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Isolation of Iron-Oxidizing Bacteria from Corroded Concretes of Sewage Treatment Plants

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Thirty-six strains of iron-oxidizing bacteria were isolated from corroded concrete samples obtained at eight sewage treatment plants in Japan. All of the strains isolated grew autotrophically in ferrous sulfate (3.0%), elemental sulfur (1.0%) and FeS (1.0%) media (pH 1.5). Washed intact cells of the 36 isolates had activities to oxidize both ferrous iron and elemental sulfur. Strain SNA-5, a representative of the isolated strains, was a gram-negative, rod-shaped bacterium (0.5–0.6 × 0.9–1.5 μm). The mean G+C content of its DNA was 55.9 mol%. The pH and temperature optima for growth were 1.5 and 30°C, and the bacterium had activity to assimilate $^{14}CO_2$ into the cells when ferrous iron or elemental sulfur was used as a sole source of energy. These results suggest that SNA-5 is *Thiobacillus ferrooxidans* strain. The pHs and numbers of iron-oxidizing bacteria in corroded concrete samples obtained by boring to depths of 0–1, 1–3, and 3–5 cm below the concrete surface were respectively 1.4, 1.7, and 2.0, and 1.2×10^8, 5×10^7, and 5×10^6 cells/g concrete. The degree of corrosion in the sample obtained nearest to the surface was more severe than in the deeper samples. The findings indicated that the levels of acidification and corrosion of the concrete structure corresponded with the number of iron-oxidizing bacteria in a concrete sample. Sulfuric acid produced by the chemolithoautotrophic sulfur-oxidizing bacterium *Thiobacillus thiooxidansis* known to induce concrete corrosion. Since not only *T. thiooxidans* but also *T. ferrooxidans* can oxidize reduced sulfur compounds and produce sulfuric acid, the results strongly suggest that *T. ferrooxidans* as well as *T. thiooxidans* is involved in concrete corrosion.

[**Key words:** iron-oxidizing bacterium, *Thiobacillus ferrooxidans*, sulfur-oxidizing bacterium, concrete, corrosion]

Parker first presented evidence that concrete structures are corroded by sulfuric acid produced by the sulfur-oxidizing bacterium *Thiobacillus concretivourus* (1–3). Concrete in its main structure is composed of calcium oxides, hydroxides, and carbonates, and these compounds can react with sulfuric acid to give calcium sulfate (gypsum) (4). This resultant calcium loss greatly diminishes the strength of concrete structures. The bacterium isolated by Parker was later identified as *Thiobacillus thiooxidans*, and thiobacilli other than *T. thiooxidans* (5, 6), namely *Thiobacillus neapolitanus, T. intermedius, Thiobacillus novellus*, and *Thiobacillus versutus* have also been isolated from sewer systems and corroded concrete (7–10). The iron-oxidizing bacterium *Thiobacillus ferrooxidans* mainly inhabits drainage areas in acid mines and plays a crucial role in the bacterial leaching of sulfide ores (11–15). As well as *T. thiooxidans, T. ferrooxidans* is also known to oxidize reduced inorganic sulfur compounds and produce sulfuric acid. The enzymes involved in the oxidation of inorganic sulfur compounds have been purified from the bacterium (16–19). The ability of *T. ferrooxidans* to produce sulfuric acid suggested that in addition to *T. thiooxidans, T. ferrooxidans* strains are also likely to be present in corroded concrete and play a role in concrete corrosion. We were able to confirm that this is the case, as well as to clarify the mechanism of concrete corrosion more precisely, by isolating iron-oxidizing bacteria from corroded concrete samples obtained from eight sewage treatment plants in Japan. To our knowledge, there have been no previous reports on concrete corrosion by microorganisms that have focussed on iron-oxidizing bacteria.

