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EXPERIMENTAL ARTICLES

The Detection of Cyanobacterial Cells by a Non-Fluorescent *in situ* Hybridization Method

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Abstract—Seven cyanobacterial strains (*Anabaena macrospora* NIER10016, *Oscillatoria* sp. NIER10042, *Microcystis aeruginosa* NIER10015, *M. ichtyoblabe* NIER10025 and NIER10040, *M. novacekii* NIER10029, and *M. wesenbergii* NIER10068) were tested by a nonfluorescent in situ hybridization method using two specific horseradish peroxidase–labeled oligonucleotide probes and two chromogenic substrates. This approach was shown to be appropriate for the analysis of natural samples.

Key words: cyanobacteria, in situ hybridization, horseradish peroxidase.

Cyanobacteria, such as *Anabaena, Oscillatoria,* and *Microcystis,* are widely spread in lakes and rivers. Summer cyanobacterial bloom, which is typical of many eutrophic bodies of freshwater, may lead to poisoning of the water with cyanobacterial toxins [1, 2]. The detection of toxin-producing cyanobacteria in the early terms of the bloom is very important in environmental monitoring. The detection of cyanobacteria by modern molecular biological methods is based on the analysis of ribosomal [3] or structural genes, including those which control toxin production [4–7]. With these methods, either the total cyanobacterial DNA or the DNA of particular cyanobacterial strains is used. Cyanobacterial cells in natural samples are commonly detected by the fluorescent in situ hybridization (FISH) method, although this can also be accomplished by a nonfluorescent hybridization method with the use of horseradish peroxidase–labeled oligonucleotide probes and chromogenic substrates [8].

The aim of the present work was to optimize the conditions of cell fixation, prehybridization treatment, and DNA hybridization, as well as to investigate the appropriateness of the non-fluorescent in situ hybridization method for detecting cyanobacteria in natural samples.

MATERIALS AND METHODS

Strains and cultivation conditions. The seven cyanobacterial strains (*Anabaena macrospora* NIER10016, *Oscillatoria* sp. NIER10042, *Microcystis aeruginosa* NIER10015, *M. ichtyoblabe* NIER10025 and NIER10040, *M. novacekii* NIER10029, and *M. wesenbergii* NIER10068) used in this work were obtained from the National Institute of Environmental Research (NIER), Seoul, South Korea. The strains were grown in BG-11 medium with continuous stirring and variable illumination [9].

Oligonucleotide probes. The horseradish peroxidase–labeled oligonucleotide probes CYA762 and CYA664, whose sequences are presented in Table 1, were purchased from Interactiva (Ulm, Germany).

Cell fixation. Cells were fixed by the two methods described by Schoenhuber *et al.* [8]. With the first method, cells precipitated from a culture (3 ml) by centrifugation at 10 000 *g* for 5 min (4°C) were fixed by adding the equal volume of absolute ethanol and stored at –20°C for future use. With the second method, cells precipitated from the culture as in the first method were washed with phosphate-buffered saline (PBS) containing 130 mM NaCl in 10 mM sodium phosphate buffer, pH 7.2, and resuspended in 50–100 μl of PBS. Cells were fixed by mixing this suspension with 3 volumes of a cold fixation solution (4% paraformaldehyde in PBS) prepared no earlier than 24 h before fixation and incubating the mixture at 4°C for 16 h [10]. The fixed cells were precipitated by centrifugation, washed with PBS, and resuspended in 1 ml of PBS to a concentration of 10^8–10^9 cells/ml. This cell suspension was mixed with an equal volume of cold absolute ethanol, and the mixture was stored at –20°C until needed.

Immobilization of fixed cells on specimen slides. Aliquots of fixed cell suspensions 3 to 5 μl in volume were placed onto gelatin-covered specimen slides [10], dried in the air for 2 h, and then dehydrated in a series of alcohol solutions of increasing concentration (50, 80, and 96% ethanol) for 3 min in each step.

