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Seasonal dynamics of dimethylarsinic-acid-decomposing bacteria dominating in Lake Kahokugata

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Decomposition processes of organoarsenic compounds significantly influence arsenic cycles in aquatic environments, and such processes depend on bacterial activity. However, the bacterial characteristics in these environments are obscure. Accordingly, we observed seasonal variations of arsenic species and the bacterial population decomposing dimethylarsinic acid (DMAA) in Lake Kahokugata from April 2002 to January 2003. Monitoring of bacterial biomass involving DMAA decomposition using the most probable number procedure showed that the bacterial cell densities ranged from 36 to 3600 ml^{-1}. On the other hand, methylated arsenic was not detected during the experimental period, although the inorganic arsenic concentration was over 4 nM. This suggests that bacteria remineralized methylated arsenic species to inorganic arsenic. Furthermore, the composition of bacterial communities involving DMAA decomposition was examined by restriction-fragment-length polymorphism analysis of the 16S rDNA nucleotide. As a result, a total of 49 isolates were classified into 10 type groups, and 32 of these isolates belonged to three dominant type groups. Phylogenetic analysis using 16S rDNA partial sequences (*ca* 320 bp) suggests that the representative isolates of the dominant type groups are specific to the summer or winter season. Moreover, as a result of the culture experiments to examine DMAA decomposition activity, the representative isolates decomposed 1 μM DMAA at a decomposition percentage of below 80%. In conclusion, some bacterial communities in a specific season can decompose DMAA to varying degrees, contributing to the annual cycle of arsenic species.

KEYWORDS: organoarsenic; dimethylarsinic acid; DMAA decomposition; bacterial population; MPN; RFLP

INTRODUCTION

Arsenic is widely distributed in aquatic environments in a variety of forms. In natural water, arsenate, arsenite and methylated arsenic compounds are found as the dominant arsenic species.[1,2] The cycles of the arsenic species depend on the bioactivities of microorganisms.[1,3] Arsenate-reducing or arsenite-oxidizing bacteria are known to contribute to the mobilization of arsenic from aquatic sediment into water.[4,5] In Bangladesh, arsenite released into the groundwater contaminated drinking water, causing serious water problems.[6] Arsenate is a chemical analogue of phosphate and may interfere with oxidative phosphorylation.[1] Accordingly, under phosphate-limited conditions, some microorganisms, such as phytoplankton and bacteria, accumulate dissolved arsenate through their phosphate-concentrating mechanisms.[7] Then, arsenate within the microorganism cells is reduced to arsenite or methylated to monomethylarsonic acid ($CH_3AsO(OH)_2$; MMAA(V)) and dimethylarsinic acid ($(CH_3)_2AsO(OH)$; DMAA(V)).[2] Next, the methylated arsenic compounds are converted to more complex organoarsenic compounds, such as arsenobetaine or arsenosugars.[2]

The decomposition of organoarsenic compounds is thought to depend mostly on bacterial activities influencing the arsenic cycles in aquatic environments. When marine bottom

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sediments were incubated with medium containing arsenobetaine originating from marine animals, marine bacteria in the sediments decomposed the arsenobetaine to trimethylarsine oxide.[8] Natural seawater, in which bacterial activity was induced by bacterial culture medium, caused decomposition of DMAA.[9] Some bacterial populations associated with marine animals, such as crabs[10] and a mussel,[11] were demonstrated to decompose arsenobetaine to dimethylarsinate. On the other hand, only two isolates were reported to convert organoarsenic to inorganic arsenic. An isolate obtained from sludge water, strain ASV2, decomposes arsenobetaine, metabolizing it as carbonic source,[12] and *Mycobacterium neoaurum* demethylates MMAA(V) to mixtures of arsenate and arsenite.[13] The ecophysiological characteristics of organoarsenic-decomposing bacteria have not been investigated in detail and the decomposition processes in aquatic environments are obscure.

The decomposition process of organoarsenic compounds cannot be neglected in considering field data of the arsenic cycle in aquatic environments.[14] Although phytoplanktons are known to produce organoarsenic compounds, the amounts of chlorophyll *a* often did not relate to the abundance of organoarsenic compounds in Tosa Bay[15] and Lake Biwa.[16] Sohrin *et al*.[16] suggested that the abundance of organoarsenic compounds would be underestimated by bacterial metabolizing activities for organoarsenic compounds. For correctly elucidating the decomposition mechanism of organoarsenic compounds in aquatic environments, the dynamics of the decomposing bacterial population should be monitored and the composition of the bacterial population needs to be investigated.

