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The Graduate School

CERTIFICATE FOR APPROVING THE DISSERTATION

We hereby approve the Dissertation

of

Hongchen Jiang

Candidate for the Degree

Doctor of Philosophy

Dr. Hailiang Dong, Director

Dr. Chuanlun Zhang, Reader

Dr. Yildirim Dilek, Reader

Dr. Jonathan Levy, Reader

Dr. Q. Quinn Li, Graduate School Representative

ABSTRACT

GEOMICROBIOLOGICAL STUDIES OF SALINE LAKES ON THE TIBETAN PLATEAU, NW CHINA: LINKING GEOLOGICAL AND MICROBIAL PROCESSES

By

Hongchen Jiang

Lakes constitute an important part of the global ecosystem as habitats in these environments play an important role in biogeochemical cycles of life-essential elements. The cycles of carbon, nitrogen and sulfur in these ecosystems are intimately linked to global phenomena such as climate change. Microorganisms are at the base of the food chain in these environments and drive the cycling of carbon and nitrogen in water columns and the sediments. Despite many studies on microbial ecology of lake ecosystems, significant gaps exist in our knowledge of how microbial and geological processes interact with each other.

In this dissertation, I have studied the ecology and biogeochemistry of lakes on the Tibetan Plateau, NW China. The Tibetan lakes are pristine and stable with multiple environmental gradients (among which are salinity, pH, and ammonia concentration). These characteristics allow an assessment of mutual interactions of microorganisms and geochemical conditions in these lakes. Two lakes were chosen for this project: Lake Chaka and Qinghai Lake. These two lakes have contrasting salinity and pH: slightly saline (12 g/L) and alkaline (9.3) for Qinghai Lake and hypersaline (325 g/L) but neutral pH (7.4) for Chaka Lake. We have taken an integrated approach combining geochemistry, molecular phylogeny (both DNA and RNA based, both 16S rRNA and amoA gene), quantitative PCR (total Bacteria, Archaea, total crenarchaeota, AOA, and AOB), and cultivation and isolation. Both lake water and sediments have been analyzed. The results are divided into four chapters and they are summarized below. In addition, I

also studied microbial communities and functions in sediments from South China Sea, a potential site for gas hydrate deposits. This work was done as extra add-on to the microbial ecology studies in Titeban lakes.

GEOMICROBIOLOGICAL STUDIES OF SALINE LAKES ON THE TIBETAN PLATEAU, NW CHINA: LINKING GEOLOGICAL AND MICROBIAL PROCESSES

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FIELDS OF STUDY

Major Field: GEOMICROBIOLOGY

CHAPTER 1:

INTRODUCTION

Lakes and oceans constitute an important part of the global aquatic ecosystem as habitats in these environments play an important role in biogeochemical cycles of life-essential elements (Dean and Gorham, 1998, Kirchman, 2000). The cycles of carbon, nitrogen and sulfur in these ecosystems are intimately linked to global phenomena such as climate change. For example, emission of carbon and nitrogen compounds from aquatic environments into the atmosphere causes the greenhouse effect and affects the ozone layer. Global warming, as a result of the greenhouse effect, can accelerate the desertification process by increasing net evaporation and can augment the release of carbon and nitrogen from terrestrial organic matter to the atmosphere. Microorganisms are the major players mediating the cycling of carbon and nitrogen, primary production, and decomposition of organic matter in water columns and sediments of aquatic ecosyctems (Madigan, et al., 2004). So it is important to understand the role of microorganisms in these environments.

Saline and alkaline lakes account for almost half of the total volume of all terrestrial aquatic ecosyctems (William, 1996). Both cultivation and molecular-based approaches (SSU rRNA gene analysis) have been employed to reveal diverse bacterial and archaeal communities over a range of environmental conditions such as salinity, pH, and temperature. Various lakes from around the world have been studied, including the Great Salt Lake in Utah, the Great Salt Plains of Oklahoma, the Dead Sea, the Mediterranean Sea, the Solar Lake in Sinai, Egypt, Antarctic hypersaline lakes, deep-sea brine sediments, and various salterns (evaporation ponds for salt recovery), Mono Lake in California, saline meromictic Lake Kaiike in Japan, soda lakes in Mongolia, soda lakes in the Kenyan-Tanzanian Rify Valley, saline and alkaline lakes of the Wadi el Natrun in the Libyan Desert, Egypt, (Mancinelli, 2005, Oren, 2002), athalassohaline lakes (saline lake not of marine origin but evolved from evaporation of fresh water) of the Atacama Desert, Chile (Zelles, 1999). Compared to the recent interest in microbial ecology in the water column of many lakes, the number of investigations related to lake sediments is relatively

low, and let alone microbial investigation in sediments of athalassohaline lakes at high elevation.

