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Activities of Iron Oxidase and Hydrogen Sulfide: ferric Ion Oxidoreductase of *Thiobacillus ferrooxidans* isolated from natural environments

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It has been reported that both iron oxidase and hydrogen sulfide: ferric ion oxidoreductase (SFORase) were involved in bacterial leaching of metal ions from sulfide ores, and the amount of Cu^{2+} solubilized from copper ore by iron-oxidizing bacterium differed from strain to strain. The activities of iron oxidase and SFORase of iron-oxidizing bacteria isolated from the natural environments were determined. Iron-oxidizing activity and SFORase activity of 200 strains ranged from 1.20-1.65 μmol/mg/min and from 0.11-2.80 μmol/mg/min, respectively. The findings that a remarkable difference was observed in the levels of SFORase activity, but not in iron-oxidizing activity, suggest that SFORase, but not iron oxidase, is the enzyme that determines the bacterial leaching activity of this bacterium.

Key words : iron oxidase, sulfur oxidase, iron-oxidizing bacterium, bacterial leaching

Introduction

Copper and uranium are two minerals recovered on a commercial scale using bioleaching[1]. The amount of copper produced by this method was estimated to be more than 10 % of the total U.S. production[2]. The iron-oxidizing bacterium *Thiobacillus ferrooxidans* has been considered one of the most important microorganisms for bacterial leaching of sulfide ores. This may be due to the ability of this bacterium to oxidize both ferrous iron (Fe^{2+}) and inorganic sulfur compound in sulfide ores to ferric iron (Fe^{3+}) and sulfate. The ferric iron thus produced by iron-oxidizing bacterium has been known to be a potent oxidant for various metal ions. To breed valuable strains of *T. ferrooxidans* for bacterial leaching, it is important to identify in this bacterium the enzyme that is involved directly in the solubilization of metal ions from sulfides ores. The importance of iron oxidase in bacterial leaching is well known[3]. However, there have been few reports on enzymes apart from iron oxidase that are involved in bacterial leaching. We have proposed a sulfur oxidation route other than that previously reported for *T. ferrooxidans*[2-5], in which elemental sulfur is oxidized by three enzymes, namely, hydrogen sulfide: ferric ion oxidoreductase (SFORase), sulfite: ferric ion oxidoreductase, and iron oxidase[8-15]. The following results obtained by using 8 pure cultures of iron-oxidizing bacteria isolated from natural environments suggest that SFORase compared to iron oxidase is more important in solubilization of Cu^{2+} from copper concentrate: (1) *T. ferrooxidans* strains that showed high Cu^{2+} solubilization activity had a high SFORase activity; (2) the strains that showed low Cu^{2+} solubilization activity had a low SFORase activity; (3) no remarkable difference in the levels of cellular iron-

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oxidizing activity were observed in all of the strains tested[16,17]. To clarify more precisely the roles of SFORase and iron oxidase in bioleaching, it is quite important to compare the levels of iron and SFORase activities with much more different cultures of iron-oxidizing bacteria. In this work we compare the levels of iron and SFORase activities among 200 iron-oxidizing bacterial strains isolated from natural environments.

Materials and Methods

Microorganisms, media, and conditions of cultivation 200 strains of iron-oxidizing bacteria isolated from streams and soils were used in this study. To isolate the bacteria, stream water or soil samples were incubated at 30 °C under aerobic conditions in a Fe^{2+} medium (pH 2.5) containing $FeSO_4 \cdot 7H_2O$ (3.0 %), $(NH_4)_2SO_4$ (0.3 %), K_2HPO_4 (0.5 %), $MgSO_4 \cdot 7H_2O$ (0.5 %), KCl (0.1 %), $Ca(NO_3)_2$ (0.01 %). When the culture medium turned rusty, i.e., Fe^{2+} in the medium was oxidized by iron-oxidizing bacteria to Fe^{3+}, aliquots of the culture medium were plated on gellan gum plates containing $FeSO_4 \cdot 7H_2O$ (2.0 %), yeast extract (0.03 %), and the same concentrations of salts as described above. Rusty colonies appearing on the plate were picked. This process was repeated more than three times and the final isolates were preserved on the the Fe^{2+} medium (pH 2.5) and used throughout this study. The *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NG) treated *T. ferrooxidans* AP19-3 was prepared as follow. Washed intact cells of *T. ferrooxidans* AP19-3 in 0.1 M citrate buffer (pH 5.0) was incubated with 50 μmol/ml NG for 1 h at 30 °C, washed with 0.1 M sodium phosphate buffer (pH 7.0), and cultured in a Fe^{2+} medium (pH 2.5) for 5 days. Aliquots of the culture medium were plated on the gellan gum plate (pH 2.5) containing $FeSO_4 \cdot 7H_2O$ (2.0 %), $(NH_4)_2SO_4$ (0.3 %), K_2HPO_4 (0.5 %), $MgSO_4 \cdot 7H_2O$ (0.5 %), KCl (0.1 %), $Ca(NO_3)_2$ (0.01 %). Rusty colonies appearing on the plate were picked. The isolates were preserved on the Fe^{2+} medium (pH 2.5) and used throughout this study.

