Low Taxon Richness of Bacterioplankton in High-Altitude Lakes of the Eastern Tibetan Plateau, with a Predominance of *Bacteroidetes* and *Synechococcus* spp.^{▽†}

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Plankton samples were collected from six remote freshwater and saline lakes located at altitudes of 3,204 to 4,718 m and 1,000 km apart within an area of ca. 1 million km² on the eastern Tibetan Plateau to comparatively assess how environmental factors influence the diversity of bacterial communities in high-altitude lakes. The composition of the bacterioplankton was investigated by analysis of large clone libraries of 16S rRNA genes. Comparison of bacterioplankton diversities estimated for the six Tibetan lakes with reference data previously published for lakes located at lower altitudes indicated relatively low taxon richness in the Tibetan lakes. The estimated average taxon richness in the four Tibetan freshwater lakes was only one-fifth of the average taxon richness estimated for seven low-altitude reference lakes. This cannot be explained by low coverage of communities in the Tibetan lakes by the established libraries or by differences in habitat size. Furthermore, a comparison of the taxonomic compositions of bacterioplankton across the six Tibetan lakes revealed low overlap between their community compositions. About 70.9% of the operational taxonomic units (99% similarity) were specific to single lakes, and a relatively high percentage (11%) of sequences were <95% similar to publicly deposited sequences of cultured or uncultured bacteria. This beta diversity was explained by differences in salinity between lakes rather than by distance effects. Another characteristic of the investigated lakes was the predominance of *Cyanobacteria* (*Synechococcus*) and *Bacteroidetes*. These features of bacterioplankton diversity may reflect specific adaptation of various lineages to the environmental conditions in these high-altitude lakes.

Bacterioplankton is a major component of aquatic ecosystems (13). The advent of molecular techniques during the past 2 decades has advanced our knowledge of the diversity and ecology of bacterioplankton in various aquatic habitats (7, 14, 19, 20, 39, 63, 64, 67). Based on comparative analyses of bacterial 16S rRNA sequences obtained from diverse freshwater habitats, typical freshwater bacterial clusters have been proposed (67), and this list of clusters was subsequently expanded (e.g., 2, 15, 23, 57). Many of these freshwater bacterial clusters appeared to be widely distributed in lakes with different ecological characteristics and in different geographic regions (34, 38, 44, 57, 67). However, most of the knowledge about freshwater bacterioplankton diversity originated from investigations of low-altitude lake systems. Differences in elevation result in pronounced differences in environmental conditions in lakes (59). Lakes at high altitudes are often characterized by oligotrophy, low temperature, low productivity, simple food web structure, and strong UV radiation in surface water layers. All these factors have been shown to strongly influence bacterial

community composition and bacterial taxon richness (e.g., 25, 32, 33, 35, 48, 58, 60). These in situ environmental conditions, which change with the elevation, may result in a change in the bacterioplankton community and taxon richness at high elevations. Since lake ecosystems at high altitude are quite sensitive to the impacts of climate change, i.e., global warming, and these rarely explored lakes may harbor new microbial species, our knowledge of the bacterial diversity in high-altitude lakes is also crucial to species protection and ecosystem conservation.

In this study, sequence analysis of large 16S rRNA gene clone libraries was applied in order to obtain deep insights into the phylogenetic diversity of bacterioplankton in six permanent high altitude lakes located ca. 1,000 km apart within an area of ca. 1 million km² on the eastern Tibetan Plateau (Fig. 1). We selected these lakes because (i) they are permanent lakes that have existed for thousands of years and lack direct anthropogenic influences (66); (ii) they possess typical characteristics of high-altitude lakes, like low nutrient concentration, low temperature, low primary productivity, and transparent water; (iii) they are located distant from each other in three different regions of the plateau; and (iv) they cover gradients in salinity and chemical composition that are typical of the ecologically broad spectrum of Tibetan lakes (62). By performing interlake comparisons across these Tibetan lakes and comparisons with the available information on bacterioplankton diversity in low-altitude lakes, we aimed to (i) evaluate the taxon richness of bacterioplankton in lakes in relation to the indirect influence

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FIG. 1. Map of central Tibet indicating the locations of the investigated lakes. 1, Lake Qinghai; 2, Lake Zhaling; 3, Lake Tuosuhai; 4, Lake E'ling; 5, Lake Xinxinhai; 6, Lake Namucuo. (Adapted from reference 61a, with kind permission from Springer Science+Business Media.)

of elevation via the environmental conditions, (ii) test the importance of spatial and local environmental factors for bacterioplankton community composition (BCC) in these remote high-altitude lakes, and (iii) characterize the phylogenetic diversity of bacterioplankton and assess the community features in these remote ecosystems.

