

Comparative 16S rRNA Analysis of Lake Bacterioplankton Reveals Globally Distributed Phylogenetic Clusters Including an Abundant Group of Actinobacteria

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In a search for cosmopolitan phylogenetic clusters of freshwater bacteria, we recovered a total of 190 full and partial 16S ribosomal DNA (rDNA) sequences from three different lakes (Lake Gossenköllesee, Austria; Lake Fuchskuhle, Germany; and Lake Baikal, Russia). The phylogenetic comparison with the currently available rDNA data set showed that our sequences fall into 16 clusters, which otherwise include bacterial rDNA sequences of primarily freshwater and soil, but not marine, origin. Six of the clusters were affiliated with the α , four were affiliated with the β , and one was affiliated with the γ subclass of the *Proteobacteria*; four were affiliated with the *Cytophaga-Flavobacterium-Bacteroides* group; and one was affiliated with the class *Actinobacteria* (formerly known as the high-G+C gram-positive bacteria). The latter cluster (hgcl) is monophyletic and so far includes only sequences directly retrieved from aquatic environments. Fluorescence in situ hybridization (FISH) with probes specific for the hgcl cluster showed abundances of up to 1.7×10^5 cells ml⁻¹ in Lake Gossenköllesee, with strong seasonal fluctuations, and high abundances in the two other lakes investigated. Cell size measurements revealed that *Actinobacteria* in Lake Gossenköllesee can account for up to 63% of the bacterioplankton biomass. A combination of phylogenetic analysis and FISH was used to reveal 16 globally distributed sequence clusters and to confirm the broad distribution, abundance, and high biomass of members of the class *Actinobacteria* in freshwater ecosystems.

During the last decade, rRNA-based methods have allowed studies of microbial diversity to move from a solely cultivation-based perspective to one that encompasses as yet uncultured microorganisms (42, 44). This development was promoted by the discovery of the potential of 16S rRNA for phylogenetic reconstructions and microbial diversity (42, 65). Global efforts have led to the 16S ribosomal DNA (rDNA) sequencing of nearly all validly described prokaryotes. This, plus the collection of environmental 16S rDNA clone libraries, has created one of the largest data sets for any individual gene (38).

Most 16S rRNA surveys so far have been performed in marine and soil habitats. Only a few are available for freshwater systems (33). Phylogenetic analysis of sequences from marine environments has revealed habitat-specific phylogenetic clusters. The most prominent are the SAR clusters, monophyletic lineages of solely marine 16S rDNA sequences (26, 41). Despite the smaller data set, some freshwater-specific clusters have been proposed, such as the freshwater clusters A, B, and C (66). All these marine and freshwater studies give good evidence for the postulated phyla or clusters based on careful phylogenetic analysis of the rDNA data sets, but little effort has been made to link these in silico results to the real environment by in situ methods. It is now necessary to bridge the gap between comparative sequence analysis and community composition with in situ detection methods like fluorescence in situ hybridization (FISH). FISH with rRNA-targeted oligonucleotide probes allows selective visualization of bacterial cells with defined phylogenetic affiliations (1, 5). In contrast to other

quantitative methods, such as slot blot hybridization, FISH conserves the morphology and cell sizes of the targeted organisms (48, 50). In combination with image cytometry, this not only allows cell counts of defined phylogenetic groups but links them to biomass, an ecologically relevant measure (48). In this study, first the 16S rDNA data set was extended with 190 sequences retrieved from three different lakes (Lake Gossenköllesee, Lake Fuchskuhle, and Lake Baikal), and then a comparative sequence analysis was performed to identify widely distributed phylogenetic clusters of freshwater bacteria. Probes for one conspicuous new cluster within the class *Actinobacteria* were designed and successfully applied, linking phylogeny with community composition and succession.

MATERIALS AND METHODS

Description of sampling sites and sampling. Lake Gossenköllesee is a small oligotrophic high-mountain lake in the Central Alps (Tyrol, Austria), situated at 2,417 m above mean sea level (AMSL) (20). Samples for FISH were collected monthly between 4 July 1996 and 25 June 1997 at the surface and at a depth of 4 m. Filtration, fixation, and storage were done as described earlier (27, 50). For DNA extraction, a sample was taken with sterile bottles from a 3-m depth in December 1995. In September 1997, a mixed sample from an 0- to 8-m depth was taken.

Lake Fuchskuhle is a meso- to acidotrophic forest lake in the Brandenburg-Mecklenburg lake district (Germany) at an altitude of 59 m AMSL. In 1990, it was artificially divided into four basins with different catchment areas (56). In May 1998, two independent samples for DNA extraction were taken with sterile bottles from the northeast and the southwest part at an 0.5-m depth.

Lake Baikal is the deepest (1,637 m) and, by volume, largest (23,000 km³) of all freshwater lakes. It is located in East Siberia between 51°29'N and 55°46'N latitude and 103°41'E and 109°57'E longitude at an altitude of 456 m AMSL (36). Samples for DNA extraction were collected from the South Basin at a 400- and a 1,200-m depth in December 1995 and at 25, 400, 1,200, and 1,400 m in August 1997. The Central Basin was sampled at a 25-, a 1,400-, and a 1,600-m depth in August 1997. Sampling was done with a metallic 10-liter sampler (batometer) pre-rinsed with lake water.

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DNA extraction, amplification, and cloning. For DNA extraction, water samples (100 ml to 1 liter) taken at Lake Gossenköllesee and Lake Fuchskuhle were prescreened (50- μ m pore size) and subsequently filtered on hydrophilic filters (Durapore [pore size, 0.2 μ m; diameter, 47 mm]; Millipore Corp., Bedford, Mass.) until the filters were completely clogged. During filtration, the samples were kept at ambient water temperature. The filters were cut into sections and stored at -20°C until further processing. DNA extraction was done according to the protocol of Fuhrman et al. (24). The December 1995 Lake Gossenköllesee sample was further processed for DNA extraction as described previously (50). Nearly full-length bacterial 16S rDNA fragments were amplified by PCR from the Lake Fuchskuhle and the September 1997 Lake Gossenköllesee samples with the general bacterial 16S rDNA primers 8F and 1492R (12). Amplification and cloning were performed as described elsewhere (29).

Treatment of the Lake Baikal samples. Three liters of the water sample was immediately filtered through a 0.22- μ m-pore-size polycarbonate filter (Millipore Corp.). Bacterioplankton were washed off the filters with 5 ml of a solution containing 10 mM Tris-HCl (pH 7.5) and 0.15 M NaCl and centrifuged. The pellet was frozen and transported to the laboratory. Chromosomal DNA was isolated using the QIAamp blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Isolated DNA was amplified by PCR with the following primers: 500L (5'-CGTGCCAGCAGCCGCGTAA-3') and 1350R (5'-GACGGGCGGTGTGTACAAG-3'). PCR amplification and cloning were done as described in the work of Denisova et al. (15).

16S rDNA sequencing. Most of the DNA sequencing was conducted on automated sequencers (ABI 377; Perkin-Elmer, Langen, Germany) by cycle sequencing with the chain termination technique using dye-labeled dideoxynucleotides (13), following the manufacturer's instructions. Manual sequencing of selected Lake Baikal clones was performed using a PCR Cyclist kit (Stratagene). Sequencing was done as recommended by the manufacturer. To screen out identical sequences, "single-letter" sequencing (ddGTP reactions only) was performed for all clones using primer 500L. For full sequencing, subfragments were amplified from the primary PCR products with the primer pairs 500L-1000R and 800L-1350R (15) and purified by agarose gel electrophoresis.

Data analysis. The retrieved 16S rDNA sequences were added to the rDNA sequence database of the Technical University of Munich (release December 1998) using the program package ARB (Lehrstuhl für Mikrobiologie, Technische Universität München [http://www.mikro.biologie.tu-muenchen.de]). The tool ARB_ALIGN was used for automatic sequence alignment. The resulting alignments were checked and corrected manually, considering the secondary structure of the rRNA molecule. For a general phylogenetic comparison, the ARB database was supplemented by importing most of the available 16S rDNA sequences from freshwater habitats, including estuarine and coastal marine systems (February 2000). Phylogenetic trees were reconstructed based on distance matrix analyses of all rRNA primary structures in the database (more than 13,000). Tree topologies were evaluated by maximum parsimony, neighbor joining, and maximum likelihood analyses in combination with different filters excluding highly variable positions on a subset of 152 (FastDNAml) (43) and 269 (neighbor joining) (54) nearly full-length sequences. A consensus tree was constructed with topology corrected by consideration of the results of the various tree reconstruction algorithms. Partial sequences were inserted into the reconstructed tree by parsimony criteria, without allowing changes in the overall tree topology. The clone identifications, closest relatives, and similarity values are given in Table 1.

