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RESEARCH PAPER

Microbial diversity in two cold springs on the Qinghai-Tibetan Plateau

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Abstract The microbial diversity in Wuli Area, Qinghai-Tibetan Plateau was investigated using 16S rRNA gene phylogenetic analyses. A total of 117 bacterial and 66 archaeal 16S rRNA gene clones were obtained from the Wuli cold springs. The bacterial clones could be classified into *Proteobacteria*, *Acidobacteria*, *Deinococci*, *Sphingobacteria*, *Flavobacteria*, *Nitrospirae*, *Actinobacteria*, *Gemmatimonadetes*, and unclassified-bacteria; and the archaeal clones could be classified into *Crenarchaeota* and *Thaumarchaeota*. Among the major groups, *Proteobacteria* and *Crenarchaeota* were dominant in the bacterial and archaeal 16S rRNA gene clone libraries, respectively. The clone sequences obtained in Wuli cold springs were closely related to those from cold habitats, such as snow/ice/soils on high mountains or at high latitude. Especially, the microbial community composition of Wuli Area was more similar to that in Tibetan glaciers than cold environments of other locations. The data presented in this study have implications for a better understanding of microbial diversity in cold springs on the Qinghai-Tibetan Plateau.

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1. Introduction

Microbial communities in terrestrial cold springs at low elevation and high latitude have been sporadically reported, such as cold sulfurous springs in Regensburg, Bavaria, Germany (Rudolph et al., 2001), a cold sulfurous spring in the province of Valencia, East Spain (Camacho et al., 2005), cold perennial springs of the High Arctic (Perreault et al., 2007; Perreault et al., 2008), a cold sulfur-rich-spring on the shoreline of Lake Erie, Michigan (Chaudhary et al., 2009), and a cold spring in Shawan, Xinjiang, China (Zeng et al., 2010). These studies reveal that diverse microbial communities are present in cold springs; and the diverse microbial communities were mainly psychrotolerant or psychrophilic (Perreault et al., 2008).

Despite these limited microbial studies of cold springs, almost little is known about the microbial diversity in cold springs at high elevations, especially on the Qinghai-Tibetan Plateau. The Qinghai-Tibetan Plateau is located in the Mediterranean-Himalayas tectonic zone, and most of this area is underlain by deep, continuous permafrost and it hosts many active faults, along which cold springs are often distributed (Wu et al., 2003). However, nothing is known about the bacterial and archaeal diversity in these cold springs. The objective of this study was therefore to investigate the bacterial and archaeal diversity in two cold springs in Wuli Area, located in the west of Qinghai Province and elevated at 4600 m above sea level, on the Qinghai-Tibetan Plateau by using 16S rRNA gene phylogenetic analysis.

2. Materials and methods

2.1. Sample collection

In July 2010, two cold springs (34°20′N/94°38′E) adjacent to each other were selected for sampling in Wuli Area (Fig. 1). At the sampling location (around noon), the ambient temperature was 15–17 °C, whereas the water temperature of the sampled cold spring was around 4 °C. Sediments in the two cold springs (designated as QCS1 and QCS2, respectively) were collected into 50 mL sterile Falcon tubes using a sterile spatula, and were subsequently stored at −20 °C on the field and during transportation. The samples were stored at −80 °C on arrival in the laboratory until further analysis.

2.2. Pore water chemistry and sediment mineralogy

Anion compositions of the pore water in the sediment were analyzed using ionic chromatograph (IC) on a Dionex ISC90 equipped with a conductivity detector and an AS14A column (Eluent, 10 μmol/L Na_2CO_3/$NaHCO_3$; flow rate, 1.0 mL/min). Cation compositions of the pore water in the sediment were analyzed using inductively coupled plasma-optical emission spectrometry (ICP-OES; Varian Vista MPX, Varian, Palo Alto, CA, USA). The sediment mineralogy was analyzed by using powder X-ray diffraction (XRD) with a D/max-2550/PC diffractometer (Rigaku) using a Cu KR as X-radiation source (40 kV; 40 mA).