MATERIALS AND METHODS

Study sites and sampling. Six lakes located on the eastern and central Tibetan Plateau at altitudes ranging from 3,204 to 4,718 m above sea level were investigated (Table 1). The six lakes, which cover a gradient of total dissolved solids (TDS) from 0.4 to 22.6 g liter⁻¹, are located in three different regions of the Tibetan plateau, with four lakes located less than 50 km apart and the other two

TABLE 1. Limnological and geographical characteristics of the investigated lakes^a

Parameter	Value for Lake:					
	Qinghai ^b	Namucuo ^b	Zhaling	E'ling	Tuosuhai	Xinxinhai
Sampling time (day/mo/yr)	16/07/2004	26/07/2004	8/07/2005	10/07/2005	14/07/2005	7/07/2005
Latitude (°N)	36.607	30.707	34.514	35.033	35.154	34.505
Longitude (°E)	100.509	90.869	97.213	97.426	98.306	98.075
Altitude (m above sea level)	3,204	4,718	4,298	4,279	4,082	4,219
Lake area (km ²)	4,340	1,984	232.2	526	610.7	29.3
Maximum depth (m)	23	70	ND ^c	21	20	ND
TDS (mg/liter)	22,633.3	1,958.2	616.4	428.2	566.3	673.2
Lake type	Polysaline	Oligosaline	Freshwater	Freshwater	Freshwater	Freshwater
Temp (°C)	13.5	10.8	9.5	9.5	12.5	13
pH	9.3	9.4	8.42	8.74	8.83	8.47
TN (mg/liter)	0.94	0.025	0.352	0.384	0.252	0.723
TP (mg/liter) ^d	0.015	0.025	0	0	0	0
K ⁺ (mg/liter)	219.4	44.9	5.0	3.2	5.0	5.2
Na ⁺ (mg/liter)	6,199.9	435.1	108.8	63.2	85.2	131.2
Ca ²⁺ (mg/liter)	6.9	9.1	23.8	30.3	28.2	22.1
Mg ²⁺ (mg/liter)	1,087.0	99.7	38.1	22.9	38.1	48.1
Cl ⁻ (mg/liter)	6,255.5	125.1	113.9	55.1	77.5	141.2
SO ₄ ²⁻ (mg/liter)	7,477.9	363.5	26.5	22.8	55.0	21.8
CO ₃ ²⁻ (mg/liter)	593.5	188.0	45.3	26.7	36.0	90.7
HCO ₃ ⁻ (mg/liter)	793.2	692.8	254.9	206.0	241.3	212.8

^a Sampling depth was 0.5 m.

^b Partial data were adopted from reference 60.

^c ND, no data available.

^d Zero means below the detection limit.

lakes located more than 300 km apart (Fig. 1). All of the lakes are oligotrophic, which is typical for Tibetan lakes. Water samples were collected from surface waters (the top 0.5 m) with a 5-liter Schindler sampler in the pelagic zone (ca. 2 km from the shorelines). The water samples used for determination of prokaryote numbers were fixed on location with 2% formaldehyde (final concentration) and analyzed within 2 months. Bacterioplankton samples (250 to 500 ml water) for 16S rRNA gene-cloning analyses were collected on 0.2- μ m-pore-size Isopore filters in the field by using a hand-driven vacuum pump. The filters were stored in liquid nitrogen during the field campaign and at -20°C in the laboratory until analyses were performed. Untreated water samples (2 to 3 liters) were transported to the laboratory for immediate chemical analysis.

Measurement of physical and chemical parameters. Water temperature, pH, conductivity, and Secchi depth were measured on location. The concentrations of the eight major ions, potassium (K^+), sodium (Na^+), calcium (Ca^{2+}), magnesium (Mg^{2+}), chloride (Cl^-), sulfate (SO_4^{2-}), carbonate (CO_3^{2-}), and bicarbonate (HCO_3^-), as well as the concentrations of total nitrogen (TN) and total phosphorus (TP), were measured according to standard methods after transportation of the samples to the laboratory (21). The TDS in the investigated habitats was determined by summation of the concentrations of the eight major ions and was regarded as the salinity (59).

DNA extraction and purification. DNA was extracted from biomass collected on filters using a standard phenol-chloroform extraction method. In brief, the filters containing the microbial cells were lysed using sodium dodecyl sulfate and proteinase K for 3 h. The lysate was extracted sequentially with phenol-chloroform and chloroform. The DNA was precipitated in ethanol, and the precipitant was collected by centrifugation. The pellet was dissolved in water (molecular grade), and the volume was adjusted to a final volume of 50 μl (45).