In this study, we focus on DMAA as a model organoarsenic compound, as it is widely distributed in freshwater,[16] and we examined the seasonal dynamics of DMAA-decomposing bacteria of Lake Kahokugata using the most probable number (MPN) procedure. The MPN procedure is a powerful tool for monitoring and isolating bacteria in aquatic environments. By combining the MPN procedure with the chemical reaction of arsenite and sulfate we could easily determine arsenate-reducing bacteria in the field.[17] Moreover, compositions of the bacterial communities were estimated using restriction-fragment-length polymorphism (RFLP) analysis of 16S rDNA sequences. RFLP analysis of 16S rDNA sequences has been widely used for classifying several bacterial populations, such as cellulolytic bacterial isolates,[18,19] infectious bacteria to plants,[20] and *Pseudomonas* strains.[21] We tried to elucidate the dynamics of bacterial communities related to the DMAA decomposition process in Lake Kahokugata.

EXPERIMENTAL

Sampling

Water samples were collected from the coast of Lake Kahokugata in Ishikawa Prefecture, Japan, once a month from May 2002 to January 2003, using 1 l polycarbonate vials. The water was brought to the laboratory and used in the experiments within 2 h. 50 ml water samples were used directly to determine the cell densities of DMAA-decomposing bacteria with the MPN procedure. For measurement of arsenic species and chlorophyll *a*, 50 ml water samples were filtered with a GF/C glass-fiber filter (Advantec, Tokyo, Japan). The concentrations of arsenic species in the filtrate were determined using the cold-trap hydride generation atomic absorption (HG-AA) speciation procedure. Chlorophyll *a* was extracted from the GF/C glass-fiber filter with acetone and assessed colorimetrically.[22]

Bacterial cell number determination using MPN procedure

Cell densities of bacteria involved in DMAA decomposition were determined using the MPN procedure with a modified CD minimal medium, which contained 10 μM DMAA as the sole carbon source instead of peptone. The medium components were $NaNO_3$ (2 g l^{-1}), K_2HPO_4 (1 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (0.5 g l^{-1}), KCl (0.5 g l^{-1}), $FeSO_4$, thiamine hydrochloride (0.005 g l^{-1}), and DMAA (0.214 mg l^{-1}). The bacteria grown in this culture medium metabolize DMAA as the sole carbon source and are defined as 'DMAA-decomposing bacteria'. 10 ml of the medium was dispensed to tubes used for the MPN method and autoclaved. Environmental samples were 10-fold serially diluted to 10^{-5} in sterile water. 10 ml of growth medium including DMAA were added to 10 ml aliquots of each dilution. At each dilution step from 10^{-1} to 10^{-5} dilution, 200 μl of diluted sample water were transferred to 24 wells in a 96-well microtiter plate, which was incubated at room temperature. The bacterial growth was evaluated after 14 days by turbidity based on visual inspection.

Bacterial classification using RFLP analysis

All isolates were classified by RFLP analysis of 16S rDNA sequences. The isolates cultivated in ST 10^{-1} culture medium overnight were pelleted by centrifugation at 2600 *g* for 15 min. The ST 10^{-1} culture medium is composed of 0.5 g l^{-1} peptone and 0.05 g l^{-1} yeast extract. The bacterial cell pellets were stored at −70 °C until required for experiments. The bacterial cell pellets were resuspended in 500 μl of TE buffer (10 mM Tris–HCl, pH 8.0; 1 mM EDTA), and lysed with 1 mg ml^{-1} sodium dodecyl sulfate, 0.1 mg ml^{-1} proteinase K, and 1 mg ml^{-1} lysozyme at 50 °C for 30 min. Genomic DNAs were purified by phenol–chloroform extraction, chloroform extraction and 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 polymerase chain reaction (PCR). Reaction mixtures (final volume 100 μl) contained 200 μM of deoxyribonucleoside triphosphates, 0.5 units of Ex Taq polymerase (Takara Bio Inc., Ohotsu, Japan), and 0.2 μM of each oligonucleotide primer, 27F and 1492R. These primers specifically bind to eubacterial 16S rDNA.[23] Genomic DNA

