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Growth Inhibition by Tungsten in the Sulfur-Oxidizing Bacterium *Acidithiobacillus thiooxidans*

Atsunori NEGISHI,[1] Tadashi MURAOKA,[2] Terunobu MAEDA,[3] Fumiaki TAKEUCHI,[4] Tadayoshi KANAO,[5] Kazuo KAMIMURA,[5] and Tsuyoshi SUGIO[2,†]

[1]*Technical Research Institute, Hazama Corporation, 515-1 Nishimukai, Karima, Tsukuba 305-0822, Japan*
[2]*Division of Science and Technology for Energy Conversion, Graduate School of Natural Science and Technology, Okayama University, 3-1-1 Tsushima-Naka, Okayama 700-8530, Japan*
[3]*Civil Chemical Engineering Corporation, 3411 Sanuki-machi Ryugasaki, Ibaragi 301-0033, Japan*
[4]*Environmental Management and Safety Section, Health and Environment Center, Okayama University, 3-1-1 Tsushima-naka, Okayama 700-8530, Japan*
[5]*Faculty of Agriculture, Okayama University, 1-1-1 Tsushima-naka, Okayama 700-8530, Japan*

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Growth of five strains of sulfur-oxidizing bacteria *Acidithiobacillus thiooxidans,* including strain NB1-3, was inhibited completely by 50 μM of sodium tungstate (Na_2WO_4). When the cells of NB1-3 were incubated in 0.1 M β-alanine–SO_4^{2-} buffer (pH 3.0) with 100 μM Na_2WO_4 for 1 h, the amount of tungsten bound to the cells was 33 μg/mg protein. Approximately 10 times more tungsten was bound to the cells at pH 3.0 than at pH 7.0. The tungsten binding to NB1-3 cells was inhibited by oxyanions such as sodium molybdenum and ammonium vanadate. The activities of enzymes involved in elemental sulfur oxidation of NB1-3 cells such as sulfur oxidase, sulfur dioxygenase, and sulfite oxidase were strongly inhibited by Na_2WO_4. These results indicate that tungsten binds to NB1-3 cells and inhibits the sulfur oxidation enzyme system of the cells, and as a result, inhibits cell growth. When portland cement bars supplemented with 0.075% metal nickel and with 0.075% metal nickel and 0.075% calcium tungstate were exposed to the atmosphere of a sewage treatment plant containing 28 ppm of H_2S for 2 years, the weight loss of the portland cement bar with metal nickel and calcium tungstate was much lower than the cement bar containing 0.075% metal nickel.

Key words: tungsten; *Acidithiobacillus thiooxidans*; concrete corrosion; bacteriostatic agent; sulfur oxidation

Corrosion of concrete structures, especially, in sewer systems and sewage treatment plants, has become a big social problem. In 1945, Parker first presented evidence that concrete structures are corroded by sulfuric acid produced by the sulfur-oxidizing bacterium *Thiobacillus concretivourus*.[1–3] The bacterium isolated by Parker was later identified as *Acidithiobacillus thiooxidans*. In the process of concrete corrosion, hydrogen sulfide produced in sewage pipes by the action of sulfate-reducing bacteria is oxidized by sulfur-oxidizing bacteria to give sulfuric acid.[1–7] Concrete contains calcium oxide, calcium hydroxide, and calcium carbonate, and these compounds react with sulfuric acid to give a sandy calcium sulfate (gypsum).[7] After the studies done by Parker, many kinds of thiobacilli such as *Halothiobacillus neapolitanus*[8] (formerly *Thiobacillus neapolitanus*), *Thiomonas intermedius*[8] (formerly *Thiobacillus intermedius*), and *Thiobacillus novellus*, *Paracoccus versutus*[8] (formerly *Thiobacillus versutus*), and *Acidithiobacillus ferrooxidans*[8] (formerly *Thiobacillus ferrooxidans*) were isolated from corroded concrete and more information was accumulated about the process of concrete corrosion. A concrete structure just after construction contains calcium hydroxide and thus has a pH of 12–13. The initial step in concrete corrosion is the neutralization of the concrete structure mainly by atmospheric carbon dioxide or by sulfur-oxidizing bacteria that can grow under alkaline pH.[9] The second step is a rapid acidification of the neutralized concrete structure by sulfuric acid produced by obligately chemolithotrophic sulfur oxidizers *A. thiooxidans* and *A. ferrooxidans*.[10]

It is known that oxidation of elemental sulfur by *A. thiooxidans* is a complex process involving the contact of cells with sulfur particles, the oxidation of sulfur to sulfite, and the oxidation of sulfite to sulfate. The first step in sulfur oxidation from elemental sulfur to sulfite is catalyzed by sulfur dioxygenase.[11–13] Sulfite is thought to be the key intermediate in the oxidation of inorganic sulfur compounds in thiobacilli.[14–18] Sulfur is nearly quantitatively oxidized to sulfite by *A. thioox-*

