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Nickel Inhibition of the Growth of a Sulfur-oxidizing Bacterium Isolated from Corroded Concrete

Terunobu MAEDA, Atsunori NEGISHI, Yasuo NOGAMI,* and Tsuyoshi SUGIO*,†

Hazama Corporation, Technical Research Institute, 515–1 Nishimukai, Karima, Tsukuba 305, Japan
**Department of Biological Function and Genetic Resources Science, Faculty of Agriculture, Okayama University, 1–1–1 Tsushima Naka, Okayama 700, Japan*
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A sulfur-oxidizing bacterium strain NB1-3 isolated from corroded concrete was a Gram negative, non-spore-forming, and rod-shaped bacterium (0.5–1.0 × 1.5–2.0 μm) with a polar flagellum. Strain NB1-3 had its optimum temperature and pH for growth at 30°C and 3.0–4.0, respectively. Strain NB1-3 had enzyme activities that oxidized elemental sulfur, thiosulfate, tetrathionate, and sulfide and the activity to incorporate $^{14}CO_2$ into the cells. The mean G+C content of the DNA was 52.9 mol%. These results indicate that strain NB1-3 is *Thiobacillus thiooxidans*. Since nickel has been known to protect concrete from corrosion, the effect of Ni on the growth of strain NB1-3 was studied. The cell growth on tiosulfate-, elemental sulfur-, or tetrathionate-medium was completely inhibited by 0.1% metal nickel or 5 mM $NiSO_4$. Both cellular activities of elemental sulfur oxidation and CO_2 incorporation were strongly inhibited by 5 mM $NiSO_4$. The amounts of Ni in cells with or without nickel treatment were 1.7 and 160.0 nmol/mg protein, respectively. These results indicate that nickel binds to strain NB1-3 cells and inhibits enzymes involved in sulfur oxidation of this bacterium, and as a result, inhibits cell growth.

Key words: sulfur-oxidizing bacterium; *Thiobacillus thiooxidans*; corroded concrete; inhibition by Ni

Serious corrosion was noted in some concrete structures constructed for sewage treatment.[1–7] Two kinds of bacteria, namely sulfate-reducing and sulfur-oxidizing bacteria, have been known to be mainly involved in the corrosion of concrete.[2–5] In the process of concrete corrosion, sulfate-reducing bacteria produce hydrogen sulfide from organic compounds and sulfate ion in the sewage and the hydrogen sulfide thus formed is oxidized by sulfur-oxidizing bacteria on the surface of sewer pipes to give sulfuric acid. In addition to the production of hydrogen sulfide by sulfate-reducing bacteria, heterotrophic bacteria can also produce hydrogen sulfide from sulfur-containing organic compounds under anaerobic conditions. The importance of hydrogen sulfide in concrete corrosion has been stressed.[4,8] The concrete structure just after construction contains calcium hydroxide and thus has a pH of 12–13. Carbon dioxide in the atmosphere neutralizes the concrete structure and prompts concrete corrosion. However, sulfuric acid produced by sulfur-oxidizing bacteria also prompts the neutralization of the concrete and damages the concrete structures.

Parker first isolated a sulfur-oxidizing bacterium from the corroded concrete and named the bacterium *Thiobacillus concretivourus*, which was later identified as *Thiobacillus thiooxidans*.[1,2] Thiobacilli other than *T. thiooxidans* were also isolated from sewer systems and corroded concretes, and identified as *Thiobacillus neapolitanus*, *T. intermedius*, and *T. novellus*.[5,9] However, there have been few reports on practical methods to protect concrete from this corrosion. Duecker *et al.* reported that sulfur cement with β-naphthol and selenium in it resisted a sulfur-oxidizing bacterium[10] and Emmel *et al.* described biocides that inhibit the corrosion by biogenic sulfuric acid in sewage pipelines.[11] Recently, we have prepared portland cement bars (4 × 4 × 16 cm) containing various kinds of inorganic and organic compounds, and these bars were hung from a lid of a sludge tank manhole and exposed to 5–10 ppm hydrogen sulfide atmosphere.[12] After 2 years of exposure, it was found that the surface of the portland cement containing nickel was especially smooth, while that without nickel was brittle and rough,[12] However, the precise mechanism of this protection from concrete corrosion by nickel addition were not known. To study the biological bases of this protection of portland cement corrosion by nickel, we isolated a sulfur-oxidizing bacterium from corroded concrete, identified it, and studied the effects of nickel on the cell growth and the activities of both sulfur oxidation and $^{14}CO_2$ fixation in the cells. The amounts of nickel in the cells after nickel treatment were also measured.