of bacteria was added at a final concentration of 1–10 ng μl^{-1}. 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, for a total of 30 cycles. 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 (Toyobo Co. Ltd, Osaka, Japan). The DNA fragments were separated in 1.5% w/v agarose gel-LE classic type (nacalai-tesque, Kyoto, Japan), depending on the fragment sizes to be distinguished. DNA molecular weight marker 100 bp ladders were used as standards with a size range of 100–2000 bp (Toyobo Co. Ltd, Osaka, Japan). The gels were stained with ethidium bromide and documented with a Polaroid camera DS-34M (Ultra Lum, Inc., CA, USA).

Sequencing of 16S rDNA and phylogenetic analysis

Representative isolates were selected from each dominant RFLP type group and their 16S rDNA partial sequences were determined. 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, chloroform extraction, followed by ethanol precipitation. Finally, the DNA fragments were resuspended in 10 μl of sterile distilled water. Partial sequences (*ca* 320 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 sequences determined were compared with a DDBJ database using BLASTA and FASTA SEARCH program.[24]

A phylogenetic tree including all isolates was constructed according to the neighbor-joining algorithmic method,[25] using the partial sequences of 16S rDNA. The nearly complete 16S rDNA sequences were compared with those available from the Ribosomal Database Project and EMBL/GenBank databases. The similarity values were based on a pairwise comparison of sequences. For phylogenetic analyses, the DNA sequences were aligned using the CLUSTAL W ver. 1.7 (European Bioinformatics Institute).[26] The trees were constructed using neighbor 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 DNADIST with a transition/transversion ratio of 2.0. The tree topologies were evaluated by bootstrap analysis of the neighbor-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* as an outgroup.

Measurements of arsenic species

The cold trap HG-AA speciation procedure was employed.[27,28] The water samples or bacterial cultures were filtered with a 0.45 μm nuclepore filter (Advantec, Tokyo, Japan). The filtrate was acidified and reacted with sodium tetrahydroborate and the arsines produced were swept by a flow of nitrogen into a cold trap. This trap was cooled by liquid nitrogen, before being gently warmed by electrical heating. The arsines, such as inorganic arsine, MMAA and DMAA, released into a quartz-T tube held at *ca* 900 °C in an electrically heated furnace, were monitored by a Z-8100 atomic absorption spectrometer (Hitachi, Chiba, Japan).

Detection of DMAA decomposition activity

The representative isolates were incubated in liquid ST 10^{-1} culture medium with 1 μM DMAA for about 1 week. For evaluation of methylarsenic decomposition activities, 1 ml of each isolate culture was inoculated into 19 ml of liquid ST 10^{-1} culture medium including 1 μM final concentrations of DMAA (Roth, Karlsruhe, Germany). After 1 and 2 weeks incubation, 5 ml of bacterial culture was applied to measurements of arsenic species as described. DMAA decreasing percentage was calculated by dividing the decreasing amounts of DMAA for 1 and 2 weeks by the initial amounts of DMAA.

RESULTS

Seasonal variation in Lake Kahokugata

The MPN method using culture medium including 1 μM DMAA as the sole carbon source was used to determine the abundance of DMAA-decomposing bacteria in Lake Kahokugata at bacterial cell densities from 36 and 3600 ml^{-1} during the experimental period (Fig. 1). The bacterial cell density increased from 74 ml to 1600 ml^{-1} between 10 May and 18 July, then decreased to 36 ml^{-1} by 11 September, and increased again to 3300 ml^{-1} until 20 November, then remaining or the order of 1000 ml^{-1} to 16 January. Chlorophyll *a* was also detected at amounts ranging from 3 to 58 $\mu g\ l^{-1}$, indicating high phytoplankton bioactivity during the experimental period. Inorganic arsenic concentrations in Lake Kahokugata ranged from 5 to 17 nM, showing particularly high values of over 14 nM between 7 August and 17 October. However, methylated arsenic species were not detected from water samples during the experimental period.

