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Strain Identification and Comparative Analysis of Toxigenic Cyanobacteria Determined by PCR

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Microcystis aeruginosa is common form of cyanobacteria (blue-green algae) capable of producing toxic heptapeptide (microcystin) that cause illness or death. The comparison of molecular genetic method with the morphological characteristics of cyanobacteria was conducted. We have designed PCR primers (JJM98F, JJM1141R) for cyanobacterial 16S rRNA and phycocyanin intergenic spacer (PC-IGS) gene domain. To confirm the production of microcystins, PCR primers for the *N*-methyltransferase (NMT) domain of microcystin synthetase gene *mcyA* were designed using 21 cyanobacteria strains Most of isolated strains from the Nakdong River was classified as *Microcystis aeruginosa* and the similarities were 99% with *M. aeruginosa* AF 139292. 38.1% of isolated strains contained microcystin synthesis gene. NMT (N-methyltransferase) were not detected in isolated strain in several strains, which means non-toxic. However, the NMTs of the strains were detected during the cultivation.

Key Words : Cyanobacteria, Microcystins, 16S rRNA, N-methyltransferase (NMT) domain

1. Introduction

Water-blooms of the cyanobacteria are ubiquitous in eutrophic lakes and rivers. These caused many matters in use of pH rising, bad taste · odor and filter clogging in the water treatment process[1]. *Microcystis*, the dominant cyanobacteria in the species causing water blooms, is widely spreaded in many countries of the world[1]. It is reported that blooms of *Microcystis* have shown in the lower Nakdong River[2], Daechung Reservoir[3], Paldang Reservoir and so on[4]. Some cyanobacteria produce the toxin, broadly divided into the hepatotoxin called microcystin and neurotoxin called anatoxin, saxitoxin.

Currently, cyanobacteria is classified over 1,500 species according to morphological characteristics[5]. Classification of cyanobacteria is divided into morphology based on the cell size, shape, buoyancy, toxin and functional characteristics[6]. It is formed to the variety of populations in fresh water and coastal water. However, it is difficult to exactly be classified and identified by habitat use[7].

It was supposed to be one of the 11 bacteria groups from the phylogeny and has begun to molecular approaches of the possibility of growing prokaryotic chromosome from the ancient of cyanobacteria. Identification of unclear cyanobacteria genus or species was developed by modern molecular biology techniques[8~10]. It is known that phycocyanin is consisted of two bilin subunit and three linker polypeptide. Intergenic spacer (IGS) region between bilin subunit genes is very available and is useful to classification of cyanobacteria[11]. Microcytins may activate phospholipase A_2 and cyclooxygenase in hepatocytes and induce tumor necrosis factor alpha (TNF-α) and interleukin 1. These functions with hyperphosphorylation of DNA have implicated microcystins as agents promoting hepatocellular carcinoma[8]. The recent identification of the genetic locus responsible for microcystin synthesis in *Microcystis aeruginosa* allows the question of toxigenicity to detect microcystin-producing cyanobacterial strains. Neilan *et al.*[11] have developed genetic probes directed, to the *mcyB* gene and to adenylation domains within the microcystin synthetase gene cluster. Phylogeny from the

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ability to produce cyanobacterial toxin is challenged for this purpose[12].

Therefore, in this study, we have identified species of cyanobacteria in Nakdong River using molecular genetic method, and designed primer as comparing and analyzing base sequences of 16S rRNA from environmental isolates. We compared gene amplification, cloning and base sequences with morphology, using intergenic spacer (IGS) region genes from *Microcystis* species and synthesized 16S rRNA. Correlation between peptide synthetase genes and the microcystin production was fast conducted for analyzing toxic species.

2. Materials and methods

2.1. Sampling and pure culture of cyanobacteria

Sixteen cyanobacterial strains were used for this study (Table 2). Six strains of *Microcystis* and 1 strains of *Aphanizomenon* were obtained from NIES (National Institute for Environmental Studies) of Japan. Nine strains of *Microcystis* or *Aphanizomenon* were purely divided from the Nakdong River, West-Nakdong River, Habchun Lake and Hoidong Reservoir, South Korea (Fig. 1).