† To whom correspondence should be addressed. Tel/Fax: +81-86-251-8306; E-mail: sugio@cc.okayama-u.ac.jp

idans cells when the sulfite oxidation is inhibited by 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HOQNO).[19] The sulfite produced by sulfur dioxygenase is further oxidized to sulfate either through sulfite:cytochrome *c* oxidoreductase,[20] or through adenosine phosphosulfate (APS) reductase.[21] *A. thiooxidans* JCM 7814 has only a AMP-independent sulfur oxidation pathway, and the membrane-bound sulfite dehydrogenase was purified from the bacterium.[22,23]

Previously we showed that concrete supplemented with nickel is resistant to corrosion,[24] and that nickel bound to the *A. thiooxidans* NB1-3 cells inhibits both sulfur dioxygenase and sulfite oxidase, and as a result, inhibits cell growth.[9,25,26] We prepared portland cement bars ($40 \times 40 \times 160$ mm) supplemented with various kinds of inorganic and organic compounds, and these bars were hung from the lid of a sludge tank manhole and exposed to 5–10 ppm hydrogen sulfide atmosphere.[24] After 2 years of exposure, it was found that the surface of the portland cement bar supplemented with nickel was especially smooth, while that without nickel was brittle and rough,[24] suggesting the possibility that nickel can be used as a bacteriostatic agent for the prevention of concrete corrosion. Among the inorganic and organic compounds tested, nickel was the most effective for the prevention of concrete corrosion. But, nickel is not the best bacteriostatic agent for prevention of the growth of *A. thiooxidans* cells, which have an optimum growth pH at 2–3. We found that the growth of NB1-3 cells was more strongly inhibited by Ni^{2+} at pH 7.0 than at pH 3.0, probably because more nickel binds to NB1-3 cells at neutral pH than at acidic pH. Strain NB1-3 cells without nickel treatment contained 1.7 nmol nickel per mg protein, and the cells treated with 10 mM $NiSO_4$ at pH 3.0 and 7.0 contained 35 and 160 nmol nickel per mg of protein respectively.[25] Therefore, to make a concrete structure that does not corrode easily under attack by *A. thiooxidans* cells, it is important to look for a bacteriostatic agent that can bind to *A. thiooxidans* cells more tightly under acidic pH than under neutral and/or alkaline pH and thus can inhibit more strongly the sulfur oxidation enzyme system of the bacterium.

In this report we show for the first time that a low concentration of sodium tungstate (50 μM) completely inhibits the growth and sulfur-oxidation enzyme system of *A. thiooxidans* NB1-3 cells, and that in contrast to Ni^{2+}, which inhibits completely the growth of *A. thiooxidans* cells at 5 mM and binds more to NB1-3 cell at pH 7.0 than at pH 3.0,[25] approximately 10 times more tungsten binds to the NB1-3 cells at pH 3.0 than at pH 7.0. These results indicate that tungsten can cover the weak point of Ni^{2+} as a bacteriostatic agent for the prevention of concrete corrosion caused by *A. thiooxidans*. The rate of concrete corrosion decreased markedly when the portland cement bars supplemented with both 0.075% calcium tungstate and 0.075% metal nickel were incubated in the atmosphere of sewage treatment plants containing 28 ppm H_2S for 2 years as compared with the cement bar supplemented with only 0.075% metal nickel.

Materials and Methods

Microorganism, medium, and conditions of cultivation. The sulfur-oxidizing bacteria *Acidithiobacillus thiooxidans* strain NB1-3, *A. thiooxidans* JCM 3867, *A. thiooxidans* ON106, *A. thiooxidans* NBRC 13701, and *A. thiooxidans* NBRC 137724 were used throughout this study. Strain NB1-3 was isolated from corroded concrete in Fukuyama, Japan.[25] A 1,000 ml of salt solution contained 3 g $(NH_4)_2SO_4$, 0.1 g KCl, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, and 0.01 g $Ca(NO_3)_2$. The pH of the salt solution was adjusted at pH 2.5 with sulfuric acid. Elemental sulfur was autoclaved by heating for 30 min at 110 °C and further incubated for 24 h at 30 °C. This sterilization process of elemental sulfur was repeated three times. Sulfur-salt medium used for the cultivation of NB1-3 cells composed of 20 ml of the autoclaved-salt solution (pH 2.5) and the sterilized elemental sulfur (0.2 g). Growth experiments were done by shaking 20 ml of sulfur-salt medium inoculated with NB1-3 culture in a 50-ml flask at 30 °C. A large mass of NB1-3 cells used for the isolation of sulfur dioxygenase was obtained by culturing the strain in 72-liter sulfur-salt medium (pH 2.5) under forced aeration. After cultivation of the strain for one week at 30 °C, the culture fluid obtained was filtered with Toyo no. 2 filter paper to remove sulfur particles and centrifuged at $10{,}000 \times g$ to obtain cell pellets. The harvested cells were washed three times with 50 mM Tris–HCl buffer (pH 7.5).