2.3. DNA isolation, PCR amplification, and phylogenetic analysis

The collected samples were subjected to DNA extraction using FastDNA® SPIN Kit for soil (MP Biomedicals, LLC, Ohio, USA) according to the manufacturer's protocols. Total community DNA was amplified using 16S rRNA gene primer sets, Bac27F-YM (5′-AGA GTTTGATYMTGGCTCAG-3′)/Univ1492R (5′-CGG TTACCTTGTTACGACTT-3′) and Arch21F (5′-TTCYGGTT GATCCYGCCRGA-3′)/Arch958R (5′-YCCGGCGTTGAMTCC ATTT-3′) for bacteria and archaea, respectively (Jiang et al., 2007; Jiang et al., 2009). PCR conditions were established according to Jiang et al. (2007) and Jiang et al. (2009). The PCR products were purified using an Agarose Gel DNA Fragment Recovery Kit Ver. 2.0 (TaKaRa, Dalian, China) according to the manufacturer's instructions. Purified PCR products were ligated into pGEM®-T Easy Vector system (Promega, Madison, WI, USA) and transformed into competent *Escherichia coli* JM109 cells according to the manufacturer's instructions, and 16S rRNA gene clone libraries were constructed. Approximately 30 and 60 positive clones per library were randomly selected for sequencing. The 16S rRNA gene inserts were sequenced at Shanghai Sangon Biotech with an ABI 3100 automated sequencer using primers of Arch21F and Bac27F-YM for archaea and bacteria, respectively. The raw sequences were trimmed by

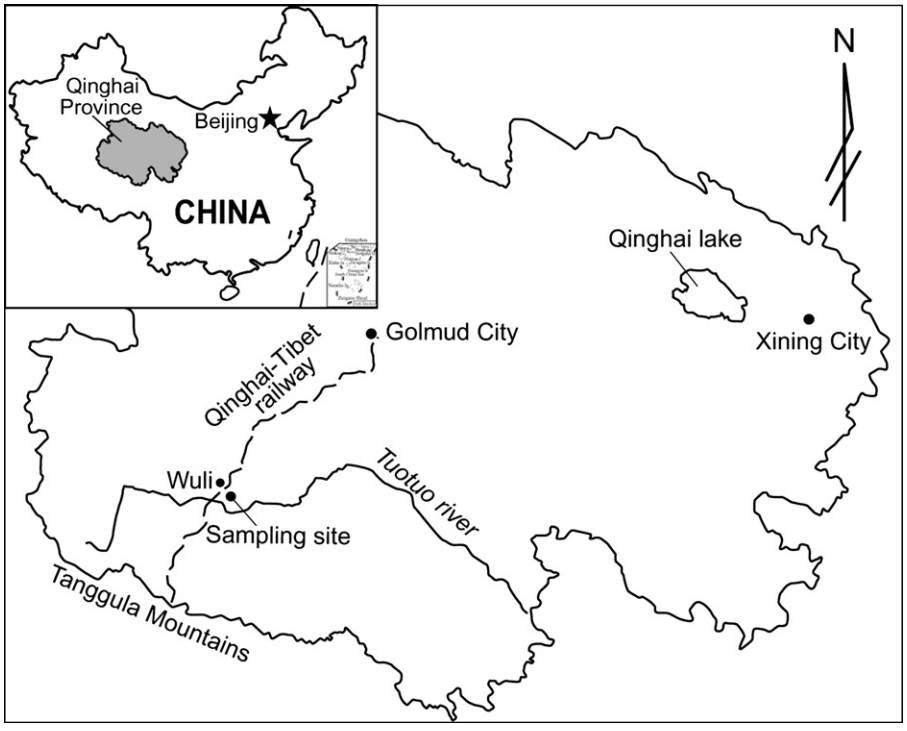

Figure 1 A geographic map showing the locations of sampling site in Wuli, Qinghai Province, NW China.

using Sequencher 4.8, and were examined for chimera with Bellerophon (http://foo.maths.uq.edu.au/~huber/bellerophon.pl). Potential chimeric sequences were removed. Operational taxonomic units (OTUs) were determined using DOTUR (Schloss and Handelsman, 2005) with a 97% cutoff value. Rarefaction analysis (Schloss and Handelsman, 2005) was performed to evaluate the saturation of the sampled clones. The clone sequencing was stopped when the rarefaction curves were (or almost) saturated. Representative clones (one from each OTU) were BLASTed in the GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis was performed using distance-based Neighbor-Joining method with MEGA version 4.0 (Tamura et al., 2007). Bootstrap replications of 1000 were assessed. The representative clone sequences determined in this study have been deposited in the GenBank database under accession numbers JF776281-JF776296 and JF776297-JF776355 for archaea and bacteria, respectively.

2.4. Statistical analysis and sequence population diversity

Clone sequences with similarities of greater than 97% were considered to represent the same phylotypes (Jiang et al., 2007). Coverage (C) was calculated as follows: $C = 1 - (n_1/N)$, where n_1 is the number of phylotypes that occurred only once in the clone library and N is the total number of clones analyzed.

3. Results

3.1. Pore water chemistry and sediment mineralogy

The concentrations of major anions and cations in the pore water are as follows (ppm): Cl^- (132.94), SO_4^{2-} (159.06), NO_3^- (3.57), PO_4^{3-} (0.42), K^+ (5.19), Na^+ (104.15), Ca^{2+} (50.14), and Mg^{2+} (40.75). X-ray diffraction results indicated that the sediment samples were dominated with quartz (70%), plagioclase (10%), calcite (3%–4%), montmorillonite, illite and kaolinite in decreasing order of abundance.

