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Microheterogeneity in 16S Ribosomal DNA-Defined Bacterial Populations from a Stratified Planktonic Environment Is Related to Temporal Changes and to Ecological Adaptations

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Temporal changes of the bacterioplankton from a meromictic lake (Lake Vilar, Banyoles, Spain) were analyzed with four culture-independent techniques: epifluorescence microscopy, PCR-denaturing gradient gel electrophoresis (DGGE) fingerprinting, fluorescence in situ whole-cell hybridization and flow cytometry sorting. Microscopically, blooms of one cyanobacterium (*Synechococcus* sp.-like), one green sulfur bacterium (*Chlorobium phaeobacteroides*-like), and one purple sulfur bacterium (*Thiocystis minor*-like) were observed at different depths and times. DGGE retrieved these populations and, additionally, populations related to the *Cytophaga-Flavobacterium-Bacteroides* phylum as predominant community members. The analyses of partial 16S ribosomal DNA sequences from the DGGE fingerprints (550 bp analyzed) revealed higher genetic diversity than expected from microscopic observation for most of these groups. Thus, the sequences of two *Synechococcus* spp. (both had a similarity of 97% to *Synechococcus* sp. strain PCC6307 in 16S rRNA), two *Thiocystis* spp. (similarities to *Thiocystis minor* of 93 and 94%, respectively), and three *Cytophaga* spp. (similarities to *Cytophaga fermentans* of 88 and 89% and to *Cytophaga* sp. of 93%, respectively) were obtained. The two populations of *Synechococcus* exhibited different pigment compositions and temporal distributions and their 16S rRNA sequences were 97.3% similar. The two *Thiocystis* populations differed neither in pigment composition nor in morphology, but their 16S rRNA sequences were only 92.3% similar and they also showed different distributions over time. Finally, two of the *Cytophaga* spp. showed 96.2% similarity between the 16S rRNA sequences, but one of them was found to be mostly attached to particles and only in winter. Thus, the identity of the main populations changed over time, but the function of the microbial guilds was maintained. Our data showed that temporal shifts in the identity of the predominant population is a new explanation for the environmental 16S rRNA microdiversity retrieved from microbial assemblages and support the hypothesis that clusters of closely related 16S rRNA environmental sequences may actually represent numerous closely related, yet ecologically distinct, populations.

The number of prokaryotes on earth is estimated to be 4×10^{30} to 6×10^{30} cells (56). They constitute, by far, the largest reservoir of life and encompass the major part of earth's physiological and phylogenetic diversity (58). Characterization of the diversity hidden behind this tremendous population size has been, however, chronically limited by the small cell size, cryptic morphology, and low cultivability of microorganisms (39, 41). Fortunately, our perception of microbial diversity has dramatically changed in the last decade by retrieving rRNA genes (mostly 16S ribosomal DNA [rDNA] and 18S rDNA) from the environment, without the need to have pure cultures to examine (for a review, see references 3 and 34). As a result, new rDNA sequences, substantially different from any previously known sequences, have led to the discovery of new phyla of still uncultured microorganisms (26, 31, 38). However, groups of closely related rRNA sequences (with similarities ranging from 90 to 99.9%) also indicate high diversity but at a much smaller scale (microdiversity) (18, 32). Such microheterogeneity in the ribosomal sequences has been observed in a wide range of environments and in genetic libraries obtained from bacteria, archaea, and eukaryotic microorganisms (2, 5, 6, 10, 12, 16, 19, 25, 29, 31, 33). Therefore, this might be a widespread characteristic of microbial populations. However, only a few recent studies focused on the marine cyanobacterium *Prochlorococcus* and on freshwater *Synechococcus* provide some clues about the ecological significance of microdiversity in natural populations (32, 43, 44, 54, 55).

The ecological relevance of this small-scale diversity is difficult to establish essentially for two reasons. First, because methodological difficulties that arise in the long process from natural samples to sequences may give a biased view of the microbial assemblage (57). In the worst case, the small-scale diversity in the ribosomal sequences could be due to PCR artifacts or to rRNA multioperons belonging to a single population. In order to circumvent this problem, fluorescence in situ hybridization (FISH) and flow cytometry can be used to "visualize" cells in situ (2, 3, 32). The second caveat is that most of the sequences deposited in databases have been obtained from single samples and, therefore, do not reflect time-depth dynamics of microbial assemblages. In order to compare microbial assemblages and to assess temporal and spatial changes, fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE) (36), offer the best alternative.

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Finally, testing the ecological significance of environmental rDNA-defined populations requires the selection of an appropriate environment to study (see, for example, reference 52).

In the present work, we selected a small, well-known, stratified, and sulfide-rich lake (Lake Vilar) for a survey of the temporal changes of microbial populations with limited 16S rRNA sequence variability. Detailed and frequent long-term sampling can be easily carried out in lakes (42), and many studies have taken place in Lake Vilar over many years by traditional (4, 22–24, 30) and, recently, by molecular RNA-based methods (7–9). These studies have shown that microbial populations are finely adapted to vertical and temporal gradients of physicochemical conditions. These fine gradients are very difficult to mimic in the laboratory, and microorganisms inhabiting these systems are difficult to culture (8, 9). In addition, closely related 16S rDNA sequences had been previously detected in Lake Vilar, providing us with an appropriate natural model system for this study.

