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Full Paper

Determination of microcystin-LR degrading gene *mlrA* in biofilms at a biological drinking water treatment facility

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Abstract: Biological treatment methods used in water purification plants help degrade the cyanotoxin microcystin. The microcystin degraders in the aggregated microbial communities of biofilms in biological treatment facilities have not been previously investigated by molecular analysis. In the present study, the bacteria relevant to microcystin degradation and the gene encoding the microcystin-degrading enzyme MlrA were investigated in the biofilms of a biological drinking water treatment facility. Based on phylogenetic analyses of the 16S rRNA genes and *mlrA*, microcystin-degrading bacterial species were present in these biofilms and these bacteria possessed *mlrA* homologues. The deduced MlrA amino acid sequences had high sequence homology with previously reported MlrA enzymes. Non-culturable microcystin-degrading bacteria were found in the biofilms throughout the sampling period. This biological water treatment seems be a suitable method for the removal of microcystin by microcystin-degrading bacteria.

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Keywords: biofilm, biological potable water treatment plant, microcystin-degrading bacteria, microcystin

INTRODUCTION

In recent years, many drinking water reservoirs have experienced problems with cyanotoxins, especially microcystin (MC) and cylindrospermopsin, from toxic cyanobacterial blooms [1, 2]. It has also been predicted that climate change and global warming are likely to increase the occurrence and intensity of toxic cyanobacterial blooms [3]. Toxic cyanobacterial blooms consisting of organisms from the genera *Microcystis*, *Anabaena*, *Planktothrix* and *Nostoc* can occur in lakes and reservoirs. These cyanobacteria produce microcystins (MCs), potent hepatotoxic compounds with cyclic heptapeptides, which can kill wild and domestic animals as well as humans. MC contamination of treated water resulted in the death of hemodialysis patients in Caruaru, Brazil in 1996 [4]. MCs inhibit serine/threonine phosphatases 1 and 2A leading to tumour-promoting activity [5]. The main structure of MCs is cyclo(-D-Ala-L-X-D-MeAsp-L-Y-Adda-D-Glu-Mdha-), where X and Y are variable amino acids called MC-XY, Adda is (2*S*,3*S*,8*S*,9*S*)-2-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid, and Mdha is *N*-methyldehydroalanine [6]. Due to the toxicity of MCs, a regulatory guideline limit for MC-LR, the most common toxic MC found in potable water, of 1.0 μg/L has been set by the World Health Organisation [7]. MC-producing cyanobacterial blooms that occur after surface water storage in water facilities are the main concern for water supply safety. Conventional drinking water treatment processes do not effectively remove dissolved or extracellular MCs [8-10] because they are stable in water [11-14]. More advanced treatment methods, such as activated carbon absorption or ozonation and oxidation applications, have been used to remove MC from drinking water [15-17]. However, these methods are expensive in terms of development and operation, and their removal efficiency is often diminished by the presence of natural organic matter [16-18].

Biological treatment methods for water purification have numerous benefits. These methods are generally low technology processes that remove micropollutants, improve treatment processes, enhance the taste and odour, and reduce the chlorine demand of treated water [19]. Biological removal of MCs is now recognised as an alternative treatment method [20-25]. Biofilms that develop in the biological water treatment facility of water purification plants have been shown to effectively degrade MCs [24]. Although there are many reports on the isolation of MC-degrading bacteria from natural lakes, reservoirs and drinking water at purification plants [25-36], little is known about the bacterial communities coexisting with the MC-degrading bacteria in the biofilms on sand filters or biofilters at water purification plants [20-22]. In addition, little is known about the mechanisms underlying MC degradation and the genetic make-up of the MC-degrading bacterial isolates from actual biofilms [21, 25, 26]. Consequently, there is little data linking bacterial isolates and the microbial communities found in biofilms that would facilitate a better understanding of MC degradation for public health purposes. In some MC-degrading bacteria, the *mlr* gene cluster is known to encode an enzyme involved in MC degradation [37]. MlrA is the enzyme that catalyses the initial hydrolytic cleavage of the cyclic MC structure [37]. There have been few reports of *mlrA*

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homologues in biofilms at water purification plants [23, 38, 39]. The genetic information related to MC biodegradation and the microbial community is therefore very limited. MC-degrading bacteria are known to be present in biofilms throughout the year [39] and can survive the winter season. So they are likely to grow during toxic cyanobacterial blooms and degrade MCs [23].

The objective of the present study is to obtain evidence for and homologues of the MC-degrading gene *mlrA* in biofilms at a drinking water treatment facility. Any seasonal changes in the bacterial community that may affect MC degradation by biofilms in an actual drinking water treatment facility are also investigated.

