Vol. 66, No. 2

Identification of and Spatio-Temporal Differences between Microbial Assemblages from Two Neighboring Sulfurous Lakes: Comparison by Microscopy and Denaturing Gradient Gel Electrophoresis

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The microbial assemblages of Lake Cisó and Lake Vilar (Banyoles, northeast Spain) were analyzed in space and time by microscopy and by performing PCR-denaturing gradient gel electrophoresis (DGGE) and sequence analysis of 16S rRNA gene fragments. Samples obtained from different water depths and at two different times of the year (in the winter during holomixis and in the early spring during a phytoplankton bloom) were analyzed. Although the lakes have the same climatic conditions and the same water source, the limnological parameters were different, as were most of the morphologically distinguishable photosynthetic bacteria enumerated by microscopy. The phylogenetic affiliations of the predominant DGGE bands were inferred by performing a comparative 16S rRNA sequence analysis. Sequences obtained from Lake Cisó samples were related to gram-positive bacteria and to members of the division *Proteobacteria*. Sequences obtained from Lake Vilar samples were related to members of the *Cytophaga-Flavobacterium-Bacteroides* phylum and to cyanobacteria. Thus, we found that like the previously reported differences between morphologically distinct inhabitants of the two lakes, there were also differences among the community members whose morphologies did not differ conspicuously. The changes in the species composition from winter to spring were also marked. The two lakes both contained sequences belonging to phototrophic green sulfur bacteria, which is consistent with microscopic observations, but these sequences were different from the sequences of cultured strains previously isolated from the lakes. Euryarchaeal sequences (i.e., methanogen- and thermoplasma-related sequences) also were present in both lakes. These euryarchaeal group sequences dominated the archaeal sequences in Lake Cisó but not in Lake Vilar. In Lake Vilar, a new planktonic population related to the crenarchaeota produced the dominant archaeal band. The phylogenetic analysis indicated that new bacterial and archaeal lineages were present and that the microbial diversity of these assemblages was greater than previously known. We evaluated the correspondence between the abundances of several morphotypes and DGGE bands by comparing microscopy and sequencing results. Our data provide evidence that the sequences obtained from the DGGE fingerprints correspond to the microorganisms that are actually present at higher concentrations in the natural system.

Identification and quantification of organisms, which provide the key parameters in diversity studies, are routinely performed operations in macroecology but are still difficult tasks in microbial ecology (5, 38). Measurements of bacterial metabolic processes yield valuable ecological information but, most of the time, give no clue as to which species are involved (15). As a result, our knowledge of the taxonomic compositions of microbial communities and of the factors which control the abundance and distribution of microbial populations is extremely limited.

Over the last 10 years several molecular techniques have been developed in order to study natural samples (33). These techniques can help identify microorganisms without isolation (2, 22) and have revealed the enormous extent of microbial diversity (43). Moreover, new molecular approaches have been proposed recently in order to link microbial processes with the organisms involved (3, 9). However, it is very probable that molecular techniques provide a biased view of microbial diversity. For example, many of the procedures rely on PCR, a technique in which biases have been shown to exist, and on cloning, which can act in a selective way (58). Likewise, it is not clear whether bacterial cells in nature exhibit different degrees of resistance to cell breakage, which is necessary for nucleic acid extraction. Altogether, it is difficult to ascertain whether the collection of sequences obtained from an environment represents the natural assemblage accurately. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA genes is a molecular technique that is used to study the dynamic behavior of complex microbial assemblages (33, 36) and to isolate microorganisms in pure culture (53, 57). In this study we tested the performance of a widely used technique, DGGE, in an environment for which double checking with microscopic techniques is at least partially possible.

To do this, we used microbial assemblages that inhabit sulfurous karstic lakes. These assemblages include a few photo-

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TABLE 1. Sequences, target sites, and specificities of the primers used in this study

Primer[a]	Target site[b]	Sequence (5′ to 3′)	Specificity	Reference
341F-GC[c]	341-357	CCT ACG GGA GGC AGC AG	Most *Bacteria*	35
907R	907-926	CCG TCA ATT CMT TTG AGT TT	Most known organisms	37
CYA359F-GC	359-378	GGG GAA TYT TCC GCA ATG GG	Cyanobacteria and chloroplasts	39
CYA781R(a)[d]	781-805	GAC TAC TGG GGT ATC TAA TCC CAT T	Cyanobacteria and chloroplasts	39
CYA781R(b)[d]	781-805	GAC TAC AGG GGT ATC TAA TCC CTT T	Cyanobacteria and chloroplasts	39
ARC344F-GC	344-363	ACG GGG YGC AGC AGG CGC GA	Most *Archaea*	49
ARC915R	915-934	GTG CTC CCC CGC CAA TTC CT	Most *Archaea*	55

[a] F (forward) and R (reverse) indicate the orientation of the primers in relation to the rRNA sequence.
[b] *Escherichia coli* numbering of Brosius et al. (6).
[c] GC is a 40-nucleotide GC-rich sequence attached to the 5′ end of the primer as described by Muyzer et al. (34). The GC sequence is 5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3′.
[d] Reverse primer CYA781R is an equimolar mixture of CYA781R(a) and CYA781R(b).

synthetic populations that are very large (44) and are easily distinguished and quantified on the basis of morphology and pigment content at the genus level and even at the species level (47). Lake Cisó and Lake Vilar are two karstic lakes that have been studied over the last 20 years by using classical methods (46). These two lakes are about 1 km apart and have the same climatic conditions and groundwater sources (8). However, the phototrophic bacteria, algae, ciliates, rotifers, and crustaceans in Lake Vilar differ markedly from those in Lake Cisó (15, 16, 20, 29, 45, 46). The differences have tentatively been attributed to the different limnological parameters and light environments in the two lakes (18–20). The identities of other populations of the microbial assemblages were not known previously.

Here we describe the identities of such populations as determined by sequencing of PCR-amplified, DGGE separated, 16S rRNA gene fragments. A detailed microscopic quantification analysis of photosynthetic microorganisms was performed, and the results obtained were compared to the 16S rRNA gene sequences recovered. This analysis provided a double check for the possible biases and sensitivity of the molecular approach. Moreover, the information obtained directly from the DGGE band patterns was validated after we performed a sequence analysis of single DGGE bands; this study addressed the potential and limitations of fingerprint analysis for studying natural communities.