3.2. Bacterial diversity in sediments

A total of 52 and 65 bacterial 16S rRNA gene clone sequences were obtained from the QCSB1 and QCSB2 clone libraries, respectively. These sequences could be classified into the following groups: *Proteobacteria*, *Acidobacteria*, *Deinococci*, *Sphingobacteria*, *Flavobacteria*, *Nitrospirae*, *Actinobacteria*, *Gemmatimonadetes*, and unclassified-bacteria (Table 1 and Fig. 2). Among these groups, the *Proteobacteria* was the dominant component for the bacterial 16S rRNA gene clone libraries, and they accounted for 61.5% and 58.5% of the bacterial 16S rRNA gene clone sequences retrieved from QCSB1 and QCSB2 clone libraries, respectively.

The *Proteobacteria* sequences were classified into four subgroups: *Betaproteobacteria*, *Gammaproteobacteria*, *Alphaproteobacteria*, and *Deltaproteobacteria* (Fig. 2a and Table 1). The *Alphaproteobacteria* contained nine OTUs, representing 17 sequences (5.8% and 21.5% of the total sequences in QCSB1 and QCSB2, respectively), and most of these sequences were closely related to clones retrieved from cold habitats, such as glaciers and lakes on the Tibetan Plateau (Liu et al., 2006; Liu et al., 2009; Xiang et al., 2009; Xing et al., 2009), soils and ice in Arctic and Antarctic (Mosier et al., 2007; Wallenstein et al., 2007; Simon et al., 2009). One clone (QCSB1-009) is also closely related (identity: 100%) to *Ochrobactrum tritici* strain Y13 isolated from a petroleum-oil contaminated soil and to a clone sequence retrieved from the cleaning seawater from Tunisian oil reservoirs (Zrafi-Nouira et al., 2009).

The *Betaproteobacteria* included 12 OTUs, representing 22 clone sequences (5.8% and 29.2% of the total sequences in QCSB1 and QCSB2, respectively), and most of these sequences were also closely related to clones retrieved from cold habitats, such as high-mountain lakes and snow/ice (Xiang et al., 2009) and Arctic/Antarctic ice/snow (Sattley and Madigan, 2006; Cheng and Foght, 2007; Harding et al., 2011). Among these clone sequences, one clone (QCSB2-068) was also closely related (identity: 98%)

Table 1 Ecological estimates and major group affiliation of bacterial 16S rRNA gene clone sequences retrieved from the two cold springs on the Tibetan Plateau.

Clone library	QCSB1	QCSB2
Library size (No. of clones)	52	65
Coverage (%)	69.2	60.0
No. of observed OTUs	22	37
Simpson' s diversity index (D)	0.785	0.953
Shannon–Wiener's diversity index (H)	3.339	4.821
Shannon–Wiener's evenness index (E)	0.586	0.801
Major group affiliation	Number of clones (relative percentage in each clone library)	
Alphaproteobacteria	3 (5.8%)	14 (21.57%)
Betaproteobacteria	3 (5.8)	19 (29.2%)
Deltaproteobacteria	2 (3.8%)	2 (3.1%)
Gammaproteobacteria	24 (46.2%)	3 (4.6%)
Acidobacteria	7 (13.8%)	9 (13.8%)
Actinobacteria	3 (5.8%)	2 (3.1%)
Deinococci	0 (0%)	1 (1.5%)
Flavobacteria	3 (5.8%)	1 (1.5%)
Gemmatimonadetes	1 (1.9%)	2 (3.1%)
Nitrospirae	5 (9.6%)	2 (3.1%)
Sphingobacteria	0 (0%)	9 (13.8%)
Unclassified-bacteria	1 (1.9%)	1 (1.5%)

