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Strain Characterization and Classification of Oxyphotobacteria in Clone Cultures on the Basis of 16S rRNA Sequences from the Variable Regions V6, V7, and V8

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A major problem in development of a polyphasic taxonomy is that the identification of oxyphotobacterial strains (cyanobacteria and prochlorophytes) in culture collections may be incorrect. We have therefore developed a diagnostic system using the DNA sequence polymorphism in the 16S rRNA regions V6 to V8 for individual strain characterization and identification. PCR primers amplifying V6 to V8 from oxyphotobacteria in unialgal cultures were constructed. Direct solid-phase or cyclic sequencing was used to determine the sequences from the amplified DNA. This survey includes 10 strains of *Nostoc*/*Anabaena*/*Aphanizomenon* (*Nostoc* category), 5 strains of *Microcystis* (*Microcystis* category), and 4 strains of *Planktothrix* (*Planktothrix* category). Fifteen additional strains of cyanobacteria and two strains of prochlorophytes were included such that the major phyletic groups were represented. One of the strains, *Phormidium* sp. NIVA-CYA 203, contained an 11-nucleotide insertion with no homology to other known 16S rRNA sequences. Based on parsimony and neighbor-joining trees, the phyletic relationships of the strains were investigated. Thirteen major branches were found, with *Pseudanabaena limnetica* NIVA-CYA 276/6 as the most divergent strain. The strain categories *Nostoc*, *Planktothrix*, and *Microcystis* were all monophyletic. The sequence polymorphism within *Nostoc* was higher than that in *Planktothrix* and *Microcystis*. Based on the sequence and phyletic information, group-specific PCR primers for the categories *Nostoc*, *Planktothrix*, and *Microcystis* were constructed. For the strains included in this work, the amplifications were specific for the relevant groups. By combination of magnetic solid-phase DNA isolation and group-specific PCR amplifications, an accurate method for characterization, classification and identification of oxyphotobacterial clone cultures has been developed.

The oxyphotobacteria comprise two systematic groups, the cyanobacteria and the prochlorophytes. The most thoroughly studied and understood group is the cyanobacteria, a widely distributed and diverse assemblage of unicellular or multicellular photosynthetic prokaryotes that possess chlorophyll *a* and perform oxygenic photosynthesis. Cyanobacteria are traditionally classified into five orders, *Chroococcales*, *Pleurocapsales*, *Oscillatoriales*, *Nostocales*, and *Stigonematales* (31). A more recently investigated group of photosynthetic prokaryotes is placed in the order *Prochlorales* (15). These organisms share many features with the cyanobacteria, but they contain chlorophyll *b* in addition to chlorophyll *a* (3).

Several taxonomic approaches are made to classify the oxyphotobacteria. These organisms were placed by botanists in the plant kingdom and systematically handled according to the International Code of Botanical Nomenclature (7, 31). The bacteriological classification system is based on the organisms in culture; like the botanical classification, it utilizes properties connected with morphology, mode of reproduction, ultrastructure, etc., but emphasizes genetics, physiological, and biochemical parameters (3, 24, 25). A new classification based on molecular biology, including DNA sequence variation, is presently emerging. The small subunit rRNA (16S rRNA) represents the best-studied sequences (1, 8, 9, 18–20, 29, 35, 36, 38).

A major problem in the development of a polyphasic taxonomy (17) is that the identifications of strains in culture collections may be incorrect. Komárek and Anagnostidis (14) concluded that the features of more than 50% of strains in collections do not correspond to the diagnoses of the taxa to which they are assigned. Thus, there is a real need for reviewed characterization of the numerous cyanobacterial strains available in laboratories and culture collections worldwide.

The objective of this work was to develop a DNA-based method for characterization, classification, and identification of cyanobacterial strains. This has been achieved by determining the sequence polymorphism within the 16S rRNA variable regions V6, V7, and V8 (10) and by the use of the DNA regions displaying phyletically informative characters (i.e., DNA sequences unique for a group) to design group-specific PCR primers. We focused the investigation on three categories including organisms associated with toxin production: *Nostoc*, *Microcystis*, and *Planktothrix* (31, 32). Sequences from 36 different isolates, with an emphasis on strains of species with a wide geographical distribution, were determined. To obtain a representative data set, we included strains from four cyanobacterial orders along with two strains of prochlorophytes.

MATERIALS AND METHODS

Organisms and cultivation procedure. The strains investigated are listed in Table 1. The majority of the organisms were isolated at the Norwegian Institute for Water Research (34). Strains were cultivated in a constant-temperature room at 17 ± 2°C. Glass flasks containing 50 ml of culture solution were used. The main growth medium used for the freshwater species is medium Z8. A medium based on equal parts of Z8 and seawater was used for the marine species (21). Cultivation took place under illumination provided by fluorescent lamps (Philips TL 65 W/33). The different strains were exposed to light in the range of 10 to 60 microeinsteins m^{-2} s^{-1}, depending on their particular demands.

