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ORIGINAL PAPER

Characterization of the prokaryotic diversity through a stratigraphic permafrost core profile from the Qinghai-Tibet Plateau

Weigang Hu[1] · Qi Zhang[1] · Tian Tian[1] · Dingyao Li[1] · Gang Cheng[1] · Jing Mu[1] · Qingbai Wu[2] · Fujun Niu[2] · Lizhe An[1] · Huyuan Feng[1]

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Abstract Permafrost on the Qinghai-Tibet Plateau is one of the most sensitive regions to climate warming, thus characterizing its microbial diversity and community composition may be important for understanding their potential responses to climate changes. Here, we investigated the prokaryotic diversity in a 10-m-long permafrost core from the Qinghai-Tibet Plateau by restriction fragment length polymorphism analysis targeting the 16S rRNA gene. We detected 191 and 17 bacterial and archaeal phylotypes representing 14 and 2 distinct phyla, respectively. Proteobacteria was the dominant bacterial phylum, while archaeal communities were characterized by a preponderance of Thaumarchaeota. Some of prokaryotic phylotypes were closely related to characterized species involved in carbon and nitrogen cycles, including nitrogen fixation, methane oxidation and nitrification. However, the majority of the phylotypes were only distantly related to known taxa at order or species level, suggesting the potential of novel diversity. Additionally, both bacterial α diversity and community composition changed significantly with sampling depth, where these communities mainly distributed according to core horizons. Arthrobacter-related phylotypes presented at high relative abundance in two active layer soils, while the deeper permafrost soils were dominated by *Psychrobacter*-related clones. Changes in bacterial community composition were correlated with most measured soil variables, such as carbon and nitrogen contents, pH, and conductivity.

Keywords Permafrost · Bacteria · Archaea · Prokaryotic diversity · Community structure · Qinghai-Tibet Plateau

Introduction

Permafrost (ground that has been continuously frozen for at least 2 years) represents a major component of the Earth's terrestrial cryosphere and is a key reservoir of belowground organic carbon (Tarnocai et al. 2009). Recently, there are significant scientific concerns about the impacts of climate warming on permafrost thaw and degradation of long-stored organic matter, potentially leading to the release of greenhouse gasses (primarily CO_2, CH_4 and N_2O), which further warm the atmosphere and accelerate permafrost thaw in a positive feedback cycle (Schuur et al. 2009, 2015; Koven et al. 2011). The transfer of carbon or nitrogen from permafrost system to the atmosphere is largely considered to be dependent on microbial activity, through processes such as respiration, fermentation, methanogenesis, methane oxidation, nitrification and assimilatory nitrate reduction (Mackelprang et al. 2011; Graham et al. 2012; Jansson and

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✉ Qingbai Wu
qbwu@lzb.ac.cn

✉ Huyuan Feng
fenghy@lzu.edu.cn

[1] Ministry of Education Key Laboratory of Cell Activities and Stress Adaptations, School of Life Sciences, Lanzhou University, Lanzhou 730000, China

[2] State Key Laboratory of Frozen Soil Engineering (SKLFSE), Cold and Arid Regions Environmental and Engineering Research Institute (CAREERI), Chinese Academy of Sciences, Lanzhou 730000, China

Taş 2014; McCalley et al. 2014; Hultman et al. 2015; Tveit et al. 2015). Therefore, characterizing the diversity and structure of microbial communities in this cold habitat is essential to our understanding of their functional roles and how they could respond and contribute to climate change and subsequently permafrost thaw.

Although permafrost environment is considered inhospitable, a high diversity of microorganisms, including bacteria, archaea, photoautotrophic cyanobacteria and green algae, fungi and protozoa, are present in it (Margesin and Miteva 2011). Furthermore, cultivation-dependent surveys in combination with 16S rRNA gene sequence data and 'omics' technologies have also revealed diverse microbial taxa associated with different functional guilds in permafrost, including acetoclastic methanogens, hydrogenotrophic methanogens, iron reducers, sulfate reducers, methanotrophs, ammonia oxidizers and denitrifiers (Gilichinsky et al. 2007; Liebner et al. 2009; Zhang et al. 2009; Mondav et al. 2014; Hultman et al. 2015). The permafrost microbial communities have been extensively investigated in the polar region (Cowan et al. 2014; Jansson and Taş 2014). Collectively, these studies have suggested that permafrost from different geographical locations could share a core set of microorganisms with a distinct relative abundance and composition (Jansson and Taş 2014). Unlike unfrozen soil and other habitats, permafrost is overlain by a soil horizon, called active layer, which experiences seasonal freeze–thaw cycles and suffers frequent environmental disturbances. Some previous findings have shown that microbial abundance, diversity, and community composition in the active layer and permafrost were significantly different (Steven et al. 2008; Waldrop et al. 2010; Yergeau et al. 2010; Mackelprang et al. 2011; Taş et al. 2014; Hultman et al. 2015). Several reports have demonstrated the relationship between permafrost microbial communities and a number of abiotic factors. For example, microbial communities are correlated with elevation, latitude, vegetation type, UV radiation, profile depth, nutrient availability, electrical conductivity, soluble salts, pH, moisture, etc. (Tosi et al. 2004; Wallenstein et al. 2007; Yergeau et al. 2009; Zhang et al. 2009, 2014; Chu et al. 2010; Stomeo et al. 2012; Koyama et al. 2014; Shen et al. 2015).

