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Abundances, Identity, and Growth State of Actinobacteria in Mountain Lakes of Different UV Transparency

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The occurrence, identity, and activity of microbes from the class *Actinobacteria* was studied in the surface waters of 10 oligo- to mesotrophic mountain lakes located between 913 m and 2,799 m above sea level. Oligonucleotide probes were designed to distinguish between individual lineages within this group by means of fluorescence in situ hybridization (FISH). Bacteria of a single phylogenetic lineage (acI) represented >90% of all *Actinobacteria* in the studied lakes, and they constituted up to 70% of the total bacterial abundances. In the subset of eight lakes situated above the treeline, the community contribution of bacteria from the acI lineage was significantly correlated with the ambient levels of solar UV radiation (UV transparency, $r^2 = 0.72$; $P < 0.01$). Three distinct genotypic subpopulations were distinguished within acI that constituted varying fractions of all *Actinobacteria* in the different lakes. The abundance of growing actinobacterial cells was estimated by FISH and immunocytochemical detection of bromodeoxyuridine (BrdU) incorporation into de novo-synthesized DNA. The percentages of *Actinobacteria* with visible DNA synthesis approximately corresponded to the average percentages of BrdU-positive cells in the total assemblages. *Actinobacteria* from different subclades of the acI lineage, therefore, constituted an important autochthonous element of the aquatic microbial communities in many of the studied lakes, potentially also due to their higher UV resistance.

Members of the class *Actinobacteria* (44) are regarded as typical inhabitants of soil environments (14). Evidence is accumulating that these bacteria are also present in a variety of freshwater habitats (7, 10, 17, 48, 49). Bacteria from this group may in fact constitute a large fraction of the bacterioplankton in oligo- to mesotrophic lakes or in humic lakes (8, 13, 36). In order to understand the role of *Actinobacteria* in different freshwater habitats, it is important to distinguish between the various phylogenetic groups within this lineage (13, 47, 48) and to determine their population sizes in situ. This can be achieved by fluorescence in situ hybridization (FISH) with specific rRNA-targeted oligonucleotide probes (36).

However, the occurrence of particular phylogenetic groups of *Actinobacteria* in the plankton of lakes does not necessarily imply that these bacteria are growing in this environment. Many cultivated *Actinobacteria* perform a life cycle which may involve a vegetative and a resting stage (i.e., spores) (11). Spores are highly resistant to unfavorable environmental conditions and can survive for extended periods of time. This life strategy promotes the survival and dispersal of *Actinobacteria* in soils. Thus, actinobacterial phylotypes that are found in freshwater might potentially be resting stages of predominently soil-dwelling allochthonous microorganisms. In order to prove that this phylogenetic group is an indigenous component of freshwater planktonic microbial assemblages, it is necessary to demonstrate cell growth in situ, e.g., by visualization of DNA de novo synthesis at the single-cell level (31).

Our knowledge on the environmental conditions that favor the occurrence of *Actinobacteria* in lakes is scarce. The first sequence types from this group originate from acidified lakes in the Adirondack Mountains (17). *Actinobacteria* were conspicuously abundant in the water column of an alpine lake that is characterized by high levels of incident solar UV radiation and by high water transparency (13, 42). Solar UV radiation is harmful to various aquatic microorganisms, including viruses, bacteria, and unicellular eukaryotes (19, 40, 45). Furthermore, UV radiation is known to inhibit bacteria-mediated processes (41), and picoplankton organisms from clear-water ecosystems accumulate significant UV-induced DNA damage (6). DNA rich in A+T content can be particularly susceptible due to the higher probability of production of cyclobutane thymine dimers. One typical feature of the *Actinobacteria* is their high genomic G+C content (5). It is thus conceivable that such bacteria might be favored in the bacterioplankton assemblages of UV-transparent lakes.

In order to test this hypothesis, the relative abundances of *Actinobacteria* were determined in the bacterial assemblages of 10 mountain lakes. These lakes are located along an altitude gradient, and they cover a wide range of water column UV transparencies. The composition of the microbial assemblages in these lakes was analyzed using a set of available and newly designed FISH probes. Specifically, we attempted to identify the numerically important taxa of freshwater *Actinobacteria*. In addition, the proportions of growing *Actinobacteria* were determined by pulse-labeling with bromodeoxyuridine (31).

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TABLE 1. Characteristics of sampled lakes

Lake	Latitude	Longitude	Altitude (m a.s.l.)	Maximum depth (m)	Lake area (ha)	Catchment		Temp (°C)	pH	Alkalinity (μeq liter^{-1})	Concn		No. of cells[b] (10^5 ml^{-1})	FISH detection rate[c] (%)
						Area (ha)	Vegetation				DOC (mg liter^{-1})	Chorophyll *a* (μg liter^{-1})		
SOS	46°57'N	10°56'E	2,799	18	3.5	18	Exposed rocks	11.7	6.25	8	0.54	1.1	4.6 ± 0.8	91
ROT	47°14'N	11°00'E	2,485	5.5	0.9	33	Exposed rocks	13.2	7.54	126	0.45	0.81	12.2 ± 0.4	91
GKS	47°13'N	11°00'E	2,417	9.9	1.7	30	Exposed rocks	15.6	7.34	93	0.29	0.32	3.1 ± 0.6	81
GEI	47°13'N	11°00'E	2,410	1.0	0.4	NA[a]	Exposed rocks	15.6	7.42	135	0.34	2.36	14.1 ± 1.4	89
OPL	47°12'N	11°02'E	2,344	7.5	2.1	97	Exposed rocks	12.6	7.26	95	0.31	1.46	5.4 ± 0.1	86
MPL	47°12'N	11°03'E	2,317	5.7	1.6	8	Exposed rocks	16.1	6.91	39	0.25	2.53	6.6 ± 0.2	95
DRA	47°02'N	10°56'E	1,874	24	4.5	188	Exposed rocks	14.9	8.33	1,390	0.51	0.62	2.7 ± 0.3	82
SEE	47°21'N	10°56'E	1,650	14	6.4	NA	Shrubs	14.5	8.41	1,550	0.77	0.86	4.0 ± <0.1	76
ACH	47°27'N	11°42'E	929	133	680	10,500	Trees	20.3	8.41	2,640	1.10	0.49	13.0 ± <0.1	64
PIB	47°11'N	10°53'E	913	24.6	13.4	265	Trees	23.4	8.15	485	2.49	1.74	38.6 ± 0.3	63

