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ORIGINAL PAPER

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Bacterial diversity in permanently cold and alkaline ikaite columns from Greenland

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Abstract Bacterial diversity in alkaline (pH 10.4) and permanently cold (4°C) ikaite tufa columns from the Ikka Fjord, SW Greenland, was investigated using growth characterization of cultured bacterial isolates with Terminal-restriction fragment length polymorphism (T-RFLP) and sequence analysis of bacterial 16S rRNA gene fragments. More than 200 bacterial isolates were characterized with respect to pH and temperature tolerance, and it was shown that the majority were cold-active alkaliphiles. T-RFLP analysis revealed distinct bacterial communities in different fractions of three ikaite columns, and, along with sequence analysis, it showed the presence of rich and diverse bacterial communities. Rarefaction analysis showed that the 109 sequenced clones in the 16S rRNA gene library represented between 25 and 65% of the predicted species richness in the three ikaite columns investigated. Phylogenetic analysis of the 16S rRNA gene sequences revealed many sequences with similarity to alkaliphilic or psychrophilic bacteria, and showed that 33% of the cloned sequences and 33% of the cultured bacteria showed less than 97% sequence identity to known sequences in databases, and may therefore represent yet unknown species.

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Introduction

Microbial communities in alkaline environments have recently been thoroughly studied due to the possible biotechnological and industrial applications of alkaliphilic enzymes.

But natural alkaline environments are rather rare. The best-studied alkaline environments are temperate and subtropical alkaline soda lakes with pH values ranging from 8 to more than 12 and which contain large amounts of sodium carbonates in combination with low concentrations of Mg^{2+} and Ca^{2+} (Duckworth et al. 1996; Jones et al. 1998). These extreme conditions are reflected in the adaptation of the microorganisms found in the lakes, many of which are both alkaliphilic and halophilic or extremely halotolerant species (Sorokin et al. 2002, 2003; Hoover et al. 2003; Ma et al. 2004). These microorganisms often represent separate lineages within accepted taxa or show no strong relationship to any other known species.

Natural, stable, low-saline, alkaline environments are rarer than the soda lake environments, but some have recently been discovered and investigated. Among these are the Lost City Hydrothermal Field in the Mid-Atlantic Ocean (Schrenk et al. 2004) and the groundwater of Cabeço de Vide in Southern Portugal (Tiago et al. 2004), both with pH values above 10. The Lost City Field is home to several column structures composed of calcium carbonate and magnesium hydroxide minerals, which are associated with hydrothermal vents with temperatures in the range of 40–93°C (Kelley et al. 2001). The groundwater of Cabeço de Vide is borehole water that contains very low amounts of salts and has a temperature of 20.5°C (António Veríssimo, personal communication). Both these environments have been shown to contain alkaline microbial communities.

Ikaite tufa columns from the Ikka Fjord in South West Greenland constitute the only known alkaline environment on Earth, which is both low-temperature and low-saline (Pauly 1963; Buchardt et al. 1997). These vertical chimney-like columns are composed of the rare carbonate mineral ikaite, which is a metastable hexahydrate of calcium carbonate ($CaCO_3 \cdot 6H_2O$). An important difference between the ikaite columns and the columns of the Lost City is the way they are formed. In contrast to ikaite columns, Lost City columns are formed by exothermic serpentinization where low-silica rocks are oxidized and hydrolyzed with water into serpentinite and contain the minerals calcite, aragonite, and brucite (Kelley et al. 2001). Ikaite columns are formed when alkaline freshwater springs rich in sodium, carbonate, and bicarbonate meet the cold, calcium-rich seawater, which leads to an immediate oversaturation of calcium and carbonate ions and, due to the high level of phosphate (0.26 mmol l^{-1}) in the seep water, a subsequent precipitation of ikaite rather than of the anhydrous calcium carbonate minerals calcite or aragonite (Buchardt et al. 1997, 2001). The ikaite columns grow up to 50 cm yr^{-1} and can reach a height of up to 20 m while the diameter varies from a few centimeters to several meters. Ikaite is only stable at temperatures below 6°C and therefore, an increase in sea water temperature of only 2°C in the Ikka Fjord due to global warming may result in the disintegration of this unique ecological niche.

The interior of the ikaite columns is filled with alkaline (pH 10.4) and cold (4°C) seeping spring water with a salinity of 0.9%, whereas the temperature, pH, and salinity of the exterior of the columns are similar to the surrounding seawater (Buchardt et al. 1997). Previous investigations have shown that ikaite tufa columns contain a wide variety of algae and other eukaryotic organisms (Buchardt et al. 1997; Kristiansen and Kristiansen 1999; Sørensen and Kristensen 2000; Stockman et al. 2000; Thorbjørn and Petersen 2003), and we have in a preliminary report shown that the columns may also harbor a large diversity of microbial species (Stougaard et al. 2002). Our previous results indicated that the microbial diversity in ikaite columns showed some resemblance to the diversity observed in temperate and subtropical alkaline environments. However, the similarity of the previous 16S rRNA gene sequences to known sequences was shown to be very low with approximately 50% of the phylotypes showing less than 90% identity to known 16S rRNA gene sequences in databases. Previous attempts to cultivate bacteria from the ikaite samples resulted in only ten cultured bacterial strains.

The preliminary results were based on samples of ikaite tufa columns, which were not sampled for microbiological purposes, and which were stored in a −20°C freezer for approximately 6 years before investigation. The limited success in cultivating live bacteria from these ikaite samples might be due to improper handling and storing, and therefore, during the summer of 2002, we sampled new ikaite material and preserved the columns for analysis of microbial diversity. In this paper, we describe the bacterial diversity and species richness in these ikaite columns. Furthermore, we compare separate microbial communities from individual ikaite columns, and briefly address some general growth characteristics of the bacteria living inside the ikaite columns.

Materials and methods

Samples

Ikaite tufa columns and seawater samples were collected from the Ikka Fjord in South WestGreenland (61°11′N, 48°01′W) in August 2002. Scuba divers collected three columns from a depth of approximately 10 m in the fjord within an area of 10 m^2. The columns were between 1 and 2 m in height and between 10 and 30 cm in diameter. The columns were kept cold during transportation to the laboratory, where they were cut into slices and stored either at 5 or at −20°C in 20% glycerol until further use. The pH of the water drained from the interior of the column was measured to be 10.4–10.5. In the laboratory, ikaite material was drilled out as described previously (Stougaard et al. 2002). The ikaite material was analyzed for cultivable microorganisms and subjected to DNA isolation. Three different ikaite columns (columns A, B, and C) were used for the analysis. Samples of sea water surrounding the ikaite columns were frozen in 20% glycerol and stored for further cultivation purposes.