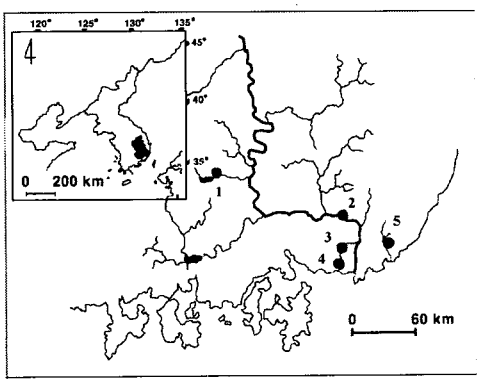

Fig. 1. Study area showing the location of the sampling sites. (1. Habchun Lake 2. Mulgum 3. Kangdong 4. Noksan 5. Hoidong Reservoir)

The cyanobacterial strains were maintained in CB medium at 25℃ with a light intensity of approximately 40 μmol/$m^2 \cdot s$[3]. At 28 days of growth, 2 mℓ of each culture was transferred to a serum vial and lyophilized for 48 h in a freeze-drier (Samwon model SFDSF12, Korea). Freeze-dry cells were used for this experiment.

2.2. DNA extraction

For genomic DNA extraction from cyanobacteria strain, a 1 mℓ aliquot of mid- to late-exponential-phase culture was pellected by centrifugation (12,000 × g for 5 min), the pellets were resuspended in 500 μℓ of 10 mM Tris-HCl (pH 7.8), 5 mM EDTA (pH 8.0), 0.5% SDS. Proteinase K (20 mg/mℓ) was added to give a final concentration of 50 μg/mℓ, and the solution was incubate at 56℃ for 2 h. The solution was chilled on ice and extracted with an equal volume of phenol-chloroform-isoamyl alcohol. The organic extraction was repeated, and the supernatant was added to an equal volume of 4 M ammonium acetate. Total genomic DNA was precipitated by the addition of 2.5 volumes of ethanol and centrifugation (12000 × g for 5 min) at room temperature. The supernatant of this cell lysate has been found to retain sufficient DNA to act as a template for PCR amplification. The DNA pellets were washed twice with 200 μℓ of 70% ethanol.

2.3. PCR amplification and DNA sequence

PC-IGS[11], 16S rRNA primer and NMT region primer were based on an alignment of sequences from cyanobacteria available from the GenBank. Designed primer sequences are listed in Table 1. The PCR mixture contained 10 μℓ of 10×PCR buffer (Takara Shuzo co., LTD, Japan), 5 μℓ of 1.5 mM $MgCl_2$, 50 pmol of primers (PCβF, PCαR, JJM198F, JJM1141R (Table 1). 1-100 ng of genomic DNA, 5 U of *Taq* DNA polymerase (Takara Shuzo co., LTD, Japan), and water to a final volume of 100 μℓ. PCR conditions were

Table 1. Primer pairs used for the detection of cyanobacteria

Target	Sequence(5'-3')
PC-IGS	PCβF 5'-ggctgcttgtttacgcgaca-3' PCαR 5'-ccagtaccaccagcaactaa-3'
16S rRNA of cyanobacteria	JJM 198F 5'-cgatcggtagctggtctgaga-3' JJM 1141R 5'-cattgtagtacgtgtgtagccca-3'
microcystin synthetase A gene	NMTF 5'-atccagcagttgagcaagc-3' NMTR 5'-tgcagataactccgcagttg-3'

40 cycles of 95℃ for 30 s, 56℃ for 45 s, and 72℃ for 60 s. PCR conditions of NMT F and NMT R were 35 cycles of 95℃ for 10 s, 50℃ for 10 s, and 72℃ for 60 s in a Perkin-Elmer 9600 PCR thermocycler. PCR products were analyzed by electrophoresis in 1% (wt/vol) agarose gels containing of ethidium bromide.

For the DNA sequencing by autosequencer ABI PRISM 3700 DNA Analyzer, amplified products were purified and performed cloning in pGEM-T vector (promega).

Nucleotide sequences were determined using an automated DNA sequencer (Model 373A) and Dye Deoxy teminator kit (Applied Biosystem) with M13 reverse primer.

