PCR Primers To Amplify 16S rRNA Genes from Cyanobacteria

ULRICH NÜBEL, FERRAN GARCIA-PICHÉL, AND GERARD MUYZER*
Max Planck Institute for Marine Microbiology, D-28359 Bremen, Germany

Received 26 February 1997/Accepted 20 May 1997

We developed and tested a set of oligonucleotide primers for the specific amplification of 16S rRNA gene segments from cyanobacteria and diatoms by PCR. PCR products recovered from all cultures of cyanobacteria and diatoms that were checked but not from other bacteria and archaea. Gene segments selectively retrieved from cyanobacteria and diatoms in unialgal but nonaxenic cultures and from cyanobionts in lichens could be directly sequenced. In the context of growing sequence databases, this procedure allows rapid and phylogenetically meaningful identification without pure cultures or molecular cloning. We demonstrate the use of this specific PCR in combination with denaturing gradient gel electrophoresis to probe the diversity of oxygenic phototrophic microorganisms in cultures, lichens, and complex microbial communities.

To understand the ecology of cyanobacteria, it is desirable to match isolated strains and their counterparts in nature. Only then can physiological data gained from culture studies begin to be confidently extrapolated to natural conditions (9, 16). Inadequate culture conditions leading to the loss of various morphological characteristics (9), researchers’ inability to grow certain organisms in the laboratory (12, 43), and misidentifications of strains in culture collections (16, 47) make it difficult in many cases to apply taxonomic assignments based on cultures to field populations. Both classification systems for the cyanobacteria—the bacteriological approach (9, 20, 34) as well as the traditional botanical approach (2, 17)—rely primarily on morphological characteristics of cells and colonies and do not necessarily lead to the identification of phylogenetically coherent taxa (10, 45). At all taxonomic levels above species, the sequence analysis of genes encoding small-subunit ribosomal RNA (16S rRNA) is currently the most promising approach for the phylogenetic classification of cyanobacteria (47). Furthermore, the comparative analysis of 16S rRNA gene sequences provides a new means to investigate the discrepancy between strain collections and natural communities (11, 44). Sequences of 16S rRNA genes are independent from cultivation or growth conditions and can be retrieved by PCR from small amounts of DNA extracted from laboratory cultures or natural environments (18). Several different approaches to extend the analysis of 16S rRNA from cyanobacteria beyond axenic cultures have been described. These approaches include antibiotic treatments to suppress heterotrophic bacteria in nonaxenic cultures (46), physical cleaning of cyanobacteria by micromanipulation (16), and molecular cloning followed by screening for plasmid inserts of interest (29, 44).

On the basis of published 16S rRNA sequences, we developed a PCR procedure for the selective retrieval of cyanobacterial rRNA gene fragments from a variety of natural and artificial settings. The combination of this procedure with denaturing gradient gel electrophoresis (DGGE), a technique for the sequence-dependent separation of DNA molecules (19, 23), proved useful to visualize the diversity of cyanobacterial 16S rRNA genes in environmental samples, to detect the uniqueness of isolated strains, and to assign PCR products derived from cultures to populations in the field. PCR products containing a single homogeneous population of DNA molecules are recognized as single bands after DGGE and can be directly sequenced, yielding information about approximately 700 nucleotides of the 16S rRNA genes. Sequence data, therefore, can be generated without time-consuming molecular cloning procedures from cyanobacterial cultures containing heterotrophic bacteria, allowing the rapid survey of a collection of strains for genetic diversity.

Specificity of PCR. Primer design was based on an alignment of all 16S rRNA sequences from cyanobacteria available from the Ribosomal Database Project (22) and GenBank (4). Primer sequences and target regions within the 16S rRNA gene are listed in Table 1. The forward primers CYA106F and CYA359F were used alternatively, whereas the reverse primer, referred to hereafter as CYA781R, was an equimolar mixture of CYA781R(a) and CYA781R(b). Forty-nucleotide GC-rich sequences at the 5’ end of the forward primers improved the detection of sequence variations in the amplified DNA fragments in subsequent DGGE (37). The primers were synthesized commercially (Biometra, Göttingen, Germany).

