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Development of a Universal Microarray Based on the Ligation Detection Reaction and 16S rRNA Gene Polymorphism To Target Diversity of Cyanobacteria

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The cyanobacteria are photosynthetic prokaryotes of significant ecological and biotechnological interest, since they strongly contribute to primary production and are a rich source of bioactive compounds. In eutrophic fresh and brackish waters, their mass occurrences (water blooms) are often toxic and constitute a high potential risk for human health. Therefore, rapid and reliable identification of cyanobacterial species in complex environmental samples is important. Here we describe the development and validation of a microarray for the identification of cyanobacteria in aquatic environments. Our approach is based on the use of a ligation detection reaction coupled to a universal array. Probes were designed for detecting 19 cyanobacterial groups including *Anabaena*/*Aphanizomenon*, *Calothrix*, *Cylindrospermopsis*, *Cylindrospermum*, *Gloeothece*, halotolerants, *Leptolyngbya*, Palau *Lyngbya*, *Microcystis*, *Nodularia*, *Nostoc*, *Planktothrix*, Antarctic *Phormidium*, *Prochlorococcus*, *Spirulina*, *Synechococcus*, *Synechocystis*, *Trichodesmium*, and *Woronichinia*. These groups were identified based on an alignment of over 300 cyanobacterial 16S rRNA sequences. For validation of the microarrays, 95 samples (24 axenic strains from culture collections, 27 isolated strains, and 44 cloned fragments recovered from environmental samples) were tested. The results demonstrated a high discriminative power and sensitivity to 1 fmol of the PCR-amplified 16S rRNA gene. Accurate identification of target strains was also achieved with unbalanced mixes of PCR amplicons from different cyanobacteria and an environmental sample. Our universal array method shows great potential for rapid and reliable identification of cyanobacteria. It can be easily adapted to future development and could thus be applied both in research and environmental monitoring.

The cyanobacteria are photosynthetic prokaryotes that form a monophyletic group among the eubacteria (29). They are primary producers (3) and a rich source of bioactive compounds (1), and thus, they are ecologically and biotechnologically significant organisms. The cyanobacteria are distributed over a wide range of habitats. In eutrophic fresh and brackish waters, cyanobacteria frequently form toxic water blooms (23) that constitute high potential risks for animal and human health (13).

Traditionally, the identification of cyanobacteria has relied on morphological, physiological, and ecological characteristics that can vary under different environmental or growth conditions (3). Currently, the classification of cyanobacteria is based on a polyphasic approach that considers different phenotypic and genotypic features (29, 30). The molecular classification of cyanobacteria is based on 16S rRNA gene sequences obtained from pure cultures (30). Using this molecular information, several techniques can be employed to determine the cyanobacterial composition of an environmental sample. One of the most informative methods is based on amplification, cloning, sequencing, and phylogenetic reconstruction based on the entire 16S rRNA gene (8, 10). This strategy is very time-consuming and is therefore not suitable for large-scale screenings. Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis have been widely applied to molecular ecological research (18). However, band excision, reamplification, and sequencing are necessary to identify community members.

Therefore, new approaches to the genetic analysis of complex cyanobacterial communities are needed. Recently, oligonucleotide microarrays (microchips) have been used widely in molecular biological studies and have shown great potential for environmental diagnostics. DNA microarray technology has already been applied for the detection of microbial diversity. Microarrays were used for analysis of cultured nitrifying bac-

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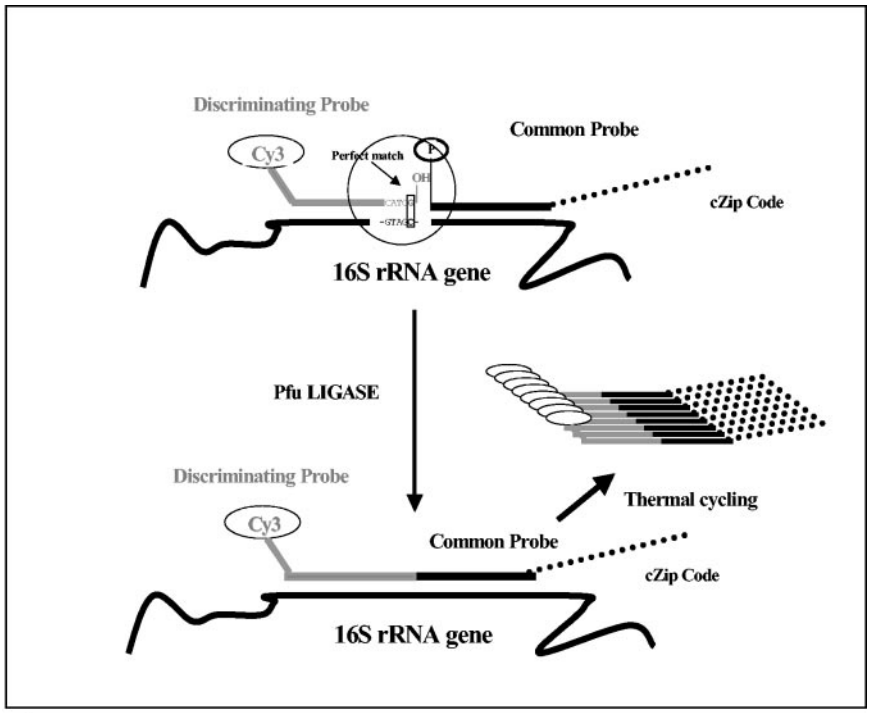

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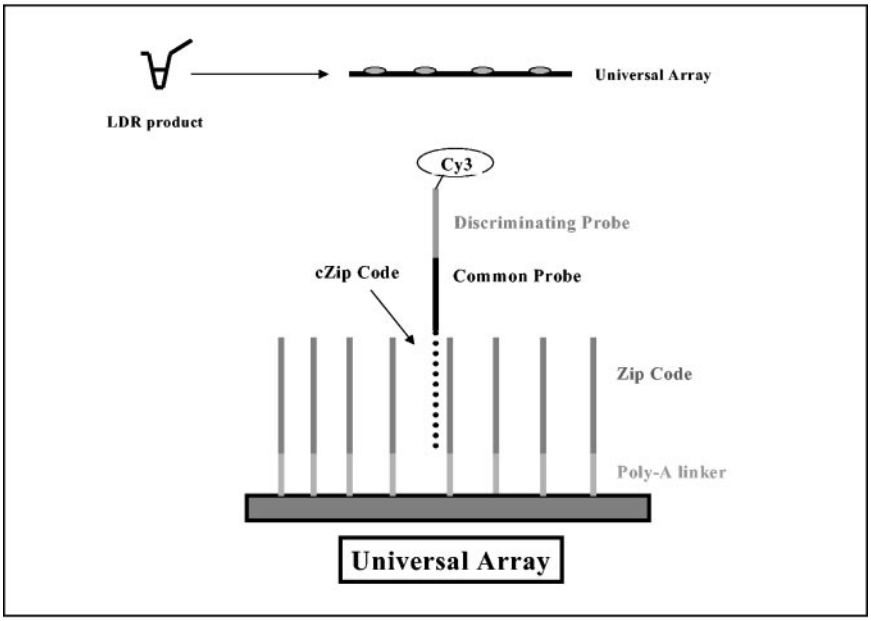

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FIG. 1. Main features of LDR method coupled with a universal microarray. After hybridization of a discriminating probe and a common probe to the target sequence (16S rRNA gene), ligation occurs only if there is perfect complementarity between the two probes and the template (A). The reaction is thermally cycled, generating single-stranded DNA fragments bearing a 5′ Cy3 fluorescent moiety and a 3′ czip code sequence. The cycling allows more common probe (and the corresponding czip code) to ligate to the discriminating probe, given a fixed amount of PCR target. (B) The LDR product is hybridized to a universal microarray, where unique zip code sequences have been spotted.

teria (11) and for the direct detection of 16S rRNA in unpurified soil extracts (24), indicating their applicability for environmental studies. Loy and coworkers (15) and Wu and coworkers (32) tested the microarray method for actual environmental samples, the former for sulfate-reducing prokaryotes and the latter for functional genes of the nitrogen cycle. An oligonucleotide microarray method was also developed for the detection of 20 predominant human intestinal

