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Development of Oligonucleotide Primers for the Detection of Harmful *Microcystis* in Water

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Microcystis is one of the dominant genus of cyanobacteria (blue-green algae) that cause water blooms in an eutrophic lake and reservoir. Some members of the genus *Microcystis* produce cyclic heptapeptide hepatotoxins called microcystins. Microcystins are members of a family of more than 65 heptapeptides and share a common structure of cyclo ($_D$-Ala-$_L$-X-$_D$-MeAsp-$_L$-Z-Adda-$_D$-Glu-Mdha) (Rinehart et al. 1988). Toxicity is mediated through active transport of microcystin into hepatocytes by the bile acid organic anion transport system, followed by inhibition of eukaryotic serine/threonine protein phosphatases 1 and 2A (MacKintosh et al. 1990; Carmichael 1994). Due to its toxicity, the microcystins have posed a considerable threat to humans and animals (Dawson 1998). According to WHO guideline for drinking water quality (1998) the provisional guideline value of microcystin-LR (total microcystin-LR, free plus cell-bound) is 1 ppb. Therefore, a rapid and reliable method to detect and monitor populations of microcystin-producing *Microcystis* species is needed in drinking water management.

Microcystin-producing *Microcystis* species can hardly be distinguished from non toxin-producing *Microcystis* unless isolated and tested for toxin production. Microcystins are synthesized by the genes that construct an unusual structure and by forming a large complex through assemblage of cyclic peptides. Technically, it is assumed that the toxin-producers can be detected by molecular techniques using the genes coding for cyclic polypeptides. A recent finding of the genetic locus responsible for microcystin synthesis from *Microcystis aeruginosa* allows the question of toxigenicity of *Microcystis* to be reexamined. In an attempt to detect toxin-producing cyanobacteria, Neilan et al. (1999) and Nishizawa et al. (1999) developed the sets of oligonucleotide primers derived from *mcyB* (microcystin synthetase B gene) and the adenylation domains of microcystin synthetase gene clusters. Although they were able to detect the toxin-producers with the oligonucleotide primers, a number of anomalies still existed. In another study, Tillett et al. (2001) developed oligonucleotide primers derived from *mcyA* (microcystin synthetase A gene) and were able to identify toxin-producing *Microcystis* with the primers as well as several non toxin-producing *Microcystis.*

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In this study, we developed a set of oligonucleotide primers derived from the *N*-methyltransferase domain of *mcyA* and were able to detect and monitor harmful *Microcystis* strains from other cyanobacteria tested. This method may be suitable, in drinking water management, to detect and monitor harmful *Microcystis* blooms in advance.

MATERIALS AND METHODS

The strains used in this study are *Microcystis*, *Anabaena*, *Oscillatoria, Nostoc* and *Synechococcus* (Table 1). The strains were commonly found in cyanobacterial blooms. They were obtained from various sources: National Institute of Environmental Research Culture Collection for Environmental Microorganism (NIER, Korea), Korean Collection for Type Cultures (KCTC, Korea), Pasteur Culture Collection (PCC, France) and the Culture Collection of Algae and Protozoa (CCAP, UK). All cyanobacteria were cultured in BG11 medium (Stanier et al. 1971) at 28℃ under illumination of approximately 60 μ $Em^{-2}s^{-1}$ with a 12h:12h light-dark cycle for two weeks or so. Bacterial strains (*Comamonas acidovorans, Pseudomonas aeruginosa* CW961 and *Pseudomonas putida* KT2442) were cultured in LB agar (Difco, USA).

Cyanobacterial genomic DNA was extracted with DNeasy Plant Mini Kit (Qiagen, Germany). Bacterial genomic DNA was obtained with Genomic-tip 100/G and Genomic DNA Buffer Set (Qiagen, Germany).

In order to design oligonucleotide primers to detect microcystin-producing *Microcystis*, a genomic DNA of five *Microcystis* strains (*M. aeruginosa* NIER-10039, *M. aeruginosa* PCC7806, *M. aeruginosa* PCC7820, *M. aeruginosa* CCAP1450/6 and *M. aeruginosa* NIER-10004) was first amplified with the primers developed by Tillett et al. (2001). Then, the sequences of the PCR products were analyzed. Nucleotide sequences from the 1.3 kb *N*-methyltransferase domain of the five *Microcystis* strains were aligned along with those of seventeen other *Microcystis* strains pulled out from GenBank. The sequence alignment was performed with a ClustalX (Thompson et al. 1997) of the BioEdit version 5.0.9 (Hall 1999). The most conserved regions of the sequences were then extracted and trimmed for oligonucleotide primers.

