

*Ferrobacillus ferrooxidans*와 *Thiobacillus thiooxidans*

의 免疫學的 同定研究

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Immunological Identification of *Ferrobacillus ferrooxidans*  
and *Thiobacillus thiooxidans*

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ABSTRACT

The chemolithoautotrophic bacteria, *Ferrobacillus ferrooxidans* and *Thiobacillus thiooxidans* were identified according to their immunological and serological properties.

The antibody to these organisms was easily elicited in experimental animals, however, the overall serological reactivities were low according to different kinds of titration methods.

By means of the quantitative and qualitative analysis such as hemagglutination or ouchterlony tests, *F. ferrooxidans* and *T. thiooxidans* were different in their immunoreactivities, whereas the strains among the *F. ferrooxidans* were possessed, in some extent, the sharing antigenic determinants.

In the results of the polyacrylamide gel electrophoresis/radioimmunometric method, the major antigenic determinants of the organisms illustrated the type specificities in the fraction number of 20-30 in their gel electrophoretograms with some modifications of the antigenic moieties.

INTRODUCTION

On the basis of morphological, physico-chemical, and microbiological characteristics, the chemolithoautotrophic bacteria are divided into two genus *Ferrobacillus* and *Thiobacillus*.

These groups are further subdivided into several species with their properties.

Recently, these organisms are known as an useful bacteria for the industrial purpose particularly in the process on bacterial leaching of low-grade ores such

as copper (Woodcock, 1967) and uranium (MacGregor, 1966).

However, the overall researches on *Ferrobacillus* and *Thiobacillus* are not much studied except the several studies which have been reported on the metabolism of phospholipid (Short, 1969), the role of peptidoglycan (Wang, 1968) and ultrastructure in these organisms (Taylor, 1969; Shively, 1973; and Murphy, 1974).

On the other hand, it is well known that the classification of related organisms with immunological method is a

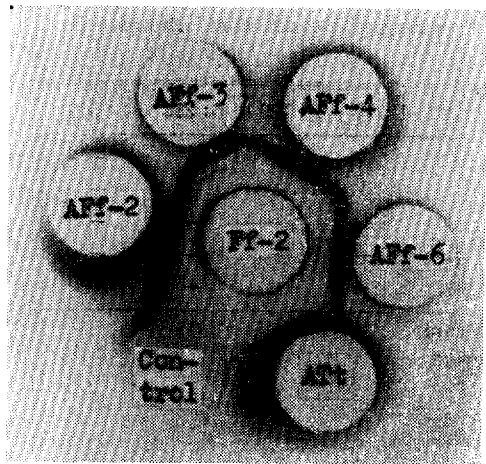


Fig. 2. Double gel diffusion analysis for antigenic identity between antisera to each strains of *F. ferrooxidans*(AFF-) and *T. thiooxidans*(ATt) with *F. ferrooxidans* strain 2(Ff-2) antigen.

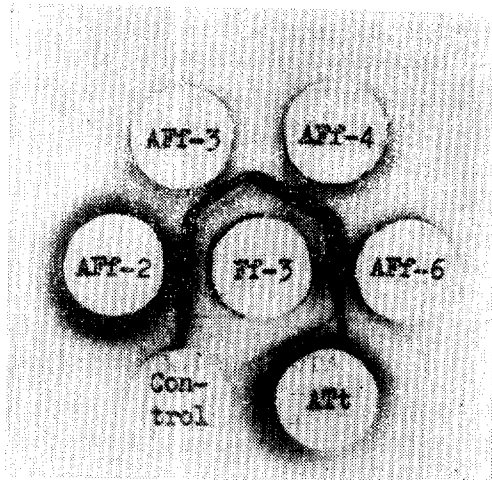


Fig. 3. Double gel diffusion analysis for antigenic identity between antisera to each strains of *F. ferrooxidans*(AFF-) and *T. thiooxidans*(ATt) with *F. ferrooxidans* strain 3 antigen.

strain 2 and 3, showed that these antigens were very reactive and wide cross-reactivities were observed in antisera to *F. ferrooxidans* strain 2, 3, 4 and 6, whereas the antiserum to *T. thiooxidans* was very weak in immunoreactivity (Figs. 2 and 3).

