内湖底泥における鉄可溶化細菌の分布

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Distribution of Iron-Solubilizing Bacteria in the Sediment of a Small Lagoon

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Iron-solubilizing activity of heterotrophic bacteria isolated from sediment of the Komatsunuma Lagoon was examined by stabbing bacteria on agar plates contained granules of ferric phosphate.

Iron-solubilizing ability was judged from clear zones caused by solubilization of granules around the colony. Both because about one third of the heterotrophic bacteria exhibited iron-solubilizing activity and because their flora were not so much different from those of total heterotrophic bacteria, the character seems common to bacteria living in aerobic environments. The ratio of iron-solubilizing bacteria appeared to be low when Eh and/or pH in sediment were low. However, the ability of bacteria to solubilize iron could not be explained only in terms of a reduction of iron or in terms of a decrease in pH by organic acid production.

Iron is an essential element for organisms, because it is indispensable for many enzymes concerning oxidation reduction reaction in cytoplasms. In aerobic environments, however, iron is present in a ferric form which is very hard to dissolve and the solubilization of precipitated ferric compounds is necessary to be taken up by microorganisms living in such an environment.
Microorganisms in sediments play important roles in solubilization of iron in many ways, though dissolution of iron mainly depends on the physicochemical conditions such as pH and/or oxidation reduction potential (ORP). Some heterotrophs produce organic acids in decomposing organic matter and reduce pH value in the sediment. Some heterotrophic anaerobes reduce ferric iron as electron acceptor to a ferrous state directly in catabolising organic matter or indirectly by forming a reductant such as hydrogensulfide. Some recent studies denied the indirect reduction of iron by a reductant,[1,2] and direct reduction of Fe(III) by bacteria associated with nitrate reductase has been shown in waterlogged soil,[3,4] anaerobic lake sediments,[5,6] river sediments,[7,8] and in anaerobic marine sediments.[9]

In an aerobic environment, however, it is probable that microbial iron reduction does not work and that lowering local pH and ORP in the sediment pore is not effective for liberation of iron because ferrous iron dissolved under such conditions is readily oxidized when it diffuses from sediment pore to the overlying water where the pH is neutral and the ORP is high. In order to maintain ferric iron in a stable state in aerobic environments, iron should be chelated with some kinds of organic compounds of microbial origin, such as organic acids or siderophores. Siderophore is a chelator excreted by many kinds of organisms specifically designed to form complexes with ferric iron.[10,11] In a shallow lagoon like Komatsunuma, microorganisms which produce such compounds may play important roles in the cycling of iron.

In this study, distribution of iron-solubilizing bacteria in the sediments of Komatsunuma Lagoon by Lake Biwa, Japan were examined and its relationship with environmental conditions were discussed.

Materials and Methods

Sampling

Komatsunuma is a small and shallow lagoon whose area is about 78,000 m^2 and depth is ca. 2 m at the sampling site. Sediment sample were collected with a KK-type core sampler[12] from Komatsunuma Lagoon by Lake Biwa between June 1982 and December 1983. The core sediments were cut off at intervals of 0–1 and 8–10 cm form the surface. Each sample was kept separate

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in a plastic container and chilled in an ice box.

Isolation of Heterotrophic Bacteria from the Sediments

Samples for 0–1 and 8–10 cm core sediment were serially diluted and plated onto 1/10 strength of LT agar plates (LT 10^{-1}) in triplicate. The LT 10^{-1} medium contained 0.5 g of Tripticase peptone (BBL), 0.05 g of yeast extract (Difco), and 15 g of agar (Nacalai) in 1 *l* of tap water. Colonies formed on the plates were counted after 15 days incubation at 20°C and about 50 colonies were isolated and purified as a single strain on the LT 10^{-1} plates.