Although microbial communities have long been studied in high-latitude permafrost, meticulous study of those in low-middle latitude, high-altitude permafrost in China has emerged only in the past decade (Hu et al. 2015). The altitudinal permafrost in China covers an area of approximately 1.35×10^6 km^2, which ranks first in the world, and is mainly distributed on the Qinghai-Tibet Plateau (Ran et al. 2012). The Qinghai-Tibet Plateau, with an average elevation of over 4 km above sea level, has long been known as the 'roof of the world' and the 'third pole' (Qiu 2008). It is characterized by unique and complex topography, climate, and history, being a consequence of land uplift on the order of 3 km over the last 2 million years (Wang and French 1995). Due to relatively young geological feature and steep geothermal gradient, permafrost on the Qinghai-Tibet Plateau is thin and warm in comparison with the cold permafrost at high latitudes. The region also experiences a high solar radiation load, rendering altitudinal permafrost more sensitive to surface disturbance and climate warming (Yang et al. 2010). Recent measurements indicated that the regional climate exhibited a tendency toward significant warming over the last 50 years with the air temperature increased by 0.3 °C a decade—approximately three times the global warming rate (Qiu 2008). If regional warming continues, one-third to one-half of the permafrost on the Qinghai-Tibet Plateau is anticipated to degrade by 2100 (Li et al. 2008). Considering the crucial role of microbial processes in the event of climate change, therefore, it is of great importance and urgency to understand the microbial ecology of this unique permafrost region.

In general, Bacteria, Archaea and Fungi are the most pivotal actors interacting in the terrestrial ecosystems, and their functional roles and major determinants might differ. Although many Fungi and Bacteria compete for the same organic material in soil (Rousk et al. 2008), Fungi could utilize complex substrates that are unavailable for most bacteria and then release labile substrates to support other microbial communities (Coolen et al. 2011). In addition, several studies have found that prokaryotes are more closely related to soil characteristics than Fungi; however, the fungal communities being more influenced by plants (Zinger et al. 2011). Previously, we characterized the fungal diversity and community composition in a 10-m-long soil core taken within permafrost of the Qinghai-Tibet Plateau (Hu et al. 2014). The objectives of this research are to describe the prokaryotic diversity in the same permafrost core as Hu et al. (2014) and to determine how this diversity varies with sampling depth.

Materials and methods

Study site and sampling

The study site is located in the Kunlun Mountain Pass (35°39′28.6″N, 94°03′17.3″E), Qinghai-Tibet Plateau, which is a typical plateau permafrost region. Three replicate soil subsamples (~50 g each) were obtained from each of sixteen depth intervals within a 10-m-long permafrost core in August 2010. The permafrost core was collected by a rotary coring device without the use of drilling fluid. To prevent external contamination, the sampling process was performed using the previously described method (Hu et al. 2014). The collected soil samples were sealed in autoclaved

aluminum tins, and returned frozen to the laboratory. Overall, a total of forty-eight soil subsamples were collected for this study. These samples were stored at −20 °C until further processing.

Soil physical and chemical analyses

Soil physical and chemical analyses were carried out as previously described (Hu et al. 2014). Soil organic carbon and total nitrogen were determined in air-dried soil by combustion at 450 and 1250 °C, respectively, with the CHNS-analyzer system (Elementar Vario EL, Elementar Analysensysteme GmbH, Hanau, Germany). Soil pH and conductivity were measured in 1:5 soil/KCl solutions and 1:2 soil/deionized water slurry, respectively. Soil moisture was based on the differences in mass of fresh soil following drying at 105 °C for 24 h.

Community DNA extraction and 16S rRNA gene amplification

Total community DNA was isolated from each soil subsample using the modified method described in previous study (Hu et al. 2014). The DNA extractions from three replicates at each sampling depth were pooled to yield enough DNA for PCR amplification, which ultimately resulted in sixteen pooled DNA samples. The crude community DNA was then purified using the Universal DNA Purification Kit (Tiangen Biotech, China) according to the manufacturer's recommended protocol. Negative controls (deionized H_2O in place of DNA or soil) underwent identical handling during the extraction procedure and purification steps to ensure zero contamination in downstream analyses.

The primer pair 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′) was used for amplification of bacterial 16S rRNA genes for clone library production: thermocycling conditions consisted of 5 min at 94 °C and then subjected to 35 amplification cycles of 1 min denaturation at 94 °C, 1 min annealing at 58 °C, followed by 72 °C for 1 min 30 s and a final extension of 72 °C for 10 min. Archaeal 16S rRNA genes were amplified using the primer pair 4F (5′-TCCGGTTGATCCTGCCRG-3′) and 958R (5′-YCCGGCGTTGAMTCCAATT-3′): thermal conditions were composed of an initial denaturation of 4 min at 95 °C, followed by 35 cycles at 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min; with a final extension at 72 °C for 10 min. Polymerase chain reaction reagents and their final concentrations in a 25-μL reaction mixture were as follows: 1 U of *Taq* polymerase (New England Biolabs, USA), 1 × PCR buffer, 2 mM $MgCl_2$, 0.3 μL of 10 mg/mL bovine serum albumin, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 0.5 μM of each primer, and 3 μL of template DNA. To minimize PCR drifting, all samples were amplified in triplicate and combined together. All PCRs were performed using the Applied Biosystems® 2700 Thermal Cycler. To ensure correct amplicon length, the PCR product was evaluated on 1.0 % agarose gel with ethidium bromide staining.

Construction of 16S rRNA gene clone libraries and restriction fragment length polymorphism (RFLP) analysis

DNA sufficient for producing bacterial 16S rRNA gene clone library was extracted from all of the samples but amplification of archaea was only successful from 6 of the 16 pooled DNA samples. Thus, a total of twenty-two clone libraries were produced to analyze the prokaryotic communities in this study. Amplified 16S rRNA genes were purified using the TIANgel Midi Purification Kit (Tiangen Biotech, China). The cleaned PCR products of ~1465 bp for Bacteria and ~954 bp for Archaea were cloned into pGEM-T vector system as described by the manufacturer (Promega, Madison, USA) and then transformed into *Escherichia coli* strain DH5α. 250 and 450 putative positive transformants were picked randomly from each archaeal and bacterial clone library, respectively, and plasmid templates were prepared as previously described (Hu et al. 2014). Cloned inserts were re-amplified with the pGEM-T-targeted primer pair T7/SP6 using the same PCR conditions as amplification of bacterial community DNA, with the exception that only 30 cycles were performed.