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Table 1. Oligonucleotide probes used in the work

Probe	Sequence (3'–5')	Nucleotide positions *E. coli*	Formamide concentration, %	NaCl concentration, M
CYA762	CTGCTTTCGATCCCCTCGC	762–780	65	0
CYA664	CCCCGTCTCCCTTAAGG	664–680	55	0.013

Prehybridization treatment. Before hybridization, cells immobilized on specimen slides were treated with a solution containing 1–5 mg/ml lysozyme and 0.05 M EDTA in 0.1 M Tris–HCl buffer, pH 8.0 [11]. The preparations were incubated at 4 or 37°C for 10 to 30 min, washed with sterile water, and dehydrated as described above.

In situ hybridization with horseradish peroxidase–labeled oligonucleotide probes. In situ hybridization was performed by the Amann *et al.* method [12]. Each hybridization well on the slides was filled with 10 μl of a hybridization solution containing 0.9 M NaCl and 0.01% SDS in 20 mM Tris–HCl buffer (pH 7.2) and one of the two oligonucleotide probes taken in an amount to give an optical density of 0.002 unit. The hybridization solution also contained formamide at a concentration of either 55 or 65% (Table 1). The preparations were incubated in a humid chamber at 46°C for 2 h. The probe that remained unbound was removed by washing the preparations at 48°C for 20 min with 20 mM Tris–HCl buffer (pH 7.2) containing 0.01% SDS and, in the case of CYA664 probe, 0.013 M NaCl (Table 1) [12]. Then the preparations were additionally washed with distilled water and dried in the air.

The evaluation of hybridization with nonfluorescent substrates. Hybridization was evaluated with two nonfluorescent substrates, diaminobenzidine (DAB), purchased from Sigma, and BM TETON POD, purchased from Boehringer Mannheim [8]. The working solution of the first substrate contained 150 mM NaCl, 0.5 mg/ml DAB, and 0.003% H_2O_2 in 50 mM Tris–HCl buffer [12]. To prepare the working solution of the second substrate, 5 μl of BM TETON and 0.6 μl of 30% H_2O_2 were added to 1 ml of 0.1 M Tris–HCl buffer (pH 8.0) containing 0.1 M NaCl and 0.05 M $MgCl_2$ [8]. Each hybridization well was filled with 30 μl of one of the freshly prepared substrate solutions and incubated in the humid chamber for 2 h (DAB) or 10 min (BM TETON). The substrate that remained unreacted was removed by washing the preparations with distilled water, after which they were dried in the air. Mixed cyanobacterial cultures were analyzed with the use of DAB and then BM TETON. Each slide was mounted with a drop of Citifluor (Citifluor Ltd., Canterbury, United Kingdom) antifadent, overlaid with a coverslip, and examined under an Olympus microscope. Micrographs were made using a KODAK100 film.

RESULTS AND DISCUSSION

Cell fixation and prehybridization treatment. The permeability of the cell wall of some gram-negative bacteria fixed with paraformaldehyde is insufficient to permit the penetration of oligonucleotide probes to cells [10]. The permeability of the cell wall of such cells can be increased by their fixing with absolute ethanol or by pretreating them with lysozyme [10, 11]. Our tentative studies showed that the permeability of cyanobacterial cells can be improved by both absolute ethanol and paraformaldehyde (PFA).

To permit the hybridization of *Oscillatoria* sp. NIER10042 cells, which are extremely sensitive to lysozyme, they can be either fixed with absolute ethanol alone or fixed with PFA and then mildly treated with 1 mg/ml lysozyme at 4°C for 10 min. Experiments showed that to obtain the best results, four strains (*A. macrospora* NIER10016, *M. ichtyoblabe* NIER10025 and NIER10040, and *M. novacekii* NIER10029) should be fixed with PFA and then treated with 1 mg/ml lysozyme at 4°C for 30 min, and the two other strains (*M. aeruginosa* NIER10015 and *M. wesenbergii* NIER10068) should be fixed with PFA and then treated with lysozyme at concentrations of 1 to 5 mg/ml at 37°C for 30 min.

Hybridization conditions. The permeability of bacterial cells is a crucial factor in hybridization with oligonucleotide probes labeled with horseradish peroxidase (HRP) [11, 12], since the molecular mass of HRP (ca. 40 kDa) is two orders of magnitude higher than that of fluorescent probe markers (fluorescein and tetramethylrhodamine isothiocyanate) [12]. To enhance the permeability of *Methanococcus* cells to enzyme-labeled oligonucleotide probes, Amann *et al.* raised the concentration of SDS in the hybridization buffer to 1% [12]. However, our studies with *M. ichtyoblabe* NIER10025 cells and the CYA762 oligonucleotide probe did not reveal considerable differences in the intensity of hybridization at SDS concentrations of 0.01, 0.1, and 1%.

