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Classification for Dimethylarsenate-decomposing Bacteria Using a Restrict Fragment Length Polymorphism Analysis of 16S rRNA Genes

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A new monitoring system for bacterial communities involving dimethylarsinic acid (DMAA) decomposition was provided by combining the MPN (Most Probable Number) method and RFLP (restriction-fragment-length polymorphism analysis). The abundance of DMAA decomposing bacteria was estimated by the MPN method using a bacterial culture medium, which included DMAA as the sole carbon source, indicating bacterial cell densities of 1700 cells/ml in Lake Kahokugata and 330 cells/ml in Lake Kibagata. After isolating the dominant bacteria using agar plates, the isolates were classified into some genotype groups by RFLP analysis using 16S rDNA sequences. Classification of the RFLP analysis indicated that 14 isolates of Lake Kahokugata were classified into 6 types, which included 2 dominant types related to genus *Pseudomonas*, while 8 isolates of Lake Kibagata displayed 6 types including one or two isolates. Moreover, the RFLP types were unique for each lake, suggesting that DMAA decomposing bacteria were specific for the aquatic environment related to the arsenic cycle. The activities of DMAA decomposition mostly matched with the RFLP type category of the isolates. Accordingly, combining the MPN method with the RFLP analysis will play an important role in elucidating the distributions and dynamics of the DMAA-decomposing bacterial community.

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Introduction

Arsenic, the 20th most common element in the earth crust, is widely distributed in nature. In aquatic environments, arsenate, arsenite, and methylate arsenic species are found as dominant arsenic speciation.[1,2] The arsenic cycles depend on the bioactivities of microorganisms.[1,3] The mobilization of arsenic from aquatic sediment into aquatic water would contribute to arsenic reducing or oxidizing bacteria.[4,5] For example, in Bangladesh, the arsenite released into the ground water contaminates drinking water, thus causing serious water problems.[6] Arsenate is a chemical analogue of phosphate, and may interfere with oxidative phosphorylation.[1] Accordingly, under the phosphate limited condition, some microorganisms, such as phytoplankton and bacteria, uptake dissolved arsenate through their phosphate-concentrating mechanisms.[7] Then, arsenate within the microorganism cells would be reduced to arsenite, and methylated to monomethylarsonic acid ($CH_3AsO(OH)_2$; MMAA(V)) and dimethylarsinic acid ($(CH_3)_2AsO(OH)$; DMAA(V)).[2] The methylated arsenic compounds are converted to more complex organoarsenic compounds, such as arsenobetaine or arsenosugar.[2]

The decomposition of organoarsenic compounds is considered to depend mostly on bacterial activities influencing the arsenic cycles in aquatic environments. When marine bottom sediments were incubated with a medium containing arsenobetaine originating from marine fish or marine bacteria in the sediments, microorganisms would decompose the arsenobetaine to trimethylarsine oxide.[8] Natural seawater, in which bacterial activities were induced by the bacterial culture, indicates the decomposition of DMAA.[9] However, most of studies have demonstrated the organoarsenic decomposition activities of water samples or sediment samples, but nobody has monitored and isolated the organoarsenic decomposing bacteria in fields. Only one report on the isolate, arsenobetaine-degrading bacteria, strain ASV2, was reported by Quion, showing that the bacteria metabolize the organoarsenic compounds as a carbonic source.[10] As mentioned above, the organoarsenic decomposition process in aquatic environments is still veiled, and the ecophysiological characteristics of organoarsenic decomposing bacteria have not been investigated.

The organoarsenic decomposition process can not be neglected when considering field data of the arsenic cycle in aquatic environments.[11] Although phytoplankton is known to produce organoarsenic compounds, as described above, the amounts of chlorophyll *a* did not show any relationship with the organoarsenic abundance in Tosa Bay[12] and Lake Biwa.[13] Sohrin suggested that the abundance of organoarsenic compounds would be underestimated by organoarsenic decomposing bacterial activities.[13] In order to know the correct relationship between the bioactivities and organoarsenic compounds in the fields, the organoarsenic decomposition mechanisms need to be elucidated by monitoring organoarsenic decomposing bacteria.

We have recently tried to combine the MPN (Most Probable Number) method with RFLP (restriction-fragment-length polymorphism analysis) analysis to investigate the biomass and composition of bacterial communities decomposing organoarsenic compounds in aquatic environments. The MPN method is a powerful tool for monitoring and isolating bacteria

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in the aquatic environments. Arsenate-reducing bacteria in the field were easily monitored by the MPN methods using the chemical reactions of arsenite and sulfate.[14] RFLP analysis of 16S rDNA sequences was widely used for classifying several bacterial populations, such as cellulolytic bacterial isolates,[15,16] infectious bacteria to plants,[17] and *Pseudomonas* strains.[18] In Japanese lakes, Lake Kahokugata and Lake Kibagata, the cell densities of DMAA decomposing bacteria were estimated using the MPN method. As a model of organoarsenic compounds, we focused on DMAA which is widely distributed in freshwater. Moreover, the bacterial compositions were determined using RFLP analysis of 16S rDNA sequences.

