The Journal of Microbiology (2012) Vol. 50, No. 2, pp. 186–190
DOI 10.1007/s12275-012-1418-9

Identification and Enumeration of *Microcystis* Using a Sandwich Hybridization Assay

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(Received August 24, 2011 / Accepted November 18, 2011)

Based on sequence analyses of phycocyanin intergenic spacers (PC-IGS) from *Microcystis*, *Anabaena*, *Aphanizomenon*, and *Planktothrix* (*Oscillatoria*) strains, a genus-specific probe pair TF/TR was designed, and a sandwich hybridization assay was established to quantitatively detect *Microcystis*. Through BLAST and cyanobacterial culture tests, TF/TR was demonstrated to be specific for *Microcystis*. A calibration curve for the sandwich hybridization assay was established, and the lowest detected concentration was 100 cell/ml. Laboratory and field samples were analyzed with both sandwich hybridization assay and microscopy. The biotic and abiotic components of the samples were of little disturbance to the sandwich hybridization assay. The results showed no distinct difference between the two methods. In this study, a sandwich hybridization assay was established to detect *Microcystis*, providing an alternative to traditional microscopic, morphology-based methods.

Keywords: *Microcystis*, molecular probes, sandwich hybridization assay, PC-IGS

Introduction

In eutrophic freshwater lakes, cyanobacterial biomass occurrences are generally formed by *Anabaena*, *Microcystis*, and *Planktothrix*. Rapid and reliable genus identification and enumeration are necessary for water monitoring programs. Taxonomists have traditionally used morphology as the basis for identification of cyanobacteria. Microscopic analysis is the most popular method for observing abundant cyanobacteria. With the development of the GenBank, EMBL and DDBJ databases, many sequences of cyanobacteria have become available. Molecular techniques have been used to detect cyanobacteria within the phytoplankton community. Based on phycocyanin noncoding intergenic spacers (PC-IGS) and other special gene (such as 16S rRNA and 16S-23S rRNA internally transcribed spacer regions) analysis techniques, a number of detection methods such as polymerase chain reaction (PCR) technology have been developed for identification of cyanobacteria (Neilan *et al.*, 1997; Otsuka *et al.*, 1999; Rudi *et al.*, 2000; Baker *et al.*, 2001; Matsunaga *et al.*, 2001; Tillett *et al.*, 2001; Pan *et al.*, 2002; Kurmayer and Kutzenberger, 2003; Vaitomaa *et al.*, 2003; Rantala *et al.*, 2006; Tomioka *et al.*, 2008; Li *et al.*, 2009; Al-Tebrineh *et al.*, 2010; Baxa *et al.*, 2010; Semary and Adel, 2010).

The sandwich hybridization assay (SHA) established by Scholin *et al.* (1996) has been used to quantitatively detect several microalgae, for example, *Pseudo-nitzschia pungens*, *Heterosigma akashiwo*, *Fibrocapsa japonica*, and *Alexandrium fundyense* (Scholin *et al.*, 1996; Tyrrell *et al.*, 2001; Anderson *et al.*, 2005; Mikulski *et al.*, 2008; Preston *et al.*, 2009; Zhen *et al.*, 2009). The SHA method shows high potential for analysis of microalgae species. The SHA method detects the presence of target genes in a sample by employing a 'sandwich' of probes. For example, one probe is 'taxon-specific' and captures the target molecules on a solid support. A second 'signal' probe that binds to another site on the same molecule facilitates quantification based on colorimetric detection (Scholin *et al.*, 1996; Anderson *et al.*, 2005). The advantages of SHA include good specificity and sensitivity as well as the convenience of automation. Additionally, SHA has the potential to identify and quantify phytoplankton quickly for routine monitoring in field surveys.

Cyanobacteria of the genus *Microcystis* are among the most commonly reported bloom-forming species in lakes and reservoirs worldwide (Charmichael, 1996; Codd *et al.*, 1999). Detection of *Microcystis* through molecular markers may have great use-potential in routine analysis of aquatic ecosystems (Tan *et al.*, 2010). The purpose of this study was to develop a sandwich hybridization assay with genus-specific probes targeted at PC-IGS sequences to quantitatively detect *Microcystis*.