Nonfluorescent detection of cells. Nonfluorescent hybridization is based on the ability of horseradish peroxidase to produce insoluble colored products from chromogenic substrates. We tested two such substrates (DAB and BM TETON) and found that DAB gives rise to stable brown products and can be used as the HRP substrate at the first step of two-step hybridization procedures [8]. As for BM TETON, it gives a deep blue color and is a more sensitive and more stable substrate than DAB during the long-term storage of preparations.

Table 2. The nucleotide sequences of the cyanobacterial probes CYA762 and CYA664 and the respective marker fragments of the 16S rRNA genes of cyanobacterial strains of the genera *Anabaena*, *Oscillatoria*, and *Microcystis* available from the EMBL database

CYA762	CTGCTTTCGATCCCCTCGC
AJ133165 *Oscillatoria* sp.	GACGAAAGCTAGGGGAGCG
AJ133166 *Oscillatoria* sp.	GACGAAAGCTAGGGGAGCG
AJ133185 *Oscillatoria* sp.	GACGAAAGCTAGGGGAGCG
AJ133106 *Oscillatoria* sp.	GACGAAAGCTAGGGGAGCG
AB047103 *Anabaena macrospora*	GACGAAAGCTAGGGGAGCG
AF139292 *Microcystis aeruginosa*	GACGAAAGCTAGGGGAGCG
U03402 *Microcystis aeruginosa*	GACGAAAGCTAGGGGAGCG
U40337 *Microcystis aeruginosa*	GACGAAAGCTAGGGGAGCG
D89031 *Microcystis aeruginosa*	GACGAAAGCTAGGGGAGCG
AB035551 *Microcystis novacekii*	GACGAAAGCTAGGGGAGCG
AB012336 *Microcystis novacekii*	GACGAAAGCTAGGGGAGCG
AB012337 *Microcystis novacekii*	GACGAAAGCTAGGGGAGCG
AB012334 *Microcystis wesenbergii*	GACGAAAGCTAGGGGAGCG
AB035553 *Microcystis wesenbergii*	GACGAAAGCTAGGGGAGCG
D89034 *Microcystis wesenbergii*	GACGAA**G**GCTAGGGGAGCG
U40333 *Microcystis wesenbergii*	GACGAAAGCTAGGGGAGCG
Y12611 *Microcystis* cf. *ichthyoblabe*	GACGAAAGCTAGGGGAGCG
CYA664	**CCCCGTCTCCCTTAAGG**
AJ133165 *Oscillatoria* sp.	GGGGCAGAGGGAATTCC
AJ133166 *Oscillatoria* sp.	GGGGCAGAGGGAATTCC
AJ133185 *Oscillatoria* sp.	GGGGCAGAGGGAATTCC
AJ133106 *Oscillatoria* sp.	GGGG**T**AGAGGGAATTCC
AB047103 *Anabaena macrospora*	GGGGCAGA**A**GGAATTCC
AF139292 *Microcystis aeruginosa*	GGGG**T**AG**CA**GGAATTCC
U03402 *Microcystis aeruginosa*	GGGG**T**AG**CA**GGAATTCC
U40337 *Microcystis aeruginosa*	GGGG**T**AG**CA**GGAATTCC
D89031 *Microcystis aeruginosa*	GGGG**T**AG**CA**GGAATTCC
AB035551 *Microcystis novacekii*	GGGG**T**AG**CA**GGAATTCC
AB012336 *Microcystis novacekii*	GGGG**T**AG**CA**GGAATTCC
AB012337 *Microcystis novacekii*	GGGG**T**AG**CA**GGAATTCC
AB012334 *Microcystis wesenbergii*	GGGG**T**AG**CA**GGAATTCC
AB035553 *Microcystis wesenbergii*	GGGG**T**AG**CA**GGAATTCC
D89034 *Microcystis wesenbergii*	GGGG**T**AG**CA**GGAATTCC
U40333 *Microcystis wesenbergii*	GGGG**T**AG**CA**GGAATTCC
Y12611 *Microcystis* cf. *ichthyoblabe*	GGGG**T**AG**CA**GGAATTCC

Note: Bold underlined letters indicate the nucleotides that differ from the respective nucleotides of the probes.

