

Detection and sequencing of the microcystin LR-degrading gene, *mlrA*, from new bacteria isolated from Japanese lakes

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Abstract

mlrA is the only microcystin-degrading gene detected in *Sphingomonas* sp. MJ-PV. The gene has an extremely rare nucleotide sequence and homologous genes have not yet been discovered in the DNA database. We discovered the existence of a gene homologous to *mlrA* in new microcystin-degrading bacteria, MD-1 and Y2. These strains possessed *mlrA* homologues, and the identities of the genes of MD-1 and Y2 with the corresponding MJ-PV exceeded 98% and 84%, respectively. On the other hand, the *mlrA* gene was not detected in laboratory strains of the closely related *Sphingomonas* spp. strains employing hemi-nested polymerase chain reaction detection using two primer sets. Although the microcystin-degrading bacteria were closely related strains, they did not cluster together as the same species. We can conclude that the *mlrA* gene is conserved in three different bacterial species, and it is unique to microcystin degraders but not to the genus *Sphingomonas*.

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1. Introduction

Microcystins are a group of cyclic heptapeptide hepatotoxins produced by cyanobacteria belonging to the genera *Microcystis*, *Anabaena*, *Oscillatoria* and *Nostoc* [1,2]. Microcystins are fatal to fish, birds, domestic animals and even humans [3,4]. In 1996 in Brazil, many people died from using water that was contaminated with microcystins introduced during water treatment [5]. Moreover, recent studies have reported the carcinogenicity of microcystins through tumor promotion [6].

As microcystins are stable against physico-chemical factors, biodegradation is the main factor that leads to the decrease of microcystins in natural water [7–11]. Until now, relatively few microcystin-degrading bacteria isolated from different areas have been reported. In 1994, Jones et

al. [12] isolated a microcystin-degrading bacterium, MJ-PV, from Australian water bodies for the first time. The strain was identified as *Sphingomonas* sp. based on 16S rRNA gene sequence. Bourne et al. [13] performed cloning and gene library screening of the *Sphingomonas* sp. strain and detected the microcystin-degrading gene cluster, *mlrA*, *B*, *C* and *D*. The enzyme encoded by the *mlrA* gene can cleave the ADDA–Arg peptide bond in microcystin LR and open the cyclic structure. After opening the cyclic structure, linear microcystin LR is degraded by the peptidases encoded by *mlrB* and *mlrC*, and divided into each amino acid. The *mlrD* encodes the transporter protein that allows the uptake of microcystins into the cell [13,14].

MlrA is the most important enzyme for the microcystin metabolism mechanism because the cyclic structure promotes microcystin stability against other protease and chemical factors. As the *mlrA* gene has a very rare sequence and there are almost no homologous genes in the DNA database, we could not predict its origin or family. Since there are no other reports of an *mlrA* homologous gene, we have very limited genetic information on the

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biodegradation of microcystin [7,13]. In previous studies, we have however isolated a microcystin-degrading bacterium, strain MD-1, from Lake Kasumigaura in Japan [15]. The bacterium could degrade microcystin RR, YR and LR very rapidly. Park et al. [16] also isolated a microcystin-degrading bacterium, strain Y2, from Lake Suwa in Japan. Although these strains were identified based on the 16S rRNA sequences in the former study, there has been to date no investigation of the microcystin-degrading genes they possess.

The objective of this study was to obtain evidence of the distribution of the microcystin-degrading gene, *mlrA*, in several lakes situated geographically distant from each other in order to determine their diversity. We performed *mlrA* detection using nested polymerase chain reaction (PCR) from two microcystin-degrading bacteria, MD-1 and Y2, and several non-degrading bacteria (strains of NBRC). Furthermore, the sequences of *mlrA* homologues in MD-1 and Y2 were determined and aligned with the corresponding *mlrA* of the *Sphingomonas* strain MJ-PV.