MATERIALS AND METHODS

Biological Treatment Facility

The biological treatment facility and the honeycomb tube, a carrier made of polyvinyl chloride (0.1-mm thick), were described previously [19]. A water purification plant equipped with this biological treatment facility, which provides a maximum water supply quantity of 160,000 $m^3 \bullet day^{-1}$, is located near Lake Kasumigaura, Japan. The experiment was conducted from September 2006 to March 2007. Samples were obtained once every 2 months.

DNA Extraction, Sequencing of 16S rRNA Genes and the *mlrA* Gene, and Phylogenetic Analysis

Total DNA was extracted using ISOIL for Bead Beating (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. A phylogenetic tree was constructed using 16S rRNA gene and *mlrA* homologue sequences. A polymerase chain reaction (PCR) was performed using *Ex Taq* DNA polymerase (Takara Bio, Japan) and 27F-1492R primer set for the 16S rRNA gene and primers MF (5'-GACCCGATGTTCAAGATGCT-3') and MR (5'-CTCCTCCCACAAATCA GGAC-3') for the *mlrA* gene [40]. TA cloning was performed using the TOPO TA Cloning Kit (Invitrogen, USA) before sequencing. Restriction enzyme analysis was done using *Eco*RI, *Eco*RV and *Sma*I (Nippon Gene, Japan) for the 16S rRNA gene and *Hae*III for the *mlrA* gene. Two hundred clones were selected for 16S rRNA gene sequence analysis and all clones were selected for *mlrA* sequence analysis. Plasmid DNA was extracted using the Perfectprep Plasmid 96 Vac Direct Bind Kit (Eppendorf, Germany). The Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) was used for sequencing with T7 or T3 primers for the 16S rRNA gene and MF or MR primer for the *mlrA* homologues [40].

The obtained 16S rRNA genes and *mlrA* homologues sequences were used in BLAST searches [41] of GenBank [42]. The 16S rRNA and *mlrA* sequences of related species obtained from the BLAST searches were aligned using Clustal X version 2.0 [43] to produce a dendrogram. The data were subjected to a neighbour-joining (NJ) analysis with 1,000 bootstrap replicates using NJ plot [44].

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Microcystin Analysis

The MCs MC-LR, MC-RR and MC-YR were analysed by HPLC as previously reported [32]. Dissolved MCs in bodies of water were analysed after filtration using a GF/C filter (Whatman, Japan).

RESULTS AND DISCUSSION

Water purification plants around the world must deal with toxic cyanobacterial blooms, and MC-producing cyanobacterial blooms in particular are a serious public health risk. Recently, biological treatment methods have been developed for use in water purification plants because these methods are low technology, low cost and have a low environmental burden [19, 25]. Previous reports [20, 23] on biological water treatment methods have suggested that MlrA is the enzyme that degrades MCs on biologically active sand filters equipped with an experimental water treatment apparatus and on a honeycomb tube as a carrier similar to that in the facility investigated in the present study. Moreover, the *mlrA* gene levels are positively correlated with MC degradative activity [23]. At the biological treatment facility investigated in the present study, these MC-degrading bacteria are present in the biofilms throughout the year [23, 39]. However, little is known about the MC-degrading genes, specifically the *mlrA* homologues, within this bacterial community.

To understand the bacterial community profile involved in MC-degradation, we analysed the 16S rRNA and *mlrA* genes *in situ* using a phylogenetic tree. The phyla and classes of bacteria detected are shown in Table 1 (Planctomycetes, Verrucomicrobia, Cyanobacteria, Firmicutes, Bacteroides, Nitrospira, α-Proteobacteria, β-Proteobacteria and γ-Proteobacteria). Over 80% of these bacteria were detected in every sampling during the study period, which indicated that these bacteria could grow at low water temperatures and in alkaline conditions. The abundance of bacteria in these phyla and classes varied depending on the water temperature and alkalinity in March 2007. Acidobacteria and ε-Proteobacteria might be particularly sensitive to water temperature, and Actinobacteria might be sensitive to alkaline conditions. MC-degrading bacteria were found in the genera *Sphingomonas* [29, 34, 45], *Novosphingobium* [25], *Sphingopyxis* [26, 32], *Paucibacter* [33] and *Burkholderia* [46]. Only *Sphingomonas* spp. were detected every month (Figures 2 and 3). *Novosphingobium* spp. were detected in September and January (Figure 2). *Paucibacter* spp. were detected in November (Figure 3). *Burkholderia* spp. were detected in January and March (Figures 2 and 3). *Sphingopyxis* spp. were not detected every month (Figures 2 and 3). Several gram-positive MC-degrading bacteria, *Rhodococcus* spp., *Arthrobacter* spp. and *Brevibacterium* spp., were recently reported [30]; however, these bacteria were not detected every month (data not shown). These results suggest that *Sphingomonas* spp. can grow at low water temperatures and in alkaline conditions (Figure 1), whereas other species are strongly affected by environmental fluctuation. Therefore, *Sphingomonas* spp. and related species growing in biofilms could degrade MC throughout the year. Most of the species involved in MC degradation, such as *Novosphingobium* spp. and *Paucibacter* spp., degraded MC only after the appearance of a toxic cyanobacterial bloom. Although the water temperature was low, some bacteria that could degrade