Growth rate. Cells were separated from elemental sulfur particles by filtering cultures with Toyo paper filter no. 5B. Almost all of the cells in the culture fluid passed through the paper filter. The numbers of cells after filtration were counted with a hemacytometer (Kayagaki Irika Kogyo, Tokyo, Japan) after dilution with 0.1 N sulfuric acid, when necessary.

Sulfur oxidase activity. Sulfur oxidase activity was measured at pH 3.0 with resting cells of NB1-3 by the oxygen uptake caused by the oxidation of elemental sulfur in a Warburg manometer. The reaction vessel contained 3.0 ml of reaction mixture plus 0.2 ml of 20% potassium hydroxide in the center well. The gas phase was air and the temperature was kept at 30 °C. The reaction mixture (pH 3.0) was composed of β-alanine (100 μmol), resting cells (5 mg protein), and elemental sulfur (100 mg).

Sulfur dioxygenase activity. Sulfur dioxygenase activity was measured at pH 6.0 with the cytosol of NB1-3 by the oxygen uptake caused by the oxidation of elemental sulfur in a Warburg manometer.[25] Each

vessel contained 3.0 ml of reaction mixture plus 0.2 ml of 20% KOH in the center well. The composition of the reaction mixture was as follows (in 3.0 ml): 2 mg of protein from the 105,000 × *g* soluble fraction (the cytosol) of NB1-3, 200 μmol of MES buffer (pH 6.0), 0.5 μmol of HOQNO, 300 mg of glycerol, 100 mg of elemental sulfur, and 50 μmol of GSH. The gas phase was air, and the temperature was 30 °C.

Sulfite oxidase activity. Sulfite oxidase activity was manometrically measured at pH 7.5 with the plasma membrane by the oxygen uptake caused by the oxidation of SO_3^{2-} in a Warburg manometer.[25] Each vessel contained 3.0 ml of reaction mixture plus 0.2 ml of 20% KOH 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): 2 mg of plasma membrane, 200 μmol of MOPS buffer (pH 7.5), and 20 μmol of $NaHSO_3$.

Analysis of tungsten bound to A. thiooxidans NB1-3 cells. The amounts of tungsten bound to resting cells of *A. thiooxidans* NB1-3 cells were measured by the thiocyanate method.[27,28]

Endurance test of portland cement bar containing nickel and tungsten. The portland cement bar (40 × 40 × 160 mm) containing 0.075% metal nickel and 0.075% calcium tungstate were prepared with a mortar mixer. After 28 d of standard curing, the portland cement bars prepared were hung from the lid of a sludge tank manhole and exposed to the atmosphere, containing hydrogen sulfide (H_2S).[24] The concentrations of H_2S in sewage treatment plants A and B were 42 and 28 ppm respectively. After 2 years of exposure in the sludge tank manhole, a sandy calcium sulfate on the cement bar was taken away, and the weights of the concrete bars were measured.[24]

Protein content. Protein was measured by the method of Lowry *et al.* with crystalline bovine serum albumin as the reference protein.[29]

Results

Growth of sulfur-oxidizing bacteria in sulfur medium with sodium tungstate

A. thiooxidans NB1-3 was grown in 20 ml of sulfur medium (pH 2.5) with or without sodium tungstate (Na_2WO_4). Growth of NB1-3 was strongly inhibited by 20 μM Na_2WO_4, and completely by 50 μM Na_2WO_4 (Fig. 1). Five strains of *A. thiooxidans*, including strain NB1-3, were cultured in sulfur medium with or without 50 μM Na_2WO_4. None of the strains tested grew in sulfur medium (pH 2.5) with 50 μM Na_2WO_4 (data not shown), indicating that Na_2WO_4 is a strong growth inhibitor common to *A. thiooxidans* cells.

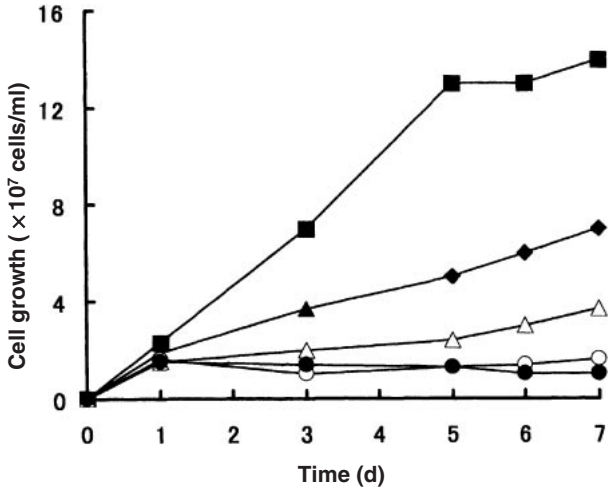

Fig. 1. Effect of Sodium Tungstate on the Growth of *A. thiooxidans* NB1-3.