Materials and Methods

Microorganism, medium, and conditions of cultivation. The bacterium used in this study was isolated from corroded concrete in Fukuyama City, Japan. The methods for the isolation of sulfur-oxidizing bacterium were as follows. The corroded concrete (1.0 g) was incubated under aerobic conditions at 30°C in 20 ml of thiosulfate-salt medium (pH 7.0) containing sodium thiosulfate (0.2%), yeast extract (0.03%), $(NH_4)_2SO_4$ (0.3%), $MgSO_4 \cdot 7H_2O$ (0.05%), K_2HPO_4 (0.05%), KCl (0.01%), and $Ca(NO_3)_2$ (0.001%). Thiosulfate is unstable at low pH, but stable at neutral pH. Thus, the pH of the medium was adjusted to 7.0. When sulfur-oxidizing bacteria grew on the thiosulfate-salt medium, the pH of the medium decreased to 2.0 because of the production of sulfuric acid by the bacteria. After culturing 5 times in the thiosulfate-salt medium, the culture medium were spread over the thiosulfate-salt medium with gellan gum (pH 7.0). A cream-colored colony appearing on the plate was isolated. This isolation process was repeated three times and the final isolate (strain NB1-3) were maintained on the thiosulfate-salt medium and used throughout this study.

Growth rate. Cells were separated from a solid elemental sulfur by

† Corresponding author.

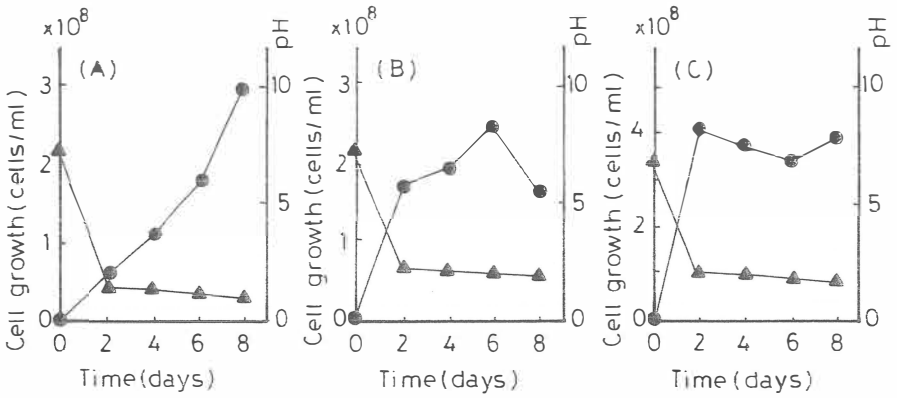

Fig. 1. Cell Growth of Strain NB1-3 on 20 ml of (A) Elemental Sulfur-, (B) Thiosulfate-, and (C) Tetrathionate-salt Media.
Symbols: ▲, pH of the medium; ●, cell growth.

filtering cultures with a Toyo paper filter No. 5B. The numbers of cells in the filtrates were counted with a hemacytometer (Kayagaki Irika Kogyo Co., Ltd.) after dilution with 0.1 N sulfuric acid, when necessary.

Oxidative activities on sulfur, thiosulfate, sulfite, tetrathionate, and sulfide. The oxidative activities on sulfur, thiosulfate, sulfite, tetrathionate, and sulfide were measured by the oxygen uptake caused by the oxidation of these inorganic sulfur compounds in a Warburg manometer. Each vessel contained 3.0 ml of reaction mixture plus 0.2 ml of 20% potassium hydrooxide 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-SO_4^{2-} (100 μmol), washed intact cells (5 mg of protein), and substrate. As substrates, sulfur (100 mg), thiosulfate (20 μmol), sodium sulfite (20 μmol), potassium tetrathionate (20 μmol), and sodium sulfide (5 μmol) were added to the reaction mixture. The total volume of the reaction mixture was 3.0 ml.