PCR and cloning. The almost-complete 16S rRNA genes (longer than 1,400 nucleotides) were amplified using the primer pair 8f (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492r (5'-GGY TAC CTT GTT ACG ACT T-3') (31). Reaction mixtures for PCR contained 1 $\times$ PCR buffer, 200 μM of each deoxynucleotide triphosphate, 1.5 mM MgCl_2 , 0.1 mM of each primer, and 2.5 U of *Taq* DNA polymerase (MBI Fermentas) in a final volume of 50 μl . The PCRs were performed in a PT-200 gradient cycler (MJ Research) using an initial denaturation step at 95°C (5 min), followed by 30 cycles of denaturation at 94°C (1 min), annealing at 53°C (1 min), and extension at 72°C (2 min). A final extension at 72°C (10 min) and subsequent cooling at 10°C completed the reaction. The amplified DNA was verified by electrophoresis of PCR mixture aliquots (3 μl) in 1.2% agarose gels in $0.5\times$ Tris-borate-EDTA buffer. Replicate PCR products were pooled and purified using the NucleoSpin Extract II PCR purification kit (Macherey-Nagel) and stored at -20°C . Cloning of the almost-complete 16S rRNA genes was performed using the pGEM-T Easy Vector System II (Promega) according to the manufacturer's protocol.

Clone library screening by RFLP. Positive clones from each library were screened by reamplifying the 16S rRNA genes as described above using the vector primers, except that 1 μl of culture of *Escherichia coli* containing the insert was used as the template. Ten microliters of PCR product was digested for 2 h at 37°C with 5 U each of the restriction endonucleases *HhaI* and *XbaI* (New England Biolabs) simultaneously. The restriction fragments were separated by gel electrophoresis in 2.5% (wt/vol) Metaphor agarose (FMC Bioproducts) gels in $0.5\times$ Tris-acetate-EDTA buffer. The gel was stained with ethidium bromide (0.5 mg ml^{-1}) and visualized by UV excitation. Restriction profiles were compared using Fragment Analysis software (Amersham BioSciences), and clones were classified according to distinct restriction fragment length polymorphism (RFLP) patterns (36).

Sequencing and phylogenetic analysis. At least one clone was sequenced for each RFLP pattern. Additional clones, chosen randomly, were also sequenced to obtain more abundant patterns. Plasmids were isolated from the clones with the Wizard plus SV miniprep DNA purification system (Promega). Sequencing reactions were performed by the Invitrogen Company (Shanghai Branch) using the BigDye Terminator v3.1 cycle-sequencing kit (Applied Biosystems) and an ABI Prism 3730 Avant genetic analyzer (Applied Biosystems) according to the manufacturer's instructions. Primers 8f and 1492r were used to sequence the cloned 16S rRNA gene fragments from opposite directions. Partial sequences were assembled and manually corrected using Chromas version 1.45 software (Technelysium Pty Ltd).

The RDPII Chimera Check program (<http://rdp8.cme.msu.edu/cgis/chimera.cgi?su=ssu>) and the Mallard program (4) were used to identify sequences with a high probability of being chimeric. The suspicious sequences were excluded from further analyses. The remaining sequences were compared to GenBank entries using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) in order to select reference sequences and obtain a preliminary phylogenetic affiliation of the clones. Phylogenetic affiliation was performed using the Ribosomal Database

Project classifier (55) to assign 16S rRNA gene sequences to the taxonomical hierarchy proposed in *Bergey's Manual of Systematic Bacteriology*, 2nd ed. (17a). All sequences were imported into ARB (34a) and automatically aligned using the integrated aligner tool and the fast-aligner option, followed by manual alignment of closely related sequence, taking into account the secondary structure of the rRNA. Poorly aligned and very variable regions of the alignments were automatically removed with Gblocks (11) using the following parameters: allowing gaps in half position, with the minimum length of a block equal to 5. Separate trees were constructed for *Bacteroidetes*, *Cyanobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, *Actinobacteria*, and other phyla. Each alignment was analyzed by maximum likelihood (ML) using Paup 4.0b10 (50). Different nested models of DNA substitution and associated parameters were estimated using Modeltest (40). The model selected was the GTR plus G plus I for *Bacteroidetes*; TrN plus G plus I for *Alphaproteobacteria*, *Betaproteobacteria*, and *Actinobacteria*; and TrN plus G for *Cyanobacteria* and other phyla. Settings given by Modeltest were used to perform ML analysis. The robustness of the tree topology was confirmed by maximum parsimony with 1,000 bootstrap replications (16) and neighbor-joining with Jukes-Cantor distance correction (30). When there was a discrepancy between the ML topology and bootstrap analyses in NJ and MP, ML topology was favored, as it is considered more robust (51).