Microcystin production was determined by a commercially available ELISA kit (EnviroLogix, USA). All procedures were done by the recommendations of the manufacturer. A darker color developed in the samples indicated low microcystin production, while a lighter color indicated high microcystin production. After addition of a stop solution, optical density of the samples and the calibrators (0.5 and 3 ppb) was measured at 450 nm with a Lightwave UV/VIS Diode-Array Spectrophotometer (WPA, UK). The raw OD measurements of the samples and the calibrators are listed in Table 1. A linear regression was obtained at each series of OD measurements with the calibrators. The concentration of each sample was calculated by regression (Table 1).

Table 1. A list of strains used in this study and their toxin production.

Strains	Origin	Microcystin Production	
		OD*	Cal. Conc. (ppb)**
M. aeruginosa NIER-10001	Lake Daechung, Korea	ND§	NA§§
M. aeruginosa NIER-10008	Lake Kasumigaura, Japan	ND	NA
M. aeruginosa NIER-10010	Lake Sapgyo, Korea	0.152	6.60
M. aeruginosa NIER-10015	Paldang Reservoir, Korea	0.378	5.34
M. aeruginosa NIER-10037	Paldang Reservoir, Korea	1.023	1.77
M. aeruginosa NIER-10039	Paldang Reservoir, Korea	0.064	7.09
M. aeruginosa NIER-10051	Lake Daechung, Korea	1.555	NA (-1.18)
M. aeruginosa KCTC-AG10073	Not known	1.643	NA (-1.67)
M. aeruginosa KOWACO	Not known	ND	NA
M. aeruginosa Inje	Not known	1.907	NA (-2.23)
M. aeruginosa BC10	Scotland, UK	1.902	NA (-2.22)
M. aeruginosa PCC7005	Lake Mendota, USA	1.719	NA (-1.68)
M. aeruginosa PCC7806[a]	Braakman Reservoir, The Netherlands	0.062	3.23
M. aeruginosa PCC7820[b]	Loch Balgavies, UK	-0.04	3.46
M. aeruginosa PCC7941	Little Rideau Lake, Canada	-0.149	3.78
M. aeruginosa CCAP1450/1	Lake Mendota, USA	1.422	NA (-0.82)
M. aeruginosa CCAP1450/3	Cumbria, UK	1.406	NA (-0.77)
M. aeruginosa CCAP1450/4	Little Rideau Lake, Canada	1.581	NA (-1.28)
M. aeruginosa CCAP1450/6	Little Rideau Lake, Canada	0.142	2.93
M. ichthyoblabe NIER-10021	SeoNakdong River, Korea	1.568	NA (-1.24)
M. ichthyoblabe NIER-10030	Lake Youngsan, Korea	1.522	NA (-1.11)
M. ichthyoblabe NIER-10045	SeoNakdong River, Korea	1.639	NA (-1.45)
M. novacekii NIER-10022	SeoNakdong River, Korea	1.650	NA (-1.48)
M. novacekii NIER-10029	Lake Youngsan, Korea	1.540	NA (-1.16)
M. novacekii BC18	Scotland, UK	1.937	NA (-1.47)
M. sp. NIER-10004	Lake Soyang, Korea	0.197	3.54
M. sp. NIER-10025	Lake Hapcheon, Korea	1.859	NA (-1.25)
M. sp. NIER-10056	Lake Daechung, Korea	1.913	NA (-1.40)

Table 1. (cont.)