[a] NA, data not available.
[b] Total cell numbers as determined by DAPI staining.
[c] Percent of DAPI-stained cells which were hybridized with the eubacterial probe EUBI-III by FISH.

MATERIALS AND METHODS

Sampling sites and scheme. A set of 10 mountain lakes in the Tyrolean Alps (Austria) located at altitudes between 913 and 2,799 m above sea level (a.s.l.) were selected for sampling. Abbreviations of lake names are given in Table 1. Background information on their main limnological characteristics as well as on their UV transparency is found elsewhere (21, 42, 43). Samples of lake surface water were collected between 16 and 22 July 2003 with a 5-liter Schindler-Patalas sampler. Water was stored in the dark and at in situ temperature until further processing (usually within 1 h after sampling). Subsamples for FISH analysis (50 ml) were fixed with an equal volume of ethanol (final concentration, 50%) for 24 h, filtered onto white polycarbonate membrane filters (type GTTP; pore size, 0.2 μm; diameter, 47 mm; Millipore, Eschborn, Germany), and washed with 10 ml of deionized particle-free water (MilliQ; Millipore). Filters were stored at −20°C until further processing.

Physical and chemical parameters. Water samples were analyzed for basic parameters such as pH, conductivity, alkalinity, and temperature. The chromophoric dissolved organic matter characteristics were determined by measurements of dissolved organic carbon (DOC) concentration and spectral absorptivity of filtered lake water. The DOC-specific absorption at 320 nm was estimated as the absorptivity at 320 nm normalized to the DOC concentration. For these analyses, the lake water was filtered on the same day (within 6 h) through glass fiber filters (Whatman GF/F; precombusted for 1 h at 450°C, rinsed with 20 ml of Milli-Q water and 10 ml of lake water) and stored in clean precombusted glass bottles (Teflon capped) in the dark at 4°C until analysis (within 24 h). The DOC was measured in a model 5000 TOC analyzer (Shimadzu, Duisburg, Germany) equipped with a platinized-quartz catalyst for high sensitivity analysis. Absorption by chromophoric dissolved organic matter was determined by spectrophotometry (scans between 250 and 750 nm; Hitachi U-2000 double-beam spectrophotometer) using an acid-cleaned 10-cm quartz cuvette (Suprasil I) prerinsed two times with the sample. Pigments were extracted with 90% acetone for 24 h in the dark at 4°C. Filters were briefly sonicated with a tip sonicator (1 min, 4 W) and the extracts cleared using an Anodisc filter (pore size, 0.1 μm; Whatman). The extracts were scanned in a spectrophotometer (same as above; scans between 400 and 750 nm) against an acetone reference using 5-cm glass cuvettes. The diffuse vertical attenuation coefficients of UVB radiation at 320 nm (K_d320) were estimated either from direct measurements in the water column (Gossenköllesee [GKS], Piburgersee [PIB], Achensee [ACH]) done with a PUV-501 radiometer (Biospherical Instruments Inc., San Diego, CA) or with an empirical model established for mountain lakes (including the ones investigated here) that uses the absorptivity at 320 nm as an independent variable (21).

Oligonucleotide probe design and testing. Based on a previously published phylogenetic analysis of 16S rRNA sequences (47) oligonucleotide probes for FISH were designed for four lineages of freshwater *Actinobacteria* and for subgroups within these lineages. Discriminatory oligonucleotide sequences were discovered using the software packages ARB (22) and Primrose (2). In Primrose, 2,000 sequences from other actinobacterial groups were imported from the ARB database as a non-target-background data set. Subsequently, groups of sequences from the four actinobacterial clusters acI to acIV (47) or from subgroups within these clusters were imported as targets for the automatic probe design. Primrose—as opposed to ARB—is able to design probes that contain "wobbles," i.e., alternative bases at specific positions. Probe candidates were checked for specificity against the ARB database and against sequences in GenBank via a BLAST search. Probes were ranked according to their coverage of target groups, the number and location of mismatching nucleotides to nontarget hits, and the potential in situ accessibility of their respective target sites on the 16S rRNA (4). A list of newly designed (and other, previously published) probes that were used for the analysis of bacterioplankton communities in the studied lakes is given in Table 2.

Due to a lack of cultivated representatives from the actinobacterial acI lineage, the following strategies were applied for the establishment of specific hybridization conditions. For probe AcI-852, a series of hybridizations with increasing formamide concentrations in the hybridization buffer (formamide series, 30 to 70%) were performed on an environmental sample (Schwarzsee ob Sölden [SOS]) and in parallel with cells from a culture of *Actinoplanes philippinensis* (DSMZ 43019). This organism features a single-base mismatch to the probe at a central location of the probe target site on the 16S rRNA. It was therefore comparable to other database entries of uncultured microbes outside the acI group that feature such a mismatch. Probe AcI-1214 is targeted to the same group as AcI-852, i.e., the acI lineage (Table 2). Stringent hybridization conditions for AcI-1214 were established by comparative analysis of the fraction of hybridized cells in several environmental samples at increasing stringency (i.e., concentrations of formamide in the hybridization buffer). The hybridization

