Note

Biological Reduction of Ferric Iron by Iron- and Sulfur-oxidizing Bacteria

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The ability of ferric iron reduction has been observed for some fungi and bacteria possessing with the enzyme, nitrate reductase.¹⁾ Ottow and Klopotek²⁾ have concluded that iron reduction is an enzymatic process and may be mediated by nitrate reductase. However, *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* are unable to utilize nitrite salts as a nitrogen source. Therefore it is unlikely that nitrate reductase is involved in ferric iron reduction by iron- and sulfur-oxidizing bacteria. The present paper deals with the ability of ferric iron reduction by thiobacilli cultures when grown on an elemental sulfur medium.

Acidophilic bacterium strain WU-66B, capable of oxidizing sulfur as well as ferrous iron, was isolated from waste water of Dowa Kosaka Mine, Akita Prefecture, Japan.³⁾ Sulfur-oxidizing bacterium strain WU-79A was isolated from soil at Manza Spa, Japan.⁴⁾ These strains have been identified as *T. ferrooxidans* and *T. thiooxidans*, respectively, on the basis of biochemical criteria.⁵⁾

Microorganisms were cultivated aerobically at 30°C in a 500 ml Erlenmeyer flask containing 100 ml of the medium and were shaken at 200 rpm on a rotary shaker-incubator. The composition of the culture medium was as follows (g/liter of distilled water): (NH₄)₂SO₄, 2.0; KH₂PO₄, 3.0; MgSO₄·7H₂O, 0.2; CaCl₂, 0.25. The initial pH was adjusted to 2.5 with 2N H₂SO₄ for *T. ferrooxidans*, and to 4.0 with 1N HCl for *T. thiooxidans*. Elemental sulfur was added at the level of 10 g per liter.

The amount of formed sulfate was determined by the titration method of Fritz and Freeland.⁶⁾ Cell growth was quantified by measuring optical density at 470 nm (UOD) as described previously.⁷⁾ Ferric iron reduction was measured by assaying the formation of ferrous iron, using *o*-phenanthroline as a specific reagent. The amount of dissolved ferrous iron was expressed in micromoles of Fe²⁺ per ml.

The supernatant fraction containing the crude cell-free extract (fraction S) and the pellet fraction (fraction P) used

for determination of enzyme activity of ferric iron reduction were prepared as described previously.⁸⁾ The enzyme activity of ferric iron reduction was determined by a modification of the α,α' -dipyridyl method, which was used in the measurement of iron oxidase activity.⁷⁾ After 5-minute preincubation of 0.5 ml of cell-free extracts with 1.3 ml of 2.5 mM citrate-5 mM phosphate buffer (pH 5.0), the reaction was initiated by addition of 0.1 ml of 10 mM FeCl₃·6H₂O acidified to pH 2.5 with 2N H₂SO₄ and 0.1 ml of 5 mM ascorbic acid as an electron donor, and incubation was carried out for 15 min at 30°C. The reaction was terminated by addition of 2.0 ml of a solution composed of 1 part 1:10 H₂SO₄, 1 part 20% ammonium acetate and 3 parts 0.1 M sodium phosphate. The amount of ferrous iron formed was determined by the α,α' -dipyridyl method since ferric iron does not interfere with this assay. After adding 1.0 ml of 0.5% α,α' -dipyridyl solution, the total volume of the reaction mixture was adjusted to 15 ml with distilled water. After 20 min in the dark, the reaction mixture was centrifuged at 11,000×g for 10 min to eliminate the turbidity caused by the preparation of cell-free extracts. The absorbance was measured at 520 nm. Protein content was determined by the method of Lowry *et al.*⁹⁾ using bovine serum albumin as a standard.

It was found that *T. ferrooxidans* and *T. thiooxidans* were able to reduce ferric iron to the ferrous state when aerobically grown on elemental sulfur as an energy source. Figure 1 shows reduction of ferric iron as demonstrated by

FIG. 1. Reduction of Ferric Iron and Growth of *T. thiooxidans* WU-79A on Elemental Sulfur Medium.

T. thiooxidans was cultivated aerobically at 30°C in a 500 ml Erlenmeyer flask containing 100 ml of the medium, ferric chloride was added from a stock solution of FeCl₃·6H₂O to a final concentration of 7.16 μmol Fe³⁺ per ml. Ferric iron reduction was measured by assaying the formation of ferrous iron, using *o*-phenanthroline reagent.

○—○, pH change; △—△, formed SO₄²⁻; ▲—▲, cell growth (UOD); ●—●, formed Fe²⁺ (inoculated); ●---●, formed Fe²⁺ (uninoculated).

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TABLE I. COMPARISON OF THE ABILITY OF FERRIC IRON REDUCTION IN *T. ferrooxidans* AND *T. thiooxidans*Figures show values relative to the 100% of *T. thiooxidans* after 25 day-cultivation.