Materials and Methods

Cyanobacterial strains and culturing

11 cyanobacterial strains, including *Microcystis*, *Anabaena*, *Aphanizomenon*, and *Planktothrix* (*Oscillatoria*) strains (Table 1), were obtained from the Freshwater Algae Culture Collection, Institute of Hydrobiology, Chinese Academy. These strains were cultured in BG11 medium (Rippa *et al.*, 1979) at 25°C under continuous white light (25 μM/m^2/sec). Cells were harvested in the stationary phase.

DNA extraction and PCR

Each culture (10 ml) of the above cyanobacterial strains was pelleted by centrifugation and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Quartz sand

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Table 1. Cyanobacterial strains used in this study and SHA specificity

Genus	Strains	PC-IGS accession number	Absorbance at 405 nm[a, b, c]
Microcystis	*Microcystis aeruginosa* FACHB 905	HM243151	0.2417±0.0266
	Microcystis aeruginosa FACHB 915	HM243150	0.2590±0.0299
	Microcystis aeruginosa FACHB 942	HM243152	0.2430±0.0239
	Microcystis aeruginosa FACHB 469	HM243153	0.2505±0.0052
	Microcystis wesenbergi FACHB 1112	JF805723	0.2579±0.0262
	Microcystis viridis FACHB 979	JF798342	0.2581±0.0137
	Microcystis flos-aquae FACHB 1028	JF798343	0.2412±0.0098
Planktothrix (*Oscillatoria*)	*Planktothrix agardhii* FACHB 1166	JF923653	0.0705±0.0099
	Oscillatoria planctonica FACHB 708	JF798345	0.0308±0.0051
Aphanizomenon	*Aphanizomenon issatschenkoi* FACHB 1247	JF916568	0.0173±0.0056
Anabaena	*Anabaena flos-aquae* FACHB 245	JF798344	0.0646±0.0062

[a] In this SHA assay, the cyanobacterial strains used were at the similar concentration (approximately 7,000 cell/ml).
[b] data was mean±SD, n=3.
[c] Absorbance at 405 nm for negative control was 0.0345±0.0089.

(analytical grade) was added and shaken with the rapid mixing device (Touch Mixed Model SK-1, China) to break cells. After centrifugation, the solution was extracted twice with equal volumes of phenol-chloroform-isoamyl alcohol (25:24:1) followed by a chloroform-isoamyl alcohol (24:1) extraction. The supernatant was collected and DNA was precipitated by addition of two volumes of ethanol. The precipitated DNA was washed with 70% (v/v) ethanol, dried, and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Primers PCβF (5′-GGCTGCTTGTTTACGCGAC-3′) and PCαR (5′-CCAGTACCACCAGCAACTAA-3′) (Neilan, 1995) for PCR amplification of PC-IGS segments were synthesized by SBS Genetech Co. Ltd (China). The PCR mixture (25 ml) was comprised of 1×PCR buffer, 2.0 mM $MgCl_2$, 0.4 mM of each PCR primer, 300 mM of each deoxynucleotide triphosphate, 1 U DNA polymerase (Bioer, China), and 10 ng DNA from the cultured cyanobacteria. PCR amplification was performed in an Eppendorf PCR thermocycler (Germany) as follows: an initial denaturation at 95°C for 5 min; 30 cycles of 95°C for 30 sec, 50°C for 30 sec, 72°C for 1 min; and the final extension at 72°C for 5 min.

PCR products of PC-IGS segments were purified with the PCR purification kit (Bioer, China) according to the manufacturer's protocol, and were directly sequenced by GenScript Biotech Co. Ltd. (China). The sequences obtained were deposited into the GenBank database (accession numbers were shown in Table 1).

Probe design for sandwich hybridization assay

The sequences of PC-IGS obtained in this study, were combined with 270 *Microcystis*, 100 *Anabaena*, 19 *Aphanizomenon*, and 18 *Planktothrix* strains available in GenBank. These sequences were all aligned using program Cluster W. The highly conserved region of PC-IGS in the *Microcystis* strains was selected for probe design, which would be used for specific identification of *Microcystis* from other cyanobacteria genera. The hybridization probes TF/TR were designed through program Primer 6.0 (PREMIER Biosoft International, USA).