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MATERIALS AND METHODS

Microorganisms, media, conditions of cultivation, and isolation procedures *T. ferrooxidans* AP19-3 (16, 19) and 36 strains of iron-oxidizing bacteria isolated from corroded concrete samples obtained at eight sewage treatment plants in Japan were used throughout this study. Among the 36 strains, strains SNA-1, SNA-3, and SNA-5 were isolated from samples respectively obtained by boring 0–1, 1–3, and 3–5 cm in depth from the surface of corroded concrete at a sewage treatment plant in Saitama prefecture, and strains SYU-2 and SYU-4 from samples respectively obtained by boring 0–2 and 2–4 cm into corroded concrete obtained at a sewage treatment plant in Shizuoka prefecture. Strain OIT 5-10 was isolated from corroded concrete obtained at a sewage treatment plant in Okinawa prefecture. To isolate the bacteria, 1 g of corroded concrete was incubated at 30°C under aerobic conditions in an Fe^{2+} medium (pH 2.5) containing $FeSO_4 \cdot 7H_2O$ (3%), $(NH_4)_2SO_4$ (0.3%), K_2HPO_4 (0.05%), $MgSO_4 \cdot 7H_2O$ (0.05%), KCl (0.01%), and $Ca(NO_3)_2$ (0.001%). When the culture medium turned rusty, *i.e.*, when the 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%), $(NH_4)_2SO_4$ (0.3%), K_2HPO_4 (0.05%), $MgSO_4 \cdot 7H_2O$ (0.05%), KCl (0.01%), $Ca(NO_3)_2$

(0.001%), and yeast extract (0.03%). Rusty colonies appearing on the plate were picked up. This process was repeated more than three times and the final isolates were preserved on the Fe^{2+} medium (pH 2.5) and used throughout this study. Iron-oxidizing bacteria isolated were cultured in an elemental sulfur medium (pH 1.5) containing elemental sulfur (1.0%), $(NH_4)_2SO_4$ (0.3%), K_2HPO_4 (0.05%), $MgSO_4 \cdot 7H_2O$ (0.05%), KCl (0.01%), and $Ca(NO_3)_2$ (0.001%) or in an FeS medium (pH 1.5) containing FeS (1.0%), $(NH_4)_2SO_4$ (0.3%), K_2HPO_4 (0.05%), $MgSO_4 \cdot 7H_2O$ (0.05%), KCl (0.01%), and $Ca(NO_3)_2$ (0.001%).

Growth rate Cells in the medium were counted with a hemacytometer (Kayagaki Irika Kogyo, Tokyo) after dilution with 0.01 N H_2SO_4 when necessary.

Iron-oxidizing activity The iron-oxidizing activity of washed intact cells of iron-oxidizing bacteria was estimated from the oxygen uptake rate in a biological oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, OH, USA). The reaction mixture was composed of 0.1 M β-alanine-SO_4^{2-} buffer, pH 3.0, (2.5 ml), washed intact cells, and $FeSO_4 \cdot 7H_2O$ (100 μmol). The reaction was done at 30°C. The washed intact cells used were prepared as follows. Each strain of iron-oxidizing bacteria was grown in 20 *l* of the Fe^{2+} medium (pH 1.5) under aeration at 28°C for a week. The culture medium was filtered with Toyo no. 2 filter paper to remove the bulk of the ferric precipitates and then centrifuged using a Hitachi 18PR-52 continuous-flow rotor at 15,000 × *g* and a flow rate of 200 ml/min. Harvested cells were washed three times with 0.1 M β-alanine-O_4^{2-} buffer (pH 3.0).

Fe^{2+}-dependent oxygen uptake activity of corroded concrete The Fe^{2+}-dependent oxygen uptake activity of corroded concrete was estimated in a Warburg manometer (Yanagimoto Mfg, Kyoto). A Warburg flask contained the reaction mixture (3.0 ml) in the reaction chamber and 20% potassium hydroxide (0.2 ml) in the center well. The gas phase was air and the temperature was kept at 30°C. The reaction mixture in the Warburg flask was composed of 0.1 M β-alanine-SO_4^{2-} buffer, pH 3.0, (2.5 ml), corroded concrete (1.0 g), and $FeSO_4 \cdot 7H_2O$ (100 μmol). To eliminate any endogenous oxygen uptake by microorganisms in the concrete sample other than iron-oxidizing bacteria, the oxygen uptake activity was also measured using the above reaction mixture without Fe^{2+}. The reaction was started by adding Fe^{2+} to the reaction mixture and carried out at 30°C.