MATERIALS AND METHODS

Description of the lakes. Lake Cisó and Lake Vilar belong to the Banyoles karstic system located in Girona in northeast Spain (42°8′N, 2°45′E). The high concentrations of dissolved sulfate in the lake water (up to 10 mM) result in high concentrations of sulfide after microbial activity (20). Lake Cisó is a small holomictic lake with a surface area of approximately 650 m² and a maximum depth of 6.5 m. Physicochemical properties of this lake and the distribution and activity of microorganisms in it have been extensively studied by using classical limnological and microbiological methods (46). Lake Cisó becomes anoxic during winter holomixis (complete mixing), and high sulfide concentrations (up to 0.5 mM) are present in the entire water column from the bottom of the lake to the surface. Under these conditions all eukaryotic organisms disappear, and the microbial assemblage is almost exclusively prokaryotic; dense populations of photosynthetic sulfur bacteria are distributed throughout the lake. Light penetration is severely limited by the abundant populations, and light is extinguished at a depth of only a few centimeters. At the beginning of thermal stratification (which lasts from April until September), the epilimnion becomes oxic, and a morphologically more diverse microbial assemblage (including eukaryotic microorganisms) develops. In the hypolimnion, the sulfide concentrations increase (up to 1.2 mM), and a discrete zone where oxygen and sulfide coexist is established in the metalimnion (depth, about 1.5 m). Here, a stratified microbial community develops coinciding with the well-established gradient of physicochemical conditions (29, 46).

Lake Vilar is about 1 km away from Lake Cisó and consists of two basins with a maximum depth of 9 m and a surface area of approximately 11,000 m². In contrast to holomictic Lake Cisó, Lake Vilar is meromictic. Sulfide is present during the entire year, although it is restricted to the deeper, high-conductivity waters. A stable chemocline exists at 4.5 m between the oxygenated surface and the sulfide-rich bottom water. Here, dense populations of photosynthetic sulfur bacteria can develop when enough light penetrates (18, 31).

Sampling procedure. The two lakes were sampled on the same day within a 2- to 3-h interval. Samples were collected on 19 February 1996 during the winter mixing and on 9 April 1996 during a phytoplankton spring bloom. Depth profiles for water temperature, conductivity, and oxygen concentration were determined in situ by using a portable multisensor probe (model Hydrolab DS-3; Hydrolab Instruments). Light penetration was measured with a submersible spherical quantum meter (model QSP-170; Biospherical Instruments). Samples used for biological and chemical analyses were taken from different depths by using a battery-driven pump connected with tubing to a conical polyvinyl chloride structure to improve laminar sampling at the interface. For sulfide measurements, 10-ml subsamples were first alkalinized by adding 100 μl of 10 N NaOH and then chemically fixed by adding zinc acetate to a final concentration of 0.1 M (17). To determine total cells counts, 10-ml subsamples were fixed by adding formaldehyde to a final concentration of 4% (vol/vol). Samples used for molecular biological analyses were kept in the dark on ice until further processing in the laboratory, which was always started within 2 to 6 h after collection.

Chemical and biological analyses. Sulfide contents were measured spectrophotometrically by the methylene blue colorimetric method (17). DAPI (4′,6′-diamidino-2-phenylindole)-stained cells (48) were counted by using an epifluorescence Axiophot II microscope (Zeiss, Jena, Germany) and previously described statistical recommendations (23). In each case the standard deviation was less than 10% of the cell count. Morphologically distinguishable phototrophic bacteria were identified as described by Pfennig and Trüper (47). Some taxonomically valuable characteristics, such as motility and the presence of gas vesicles, were observed by using phase-contrast microscopy and live samples. For nucleic acid analysis, 1 to 5 liters of lake water was concentrated by using a refrigerated centrifuge operated at 8,000 × g (Sorvall Instruments). The cell pellet was kept frozen at −80°C until it was used. Microscopic observation of the supernatants revealed that between 1 and 3% of the cells were not recovered by this method.

Nucleic acid extraction and PCR amplification of 16S rDNA. DNA was extracted with hot sodium dodecyl-sulfate-phenol and was purified by using phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol), followed by ethanol precipitation; about 50 mg (fresh weight) of bacterial cells and the protocol of Oelmüller et al. (40) were used. We determined microscopically that phototrophic bacteria were effectively broken by this method. Fragments of the 16S ribosomal DNA (rDNA) suitable for DGGE analysis were obtained by using three different primer combinations for different phylogenetic lineages (Table 1); one combination was used for members of the domain *Bacteria* (34, 35), another combination was used for the oxygenic phototrophs and targeted the 16S rRNA genes of cyanobacteria and algal chloroplasts (39), and the third combination was used for members of the domain *Archaea* (49, 55). The lengths of the PCR products were 585, 446, and 624 bp, respectively. Recently, researchers have shown that the bacterial primer combination consisting of primers 341F and 907R used here provides the most reliable results (21, 24). The PCR conditions used for the bacterial and cyanobacterial primer sets have been described previously by Muyzer et al. (34) and Nübel et al. (39), respectively. To amplify the archaeal genes, we used a touchdown protocol for 20 cycles with temperatures ranging from 71 to 61°C; the annealing temperature was reduced 1°C every two cycles. This procedure was followed by 15 additional cycles at an annealing temperature of 61°C. Except for the initial denaturation step (94°C, 5 min), denaturation and annealing phase steps were 1 min long, while most of the polymerization phase steps were 3 min long (the only exception was the final cycle, which was 10 min long).

DGGE analysis of PCR products and sequencing. DGGE was performed as described previously (34) for 3.5 h at a constant voltage of 200 V. The archaeal primer set gave unsatisfactory melting behavior results with one of the four cultures of methanogens tested. The PCR product obtained from this culture did

not stop at a fixed position in the gel but kept migrating until electrophoresis was stopped. If this also happened with the PCR products from a complex community, the faster DGGE band would include a mixture of sequences. However, sequencing of this band showed that the problem did not occur with our natural samples. About 600 ng of PCR product was deposited in each well. After electrophoresis, the gels were stained with ethidium bromide and photographed with UV transillumination (wavelength, 314 nm) with a Polaroid camera. The photographs were scanned, and the digitized images were processed with the NIH Image software (National Institutes of Health, Bethesda, Md.) in order to measure relative band intensities. A band was defined as an ethidium bromide signal whose intensity was more than 0.2% of the total intensity for each lane.