Experimental

Sampling

Water samples were collected from the coast of Lake Kahokugata and Lake Kibagata in Ishikawa prefecture of Japan, in December and September of 2002, respectively, using 1 l polycarbonate vials. The water samples were brought to the laboratory and applied to experiments within 2 h. A 50-ml volume of sample water was directly used for the MPN (Most Probable Number) method, determining the cell densities of DMAA decomposing bacteria; another 50 ml portion of the sample water was filtrated with a GF/C filter (ADVANTEC, Tokyo, Japan) for measuring the arsenic species.

Determination of bacterial cell number using the MPN procedure

The cell densities of bacteria involving DMAA decomposition were determined using the culture dilution method, *i.e*., the MPN method. A CD (Czapec-Dox) minimal medium containing 10 μM DMAA as the sole carbon source, instead of peptone, was used for the MPN procedure. The medium components were $NaNO_3$ (2 g/l), K_2HPO_4 (1 g/l), $MgSO_4 \cdot 7H_2O$ (0.5 g/l), KCl (0.5 g/l), $FeSO_4$, thiamine-HCl (0.005 g/l), and DMAA (0.214 g/l). The bacteria can grow in this culture medium, using DMAA as the sole carbon source, and are thus defined as "DMAA decomposing bacteria". A 10 ml portion of medium was dispensed to the autoclaved tubes used for the MPN method. Environmental samples were diluted 10-fold serially to 105 in sterile water. A 10-ml portion of the growth medium including DMAA was added to 10 ml aliquots of each dilution. For each dilution step, 200 μl of diluted environmental samples were transferred to 24 wells in a 96-well microtiter plate. These plates were then incubated at room temperature. The bacterial growth was evaluated after 14 days by the appearance of well-dense upon bacterial biomass.

Bacterial classification using RFLP analysis

All isolates were classified by the RFLP analysis of 16S rDNA sequences. A total of 24 isolates cultivated in an ST 10^{-1} culture medium overnight were pelleted by centrifugation at 3000 rpm for 15 min. The ST 10^{-1} culture medium was composed of 0.5 g/l peptone and 0.05 g/l yeast extract. The bacterial cell pellets were stored at –70˚C until the following experiments. The bacterial cell pellets were resuspended in 500 μl of TE buffer (10 mmol/l Tris-HCl, pH 8.0; 1 mmol/l EDTA). The cells were lysed with SDS, protainase K, and lysozyme at 50˚C for 30 min. Furthermore, genomic DNAs were purified by phenol-chloroform extraction, and chloroform extraction, followed by ethanol precipitation. Finally, the DNA fragments were resuspended in 10 μl of sterile distilled water.

The 16S rDNA fragments (*ca*. 1450 bp) of bacteria were amplified by the polymerase chain reaction (PCR). Reaction mixtures (final volume, 100 μl) contained 200 μM of dNTPs, 0.5 units of Ex Taq polymerase (TAKARA BIO INC, Shiga, Japan), and 0.2 μM of each oligonucleotide primer, 27F and 1492R. These primers especifically bind to eubacterial 16S rDNA.[19] Genomic DNA of bacteria was added at the final concentration of 1 – 10 ng/μl. Thermal cycling was performed using a Program Temp Control System (PC-700; ASTEC, Fukuoka, Japan) under the following conditions: denaturation at 95˚C for 1 min, annealing at 55˚C for 2 min, extension at 72˚C for 2 min, as a total of 30 cycles. The PCR products (1 – 9 μl) were digested with each of the following enzyme mixtures: series 1, *EcoR* I and *Hind* III; series 2, *BamH* I and *Sal* I; series 3, *Sac* I; series 4, *Pst* I and *Hinf* I (TOYOBO). In order to detect minor differences between bands, the DNA fragments were separated in 1.5% w/v agarose gel-LE classic type (Nacalai-Tesque, Kyoto, Japan), depending on the distinguishable fragment sizes. A DNA molecular weight marker, 100 bp ladder, was used as a standard with a size range of 100 – 2000 bp (TOYOBO). The gels were stained with ethidium bromide and documented with a Polaroid camera (DS-34M; ULTRA LUM, CA, USA). The restriction profiles were classified according to the presence or absence of fragments for each enzyme.

Sequencing of 16S rDNA and phylogenetic analysis

The PCR amplicons were separated by 1.5% agarose gel electrophoresis, and about 1450 bp of DNA bands (16S rDNA fragments) were excised and purified by phenol-chloroform extraction and chloroform extraction, followed by ethanol precipitation. Finally, the DNA fragments were resuspended in 10 μl of sterile distilled water. Partial sequences (*ca*. 460 bp) of 16S rDNA were determined using a Dye Deoxy™ Terminator Cycle Sequencing Kit (ABI, CA, USA) with the 27F sequencing primer and a DNA auto-sequencing system (Model 373A) according to the recommended protocol. The determined sequences were compared with a DDBJ database using BLASTA and FASTA SEARCH programs.[20]

A phylogenetic tree including all isolates was constructed according to the neighbor-joining algorithmic method,[21] using the *ca*. 460 bp sequences of 16S rDNA. The nearly complete 16S rDNA sequences were compared to sequences available from the Ribosomal Database Project and EMBL/GenBank databases. The similarity values were based on a pairwise comparison of the sequences. For phylogenetic analyses, the DNA sequences were aligned using the CLUSTAL W ver. 1.7 (European Bioinformatics Institute).[22] The trees were constructed using the Neighbour-Joining (PHYLIP computer program package, version 3.6a2). The Neighbor-Joining algorithm NEIGHBOR was based on a matrix of pairwise distances corrected for multiple base substitutions using a DNADIST with a transition/transversion ratio of 2.0. The tree topologies were evaluated by a bootstrap analysis of the Neighbour-Joining tree using the original data set and 1000 bootstrap data sets. The root position was estimated by using the 16S rDNA sequence of *Thermotoga maritima* (M21774) as the outgroup.

