Real-time PCR and Melting Curve Analysis for Rapid Detection of Dissimilatory Iron-Reducing *deltaproteobacteria* in River Sediments

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Abstract

Geobacter species are dissimilatory iron-reducing *deltaproteobacteria* (DIRD) that have various capabilities for bioremediation and microbial fuel cells, including, but not limited to, dissimilatory iron reduction. Furthermore, novel DIRD have been isolated and studied worldwide. However, these bacteria have not been isolated in Japan. In this study, we attempted to develop a rapid detection method of DIRD by real-time PCR and melting curve analysis to make DIRD detection more efficient. Sediments samples were collected from four sites of the Tomoe River and one of its tributaries in the urban area of Shizuoka City, Japan. When sediments from all the sites were incubated in a ferrihydrite-enriched medium, the medium turned black after 14 days of incubation and showed a magnetic response, indicating the presence of DIRD. Denaturing gradient gel electrophoresis profiles of the samples after incubation revealed that species closely related to *Geobacter sulfurreducens* PCA exist in all the samples. Furthermore, we found a species closely related to *Desulfuromonas alkalicophilus* Z-0531 in the midstream sample of the Tomoe River. Consequently, we succeeded in detecting DIRD in all the samples by melting curve analysis without ferrihydrite-enriched cultures.

Key words: dissimilatory iron-reducing bacteria, *Geobacter*, river sediment, PCR-DGGE, real-time PCR, melting curve analysis

INTRODUCTION

Geobacter species are dissimilatory iron-reducing *deltaproteobacteria* (DIRD) that have been known to produce magnetite ($\text{Fe}^2+\text{Fe}^{3+}_2\text{O}_4$) in media containing amorphous iron oxide as the electron acceptor and acetate as the electron donor¹⁻³. They have various capabilities, including, but not limited to, dissimilatory iron reduction. *G. sulfurreducens* PCA, isolated from the surface sediments of a hydrocarbon-contaminated ditch in Norman, Oklahoma², is a well-known current-producing microorganism in microbial fuel cells that is able to use acetate and hydrogen as electron donors^{4,5}. *G.*

thiogenes K1 that grows through the process of reductive dechlorination of trichloroacetate, was isolated from subsoil from a site located along Mill Creek in Muskegon County, Michigan, was formerly a Koch Industries chemical processing plant and is a Michigan Superfund site^{6,7}. *G. lovleyi* SZ reduces hexavalent uranium, U(VI), to U(IV) and is the first member of the metal-reducing *Geobacter* group that use tetrachloroethene as the growth-supporting electron acceptor⁸.

Thus, DIRD that include the genera *Geobacter*, *Pelobacter* and *Desulfuromonas* have various capabilities and have been studied worldwide⁹⁻¹³. However, DIRD have not been isolated from freshwater sediments

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in Japan. In our previous study¹⁴⁾, ferrihydrite-enriched medium and PCR-denaturing gradient gel electrophoresis (PCR-DGGE) were used to detect DIRD in freshwater sediments; however, it took more than 14 days to determine whether DIRD were present in the sediments. These studies are not sufficient for investigating the diversity of DIRD. Hence, we considered that it is important to develop a rapid method to make DIRD detection more efficient.

In this study, we attempted to develop a rapid detection method of DIRD by real-time PCR and melting curve analysis. Sediment samples were collected from four sites of the Tomoe River, and one of its tributaries in the urban area of Shizuoka City, Japan. Also, we tried to detect DIRD cultured in a ferrihydrite-enriched medium by PCR-DGGE and identify species of these bacteria by DNA sequence analysis.

MATERIALS AND METHODS

Sample collection and preparation In June 2008, sediment samples were collected from four sites in the Tomoe River and one of its tributaries in Shizuoka City, Japan (Fig. 1). The samples were collected in 50-ml sterile tubes and stored in a refrigerator until analysis. The Tomoe River flows through the urban areas of Shimizu-ku in Shizuoka City, into the port of Shimizu. Site A (35°04'72"N, 138°41'00"E) was located upstream of the Tomoe River, sites B (35°02'23"N, 138°45'57"E) and C (35°02'54"N, 138°46'64"E) were located midstream of the Tomoe River, and site D (35°01'25"N, 138°47'04"E) was located midstream of the Osawa River, a tributary of the Tomoe River.