Prominent bands were excised from the gels, reamplified, and electrophoresed again in a DGGE gel as previously described (34). The new PCR products were purified by using a Qiaquick PCR purification kit (Qiagen Inc.). A *Taq* Dyedeoxy terminator cycle sequencing Ready Reaction kit (Applied Biosystems, Forster City, Calif.) was used to sequence the 16S rDNA fragments with the appropriate forward PCR primer without the GC clamp. The sequencing reactions were performed with an Applied Biosystems model 373S DNA sequencer.

Comparative sequence analysis and construction of phylogenetic trees. A BLAST search (1) was used to get a first indication of what sequences were retrieved. New sequences were aligned with about 5,400 homologous complete prokaryotic 16S rRNA primary structures (28) by using the automated aligning tool of the ARB program package (http://www.mikro.biologie.tu-muenchen.de). A similarity matrix was constructed in order to obtain sequence similarity values. Then partial sequences were inserted into the optimized tree derived from the complete sequence data by using the maximum-parsimony criterion and a special ARB parsimony tool that did not affect the initial tree topology (27). The resulting tree was pruned to save space, and the closest relatives were retained.

Nucleotide sequence accession numbers. A total of 26 partial sequences (lengths, 500 to 600 bp) have been deposited in the EMBL nucleotide sequence database under the following accession numbers: AJ239988 to AJ239990 (CIARC-1 to CIARC-3), AJ239991 to AJ240001 (CIBAC-1, -2, -3, -6, -7, -9, -10, -11, -12, -13, and -15), AJ240002 and AJ240003 (CICYA-1 and CICYA-2), AJ240004 to AJ240006 (VIARC-1 to VIARC-3), AJ240007 to AJ240011 (VIBAC-1, -3, -4, -6, and -7), and AJ240012 and AJ240013 (VICYA-1 and VICYA-2). These sequences are the sequences shown in Fig. 4 and 5. Each band designation includes, in addition to a number (see Fig. 3), a code for the lake (CI, Lake Cisó; VI, Lake Vilar) and a code for the primer set used (ARC, *Archaea*; BAC, *Bacteria*; CYA, cyanobacteria).

RESULTS

Physicochemical parameters of the lakes. Figures 1 and 2 show the vertical profiles for temperature, conductivity, light, and concentrations of oxygen and sulfide for Lake Cisó and Lake Vilar, respectively. Figures 1A and 2A show the data obtained in February 1996 during the winter holomixis, and Fig. 1B and 2B show the data obtained in April 1996 during the early spring. In Lake Cisó the conductivity did not differ along the vertical profiles, whereas in Lake Vilar it did, reflecting the meromictic nature of this lake. The water temperature in both lakes ranged from 8 to 15°C, but due to its smaller volume and sheltered environment, Lake Cisó became thermally stratified during the first weeks of spring. The patterns of incident light extinction differed between lakes and between seasons. No light penetrated further than a depth of 2 m in Lake Cisó, whereas light penetrated to a depth of 5 m in Lake Vilar. Opposite oxygen and sulfide gradients were observed in both lakes. During the winter, the water column in Lake Cisó was anaerobic (Fig. 1A), whereas the maximal oxygen concentration was found in the spring (Fig. 1B), mainly due to the photosynthetic activity of the alga *Cryptomonas* sp. However, no oxygen was detected at depths below 1.5 m. In Lake Vilar, the maximal oxygen concentration was also found in the spring due to the activity of different algae and cyanobacteria. High sulfide concentrations were found in both lakes, and these concentrations ranged from 0.4 mM in Lake Cisó to 1.0 mM in Lake Vilar.

Vertical distribution of microbial populations. The vertical distribution of microorganisms was assessed by microscopy. The most abundant group in all of the samples was the group designated "other prokaryotes" (Fig. 1 and 2). This group included all of the morphologically indistinguishable prokaryotes. The bacteria with conspicuous morphologies are
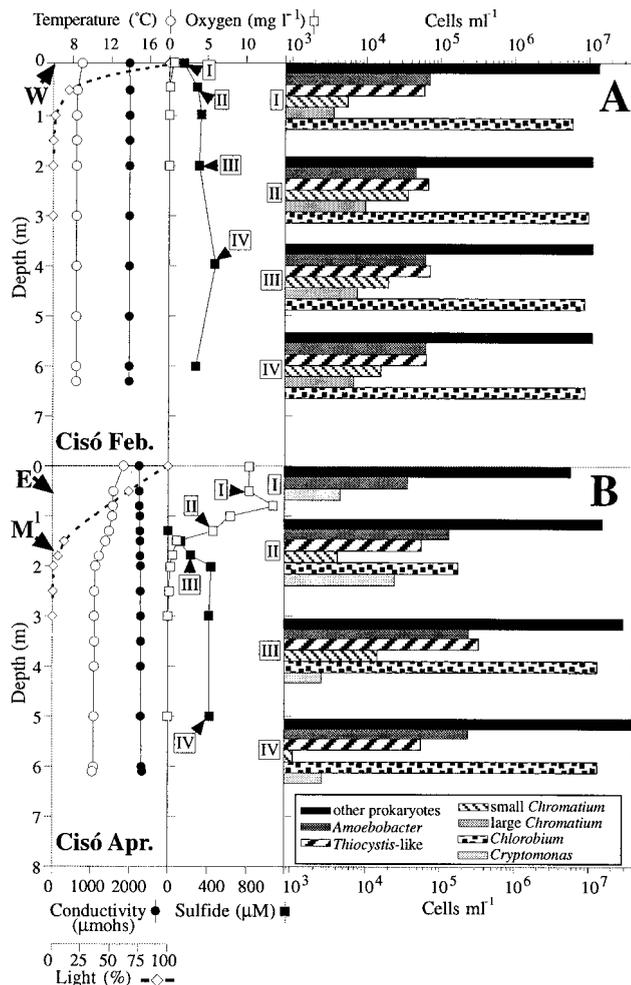
FIG. 1. Vertical profiles for temperature, conductivity, oxygen concentration, sulfide concentration, light penetration, and cell counts on two different days in Lake Cisó. (A) Winter mixing (19 February 1996). (B) Spring stratification (9 April 1996). The sampling depths used for the molecular survey are indicated by arrows. Cell counts are on a logarithmic scale.