TABLE 2. Oligonucleotide probes used in this study

Probe	Specificity	Sequence (5′ to 3′)	Target site (rRNA, 5′ position)	% FA[a]	Reference
EUBI-III	*Bacteria*	GCW GCC WCC CGT AGG WGT	16S, 338	55	9
HGC69a	*Actinobacteria*	TAT AGT TAC CAC CGC CGT	23S, 1907	30	35
AcI-852	acI lineage of limnic *Actinobacteria*[b]	AAT GCG TTA GCT GCG TCG CA	16S, 852	55	This study
AcI-852-H1	Helper for AcI-852	AAA CCG TGG AAG GTY CSC ACA ACT AG	16S, 826	55	This study
AcI-852-H2	Helper for AcI-852	TCC CCA GGC GGG GCR CTT	16S, 872	55	This study
AcI-1214	acI lineage of limnic *Actinobacteria*[b]	CAT GCG TGC AGC CCA AGA CA	16S, 1214	55	This study
AcI-840-1	Subgroup of acI lineage	TCG CAC AAA CCG TGG AAG	16S, 840	30	This study
AcI-840-2	Subgroup of acI lineage	TCG CAG AAA CCG TGG AAG	16S, 840	30	This study
AcI-840-3	Subgroup of acI lineage	TCG CAG AGA CCG TGG AAG	16S, 840	30	This study
AcI-840-H1	Helper for AcI-840 probes	CTA GYG CCC AYC GTT TAC GG	16S, 810	30	13
AcI-840-H2	Helper for AcI-840 probes	GTT CSC ACA ACT AGY GCC CA	16S, 820	30	13
AcI-840-H3	Helper for AcI-840 probes	GGG GCR CTT AAT GCG TTA GCT G	16S, 859	30	13
Bet42a	β-Proteobacteria	GCC TTC CCA CTT CGT TT	23S, 1033	55	24
R-BT065	Beta I lineage of limnic β-proteobacteria[c]	GTT GCC CCC TCT ACC GTT	16S, 65	55	38
Bet2-870	Beta II lineage of limnic β-proteobacteria[c]	CCC AGG CGG CTG ACT TCA	16S, 870	55	8

[a] Percent formamide (FA) in hybridization buffer for hybridizations at 35°C.
[b] Proposed in reference 47.
[c] Proposed in reference 13.

conditions for probes AcI-840-1 to AcI-840-3 were derived from those reported by Glöckner et al. (13) for probe HG1-840 and recalculated for hybridization at 35°C (i.e., 30% of formamide instead of 10%).

CARD-FISH. Fluorescence in situ hybridization with horseradish-peroxidase-labeled probes and tyramide signal amplification was performed as described previously (30) using the modified permeabilization protocol developed for freshwater bacterioplankton (36). Specificity of hybridizations at 35°C was established by the increased addition of formamide to the hybridization buffers (Table 2). Probe AcI-852 and the set of AcI-840-1 to AcI-840-3 probes required the presence of unlabeled helper oligonucleotides to improve the accessibility of the probe target site on the 16S rRNA (12). Helpers were added to the hybridization buffer at the same concentration as the labeled probes (0.5 ng μl^{-1}). Counterstaining of catalyzed reporter deposition (CARD)-FISH preparations with 4′,6′-diamidino-2-phenylindole (DAPI; 1 $\mu g\ ml^{-1}$), mounting, and microscopic evaluations were performed as described previously (29). Total bacterial abundances and the fractions of FISH-stained bacteria in at least 1,000 DAPI-stained cells per sample were quantified at a 1,000× magnification on a Zeiss Axioplan II epifluorescence microscope (Carl Zeiss, Jena, Germany).

Leucine and BrdU incorporation. Bacterial production was estimated via the incorporation of [^{14}C]leucine (Amersham, Little Chalfont, England). Radiolabeled leucine was added at saturating concentrations (20 nM) to triplicate subsamples and to one formaldehyde-killed control (3% final concentration). The treatments were incubated at in situ temperature in the dark for 1 h and fixed by the addition of formaldehyde (3%). Macromolecule extractions were performed as previously described (41). Measurements of leucine incorporation into bacterial biomass were done using a Beckman LS5000TD liquid scintillation counter (Beckman, Fullerton, CA).

For the determination of the fraction of cells with DNA de novo synthesis (31), subsamples of 50 ml were incubated in triplicate at in situ temperature with 5-bromo-2′-deoxyuridine (BrdU; final concentration, 20 μM; Roche Diagnostics, Mannheim, Germany) and thymidine (final concentration, 33 nM) for 4 h in the dark. Subsequently, the samples were fixed with equal volumes of ethanol (final concentration, 50%) and filtered onto polycarbonate membrane filters (type GTTP; diameter, 47 mm) as described above for CARD-FISH. One sample that was fixed prior to BrdU and thymidine addition served as the control. The immunocytochemical detection of incorporated BrdU in bacterial cells and in members of the *Actinobacteria* was performed using an improved version of the original protocol (28). Subsequently, samples were hybridized with probe EUBI-III (9) and HGC69a (35) by the modified CARD-FISH procedure of Sekar et al. (36). The fractions of BrdU-incorporating cells were determined in at least 1,000 probe-positive cells per sample on an Axioplan II epifluorescence microscope (Carl Zeiss) at a 1,000× magnification.