Measurements of arsenic species

The cold-trap HG-AA speciation procedure was employed for measurements of arsenic compounds.[23,24] The water samples or bacterial culture were filtrated with a 0.45 μm nuclepore filter (ADVANTEC). The sample was acidified and reacted with sodium tetrahydroborate, producing arsines, which were then swept by a nitrogen flow into a cold trap. This trap was cooled

Table 1 DMAA decomposing bacterial strains isolated from water samples of Lake Kahokugata and Lake Kibagata

	Kahokugata	Kibagata
DMAA decomposing bacteria (cells/ml)	1700	330
Isolate	12M2, 12M3, 12M5, 12M6, 12M7, 12M8, 12M9, 12M10, 12M11, 12M14, 12M15, 12M16, 12M17, 12M18, 12M19, 12M20	K1, K3, K5, K8, K9, K10, K12, K14
Methylated arsenic concn. (nM)	0	0
Inorganic arsenic concn. (nM)	5.13	7.65
Chlorophyll *a* concn. (μg/l)	7.31	54.5

Abundance of DMAA decomposing bacteria was determined by the MPN method.

by liquid nitrogen before it was gently warmed by electrical heating. The arsines, such as inorganic arsine, MMAA and DMAA, sequentially released into a quartz-T tube held at *ca*. 900˚C in an electrically heated furnace were monitored by an atomic-absorption spectrometer.

Detection of DMAA decomposition activity

As culture preincubation, the representative isolates were incubated in a liquid ST 10^{-1} culture medium with DMAA for about one week. For evaluating the methylarsenic decomposition activities, each isolate culture was inoculated into 20 ml of liquid ST 10^{-1} culture medium, including 10 μmol/l of DMAA (Roth, Karlsruhe, Germany), as the final concentrations at initial bacterial cell density of 10^5 cells/ml. After 2 weeks of incubation, 2 ml of bacterial culture was applied to measurements of the arsenic species, as described above. Inorganic arsine and MMAA formed in the bacterial culture indicate the products converted from DMAA. The DMAA decreasing rate was calculated by dividing the decreased amounts of DMAA for two weeks by the initial amounts of DMAA.

Nucleotide sequence accession numbers

The DDBJ accession numbers for the new 16S rDNA sequences of 12M2, 12M3, 12M5, 12M7, 12M8, 12M9, 12M10, 12M11, 12M12, 12M14, 12M15, 12M16, 12M17, 12M18, 12M19, K1, K3, K5, K8, K9, K10, K12 and K14 are from AB120335 to AB120358, respectively.

Results

Abundance of DMAA decomposing bacteria

The abundances of DMAA decomposing bacteria in Lake Kahokugata and Lake Kibagata could be determined by the MPN methods using a culture medium including 1 μmol/l DMAA as the sole carbon source (Table 1). The bacterial cell densities were 1700 cells/ml and 330 cells/ml in Lake Kahokugata and Lake Kibagata, respectively. The inorganic arsenic concentration in Lake Kahokugata and Lake Kibagata were 5.13 nmol/l and 7.65 nmol/l, respectively, while methylarsenic species were not detected from water samples of those lakes. The amounts of chlorophyll *a* were 7.31 μg/l in Lake Kahokugata and 54.5 μg/l in Lake Kibagata, indicating high bioactivities of phytoplankton. The bacterial culture in the highest dilution degree of wells, where the bacterial densities were observed by the MPN procedure counts, were spread on the agar ST 10^{-1} culture medium. After incubation for 1 – 5 days, DMAA-decomposing bacterial isolates displayed a broad variability of morphological features. Randomly selected bacterial colonies were subcultured and purified. As a result, 16 isolates were obtained from a water sample of Lake Kahokugata, and 8 isolates were obtained from Lake Kibagata.

Table 2 Band sizes within the RFLP pattern group for DMAA decomposing bacterial isolates obtained from Lake Kahokugata and Lake Kibagata

Endonuclease mixture	RFLP pattern	Size of restriction fragments/bp				
Series 1	A	1500				
EcoR I, *Hind* III	B	850	400	150	<100	
	C	850	400	250		
	D	800	700			
	E	850	450	200		
Series 2	A	1500				
BamH I, *Sal* I	B	1200	300			
	C	850	650			
Series 3	A	1500				
Sac I	B	800	650	<100		
	C	950	550			
Series 4	A	1100	250	150		
Pst I, *Hinf* I	B	350	250	200	150	<100
	C	700	350	200	<100	
	D	950	350	200		
	E	350	250	150	<100	
	F	500	350	250	150	<100
	G	650	300	250	<100	
	H	500	200	150	<100	
	I	650	350	250	<100	
	J	650	400	300	150	
	K	1350	150			