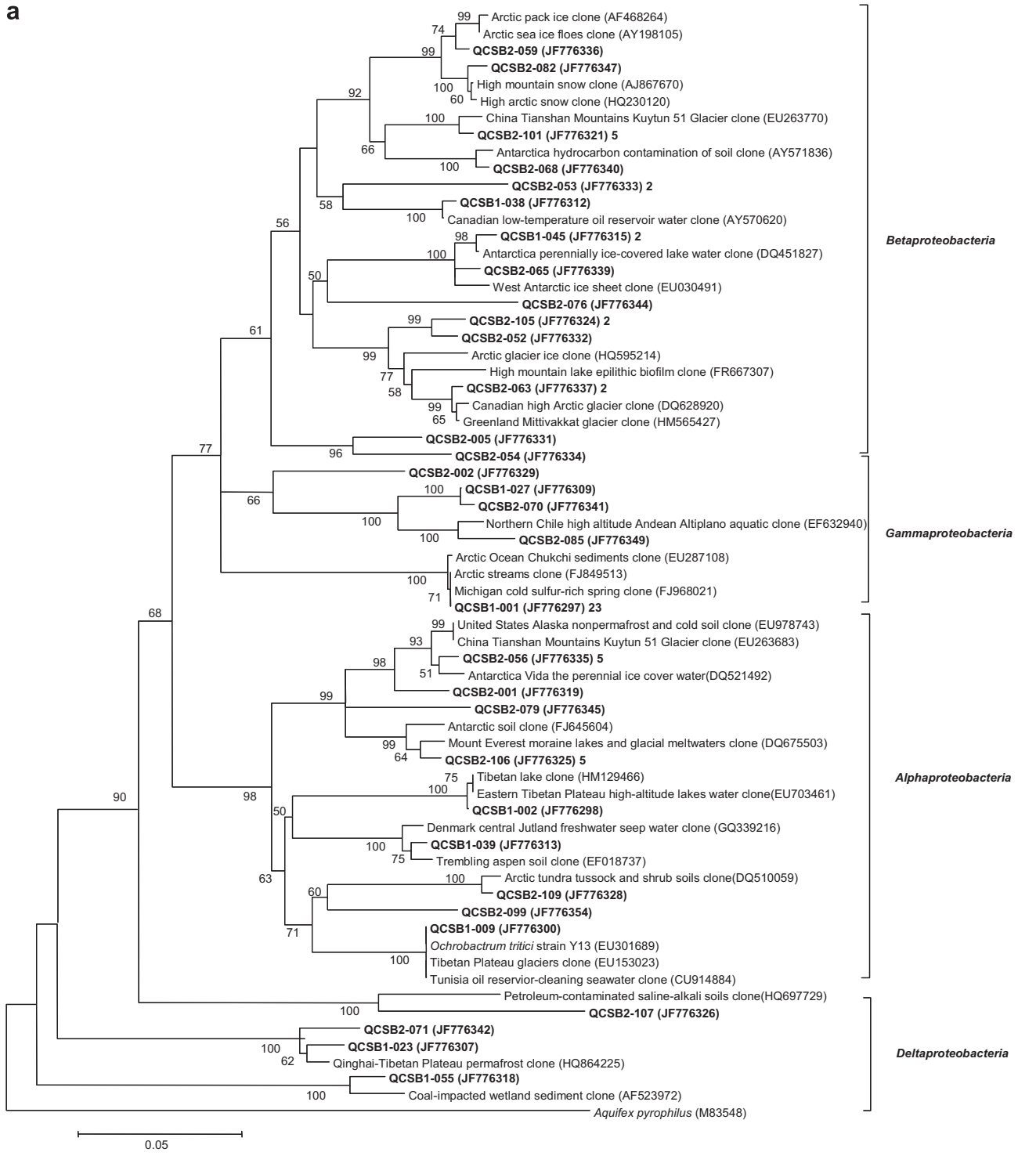

Figure 2 Neighbor-Joining tree (partial sequences, ~700 bp) showing the phylogenetic relationships of bacterial 16S rRNA gene sequences cloned from the studied samples to closely related sequences from the GenBank database. One representative clone type within each OTU is shown, and the number of clones within each OTU is shown at the end (After the GenBank accession number). If there is only one clone sequence within a given OTU, the number '1' is omitted. Clone sequences from this study are coded as follows for the example of QCSB1-001: cold spring QCS1 bacterial clone number 001. Scale bars indicate the Jukes–Cantor distances. Bootstrap values of >50% (for 1000 iterations) are shown. Clone sequences obtained in this study are in bold. *Aquifex pyrophilus* is used as an outer group. Fig. 2a, b is for *Proteobacteria* and non-Proteobacteria, respectively.