On the other hand, the whole bacterial antigen from *T. thiooxidans* was strongly reactive to homologous, however, the immunoreactivities between *T. thiooxidans* antigen and antisera to *F. ferrooxidans* strains were weak, whereas they possessed small quantities of common antigenic substances by the process of immunodiffusion analysis(Fig.4).

The serological relationship was very similar in *F. ferrooxidans* strains, however, *F. ferrooxidans* and *T. thiooxidans* were clearly different in their serological specificities.

According to the results of hemagglutination and ouchterlony test, the immunoreactivity between *F. ferrooxidans* and

*T. thiooxidans* were different clearly to their homologous and heterologous antibodies.

However, the serological differences between two organisms were expressed as quantitatively in hemagglutination test

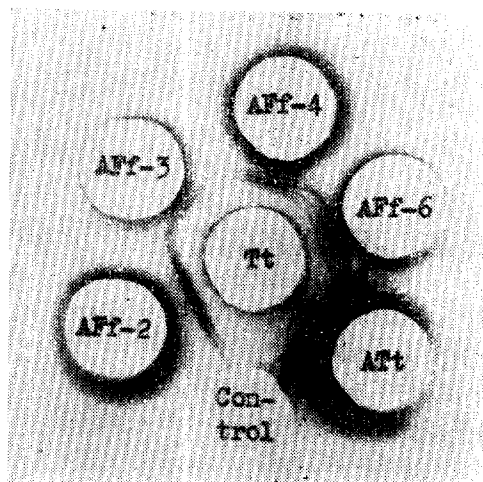


Fig. 4. Double gel diffusion analysis for antigenic identity between antisera to each strains of *F. ferrooxidans*(AFF-) and *T. thiooxidans*(ATt) with *T. thiooxidans* (Tt) antigen.

at 37°C moisture chamber and let stand further at room temperature.

#### 4. Agar Gel Diffusion

1gm of purified agar(Difco, USA) was dissolved in 100ml of 0.14M NaCl containing merthiolate(1:10,000). The melted and deaerated agar was dispensed glass Petri dish.

The sonicated whole suspensions of *F. ferrooxidans* and *T. thiooxidans* were used as an antigen at the concentrations of 10—15 mg protein per ml with undiluted each antisera.

The double gel diffusion test was carried out in moisture chamber at 37°C for 24hr and consecutively for 2 days at 4°C.

#### 5. Polyacrylamide Gel Electrophoresis and Radioimmunometric Analysis

The polyacrylamide gel electrophoresis of the whole cells of *F. ferrooxidans* and *T. thiooxidans* were performed according to Takayama(1964).

The packed cells were lysed with phenol:acetic acid:D.W. (2:1:1, w/v/v) and 500µg protein per 0.1ml of samples were subjected to electrophoresis.

The gel concentration was 7.5% and 10% acetic acid was used for running buffer.

Electrophoretograms were stained with amidoblack 10B and destained with 7% acetic acid.

The polyacrylamide gel electrophoresis/radioimmunometric analysis was conducted and the overall procedure was illustrated in Fig. 1.

The antisera to *F. ferrooxidans* strain 2,3, and *T. thiooxidans* were iodinated with radioactive  $^{131}\text{I}$  (carrier free, KAE-RI) by the method of chloramine-T (Hunter and Greenwood, 1962).

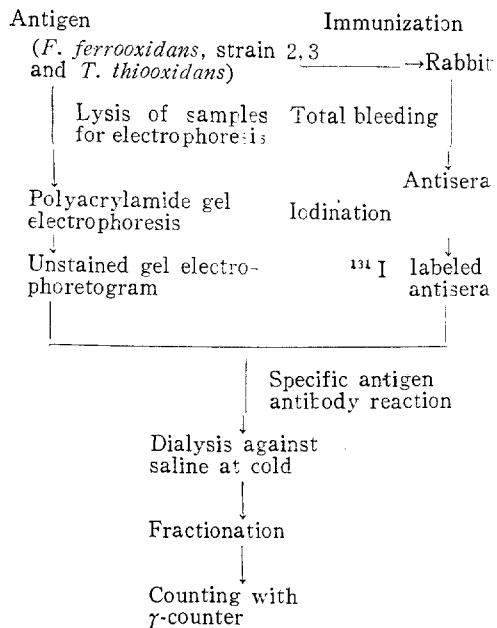


Fig. 1. Schematic representation of polyacrylamide gel electrophoresis/radioimmunometric method

folds with 0.14M NaCl and placed into the screw cap test tube.