A. thiooxidans NB1-3 was grown in 20 ml of sulfur medium (pH 2.5) with 1 μM (◆), 10 μM (△), 20 μM (○), and 50 μM (●) sodium tungstate, or without sodium tungstate (■). Values shown are means of duplicate experiments.

Amounts of tungsten bound to A. thiooxidans NB1-3 cells

To clarify the mechanism of tungsten inhibition more precisely, the amounts of tungsten bound to *A. thiooxidans* NB1-3 cells were measured. Resting cells of NB1-3 were incubated for 1 h in 2.0 ml of 0.1 M β-alanine–SO_4^{2-} buffer (pH 3.0) containing 100 μM Na_2WO_4. After incubation of the cells with 100 μM Na_2WO_4, the cells were washed three times with 0.1 M β-alanine–SO_4^{2-} buffer (pH 3.0), and then the concentrations of tungsten bound to the cells were measured. The amounts of tungsten bound to the resting cells increased with the amounts of resting cells used (Fig. 2). When the resting cells were incubated with 2.0 ml of 100 μM Na_2WO_4 at pH 3.0 for 1 h, the maximum amount of tungsten bound to NB1-3 cells was 33 μg/mg protein. The amounts of tungsten bound to the cytosol and plasma membrane fractions of NB1-3 were 90 and 88 μg/mg protein respectively.

Effects of pH on the binding of tungsten to A. thiooxidans NB1-3 cells

The effects of pH on the binding of tungsten to *A. thiooxidans* cells were studied with resting cells of strain NB1-3. The amount of tungsten bound to NB1-3 cells increased concomitant with pH decrease (Fig. 3). Approximately 10 times more tungsten bound to the NB1-3 cells at pH 3.0 than at pH 7.0.

Effect of heavy metal ions on the binding of tungsten to A. thiooxidans NB1-3 cells

To study the specificity of tungsten binding to resting cells of *A. thiooxidans*, the amounts of tungsten bound to the NB1-3 cells was measured in the presence of 1.0 mM Na_2WO_4 and 1.0 mM metal ions other than

Na_2WO_4. The same concentration of cupric, cadmium, zinc, and manganese ions as that of sodium tungstate (1.0 mM) did not disturb tungsten binding to the cells (Fig. 4). Ferric ions slightly increased the amounts of tungsten bound to the cells. Vanadate and molybdenum, which are oxyanions, inhibited tungsten binding 42 and 68% respectively. These results suggest the existence of a protein to which oxyanion binds specifically in *A. thiooxidans* NB1-3 cells.

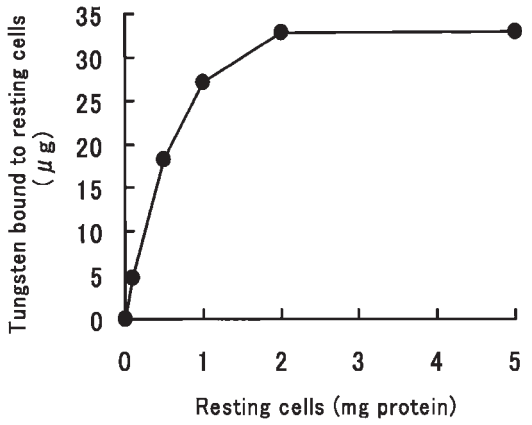

Fig. 2. Effects of Concentration of *A. thiooxidans* NB1-3 Cells on Tungsten Binding.

Various concentration of *A. thiooxidans* NB1-3 cells was incubated in 2.0 ml of 0.1 M β-alanine–SO_4^{2-} buffer (pH 3.0) with 100 μM Na_2WO_4 for 1 h at 30 °C. After treatment, the resting cells were washed three times with 0.1 M β-alanine–SO_4^{2-} buffer (pH 3.0) to remove free Na_2WO_4, and the amounts of tungsten bound to the cells were measured, as described in "Materials and Methods". Values shown are means of duplicate experiments.