Phylogenetic classification of DMAA decomposing bacteria

The rDNA of 24 isolates could be amplified using the primers 27F and 1492R, resulting in a characteristic single band of about 1500 bp. An RFLP analysis of the PCR products of 24 isolates using 4 series of endonuclease mixtures (series 1, *EcoR* I and *Hind* III; series 2, *BamH* I and *Sal* I; series 3, *Sac* I; series 4, *Pst* I and *Hinf* I) revealed various restriction patterns, as demonstrated in Figs. 1 and 2. DNA fragments smaller than 100 bp were not properly resolved by electrophoresis, and therefore were not used to compose the patterns. The PCR products of 24 isolates digested with series 2 and 3 provided a single band or two bands on the gels, while the PCR products digested by series 1 displayed a single band to three bands (Table 2). In the case of series 4, two to four bands were obtained. Ultimately, six to seven restriction fragments per pattern were used to classify isolates. A comparison of all the restriction patterns resulted in 12 distinct RFLP pattern groups (Table 3).

The isolates belonged to one RFLP pattern group, such as AABA type, AAAA type, AAAD type, DAAE type, DBCF type or DAAG type. These displayed high similarity of 16S rDNA partial sequences ranging from 94.8% to 100% (Table 4), forming each single cluster on the phylogenetic tree (Fig. 3). However, the similarities of the isolates among the RFLP pattern groups were less than 91.2%, except for comparisons

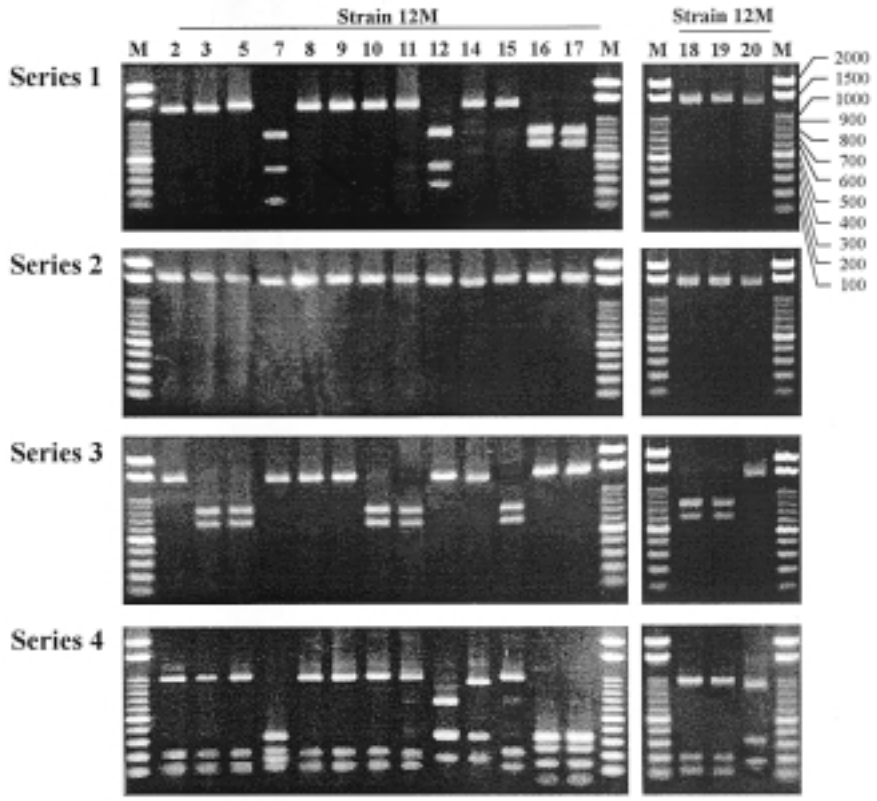

Fig. 1 Restriction patterns of amplified 16S rDNA digested with series 1, *Eco R* I and *Hind* III; series 2, *BamH* I and *Sal* I; series 3, *Sac* I; and series 4, *Pst* I and *Hinf* I. The pattern of 14 isolates obtained from Lake Kahokugata is demonstrated. The first and last lanes represent a molecular weight 100 bp marker (TOYOBO).

Table 3 RFLP pattern of amplified 16S rDNA of DMAA decomposing bacterial isolates obtained from Lake Kahokugata and Lake Kibagata

Site	Isolate	RFLP pattern of amplified 16S rDNA digested with[a]			
		Series 1 *EcoR* I, *Hind* III	Series 2 *BamH* I, *Sal* I	Series 3 *Sac* I	Series 4 *Pst* I, *Hinf* I
Kahokugata	12M3, 12M5, 12M10, 12M11, 12M15, 12M18, 12M19	A	A	B	A
	12M2, 12M8, 12M9	A	A	A	A
	12M14, 12M20	A	A	A	D
	12M16, 12M17	D	A	A	E
	12M7	B	A	A	B
	12M12	C	A	A	C
Kibagata	K1, K3	D	B	C	F
	K5, K12	D	A	A	G
	K8	E	A	C	H
	K9	D	A	A	I
	K10	D	C	A	J
	K14	A	A	C	K

a. Different restriction patterns were indicated with different letters of each endonuclease used.

between the AABA type and the AAAA type, which were dominant in Lake Kahokugata, including 7 isolates and 3 isolates, respectively (Table 4). The 10 isolates belonged to the AABA type and the AAAA type, which were similar to each other at more than 98.1%. On the phylogenetic tree, the AAAA type isolates displayed a closer relationship with the AABA type isolates in gamma proteobacteria (Fig. 3). Moreover, 10 isolates belonged to the AABA type or the AAAA type most closely related to genus *Pseudomonas* at high similarities ranging from 99.7% to 100% (Table 5). The RFLP analysis with *Sac* I was able to distinguish the AABA type isolates from AAAA type isolates, classifying the closely related bacterial population.