2. Materials and methods

2.1. Bacterial strains

We used two microcystin-degrading strains, MD-1 and Y2, and several closely related strains in the present study. *Sphingomonas* sp. MD-1 was isolated from Lake Kasumigaura in Japan. The strain can degrade microcystin RR, YR and LR, but not another hepatotoxin, nodularin. The bacterium was identified as *Sphingomonas* sp. based on 16S rRNA sequence (accession number AB110635). Another microcystin-degrading strain, Y2, was provided by Dr. Park from Shinshu University. The strain was isolated in 1997 from Lake Suwa [16], which is located 300 km away from Lake Kasumigaura. The strain is also included in the genus *Sphingomonas* (accession number AB084247). Other strains of *Sphingomonas* spp. that we used in this

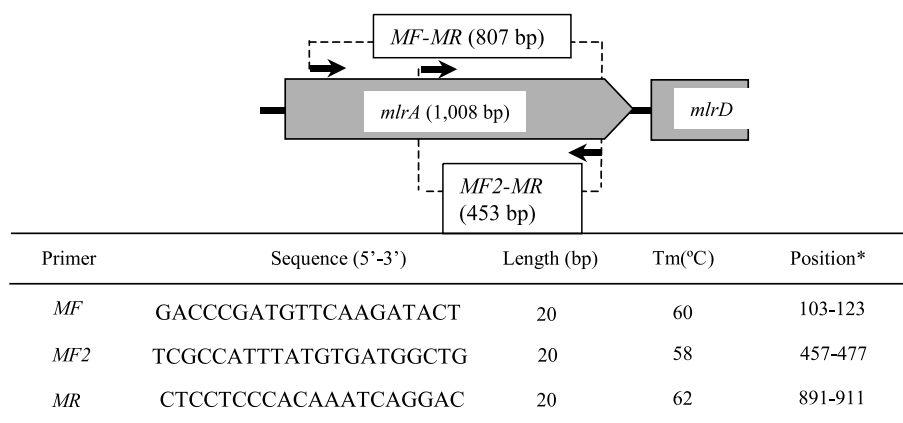
study were purchased from the NITE Biological Resource Center in Japan (previously named IFO). We used strains for which the 16S rRNA sequence was known and published in the database. The strain names, strain numbers and accession numbers are described in Section 3.

2.2. Bacterial cultivation and DNA extraction

The bacteria we used in this study were preserved at -30°C , and pre-incubated by inoculation in PY medium (5 g of peptone, 2.5 g of yeast extract per 1000 ml at pH 7.2) then pelleted by centrifugation ($15\,000\times g$, 5 min) prior to DNA extraction. For DNA extractions, we employed the ISOPLANT DNA extraction kit according to the manufacturer's instructions (Nippon Gene) [17]. A 50-mg aliquot of wet bacteria in 1.5-ml tubes was supplemented with 300 μl of sodium dodecyl sulfate solution and 150 μl of benzyl chloride solution. The solution was incubated for 15 min at 50°C and then 150 μl of sodium acetate solution was added. After setting on ice for 15 min, the solution was centrifuged ($12\,000\times g$, 15 min). The supernatant sample was removed and added to a tube containing about 1 ml of 100% methanol. The sample was centrifuged, the supernatant discarded, and the total precipitated DNA dissolved in 50 μl of TE Buffer (pH 8.0).

2.3. PCR and sequencing

Small volume PCR was performed for rapid and highly specific generation of DNA molecules. Fig. 1 shows the details of the primers employed, which were designed using PRIMER3 software referring to the *mlrA* gene sequence of MJ-PV (accession number AF411068). To ensure that fragments detected using primers MF-MR were *mlrA* homologues, we also performed hemi-nested PCR with the primer set MF2-MR using the product of the first PCR as template. PCR reactions were performed in a 20- μl reaction volume and thermal cycling conducted using a



* On *mlrA* gene of MJ-PV strain

Fig. 1. Schematic showing target regions in the *mlrA* gene for amplification by first round and hemi-nested PCR. Below are the primer sequences used in this study and their relevant information.