Restriction fragment length polymorphism (RFLP) analysis was used to group cloned 16S rRNA gene sequences. Bacterial and archaeal PCR fragments were digested using the restriction endonucleases *Hin*fI/*Csp*6 (MBI Fermentas, Vilnius, Lithuania) and *Msp*I/*Hha*I (MBI Fermentas, Vilnius, Lithuania), respectively, at 37 °C for 3.5 h. Sequences with identical RFLP patterns were visually regrouped, and representatives of each RFLP pattern were chosen for sequencing. Sequencing was undertaken using vector primer pair T7 and SP6 by the Major Biotech Co., Ltd (Shanghai, China).

Phylogenetic analyses

All DNA sequences were edited and checked for chimeras using the CHIMERA CHECK program on the RDP II database (http://rdp.cme.msu.edu/index.jsp). Identified chimeric sequences were eliminated from future analyses, ultimately resulting in a total of 373 bacterial sequences and 71 archaeal sequences. The remaining sequences were then aligned using CLUSTAL W (Thompson et al. 1994) and assigned to species-level groups according to 97 % pairwise identity using the furthest neighbor algorithm in the MOTHUR program (Schloss et al. 2009).

Each species-level group was regarded as a prokaryotic phylotype. Representative sequences from each phylotype were submitted for comparison to the GenBank database using the BLAST (Altschul et al. 1990) to determine their closest relatives. Phylogenetic classification was inferred by submitting the sequences to the EzTaxone online database (Kim et al. 2012). The coverage of the clone libraries was estimated using the following formula: $C\ (\%) = (1 - n/N) \times 100$, where n is the number of sequences represented by a single clone and N is the total number of sequences analyzed. The Chao1 estimator of phylotype richness and Shannon–Wiener index (H') of species diversity were calculated with EstimateS version 9 (http://viceroy.eeb.uconn.edu/estimateS).

To identify novel orders, neighbor-joining trees were constructed with the Jukes–Cantor distance model using the MEGA 5.0 (Tamura et al. 2011) program and the reliability of the tree branch points was assessed by bootstrap analysis of 1000 replicates. Novel orders were defined as a bootstrap-supported (>50 %) group of at least two clone sequences sharing >90 % sequence similarity with each other but <90 % sequence similarity to sequences from a recognized order (Spain et al. 2009).

Habitat affiliations of prokaryotic phylotypes were estimated using a nearest-neighbor analysis described by Souza et al. (2006). Briefly, representative sequences from each phylotype were compared with the most closely related reference sequences in databases, and then the most frequent environmental affiliation in the first five nearest neighbors was assigned to each phylotype.

Statistical analyses

Microbial community composition was estimated from the clone numbers of each phylotype in a soil sample. Soil physicochemical variables were calculated on the basis of the measures on three replicates in each sampling depth. Before analysis, all data were tested for normality and verified homogeneity of variance using Levene's test. Raw community data for bacteria were Hellinger-transformed to down-weight the importance of abundant phylotypes.

Analysis of similarity (ANOSIM) with 999 permutations and non-metric multidimensional scaling (NMDS) were performed to compare microbial community structure. Furthermore, to elucidate the correlations between microbial communities and environmental variables, depth and all measured soil variables were fitted as vectors onto the NMDS plot using the function 'envfit' from the 'vegan' (Oksanen 2013) library of the R package (version 3.0.2; http://www.r-project.org).

The phylotypes present at the highest relative abundance were extracted and also fitted as vectors onto the NMDS plot of community dissimilarities. Phylotypes in which the goodness of fit statistic (r^2) was >0.5 were displayed in NMDS analysis (Lin et al. 2012). Vector length and direction represent the strength and sign of the relationship between phylotypes and the NMDS axes, respectively. It is important to note, however, that the significant vector based on fitting analysis should be only considered as an exploratory tool rather than causation of community dissimilarities (Lin et al. 2012).

Nucleotide accession numbers

GenBank accession numbers for the nucleotide sequences deposited in this study are as follows: Archaea clone library, KC505253-KC505323; Bacteria clone library, KF494429-KF494801.

Results

Physical and chemical properties of soil

The annual mean air temperature and precipitation at Kunlun Mountain Pass are −5.0 to −7.0 °C and 400 mm, respectively. The site is approximately 4780 m above sea level, and dominated by alpine meadow consisting primarily of *Oxytropis glacialis*, *Stipa purpurea* and *Kobresia pygmaea*. At the time of sampling, the depth of the active layer was about 2.6 m. The sampling depth and physicochemical characteristics of soil samples are given in Table 1. The soil was characterized by relatively low organic carbon [0.40 to 0.82 % (dry weight)] and total nitrogen content [0.027–0.067 % (dry weight)], which were in the range described for Antarctic permafrost (Vorobyova et al. 1997; Gilichinsky et al. 2007) but were lower than in Siberian, Canadian, and Spitsbergen permafrost (Steven et al. 2006, 2008; Hansen et al. 2007). The alkaline soils with pH ranging from 7.95 to 8.95 were similar to Antarctic permafrost (Steven et al. 2006), which may be attributed to soil salts being largely chlorides and sulfate (Campbell and Claridge 2009). Furthermore, Pearson correlation analysis suggested that soil moisture and conductivity increase significantly ($P \leq 0.003$ for both) with sampling depth. No detectable correlations were observed between the other variables and depth.