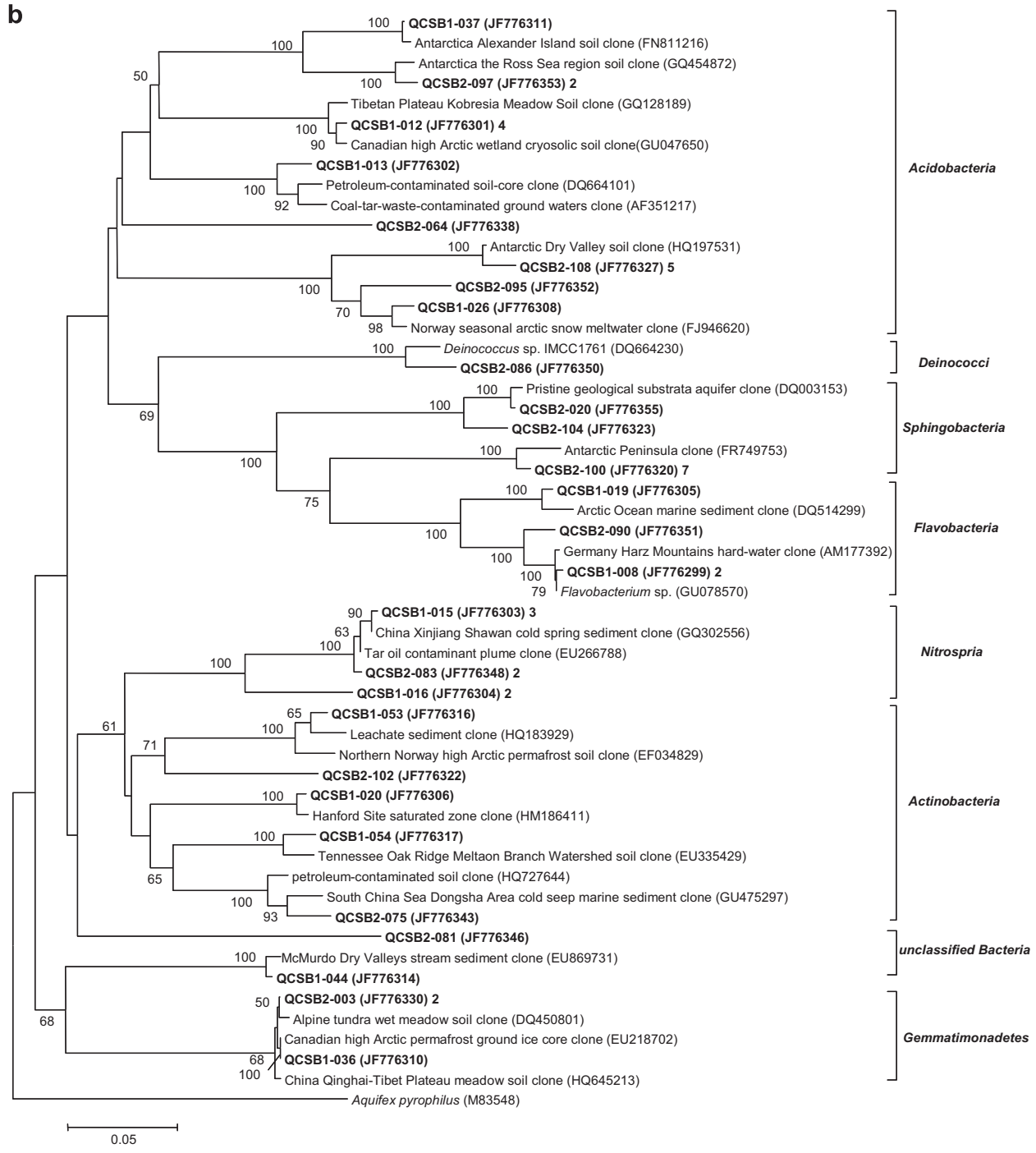

Fig. 2 *(Continued).*

to a clone retrieved from hydrocarbon-contaminated soil in Scott Base, Antarctica (Saul et al., 2005); another clone (QCSB1-038) was related (identity: 97%) to a clone sequence obtained from production waters in a low-temperature biodegraded oil reservoir in the Pelican Lake oilfield located in the Western Canadian Sedimentary Basin (Grabowski et al., 2005).

The *Deltaproteobacteria* included 4 OTUs, representing four clone sequences, two of which (QCSB1-023 and QCSB2-071) showed 97% similarity to a clone obtained from permafrost soil on the Qinghai-Tibetan Plateau (GenBank description), and the other two (QCSB1-055 and QCSB2-107) were related to clones retrieved from coal-impacted wetland sediment (Brofft et al., 2002) and petroleum-contaminated saline-alkali soils (GenBank description).

The *Gammaproteobacteria* included twenty-seven sequences (46.2% and 4.6% of the total sequences in QCSB1 and QCSB2, respectively) grouped into five OTUs, among which the QCSB1-001 (representing 23 clone sequences) was closely related (99%–100%) to clones the 447 m-depth sediment on the submarine plateau in the Arctic ocean (Li et al., 2009) and cold sulfur-rich-spring water near Woodtick Peninsula on the shoreline of Lake Erie, Michigan

(Chaudhary et al., 2009). The other four OTUs (QCSB2-002, CSB1-027, QCSB2-070, and QCSB2-085) showed high similarity to a clone retrieved from aquatic environments of the high altitude Andean Altiplano in northern Chile (GenBank description).