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MCs, such as *Burkholderia* spp. and *Novosphingobium* spp., were present. Therefore, MCs could be degraded throughout the year due to the presence of these bacteria in the biofilms [23, 24].

Table 1. Bacterial diversity (phyla and classes) in water treatment plant biofilms at various sampling times as determined by 16S rRNA gene sequencing

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
September 2006	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
November 2006	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+
January 2007	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-
March 2007	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-

Note:
Number of phyla and selected clones/total clones are as follows; September, 14, 127/661; November, 11, 92/682; January 9, 83/634; and March 7, 89/715.
A: phylum Nitrospira, B: phylum Firmicutes, C: phylum Bacteroidetes, D: phylum Planctomycetes, E: phylum Cyanobacteria, F: phylum Verrucomicrobia, G: class α-proteobacteria, H: class β-proteobacteria, I: class γ-proteobacteria, J: δ-proteobacteria, K: phylum Actinobacteria, L: phylum Acidobacteria, M: ε-proteobacteria, N: phylum Chloroflexi, O: phylum Chlamydiae, P: phylum Lentisphaerae, Q: phylum Fibrobacteres, R: phylum Chlorobi, S: phylum Spirochaetes

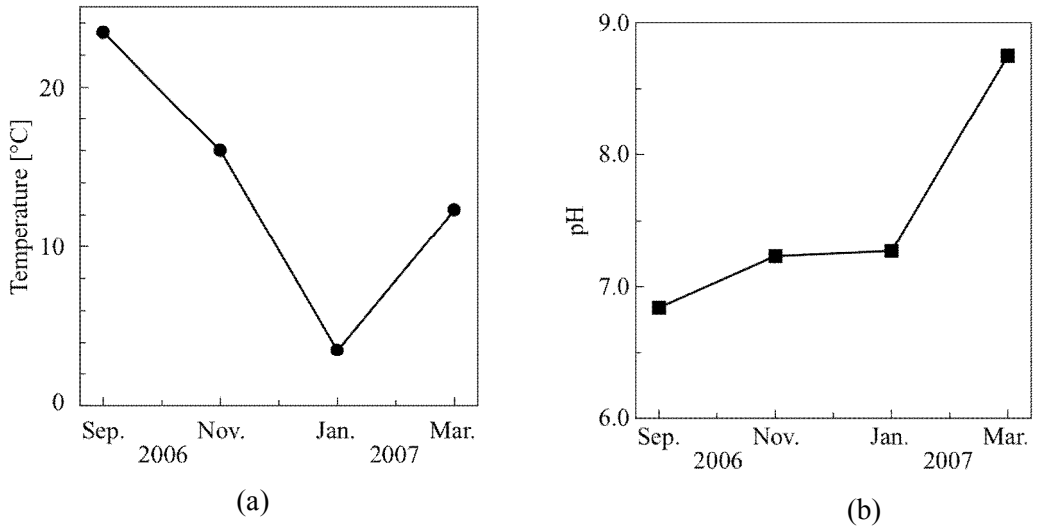

Figure 1. Environmental conditions at sampling times: (a) water temperature; (b) pH