Probes TF/TR were synthesized by SBS Genetech Co. Ltd (China). For SHA, the capture probe was modified with a 3′ amino modifier C6, which reacted with the isothiocyanate groups on the magnetic beads' surface, and the signal probe was modified with a 5′ biotin modifier for immunosorbent detection.

Coupling reaction and sandwich hybridization assay for sample analysis

The 11 cyanobacterial cultures were pelleted by centrifugation and resuspended in distilled H_2O. Freeze-thawing cycle was repeated five times to break cells. After centrifugation, the supernatant was directly used in SHA.

Magnetic beads modified with isothiocyanate groups (average size 5 μm) were purchased from Shanxi Lifegen Co. Ltd. (China). According to the manufacturer's protocol, the capture probe with a 3′ amino modifier C6 was coupled to the isothiocyanate groups on the magnetic beads' surface. The capture probe was used to immobilize target DNA sequences from the above supernatant, and the solid support transferred the immobilized DNA to the biotinylated signal probe, thus forming the sandwich hybrid complex. The total incubation time for capture and signaling steps was 2 h at 47°C. Subsequently the magnetic beads were washed and resuspended in HEPES buffer (10 mM HEPES, 0.15 M NaCl, 1 mM Mg^{2+}, 0.1 mM Zn^{2+}). The hybridization product was detected by the immunosorbent method. Alkaline phosphatase streptavidin was added into the mixture and incubated at 37°C for 20 min. The magnetic beads were then washed and enzymatic substrate p-nitrophenyl phosphate sodium was added. After incubation, the color reaction was ended by 1M NaOH and absorbance was detected at 405 nm (A_{405}) (Cary 50 UV-Vis Spectrophotometer, Varian, USA).

Specificity testing

The newly developed probe pair TF/TR was tested using 11 reference strains of representative cyanobacteria, including *Microcystis*, *Anabaena*, *Planktothrix* (*Oscillatoria*), and *Aphanizomenon* to determine whether the probes demonstrated an adequate sensitivity and specificity to discriminate *Microcystis* strains. Triplicate tests were done for each sample. Probe specificity could be judged from the absorbance values (Table 1).

Table 2. PC-IGS sequences from several cyanobacterial strains

Genus	Sequences[a]
Anabaena	AAATTACAAA TTAGCTCCCG TCTAAATTGA GCTTTTTTCA TAACCTGCATGGCGTAGCCA TACAAAACAA GCAAGAAACA ATCTAGGAGA TTTTCACCA
Aphanizomenon	TAATTCATTA CAAATAGCTC AGAACCTAAA AGAGCTTATT CTTCTGAAAA CAAGCCACTA ACAATCTAGG AGATTTTCAA CA
Planktothrix (*Oscillatoria*)	TTTTTCTAAA ATCTGAATTT TCATAATTCC ATAGAAGTCG GATTAAATGA CTTCAATTTG TACCTCAACT TTGTAAACCC AATCGGGAGA TAATTCAAGC
Microcystis	TCCCCAGGGC TAGTCTCAAT TAAACCGTAG GAAACTTATT GCAAGTTAT TGGGAGATAC CAAACA

[a] The consensus bases in 101 *Anabaena*, 20 *Aphanizomenon*, 20 *Planktothrix* (*Oscillatoria*) and 277 *Microcystis* strains, separately, were marked red. The region for *Microcystis* probes design was underlined.

Calibration curve

Microcystis aeruginosa FACHB 905 cells at stationary phase were collected. The number of cells was counted using microscopy. Then the cells were broken through five rounds of freeze-thawing cycles. After centrifugation, the supernatant was directly used in SHA. SHA was performed and the absorbance at 405 nm was plotted against the cell number to create the calibration curve.

Field samples assay

Surface water samples were collected from Lake Yujia (30°51′89.42″N, 114°43′08.47″E) and Lake Yuan (30°51′31.19″N, 114°40′96.68″E), Wuhan from June to December in 2010. For each lake, each sample (about 500 ml) was taken at the same position. PCR amplification for PC-IGS was done for each sample. Prior to analysis, each sample was divided into two aliquots. One was analyzed by SHA, and the other was simultaneously observed and enumerated with microscope. Each assay was performed in triplicates.