The unstained electrophoretograms were dipped into the screw cap test tube and reacted for 16 hr at 4°C.

After completed the immune reaction, they were sliced into 40 fractions with cutter and counted the radioactivity on the well type  $\gamma$ -counter.(Model EA-14, Fujitsu, Japan).

## RESULTS AND DISCUSSIONS

Table 1 and 2 record data concerned with the titers according to the various kinds of titration methods.

In the results of paper electrophoresis, most of the antibodies to the whole cell homogenates of *F. ferrooxidans* strains and *T. thiooxidans* were located mainly in  $\gamma$ -globulin of the antisera fractions.

The increment of the  $\gamma$ -globulin of the

useful tool because of its specificity and accuracy.

In this point of view, *Ferrobacillus ferrooxidans* and *Thiobacillus thiooxidans* are so similar in their characteristics, life-cycles that the classification is difficult and very obscure.

Furthermore, there has been no previous report on the identification of *Ferrobacillus ferrooxidans* or *Thiobacillus thiooxidans* with immunochemical and serological properties.

The present investigation deals with the identification of *Ferrobacillus ferrooxidans* and *Thiobacillus thiooxidans* by the immunological and radioimmunometric/polyacrylamide gel electrophoretic methods which have been used in our laboratory.

## MATERIALS AND METHODS

### 1. Bacterial Strains and Growth

The chemosynthetic autolithotrophic bacteria, i.e., *Ferrobacillus ferrooxidans* (designated as *F. ferrooxidans*) and *Thiobacillus thiooxidans* (designated as *T. thiooxidans*) were isolated from acid strip mine effluents in Korea and identified according to the method of Sutton and Corrick (1961).

The bacterial strains of *F. ferrooxidans* were designated as strain 2, 3, 4 and 6, respectively.

*F. ferrooxidans* and *T. thiooxidans* were propagated in 9K and Waksman media (Silverman, 1959; Waksman, 1922) in 20l carboy under forced aeration at 28°C for 5–6 days. The cells were harvested by centrifugation (I.E.C. B-20 centrifuge, Rotor 872) at 9,000rpm for 15min, the resulting paste of cells were suspended in cold 0.14M NaCl and washed for

3 times.

### 2. Preparation of Antisera and Titration

Antisera to the whole organisms of *F. ferrooxidans* and *T. thiooxidans* were raised in rabbits, weighing 2.0 to 2.5kg, using 3 animals for each organism (antigen).

Injections of antigens with Freund's complete adjuvant were given intradermally, subcutaneously and intramuscularly at 10 days intervals for a month using 1.8 mgN of sonicated bacterial suspensions for each injection.

The rabbits were bled by cardiac puncture 10 days after the last immunization; the antisera were collected, millipore filtered, and stored at 60°C.

The antibody titrations were done by the procedures of paper electrophoresis, agglutination and hemagglutination tests, described respectively in elsewhere.

### 3. Passive-Hemagglutination Test

The tests were performed according to the method of Mergenhagen (1962).

Sheep erythrocytes were washed twice with PBS, pH 7.2 and adjusted to 0.5% suspension.

The sensitization were carried out with the whole bacterial suspensions (*F. ferrooxidans* and *T. thiooxidans*) in proper dilution.

The mixtures were incubated at 4°C for 16 hr. and afterward for 2hr. at 37°C.

The erythrocytes were washed twice to remove excess antigen by centrifugation.

The hemagglutination tests were done with microtiter apparatus (Cooke Inst. Co., USA); portions (50  $\mu$ ) of the 0.5% sensitized erythrocyte suspensions were added to 50  $\mu$  portions of serial dilutions of each antisera.