Diversity and phylogenetic composition of bacterial clone libraries

After chimera editing, bacterial clone libraries constructed from 16 community DNA samples consisted of 6506 clones and comprised 373 near full-length bacterial 16S rRNA gene sequences. These sequences clustering at the 3 % difference level resulted in 191 phylotypes of which

Table 1 Physical and chemical properties of different-depth soil samples collected from the Qinghai-Tibet Plateau

Sample	Depth (m)	Conductivity (μS/cm)[a]	Moisture (%)[a]	pH[a]	Organic C (%)[a]	Total N (%)[a]	C/N ratio[a]
KLM50	0.50	147.0 ± 2.5	4.3 ± 0.1	8.72 ± 0.02	0.48 ± 0.05	0.027 ± 0.001	18.4 ± 2.3
KLM100	1.00	171.0 ± 6.6	5.0 ± 0.2	8.76 ± 0.01	0.50 ± 0.01	0.037 ± 0.003	13.6 ± 1.0
KLM150	1.50	376.7 ± 11.8	13.4 ± 0.1	8.77 ± 0.01	0.55 ± 0.03	0.042 ± 0.003	13.2 ± 0.2
KLM175	1.75	290.7 ± 4.1	12.1 ± 0.4	8.39 ± 0.03	0.40 ± 0.01	0.041 ± 0.001	9.7 ± 0.4
KLM200	2.00	249.7 ± 2.9	14.7 ± 0.4	8.18 ± 0.04	0.82 ± 0.01	0.067 ± 0.001	12.3 ± 0.4
KLM225	2.25	160.3 ± 5.2	4.8 ± 0.4	8.83 ± 0.04	0.47 ± 0.02	0.032 ± 0.002	14.7 ± 0.9
KLM250	2.50	285.7 ± 25.5	14.0 ± 0.3	8.81 ± 0.03	0.43 ± 0.03	0.033 ± 0.002	13.2 ± 0.8
KLM275	2.75	251.3 ± 16.2	14.8 ± 0.6	8.92 ± 0.03	0.45 ± 0.02	0.035 ± 0.003	13.1 ± 1.6
KLM300	3.00	261.0 ± 12.0	18.5 ± 0.4	8.66 ± 0.03	0.40 ± 0.04	0.034 ± 0.003	12.1 ± 1.2
KLM400	4.00	349.7 ± 12.7	14.4 ± 0.5	8.85 ± 0.02	0.47 ± 0.01	0.038 ± 0.001	12.5 ± 0.5
KLM500	5.00	568.7 ± 8.4	16.9 ± 1.4	8.90 ± 0.03	0.40 ± 0.02	0.039 ± 0.001	10.4 ± 0.3
KLM600	6.00	396.3 ± 14.8	17.6 ± 0.2	8.86 ± 0.02	0.45 ± 0.01	0.036 ± 0.004	12.9 ± 1.4
KLM700	7.00	515.7 ± 4.8	13.4 ± 0.1	8.91 ± 0.04	0.41 ± 0.02	0.034 ± 0.003	12.3 ± 0.7
KLM775	7.75	464.7 ± 7.2	17.6 ± 0.3	8.95 ± 0.03	0.49 ± 0.04	0.037 ± 0.001	13.5 ± 1.6
KLM900	9.00	666.7 ± 26.7	19.5 ± 0.8	8.84 ± 0.01	0.43 ± 0.02	0.035 ± 0.003	12.3 ± 0.8
KLM975	9.75	2446.7 ± 8.8	27.7 ± 0.9	7.95 ± 0.03	0.79 ± 0.01	0.062 ± 0.001	12.8 ± 0.2

[a] Values represent means ± the standard error ($n = 3$)

139 were represented by a single sequence and 52 by clusters of similar sequences. The numbers of clones and phylotypes in the different-depth and combined clone libraries are presented in Table 2. Observed phylotype richness varied largely among samples. The diversity of bacterial clone libraries was characterized by Chao1-richness estimator and Shannon–Wiener index (Table 2), which varied significantly with sampling depth. Spearman correlation analyses suggested that depth was highly negatively correlated with Chao1 estimator ($\rho = -0.855$, $P < 0.001$) and Shannon–Wiener index ($\rho = -0.648$, $P = 0.007$). Analysis of rarefaction curve (Fig. S1) combined with the estimates of library coverage (Table 2) showed that ~71 % of the bacterial diversity was described in the soil samples, indicating that the sequences analyzed in our study represented a significant proportion of total population.

The bacterial phylotypes could be divided into 14 phyla, including 1 unclassified phylum (Fig. 1). Proteobacteria was the most abundant (72 % of the total clones) and heterogeneous (44 % of the total phylotypes) phylum in the libraries. Proteobacteria-related sequences were distributed among the Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Deltaproteobacteria, with the class Gammaproteobacteria being most abundant (87 % of the proteobacterial clones). The Gammaproteobacteria dominance was largely attributed to the high proportion of phylotype KL_B6-130 (Fig. S2), which alone represented 68 % of Proteobacteria clones. The Alphaproteobacteria showed the highest diversity among the Proteobacteria, comprising 46 phylotypes (Fig. 1); several phylotypes were closely related (98–99 % sequence similarity) to nitrogen-fixing rhizobial symbionts that belonged to the genera Rhizobium, Phyllobacterium, Mesorhizobium, Devosia and Bradyrhizobium, within the order of Rhizobiales (Fig. S2). In addition, two phylotypes (KL_B24-13 and KL_B24-381) were highly related (>99 % sequence similarity for both) to the facultatively methanotrophs *Methylobacterium bullatum* and *Methylobacterium podarium* (Fig. S2). The *Betaproteobacteria* were represented by 17 phylotypes; one phylotype (KL_B11-44) shared 99 % sequence identity with the characterized species *Thiobacillus thiophilus*, an obligately chemolithotrophic, facultatively anaerobic, thiosulfate-oxidizing bacterium with an ability to couple the oxidation of thiosulfate to the reduction of nitrate (Fig. S2; Kellermann and Griebler 2009). The Deltaproteobacteria showed the lowest diversity and abundance among the Proteobacteria, consisting of only four phylotypes (Figs. 1, S2).