MATERIALS AND METHODS

Site and sampling. Lake Vilar is a small freshwater meromictic lake in the Banyoles karstic system in Girona in northeastern Spain (42°8′N, 2°45′E). The lake consists of two basins with a total surface area of ca. 11,000 m^2 and a maximum depth of 9 m. Sulfide (up to 1.5 mM) is present during the whole year, although it is restricted to the deeper, higher conductivity waters. The chemocline is found at 4.5 m. Here, dense populations of photosynthetic sulfur bacteria can develop, provided that sufficient light penetrates. Blooms of phototrophic populations (both oxygenic and anoxygenic) are segregated in depth, along gradients of light and sulfide, and in time. Autofluorescent cyanobacteria (*Synechococcus* sp.-like), green sulfur bacteria (*Chlorobium phaeobacteroides*-like), and purple sulfur bacteria (*Thiocystis minor*-like) were counted by epifluorescence microscopy. Total cells were counted by using DAPI (4′,6′-diamidino-2-phenylindole) staining. In each case, the standard deviation was <10% of the cell count. The lake was sampled fortnightly from 19 February to 5 June 1996, and physicochemical analyses were carried out as described previously (9). Samples for molecular biological analyses were kept in the dark, on ice, for processing in the laboratory 2 to 4 h later.

Nucleic acid analyses, DGGE, and sequencing. One to five liters of lake water were concentrated by using a refrigerated centrifuge (Sorvall Instruments, DuPont, Del.) at 8,000 × *g* for 20 min and then stored at −80°C. This method recovered most of the cells (9). Total DNA was extracted and purified by using the hot phenol-sodium dodecyl sulfate method as described previously (9) and was used as target in the PCR to amplify bacterial 16S rRNA genes. Bacterial 550-bp fragments suitable for subsequent DGGE analysis were obtained with the primer combination of 341f with a GC clamp (40-nucleotide GC-rich sequence, 5′-CCT ACG GGA GGC AGC AG-3′) and 907r (5′-CCG TCA ATT CMT TTG AGT TT-3′) (35). Specific primers targeting cyanobacteria and chloroplasts from algae were also used with DGGE conditions reported elsewhere (37). DGGE for bacteria was run as described before (9) for 3.5 h at a constant voltage of 200 V and at 60°C in a 20 to 80% vertical denaturant gradient (the 100% denaturant agent is 7 M urea and 40% deionized formamide). Gels were photographed with UV transillumination after ethidium bromide staining. The pictures were digitalized and analyzed by using the gel plotting macro tool of the NIH Image software package version 1.62 (National Institutes of Health, Bethesda, Md.). After background subtraction, the intensity of each band was measured integrating the area under the peak and was expressed as a percentage of the total area in the profile. The error among replicates was <4%. Several bands were excised from the denaturing gradient gels, reamplified, and purified for sequencing as reported earlier (9). Partial sequences were evaluated by using the basic local alignment search tool (BLAST) (1) on the Internet (http://www.ncbi.nlm.nih.gov) to determine the closest relatives in the database. The new sequences were added to an alignment of full prokaryotic 16S rRNA sequences by using the automated aligning tool of the ARB program package (Technical University of Munich, Munich, Germany [http://www.arb-home.de]). Analysis of sequence similarity was further done by constructing a similarity matrix with the ARB facilities.

Oligonucleotide probes and in situ hybridization. *Thiocystis*- and *Cytophaga*-related populations were counted by FISH. An 18-nucleotide specific sequence was chosen for each of the two *Thiocystis* populations as a target site by using the appropriate tools of the ARB software package. Probe CHR-452 (5′-GTA TTC GCC ACG CGC TTT-3′) targeted the *Thiocystis* sp. that was represented by sequence DGGE-6. Optimum hybridization was found at 35% formamide. Probe CHR-626 (5′-GTA TCC ACT GCC GTT CCC-3′) targeted the *Thiocystis* sp. that was represented by sequence DGGE-8. Optimum hybridization was found at 20% formamide. For *Cytophaga*-like bacteria, we used probes described in the literature (27, 53). Some of the cytophagas did not give good hybridization signals with the specific probes designed and were targeted with the phylum probe CF319a. Oligonucleotide probes were synthesized with Cy3 fluorochrome at the 5′ end (Interactiva Biotechnologie GmbH, Ulm, Germany). Hybridization and microscopic counts were performed as previously described (21) with an Axiophot II microscope (Zeiss, Jena, Germany).

Flow cytometry and sorting. Cell sorting for the *Synechococcus*-like cells was performed as described previously (50). Sorted cells were PCR amplified by using cyanobacterium-specific DGGE primers (37). PCR products were separated in a DGGE gel and sequenced as described above.

The sequences reported for Lake Vilar have been deposited in the EMBL database under the accession numbers AJ240007 to AJ240013 and AJ422234 to AJ422239.

RESULTS

Characterization of the environment and distribution of microbial populations by microscopy. Time-depth changes in temperature, conductivity, oxygen, and sulfide are shown in Fig. 1 for three representative dates in Lake Vilar (in winter, spring, and summer). Additional data are presented in Table 1. During winter the lake had colder water (9°C at surface) overlying warmer, higher-conductivity, bottom water (13°C). Light did not penetrate further than 4.5 m. Oxygen and sulfide showed opposite gradients with coexistence at ca. 4.5 m. In spring, the surface temperature increased and the inverse thermal stratification disappeared. This favored the coexistence of sulfide and light at 4.0 m. High oxygen concentrations were measured during this period in the first 2 m of the lake (14 mg/liter), produced by the photosynthetic activity of algae and cyanobacteria. In June, however, the oxygen concentration decreased (9 mg/liter), the surface water was more transparent, and the sulfide concentration increased up to 1.2 mM in the anoxic compartment.