Activity of $^{14}CO_2$ uptake into washed intact cells. The activity of carbon dioxide (CO_2) fixation was measured by the amount of $Na_2{}^{14}CO_3$ incorported into the cells. The composition of the reaction mixture was as follows: 4.0 ml of 0.1 M β-alanine-SO_4^{2-} buffer, pH 3.0; washed intact cells of strain NB1-3 grown on sulfur-salt medium, 1 mg of protein; carrier Na_2CO_3, 1 μmol; $Na_2{}^{14}CO_3$, 1 μCi; elemental sulfur (S^0), 200 mg. The total volume of the reaction mixture was 5.0 ml. The reaction mixture, except sodium carbonate and S^0, was incubated at 30°C for 10 min. The reaction was started by adding $Na_2{}^{14}CO_3$, carrier Na_2CO_3, and S^0. After 1 h of incubation at 30°C, the reaction was stopped by adding 0.5 ml of 20 mM mercuric chloride. The reaction mixture (0.55 ml) was withdrawn and then passed through a 0.45-μm membrane filter. The filter with cells was washed three times with 10 ml of water and then put into 4 ml of a counting sol (Scintisol EX-H). After the filter had been completely solubilized in the counting mixture, the radioactivity was measured with an Aloka LSC-635 liquid scintillation system.

Nickel measurement. Strain NB1-3 cells (20 mg protein) were incubated in 5 ml of 0.1 M 3-(*N*-morpholino)propanesulfonic acid buffer (MOPS buffer), pH 7.0 or 0.1 M β-alanine-SO_4^{2-} buffer, pH 3.0, with 10 mM $NiSO_4$ for 10 h at 30°C. The cells treated with $NiSO_4$ were washed three times with 20 ml of MOPS buffer, pH 7.0, and ashed in a crucible at about 800°C for 2 h. The ashes were dissolved in 1 N HCl. The amount of nickel was measured by atomic absorption spectroscopy with a Shimadzu AA-625-01 spectrophotometer using an air-acetylene flame. The spectral line chosen was 2320 Å. The standard solution of nickel ion was prepared by dilution of a 1000 μg/ml standard nickel solution for atomic absorption spectroscopy (Ishizu Pharmaceutical Co., Ltd.) with 1 N HCl.

Analysis of DNA base composition. The DNA base composition was measured by reversed-phase high-pressure liquid chromatography after the DNA was hydrolyzed into nucleosides with enzymes.[13]

Results and Discussion

Morphology and physiological characteristics of sulfur-oxidizing bacterium isolated from the corroded concrete

Sulfur-oxidizing bacterium (strain NB1-3) was isolated from the corroded concrete of a sewage treatment plant in Fukuyama City, Japan. Strain NB1-3 was a Gram-negative, non-spore-forming, rod-shaped bacterium (0.5–1.0 × 1.5–2.0 μm) with a polar flagellum. The mean G+C content of the DNA of strain NB1-3 was 52.9 mol%. The bacterium had the optimum temperature and pH for the growth on elemental sulfur-salt medium at 30°C and 3–4, respectively. Though elemental sulfur, thiosulfate, and tetrathionate could be used as a sole source of energy for growth (Fig. 1), the bacterium could not use Fe^{2+} or glucose. Washed intact cells of strain NB1-3 had enzyme activities to oxidize elemental sulfur, thiosulfate, tetrathionate, and sulfide (Table I). However, Fe^{2+} and glucose were not oxidized by the strain. The bacterium had a high sulfur-oxidizing activity under the pH range between 2–8 (Fig. 2). Approximately 46% of sulfur-oxidizing activity still remained at pH 9.0.

Table I. Specific Activity of Sulfur Compound Oxidation by Strain NB1-3

Substrate	Activity of sulfur compound oxidation O_2 uptake (μl/mg protein/min)
Sodium sulfide	1.30
Elemental sulfur	0.43
Sodium thiosulfate	2.0
Potassium tetrathionate	2.0
Sodium sulfide	2.8
Ferrous sulfate	0.0
Glucose	0.0

Strain NB1-3 had the activity to incorporate $^{14}CO_2$ into the cells. Optimum pH for CO_2 fixation with elemental sulfur as an electron donor was 5.0 (data not shown). And approximately 46% of CO_2 fixation activity still remained at pH 8.0. $^{14}CO_2$ uptake activity was not observed when washed intact cells were incubated in the reaction mixture without elemental sulfur (Fig. 3) and strongly inhibited by 0.1 mM 2,4-dinitrophenol and 10 μM carbonylcyanide *m*-chlorophenylhydrazone (CCCP), suggesting that the incorporation of $^{14}CO_2$ into the cells is energy dependent. The results described above strongly indicate that sulfur-oxidizing bacterium strain NB1-3 is a chemolithotrophic, sulfur-oxidizing bacterium *Thiobacillus thiooxidans* (Table II).