TABLE 1. Cyanobacterial strains used to validate the LDR procedure

Group	Strain	Geographic origin
Anabaena/Aphanizomenon	*Anabaena cylindrica* PCC 7122	Pond water, Cambridge, England
	Anabaena sp. strain PCC 73105	Pond water, Cambridge, England
	Anabaena sp. strain PCC 7108	Intertidal zone, Moss Beach, Calif.
	Anabaena sp. strain 90	Lake Vesijärvi, Lahti, Finland
	Anabaena sp. strain 202A1	Lake Vesijärvi, Lahti, Finland
	Aphanizomenon sp. strain 202	Lake Vesijärvi, Lahti, Finland
	Aphanizomenon sp. strain PCC 7905	Lake Brielse Meer, The Netherlands
Nostoc	*Nostoc* sp. strain PCC 7107	Shallow pond, Point Reyes, Calif.
	Nostoc sp. strain PCC 8114	Water bloom, Lake Hepetcon, Morris Co., N.J.
	Nostoc punctiforme Hegewald 1971-108	Fish pond, Babat, Hungary
	Nostoc linckia Hegewald 1971-144	Fish pond, Szeged, Feher-tó, Hungary
	Nostoc sp. strain 152	Lake Säaksjärvi, Iitti, Finland
Microcystis	*Microcystis aeruginosa* PCC 9354	Little Rideau Lake, Ontario, Canada
	Microcystis sp. strain PCC 7005	Lake Mendota, Wis.
	Microcystis aeruginosa 1BB38S07	Bubano Basin, Imola, Italy
	Microcystis aeruginosa 0BF29S03	Finissaggio Basin, Imola, Italy
	Microcystis sp. strain 0BB35S01	Bubano Basin, Imola, Italy
	Microcystis ichthyoblabe 0BB39S02	Bubano Basin, Imola, Italy
	Microcystis wesenbergii NIES104	Freshwater lake, Chiyoda-ku, Tokyo, Japan
Synechococcus	*Synechococcus* sp. strain Hegewald 1974-30	Lake Kuusjärvi, Saukkolahti, Finland
	Synechococcus sp. strain 0BB26S03	Bubano Basin, Imola, Italy
	Synechococcus sp. strain WH 7803	Sargasso Sea
	Synechococcus sp. strain WH 8103	Sargasso Sea
	Synechococcus sp. strain 0BB42S04	Bubano Basin, Imola, Italy
Prochlorococcus marinus	*Prochlorococcus marinus* SS120	Sargasso Sea
	Prochlorococcus marinus PCC 9511	Mediterranean Sea
Planktothrix	*Planktothrix* sp. strain 1LT27S08	Trasimeno Lake, Italy
	Planktothrix sp. strain 2	Lake Markusbölefjärden, Åland Islands, Finland
	Planktothrix sp. strain 28	Lake Markusbölefjärden, Åland Islands, Finland
	Planktothrix sp. strain NIVA-CYA 126	Lake Längsjön, Åland Islands, Finland
	Oscillatoria amphibia AGARDH Bai 1971-60	Pond Kakasszeg-tó, Hungary
Spirulina	*Spirulina major* PCC 6313	Brackish water, Berkeley, Calif.
	Spirulina major 0BB22S09	Bubano Basin, Imola, Italy
	Spirulina major 0BB36S18	Bubano Basin, Imola, Italy
Halotolerants	*Cyanothece* sp. strain PCC 7418	Solar Lake, Israel
Nodularia	*Nodularia* sp. strain PCC 73104/1	Alkaline soil, Spotted Lake, British Columbia, Canada
	Nodularia sp. strain BY1	Baltic Sea
	Nodularia sp. strain NSPI-05	Coastal water, Peel Inlet, Australia
	Nodularia sp. strain HKVV	Baltic Sea
	Nodularia sp. strain NSOR-12	Coastal water, Oriental Lagoon, Tasmania, Australia
Cylindrospermum	*Cylindrospermum stagnale* PCC 7417	Soil, greenhouse, Stockholm, Sweden
Synechocystis	*Synechocystis* sp. strain PCC 6905	Low-salinity brine pond, Newark, Calif.
	Synechocystis sp. strain PCC 7008	Shallow pond, Point Reyes Peninsula, Calif.
Calothrix	*Calothrix* sp. strain PCC 7714	Small pool, Aldabra Atoll, India
	Calothrix marchica LEMM. Bai 1971-96.	Pond Belsö-tó, Tihany, Hungary
Leptolyngbya	*Leptolyngbya* sp. strain 0BB24S04	Bubano Basin, Imola, Italy
	Leptolyngbya sp. strain 0BB30S02	Bubano Basin, Imola, Italy
	Leptolyngbya sp. strain 0BB19S12	Bubano Basin, Imola, Italy
	Leptolyngbya sp. strain 0BB32S02	Bubano Basin, Imola, Italy
	Leptolyngbya sp. strain SCHMIDLE Bai 1971-66	Fish pond, Szeged, Feher-tó, Hungary
Lyngbya	*Lyngbya* sp. strain 0BB32S04	Bubano Basin, Imola, Italy

bacterial species (28). Wilson and coworkers (31) used a method based on Affymetrix GeneChip technology to study pure bacterial cultures.

The use of microarrays to specifically characterize cyanobacterial diversity is quite recent. Rudi and coworkers (21) designed a small cyanobacterium-specific microarray for the genera *Microcystis*, *Planktothrix*, *Anabaena*, *Aphanizomenon*, *Nostoc*, and *Phormidium*. Using this assay, the compositions of cyanobacteria in eight lake communities were determined. The DNA microarray and the magnetic-capture hybridization technique have been combined to form a new technology named MAG microarray. Bacterial magnetic particles on a MAG microarray were used for the identification of cyanobacterial DNA (17). Genus-specific oligonucleotide probes for the detection of *Anabaena* spp., *Microcystis* spp., *Nostoc* spp., *Oscillatoria* spp., and *Synechococcus* spp. have been designed from the variable region of the cyanobacterial 16S rRNA gene of 148 strains. All five cyanobacterial genera were successfully discriminated by using both axenic strains and unknown cultured cyanobacteria.

We applied a universal DNA array method to discriminate some groups of bacteria (2). This procedure is based on the discriminative properties of the DNA ligation detection reaction (LDR) and requires two probes specific for each target sequence, as described by Gerry et al. (7). A fluorescent label is coupled to one of the probes, and a complementary zip code

TABLE 2. Clones of 16S rRNA gene libraries obtained from environmental samples and used for validating the LDR

Group	Name	Environmental source
Anabaena/ Aphanizomenon	0TU23C120	Lake Tuusulanjärvi (Finland)
	0TU23C167	Lake Tuusulanjärvi (Finland)
	0TU27CN57	Lake Tuusulanjärvi (Finland)
	0TU34C45	Lake Tuusulanjärvi (Finland)
	0TU34C47	Lake Tuusulanjärvi (Finland)
	0TU34C86	Lake Tuusulanjärvi (Finland)
	0TU34C109	Lake Tuusulanjärvi (Finland)
	0TU34C175	Lake Tuusulanjärvi (Finland)
	0ES24F8	Lake Esch-sur-Sure (Luxembourg)
	0ES24E16	Lake Esch-sur-Sure (Luxembourg)
Microcystis	0TU23C141	Lake Tuusulanjärvi (Finland)
	0TU27C97	Lake Tuusulanjärvi (Finland)
	0TU27CN214	Lake Tuusulanjärvi (Finland)
	0TU27CN235	Lake Tuusulanjärvi (Finland)
	0TU27CN255	Lake Tuusulanjärvi (Finland)
	0TU27CN258	Lake Tuusulanjärvi (Finland)
	0TU27CN297	Lake Tuusulanjärvi (Finland)
	0TU27CN318	Lake Tuusulanjärvi (Finland)
	0TU27CN324	Lake Tuusulanjärvi (Finland)
	0TU27CN329	Lake Tuusulanjärvi (Finland)
	0ES46B58	Lake Esch-sur-Sure (Luxembourg)
Synechococcus	0TU34C70	Lake Tuusulanjärvi (Finland)
	0TU34C89	Lake Tuusulanjärvi (Finland)
	0TU34C113	Lake Tuusulanjärvi (Finland)
	0TU34C129	Lake Tuusulanjärvi (Finland)
	0TU34C134	Lake Tuusulanjärvi (Finland)
	0TU34C148	Lake Tuusulanjärvi (Finland)
	0TU34C154	Lake Tuusulanjärvi (Finland)
	0TU34C157	Lake Tuusulanjärvi (Finland)
	0TU34C176	Lake Tuusulanjärvi (Finland)
	0TU34C189	Lake Tuusulanjärvi (Finland)
Planktothrix	0ES28C14	Lake Esch-sur-Sure (Luxembourg)
	0ES28C10	Lake Esch-sur-Sure (Luxembourg)
	0ES28C20	Lake Esch-sur-Sure (Luxembourg)
	0ES28D25	Lake Esch-sur-Sure (Luxembourg)
	0ES28A2	Lake Esch-sur-Sure (Luxembourg)
	0ES28C18	Lake Esch-sur-Sure (Luxembourg)
	0ES28D3	Lake Esch-sur-Sure (Luxembourg)
	0ES28C8	Lake Esch-sur-Sure (Luxembourg)
	0ES28A5	Lake Esch-sur-Sure (Luxembourg)
Woronichinia	0ES24A3	Lake Esch-sur-Sure (Luxembourg)
	0ES46C21	Lake Esch-sur-Sure (Luxembourg)
	0ES46C32	Lake Esch-sur-Sure (Luxembourg)
	0ES46B48	Lake Esch-sur-Sure (Luxembourg)