Iron-solubilizing Activity of Heterotrophic Bacteria

About 40 isolates from surface (0–1 cm) and subsurface (8–10 cm) layers of the sediments were tested for iron-solubilizing activity. Each isolate was stabbed on the agar plates contained 1 g of $FePO_4 \cdot 4H_2O$ granule and 20 g of glucose in a 1 *l* of LT 10^{-1} medium. After 4 weeks of incubation at 20°C, colonies which solubilized the granule of ferric phosphate were judged as positive under a stereoscopic microscope (20 × magnification) (Fig. 1).

Identification of Heterotrophic Bacteria

Each thirty strains from both layers in April and October in 1983 were gathered and identified at a generic level. Each isolate was characterized by the methods described by Cowan[13] on cell shape; Gram stain; motility; flagella stain; pigment production; activities of catalase and oxidase; OF test on the Hugh-Leifson's medium. Identification of bacterial isolates were made according to Bergey's Manual of Systematic Bacteriology vol. 1.[14]

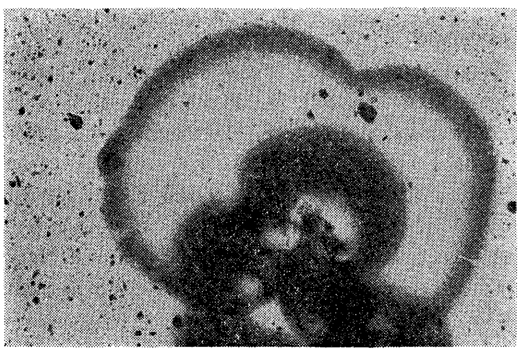

Fig. 1. Iron-solubilizing bacteria. Precipitates of ferric phosphate were dissolved and disappeared around the colony.

Determination of Reducing Sugar

Reducing sugar in the dried sediment sample was extracted by boiling with distilled water for 10 minutes. The reducing sugar in the supernatant of the extract was determined colorimetrically with 3,6-dinitrophthalic acid.[15]

Determination of Organic Acids

Standing cultures of the LT medium supplemented with 10 g of glucose were filtrated atfer three weeks incubation at 20°C to remove bacterial cells. Organic acids in the culture filtrate were extracted with diethylether, esterized with n-butyl alcohol and analyzed on Shimadzu GC-7A gas chromatography as described in Maeda and Kawai.[16]

Dissolution of Ferric Iron by Culture Fluid

A 100 μg of $FePO_4$ or $Fe(OH)_3$ was added in 30 m*l* of each culture fluid and incubated for 3 hours. Each sample was filtrated with 0.1 μm pore size membrane filter (Millipore) and iron in the filtrate was analyzed using a Hitachi atomic absorption spectrometer 170–30.

Results

Flora and Numbers of Heterotrophic Bacteria

Numbers of heterotrophic bacteria counted in the sediments of Komatsunuma Lagoon were shown in Table 1. Bacterial numbers in the surface layer of the sediment (0–1 cm) were one order of magnitude higher than those in 8–10 cm layer and ranged from 10^5 to 10^6 CFU/g wet wt. through the successive two years with some exceptions. About a half of the isolated starins were found Gram negative and genera *Bacillus* (23.5%), *Pseudomonas* (16.2%), *Flavobacterium* (14.7%) were dominant in the 0–1 cm layer. In the 8–10 cm layer, about 80% of the strain were found Gram positive and the dominant genera were *Bacillus* (42.0%), *Corynebacterium* (26.0%), and *Lactobacillus* (5.8%) (Table 2).