Based on these data, sequences were grouped as follows: sequences with similarities greater than 99% were considered to belong to the same phylotype and were defined as operational taxonomic units (OTUs) (46, 47, 49). To investigate the similarities between clone libraries from the six lakes, the absence or presence of different OTUs was converted into a binary matrix. Distance matrices were calculated using Sorenson's coefficient, where N_x and N_y represent the number of OTUs in either lake x or y , respectively, and N_{xy} is the number of OTUs present in both lakes.

Estimation of richness, evenness, and coverage. In order to facilitate comparison with other studies, richness, evenness, and coverage were calculated as sequence similarity with a 97% cutoff. Richness was estimated according to the nonparametric model of Chao (12): $S = S_{\text{obs}} + (a^2/2b)$, where S is the Chao1 richness estimator, S_{obs} is the observed number of OTUs, a is the number of OTUs observed only once, and b is the number of OTUs observed only twice. The reciprocal Simpson's index was also estimated to account for both the abundance and richness of OTUs (24): $D = \sum n_i(n_i - 1)/(N(N - 1))$, where n_i is the number of sequences in the i th OTU and N is the total number of sequenced clones in the sample. The coverage index of the clone library was estimated by the equation $C = (1 - n_i/N) \times 100$, where N represents the number of sequences in the sample and n_i is the number of OTUs classified.

Considering the high fraction of *Bacteroidetes* and *Cyanobacteria* (exclusively *Synechococcus* spp.) obtained in this work, the local diversity of the two groups was compared using FastGroup II software (<http://phage.sdsu.edu/research/tools/fastgroup/>), which allows grouping of sequences according to a defined level of similarity (65). The grouping criteria were set to compare sequence lengths of at least 1,000 bp covering the highly variable regions V3 to V7 of bacterial 16S rRNA genes (37). An algorithm was then developed to group the sequences into percentage similarity clusters (100%, 99%, 98%, and so on) (1). Subsequently, the diversity index, Chao1, was calculated for *Bacteroidetes* and *Cyanobacteria* at three similarity levels of 95%, 97%, and 99%, as mentioned above. These values were selected to approximate the taxonomic categories of genus (46) and species (49), as well as to set a conservative threshold for unique sequence types (3).

Statistics. Principal-component analysis (PCA) was applied to determine the primary differences in environmental variables between lakes. The tested factors included altitude, latitude, area, water temperature, pH, TN, TP, the concentrations of the eight major ions mentioned above, TDS, and total prokaryote number.

Canonical correspondence analysis (CCA) was used to investigate the relationship between BCC and explanatory variables for the absence or presence of different OTUs at a 99% similarity cutoff using the software package CANOCO 4.0 for Windows (52). The environmental parameters included in the PCA were also used here. Explanatory variables were $\log(x + 1)$ transformed, except for temperature and pH. The suitability of weighted-averaging techniques as opposed to linear methods was tested by performing a detrended correspondence analysis with detrending by segments. Exploratory detrended correspondence analyses of the data sets for the six lakes showed that the gradient length of the first axis in standard deviation units always exceeded two units, confirming the suitability of weighted-averaging-based techniques for analyzing our data. The manual forward-selection procedure available in CANOCO was used to estimate the best minimum set of explanatory variables from the full set of explanatory variables. The significance of the relationship between explanatory variables (or sets thereof) and community composition was tested using Monte Carlo permutation tests (499 unrestricted permutations; $P < 0.05$).

Nucleotide sequence accession numbers. The partial sequences of 16S rRNA genes obtained in this study were deposited in GenBank under accession numbers EU703153 to EU703156, EU703158 to EU703185, EU703187 to EU703191, EU703193 to EU703196, EU703199 to EU703205, EU703207 to EU703211, EU703213 and EU703214, EU703216 to EU703223, EU703225 to EU703229, EU703231 and EU703232, EU703234, EU703236 to EU703244, EU703246 to EU703250, EU703252 to EU703258, EU703260 to EU703289, EU703291 to EU703297, EU703299, EU703302 to EU703310, EU703312 to EU703319, EU703322 to EU703339, EU703341 to EU703348, EU703351 to EU703353, EU703355 to EU703360, EU703362 to EU703377, EU703379 to EU703385, EU703387 and EU703388, EU703390 to EU703395, EU703397 to EU703412, EU703414 to EU703416, EU703418 to EU703428, EU703430 to EU703439, EU703442 to EU703468, EU703470 to EU703483, and EU703485 to EU703498. These accession numbers are shown in Fig. S1 in the supplemental material.