(czip code) is coupled to the other. When the proper template is present, the two probes are ligated by the activity of a DNA ligase and are hybridized to the microarray spot that contains the corresponding zip code (Fig. 1). Such an array is called universal, because these zip code pairs could be used with any other probe set.

Here we present the universal DNA array method applied to the detection of cyanobacterial diversity. We designed probes specific for 19 different cyanobacterial groups identified from a phylogenetic tree built with the ARB program (16). The microarrays were validated with axenic and nonaxenic strains of cyanobacteria and an environmental sample.

MATERIALS AND METHODS

All chemicals and solvents were purchased from Sigma-Aldrich (Milan, Italy) and used without further purification. The oligonucleotides were purchased from Thermo Electron GmbH (Ulm, Germany).

DNA samples. The samples used to validate the probes included axenic strains kept in our culture collections, strains isolated from European lakes and a reservoir during this study (Table 1), and clones of environmental DNA libraries obtained from Lake Esch-sur-Sûre (Luxembourg) and Lake Tuusulanjärvi (Finland) (Table 2). The 16S rRNA gene of the cultured strains and clones was sequenced (unpublished data). In addition, the array was tested with an environmental DNA sample (Lake Tuusulanjärvi), which was isolated by the hot-phenol method (9). To verify the microarray results, the same environmental sample was analyzed by DGGE and cloning of the 16S rRNA gene.

Ligation probe design. For the LDR, we designed specific probes for the 16S rRNA gene sequences of 19 different cyanobacterial groups. These groups were identified by using a cyanobacterial 16S rRNA gene alignment built with ARB software, version Beta 011107 (16). The alignment contained 281 sequences from public databases and 57 from this study in addition to the out-group *Escherichia coli*. All of these sequences were longer than 1,400 bp, except the two sequences of Antarctic *Phormidium* (about 1,350 bp) and 21 (of 42) sequences of *Prochlorococcus marinus* (about 1,250 bp). All sequences were aligned with CLUSTAL W (26) and ARB. The sequence alignment is available upon request. The phylogenetic analysis was performed with ARB by using the neighbor-joining (NJ) algorithm (22). From the sequence alignment, group-specific consensus sequences were obtained with a cutoff percentage of 75%. If a base at a given position occurred at a lower frequency than the cutoff percentage, it was replaced by an appropriate International Union of Pure and Applied Chemistry ambiguity code in the consensus sequence. The group-specific consensus sequences were imported to GCG Omiga, version 2.0 (Oxford Molecular Ltd.), for group-specific probe design. The probes were designed by following the LDR approach. After hybridization of a discriminating probe and a common probe to the target sequence, ligation occurs only if there is perfect complementarity between the two probes and the template, in this case, an amplified fragment of the 16S rRNA gene (Fig. 1). For this reason, the discriminating probes were designed to have 3′ ends unique to each of the 19 cyanobacterial groups. The common probes were located immediately after the discriminating probes according to the group-specific consensus sequences. An example of selection is shown in Fig. 2. To discard potentially unspecific probe pairs, we checked each probe pair (discriminating probe and common probe) by using the probe match tool of the ARB program. We also designed a probe pair (named UNICYANO) to detect the presence of any cyanobacteria in the sample. No significant self-annealing of the probe sequences was detected by computer analysis (data not shown). All probes were designed to have a theoretical melting temperature (T_m) between 63 and 68°C, calculated by using the Oligonucleotide Properties Calculator program (http://www.basic.nwu.edu/biotools/oligocalc.html).

We randomly selected 21 czip code sequences from those described by Gerry et al. (7) and Chen et al. (4). These czip codes were randomly assigned to the UNICYANO probe pair, the 19 group-specific probe pairs, and a positive control for the hybridization reaction. The latter was a Cy3-labeled czip code that has its own corresponding zip code in the universal array. As a negative control for the hybridization and LDR, double-distilled water was used instead of genomic DNA as the PCR template. The discriminating probes were labeled with Cy3 at the 5′ end. The common probes were phosphorylated at the 5′ end and carried the czip code at the 3′ end. When a probe sequence contained an ambiguity code, this base was replaced with inosine during oligonucleotide synthesis.

Universal array preparation. The microarrays were prepared by using CodeLink slides (Amersham Biosciences, Piscataway, N.J.), designed to covalently immobilize amino (NH_2)-modified oligonucleotides. The 5′ NH_2-modified zip code oligonucleotides, carrying an additional poly(dA)$_{10}$ tail at their 5′ ends, were diluted to 25 μM in 100 mM phosphate buffer (pH 8.5). Spotting was performed by using a contact-dispensing system (MicroGrid II; BioRobotics, Huntingdon, United Kingdom). The printed slides were processed according to the manufacturer's protocols. Eight arrays per slide were generated. Quality control of the printed surfaces was performed by sampling one slide from each deposition batch. This slide was hybridized with 1 μM 5′ Cy3-labeled poly(dT)$_{10}$ in a solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1 mg of salmon sperm DNA/ml at room temperature for 2 h and then washed for 15 min in 1× SSC. The fluorescent signal was controlled by laser scanning, as described below.

PCR amplifications from DNA samples. The 16S rRNA gene and the internal transcribed spacer region were amplified with universal primer 16S27F (5′-AG AGTTTGATCMTGGCTCAG-3′) (6) and cyanobacterium-specific primer 23S30R (5′-CCTCGCCTCTGTGTGCCTAGGT-3′) (14, 25). The PCR amplifications were performed with a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, Calif.). The reaction mixtures included 500 nM concentrations of each primer, 200 μM concentrations of each deoxynucleoside triphosphate, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1%