Actinobacteria was also dominated in the libraries and represented 19 % of the total clones and 24 % of the total phylotypes (Fig. 1). Arthrobacter-affiliated clones were the most abundant among the Actinobacteria (Fig. S3), making up 78 % of the Actinobacteria-related clones. Of the 16 phylotypes that represented the phylum Firmicutes, some were classified to the endospore-forming genera *Paenibacillus*, *Cohnella*, *Bacillus*, *Oceanobacillus* and *Paenisporosarcina* within the order *Bacillales*; the phylotype KL_B4-39 was 99 % identical to *Paenisporosarcina macmurdoensis*, a psychrophilic bacterium isolated from an Antarctic cyanobacterial mat sample (Fig. S3; Reddy et al. 2003). Furthermore, phylotypes grouping within the phyla Acidobacteria, Bacteroidetes, Gemmatimonadetes,

Table 2 Prokaryotic clone library statistics and diversity estimates (based on phylotypes defined at 97 % similarity)

Sample	Clones[a]	Phylotypes richness	Chao1[b]	H'[c]	Coverage (%)
Bacteria clone library					
KLM50	721	28	34	1.4	64.3
KLM100	423	57	76	3.0	61.4
KLM150	384	72	106	3.4	58.3
KLM175	369	19	22	1.0	68.4
KLM200	335	104	152	4.1	58.7
KLM225	436	21	22	1.8	81.0
KLM250	385	20	27	1.2	60.0
KLM275	347	22	26	1.4	68.2
KLM300	376	13	16	0.9	61.5
KLM400	378	19	22	1.4	73.7
KLM500	438	12	15	1.1	58.3
KLM600	437	16	19	0.7	68.8
KLM700	377	10	11	0.7	70.0
KLM775	429	7	7	0.2	85.7
KLM900	384	14	17	1.5	71.4
KLM975	287	7	8	0.3	57.1
Combined	6506	191	250	2.5	70.7
Archaea clone library					
KLM50	249	7	7	1.3	85.7
KLM100	222	7	7	0.6	85.7
KLM150	248	11	12	1.3	81.8
KLM175	224	13	15	1.7	76.9
KLM200	238	8	8	0.8	87.5
KLM250	222	7	7	0.8	85.7
Combined	1403	17	20	1.4	76.5

[a] Number of clones retained after chimera editing

[b] Chao1-richness estimator

[c] Shannon–Wiener index

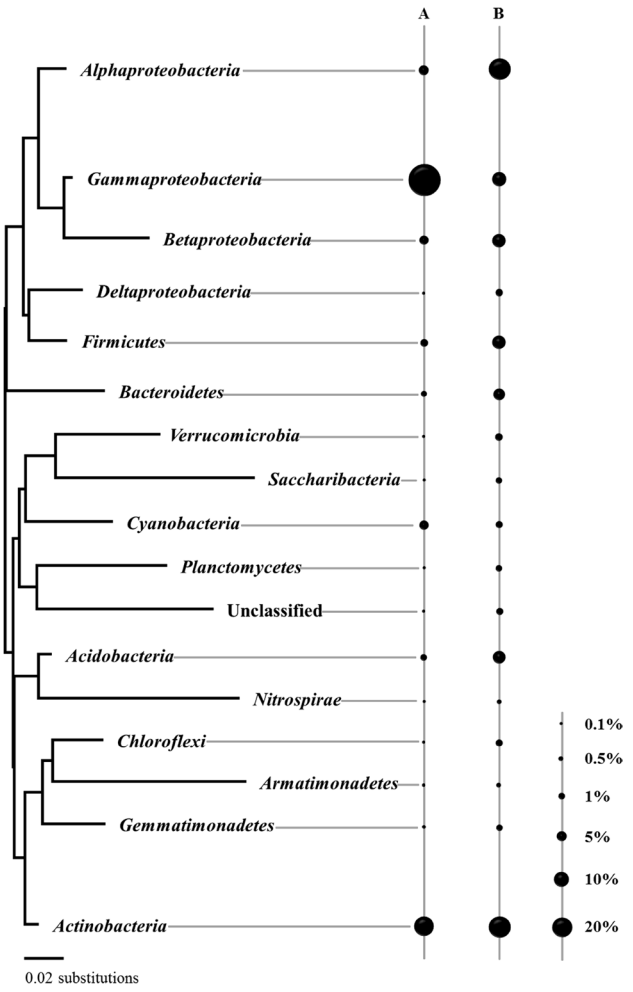


Fig. 1 Bacterial neighbor-joining tree and relative abundance (**A**) and proportion of phylotypes (**B**) of phylum or class-level clades. The *scale* represents the number of mutations per nucleotide position. The relative abundance and proportion of phylotypes of a division are proportional to the circular area, as illustrated by the *scale on the right*

Planctomycetes, Verrucomicrobia, Cyanobacteria, Saccharibacteria (former candidate division TM7), Armatimonadetes (former candidate division OP10), Chloroflexi and Nitrospirae were also detected in the libraries but represented low abundance with clone frequencies of less than 5 % (Figs. 1, S4).

Diversity and phylogenetic composition of archaeal clone libraries

Seventy-one non-chimeric sequences were obtained by RFLP analysis of 1403 archaeal clones from six active layer samples and grouped into 17 phylotypes at 97 % similarity level, representing an estimated coverage of approximately 77 % of the combined clone library (Table 2). The Chao1 estimator, *H'* diversity index (Table 2) and rarefaction curve (Fig. S1) demonstrated that the archaeal communities were less diverse than the bacterial communities. Phylogenetic grouping of the archaeal phylotypes revealed that they were assigned to two phyla, Thaumarchaeota and Euryarchaeota (Fig. 2). The Thaumarchaeota accounted for an overwhelming majority of the archaeal clones (>99 %) and consisted of 16 phylotypes, with the phylotype KL_AR6-132 being most abundant (59 % of the thaumarchaeotal clones). Thaumarchaeota phylotypes fell into two groups: ″Candidatus *Nitrosopumilales*″ of Group I.1a and *Nitrososphaerales* of Group I.1b. The single phylotype (KL_AR7-226) assigned to Group I.1a was closely related (99 % rRNA gene sequence identity) to the ammonia-oxidizing archaeon ″Candidatus *Nitrosoarchaeum limnia*″ (Blainey et al. 2011). The remaining Group I.1b