Pretreatment and controls. Aliquots of dense culture (1 ml containing approximately 10^7 cells/ml) were pelleted in a microcentrifuge at 5,000 rpm for 10 min

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TABLE 1. Strains of oxyphotobacteria used in this study and databank accession numbers for 16S rRNA sequences

Strain	Geographical origin	EMBL accession no.
Cyanobacteria		
Chroococcales		
Microcystis sp. NIVA-CYA 324/1	Lake Tørråssjøen, Norway	z82808
Microcystis aeruginosa NIVA-CYA 43	Wisconsin (strain ATCC 22663)	z82784
Microcystis aeruginosa NIVA-CYA 57	Lake Frøylandsvatnet, Norway	z82785
Microcystis aeruginosa NIVA-CYA 143	Lake Akersvatnet, Norway	z82786
Microcystis aeruginosa NIVA-CYA 228/1	Lake Akersvatnet, Norway	z82783
Cyanothece aeruginosa NIVA-CYA 258/2	Dronning Mauds Land, Antarctica	z82775
Synechococcus leopoliensis NIVA-CYA 20	Freshwater, Texas (strain PCC 6301) (24)	z82780
Synechococcus sp. NIVA-CYA 328	Holmestrand, Oslofjord, Norway	z82779
Oscillatoriales		
Planktothrix agardhii NIVA-CYA 29	Lake Gjersjøen, Norway	z82796
Planktothrix agardhii NIVA-CYA 299	Lake Kalvsjøtjernet, Norway	z82799
Planktothrix mougeotii NIVA-CYA 11	Lake Akersvatnet, Norway	z82795
Planktothrix prolifica NIVA-CYA 320	Lake Kolbotnvatnet, Norway	z82798
Phormidium sp. NIVA-CYA 203	Coraholmen, Spitsbergen, Svalbard	z82792
Phormidium sp. NIVA-CYA 202	Svea, Spitsbergen, Svalbard	z82794
Phormidium sp. NIVA-CYA 177	Dronning Mauds Land, Antarctica	z82790
Arthrospira fusiformis NIVA-CYA 136/2	Crater Lake, Central Island, Lake Turkana, Kenya	z82793
Tychonema bourrellyi NIVA-CYA 261/1	River Glåma, Fredrikstad, Norway	z82791
Pseudanabaena limnetica NIVA-CYA 276/6	Lake Mälaren, Sweden	z82778
Spirulina subsalsa NIVA-CYA 163	Drøbak, Oslofjord, Norway	z82787
Spirulina subsalsa NIVA-CYA 164	Drøbak, Oslofjord, Norway	z82788
Nostocales		
Anabaena sp. NIVA-CYA 267/4	Lake Fammestadtjønni, Norway	z82802
Anabaena lemmermannii NIVA-CYA 281/1	Lake Storavatnet (Lindås), Norway	z82797
Anabaena lemmermannii NIVA-CYA 83/1	Lake Edlandsvatnet, Norway	z82801
Anabaena lemmermannii NIVA-CYA 266/1	Lake Bergevatnet, Norway	z82800
Aphanizomenon gracile NIVA-CYA 103	Pond, Vingrom, Norway	z82806
Aphanizomenon flos-aquae NIVA-CYA 142	Buffalo Pound Lake, Canada (strain NRC-566)	z82809
Nostoc sp. NIVA-CYA 246	United States (strain PCC 7120)	z82803
Nostoc sp. NIVA-CYA 124	Lake Steinsfjorden, Norway	z82776
Nostoc sp. NIVA-CYA 194	Jutulsessen, Dronning Mauds Land, Antarctica	z82805
Nostoc sp. NIVA-CYA 308	Ny Ålesund, Spitsbergen, Svalbard	z82804
Pleurocapsales		
Chroococcidiopsis thermalis PCC 7203	Soil sample, Greifswald, Germany (24)	z82789
Dermocarpella incrassata PCC 7326	Snail shell, Puerto Penasco, Mexico (24)	z82807
Pleurocapsa minor PCC 7327	Hunter's Hot Spring, Oregon (24)	z82810
Dermocarpa violacea PCC 7301	Marine aquarium, La Jolla, Calif. (24)	z82777
Prochlorophytes		
Prochlorales		
Prochlorothrix hollandica NIVA-5/89	Lake Loosdrecht, The Netherlands (strain CCAP 1490/1)	z82782
Prochlorothrix sp. NIVA-8/90	Lake Mälaren, Sweden	z82781

and immediately frozen at −80°C. Organisms with gas vacuoles were heated at 65°C for 2 min in order to break the vesicles before pelleting.

Commensals ordinarily present in natural waters (e.g., *Rhodobacter* sp., *Chlorobium* sp., *Cytophaga* sp., and *Escherichia coli*), along with *Bacillus cereus* and *Agrobacterium tumefaciens*, were included as controls for the specificity of the PCR amplifications.