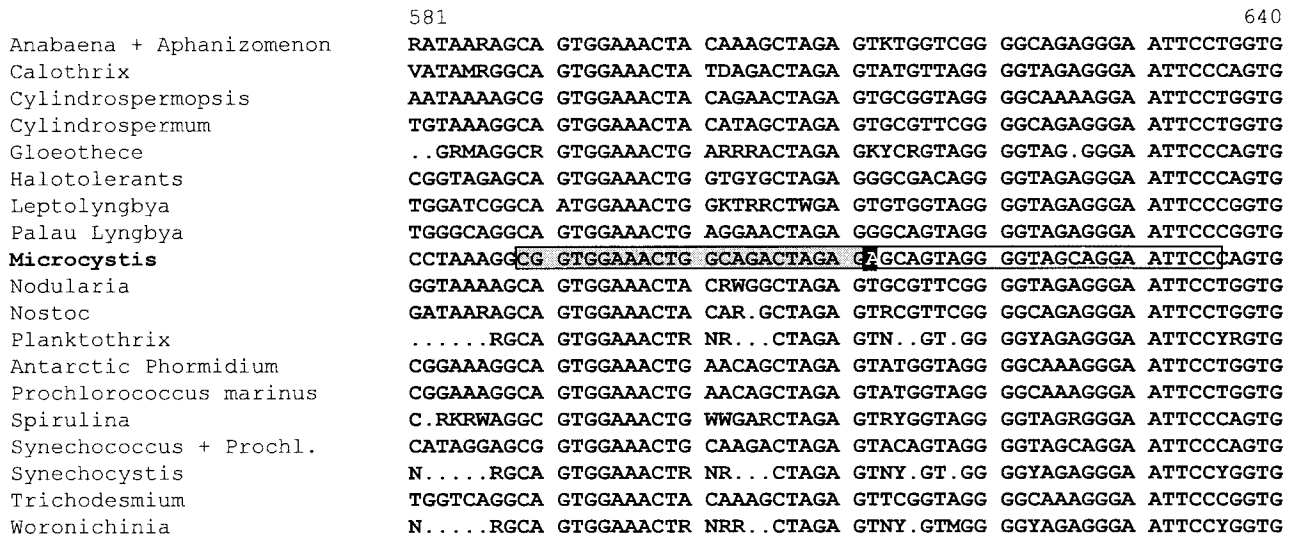


FIG. 2. Partial alignment of group-specific consensus sequences and an example of probe selection for *Microcystis*. The discriminating probe is indicated by light gray box, and the common probe is indicated by an unshaded box. The important base (A) at the 3′ end of the discriminating probe is underlined.

(wt/vol) Triton X-100, 1 U of DyNAzyme DNA polymerase II (Finnzymes, Espoo, Finland), and 5 to 8 ng of genomic DNA in a final volume of 50 μl. Prior to amplification, the DNA was denatured for 5 min at 95°C. Amplification consisted of 30 cycles at 94°C for 45 s, 57°C for 45 s, and 72°C for 2 min. After the cycles, an extension step (10 min at 72°C) was performed. The PCR products were purified by using a GFX PCR DNA purification kit (Amersham), eluted in 50 μl of autoclaved water, and quantified with a BioAnalyzer 2100 (Agilent Technologies, Palo Alto, Calif.).

LDR. The LDR was carried out in a final volume of 20 μl containing 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM $MgCl_2$, 0.1% NP-40, 0.01 mM ATP, 1 mM dithiothreitol, 250 fmol of each discriminating probe, 250 fmol of each common probe, 10 fmol of the hybridization control, and from 0.5 to 100 fmol of purified PCR products. After the reaction mixture was preheated for 2 min at 94°C and centrifuged for 1 min, 4 U of *Pfu* DNA ligase (Stratagene, La Jolla, Calif.) was added. The LDR was cycled for 30 rounds at 90°C for 30 s and at 60°C for 4 min in a GeneAmp PCR system 9700 thermal cycler.

Array hybridization, detection, and data analysis. The hybridization mixture had a total volume of 65 μl and contained 20 μl of LDR mixture, 5× SSC, and 0.1 mg of salmon sperm DNA/ml. After heating at 94°C for 2 min and chilling on ice, the hybridization mixture was applied to the slide, on which the eight arrays were separated by Press-To-Seal silicone isolators (1.0 × 9 mm; Schleicher & Schuell BioScience, Dassel, Germany). Hybridization was carried out in a chamber in the dark at 65°C for 1 h in a temperature-controlled water bath. After hybridization, the slide was washed at 65°C for 15 min in prewarmed 1× SSC and 0.1% sodium dodecyl sulfate. Finally, the slide was dried by spinning at 80 × *g* for 3 min. The fluorescent signals were acquired at a 5-μm resolution by using a ScanArray 4000 laser-scanning system (PerkinElmer Life and Analytical Sciences, Boston, Mass.) with a green laser for Cy3 dye (λ_{ex}, 543 nm; λ_{em}, 570 nm). Both the laser and the photomultiplier (PMT) tube power were set between 70 and 95%, depending on the signal intensities. QuantArray quantitative microarray analysis software (PerkinElmer) was used to quantitate the fluorescent intensity of the spots. The fluorescent intensity values obtained from the replicated spots (four replicate spots for each group-specific probe and eight replicates for the universal probe) and replicated experiment sets (three separate LDR-universal array experiments) were analyzed, and the means and standard deviations were calculated.

Concerning the method used to calculate nonspecific hybridization values, data analysis for each target was performed as follows: (i) the hybridization fluorescent intensities from nonspecific zip codes were calculated and averaged; (ii) the mean of these nonspecific hybridization values was compared with that of the expected positive zip code.

RESULTS

Sequence analysis of cyanobacterial 16S rRNA genes and design of ligation probes. The cyanobacterial groups identified with ARB by using the NJ alghorithm (as described in Materials and Methods) were named after the genus designations of their components: *Anabaena*/*Aphanizomenon*, *Calothrix*, *Cylindrospermopsis*, *Cylindrospermum*, *Gloeothece*, halotolerants, *Leptolyngbya*, Palau *Lyngbya*, *Microcystis*, *Nodularia*, *Nostoc*, *Planktothrix*, Antarctic *Phormidium*, *P. marinus*, *Spirulina*, *Synechococcus*, *Synechocystis*, *Trichodesmium*, and *Woronichinia* (Fig. 3). The list of strains present in each group is available at http://www.ulg.ac.be/cingprot/midichip/output/publications/Castiglioni_Tree.htm. For all of these, a group-specific consensus sequence was determined and used for probe design. The probes were designed to be complementary to the polymorphic regions of the group-specific consensus sequence alignment. We selected 19 group-specific probe pairs and a universal control probe matching all of the cyanobacteria. All of the probes had theoretical melting temperatures between 63 and 68°C. Table 3 lists all of the selected group-specific and universal probes and randomly chosen czip code sequences. Although DNA samples for some of the 19 selected groups (*Gloeothece*, Antarctic *Phormidium*, *Trichodesmium*, and *Cylindrospermopsis*) were not available, they were included to allow future applications of this cyanobacterial microarray.

Validation of universal array designed for cyanobacteria. (i) Specificity of probes. In the presence of a proper DNA template, only group-specific spots, universal spots, and those spots corresponding to the hybridization control showed positive signals. Several examples of the results are shown in Fig. 4. The specificity of the probes for freshwater cyanobacterial groups was tested by using PCR-amplified 16S rRNA genes originating either from 52 cyanobacterial strains (both axenic and isolated in this study) or from 44 clones. Three replicated LDR-universal array experiments showed good reproducibility of the results.

The intensities of signals of nonspecific hybridization for the cyanobacterial groups examined never exceeded 6% with respect to the expected positive signals (Table 4), and this value