The bacterial culture in the highest dilution degree of wells, where dense bacteria were observed in MPN procedure counts, were spread on the agar ST 10^{-1} culture plates. 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. A total of 49 strains

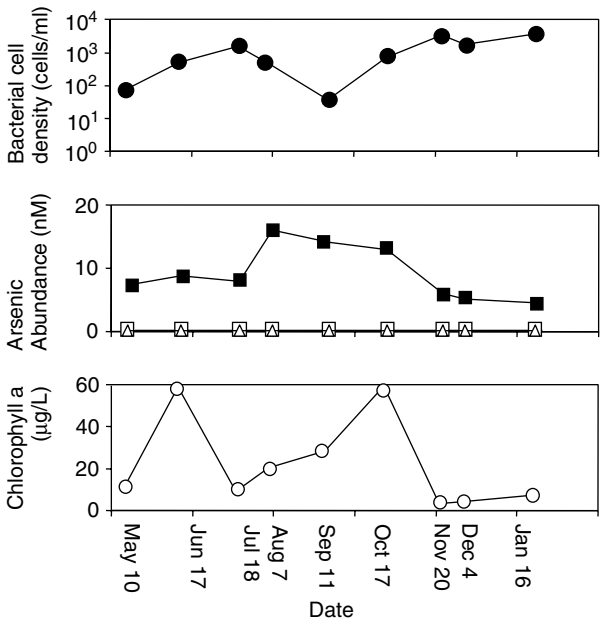

Figure 1. Seasonal variation in the cell density of DMAA-decomposing bacteria and the concentrations of arsenic species and chlorophyll *a*. Filled circles indicate the cell density of DMAA-decomposing bacteria estimated by the MPN procedure. Filled squares, open squares and open triangles indicate the abundance of inorganic arsenic, DMAA and MMAA respectively. Open circles show the amount of chlorophyll *a*.

were isolated during this experimental period; 5 to 16 isolates were obtained in each month.

Phylogenetic classification of DMAA-decomposing bacteria

The rDNA of 49 isolates could be amplified using primers 27F and 1492R, resulting in a characteristic single band of about 1450 bp. RFLP analysis of the PCR products of 49 isolates using three series of endonuclease mixture (series 1: *EcoR* I and *Hind* III; series 2: *BamH* I and *Sal* I; series 3: *Sac* I) revealed various restriction types (Table 1). Since the fragments under 100 bp did not indicate sufficiently sized bands on the agarose gel, the sizes of fragments under 100 bp were neglected for RFLP analysis. The PCR products of 49 isolates digested with series 1 indicated one to three bands on the gels, whereas those with series 2 or 3 displayed one and two bands. Ultimately, three to five restriction types per series were used for classification of isolates. Comparing all the restriction patterns of the 49 isolates resulted in 10 distinct RFLP type groups (Table 2). Among the total 49 isolates, 19 isolates belonged to the BAA type group, 14 isolates were in the AAC type group, and four isolates were in the BBA type group. Although the AAA type group included seven isolates, the restriction types of this group showed only a single band, *ca* 1450 bp, which was not digested with any endonuclease mixture. Comparison of the 16S rDNA sequences demonstrated that the seven isolates of the AAA type group are not identical with each other (date not shown). Therefore, the three type groups BAA, AAC and BBA were regarded as the dominant RFLP type groups.

Table 1. Band sizes within RFLP type for DMAA-decomposing bacterial isolates obtained from Lake Kahokugata

Endonuclease mixture	RFLP type	Size of restriction fragment (bp)			
Series 1	A	1500			
EcoR I, *Hind* III	B	800	700		
	C	750	550	<100	
	D	850	400	150	<100
	E	850	400	250	
Series 2	A	1500			
BamH I, *Sal* I	B	800	700		
	C	1200	300		
Series 3	A	1500			
Sac I	B	1200	300		
	C	800	650	<100	