DNA extraction. DNA was isolated from frozen pellets, using the magnetic bead-based DNA Direct DNA isolation kit (Dynal A/S, Oslo, Norway). This DNA isolation system, originally designed for DNA isolation from blood (4), has been modified for isolation of DNA from cyanobacteria by Rudi et al. (26). The procedure used was as follows: Dynabeads DNA Direct (200 μl) was added to the bacterial pellet, mixed gently, and incubated at 65°C for 15 min, followed by a 5-min storage at room temperature. Beads bound to DNA were drawn to the side of a microcentrifuge tube by an MPC-E magnet (Dynal A/S). While bound to the beads, the DNA was washed twice with the washing buffer supplied with the kit. Finally, the Dynabeads-DNA complex was broken up by thorough resuspension in 50 μl of water. Either the DNA and the beads were used directly for amplification or the DNA was eluted at 65°C for 5 min, the beads were collected by a magnet, and the DNA-containing supernatant was transferred to a new tube.

Construction of PCR primers. Small subunit rRNA sequences aligned by using the multisequence alignment algorithm PILEUP (11) in the Wisconsin package version 8.1 for UNIX (Genetic Computer Group, Madison, Wis.) were inspected for regions with suitable polymorphism for construction of selective PCR primers. The Oligo program version 3.3 (National Bioscience, Minn.) was used to select PCR primers from these regions. Finally, the putative PCR primer sequences were subjected to a FASTA search (23) in the database to determine the similarity to other sequences. Construction of eubacterium- and oxyphotobacterium-specific PCR primers was based on the alignment of sequences from the EMBL nucleotide sequence database (release 45, December 1995; Cambridge, England). Primer pair CC-CD (Fig. 1A) is eubacterium specific, amplifying both gram-positive and gram-negative eubacteria (26). Primer CC has an M13 −21 tail suited for automatic sequencing. The primer pair CC-CG (Fig. 1A) was designed for amplifying oxyphotobacteria in unialgal cultures.

An alignment of the 16S rRNA regions, used for design of group-specific primers for the *Microcystis*, *Planktothrix*, and *Nostoc* categories, is shown in Fig. 1B. Strains NIVA-CYA 43, 143, 228/1, 57, and 324/1 are included in the *Microcystis* category, strains NIVA-CYA 29, 299, 11, and 320 are included in the *Planktothrix* category, and strains NIVA-CYA 267/4, 281/1, 83/1, 266/1, 103, 142, 246, 124, 194, and 308 are included in the *Nostoc* category.

PCR amplification and sequencing. Amplification of genomic DNA was performed with 30 to 35 PCR cycles; for the nested amplifications, 15 to 20 cycles were used. Prior to amplification, the DNA was denatured for 4 min at 94°C; after amplification, an extension step for 7 min at 72°C was included. For primer pairs CC-CD and CC-CG, cycling parameters were 96°C for 15 s, 70°C for 30 s, and

