolation and some properties of silver ion-resistant iron-oxidizing bacterium *Thiobacillus ferrooxidans*. Agric. Biol. Chem. **45**:2037–2051.
18. **Torma, A. E., C. C. Walden, and R. M. R. Branion.** 1970. Microbiological leaching of a zinc sulfide concentrate. Biotechnol. Bioeng. **12**:501–517.
19. **Torma, A. E., C. C. Walden, D. W. Duncan, and R. M. R. Branion.** 1972. The effect of carbon dioxide and particle surface area on the microbiological leaching of a zinc sulfide concentrate. Biotechnol. Bioeng. **14**:777–786.
20. **Tuovinen, O. H., S. I. Niemelä, and H. G. Gyllenberg.** 1971. Tolerance of *Thiobacillus ferrooxidans* to some metals. Antonie van Leeuwenhoek J. Microbiol. **37**:489–496.
21. **Tuttle, J. H., and R. R. Dugan.** 1976. Inhibition of growth, iron, and sulfur oxidation in *Thiobacillus ferrooxidans* by simple organic compounds. Can. J. Microbiol. **22**:719–730.