RESULTS

Environmental and geographical aspects of the investigated lakes. The major geographical and physicochemical characteristics of the investigated lakes are summarized in Table 1. All of the lakes are oligotrophic and alkaline, as indicated by the concentration of TP and the pH. The TDS of the six lakes ranged from 0.04 g liter⁻¹ to 22.6 g liter⁻¹. The six lakes are located in three different regions, with a minimum distance of ca. 300 kilometers between them (Fig. 1). Lake Qinghai is located in the northern part of the Tibetan plateau, where less precipitation and high evaporation have resulted in saline conditions. Lake Namucuo is located in the central part of the Tibetan plateau, while the other four lakes are located close to one another in the eastern part of the Tibetan plateau but have physical and chemical conditions similar to those of Lake Namucuo (Table 1). PCA of lake environmental factors indicated that the first two components extracted could account for 88.5% of the total variation (PC1, 69.0%; PC2, 19.5%). The concentrations of eight major ions (i.e., K⁺, Na⁺, Ca²⁺, Mg²⁺, Cl⁻, SO₄²⁻, CO₃²⁻, and HCO₃⁻) were all highly correlated with PC1. In addition, the conductivity and total prokaryote number were also closely related to PC1. TP, temperature, and altitude were the main contributors to PC2.

Diversity patterns inferred from clone libraries. After initial screening of 766 clones (154 clones from Qinghai, 96 from Namucuo, 94 from Zhaling, 150 from E'ling, 99 from Tuosuhai, and 173 from Xinxinhai clone libraries) by RFLP analysis, 126 RFLP patterns were identified. Only one pattern was present in all six libraries. A total of 363 representative clones from the six independent 16S rRNA gene libraries were fully sequenced (~1,400 nucleotides) and phylogenetically analyzed. Fifty-five of these sequences were identified as likely chimeras and excluded from further analyses. The remaining 308 cloned sequences (59 from Lake Qinghai, 51 from Lake Namucuo, 35 from Lake Zhaling, 48 from Lake E'ling, 44 from Lake Tuosuhai, and 71 from Lake Xinxinhai) revealed 87 and 151 ribosomal OTUs with similarity cutoffs of 97% and 99% sequence similarity, respectively. About 57.5% (97% similarity cutoff) and 70.9% (99% similarity cutoff) of the OTUs were limited to only a single lake. The Sorenson's coefficients for different clone libraries ranged from 11.7 to 25.1% at 99% sequence similarity cutoffs. Furthermore, CCA of the observed heterogeneous diversity patterns among the lakes indicated that the observed patterns were mainly separated by salinity (Fig. 2) based on manual forward selection of the best minimum set of explanatory variables.

FIG. 2. CCA of the BCCs in the six lakes in which the five factors were a minimal model of variables explaining the species data and TDS was the only significantly correlated one ($P = 0.004$). The first two axes accounted for 28.0% and 23.6% of the variations of the six clone libraries, respectively.

The Chao1 estimator was calculated to predict the total number of OTUs (richness) at a 97% similarity cutoff present in the water samples from the studied lakes. This threshold value was chosen for the sake of comparability with previously published investigations. The taxonomic richnesses estimated for the six Tibetan lakes ranged from 14.2 to 103 OTUs (Table 2), which is considerably lower than the estimated richness in water samples from low-altitude reference lakes (85 to 161 OTUs) (Table 2). The seven reference lakes representing freshwater systems (Mono Lake is a saline system) had on average a taxon richness (128.5; standard deviation, 39.1) five times higher than that of the four investigated Tibetan freshwater lakes (25.0; standard deviation, 7.6). Coverage analyses of the constructed clone libraries indicated that they represented 25.2 to 88.2% of the total number of ribosomal OTUs present in the original water sample from which the libraries were prepared (Table 2).

Taxonomic groups and their distributions. Most sequences from the studied libraries were affiliated with the phyla *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Cyanobacteria*, *Firmicutes*, *Chloroflexi*, *Planctomycetes*, and *Verrucomicrobia*. Only one sequence, from Lake Qinghai (XZQH43), was affiliated with candidate phylum TM7. Eight sequences obtained from three different lakes, i.e., Lakes Qinghai (three sequences), Namucuo (three sequences), and Tuosuhai (two sequences), were defined as unclassified bacteria by the Ribosomal Database Project classifier. *Bacteroidetes* and *Cyanobacteria* (exclusively *Synechococcus* spp.) were the two most common phyla in the analyzed libraries, accounting for 36.4% and 28.2% of the total number of sequences, respectively (Fig. 3). The next most numerically prevalent phyla were *Proteobacteria* (15.3%) and *Actinobacteria* (9.1% of the clones). *Firmicutes*, *Chloroflexi*, *Planctomycetes*, *Verrucomicrobia*, candidate phylum TM7, and unclassified bacterial sequences together accounted for 11.0% of the total number of clones.