Activity of hydrogen sulfide: ferric ion oxidoreductase (SFORase) The SFORase activity was determined by measuring the Fe^{2+} produced in a reaction mixture under aerobic conditions in the presence of sodium cyanide, an inhibitor of iron oxidase (16). The reaction mixture was composed of 0.1 M β-alanine-SO_4^{2-} buffer, pH 3.0, (8.0 ml), elemental sulfur (200 mg), Fe^{3+} (50 μmol), washed intact cells, and sodium cyanide (50 μmol). The total volume was 10 ml. The reaction was carried out by shaking the mixture at 30°C. A sample 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).

Activity of $^{14}CO_2$ uptake into washed intact cells The carbon dioxide (CO_2) fixation activity was estimated from the amount of $Na_2{}^{14}CO_3$ assimilated by 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 an iron-oxidizing bacterium grown in a Fe^{2+} medium (pH 2.5), 1 mg of protein; carrier Na_2CO_3, 1 μmol; $Na_2{}^{14}CO_3$, 1 μCi; and $FeSO_4 \cdot 7H_2O$ (20 μmol) or elemental sulfur (200 mg). The total volume of the reaction mixture was 5.0 ml. The reaction mixture without sodium carbonate and $FeSO_4 \cdot 7H_2O$ or elemental sulfur was incubated at 30°C for 10 min. The reaction was started by adding $Na_2{}^{14}CO_3$, carrier Na_2CO_3, and $FeSO_4 \cdot 7H_2O$ or elemental sulfur. 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 water and then put into 4.0 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.

TABLE 1. Growth of cells in elemental sulfur and FeS media, iron-oxidizing activity, and SFORase activity of iron-oxidizing bacteria isolated from sewage plant corroded concrete

Strain	Growth of cells in medium with		Iron-oxidizing activity O_2 uptake (μl/mg/min)	SFORase activity[a] (μmol/mg/min)
	elemental sulfur (× 10^6 cells/ml/20 d)	FeS (× 10^6 cells/ml/20 d)		
Strains isolated from corroded concrete				
OIT 4-1	11	33	63	0.13
OIT 4-10	210	230	60	0.16
OIT 5-10	162	205	85	0.22
OMO 4-1	52	37	54	0.07
OMO 4-2	48	11	56	0.09
OIS 3-10	174	141	149	0.07
OIS 3-100	96	93	120	0.15
ONA 4-1	224	125	84	0.37
HNI 4-1	16	9	132	0.09
HNI 4-10	148	80	86	0.15
SYU 3-2	250	92	279	0.35
SNA-1	51	42	246	0.05
SNA-3	288	120	124	0.11
SNA-5	88	33	132	0.20
NAB 3-1	70	27	242	0.50
Thiobacillus ferrooxidans AP19-3	152	58	182	0.51

[a] The activity of hydrogen sulfide: ferric ion oxidoreductase (SFORase) (16) was determined by measuring the Fe^{2+} produced in the reaction mixture as described in Materials and Methods.

RESULTS

Isolation of iron-oxidizing bacteria from corroded concrete Thirty-six strains of iron-oxidizing bacteria were isolated from corroded concrete samples obtained at eight sewage treatment plants in Japan. When grown statically in Fe^{2+} medium (pH 1.5), the isolates gave cell yields ranging from $5–13 \times 10^8$ cells/ml. The strains grew autotrophically not only in Fe^{2+} medium but also in sulfur and FeS media. The growth of 15 representative strains in sulfur and FeS media is shown in Table 1. The growth yields in the two media differed from strain to strain. Two kinds of mesophilic iron-oxidizing bacteria are known, *T. ferrooxidans* and *Leptospirillum ferrooxidans*. *T. ferrooxidans* can use either ferrous iron or elemental sulfur as a sole source of energy, whereas *L. ferrooxidans* is able to use only ferrous iron. Our experimental results on energy source utilization thus suggest that the 36 isolates were *T. ferrooxidans* strains. Their iron-oxidizing activity ranged from 54 to 279 μl/mg/min (Table 1). *T. ferrooxidans* are known to possess hydrogen sulfide: ferric ion oxidoreductase (SFORase), which catalyzes the oxidation of elemental sulfur with ferric ion as an electron acceptor to give sulfite and ferrous ion (16, 19). SFORase plays a crucial role in sulfur oxidation by *T. ferrooxidans*. All the iron-oxidizing bacteria isolated from corroded concrete had SFORase activity, ranging from 0.05 to 0.50 μmol/mg/min (Table 1), which accords with their ability to grow in a sulfur medium.