RESULTS

Sampled lakes. The physical and chemical characteristics of the sampled lakes reflected a wide range of environmental conditions (Table 1). For example, the surface water temperature ranged from 11.7 to 23.4°C, the pH from 6.25 to 8.41, and the DOC concentrations from 0.5 to 2.5 mg $liter^{-1}$. The highest DOC concentrations were measured in lakes situated <1,000 m a.s.l. within a forested catchment area (ACH, PIB). Bacterial cell numbers varied over 1 order of magnitude (Table 1). The highest cell densities were found in mesotrophic PIB and the lowest ones in Drachensee (DRA) and GKS. The FISH detection rate of bacteria as determined with the probe EUBI-III ranged from 63% to 95% and showed a significant trend to increase in lakes situated at higher altitudes ($r^2 = 0.87$; $P < 0.01$) (Table 1).

Design of FISH probes targeting freshwater *Actinobacteria*. Two probes targeting the whole acI lineage (47) were designed and tested during this study (Table 2). Both probes were found using the Primrose software. Cells from the *A. philippinensis* culture hybridized with probe AcI-852 were no longer visible at 55% formamide or above in the hybridization buffer (data not shown). This concentration was therefore used to determine the abundance of members of the acI lineage in environmental samples (Fig. 1A). Since the counts with both probes for the acI lineage, AcI-852 and AcI-1214, were very similar, it is likely that equivalent populations were targeted (i.e., that specific hybridization conditions were correctly established). The observed differences between probes (±2.5%) were within the typical error margin of the FISH counting method (33).

The previously published probe HG1-840 is targeted to many but not all sequences of the acI lineage of *Actinobacteria* (13). It was originally designed with a wobble at position 6 (G or C). Since we strived for a higher phylogenetic resolution of the actinobacterial community structure, these two sequence

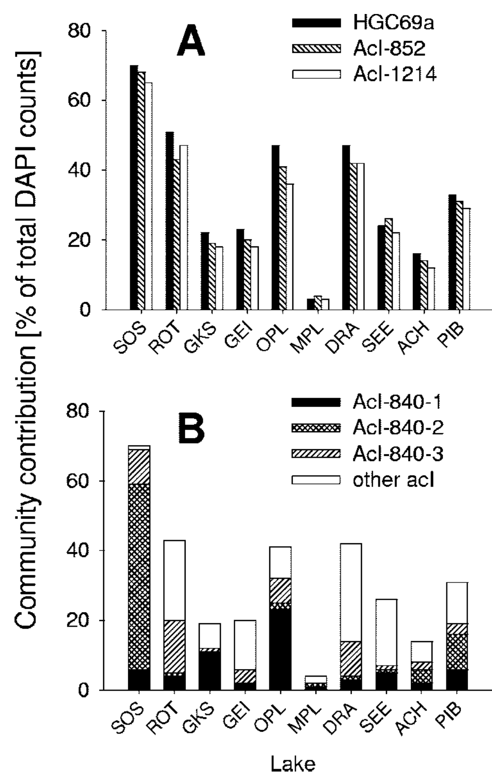

FIG. 1. (A) Relative abundances of members of the class *Actinobacteria* and of the acI lineage in the surface waters of 10 mountain lakes. (B) Relative abundances of different subgroups of *Actinobacteria* from the acI lineage. A detailed description of the different probes is given in Table 1.

variants were applied separately (probes AcI-840-1 and AcI-840-2) (Table 2). The two probes approximately cover the subclusters acI-A and acI-B as defined by Warnecke et al. (47). A third variant (AcI-840-3) was designed by manual sequence comparison. It is targeted to a few sequences inside the acI-B subcluster. In order to depict the phylogenetic affiliations of the targets for the AcI-840 probe set, a phylogenetic tree from all currently available nearly complete 16S rRNA gene sequences from the acI clade was calculated as described previously (47).

Evidence for the high specificity of these three probes was provided by the contrasting counting results found in the environmental samples (Fig. 1B). For example, 23% of total bacterial counts were detected with probe AcI-840-1 in Oberer Plenderlesee (OPL), but only 2% with probe AcI-840-2 (Fig. 1B). In contrast, the majority of bacterial cells in SOS (53%) was detected with probe AcI-840-2, but only 5% with AcI-840-1. These observations suggest that most probably, there was no unspecific cross-hybridization between the two probes. Similar unequivocal differences between the fractions of hybridized cells were also obtained with the probe pair AcI-840-1 and AcI-840-3 (e.g., in Rotfelssee [ROT] and DRA) (Fig. 1B).

Application of probe AcI-852 and the AcI-840-1 to AcI-840-3 probe set was hampered by a low in situ accessibility of the target site, as indicated by their respective positions in the lowest 16S rRNA accessibility classes (IV and V, respectively), as defined by Behrens et al. (4). Consequently, two helper oligonucleotides (Table 2) were required to enhance the signal intensities of probe AcI-852. The three helper oligonucleotides designed by Glöckner et al. (13) were sufficient to perform FISH with the AcI-840-1 to AcI-840-3 probe set.

Abundances of *Actinobacteria*. The contribution of *Actinobacteria* to the bacterioplankton communities varied from $<5\%$ in Mittlerer Plenderlesee (MPL) to 70% in SOS, as determined with the general actinobacterial probe HGC69a (Fig. 1A). FISH with two specific probes for the same subgroup (AcI-852, AcI-1214) revealed that the vast majority of actinobacterial phylotypes in the studied habitats belonged to the acI lineage (Fig. 1A). This was also supported by low counts obtained with other probes targeting subclades A through C of the acII lineage, the acII-D clade, or "Luna" cluster (16), and the A and B subclusters of the acIV clade (data not shown).