GeneAmp® PCR System 2400 (Perkin Elmer). The reaction mix consisted of reaction buffer (67 mM Tris–HCl, 16 mM (NH₄)SO₄, 0.45% Triton X-100, 0.2% gelatin), 2.5 mM MgCl₂, 200 μM deoxyribonucleotide triphosphates, ~1 ng chromosomal DNA template, 1 pmol forward primer and reverse primer, and 0.5 U *Taq* recombinant polymerase (Toyobo, Japan). After an initial denaturation step of 94°C for 1 min, 30 cycles were performed for the first PCR, consisting of denaturation at 94°C for 20 s, primer annealing at 60°C for 10 s, and elongation at 72°C for 30 s. The second hemi-nested PCR was performed for 27 cycles, consisting of denaturation at 94°C for 20 s, primer annealing at 58°C for 10 s, and elongation at 72°C for 20 s. Eight μl of the PCR products were separated by agarose gel electrophoresis. The bands on the agarose gel were stained with ethidium bromide and detected using a UV illuminator.

The PCR products amplified using the primer set MF-MR (approximately 800 bp) were cloned and sequenced. Samples of DNA from PCR amplifications were purified using the QIA Quick PCR Purification Kit (Qiagen) and sequenced using the ABI Prism BigDye Terminator v.3.0 Ready Reaction Cycle Sequence Kit (Perkin-Elmer Applied Biosystems) and an ABI Prism 377 DNA Analyzer (Perkin-Elmer Applied Biosystems). DNA sequences were compiled and aligned with GENETYX-MAC (Software Development, Japan). BLAST searches (<http://www.ncbi.nlm.nih.gov/BLAST>) were used to identify similar sequences from GenBank (<http://www.ncbi.nlm.nih.gov/Genbank>). The *mlrAMD-1* and *mlrAY2* gene sequen-

ces are available under GenBank accession numbers AB114202 and AB114203, respectively.

2.4. Analysis of microcystin concentration

For elucidation of the microcystin-degrading ability of *Sphingomonas* spp., we performed a microcystin-degrading test on each strain. A 250-μl sample was drawn from each test tube and filtrated with a 0.45-μm filter for microcystin analysis. Microcystins were analyzed by high performance liquid chromatography (Shimadzu) using a reverse phase column Cosmosil C18, 4.6 mm diameter × 250 mm (Nacalai Tesque, Japan). The mobile phase was 60% methanol adjusted to pH 3 with phosphate (0.05 M NaH₂PO₄+0.05 M H₃PO₄) buffer. The flow rate was 1 ml min⁻¹. The column oven was set at 40°C and the wavelength was set at 238 nm [18].

3. Results

3.1. Phylogenetic position of microcystin-degrading bacteria based on 16S rRNA gene sequences

Fig. 2 shows the phylogenetic position of microcystin-degrading bacteria based on 16S rRNA gene sequences. MD-1 was most similar to *Sphingomonas stygia* in the database. The MJ-PV strain, which was isolated from an Australian lake, was of the same genus, and the similarity was about 96% using BLAST analysis. Interestingly,