Enrichment conditions The ferrihydrite medium consisted of $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$ (2.2 g/l), NaHCO_3 (2.5 g/l), NH_4Cl (1.5 g/l), KCl (0.1 g/l), NaCl (0.1 g/l), $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (0.8 g/l), yeast extract (0.05 g/l, Nacalai Tesque), chemically synthesized ferrihydrite ($\text{Fe}_5\text{HO}_8 \cdot 4\text{H}_2\text{O}$ [11.2 g/l]) as amorphous iron oxide, and a stock solution [$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.1 g/l), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.1 g/l), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.005 g/l), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.001 g/l)]¹⁴⁾. Ferrihydrite was synthesized by neutralizing a 0.4 M solution of FeCl_3 to a pH of 7 with NaOH ¹⁴⁾. The reagents $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$, NaHCO_3 ,

NH_4Cl , $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, KCl , NaCl , and yeast extract were dissolved in distilled water and sterilized by passing through a 0.2- μm membrane filter (Advantec). The ferrihydrite medium and stock solution were autoclaved at 121°C for 20 min.

Under sterile conditions on a clean bench, 27 ml of ferrihydrite medium and 3 ml of each sediment sample were added to 50-ml vials that had been sterilized by dry heat (180°C, 2 h). The vials were sealed with butyl rubber stoppers and aluminum seals, and the air inside was replaced with nitrogen gas to produce anaerobic conditions. The vials were incubated at 30°C for 14 days

Fig. 1. Sampling sites in Shizuoka City (b) in Shizuoka Prefecture (a). Site A, upstream of the Tomoe River; sites B and C, midstream of the Tomoe River; and site D, midstream of the Osawa River.

under dark and static conditions. The formation of magnetite was confirmed visually using a neodymium magnet.

PCR-DGGE analysis Adequate amount of oxalate solution (oxalate acid 15 g/l and ammonium oxalate 28 g/l, pH 3.2) was added to 400 μ l of each vial to completely dissolve insoluble iron oxides in the sample¹⁴. The treated samples were then centrifuged at 4,000 $\times g$ for 10 min, and the supernatant was discarded. The samples were then resuspended in sterile water (Invitrogen) and centrifuged at 4,000 $\times g$ for 10 min. Bacterial cells were collected from the sample by repeating the above procedure three times. DNA was extracted and purified from the bacterial cells using the DNeasy Tissue Kit (Qiagen).

Extracted DNA was amplified using the universal primer set of 341F-GC (5'-GC clamp [5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG-3']-CCT ACG GGA GGC AGC AG-3') and 907R (5'-CCG TCA ATT CCT TTG AGT TT-3') for the 16S rRNA V3-V5 region (586 bp) using the GeneAmp PCR System 9700 (Applied Biosystems). PCR was performed using the FastStart *Taq* DNA Polymerase, dNTPack (Roche Diagnostics) that contained FastStart *Taq* DNA polymerase (5 U/ μ l), PCR Grade Nucleotide Mix, and 10 $\times$ PCR buffer (20 mM MgCl and distilled water). Each 25- μ l reaction mixture contained 0.2 μ l of FastStart *Taq* DNA polymerase, 0.5 μ l of PCR Grade Nucleotide Mix, 2.5 μ l of 10 $\times$ PCR buffer, 20.3 μ l of distilled water, 0.5 μ l of each forward and reverse primer (10 μ M), and 0.5 μ l of DNA template. The PCR conditions were as follows: preheating at 95°C for 4 min for hot-start PCR; 35 cycles of denaturing at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min; and final extension at 72°C for 7 min.

Amplified 16S rRNA gene fragments were analyzed by DGGE using the Dcode Universal Mutation Detection System (Bio-Rad) with a Power Station 1000XP (Atto) as the power supply. PCR products were separated on an 8% polyacrylamide gel, and a linear gradient of urea and formamide increasing from 30% at the top of the gel to 60% at the bottom was applied for the separation of the 16S

rRNA gene fragments. For each sample, 40 μ l of the PCR product was loaded, and the gel was run for 12 h at 100 V and 60°C. After electrophoresis, the gel was incubated for 30 min in Milli-Q (Millipore) water containing ethidium bromide (1.0 mg/l) and was removed from the plastic plate to a UV-transparent gel scoop.