Among the non-Proteobacteria groups, the *Acidobacteria* was most abundant, and contains 8 OTUs, representing 16 clone sequences (13.5% and 13.8% of the total sequences in QCSB1 and QCSB2, respectively) (Fig. 2b and Table 1). Most of the clone sequences (7 OTUs and 15 clone sequences) were related to clones retrieved from cold environments, such as meadow soil from Mount Mila on the Qinghai-Tibetan Plateau (GenBank Description) and soil/ice/snow from Antarctic/Arctic (Larose et al., 2010). One OTU (QCSB1-013) from the QCSB1 clone library was related (identity: 96%) to a clone from petroleum-contaminated sediments in Michigan (Allen et al., 2007) and to a clone from coal tar waste-contaminated groundwater located in South Glens Falls, NY (Bakermans and Madsen, 2002).

A total of thirty-one clones (25.0% and 27.7% of the total sequences in QCSB1 and QCSB2, respectively) were affiliated with some minor non-Proteobacteria groups, such as *Actinobacteria*, *Deinococci*, *Flavobacteria*, *Gemmatimonadetes*, *Nitrospirae*, *Sphingobacteria*, and unclassified-Bacteria (Fig. 2b and Table 1). Most of these clone sequences were also related to clones retrieved from cold environments, such as tundra/permafrost/wetland soils and snow meltwater/ice in Arctic/Antarctic. In addition, two OTUs in the QCSB2 library were related to clones from tar-oil contaminated aquifer sediments (Winderl et al., 2008) and marine sediments of cold seeps in Dongsha Area of South China Sea (GenBank description).

3.3. Archaeal diversity in sediments

A total of 43 and 23 archaeal 16S rRNA gene clone sequences were obtained from the QCSA1 and QCSA2 clone libraries, respectively. These clone sequences could be classified into *Crenarchaeota* and *Thaumarchaeota* (Spang et al., 2010). The *Crenarchaeota* was the dominant component, accounting for 100.0% and 91.3% of the archaeal 16S rRNA gene clone sequences retrieved from QCSA1 and QCSA2 clone libraries, respectively (Fig. 3 and Table 2). The *Crenarchaeota* contained fifteen OTUs, and they were related to clones retrieved from cold habitats, such as river water in Arctic (Galand et al., 2008), snow