Conspicuous photosynthetic populations were identified with the microscope (Table 2). In the oxic zone they mostly corresponded to cyanobacteria of the *Synechococcus* sp. type (autofluorescent, nonmotile, small cocci; concentration up to 10^6 cells/ml) and algae (*Cryptomonas* and *Crucigenia* spp. among others; concentrations up to 10^3 cells/ml each). In the anoxic zone conspicuous phototrophic organisms were brown-pigmented green sulfur bacteria (*Chlorobium phaeobacteroides*-like cells; concentration up to 10^7 cells/ml) and okenone-containing purple sulfur bacteria of the family *Chromatiaceae* (mainly *Thiocystis minor*-like cells, concentration up to 10^6 cells/ml, but also *Chromatium weissei*- and *Chromatium okenii*-like cells, concentration 10^3 cells/ml). These anaerobic photosynthetic populations were vertically segregated along gradients of light and sulfide, i.e., purple sulfur bacteria bloomed above green sulfur bacteria (Table 1). In addition, blooms of each photosynthetic populations were separated in time. Thus, the bloom of *Synechococcus* sp. occurred in March, followed by the bloom of *Chlorobium* sp. in April and, finally, followed by the bloom of *Thiocystis* sp. in May. Therefore, microscopy revealed successive changes in the photosynthetic populations of Lake Vilar involving three distantly related phylogenetic

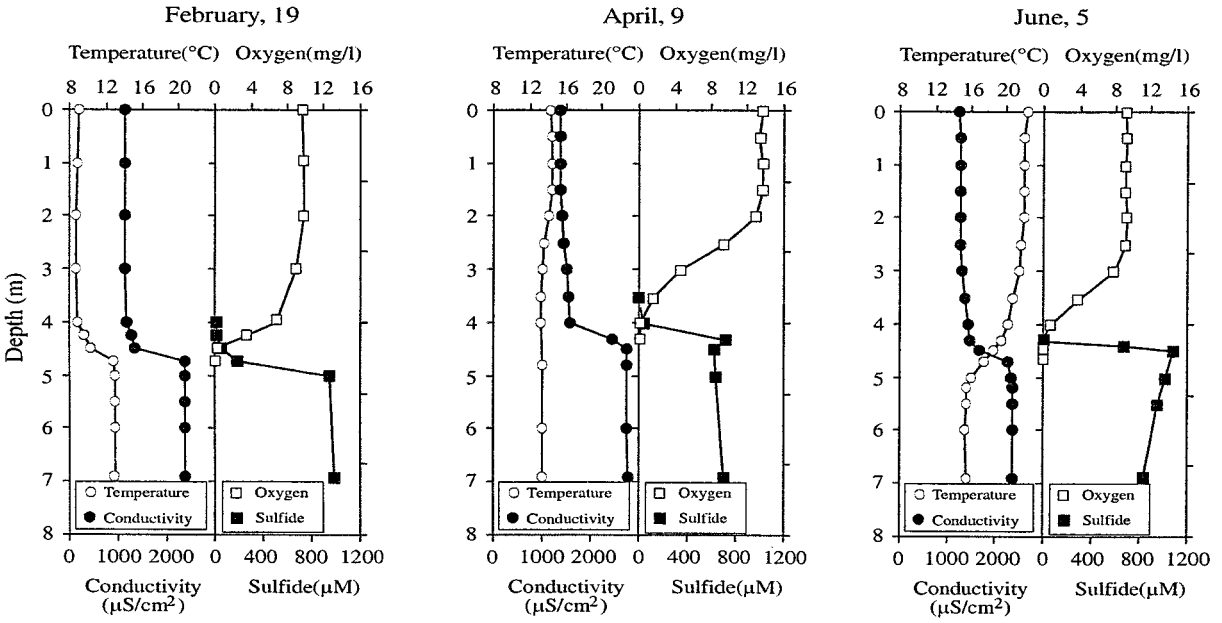

FIG. 1. Depth profiles of temperature, conductivity, oxygen, and sulfide from Lake Vilar for three selected dates in 1996.

groups: cyanobacteria, green sulfur bacteria, and purple sulfur bacteria.

DGGE fingerprints and 16S rDNA sequences. As expected from microscopic observations, the fingerprints showed different banding patterns when the aerobic and the anaerobic assemblages were compared (Fig. 2). Up to 17 bands were observed in the aerobic epilimnion, whereas in the sulfide-rich depths no more than 8 bands were detected. In the hypolimnion, only a few bands (one to three) were very strong. Finally, changes with time in the number and intensity of the bands were more marked in the aerobic than in the anaerobic depths.