Growth rate Cells were separated from ore particles by filtration of culture through a Toyo no. 5C paper filter. The number of cells in the filtrates was counted with a hemacytometer (Kayagaki Irika Kogyo Co. Ltd., Tokyo) after dilution with 0.1 N sulfuric acid, when necessary.

Iron-xidizing activity Each of the 200 strains of iron-oxidizing bacteria was grown statically in 20 ml of Fe^{2+} medium (pH 2.5) at 30 °C. After 7 days cultivation, all of the medium (20 ml) was centrifuged at 12,000 × *g* for 10 min to obtain a rusty precipitate containing cells and ferric hydroxide. The precipitate was washed once with 20 ml of 0.1 M β-alanine-SO_4^{2-} buffer (pH 3.0) and suspended in 9.5 ml of 0.1 M β-alanine-SO_4^{2-} buffer (pH 3.0). The activity was determined by measuring the Fe^{2+} oxidized in the reaction mixture under aerobic conditions. The reaction mixture was composed of 0.1 M β-alanine-SO_4^{2-} buffer (pH 3.0), washed intact cells, and $FeSO_4 \cdot 7H_2O$ (180 μmol). The total volume was 10.0 ml. The reaction was carried out by shaking the reaction mixture at 30 °C. A sample of the reaction mixture (0.5 ml) was withdrawn and centrifuged at 12,000 × *g* for 2 min, and the concentration of Fe^{2+} in the supernatant (80 μl) was determined spectrophotometriaclly by the o-phenanthroline method[18]. Iron-oxidizing activity was also measured by oxygen uptake, caused by the oxidation of Fe^{2+}, in a Warburg manometer[19].

Hydrogen sulfide: ferric ion oxidoreductase activity Activity was determined by measuring the Fe^{2+} produced in the reaction mixture under aerobic conditions in the presence of sodium cyanide or an inhibitor of iron oxidase. The method used for the preparation of washed intact cells from 20 ml of Fe^{2+} medium (pH 2.5) was the same as that used for the determination of iron oxidase. The reaction mixture was composed of

0.1 M β-alanine-SO_4^{2-} buffer (pH 3.0, 8 ml), elemental sulfur (200 mg), Fe^{3+} (50 μmol), washed intact cells and sodium cyanide (50 μmol). Total volume was 10.0 ml. The reaction was carried out by shaking the reaction mixture at 30 °C. A sample of the reaction mixture was withdrawn and centrifuged at 12,000 × *g* for 2 min to remove elemental sulfur and cells, and the concentration of Fe^{2+} produced in the supernatant was measured by the *o*-phenanthroline method[19].

Protein concentration The amount of cell protein produced in 20 ml of Fe^{2+} medium was measured by the following method. Each of the strains of iron-oxidizing bacteria was grown statically on 20 ml of Fe^{2+} medium (pH 2.5) at 30 °C for 7 days. After 7-day cultivation, all of the medium (20 ml) was centrifuged at 12,000 × *g* for 10 min to obtain a rusty precipitate containing cells and a solid ferric hydroxide. Concentrated hydrochloric acid (1.0 ml) was added to the precipitate to solubilize a solid ferric hydroxide. The yellow solution produced was centrifuged at 12,000 × *g* for 10 min to remove cells from ferric ion. Yellow clear supernatant containing ferric ion was discarded. To the cell pellet obtained, 1.0 ml of 2N sodium hydroxide was added and this was incubated for 5 min at 100 °C to dissolve the cells completely in the alkaline solution. A rusty ferric hydroxide which appeared by this alkaline treatment was removed by centrifugation at 12,000 × *g* for 10 min. Alkaline copper solution (1.0 ml) and phenol reagent (0.1 ml) were added to the 0.2 ml of supernatant obtained. After incubation of the mixture for 2 min, an absorbance at 660 nm was measured.