Sequencing and phylogenetic analysis DGGE bands containing 16S rRNA gene fragments were punched from the gel using a sterile pipette tip. The gene fragments were extracted from the DGGE band using the High Pure PCR Product Purification Kit (Roche Diagnostics). The purified gene fragments were amplified with 341F and 907R primers for 30 PCR cycles, and the amplified gene fragments were purified using the DNA Purification Kit. The sequencing reactions were performed by MacroGen Japan Corporation. The sequences of 16S rRNA gene fragments were determined based on the nucleotide sequence in DNA DataBank of Japan (<http://www.ddbj.nig.ac.jp>). High sequence similarity with known bacteria was investigated using the BLAST gene sequence database (<http://blast.ddbj.nig.ac.jp/top-j.html>). Sequences from bacteria closely related to the cultured types were estimated from the results of the similarity search, and alignment was performed by MEGA 5.0 (<http://www.megasoftware.net/beta/index.php>), which was used to create a phylogenetic tree. Estimation of the phylogenetic tree was performed by the neighbor-joining method.

Analytical procedures As a pretreatment for the measurement of metal concentrations, the supernatant of each sediment sample was passed through a 0.45- μ m membrane filter (DISMIC-13HP, Advantec). Standard curves were generated using 1,000-ppm standard solutions (Wako) of Ca, Fe, K, Mg, Mn, and Na. Each sample was diluted with 2% nitric acid, and metal concentrations were measured with an inductively coupled plasma optical emission spectrometer (730-ES, Varian). Total organic carbon (TOC) was measured using a total carbon analyzer (TOC-5000A, Shimadzu). The Japanese Industrial Standard protocol K0100 was employed for the determination of total nitrogen (TN) and total phosphate (TP)¹⁵.

Real-time PCR reaction and melting curve analysis The primers correspond to the following positions on the *Escherichia coli* 16S rRNA gene sequence (GenBank accession number J01859): the Geo564F primer (5'-AA GCGTTGTTTCGGAWTTAT-3')¹⁶⁾, bp 566–584 and the GeY840R primer, bp 860–878. The positions were determined using the MegAlign program of the MEGA 5 software. Mismatches are indicated by underlining of the bases. The GeY840R primer (5'-GGCACYGCAGGGGTCAATA-3') was designed with reference to the Geo840R primer (5'-GGCACTGCAGGGGTCAATA-3')¹⁶⁾ used to target the 16S rRNA genes of *Geobacter*, *Pelobacter*, *Desulfuromonas*, and *Desulfuromusa*, because the Geo840R primer without a mixed base of Y (T + G) is not suitable for detecting some species of *Pelobacter* and *Desulfuromonas* (Table 1).

The PCR reaction was performed in a total volume of 20 μ l containing 1 μ l of 10 $\times$ each primer, 4 μ l of 5 $\times$ master mix (LightCycler FastStart DNA MasterPLUS SYBR Green I, Roche Diagnostic), 12 μ l of PCR grade water (Roche Diagnostic), and 2 μ l of template DNA. The PCR temperature program was as follows: preheating at 95°C for 10 min for hot-start PCR; 55 cycles of denaturing at 95°C for 10 s, annealing at 50°C for 10 s, and extension at 72°C for 13 s. Real-time PCR was performed on the LightCycler[®] 1.5 (Roche Diagnostic) and melting curve analysis was

performed with the default settings of LightCycler[®] software (Roche Diagnostic) from 65°C to 95°C. The average melting temperatures (T_m) were calculated from triplicate reactions for each sample. The DNA extracted from *G. sulfurreducens* PCA (ATCC 51573) was used as the positive control.

Nucleotide sequence accession numbers

The sequences generated in this study have been deposited in the GenBank database under accession numbers AB568586 and AB630332 to AB630336.