Predominant bands from the gel were excised and sequenced. Bands that were in the same position in the DGGE gel were excised from different lanes and sequenced to confirm that the same position corresponded to the same sequence all along the temporal survey (bands marked with a small round circle in Fig. 2). The 16S rRNA fingerprinting and sequencing approach recovered the predominant microorganisms identified under the microscope (*Synechococcus*, *Chlorobium*, and *Thiocystis* spp.), as well as three sequences belonging to the *Cytophaga-Flavobacterium-Bacteroides* phylum, as the most abundant sequences (Table 2). In most cases, however, more

TABLE 1. Temperature, conductivity, irradiance, oxygen, sulfide, and DAPI counts from the depths studied in Lake Vilar

Date (day.mo)	Depth (m)	Temp (°C)	Conductivity ($\mu S\ cm^{-2}$)	Light ($\mu E\ m^{-2}\ s^{-1}$)	Concn of:		DAPI counts (10^5 cells/ml)			
					O_2 (mg/liter)	H_2S (μM)	*Synechococcus* sp.	*Chlorobiaceae*	*Chromatiaceae*	Other prokaryotes
19.02	0.20	9	1,121	800	9.7	0	6.20	BD	BD	250
	4.75	13	2,380	10	0.2	190	0.32	11.3	BD	69.3
	7.00	13	2,370	0	0.0	986	0.28	7.0	BD	98.2
09.04	2.00	14	1,397	65	13.1	0	13.2	BD	BD	31.4
	4.25	13	2,430	<5	0.0	720	4.00	41.0	BD	130
	7.00	13	2,770	0	0.0	707	0.61	14.2	BD	140
23.04	3.75	15	1,180	16	1.4	4	ND	0.4	0.85	82.1
	4.25	14	1,660	<5	0.0	709	ND	260	BD	160
	7.00	14	2,040	0	0.0	837	ND	43.1	BD	122
08.05	4.15	16	1,240	<5	1.0	140	ND	96.3	0.96	131
	4.40	15	1,600	0	0.0	550	ND	121	0.17	161
21.05	4.20	18	1,480	ND	0.2	104	ND	2.3	13.3	64.2
	4.40	17	1,910	ND	0.0	844	ND	180	0.17	131
05.06	2.00	22	1,283	ND	9.4	0	8.61	BD	BD	51.3
	4.30	19	1,467	ND	0.1	13	ND	0.9	16.1	97.4

[a] ND, not determined; BD, below detection limits.

TABLE 2. Microbial morphotypes observed microscopically and sequences retrieved which might correspond to them

Population (genus)	Morphology	Cell size (μm)	DGGE sequence	Closest relative in database	% Similarity[a]
Synechococcus	Coccus	1.5–2.0	DGGE-2, CYA-1	*Synechococcus* sp. strain PCC6307	97.1
Synechococcus	Coccus	1.5–2.0	DGGE-7, CYA-2	*Synechococcus* sp. strain PCC6307	98.1
Chlorobium	Rod	0.8 by 2.0–2.5	DGGE-5	*Chlorobium phaeobacteroides*	95.2
Thiocystis	Rod	2.0–4.0 by 4.0–6.0	DGGE-6	*Thiocystis minor*	93.7
Thiocystis	Rod	2.0–4.0 by 4.0–6.0	DGGE-8	*Thiocystis minor*	93.0
Cryptomonas	Chloroplast	15 by 30	CYA-3	*Cryptomonas* sp.	95.1
Cryptomonas	Chloroplast	15 by 30	CYA-4	*Cryptomonas* sp.	95.2
Pseudanabaena	Filaments	2.5 by 35	CYA-5	*Pseudanabaena limnetica*	97.0
Cytophaga	Unknown	Unknown	DGGE-3	*Cytophaga* WCHB	93.3
Cytophaga	Small rod, coccus	0.5–0.8	DGGE-1	*Cytophaga fermentans*	88.2
Cytophaga	Unknown	Unknown	DGGE-4	*Cytophaga fermentans*	88.6

[a] The percent similarity of the 16S rRNA sequence to the closest relative in the database.

than one sequence was recovered that was affiliated with the same morphotype. Thus, two different sequences (DGGE-2 and DGGE-7, 97.3% similarity to each other) were related to *Synechococcus* sp., and two sequences (DGGE-6 and DGGE-8, 92.3% similarity to each other) were related to *Thiocystis minor*. In addition, two sequences (DGGE-1 and DGGE-4, 96.2% similarity to each other) were related to *Cytophaga fermentans* and a third sequence (DGGE-3, 91.1% similarity to the former) was related to *Cytophaga* sp. Only one sequence related to *Chlorobium phaeobacteroides* (DGGE-5) was recovered. Changes in the relative abundance of such sequences were monitored in time by changes in the intensity of the DGGE bands. Thus, temporal changes for the different sequences of *Synechococcus*, *Thiocystis*, and *Cytophaga* spp. were observed in the aerobic epilimnion, the oxic-anoxic interface, and the anaerobic hipolimnion, respectively (Fig. 3). These data indicated a shift with time in the relative abundance of the different sequences of each morphotype. In order to reject PCR artifacts or the presence of 16S rRNA multioperons in these populations, PCR-independent techniques (i.e., FISH with specific probes and flow cytometry coupled to sorting and further sequencing) were subsequently used.

FISH counts and flow cytometry sorting. Two different approaches were carried out depending on the population targeted. For the *Cytophaga*- and *Thiocystis*-related populations, FISH with specific probes was used. Between 55 and 75% of all prokaryote-shaped particles that were stained with DAPI hybridized with the general bacterial probe EUB338. Fewer than

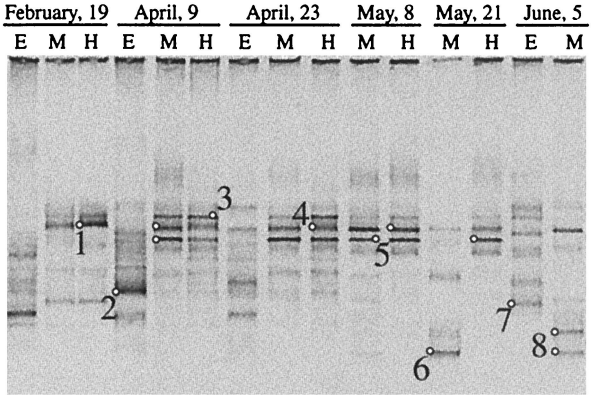

FIG. 2. Negative image of ethidium bromide-stained DGGE gel containing PCR-amplified segments of 16S rRNA genes obtained by using universal bacterial primers. Lanes E, M, and H correspond to the aerobic epilimnion, the oxic-anoxic metalimnion, and the anaerobic rich-sulfide hypolimnion, respectively. Small circles correspond to the bands excised from the gel and then sequenced, and numbers are given for these sequences.