As the result of an RFLP analysis for DMAA decomposing bacterial strains obtained from two lakes, 16 isolates of Lake Kahokugata were classified into 6 types, and 8 isolates of Lake

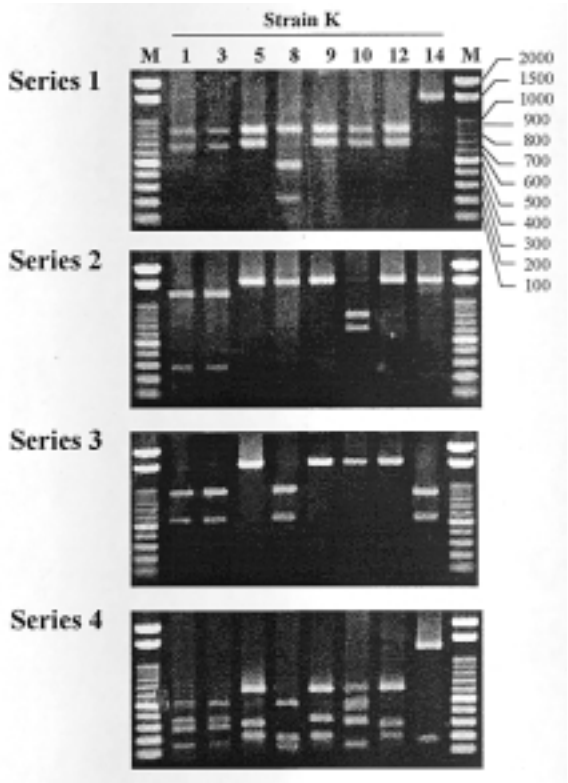

Fig. 2 Restriction patterns of amplified 16S rDNA digested with series 1, *Eco R* I and *Hind* III; series 2, *BamH* I and *Sal* I; series 3, *Sac* I; and series 4, *Pst* I and *Hinf* I. The pattern of 8 isolates obtained from Lake Kibagata is demonstrated. The first and last lanes represent a molecular weight 100 bp marker (TOYOBO).

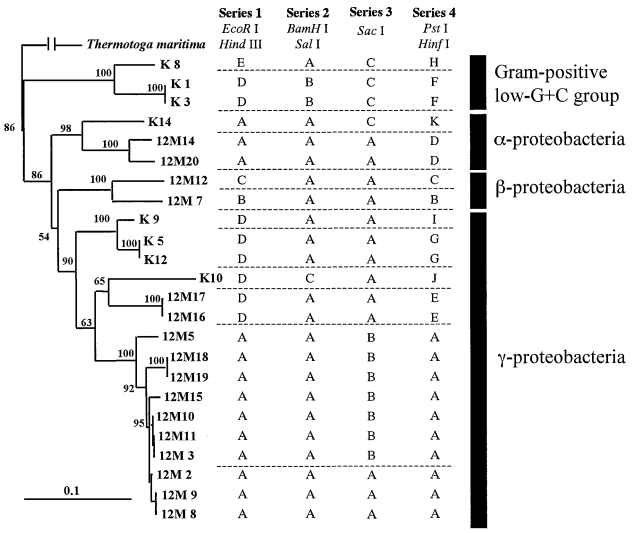

Fig. 3 Phylogenetic tree of the relationships between 24 isolates obtained from Lake Kahokugata and Lake Kibagata. The tree was calculated from a dissimilarity matrix of *ca*. 460 bp alignment using a neighbor-joining algorithm. The number of types are indicated in Table 2.

Kibagata indicated 6 types (Table 3). Each RFLP pattern group was unique for Lake Kahokugata and Lake Kibagata, without being observed across the two lakes. Moreover, upon comparing the partial 16S rDNA sequences, the isolates of Lake Kahokugata were different from those of Lake Kibagata at low similarities ranging from 65.4% to 87.3% (Table 4). Furthermore, phylogenetic analyses also indicated that isolates of Lake Kahokugata did not show a close relationship with those of Lake Kibagata. The isolates of Lake Kahokugata formed clusters in three bacterial groups: alpha-, beta- and gamma-proteobacteria divisions. Then, 12 isolates indicating high proportion of 75% (12/16) belonged to the gamma-proteobacteria division. Moreover, 10 isolates of the 12 isolates belonging to the AABA type and the AAAA type were, closely related to the genus *Pseudomonas* in the gamma-proteobacteria division. In the case of Lake Kibagata, 8 isolates randomly formed clusters in four bacterial groups: alpha-, beta-, gamma-proteobacteria divisions and gram-positive low-G+C group. An RFLP analysis indicated that the DBCF type and the DAAG type, including two isolates, and each group of the other patterns contained one isolate. The isolates belonged to EACH type and DBCF type, closely related to *Aureobacterium resistens* and *Arthrobacter* sp. M4, respectively, in the gram-positive bacteria group. Gram-positive bacteria were specific for Lake Kibagata.

