[SiV-5]

In Situ Identification of Cyanobacteria

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Abstract

Seven cyanobacteria strains (*Anabaena macrospora* NIER10016, *Oscillatoria sp.* NIER10042, *Microcystis aeruginosa* NIER10015, *M. ichtyoblabe* BIER10025, BIER10040, *M. novacekii* NIER10029, *M. wesenbergii* NIER10068) were tested with four rRNA - targeted oligonucleotide probes labelled with horseradish peroxidase (HRP) and specific for cyanobacteria. Non-fluorescent detection of hybridization signal was used. The hybridization with artificial mixture of cyanobacteria have shown the possibility to use 2 species-specific probes in duplicate hybridization and detection with different colored substrates.

Introduction

Cyanobacteria, such as *Anabaena*, *Oscillatoria*, and *Microcystis* genera, are widely distributed in lakes, rivers, and soil surfaces thoughout the world. Summer blooming are common in eutrophic freshwater, and sometimes is toxic and poison (5, 6). Therefore, early detection of water blooms caused by potential toxin-producing cyanobacteria is very important in environmental monitoring public and health. Toxic strains of cyanobacteria cannot be distinguished from nontoxic strains without isolation and testing on toxin production (7). Molecular biological techniques are widely spread recently, and suggest new approaches to investigation of cyanobacteria. Some of them are based on PCR application to test cyanobacteria with repeated primer sets (8), 16S rRNA sequencing (9, 10, 11, 12). But only *in situ* hybridization allows to detect single cells (13). And unfortunately, the fluorescent labels that are used in standard protocol of *in situ* hybridization can hardly be used for detection of cyanobacteria due to the strong autofluorescent signal of the cells (1). By Schonhuber and coauthor (1) a new approach was suggested. Nonfluorescent assay is based on using horseradish peroxidase-labelled rRNA-targeted oligonucleotide probes for identification of single cells of cyanobacteria. The molecular weight of horseradish peroxidase (40,000) is approximately 100 times greater than that of fluoresceine or tetramethyllrhodamine (4), so the permeability of cell wall and probe penetration into the cells are the most important steps in this approach. After the hybridization, enzyme marker can be easily detected by its ability to precipitate a colored, nonfluorescent substrates, such as diaminobansidine (DAB) or BM TETON. TETON is more perspective due to its sensitivity and low bleaching compared to

DAB, but the second one is the only stable during hybridization and can be used for the detection of a first signal during the duplicate hybridization. For definition the set-up condition different kinds of cell fixation procedure, prehybridization treatment and hybridization condition were tested in this study.

Materials and Methods

Strains and cultivation. Seven non-axenic cyanobacterial strains were received from National Institute of Environmental Research (NIER), Korea: *Anabaena macrospora* NIER10016, *Oscillatoria sp.* NIER10042, *Microcystis aeruginosa* NIER10015, *M. ichtyoblabe* NIER10025, NIER10040, *M. novacekii* NIER10029, *M. wesenbergii* NIER10068 (Table 1). All of them were cultivated on BG-11 medium with shaker and light-dark cycle.

Table 1. Used Probe

Probe name	Sequencing	Target Site
CYA762	3′-CTGCTTTCGATCCCCTCGC-5′	*E. coli* position 762-780
CYA664	3′-CCCCGTCTCCCTTAAGG-5′	*E. coli* position 664-680
CYA361	3′-CCTTAAAAGGCGTTACCC-5′	*E. coli* position 361-378
CIV/V1342	3′-GCGATCATTAACGTCCAG-5′	*E. coli* position 1342-1359

Probes. Oligonucleotide probes labelled with HRP were obtained from Interactive (Ulm, Germany), and probe sequences are given in the Table 2.