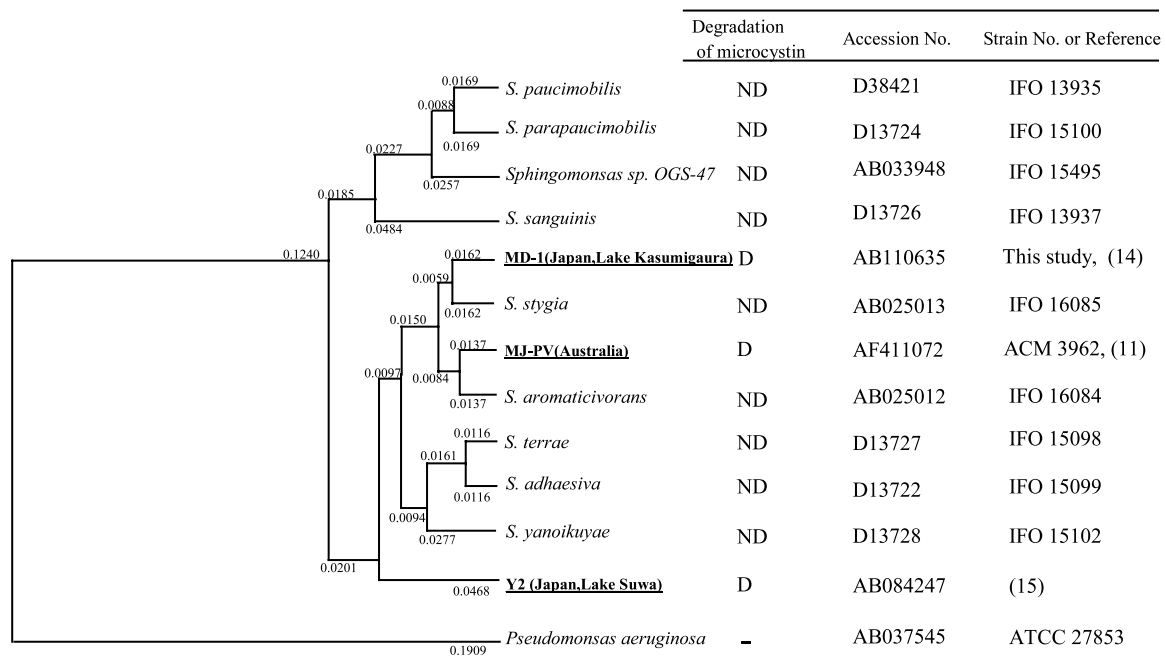


Fig. 2. Phylogenetic tree of microcystin-degrading bacteria and closely related strains, and their related information. This tree was analyzed by the neighbor-joining method. D and ND denote the strains which can and cannot degrade microcystin LR during 17 days respectively. Accession numbers correspond to partial sequences of 16S rRNA.

Table 1
Identity of the *mlrA* gene and 16S rRNA between microcystin-degrading bacteria

Strain	Identity (%)		
	MJ-PV	MD-1	Y2
MJ-PV	–	96 ^a	94 ^a
MD-1	98 ^b	–	92 ^a
Y2	84 ^b	84 ^b	–

^aPartial sequence of 16S rRNA.

^bPartial sequence of the *mlrA* gene.

although the three microcystin-degrading bacteria were closely related strains they did not cluster together. Though also relatively closely positioned, the Y2 strain branched the deepest in this phylogenetic tree. The similarities of Y2 to MD-1 and MJ-PV were 92% and 94%, respectively, based on partial sequences of 16S rRNA (Table 1). Several strains of *Sphingomonas* obtained from NBRC were also examined for microcystin LR-degrading ability. Fig. 2 also shows the result of microcystin degradation testing. Each strain was incubated with 1 mg l⁻¹ microcystin LR and inorganic medium for a maximum of 17 days and monitored for changes in microcystin concentration. All strains except MD-1 and Y2 showed no decrease in microcystin LR during the test period. MD-1 and Y2 were able to completely degrade microcystin LR within 24 h. Although we also checked the microcystin LR degradation ability of other strains (*S. capsulata*, *S. puri*, *S. mali* and *S. macrogobilis*), none of the strains was able to degrade microcystin LR during the experimental period.

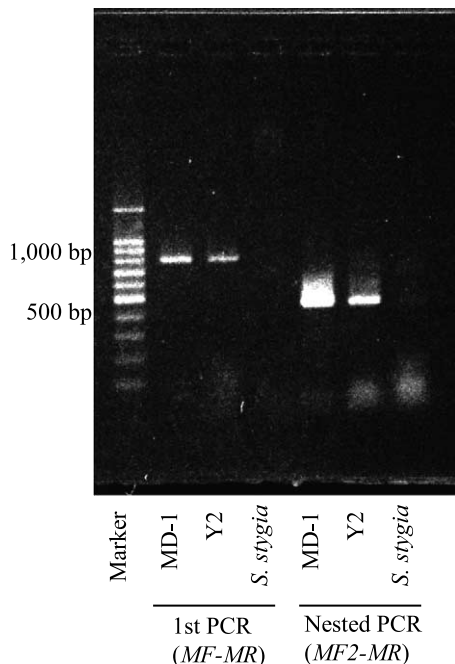


Fig. 3. Detection of *mlrA* gene fragments by first round and hemi-nested PCR in microcystin-degrading strains and non-degrading strains. Amplification of first PCR product using the primer set MF-MR; amplification of nested PCR product with primers MF2-MR as described in Section 2.