Primer specificities with reference to published sequences were checked with the CHECK_PROBE program supported by the Ribosomal Database Project and the BLAST program (1) at the National Center for Biotechnology Information, Washington, D.C. The primers CYA359F and CYA781R match virtually all 174 16S rRNA sequences from cyanobacteria currently deposited in public databases as of June 1997. There is evidence that the apparent mismatches in the few exceptions correspond to sequencing errors for one or more of the following reasons. The nonmatching nucleotides (i) would disrupt the secondary structures of the corresponding RNA molecules, (ii) affect positions considered to be invariant among prokaryotic 16S rRNA sequences (41), and (iii) simply are contradictory to sequences determined for the same strains by other researchers. In contrast, CYA106F has one to three mismatches to 16S rRNA genes from many strains of cyanobacteria affiliated with various phylogenetic groups. Therefore, this primer is not recommended for the analysis of microbial community composition. However, in this study it was successfully used in combination with CYA781R to specifically generate amplification products from cyanobacteria and diatom plastids from axenic and nonaxenic unialgal cultures (Table 2).

Plastids are believed to be an early offshoot of the cyanobacterial evolutionary line (28). Probably after a single primary
endosymbiotic event, a nearly simultaneous radiation of the an-
ccestors of recent cyanelles, rhodoplasts, and chloroplasts oc-
curred and, subsequently, other plastids evolved through mul-
tiple secondary endosymbioses (6). Published 16S rRNA se-
cquences from cyanelles match primers CYA106F, CYA359F,
and CYA781F. Among the other two main plastid lineages,
there are 16S rRNA sequences with one or more mismatches
to the sequences of cyanobacterial primers. On the basis of the
limited data set currently available, a more detailed evaluation
of primer specificity for phylogenetic groups of plastids is not
yet possible. Therefore, the applicability of the PCR reported
here for the analysis of eukaryotic phototrophs must await the
advent of new 16S rRNA sequences from plastids.

All primers presented here have sequences that match some
16S rRNA sequences from bacteria not affiliated with the phy-
lum of cyanobacteria. However, none of these sequences has
fewer than two mismatches total to CYA359F and CYA781R.
Thus, the combined use of both primers results in a PCR highly
specific for cyanobacteria. CYA359F matches the 16S rRNA
sequences from a number of gram-positive bacteria with a low
G+C content of the genomic DNA, namely *Hellobacterium*
sp., several anaerobic halophiles, and relatives of *Desulfo-
tomaculum* and *Syntrophomonas*. Information about the target
site for reverse priming is also available for all these strains;
most of them have four and some have two mismatches to
CYA781R. An example of the latter is *Pectinatus frisingensis*
DSM 6306T, which we included in the experimental study of
PCR specificity for this reason (Table 2). It could be safely
discriminated against when PCR conditions were used as de-
scribed below. On the other hand, CYA781R is complemen-
tary to the 16S rRNAs from several crenarchaeota affiliated
with the order *Sulfolobales*. However, these rRNA sequences
have either ten or seven mismatches to CYA359F. *Sulfolobus
shibatae* DSM 5389T (seven mismatches to CYA359F) was also
included in the experimental study of PCR specificity (Table
2). The alternative forward primer CYA106F matches a num-
er of published 16S rRNA sequences from prokaryotes with
various phylogenetic affiliations outside the phylum of the cya-
nobacteria, not all of which contain information about the target
target for reverse priming. Therefore, and in addition to the
reasons discussed above, its applicability is limited, and it
should not be used for studies on environmental nucleic acids
of unknown composition. Yet, it has been included in the
present report because it proved useful in the generation of
sequence data from unialgal cultures. In these cases, the am-
plification specificity was checked by DGGE to investigate the
sequence homogeneity of PCR products prior to sequence
analysis. Despite its limitations, CYA106F might be preferred,
because compared to CYA359F, its use generates longer am-
plification products (approximately 700 base pairs instead of
450), therefore enabling the determination of more informa-
tive sequence data.