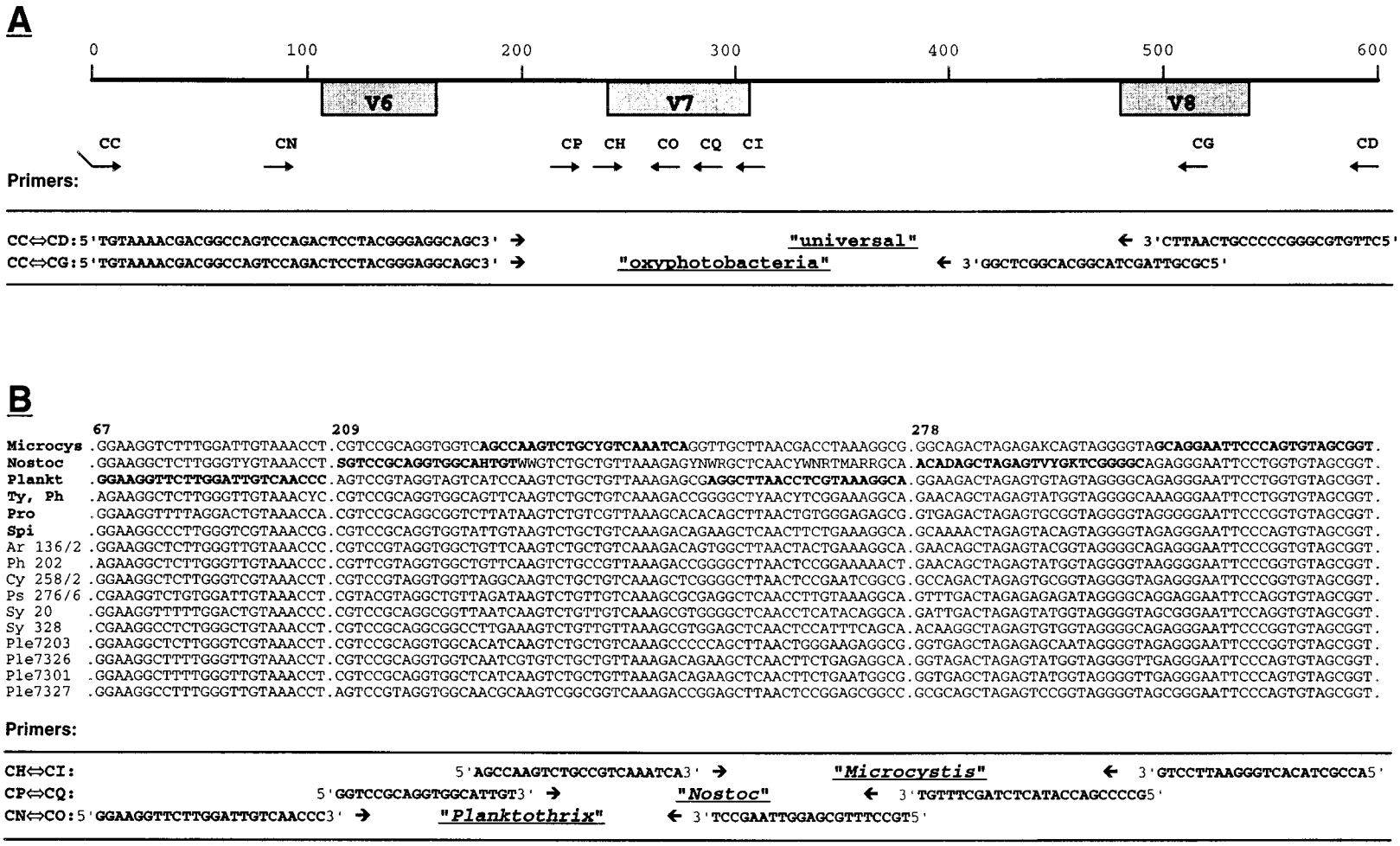

FIG. 1. Small subunit rRNA sequences and locations of PCR primers. (A) Locations of all primers relative to the CC-CD amplicon (*E. coli* 16S rRNA nt 334 to 939) and the primer sequences for the universal (CC-CD) and oxyphotobacterium-specific (CC-CG) primer pairs. (B) Alignment showing locations (relative to the CC-CD amplicon) and sequences of the group-specific primer pairs CN-CO, CP-CQ, and CH-CI. The alignment is not contiguous, and the different blocks are separated with dots. For tightly clustered isolates (indicated in boldface), only one sequence is shown. Microcys, *Microcystis aeruginosa* NIVA-CYA 43, *Microcystis aeruginosa* NIVA-CYA 143, *Microcystis aeruginosa* NIVA-CYA 57, *Microcystis aeruginosa* NIVA-CYA 228/1, and *Microcystis* sp. NIVA-CYA 324/1; Nostoc, *Anabaena lemmermannii* NIVA-CYA 83/1, *Anabaena lemmermannii* NIVA-CYA 281/1, *Anabaena lemmermannii* NIVA-CYA 266/1, *Anabaena* sp. NIVA-CYA 267/4, *Aphanizomenon gracile* NIVA-CYA 103, *Aphanizomenon flos-aquae* NIVA-CYA 142, *Nostoc* sp. NIVA-CYA 246, *Nostoc* sp. NIVA-CYA 194, *Nostoc* sp. NIVA-CYA 124, and *Nostoc* sp. NIVA-CYA 308; Plankt, *Planktothrix agardhii* NIVA-CYA 29, *Planktothrix agardhii* NIVA-CYA 299, *Planktothrix prolifica* NIVA-CYA 320, and *Planktothrix mougeotii* NIVA-CYA 11; Ty, Ph, *Phormidium* sp. NIVA-CYA 177, *Phormidium* sp. NIVA-CYA 203, and *Tychonema bourrellyi* NIVA-CYA 261/1; Pro, *Prochlorothrix hollandica* NIVA-CYA 5/89 and *Prochlorothrix* sp. NIVA-CYA 8/90; Spi, *Spirulina subsalsa* NIVA-CYA 163 and *Spirulina subsalsa* NIVA-CYA 164. Polymorphic sites are indicated with the corresponding degenerated symbols: R = A or G; Y = C or T; W = A or T; K = G or T; M = A or C; S = G or C; H = A, C, or T; V = A, C, or G; D = A, G or T; and N = A, C, G, or T. The NIVA or Pasteur culture collection clone numbers are shown for the other isolates.