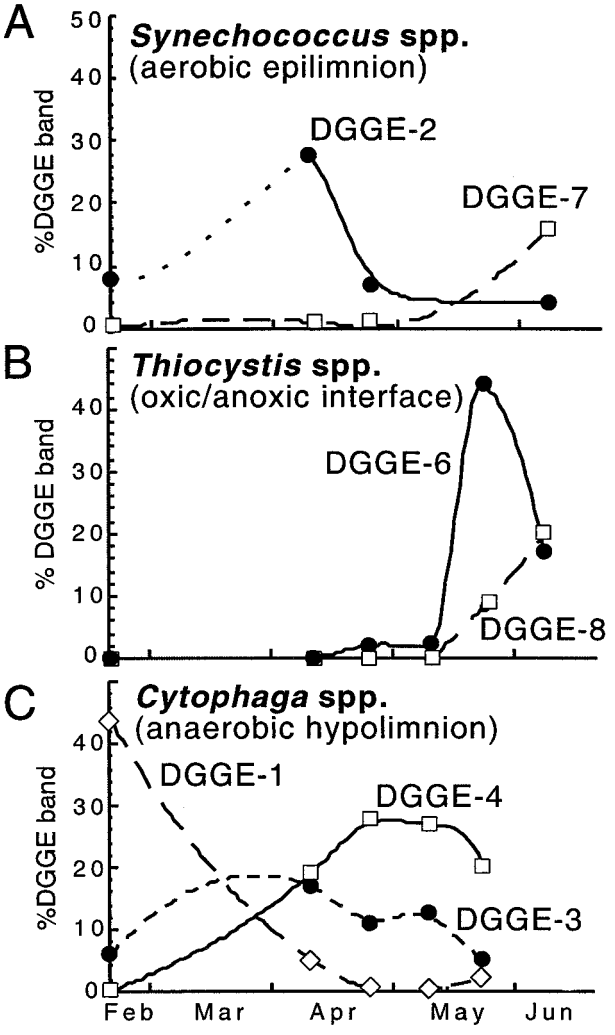

FIG. 3. Seasonal changes in the relative contributions of selected DGGE bands to the total intensity for each sample in Fig. 2. (A) Bands from *Synechococcus* spp. in the aerobic epilimnion; (B) bands from *Thiocystis* spp. in the oxic-anoxic interface; (C) bands of *Cytophaga* spp. in the anaerobic rich-sulfide hipolimnion.