Table 2. RFLP types of amplified 16S rDNA of DMAA-decomposing bacterial isolates

	RFLP type of amplified 16S rDNA digested with[b]		
Isolate[a]	*EcoR* I, *Hind* III	*BamH* I, *Sal* I	*Sac* I
7M1, 7M2, 7M3, 7M4, 7M5, 7M7, 8M2, 8M3, 8M5, 9M2, 9M3, 10M1, 10M2, 10M4, 10M6, 11M5, 11M11	B	A	A
10M5, 11M1, 11M3, 11M6, 11M9, 11M10, 12M3, 12M5, 12M10, 12M11, 12M15, 12M18, 12M19	A	A	C
7M6, 8M6, 9M1, 9M7	B	B	A
8M4	B	C	A
10M3	B	A	B
11M2	A	B	C
11M4	C	B	A
12M7	D	A	A
12M12	E	A	A
9M5, 11M7, 12M2, 12M8, 12M9, 12M14, 12M20	A	A	A

[a] The isolates of 7M, 8M, 9M, 10M, 11M and 12M were obtained on 18 July, 17 October, 20 November and 20 December respectively.
[b] Different restriction patterns a indicated with different letters of each endonuclease used.

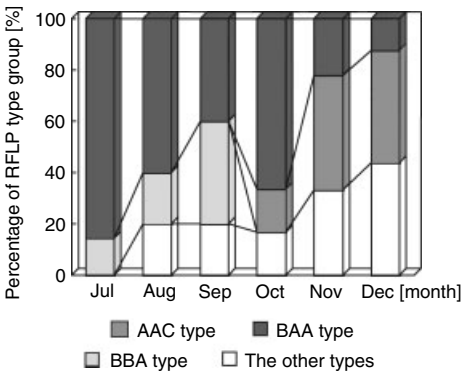

Figure 2. Seasonal change in percentage of each RFLP type groups.

The occupation rates of the three RFLP type groups to total isolates showed seasonal fluctuations in Lake Kahokugata (Fig. 2). The BAA type group constantly occupied 12 to 86% of total isolates during the experimental period. In particular, in July, August and October the BAA type group dominated, sharing more than 60% of total isolates. On the other hand, the isolates belonging to the BBA and AAC type groups were specific to the summer season and winter season respectively. The rates of the BBA type group to total isolates increased from 14 to 40% during the summer season between July and September. After disappearance of the BBA type group, the AAC type group appeared at 17% in October and increased to 44% in November, maintaining the rate of 44% until December.

The 16S rDNA partial sequences (*ca* 320 bp) of 10 representative isolates (six isolates for the BAA type group, two isolates for the BBA type group and two isolates for the AAC type group) were determined. Comparing the partial sequences determined indicated that the BBA type or AAC type group isolates were very similar to each other at over 99.4%, whereas the isolates of the BAA type group showed a wide range of similarities, from 74.1 to 100% (Table 3). The results of the phylogenetic analysis using the partial 16S rDNA sequences showed that the isolates of the BBA or AAC type group formed a distinct cluster (Fig. 3). The two isolates (8M6 and 9M7) of the BBA type group made a single cluster in the alpha-proteobacteria, indicating low similarities of below 97.0% with known bacteria such as *Ensifer adhaerens* and *Ochrobactrum* sp. The two isolates (11M3 and 12M3) of the AAC type group were closely related to the genus *Pseudomonas* with high similarities of over 99.1%. On the other hand, the six isolates of the BAA type group made five clusters. The two clusters including 8M2 and 9M3 belonged to the Gram-positive bacteria group and were placed in the genus *Bacillus*. In the two clusters placed in the gamma-proteobacteria, 12M16 related to *Aeromonas hydrophila* and 11M5 to *Pseudomonas putida*. The other cluster including two isolates, 7M5 and 10M1, was placed in the genus *Comamonas* in the beta-proteobacteria. Furthermore, the clusters of some representative isolates differed between summer season and winter season. The isolates obtained in August and September (8M6, 9M7, 8M2 and 9M3) were classified into the genus *Bacillus* or alpha-proteobacteria, whereas the isolates obtained in November and December (11M3, 11M5, 12M3 and 12M16) were in the gamma-proteobacteria. In contrast, the two isolates obtained from different seasons, such as July and October (7M5 and 10M1 respectively), formed the same cluster as the genus *Comamonas*.