The mixtures were incubated for 2hr

Table 3. Comparisons of hemagglutination titer between homologous and heterologous antisera to *F. ferrooxidans* strain

Diluted titer** Antisera*	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
AFf-2	++	++	++	++	+	+	+	+	-
AFf-3	++	++	++	+	+	+	-	-	-
AFf-4	++	++	+	+	+	-	-	-	-
AFf-6	++	++	+	+	+	-	-	-	-
ATt	++	++	+	+	+	-	-	-	-

Remarks: \*AFf-: Anti-*F. ferrooxidans* strain serumATt-: Anti-*T. thiooxidans* strain serum

\*\* ++: Strong react

+: React

-: Non react

Table 4. Comparisons of hemagglutination titer between homologous and heterologous antisera to *F. ferrooxidans* strain 3

Diluted titer Antisera	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
AFf-3	++	++	++	+	+	+	+	+	-
AFf-2	++	++	++	+	+	+	-	-	-
AFf-4	++	++	+	+	-	-	-	-	-
AFf-6	++	+	+	+	-	-	-	-	-
ATt	++	+	+	+	-	-	-	-	-

Remarks: Same as Table 3.

Table 5. Comparisons of hemagglutination titer between homologous and heterologous antisera to *T. thiooxidans*

Diluted titer Antisera	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
ATt	++	++	++	+	+	+	-	-	-
AFf-2	++	+	+	+	-	-	-	-	-
AFf-3	++	++	+	+	-	-	-	-	-
AFf-4	++	+	+	+	-	-	-	-	-
AFf-6	++	+	+	-	-	-	-	-	-

Remarks: Same as Table 3.

*oxidans* strain 2 and 3 with their own and heterologous antisera.

There were no detectable differences in hemagglutination titers of antisera to *F. ferrooxidans* strain 4 and 6, when reacted with the antigens of *F. ferrooxidans* strains 2, 3 and *T. thiooxidans*, respectively.

The wide cross-reactivities were observed in *F. ferrooxidans* strains and *T.*

*thiooxidans* in hemagglutination test and these suggested the presence of related antigenic substance which will easily be detected in hemagglutination test.

In another respect, the immunodiffusion tests were conducted, to compare the antigenic relationship between *F. ferrooxidans* strains and *T. thiooxidans*.

Ouchterlony tests, with sonicated whole bacterial homogenate of *F. ferrooxidans*

- ten constant of  $\beta$ -1,4-glucan-4-glucanohydrolase (E.C. 3.2.1.4)[C<sub>x</sub>-Cellulase Enzyme]. *Monatsh. Chem.* **100**(3), 986-97.
2. Kim, W.S., Y.M. Kim, Y.H. Kang, and T.J. Choi, 1965. Studies on the cellulolytic enzymes of *Stachybotrys atra*. I. Effects of temperature and pH on the enzyme activities. *Kor. Jour. Microbiol.* **13**, 164-168
  3. Marshall, J.J., 1974. Application of enzymic methods to the structural analysis of polysaccharides. *Advances in Carbohydrate; Chemistry and Biochemistry.* **30**. p. 257-370, Academic Press, New York.
  4. Okada, G., 1975. Enzymic studies on a cellulase system of *Trichoderma viride*. II. Purification and properties of two cellulases. *J. Biochem.* **77**, 33-42.
  5. Pettersson, G., 1968. Structure and function of a cellulase from *Penicillium notatum* as studied by chemical modification and solvent accessibility. *Arch. Biochem. Biophys.* **126**, 776.
  6. Whitaker, D.R., 1971. Cellulases. *The enzymes.* 3rd., 5. p. 273-290, Academic Press, New York.
  7. Youatt, G., 1958. Fungal Cellulase. IX. Growth of *Stachybotrys atra* on cellulose and production of a  $\beta$ -glucosidase hydrolyzing cellobiose. *Aust. J. Biol. Sci.* **20**(1), 193-220.

and furthermore the antigen moiety employed in this test was crude form that it was very difficult to clarify the differences between them evidently.