Large cell populations were detected by the subgroup-specific probes AcI-840-1 and AcI-840-2 in several of the investigated lakes (Fig. 1B). For example, in SOS, the AcI-840-2 sequence type was dominant. In contrast, large cell populations were detected by probe AcI-840-1 in GKS and OPL, whereas *Actinobacteria* of sequence type AcI-840-2 were rare. In some lakes (e.g., ROT and DRA), a substantial proportion of the acI lineage, as determined with AcI-852, could not be detected with either of the two probes (Fig. 1B). This led to a reinvestigation of sequence data within the acI lineage. Only three of the nearly complete sequences in this cluster were not detected by either probe AcI-840-1 or AcI-840-2 because of a single-base mismatch with probe AcI-840-2 (Fig. 2). Additionally, 17 partial sequences in the data set carried the same single-nucleotide exchange. These sequences originate from three independent studies from the River Weser (37), the eutrophic Lake Zwischenahner Meer (7), and a Siberian reservoir (46). Thus, a sequencing artifact appeared unlikely. Counts with the corresponding probe AcI-840-3 (Table 2) revealed that this sequence type represented, e.g., 15% of total cell numbers in ROT and 10% in DRA and SOS (Fig. 1B).

Abundances of β-proteobacteria. β-Proteobacteria also represented an abundant lineage in some of the bacterial communities (Fig. 3). Counts with the general probe Bet42a varied between 7% and 38%, with the lowest contribution found in mesotrophic PIB and the highest in oligotrophic Seebensee (SEE). Only a relatively low fraction of β-proteobacteria could be assigned to two freshwater subgroups (13) with specific probes targeted to the beta I (38) and beta II lineages (8).

Growth state of the bacterial populations. Bulk incorporation of [^{14}C]leucine varied by about 1 order of magnitude across the studied lakes (Fig. 5). Bacteria from the mesotrophic PIB showed the highest uptake rate (ca. 330 pmol liter^{-1} h^{-1}), whereas the lowest values were found in the oligotrophic DRA (ca. 33 pmol liter^{-1} h^{-1}). DNA de novo synthesis was detected in freshwater *Actinobacteria* via BrdU incorporation (Fig. 4). The incubation experiments revealed total densities of ca. 0.2×10^6 to 0.8×10^6 DNA-synthesizing bacteria ml^{-1} in lakes Geirneggsee (GEI), MPL, and PIB (Fig. 5). The smallest population of cells with visible DNA de novo synthesis was again detected in DRA. There was no significant correlation between the incorporation of radiolabeled leucine and the total abundances of BrdU-active bacteria ($r^2 = 0.35$; $P > 0.05$) (Fig. 5).

A comparison of the fractions of *Actinobacteria* with visible

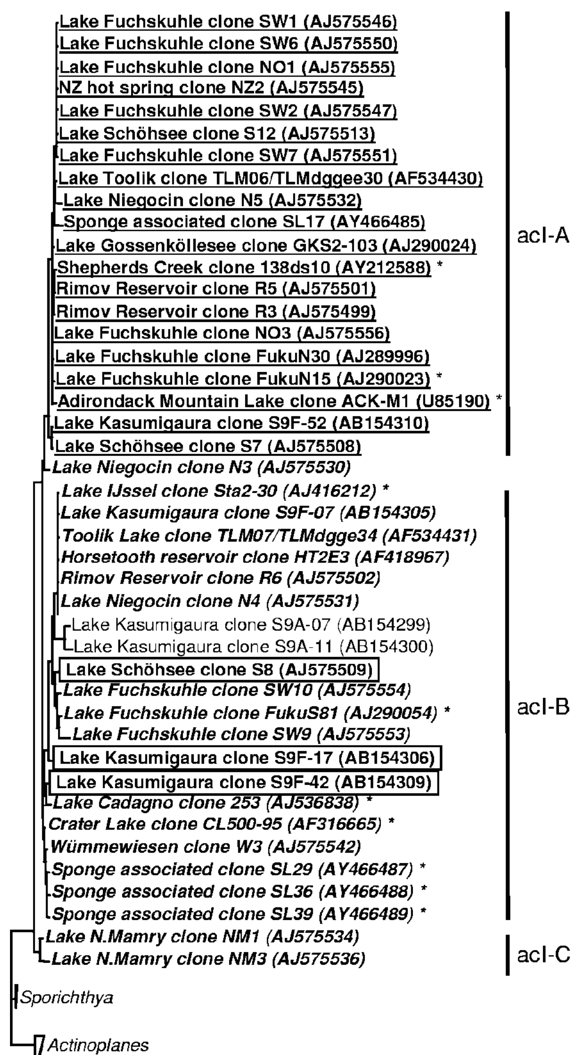

FIG. 2. Phylogenetic tree of all full (>1400 nucleotides) and selected partial (>1,000 nucleotides) actinobacterial 16S rRNA sequences from the acI lineage. Sequences depicted in boldface represent the targets of the three probes AcI-840-1 (underlined), AcI-840-2 (italics), and AcI-840-3 (framed in box). Partial sequences are marked by asterisks. The bar represents 10% of the estimated sequence divergence.

DNA de novo synthesis with all BrdU-positive bacteria revealed that there was no significant deviation from the 1:1 line in the majority of studied lakes (Fig. 6). In SEE and ACH, a fraction of dividing *Actinobacteria* significantly higher than the community average was observed. A smaller than average amount of active *Actinobacteria* was found in the surface water microbial assemblage of PIB.

Actinobacterial abundances and water UV transparency. In 3 of the 10 lakes (GKS, ACH, and PIB), the K_d320 was measured in situ. The measured values were very similar to those estimated using the empirical model of Laurion et al. (21) (for GKS, modeled, 0.22 m^{-1}, and measured, 0.25 m^{-1}; for ACH, modeled, 1.71 m^{-1}, and measured, 1.72 m^{-1}; for PIB, modeled, 2.98 m^{-1}, and measured, 3.05 m^{-1}). The relative contribution of *Actinobacteria* from the acI lineage to the total bacterial abundance was negatively correlated with the estimated K_d320 in lakes above the treeline. Seventy-two percent of the observed variation in community contribution could be explained by this single parameter if the lakes from <1,000 m a.s.l. (ACH and PIB) were excluded from the data set (Fig. 7A). A significant correlation was also observed with the DOC-specific UV absorption across the whole data set (Fig. 7B). No correlations were found with other measured environmental parameters, e.g., altitude or the ratio of lake-to-catchment area (a proxy for the importance of the watershed as a source of *Actinobacteria*).