We also used sequences from the following sources: Columbia River and estuary, Coastal Ocean, Oregon (14); Adirondack mountain lakes, New York (32); groundwater bacteria (A), Sweden (45); Toolik Lake, Alaska (6); gas vacuolate bacteria, Antarctica (31); activated sludge (T), Germany (58); negative controls (MT), California (61); Lake Loosdrecht (LD), The Netherlands (66); activated sludge (SMK), Germany (55); drinking water (B), Germany (35); activated sludge (Ben), Australia (10); subterranean groundwater bacteria (G), Africa (46); sequencing batch reactors (SBR), Australia (7); activated sludge (LDI), Germany (63); Stripa groundwater, Sweden (18); peat bog (TM), Germany (52); Carolina Bay, South Carolina (64); grassland soil, The Netherlands (21); blanket bog peat (MHP), United Kingdom (39); Mariana Trench mud (HTA), (60); freshwater enrichment (NO), Germany (28); borehole isolate (S), Sweden (47); subtropical soil (MC), Australia (59); contaminated aquifer (WCHB), Michigan (16); Wisconsin soil, Wisconsin (8); and Eastern Amazonian soil, Amazonia (9). All sequences are available in aligned ARB and GenBank format via our anonymous FTP server: ftp.mpi-bremen.de/pub/molecol_p/fog-aem/.

Probe design and FISH. Using the PROBE DESIGN tool of ARB (see above), two oligonucleotide probes and helper oligonucleotides (helper) (22) were constructed for the hgcI cluster (Fig. 1C). For the probes HGC236 and HGC664 (19), additional helpers were designed to enhance the signal intensities. Probes labeled with the indocarbocyanine dye Cy3 and unlabeled helpers were purchased from Interactiva (Ulm, Germany). All sequences and target positions of the probes and helpers are given in Table 2.

FISH of filter sections with oligonucleotide probes, counterstaining with DAPI (4',6'-diamidino-2-phenylindole), and mounting for microscopic evaluation were performed as described previously (27, 30). Epifluorescence microscopy was done on Zeiss Axioplan microscopes, equipped with HBO 100-W mercury lamps and 100 \times Plan Aplan objectives. Between 500 and 1,000 DAPI-stained objects were evaluated per sample. Absolute densities of hybridized bacteria

were calculated as the product of their relative abundances on filter sections (percentage of DAPI-stained objects) and the DAPI-stained direct cell counts. Since parallel counting on independent filters was not practicable, a general counting error of $\pm 10\%$ was calculated (49), which applies to all counts shown. Hybridization conditions for the probes HG1-654 and HG1-840 were adjusted by formamide series applied to different filters from Lake Gossenköllesee and evaluated by eye. Probes were also checked against the next nontarget organisms (*Actinomadura glomerata*, *Terracoccus luteus*, and *Micromonospora aurantiaca*) in the database which had one or two mismatches in the probe target region. Optimal formamide concentrations for all probes are given in Table 2.

Bacterial cell size determination. On Lake Gossenköllesee samples from August 1996, November 1996, April 1997, and June 1997, size measurements of all DAPI-stained bacteria and of cells hybridizing with the probe HG1-840 were carried out as described earlier (34, 48). Double images of the Cy3 and DAPI fluorescence of individual cells in microscopic preparations were captured using a PC-based image analysis system. Between 500 and 1,000 hybridized cells were analyzed per sample (corresponding to 5 to 30 image pairs). Biomass was calculated as the product of mean cell size and abundance.

Nucleotide sequence accession numbers. Accession numbers of the sequences determined in this study are AJ224988-90, AJ289926-61/63-65/80/82-99, AJ290000-80, AJ007641-42/44-58, and X99983-89.

RESULTS

Clone libraries. A total of 190 clones from 13 independent 16S rDNA clone libraries were partially sequenced (~ 400 to 500 nucleotides) and phylogenetically analyzed. Sequences with similarities greater than 97% were grouped together. Fifteen clones belonging to the *Cyanobacteria* phylum were rejected from further analysis because our focus was on heterotrophic bacteria. Furthermore, five chimeric sequences have been omitted. The value for sequence similarity to the next closest published sequence and phylogenetic affiliation of the remaining 170 clones are summarized in Table 1. From the Lake Gossenköllesee and Lake Fuchskuhle libraries, 43 clones representative of the different groups were selected for nearly full-length sequencing of 16S rDNA ($\sim 1,400$ nucleotides), whereas for 28 of the Lake Baikal clones 800- to 1,200-nucleotide-long sequences were determined. Although most of the clones fall into well-established groups, only 19 of the 71 long 16S rDNA sequences are more than 97% similar to published sequences. Clones affiliated with the class *Actinobacteria* ($n = 37$), the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group ($n = 35$), the β subclass of the class *Proteobacteria* ($n = 37$), and the α subclass of *Proteobacteria* ($n = 30$) were about equally abundant and together accounted for almost three quarters of the 190 retrieved sequences. The sequences of 31 clones were affiliated with other groups: nine with γ -*Proteobacteria*, six each with the δ -*Proteobacteria* and the *Holophaga-Acidobacterium* group, five with the *Verrucomicrobia*, two each with the *Planctomycetales* and the candidate division OP11, and one with the low-G+C gram-positive bacteria.

Phylogenetic tree. A phylogenetic comparison of the new sequences with published 16S rDNA sequences revealed 16 clusters. Five included only sequences from freshwater samples, seven included sequences from freshwater and freshwater-influenced coastal marine systems, and four clusters also contained sequences originating from soil samples. The criterion for defining a cluster was that at least three sequences from independent sources cluster together in all tree reconstructions. The clusters are based on phylogenetic affiliations and not on similarity thresholds because, in most cases, due to the sequencing of different 16S rDNA regions by various authors, no reasonable similarity values could be determined. With the exception of cluster α II, all described clusters are clearly separated from sequences found in the open ocean or marine sediments (Fig. 1).

α -proteobacterial 16S rDNA sequences. Six clusters, α I to α VI, can be described in the α -*Proteobacteria* (Fig. 1A). The α V cluster has already been described as freshwater cluster A

TABLE 1. Clones of the different libraries, their affiliations, nearest relatives, percents similarity, and accession numbers