Effects of sodium tungstate on the enzymes involved in elemental sulfur oxidation

To clarify the mechanism of growth inhibition of strain NB1-3 by Na_2WO_4, the effect of tungsten on the activity of sulfur oxidase, which catalyzes oxidation of elemental sulfur to yield sulfate, was studied at pH 3.0 with resting cells of NB1-3. The activity of sulfur oxidase of NB1-3 cells was inhibited 33, 68, and 95% by 0.1, 1.0, and 10 mM of Na_2WO_4 respectively (Fig. 5A). It is known that elemental sulfur is first oxidized by sulfur dioxygenase to yield sulfite, and that the sulfite produced is further oxidized by sulfite oxidase to yield sulfate in *A. thiooxidans*.[11–20] Sulfur dioxygenase activity is located in the 105,000 × *g* soluble fraction of *A. thiooxidans* NB1-3 cells. The activity measured at pH 6.0 with 105,000 × *g* soluble fraction was inhibited 8, 63, and 64% by 1.0, 3.0, and 10 mM Na_2WO_4 respectively (Fig. 5B). Sulfite oxidase was located in the plasma membrane of NB1-3. Activity measured at pH 7.5 with the plasma membrane was inhibited 8, 36, and 49% by 1.0, 3.0 and 10 mM Na_2WO_4 respectively (Fig. 5C). These results indicate that tungsten bound to NB1-3 cells and inhibited the sulfur oxidation enzyme system of the cells, and as a result, inhibited cell growth.

Endurance test of portland cement bar supplemented with nickel and tungsten

Portland cement bars (40 × 40 × 160 mm) without metal, with 0.075% metal nickel, and with 0.075% metal nickel plus 0.075% calcium tungstate were prepared. The portland cement bars thus prepared were hung from the lids of sludge tank manholes of sewage treatment

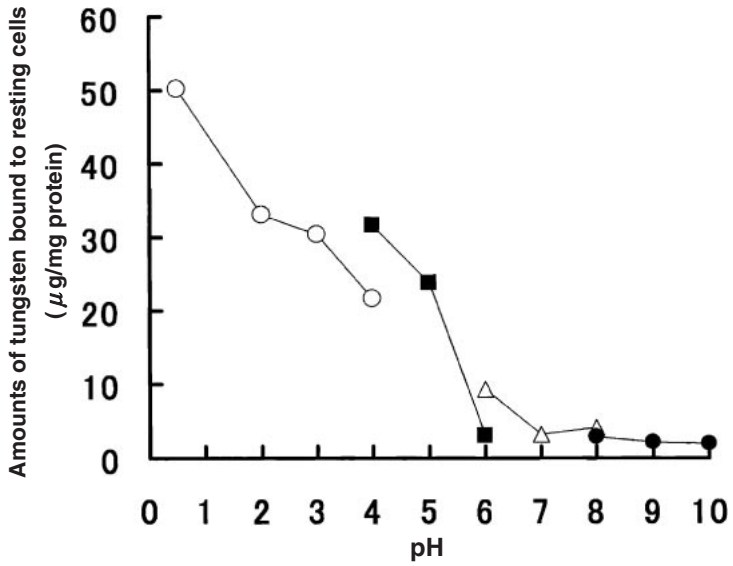

Fig. 3. Effects of pH on Tungsten Binding to Resting Cells of *A. thiooxidans* NB1-3.

Resting cells (1 mg of protein) of *A. thiooxidans* NB1-3 were incubated in 2.0 ml of the buffers indicated in this figure (○, 0.1 M β-alanine–SO_4^{2-} buffer; ■, 0.1 M sodium acetate buffer; △, 0.1 M sodium phosphate buffer; ●, 0.1 M H_3BO_4–KCl–NaOH buffer) with 100 μM Na_2WO_4 for 1 h at 30 °C. After the treatment, the cells were washed three times with 0.1 M β-alanine–SO_4^{2-} buffer (pH 3.0) to remove free Na_2WO_4 and the amounts of tungsten bound to the cells were measured, as described in "Materials and Methods". Values shown are means of duplicate experiments.