Electron micrographs of strains SNA-1, SNA-5, OIT 5-10, and *T. ferrooxidans* AP19-3 are shown in Fig. 1. Like a control strain *T. ferrooxidans* AP19-3 (16), the isolates were all strait rods (Fig. 1). Strain SNA-5 ($0.5–0.6 \times 0.9–1.5$ μm) was gram-negative and non-spore-forming. The mean G+C content of its DNA was 55.9 mol% (data not shown). Kuenen and Tuovinen reported the mean G+C content of *T. ferrooxidans* DNA to be 53.6–60.1 mol% (20), which is further evidence that strain SNA-5 is a *T. ferrooxidans* strain. SNA-5 and SNA-1 had optimum pH and temperature optima for growth of 1.5 and 30°C (data not shown), indicating that they are acidophilic and mesophilic iron-oxidizing bacteria.

Carbon dioxide fixation activity The $^{14}CO_2$ fixation activities of strains SNA-5 and *T. ferrooxidans* AP19-3 were studied with washed intact cells grown in Fe^{2+} medium (pH 2.5). Strain SNA-5 as well as *T. ferrooxidans* AP19-3 assimilated $^{14}CO_2$ when ferrous iron or elemental sulfur was used as an energy source (Fig. 2). The specific activities of $^{14}CO_2$ fixation of strain SNA-5 and *T. ferrooxidans* AP19-3 were 56 and 171 nmol/mg/60 min, respectively. This result supports that strain SNA-5 grew autotrophically in Fe^{2+}, sulfur and FeS media.

Estimating the number of iron-oxidizing bacteria in corroded concrete samples Since iron-oxidizing bacteria were isolated from all of the corroded concrete samples tested, we considered it important to estimate the numbers of bacteria in 1-g samples taken at various depths in the concrete in order to better understand the role of iron-oxidizing bacteria in concrete corrosion. To our knowledge, this is the first time that such an estimate has been made. Samples were obtained from different depth by boring into the concrete structure at sewage treatment plants. Samples SNA (0–1 cm) SNA (1–3 cm) and SNA (3–5 cm) were obtained from corroded concrete at a plant in Saitama prefecture at depths 0–1, 1–3 and 3–5 cm, respectively, below the concrete surface.