RESULTS AND DISCUSSION

Water quality of river Physicochemical properties of the supernatants obtained from centrifugation of sediment samples are shown in Table 2. TOC content at site A was less than 0.05 mg/l, however, that at site B, C, and D was 4.3, 11, and 5.9, respectively. As there are houses that are not connected to public sewer systems around the midstream of the Tomoe River, untreated domestic wastewater discharged into the river may influence the concentration of TOC. A difference in metal concentration was observed between the sites. Total Mg and Na concentrations at site C were significantly higher than those at the other sites. Seawater is considered to affect the concentration of Mg and Na in the river. It is not obvious why the Ca concentration at site A was as

Table 1. Alignment of corresponding regions of primers for detecting *Geobacter*, *Desulfuromonas*, and *Pelobacter* species

Organism	Alignment of 16S rRNA fragments (5'→3') ^a		Accession No.
	566–584	860–878	
<i>Geobacter chapelleii</i> 172 ^T	AAGCGTTGTTTCGGATTTAT	TATTGACCCCTGCAGTGCC	U41561
<i>Geobacter lovleyi</i> SZ ^T	AAGCGTTGTTTCGGATTTAT	TATTGACCCCTGCAGTGCC	CP001089
<i>Geobacter metallireducens</i> GS-15 ^T	AAGCGTTGTTTCGGAATTAT	TATTGACCCCTGCAGTGCC	CP000148
<i>Geobacter sulfurreducens</i> PCA ^T	AAGCGTTGTTTCGGAATTAT	TATTGACCCCTGCAGTGCC	AE017180
<i>Desulfuromonas alkaliphilus</i> Z-0531 ^T	AAGCGTTGTTTCGGAATTAT	TATTGACCCCTGCAGTGCC	DQ309326
<i>Desulfuromonas palmitatis</i> SDBY1 ^T	AAGCGTTGTTTCGGAATTAT	TATTGACCCCTGCAGTGCC	U28172
<i>Desulfuromonas thiophila</i> DSM 8987 ^T	AAGCGTTGTTTCGGAATTAT	TATTGACCCCTGCAGTGCC	Y11560
<i>Pelobacter carbinolicus</i> DSM 2380 ^T	AAGCGTTGTTTCGGAATTAT	TATTGACCCCTGCAGTGCC	CP000142
<i>Pelobacter propionicus</i> DSM 2379 ^T	AAGCGTTGTTTCGGAATTAT	TATTGACCCCTGCAGTGCC	CP000482
<i>Pelobacter venetianus</i> Gra PEG1 ^T	AAGCGTTGTTTCGGAATTAT	TATTGACCCCTGCAGTGCC	U41562

a) Underline indicates mismatches. The primers correspond to the following positions on the *E. coli* 16S rRNA gene sequence (GenBank accession number J01859): The Geo564F primer, bp 566–584 and the GeY840R primer, bp 860–878. The positions were determined using the MegAlign program of the MEGA 5 software.

Table 2. Physicochemical properties of the river water

Sample name	pH	TOC (mg/l)	TN (mg/l)	TP (mg/l)	Metals (mg/l)					
					Ca	Fe	K	Mg	Na	Mn
Site A	7.14	<0.05	5.6	0.11	130	<0.01	3.5	5.5	5.6	<0.01
Site B	7.48	4.3	1.3	0.59	30	<0.01	1.2	5.5	26	0.77
Site C	6.98	11	2.9	1.2	46	<0.01	4.7	24	26	<0.01
Site D	7.36	5.9	1.5	0.59	52	<0.01	1.9	10	4.5	<0.01

Fig. 2. The magnetic response of the ferrihydrite medium before (a) and after (b) the 14-day incubation with the sediment sample from site A (upstream of the Tomoe River) exposed to a neodymium magnet. The image shows the magnetic response immediately after shaking the vials.

high as 130 mg/l and site B only contained Mn (0.77 mg/l).

Magnetite production by bacteria Figure 2 illustrates the ferrihydrite medium containing the sediment sample from site A before (a) and after (b) incubation exposed to a neodymium magnet. Images of the magnetic response were taken immediately after shaking the vials. Before incubation, brown amorphous ferrihydrite precipitated at the bottom of the vial in the stationary phase, and the ferrihydrite showed no magnetic response. After 14 days of incubation, however, the ferrihydrite medium turned black and showed a magnetic response in vials containing the sediment samples from all sites (Fig. 2(b)), indicating that the black substance was magnetite produced by DIRD.