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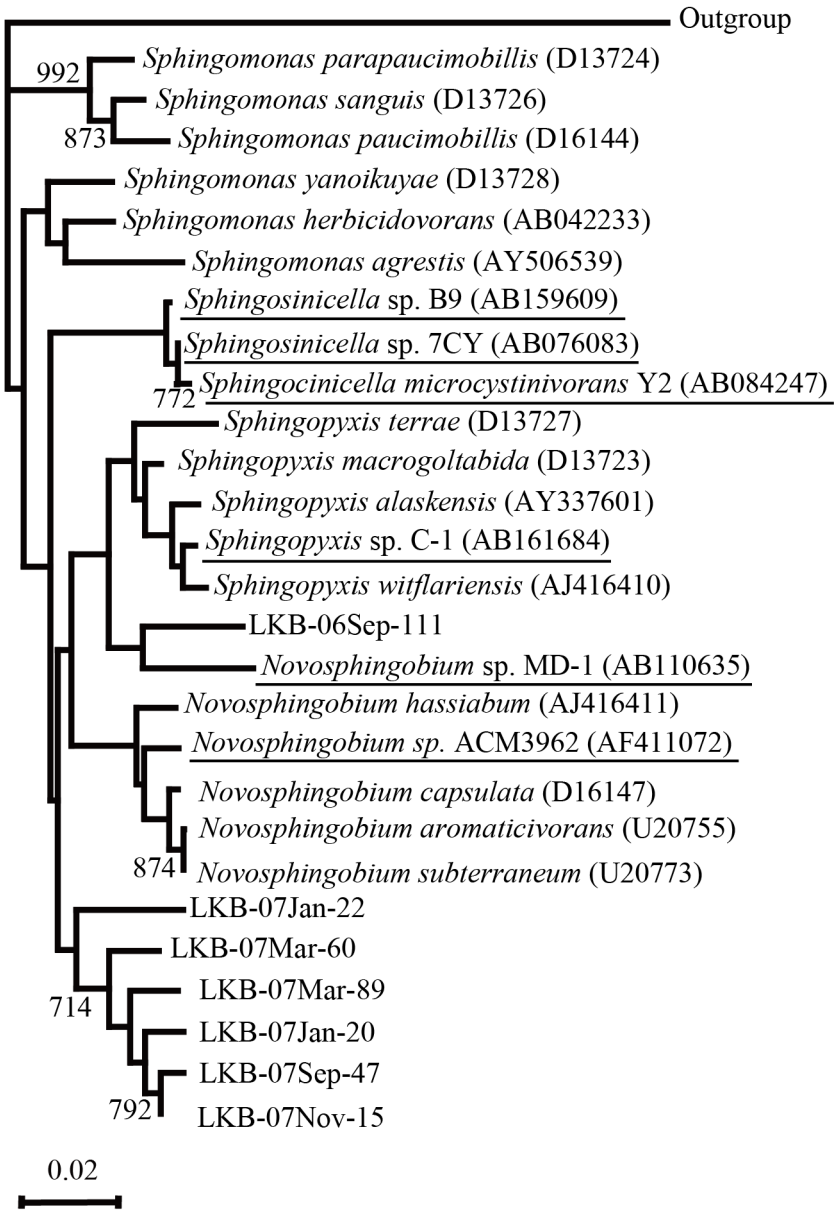

Figure 2. Phylogenetic tree of 16S rRNA gene sequences focused on the Sphingomonadales using the neighbour joining method. Numerical values shown are the bootstrap values. The scale bar represents the expected changes per site. The number on the scale bar shows evolutionary distance. MC-degrading bacteria are underlined. The sample number is the month-clone number; for example, 3-69 means clone number 69 isolated in March.