shown in Table 2. In Lake Cisó five such bacterial populations were distinguished on the basis of shape, size, and autofluorescence (Fig. 1). These populations were populations of green sulfur bacteria of the *Chlorobium* type (autofluorescent, nonmotile, small rods), whose concentrations reached 10^7 cells ml^{-1}, and purple sulfur bacteria similar to both *Amoebobacter* and *Thiocystis*-like cells, whose concentrations reached 10^5 cells ml^{-1}. Other populations identified in Lake Cisó were populations of small and large *Chromatium* cells (Table 2) whose concentrations were between 10^3 and 10^4 cells ml^{-1}. In Lake Vilar, purple sulfur bacteria were not detected during the time that samples were collected, and the sulfur bacteria present were similar to *Chlorobium phaeobacteroides* (maximum concentration, 7×10^6 cells ml^{-1}).

Oxygenic phototrophic populations were also identified. In Lake Vilar, populations of algae (*Cryptomonas* and *Crucigenia* species; concentrations, up to 2×10^3 cells ml^{-1} each) and cyanobacteria (*Synechococcus*-shaped cells; concentration, 10^6 cells ml^{-1}) were detected, whereas *Cryptomonas* cells (concentrations, up to 2.4×10^4 cells ml^{-1}) were the dominant algae in Lake Cisó. The cyanobacterium *Pseudanabaena* sp. was observed sporadically in Lake Cisó but not in Lake Vilar. Several

FIG. 2. Vertical profiles for temperature, conductivity, oxygen concentration, sulfide concentration, light penetration, and cell counts on two different days in Lake Vilar. (A) Winter (19 February 1996). (B) Spring (9 April 1996). The sampling depths used for the molecular survey are indicated by arrows. Cell counts are on a logarithmic scale.

respectively). Thus, we were able to monitor qualitative differences in the communities between lakes, between two seasons, and among depths.

First, we found that there were marked differences in the mobilities of the DGGE bands when we compared lakes; the most intense bands for the two lakes did not coincide with any of the primer sets tested (Fig. 3). Therefore, we expected the PCR-recovered populations in Lake Cisó and Lake Vilar to be different. Despite the difference in composition, the overall numbers and relative intensities of the bands were similar for the two lakes; i.e., the total number of bands detected ranged from 8 to 17 for both lakes, and just a few bands (one to three bands) appeared to be the most intense bands in all cases.

Second, when we compared seasons, the winter bacterial community in Lake Cisó (Fig. 3A, lane W) shared more bands with the anaerobic spring community (Fig. 3A, lane M) than with the aerobic spring community (Fig. 3A, lane E). This shift in the community fingerprint was expected as a result of growth of cyanobacteria or algae (the bacterial primer recovered DNA from chloroplasts) in the epilimnion. However, no changes in the fingerprint resulting from oxygenic phototrophs were detected (Fig. 3B, lanes W, M, and E). Therefore, the shift was probably due to the appearance of new populations of members of the *Bacteria*. A very weak PCR product was obtained for members of the *Archaea* in the aerobic samples, and this product did not yield a DGGE band pattern (data not shown). Archaeal bands from the anaerobic zone of Lake Cisó migrated to the same position in the winter and in the spring (Fig. 3C, lanes W and M), indicating that the archaeal assemblages were very similar.

In Lake Vilar, the change of season resulted in a marked shift in the bacterial populations both in the aerobic zones and in the anaerobic zones (Fig. 3A). Bands that appeared to be dominant in the winter were less intense or disappeared completely in the spring (Fig. 3A, lanes E1 and E2). Moreover, new dominant DGGE bands were obtained with the spring samples (Fig. 3A, lane M2, bands 4 and 5). When the oxygenic phototrophs were analyzed, new bands were detected in the spring (Fig. 3B, lane E2), and these bands might have been partially responsible for the shift observed with the bacterial primers. As occurred in Lake Cisó, the archaeal pattern did not change markedly (Fig. 3C).

Finally, the DGGE patterns of samples obtained from different depths reflected substantial differences between the aerobic and anaerobic assemblages.

Phylogenetic affiliations of predominant community members. Most bands (40 bands) in the DGGE fingerprints were excised, reamplified, purified, and sequenced. A total of 26 of the 40 bands yielded sequences without ambiguous positions and were included in phylogenetic trees (Fig. 4 for members of the *Bacteria* and Fig. 5 for members of the *Archaea*). These bands were designated as described above. Of the remaining 14 bands, 6 bands (mainly weak bands) did not yield a reamplification product or sequence, and 2 did yield such a product but there were many ambiguous positions (more than 20% of the partial sequence). For the last six bands (labeled in Fig. 3 but not included in Fig. 4 and 5) some positions (about 10% of the partial sequence) were ambiguous, and these bands were not included in the tree. However, the affiliations of some of these sequences provided results that were consistent with microscopic observations (e.g., VIBAC-2 with *Cryptomonas* sp. and CIBAC-4 with *Chlorobium limicola*) and with the affiliations of bands exhibiting the same melting behavior in the gels (CIBAC-14 with CIBAC-7 and CIBAC-8 with CIBAC-15).

The resulting parsimony trees are shown in Fig. 4 (*Bacteria*) and Fig. 5 (*Archaea*). Parallel analyses in which we used the

ciliates previously reported to occur in these ecosystems, such as *Plagiopyla* sp. at anaerobic depths and *Coleps* sp. at aerobic depths (30), were detected microscopically as well.