Iron-solubilizing Bacteria

Six to fortysix percent of the isolates were found to have iron-solubilizing activity. The percentage occurrence of iron-solubilizing bacteria in the total heterotroph in the surface layer (0–1 cm) did not differ so much from those of the subsurface layer (8–10 cm) (Table 1). Of the 105 strains tested, 33% were found to possess iron-solubilizing activity. The dominant genera which showed the strong activity in solubilizing iron were *Bacillus* (40%),

Table 1. Number of heterotrophic bacteria and the ratios of iron-solubilizing bacteria to the heterotrophic bacteria in the sediment of Komatsunuma Lagoon in 1982 (A) and in 1983 (B)

(A) 1982

	Depth (cm)	Date					
		Jun. 8	Jul. 6	Jul. 28	Sep. 14	Oct. 5	Nov. 6
Heterotrophic	0–1	0.85	9.8	8.6	3.5	3.8	4.2
bacteria	8–10	1.2	1.8	0.98	0.34	0.62	0.54
($\times 10^5$ CFU/g wet wt.)							
Iron-solubilizing	0–1		27.1		23.3		23.3
bacteria	8–10		43.8		20.0		16.7
(%)							

(B) 1983

	Depth (cm)	Date				
		Apr. 22	Jun. 22	Aug. 30	Oct. 21	Dec. 14
Heterotrophic	0–1	11	6.8	4.0	3.7	0.45
bacteria	8–10	0.57	0.50	0.29	0.69	0.81
($\times 10^5$ CFU/g wet wt.)						
Iron-solubilizing	0–1	43.7	16.7	23.0	18.8	33.3
bacteria	8–10	45.9	27.1	18.8	6.3	39.6
(%)						

Table 2. Generic composition (%) of heterotrophic bacteria and iron-solubilizing bacteria in the sediment of Komatsunuma Lagoon in 1983

Genera	Total hetetrotrophic bacteria	Depth (cm)		Iron-solubilizing activity	
		0–1	8–10	Iron$^+$	Iron$^-$
Bacillus	32.8	23.5	52.0	40.0	34.3
Corynebacterium	15.3	4.4	26.0	20.0	17.1
Lactobacillus	8.8	11.8	5.8	8.6	8.5
Arthrobacter	1.5	0	1.5	5.7	0
Staphylococcus	2.9	2.9	2.9	0	4.3
Flavobacterium	8.8	14.7	2.9	8.6	2.9
Pseudomonas	8.8	16.2	1.4	2.9	11.4
Acinetobacter	8.0	10.3	5.8	5.7	7.1
Vibrio-Aeromonas	5.1	7.4	2.9	2.9	5.7
Alcaligenes	5.8	5.9	5.8	2.9	5.7
Enterobacteriaceae	2.2	0	4.3	2.9	2.9

Of the **120** strains identified, **15** strains died off. Among 105 strains of the heterotrophic bacteria identified, 33% of the strains exhibited iron-solubilizing activity.
Iron$^+$: iron-solubilizing bacteria
Iron$^-$: non iron-solubilizing bacteria

Corynebacterum (20%), and *Lactobacillus* (8.6%). The generic composition of the iron-solubilizing bacteria was similar to that of non iron-solubilizing bacteria (Table 2). Seasonal changes in the ratio of iron-solubilizing bacteria to total heterotrophic bacteria, pH and ORP in the sediment were shown in Fig. 2. Bacteria which showed iron-solubilizing activity were generally found at low ratios when Eh value and pH value was low in 0–1 cm and 8–10 cm, respectively.

Production of Organic Acids and the Dissolution of Iron

Organic acids produced by twelve strains were determined. Major components of organic acids produced by most of the strains were acetate and succinate (Table 3). Some strains also produced lactate, citrate and butylate. Culture fluids of

Table 3. Concentration of organic acids in the culture fluid of bacteria isolated from the sediment of Komatsunuma Lagoon