Seven phyla (*Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Cyanobacteria*, *Chloroflexi*, *Firmicutes*, and *Planctomycetes*) were subjected to further phylogenetic analyses. A detailed

TABLE 2. Comparison of bacterial diversities in six clone libraries, by means of the Chao1 richness estimator and the reciprocal Simpson's dominance index^a

System	Altitude (m above sea level)	Total no. of sequences analyzed	No. of OTUs detected ^b	Chao1 estimate ^c	Reciprocal Simpson index	Coverage (%)	Reference
Lake Qinghai	3,204	59	32	74.0 (26.0)	28.5	43.2	This study
Namucuo	4,718	51	26	103.0 (50.2)	6.3	25.2	This study
Zhaling	4,298	35	10	14.2 (4.9)	2.7	70.4	This study
E'ling	4,279	48	17	25.1 (7.1)	6.6	67.7	This study
Tuosuhai	4,082	44	18	30.1 (9.7)	6.1	59.8	This study
Xinxinhai	4,219	71	27	30.6 (3.4)	25.1	88.2	This study
Mono Lake, CA	1,945	186	59	111.5 (18.8)	18.5	53.0	26
Lake Limnaren, Sweden	3.9	211	66	161.1 (31.5)	25.4	41.0	15
Lake Erken, Sweden	11	195	55	86.3 (13.2)	23.5	63.7	15
Lake Cadagno, Switzerland	1,921	45	28	138.3 (49.7)	29.4	20.2	6
Lake Stechlin, Germany	59.7	29	29	130	6.1	22.3	2
Lake Visvijver, Belgium	23	71	44	108	47.8	40.7	54
Lake Maten 13, Belgium	55	128	79	191	93.4	41.4	54
Bugach, Siberia, Russia	223	105	32	85	26.3	37.6	53

^a All samples for this comparison were from oxic layers of the respective lakes.

^b OTUs were defined at a sequence similarity cutoff of 97% in order to facilitate comparison with other studies.

^c The standard deviation for the Chao1 estimator is given in parentheses.

description of the revealed phylogenetic diversity is presented in the supplemental material. An unusual result, found throughout the major detected phyla, is the presence of ribotypes lacking close relatives in public databases. As a whole, 34 new sequence types showing <95% identity to the closest related sequences deposited in GenBank were detected. In other words, about 10% (*Actinobacteria*) to 30% (*Betaproteobacteria*) of the obtained sequences demonstrated <95% sequence identity to any GenBank entry (Fig. 4), except *Cyanobacteria*, for which no new sequence with such a low simi-

larity value was detected. At least nine new clusters or new subclusters were identified within the above seven phyla (see Fig. S1 in the supplemental material). Here, only monophyletic groups that had nearly full-length 16S rRNA sequences that were at least 95% identical, contained sequences from at least two of the studied lakes, and were supported by high bootstrap values were considered new clusters or subclusters (67). Three out of these nine new clusters were affiliated with the

FIG. 3. Relative contributions of major phyla to the total number of OTUs (99% similarity cutoff) detected in each investigated Tibetan lake.

FIG. 4. Identities of sequences and OTUs (defined by 99% similarity cutoffs) found in the six Tibetan habitats to sequences deposited in GenBank (analyzed by BLAST searches on 30 April 2008). For each phylum, sequences and OTUs were classified in three relatedness categories, i.e., identity $\geq 99\%$, identity <99% but $\geq 95\%$, and identity <95% to the top BLAST hit. The upper stacked bar (I) shows the sequence contribution in each classification, and the lower stacked bar (II) shows the contribution of OTUs.

FIG. 5. Numbers of OTUs plotted against changing degrees of cutoffs in 1% increments for sequences of the taxa *Bacteroidetes* and *Cyanobacteria* and all bacteria. The numbers of OTUs detected at a 99% similarity cutoff for *Bacteroidetes*, *Cyanobacteria*, and all bacteria were 67, 30, and 189, respectively.

phylum *Bacteroidetes*. One of the nine newly identified clusters (XZNMC16; *Alphaproteobacteria*) had no closely related sequences in GenBank, i.e., it showed <95% sequence identity to the closest database entry.