Cell fixation. The cells were fixed either with absolute ethanol, or with paraformaldehyde solution (PFA) (1, 2). For ethanol fixation 3-ml aliquots of mid- to late-log-phase cell cultures were concentrated by centrifugation (10,000 x *g*, 5 min, 4℃), and fixed by addition of the same volume of absolute ethanol. Cells were stored at -20℃. For fixation with PFA solution (2), cells were pelleted by centrifugation, washed once in phosphate buffered saline (PBS) solution (130 mM sodium chloride, 10 mM sodium phosphate buffer [pH 7.2]), centrifuged again, resuspended in 50 ml to 100 ml of 1xPBS, and fixed with fresh (not older than 24 hours), cold PFA solution (4% in PBS). One volume of cell suspention was mixed with 3 volumes of fixative, and the mixture was incubated at 4℃ for 16 hours. The cells were pelleted by centrifugation, washed with PBS solution to remove residual fixative, pelleted again, and resuspended at a concentration of 10^8 to 10^9 cells per ml in PBS solution. One volume of fixed cell suspention was added to 1 volume of cold absolute ethanol, and the mixture was stored at -20℃.

Immobilization of fixed cells on microscope slides. 3-5 $\mu\ell$ of fixed cell suspention was spread on a gelatine-coated slide (3, 4), air dried at least 2 hours and dehydrated in 50, 80,

and 96% ethanol for 3 min each. Prior to hybridization, wells were covered with lysozyme-EDTA solution. Different concentrations and incubation conditions were tested. After lysozyme treatment, slides were washed with distilled water, followed by a second dehydration with ethanol.

***In situ* hybridization with HRP-labelled oligonucleotide probes.** Hybridization was done as recommended by Amman et al. (4). Each hybridization wells were covered with 10 ml of hybridization solution (0.9 M NaCl, 0.01% sodium dodecyl sulphate [SDS], 20 mM Tris HCl [pH 7.2]), contained corresponding concentration of formamide (Table 2) and 0.002 optical density unit of HRP-labelled probe. Slides were incubated at 46℃ in humid chamber during 2 hours, after incubation the slides were washed in hybridization solution with NaCl concentration as recommended Table 2 at 48℃ for 20 min (1). Slides were washed with distilled water and air dried.

Table 2. Concentration of formamide and NaCl

	CYA762	CYA664	CYA361	CIV/V1342
Formamide	65%	55%	50%	50%
NaCl	0 M	0.019 M	0.037 M	0.037 M

In situ detection with non-fluorescent substrates. Two non-fluorescent substrates: diaminobensidine (DAB) (Sigma) and BM TETON POD substrate, precipitating (Boehrenger Mannheim) were used for detection. DAB (4) was dissolved in 150 mM sodium chloride - 50 mM Tris-HCl at a concentration of 0.5 mg/ml and then made 0.003% H_2O_2. BM TETON (1) was prepared by mixing 1 ml of incubation buffer (0.1 M Tris HCl, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 8.0); 5 ml of substrate, and 0.6 ml of 30% H_2O_2 (vol/vol). Each hybridization wells were covered with 30 ml of freshly prepared substrate solution and incubated in humid chamber at room temperature for 2 hours with DAB and 10 min with TETON. The substrate solution was rinsed away with distilled water and slides were air dried. As for analysis of artificial mixture of cyanobacterial cells for the first detection DAB was used as a substrate, and TETON was used for a second detection of hybridization signal. Citifluor was used as the mounting medium for microscopy with Olympus BX 60· Bright-field photomicrographs were done on KODAK 100 film.

Results and discussion

Cell fixation. It was shown that cell suspention fixed with absolute ethanol could be easily used for hybridization without lysozyme treatment for *Oscillatoria sp.* NIER10042. This strain

is too sensitive to lysozyme treatment. For PFA-fixed cells good results were obtained when lysozyme concentration 1 mg/ml and time incubation 10 min at 4℃.