Enrichment, media, and isolation procedures

Samples for isolation of live microorganisms were taken from fresh ikaite material stored at 5°C or from ikaite or sea water frozen in 20% glycerol after sampling. Small pieces of ikaite material were crushed and the grains were either suspended in liquid medium or plated directly onto solid media. Four different media were used for isolation: a diluted and modified R2 medium adjusted to pH 10, called AeIB10, containing the following ingredients (per liter): 0.05 g yeast extract (Difco, Sparks, MD, USA), 0.05 g bacto peptone (Difco), 0.05 g casamino acids, 0.05 g glucose, 0.005 g $MgSO_4 \cdot 7\ H_2O$, 0.01 g NaCl, 0.05 g soluble starch, 0.03 g sodium pyruvate, 0.03 g KH_2PO_4, 100 ml 1 M Na_2CO_3/$NaHCO_3$-buffer (pH 10); R2A10 medium, pH 10 (per liter): 18.2 g R2A agar (Difco), 200 ml 0.5 M $Na_2CO_3 \cdot H_2O$ (pH 10); G2 medium, pH 6.8 (per liter): 10 g bacto peptone, 10 g casamino acids, 10 g yeast extract, 2.5 g beef extract powder, 100 ml 10× phosphate buffer (pH 6.8) (90 g l^{-1} KH_2PO_4, 68 g l^{-1} K_2HPO_4), 2 mM $MgSO_4$, 0.2 mM $MnSO_4$, 0.5% (w/v) glucose, 1.5% agar; and Marine Agar medium: 55.1 g l^{-1} Marine Agar 2216 (Difco).

Bacteria for phylogenetic analysis were either grown in AeIB10, where they were incubated at 5°C for

6 weeks at 200 rpm on a rotary shaker prior to plating on AeIA10 agar medium, which is AeIB10 medium solidified with 1.5% (w/v) agar, or plated directly from crushed ikaite material onto AeIA10, R2A10, or G2 media. Plates were incubated at 5°C for 1–3 months, and distinct colonies were purified and maintained on agar media.

Growth requirements of cultured bacterial isolates from the ikaite columns were analyzed in microtiter plates. Isolates were selected on R2A10, G2, and Marine Agar media and transferred to microtiter plates and resuspended in R2 broth. After resuspension, the isolates were replicated onto R2A10, G2, and Marine Agar media buffered to different pH values (pH 7–10) and incubated at temperatures ranging from 10 to 37°C. pH was measured both at the beginning and at the end of the experiment.

Sea water samples of approximately 100 µl each were spread on R2A10 agar plates and incubated for 1 month at 5°C to isolate bacteria able to grow on the alkaline medium from the sea water surrounding the ikaite columns.

DNA isolation, 16S rRNA gene clone library construction, and phylogenetic analysis

Samples for DNA analysis were drilled out from ikaite material frozen at −20°C immediately after sampling. Samples from top, middle, and bottom in each of the three ikaite columns were analyzed with four to six replicas of each sample.

DNA was extracted using FastDNA® SPIN Kit for Soil as described by the manufacturer (BIO 101, Irvine, CA, USA) with the following modifications: The samples were processed in the FastPrep® Instrument three times for 30 s at speed 5.5. Between processing, the samples were placed on ice for 30 s. Furthermore, all centrifugation times were doubled and DNA was eluted in 100 µl DES (DNA Elution Solution BIO 101). Other methods were tried for the extraction of DNA from ikaite (Marmur 1961; Zhou et al. 1996), but since the highest diversity in 16S rRNA gene sequences was obtained with FastDNA SPIN Kit for Soil, this method was selected for the subsequent 16S rRNA gene clone library constructions and Terminal-restriction fragment length polymorphism (T-RFLP) analysis. PCR reactions were carried out in 100 µl volumes containing 2 µl DNA extract, 0.2 µM of each primer, and 2.5 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). The primers for amplification of bacterial 16S rRNA gene sequences were 610V (5′-GTGCCAGCAGCCGCGGT-3′) (position 515-531, *Escherichia coli* numbering) and 606R (5′-GGTGTGACGGGCGGT-3′) (position 1,398–1,412, *E. coli* numbering). The PCR program was an initial denaturation step at 94°C for 5 min followed by 35 cycles each of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C followed by a final extension cycle at 72°C for 7 min. PCR reactions were analyzed by agarose gel electrophoresis. PCR products from the top, middle, and bottom sections of one of the ikaite columns were cloned in three separate cloning reactions. Bands were cut out and subjected to purification using GFX PCR DNA and Gel Band Purification Kit according to the manufacturer's manual (Amersham Biosciences, Uppsala, Sweden). Purified bands were ligated into pCRII-TOPO vector and transformed into *E. coli* TOP10F' using the TOPO TA Cloning Kit (Invitrogen). Transformants were analyzed for the presence of inserts in the cloning vector by PCR using standard primers (M13 forward and M13 reverse) and agarose gel electrophoresis. Reactions with inserts of the expected size were subjected to further purification with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) before sequence analysis.

We furthermore tried to amplify archaeal 16S rRNA gene fragments using the Archaea-specific primers 21F (5′-TTCCGGTTGATCCYGCCGGA-3′) and 958R (5′-YCCGGCGTTGAMTCCAATT-3′) (Bano et al. 2004) under the same PCR conditions as described above.

Sequence analysis of 16S rRNA gene fragments was carried out on a MegaBACE 1000 DNA Sequencing system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) DNA sequencer using DYEnamic™ ET Terminator Sequencing Premix (Amersham Pharmacia Biotech). Sequences were handled using MegaBACE Sequence Analyzer, Version 3.0 (Amersham Biosciences). Alignment of sequences was carried out using the ClustalW algorithm from the EMBL-EBI homepage (Chenna et al. 2003, http://www.ebi.ac.uk/clustalw) and the BioEdit Version 7.0.4.1 software (Hall 1999). Phylogenetic trees were created using the Treecon for Windows Version 1.3b software (Van de Peer and De Wachter 1994).

Nucleotide accession numbers

The phylogenetic relationship was determined as described previously by Stougaard et al. (2002). Accession numbers for sequences deposited in the NCBI GenBank database are numbers DQ028333–DQ028358 for cultured isolates and DQ028359–DQ028410 for phylotype sequences. All sequences were checked for chimeras by the ribosomal database project (RDP) program CHECK_CHIMERA (Maidak et al. 1997).

Rarefaction analysis

Rarefaction analysis was performed on a total of 109 16S rRNA gene sequences from the clone library using the EstimateS software, Version 7.5 (Colwell 2005), and SigmaPlot, Version 8.0 (SPSS Inc., Chicago, IL, USA). The statistical outputs reported here are the ChaoI (Chao 1984) and the Coleman rarefaction analysis (Coleman et al. 1982).