By ouchterlony test, with anti *F. ferrooxidans* strains rabbit sera showed that the *F. ferrooxidans* strains were composed of major precipitation component, whereas minor precipitin components were also observed in *F. ferrooxidans* and *T. thiooxidans*, respectively.

The serological and phylogenetic relationship between *F. ferrooxidans* and *T. thiooxidans* were distinguished distinctly, however, in *F. ferrooxidans* strains, they were very similar or same kinds of serological specificities were represented in each other.

The O antigen(endotoxins and lipopoly-

saccharides) of Gram-negative bacteria generally are composed of polysaccharide, lipid, and a small amount of protein(De Araujo, 1963).

Especially, the polysaccharide moiety endows the antigen with serological specificity.

In this respect, it will be required to introduce a methodology with more defined fractionation procedures of the cell component such as lipopolysaccharide complex.

In heterotrophic bacteria, many authors (Mergenhagen, 1963; Raff, 1968 and Holingdale, 1973) had reported that the serological specificities were closely related with LPS.

In order to conform the immunological specific antigen substance in cell components of the *F. ferrooxidans* and *T. thi-*

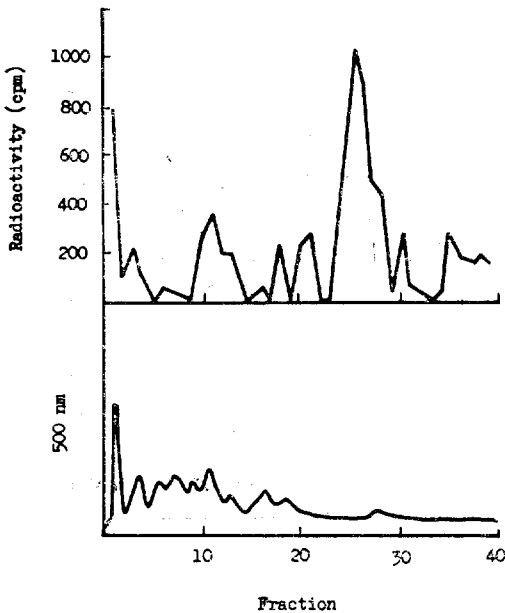


Fig. 5. Polyacrylamide gel electrophoresis/radioimmunometric analysis of *F. ferrooxidans*, St-2.

The unstained electrophoretogram was reacted with <sup>131</sup>I labeled homologous antiserum(upper) and its densitometric representation of lysed whole cell protein (lower).

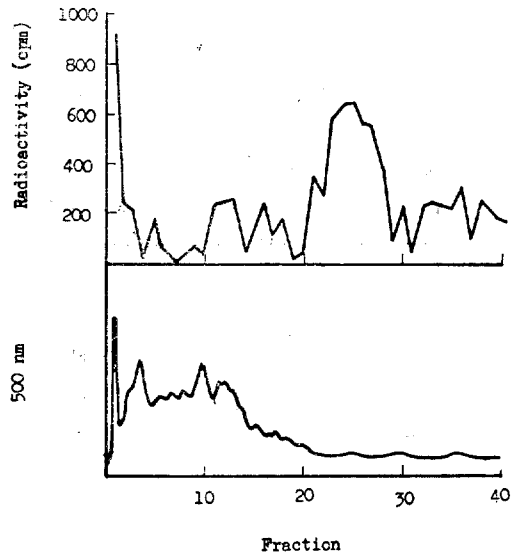


Fig. 6. Polyacrylamide gel electrophoresis/radioimmunometric analysis of *F. ferrooxidans*, St-3

The unstained electrophoretogram was reacted with <sup>131</sup>I labeled homologous antiserum(upper) and its densitometric representation of lysed whole cell protein (lower).