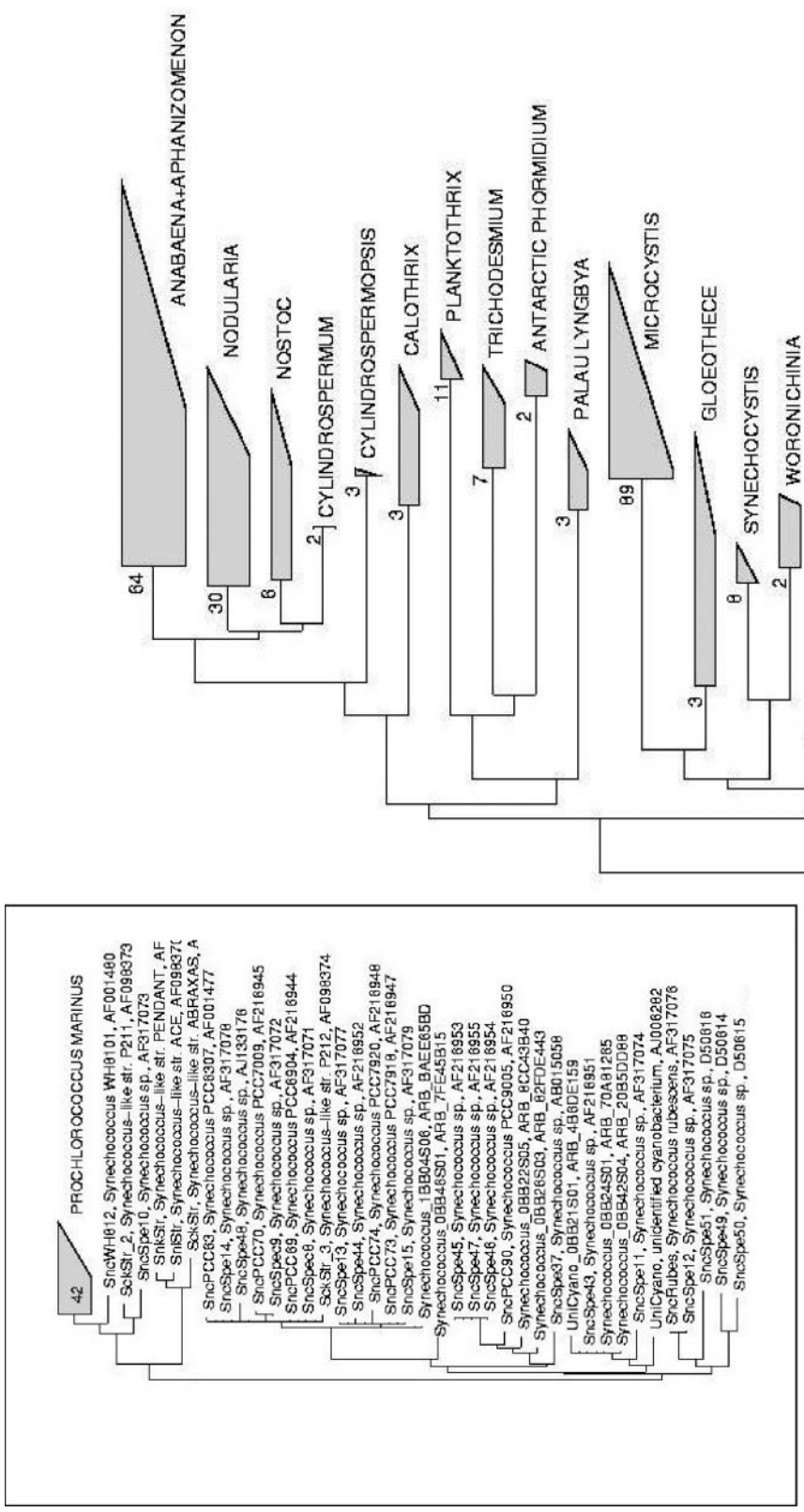


FIG. 3. NJ tree based on 16S rRNA gene sequences showing the 19 cyanobacterial groups. The probes used in the microarray were designed according to these groups. The *P. marinus* group is embedded in the large *Synechococcus* group. The tree contained 338 cyanobacterial 16S rRNA gene sequences. This phylogenetic tree stability has been supported by bootstrap analysis. For bootstrap analysis, 500 resamplings were performed by using the NJ algorithm in ARB. The bootstrap tree is available at http://www.ulg.ac.be/cingprot/midichip/output/publications/Castiglioni_Tree.htm.

was used as the lower limit for positive signals in subsequent microarray analyses.

To evaluate the relative ligation efficiency of the probes, the mean signal intensity values of the group-specific spots for each target were measured and normalized with respect to the signal intensity values of the universal spot. The hybridization intensities of the probes differed and ranged from 92 to 155% (Table 4).

A negative control of the entire process was performed by using double-distilled water instead of genomic DNA as the PCR template. Following hybridization on the universal chip, no signal was detected even after setting the PMT and laser to 95% power (data not shown).

(ii) LDR sensitivity. To establish the detection limit of the method and the correlation between signal intensity and template concentration, we tested various template concentrations (0.5 to 100 fmol) in the LDR. The PCR products originated from the strains *Planktothrix* sp. strain 1LT27S08, *Calothrix* sp. strain PCC 7714, and *Microcystis aeruginosa* PCC 9354. The detected signals progressively decreased, and signal was detectable in up to 1 fmol of the PCR product, corresponding to 1 ng of amplified DNA. No signals were detected with 0.5 fmol of the PCR product, even after setting the PMT and laser to 95% power (data not shown). We found a linear correlation (R^2 = 0.98, 0.94, and 0.96, respectively) between signal intensity and template concentration (Fig. 5A). Nevertheless, the signal-to-noise ratio also decreased with gradually reducing template concentration (Fig. 5B). This ratio was obtained from the signal intensities of the target-specific spots divided by the mean signal intensity of the nonspecific spots. The signal-to-noise ratio was clearly higher at template quantities above 25 fmol than at lower quantities (Fig. 5B). Essentially the same results were obtained with similar concentrations of PCR products derived from *Calothrix* sp. strain PCC 7714 and *M. aeruginosa* PCC 9354 as the LDR substrate (data not shown). Therefore, the target concentration of 25 fmol of each strain or clone was used in the LDR.

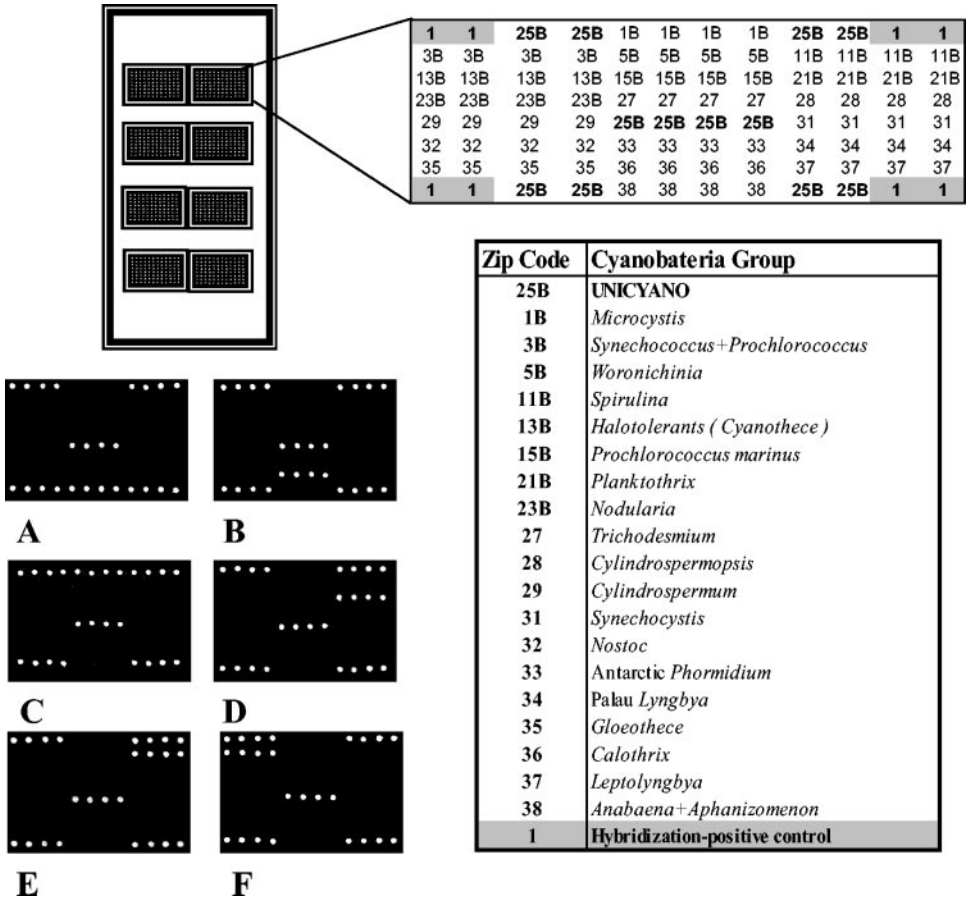
Use of artificial mixes of PCR products from different strains. To determine the efficiency of the LDR method in the presence of complex molecular targets, we used artificial mixes with unequal amounts of PCR products derived from the following cyanobacterial strains: *M. aeruginosa* PCC 9354, *Aphanizomenon* sp. strain 202, *Planktothrix* sp. strain 1LT27S08, *Spirulina major* sp. strain PCC 6313, and *Calothrix* sp. strain PCC 7714. After separate PCRs, the amplified fragments were pooled in the unbalanced mixes. In all of these experiments, the unbalanced mixes were 5 fmol of both *S. major* and *Calothrix* versus 100 fmol of both *Aphanizomenon* and *Microcystis* or *Planktothrix*. After hybridization of the LDR products on the universal array, all of the expected signals were detected and easily discriminated from the nonspecific signals. An example of these experiments is shown in Fig. 6A. The amplicon concentrations were reflected in the signal intensities (Fig. 6A).