Sample location (no. of clones)	Clone	Category	Nearest published relative (accession no.)	Similarity (%)	Accession no. ^a
Lake Gossenköllesee, December 1995 (8)	GKS59	α-Proteobacteria	<i>Rhodobacter sphaeroides</i> (D16424)	95.5	AJ224988
	GKS69	α-Proteobacteria	Clone T28 (Z93995); <i>Sphingomonas</i> sp.	97.4	AJ224989
	GKS16, -70, -71	β-Proteobacteria	Isolate 34-P (U14585); <i>Rhodoferrax</i> sp.	97.2	AJ224987
	GKS98, -35	β-Proteobacteria	<i>Alcaligenes denitrificans</i> (D88005)	96.5	AJ224990
	GKS61	<i>Actinobacteria</i>	Clone ACK-M1 (U85190)	92.5	AJ290036
Lake Gossenköllesee, September 1997 (35)	GKS2-124	α-Proteobacteria	Clone NO22 (AJ001344); <i>Blastobacter</i> sp.	99.7	AJ290027
	GKS2-215	α-Proteobacteria	<i>Beijerinckia indica</i> (M59060)	96.5	AJ290032
	GKS2-218	α-Proteobacteria	<i>Roseococcus thiosulfatophilus</i> (X72908)	92.7	AJ290043
	GKS2-122, -77, -187	β-Proteobacteria	Clone T3 (Z93975); <i>Rhodoferrax</i> sp.	95.0	AJ290026
	GKS2-91	γ-Proteobacteria	Isolate CDC658-79 (U88437); <i>Rahnella</i> sp.	92.7	AJ290038
	GKS2-103, -38, -79, -176, -189	<i>Actinobacteria</i>	Clone ACK-M1 (U85190)	98.6	AJ290024
	GKS2-33, -7, -15, -19, -63, -88, -111, -112, -120, -121, -142, -155, -205, -207, -208, -232	CFB	<i>Spirosoma linguale</i> (M27802)	84.4	AJ290035
	GKS2-70	CFB	<i>Spirosoma linguale</i> (M27802)	87.7	AJ290037
	GKS2-106, -164	CFB	<i>Flavobacterium ferrugineum</i> (M28237)	92.2	AJ290025
	GKS2-216	CFB	<i>Flectobacillus major</i> (M27800)	93.5	AJ290033
	GKS2-217	CFB	<i>Haliscomenobacter hydrossis</i> (M58790)	94.6	AJ290034
	GKS2-30, -174	Candidate division OP11	Isolate Koll6 (AJ224539)	78.4	AJ290044
	Lake Fuchskuhle, northeastern part (34)	FukuN22	α-Proteobacteria	Clone T28 (Z93995); <i>Sphingomonas</i> sp.	96.9
FukuN57		α-Proteobacteria	<i>Afipia</i> genosp. 9 (U87777)	94.0	AJ290000
FukuN33, -2, -60, -66, -109		β-Proteobacteria	Clone ACK-C4 (U85124); <i>Polynucleobacter</i> sp.	99.7	AJ289997
FukuN55, -107		β-Proteobacteria	Clone T3 (Z93957); <i>Rhodoferrax</i> sp.	94.7	AJ289999
FukuN65		β-Proteobacteria	<i>Alcaligenes denitrificans</i> (D88005)	96.4	AJ290001
FukuN108		β-Proteobacteria	<i>Eikenella corrodens</i> (M22467)	89.1	AJ289984
FukuN13		γ-Proteobacteria	Isolate BB5.1 (AF016981); <i>Methylo-</i> <i>bacter</i> sp.	96.6	AJ290055
FukuN9, -97, -110		δ-Proteobacteria	<i>Bdellovibrio stolpii</i> (M34125)	96.1	AJ290009
FukuN30, -8, -15, -44, -105		<i>Actinobacteria</i>	Clone ACK-M1 (U85190)	98.3	AJ289996
FukuN101		<i>Actinobacteria</i>	<i>Rathayibacter tritici</i> (X77438)	95.5	AJ289982
FukuN21		CFB	<i>Flavobacterium ferrugineum</i> (M28237)	90.8	AJ289993
FukuN24, -3, -47, -50, -54, -104		CFB	<i>Flavobacterium ferrugineum</i> (M28237)	91.1	AJ289995
FukuN36		CFB	<i>Flavobacterium aquatile</i> (M28236)	95.2	AJ289998
FukuN63		CFB	Clone WCHB1-53 (AF050539); <i>Cytophaga</i> sp.	87.9	AJ290056
FukuN18, -43, -106		<i>Verrucomicrobia</i>	Clone LD29 (AF009975); <i>Verrucomicrobium</i> sp.	92.2	AJ289992
FukuN111		<i>Verrucomicrobia</i>	Isolate 2 (X99390); <i>Verruco-</i> <i>microbium</i> sp.	94.6	AJ289985
Lake Fuchskuhle, southwestern part (29)		FukuS56, -132, -182	α-Proteobacteria	<i>Sphingomonas</i> sp. (AJ001051)	97.5
	FukuS110, -187	α-Proteobacteria	<i>Beijerinckia indica</i> (M59060)	96.5	AJ289986
	FukuS35, -12, -21, -36, -89, -99, -129, -183, -191	β-Proteobacteria	Clone ACK-C4 (U85124); <i>Polynucleobacter</i> sp.	99.4	AJ290013
	FukuS93, -68, -75, -135	β-Proteobacteria	Isolate R6 (AJ002809); <i>Alcaligenes</i> sp.	96.9	AJ290018
	FukuS94	β-Proteobacteria	<i>Rhodoferrax fermentans</i> (D16211)	92.7	AJ290057
	FukuS5, -24, -98	<i>Actinobacteria</i>	Isolate TM177 (X92701); <i>Actinomycetales</i>	96.7	AJ290022
	FukuS81	<i>Actinobacteria</i>	Clone ACK-M1 (U85190)	92.5	AJ290054
	FukuS23	CFB	<i>Flexibacter sancti</i> (M28057)	89.2	AJ290011
	FukuS59, -20, -140, -188	CFB	<i>Flavobacterium ferrugineum</i> (M28237)	91.7	AJ290042
	FukuS27	<i>Verrucomicrobia</i>	Clone T62 (Z94005); <i>Prosthecobacter</i> sp.	98.0	AJ290012
Lake Baikal, South Basin, 25 m (3)	B25-4-32	β-Proteobacteria	<i>Variovorax</i> sp. strain WFF52 (AB003627)	97.0	AJ007642
	B25-4-10	<i>Actinobacteria</i>	" <i>Microthrix parvicella</i> " (X89774)	89.3	AJ007641
	B25-4-33	<i>Actinobacteria</i>	Clone ACK-M1 (U85190)	95.9	AJ289950

Continued on following page

TABLE 1—Continued

Sample location (no. of clones)	Clone	Category	Nearest published relative (accession no.)	Similarity (%)	Accession no. ^a
Lake Baikal, Central Basin, 25 m (4)	B25-5-20	β -Proteobacteria	Clone T3 (Z93957); <i>Rhodoferax</i> sp.	98.6	AJ289951
	B25-5-66, -64, -69	<i>Actinobacteria</i>	Clone ACK-M1 (U85190)	95.7	AJ007644
Lake Baikal, South Basin, 400 m, 1995 (5)	B400-145	α -Proteobacteria	<i>Rhodobacter sphaeroides</i> (D16425)	96.4	AJ001425
	B400-148	α -Proteobacteria	<i>Azospirillum lipoferum</i> (M59061)	87.0	AJ001426
	B400-149	α -Proteobacteria	<i>Acetobacter liquefaciens</i> (X75617)	94.4	AJ001427
	B400-1411	<i>Actinobacteria</i>	<i>Nocardioides</i> sp. strain 4 (X94145)	94.8	AJ289954
	B400-138	<i>Holophaga-Acido-</i> <i>bacterium</i> division	Clone II3-36 (Z95726)	95.0	Z95731
Lake Baikal, South Basin, 400 m, 1997 (9)	B404-5	α -Proteobacteria	<i>Acetobacter liquefaciens</i> (X75617)	92.3	AJ289960
	B404-2	<i>Actinobacteria</i>	<i>Aeromicrobium erythreum</i> (M37200)	97.4	AJ007645
	B404-11, -23, -29	<i>Actinobacteria</i>	" <i>Microthrix parvicella</i> " (X89560)	85.3	AJ289955
	B404-57, -19, -451, -50	<i>Actinobacteria</i>	Clone ACK-M1 (AJ007646)	91.6	AJ007646
Lake Baikal, South Basin, 1,200 m, 1995 (7)	B1200-9	α -Proteobacteria	<i>Sphingomonas macrogoltabidus</i> (D13723)	97.3	X99989
	B1200-25	α -Proteobacteria	<i>Caulobacter subvibrioides</i> (M83797)	99.5	X99988
	B1200-63	α -Proteobacteria	Clone NO22 (AJ001344); <i>Blastobacter</i> sp.	99.5	X99983
	B1200-64	α -Proteobacteria	<i>Methylobacterium</i> sp. (D32231)	99.1	X99984
	B1200-34	<i>Planctomycetales</i>	<i>Planctomyces</i> strain 130 (X81952)	89.6	X99985
	B1200-12	GPLGC ^b	<i>Paenibacillus curdianolyticus</i> (D78466)	98.3	X99987
	B1200-56	<i>Holophaga-Acido-</i> <i>bacterium</i> division	Clone MB1228 (Z95733)	96.6	X99986
Lake Baikal, South Basin, 1,200 m, 1997 (7)	B1204-15L	α -Proteobacteria	Clone OM75 (U70683); <i>Rhodospirillum</i> sp.	88.8	AJ289926
	B1204-53L	α -Proteobacteria	<i>Beijerinckia indica</i> (M59060)	95.3	AJ289931
	B1204-33L	γ -Proteobacteria	<i>Methylobacterium methanica</i> (L20840)	92.1	AJ289928
	B1204-51L, -61L	δ -Proteobacteria	<i>Desulfomonile tiedjei</i> (M26635)	88.2	AJ289930
	B1204-20L	<i>Actinobacteria</i>	Isolate TM213 (X92705)	92.2	AJ289927
B1204-39L	<i>Actinobacteria</i>	" <i>Microthrix parvicella</i> " (X89560)	89.7	AJ289929	
Lake Baikal, South Basin, 1,400 m, 1997 (7)	B1404-8	α -Proteobacteria	Isolate 23 (Y12598); <i>Rhodopseudomonas</i> sp.	94.5	AJ007647
	B1404-16	α -Proteobacteria	" <i>Nitrospira moscoviensis</i> " (X82558)	94.5	AJ007648
	B1404-44	α -Proteobacteria	<i>Acetobacter liquefaciens</i> (X75617)	94.5	AJ289935
	B1404-59	α -Proteobacteria	Unidentified bacterium 22 (Y12596); <i>Rhodospirillum</i> sp.	89.1	AJ007657
	B1404-25L	<i>Actinobacteria</i>	Clone ACK-M1 (U85190)	88.4	AJ289933
	B1404-40	<i>Planctomycetales</i>	<i>Gemmata obscuriglobus</i> (S39802)	85.8	AJ007656
	B1404-27	<i>Holophaga-Acido-</i> <i>bacterium</i> division	<i>Acidobacterium capsulatum</i> (D26171)	81.8	AJ289934
Lake Baikal, Central Basin, 1,400 m, 1997 (15)	B1405-19	α -Proteobacteria	<i>Nitrospira</i> sp. (AF35813)	95.1	AJ007652
	B1405-56, -60	α -Proteobacteria	Isolate BF14 (Z23157); <i>Blastobacter</i> sp.	98.6	AJ289940
	B1405-9, -79	β -Proteobacteria	" <i>Chromobacterium indigoferum</i> " (U45995)	91.0	AJ007650
	B1405-1, -2	γ -Proteobacteria	<i>Methylobacterium methanica</i> (L20840)	93.3	AJ007649
	B1405-20	γ -Proteobacteria	Isolate HTA580 (AB002659); <i>Acinetobacter</i> sp.	99.3	AJ007653
	B1405-48	γ -Proteobacteria	<i>Methylobacter luteus</i> (M95657)	94.1	AJ289939
	B1405-10	δ -Proteobacteria	<i>Desulfomonile tiedjei</i> (M26635)	86.2	AJ007651
	B1405-72, -41	<i>Actinobacteria</i>	Clone ACK-M1 (U85190)	96.5	AJ007655
	B1405-49, -22	<i>Holophaga-Acido-</i> <i>bacterium</i> division	Clone C112 (AF013534)	96.8	AJ007654
	B1405-62	<i>Holophaga-Acido-</i> <i>bacterium</i> division	Clone RB27 (Z95719)	98.1	AJ289942
Lake Baikal, Central Basin, 1,600 m, 1997 (7)	B1605-3	α -Proteobacteria	<i>Azospirillum irakense</i> (Z29583)	88.6	AJ289947
	B1605-17	β -Proteobacteria	<i>Thiobacillus aquaesulis</i> (U58019)	92.8	AJ289945
	B1605-22	β -Proteobacteria	<i>Chromobacterium violaceum</i> (M22510)	89.1	AJ289946
	B1605-43	γ -Proteobacteria	<i>Methylobacter luteus</i> (M95657)	93.9	AJ289949
	B1605-59	γ -Proteobacteria	<i>Methylobacter</i> sp. strain BB5.1 (AF016981)	93.8	AJ007658
	B1605-35	<i>Actinobacteria</i>	" <i>Microthrix parvicella</i> " (X89560)	85.8	AJ289948
	B1605-10	<i>Actinobacteria</i>	Clone ACK-M1 (U85190)	94.3	AJ289944