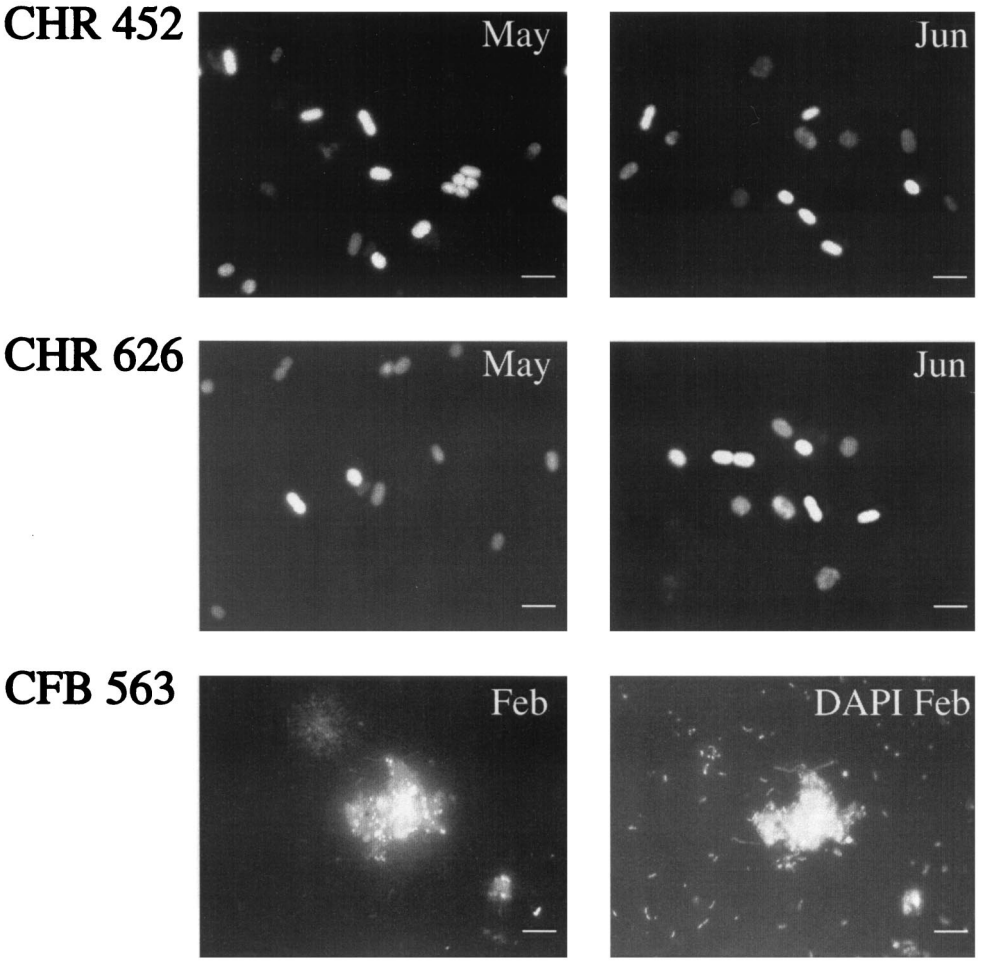

FIG. 4. FISH analysis with specific probes for both *Thiocystis* spp. (CHR-452 and CHR-626; upper and middle panels, respectively) and one *Cytophaga* sp. (CFB563, lower panels). Bars, 5 μm. Positive *Thiocystis* cells appear white; autofluorescent negative cells of *Thiocystis* appear gray. Cytophagas are shown to correspond to the DAPI-stained cells attached to particles.

0.1% of DAPI-stained nontargeted cells (i.e., nontypical *Chromatiaceae*-like cells) were found to cross-react with the specific probes designed for *Thiocystis*, and these cells were not considered. FISH demonstrated that the different *Thiocystis*-related sequences corresponded to different populations and were not a result of methodological problems (Fig. 4). The two *Thiocystis* populations were rod shaped with similar ranges of cell sizes (Table 2). No shifts in pigment composition (bacteriochlorophyll *a* and the carotenoid okenone, both found in *Thiocystis minor*) were detected in spring or in summer after high-pressure liquid chromatography analyses (L. Bañeras, unpublished data). Therefore, both *Thiocystis* spp. did not differ either in morphology or in pigment composition. The *Cytophaga* population present in the winter (band DGGE-1) was specifically targeted by probe CFB563, and most of the cells were coccoid and mostly attached to particles (Fig. 4) whereas the cytophagas, which hybridized with the phylum probe CF319a, showed more variable morphology. For instance, filamentous cytophagas were observed to hybridize with probe CF319a but not with probe CFB563 (data not shown).

Temporal shifts detected by DGGE were confirmed after FISH counts. Thus, in the bloom of May ca. 70% of the total *Chromatiaceae*-shaped cells (i.e., 9×10^5 cells/ml) hybridized with probe CHR-452 (specific for DGGE-6) and ca. 20% (i.e., 2×10^5 cells/ml) hybridized with probe CHR-626 (specific for DGGE-8). In June, however, both *Thiocystis* spp. contributed to the *Chromatiaceae*-like assemblage in similar percentages (Table 3). In contrast, the winter *Cytophaga* population detected in sulfide-rich depths by probe CFB563 (DGGE-1) accounted for 23% (i.e., 2×10^6 cells/ml) of the total cell counts in February but decreased to below detection limits in May (Table 4), in agreement with the decrease in DGGE band intensity (Fig. 3).

Characterization of *Synechococcus* spp. was carried out by using flow cytometry and cell sorting based on autofluorescence. Both *Synechococcus*-like cells showed autofluorescence in the red channel (presence of chlorophyll *a*), but the summer population (DGGE-7) lacked autofluorescence in the orange channel (phycoerythrin either absent or present in a low concentration). The sorted populations were PCR amplified with specific chloroplast-cyanobacterium primers and were compared by DGGE to the natural oxygenic photosynthetic assemblage of Lake Vilar over time (Fig. 5). The sorted *Synechococcus* populations of spring and summer yielded a single band

TABLE 3. *Thiocystis* sp.-related FISH counts in the oxic-anoxic interface of Lake Vilar as determined with the specific probes CHR-452 (DGGE-6) and CHR-626 (DGGE-8)

Date (day.mo)	Depth (m)	Mean FISH count (10^4 cells/ml) ± SD (% DAPI)[a] with:	
		CHR-452	CHR-626
19.02	4.75	BD	BD
09.04	4.25	BD	BD
23.04	3.75	6.0 ± 0.3 (0.7)	BD
08.05	4.15	8.3 ± 0.2 (0.4)	0.77 ± 0.03 (0.03)
21.05	4.20	91.1 ± 3.5 (11.5)	26.3 ± 4.2 (3.3)
05.06	4.30	74.2 ± 3.8 (6.5)	61.2 ± 3.3 (5.4)

[a] BD, below detection limits. The % DAPI concentration is given in parentheses.

each in the DGGE (CYA-1 and CYA-2, respectively) that matched DGGE bands from the natural assemblage. CYA-1 (positive in both the red and the orange channels) was present in winter and spring, and CYA-2 (positive in the red channel and negative in the orange channel) was present mostly in the summer. In addition, the sequence CYA-1 matched the sequence of band DGGE-2, whereas the sequence CYA-2 matched the sequence of band DGGE-7. Thus, the primer combinations 341f-907r for bacteria and 359f-781r for cyanobacteria recovered the same populations. Interestingly, different but closely related 16S rRNA chloroplast sequences were also observed for the alga *Cryptomonas* sp. in spring (CYA-3) and in summer (CYA-4) (Fig. 5 and Table 2), and the cyanobacterium *Pseudanabaena* sp. appeared in summer as an important DGGE band (CYA-5) but not in winter or early spring. Therefore, the temporal shift in *Synechococcus* populations was accompanied also by changes in other members of the oxygenic photosynthetic assemblage.