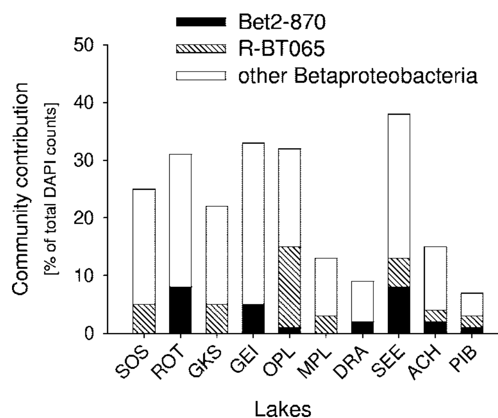

FIG. 3. Relative abundances of members of the β-proteobacteria and freshwater beta I (probe R-Bt065) and beta II (probe Bet2-870) lineages in the surface waters of 10 mountain lakes.

DISCUSSION

Bacterioplankton populations in mountain lakes. As illustrated in earlier investigations, oligotrophic alpine lakes are habitats that can be readily studied by FISH (13, 32) or CARD-FISH (36). In our investigation, the detection rates of hybridized bacteria (i.e., the fraction of cells hybridized by probe EUBI-III) were positively correlated with lake altitude ($r^2 = 0.87$; $P < 0.001$) (Table 1). At present, we can only speculate about this result. It is, for example, conceivable that high mountain lakes contain smaller fractions of objects that typically lead to the overestimation of bacterial abundances when using DNA staining and epifluorescence microscopy. Such objects could be dead bacterial cells (50), bacteria-sized DNA-containing detrital particles (26), or large viruses (39).

Members of the acI clade (47) were the most prominent phylogenetic group of *Actinobacteria* in all studied mountain lakes (Fig. 1A). This was confirmed by two newly designed probes (AcI-1214, AcI-852). At the selected hybridization conditions, AcI-852 could readily discriminate a single mismatching base in *A. philippinensis*. Since a similarly powerful testing system was not available for AcI-1214, we recommend using probe AcI-852 to quantify bacteria affiliated with the acI clade in freshwater by FISH.

The newly designed probes covered the acI clade much more completely than the previously published probe HG1-840 (13) (Fig. 1B). The original probe for this lineage designed by Glöckner et al. (13) features a G-C wobble at probe position 6

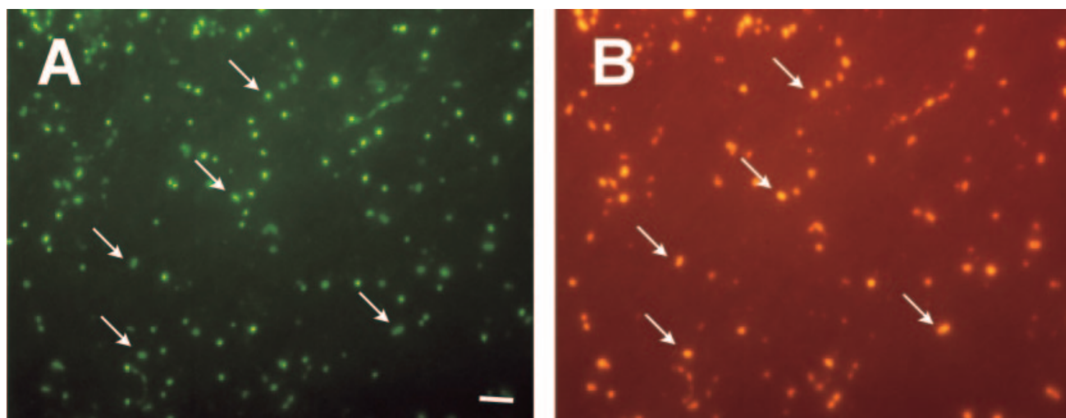

FIG. 4. Photomicrographs of hybridized *Actinobacteria* (A) and of all cells with incorporated BrdU (B) (same microscopic field) in the surface waters of GEI. Arrows highlight examples of double-stained cells. Bar, 10 μm.

(5′-3′). If the probe was split into its two components (AcI-840-1, AcI-840-2) (Table 1), then the sums of cells that hybridized with these two probe variants were significantly smaller than the total fraction of acI-related bacteria (as detected by probe AcI-852) in 8 out of the 10 lakes studied (Fig. 1B). We therefore constructed a third probe modification, AcI-840-3 (G at position 8) (Table 1). In several of the lakes studied, a prominent fraction of cells was hybridized by this probe (Fig. 1B). For example, in ROT, >15% of hybridized cells belonged to this group, which corresponded to more than one-third of all *Actinobacteria* in this lake. Therefore, at least three closely related subpopulations of the cosmopolitan acI clade may coexist in the water column of lakes. Interestingly, a varying fraction of members of the acI clade (as determined by FISH with two cluster-specific probes, AcI-852 and AcI-1214) (Fig. 1A) could not be detected by any of the AcI-840 probe variants (Fig. 1B). The three AcI-840 probes cover all but two of the published acI sequence types consisting of >1,000 nucleotides (Fig. 2). Our FISH data thus hint at additional 16S rRNA diversity within the actinobacterial acI group in mountain lakes.