DMAA decomposing activity

The isolates were assayed for *in vitro* methylarsenic decomposing activity using the ST 10^{-1} culture medium with 1 μmol/l DMAA (Table 6). The DMAA-decomposing activity of isolates was evaluated according to the measurement of inorganic arsine, MMAA and the decreasing rate of DMAA. Out of 24 isolates tested for methylarsenic decomposition, 16 isolates decreased the amount of DMAA at rates ranging from 14.3% to 70.1%, indicating that 140 nmol/l and 700 nmol/l of DMAA were decomposed to MMAA and inorganic arsine, or converted to complex organoarsenic compounds. Synthesized inorganic arsine could be detected in the culture media of 18 isolates in a total of 24 isolates at concentrations ranging from 32 nmol/l to 88 nmol/l. The concentrations of inorganic arsenic originated form the other 6 isolates were under 13 nmol/l, which was too low to be detected by the measurement method used in this study. MMAA could also be detected at concentrations over 40 nmol/l, and 14 isolates were found to convert DMAA to MMAA at concentrations ranging from 45 nmol/l to 234 nmol/l. Inorganic arsenic and MMAA in bacterial culture incubated for two weeks indicated the relatively low decreasing rates of DMAA.

In Lake Kahokugata, the isolates indicating high activities of DMAA decomposition belonged to the AABA type, the DAAE type, and the AAAA type in gamma-proteobacteria division. The isolates belonged to the AABA type and the DAAE type decreased the DMAA amounts in the culture medium at rates ranging from 14.3% to 68.2%, while the isolate of the AAAA type produced inorganic arsenic and MMAA at high concentrations. In contrast, the AAAD type, the CAAC type and the BAAB type isolates belonging to the alpha- or beta-proteobacteria division showed low DMAA decrease rates of below 10%, and a low production of inorganic arsine and MMAA at below 10 nmol/l.

In the case of Lake Kibagata, the high DMAA decrease rates (32.3 – 70.1%) were found in the RFLP types, the EACH type and the DBCF type belonging to the gram-positive group, and also in the DCAJ type belonging to the gamma-proteobacteria division. However the AACK type isolates belonging to the alpha-proteobacteria division showed a low DMAA decomposition at below 10%, similar to the case of Lake Kahokugata.

Table 4 Simirality of 16S rDNA partial sequences (*ca*. 460 bp) among DMAA decomposing bacterial isolate obtained from Lake Kahokugata and Lake Kibagata

	Similarity, %																							
RFLP pattern group	AABA							AAAA			AAAD		DAAE		BAAB	CAAC	DBCG			DAAH		EACH	DAAI	DCAJ	AACK
Isolate	12M3	12M5	12M10	12M11	12M15	12M18	12M19	12M2	12M8	12M9	12M14	12M20	12M16	12M17	12M7	12M12	K1	K3	K5	K12	K8	K9	K10	K14
Kahokugata																								
12M3	—																							
12M5	96.6	—																						
12M10	100	96.6	—																					
12M11	100	96.6	100	—																				
12M15	98.5	95.3	98.5	98.5	—																			
12M18	97.8	94.8	97.8	97.8	96.3	—																		
12M19	97.8	94.8	97.8	97.8	96.3	100	—																	
12M2	99.6	96.3	99.6	99.6	98.5	97.4	97.4	—																
12M8	99.1	96.6	99.1	99.1	98.1	97.0	97.0	99.6	—															
12M9	99.1	96.6	99.1	99.1	98.1	97.0	97.0	99.6	100	—														
12M14	67.4	66.4	67.4	67.4	66.9	67.4	67.4	67.8	68.0	68.0	—													
12M20	68.0	68.0	68.0	68.0	67.2	68.3	68.3	68.0	68.3	68.3	95.3	—												
12M16	88.6	87.7	88.6	88.6	87.5	87.5	87.5	88.6	89.0	89.0	69.2	69.9	—											
12M17	88.6	87.7	88.6	88.6	87.5	87.5	87.5	88.6	89.0	89.0	69.2	69.9	100	—										
12M7	80.6	81.5	80.6	80.6	81.1	78.9	78.9	80.6	80.6	80.6	75.1	73.4	80.4	80.4	—									
12M12	81.3	81.3	81.3	81.3	81.3	80.6	80.6	81.3	81.0	81.0	67.8	68.0	79.1	79.1	91.2	—								
Kibagata																								
K1	66.3	68.9	66.3	66.3	68.4	66.1	66.1	68.4	66.1	66.1	78.0	78.5	68.0	68.0	65.8	66.1	—							
K3	66.3	68.9	66.3	66.3	68.4	66.1	66.1	68.4	66.1	66.1	78.0	78.6	68.0	68.0	65.8	66.1	100	—						
K5	87.3	86.0	87.3	87.3	86.7	86.0	86.0	87.3	87.1	87.1	75.3	74.1	84.9	84.9	85.1	82.1	65.7	65.7	—					
K12	87.3	86.0	87.3	87.3	86.7	86.0	86.0	87.3	87.1	87.1	75.7	74.1	84.9	84.9	85.1	82.1	65.7	65.7	100	—				
K8	65.8	68.4	65.8	65.8	68.1	66.1	66.1	66.3	66.3	6.3	76.9	75.2	66.7	66.7	66.7	65.4	91.1	91.1	67.1	67.1	—			
K9	88.1	86.6	88.1	88.1	87.5	86.9	86.9	88.1	87.7	87.7	76.5	74.9	84.3	84.3	84.5	83.2	65.9	65.9	96.6	96.6	67.1	—		
K10	84.5	84.7	84.5	84.5	84.5	84.3	84.3	84.7	87.4	87.4	68.3	70.6	86.9	86.9	78.9	79.4	66.4	66.4	80.4	80.4	65.0	80.8	—	
K14	67.5	66.8	67.5	67.5	66.8	67.7	67.7	68.0	68.2	68.2	87.8	87.3	70.9	70.9	67.4	66.5	75.1	75.1	77.6	77.6	82.8	73.7	68.7	—