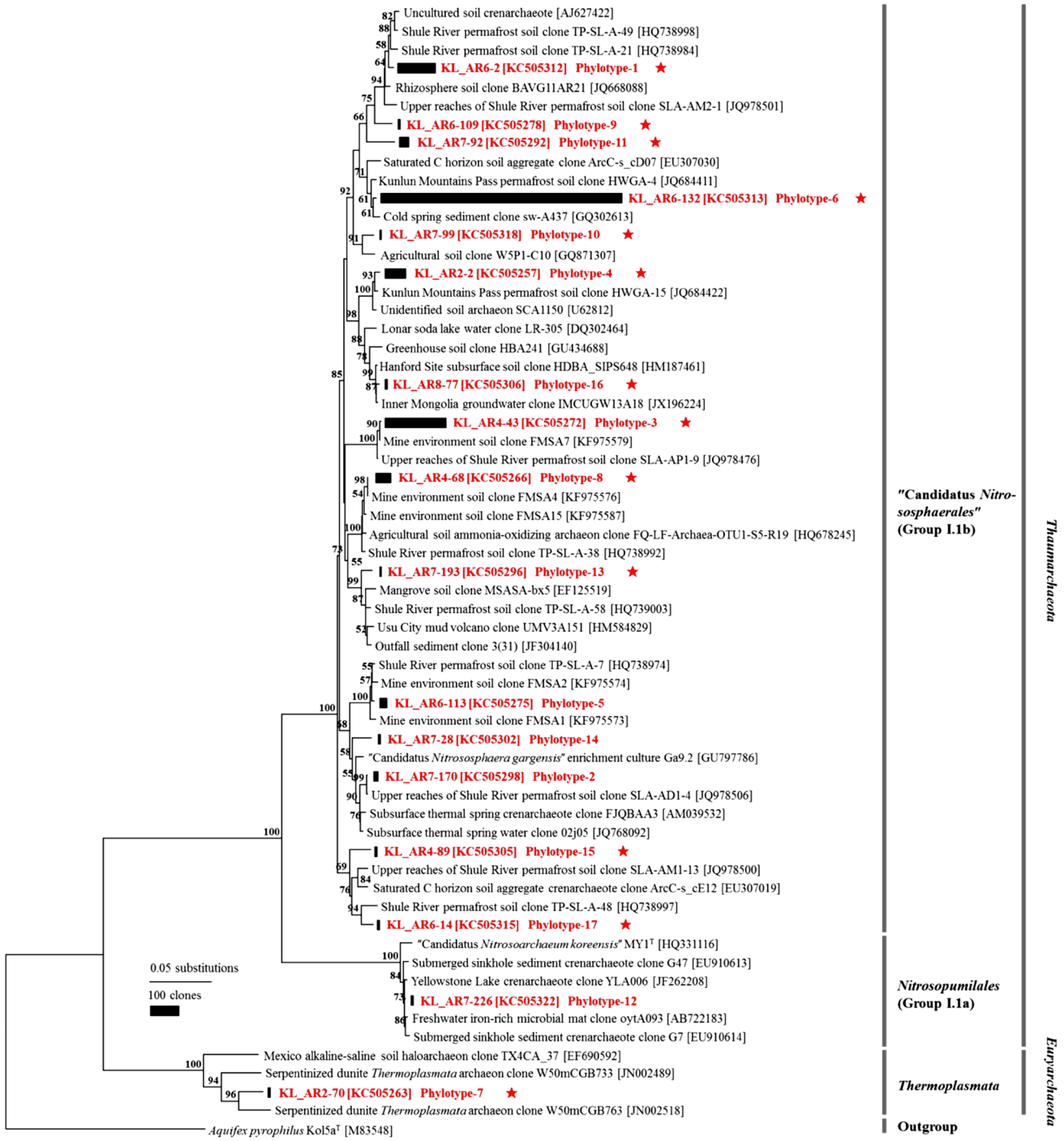


Fig. 2 Phylogenetic analysis of representative 16S rRNA gene sequences of archaeal phylotypes. Sequences sharing >97 % sequence identity to each other were grouped as a phylotype. Phylogenetic relationships were constructed by neighbor-joining analysis using the Jukes–Cantor model, and rooted to the outgroup *Aquifex pyrophilus* strain Kol5A^T (M83548). Bootstrap values above 50 % are shown as a percentage of 1000 replicates. Sequences obtained in this study were highlighted. The *scale* represents the number of mutations per nucleotide position. Frequency of clones was designated as lengths of *black squares on the left side* of phylotype names. New sequence types are marked with *stars*

phylotypes were all related (93–98 % sequence similarity) to the ammonia-oxidizing archaea "Candidatus *Nitrososphaera gargensis*" (Hatzenpichler et al. 2008) and *N. viennensis* (Stieglmeier et al. 2014). The Euryarchaeota was composed of only a single phylotype (KL_AR2-70), which clustered with Thermoplasmata-related

Table 3 Novel (uncharacterized) orders and phylotypes in prokaryotic divisions

	Total phylotypes	Total orders	Novel orders	Novel phylotypes	Phylotypes affiliated with cold habitats
Bacterial divisions					
Alphaproteobacteria	46	6	1	21	11
Betaproteobacteria	16	4	0	4	7
Gammaproteobacteria	18	3	0	7	8
Deltaproteobacteria	4	2	1	3	0
Firmicutes	16	3	0	5	3
Bacteroidetes	11	3	0	9	4
Verrucomicrobia	4	3	1	3	0
Saccharibacteria	2	1	1	2	0
Cyanobacteria	3	3	1	2	0
Planctomycetes	2	2	1	2	0
Acidobacteria	13	6	4	13	7
Chloroflexi	3	3	3	3	0
Armatimonadetes	1	1	1	1	0
Gemmatimonadetes	2	2	1	2	1
Actinobacteria	46	7	2	27	15
Nitrospirae	1	1	0	1	0
Unclassified	3	2	2	3	1
Total	191	52	19	108	57
Archaeal divisions					
Thaumarchaeota	16	2	0	12	10
Euryarchaeota	1	1	1	1	1
Total	17	3	1	13	11