Results

Iron-oxidizing activities of 200 strains of iron-oxidizing bacteria Iron-oxidizing activities of iron-oxidizing bacteria grown in Fe^{2+}-medium were determined by a rapid method using 20 ml of culture medium. The 150 pure strains of iron-oxidizing bacteria isolated from stream waters and soils and 50 strains of *T. ferrooxidans* AP19-3 that were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) were grown statically on 20 ml of Fe^{2+} medium (pH 2.5) at 30 °C. After 7 day cultivation, all of the media (20 ml) were centrifuged at 12,000 × *g* for 10 min to obtain a rusty precipitate. The rusty precipitate that contained both cells and ferric hydroxide was washed once with 20 ml of 0.1 M β-alanine-SO_4^{2-} buffer (pH 3.0) and used as an enzyme source for determining iron-oxidizing activity in 20 ml of culture medium. No remarkable difference was observed in the levels of iron-oxidizing activity among the 200 strains of iron-oxidizing bacteria tested. The results obtained for 18 representative strains are shown in Table 1. Among them NT-103 was a representative strain of NG treated *T. ferrooxidans* AP19-3. We could not find a noticeable difference in the levels of iron-oxidizing activity among 50 NG treated strains of *T. ferrooxidans* (data not shown). To estimate the accuracy of our rapid method used for determining iron-oxidizing activity, the activity was also measured by a conventional method using a Warburg manometer[19]. Washed intact cells of strains AP19-3, OK-5, and NT-103 were obtained culturing each of the three strains for 7 days in 10 liters of Fe^{2+} medium (pH 2.5) under the forced airation at 30 °C, collecting cells with centrifugation at 12,000 × *g*, and washing with 0.1 M β-alanine-SO_4^{2-} buffer (pH 3.0) three times. Iron-oxidizing activities determined by a conventional Warburg method were 1.22, 1.29, and 1.28 μmol/mg/min for strains AP19-3, OK-5, or NT-103, respectively (Fig. 1B), indicating that iron-oxidizing activity determined by a Warburg method corresponded with those obtained by the rapid method developed in this study (Fig. 1A and Table 1).

The effects of culture time on iron-oxidizing activity were studied with strains AP19-3, OK-5, and NT-103. Iron-oxidizing activities were measured after these strains were cultured in 20 ml of