Results and Discussion

Specificity of the probes

Studies have shown that the PC-IGS sequences can be used to differentiate cyanobacterial species (Neilan *et al.*, 1995; Bolch *et al.*, 1996, 1999; Tan *et al.*, 2010). It has been reported that PC-IGS sequences are relatively conserved in a genus.

PC-IGS sequences from *Microcystis*, *Anabaena*, *Planktothrix*, and *Aphanizomenon* strains were analyzed. The conserved regions in PC-IGS sequences were found in 277 *Microcystis*, 101 *Anabaena*, 20 *Planktothrix* (*Oscillatoria*), and 20 *Aphanizomenon* strains, respectively (Table 2). However no significant similarity of PC-IGS sequences was found among these genera.

PC-IGS sequences from 277 *Microcystis* strains were highly conserved. The probe pair TF/TR (Table 3) was designed based on the highly conserved PC-IGS sequences of these *Microcystis* strains. The results of BLAST showed that the region of *Microcystis*-specific TF/TR probes targeted was identical in *Microcystis wesenbergii*, *Microcystis aeruginosa*, *Microcystis flos-aquae*, *Microcystis botrys*, and *Microcystis viridis* strains. Probes TF/TR had high specificity to *Microcystis* strains. No similarities between TF/TR and other cyanobacterial genera were found.

The specificity of TF/TR probes was tested in several cyanobacterial strains at similar concentrations (approximately 7,000 cell/ml). When probes matched the cyanobacterium, distinct absorbance values were observed (t-test, $P<0.05$). SHA signals generated by other cyanobacteria genera showed significantly lower intensity than those of the target *Microcystis*, similar to the negative control (no cell additions). When *M. aeruginosa* FACHB 905 (7,000 cell/ml) was mixed with *Anabaena flos-aquae* FACHB 245 (0 cell/ml, 7,000 cell/ml or 14,000 cell/ml), or *Oscillatoria planctonica* FACHB 708 (0 cell/ml, 7,000 cell/ml or 14,000 cell/ml), no distinct SHA signal difference was observed (data not shown). From this, we can infer that the existence of other cyanobacteria genera did not disturb *Microcystis* detection by SHA.

Table 3. Molecular probes used in this study

Probes	Sequences	T_m (°C)
TF (capture probe)	5'GCAATAAGTTTCCTACGG-NH_2	48.0
TR (signal probe)	5'Biotin-GGTATCTCCCAATAATCT	42.6

Calibration curves

SHA absorbance of *M. aeruginosa* FACHB 905 at 405 nm was plotted against microscope-based cell number (Fig. 1). The linear regression equation was listed and the linear range was $5.0\times10^2 - 2.5\times10^4$ cell/ml.

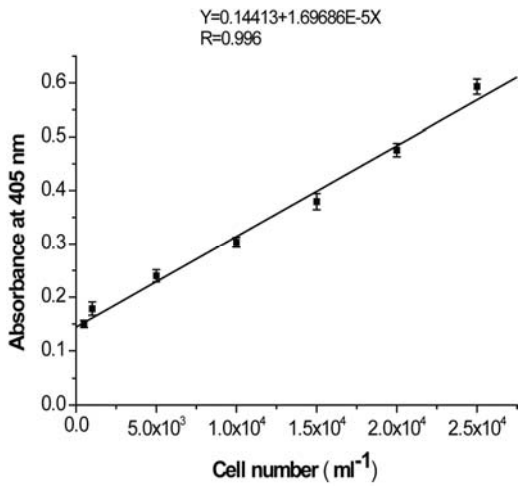

Fig. 1. Calibration curve for absorbance at 405 nm against microscopic cell counts. The experiment was repeated in triplicate tests. All plots represented the mean of triplicate tests, with the error bars showing the standard deviation.

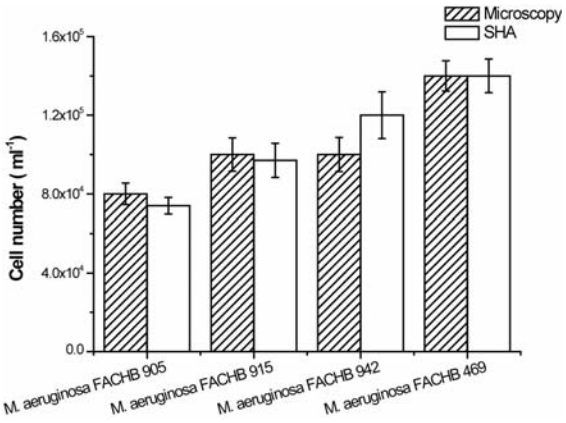

Fig. 2. Comparison of SHA and microscopy data for cultured *Microcystis* (= *M.*) strains. All data represented the mean of triplicate tests, with the error bars showing the standard deviation.