Discussion

Methylarsenic species would be produced by phytoplankton uptaking inorganic arsenic with phosphorus, according to previous field data[25–27] and laboratory experiments.[28–30] However, in Lake Kahokugata and Lake Kibagata, where chlorophyll *a* was present, methylarsenic species were not detected, while only inorganic arsenic was detected at the nM order in the two lakes. Some researches also reported that the densities of chlorophyll *a* did not relate to those of the methylarsenic species in marine[12] and freshwater environments.[13] In the present study, bacteria growing in the culture medium with DMAA as a carbon source were isolated from Lake Kahokugata and Lake Kibagata. Sunders demonstrated that DMAA added into environmental freshwater was decomposed by microorganism activities.[9] Accordingly, it was thought that bacteria metabolize the methylarsenic species to inorganic arsenic and decrease the methylarsenic species in aquatic environments.

It is known that organoarsenic decomposing bacteria metabolize DMAA,[9] arsenobetaine,[8] and monosodium methanearsonic acid.[31] Furthermore, methylarsenic decomposing bacteria would contribute to seasonal abundance changes of methylarsenic species in natural water.[11] However, the composition of organoarsenic decomposing bacteria has been veiled. The RFLP analysis of 16S rDNA sequences was used to classify various bacterial groups, such as celluloselytic bacteria,[15,16] bacterioplankton,[32] and nitrogen-fixation bacteria isolated from solids.[33,34] In the present study, the RFLP analysis of DMAA decomposing bacteria revealed that 27 isolates obtained from Lake Kahokugata and Lake Kibagata were classified into 12 types. Moreover, no RFLP type was common between Lake Kibagata and Lake Kahokugata, suggesting that the composition of DMAA decomposing bacteria would be specific for each lake. The classification of RFLP analysis was supported by a phylogenetic analysis using 16S rDNA partial sequences (Fig. 3). On the phylogenetic tree, the isolates belonged to one RFLP type that formed a distinct cluster, indicating sequence similarities of less than 91.2% except for the similarity between the AAAA type and the AABA type (Table 3). The present paper is the first report demonstrating that the DMAA decomposition in aquatic environments would depend on various bacterial populations, and that the bacterial composition would be specific for each aquatic environment. Moreover, an RFLP analysis using four endonuclease mixtures would be useful for classifying the bacterial types of decomposing DMAA in aquatic environments.

In Lake Kahokugata, 10 isolates having a total of 16 isolates belonged to type AABA or type AAAA, suggesting that some dominant bacteria decompose DMAA. The phylogenetic tree revealed that the dominant bacterial population in Lake Kahokugata was closely related to the bacteria belonging to genus *Pseudomonas*. However, in Lake Kibagata, no isolate belonged to genus *Pseudomonas*, and 8 isolates randomly formed clusters in three bacterial groups: alpha- and gamma-proteobacteria divisions and gram-positive low-G+C. These results suggested that DMAA decomposition can be ascribed to

Table 5 Most closely related bacteria for DMAA decomposing bacterial isolate obtained from Lake Kahokugata and Lake Kibagata

RFLP pattern group	Isolate	Relative bacteria	Accession No.	Similarity, %
AABA	12M3	*Pseudomonas putida*	D86003	100
	12M5	*Pseudomonas* sp. Ps 3-1	AF468453	99.6
	12M10	*Pseudomonas putida*	D86003	100
	12M11	*Pseudomonas putida*	D86003	100
	12M15	*Pseudomonas* sp. pfB13	AY336542	99.4
	12M18	*Pseudomonas* sp. NZ102	AY014820	100
	12M19	*Pseudomonas* sp. NZ102	AY014820	100
AAAA	12M2	*Pseudomonas* sp. MWH1	AJ556801	100
	12M8	*Pseudomonas* sp. NZ009	AY014802	100
	12M9	*Pseudomonas* sp. NZ009	AY014802	100
AAAD	12M14	Blackwater bioreactor bacterium	AF394174	100
	12M20	*Brevundimonas* sp. KIN166	AY136095	99.3
DAAE	12M16	*Aeromonas hydrophila*	AJ416907	100
	12M17	*Aeromonas hydrophila*	AJ416907	100
BAAB	12M7	*Acidovorax* sp.	AJ534865	100
CAAC	12M12	*Comamonas* sp.	AF532869	99.7
DBCF	K1	*Aureobacterium resistens*	Y14699	98.4
	K3	*Aureobacterium resistens*	Y14699	98.4
DAAG	K5	*Stenotrophomonas maltophilia*	AF417866	100
	K12	*Stenotrophomonas maltophilia*	AF417866	100
EACH	K8	*Arthrobacter* sp. M4 16S	AY177360	99.1
DAAI	K9	*Stenotrophomonas maltophilia*	AJ131117	100
DCAJ	K10	*Pantoea* sp. 77000	AF227846	100
AACK	K14	*Agrobacterium larrymoorei*	Z30542	100