Strains	Origin	Microcystin Production	
		OD*	Cal. Conc. (ppb)**
M. viridis NIER-10017	NIES-strain	0.257	3.37
M. viridis NIER-10020	SeoNakdong River, Korea	0.139	3.71
M. viridis PUCC-1002	Nakdong River, Korea	ND	NA
M. wesenbergii NC-5	Lake Biwa, Japan	ND	NA
A. affinis KCTC-AG10008	Lake Kasumigaura, Japan	ND	NA
A. flos-aquae KCTC-AG10011	Lake Daechung, Korea	1.771	NA (-0.99)
A. flos-aquae KCTC-AG10082	UTEX-strain	1.765	NA (-0.97)
A. flos-aquae KCTC-AG10083	UTEX-strain	ND	NA
A. macrospora NIER-10016	Paldang Reservoir, Korea	1.705	NA (-0.80)
A. macrospora ATCC-22664	Not known	1.809	NA (-1.10)
A. sp. KCTC-AG10059	Not known	1.593	NA (-2.19)
A. special KCTC-AG10064	Not known	1.522	NA (-1.91)
O. sancta NIER-10027	Lake Daecuhung, Korea	1.642	NA (-2.39)
O. sp. NIER-10042	Lake Juam, Korea	1.444	NA (-1.61)
N. ellipsosporum CCAP1453/18	Wisconsin, USA	1.837	NA (-3.16)
S. bacillaris CCAP1479/7	Connecticut, USA	1.885	NA (-3.35)
S. elongatus CCAP1479/1B	France	1.543	NA (-1.97)
Mix A	In this study	0.215	3.85
Mix B	In this study	0.015	4.49
Mix C	In this study	0.025	4.45
Mix D	In this study	0.562	2.76
Mix E	In this study	0.393	3.29

* OD was determined at A_{450} (raw value).
** Cal. Conc. calculated concentration, by linear regression obtained during each series of measurements.
§ ND (not determined. The reading was over the detection limit of spectrophotometer)
§§ NA [not available. This indicates that the strain is either a non-toxin producer or very little toxin producer (less than 0.5 ppb). The negative value in parenthesis is the calculated concentration by the regressions]
a. Wiedner et al. (2003)
b. Ishii and Abe (2000)

PCR amplification was performed with a primer set of cho09F (5'-TGGCGACT TCAGAAAAAGGGC-3') and cho09R (5'-GCGGGGCAAAAATCACATC-3') in total volume of 50 μL containing 1 μL of genomic DNA, 2 μL each of 100 pmol of cho09F primer and cho09R, 2 μL of 10 m*M* dNTPs (Gibco, USA), 5 μL of 10× Han-OMNI PCR buffer with $MgSO_4$ and 0.2 μL of Han-*Taq* DNA polymerase (Genenmed, Korea). Thermal cycling was performed in a PTC-100 Programmable Thermal Controller (MJ Research, USA) with a program setting: at 95℃ for 4 min; 25 cycles of 95℃ for 20 sec, 63℃ for 30 sec, 72℃ for 1 min and a final extension at 72℃ for 7 min.

In order to confirm the specificity of the oligonucleotide primers, several mix and match batches of cyanobacteria were prepared. A negative group (either non microcystin-producers or very little toxin producers) was a mixture of NIER-10001, KCTC-AG10073, Inje, CCAP1450/1 and BC18. A positive group (microcystin-producers) was a mixture of NIER-10004, NIER-10039, PCC7806, PCC7820 and CCAP1450/6. The MixA was a mixture of either no microcystin-producing or very little toxin producing Inje and microcystin-producing NIER-10004, NIER-10039, PCRR7806 and PCC7820. The MixB was a mixture of either no microcystin-producing or very little toxin producing Inje and KCTC-AG10073 and microcystin-producing NIER-10039, PCC7806 and PCC7820. The MixC was a mixture of either no microcystin-producing or very little toxin producing Inje, KCTC-AG10073 and CCAP1450/1 and microcystin-producing PCC7806 and PCC7820. The MixD was a mixture of either no microcystin-producing or very little toxin producing Inje, KCTC-AG10073, CCAP1450/1 and BC18 and microcystin-producing PCC7820. The MixE was a mixture of no microcystin-producing *Anabaena* KCTC-AG10082, *Oscillatoria* NIER-10027, *Nostoc* CCAP1453/18 and *Synechococcus* CCAP1479/1B and microcystin-producing *Microcystis* PCC7806. The final mixture volume was 50 mL (containing 10 mL of each batch). Genomic DNA extraction and PCR amplification were performed as described previously.