Strain	Concentrations of orgainc acid (mM)							
	For	Ace	Pro	But	Lac	Suc	Cit	Total
A 0	—	—	—	2.1	—	8.0	—	10.1
A 2	—	10.8	0.1	5.4	3.0	8.5	—	27.8
A 3	—	10.3	—	—	18.6	0.5	—	29.4
A 4	—	0.8	—	0.2	—	1.5	2.9	5.4
B 1	19.0	12.8	—	—	17.5	2.3	0.3	51.9
B 2	—	32.8	—	—	28.5	1.0	0.2	62.5
B 3	—	23.0	—	1.4	—	2.8	1.7	28.9
C 1	—	28.0	—	—	22.5	0.8	—	51.3
C 2	—	4.5	—	—	—	2.3	—	6.8
D 1	—	14.0	—	3.6	—	5.8	—	23.4
D 2	—	—	—	—	—	0.8	—	0.8
D 3	—	20.3	—	—	—	2.3	—	22.6

A, B: strains which showed iron-solubilizing activity.
C, D: strains which did not show iron-solublizing activity.
Abbreviations: For, formate; Ace, acetate; Pro, propionate; But butylate; Lac, lactate; Suc, succinate; Cit, citrate —: not detected

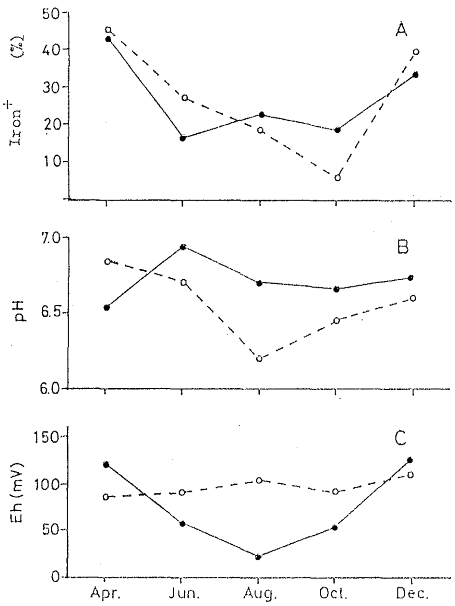

Fig. 2. Seasonal changes in percentage occurrence of iron-solubilizing bacteria (A), pH (B), and Eh (C) in 0–1 cm core (solid line) and 8–10 cm core (broken line) sediment of Komatsunuma Lagoon. Ratios of iron-solubilizing bacteria (Iron$^+$) to total heterotrophic bacteria were expressed as a percentage.

nine strains dissolved both precipitates of ferric phosphate and ferric hydroxide (Table 4), but there was no relationship between the amounts of organic acids and those of dissolved iron.

Reducing Sugar

Concentrations of reducing sugar in the surface of the sediment were lower in April and June and higher in August, October, and December than those in the subsurface layer in 1983 (Table 5).

Table 4. Concentration of iron dissolved by culture fluid of bacteria

Strain	Concentration of iron ($\mu g/l$)	
	$FePO_4$	$Fe(OH)_3$
A 1	32	32
A 2	35	49
A 3	103	36
A 4	44	58
B 1	121	94
B 2	76	76
B 3	42	56
C 1	40	27
C 2	—	9
D 1	—	—
D 2	36	62
D 3	17	—

Strains A, B, C, and D: same as in Table 3.
—: not detected.

Table 5. Concentration of reducing sugar in the sediment of Komatsunuma Lagoon

month	Concentration of reducing sugar ($\mu g/g$ dry wt.)	
	0–1 cm	8–10 cm
Apr.	136	417
Jun.	175	375
Aug.	451	287
Oct.	399	328
Dec.	262	228