^a The accession number is that of the sequence of the first clone listed in each row. This corresponds generally with the full-length or long sequences determined.

^b GPLGC, gram-positive, low-G+C group.

TABLE 2. Oligonucleotide probes used in this study

Probe	Specificity	Sequence (5' to 3') of probe	Target site (rRNA positions) ^a	% FA in situ ^b	Reference
EUB338	Bacteria	GCTGCCTCCCGTAGGAGT	16S, 338–355	0–35	4
NON338		ACTCCTACGGGAGGCGAGC	16S, 338–355	0–35	62
HGC69a	Actinobacteria	TATAGTTACCACCGCCGT	23S, 1901–1981	20	53
HGC236	Actinobacteria	AACAAGCTGATAGCCGCG	16S, 235–253	10	19
HGC664	Actinobacteria	AGGAATTCCAGTCTCCCC	16S, 663–681	30	19
HG1-840	HG1 cluster of the class <i>Actinobacteria</i>	TCGCA(C/G)AAACCGTGGAAAG	16S, 840–846	10	This study
HG1-654	HG1 cluster of the class <i>Actinobacteria</i> (without FukuN105, 1605-10, 1405-72, and 25-4-33); “cross-reaction” with some CFB ^{***}	AGTCTCCCCTACCAAATC	16S, 654–672	35	This study
HGC270H	Helper for HGC236	ACCCGTCG(T/A/C)(C/A)GCCTTGGT	16S, 270–287	10	This study
HGC697H	Helper for HGC664	GTTCCCTCTGATATCTGCGCA	16S, 697–717	30	This study
HG1-810H	Helper for HGC840	CTAG(C/T)GCCCA(C/T)CGTTTACGG	16S, 810–829	10	This study
HG1-820H	Helper for HGC840	GTTC(G/C)CACAACCTAG(C/T)GCCCA	16S, 820–839	10	This study
HG1-859H	Helper for HGC840	GGGGC(G/A)CTTAATGCGTTAGCTG	16S, 859–880	10	This study

^a *E. coli* numbering (11).

^b Percent formamide (FA) in in situ hybridization buffer.

(66). The sequence similarity values within clusters range from 91.0 to 92.7% for α VI to 94.0 to 99.7% in α III. These values were calculated only on sequences longer than 1,200 nucleotides, whereas the similarity value given in brackets for α V had to be calculated with truncated sequences (282 nucleotides). Based on the current data set, three of the clusters appear to be true freshwater sequence clusters (α II, α IV, and α V), although cluster α II includes a sequence derived from the Mariana Trench deep-sea mud (60) and a negative control clone sequence described as a common PCR contaminant (61). Cluster α III also contains one sequence each from the Columbia River estuary and from paddy field soil, and clusters α I and α VI are intermingled with sequences originating from marine coastal regions and soil habitats. All clusters except α V include cultivated strains.

β -proteobacterial 16S rDNA sequences. Among the β -proteobacterial sequences, four stable clusters could be identified, with in-cluster similarities ranging from 91.2 to 99.5% (β I) to 97.7 to 99.7% (β III), calculated on nearly full-length sequences only (Fig. 1B). For β IV, the similarity of 99.1 to 99.4% is based on the sequences longer than 900 nucleotides. Sequences from coastal regions or estuaries are in all clusters, but no sequences from soil ecosystems can be found. With 35 sequences, β I is the largest cluster defined in this study. As sequences from additional environmental studies become available, subgroups already visible in the current topology might stabilize. Cluster β I contains numerous cultured bacteria, most formerly classified as pseudomonads, but now assembled in the genera *Acidovorax*, *Comamonas*, *Hydrogenophaga*, and *Variovorax*. β II, β III, and β IV are comprised only of cloned sequences from environmental samples. The closest cultivated relatives of these clusters are *Ralstonia eutropha*, *Alcaligenes denitrificans*, and *Methylophilus methylotrophus*, with similarities of 91.9 to 93.4, 96.2 to 96.6, and 91.8 to 95.5%, respectively. β II and β IV have already been described by Zwart and coworkers as freshwater clusters B and C (66).

γ -proteobacterial 16S rDNA sequences. Only one cluster was identified within the γ -Proteobacteria (γ I), consisting currently only of cloned sequences (Fig. 1C). The in-cluster similarity is 93.3 to 99.2%, based on all sequences. The similarity to the next cultivated relative, *Methylobacter luteus*, ranges between 91.7 and 96.0%.

***Actinobacteria*-related 16S rDNA sequences.** The cluster hgcI is exceptional (Fig. 1C). It forms a stable monophyletic new

branch within the class *Actinobacteria*, with a similarity of only 87.2 to 89.0% to the next cultivated relative, *Rathayibacter rathayi*. The similarity within the cluster ranges from 91.5 to 99.0%, calculated for the sequences which overlap at least in a 400-nucleotide-long, central region of the 16S rRNA (*Escherichia coli* position 502 to 961 [11]). Twenty-four of the 37 actinobacterial sequences retrieved in this study can be assigned to the hgcI cluster. This cluster consists mostly of cloned sequences from different freshwater habitats but also two sequences from estuary and coastal ocean systems. The uneven topology and the low similarity values within the cluster suggest that subgroups may be identifiable when more sequences become available. Remarkably, 16 out of 17 actinobacterial clone sequences from Adirondack mountain lakes, formerly described as the ACK-4 group by Hiorns et al. (32), belong to this cluster.

CFB sequences. The four clusters proposed in the CFB phylum have a low in-cluster similarity of only 87.7 to 99.7% to 93.5 to 96.8% (Fig. 1D). The similarity values given for cluster cflI are calculated from an alignment truncated to the shortest sequence. Clusters cflI and cflII include sequences from different soil habitats and from the Columbia River estuary. In cluster cflI, the negative control clone MT10 described previously (61) is also present. Cluster cflIII consists mostly of freshwater sequences, with only one sequence originating from the Columbia River estuary, and cflIV consists only of freshwater sequences with low similarities ranging from 89.8 to 94.6% (calculated only on the sequences with more than 900 nucleotides). The low similarities within the whole CFB phylum imply a high unknown diversity and therefore affect the stability of the designated clusters. All clusters contain at least one pure culture.