RESULTS AND DISCUSSION

Forty-five strains of *Microcystis* and other cyanobacteria were tested for their microcystin production. After addition of a substrate, a white color was developed in the reactions of eight *Microcystis* strains (*M. aeruginosa* NIER-10010, *M. aeruginosa* NIER-10039, *M. aeruginosa* PCC7806, *M. aeruginosa* PCC7820, *M. aeruginosa* PCC7941, *M. aeruginosa* CCAP1450/6, *M.* sp. NIER-10004 and *M. viridis* NIER-10017), a light blue color in two *Microcystis* strains (*M. aeruginosa* NIER-10015 and *M. viridis* NIER-10020), a blue color in *M. aeruginosa* NIER-10037, and a dark blue color in the rest of other strains. After stop solution treatment, OD values of the samples and the 0.5 and 3.0 ppb calibrators were measured. OD values of the calibrators were standardized and a linear regression was obtained at each series of measurements. There were eleven *Microcystis* species showing microcystin production of more than 0.5 ppb

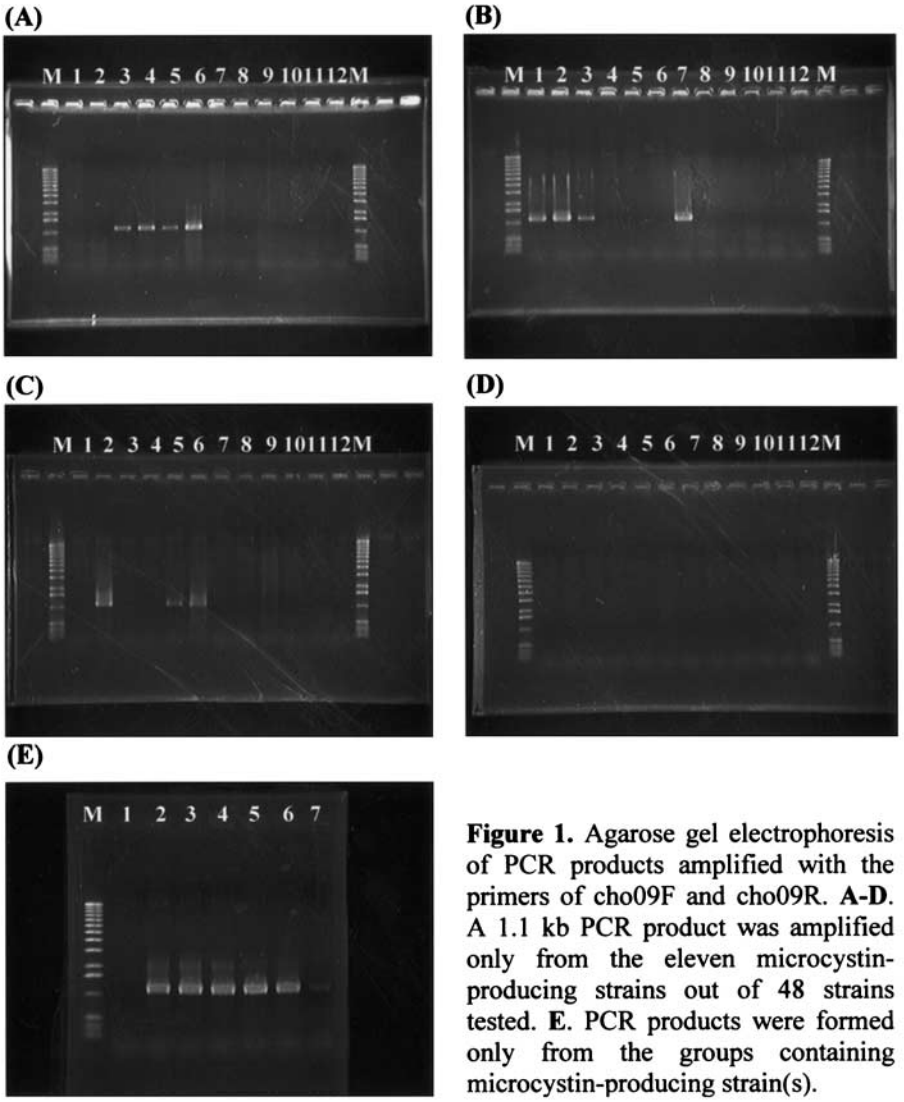

Figure 1. Agarose gel electrophoresis of PCR products amplified with the primers of cho09F and cho09R. **A-D**. A 1.1 kb PCR product was amplified only from the eleven microcystin-producing strains out of 48 strains tested. **E**. PCR products were formed only from the groups containing microcystin-producing strain(s).