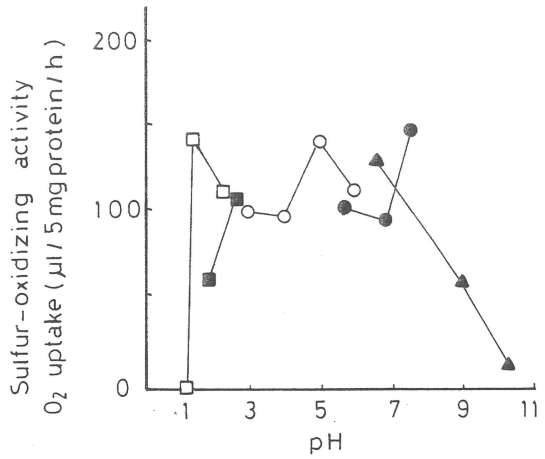

Fig. 2. Effects of pH on the Sulfur-oxidizing Activity of Strain NB1-3.

The methods for analysis and the composition of the reaction mixture for measuring sulfur-oxidizing activity were shown in Materials and Methods. Symbols: □, 0.1 M HCl–KCl buffer; ■, 0.1 M glycin–HCl buffer; ○, 0.1 M citrate buffer; ●, 0.1 M sodium phosphate buffer; ▲, 0.1 M H_3BO_3–NaOH buffer.

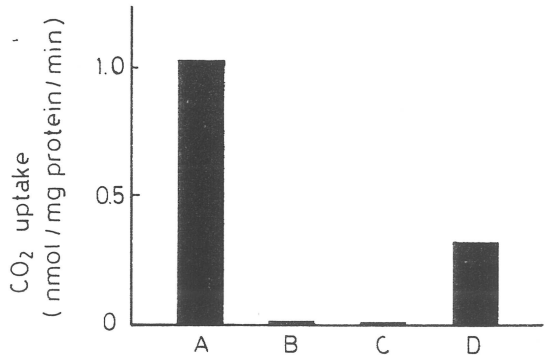

Fig. 3. Effects of Elemental Sulfur and Uncoupler on the Activity of CO_2 Incorporation into Strain NB1-3 Cells.

The methods for analysis and the composition of the reaction mixture for measuring CO_2 fixation are shown in Materials and Methods. Symbols: A, complete reaction mixture with 200 mg of elemental sulfur; B, incomplete reaction mixture without elemental sulfur; C, complete reaction mixture with 0.1 mM of 2,4-dinitrophenol; D, complete reaction mixture with 10 μM of carbonylcyanide *m*-chlorophenylhydrazone (CCCP).

Table II. Characteristics of Sulfur-oxidizing Bacterium Strain NB1-3 Isolated from Corroded Concrete

Characteristics	Sulfur-oxidizing bacteria	
	Strain NB1-3	*Thiobacillus thiooxidans*
Morphology	Rod-shaped	Rod-shaped
Flagellum	Single polar	Single polar
Gram stain	Negative	Negative
Energy source	S^0, $S_2O_3^{2-}$, $S_4O_6^{2-}$	S^0, $S_2O_3^{2-}$, $S_4O_6^{2-}$
Carbon source	CO_2	CO_2
Optimum pH for growth	3–4	2–4
Optimum temperature for growth	30°C	25–30°C
G+C content (mol%)	52.9	51–53

Effects of nickel and $NiSO_4$ on the growth of strain NB1-3 in sulfur media

The growth of strain NB1-3 on thiosulfate-salt medium was monitored by the pH decrease caused by the production of sulfuric acid from thiosulfate by the catalytic action of this bacterium. Cell growth was completely inhibited by 0.1% metal nickel and 5 mM $NiSO_4$ (Fig. 4). Similar results were also obtained when strain NB1-3 was grown on elemental sulfur- or tetrathionate-salt medium containing 0.1% metal nickel or 5 mM $NiSO_4$ (data not shown). To investigate the mechanism of growth inhibition by Ni^{2+}, the effects of Ni^{2+} on the activities of sulfur oxidation and $^{14}CO_2$ incorporation into the cells were studied with washed intact cells. When elemental sulfur oxidation was measured at pH 7.0, the activity was completely inhibited by 10 mM $NiSO_4$ (Fig. 5A), supporting the observation that Ni^{2+} strongly inhibited cells growth on elemental sulfur-salt media. Approximately 41% of the activity still remained when it was measured in the presence of 10 mM $NiSO_4$ at pH 3.0. CO_2 uptake activity, when measured at pH 7.0, was completely inhibited by 5 mM $NiSO_4$ (Fig. 5B). But 68% of CO_2 uptake activity still remained when it was measured in the presence of 10 mM $NiSO_4$ at pH 3.0. The strong inhibition by Ni^{2+} observed in both sulfur oxidation and CO_2 fixation at pH 7.0 suggests the possibility that