DMAA decomposition activity

The representative 10 isolates of RFLP type groups were assayed for *in vitro* DMAA decomposition activity using ST 10^{-1} culture medium with 1 μM DMAA (Table 4). When one isolate (12M16) of BAA type group was incubated in the culture medium with DMAA, the DMAA decreased remarkably at a decomposition percentage of 78% after 2 weeks, and the inorganic arsenic concentration increased to 810 nM in the second week. In the cultures of the 7M5 and 10M1 isolates of the BAA type group after 2 weeks

Table 3. Similarities of 16S rDNA partial sequences (*ca* 320 bp) among representative isolates of dominant RFLP type groups

	Similarity (%)									
	BAA						BBA		AAC	
Isolates	7M5	8M2	9M3	10M1	11M5	12M16	9M7	8M6	11M3	12M3
7M5	—									
8M2	70.8	—								
9M3	74.1	87.2	—							
10M1	100	70.8	74.1	—						
11M5	83.5	74.4	76.5	83.5	—					
12M16	81.0	72.3	73.8	81.0	89.6	—				
8M6	74.9	70.3	71.3	74.9	78.0	78.3	—			
9M7	74.9	70.3	71.3	74.9	78.0	78.3	100	—		
11M3	82.6	74.4	76.8	82.6	97.9	90.5	78.9	78.9	—	
12M3	82.6	75.0	76.8	82.6	98.5	90.5	79.2	79.2	99.4	—

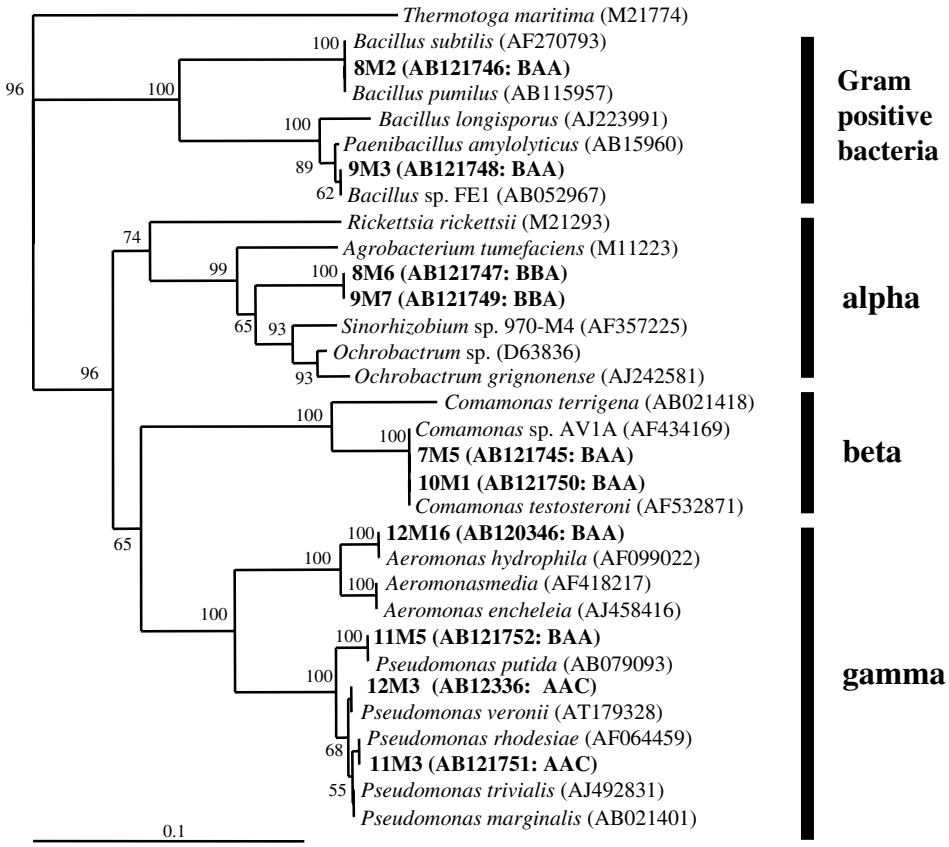

Figure 3. Phylogenetic tree of the representative isolates belonging to the dominant RFLP type groups and reference bacteria. The tree was calculated from a dissimilarity matrix of *ca* 320 bp alignment using a neighbor-joining algorithm. Bootstrap values larger than 50% (after 1000 resamplings) are indicated on the branch. The accession number of each reference sequence is also given.