Terminal-restriction fragment length polymorphism analysis

Terminal-restriction fragment length polymorphism analysis of 16S rRNA gene fragments was performed on DNA extracted from ikaite columns using the primers and the PCR conditions described above, except for the 610V forward primer being HEX-labeled. The amplified 16S rRNA gene fragments from the PCR reactions were purified with the GFX PCR DNA and Gel Band Purification Kit. DNA was eluted in 50 µl TE buffer (pH 8) and concentrated by ethanol precipitation prior to restriction enzyme digestion and T-RFLP analysis. The concentrated 16S rRNA gene fragments were digested with restriction enzymes *Alu*I and *Rsa*I (New England Biolabs, Beverly, MA, USA) at 37°C for 4 h followed by thermal inactivation at 65°C for 20 min. MegaBACE ET900-R size standards labeled with Texas Red (Amersham Biosciences) were added to the digested samples, which were then desalted using Sephadex G-50 (Amersham Biosciences), denatured at 94°C for 1 min, and kept on ice before injection into the MegaBace 1000 DNA Sequencing system. Samples were injected at 3 kV for 3 min, and separation was performed at 7 kV for 180 min in MegaBACE Long Read Matrix (Amersham Biosciences).

Terminal-restriction fragment length polymorphism data were analyzed using the MegaBACE Genetic Profiler Version 1.5 (Amersham Biosciences) and the Primer 5 Version 5.2.9 software (Primer-E Ltd., Plymouth, UK). Analyses were based on a non-transformed Bray–Curtis similarity and the statistics applied were ANOSIM one-way and two-way crossed analysis using 10,000 permutations, while data were visualized using non-metric MDS (multidimensional scaling) analysis with 100 restarts (Clarke and Warwick 2001).

Results

Growth of cultured bacteria

Isolates were grown on three different media adjusted to different pH values and at different temperatures to investigate the temperature and pH tolerance of the ikaite bacteria. Table 1 shows that the majority of bacteria isolated on R2A10 plates (115 out of 158 isolates) were able to grow on R2A plates buffered to pH 8–10. Nineteen of the 158 isolates were able to grow at all pH values tested, and a small minority, 15 and 9 isolates, were able to grow only at pH 9–10 or only at pH 10, respectively. Temperature tolerance was measured in a similar manner. Two of the 158 isolates cultivated on R2A10 plates were able to grow only at 10°C but not at 20°C or higher, whereas the majority, 156 isolates, grew both at 10 and 20°C. No growth was observed at 30 or 37°C. Seventy-three bacterial isolates were isolated on marine agar and all of them only grew on media buffered to pH 7 or pH 8 (Table 1). The temperature tolerance assayed on marine agar plates showed that the majority (52 out of 73 isolates) was able to grow up to 20°C and a small fraction, 18 out of 73 isolates, could grow at temperatures up to 30°C. In a similar experiment with bacteria isolated on G2 medium buffered to pH 7, approximately half of the isolates, 29 out of 53, were able to grow at temperatures up to 20°C, 19 isolates grew up to 30°C, and five isolates were able to grow at all temperatures from 10 to 37°C.

Table 1 Number of ikaite isolates cultured on three different media: R2A10, Marine Agar, and G2 agar growing at different pH and temperatures

	R2A10	Marine Agar	G2
PH			
pH 7	0	0	53
pH 7 and 8	0	73	nt
pH 7–9	0	0	nt
pH 7–10	19	0	nt
pH 8–10	115	0	nt
pH 9 and 10	15	0	nt
pH 10	9	0	nt
Temperature			
10°C	2	3	0
10 and 20°C	156	52	29
10, 20, and 30°C	0	18	19
10, 20, 30, and 37°C	0	0	5

nt not tested

Phylogenetic analysis of cultured bacteria

Sixty-seven cultured isolates from ikaite samples were subjected to phylogenetic analysis. The isolates, two to five from each agar plate, were selected based on their different phenotypical appearance. The 16S rRNA gene sequences showed similarity to phylotypes of six bacterial classes (Fig. 1), and 22 of the 67 ikaite isolates showed less than 97% sequence similarity to sequences from known species.

The majority of the ikaite isolates (63 out of 67 isolates) were affiliated to Gram negative bacteria. Of the 63 Gram negative isolates, 54 showed similarity to *Proteobacteria*, of which 26, 1, and 27 isolates were affiliated to the α-, *β*-, and *γ*-*Proteobacteria*, respectively (Table 2).

All isolates affiliated to the α-*Proteobacteria* showed sequence similarity to bacteria within the *Rhodobacteraceae* family and the three genera *Rhodobaca*, *Rhodobacter*, or *Loktanella*. Nine isolates showed resemblance to the soda lake bacterium *Rhodobaca bogoriensis* (Milford et al. 2000), while 16 isolates all shared a 98% sequence similarity to the Antarctic bacterium *Loktanella vestfoldensis* (Van Trappen et al. 2004) (Table 2). Seventeen isolates were affiliated with 95–99% sequence similarity to species of the *Pseudomonadaceae* family.

Nine ikaite isolates were related to bacteria within the phylum *Bacteroidetes*, of which seven showed sequence similarity to the unclassified CFB group bacterium

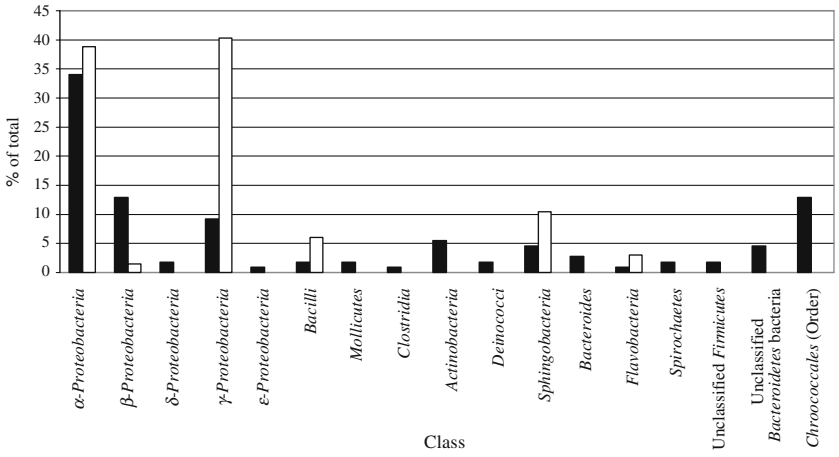

Fig. 1 Phylogenetic affiliation of partial 16S rRNA gene sequences from cultured isolates ($n = 67$) and clone library phylotypes ($n = 109$) from ikaite columns. Clone library phylotypes (*black bars*), cultured isolates (*white bars*)

Ikaite c9 (Stougaard et al. 2002) and *Rhodonellum psychrophilum* (unpublished), whose closest relative is *Belliella baltica* (Brettar et al. 2004) of the *Flexibacteraceae* family.

Only four isolates showed similarity to Gram positive bacteria, and they were all related to species within the class *Bacilli* in the *Firmicutes*. One isolate showed a 97% sequence identity to *Bacillus alcaliphilus* (Nielsen et al. 1995), while the remaining three were related to species in the family *Carnobacteriaceae*.