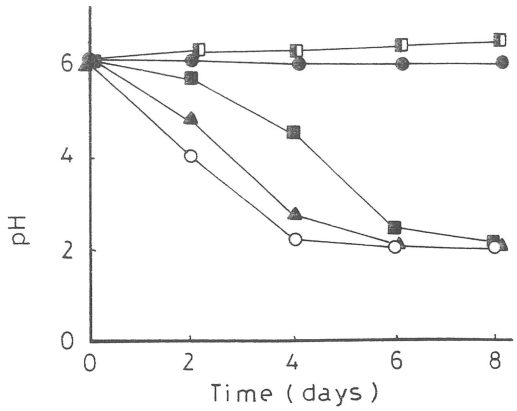

Fig. 4. Effects of Metal Nickel and Nickel Sulfate on the Growth of Strain NB1-3 in Thiosulfate-salt Medium.

The growth of strain NB1-3 on thiosulfate-salt medium was monitored by pH decrease caused by the production of sulfuric acid from thiosulfate by the catalytic action of this bacterium. Symbols: ○, thiosulfate-salt medium without nickel; ▲, thiosulfate-salt medium with 0.5 mM $NiSO_4$; ■, thiosulfate-salt medium with 1.0 mM $NiSO_4$; ●, thiosulfate-salt medium with 5.0 mM $NiSO_4$; □, thiosulfate-salt medium with 0.1% metal nickel.

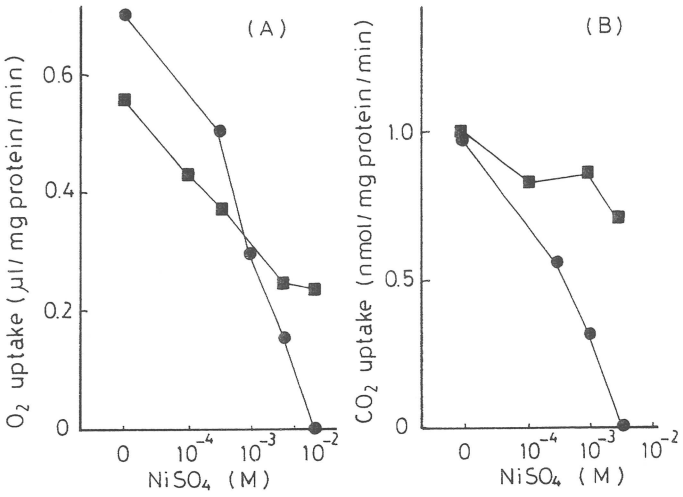

Fig. 5. Effects of Concentration of Nickel Sulfate on the Activity of Sulfur Oxidation (A) and CO_2 Fixation (B).

The activities of sulfur oxidation and CO_2 fixation were measured in the presence of different concentrations of nickel sulfate at pH 3.0 (■) or 7.0 (●).

much more nickel binds to the cells at higher pH than a lower pH.

Binding of nickel to strain NB1-3 cells

To investigate whether nickel binds to strain NB1-3 cells or not, the amount of nickel in the cells after nickel treatment was measured by the methods shown in Materials and Methods. Strain NB1-3 cells without nickel treatment contained 1.7 nmol nickel per mg protein. In contrast, 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, indicating that nickel can bind to strain NB1-3 cells and much more nickel binds to the cells at neutral pH than acidic pH. This findings correspond with the results that the bacterium was much more strongly inhibited by Ni^{2+} at pH 7.0 than pH 3.0.

We previously showed that the portland cement containing nickel was especially effective for protecting concrete from corrosion. From the results obtained in this report, the protective effect of Ni^{2+} on concrete corrosion can be explained as follows: Ni^{2+} binds to sulfur-oxidizing bacterium *Thiobacillus thiooxidans* cells and inhibits the enzyme system involved in sulfur oxidation of this bacterium, and as a result, inhibits cell growth and sulfuric acid production. To purify the protein(s) that is involved in nickel binding from *T. thiooxidans* strain NB1-3 and to clarify the mechanism of inhibition by Ni of sulfur oxidation of this bacterium are quite important and now under investigation.

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