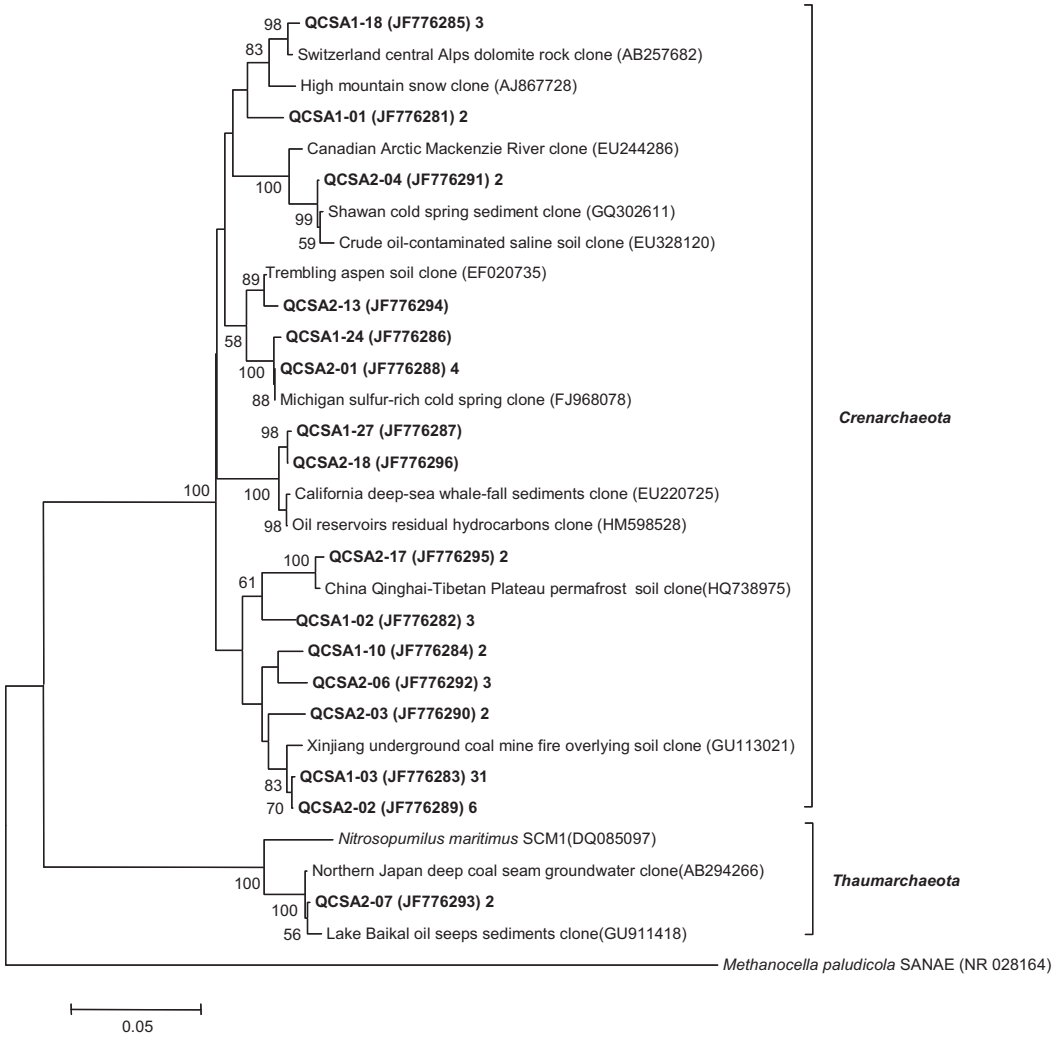

Figure 3 Neighbor-Joining tree (partial sequences, ~750 bp) showing the phylogenetic relationships of archaeal 16S rRNA gene sequences cloned from the studied samples to closely related sequences from the GenBank database. The same algorithms as those for the archaeal tree (Fig. 2a, b) were used. *Methanocella paludicola* is used as the outgroup.

Table 2 Ecological estimates and major group affiliation of archaeal 16S rRNA gene clone sequences retrieved from the two cold springs on the Tibetan Plateau.

Clone library	QCSA1	QCSA2
Library size (No. of clones)	43	23
Coverage (%)	95.4	91.3
No. of observed OTUs	7	9
Simpson' s diversity index (*D*)	0.465	0.851
Shannon–Wiener' s diversity index (*H*)	1.540	2.947
Shannon–Wiener's evenness index (*E*)	0.284	0651
Major group affiliation	Number of clones (relative percentage in each clone library)	
Crenarchaeota	43 (100.0%)	21 (91.3%)
Thaumarchaeota	0 (0%)	2 (8.7%)

and permafrost soil on the Qinghai-Tibetan Plateau (GenBank description), and terrestrial cold springs (Chaudhary et al., 2009; Zeng et al., 2010). Among the *Crenarchaeota* clone sequences, two OTUs were also closely related to clones from crude oil-contaminated soil (GenBank description) and residual hydrocarbon in oil reservoirs (Siegert et al., 2011).

Only one OTU (QCSA2-07, representing two clone sequences) fell in the *Thaumarchaeota*. This OTU was related to clones from deep coal seam groundwater in Northern Japan (Shimizu et al., 2007) and bottom sediments at Lake Baikal sites of natural oil seeps (GenBank description). This OTU and the two references were closely affiliated with ammonia-oxidizing *Nitrosopumilus maritimus* SCM1 (Konneke et al., 2005).

4. Discussion

4.1. Microbial diversity in sediments of cold springs in Wuli Area of the Qinghai-Tibetan Plateau

To our knowledge, this research was the first to specifically study microbial diversity in cold springs on the Qinghai-Tibetan Plateau. Previously, several studies have investigated the microbial diversity in cold springs at low elevation or high latitude (Rudolph et al., 2001; Grabowski et al., 2005; Perreault et al., 2008; Chaudhary et al., 2009; Zeng et al., 2010). The microbial communities of the Wuli cold springs were characteristic of predominance of *Proteobacteria* and *Crenarchaeota* for the bacterial and archaeal 16S