Cells from the actinobacterial lineages acII to acIV were rare or absent in the studied lakes, as determined by other specific FISH probes (data not shown). Specifically, we found no or only very few bacteria related to the Luna cluster (16) (the D subclade) of the acII *Actinobacteria*. Recently, several isolates from this clade have been described (16), and in situ studies have been called for "to reveal the contributions of these bacteria to the bacterioplankton of different freshwater ecosystems" (16). In view of our results, it is likely that members of the acII clade play a minor role in the plankton of oligo- to mesotrophic mountain lakes (Fig. 1A).

Since our study was focused mainly on *Actinobacteria*, we did not attempt an exhaustive community analysis by FISH. Nevertheless, we also determined the abundances of two groups of β-proteobacteria known to form prominent populations in a eutrophic reservoir (probe R-Bt065 for the freshwater beta I clade) (38) and in an acidified forest lake (probe BET2-870 for the freshwater beta II clade) (8). We found evidence for the occurrence of both groups in several lakes. For example, 5% and >10% of all bacteria were members of the freshwater beta I lineage in GKS and OPL, respectively (Fig. 3). In a previous study, bacterial populations affiliated with the beta I clade were abundant in the water column of GKS over periods of several

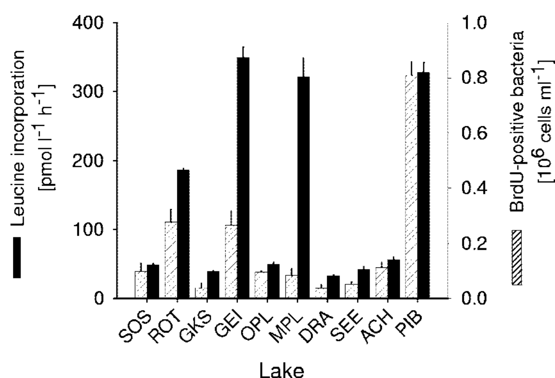

FIG. 5. Incorporation rate of [^{14}C]leucine into bacterial biomass and fractions of cells with detectable DNA synthesis (BrdU-positive cells).

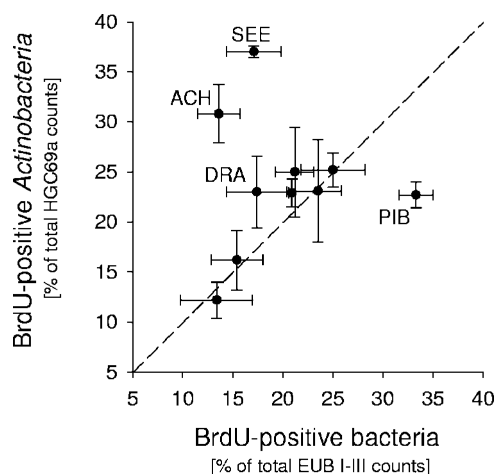

FIG. 6. Comparison of the relative abundances of BrdU-positive *Actinobacteria* with the total fraction of BrdU-positive bacteria in the different lakes. Lakes with significant deviation from a 1:1 relationship (broken line) are labeled.

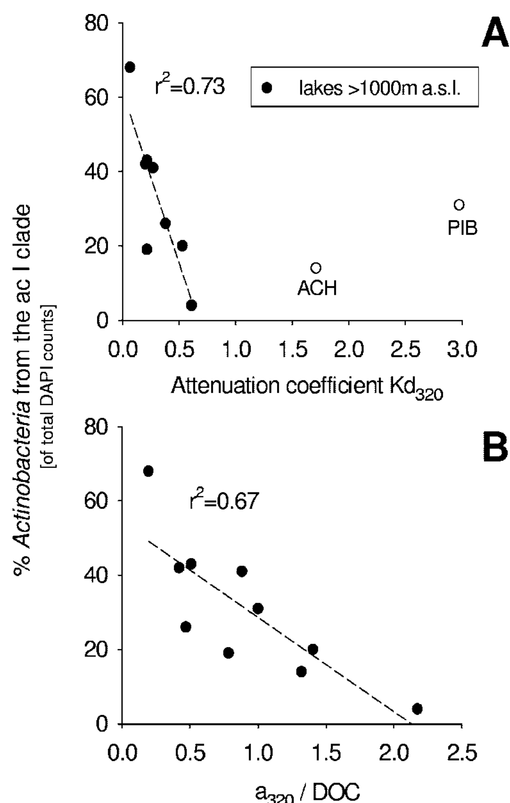

FIG. 7. Relationships between the fraction of *Actinobacteria* from the acI lineage (as determined by probe AcI-852) and the modeled diffuse UV-B attenuation coefficient at 320 nm (A) or the UV absorption at 320 nm normalized to the respective concentrations of DOC (B). In panel A, the two lakes situated below the timberline (open symbols) were excluded from the regression.

months (32). Interestingly, sequences of the *puf* operon have been found in an isolate from the beta I clade that is closely related to a 16S rRNA gene sequence type from GKS (27). We thus speculate that some freshwater β-proteobacteria in oligotrophic mountain lakes might be capable of photosynthesis. Currently, it is unknown if bacteria from the culturable beta II (*Polynucleobacter necessarius*) lineage (15) might also be capable of photosynthesis. In contrast to the predominance of members of the acI lineage within the freshwater *Actinobacteria* (Fig. 1A), bacteria of the beta I and beta II subgroups typically represented only a minor fraction of all β-proteobacteria in the studied systems (Fig. 2). At present, it remains unknown which are the numerically important phylogenetic groups of β-proteobacteria in these mountain lakes.