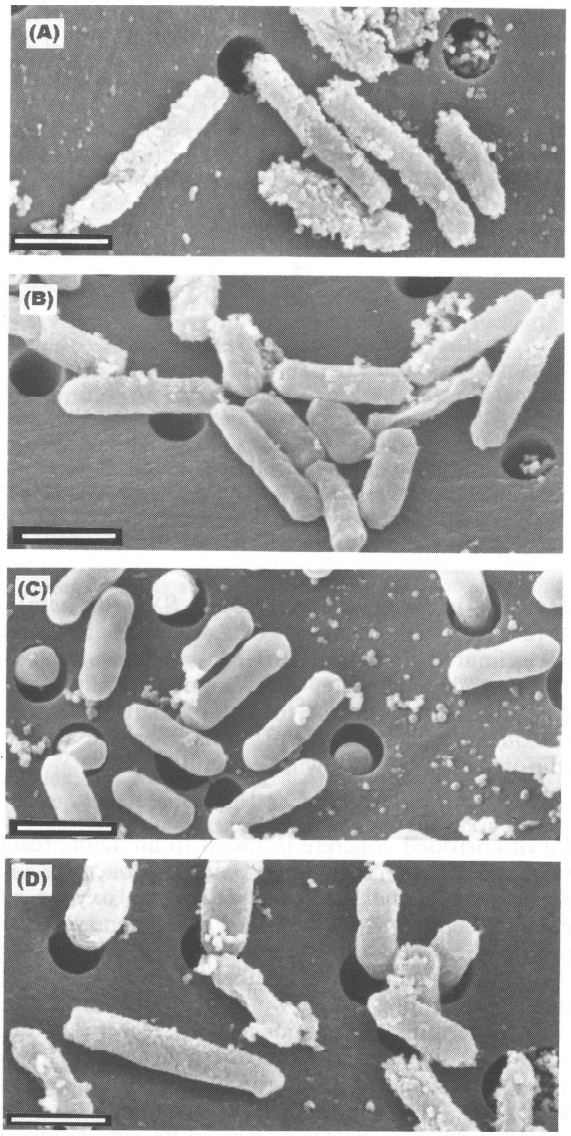

FIG. 1. Electron micrographs of iron-oxidizing bacteria isolated from corroded concrete and of *T. ferrooxidans* AP19-3. (A) Strain SNA-1; (B) strain SNA-5; (C) strain OIT 5-10; (D) *T. ferrooxidans* AP19-3. The bar represents 1.0 μm.

The concentrations of oxygen, hydrogen sulfide, and carbon monoxide in the atmosphere above the sampling site were 17.7%, 96 ppm, and 1 ppm, respectively. The level of corrosion in sample SNA (0–1 cm) was much more severe than in SNA (1–3 cm) and SNA (3–5 cm). The pHs of samples SNA (0–1 cm), SNA (1–3 cm) and SNA (3–5 cm) were 1.4, 1.7, and 2.0, respectively (Table 2). The fact that high concentrations of hydrogen sulfide (96 ppm) and molecular oxygen (17.7%) were observed in the atmosphere above the sampling site suggests that the strong acid (pH 1.4) produced at the surface of the corroded concrete is highly likely to be sulfuric acid rather than hydrochloric, nitric, or carbonic acid, because it is known that *T. thiooxidans* and *T. ferrooxidans* can oxidize hydrogen sulfide with molecular oxygen as an electron acceptor to give sulfuric acid. Similar pH and corrosion level trends were observed in the corroded concrete samples obtained from a sewage treat-

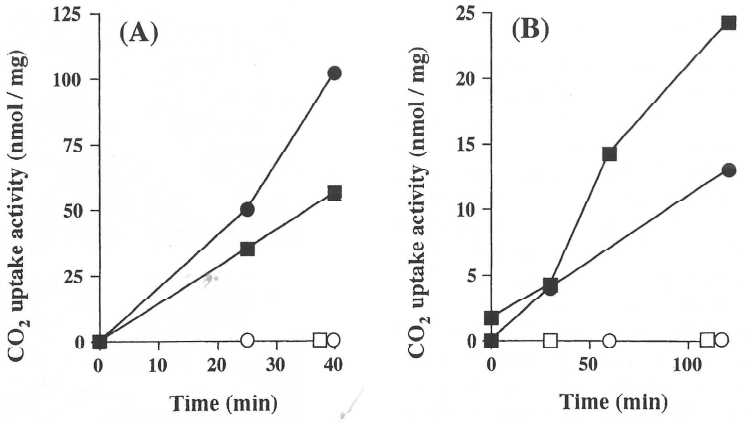

FIG. 2. $^{14}CO_2$ uptake activities of an iron-oxidizing bacterium isolated from corroded concrete and of *T. ferrooxidans* AP19-3. The $^{14}CO_2$ uptake activities of washed intact cells of *T. ferrooxidans* AP19-3 (■) and strain SNA-5 (●) isolated from corroded concrete obtained from a sewage treatment plant in Saitama prefecture were measured in the presence of ferrous iron (A) or elemental sulfur (B) as an electron donor for the $^{14}CO_2$ assimilation reaction. The $^{14}CO_2$ uptake activities of washed intact cells of *T. ferrooxidans* AP19-3 (□) and strain SNA-5 (○) were also measured in the reaction mixture with 20 mM $HgCl_2$.

ment plant in Shizuoka prefecture, SYU (0–2 cm) and SYU (2–4 cm), which had pHs of 1.4 and 1.7, respectively.