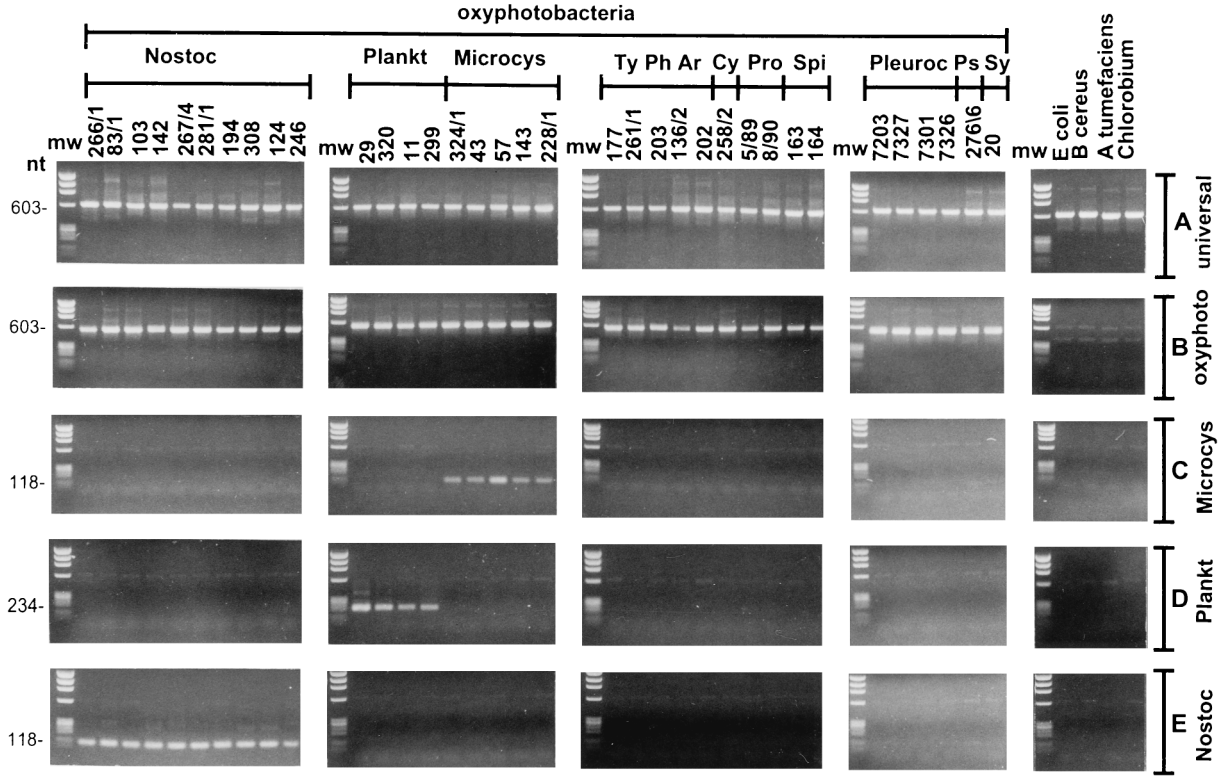

FIG. 2. Amplification products for the primer pairs and bacterial strains used in this work. All samples were electrophoresed in 1.5% agarose gels for 30 min at 100 V. Twenty percent of the amplification products was loaded in each lane. Abbreviations for the phyletic groups: Plankt, *Planktothrix*; Microcys, *Microcystis*; Ar, *Arthrospira*; Ph, *Phormidium*; Ty, *Tychonema*; Cy, *Cyanothece*; Pro, *Prochlorothrix*; Spi, *Spirulina*; Pleuroc, *Pleurocapsales*; Ps, *Pseudanabaena*; Sy, *Synechococcus*. The culture collection (NIVA and Pasteur) clone numbers are shown for each lane for the oxyphotobacteria. The first column (A), designated universal, shows amplification products for the primer pair CC-CD. Genomic DNA was used in these amplifications. In the other amplifications, 0.5 μl of PCR products from the CC and CD amplifications were used. The second column (B), designated oxyphoto, shows amplification products from primers specific for oxyphotobacteria, CC and CG. The third column (C), designated Microcys, shows amplification products for the *Microcystis*-specific primers, CH and CI. The fourth column (D), designated Plankt, shows amplification products for the *Planktothrix*-specific primers, CN and CO. The fifth column (E), designated Nostoc, shows amplification products for the *Nostoc*-specific primers, CP and CQ. mw, molecular weight markers.

72°C for 1 min. For the group-specific primer pairs (CP-CQ, CH-CI, and CN-CO), the cycling parameters were 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, respectively. Amplification reactions using the GeneAmp 2400 PCR system (Perkin-Elmer, Norwalk, Conn.) contained 10 pmol of primers, 200 μM deoxynucleoside triphosphate, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 1 U of DynaZyme thermostable DNA polymerase (Finnzymes Oy, Espoo, Finland), and 0.1 to 2 μl of purified DNA in a final volume of 50 μl.

Sequencing was performed manually by magnetic solid-phase DNA sequencing (12), sequencing both strands separately, using a Sequenase 2.0 sequencing kit (United States Biochemical, Cleveland, Ohio). The process was also done automatically on an ABI 373A DNA sequencer, using cyclic sequencing with dye primer or dye terminator, as recommended by the manufacturer (Perkin-Elmer, Foster City, Calif.).

Phylogenetic reconstruction. Phylogenetic trees were constructed with the neighbor-joining method (28) and the parsimony analysis (6) from the Phylogeny Inference Package (PHYLIP; version 3.5) developed by J. Felsenstein (Department of Genetics, University of Washington). A two-parameter model (13) was used to compute the distance matrices for the neighbor-joining analysis. To infer confidence of the branchpoints in the constructed tree, bootstrap analysis (5) was used. Consensus trees were constructed from 500 bootstrap replicates.