Bacterial growth state and activity. One major goal of this study was to determine whether freshwater *Actinobacteria* represent an active component within the picoplankton assemblages of mountain lakes. Soil is considered to be a typical habitat of many actinobacterial groups (14), so theoretically, such bacteria might be introduced to lakes from the catchment as dormant cells. Moreover, some gram-positive freshwater *Actinobacteria* are highly resistant to protistan grazing (16). If such bacteria thus experience significantly lower mortality rates than representatives from other bacterial lineages, they might not require rapid growth to maintain high densities in the water column (34). We therefore hypothesized that the fraction of growing *Actinobacteria* would be significantly lower than the community average (i.e., the total fraction of growing bacteria). However, this was not the case. Between 14 and 33% of bacterial cells (mean, 20%) exhibited DNA de novo synthesis (Fig. 6), as detected by the visible incorporation of BrdU (31) (Fig. 4). The percentage of BrdU-positive *Actinobacteria* was statistically indistinguishable from the total fraction of DNA-synthesizing bacteria across the set of studied lakes, and it was even significantly higher for two lakes (ACH and SEE) (Fig. 6). Therefore, it cannot be concluded that *Actinobacteria* in the studied lakes were dormant or that they were more slowly growing than the community average.

There was no significant correlation between the incorporation of radiolabeled leucine and the total abundances of BrdU-active bacteria. These two parameters address conceptually very different aspects of microbial activity. Leucine uptake is a proxy for the rate of total biomass production, whereas the number of BrdU-positive cells reflects the fraction of cells that are in a particular phase of their cell cycle (DNA synthesis). Interestingly, the correlation between the two parameters drastically improved (r^2 = 0.90; $P < 0.001$) if the two lakes that exhibited the highest chlorophyll *a* concentrations (GEI and MPL) were excluded (Table 1). Substantially high rates of leucine incorporation per BrdU-positive (i.e., growing) cell were observed in these two lakes, compared to all other study sites (for GEI, 1.3 fmol liter^{-1} h^{-1}, and for MPL, 3.8 fmol liter^{-1} h^{-1}; average for other eight lakes, 0.6 fmol liter^{-1} h^{-1}). One might speculate that the presence of fewer, but more highly active, microbial cells in GEI and MPL is related to the higher levels of phytoplankton in these lakes (Table 1).

Are *Actinobacteria* favored in UV-transparent lakes? Another aim of this study was to explore whether there exists a relationship between the fraction of *Actinobacteria* in the surface bacterioplankton assemblages of mountain lakes and the ambient levels of solar UV irradiation in the water column. The sites under investigation were therefore selected to cover a wide range of incident UV radiation and UV transparency conditions, two factors that increase with altitude (21).

The highly significant correlation between the community contribution of *Actinobacteria* and the UV transparency in lakes above the treeline (Fig. 7A) suggest that this group may be particularly well adapted to the UV stress that is characteristic of clear, high-altitude mountain lakes. However, the observed relationship is correlative rather than causal. Other, unknown parameters that covary with UV transparency might be ultimately responsible for the contrasting abundances of *Actinobacteria* in the different lakes. In addition, it is unknown if actinobacterial population sizes in the study sites are stable or if they are subject to rapid fluctuations. Thus, our results need to be extended by more direct experimental evidence, such as in situ incubation experiments in the presence and absence of UV radiation (40, 41).

Many of the genetic mechanisms involved in DNA repair, e.g., *recA*, are virtually ubiquitous in bacteria, and there is extensive regulatory overlap of DNA repair and other stress-induced responses (25). Nevertheless, there is a wide variation in the phenotypic expression of UV radiation survival among different bacterial species and strains. Some pigmented bacteria tolerate significantly higher levels of UV radiation. Pigment

production is widespread within *Actinobacteria*, e.g., yellow-pigmented ultramicrobacteria from the acII-D (Luna) cluster have recently been isolated from various lakes (16). The majority of UV-resistant bacterial isolates obtained from surface and subsurface soil habitats were gram positive (1). The higher resistance of such bacteria has also been assigned to components in gram-positive cell walls that may help deflect UV photons (18). Enhanced UV resistence is furthermore observed in species with unusually efficient DNA repair mechanisms, such as *Deinococcus radiodurans* (3). *D. radiodurans* or the anoxygenic phototroph *Rhodobacter sphaeroides* has a 65% G+C genomic base composition (3, 23). The genomes of these bacteria feature a significantly higher frequency of CC (rather than TT) dimers than *Escherichia coli*, which has been interpreted as one possible cause of higher UV resistance (23). The high genomic G+C content of *Actinobacteria* (20) may therefore potentially also play a role in enhanced UV resistance.

Unfortunately, there are currently no isolated representatives of *Actinobacteria* from the acI clade. The group is phylogenetically rather distanced from the most closely related genera of cultured representatives (*Sporichthya*, *Kineosporia*, *Cellulomonas*) (see Fig. 3 in reference 47). Thus, it is currently impossible to draw conclusions about the physiological properties of acI *Actinobacteria* that might promote higher UV resistance.

In any case, a higher UV resistance of *Actinobacteria* would likely be only one among several reasons that explain the apparent success of this group in freshwater bacterioplankton. This is suggested by the significant correlation between acI *Actinobacteria* and the DOC-specific absorption at 320 nm (Fig. 7B), a proxy for DOC quality. Moreover, high abundances of such bacteria have also been found, e.g., in a humic forest lake (8). Since *Actinobacteria* appear to be an autochthonous component of these lakes (Fig. 6), it is moreover likely that their fate is related to organic carbon released by nutrient-limited primary producers. However, the importance of such interactions cannot be assessed from our data set, e.g., we could not find a statistical relationship between chlorophyll *a* concentrations and actinobacterial abundance.

In summary, our data provide evidence that *Actinobacteria* from different lineages of the acI clade (47) are active members of the microbial assemblages in mountain lakes. Moreover, there appears to be a relationship between their community contribution in lakes above the treeline and water transparency (Fig. 7). Currently, it remains unresolved whether acI is indeed the only numerically dominant freshwater actinobacterial lineage or if groups such as the acII-D (Luna) cluster (16) might be abundant in other types of lakes.

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