2.4. Microcystin analysis

Intracellular microcystin content was measured using HPLC (Waters 2690, USA). The cells were harvested by centrifugation at 8,000 rpm at 4℃, freeze-dried, and stored at -70℃ until analysis. Microcystins in cyanobacterial cells were extracted using a slightly modified method of Harada[13]. A HPLC (Waters 2690, waters 996 Photodiode Array Detector) was equipped with a constant-flow pump and variable-wavelength u.v. detector operated at 238 nm. The seperation of microcystins were performed on a Capcellpak C_{18} (4.6 ×150 mm, Ø 5.0, SHISEIDO, Tyokyo, Japan) reverse-phase column and the mobile phase was a methanol-0.05 M phosphate buffer (58:42, pH 3.0; Harada *et al.*[13], at a flow rate of 1 mℓ/min. The microcystins were identified by their u.v. spectra and retention times, and by spiking the sample with a purified standard of microcystin (MC)-RR (M1537, Sigma, USA) and MC-LR (M2912, Sigma, USA).

3. Results

Cyanobacterial water bloom samples were obtained and frozen at various times during a large toxic cyanobacterial bloom in Nakdong River, West-Nakdong River, Habchun Lake and Hoidong Reservoir, South Korea (Fig. 1). These bloom, in which *Microcystis* sp. and *Aphanizomenon* sp. were predominated, underwent several complex population successions as determined by microscopy and microcystin assay.

Cyanobacteria was observed and indicated in morphology by microscopic examination, that was pure cultured. Genomic DNA was isolated these samples, PCR was performed by synthesized two pairs of primer, 16S rRNA, PC-IGS and NMT region which primer of NMT regions to amplify gene for producing liver toxin was designed.

The results of the agarose gel (1%) electrophoresis of PCR amplified products were observed. 16S rRNA primer set yields a 943 bp product, PC-IGS primer set yields a 700 bp and NMT primer set yields a 1300 bp (Fig. 2).

For the DNA sequencing, amplified products were purified and performed cloning in pGEM-T vector (promega). By comparison with designed 16S rRNA sequences and known PC-IGS gene regions in all of the cyanobacteria, it was tried to study identification in this study (Fig. 3). From cultured samples, after DNA purification, it could be performed PCR reaction using each pairs of primer. As the result of DNA sequencing in designed 16S rRNA and PC-IGS regions, seven standard strains is homological to 100% in species character. On the basis of this information, cyanobacteria in environmental samples were identified by using designed 16S rRNA and PC-IGS primer (Table 1). As the result, the DNA sequence analysis of PC-IGS gene is more identical than that of 16S rRNA. Seven strains of NIES matched to 100% by two pairs of primer in species identification. The six of nine samples were identified to *Microcystis aeuroginosa*, the rest three samples to *Aphanizomenon* sp. (Table 2).

The DNA sequencing analysis of the synthesized gene, 54.5% of standard samples and 56% of samples

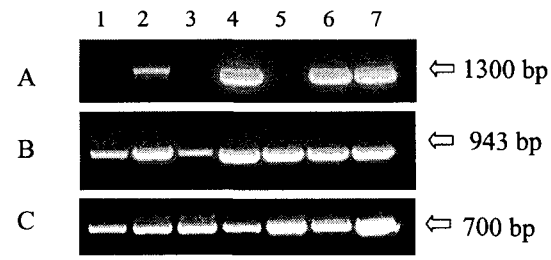

Fig. 2. PCR amplification of cyanobacteria from water bloom. PCR mixture were run on a 1% agarose gel in 1 × TAE. The gel was stained with ethidium bromide and photographed under UV transillumination.
A. NMT region (1300 bp), B. 16s rRNA (943 bp), C. PC-IGS region (700 bp)
(1. Sample'No.5, 2. No.6, 3. No.10, 4. No.11, 5. No.14, 6. No.15, 7. No.16)