The linearity of the regression equation indicated that it could be used to transform SHA absorbance values to a cell number. Cultured *Microcystis* samples were analyzed with both SHA and microscopy. Cell counts with SHA, calculated according to the calibration curve, were similar to those obtained by microscopy (Fig. 2).

Identification and enumeration of *Microcystis* in water samples

PCR primers PCβF and PCαR were specific for all cyanobacteria. The results of PCR amplification of PC-IGS for each sample showed the existence of cyanobacteria (data not shown). Analysis of field samples by SHA revealed the presence of *Microcystis*.

Through microscopic observation, in field samples from Lake Yujia and Lake Yuan, biotic components (such as rotifers) and abiotic components (such as plant residue) were discovered. *Microcystis* was the dominant cyanobacterium found in these field samples. No disturbances due to cyanobacteria genera were observed during microscopy or SHA assays.

The cell number of *Microcystis* in samples, calculated according to the calibration curve, was compared with the results of microscopic observation (Fig. 3). The calibration-curve cell number showed no distinct difference from the microscopic observation results.

The population of *Microcystis* in Lake Yujia and Yuan was the highest in August. The results agreed with previous reports. Lake Yujia and Yuan have both been identified as eutrophic lakes. The temperature in August was at its annual peak in Wuhan and water blooms were often reported. For example, *Microcystis* bloom broke out in Guanqiao Lake (30°30′47.33″N, 114°23′24.28″E) in August 2009.

In this study, SHA was established to detect *Microcystis*, providing an alternative to microscopy. Though SHA and PCR-ELISA are similar in enzyme-linked assays, SHA is more convenient to perform without PCR amplification. Although real-time PCR is more sensitive than SHA, SHA assays are more cost-effective. The lowest detected concentration of SHA was 100 cell/ml, which was below the concentration observed during blooms. SHA could therefore be applied to quantitative analysis of *Microcystis*.

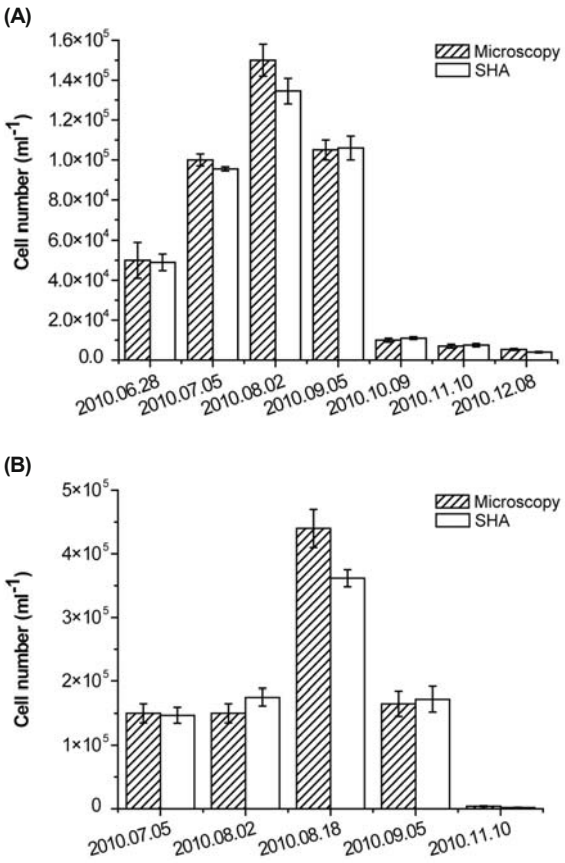

Fig. 3. Comparison of SHA and microscopy data for field samples from Lake Yujia (A) and Lake Yuan (B), with the error bars showing the standard deviation.

Acknowledgements

This work was supported by National Natural Science Foundation of China (20807016) and Central University Special Fund Operational Costs of Basic Research of China (2010MS141).

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