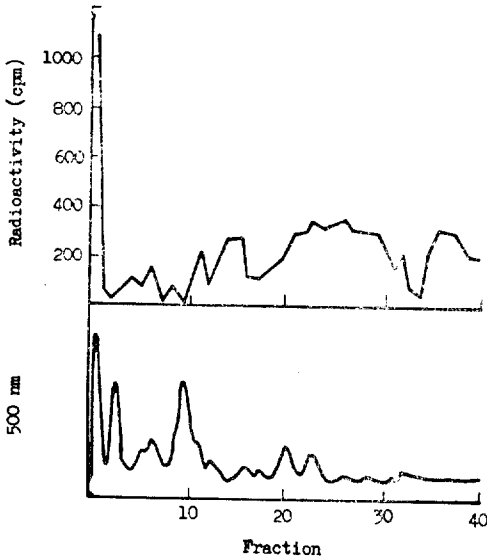


Fig. 7. Polyacrylamide gel electrophoresis/radioimmunometric analysis of *T. thiooxidans*. The unstained electrophoretogram was reacted with  $^{131}\text{I}$  labeled homologous antiserum (upper) and its densitometric representation of lysed whole cell protein (lower).

*ooxidans*, the whole cells were lysed and subjected to gel electrophoresis.

After the electrophoresis, the unstained electrophoretograms were directly reacted with  $^{131}\text{I}$  labeled each homologous antiserum and counted the radioactivity.

The results were illustrated in Figs. 5, 6, and 7, respectively.

According to the data from the radioimmunometric/polyacrylamide gel electrophoresis, the antigen-antibody complexes of *F. ferrooxidans* strain 2 and 3 which were reacted with their homologous antisera, showed the specific immune reaction on the fraction number of 20

to 30 in gel, whereas the densitometric analysis demonstrated that the protein band of that regions were very low to compare with the remaining other part of the gel.

In the case of *T. thiooxidans*, the major specific immune reaction in gel was also occurred at the same region comparing with that of *F. ferrooxidans*, however, more broad immunoreactive region was observed.

At the results of radioimmunometric/polyacrylamide gel electrophoretic analysis, the major specific immuno-reactive regions in gel electrophoretograms of the *F. ferrooxidans* strain 2 and 3 are the sharing antigenic determinants in *F. ferrooxidans*, however, it is assumed that the remaining regions were represented the type specificity in *F. ferrooxidans*.

According to our data (unpublished), the specific immuno-reactive region in gel electrophoretograms of the *F. ferrooxidans* strains and *T. thiooxidans* showed the molecular weight in a range of 75,000–60,000 to compare the electrophoretic mobility with references.

From these results, we suggest the major antigenic determinants of the *F. ferrooxidans* and *T. thiooxidans* are mainly polysaccharide, while the specificities are expressed, in some less extent, as the modification of carbohydrate moiety with lipid or protein.

Another aspects of immunochemical approaches in serologic typing of the bacteria are now in progress.

## 摘 要

국내 각 동광산의 갱내수에서 분리한 무기영양성 세균인 *F. ferrooxidans* 네 균주와 *T. thiooxidans* 균주를 면역학적인 방법으로 동정하기 위하여 각 세균에 대한 항원성으로 혈구 응집반응, 한천 침강반응 및 전 균체를 polyacrylamide gel 전기영동 후 屬 및 種間的 면역학적인 특성을 고찰한 결과 혈구 응



집반응과 한천 침강반응에서 *F. ferrooxidans* 및 *T. thiooxidans*는 약간의 공통 항원성을 공유하고 있으나 정량 및 정성적인 차이점을 나타냈고 radioimmunometric 방법으로 분석한 결과 *F. ferrooxidans* 및 *T. thiooxidans*간의 공통 항원 내지 균체특이 항원-항체 complex는 gel 분획 20-30번째에서 다같이 나타났다.