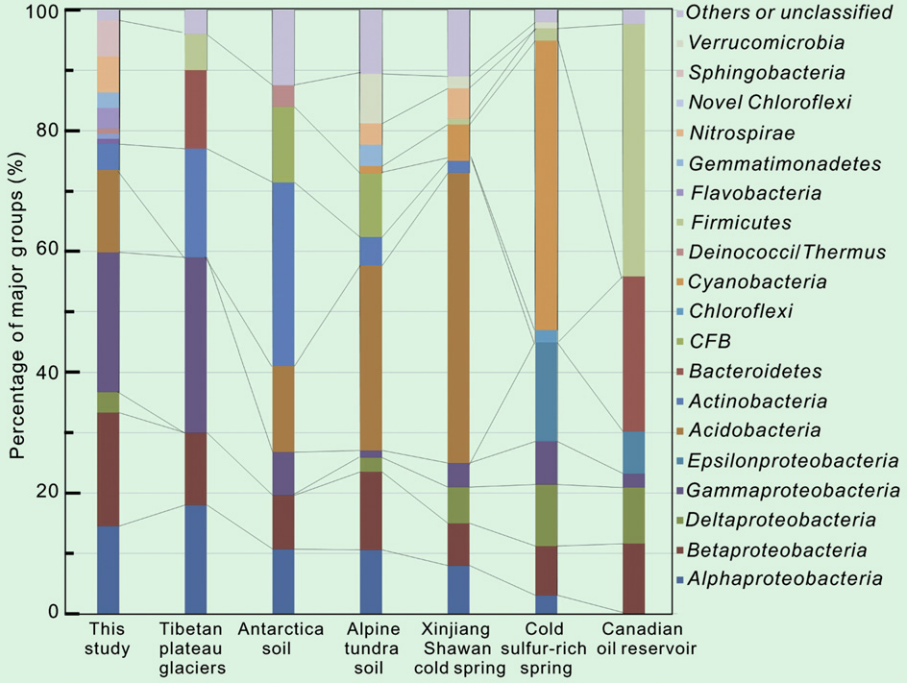

Figure 4 Histogram showing frequencies of OTUs affiliated with major phylogenetic groups in the bacterial clone libraries for Wuli Cold Springs (This study), glaciers on the Tibetan Plateau (Liu et al., 2009), hydrocarbon-contaminated soil in Antarctica (Saul et al., 2005), saturated meadow soil in alpine tundra in Rocky Mountain Front Range, Colorado, USA (Costello and Schmidt, 2006), Shawan cold spring in Xinjiang (Zeng et al., 2010), a cold sulfur-rich-spring near to Woodtick Peninsula (41°46′N/83°27′E) on the shoreline of Lake Erie, Michigan (Chaudhary et al., 2009), and a low-temperature biodegraded oil reservoir in the Pelican Lake oilfield located in the Western Canadian Sedimentary Basin (Grabowski et al., 2005).

rRNA gene clone libraries, respectively. Such microbial predominance has been found for the bacterial community in a cold sulfur-rich-spring on the shoreline of Lake Erie, Michigan (Chaudhary et al., 2009) and for the archaeal community in Shawan cold spring of Xinjiang, China (Zeng et al., 2010), respectively.

In addition, the bacterial communities of the Wuli cold springs were composed of microorganisms related to clone sequences retrieved from cold habitats, such as snow/ice and soils in Qinghai-Tibetan Plateau and Arctic/Antarctic. In order to make comparisons among the bacterial communities of different cold habitats, bacterial 16S rRNA gene clone libraries of several cold environments were selected based on the phylogenetic analysis for making a histogram showing frequencies of OTUs affiliated with major phylogenetic groups for Wuli Cold Springs (This study), glaciers on the Qinghai-Tibetan Plateau (Liu et al., 2009), hydrocarbon-contaminated soil in Antarctica (Saul et al., 2005), saturated meadow soil in alpine tundra in Rocky Mountain Front Range, Colorado, USA (Costello and Schmidt, 2006), Shawan cold spring in Xinjiang (Zeng et al., 2010), a cold sulfur-rich-spring near to Woodtick Peninsula (41°46′N/83°27′E) on the shoreline of Lake Erie, Michigan (Chaudhary et al., 2009), and a low-temperature biodegraded oil reservoir in the Pelican Lake oilfield located in the Western Canadian Sedimentary Basin (Grabowski et al., 2005) (Fig. 4). The comparison showed the difference of the bacterial diversities between Wuli cold springs and Qinghai-Tibetan glaciers was much smaller than that between Wuli cold springs and other cold habitats of low elevation or high altitude. For example, the bacterial communities of Wuli cold springs and Qinghai-Tibetan glaciers were mainly composed of *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* (Fig. 4). This indicates that microbial community in cold springs on the Qinghai-Tibetan Plateau may be distinct from that of cold habitats in other locations; and biogeography may play an important role in shaping the microbial distribution in cold springs. However, the latter must await further investigation.

4.2. Implication for the presence of underlying coal or gas hydrate in Wuli Area

It is remarkable that some of bacterial and archaeal 16S rRNA gene clone sequences of this study showed close affiliation with those retrieved from petroleum (oil)- or coal-related environments. Previous studies showed that the methane-related microbial communities in the marine cold seeps may be indicative of underlying gas hydrate (Hovland, 2000; Reed et al., 2006). The sampling site of this study is located in one part of Wuli-Daha coal-bearing belt of the southern Qinghai province, and coal seams are relatively developed in this region (Zhou, 2004), which could account for the observed relatedness of clone sequences between Wuli cold springs and other petroleum (oil)- or coal-related environments. So with the limited data, it is hard to conclude there is gas hydrate underneath the sampling site in this study. However, our results at least suggested that microbial analysis might be an accessorial tool for prospecting for coal and oil deposits in extreme environments.

5. Conclusions

The microbial communities of Wuli Area in Qinghai-Tibetan Plateau were mainly composed of *Proteobacteria* and *Crenarchaeota* for bacteria and archaea, respectively, and the retrieved bacterial and archaeal 16S rRNA gene clone sequences were closely related to those from cold habitats, such as snow/ice and permafrost soils in high mountains and snow/ice/soil in Arctic/Antarctic. The microbial community composition of Wuli Area was more similar to that in Tibetan glaciers than cold environments of other locations.

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