M:1 kb DNA ladder **(A)**1:NIER-10001, 2:NIER-10008, 3:NIER-10010, 4:NIER-10015, 5:NIER-10037, 6:NIER-10039, 7:NIER-10051, 8:K-AG10073, 9:KOWACO, 10:Inje, 11:BC10, 12:PCC7005 **(B)**1:PCC7806, 2:PCC7820, 3:PCC7941, 4:CCAP1450/1 5:CCAP1450/3, 6:CCAP1450/4, 7:CCAP1450/6, 8:NIER-10021, 9:NIER-10030, 10:NIER-10045, 11:NIER-10022, 12:NIER-10029 **(C)**1:BC18, 2:NIER-10004, 3:NIER-10025, 4:NIER-10056, 5:NIER-10017, 6:NIER-10020, 7:PUCC-1002 8:NC5, 9:K-AG10008, 10:K-AG10011, 11:K-AG10082, 12:K-AG10083 **(D)**1:NIER-10016, 2:ATCC-22664, 3:K-AG10059, 4:K-AG10064, 5:NIER-10027, 6:NIER-10042, 7:CCAP1453/18, 8:CCAP1479/7, 9:CCAP1479/1B, 10:*C. acidovorans,* 11:*P. aeruginosa,* 12:*P. putida* **(E)**1:Negative, 2:Positive, 3:MixA, 4:MixB, 5:MixC, 6:MixD, 7:MixE.

: more than 3 ppb microcystin production in ten *Microcystis* species (*M. aeruginosa* NIER-10010, *M. aeruginosa* NIER-10015, *M. aeruginosa* NIER-10039, *M. aeruginosa* PCC7806, *M. aeruginosa* PCC7820, *M. aeruginosa* PCC7941, *M. aeruginosa* CCAP1450/6, *M.* sp. NIER-10004, *M. viridis* NIER-10017 and *M. viridis* NIER-10020) and 1.77 ppb microcystin production in *M. aeruginosa* NIER-10037 (Table 1). PCR amplification was performed with the oligonucleotide primers of cho09F and cho09R derived from *N*-methyl transferase domain of *mcyA*. A 1.1 kb single PCR product was successfully amplified only from the genomic DNAs of eleven *Microcystis* strains that produced microcystin of more than 0.5 ppb (Table 1). The strains were *M. aeruginosa* NIER-10010, *M. aeruginosa* NIER-10015, *M. aeruginosa* NIER-10037, *M. aeruginosa* NIER-10039, *M. aeruginosa* PCC7806, *M. aeruginosa* PCC7820, *M. aeruginosa* PCC7941, *M. aeruginosa* CCAP1450/6, *M.* sp. NIER-10004, *M. viridis* NIER-10017 and *M. viridis* NIER-10020 (Figure 1 A to D). On the other hand, there were no PCR products formed from the rest of the strains tested (Figure 1 A to D). These strains were the ones that either did not produce microcystins or produce very little microcystins (less than 0.5 ppb in our assay) as shown in Table 1.

In order to confirm the specificity of the oligonucleotide primers in detecting harmful *Microcystis*, several mix and match tests were carried out. As shown in figure 1 E, there were no PCR products formed from a negative group containing the genomic DNAs of non microcystin-producers or very little microcystin producers (less than 0.5 ppb). On the other hand, a 1.1 kb PCR product was amplified from the rest of the groups containing microcystin-producers, although the band intensity of MixE was faint.

The results from the PCR amplification with the primer set of cho9F and cho9R (Figure 1) along with the microcystin production (Table 1) clearly shows that the primers have specificity in detecting harmful *Microcystis* of concern, especially in drinking water quality management suggested by WHO.

Recently, several research groups developed oligonucleotide primers for detecting microcystin-producing *Microcystis* (Tillett et al. 2001; Pan et al. 2002). However, their results showed that the primers detected not only microcystin-producing *Microcystis* but also a few non microcystin-producing *Microcystis*.

In this study, we developed a set of oligonucleotide primers derived from the *N*-methyltransferase domain of *mcyA* and were able to detect harmful microcystin-producing *Microcystis* strains. The method presented here may have a potential application in detecting and monitoring harmful microcystin-producing *Microcystis* in drinking water before the bloom occurs.

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