The sea water surrounding the ikaite columns was analyzed for bacteria able to grow on the high pH medium. Only four isolates were obtained from sea water samples when isolation took place on R2A10. All four isolates were affiliated to *Carnobacterium maltaromaticum* (approximately 99% similarity).

Analysis of 16S rRNA gene clone library

A 16S rRNA gene clone library was constructed from DNA extracted from top, middle, and bottom fractions of a single ikaite column. Sequence analysis of 16S rRNA gene sequences from the clone library revealed a large prokaryotic diversity within the ikaite column. A total of 109 clones were sequenced (32 clones from the

Table 2 Cultured isolates from ikaite and their closest relatives inferred from 16S rRNA gene sequence similarity

Taxonomic group[a]	Class	Closest cultured relative	Similarity (%)	No. of sequences[b]
GCM51	*Bacilli*	*Bacillus alcalophilus* (X76436)	97	1
GCM1	*Bacilli*	*C. maltaromaticum* (AF184247)	99	3
GCM9	*Flavobacteria*	*Flavobacterium hydatis* (M58764)	99	1
GCM6	*Flavobacteria*	*Flavobacterium psychrolimnae* (AJ585428)	98	1
GCM63	*Sphingobacteria*	*B. baltica* (AJ564643)	80	1
GCM65	*Sphingobacteria*	*B. baltica* (AJ564643)	82	1
GCM36	*Sphingobacteria*	*R. psychrophila* (DQ112660)	99	5
GCM53	α-*Proteobacteria*	*L. vestfoldensis* (AJ582227)	98	16
GCM19	α-*Proteobacteria*	*Rhodobaca bogoriense*s (AF248638)	95	2
GCM24	α-*Proteobacteria*	*R. bogoriense*s (AF248638)	99	1
GCM64	α-*Proteobacteria*	*R. bogoriense*s (AF248638)	98	3
GCM67	α-*Proteobacteria*	*R. bogoriense*s (AF248638)	92	1
GCM49	α-*Proteobacteria*	*R. bogoriensis* (AF248638)	96	2
GCM28	α-*Proteobacteria*	*Rhodobacter veldkampii* (Y14150)	97	1
GCM11	β-*Proteobacteria*	*Collimonas fungivorans* (AJ496445)	99	1
GCM35	γ-*Proteobacteria*	*Marinobacterium halophilum* (AY563030)	93	2
GCM39	γ-*Proteobacteria*	*Pseudomonas alcaligenes* (AY651923)	95	6
GCM50	γ-*Proteobacteria*	*P. alcaligenes* (AY651923)	95	2
GCM56	γ-*Proteobacteria*	*P. alcaligenes* (AY651923)	96	1
GCM3	γ-*Proteobacteria*	*Pseudomonas antarctica* (AJ537601)	99	6
GCM14	γ-*Proteobacteria*	*P. antarctica* (AJ537601)	99	2
GCM8	γ-*Proteobacteria*	*Psychrobacter glacialis* (AJ539102)	97	1
GCM26	γ-*Proteobacteria*	*Rheinheimera baltica* (AJ441082)	96	1
GCM59	γ-*Proteobacteria*	*R. baltica* (AJ441082)	96	3
GCM10	γ-*Proteobacteria*	*Serratia fonticola* (AJ279002)	100	1
GCM7	γ-*Proteobacteria*	*Shewanella arctica* (AJ877256)	100	2

GCM Greenland collection of microorganisms[a]Sequence lengths were between 306 and 1,076 bases[b]Identical sequences were defined as sequences with 97% or more similarity

top, 42 from the middle, and 35 from the bottom section), and phylotypes representing 14 bacterial classes were identified. In addition, we identified phylotypes belonging to a group of unclassified *Bacteroidetes* bacteria, a group of unclassified *Firmicutes*, and to the order *Chroococcales* of the *Cyanobacteria* (Fig. 1).

Analysis of the distribution of phylotypes throughout the ikaite column showed that the bacterial classes were not evenly distributed in the top, middle, and bottom fractions of the column (Fig. 2). The top fraction contained eight identified classes of bacteria dominated by the α-*Proteobacteria* (50%), followed by the β-*Proteobacteria* (13%), the γ-*Proteobacteria* (10%), and the *Bacteroides* (10%). In the middle fraction, we found representatives from 12 of the 14 identified classes, the dominating class again being the α-*Proteobacteria* (30%), while the remaining of the identified bacterial classes each represented 2–10% of the total number of sequences in this fraction. The bottom fraction was dominated by cyanobacteria of the order *Chroococcales* (36%), followed by the α-*Proteobacteria* (25%), the β-*Proteobacteria* (17%), and the γ-*Proteobacteria* (8%).

A closer look into the sequences of the dominating class, the α-*Proteobacteria*, from top, middle, and bottom fractions revealed that 33 of 37 phylotypes affiliated to this class showed sequence similarity to species or phylotypes belonging to the *Rhodobacteraceae* family (genera: *Rhodobaca*, *Loktanella*, *Antarctobacter*).

We were not able to amplify archaeal 16S rRNA gene fragments from ikaite columns with the Archaea-specific primers used in this investigation.

Rarefaction analysis

Rarefaction analysis allowed us to estimate the number of distinct phylotypes in the ikaite column based on the 16S rRNA gene sequences from the clone library. In the analysis, we wanted to show if the estimate of species richness was affected by different definitions of unique phylotypes based on sequence identity between DNA sequences. We therefore made the analysis using four different definitions of distinct phylotypes: the 16S rRNA gene sequence having ≤ 96, ≤ 97, ≤ 98, or ≤ 99% identity to the closest relative in the clone library. We applied both ChaoI and Coleman rarefaction analyses for estimating species richness. Independent of the statistical method applied, the estimated number of species in the column was rather constant no matter what definition of phylotype we included in the analysis (Fig. 3). Coleman rarefaction estimated the total number of species to 79–90 [Standard error (SE): ±2], while ChaoI estimated a total of 208–227 species (95% confidence intervals: Lower: 79–95; Upper: 723–824).