Table 4. DMAA-decomposing activities of isolates obtained from Lake Kahokugata. The concentrations of inorganic arsenic and MMAA, and the decomposition percentages of DMAA were measured in culture medium incubated with the bacterial isolates and 1 μM DMAA in the first week and the second week

		Concentration (nM)					
		Inorganic arsenic		MMAA		Decomposing percentage of	
RFLP type	Isolate	1st week	2nd week	1st week	2nd week	1st week	2nd week
BAA	7M5	83	91	27	25	15	26
	8M2	25	28	<10	<10	<10	16
	9M3	<10	20	<10	<10	<10	<10
	10M1	57	110	37	33	<10	34
	11M5	<10	13	<10	<10	<10	<10
	12M16	520	810	61	19	69	78
BBA	8M6	17	28	<10	<10	<10	<10
	9M7	<10	14	<10	<10	<10	<10
AAC	11M3	<10	71	<10	<10	<10	<10
	12M3	50	71	<10	<10	<10	<10

incubation, inorganic arsenic was detected at concentrations of 91 nM and 110 nM respectively, and the DMAA decreased to 26% and 34% respectively. The 8M2, 9M3 and 11M5 isolates of the BAA type group indicated a low concentration of inorganic arsenic below 30 nM and low percentages of DMAA decrease below 20% after 2 weeks incubation. The 8M6 and 9M7 isolates of the BBA type group also showed low inorganic arsenic concentrations of 28 nM and 14 nM respectively and low DMAA decomposition percentages below the level of detection. In the culture of the two isolates 11M3 and 12M3 of the AAC type group, the inorganic arsenic concentration increased to 71 nM, but the DMAA decomposition percentages were below the level of detection. The MMAA concentrations showed high values in the culture of 7M5, 10M1, and 12M16 with 27 nM, 37 nM and 61 nM in the first week respectively, then tended to decrease to the second week. Additionally, all the isolates grew in the culture medium that included DMAA as the sole carbon source.

DISCUSSION

Methylarsenic species can be synthesized by phytoplankton, which accumulate inorganic arsenic as an alternative to phosphorus, according to previous field data[29–31] and laboratory experiments.[32–34] In Lake Kahokugata, the amount of chlorophyll *a* in the water samples ranged from 3 to 58 $\mu g\ l^{-1}$, which is indicative phytoplanktons activity (Fig. 1). Methylarsenic species were not detected; only inorganic arsenic was detected, in the range from 4 to 15 nM. Some researchers also reported that the densities of chlorophyll *a* did not relate to those of methylarsenic species in marine[15] and freshwater environments.[16] In this study, the MPN method using the culture medium with DMAA as the carbon source revealed that the DMAA-metabolizing and -decomposing bacteria would be present at cell densities from 36 to 3600 ml^{-1}. In a previous study, DMAA added into environmental freshwater was decomposed by microorganism activities.[9] Because of bacterial conversion of methylarsenic species to inorganic arsenic, methylarsenic species can disappear in Lake Kahokugata. Among the 10 representative isolates, some isolates, such as 7M5, 10M1 and 12M16, belonging to the BAA type group significantly decomposed DMAA to inorganic arsenic, but the other isolates belonging to the dominant RFLP type groups showed low activities of DMAA decomposition (Table 4). Sanders[9] suggested that microorganisms in natural water would decompose DMAA at a slow rate of approximately 1.1 $ng\ l^{-1}\ day^{-1}$. Presumably, several bacterial populations, which decompose methylarsenic species to various degrees, contribute to the arsenic cycle and in total display low DMAA decomposition percentages in aquatic environments.