Discussion

About one third of heterotrophic bacteria isolated from sediment of Komatsunuma Lagoon exhibited iron-solubilizing activity. As iron-solubilizing activity was examined under oxic conditions different from that in the sediment, it can not necessarily be correlated to in situ activity exhibited in the sediment of Komatsunuma Lagoon. However, apparent correlation between the occurrence of iron-solubilizing bacteria and those of pH or Eh in the sediment was observed. The ratio of iron-solubilizing bacteria to the total heterotrophic bacteria was low when pH or Eh values in sediment were low in summer. It is probable that the lower pH is caused by the formation of organic acids in the process of decomposing organic matters supplied from overlying water during the season, and then the ORP is also reduced by depletion of oxygen during the process. Redox potential of Fe(II)/Fe(III) is a function of ORP and pH. Stability constants of ferrous compounds are relatively lower than those of ferric compounds and ferrous compounds are present stably at low pH or low ORP conditions. As bacteria can take up iron easily which is present in a ferrous state in such condition in the sediment, the ability to solubilize iron do not make advantages in their proliferation during that period. In the subsurface layer (8–10 cm), however, ferrous iron was present in a high concentration (60–200 μmol/l) throughout the year as compared with that in the surface layer (2–30 μmol/l), and no relationship was observed between the redox state in the sediment and those in the occurrence of iron-solubilizing bacteria. There may be factors other than pH and Eh condition that influence the existence of an iron-solubilizer.

Many studies[1,4,5,9] suggested the direct reduction of ferric iron as an electron acceptor of nitrate reductase by anaerobic bacteria and indirect reduction by some reductant is denied. Lovley *et al.*[7] and Jones *et al.*[6] suggested that reducing sugars and organic acids such as acetate, propionate, and malate serve as electron donors for nitrate reductase. There is a possibility that these compounds played some roles in reducing iron as electron donors in our study. Reducing sugars were found in the sediments of Komatsunuma Lagoon. Though we did not examined organic acids in the sediment in this study, organic acids such as acetate and formate are common organic acids in anaerobic sediment as reported by Maeda and Kawai.[16] They also reported that facultative anaerobic bacteria isolated from sediment produced almost the same organic acids as our isolates did.[17] However, there may be factors to solubilize iron other than reducing iron, because iron-solubilizing activity was examined under oxic condition where nitrate reductase does not work and because it was aerobic at the very surface of the sediment of Komatsunuma Lagoon.

Organic acids play other roles in solubilizing iron besides acting as substrates for nitrate reductase. They may act as chelating agents for iron or contribute to lower pH in the sediment. Harrison *et al.*[18] examined the phosphate- (including ferric phosphate-) solubilizing activity of bacteria in a medium containing glucose. They found organic acids such as lactate, glycolate, and gluconate in the culture fluids of phosphate-solubilizer cultivated in glucose containing media or in marine sediments, and suggested that calcium phosphate was solubilized directly by organic acids and that it may due to their ability to form five or six-membered coordination compounds with metals. Craven and Hayasaka[19] also found acetate in the culture fluid of phosphate-solubilizing bacteria. In this study, acetate, lactate, or succinate were the major end products of fermentation in bacterial culture fluids. However, there was no relationship between the amount of iron solubilized by the culture fluids and the total concentrations of organic acids or those of which have chelating activity such as citrate or succinate. Organic acids were produced not only by bacteria which exhibited iron-solubilizing activity but also by those which did not. Furthermore, the concentrations of reducing sugar in the sediments were very low relative to those in the culture medium tested and contribution of organic acids to the solubilization of iron might be very little in the sediment. Possibilities of solubilizing iron by other factors such as chelators of a microorganism origin should be investigated further.

Many bacterial genera are reported to be responsible for the solubization of metal phosphates or iron precipitates. Banik and Dey[20] found one third of the bacteria in manure sediments have metal phosphate solublizing activity and most of them belonged to genus *Bacillus*. Ottow[3] reported that iron reducers belownged to family Enterobacteriaceae, Bacillaceae, methanogenic clostridia, or sulfate reducing bacteria. Jones *et al.*[5,6] found a strain of *Vibrio* has high activity in producting acetic acid and in reducing iron. In this study, the dominant genera of iron-solubilizing bacteria were *Bacillus*, *Lactobacillus*, and

Corynebacterium. Gram negative bacteria were also found to have iron-solubilizing activity and there were not so much differences in the flora of iron-solubilizing bacteria and total heterotrophic bacteria.

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