To estimate the number of iron-oxidizing bacteria in 1 g of corroded concrete, we used, for the first time, a biological reaction specific to iron-oxidizing bacteria. *T. ferrooxidans* cells oxidizes Fe^{2+} under aerobic and acidic conditions as in the following equation:

$$4FeSO_4 + O_2 + 10H_2O \rightarrow 4Fe(OH)_3 + 4H_2SO_4$$

If 1 g of corroded concrete is added to an acidic reaction mixture containing Fe^{2+}, iron-oxidizing bacteria in the concrete oxidize the Fe^{2+} with molecular oxygen as an electron acceptor. As a result, Fe^{2+}-dependent oxygen uptake is observed, with the level of oxygen uptake activity being dependent on the number of iron-oxidizing bacteria in the sample. Thus, we first plotted an authentic curve to determine the relationship between the cell number and Fe^{2+}-dependent iron-oxidizing activity for each of the strains isolated from the corroded concrete samples (Fig. 3). The iron-oxidizing activity per 10^8 cells of the iron-oxidizing bacteria was different from each strain. The activity of strain SNA-1 was approximately 4 times higher than that of strain SNA-3. We next measured the oxygen uptake of 1 g of each corroded concrete samples in the reaction mixture with Fe^{2+} (Fig. 4). The oxygen uptake activity was also measured in the reaction mixture with 1 g of each samples but without Fe^{2+} to eliminate any oxygen uptake due to the endogenous respiration of heterotrophic microorganisms in the samples. The amount of oxygen taken up in the reaction mixture with Fe^{2+} was higher than the uptake without Fe^{2+}. The difference in oxygen uptake activity between reaction mixtures with or without Fe^{2+} was considered to be Fe^{2+}-dependent oxygen uptake activity caused by iron-oxidizing bacteria in the corroded concrete sample. Using this Fe^{2+}-dependent oxygen uptake activity (Fig. 4) and the previously determined relationship between the cell number and iron-oxidizing activity (Fig. 3), the number of iron-oxidizing bacterial cells in 1 g of the corroded concrete samples SNA (0–1 cm), SNA (1–3 cm), SNA (3–5 cm), SYU (0–2 cm), and SYU (2–4 cm) was calculated to be 1.2×10^8, 5×10^7, 5×10^6, 3.1×10^7 and 2.0×10^7 cells, respectively (Table 2). Among the SNA samples, the one with the lowest pH contained many more iron-oxidizing bacterial cells than that whose pH was the highest.

DISCUSSION

Iron-oxidizing bacteria were isolated from all the corroded concrete samples obtained at eight sewage treatment plants located in different regions of Japan. A close relationship was observed among three parameters: the degree of concrete corrosion, the number of iron-oxidizing bacteria in the sample, and the level of acidity produced in the corroded concrete. The degree of concrete corrosion was shown to be much more severe at the surface of a concrete structure than in the interior. The pH of samples obtained at or near the surface of corroded concrete was lower than that of deeper samples, indicating that the level of acidification was higher at the surface than in interior. Since both hydrogen

TABLE 2. Number of iron-oxidizing bacteria in corroded concrete from sewage treatment plants

Corroded concrete	Depth[a]	pH	Fe^{2+}-dependent O_2 uptake activity (μmol/min/g concrete)	Number of cells in corroded concrete ($\times 10^7$ cells/g concrete)
SNA (0–1 cm)[b]	0–1 cm	1.4	0.67	12.0
SNA (1–3 cm)[b]	1–3 cm	1.7	0.07	5.0
SNA (3–5 cm)[b]	3–5 cm	2.0	0.04	0.5
SYU (0–2 cm)[c]	0–2 cm	1.4	0.08	3.1
SYU (2–4 cm)[c]	2–4 cm	1.7	0.05	2.0

[a] Depth from the surface of corroded concrete.
[b] Corroded concrete samples from a sewage treatment plant in Saitama prefecture.
[c] Corroded concrete samples from a sewage treatment plant in Shizuoka prefecture.