Terminal-restriction fragment length polymorphism analysis

Terminal-restriction fragment length polymorphism analysis was performed on 16S rRNA gene fragments from three ikaite columns. MDS analysis revealed three

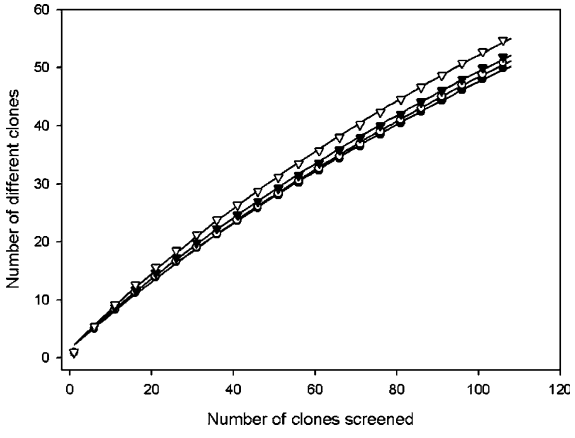

Fig. 3 Rarefaction curves estimating total species richness in the ikaite columns. Data are based on 109 clones. *Curves* represent non-linear fit to the logarithmic equation $y = y_o + a \ln(x\text{-}x_o)$ where x is the number of clones screened and y is the number of different clones ($r^2 = 0.9996$ for each fit). The four curves are based on four different definitions of phylotypes: ≤ 96% (*filled circle*), ≤ 97% (*open circle*), ≤ 98% (*filled triangle*), and ≤ 99% (*open triangle*) similarity to closest relative

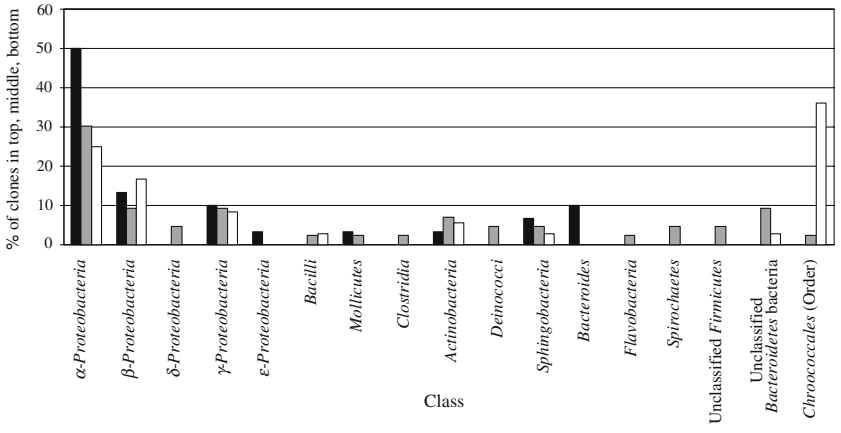

Fig. 2 Relative distribution of 16S rRNA gene sequences in top, middle, and bottom fractions of ikaite column C. Top (*black bars*), Middle (*gray bars*), and bottom (*white bars*)

distinct groups of samples when 16S rRNA gene fragments were digested with *Alu*I (Fig. 4) and *Rsa*I (data not shown). Each of these three groups represents each individual ikaite column used for the analysis. For statistical analysis of the T-RFLP data, we applied the ANOSIM two-way crossed analysis for analysis of differences between the three ikaite columns and between three locations in top, middle, and bottom within each column. Data from digestions with the restriction enzyme *Alu*I revealed distinct microbial communities in the three ikaite columns (Global R: 0.8; $P < 0.01\%$) and in the three locations within each column (Global R: 0.504; $P < 0.01\%$). Data from digestion with the enzyme *Rsa*I confirmed these results (Column: Global R: 0.694; $P < 0.01\%$, location within column: Global R: 0.493; $P < 0.01\%$).

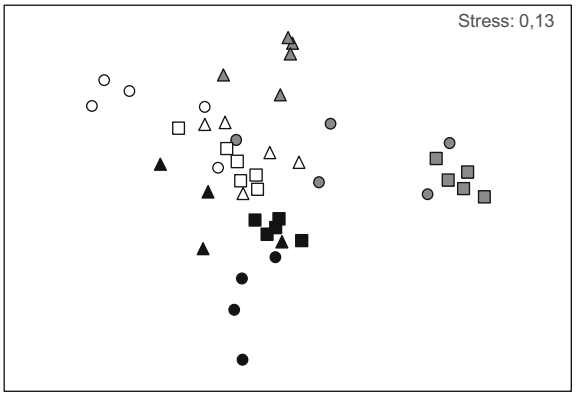

Fig. 4 MDS plot based on Bray–Curtis similarities of T-RFLP patterns from 16S rRNA gene fragments digested with *Alu*I. T-RFLP was performed on 16S rRNA gene fragments sampled from three locations within three individual ikaite columns. Top (*triangle*), middle (*square*), and bottom (*circle*). Column A (*black*), column B (*gray*), and column C (*white*)

Furthermore, we tested the data with an ANOSIM one-way analysis for differences between the individual samples. This test showed a significant difference between all nine sampling locations (*Alu*I: Global R: 0.716; $P < 0.01\%$, *Rsa*I: Global R: 0.675; $P < 0.01\%$).

The electropherograms in Fig. 5 show that most of the 16S rRNA gene fragments were represented in all samples from top, middle, and bottom fractions of the ikaite columns, which implies that the difference in community structure between the three locations is due to differences in peak intensities, i.e., the number of bacteria represented by each terminal fragment.

Identification of Terminal-restriction fragment length polymorphism peaks

Combining the sequence information from the 16S rRNA gene clone library and the data from the T-RFLP analysis, we were able to assign possible phylogenetic relationship to all major peaks in the T-RFLP electropherograms obtained with restriction enzyme *Alu*I (Fig. 5) and *Rsa*I (data not shown), even though each peak may represent several bacterial classes, and different phylotypes within one class can have different fragment patterns and therefore be represented by several different peaks. Hence, we believe that the clone library is representative of the actual biodiversity in the ikaite columns. The dominating classes from the clone library, the α-*Proteobacteria* and γ-*Proteobacteria*, were represented by different fragment lengths in the T-RFLP analysis (Fig. 5 and Table 3) and hence contain a variety of different species. Examples of phylotypes that can be identified in the T-RFLP peaks by in silico digestion of the 16S rRNA gene clone library sequences are phylotypes showing sequence similarity to *R. bogoriensis*, which can be identified in the 553–558 basepair (bp)