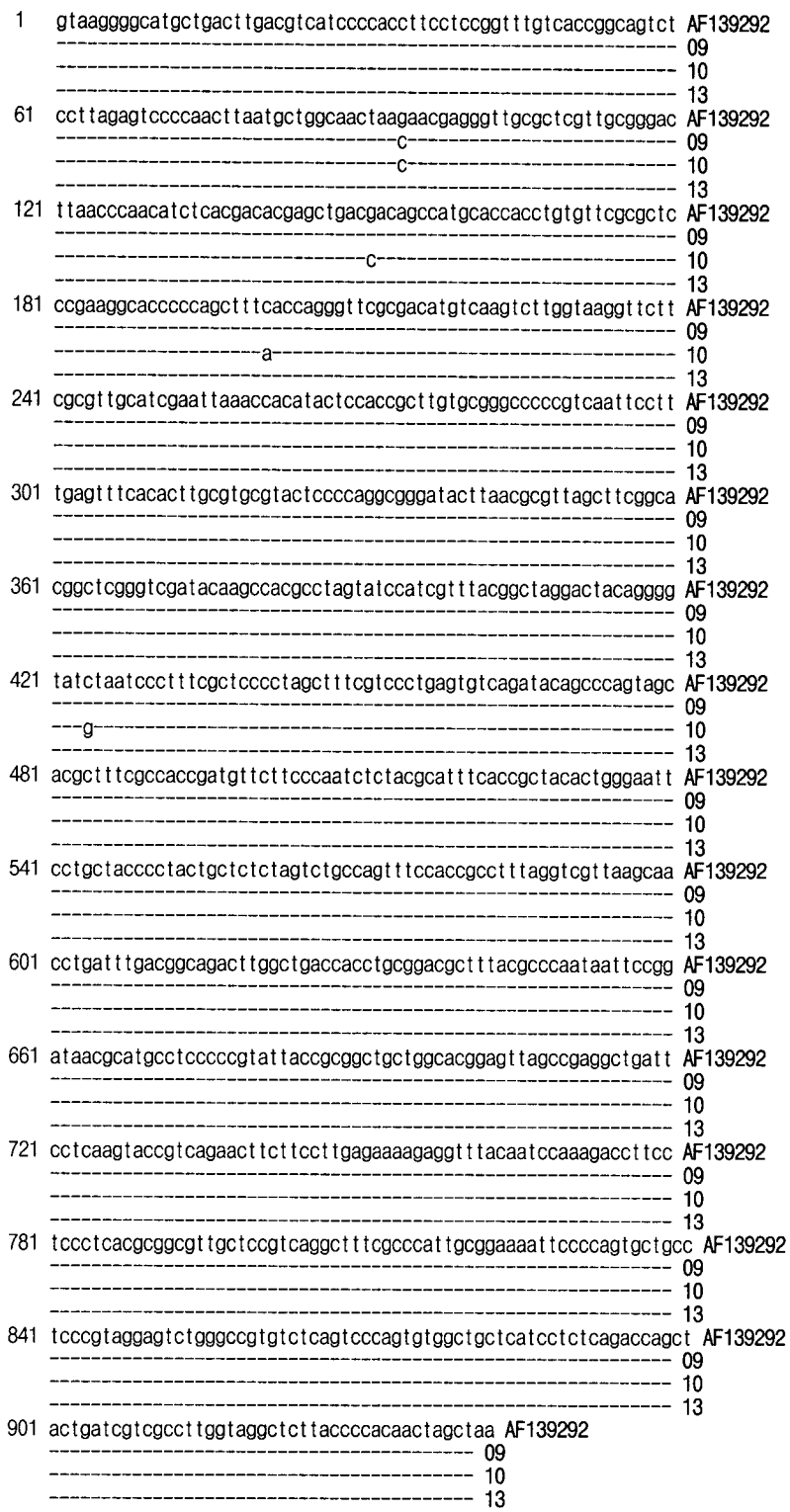

Fig. 3. 16S rRNA sequence of *Microcystis* sp. from Nakdong River aligned with that of *M. aeruginosa.* (AF 139292)

Table 2. Cyanobacterial strains used in this study

No.	Strains	NMT	Source & Culture collection
1	*Microcystis aeruginosa*	-	NIES 44
2	*Aphanizomenon flos-aquae*	+	NIES 81
3	*Microcystis aeruginosa*	-	NIES 87
4	*Microcystis aeruginosa*	+	NIES 88
5	*Microcystis aeruginosa*	-	NIES 99
6	*Microcystis viridis*	+	NIES 102
7	*Microcystis viridis*	+	NIES 103
8	*Microcystis aeruginosa*	+	Kangdong (4 Jun. 1999)
9	*Microcystis aeruginosa*	+	Noksan (20 Aug. 1999)
10	*Microcystis aeruginosa*	-	Habchun (20 Aug. 1999)
11	*Aphanizomenon* sp.	+	Mulgum (19 Jul. 2000)
12	*Aphanizomenon flos-aquae*	+	Hoidong (20 Jan. 2000)
13	*Microcystis aeruginosa*	-	Mulgum (21 Jun. 2000)
14	*Microcystis aeruginosa*	-	Noksan (28 May 1999)
15	*Microcystis aeruginosa*	-	Kangdong (9 Sep. 1998)
16	*Aphanizomenon* sp.	+	Hoidong R.(29 Jun. 2000)

a. Identified strains by PCR product of 16S rRNA and PC-IGS.
b. Presence(+) or absence(-) of the microcystin synthetase NMT domain.