PCR amplifications were performed with a Cyclogene Tem-
perature Cycler (Techne, Cambridge, United Kingdom). Fifty
picomoles of each primer, 25 nmol of each deoxynucleoside
triphosphate, 200 μg of bovine serum albumin (35), 10 μl of
10× PCR buffer (100 mM Tris-HCl [pH 9.0], 15 mM MgCl2,
500 mM KCl, 1% [vol/vol] Triton X-100, 0.1% [wt/vol] gelatin),
and 10 ng of template DNA were combined with H2O to
a volume of 100 μl in a 0.5-ml test tube and overlaid with 2 drops
of mineral oil (Sigma Chemical Co., Ltd.). To minimize non-
specific annealing of the primers to nontarget DNA, 0.5 U of
SuperTag DNA polymerase (HT Biotechnology Ltd., Cam-
bridge, United Kingdom) was added to the reaction mixture
after the initial denaturation step (5 min at 94°C), at 80°C.
Thirty-five incubation cycles followed, each consisting of 1 min
at 94°C, 1 min at 60°C, and 1 min at 72°C. The annealing
temperature for PCR was optimized empirically by the per-
formance of PCRs with a nondegenerate reverse primer (50
pmol/100 μl of CYA781R(a) instead of an equimolar mixture
of CYA781R(a) and CYA781R(b) and with amplification
products of the complete 16S RNA genes (24) from various
cyanobacteria with known sequences as templates. With an
annealing temperature of 60°C, no amplification product was
generated from templates with two mismatches to the reverse
primer, as determined by agarose gel electrophoresis. When
the degenerate reverse primer was applied in combination with
either one of the forward primers, gene fragments from chro-
mosomal DNAs were amplified from all cyanobacteria
checked, spanning a broad range of cyanobacterial phylogeny,
as is currently established, whereas other bacteria and archaea
(Table 2) were discriminated against.

**Nonaxenic cultures.** The purification of cyanobacteria can
be a difficult and time-consuming procedure, and often they
are cultivated more easily when accompanied by heterotrophic
bacteria (9, 13). Therefore, some strains growing as unicya-
nobacterial but nonaxenic cultures have been included in the
bacteriological classification system (9). If conventional prim-
ers are used, targeting highly conserved regions of 16S RNA
genes, PCR yields a heterogeneous mixture of amplification
products from such cultures (Fig. 1C, lane 6), the direct se-
quence analysis of which is not possible. Prior to sequence
determination, different DNA molecules need to be separated
by cloning and the clone library has to be screened for the
plasmid inserts of interest by multiple partial sequencing reac-
tions (29). In contrast, the PCR reported here has been used to
amplify cyanobacterial 16S RNA gene fragments exclusively
(nucleotide positions 106 to 805; *Escherichia coli* numbering
system [7]) and thereby retrieve them from impure cultures.

Twenty to one hundred micrograms of cells from cultures of

### Table 1. Primer sequences and target sites

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Target site</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYA106F</td>
<td>CGG ACG GGT GAG TAA CGC GGT G</td>
<td>106–127</td>
</tr>
<tr>
<td>CYA359F</td>
<td>GGG GAA TTT TCC GCA ATG GGG</td>
<td>359–378</td>
</tr>
<tr>
<td>CYA781R(a)</td>
<td>GAC TAC TGG GGT ATC TAA TCC CAT T</td>
<td>781–805</td>
</tr>
<tr>
<td>CYA781R(b)</td>
<td>GAC TAC AGG GGT ATC TAA TCC CTT T</td>
<td>781–805</td>
</tr>
</tbody>
</table>

* R (reverse) and F (forward) designations refer to primer orientation in relation to the rRNA. A 40-nucleotide GC-rich sequence (5’-CGC CCG CCG CCG CCC GCC GCG CCC GTC CCG CCC GCG GCG CCC G-3’) is attached to the 5’ end of the forward primers.

* E. coli numbering of 16S rRNA nucleotides (7).

* Forward primers CYA106F and CYA359F were used in alternative reactions.

* Y, a C/T nucleotide degeneracy.

* Reverse primer CYA781R was an equimolar mixture of CYA781R(a) and CYA781R(b).
DGGE analysis can be performed as described previously (25), with the following modifications: 1-mm-thick polyacrylamide gels with a denaturant gradient from 20 to 60% were used, and electrophoresis was run in 1x TAE (40 mM Tris-HCl [pH 8.3], 20 mM acetic acid, 1 mM EDTA) for 3.5 h at 200 V. DGGE analysis can be used to detect sequence differences among the PCR products and therefore allows the identification of unique strains in a collection, which then may be selected for more detailed studies. Furthermore, interoperon sequence heterogeneities of 16S rRNA genes within single genomes potentially leading to ambiguities in the sequence data can be detected (30). In this study, the amplification products that contained a single sequence, confirmed by DGGE, were sequenced directly. After amplification, products of sequencing reactions were strands were determined by using the same primers as those with AmpliTaq DNA polymerase. Sequences of both DNA strands were determined by using the same primers as those used for amplification. Products of sequencing reactions were