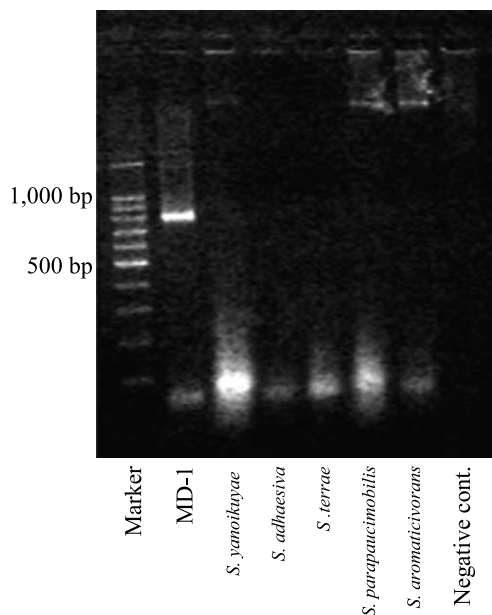


Fig. 4. PCR screen of different *Sphingomonas* strains closely related to MD-1 for the presence of the *mlrA* gene. PCR performed with the primers MF-MR as described in Section 2.

3.2. Detection of the *mlrA* gene from the microcystin-degrading bacteria MD-1 and Y2

Fig. 3 shows the results of detection of the *mlrA* gene by nested PCR. Bands of the expected size (approximately 800 bp) were detected in the strains MD-1 and Y2 by the first PCR employing the primer set MF-MR. In the subsequent hemi-nested PCR with the primer set MF2-MR, bands were also detected using the products of the first PCR as a template, which resulted in the amplification of an approximately 480-bp product (Fig. 3). This confirmed that *mlrA* homologues exist in these strains. Employing the same PCR conditions we were not able to obtain PCR products in either the first or second hemi-nested PCR in *S. stygia*, which is the most closely related strain to the MD-1 strain but does not degrade microcystin LR.

Fig. 4 shows the results of screening for *mlrA* homologues with a primer set MF-MR for each strain obtained from NBRC. Bands of the target length (807 bp) were not detected in any of the strains. We found *mlrA* homologous genes in just two different bacteria, MD-1 and Y2. This indicates that the *mlrA* gene is a peculiar and essential gene conserved among microcystin-degrading bacteria. Nucleotide sequence determination was performed on the PCR products of MD-1 and Y2. We found a high similarity between the genes. The similarities of the *mlrA* gene of MJ-PV with MD-1 and Y2 were over 98% and 84%, respectively, using BLAST searches (Table 1). Putative translated amino acids sequences were also close, with similarities of 91% and 87%, respectively, and positive sites of 97% and 91%, respectively, using BLAST searches.

From PCR detection and sequencing, we concluded that MD-1 and Y2 conserve *mlrA* homologues.

4. Discussion

This study is the first to examine the distribution of the microcystin-degrading gene, *mlrA*, in several geographically distant areas. The results of previous studies indicate that biodegradation of microcystin LR is performed by a specific enzyme. Indigenous aquatic bacteria required several days of lag time to begin degrading microcystin LR in laboratory experiments [8,10]. However, after acclimatization with microcystin LR, the indigenous bacteria did not require lag time to start degrading microcystin LR [11,12]. This suggests that minor succession occurred in indigenous mixed bacteria to a microcystin degrader possessing specific enzyme activities. Furthermore, microcystin was stable against several existing proteases, trypsin, chymotrypsin, elastase, thrombin, papain, collagenase, carboxypeptidase and pepsin, despite the proteases having a specific reaction with the peptide bond in microcystin LR [19]. It seems therefore that specific structures in proteases are needed to catalyze degradation of microcystin LR. In previous studies employing neprilysin, it is believed that small cyclic peptides have poor flexibility and are difficult to fit into the enzyme pocket for reaction [20]. Since microcystin LR is also a small cyclic peptide (seven amino acids), it would be difficult to degrade by either enzymatic or physico-chemical factors.