PCR-DGGE analysis for identification of DIRD In the DGGE profiles, characteristic bands appeared in all samples before and after incubation (Fig. 3). Band 5 (Fig. 3 (g)) was the only clear band in the DGGE profile

Fig. 3. DGGE profiles of 16S rRNA gene fragments amplified from DNA extracts from the sediment samples (a, c, e, g) and after samples were incubated for 14 days in ferrihydrite-enriched medium (b, d, f, h)

before incubation. Significant changes occurred in the microbial community after incubation in the ferrihydrite-enriched medium. Bands 1, 2, 3, 4, and 6 were found in sediment samples from different sites after incubation. The closest matches to the excised and sequenced 16S rRNA-derived DGGE bands 1-6 (Fig. 3) are listed in Table 3. Bacteria closely related to *G. sulfurreducens* PCA were found in all the sediment samples after incubation. Band 4 showed 97% similarity with *Desulfuromonas alkaliphilus* Z-0531. *D. alkaliphilus* Z-0531 isolated from salt lakes in Russia is able to grow at NaCl concentrations of 0-50 g/l, and has an optimum pH of 8.6, and produce magnetite from ferrihydrite¹¹. Bands 1, 2, 3, and 6 after incubation belonged to *G. sulfurreducens* PCA, while Band 4 belonged to a different phylogenetic cluster (Fig. 4). Band 5 showed 97% similarity with *Janthinobacterium lividum* NBGD31 before incubation. *J. lividum* is a gram negative, motile, aerobic

Table 3. Closest matches to the excised and sequenced 16S rRNA-derived DGGE bands

Band	Length of sequence (bp)	Closest match in Genbank database (Accession No.)	Similarity (%)	Accession No.
1	578	<i>Geobacter sulfurreducens</i> PCA ^T (AE017180)	100	AB568596
2	559	<i>Geobacter sulfurreducens</i> PCA ^T (AE017180)	100	AB630332
3	567	<i>Geobacter sulfurreducens</i> PCA ^T (AE017180)	99	AB630333
4	575	<i>Desulfuromonas alkaliphilus</i> Z-0531 ^T (DQ309326)	97	AB630334
5	529	<i>Janthinobacterium lividum</i> NBGD31 ^T (HQ003440)	97	AB630335
6	564	<i>Geobacter sulfurreducens</i> PCA ^T (AE017180)	99	AB630336

Fig. 4. Phylogenetic tree of 16S rRNA gene sequences of the DNA extracts from samples incubated for 14 days in ferrihydrite-enriched medium. A phylogenetic tree was constructed for 446-bp aligned sequences with neighbor-joining method-established positions using MEGA 5.0 software. Robustness of derived groupings was examined by bootstrap using 1,000 replications. Numbers show percentage bootstrap support of tree branch points. The scale bar represents 5 substitutions per nucleotide site.

bacterium commonly isolated from the soil and water of rivers, lakes, and springs, and it is one of the most common species showing purple pigmentation¹⁷⁾.

A previous study reported that DIRD such as bacteria closely related to *G. sulfurreducens* PCA, *G. thiogenes* K1 and *D. alkaliphilus*

Z-0531 were found in rivers in Shizuoka prefecture regardless of the TOC and metal content¹⁴⁾. Also, no correlation was observed between the existence of bacteria closely related to *G. sulfurreducens* PCA and physicochemical properties of the river water analyzed in this study. However, we

considered that the salinity of the river water influences DIRD habitats because *D. alkaliphilus* Z-0531 was found in only one sample containing high concentrations of Mg and Na.

Detection of DIRD by melting curve analysis As seen in Table 1, the Geo564F primer (5'-AAGCGTTGTTTCGGAWTTAT-3') and the 566-584 bp segments of the 16S rRNA gene of *Geobacter* species are complementary to each other because the primer contains the mixed base of W (A + T). However, the Geo840R primer (5'-GGCACTGCAGGGGTCAATA-3') is complementary to the 860-878 bp segments of the 16S rRNA gene of not only *Geobacter* species but also some *Desulfromonas* and *Pelobacter* species because a difference exists at the bp number 873 of the 16S rRNA gene as indicated in Table 1. Therefore, the GeY840R primer (5'-GGCACYG CAGGGGTCAATA-3') was designed from the alignment of the 16S rRNA gene of *Geobacter*, *Desulfromonas*, and *Pelobacter* species with reference to Geo840R in order to cover the difference at the bp number 873 and detect many species of DIRD in freshwater sediment samples. The GeY840R primer contains a mixed base of Y (T + G).