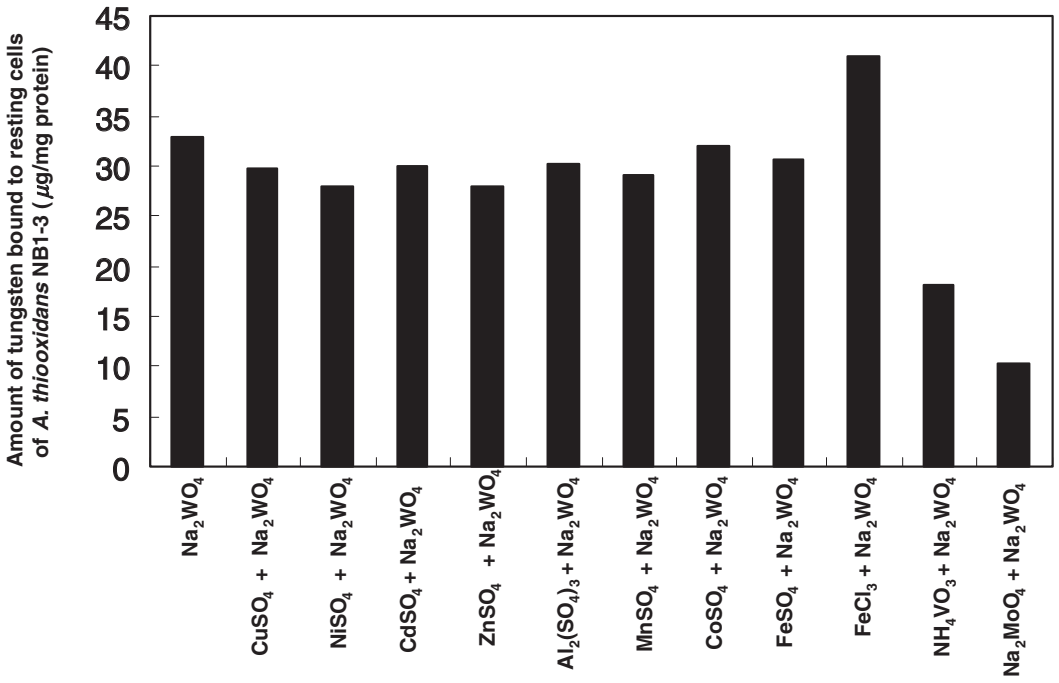

Fig. 4. Effect of Heavy Metal Ions on the Amounts of Tungsten Binding to Resting Cells of *A. thiooxidans* NB1-3.

Resting cells (1 mg of protein) of NB1-3 were incubated with 1.0 mM Na_2WO_4 for 1 h at 30 °C, or with 1.0 mM Na_2WO_4 plus 1.0 mM metal ion such as $CuSO_4$, $NiSO_4$, $CdSO_4$, $ZnSO_4$, $Al_2(SO_4)_3$, $MnSO_4$, $CoSO_4$, $FeSO_4$, $FeCl_3$, NH_4VO_3, or Na_2MoO_4 for 1 h at 30 °C. After treatment, the cells were washed three times with 0.1 M β-alanine–SO_4^{2-} buffer (pH 3.0) to remove free metal ion and Na_2WO_4. The amount of tungsten bound to the cells were measured as described in "Materials and Methods". Values shown are means of duplicate experiments.

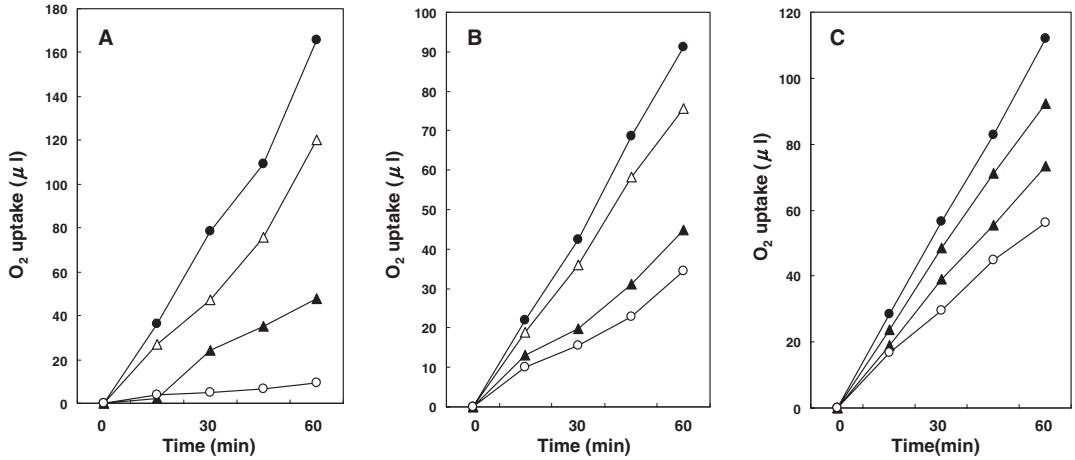

Fig. 5. Effect of Sodium Tungstate on the Activities of Sulfur Oxidase, Sulfur Dioxygenase, and Sulfite Oxidase of *A. thiooxidans* NB1-3.