A large fraction of the overall set of clones obtained from the six investigated lakes was attributed to the phyla *Bacteroidetes* (15% to 66% of 99% similar OTUs) and *Cyanobacteria* (3% to 33% of 99% similar OTUs) (Fig. 3 and 5). While a broad diversity of ribotypes and OTUs contributed to the observed *Bacteroidetes* diversity (see Fig. S1A in the supplemental material), the phylogenetically rather narrow taxon *Synechococcus* spp. comprised almost exclusively the revealed diversity of *Cyanobacteria* (Fig. 3 and 5). Consequently, about 70% of the cyanobacterial diversity is only apparent when OTUs are defined by similarity cutoffs of >99%, while the proportional increase in the *Bacteroidetes* diversity is much weaker when the similarity cutoff is raised to values above 99% (Fig. 5).

DISCUSSION

The investigation presented above revealed unusual diversity patterns across the six investigated lakes, as well as in comparison to the previously investigated lowland lakes. All six investigated lakes showed relatively low taxon richness in comparison to low-altitude freshwater habitats and less elevated mountain lakes (Table 2). This feature was combined with the detection of a relatively high proportion of taxa lacking closely related sequences in GenBank (Fig. 3). A third trait is the numerical dominance of ribotypes in the libraries affiliated with the phyla *Bacteroidetes* and *Cyanobacteria* (exclusively *Synechococcus* spp.) (Fig. 3). Furthermore, our study confirmed previously demonstrated distinct differences in the diversities of bacterioplankton in freshwater and saline lakes located at high altitudes on the eastern Tibetan Plateau.

Low taxonomic richness of bacterioplankton in high-altitude lakes. For macroorganisms (animals and plants), a gen-

FIG. 6. Numbers of bacterial taxa as determined by the Chao1 estimator for the six Tibetan lakes (squares) and reference lakes (dots) (Table 2) plotted against the surface area (A) and altitude (B) of the respective lakes. a.s.l., above sea level.

eral decrease in species richness and an increased percentage of unique species with increasing altitudes have been suggested (8, 18, 27, 42). In the case of microorganisms, it is not clear whether such a rule is also applicable (9, 41). Our investigation indicated lower taxon richness in the investigated high-altitude lakes compared to reference lakes located at lower elevations (Fig. 6). Low taxon richness of bacterioplankton was also observed for high-mountain lakes in the Sierra Nevada (Spain), although this was explained by small ecosystem size (43). The large size of some of the investigated Tibetan habitats clearly argues against a pronounced influence of habitat size on the low observed taxon richness (Fig. 6). Tibetan lakes are characterized by strong UV radiation, oligotrophy, low primary productivity, low temperature, and low terrestrial input of organic carbon resources (56). Most of these environmental parameters are related to elevation. For instance, it is generally accepted that with elevation, the temperature decreases by 6 centidegrees per 1,000 m and solar UV radiation increases by about 20 to 30% per 1,000 m. Low concentrations of nutrients and low temperature usually also result in low primary productivity (59). Interestingly, several of the above-mentioned parameters were linked in other studies to the diversity of bacterioplankton. For instance, a bell curve relationship between primary productivity and the taxon richness of aquatic *Bacteroidetes* has been observed (25). Recent observations

have indicated that the richness of marine bacterioplankton is positively related to water temperature (17). External carbon resources have been identified as important factors that strongly influence the BCCs of lakes (14). Combined effects resulting from such altitude-related environmental parameters may cause a reduced number of ecological niches to be available in the investigated Tibetan habitats, resulting in a lower taxonomic diversity of bacterioplankton. Thus, elevation may indirectly influence the bacterial taxon richness in the investigated lakes by directly influencing the environmental conditions in the lakes; however, detailed comparative investigations of bacterial diversities in low- and high-altitude lakes employing identical methodologies are needed to gain deeper insights into the influence of elevation on bacterial diversity in aquatic systems.

Presence of cosmopolitan versus local bacterial taxa. In a recent study, the BCCs of 16 Tibetan lakes were investigated by means of denaturing gradient gel electrophoresis and reverse line blot hybridization with probes targeting 17 freshwater bacterial groups that are well known in low-altitude freshwater habitats (e.g., 2, 15, 19, 67), demonstrating the presence of some typical freshwater bacterial clusters in these lakes (60). In the present study, about 60% of the obtained sequences grouped with known freshwater and marine clusters, supporting the idea that altitude may not limit the dispersal and distribution of bacterioplankton taxa. However, we do not know whether all members of such phylogenetic taxa share the same ecological adaptations. An increasing number of studies have demonstrated that significant ecophysiological diversity may be present in microdiverse clusters of bacteria characterized by almost identical 16S rRNA genes (5, 61). For instance, planktonic *Actinobacteria* with identical 16S rRNA genes have been found to differ significantly in their thermal adaptations, and the revealed ecophysiological differences reflected environmental differences of their home habitats (22). Therefore, we cannot exclude the possibility that the members of cosmopolitan clusters of freshwater or marine bacteria detected in the Tibetan habitats represent locally adapted strains whose ecological traits differ significantly from those of other members of these clusters.