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanobacteria</td>
<td>Was lysed by three sequential freezing (in liquid N₂) and thawing (at 65°C) steps followed by incubation for 20 min at 50°C in 5 ml of TESC buffer (100 mM Tris-HCl [pH 8], 100 mM EDTA, 1.5 M NaCl, 1% [wt/vol] hexadecylmethylammonium bromide) containing proteinase K at 100 μg/ml and 1% (wt/vol) sodium dodecyl sulfate. Chromosomal DNAs were extracted by applying phenol, chloroform, and isoamylalcohol (48) before they were used as templates in PCR. DGGE was performed as described previously (25), with the following modifications: 1-mm-thick polyacrylamide gels with a denaturant gradient from 20 to 60% were used, and electrophoresis was run in 1x TAE (40 mM Tris-HCl [pH 8.3], 20 mM acetic acid, 1 mM EDTA) for 3.5 h at 200 V. DGGE analysis can be used to detect sequence differences among the PCR products and therefore allows the identification of unique strains in a collection, which then may be selected for more detailed studies. Furthermore, interoperon sequence heterogeneities of 16S rRNA genes within single genomes potentially leading to ambiguities in the sequence data can be detected (30). In this study, the amplification products that contained a single sequence, confirmed by DGGE, were sequenced directly. After purification by the QIAquick PCR purification kit (Diagen, Düsseldorf, Germany), PCR products were used as templates in sequencing reactions with the Applied Biosystems PRISM Dye Terminator Cycle Sequencing Ready reaction kit supplied with AmpliTaq DNA polymerase. Sequences of both DNA strands were determined by using the same primers as those used for amplification. Products of sequencing reactions were</td>
</tr>
</tbody>
</table>
Primers CYA106F and CYA781R were used for amplification. A mixture of PCR products derived from five cyanobacterial strains was applied on each gel as a standard to allow gel-to-gel comparisons (lane 1, top to bottom: Synechocystis sp., Synechococcus leopoliensis SAG 1402-1, Microcoleus chthonoplastes MPI-NDN-1, Geitlerinema strain PCC 9452 [*Microcoleus* strain 10 mfx], Cyronothece strain PCC 7418). Primers CYA359F and CYA781R were used for amplification. (A) PCR products derived from microbial mats P4 (lane 2) and NC20 (lane 3), which had been sampled from environments of different salinities (9 and 5 to 30%, respectively). Primers CYA359F and CYA781R were used for amplification. (B) PCR products derived from marine plankton, which had been sampled from a seawater mesocosm after incubation for 65 h (lane 4) and 281 h (lane 5). Primers CYA359F and CYA781R were used for amplification. (C) PCR products derived from a nonaxenic culture of *Nostoc* strain PCC 9452 (‘strain 10 mfx’), and *Microcoleus* MPI-96P201 and *Amphora delicatissima* MPI 9452 (‘strain Scytonema strain PCC 9452 [*Microcoleus* strain 10 mfx], Cyronothece strain PCC 7418). Primers CYA359F and CYA781R were used for amplification. (D) PCR products derived from photobionts of the cyanobacterial lichens *Collema cf. coccophorum* and *Peltula lingulata*. The use of primers complementary to gene stretches highly conserved among bacteria (40) yields amplification products heterogeneous in sequence (lane 6), whereas the primers CYA106F and CYA781R enable the selective amplification of the cyanobacterial gene segment (lane 7). (D) PCR products derived from photobionts from the lichens *Collema cf. coccophorum* (lane 8) and *P. lingulata* (lane 9). Primers CYA106F and CYA781R were used for amplification.

FIG. 1. Composite figure of ethidium bromide-stained DGGE separation patterns of PCR-amplified segments of 16S rRNA genes. A mixture of PCR products derived from five cyanobacterial strains was applied on each gel as a standard to allow gel-to-gel comparisons (lane 1, top to bottom: Synechocystis sp., Synechococcus leopoliensis SAG 1402-1, Microcoleus chthonoplastes MPI-NDN-1, Geitlerinema strain PCC 9452 [*Microcoleus* strain 10 mfx], Cyronothece strain PCC 7418). Primers CYA359F and CYA781R were used for amplification. (A) PCR products derived from microbial mats P4 (lane 2) and NC20 (lane 3), which had been sampled from environments of different salinities (9 and 5 to 30%, respectively). Primers CYA359F and CYA781R were used for amplification. (B) PCR products derived from marine plankton, which had been sampled from a seawater mesocosm after incubation for 65 h (lane 4) and 281 h (lane 5). Primers CYA359F and CYA781R were used for amplification. (C) PCR products derived from a nonaxenic culture of *Nostoc* strain PCC 9452 (‘strain 10 mfx’), and *Microcoleus* MPI-96P201 and *Amphora delicatissima* MPI 9452 (‘strain Scytonema strain PCC 9452 [*Microcoleus* strain 10 mfx], Cyronothece strain PCC 7418). Primers CYA359F and CYA781R were used for amplification. (D) PCR products derived from photobionts of the cyanobacterial lichens *Collema cf. coccophorum* and *Peltula lingulata*. The use of primers complementary to gene stretches highly conserved among bacteria (40) yields amplification products heterogeneous in sequence (lane 6), whereas the primers CYA106F and CYA781R enable the selective amplification of the cyanobacterial gene segment (lane 7). (D) PCR products derived from photobionts from the lichens *Collema cf. coccophorum* (lane 8) and *P. lingulata* (lane 9). Primers CYA106F and CYA781R were used for amplification.