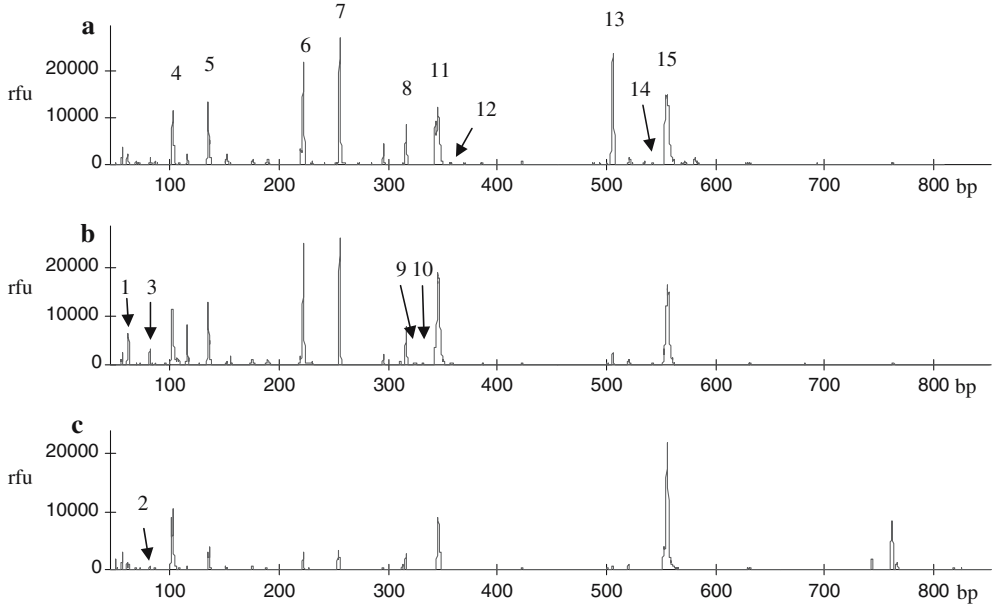

Fig. 5 Terminal-restriction fragment length polymorphism electropherograms showing the distribution of 16S rRNA gene terminal fragments in ikaite Column C, top (**a**), middle (**b**), and bottom (**c**) when digested with the restriction enzyme *Alu*I. Fragments identified from the 16S rRNA gene clone library have been numbered 1–15 (see Table 3). The electropherograms show 16S rRNA gene fragments in the size range from 40 to 850 bp. *rfu* relative fluorescence units

Table 3 Ikaite clone library phylotype sequences and their closest relatives inferred from 16S rRNA gene sequence similarity

Taxonomic group[a]	Class	Closest relative[b]	Similarity (%)	No. of types[c]
IC-T-9	*Actinobacteria*	*Arthrobacter psychrolactophilus* (AF134181)	98	1
IC-T-5	*Bacteroidetes*	*A. imshenetskii* (AJ784993)	94	3
IC-T-11	*Mollicutes*	*Mollicutes* bacterium pACH93 (AY297808)	93	1
IC-T-15	*Sphingobacteria*	*Flexibacter aggregans* (AB078038)	92	1
IC-T-12	*Sphingobacteria*	*Pedobacter piscium* (AJ438174)	96	1
IC-T-7	*α-Proteobacteria*	*L. vestfoldensis* (AJ582227)	98	8
IC-T-1	*α-Proteobacteria*	*Pelagibacter ubique* (AF510191)	99	1
IC-T-4	*α-Proteobacteria*	*R. bogoriensis* (AF248638)	97	23
IC-T-14	*β-Proteobacteria*	*A. tolulyticus* (L33694)	97	10
IC-T-13	*γ-Proteobacteria*	*Cycloclasticus spirillensus* (AY026915)	90	1
IC-T-17	*γ-Proteobacteria*	*M. alcalica* (AF384373)	97	1
IC-T-10	*γ-Proteobacteria*	*Saccharospirillum impatiens* (AJ315983)	96	1
IC-T-8	*ε-Proteobacteria*	Uncultured *ε-Proteobacterium* 49MY (AB091293)	98	1
IC-M-23	*Actinobacteria* (unclass.)	Uncultured *Actinobacterium* (AF468297)	99	1
IC-M-22	*Actinobacteria* (unclass.)	Uncultured *Actinobacterium* KY50 (AB116393)	93	1
IC-M-27	*Actinobacteria* (unclass.)	Uncultured *Actinobacterium* Nubeena304 (AY500119)	97	1
IC-M-21	*Bacilli*	*Bacillus bataviensis* (AY647284)	99	1
IC-M-10	*Bacteroidetes*	Uncultured *Bacteroidetes* bacterium ML635J-44 (AF507864)	92	2
IC-M-11a	*Bacteroidetes*	Uncultured *Bacteroidetes* bacterium ML635J-44 (AF507864)	92	1
IC-M-11b	*Bacteroidetes*	Uncultured *Bacteroidetes* bacterium ML635J-44 (AF507864)	95	1
IC-M-10	*Bacteroidetes*	Uncultured *Bacteroidetes* bacterium ML635J-44 (AF507864)	92	2
IC-M-11a	*Bacteroidetes*	Uncultured *Bacteroidetes* bacterium ML635J-44 (AF507864)	92	1
IC-M-11b	*Bacteroidetes*	Uncultured *Bacteroidetes* bacterium ML635J-44 (AF507864)	95	1
IC-M-10	*Bacteroidetes*	Uncultured *Bacteroidetes* bacterium ML635J-44 (AF507864)	92	2
IC-M-11a	*Bacteroidetes*	Uncultured *Bacteroidetes* bacterium ML635J-44 (AF507864)	92	1
IC-M-11b	*Bacteroidetes*	Uncultured *Bacteroidetes* bacterium ML635J-44 (AF507864)	95	1
IC-M-28	*Sphingobacteria*	Uncultured *Haliscomenobacter* sp. SBRT303 (AF368190)	94	2
IC-M-6	*Spirochaetes*	*Spirochaeta bajacaliforniensis* (AJ698859)	92	2
IC-M-13	*α-Proteobacteria*	*A. heliothermus* (Y11552)	95	1
IC-M-14	*α-Proteobacteria*	*A. heliothermus* (Y11552)	95	1
IC-M-26	*β-Proteobacteria*	*Matsuebacter chitosanotabidus* (AB006851)	99	1
IC-M-20	*γ-Proteobacteria*	*Lysobacter gummosus* (AB161361)	94	2
IC-M-25	*γ-Proteobacteria*	*M. buryatense* (AF096093)	97	2
IC-M-7	*γ-Proteobacteria*	*T. denitrificans* (AF126545)	98	1
IC-M-24	*γ-Proteobacteria*	Uncultured *γ-Proteobacterium* Nubeena229 (AY499950)	98	1
IC-M-19	*δ-Proteobacteria*	*D. succinoxidans* (X79415)	97	1
IC-M-18	*δ-Proteobacteria*	Uncultured *δ-Proteobacterium* AT-s3-66 (AY225609)	98	1
IC-B-15	*Actinobacteria*	Antarctic *Actinobacterium* BURTON-14 (AF142834)	98	1
IC-B-16	*Actinobacteria* (unclass.)	Uncultured *Actinobacterium* ML602J-44 (AF507852)	95	1
IC-B-8	*Bacilli*	*Staphylococcus lugdunensis* (AB009941)	99	1
IC-B-17	*Bacteroidetes*	Uncultured CFB group bacterium ML310M-18 (AF449772)	91	1
IC-B-7	*Cyanobacteria*	Antarctic bacterium CLEAR-10 (AF146234)	93	1
IC-B-3	*Cyanobacteria*	*Merismopedia glauca* (X94705)	99	12
IC-B-10	*Cyanobacteria*	*Synechococcus* sp. PS845 (AF448070)	99	1
IC-B-18	*Sphingobacteira*	*Aquiflexum balticum* (AJ744861)	96	1
IC-B-14	*α-Proteobacteria*	*Maricaulis maris* (AJ007804)	94	1
IC-B-19	*α-Proteobacteria*	Uncultured bacterium PENDANT-24 (AF142936)	75	1
IC-B-20	*α-Proteobacteria*	Uncultured bacterium PENDANT-24 (AF142936)	84	1
IC-B-12	*β-Proteobacteria*	*Burkholderia phytofirmans* (AY497470)	99	1
IC-B-5	*β-Proteobacteria*	*Janthinobacterium lividum* (AF174648)	99	2
IC-B-2	*γ-Proteobacteria*	*T. nitratireducens* (AY079010)	97	1