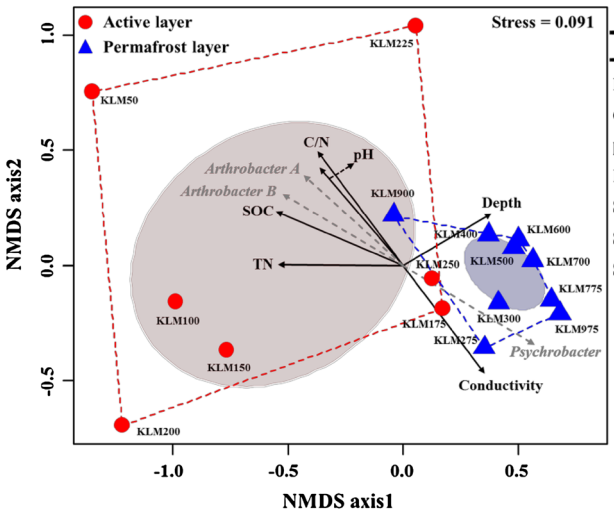
environmental clones but was only distantly related (<96 %) to any 16S rRNA gene sequences in the public databases (Fig. 2).

Novel phylogenetic taxa in prokaryotic divisions

Phylotypes affiliated with known phyla may still represent novel taxa. Previously uncharacterized phylogenetic orders (defined at 90 % sequence similarity) and phylotypes (defined at 97 % sequence similarity) were determined by constructing phylogenetic trees with the bootstrap analysis for each bacterial and archaeal division (Figs. 2, S2–S4). A total of 52 orders were identified within the 17 bacterial divisions, among which 37 % (19) of the identified orders were novel (Table 3). Using the same criteria, one novel order was identified in the archaeal divisions (Table 3). Quite unexpectedly, the vast majority of phylotypes (57 % for Bacteria and 76 % for Archaea) in these divisions had less than 97 % sequence similarity to their closest cultivated representatives in the public database, suggesting the possibility of novel species (Table 3; Figs. 2; S2–S4). Of particular interest, all of the 13 phylotypes of *Acidobacteria* represented unrecognized taxa (Table 3; Fig. S4). Moreover, a larger number of prokaryotic phylotypes were most closely affiliated with environmental sequences detected in cold habitats (Table 3), including cold soils, permafrost, ice, snow, deep sea, marine sediments, polar and alpine lakes, and cold spring.

Vertical distribution of bacterial communities

To determine whether there were differences in bacterial community composition among samples or between core horizons (active layer and permafrost layer), a NMDS analysis based on the Bray–Curtis dissimilarity metric was performed. The samples collected from active layer showed greater variability in bacterial community composition, which was indicated by the fact that the paired communities were more scattered compared with the samples collected from permafrost (Fig. 3). Compositional differences were also observed in different soil horizons of the permafrost core. Two uppermost active layer samples (KLM50 and KLM100) were dominated by actinobacterial phylotypes (*Arthrobacter* spp.). However, *Psychrobacter*-like phylotype was present at high relative abundance (63–96 %) in permafrost soils, except for the sample (KLM900) from 9.0 m (Fig. 3). Additionally, the first axis in NMDS plot reflected the changes in bacterial communities with sampling depth ($r^2 = 0.369$, $P = 0.043$), where these communities revealed significantly vertical distribution according



Variables	r^2	*P*-value
Depth	0.369	0.043*
Conductivity	0.648	0.002**
pH	0.584	0.004**
Moisture	0.163	0.328
Soil organic C (SOC)	0.681	0.001***
Soil total N (TN)	0.549	0.008**
Soil C/N ratio (C/N)	0.721	0.001***

Fig. 3 Non-metric multidimensional scaling (NMDS) analysis of community dissimilarity using Bray–Curtis metric. Environmental variables (*solid arrows*) were fitted as vectors onto the NMDS ordination plot, and significant vectors (r^2 values and *P* values are shown in the *right panels*) are depicted. For highly abundant phylotypes (*dashed arrows*), vectors representing their goodness of fit >0.5 with first two NMDS axes are indicated. Appended *A* and *B* designations connote distinct phylotypes (<97 % sequence similarity) within a genus level. *Ellipses* with *different colors* indicate 95 % confidence ellipses for each core horizon (active layer or permafrost layer)

to core horizons (Fig. 3). This pattern was confirmed by a significant ANOSIM value ($r = 0.470$, $P < 0.001$) between the two layers. The fitting analysis showed that changes in taxonomic composition of bacterial communities were significantly correlated with the majority of our measured soil variables, among which soil C/N ratio ($r^2 = 0.721$, $P < 0.001$) was the most important explanatory variable, followed by soil organic carbon ($r^2 = 0.681$, $P < 0.001$).

Discussion

Altitudinal permafrost on the Qinghai-Tibet Plateau represents a unique and fragile ecosystem. As 'a community of survivors', microorganisms play a potentially crucial role in climate changes in the region through their regulation to biogeochemical cycles of carbon and nitrogen. Here, we studied the prokaryotic (Bacteria and Archaea) communities in a vertical profile from the Kunlun Mountain Pass that proceeded from the active layer soil into a zone of permafrost soil. This record of prokaryotic diversity contributes to an emerging body of literature that document the microbial ecology of permafrost on the Qinghai-Tibet Plateau.