T_m of DIRD amplicons in all the sediment samples before and after incubation with ferrihydrite medium are shown in Fig. 5. T_m for the 315-bp amplicons generated with the primer sets of Geo564F and GeY840R were calculated using LightCycler® software (Roche Diagnostic). We found DIRD amplicons in all the sediment samples before and after incubation. T_m of DIRD amplicons obtained from sites A, B, C, and D before incubation were 87.3 ± 0.2°C, 87.4 ± 0.2°C, 87.6 ± 0.3°C, and 87.0 ± 0.3°C, respectively. T_m of the amplicons of *G. sulfurreducens* PCA (ATCC 51573) and those of DIRD obtained from sites A, B, C, and D after incubation were 87.3 ± 0.1°C, 87.4 ± 0.1°C, 87.3 ± 0.1°C, 88.0 ± 0.1°C, and 87.3 ± 0.1°C, respectively. T_m of these amplicons depended primarily on the amplicon size and GC content. T_m of DIRD amplicons from site C before and after incubation were higher than those of DIRD amplicons from the other sites. These high T_m of DIRD amplicons from site C are the reason for the higher GC content in *D.*

Fig. 5. T_m of DIRD amplicons in the sediment samples before and after incubation in ferrihydrite medium. The DNA extracted from *G. sulfurreducens* PCA (ATCC 51573) was used as positive control. Real-time PCR was performed on a LightCycler® 1.5 (Roche Diagnostic), and melting curve analysis was performed with the default settings of LightCycler® software (Roche Diagnostic) from 65°C to 95°C. T_m were calculated from triplicate reactions for each sample.

alkaliphilus Z-0531 compared to that of *G. sulfurreducens* PCA. The GC content of the PCR product of *G. sulfurreducens* PCA was 53% (166/313 bp), while that of *D. alkaliphilus* Z-0531 was 57% (178/313 bp). T_m of DIRD amplicons from site C after incubation were higher than those before incubation (Fig. 5).

The results of the melting curves in Fig. 6 showed the fluorescence intensities before incubation were lower than those after incubation, and the shapes of site C was different from those of the other sites. The shape of some overlapping peaks can be clearly seen on of the melting curves. Therefore, it is suggested that the Geo564F and GeY840R primers amplified not only 16S rRNA genes of DIRD, but also other closely related *Deltaproteobacteria* as well. As the results in this study, we succeeded in detecting DIRD in the sediment samples using the primer sets of Geo564F and GeY840R, although the species of DIRD was not identified by the difference in T_m. These results indicate that we can make the DIRD identification process shorter, since DNA sequences of a PCR product are analyzed just after getting the result of the melting curve analysis without ferrihydrite-enriched cultures.

Fig. 6. Melting curve analysis for DIRB amplicons in the sediment samples obtained from sites A, B, C, and D before and after incubation. The DNA extracted from *G. sulfurreducens* PCA (ATCC 51573) was used as the positive control. Real-time PCR was performed on the LightCycler® 1.5 (Roche Diagnostic), and melting curve analysis was performed with the default settings of LightCycler® software (Roche Diagnostic) from 65°C to 95°C.

CONCLUSION

We revealed the diversity of DIRD in the Tomoe River and one of its tributaries in Shizuoka City, Japan, and succeeded in detecting DIRD by melting curve analysis without ferrihydrite-enriched cultures. In this study, the ferrihydrite medium turned black and showed a magnetic response, and bacteria of greater than 99% similarity with *G. sulfurreducens* PCA were found in vials containing the sediment samples from all sites after 14 days of incubation. A species closely related to *G. sulfurreducens* PCA was

found to exist in the Tomoe River and one of its tributaries in Shizuoka City. Furthermore, we were able to find DIRD amplicons in all the sediment samples before and after incubation using melting curve analysis with the primer sets of Geo564F and GeY840R. However, we need more information about the diversity of DIRD in order to elucidate the correlation between their existence and environmental factors. This rapid detection method using the melting curve analysis could aid in determining whether DIRD are present in sediment samples without ferrihydrite-enriched cultures.

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