LDR detection on universal array of the 16S rRNA gene from an environmental sample. The 16S rRNA gene from a sample collected from Lake Tuusulanjärvi was analyzed to evaluate the DNA microarray applicability for environmental studies. Microarray hybridization patterns showed the presence of *Microcystis*, *Anabaena*/*Aphanizomenon*, and *Woronichinia* spp. (Fig. 6B). In DGGE, the following cyanobacterial

TABLE 3. List of group-specific probes and corresponding czip codes

Polymorphism position[a]	Group name	Discriminating probe sequence (5′–3′)	T_m (°C)	Common probe sequence (5′–3′)	T_m (°C)	czip code sequence (5′–3′)
657-A	*Microcystis*	CGGTGGAAACTGGCAGACTAGAGA	67	GCAGTAGGGGTAGCAGGAATTCCC	68	GCTGAGGTCGATGCTGAGGTCGCA
841-A	*Phochlorococcus* + *Synechococcus*	TGAACACTAGGTGTCGGGGGGA	63	ATCGACCCCTTCGGTGTCGTAG	66	GCTGCGATCGATGGTCAGGTGCTG
618-C	*Woronichinia*	CAAGTCTGTGTCAAAGAATGGAGC	66	TTAACTCCATAAAGGCIGTGGAAACTGAG	67	GCTGTACCCGATCGCAAGGTGGTC
1429-T	*Spirulina*	ACACCATGGAAGCTGGCAACAT	63	CCGAAGTCGTTACTCCAACI	63	CGCAAGGTAGGTGCTGTACCGCA
435-A	Halotolerants	GGCTCTTGGGCTGTCAACCA	63	CTTTTCTCAGGGAAGAAGICCTGACGG	67	CGCACGATAGGTGGTCTACCGCTG
748-T	*Prochlorococcus marinus*	GAAGGCGCTCTGCTGGGCCATT	68	ACTGACGCTCATGGACGAAAGCC	67	CGCATACCAGGTCGCATACCGGTC
1051-C	*Planktothrix*	CGTAAGGAACCAGAAGACAGGTGC	67	TGCATGGCTGTCGTCAGCTCGT	66	GGTCAGGTTACCGCTGCGATCGCA
747-C	*Nodularia*	GAAGGCGCTCTACTAGGCCGC	67	AACTGACACTGAGGGACGAAAGCTA	66	GGTCCGATTACCGGTCCGATGCTG
422-A	*Trichodesmium*	GCGTGGGGGAGGAAIGCTCTA	65	GGGTTGTAAACCCTTTTCTTTGGGAAG	68	GGGTATCCGTTCGGTGTTGCGTAGT
580-T	*Cylindrospermopsis*	CGGAATGATTGGGCGTAAAGGGTCT	67	GCAGGTGGAACTGAAAGTCTGCTG	67	ACCTGGTCAATGGGACCATTGGTCC
632-G	*Cylindrospermum*	GTTAAAGAGCAAGGCTCAACCTTGTAAAG	67	GCAGTGGAAACTACATAGCTAGAGTGCG	68	TATGTCAGTGACGCGCTCAGCGTTG
1262-G	*Synechocystis*	GTCGGGACAACGGCAGCGAG	67	CTCGCGAGAGTAAGCGAATCCCA	67	TGGTGCTGGCGCAGACCTTTGTCTC
1328-C	*Nostoc*	CCGGAGCTCAGTTCAGATCGC	65	AGGCTGCAACTCGCCTGC	63	ACCGCGCAAATGGACAGTGTGGCCA
484-G	Antarctic *Phormidium*	AGAAAGTTGTGAAAGCAGCCTGACG	66	GTACCAGAGGAATCAGCATGGCTA	65	GACCCCAACTTGACACGTCGCAAGG
670-GA	Palau *Lyngbya*	GAACTAGAGGGCAGTAGGGGTAGA	67	GGGAATTCCCGGTGTAGCGGTG	68	GGAGAGTTTGGCGCGACCCTAACCT
801-C	*Gloeothece*	TGTGCCGAAGCTAACGCGTTAAGTC	67	TCCCGCCTGGGGAGTACGCA	67	TGTGCTTACCGCACCTCGCAGTCGT
744-C	*Calothrix*	GTGGCGAAAGCGTTTGCTAGGA	65	CAATACTGACACTGAGGGACGAAAGC	68	GTTGGGTATATCTCCCGGCGATCGC
857-C	*Leptolyngbya*	CGTATCGACCCGTGCAGTRCC	65	GTAGCTAACGCGTTAAGTITCCCGC	67	GTATTGGTGCTCGAGTCCGGCACGA
852-T	*Anabaena* + *Aphanizomenon*	GGIGTAGCTCGTATCGACCCGAGCT	68	GTRCCGIAGCTAACGCGTTAAGTATCCC	68	GTCTACGCCATCGCGGTGCTAAAGC
359-G	UNICYANO	GACTCCTACGGGAGGCAGCAGTG	68	GGGAATTTTCCGCAATGGGC	65	GGTCTACCTACCCGCACGATGGTC

[a] With respect to *E. coli*, used as a reference sequence.



Zip Code	Cyanobateria Group
25B	UNICYANO
1B	*Microcystis*
3B	*Synechococcus*+*Prochlorococcus*
5B	*Woronichinia*
11B	*Spirulina*
13B	*Halotolerants (Cyanothece)*
15B	*Prochlorococcus marinus*
21B	*Planktothrix*
23B	*Nodularia*
27	*Trichodesmium*
28	*Cylindrospermopsis*
29	*Cylindrospermum*
31	*Synechocystis*
32	*Nostoc*
33	Antarctic *Phormidium*
34	Palau *Lyngbya*
35	*Gloeothece*
36	*Calothrix*
37	*Leptolyngbya*
38	*Anabaena*+*Aphanizomenon*
1	Hybridization-positive control

FIG. 4. Deposition scheme and several examples of LDR-universal array results. On the figure, the slide with eight arrays (top left corner), the deposition scheme of an array (top right corner), and a table specifying the cyanobacterial groups and the corresponding zip codes (bottom right corner) are shown. The hybridization-positive control is indicated by light gray shading, and the UNICYANO probe is indicated by boldface type. Each cyanobacterial group has four replicate spots. Hybridization results of the amplified 16S rRNA gene from the strains are shown in the bottom left corner. (A) *Aphanizomenon* sp. strain 202; (B) *Calothrix* sp. strain PCC 7714; (C) *M. aeruginosa* strain PCC 9354; (D) *Plankthotrix* sp. strain 1LT27S08; (E) *S. major* strain PCC 6313; (F) *Synechococcus* sp. strain Hegewald 74-30.

groups were detected: *Microcystis*, *Snowella*, and *Anabaena*/*Aphanizomenon*. Cloning revealed the following groups: *Microcystis* (the most abundant group, 62% of the cyanobacterial clones), *Anabaena*/*Aphanizomenon* (18%), and *Snowella* (15%) (A. Rantala, P. Rajaniemi, and K. Sivonen, unpublished data).

DISCUSSION

Studies in environmental microbiology are often limited by difficulties in identifying the diversity of natural populations because isolation and cultivation of microorganisms from natural environments are sometimes impossible. Molecular approaches are intended to overcome this problem. We designed and tested a microarray-based method for detection of cyanobacterial diversity. The method was based on our previous experience with a bacterial universal array (2). In that study, we developed and evaluated a molecular strategy based on amplification of the cyanobacterial 16S rRNA gene region and its molecular discrimination with the LDR-universal array approach. Using the universal array, we overcame one of the major limitations of DNA microarray approaches based on hybridization. Optimal hybridization conditions are difficult to determine for large sets of different probes, which need to be hybridized on a DNA chip at the same time (15, 21). In the universal array-based approach, the optimization of hybridization conditions for each probe set is not required. New probe pairs can be added to the array without further optimization, thus reducing costs and setup time. Furthermore, problems due to secondary structures of the target DNA or steric hindrances of differently sized nucleic acid hybrids formed on the microarrays after hybridization (20) are minimized. However, this method requires an extra step (the ligation) with respect to DNA microarray approaches based on hybridization.