FISH of hgcI. To determine the abundance, biomass, and seasonal distribution of the organisms affiliated with the hgcI cluster, specific probes were designed and applied first to Lake Gossenköllesee samples (Fig. 2). Hybridizations performed on the filter samples from June 1997 yielded weak yet detectable signals from small coccoid rods (mean, 0.5 by 0.4 μ m) with the probe HG1-654, but no signals with probe HG1-840. For the latter, oligonucleotide hybridization data on *E. coli* 16S rRNA indicate limited accessibility of this probe binding site (class IV-V according to reference 23). To enhance the FISH signal intensity, three helper oligonucleotides (HG1-810H, HG1-820H, and HG1-859H) were developed. The helpers were de-

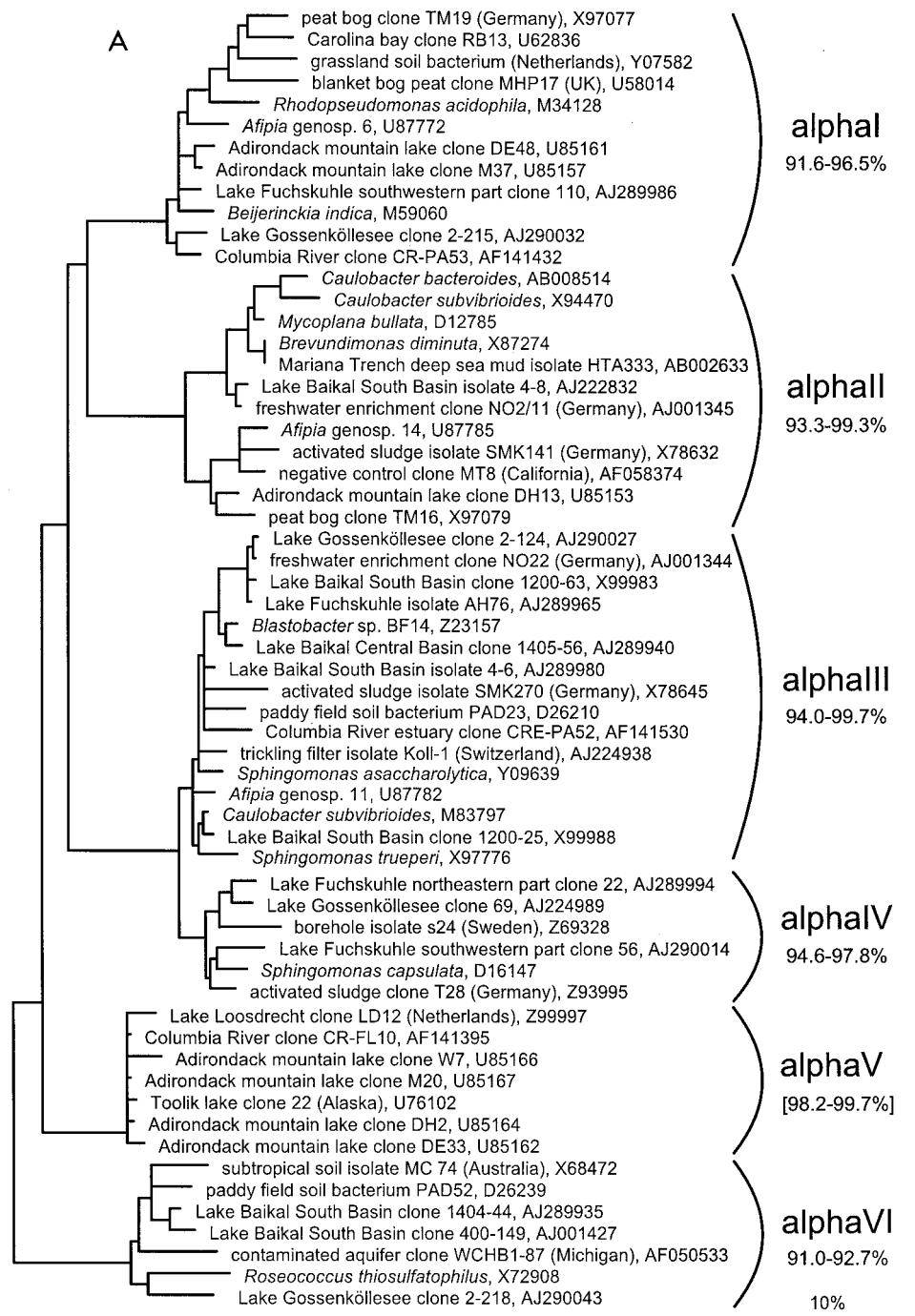


FIG. 1. (A to D) Unrooted phylogenetic consensus tree based on maximum likelihood (FastDNAmI) analysis showing the clones in the proposed clusters. (A) α -Proteobacteria; (B) β -Proteobacteria; (C) γ -Proteobacteria and *Actinobacteria*; (D) CFB phylum. Bifurcations indicate branchings which appeared stable and well separated from neighboring branchings in all cases. Multifurcations indicate tree topologies which could not be unambiguously resolved based on the available data set. For clarity, only the reported clusters are shown; all sequences between the clusters have been omitted. These are included in a more detailed version of the tree which is available at ftp.mpi-bremen.de/pub/molecol_p/fog-aem. The scale bar indicates 10% estimated sequence divergence.

signed to be complementary to sites adjacent to the probe target site and to target at least the same organisms as the probe HG1-840. In addition, they were designed with theoretical thermal stabilities above that of the probe (22). A reevaluation of the June 1997 sample revealed detectable signals for the probe HG1-840 only when combined with all three helpers.

The probe HG1-840-helper mix was checked against the cultivated nontarget organism, *Actinomadura glomerata*, which had two mismatches. No signal could be detected even under nonstringent conditions. HG1-840 probe counts were supported with counts from the more general actinobacterial probes, HGC69a, HGC236, and HGC664 (Table 3). Although

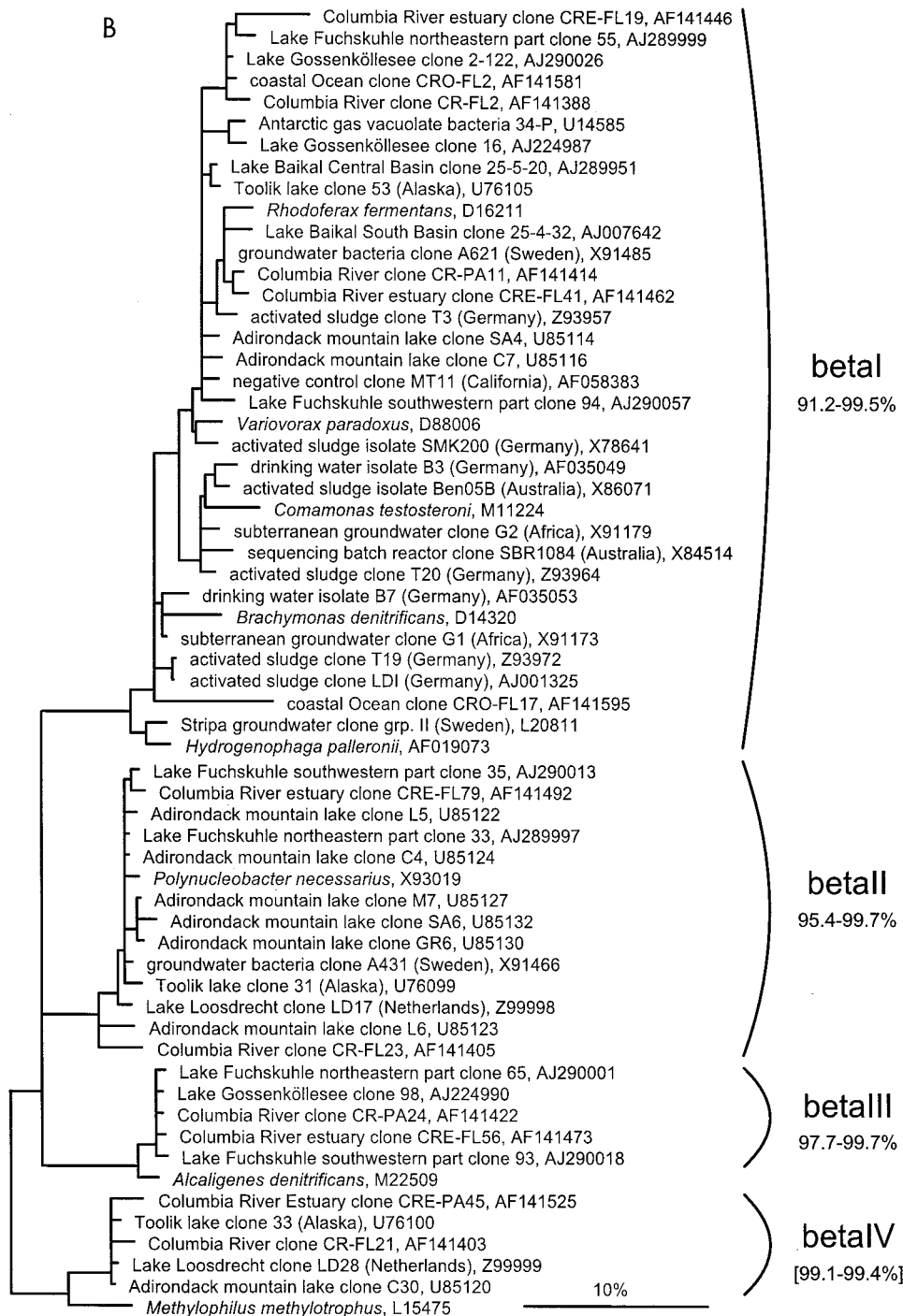


FIG. 1—Continued.

all probes showed detectable signals, helpers HGC270H and HGC697H were developed for the probes HGC236 and HGC664, respectively. A significant signal enhancement on the filters was observed for the combination of HGC236 and HGC270H, but no visible effect was seen for the pair HGC664-HGC697H.