Table 3. Microcystins concentration of samples by HPLC analysis

NO.	Site	Date	Dominance species	Microcystin cons.(μg/g)	
				MC-RR	MC-LR
8	Kandong	4 Jun. 1999	*Microcystis aeruginosa*	221.2	62.4
9	Noksan	20 Aug.1999.	*M. aeruginosa*	45.7	7.9
10	Habchun	20 Aug. 1999	*M. aeruginosa*	48.7	166.5
11	Mulgum	19 Jul. 2000	*Aphanizomenon* sp.	150.7	24.1
12	Hoidong	20 Jan. 2000	*Aphanizomenon* sp.	37.4	16.3
13	Mulgum	21 Jun. 2000	*Microcystis aeruginosa*	61.3	22.9
14	Noksan	28 May 1999	*M. aeruginosa*	179.7	115.7
15	Kangdong	9 Sep. 1998	*M. aeruginosa*	150.7	24.1
16	Hoidong	29 Jun. 2000	*Aphanizomenon* sp.	9.6	5.1

※ MC-RR : Microcystin-RR, MC-LR : Microcystin-LR

in Nakdong River were observed to possibility of toxin production and 100% of *Aphanizomenon* sp. of isolated cyanobacteria had productivity of liver toxin, too.

The microcystin concentrations in algal samples were analyzed by HPLC. Microcystins were composed of microcystin-LR and microcystin-RR. The concentration of microcystin in samples from Nakdong River was observed microcystin-RR to 9.6-221.2 μg/g and microcystin-LR to 5.1-62.4 μg/g (Table 3).

4. Discussion

The development of a molecular method for the identification of cyanobacteria is essential for the rapid and accurate analysis of cyanobacteria in Nakdong River. 16S rRNA and PC-IGS are currently the most promising approach for phylogenetic classification and identification of cyanobacteria[14]. Several previous studies of *Microcystis* have also shown no correlation between toxicity and other phenotypes or genotypes[15,16].

In this study, NIES seven strains and environmental samples were compared with 16S rRNA and PC-IGS region, each of samples were cultured purely and identified morphology. The used primer in this study were mismatched in two bases to known 16S rRNA primer but, the result of DNA sequencing is better

than that of known 16S rRNA primer. Cultured sample, 67% of the samples were identified as *Microcystis aeuroginosa.* As the result, cyanobacteria, *Microcystis aeuroginosa* may be dominant species of Nakdong River.

The differences of identification using 16S rRNA and PC-IGS were existed. If the research is performed at the many samples of cyanobacteria in involving Nakdong River, it could proceed accurately genetical classification. The data for *mcyA* are consistent with the observations of Neilan *et al.*[3] and Nubel *et al.*[16] that microcystin production by various microcystis strains is linked to the presence of the *mcyB* gene and to the occurrence of specific adenylation domains within the *mcyABC* region.[17,18].

The NMT-specific primers (MSF and MSR) reliably were identified toxigenic *Microcystis* cultures and primers of NMT region was developed by Neilan *et al.*[17].

In the paper, the presence or absence of NMT domain in strains was detected by PCR methods. The presence of NMT domain is to detect possibility of synthesizing toxin genetical method. The presence or absence of microcystin synthetase gene was observed in cyanobacteria in Nakdong River. We performed PCR amplification in sixteen samples. In the only one of standard strains, *Microcystis aeuroginosa,* and five of nine samples in Nakdong River were detected to microsystin synthetase gene. It could be detected in pure cultured *Aphanizomenon* sp., too.

The presence of microcystin synthetase gene of *Aphanizomenon* sp. indicated to produce microcystin in the condition of habit. In the before and after cultured algae, the result of PCR amplification on the NMT regions was informed to be able to produce microcystin by changing environmental condition.

And, it is considered that to prevent harm of microcystins from indicating to produce microcystin in cyanobacteria by amplification of NMT regions in the environmental samples. And if it is succeeded to study how to control transcription and expression of this NMT genes by changing environmental conditions, it is expected to be reported many methods for controlling cyanobacteria to produce toxins.