analyzed by using an Applied Biosystems 377 DNA sequencer. Many fragments were analyzed, yielding cyanobacterial sequences in all cases. Sequence data generated for the nonaxenic strains “*Oscillatoria limnetica*,” *Geitlerinema* strain PCC 9452 (‘*Microcoleus* strain 10 mfx), and *Gloeocapsa rupestris* SAG 36.87 and for the plastids from *Amphora delicatissima* MPI 96P201 and *Navicula salinicola* MPI 96P205 were deposited in GenBank. Whereas the use of complete 16S rRNA gene sequences is recommended for reliable phylogeny reconstruction, information about shorter gene segments usually is sufficient for the identification of bacteria (38).

**Symbiotic cyanobacteria in lichens.** Cyanobacteria belonging to the morphologically defined genera *Calothrix*, *Chroococcus*, *Chroococcidiopsis*, *Chroococcidiopsis*, *Cyanosarcina*, *Dichotheophyllum*, *Gloeocapsa*, *Hyella*, *Hyphomorpha*, *Mycosarcina*, *Nostoc*, *Sytonemata*, and *Stigonema* are all found in lichens as primary or secondary phototrophic symbionts (8). However, with some exceptions, the identification of the cyanobacterial photobionts in intact lichen thalli is impossible, because their morphology is modified by interactions with the fungal hyphae and because only some stages of the life cycle may be present. Thus, culti-
might not sufficiently reflect the physiological diversity of the respective organisms (14, 42). On the other hand, sequence heterogeneities due to multiple copies of rRN operons within single genomes of bacteria may complicate the interpretation of sequence data or DGGE band patterns, particularly when retrieved from natural microbial communities. The detection of slightly different 16S rRNA gene sequences is not sufficient to prove the presence of different bacterial populations in an environmental sample (30). The rpoC1 gene, encoding the γ subunit of RNA polymerase, has been described as an alternative target for analysis of cyanobacterial phylogeny (5) and community structure (31). However, the sequence data available for these genes are rather limited, whereas the determination of 16S rRNA gene sequences is a routine procedure in prokaryotic taxonomy today, resulting in large and steadily growing databases which improve the robustness of phylogenetic reconstructions, identification results, and primer specificity evaluations. Other molecular biological approaches which have been described for the identification of cyanobacteria are applicable exclusively to axenic cultures. These include multiplex randomly amplified polymorphic DNA analysis (26) and the sequence analysis of internal transcribed spacer regions of ribosomal RNA operons (47). Some other approaches apply to only certain groups of cyanobacteria. The latter include the analysis of genes encoding phycoerythrin (published primers were reported to yield no PCR products with some of the strains checked [27]) or nitrogenase (3) and the detection of a repetitive DNA sequence in toxin-producing heterocystous strains checked (27). On the other hand, sequence heterogeneities due to multiple copies of respective organisms (14, 42). 

The research described in this study was financially supported by the Max Planck Society and the Deutsche Forschungsgemeinschaft. We thank B. Biedel for the herbarium sample of P. littulata and the identification of the lichens; M. Hernández-Marine and E. Clavero for the identification of the diatoms; Y. Cohen, P. K. Hayes, and H. Jannasch for the gifts of bacterial strains; and B. Cleven and H. Schafer for the gifts of bacterial strains; and B. Cleven and H. Schafer for the identification of the lichens; M. Hernández-Marine and E. Clavero for critically reading the manuscript. We also thank Exportadora de Sal, S.A.D.C.V., Guerrero Negro, Baja California Sur, Mexico, for support in our field collections.

REFERENCES