The activity of sulfur oxidase, which catalyzes oxidation of elemental sulfur to yield sulfate (A), was measured at pH 3.0 with resting cells of NB1-3. Enzyme activity was measured in the reaction mixtures with 0.1 mM (△), 1.0 mM (▲), and 10 mM (○) of sodium tungstate, or without sodium tungstate (●). The activity of sulfur dioxygenase, which catalyzes oxidation of elemental sulfur to yield sulfite (B), was measured at pH 6.0 with the cytosol (105,000 × *g* soluble fraction) of NB1-3. Enzyme activity was measured in the reaction mixtures with 0.1 mM (△), 1.0 mM (▲), and 10 mM (○) of sodium tungstate, or without sodium tungstate (●). The activity of sulfite oxidase, which catalyzes oxidation of sodium sulfite to yield sulfate (C), was measured at pH 7.5 with the plasma membrane of NB1-3. Enzyme activity was measured in the reaction mixtures with 1.0 mM (△), 3.0 mM (▲), and 10 mM (○) of sodium tungstate, or without sodium tungstate (●). Values shown are means of duplicate experiments.

plants A and B and exposed to the atmosphere, containing hydrogen sulfide. The concentrations of hydrogen sulfide in sewage treatment plants A and B were 42 and 28 ppm respectively. After 2 years of exposure in the sludge tank manholes, the weights of the concrete bars were measured.

When portland cement bars without metals were hung for 2 years in the atmosphere of sewage treatment plant

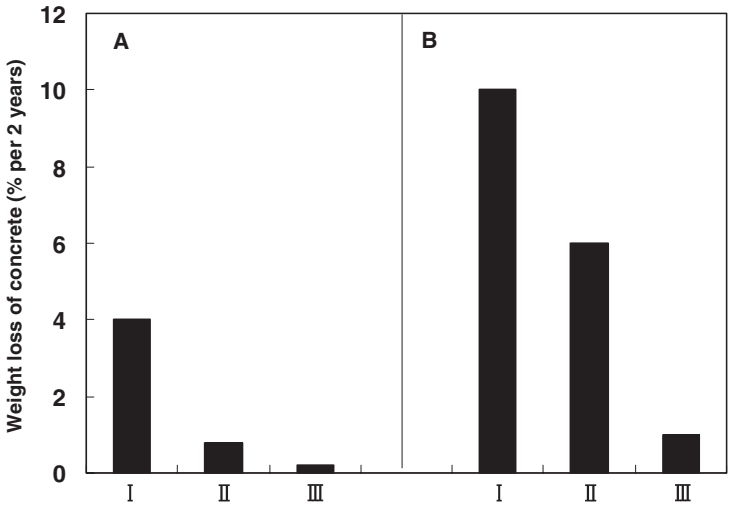

Fig. 6. Endurance Test of Portland Cement Bars Containing Nickel and Tungsten.

Portland cement bars ($40 \times 40 \times 160$ mm) without metal (I), with 0.075% metal nickel (II), and with 0.075% metal nickel plus 0.075% tungsten oxide (III) were prepared. They were hung from the rids of manholes of sewage treatment plants A and B and exposed to atmospheres containing hydrogen sulfide for 2 years. The concentrations of hydrogen sulfide in sewage treatment plants A and B were 42 and 28 ppm respectively. After 2 years of exposure in the manhole atmospheres of sewage treatment plants A and B, weight losses of the cement bars were measured. Values shown are means of duplicate experiments.

A, in which the mean concentration of H_2S per year was 42 ppm, the weight loss of the bar was 4% as compared with the bar without H_2S treatment (Fig. 6A). The addition of metal nickel to the cement bar at 0.075% decreased the weight loss by 0.8% over 2 years. Interestingly, the addition of both 0.075% metal nickel and 0.075% calcium tungstate decreased weight loss by 0.2% over 2 years. When the portland cement bars without metals, with 0.075% metal nickel, and with 0.075% metal nickel plus 0.075% calcium tungsten were also hung for 2 years in the atmosphere of sewage treatment plant B, in which mean concentration of H_2S per year was 28 ppm, the weight losses of these cement bars were 10, 6, and 1%, respectively (Fig. 6B). These results indicate that further addition of 0.075% calcium tungstate to the 0.075% metal nickel-portland cement bar can prevent weight loss in concrete bars as compared with the 0.075% metal nickel-portland cement bars.