The seasonal distribution of members of the *hgcI* cluster was determined for Lake Gossenköllesee between July 1996 and June 1997 (Table 3). The abundances determined with the

probe HG1-840-helper mix were high, up to 1.7×10^5 cells ml^{-1} , or 49% of all DAPI-stained cells (mean, 28%; $n = 13$) (Fig. 2). A biphasic population development was observed, with the first peak in August 1996 and the second peak in April 1997 (Fig. 2). The general and the specific probes applied showed identical fluctuations, within the range of the estimated counting error, which indicates that *hgcI* is the main actinobacterial group in the bacterioplankton of this lake.

Comparison of the abundance and biomass of the two dom-

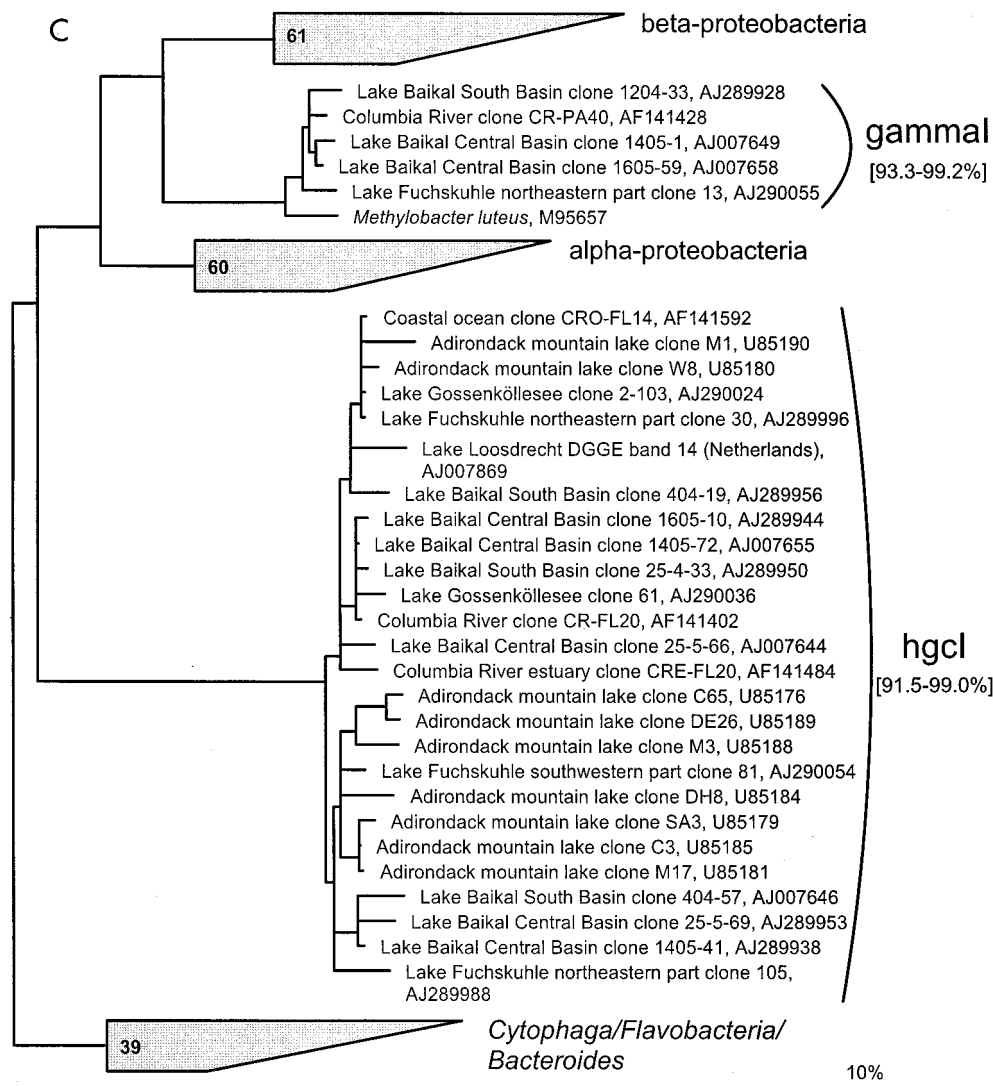


FIG. 1—Continued.

inant groups in Lake Gossenköllesee, the β subclass of *Proteobacteria* and the class *Actinobacteria*, revealed important differences over the year. While β -Proteobacteria made up 61% of the bacterioplankton biomass (biovolume, $1.2 \times 10^4 \mu\text{m}^3 \text{ml}^{-3}$) in August 1996, 63% of the April 1997 biomass (biovolume, $0.6 \times 10^4 \mu\text{m}^3 \text{ml}^{-3}$) was accounted for by the *Actinobacteria* (Fig. 2). This is consistent with the development of the total abundances of the two populations where maxima for the β -Proteobacteria ($1.6 \times 10^5 \text{ cells ml}^{-1}$) and *Actinobacteria* ($2.1 \times 10^5 \text{ cells ml}^{-1}$) were reached in September 1996 and April 1997, respectively. When applying the actinobacterial probes to bacterioplankton samples, the strongest signals were obtained on cells that had been fixed in buffered paraformaldehyde solution and stored for more than 1 year at -20°C .

Several samples from Lake Baikal and Lake Fuchskuhle, hybridized with the set of actinobacterial probes, gave positive hybridization signals with cell counts ranging from 1.3×10^4 to $1 \times 10^5 \text{ cells ml}^{-1}$ for probe HGC69a and 0 to $7.6 \times 10^4 \text{ cells ml}^{-1}$ for probe HG1-840 (Table 3). The cell counts in general were moderately lower than those in Lake Gossenköllesee,

especially in the Central Basin of Lake Baikal, where cell counts for the general actinobacterial probes ranged between 0 and 4% but no *hgcl* could be found. In this particular sample, the cells detected were large rods (2 by $1 \mu\text{m}$) rather than the typical small rods or cocci (0.5 by $0.4 \mu\text{m}$). Morphological diversity was also apparent in the Lake Fuchskuhle samples, where a fraction of bent rods could be detected with the general and specific actinobacterial probes.

DISCUSSION

The comparative analysis of 170 16S rDNA clones obtained from the bacterioplankton of three lakes showed that the vast majority affiliated in clusters that consisted only of sequences originating from freshwater, freshwater and soil, and, in some cases, from freshwater-influenced marine samples (Fig. 1). This clearly is suggestive evidence for the existence of phylogenetically coherent freshwater-continental clusters. A general bias for the selection of related sequences can be excluded, because the samples originated from spatially and ecologically diverse habitats and the strategies for retrieving the sequences