As for *A. macrospora* NIER10016 and *Microcystis* strains, they could be divided into two groups: one included *A. macrospora* NIER10016, *M. ichtyoblabe* NIER10025, NIER10040, and *M. novacekii* NIER10029. All of them were treated with 1 mg/ml of lysozyme at 4℃ for 30 min. Another ones: *M. aeruginosa* NIER10015 and *M. wesenbergii* NIER10068 could be incubated with 1 or 5 mg/ml lysozyme-EDTA solution at 37℃ for 30 min.

Hybridization condition. Probe permeability is the key point in hybridization with HRP-labelled oligonucleotide probes, because of large marker molecule is used for intracellular detection (1, 4). To increase probe penetration 0.1% SDS in hybridzation solution was used with CYA762 probe (4). No differences were found in hybridization with *M. ichtyoblabe* NIER10025.

Some strains of *Microcystis* have no hybridization signal with CYA762 and CYA664 probes, *M. ichtyoblabe* NIER10025, *M. ichtyoblabe* NIER10040. It could be caused with either not enough cell lysis, or cell penetration, or complementary secondary structure of 16S rRNA molecule (1).

Non-fluorescent cell detection. Two substrate were used to detection of hybridization signal. This detection is based on the ability of enzyme peroxidase to precipitate a colored nonfluorescent substrate. DAB is brown colored substrate and it is stable during hybridization, so could be used for the first detection of hybridization prior to the second one (1). BM TETON is dark blue colored substrate. It is more sensitive and more stable during storage the slides.

Whole-cell hybridization. Table 3 shows the hybridization results of cyanobacteria. Non-fluorescent detection of hybridization give strong signal. Probe CYA762 have full homology with targeted region on 16S rRNA *A. macrospora* and *M. aeruginosa* (1). It can be easily detected by DAB sustrate (Fig. 1). Probe CYA664 have shown low hybridization signal, followed by TETON detection with *Oscillatoria sp*, *M. novacekii* and *M. aeruginosa* (Fig. 2). It cannot be depended with low substrate detection, intracellular signal of TETON substrate could be clearly detected into cells of *A. macrospora*. It could be connected with not full homology with targeted region on 16S ribosomal RNA of these cyanobacteria. It is necessary to do sequencing of 16S rRNA gene for these strains to design species-specific probe.

As for CYA 361 and CIV/V1342 probe almost the non-detected cells could be found together with cells, markered with substrate. This might be due to targeted region on 16S ribosomal RNA.

Table 3 Hybridization Results

NIER No.	Scientific name	Hybidization results							
		CYA762		CYA664		CYA361		CIV/V1342	
		DAB	TETON	DAB	TETON	DAB	TETON	DAB	TETON
10015	*M.aeruginosa*	+	+	-	-	-	-	-	-
10025	*M. ichthyoblabe*	-	-	-	-	-	-	-	-
10016	*Anabaena macrospora*	+	+	(+)	+	-	-	+	+
10068	*M. wesenbergii*	+	+	-	-	-	-	-	-
10029	*M. novacekii*	+	+	(+)	+	-	-	-	-
10042	*Oscillatoria* sp.	NA	NA	+	+	+	+	-	-
10040	*M. ichthyoblabe*	+	+	-	-	-	-	-	-