We found at least nine new clusters or subclusters (see the supplemental material) and 34 new ribotypes, i.e., those which showed <95% identity to the closest sequences in GenBank, in our six clone libraries. Almost all of these clusters contained sequences that were exclusively reported from the investigated Tibetan lakes. The new ribotypes can be considered to represent undescribed new species, or even new genera. At least some of these new taxa could represent bacteria endemic in Tibetan lakes. Furthermore, some sequences that showed approximately 99% similarity to their closest relatives also displayed distinct phylogenetic clusters that were unique to the investigated lakes (see Fig. S1B in the supplemental material), and the formation of such clusters could have resulted from ecosystem-dependent adaptive radiation.

Dominance of *Bacteroidetes* and *Cyanobacteria*. The percentage of *Bacteroidetes* among the total number of clones exceeded by far the percentages contributed by other phyla present in the investigated Tibetan lakes. In a previous study, a predominance of *Bacteroidetes* was also found by fluorescent in situ hybridization and denaturing gradient gel electrophore-

sis fingerprinting in some other Tibetan lakes spanning a TDS gradient from 0.2 to 222.6 g liter⁻¹ (60). In addition, the percentage of *Bacteroidetes* (in contrast to other taxa, like *Betaproteobacteria* or *Gammaproteobacteria*) did not vary systematically along the salinity gradient (60), which is confirmed by the current study (Fig. 3) and another study (34). The detailed analysis of the diversity of *Bacteroidetes* in the six investigated lakes demonstrated that a broad diversity of *Bacteroidetes* consisting of many different lineages may be responsible for the overproportional contribution of this phylum to the overall diversity of bacterioplankton in these systems (see Fig. S1A in the supplemental material). Furthermore it is obvious that, with one exception (*Aquirestis* spp.), *Bacteroidetes* ribotypes in freshwater and polysaline systems represent distinct taxa. Possibly, several lineages of *Bacteroidetes* adapted to the conditions in the Tibetan lakes, resulting in the occupation of many ecological niches and numerical predominance. This seems to be in accordance with the previously suggested importance of the diversity of *Bacteroidetes* for their ecological success (28, 29). In the case of the Tibetan lakes, the trait of pigmentation found among the majority of *Bacteroidetes* (28) may also have played a crucial role. Since pigmented bacteria can usually tolerate considerably higher levels of UV radiation, *Bacteroidetes* may be favored in Tibetan lakes, where strong UV radiation and transparent water prevail.

The dominance of *Bacteroidetes* was followed by that of *Cyanobacteria*, which were exclusively represented by *Synechococcus* spp. Our results confirm the notion that *Synechococcus* tends to be abundant in oligotrophic high-mountain lakes (10). Considering the high fraction of *Bacteroidetes* and *Synechococcus* in our libraries, we compared the local diversity of the two groups. In our investigation, similar numbers of 16S rRNA gene sequences from *Bacteroidetes* and *Synechococcus* were obtained (112 and 87, respectively). In contrast to the high phylogenetic diversity of *Bacteroidetes*, we found much lower phylogenetic diversity but higher microdiversity among *Synechococcus* spp. (Fig. 5). The Chao1 estimator predicted that the expected number of genera (95% similarity), species (97%), and unique sequence types (99%) of *Bacteroidetes* (29.0, 48.1, and 121.9, respectively) were on average about seven times higher than those obtained for *Synechococcus* (2.0, 7.0, and 51.0, respectively). Obviously, different evolutionary mechanisms were responsible for the diversity of *Bacteroidetes* and *Cyanobacteria* revealed in the investigated lakes.

Importance of spatial and local environmental factors for BCC. Finally, the results presented here confirm that salinity is an environmental factor that strongly influences the taxonomic composition of bacterioplankton in inland waters (60). Salinity was the most important factor explaining variations in BCC between the investigated lakes. Sequences obtained from the saline-rich Lake Qinghai were mostly affiliated with marine bacterioplankton, while most sequences retrieved from freshwater and oligosaline lakes were related to freshwater bacterioplankton (see Fig. S1 in the supplemental material). Although the four lakes located close to each other (Fig. 1 and 2) had more similar BCC profiles than other lakes, we did not find a significant influence of spatial factors on BCC in our CCA model. It seems that a coincidence between the spatial distribution of the lakes and the salinity variation led to the BCC

variation in the investigated lakes, as the four lakes located close to one another (Fig. 1 and 2) are all freshwater lakes.

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