Bourne et al. detected the *mlrA* gene in an Australian strain for the first time in 2001 [13]. The enzyme encoded by the *mlrA* gene has the unique function of cleaving the cyclic structure of microcystin LR. Since there are no homologous genes for the *mlrA* gene in the DNA database, the origin and functional characteristics have not been identified. Until now there have been no other reports on the microcystin-degrading gene. In this study, we isolated microcystin-degrading bacteria MD-1 and Y2 from two Japanese lakes and found that the strains had an *mlrA* homologous gene. In addition, *mlrA* genes of strains MD-1 and Y2 had a high similarity to that of strain MJ-PV. The discovery may extend to that of a new protease family having the function of cleaving smaller cyclic peptides, by confirming the original functions and the fundamental structure of the *mlrA* enzyme.

Putative amino acid sequences translated from *mlrA* gene sequences obtained in the present study of the three strains were also similar. In a previous study, the MlrA enzyme was defined as a metalloprotease-like enzyme through characterization with a protein inhibitor [14]. Bourne et al. predicted an activation site of the sequence HXXHEX in putative MlrA sequences [13]. The sequence is typical in activated sites of zinc metalloproteases. The MlrA of MD-1 and Y2 identified in the present study also conserved the same motifs. Therefore, it is likely the two

strains degrade microcystin LR via the same enzyme reaction. By conducting further homology searches with putative translated amino acids using BLAST in this study, the CAAX amino-terminal protease family of *Bacillus* spp. was determined as a homologue of the translated amino acid of the *mlrA* gene [21]. The identity was 35%, and positives were 50% on putative amino acids translated from positions 421–724 of the *mlrA* gene of MD-1. The CAAX amino-terminal protease originated from a part of the DNA in the plasmid pSOL1 sequence in *Bacillus anthracis* [21], and the CAAX protease family consists of various hypothetical protein sequences for which the function is unknown [22,23]. The sequence contains a highly conserved Glu-Glu motif at the end of the alignment, plus two histidine residues that may be involved in zinc binding. As discussed above the sequences we determined in this study also have a zinc binding-like sequence.

The *mlrA* gene homologue could not be detected by PCR from the closely related *S. stygia*, which does not degrade microcystin LR. We can surmise that this strain does not contain *mlrA* homologues or that there are large differences in the sequences. In addition microcystin degradation was not detected in a range of *Sphingomonas* congeners tested (Fig. 2). We can conclude that the ability to degrade microcystin is not commonly present in the *Sphingomonas* genus, but only in specific bacterial strains. The peptide sequence CAAX from the amino-terminal protease family is found in the abortive infection protein of bacteria to bacteriophage or in the bacteriocin-like peptide plantaricin in *Lactobacillus* sp. [24]. Thus it seems that partial fragments of the *mlrA* gene consistently display a biological function in some bacteria. Closely related strains possibly have some modules, including the partial *mlrA* gene, and PCR detection with other specific primer sets or degenerate primers may allow for clarification of the origin of the *mlrA* gene.

From our results, we can conclude that the *mlrA* gene is conserved in at least three different bacterial species, and it is unique to microcystin degraders but not to the genus *Sphingomonas*. The finding that several non-degraders of microcystin were more closely related by 16S rRNA sequencing to a microcystin degrader (strain Y2) raises the intriguing possibility that the microcystin degradation trait was acquired by gene transfer at some point in the evolution of *Sphingomonas*. We expect that clarification of the evolutionary process of the *mlrA* gene may promote discovery of a new protease family having unique functions. The probes designed in this study should allow the rapid detection of microcystin-degrading bacteria in the environment, paving the way for more rational design of treatment strategies for toxic cyanobacterial blooms.

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