DGGE fingerprint analyses. DGGE analyses were performed with samples obtained at selected depths (indicated by arrows in Fig. 1 and 2) by using primer sets for members of the *Bacteria* (Fig. 3A), for oxygenic phototrophs (Fig. 3B), and for members of the *Archaea* (Fig. 3C). Each microbial assemblage produced a reproducible DGGE fingerprint in the different analyses performed. For Lake Cisó, one sample was analyzed for the winter profile and was used as a representative of the entire water column (lanes W) since the lake is homogeneously mixed at this time of the year. In the spring, a sample from the oxygenated epilimnion (lanes E) and another sample from the top of the anaerobic layer (metalimnion, lanes M) were analyzed. Samples from the bottom of the lake produced band patterns similar to those of samples from the metalimnion (data not shown). For Lake Vilar, we analyzed the epilimnion, metalimnion, and hypolimnion in the winter (lanes E1, M1, and H1, respectively) and in the spring (lanes E2, M2, and H2,

TABLE 2. Morphotypes identified by microscopy in Lake Cisó and Lake Vilar, including some phenotypic characteristics and mean cell sizes

Morphotype	Morphology	Cell size (μm)	Presence in: Lake Cisó	Presence in: Lake Vilar	Reference(s)	Related sequence(s)[a]
Amoebobacter	Coccus	1.5-2.5	+	−	46	CIBAC-7
Thiocystis class	Ovoid	$3.0\text{-}4.0 \times 5.0\text{-}6.0^b$	+	−	46	CIBAC-15
Large *Chromatium*	Rod	$4.0\text{-}6.0 \times 8.0$	+	−	47	None
Small *Chromatium*	Rod	2.0×4.0	+	−	47	None
Chlorobium	Rod	$0.8 \times 2.0\text{-}2.5$	+	+	47	CIBAC-3, VIBAC-6
Synechococcus	Coccus	1.5-2.0	−	+	This study	VIBAC-3, VICYA-1
Pseudanabaena	Long filament		+	−	This study	CICYA-2
Plagiopyla-Metopus	Autofluorescent, rod-shaped endosymbiont cells		+	+	12, 30	CIARC-1, VIARC-3
Cryptomonas	Chloroplast		+	+	46	CIBAC-9, CICYA-1, VICYA-2
Other prokaryotes	No distinctive morphological traits		+	+	29; this study	More than 20[c]

[a] Sequence(s) retrieved which could correspond to the morphotype.
[b] Includes the morphotype designated *C. minus*-like in previous papers on Lake Cisó (46).
[c] See Table 3.

neighbor-joining method with different correction coefficients (the Kimura and Jukes-Cantor coefficients) resulted in the same tree topology (data not shown). The 16S rRNA-defined groups corresponding to predominant DGGE bands were distributed throughout both phylogenetic trees, indicating that the level of genetic diversity in the lakes studied was high. Gram-positive organisms and members of the *Proteobacteria* (alpha, beta, and gamma subdivisions) dominated the PCR-amplified, 16S rRNA-defined, bacterial populations in Lake Cisó, whereas members of the *Cytophaga-Flavobacterium-Bacteroides* phylum and Cyanobacteria dominated the populations in Lake Vilar. Methanogen- and thermoplasma-related sequences were present in both lakes; however, whereas in Lake Cisó the sequences of members of these groups were the dominant recovered archaeal sequences, in Lake Vilar a planktonic crenarchaeota-like population appeared to be the predominant archaeal population.

Table 3 shows the physiology of the closest relatives for organisms that exhibit levels of rRNA sequence identity of ≥95% (genus level rRNA difference). All of the closest relatives had metabolism that was compatible with the environment. Most of the sequences from members of the *Archaea* were not included in Table 3 because they were too distantly related to any cultured organism. Sequences affiliated with the *Cytophaga* phylum or with gram-positive organisms (both high- and low-G + C-content organisms) also lacked close relatives and are not included in Table 3.

A sequence alignment performed with ARB showed that the same sequences were recovered with different primer sets and the amplification conditions employed. Thus, in Lake Cisó, bands CIBAC-9 (amplified with the bacterial primer set) and CICYA-1 (amplified with the cyanobacterium-chloroplast primer set) were identical and closely related to *Cryptomonas* bands (Fig. 4). The same thing occurred in Lake Vilar for bands VIBAC-3 and VICYA-1 in relation to *Synechococcus* bands.