DISCUSSION

The meaning of the small-scale variability in 16S rDNA sequences commonly observed in molecular surveys (such as in hot springs (5, 15), soils (6, 25), oral cavities (10), wastewater treatment plants (2), and ocean waters (16, 19, 29, 33), among others, is a controversial matter. Early evidence of widespread environmental 16S rRNA microheterogeneity was obtained from the first PCR-generated genetic libraries in marine samples. Paradigmatic examples are the SAR 11 cluster (20) and the marine archaea (11, 19). Later on, a combination of molecular and physiological studies on cultured strains and on

TABLE 4. *Cytophaga* sp.-related FISH counts in the sulfide-rich waters of Lake Vilar[a]

Date (day.mo)	Depth (m)	Mean FISH count (10^6 cells/ml) ± SD (% DAPI) with:	
		CF319a	CFB563
19.02	7.00	3.2 ± 0.7 (30.2)	2.4 ± 0.8 (22.7)
09.04	7.00	4.9 ± 0.6 (32.0)	3.1 ± 0.7 (2.0)
23.04	7.00	4.7 ± 0.4 (28.7)	1.6 ± 0.7 (0.9)
08.05	4.40	3.1 ± 0.3 (11.2)	BD

[a] CF319a refers to total *Cytophaga*-like bacteria. CFB563 is a probe specific for the *Cytophaga* population represented by the DGGE-1 sequence. BD, below detection limits. The % DAPI concentration is given in parentheses.

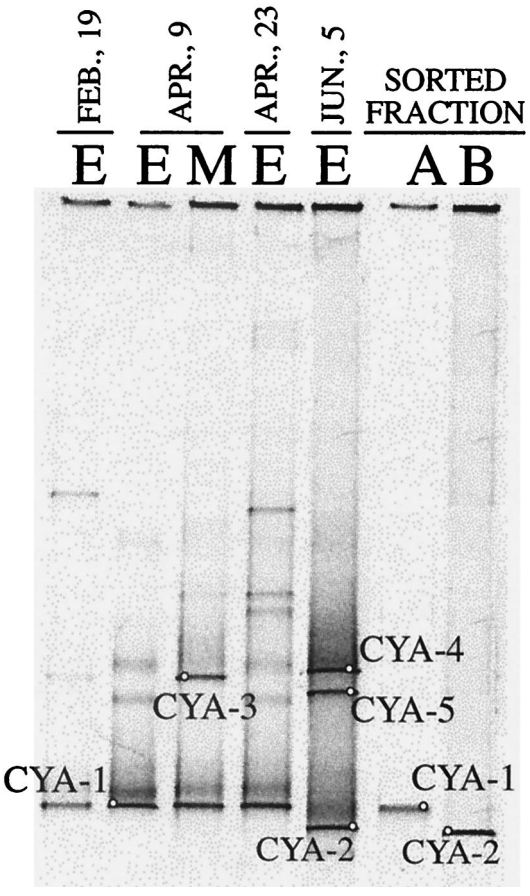

FIG. 5. Negative image of ethidium bromide-stained DGGE gel containing PCR-amplified segments of 16S rRNA genes obtained by using cyanobacterium-chloroplast primers. Lanes A and B correspond to the sorted populations from spring and summer, respectively. Identification of the bands excised from the gel is presented in Table 2.

flow cytometrically sorted field samples of *Prochlorococcus* sp. demonstrated the physiological and genetic diversity of cyanobacterial populations with a high similarity in their 16S rRNA sequences (13, 32, 43, 44, 48). Further research revealed a specific vertical distribution for such *Prochlorococcus* populations in natural samples (14, 46, 54, 55), giving some clues on the ecology of these microorganisms. In the present work, we provide new evidence for the ecological significance of environmental 16S rDNA microdiversity. We have shown that the 16S rDNA-defined populations with limited sequence diversity changed with time. Therefore, these data support the hypothesis that clusters of closely related 16S rRNA environmental sequences may actually represent numerous closely related, yet ecologically distinct, populations (18, 51).

In Lake Vilar we first assigned by traditional methods photosynthetic cells to the genus or even to the species level (i.e., *Synechococcus* sp., *Chlorobium phaeobacteroides*, and *Thiocystis minor*), and we studied their temporal dynamics. Populations with no morphologic traits, however, could not be studied in this way and required a molecular approach based on the 16S rRNA gene. DGGE identified such unknown populations and, in addition, revealed that more than one population was hidden behind some single morphotypes that we had distin-

guished with the microscope. Quantitative analysis of the DGGE band patterns indicated temporal changes in the relative abundance of these populations. However, limitations described for PCR-based techniques might prevent an accurate quantitative estimation of cell abundance (47). For that reason, we were cautious in the number of PCR cycles run, we used the same amount of template in each reaction, and the samples that we compared were all amplified in the same PCR run and were analyzed in the same DGGE gel. Thus, any biases should have been the same for all samples, and the comparison should still be valid. When these populations were counted with specifically designed FISH probes, we found good correlation between microscopic counts and DGGE fingerprints, and both methods showed the same temporal change in the populations. Because Lake Vilar is a very small lake, and one sample at the center of the basin is representative of the pelagic zone of the lake, we can rule out spatial variability due to our sampling strategy. To characterize the vertical profile, our sampling scheme first detected the depths of the chemo- or thermocline, and then we sampled these layers every few centimeters with a special laminar sampler (42). Thus, we could select the depths representative of the epilimnion (which is mixed) the oxic-anoxic interface (where oxygen and sulfide coexist at low concentrations) and the hypolimnion (which is uniform) with precision.