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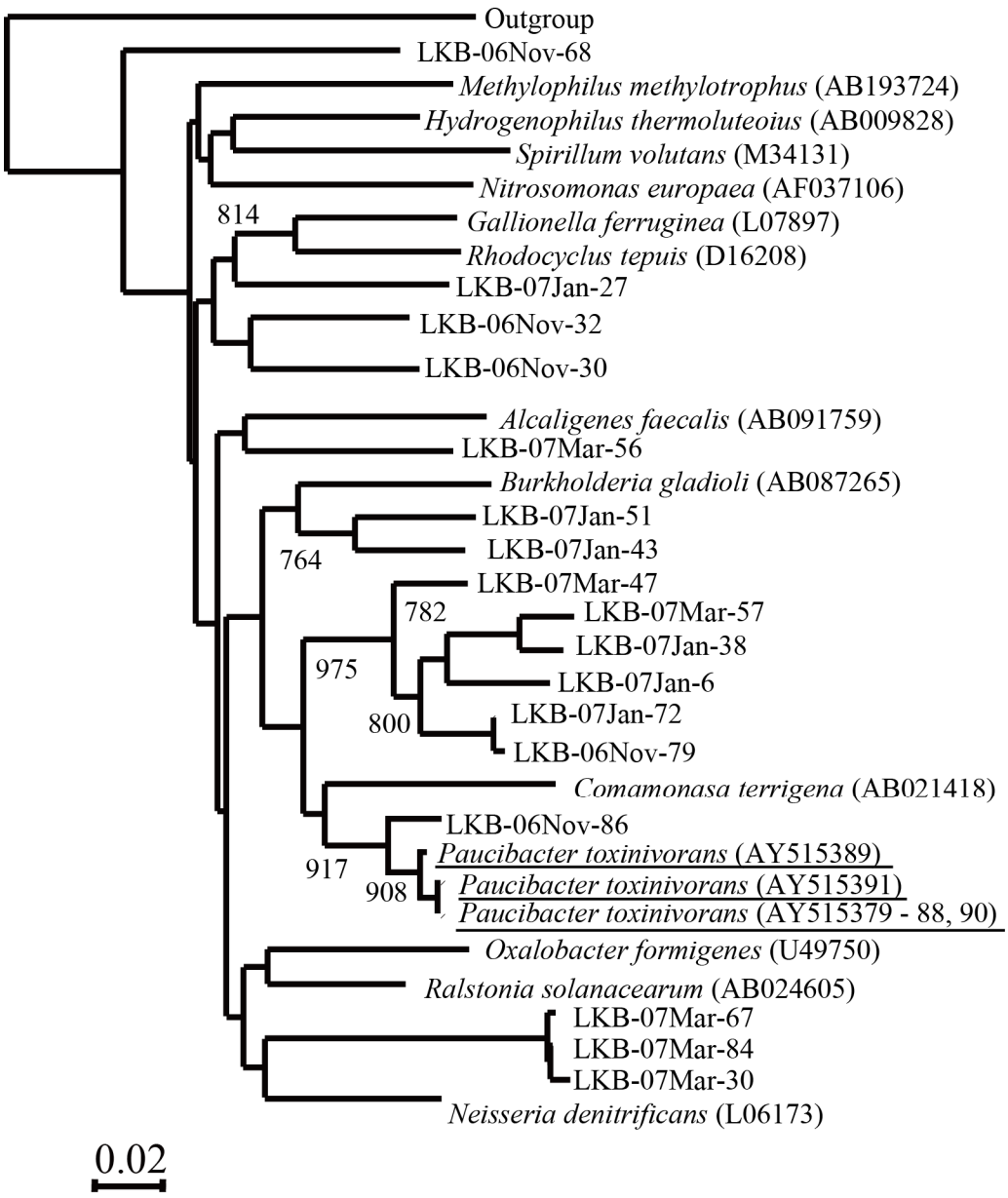

Figure 3. Phylogenetic tree of 16S rRNA gene sequences focused on the Burkholderiales using the neighbour joining method. Numerical values shown are the bootstrap values. The scale bar represents the expected changes per site. The number on the scale bar shows evolutionary distance. MC-degrading bacteria are underlined. The sample number is the month-clone number; for example, 3-69 means clone number 69 isolated in March.

To obtain evidence of MC-degrading bacteria in the biofilms, the *mlrA* homologues were analysed. Figure 4 shows that the biofilms had *mlrA* homologues that differ from those previously reported [26, 32, 37, 40]. Five clones with *mlrA* homologues were detected every month. The *mlrA* sequences from the bacteria in the biofilms were conserved because the identity of the isolated *mlrA* genes with that of *mlrA*-MD-1 was approximately 90%.

The deduced amino acid sequences of the *mlrA* genes were compared with the sequence of MD-1, a MC-degrading bacterium isolated from Lake Kasumigaura in Japan [40]. These sequences were highly conserved. Therefore, we believe that either there might have been a few MlrA

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homologues in Lake Kasumigaura or that MlrA amino acid sequences are highly conserved. Even though there are over 89 MC analogues, due to their highly conserved structure such as the Adda-Arg motif, MlrA hydrolysis can degrade numerous MC variants [37, 47, 48]. Therefore, *mlrA* is a biomarker for the MC-degrading ability of a population of bacteria in a biofilm. A quantitative PCR system using *mlrA* as a biomarker has been developed [38, 39].