Interestingly, the phylotypes related to *Cryptomonas* sp. recovered from Lake Cisó (CICYA-1) and Lake Vilar (VI-
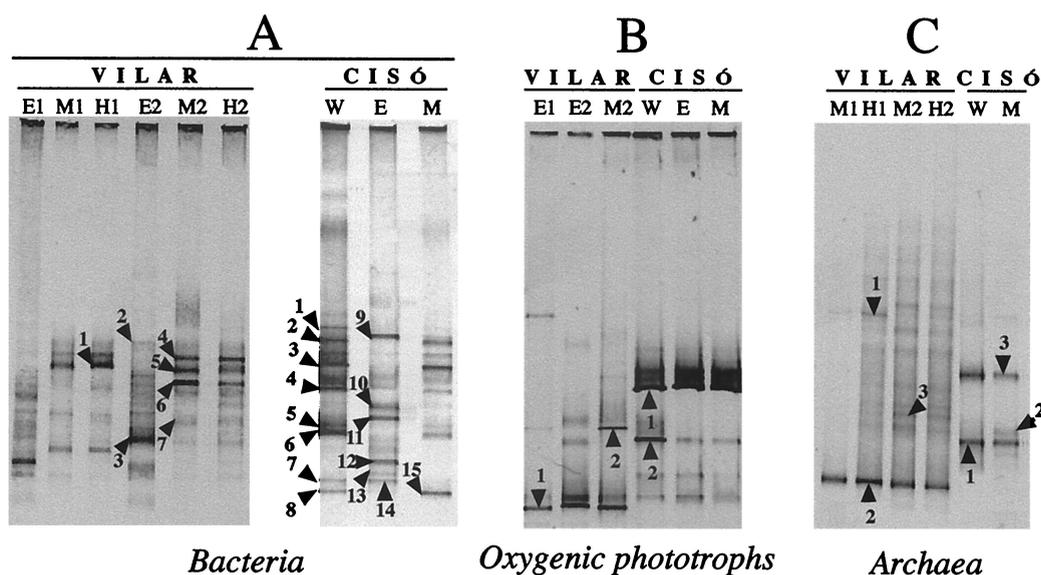
FIG. 3. Negative images of ethidium bromide-stained DGGE gels containing PCR-amplified segments of 16S rRNA genes obtained by using three primer sets. The band patterns were obtained with bacterial primers (A), cyanobacterium-chloroplast primers (B), and archaeal primers (C) from the oxic epilimnion (lanes E) and from the anoxic metalimnion (lanes M) and hypolimnion (lanes H). Lanes E1, M1, and H1 and lanes E2, M2, and H2 correspond to winter and spring, respectively, in Lake Vilar. Lane W and lanes E and M correspond to winter and spring, respectively, in Lake Cisó. Bands were designated as described in the text.
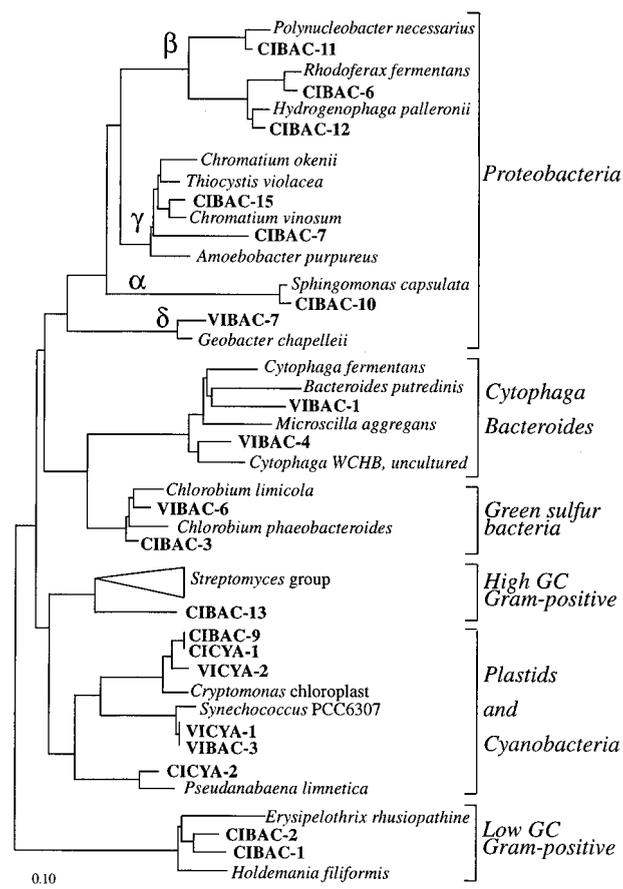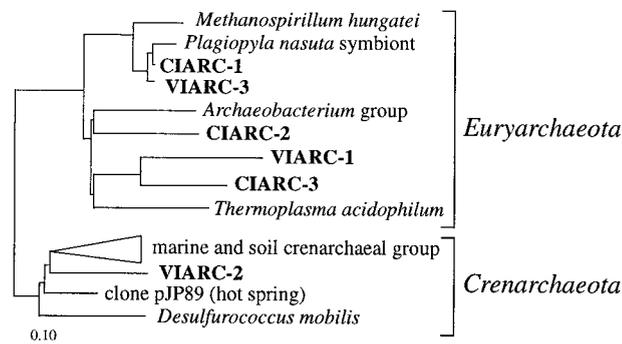
FIG. 4. Phylogenetic affiliations within the domain *Bacteria* of the excised bands obtained from the gels in Fig. 3A and 3B. The tree was constructed by adding by parsimony analysis the partial sequence data to a previously validated and optimized tree. Scale bar = 0.10 mutation per nucleotide position.

CYA-2) were not identical; they exhibited 97% similarity. Likewise, the brown-pigmented *Chlorobium*-like phylotypes from the two lakes (CIBAC-3 and VIBAC-6) exhibited 96.9% similarity. Thus, the phylogenetic affiliations revealed that different phylotypes were present in Lake Cisó and Lake Vilar for at least two different microorganisms.

Microscopic observations and recovered 16S rRNA-defined populations. Several populations recovered by PCR and sequencing confirmed microscopic observations. *Cryptomonas*-like algae and *Synechococcus*-like cyanobacteria were dominant phytoplankton components as determined by both microscopic counting (Fig. 1 and 2) and analysis of PCR products recovered with specific primers (Fig. 3B and 4). Also, *Pseudanabaena*-like cyanobacteria were recovered from Lake Cisó but not from Lake Vilar, which was in agreement with microscopic observations. Several common ciliates found previously in these ecosystems, such as *Plagiopyla* sp. at anaerobic depths and *Coleps* sp. at aerobic depths (30), were detected microscopically. It has been shown that such organisms carry endosymbiontic microbial populations (12). The CIARC-1 and VIARC-1 bands most likely corresponded to archaeal endosymbionts of the ciliate *Plagiopyla* sp. (Fig. 5). Among the photosynthetic bacteria (i.e., members of the *Chlorobiaceae* and *Chromatiaceae*), organisms present at higher cell concentrations were recovered from the sequenced DGGE bands, but organisms present at lower concentrations (e.g., large *Chroma-*

tium okenii-like cells) were not. Tables 4 and 5 show the relationship between cell counts and the signal intensities of the PCR-amplified bands.

DISCUSSION

Interpretation of data resulting from molecular approaches for studying microbial diversity in natural environments presents uncertainties, and several difficulties may arise in the long process from natural samples to sequences (11, 13, 51, 56). Some of the problems are intrinsic to PCR amplification kinetics, and, therefore, they are shared by all of the approaches that use such a step (PCR cloning and PCR fingerprinting techniques). Other difficulties are specific for the DGGE technique which we used in this study. In the present study we used independently obtained microscopy-based information for some of the organisms present in Lake Cisó and Lake Vilar to investigate the magnitude of the problems. When DGGE is used, the limitations can be probed to a certain extent by excising bands and sequencing. Heteroduplex formation (10, 13, 32) seemed not to be a significant problem in our analysis. We excised, reamplified, reelectrophoresed, and sequenced a substantial number of bands (40 bands), and only 4 of these bands (10%) yielded multiple bands after reamplification. The multiple bands (presumably representing homo- and heteroduplexes) were clearly separated in the gel when we performed a new DGGE analysis, and, therefore, they cannot be related to the quality of gel separation or the precision of excision. The possibility that in a single organism there were multiple 16S rRNA operons with different migration behaviors on DGGE gels was also eliminated after sequencing. The main problem seemed to be the presence of different sequences at the same or very similar positions in the fingerprint. A cloning step is needed in such cases in order to determine the composition of the DNA mixture, but in addition the quality of gel separation and band excision can be improved. Two of the bands resulted in a large number of sequence uncertainties (e.g., Fig. 3A, main band in lane E), and six bands resulted in a lower number of ambiguous positions (see above). The latter sequences were affiliated with some of the populations detected microscopically, reflecting the fact that these probably mixed sequences were dominated by one PCR product and provided valuable information. At any rate, most of the bands in our analysis yielded a clean sequence, indicating that each band represented a different microbial population.