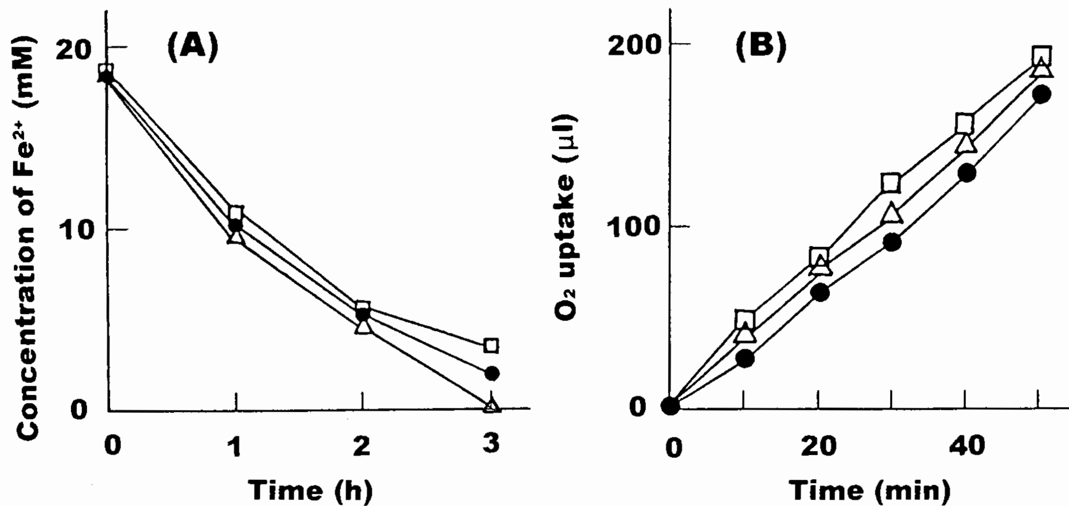

Fig. 1 Iron-oxidizing activities of iron-oxidizing bacteria strains AP19-3, OK-5 and NT1-103.
(A) Iron-oxidizing activity measured by the rapid method described in Materials and Methods.
(B) Iron-oxidizing activity measured by a coventional Warburg manometer.
Symbols: ●, AP19-3; □, OK-5; △, NT1-103.

Table 1 Iron-oxidizing activities of the strains isolated from natural environments and strains of *Thiobacillus ferrooxidans* AP19-3 treated with N-methyl-N'-nitro-N-nitrosoguanidine

Strain		Iron-oxidizing activity (μmol/mg/min)
AP19-3	Yanahara mine, Okayama Prefecture	1.52
NAS-2	Susobanagawa, Nagano Prefecture	1.49
NAS-3	Matsukawa, Nagano Prefecture	1.38
NAS-4	Chikumagawa, Nagano Prefecture	1.63
NAS-5	Nojiriko, Nagano Prefecture	1.55
HY-1	Ikunochou, Hyogo Prefecture	1.52
OK-4	Hyakkengawa, Okayama Prefecture	1.65
OK-5	Tsuchihashi mine, Okayama Prefecture	1.50
OK-6	Kurashikigawa, Okayama Prefecture	1.20
OK-8	Nariwagawa, Okayama Prefecture	1.51
SH-1	Misumigawa, Shimane Prefecture	1.20
TK-1	Yoshinogawa, Tokushima Prefecture	1.42
KO-1	Katsurahama, Kouchi Prefecture	1.46
FK-1	Iizuka City, Fukuoka Prefecture	1.44
NG-1	Unzen, Nagasaki Prefecture	1.42
KG-1	Saginosuonsen, Kagoshima Prefecture	1.31
Funis 2-1	Soil from U.S.A. mine	1.47
NT1-103	*T. ferrooxidans* AP19-3 treated with NG	1.43

Fe^{2+} medium (pH 2.5) for 5, 7, and 10 days. The levels of iron-oxidizing activities of strains AP19-3 cultured for 5, 7 and 10 days were 1.49, 1.52, and 1.12 μmol/mg/min, respectively. Nearly the same results were obtained in the case of strains OK-5 and NT-103. Growth curves of AP19-3, OK-5, and NT-103 made by measuring cell protein produced in 20 ml of Fe^{2+} medium (pH 2.5) are shown in Fig. 2. These growth curves corresponded well with those made by counting the number of cells in 20 ml of Fe^{2+} medium with a hemacytometer (data not shown).

Hydrogen sulfide: ferric ion oxidoreductase activities of 200 strains of iron-oxidizing bacteria Hydrogen sulfide: ferric ion oxidoreductase (SFORase) is present in the periplasmic space of *T. ferrooxidans* and plays a crucial role in the elemental sulfur oxidation of this bacterium[8-10].

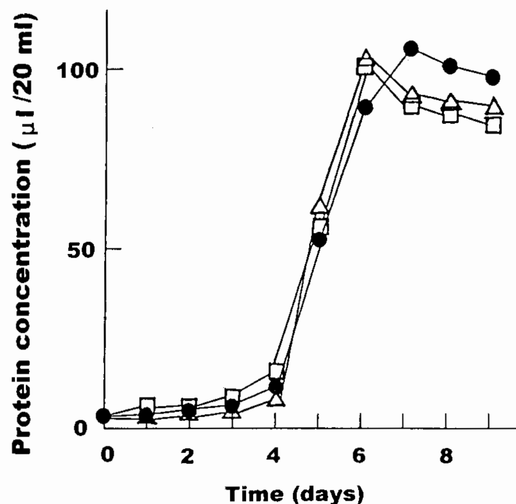

Fig. 2 Growth of iron-oxidizing bacteria strains AP19-3 (●), OK-5 (□), and NT1-103 (△) in Fe^{2+} medium (pH 2.5).