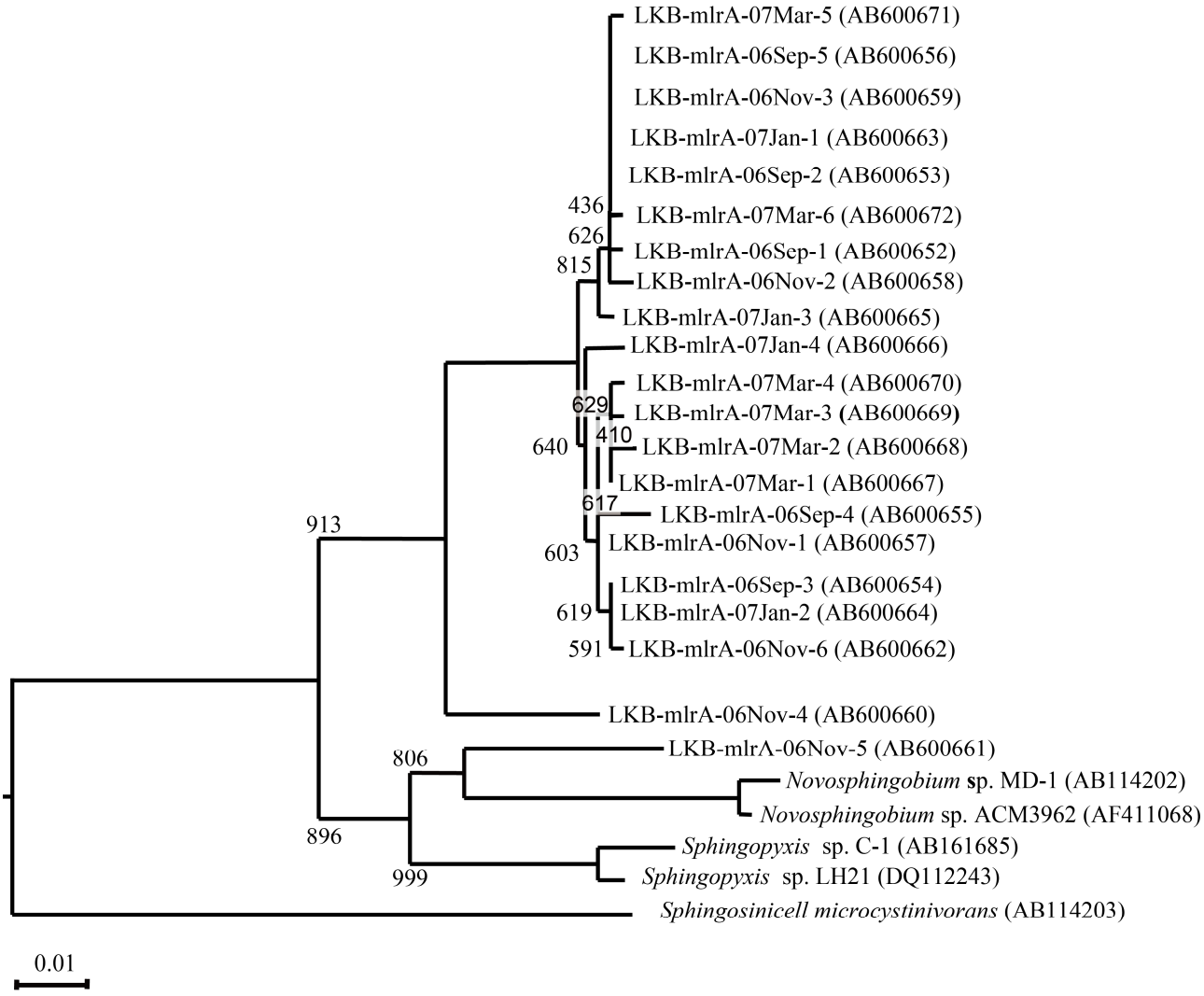

Figure 4. Dendrogram of the *mlrA* genes identified in biofilms at a drinking water facility, showing relationships with closest known relatives (underlined). The numerical values shown are the bootstrap values. The scale bar represents expected changes per site. The number on the scale bar shows evolutionary distance. The sample number is the month-clone number; for example, 3-69 is clone number 69 isolated in March.

During the summer of 2006, a toxic cyanobacterial bloom was observed in Lake Kasumigaura; however, the dissolved MC concentration in the biological water treatment facility was below the detection limit. It was thought that the density of the MC-degrading bacterial population containing *mlrA* homologues was too low to be detected by PCR. However, our results provide the first evidence that some types of MC-degrading bacteria can survive in biofilms in

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biological treatment facilities from September to March. This suggests that a bacterial group with *mlrA* homologues is closely associated with MC degradation by the biofilms (Figures 3 and 4). Microorganisms in the biofilms degraded MCs in all seasons [24] and the sand filter from the actual water purification plant was shown to degrade MCs [20, 49]. Therefore, we confirm that, even though toxic cyanobacterial blooms do not occur every year, removal of MCs via this biological treatment is sustainable. However, there is a time lag before a high-level expression of MC-degradative activity [23]. Shimizu *et al.* [50] reported that MC-degrading bacteria respond to MC-LR and its degradation products. Therefore, MC-degrading bacteria in biofilms could respond to MC-LR and its degradation products and express MC-degrading enzymes and rapidly degrade MCs. Our results indicate that non-culturable bacteria possess *mlrA* gene homologues (Figures 3-5). A constant number of MC-degrading bacteria containing *mlrA* were found in biofilms throughout the year [23, 39]. MC-degradative activity and *mlrA* gene copy number are positively correlated [23]. Therefore, quantification of the MC-degrading bacteria in biofilms attached to a carrier in a biological drinking water treatment facility is a key parameter for determining the MC-degradative activity for effective toxin removal, which ensures the safety of the drinking water supply.