The molecular methods which we used should recover the microscopically recognizable populations in Lake Cisó and

FIG. 5. Phylogenetic affiliations within the domain *Archaea* of excised bands obtained from the gel in Fig. 3C. The tree was constructed by adding by parsimony analysis the partial sequence data to a previously validated and optimized tree. Scale bar = 0.10 mutation per nucleotide position.

TABLE 3. Ecophysiology of the closest relatives of the 16S rDNA sequences that exhibited levels of similarity of ≥95% (genus level identity)

Phylogenetic group	Sequence	Related sequence	% Similarity	Ecophysiology
Algae	CIBAC-9	*Cryptomonas* chloroplast	96	Oxygenic photosynthesis
	CICYA-1	*Cryptomonas* chloroplast	96	Oxygenic photosynthesis
	VICYA-2	*Cryptomonas* chloroplast	95	Oxygenic photosynthesis
Cyanobacteria	VIBAC-3	*Synechococcus* sp. strain PCC 6307	97	Oxygenic photosynthesis
	VICYA-1	*Synechococcus* sp. strain PCC 6307	97	Oxygenic photosynthesis
	CICYA-2	*Pseudoanabaena limnetica*	97	Oxygenic photosynthesis
Green sulfur bacteria	CIBAC-3	*Chlorobium phaeobacteroides*	95	Anoxygenic sulfur photosynthesis
	VIBAC-6	*Chlorobium phaeobacteroides*	96	Anoxygenic sulfur photosynthesis
Alpha-*Proteobacteria*	CIBAC-10	*Sphingomonas*	98	Highly versatile
Beta-*Proteobacteria*	CIBAC-6	*Rhodoferax fermentans*	96	Highly versatile
	CIBAC-12	*Hydrogenophaga*	96	Fermenter
Euryarchaea	CIARC-1	Ciliate endosymbiont	95	Methanogenesis
	VIARC-3	Ciliate endosymbiont	95	Methanogenesis

Lake Vilar, and each band should have an intensity proportional to its abundance. We recovered most such populations, and, in addition, the PCR products were dominant when the populations were predominant community members. The differences between microscopic counts and signal intensity were always within a factor of 10 (Tables 4 and 5). Thus, variations in the signal intensity revealed variations in the abundance of a population in the environment at the order-of-magnitude level.

Another question to consider is what percentage of the populations present in situ can be retrieved by PCR-DGGE analysis (detectability). Working with mixtures of pure cultures, Muyzer et al. (35) found that DGGE could not detect a population whose abundance was less than approximately 1% of the total cell count. We observed a similar threshold with our natural samples. At least four morphotypes of purple sulfur bacteria were identified visually in Lake Cisó (Fig. 1 and Table 2), but only two types of sequences corresponding to members of the *Chromatiaceae* were recovered. Only sequences corresponding to the dominant members of the *Chromatiaceae* (*Amoebobacter* and *Thiocystis*-like species), whose concentrations were more than 0.3 to 1% of the total DAPI counts, were recovered. Sequences corresponding to other *Chromatium*-like cells (*Chromatium okenii*, *Chromatium weissei*, and small *Chromatium* spp.) always accounted for less than 0.1% of total DAPI counts and were not detected by DGGE. The detection threshold for *Synechococcus* spp. was 0.4% (Table 5). However, we cannot eliminate the possibility that some of the minor bands that were not excised from the gel corresponded to the other *Chromatiaceae* morphotypes, that they were masked if they exhibited the same melting behavior as other populations (41) or that their concentrations were less than the detection limit of the staining solution. More information might be obtained from the weak bands by performing Southern hybridization with specific probes (34).

The final step in the process is affiliation of the sequences obtained from the DGGE fingerprints with taxa. Although complete 16S rDNA sequences should be used for phylogenetic reconstruction (26), partial sequences may be sufficient to determine the closest relatives of unknown sequences and assign them to well-established phylogenetic groups (27, 37, 54). However, a combination of partial sequences and poor representation of a given microbial group in databases (as in the case of the *Chromatiaceae*) may prevent accurate allocation of sequences. For instance, the CIBAC-15 sequence was more closely related to *Chromatium vinosum* than to *Thiocystis* sp. Microscopy and pigment composition showed that *Thiocystis*-like organisms were present in Lake Cisó (*Thiocystis* class in Table 2). Specific pigments from *C. vinosum*, on the other hand, were not detected in the lake. Probably, the sequence recovered from Lake Cisó is the sequence of a new organism that would be easier to identify if the complete 16S rDNA sequence was available.

All of the problems described above need to be considered before conclusions concerning the natural assemblages themselves are reached. However, the following questions can be addressed with a high level of confidence.

Does the molecular approach recover the main organisms known to be present as determined by microscopy, and does this approach reveal additional differences between the assemblages in the two lakes studied for other prokaryotic populations that were not identified by microscopy? We retrieved, as PCR-amplified 16S rRNA genes, most of the algae, cyanobacteria, and photosynthetic sulfur populations detected microscopically in Lake Cisó and Lake Vilar. The only morphologies that were not retrieved by DGGE were those corresponding to *Chromatium* populations found at low levels (less than 0.3% of

TABLE 4. Cell abundance and signal intensity for bacterial populations in Lake Cisó, as determined by microscopy and DGGE sequencing, respectively

Sample pattern	% of *Amoebobacter* morphotype	CIBAC-7 signal intensity (%)	% of *Thiocystis* morphotype	CIBAC-15 signal intensity (%)
W	0.4	1.1	0.3	2.0
E	0.8	6.0	ND[a]	ND
M	1.1	1.5	1.7	16.0

[a] ND, not detected.