RESULTS

DNA isolation and generation of sequence information from 16S rRNA variable regions V6 to V8. The magnetic bead-based DNA isolation protocol used (see Materials and Methods) gave a DNA yield in the range of 50 to 500 ng/10^7 cells for the strains tested. Reproducible amplifications with the universal primer pair CC-CD (Fig. 1A), using 0.2 to 4% of the isolated DNA, were obtained from all strains (Fig. 2A). The primer pair CC-CG (Fig. 1A), specific for oxyphotobacteria, gave the best amplifications in a seminested PCR, using 0.5 μl of PCR products from CC-CD amplifications (Fig. 2B). The faint upper band for eubacteria other than the oxyphotobacteria are carryover products from the CC and CD amplifications, while the nature of the faint lower bands is not known. One strain, *Synechococcus* sp. NIVA-CYA 328, did not yield amplification products with the oxyphotobacterium-specific primers (results not shown).

Manual sequencing of the PCR products by solid-phase DNA sequencing yielded on average 350 bases of sequence. With dye primer automatic sequencing, 450 to 500 bases of sequence were obtained. Each sample was sequenced several times and edited to obtain an accuracy of 99.8% or higher (determined by known control sequences). Most of the cyanobacterial isolates could be sequenced by using PCR products from the universal primers CC and CD. However, some of the cultures contained commensals. Notably, two of the cultures gave clean sequences of the contaminating bacteria by direct sequencing of PCR products from amplifications with primers CC and CD, one with 96.5% homology to *Rhodobacter capsulatus* (EMBL accession no. D16427) and the other with 98.7% homology to *Cytophaga saccharophila* (EMBL accession no. D12671). However, the cyanobacterial strains from these cultures gave sequences of high quality, using the PCR products from the oxyphotobacterium-specific primer pair CC-CG.

Group-specific amplifications for the *Microcystis*, *Nostoc*, and *Planktothrix* categories. The partial rRNA sequences determined in this work were aligned and inspected for regions

unique for *Microcystis*, *Planktothrix*, and *Nostoc* (Fig. 1B). Group-specific primers constructed from these regions (Fig. 1B) were used in selective PCR amplifications (Fig. 2C to E). The constructed PCR primers were tested with a nested approach, using the general primers CC and CD in the first amplification (Fig. 2A) and 0.5 μl of this product in the group-specific amplifications (Fig. 2C to E). With 20 PCR cycles, the amplifications gave strong signals for the *Microcystis* and *Planktothrix* categories and no detectable signals for the other isolates (Fig. 2C and D). For the *Nostoc* category, strong signals were obtained with 15 PCR cycles (Fig. 2E), while 20 cycles gave faint signals for some other isolates not belonging to the *Nostoc* category (results not shown). The faint bands of about 600 nucleotides (nt) for the nested amplifications are the carryover products from the general CC-CD amplifications.

Phylogenetic reconstruction. The neighbor-joining and the parsimony algorithms from PHYLIP were used in phylogenetic reconstruction. Since the two methods gave congruent results for the major branching patterns of the trees, only the neighbor-joining tree is presented (Fig. 3). The differences between the two methods are that in the parsimony tree, *Prochlorothrix* spp. and *Synechococcus* spp. group with *Microcystis* spp., while they form a separate branch in the neighbor-joining tree. In the parsimony tree, species of *Pleurocapsales—Pleurocapsa minor* (PCC 7327), *Dermocarpa violacea* (PCC 7301), and *Dermocarpella incrassata* (PCC 7326)—are on the same branch and not grouped with *Microcystis* spp., as in the neighbor-joining tree.

In the neighbor-joining tree, we found 13 deep branches with 0.07 to 0.15 nucleotide substitution at each position relative to the hypothetical root of the tree. *Pseudanabaena limnetica* NIVA-CYA 276/6 is the most divergent strain, with 0.20 to 0.25 nucleotide substitution at each position from the other strains. Three of the clusters are represented by the *Nostoc*, *Planktothrix*, and *Microcystis* categories. The *Nostoc* category is further divided in three lineages, two *Nostoc* and one *Anabaena/Aphanizomenon* lineage(s) diverging with ca. 0.04 nucleotide substitution per position. The *Planktothrix* and *Microcystis* categories are very homogeneous, with less than 0.005 nucleotide substitution per position among the isolates. One cluster of *Arthrospira fusiformis*, *Tychonema bourrellyi*, and *Phormidium* spp. consists of both relatively divergent and closely related species (0.00 to 0.10 nucleotide substitution per position among the species). One of the strains (*Phormidium* sp. NIVA-CYA 203) contained an 11-nt insertion (5′-AGTTGT GAAAG-3′) with no homology to other known 16S rRNA sequences (determined by FASTA search in the EMBL database). The *Pleurocapsales* group is a collection of distantly related species (0.05 to 0.15 nucleotide substitution per position), and it is uncertain if this group is monophyletic. Interestingly, the filamentous organisms belonging to the genus *Prochlorothrix* are in 66% of the bootstrap trees on the same branch as the unicellular *Synechococcus* strains NIVA-CYA 20 and 328.

DISCUSSION

Simple and objective criteria for a phyletically relevant classification are needed for accurate identification of oxyphotobacteria. In this work, we have developed and tested group-specific PCR amplifications based on phyletically relevant characters for three oxyphotobacterial categories. The results (presence or absence of PCR fragments) gave simple and objective criteria for phyletically relevant classification.