Table 6 DMAA conversion activities of isolates obtained from Lake Kahokugata and Lake Kibagata

RFLP pattern group	Isolate	Inorganic arsenic concn./nM	MMAA concn./nM	Decomposition rate of DMAA, %
AABA	12M3	88.2	45.5	49.6
	12M5	34.5	103	49.3
	12M10	32.4	<10	33.8
	12M11	<10	<10	<10
	12M15	56.8	<10	68.2
	12M18	<10	62.5	27.3
	12M19	<10	62.5	16.7
AAAA	12M2	32.8	69.2	27.2
	12M8	50.4	91.3	<10
	12M9	41.2	87.5	<10
AAAD	12M14	38.7	<10	<10
	12M20	31.0	<10	<10
DAAE	12M16	58.8	62.5	14.3
	12M17	73.5	62.5	34.8
BAAB	12M7	47.1	<10	<10
CAAC	12M12	<10	<10	<10
DBCF	K1	33.0	193	40.2
	K3	44.4	234	62.2
DAAG	K5	60.8	<10	32.3
	K12	33.0	79.7	70.1
EACH	K8	63.0	193	60.4
DAAI	K9	63.0	<10	50.3
DCAJ	K10	<10	141	61.3
AACK	K14	<10	<10	<10

Concentrations of inorganic arsenic and MMAA, and decomposition rates of DMAA were measeured in culture medium incubated with the bacterial isolates and 1 μmol/l DMAA for two weeks.

different bacterial communities in Lake Kahokugata and Lake Kibagata. Therefore, the *Pseudomonas* dominant group contributed to the decomposition in Lake Kahokugata, whereas several bacterial groups indicating higher diversity contributed to the DMAA decomposition in Lake Kibagata. According to some field data, the abundances of DMAA were different among the water environments with no dependence on the phytoplankton activities.[12,13] Probably the DMAA decomposing bacteria, which are specific to each field, contribute to the methylarsenic cycles at some decomposing rates. The seasonal changes in the arsenic abundances in Lake Kahokugata and Lake Kibagata must be compared with each other for elucidating the relationship between the methylarsenic abundance and the phytoplankton activities.

The DMAA decomposition was remarkably observed in a culture medium of isolates belonging to the gram-positive low G+C group and gamma-proteobacteria. There are some reports that bacteria belonging to the gram-positive group decompose chemical compounds, such as antibiotics, and have a tolerance against antibiotics because of their thin cell walls.[35] However, methylated metal conversion to inorganic metal by gram-positive was the first to be reported. In gamma proteobacteria, type AABA and type AAAA, closely related to genus *Pseudomonas*, decreased the DMAA, and produced inorganic arsenic and MMAA in various degrees. Some of the bacteria belonging to genus *Pseudomonas* isolated from various environments are known to metabolize various chemical compounds.[36-39] Previous microbiological studies supported the hypothesis that the DMAA metabolism abilities would be naturally transmitted among the bacterial species of genus *Pseudomonas* by bacterial plasmids that coded the DMAA metabolism abilities.[40] Accordingly, the DMAA decomposition activities of the AABA type and the AAAA type would depend on the transformation of plasmids coding the DMAA decomposing enzymes. However, in both lakes, the isolates belonging to the alpha- and beta-proteobacteria divisions did not indicate sufficient activities to be detected by atomic absorbed spectrometry (AAS). In spite of undetectable decomposing activity, these isolates could grow in a culture medium including DMAA as the sole carbon source. Sunders suggested that microorganisms in natural water would decompose DMAA at a slow rate of approximately 1.1 ng/l/day.[9] Accordingly, the bacterial activities would be too low to be detected by the cold-trap AAS method used in the present study.

An RFLP analysis using four series of endonuclease was used to classify the DMAA decomposing bacterial isolates into bacterial divisions, which mostly reflect the activity of DMAA decomposition. However, in a dominant type, including many isolates, such as the AABA type, the decomposition rates demonstrated large changes in the activities. This result indicates that the RFLP analysis must be modified by the use of other endonuclease to establish the bacterial classification reflecting DMAA decomposing activities. The analysis using series 4 classified the isolates into smaller scale of RFLP pattern groups, compared to the use of series 1, series 2 and series 3. Additionally, classification using series 4 was identical to that using series 1 and series 2, but not that using series 3. Only series 3 among the four series could classify the AABA type and the AAAA type. Therefore, series 3 and series 4 will be useful for classifying DMAA decomposing bacteria isolated form freshwater environments.

In the future, by selecting endonuclease species used in the

RFLP analysis, the RFLP analysis will be applied to estimate the seasonal changes of the abundances and compositions of bacteria related to methylarsenic decomposition in freshwater environments. Moreover, the MPN method needs to be modified for detecting decomposition products, such as inorganic arsenic in the bacterial culture process, by applying colorimetric analysis. The combination of the MPN methods with the RFLP analysis would play important roles for elucidating of the bacterial contribution to the organoarsenic decomposing pathway in aquatic environments.

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