TABLE 5. Cell abundance and signal intensity for bacterial populations in Lake Vilar, as determined by microscopy and DGGE sequencing, respectively

Sample pattern	% of *Synechococcus* morphotype	VIBAC-3 signal intensity (%)	% of *Chlorobium* morphotype	VIBAC-6 signal intensity (%)
E1	2.4	8.1	ND[a]	ND
M1	0.4	ND	13.9	6.7
H1	0.4	2.6	7.6	3.1
E2	30.0	28.1	ND	ND
M2	3.5	0.5	30.1	15.6
H2	0.5	4.2	9.8	12.0

[a] ND, not detected.

the total cell counts). The method which we used revealed that that there are differences in the bacterial populations of the two lakes that could not be revealed by microscopy. In addition, different archaeal sequences were recovered from the two lakes. Interestingly, populations that were present in both lakes, such as the *C. phaeobacteroides* populations, belonged to different phylotypes. A similar result was derived from a study of *Chromatium minus* isolates obtained from three lakes in Spain (7). This may indicate that the anaerobic layers of stratified lakes may act as islands in an aerobic world for anaerobic microorganisms.

Are the identified sequences identical (or similar) to the sequences isolated in pure culture from the same lakes? Over time, many microorganisms have been isolated in pure culture from the two lakes in order to carry out physiological studies. From an ecological perspective, it is very important to determine whether such isolates are the same organisms that are dominant in the environment or whether a minor component of the natural assemblage was selected by the isolation procedure. Several sequences related to *Chlorobium* spp. were recovered in the present study, but they did not match exactly any of the sequences deposited in the 16S rRNA database. This is especially remarkable because most of the *Chlorobium* culture sequences available in the database were obtained from the lakes in the area around Banyoles, including Lake Cisó, Lake Vilar, and Lake Banyoles (the latter is only 10 m away from Lake Vilar) (14). Thus, the ecologically relevant *Chlorobium* populations (as detected by DGGE and sequencing) and the populations isolated in pure culture from the same environments were not the same.

Are the potential metabolic characteristics of the sequences recovered consistent with the environmental conditions? The activities and functional roles of the phylotypes recovered cannot be known until the corresponding microorganisms are isolated in pure culture and characterized. However, the known metabolic characteristics of the closest relatives were generally consistent with the environmental conditions present in the lakes (i.e., anaerobiosis, simultaneous presence of light and sulfide). Surprisingly, other potentially dominant organisms at oxic-anoxic interfaces, such as sulfur or ammonia oxidizers, were not recovered. Sulfate reducers were not recovered from the water column of Lake Cisó either. Again, some of the weak bands that were not sequenced could have corresponded to these organisms, the organisms might have been present at low levels at the time of sampling, or the organisms might be sharply stratified in the water column and thus might have escaped detection because of the sampling resolution.

The presence of several *Cytophaga*-related organisms that dominate the anaerobic, sulfide-rich water of Lake Vilar remains intriguing, because most representatives of this large group of bacteria are aerobic or microaerophilic (50). Recent molecular studies, however, have shown that *Cytophaga*-related organisms are dominant components of the microbial assemblages in anaerobic marine sediments (25, 52). These results and our results suggest that the *Cytophaga*-like bacteria available in culture collections do not account for the full range of metabolic capabilities present in the group. More extensive studies are needed to establish the ecological importance of these organisms.

Are there archaeal groups living in these environments? Culturable members of the *Archaea* found in freshwater plankton are restricted to methanogens and sulfur-metabolizing thermophiles (4). We found methanogen-related sequences in the anaerobic sulfide-rich waters of Lake Cisó and Lake Vilar, and archaeal PCR products were obtained from the aerobic depths as well. However, one of the most interesting findings of

this study was the existence of a population related to the *Crenarchaeota* in the sulfide-rich water column of Lake Vilar. Whereas all of the available pure cultures of members of the *Crenarchaeota* are sulfur-metabolizing thermophiles, sequences related to members of this group have recently been recovered from a wide variety of temperate and cold environments (4), although there have been no studies which have reported the presence of such populations in the plankton of freshwater lakes. Obviously, members of this group are able to thrive in a wide range of nonextreme environments. The phylogenetic affiliation indicates that the crenarchaeal sequence obtained from Lake Vilar (VIARC-2) does not exhibit a high level of similarity with the sequence of any cultured organism (level of similarity to *Desulfurococcus mobilis*, 81%) and that it is included in the so-called freshwater cluster (4). This finding and the ecological distribution suggest that VIARC-2 is a novel type of organism. Recently, another sequence related to crenarchaeotal sequences was recovered from the sulfide-rich waters of a Norwegian meromictic lake (42), suggesting that this potential phenotype is widely distributed. Unfortunately, the organisms have not been recovered in pure culture, and their physiology and metabolism remain unknown. Their distinct vertical distribution (they accumulate in sulfide-rich layers) suggests that they have an ecological role in the anaerobic biogeochemical activity of the lake, but their relative abundance remains to be determined. Some of the sequences recovered in our study were closely related to the sequences of endosymbiontic organisms (bacterial endosymbionts of aerobic ciliates, CIBAC-11, and methanogenic endosymbionts of the anaerobic ciliate *Plagiopyla* sp.). VIARC-2 could also correspond to the sequence of an endosymbiontic organism. In order to determine the actual environment of VIARC-2, in situ hybridization or selective prefiltration of fresh samples should be carried out.

Overall, the evidence obtained with microscopically identifiable populations indicates that the dominant DGGE bands corresponded to predominant populations in the ecosystems studied. Although the data resulting from a comparison of DGGE band patterns were limited, they allowed us to monitor community changes that were consistent with the microscopic results and with the shift in environmental conditions. The microscopic control results and the sequencing data led us to conclude that the number and intensity of DGGE bands provide valuable information concerning variations in species evenness. The number of DGGE bands is related to the number of populations that account for more than 0.3 to 0.4% of the total cell counts. Thus, the species richness or total microbial diversity in the system cannot be accurately estimated with this method. However, a general pattern was recognized when the different DGGE gels were analyzed, independent of the lake, season, or depth; there were a few very abundant populations (accounting for more than 10% of the total cell count) and a few populations that accounted for between 1 and 10% of the total cell count. There are probably many more populations that account for less than 1% of the total cell count, but they cannot be retrieved by DGGE. Thus, the ecosystems studied may be very species rich, but the evenness is certainly low. This may be a general pattern for aquatic microbial communities (44).

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