References

1) Carmichael, W. W., 1997, The cyanotoxins. In: Callow, J. A. (ED.) Advances in Botanicak research, 27, Academic press, London, 211- 256pp.
2) Ha, K., H. W. Kim and G. J. Joo, 1998, The phytoplankton succession in the lower part of hypertrophic Nakdong River (Mulgum), South Korea, Hydrobiologia, 369/370, 217-227.
3) Oh, H. M., S. J. Lee, M. H. Jang and B. D. Yoon, 2000, Microcystin production by *Microcystis aeruginosa* in a phosphorus-limited chemostat, Appl. Environ. Microbiol., 66, 176-179.
4) Park, H. D., E. B. Kim and T. Okino, 1998, Hepatotoxic microcystins and neurotoxic anatoxin-a in cyanobacterial blooms from Korean Lakes. Environ, Toxicol. Water Qual., 13, 225-234.
5) Ben-Amotz, A., T. G. Tornabene and W. H. Thomas, 1985, chemical profile of selected species of microalgae with emphasis on lipids, J. Phycol, 21, 72-81.
6) Komarék, J., 1994, Current trends and species delimination in the cyanoprokaryote taxanomy, Archiv für Hydrobiologie (suppliment: Algological Studies), 75, 11-29.
7) Tatsumi, K., M. F. Watanabe and M. Watanabe, 1991, Allozyme divergence in Anabaena(Cyanophyceae) and its taxonomic inference, Arch. Hydro biol. Suppl., 92, Algological Studies, 6, 129-140.
8) Neilan, B. A., P. T. Cox, P. R. Hawkins and A. E. Goodman, 1994, 16S ribosomal DNA gene sequence and phylogeny of toxic *Microcystis* sp. (Cyanobacteria), DNA Sequence, 4:333-337.
9) Nubel, U., F. Garcia-Pichel and G. Muyzer, 1997, PCR Primers to Amplify 16S rRNA Genes from Cyanobacteria, Appl. Environ. Microbiol., 63, 3327-3332.
10) Rudi, K., O. M. Skulberg and K. S. Jakobsen, 1998, Evolution of Cyanobacteria by Exchange of Genetic Material among Phyletically Related Strains, J. Bacteriol., 180, 3453-3461.
11) Neilan, B. A., D. Jacobs and A. E. Goodman, 1995, Genetic Diversity and Phylogeny of Toxic Cyanobacteria Determined by DNA polymorphisms within the phycocyanin Locus, Appl. Environ. Microbiol., 61, 3875-3883.
12) Wilson, K. M., M. A. Schembri, P. D. Baker and C. P. Saint, 2000, Molecular Characterization of Toxic Cyanobacterium *Cylindrospermopsis raciborskii* and Design of a Species-Specific PCR,

Appl. Environ. Microbiol., 66, 332-338.

13) Harada, K. I., K. Matsuura and M. Suzuki, 1988, Analysis and purification of toxic peptides from cyanobacteria by reversed-phase high-performance liquid chromatography, J. Chromatography, 448, 275-283.

14) Wilmotte, A., J. M. Neefs and R. D. Wachter, 1994, Evolutionary affiliation of the marine nitrogen-fixing cyanobacterium *Trichodesmium* sp. strain NIBB 1067, derived by 16S ribosomal RNA sequence analysis, Microbiology, 140, 2159-2164.

15) Otsuka, S., S. Suda, R. Li, M. Watanabe, H. Oyaizu, S. Matsumoto and M. M. Watanabe, 1998, 16S rRNA sequence and phylogenetic analysis of *Microcystis* strains with and without phycoerythrin, FEMS Microbiol. Lett., 164, 119-124.

16) Otsuka, S., S. Suda, R. Li, M. Watanabe, H. Oyaizu, S. Matsumoto and M. M. Watanabe, 1999, Phylogenetic relationships between toxic and non-toxic strains of the genus *Microcystis* based on 16S to 23S internal transcribed spacer sequence, FEMS Microbiol Lett., 172, 15-21.

17) Neilan, B. A., E. Dittmann, L. Rouhiainen, R. A. Bass, V. Schaub, K. Sivonen and T. Borner, 1999, Nonribosomal Peptide Synthesis and Toxigenicity of Cyanobacteria, J. Bacteriol., 181(13), 4089-4097.

18) Nishizawa, T., M. Asayama, K. Fujii, K. Harada and M. Shirai, 1999, Genetic analysis of the peptide synthetase genes for a cylic heptapeptide microcystin in *Microcystis,* spp. J. Biochem., 126, 520-529.