On the other hand, the use of 16S rDNAs for the identification of microorganisms calls for caution because such sequences evolve so slowly that more than one population may have the same sequence (17, 40) and, therefore, closely related 16S rDNA populations could have very different phenotypic attributes. In Lake Vilar we found differences in closely related populations ranging between 8% (*Thiocystis* spp.) and 3% (*Synechococcus* spp.). We have to consider, however, that the partial 16S rDNA sequence analyzed (positions 341 to 907, *Escherichia coli* numbering) included some of the most highly variable regions of this ribosomal gene. Thus, lower differences (close in the best case to 1 and 3%, respectively) are expected when complete 16S rDNAs are compared. In the case of *Thiocystis* sp., we found that the two genotypes had very similar physiological properties and a very characteristic morphotype (rod-shaped cells up to 6 μm in length, with internal sulfur globules and with bacteriochorophyll *a* and okenone as carotenoid). Considering the temporal patterns of occurrence for these microorganisms, they were certainly distinct populations. In the case of the two *Synechococcus* sp. and the two *Cytophaga* sp., the differences in their 16S rRNA sequences were lower but both had populations with different phenotypes and different temporal distributions. Therefore, the winter-to-summer transition study in Lake Vilar revealed that closely related 16S rDNA populations of *Synechococcus*, *Thiocystis*, and *Cytophaga* distributed differently with time and for some of them we could detect differences in the phenotype. In the case of the green sulfur bacteria, however, we detected only one population of *Chlorobium*. Nevertheless, we cannot rule out that other related populations of *Chlorobium* present in the lake were not recovered from the gel (e.g., those that corresponded to weaker bands) or that the organisms might have been present but at lower values than the detection limit of DGGE (9) (i.e., ca. 0.5% of the total DAPI counts).

Temporal population dynamics raises interesting questions about the ecology and physiology of these uncultured microdiverse microbes, and by using a combination of several methodological tools, we could answer them to a certain extent. Thus, both *Synechococcus* spp. showed different pigment compositions when they were analyzed by flow cytometry (phycoerythrin was absent or present at very low concentration in the summer population), and this might be an indication of a different light-dependent physiology. Changes in nutrient concentrations, however, could also play an important role in selecting one or the other population (43, 55). It is interesting to consider the shift detected also in other members of the oxygenic photosynthetic assemblage. In marine cyanobacteria of the genus *Prochlorococcus*, small-scale variability in 16S rRNA sequences has been clearly correlated with niche specialization of the different ecotypes (32, 54, 55), and this can also be the case for freshwater cyanobacteria. For *Cytophaga* populations, the temporal shift was also linked to changes in the habitat colonized (particle-attached versus free-living cells). The case of the purple photosynthetic *Thiocystis* bacterium is, however, more intriguing. Differences were not found either in morphology or in pigment composition. Thus, the coexistence of both ecotypes might be related to different substrate affinities (49). A better ability to grow under light-limited conditions by increasing the specific pigment content or a better response to fluctuating conditions by increasing the concentration of storage products is also a possible explanation (28). It is extremely difficult to extract further conclusions without having the appropriate pure cultures to examine. Previous molecular studies, however, have revealed that the photosynthetic sulfur bacteria isolated thus far from these environments do not match those that bloom in situ (8, 9). In addition, highly abundant *Cytophaga*-related organisms found in the anaerobic, sulfide-rich, freshwaters of Lake Vilar indicated that the organisms available in culture collections do not account for the full range of metabolic capabilities present in this group. Therefore, the phenotypic characteristics of the organisms that successfully grow in the field will remain unknown as long as the isolation process is not successful with the naturally abundant strains. Time-depth distribution patterns provided a good idea of the habitat of these microorganisms and under what physicochemical conditions they grow in situ, and this can help in the isolation process.

Overall, we can imagine a natural scenario in which an ecologically unique microbial population becomes predominant for a short period of time but that can easily be outcompeted by a close relative better adapted to newly established environmental conditions. These bacteria could represent a tremendous potential for rapid changes in community composition, since they could grow quickly and multiply over several orders of magnitude under appropriate conditions. Thus, the function carried out by the microbial guild in the environment persists, but the identity of the microbial populations may change with time as an adaptative response to environmental pressures and to fluctuating conditions. This would provide an explanation as to why so many related isolates can be obtained from a natural sample (see, for example, reference 45) or why these isolates do not match sequences of predominant organisms at a certain time (see, for example, reference 9). The successful members of the SAR 11 cluster (20) that comprises the most ubiquitous bacterial gene type recovered from sea-

water, clusters of marine archaea of cosmopolitan distribution (29), and the multiple ecotypes of *Prochlorococcus* that colonize a remarkable range of depths in the water column of the world's oceans (54, 55) might owe their ecological success to the versatility and plasticity of the closely related populations that form these groups.

We have shown here that the diversity recovered in Lake Vilar fits this scenario. The small-scale 16S rRNA diversity corresponded to ecologically significant diversity that occurred in several phylogenetically nonrelated groups. This may be a general strategy in the microbial world. Certainly, we need to bring into culture microorganisms that show 16S rRNA small-scale variability but we also have to combine molecular methods and in situ chemical measurements for a better understanding of the rules that govern temporal changes in such closely related populations.

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