Group-specific amplification for characterization groups of strains. The strains investigated consist of several phyletic lineages of tightly clustered groups (e.g., the categories *Microcystis* and *Planktothrix*). This may suggest either a strong evolutionary selection for a few strains (all intermediates are extinct), periodic selection, or some mechanism(s) of concerted evolution of closely related oxyphotobacteria.

Several regions with suitable variation for constructing group-specific PCR primers for all of the major branches in the phylogenetic tree (this work) were found. Organisms belonging to the *Microcystis*, *Planktothrix*, and *Nostoc* categories were chosen as examples because of their regional abundance and the possibility that they contain toxin-producing strains (33).

The group-specific amplification test developed in this work provides a simple method for identifying isolates. Using the described method, six samples can be run in parallel, with less than 1 h of hands-on time (the entire process takes about 5 to 6 h). The simplicity of the protocol also allows high throughput of samples. Work is in progress to adapt the process for automation, which will further simplify and increase the throughput of the test.

For the battery of strains tested, only the groups for which the primers were designed gave amplification products. In addition to the material included in this work, conserved primer sites complementary to the group-specific primers were determined for 11 strains of *Microcystis* and 6 strains of *Planktothrix* (results not shown). The *Microcystis* and *Planktothrix* amplifications are robust, while the *Nostoc* amplification required more optimization. The *Nostoc* category consists of three evolutionary different lineages; thus, fewer suitable regions for *Nostoc*-specific amplifications were found. More sequence information, i.e., complete 16S or 23S rRNA sequences, may reveal regions better suited for selective amplification of this group.

However, because mutations are random events, there may exist strains which are not classified phyletically relevantly with the primer pairs used in this work. Thus, the generic assignments need to be further confirmed by testing on a large number of strains.

Molecular classification compared to classification based on microscopic observations. Several morphological or cytological characters can change with the growth conditions (22), and morphological and cytological characters do not necessarily reflect evolution. Thus, classifications based on microscopic observations alone (morphological and cytological structures) are not always evolutionarily significant. Filamentous cyanobacteria are intricate to handle systematically; especially organisms belonging to the genera *Planktothrix* and *Tychonema* are difficult to distinguish by morphological and cytological criteria (30). Classification of unicellular cyanobacteria (*Microcystis* and *Synechococcus*) is problematic, in particular due to the size of these small organisms and the lack of easily observable characters. In contrast, the molecular differences found by sequencing 16S rRNA make it possible to accurately distinguish between *Microcystis* and *Synechococcus* (Fig. 2C and 3) and *Planktothrix* and *Tychonema* (Fig. 2D and 3).

An identification system based on sequence variation and group-specific PCR amplifications has several practical advantages: (i) the analysis is simple to perform, (ii) it reflects phylogeny and evolution, and (iii) the results are easy to interpret and allow for automation (that is, the results are represented by, e.g., presence or absence of PCR fragments and not complex morphological or cytological characters).

A newly discovered *Prochlorothrix* strain (NIVA-8/90) (16), difficult to classify by microscopic analysis, was included in the present study. The resulting sequence showed close similarity with the type species *Prochlorothrix hollandica* (NIVA-5/89). Strain NIVA-8/90 was accordingly verified as a representative of the genus *Prochlorothrix*.

Recently, a group of marine photosynthetic picoplankton was identified as cyanobacteria (37). These organisms could