[a]Sequence lengths were 878–925 bases, except IC-T-17 that was only 597 bases long. IC-M identifies fragments from the top section, IC-M identifies fragments from the middle section, and IC-B identifies fragments from the bottom section of the ikaite column examined for this study[b]The closest cultured relative is given unless there was less than 90% sequence similarity to any cultured bacterium: then the closest related sequence from database is indicated instead[c]Identical sequences were defined as sequences with 97% or more similarity

peak, and phylotypes showing sequence similarity to *L. vestfoldensis*, which can be identified in the 501–508 bp.

In addition, the in silico digest of the cloned 16S rRNA gene sequences showed that the terminal restriction fragments from all 109 sequences could be identified in the T-RFLP electropherograms (Table 3).

Discussion

The environment inside the alkaline ikaite tufa columns in the Ikka Fjord in Greenland shows superficial similarity to the well-known soda lakes in Africa, Asia, and

North America in that they all have a pH value in the range 9–11. However, a number of differences exist between the ikaite columns and the African, Asian, and American soda lakes, the most obvious being salt concentration and temperature. In the soda lakes, the water temperature is often 30°C or higher, and the content of salt is high: in general around 5–8% (w/v), but as high as 35% in the Rift Valley soda lakes (Jones et al. 1998; Yakimov et al. 2001; Sorokin et al. 2003; Rees et al. 2004;). In contrast, the seep water in the interior of the ikaite columns has a salt concentration below 1% (w/v) and a temperature below 4°C (Buchardt et al. 1997).

The ikaite columns cannot be directly compared to the columns of the Lost City either, because of the differences in both physical and chemical conditions between the two environments (Buchardt et al. 2001; Boetius 2005; Kelley et al. 2001). Therefore, it is not unexpected that our investigation revealed a unique microbial biodiversity in the ikaite columns with unknown psychrophilic and alkaliphilic species.

Phylogenetic affiliation of ikaite bacteria

The ikaite columns are habitat for a specialized microbiota which, despite the low ambient temperature, shows some similarity to microorganisms from the warmer soda lakes in North America, Africa, and Asia (Tables 2, 4). Sixty-seven percent of the ikaite phylotypes and 67% of the cultured isolates showed 97% similarity or higher to sequences of known bacterial isolates, while the remaining may represent new species, based on 16S rRNA gene sequence information alone.

Several phylotypes and cultured ikaite bacteria were related to alkaliphilic species from other alkaline environments throughout the world (Tables 2, 4). Nine cultured isolates and 23 phylotypes were related to the alkaliphilic bacterium *R. bogoriensis* (95–99% sequence similarity), an isolate from the alkaline Lake Bogoria in the African Rift Valley (Milford et al. 2000). Furthermore, phylotype IC-T-17 was affiliated to *Methylophaga alcalica* from a Transbaikal soda lake with a 97% sequence similarity (Doronina et al. 2003), phylotype IC-M-7 shared 98% sequence similarity to *Thialkalivibrio denitrificans* (Sorokin et al. 2001), and phylotype IC-T-5 shared a 94% sequence similarity to its closest relative, *Alkaliflexus imshenetskii*, an alkaliphilic, carbohydrate-fermenting CFB group bacterium from the alkaline lake Verkhneye Beloye in Central Asia (Zhilina et al. 2004).

In addition to affiliation to alkaliphilic bacteria, several ikaite bacteria showed similarity to isolates and phylotypes from Arctic and Antarctic environments. The most abundant cultured isolate GCM53 and phylotype IC-T-7 showed 98% sequence similarity to *L. vestfoldensis*, which has been isolated from Lake Ace and Lake Pendant in the Vestfold Hills of Antarctica (Van Trappen et al. 2004). Furthermore, Tables 2 and 4 show that ikaite bacteria affiliated to Arctic and Antarctic bacteria generally exhibited a higher sequence similarity to known species and sequences in databases than the potential alkaliphilic ikaite isolates.

The primers used for sequence analysis of the 16S rRNA gene clone library yield fragments of about 900 bases (bases 515–1,412, *E. coli* numbering). This region contains some of the most conserved regions of the 16S rRNA gene fragment and therefore is expected to give the largest homology to known sequences (Stackebrandt and Goebel 1994). Thus, as we have used this region for comparison to gene sequences of known organisms in databases, the estimated number of novel species in ikaite columns (see below) is a rather conservative estimate.

Diversity and species richness

A variety of statistical approaches have been developed to estimate species richness from samples of macroorganisms, e.g., rarefaction analysis (Hurlbert 1971), bootstrap (Smith and vanBelle 1984), Chao1 (Chao 1984; Chao et al. 1993), and jackknife (Burnham and Overton 1979) estimators. Only recently, rarefaction (Bills and Polishook 1994; Moyer et al. 1998) and richness estimators (Kroes et al. 1999) have been adopted by microbiologists. We used Coleman rarefaction analysis and the non-parametric Chao1 estimate to predict the number of different genotypes or operational taxonomic units (OTUs) in the ikaite columns. The ChaoI estimator predicts the point at which an accumulation curve will reach an asymptote and provides projections of the actual diversity within a sampled environment based on the distribution of species, or OTUs, captured once or twice (Chao 1984; Chao et al. 1993). Insufficient sampling often hampers the use of rarefaction analysis and richness estimators in microbial ecology as many microbial habitats harbor exceedingly large numbers of species (Curtis et al. 2002). Also, curves from rarefaction and ChaoI analyses may not reach asymptotes and may change their shape with further sampling. Usually, richness of bacterial OTUs is based on sequencing of 16S RNA gene fragments but may also involve serial analysis of ribosomal sequence tags (Neufeld and Mohn 2005) or enzyme-coding genes specific to a functional group of bacteria (Priemé et al. 2002). The cold and alkaline ikaite columns offer harsh conditions for microorganisms and we expected that only a limited number of OTUs would be found in the columns. However, the number of sequenced clones was too small to obtain reliable estimates of richness in each of the three sections of the column, and we lumped the three clone libraries into one library containing clones from all three sections. From the shape of the rarefaction (Fig. 3) and ChaoI (not shown) curves, we trust that the total number of sequenced clones is sufficient to give a reliable estimate of bacterial OTU richness in the ikaite columns. The fairly large difference between the richness estimates obtained from the rarefaction and ChaoI analyses show the importance of performing more than one analysis