Both substrates provided for a sensitivity that was sufficient for a reliable microscopic detection of cyanobacterial cells.

Hybridization data. The results of a comparative analysis of the nucleotide sequences of the probes and the marker fragments of cyanobacterial 16S rRNA genes available from the EMBL database are presented in Table 2. It can be seen that the CYA762 probe is completely homologous to almost all strains of the genera *Anabaena*, *Oscillatoria*, and *Microcystis*, except for one nucleotide in the case of *M. wesenbergii* D89034.

Figures 1a and 2a demonstrate the hybridization of *A. macrospora* NIER10016 and *Oscillatoria* sp. NIER10042 cells with the CYA762 probe using DAB

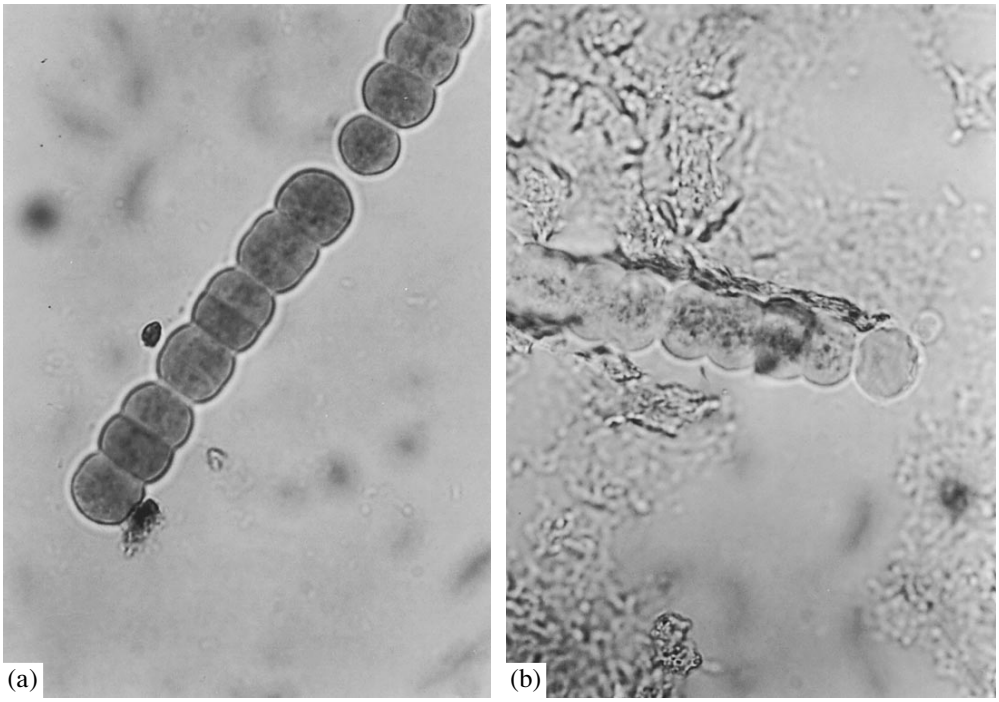

Fig. 1. Micrographs demonstrating the in situ hybridization of *Anabaena macrospora* NIER10016 cells with (a) CYA762 probe and DAB as the HRP substrate and (b) CYA664 probe and BM TETON as the HRP substrate. Magnification, 784×.