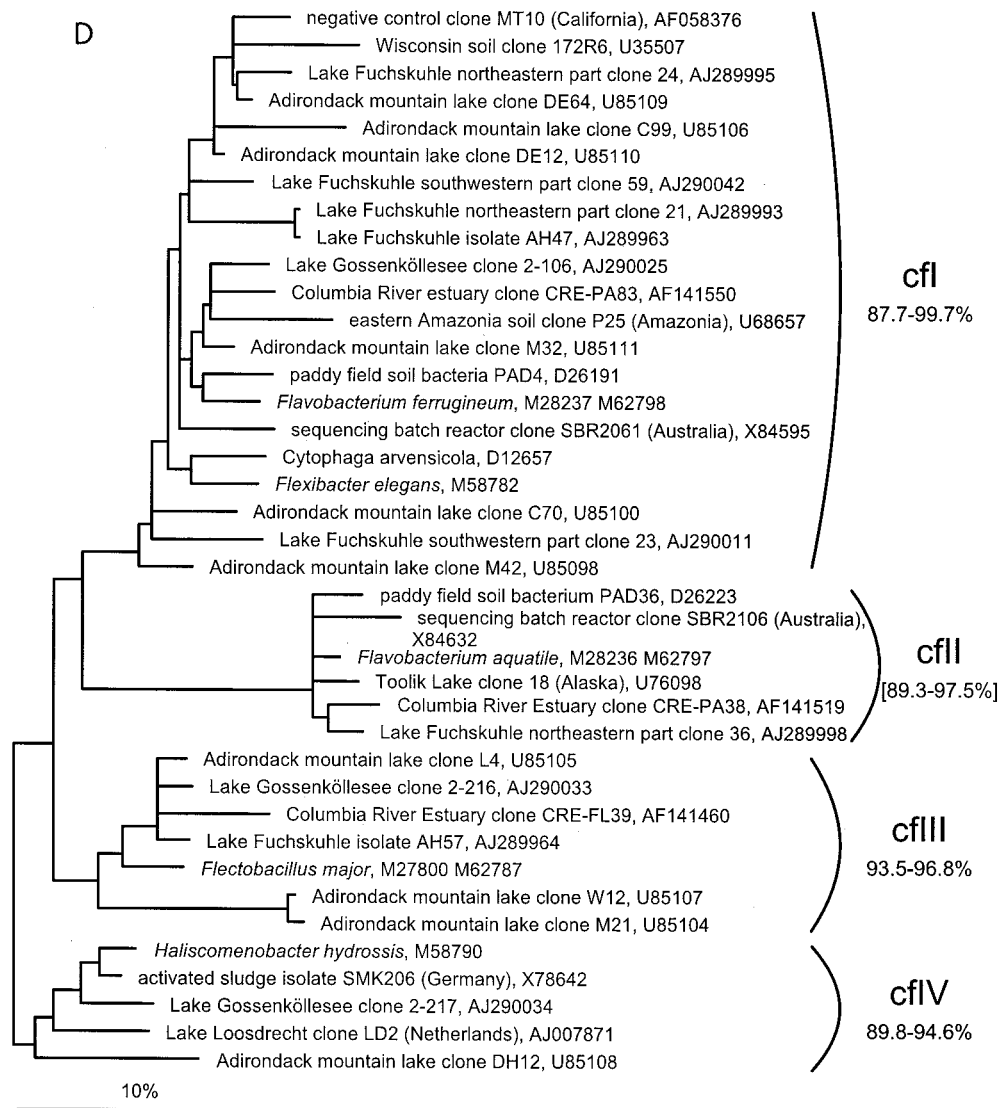


FIG. 1—Continued.

were different in many ways. All freshwater-continental clusters are stable with the phylogenetic reconstruction methods applied and clearly separated from marine clusters like the SAR clusters (26, 41). Only cluster α II contains an isolate from a true marine habitat, the Mariana Trench sediment (60). It is noteworthy that the 16S rDNA sequence of this isolate is 100% identical with the sequence of *Brevundimonas diminuta*, which, together with several *Caulobacter* spp., was recently described as a member of a phylogenetically well-supported freshwater cluster (57).

Coherent clusters might also originate from a widespread use of contaminated PCR reagents. Some of the clusters proposed in this study, e.g., β I, α II, and cfl, indeed contain sequences retrieved from negative controls by Tanner and co-workers (61). Nevertheless, considering that most of the PCR reagents are low in ion concentration (e.g., the PCR water or the nucleotide mixtures), this does not necessarily falsify the clusters proposed. However, all the clusters, especially those consisting only of cloned sequences, have to be checked for

occurrence in freshwater habitats by PCR-independent methods, e.g., FISH or slot blot hybridization.

Another intriguing observation was the presence of one sequence of different *Afipia* spp. in each of the clusters α I, α II, and α III. This organism is described as a potential animal pathogen associated with cat scratch disease. More recent data have indicated that *Bartonella henselae* is the main causative agent, and therefore the real origin of *Afipia* sp. is unclear (25).

The 16S rDNA library data suggested that, in addition to members of the β subclass of *Proteobacteria*, members of the class *Actinobacteria* are abundant in limnic ecosystems. These results were quite unexpected in the light of the general perception that gram-positive bacteria are typical for soils and less common in aquatic habitats (37). FISH was an option for checking the 16S rDNA library data. For that purpose, a set of nested 16S and 23S rRNA-targeted probes specific for the class *Actinobacteria*, together with two probes specific for the hgcI cluster, was used. The cross-reaction of probe HGC654 to members of the *Cytophaga-Flavobacterium* group (Table 2)

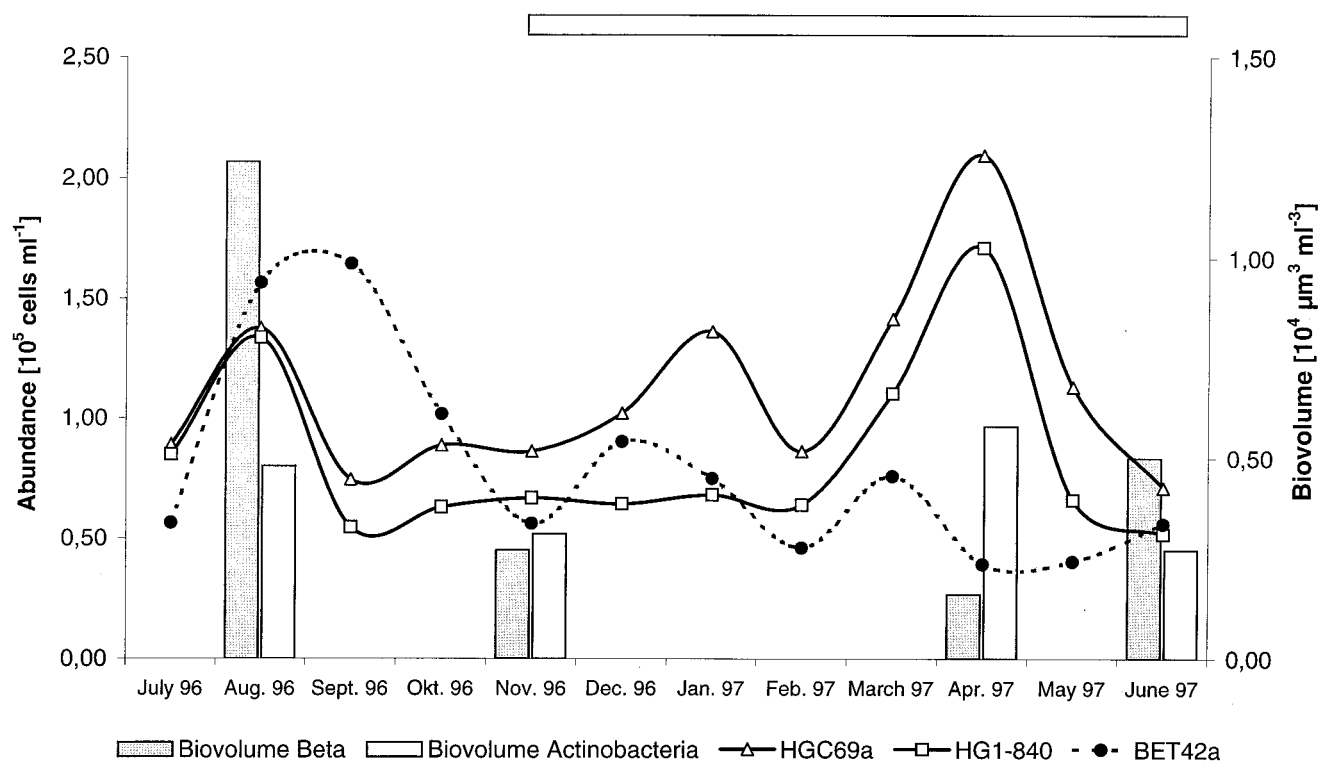


FIG. 2. Seasonal dynamics of the abundance and biomass of the β -Proteobacteria and the *Actinobacteria* counted with probes HGC69a, HG1-840, and BET42a. The horizontal bar indicates the period of ice cover.

illustrates the importance of using multiple probes (2, 3). The morphological information obtained by FISH, i.e., the detection of filaments as well as the typical small, dim cells, was the key for detecting this cross hybridization.

In Lake Gossenköllesee, the two most abundant bacterial groups, the β -Proteobacteria and the *Actinobacteria*, may have distinct temporal niches. In a previous study, the β -Proteobacteria reached their greatest biomass after icebreak, between

TABLE 3. DAPI and FISH counts

Sampling site and date	Depth (m)	Total cell count (10^5 ml^{-1})	Fraction (%) of total cells detected with probe ^a							
			EUB338	ALF1b ^b	BET42a ^b	CF319a ^b	HGC69a	HGC236	HGC664	HG1-840
Lake Gossenköllesee										
4 July 1996	4	2.07	85	10	27	ND ^c	43	44	42	41
5 August 1996	4	3.93	94	18	40	2	35	33	31	34
3 September 1996	4	4.96	47	18	33	3	15	16	14	11
15 October 1996	4	2.86	89	18	36	5	31	25	21	22
13 November 1996	0	2.78	80	11	20	10	31	29	24	24
13 December 1996	0	3.78	76	6	24	2	27	26	18	17
15 January 1997	4	3.24	76	10	23	4	42	35	26	21
13 February 1997	4	2.78	63	11	17	6	31	33	32	23
5 March 1997	4	3.45	77	10	22	8	41	38	30	32
2 April 1997	4	3.49	82	8	11	3	60	45	56	49
29 April 1997	4	3.82	90	22	27	3	44	40	40	32
29 May 1997	4	2.36	79	16	17	3	48	40	40	28
25 June 1997	4	2.15	96	5	26	2	33	36	34	24
Lake Fuchskuhle										
Northeastern part, 9 September 1997	0.5	4.00	69	ND	6	1	25	ND	21	19
Southwestern part, 23 July 1997	3.5	2.31	68	ND	31	3	19	18	14	15
Lake Baikal										
Central Basin, 13 July 1997	0	3.27	74	ND	17	21	4	3	0	0
South Basin, 18 July 1997	0	8.55	71	ND	13	7	15	10	18	10

^a Percent detection of DAPI counts. Numbers have been corrected by subtracting NON338 counts.