Table 4 Identification of bacterial classes represented in the T-RFLP electropherograms in Fig. 5 based on the 16S rRNA gene clone library

Fragment number	Fragment size (bp)	Bacterial classes within this fragment size identified from clone library	No. of clones
1	62–63	*Actinobacteria*	3
2	77–78	*Flavobacteria*	1
3	82–83	*Mollicutes, Spirochaetes, γ-Proteobacteria*	4
4	103–104	*Sphingobacteria, α-Proteobacteria, Gemmatimonadetes, Cyanobacteria, Bacteroides* (chloroplasts)	28
5	135–136	*γ-Proteobacteria*	2
6	220–225	*Deinococci*, unclassified *Bacteroidetes* bacteria	6
7	255–257	*Clostridia* (chloroplasts)	2
8	315–316	*γ-Proteobacteria*	1
9	326–328	*ε-Proteobacteria*	1
10	330–332	*γ-Proteobacteria*	1
11	346–349	*α-Proteobacteria, β-Proteobacteria, δ-Proteobacteria, γ-Proteobacteria, Actinobacteria, Bacilli*	23
12	353–354	*γ-Proteobacteria, Actinobacteria*	2
13	501–508	*α-Proteobacteria*	9
14	540–541	*Mollicutes*	1
15	553–558	*α-Proteobacteria, Sphingobacteria, Bacilli*	25
			Total: 109

when estimating microbial richness. We only have scant information about the distribution of microbial species in most habitats and hence little knowledge about the credibility of the different analyses. In our case, the SE associated with the richness estimate from the rarefaction curve is small, but one should be aware that the SE does not reflect the precision of the observed richness but merely reflects the error associated with reordering individual subsamples (Hughes et al. 2001).

Investigation of the 16S rRNA gene clone library showed that the ikaite columns contain highly diverse bacterial communities with 51 different phylotypes (sequences with less than 97% sequence similarity to known sequences in databases) representing at least seven different bacterial phyla from a single ikaite column. The estimated number of species, using ChaoI and Coleman rarefaction analyses, is between 79 and 227 different bacterial species. The comparison of isolates from ikaite with the isolates from the Ikka Fjord water samples showed that the majority of the ikaite bacteria are true ikaite bacteria in the sense that they are found at a much higher frequency in ikaite compared to the surrounding fjord water.

A total of 23% of the phylotypes showed less than 94% identity when compared to known sequences in databases and may therefore not only represent new species, but may even belong to new genera. The T-RFLP analysis showed that the three ikaite columns contained statistically different bacterial communities, but on combining the T-RFLP analysis with the information from the clone library, we found that the primary difference is not due to the presence of different species in each sampling location, but is caused by differences in the relative abundance of the species present in the ikaite columns. Therefore, the individual ikaite columns contain distinct microbial communities that are different from other known alkaline or polar environments. The communities consist of bacteria related to alkaliphilic, psychrophilic, and marine bacteria along with a variety of other known and unknown ecotypes such as nitrate reducers, sulfur reducers, and methanotrophic bacteria.

Microbial niches in ikaite columns

The seeping water inside the ikaite columns may provide several nutrients for bacterial metabolism, but due to the lack of investigations in this field, only a few measurements of chemical components of the ikaite water have been carried out so far. The ikaite water contains an enrichment of methane compared to atmospheric air (320 ppm), while the amount of SO_4^{2-} is low compared to the surrounding seawater (3–4 mmol l^{-1} vs. 26 mmol l^{-1}) (Buchardt et al. 2001). The concentrations of hydrogen, hydrogen sulfide, and N-compounds have unfortunately not been measured, but freshly sampled ikaite columns have the distinct smell of hydrogen sulfide.

Phylogenetic affiliation of 16S rRNA gene sequences from ikaite columns lead us to make tentative proposals for some of the nutrient cycles that may take place in the columns. Of bacteria with a known relationship to the N-cycle, we found phylotypes related to *Azoarcus tolulyticus* and *T. denitrificans* (Table 4) that are known denitrifiers, and to *T. nitratireducens* and *Antarctobacter heliothermus* that are known nitrate reducers. *T. denitrificans* is also capable of sulfur oxidation. Furthermore, we found phylotypes with sequence similarity to *Desulfuromusa succinoxidans*, which is a sulfur reducer that may contribute to the S-cycle along with *T. denitrificans*. We believe that at least sulfur reduction, sulfate reduction, or desulfurylation exists in the ikaite columns, as there is clear evidence of H_2S in the columns in form of a strong smell when they are collected. Also, phylogenetic relatives of *Methylomicrobium buryatense*,

a known methanotroph, and *M. alcalica*, a methylotrophic bacterium from an East Mongolian soda lake (Doronina et al. 2003), were identified among the ikaite phylotypes. Despite an effort using Archaea-specific primer sets, we were unable to obtain PCR products representing archaeal 16S rRNA gene fragments. Hence, we were unable to verify the occurrence of methanogenic organisms in the ikaite columns.

Growth of cultured bacteria

We have cultured a total of 284 bacterial isolates. The majority of these isolates were able to grow at pH 8–10, while 24 isolates could grow only on media buffered to pH 9 and 10. Irrespective of media, the majority of the isolated bacteria were cold-active (Table 1). Thus, the bacteria isolated from ikaite tufa columns seem to consist primarily of bacteria well adapted to the low temperature and alkaline environment in the interior of the columns, although some ikaite bacteria may have a marine origin and hence a preference for near-neutral pH.

Origin of ikaite bacteria

The origin of the ikaite bacteria is not known. The ikaite columns have a maximum age of 8,000 years because the area was covered with ice during the last ice age (Bjørn Buchardt, personal communication). This is too short a time for separate bacterial lineages to evolve, which means that either the alkaline springs have been active underneath the icecap and have supplied the newly formed ikaite columns with alkaliphilic microorganisms, or the ikaite bacteria have originated elsewhere and have been transported to the Ikka Fjord from other environments. The sequence similarity to bacteria from existing soda lakes and alkaline springs like the Lost City in the Atlantic Ocean may indicate that the alkaliphilic ikaite bacteria might have originated in these older, alkaline environments and may have been transported to the Ikka Fjord by sea water, air, migrating animals, or through crevices in the Earth's crust. In addition to alkaliphilic bacteria, ikaite tufa columns also harbor organisms, which show sequence similarity to known Arctic and Antarctic bacteria. These bacteria may have been transported by sea currents to the Ikka Fjord, but it is not known whether they are part of the alkaliphilic microbial community or if they form separate communities isolated from the alkaline water by, e.g., a protective biofilm (which is often observed during microscopic inspections of ikaite samples).

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