Discussion

It was found in this study that (i) 50 μM of Na_2WO_4 can inhibit completely the growth of five strains of *A. thiooxidans,* including strain NB1-3, (ii) Na_2WO_4 binds to NB1-3 cells more tightly below pH 5 than above pH 6.0, and (iii) the enzymes involved in elemental sulfur oxidation such as sulfur oxidase, sulfur dioxygenase, and sulfite oxidase are strongly inhibited by Na_2WO_4. These results suggest that inhibition of the enzyme system involved in elemental sulfur oxidation by tungsten is probably one of the main causes of the growth inhibition by tungsten in *A. thiooxidans*. Sulfur oxidase activity measured with resting NB1-3 cells showed a broad optimum pH curve from 2 to 7.5. The sulfur oxidase activity of NB1-3 cells measured at pH 3.0 was inhibited 33, 68, and 95% by 0.1, 1.0, and 10 mM of Na_2WO_4 respectively (Fig. 5A), but activity measured at pH 7.0 was inhibited 29% by 10 mM of Na_2WO_4 (data not shown). Since tungsten binds more tightly below pH 5.0 than above pH 6.0 (Fig. 3), these results suggest that the level of inhibition by Na_2WO_4 of sulfur oxidase depends on the level of tungsten bound to the cells. Sulfur dioxygenase and sulfite oxidase activities were measured at pH 6.0 and 7.5 with the cytosol and the plasma membrane as enzyme sources, respectively (Fig. 5B and C). The activities of sulfur dioxygenase and sulfite oxidase measured at the optimum pHs were inhibited 64% and 49% by 10 mM Na_2WO_4, (Fig. 5B and C). The low level of inhibition by 10 mM Na_2WO_4 of both enzyme activities is probably due to the low level of tungsten binding to these enzymes at the pHs used for enzyme analysis.

The most interesting finding obtained in this study, however, is that approximately 10 times more tungsten can bind to NB1-3 cells at pH 3.0 than at pH 7.0. This finding appears to be important for the growth prevention of *A. thiooxidans* cells by tungsten in the environment of sewage treatment plants. The pH-dependence of tungsten-binding to NB1-3 cells was quite different from that of nickel-binding to NB1-3 cells. Previously we showed that the growth of NB1-3 cells was inhibited more strongly by Ni^{2+} at pH 7.0 than at pH 3.0 and that more nickel can bind to NB1-3 cells at neutral pH than at acidic pH.[9,25,26] Since *A. thiooxidans*, which is a main sulfur-oxidizing bacterium accelerating concrete corrosion, has an optimum pH for growth between 2.0 and 3.0, the discovery of a bacteriostatic agent that can bind well to *A. thiooxidans* cells under acidic conditions and

strongly inhibit cell growth is expected to make a concrete structure that has more resistance to the attack of *A. thiooxidans* cells.

The effectiveness of the addition of tungsten to portland cement bars for the prevention of concrete corrosion was ascertained when the portland cement bars ($40 \times 40 \times 160$ mm) supplemented with both 0.075% metal nickel and 0.075% calcium tungstate were exposed to the atmosphere of sewage treatment plant B containing 28 ppm of H_2S for 2 years. But the addition of tungsten to the portland cement bars ($40 \times 40 \times 160$ mm) did not have any favorable effect on the prevention of concrete corrosion when the cement bars were exposed to the atmosphere of a sewage treatment plant containing H_2S at more than 42 ppm for 2 years. Specifically, when portland cement bars without metals, with 0.075% metal nickel, and with 0.075% metal nickel plus 0.075% calcium tungstate were hung for 2 years in the atmosphere of sewage treatment plant C, in which the mean concentration of H_2S per year was 81 ppm, the weight losses of the cement bars were 74%, 22%, and 22% respectively as compared with the cement bars without H_2S treatment (data not shown). These results suggest that the addition of tungsten to 0.075% metal nickel-portland cement bars is effective for the prevention of concrete corrosion when the cement bars are hung in the atmosphere of a sewage treatment plant containing less than 28 ppm of H_2S.

Tungstate, vanadate, and molybdenum are oxyanions. When 1.0 mM vanadate or 1.0 mM molybdenum coexisted with 1.0 mM Na_2WO_4 and NB1-3 cells, the amount of tungsten bound to the cells decreased to 42 and 68% of the control experiment without vanadate or molybdenum (Fig. 4). Metal ions other than vanadate and molybdenum did not show the same phenomena as observed with the addition of vanadate or molybdenum, suggesting the existence of a specific protein in *A. thiooxidans* NB1-3 cells that binds to oxyanions. Previously we showed existence of tungsten-binding protein in *A. ferrooxidans* cells. The protein was purified from a plasma membrane of *A. ferrooxidans* AP19-3 in an electrophoretically homogenous state.[27,28] The tungsten-binding protein was composed of two subunits with apparent molecular masses of 12 and 20.7 kDa.[28] But incubation of the tungsten-binding protein for 1 h with Na_2WO_4 plus metal ions, such as $NaVO_3$, Na_2MoO_4, $CuSO_4$, $NiSO_4$, $MnSO_4$, $CoSO_4$, or $CdCl_2$, did not markedly affect the amount of tungsten bound to the tungsten-binding protein.[28] These results suggest the possibility that the protein in *A. thiooxidans* NB1-3 cells which can bind oxyanions specifically, including Na_2WO_4 is different from the tungsten-binding protein isolated from *A. ferrooxidans*. To clarify these points and the mechanism of growth inhibition by tungsten in the genus *Acidithiobacillus* more precisely, purification of oxyanion-binding protein from *A. thiooxidans* NB1-3 cells is currently underway.

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