In the present study, all of the LDR probes selected were designed to have high theoretical melting temperatures to perform the ligation reaction at 60°C, which prevented problems caused by secondary structures of the target DNA. Additionally, the ligated products were sterically similar. In the LDR, the specificity of the hybridization probes and the selectivity of the ligation reaction are combined to increase the discrimination power. Furthermore, with the LDR it is possible to target several PCR amplicons at the same time in a single ligation reaction (multiplexing).

As described by Consolandi et al. (5), up to 54 different

TABLE 4. Evaluation of probe specificity and efficiency

Group name	Target	Signal intensity from nonspecific zip codes ± SD[a]	Probe efficiency ± SD[b]
Microcystis	*M. aeruginosa* 0BF29S03	1.45 ± 0.19	155.08 ± 5.09
	Microcystis sp. strain PCC 9354		
Prochlorococcus + *Synechococcus*	*Synechococcus* sp. strain Hegewald 74-30	0.97 ± 0.63	126.54 ± 4.18
Woronichinia	*Woronichinia* 0ES46C21 clone	2.11 ± 0.37	110.01 ± 10.38
	Woronichinia 0ES46C32 clone		
Spirulina	*S. major* PCC 6313	0.64 ± 0.58	151.23 ± 14.95
	S. major 0BB22S09		
Halotolerants	*Cyanothece* sp. strain PCC 7418	0.84 ± 0.25	124.62 ± 14.91
Planktothrix	*Planktothrix* sp. strain 1LT27508	3.56 ± 1.12	112.60 ± 1.82
	Planktothrix sp. strain 2		
Nodularia	*Nodularia* sp. strain PCC 73104/1	2.31 ± 1.62	123.57 ± 7.21
Synechocystis	*Synechocystis* sp. strain PCC 6905	1.38 ± 1.22	104.98 ± 6.48
Nostoc	*Nostoc* sp. strain 152	3.19 ± 2.58	93.90 ± 3.63
	Nostoc sp. strain PCC 7107		
Palau *Lyngbya*	*Lyngbya* sp. strain 0BB32S04	0.75 ± 0.49	136.61 ± 12.18
Calothrix	*Calothrix* sp. strain PCC 7714	2.85 ± 1.19	114.46 ± 9.28
	Calothrix marchica LEMM Bai 71-96		
Leptolyngbya	*Leptolyngbya* sp. strain 0BB19S12	3.37 ± 1.72	92.81 ± 16.15
	Leptolyngbya sp. strain 0BB32S02		
Anabaena + *Aphanizomenon*	*Anabaena* sp. strain PCC 7122	0.65 ± 0.97	125.68 ± 6.02
	Aphanizomenon sp. strain 202		

[a] Probe specificity is the measurement of intensity values from nonspecific zip codes scaled to the signal intensity of the expected positive zip codes set to a value of 100. Each value represents the mean obtained from replicated spots (four replicate spots for each group, eight replicate spots for the universal) and from three replicate experiment sets.

[b] Probe efficiency is the measurement of signal intensities of the group-specific spots for each substrate, normalized with respect to the signal intensities of the universal zip code. Each value represents the mean obtained from replicated spots (four replicate spots for each group, eight replicate spots for the universal) and from three replicate experiment sets.

discriminating probes can be used in such a multiplex ligation reaction without affecting the efficiency of the method.

We employed semi-cyanobacterium-specific PCR primers, 16S27F-23S30R (14, 25), instead of the universal primer pair, 16S27F-16S1492R (6), to eliminate the diversity of microorganisms other than cyanobacteria. The use of more specific cyanobacterial primers, such as CYA359F and CYA781R (19), which amplify only about 400 bp, would have limited the phylogenetic resolution.

We designed the probes based on a large number of cyanobacterial sequences (338) covering 19 major groups of planktonic cyanobacteria, which is more than in previous studies (17, 21). To allow for wider applicability of the array in future diversity studies, we also included groups not present in lakes, such as *Trichodesmium*.

Probe design can be considered a crucial point in the LDR approach. During definition of the group-specific consensus sequences, we set the cutoff value to preserve as much sequence information as possible. This sometimes required the inclusion of some degenerated positions in the probe sequences. A maximum of two inosine residues was included per probe (Table 3).

We evaluated our array by testing 95 samples of known 16S rRNA gene sequences: 51 strains belonging to 14 phylogenetic lineages and 44 cloned fragments from lake samples. We found perfect correspondence between the expected and actual LDR results. In fact, all samples yielded the positive signals expected without ambiguity. Nonspecific signals were always below 6% of the total signal intensities. This excellent selectivity is needed for testing complex environmental samples.

The relative probe efficiency was determined by normalizing all specific signals to the corresponding universal cyanobacterial probe. The normalized signal ranged from 92% (*Leptolyngbya*) to 155% (*Microcystis*), demonstrating a sufficient level of uniformity in the performance of the array.

Although the probes have theoretical melting temperatures varying by 5°C, this variation does not seem to be related to the relative probe efficiency.

The sensitivity of the method was investigated by using different concentrations of target DNA (amplicon of *Planktothrix* sp. strain 1LT27S08). The log plot of the signal intensity versus the total amount of amplicon showed a good linear relationship (Fig. 5). Similar results were obtained with amplicons from two other strains. The efficiency of the LDR method in the presence of complex molecular targets was assessed by means of artificial mixes composed of unbalanced amounts (100:5 fmol) of PCR products. All of the signals expected were detected and easily discriminated from nonspecific signals, indicating that a reasonable association can be found between the composition of the sample and the LDR-universal array signals. However, it should be noted that the PCR is known to introduce bias (27); therefore, caution should be taken when assuming the results of PCR-LDR-universal array as quantitative indicators of the original sample composition.

Finally, we evaluated this method by using an environmental sample; the microarray hybridization pattern showed the presence of *Microcystis*, *Anabaena*/*Aphanizomenon*, and *Woronichinia* spp. These results were compared with those of light microscopy (morphotypes) (L. Lepistö and P. Kuuppo, unpublished data) as well as cloning and DGGE analyses of the same environmental sample (unpublished results). Consistent with the microarray results, the presence of *Microcystis*