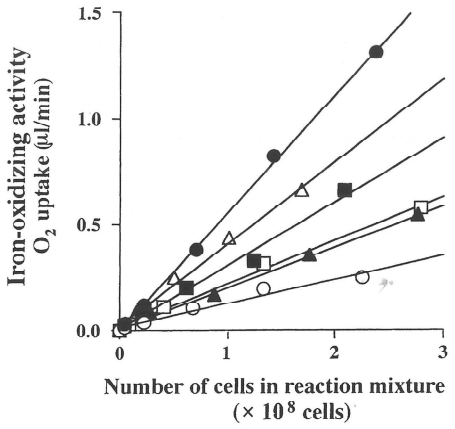

FIG. 3. Relationship between cell number and iron-oxidizing activity. The iron-oxidizing activities of *T. ferrooxidans* AP19-3 (△) and iron-oxidizing bacterial strains SNA-1 (●), SNA-3 (○), SNA-5 (■), SYU-2 (□), and SYU-4 (▲) isolated from corroded concrete were measured using an oxygen electrode. The number of cells in the reaction mixture was counted with hemacytometer.

sulfide and oxygen were observed in the atmosphere at the sewage treatment plants from which the corroded concrete samples were obtained, our findings strongly suggest that an iron-oxidizing bacterium is involved in acid production from hydrogen sulfide generated in sewage treatment plants, and consequently, in concrete corrosion because concrete is mainly composed of calcium oxides, hydroxides, and carbonates, and these compounds can react with sulfuric acid to form calcium sulfate (gypsum), thereby reducing the strength of the concrete structure (4). The findings that the pH was lower and the number of iron-oxidizing bacteria was higher at the concrete surface than in the interior seem reasonable given that the iron-oxidizing bacterium *T. ferrooxidans* is an aerobic and acidophilic chemolithotroph which produces much more sulfuric acid at sites where hydrogen sulfide and molecular oxygen are more abundant. Unfortunately, we were unable to estimate the cell number of the sulfur-oxidizing bacterium *T. thiooxidans* in 1 g of corroded concrete from the sulfur-dependent oxygen uptake activity because *T. ferrooxidans* and *T. acidophilus* (21) also have the ability to oxidize elemental sulfur at pH 2–3 with molecular oxygen as an electron acceptor, *i.e.*, microbial sulfur oxidation is not specific to *T. thiooxidans* cells.

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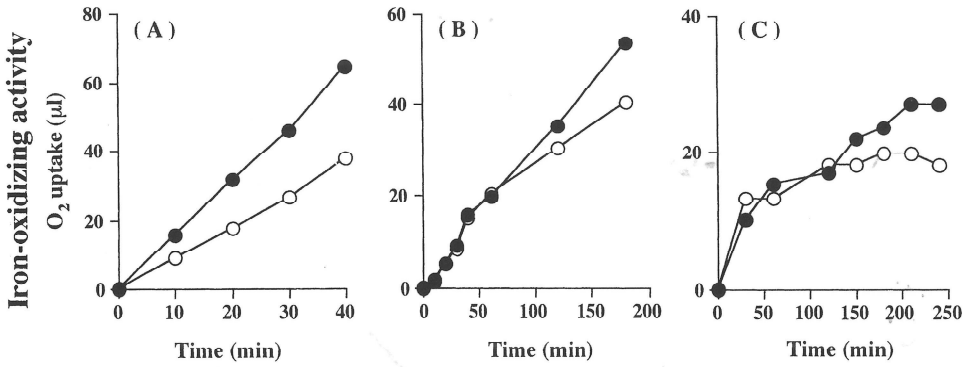

FIG. 4. Fe^{2+}-dependent oxygen uptake activities of corroded concrete samples. Iron-oxidizing bacteria in corroded concrete samples oxidized Fe^{2+} in the reaction mixture and thus showed Fe^{2+}-dependent oxygen uptake activity. The oxygen uptake activities for 1 g of corroded concrete samples (A) SNA (0–1 cm), (B) SNA (1–3 cm), and (C) SNA (3–5 cm) were measured with (●) or without (○) 100 μmol of ferrous iron. Samples SNA (0–1 cm), SNA (1–3 cm), and SNA (3–5 cm) were obtained by boring 0–1, 1–3, and 3–5 cm in depth from the surface of corroded concrete at a sewage treatment plant in Saitama prefecture.

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