Consistent with other subsurface habitats (Lehman 2007), there was a significant drop in bacterial α diversity with depth and community composition of bacteria also changed. Compositional differences were related to the presence of specific phylotypes and the physicochemical properties of soils. Deeper permafrost samples are characterized by relatively high electrical conductivity salinity, and were dominated by *Psychrobacter*-related clones. These clones were represented by a single phylotype (KL_B6-130) that showed >99 % sequence similarity to *Psychrobacter glacincola*, a psychrophilic and halotolerant bacterium isolated from Antarctic sea ice (Bowman et al. 1997). This observation is in agreement with the fact that *Psychrobacter* has been found to be an indicator genus for permafrost and other polar environments (Ayala-del-Río et al. 2010). This is also consistent with a previous report, suggesting that the distribution of *Psychrobacter* spp. correlated with salinity of their environment (Rodrigues et al. 2009). The effect of salinity on the relative abundance of *Psychrobacter*-related clones can be attributed to their adaptation to low water activity and osmotic stress in cold permafrost habitats (Rodrigues et al. 2009). Several upper active layer samples contained a high relative abundance of members of nonsporeforming *Arthrobacter* related to the high-GC Gram-positive Actinobacteria. Previous studies have suggested that the formation of endospores is the most effective strategy for bacterial survival under extremely cold conditions (Vorobyova et al. 1997). The dominancy of *Arthrobacter* spp. in our case, however, could be largely due to their metabolic activity and DNA repair mechanism at low temperatures (Johnson et al. 2007). The soil C/N ratio, organic carbon, conductivity, pH and total nitrogen were found to be the critical environmental factors influencing bacterial communities in Kunlun Mountains permafrost soils. Similar results have been reported in other studies of permafrost systems which suggested that soil carbon and nitrogen contents are key factors related to soil nutrient availability and important properties structuring

bacterial communities (Chu et al. 2011; Zhang et al. 2014; Shen et al. 2015). Previous studies have also revealed that soil pH was a good predictor for bacterial communities in permafrost soils (Männistö et al. 2007; Chu et al. 2010; Zinger et al. 2011). It is generally considered that bacterial communities are more sensitive to soil pH due to their relatively narrow growth tolerances (Rousk et al. 2010). On the other hand, differences in soil pH may reflect the different status of availability of labile organic substrates which are more accessible to Bacteria (Zinger et al. 2011). These would explain the significant relationship between soil pH and bacterial community composition.

Bacterial communities reported in polar active layer soils and permafrost have shown highly similar phylogenetic community composition, and were taken as evidence that microorganisms present in the permafrost layer are most likely originating from the overlying active layer and represent a subset of those inhabiting the active layer (Gilichinsky et al. 2007; Yergeau et al. 2010). This appears to be contradictory with our results and several previous studies (Steven et al. 2008; Mackelprang et al. 2011; Hultman et al. 2015), which indicated that bacterial communities differed significantly between active layer and permafrost. The permafrost and active layer are very different environments; the former is characterized by the prolonged exposure to subzero temperature, low water and nutrient availability; however, the latter experiences larger environmental fluctuations (Steven et al. 2006). From an ecological perspective, compositional dissimilarities could be the result of niche separation (Nemergut et al. 2013). The extreme conditions may cause abiotic stresses for indigenous microorganisms in the permafrost layer, selecting distinctive bacterial communities compared with the active layer. Nevertheless, the seasonal freeze–thaw cycles occurring in active layer are generally associated with soil physicochemical disturbances (Sawicka et al. 2010), also resulting in some differences in the bacterial community composition.

Archaeal clones classified into the phylum Thaumarchaeota were the most abundant in permafrost soils studied here. This observation is agree with a report that the ice wedge archaeal communities from Canadian High Arctic soils showed low diversity and were dominated by Thaumarchaeota-related phylotypes (Wilhelm et al. 2012). However, most of previous studies have suggested that the archaeal communities in permafrost consisted mainly of the phyla Crenarchaeota and Euryarchaeota (for example, Steven et al. 2007, 2008; Mackelprang et al. 2011; Wei et al. 2014). It should be highlighted here that thaumarchaeota were initially classified as Crenarchaeota (Brochier-Armanet et al. 2008), so some permafrost DNA sequences that were identified as crenarchaeota in previously published work may actually belong to the phylum Thaumarchaeota. For instance, Yang et al. (2008) retrieved two low-temperature crenarchaeotal sequences from Tianshan Mountains permafrost (see their Fig. 4), which actually fell within the order Nitrososphaerales, phylum Thaumarchaeota. Ayton et al. (2010) reported a predominance (>99 % of clones) of Crenarchaeota belonging to six clusters in Antarctic mineral soils, which were also assigned to the thaumarchaeotal Group I.1b. Furthermore, the phylum Thaumarchaeota thus far comprises all known archaeal ammonia oxidizers (Pester et al. 2011), which is in line with our results that all thaumarchaeotal phylotypes were related to three characterized ammonia-oxidizing archaea. Collectively, these results imply that the archaeal phylum Thaumarchaeota may play a significant role in nitrogen and carbon cycles of Kunlun Mountains permafrost and also contribute to primary production by their autotrophic lifestyle. This inference is further supported by the previous findings indicating that the thaumarchaeotes dominated ammonia oxidation in oligotrophic conditions (Pester et al. 2011).

As is often the case in molecular surveys of soil environments, our analysis of the Kunlun Mountains permafrost has identified novel taxa in both bacterial and archaeal divisions, and provides potential microbial resources for future studies. Within well-characterized phyla such as the Proteobacteria and Actinobacteria, novel lineages were identified. Of particular interest are the large number of novel orders and phylotypes within the Acidobacteria because these organisms have a major impact on the terrestrial carbon cycle and are well adapted to low-nutrient and desiccation conditions (Ward et al. 2009).

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Compliance with ethical standards

The soil samples used in this study were collected in the state-owned land which is open for scientific research. No specified permissions are required for this sampling site, which is not natural reserve and did not involve endangered or protected species.

Conflict of interest The authors have no substantial financial or commercial conflicts of interest with the current work or its publication.

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