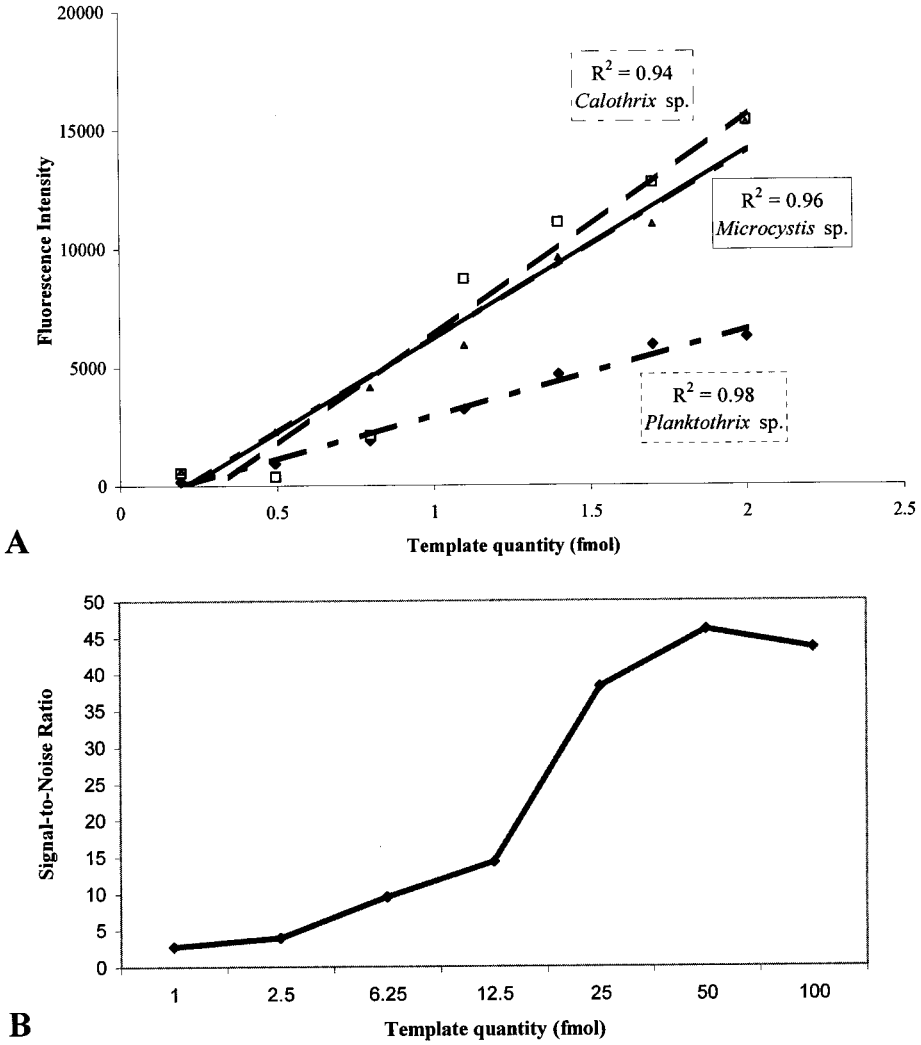


FIG. 5. Testing of LDR sensitivity. (A) Correlation between signal intensity and template concentration. The effect of template concentration on LDR was tested with PCR products of *Planktothrix* sp. strain 1LT27S08, *Calothrix* sp. strain PCC 7714, and *M. aeruginosa* strain PCC 9354, ranging from 0.5 to 100 fmol. (B) Signal-to-noise ratio plotted against template quantity. Each data point represents the ratio between the mean signal intensity of the target-specific zip codes and the mean signal intensity of nonspecific zip codes. PCR products from *Planktothrix* sp. strain 1LT27S08 (1 to 100 fmol) were used as a template. The signal-to-noise ratio increases with growing template concentrations.

and *Anabaena*/*Aphanizomenon* spp. was detected. In addition, the DGGE results showed the presence of *Snowella* but did not detect *Woronichinia* spp. This discrepancy in results can be discussed considering that the genera *Snowella* and *Woronichinia* belong to the subfamily *Gomphosphaerioideae* according to traditional botanic taxonomy (12); up to now, sequences of strains belonging to this subfamily have not been published. Recently, strains of genus *Snowella* were successfully isolated and characterized phylogenetically based on the 16S rRNA gene sequences (P. Rajaniemi, M. A. Mugnai, A. Rantala, S. Turicchia, S. Ventura, J. Komarkova, L. Lepistö, and K. Sivonen, unpublished data). The *Snowella* strains formed a highly supported cluster with the *Woronichinia* strains in all phylogenetic analyses of the 16S rRNA gene sequences. So it is possible that DGGE and microarray detect the same genotype that, lacking additional information, has been called *Woronichinia* on the basis of the only two *Woronichinia* sequences obtained during this work, which should be considered *Snowella* based on these unpublished new data.

In conclusion, we demonstrated the feasibility of the universal array-based approach, combined with the LDR for the identification of cyanobacteria. The method we established is specific, yielding unequivocal identification of the cyanobacteria, sensitive (down to 1 fmol can be detected), and amenable to the analysis of complex environmental samples. This method has wide potential for the monitoring of cyanobacteria, e.g., by water authorities and companies. This technology can be easily applied to the future study of other marker genes, one of the most interesting of which would be the combination of the array developed here with one that could detect potentially toxic cyanobacteria. This would reveal the genetic diversity of cyanobacteria as well as the presence of potentially toxic genotypes in a sample.

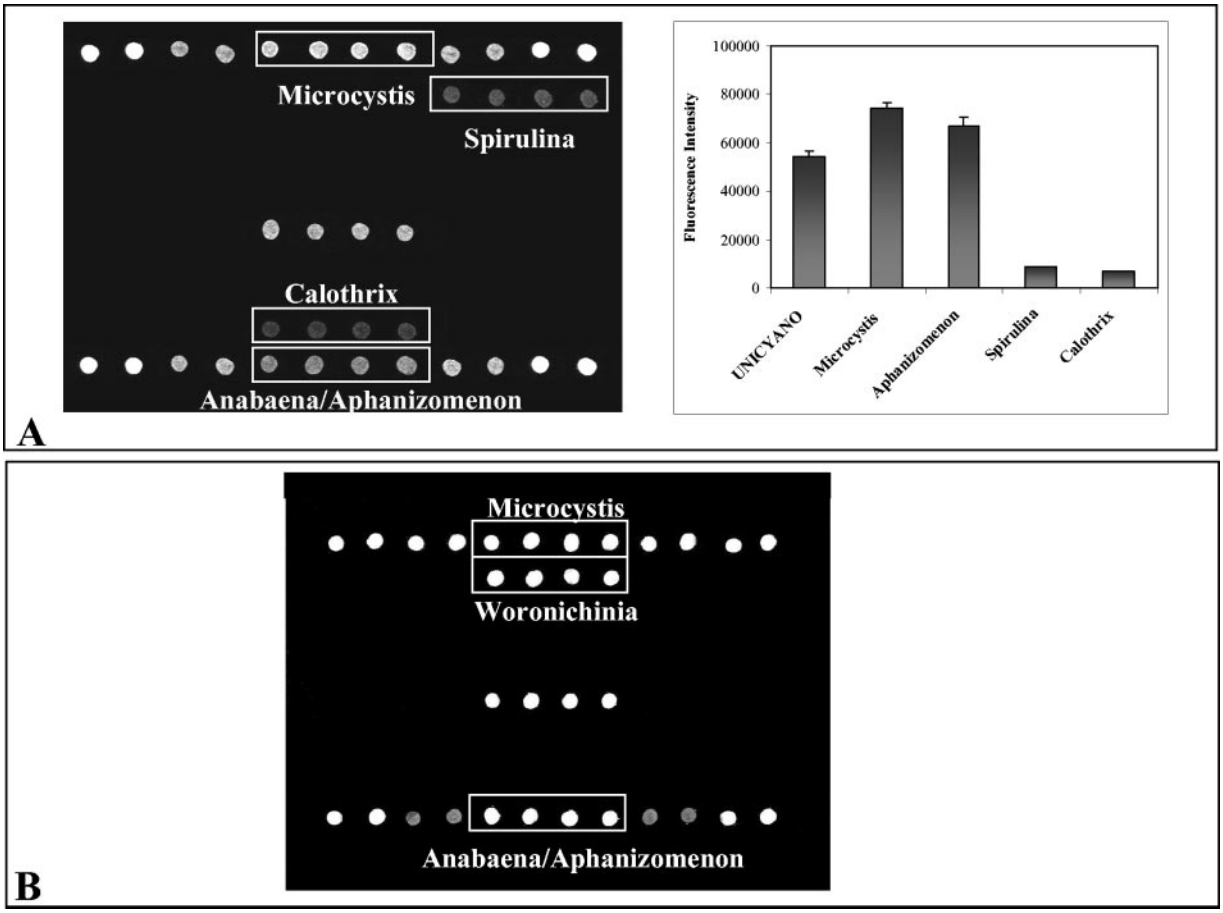


FIG. 6. Microarray analyses of complex cyanobacterial samples. (A) The hybridization result of the unbalanced LDR mix shows how spot intensity (left) and measured fluorescence intensity (right) correspond to the concentrations of targets. The LDR mix contained 100 fmol of the PCR product from both *M. aeruginosa* strain PCC 9354 and *Aphanizomenon* sp. strain 202 and 5 fmol of the PCR product of both *S. major* strain PCC 6313 and *Calothrix* sp. strain PCC 7714. (B) The environmental sample 0TU27 from Lake Tuusulanjärvi (Finland) was analyzed with the array. The hybridization pattern shows the presence of *Microcystis*, *Anabaena*/*Aphanizomenon*, and *Woronichinia* spp.

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