Microbial communities that naturally form biofilms are capable of degrading MCs. We suggest that biological removal of MCs by MC-degrading bacteria is a suitable method in developing countries that have polluted water reservoirs. In fact, some researchers have reported that biological water treatment using MC-degrading bacteria is effective for MC removal. For example, MC-RR was completely degraded by an immobilised MC-degrading bacterium, (*Sphingosinicella microcystinivorans* B-9) in polyethylene glycol for 80 days in a semi-continuous reactor [51]. In addition, immobilised *Novosphingobium* sp. ACM 3962 on a sand filter degraded 50 µg/L of MC-LR [20], while a sand filter from a water purification plant degraded approximately 20 µg/L of MC-LR and MC-LA [49]. Moreover, both *Microcystis* cells and MCs were effectively removed by microorganisms in biofilms and the hydraulic retention time was reduced by aeration control [50]. Thus, this method has been shown to be effective for the removal of MCs on a daily basis. However, the optimum operating conditions, such as the hydraulic retention time, that are suitable for forming a biofilm in a drinking water facility that rapidly degrades MCs should be determined in detail to ensure the drinking water safety, including any physicochemical factors affecting water purification [53, 54]. Further analyses are required to determine the interaction between MC-degrading bacteria and the other bacteria in the biofilm and the role of different types of carriers that can provide a steady attachment for bacteria with *mlrA* homologues to maintain effective and stable MC-degradative activity.

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MD-1    LLLFPAAPMF AALIATGIGY GQAGFRXLLS RCAPWRSPVS WRQGVTVIAV CFLAFFALTG
Sep-1   LLLFPAAPMF AALVATGIGY GRAGFRXLLS RCAPWRLPVS WRQGVTVIAV CFLAFFALTG
Sep-12  LLLFPAAPMF AALVATGIGY GRAGFRXLLS RCAPWRSPVS WRQGVTVIAV CFLAFFALTG
Sep-28  LLLFPAAPMF AALVATGIGY GRAGFRXLLS RCAPWRSPVS WRQGVTVIAV CFLAFFALTG
Sep-38  LLLFPAAPMF AALVATGIGY GRAGFRXLLS RCAPWRSPVS WRQGVTVIAV CFLAFFALTG
Sep-46  LLLFPAAPMF AALVATGIGY GRAGFRXLLS RCAPWRSPVS WRQGVTVIAV CFLAFFALTG
Nov-1   LLLFPAAPMF AALVATGIGY GRAGFRXLLS RCAPWRSPVS WRQGVTVIAV CFLAFFALTG
Nov-5   LLLFPAAPMF AALVATGIGY GRAGFRXLLS RCAPWRSPVS WRQGVTVIAV CFLAFFALTG
Nov-19  LLLFPAAPMF AALVATGIGY GRAGFRXLLS RCAPWRSPVS WRQGVTVIAV CFLAFFALTG
Nov-26  LLLFPAAPMF AALVATGIGY GRAGFRXLLS RCAPWRSPVS WRQGVTVIAV CFLAFFALTG
Nov-27  LLLFPAAPMF AALVATGIGY GRAGFRXLLS RCAPWRSPVS WRQGVTVIAV CFLAFFALTG
Jan-34  LLLFPAAPMF AALVATGIGY GRAGFRXLLS RCAPWRSPVS WRQGVTVIAV CFLAFFALTG
Jan-38  LLLFPAAPMF AALVATGIGY GRAGFRXLLS RCAPWRSPVS WRQGVTVIAV CFLAFFALTG
Jan-41  LLLFPAAPMF AALVATGIGY GRAGFRXLLS RCAPWRSPVS WRQGVTVIAV CFLAFFALTG
Mar-65  LLLFPAAPMF AALVATGIGY GRAGFRXLLS RCAPWRSPVS WRQGVTVIAV CFLAFFALTG
Mar-67  LLLFPAAPMF AALVATGIGY GRAGFRXLLS RCAPWRSPVS WRQGVTVIAV CFLAFFALTG
Mar-68  LLLFPAAPMF AALVATGIGY GRAGFRXLLS RCAPWRSPVS WRQGVTVIAV CFLAFFALTG
Mar-69  LLLFPAAPMF AALVATGIGY GRAGFRXLLS RCAPWRSPVS WRQGVTVIAV CFLAFFALTG
Mar-81  LLLFPAAPMF AALVATGIGY GRAGFRXLLS RCAPWRSPVS WRQGVTVIAV CFLAFFALTG
Mar-86  LLLFPAAPMF AALVATGIGY GRAGFRXLLS RCAPWRSPVS WRQGVTVIAV CFLAFFALTG