## REFERENCES

1. De Araujo, W.C., E. Varah, and S.E. Mergenhagen, 1963. Immunochemical analysis of human oral strains of *Fusobacterium* and *Leptotrichia*. *J. Bacteriol.* **86**, 837-844.
2. Hollingdale, M.R., 1975. Isolation of lipopolysaccharide from the walls of *Micropoly-spora faeni*. Chemical composition and serological reactivity. *J. Gen. Microbiol.* **86**, 250-258.
3. Hunter, W.H., and F.C. Greenwood, 1962. Preparation of iodine-131 labeled human growth hormone of high specific activity. *Nature* **194**, 495-496.
4. Knoche, H.W., and J.M. Shively, 1969. The identification of *cis*-11,12-methylene-2-hydroxyoctadecanoic acid from *Thiobacillus thiooxidans*. *J. Biol. Chem.* **244**, 4773-4778.
5. Knoche, H.W., and J.M. Shively, 1972. The structure of an ornithine-containing lipid from *Thiobacillus thiooxidans*. *J. Biol. Chem.* **247**, 170-178.
6. MacGregor, R.A., 1966. Recovery of  $U_3O_8$  by underground leaching. *Can. Inst. Min. Metall. Bull.* **59**, 583.
7. Mergenhagen, S.E., I. Zipkin, and E. Varah, 1962. Immunological and chemical studies on an oral *Veillonella* endotoxin. *J. Immunol.* **88**, 482-487.
8. Mergenhagen, S.E., 1965. Polysaccharide-lipid complexes from *Veillonella parvula*. *J. Bacteriol.* **99**, 1730-1734.
9. Murphy, J.R., A.E. Girard, and R.C. Tilton, 1974. Ultrastructure of a marine *Thiobacillus*. *J. Gen. Microbiol.* **85**, 130-138.
10. Raff, R.A., and R.W. Wheat, 1968. Carbohydrate composition of the phenol-soluble lipopolysaccharides of *Citrobacter freundii*. *J. Bacteriol.* **95**, 2035-2043.
11. Rhee, K.S., C.S. Chang, B.H. Min, and K. S. Lee, 1973 a b. Immunological studies on *Ferrobacillus ferrooxidans*. Chemical analysis on the bacterial cell components. *Kor. J. Microbiol.* **11**, 115-120.
12. Rhee, K.S., 1973 b. Study on the ornithine-containing lipid from *Ferrobacillus ferrooxidans*. *Kor. J. Microbiol.* **11**, 109-114.
13. Shively, J.M., and H.W. Knoche, 1969. Isolation of an ornithine-containing lipid from *Thiobacillus thiooxidans*. *J. Bacteriol.* **94**, 829.
14. Shively, J.M., F.L. Ball, and B.W. Kline, 1973. Electron microscopy of the carboxysomes (polyhedral bodies) of *Thiobacillus neapolitanus*. *J. Bacteriol.* **116**, 1405-1411.
15. Short, S.A., D.C. White, and M.I.H. Alem, 1969. Phospholipid metabolism in *Ferrobacillus ferrooxidans*. *J. Bacteriol.* **99**, 142-150.
16. Silverman, M.P., and D.G. Lundgren, 1959. Studies on the chemosynthetic iron bacterium *Ferrobacillus ferrisidans*. I. An improved medium and harvesting procedures for securing high cell yields. *J. Bacteriol.* **77**, 642-647.
17. Sutton, J.A., and J.D. Corrick, 1961. Three chemosynthetic autotrophic bacteria important to leaching operations at Arizona copper mines. U.S. Dept. of the Interior Bureau of Mines.
18. Takayama, K., D.H. MacLennan, A. Tzagoliff, and C.O. Stoner, 1964. Studies on the electron transfer system. LXVII. Polyacrylamide gel electrophoresis of the mitochondrial electron transfer complexes. *Arch. Biochem. Biophys.* **114**, 223.
19. Taylor, B.F., and D.S. Hoare, 1969. New facultative *Thiobacillus* and reevaluation of

- the heterotrophic potential of *Thiobacillus novellus*. *J. Bacteriol.* **100**, 487—497.
20. Waksman, S.A., and J.S. Joffe, 1922. Microorganisms concerned in the oxidation of sulfur in the soil. II. *Thiobacillus thiooxidans*, a new sulfur-oxidizing organism isolated from the soil. *J. Bacteriol.* **7**, 259—256.
21. Wang, W.S., and D.G. Lundgren, 1968. Peptidoglycan of a chemolithotrophic bacterium *Ferrobacillus ferrooxidans*. *J. Bacteriol.* **95**, 1851—1856.
22. Woodcock, J.T., 1967. Copper waste dump leaching. *Proc. Aust. Inst. Min. Metall.* **224**, 47.