SFORase activities of iron-oxidizing bacteria grown in Fe^{2+}-medium were measured by a rapid method using 20 ml of culture medium. The 200 pure strains of iron-oxidizing bacteria used in this experiment were the same as those described above. In striking contrast to iron-oxidizing activity, the levels of SFORase activity of 200 iron-oxidizing bacteria tested differed markedly from strain to strain, ranging from 0.11 to 2.80 μmol/mg/min. The results obtained for 18 representative strains of iron-oxidizing bacteria are shown in Table 2.

Discussion

Iron oxidase and hydrogen sulfide: ferric ion oxidoreductase are the most important enzymes for iron-oxidizing bacterium *Thiobacillus ferrooxidans* to generate energy for cell growth and cell maintenance. Rapid methods for determining both iron and SFORase activities and the concentration of cell protein in 20 ml of culture medium were developed to compare the activities of both iron oxidase and SFORase for 200 strains of iron-oxidizing bacteria isolated from the natural

Table 2 Hydrogen sulfide: ferric ion oxidoreductase activities of the strains isolated from natural environments and strains of *Thiobacillus ferrooxidans* AP19-3 treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine

Strain		Hydrogen sulfide: ferric ion oxidoreductase activity (μmol/mg/min)
AP19-3	Yanahara mine, Okayama Prefecture	0.58
NAS-2	Susobanagawa, Nagano Prefecture	0.42
NAS-3	Matsukawa, Nagano Prefecture	0.35
NAS-4	Chikumagawa, Nagano Prefecture	0.58
NAS-5	Nojiriko, Nagano Prefecture	2.50
HY-1	Ikunochou, Hyogo Prefecture	1.29
OK-4	Hyakkengawa, Okayama Prefecture	1.07
OK-5	Tsuchihashi mine, Okayama Prefecture	0.12
OK-6	Kurashikigawa, Okayama Prefecture	0.21
OK-8	Nariwagawa, Okayama Prefecture	0.50
SH-1	Misumigawa, Shimane Prefecture	0.48
TK-1	Yoshinogawa, Tokushima Prefecture	0.44
KO-1	Katsurahama, Kouchi Prefecture	0.11
FK-1	Iizuka City, Fukuoka Prefecture	0.58
NG-1	Unzen, Nagasaki Prefecture	2.36
KG-1	Saginosuonsen, Kagoshima Prefecture	0.88
Funis 2-1	Soil from U.S.A. mine	2.80
NT1-103	*T. ferrooxidans* AP19-3 treated with NG	0.58

environments. We previously suggested that the levels of Cu^{2+} solubilized from copper ore differed markedly among eight strains of iron-oxidizing bacteria isolated from the natural environments[17]. The findings that a remarkable difference was observed in the levels of SFORase activity, but not in iron-oxidizing activity among the 200 strains of iron-oxidizing bacteria tested in this experiment indicate that SFORase, but not iron oxidase, is an important enzyme that determines the iron-oxidizing bacteria to solubilize heavy metal ions from sulfide ores and also suggest that SFORase, and not iron oxidase, is the target enzyme when we try to breed an iron-oxidizing bacterium having a high leaching activity for solubilizing metal ions.

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自然環境より単離した *Thiobacillus ferrooxidans* の鉄酸化酵素及び硫化水素酸化酵素活性

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鉄酸化酵素と硫化水素酸化酵素の両方が硫化鉱石からの金属イオンのバクテリアリーチングに関与していること，銅鉱石から溶出する銅イオンの量が鉄酸化細菌の菌株間で異なることが知られている．鉄酸化酵素及び硫化水素酸化酵素活性が自然環境から単離した鉄酸化細菌に対して決定された．200株の鉄酸化細菌の鉄酸化酵素及び硫化水素酸化酵素活性は，それぞれ1.20-1.65 μmol/mg/min 及び0.11-2.80 μmol/mg/min の範囲にあった．これら菌株間において，鉄酸化酵素ではなく硫化水素酸化酵素活性に大きな違いがあるという発見は，前者ではなく後者がこの細菌のバクテリアリーチング活性を決定する酵素であることを示唆している．