MD-1    IMWVQTYLYA PPGTLDRTFL RYGSDPVAIY VMLAASLLLS PGPLLXXLGW RGFALPQLLK
Sep-1   IMWVQTYLYA PPGTLDRTFL RYGSDPLAIY VMLAASLLLS PGPLLXXLGW RGFALPQLLK
Sep-12  IMWVQTYLYA PPGTLDRTFL RYGSDPLAIY VMLAASLLLS PGPLLXXLGW RGFALPQLLK
Sep-28  IMWVQTYLYA PTGTLDRTFL RYGSDPLAIY VMLAASLLLS PGPLLXXLGW RGFALPQLLK
Sep-38  IMWVQTYLYA PPGTLDRTFL RYGSDPLAIY VMLAASLLLS PGPLLXXLGW RGFALPQLLK
Sep-46  IMWVQTYLYA PPGTLDRTFL RYGSDPLAIY VMLAASLLLS PGPLLXXLGW RGFALPQLLK
Nov-1   IMWVQTYLYA PPGTLDRTFL RYGSDPLAIY VMLAASLLLS PGPLLXXLGW RGFALPQLLK
Nov-5   IMWVQTYLYA PPGTLDRTFL RYGSDPLAIY VMLAASLLLS PGPLLXXLGW RGFALPQLLK
Nov-19  IMWVQTYLYA PPGTLDRTFL RYGSDPLAIY VMLAASLLLS PGPLLXXLGW RGFALPQLLK
Nov-26  IIWVQTYLYA PPGTLDRTFL RYGSDPVTIY VMLAASLLLS PGPLLXXLGW RGFALPQLLK
Nov-27  IMWVQTYLYA PPGTLDRTFL RYGSDPLAIY VMLAASLLLS PGPLLXXLGW RGFALPQLLK
Jan-34  IMWVQTYLYA PPGTLDRTFL RYGSDPVAIY VMLAASLLLS PGPLLXXLGW RGFALPQLLK
Jan-38  IMWVQTYLYA PPGTLDRTFL RYGSDPVAIY VMLAASLLLS PGPLLXXLGW RGFALPQLLK
Jan-41  IMWVQTYLYA PPGTLDRTFL RYGSDPVAIY VMLAASLLLS PGPLLXXSGW RGSALPQLLK
Mar-65  IMWVQTYLYA PPGTLDRTFL RYGSDPVAIY VMLAASLLLS PGPLLXXLGW RGFALPQLLK
Mar-67  IMWVQTYLYA PPGTLDRTFL RYGSDPVAIY VMLAASLLLS PGPLLXXLGW RGFALPQLLK
Mar-68  IMWVQTYLYA PPGTLDRTFL RYGSDPVAIY VMLAASLLLS PGPLLXXLGW RGFALPQLLK
Mar-69  IMWVQTYLYA PPGTLDRTFL RYGSDPVAIY VMLAASLLLS PGPLLXXLGW RGFALPQLLK
Mar-81  IMWVQTYLYA PPGTLDRTFL RYGSDPVAIY VMLAASLLLS PGPLLXXLGW RGFALPQLLK
Mar-86  IMWVQTYLYA PPGTLDRTFL RYGSDPVAIY VMGRHRCLLS PGPLLRRTGL G--AALRCRS
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Figure 5. Alignment of the deduced amino acid sequences based on the *mlrA* gene sequences. The sequences were identical in 18 of the 19 isolated *mlrA* sequences, which were identical to that of strain MD-1, an MC-degrading bacterium isolated from Lake Kasumigaura. Identical sequences are shown in dark grey and those that vary in one MlrA deduced amino acid sequences are shown in light grey.

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CONCLUSIONS

It can be concluded that biofilms attached to a carrier in the studied biological treatment facility contain a naturally developed microbial community that can facilitate MC removal in the presence of an MC-producing cyanobacterial bloom in the reservoir. This biological treatment method should be a powerful means of protecting public health in both the developing and developed countries.

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