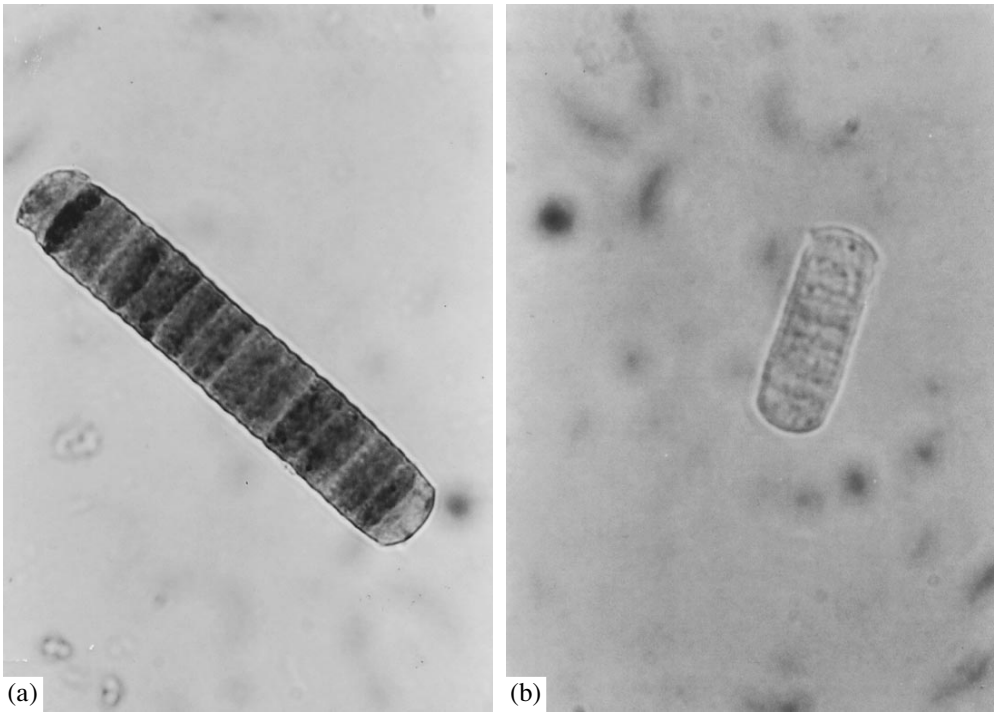

Fig. 2. Micrographs demonstrating the in situ hybridization of *Oscillatoria* sp. NIER10042 cells with (a) CYA762 probe and DAB as the HRP substrate and (b) CYA664 probe and BM TETON as the HRP substrate. Magnification, 784×.

as the HRP substrate. One can easily observe dark brown intracellular inclusions. The presence of 3–4 differing nucleotides in the sequences of the CYA664 probe and *Microcystis* strains resulted in the absence of hybridization between CYA664 and these strains. At the same time, the CYA664 probe well hybridized with the strains *A. macrospora* NIER10016 and *Oscillatoria* sp. NIER10042 with BM TETON as the HRP substrate, although the intensity of hybridization was weaker than in the previous case. This was possibly due to an incomplete homology between CYA664 and the 16S rRNA genes of these two strains. This suggestion is confirmed by the presence of a differing nucleotide in the homologous sequences of the probe and the marker fragments of the 16S rRNA genes of the *Oscillatoria* sp. AJ133106 and *A. anabaena* AB047103 strains available from the EMBL database.

Some cells of some *Microcystis* strains subjected to hybridization with the CYA762 probe did not contain colored inclusions. On the other hand, these strains were found to produce mucoid colonies. It is likely that the slime surrounding the cells of these species is responsible for their irregular fixation and prehybridization treatment, which may influence the results of hybridization analysis. Two *Microcystis* strains, *M. ichtyoblabe* NIER10025 and NIER10040, did not hybridize with either of the oligonucleotide probes used. The absence of the nucleotide sequences of the 16S rRNA genes of *Microcystis* strains in the EMBL database except for that of *M. ichtyoblabe* Y12611 intricates the analysis of homology between the probes and the NIER10025 and NIER10040 strains. In any case, the inability of the probes to hybridize with these strains may be related to the complex secondary structure of the marker fragments of their ribosomal RNA [8].

To investigate the appropriateness of the nonfluorescent in situ hybridization method for the analysis of natural samples, we performed a series of two-step hybridization procedures with the use of CYA664 and DAB at the first and CYA762 and BM TETON at the second hybridization steps. These hybridization experiments were carried out with a mixed suspension of *A. macrospora* NIER10016 and *M. aeruginosa* NIER10015 cells. The use of CYA664 at the first step was dictated by the finding that it did not hybridize with *Microcystis* strains even if the composition of the hybridization buffer was widely varied. These experiments showed that, in fact, the nonfluorescent in situ hybridization procedure can be used to rapidly detect various cyanobacterial strains in laboratory cultures and natural samples.

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