The RFLP analysis focusing on 16S rDNA sequences of DMAA-decomposing bacteria revealed that a total of 49 isolates obtained from Lake Kahokugata were classified into 10 type groups (Table 2). Each of the AAC, BBA or BAA type groups includes a number of isolates, which sequentially appeared during some months. This fact suggests that the DMAA-decomposing bacterial population is composed of various bacterial species, and that the bacteria belonging to the three RFLP type groups mainly contribute to DMAA decomposition. Moreover, the isolates belonging to the AAC and BBA type groups were obtained in the summer season (August and September) and in the winter season (November and December) respectively, and the representative isolates of each groups type formed a distinct cluster on the phylogenetic tree (Fig. 3). Although the isolates of the BAA type group were observed during all seasons from July to December (Fig. 2), the representative isolates were placed in five clusters; the isolates obtained in the summer season were related to the genus *Bacillus* and those obtained in the winter season belonged to the gamma-proteobacteria. Perhaps some populations of the DMAA decomposing bacteria mostly are classified to the summer type or the winter type. On the other hand, the two isolates belonging to the genus *Comamonas* indicated that some bacterial populations could appear in random seasons. Methylarsenic decomposition percentages depending on bacterial activities were reported to show seasonal changes of methylarsenic species in natural waters.[11] The dominant bacterial population detected in this study possibly controls the seasonal cycle between inorganic arsenic and methylarsenic species.

The representative isolates of the BBA type group clustered strongly with known bacteria belonging to alpha-proteobacteria with low similarities of under 97.0% (Fig. 3), indicating that the isolates are novel bacteria. The isolates of the AAC type group, however, belong to the genus *Pseudomonas* on the phylogenetic tree. The isolates of the two group types can grow in a culture medium that includes DMAA as the sole carbon source, but the isolates indicated low activities of DMAA decomposition in the culture experiments (Table 4). Since we used the ST 10^{-1} culture medium, which includes other carbon sources to induce the bacterial growth, perhaps the isolates mainly utilized peptone and yeast extract.

The six representative isolates of the BAA type group formed some clusters (Fig. 3). Two isolates were identical to *Comamonas* sp. AV1A in the beta-proteobacteria, and another two isolates belonged to the genus *Bacillus*. *Comamonas* sp. AV1A[35] and some bacterial species of the genus *Bacillus*[36] are known to decompose plant compounds. Presumably, the bacterial population belonging to the genus *Bacillus* or the genus *Comamonas* decompose the organoarsenic compounds originating from phytoplanktons and macroalgae.[37] The other two isolates of the BAA type group, 12M16 and 11M5, placed in the gamma-proteobacteria, were identical to *A. hydrophila* and *P. putida* respectively with 100% similarities. This study is the first report describing that two bacterial species contribute to decomposition of organometals. Most isolates of the BAA type group showed low DMAA decomposition percentages of under 10%, but the 7M5 and 10M1 isolates of the genus *Comamonas* and the 12M16

isolate identified as *A. hydrophila* remarkably decomposed DMAA at high percentages of 26%, 34% and 78% respectively (Table 4). Moreover, these isolates produced inorganic arsenic and MMAA with higher concentrations than the other representative isolates for 2 weeks (Table 4). These facts support the possibility that the bacterial populations of the genus *Comamonas* and *A hydrophila* are the main contributors to the DMAA decomposition and the arsenic cycles in Lake Kahokugata. Furthermore, the bacteria might decompose DMAA to inorganic arsenic via MMAA.

The RFLP analysis of 16S rDNA sequences has been used to classify various environmental bacterial populations into some groups, such as cellulolytic bacteria,[18,19] bacterioplankton,[38] and nitrogen-fixation-bacteria-isolated solids.[39,40] Moreover seasonal dynamics in the *Vibrio* population could be investigated using RFLP analysis.[41] On the phylogenetic tree, the representative isolates of the BBA or AAC type groups each form a single cluster relating to genus *Comamonas* or genus *Pseudomonas* respectively, whereas those of the BAA type group did not relate to each other and formed five clusters (Fig. 3). Furthermore, the culture experiments revealed that the DMAA decomposition percentages of the BAA and AAC type groups were represented by low values of under 10%, whereas those of the BAA type group showed the wide ranges from under 10 to 78%. To establish a more detailed classification reflecting the phylogenetic classification and the DMAA decomposition activities, RFLP analysis has to be improved by using other endonuclease species.

In the future, the use of other endonuclease species will be used with RFLP analysis to estimate the seasonal change of bacterial composition in relation to methylarsenic decomposition processes in freshwater environments. Moreover the possible application of colorimetric analysis in the MPN procedure for detection of inorganic arsenic[17] will allow easier monitoring of DMAA decomposing bacteria. A combination of the MPN procedure and RFLP analysis would be helpful in elucidating the bacterial contribution to the organoarsenic decomposition pathway in aquatic environments.

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