Organism	Cultivation time (day)	Formed Fe^{2+} (ΔFe^{2+})	$\Delta\text{UOD}/\Delta\text{SO}_4^{2-}$	$\Delta\text{Fe}^{2+}/\Delta\text{UOD}$	$\Delta\text{Fe}^{2+}/\Delta\text{SO}_4^{2-}$
<i>T. ferrooxidans</i> WU-66B	6	8.8	105.5	16.6	10.5
	9	13.3	117.6	21.0	14.5
	15	24.2	127.4	26.9	20.0
	25	37.9	99.0	41.6	24.0
<i>T. thiooxidans</i> WU-79A	6	21.0	103.9	39.8	24.3
	9	58.6	113.8	65.1	82.2
	15	92.6	107.7	91.8	98.2
	25	100.0	100.0	100.0	100.0

formation of ferrous iron and the cell growth of *T. thiooxidans* WU-79A. It is obvious that the cell growth, sulfate formation and ferric reduction were essentially parallel throughout the experimental period. At the end of the experiment, virtually all of the ferric iron had been reduced to the ferrous form. No reduction of ferric iron was observed in uninoculated controls even after 25 days of incubation at 30°C. Although *T. ferrooxidans* WU-66B adapted to growth on elemental sulfur grew as well as *T. thiooxidans* WU-79A, the ability of iron reduction of WU-66B was lower than that of WU-79A (Table I). We discovered that *T. ferrooxidans* grown on an elemental sulfur medium had slight iron oxidase activity.⁸⁾ On the basis of these facts, it seems likely that in the case of *T. ferrooxidans* the ferrous iron formed by the reduction process might be re-oxidized to ferric iron in the presence of O_2 , and the ability of reduction was apparently lower than that of *T. thiooxidans*.

It has generally been assumed that oxidation of sulfide minerals by ferric iron is strictly a nonbiological process.¹⁰⁾ And in the acidic environment where these bacteria occur, ferrous iron formed during the reduction by *T. thiooxidans* may be continually converted to the ferric state by bacteria such as *T. ferrooxidans*. Consequently it is possible that the rate of microbiological leaching of sulfide minerals may be promoted.

Furthermore, it can be considered that ferric iron might serve as an electron acceptor for oxidation of elemental sulfur and that reduction of ferric iron to the ferrous form is consequently detected. However, it is doubtful whether ferric iron can serve as an electron acceptor in respiration when the demand for oxygen exceeds the supply or when oxygen is absent. Nevertheless, Brock and Gustafson¹¹⁾ have reported that anaerobic growth of *Thiobacillus* cultures using ferric iron are possible. Therefore, we investigated the cell growth and ferric iron reduction of *T. thiooxidans* WU-79A when N_2 gas was substituted for O_2 in fermentors. As a result, neither ferric iron reduction nor growth could be detected. These results suggested that ferric iron can not serve as an electron acceptor in

TABLE II. STOICHIOMETRY OF FERRIC IRON REDUCTION BY *T. thiooxidans* WU-79A

The complete system contained: 1.3 ml of 2.5 mM citrate-5 mM Na_2HPO_4 buffer (pH 5.0), 1.0 μmol ferric chloride, 0.5 μmol ascorbic acid, cell-free extract. The amounts of protein used were as follows: supernatant fraction (S), 0.279 mg; pellet fraction (P), 0.028 mg.

Reaction mixture	$\mu\text{mol Fe}^{2+}$ formed per 15 min	
	S	P
Complete	0.6584	0.7366
Minus cell-free extracts	0.8007	0.8037
Minus ascorbic acid	0.0479	0.0043

respiration, and that the reduction will only occur when these bacteria are aerobically grown on elemental sulfur.

It was confirmed that ferric iron reduction was essentially parallel to the cell growth (Fig. 1). Next, we did an experiment to determine whether the reduction was an enzymatic process or not. As shown in Table II, cell-free extracts could not reduce ferric iron to the ferrous state. In the case of deletion of ascorbic acid, the electron donor, the amount of ferrous iron found after 15-minute incubation was not significantly greater than that before incubation.

These results suggested that the reduction of ferric iron by *T. thiooxidans* is a nonenzymatic process, and probably it is dependent on reducing substrates formed during the growth of cells.

REFERENCES

- 1) J. C. G. Ottow, *Z. Allg. Mikrobiol.*, **8**, 441 (1968).
- 2) J. C. G. Ottow and A. V. Klopotek, *Appl. Microbiol.*, **18**, 41 (1969).

- 3) S. Usami and S. Yumimoto, *Water Purifn. and Liq. Wastes Treatment* (Japan), **11**, 17 (1970).
 - 4) T. Sugitani, T. Oneda and S. Usami, *Hakko Kyokai-shi* (Japan), **30**, 7 (1970).
 - 5) R. E. Buchanan and N. E. Gibbons, "Bergey's Manual of Determinative Bacteriology," 8th Ed., The Williams and Wilkins Co., Baltimore, 1974, pp. 459~460.
 - 6) J. S. Fritz and M. Q. Freeland, *Anal. Chem.*, **26**, 1593 (1954).
 - 7) S. Usami, K. Kino, K. Kuroda and N. Sugimoto, *Hakkokogaku*, **58**, 123 (1980).
 - 8) K. Kino, N. Sugimoto and S. Usami, *Hakkokogaku*, **59**, 415 (1981).
 - 9) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
 - 10) P. C. Singer and W. Stumm, *Science*, **167**, 1121 (1970).
 - 11) T. D. Brock and J. Gustafson, *Appl. Environ. Microbiol.*, **32**, 567 (1976).
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