NA : not applied
+ : positive
- : negative
(+) : week signal

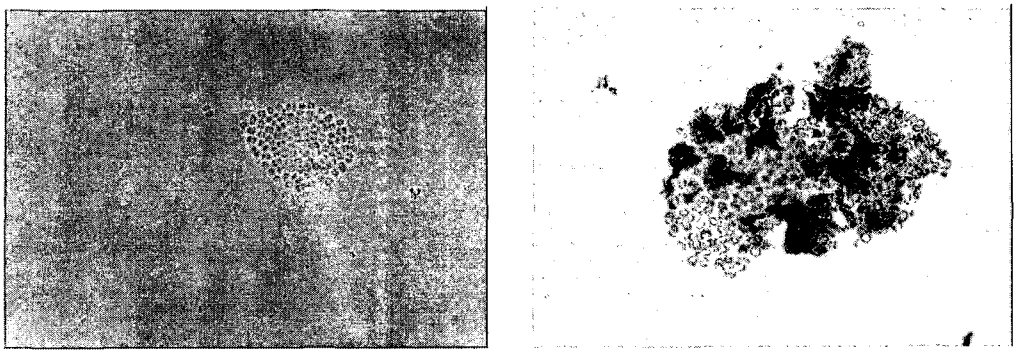

Fig. 1. *Microsystis aeruginosa.* (a) : phase contrast (×1,120), (b) : with CYA762 probe followed by detection with DAB as substrate(×448).

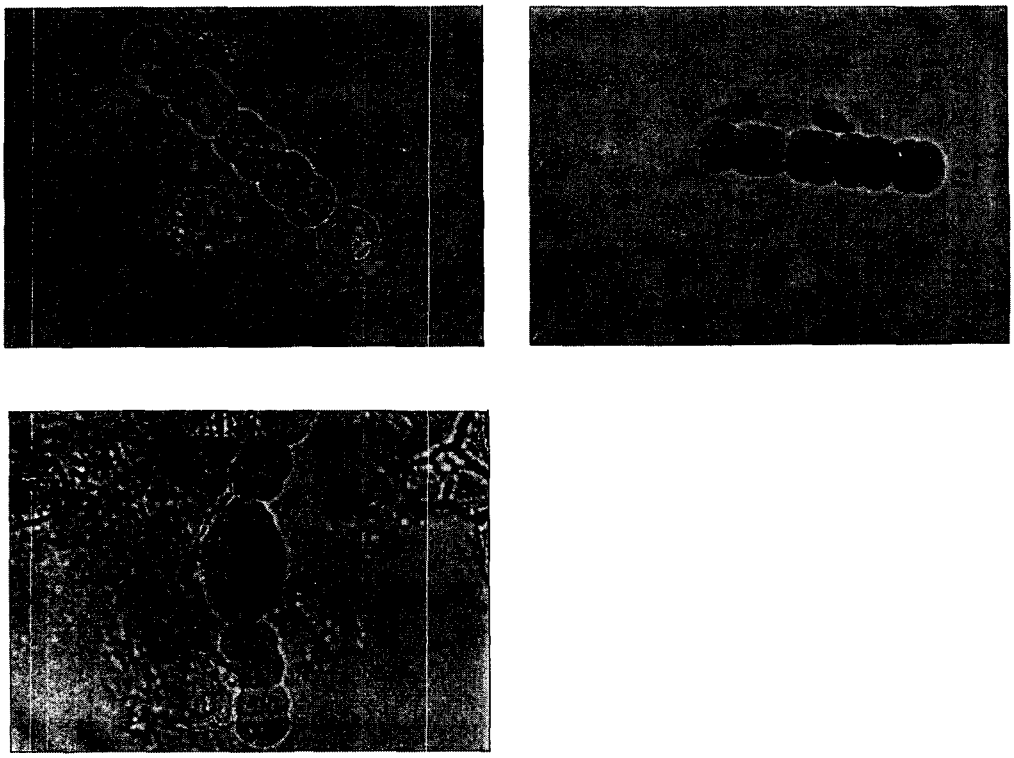

Fig. 2. ***Anabaena macrospora.*** **(a) : phase contrast (×1,120), (b) : with CYA762 probe followed by detection with DAB as substrate (×1,120), (c) : with CYA 664 probe with TETON as substrate(×1,120).**

Future perspectives. Our experience have shown the simple and powerful method for *in situ* whole-cell detection of cyanobacteria. But the problems with non signal detection for some strains of Microcystis could be connected with unknown 16S rRNA sequences for these strains. Design of new species-specific probes is necessary for precise detection of cyanobacteria from environmental samples.

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