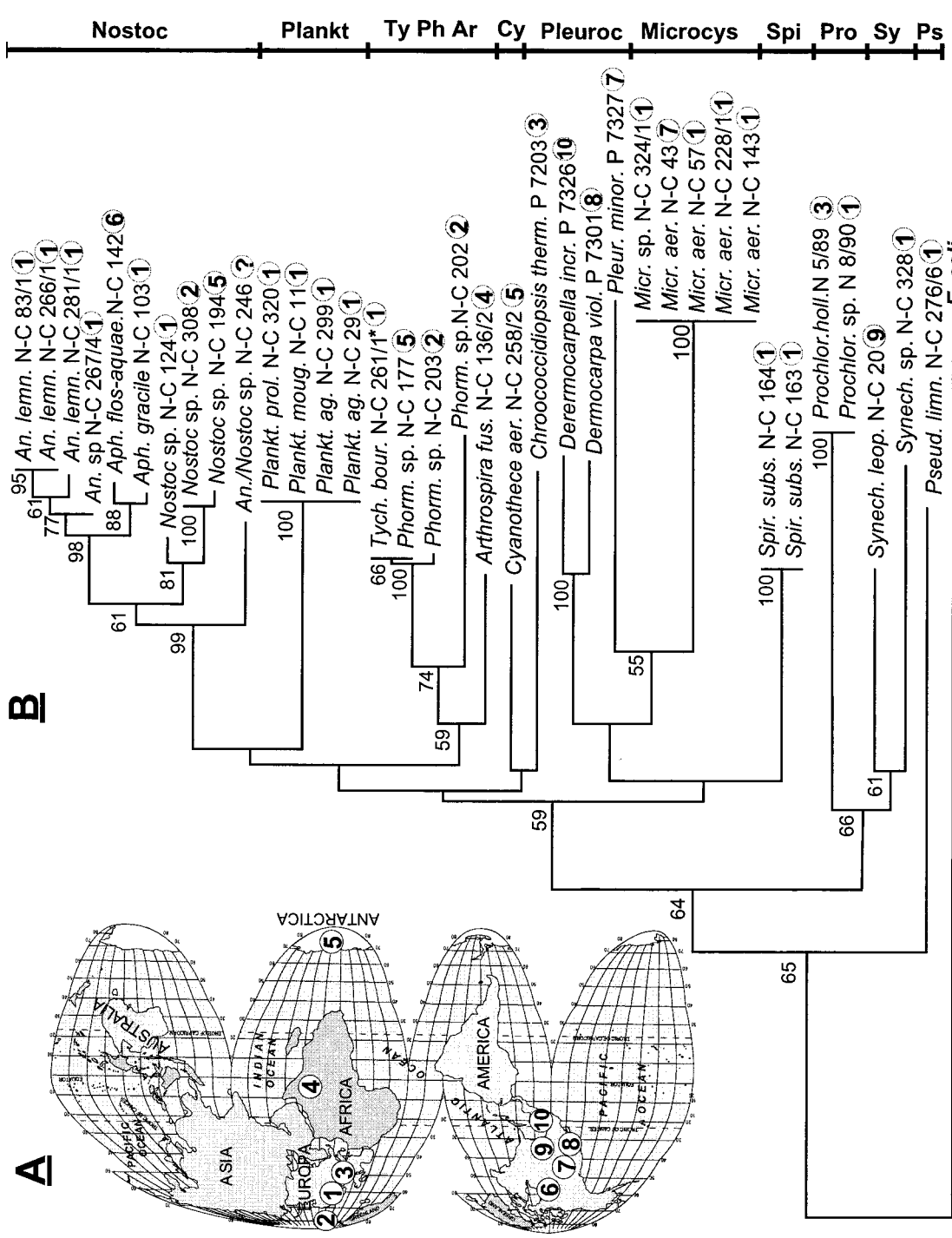

FIG. 3. Sampling location of the strains (A) and distance tree for the partial 16S rRNA sequences (B). The distance tree was constructed by the neighbor-joining method (28), using distance matrices from the Kimura two-parameter model (13) and the bootstrap analysis (5) from the software package PHYLIP. The alignment of the partial 16S rRNA sequences (nt 346 to 845) relative to the *E. coli* sequence (2) was done with the PILEUP program from the Genetics Computer Group software package. The distance between two organisms, expressed in substitutions per nucleotide, is obtained by adding the lengths of the horizontal branches connecting them. Numbers at the nodes indicate the percentage of 500 bootstrap trees in which the cluster descending from the node was found. Only bootstrap values higher than 50% are given. The position of the root was chosen to equalize the distance to the outgroup, *E. coli*, and its average distance to all other included organisms. The respective clone numbers (N-C [NIVA-CYA], N [NIVA], and P [Pasteur]) are given for each branch. Numbers in circles indicate the sampling locations of the strains shown in panel A; the origin of strain NIVA-CYA 246 is unknown. The phyletic groups are shown with the same abbreviations as in Fig. 2; full organism names are given in Table 1. *, In addition to *Tychonema bourrelly*i (*Tych. bour.*) NIVA-CYA 261/1, five other *Tychonema* isolates, NIVA-CYA 33/1, 33/6, 58, 70, and 60, were sequenced and found to have identical sequences.

not be properly identified by microscopic analysis because of the small size and lack of phyletic relevant characters. A good example is the cyanobacterial picoplankton *Synechococcus* sp. NIVA-CYA 328 isolated from the Oslofjord, Norway, showing high sequence similarity (95.7%) to the prochlorophyte *Prochlorococcus marinus* SSW5 (EMBL accession no. x63140). Sequences belonging to the same group have previously been found in the Pacific (29) and the Atlantic (9) oceans, suggesting that these organisms belong to a large and uncharacterized group of oxyphotobacteria.

Group-specific amplifications on organisms from natural habitat. Group-specific amplifications have been tested with success on samples of water from habitats containing organisms belonging to the *Nostoc* category (27). The distribution and abundance of organisms previously characterized in culture can now be determined in natural environment by group-specific amplifications.

Analysis of the natural occurrence of certain oxyphotobacterial groups in water is important both for monitoring toxin-producing species and for biological classification of water quality by indicator organisms (e.g., for evaluating pollution and eutrophication). Monitoring of relevant organisms, however, has been difficult since microscopic examination does not conveniently give enough information for accurate identification. Molecular techniques, on the other hand, are applicable to organisms in natural samples and provide a sufficient basis for correct identification. The work described here, utilizing 16S rRNA, is a first step toward a PCR-based identification of oxyphotobacteria both in nature and in culture.

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