^b Data for Lake Gossenköllesee were taken from the work of Pernthaler et al. (50).

^c ND, not done.

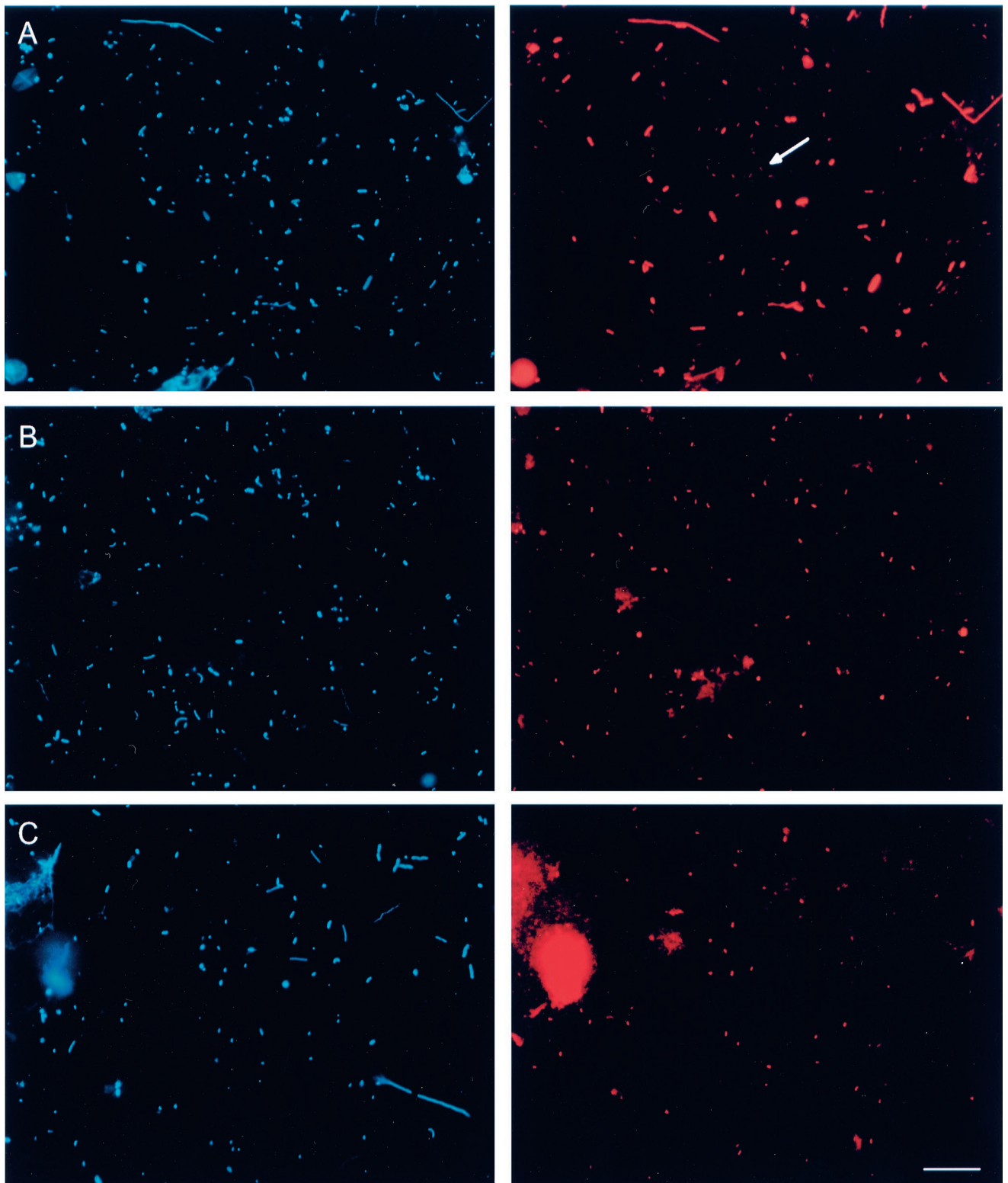


FIG. 3. FISH of samples from Lake Gossenköllesee. DAPI (left) and epifluorescence (right) micrographs are shown for identical microscopic fields. (A) In situ hybridization with probe EUB338 labeled with Cy3. The arrow indicates a representative of a population of small, dim cells. (B) In situ hybridization with probe HGC664 labeled with Cy3. (C) In situ hybridization with probe HG1-840 labeled with Cy3. Bar, 10 μ m (all panels).

June and July, suggesting that they are the first of the studied groups to respond to thermal mixing and the allochthonous input of nutrients from the ice cover and surrounding rockland (50). The same study also described a second biomass maximum in late spring, in the deeper water layers, which at the time could not be assigned to a particular microbial group. In this study, we have identified this peak as actinobacteria, which show high abundance and biomass concentration in April, as well as in August (Fig. 2). The April maximum follows the spring phytoplankton bloom, which occurs in Lake Gossenköllesee between January and March (50), and unlike the August *Actinobacteria* maximum, it is not associated with a β -proteobacterial bloom. In contrast to the spring maximum, the August peak is primarily triggered by allochthonous inputs. In addition, the range of biomass fluctuation is much lower for *Actinobacteria* (2.1-fold) than for the β -Proteobacteria (7.8-fold). This leads us to the hypothesis that in this lake the β -Proteobacteria and *Actinobacteria* members inhabit separate functional niches: the β -Proteobacteria are able to rapidly utilize the main annual input, whereas the hgcI group more efficiently consumes lower levels of organic carbon at low temperature.

First results from Lake Baikal and Lake Fuchskuhle confirm a widespread occurrence of the hgcI cluster. Recently, a marine cluster of *Actinobacteria* has been described which is clearly separated from cluster hgcI (51). This supports our view that hgcI might be a typical freshwater cluster, comparable to the β -proteobacterial clusters (30, 40). Unfortunately, despite considerable efforts it was not possible to obtain pure cultures of hgcI cluster representatives, and therefore their physiology is still unknown. A screen of more than 200 Lake Fuchskuhle and Lake Baikal isolates by partial rDNA sequencing and FISH failed to identify any hgcI strains (data not shown).

Despite the progress made with FISH, it is still a challenge to visualize small and dimly staining organisms like members of the hgcI cluster (Fig. 3A). To calculate this effect, we reevaluated the EUB338 counts reported by Perntaler and coworkers (50). Detection yields relative to DAPI ranging from 47 to 96% (mean, 79%; $n = 13$) were obtained by including the small and dim cells that had been dismissed in the previous study. This is on average one-quarter more than determined earlier (50). By combining the reanalyzed cell counts for the group-specific probes ALF1b, BET42a, and CF319a with counts for the general actinobacterial probe HGC96a from this study, nearly all of the cells detected with probe EUB338 (mean, 99%; $n = 12$) could be accounted for (Table 3). In these particular samples, it seems that the currently available group-specific probes are adequate to further classify all bacteria detected by EUB338. Furthermore, the difference between the number of particles stained by DAPI and the sum of counts with the domain-specific probes EUB338 and ARCH915 (targeting *Archaea*) was reduced from 43% (range, 18 to 55%) (50) to 20% (range, 2 to 50%).

A recent development to enhance signal intensities is the application of unlabeled helper oligonucleotides. Helpers that bind adjacent to the probe target site have been shown to enhance the fluorescence signals at nearly inaccessible sites in *E. coli* up to 25-fold (22). The general applicability of this new approach to environmental samples was demonstrated in this study. For probe HG1-840, all three helpers were needed to yield detectable signals. It remains unclear why the combination of probe HGC664 and helper HGC697H did not result in stronger signals, while the combination of probe HGC236 with helper HGC270H led to clearly visible signal enhancement. Perhaps the RNA-protein cross-linking sites at positions 695 and 703 (*E. coli* numbering) for the ribosomal proteins S11 and

S21 prevent the hybridization of the helper (17). Due to the variable character of the adjacent regions it was not possible to design more than one helper with a reasonable number (less than 4) of wobble base pairs.

In this study, we show that a distinct cluster of small *Actinobacteria* members, hgcI, can become a dominant fraction of the bacterioplankton in freshwater lakes. The phylogenetic divergence indicated by the comparative 16S rDNA sequence analysis and the biotechnological potential of this class are good reasons for further efforts, both traditional and molecular, to learn more about these bacteria.

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