Other than filling in knowledge gaps, there are other theoretical and practical reasons that stimulate us to perform microbial research in saline environments: 1) biotechnological applications of halophilic microorganisms were recognized and employed even centuries ago, with a recently renewed interest (Oren, 2002). Potential exists to find novel applications with more in-depth research; 2) because of the presence of salt deposits and saline environments on Mars (Catling, 1999, Cooper and Mustard, 2002, Gendrin, et al., 2005, Langevin, et al., 2005, Mancinelli, et al., 2004, Squyres, et al., 2004), studies of microbial diversity in terrestrial saline environments may shed light on the forms of extinct and/or extant life on Mars; 3) primordial life on earth might have started in hypersaline environments (Dundas, 1998, Knauth, 1998), thus research on microbial survivability and adaptation in saline environments bears relevance to our understanding of the early evolution of the biosphere on Earth.

Saline lakes on the Tibetan Plateau are all athalassohaline at high elevation. These lakes are thus unique and studies of geomicrobiological processes in these environments can fit the aforementioned knowledge gap. The Tibetan Plateau is Earth's largest and highest plateau (average ~4500 masl, meters above sea level), which is a result of the collision between Eurasian continent and India sub-continent ~40-50 Ma ago (Kerr, 2006, Mulch and Chamberlain, 2006, Patriat and Achache, 1984). The tectonic uplifting of the Tibetan Plateau makes its location unique with respect to climatic systems: it lies in a critical and sensitive junction of three climatic systems: the East Asian Monsoon, the cold polar airflow from the Siberian high pressure and the India monsoon (Dong, et al., 2006) (Fig. 1). Dry climate dominates the region and makes saline lakes widely distributed in this area. The sediments in these saline lakes archive environmental changes and store abundant information about the history of climatic change, especially climatic variation, vegetation succession, glacial effect, and their connection with global change. Thus geomicrobiological research in these saline lake sediments may link the global climatic changes to microbial ecosystem response.

The first part of this research will focus on two saline lakes, Lake Chaka and Qinghai Lake: two representative saline lakes on the NE Tibetan plateau (Fig. 2). These

two lakes are separated by Qinghai South Mountain. The reason for choosing these two lakes is that Qinghai Lake is the largest inland lake in China with a low salinity and alkaline pH, and Lake Chaka is a hypersaline lake with neutral pH. These two lakes have contrasting geochemical conditions and are representative of many lakes on the Tibetan Plateau.

Lake Chaka is a shallow salt lake located at an elevation of 3214 masl at the southeastern corner of Qaidam Basin where semi-arid continental climate dominates. Strong evaporation and little precipitation in this area (2264 mm evaporation versus 224 mm rainfall/year) have resulted in a nearly-dry lake with a high salinity (325 g/ L) (Liu, et al., 1996). It is an elliptic and closed drainage basin. The water depth and coverage in the lake vary seasonally. The area of liquid water in high-water period (summer) can reach 104 km^2 with an average water depth of 0.5 m. The area of liquid water and water depth significantly decrease in low-water period (winter). Average water temperature is 4.2^oC with -6 to -8^oC in winter and 6 to 20^oC in summer. Upper Pleistocene and Holocene rocks occur widely in the basin of Lake Chaka. The lake became progressively saline in the last 50,000 years in salinity from freshwater (at 10-meter depth of sediment) to near saturation of NaCl (lake water and at the top of the sediment). Accompanying this salinity gradient is a typical progressive evaporation sequence ranging from freshwater clays and fine sands at the bottom, to evaporative gypsum ($CaSO_4 \cdot 2H_2O$) and mirabilite ($Na_2SO_4 \cdot 10H_2O$) in the middle, to halite (NaCl) at the top. Most of the lake area is dry and being mined for halite deposit. The majority of water supply to the lake is from rivers and spring water in the surrounding area (Liu, et al., 2004). The source waters contain high concentrations of cations and anions.

Qinghai Lake is a perennial lake located in a structural intermontane depression on the northeastern corner of the Tibetan Plateau (Fig. 2). The lake does not have outflowing discharges and is a saline (12.5g/L silinity) and alkaline lake with a pH of 9.4. The lake has an area of 4300 km^2 with an average water depth of 21 m and lies within a catchment of limestones, sandstones, and shales. The altitude of the lake is 3196 masl and the surrounding mountains rise to above 5200 masl. The evaporation of the lake (~1400 mm/year) is in access of mean annual precipitation (~400 mm/year), resulting in the development of a saline lake. Source waters for Qinghai Lake are mainly rain and

snowmelt. Qinghai Lake possesses a continuous sedimentary sequence at least up to the Pliocene period (5 Ma) (Dong, et al., 2006).

The second part of this research will focus on the sediments collected from the Qiongdongnan Basin (QB) (Fig. 2) in the South China Sea (SCS). The SCS is a site with potential gas hydrate deposits (Guo, et al., 2004, McDonnell and Czarnecki, 2000). The SCS is located at the junction of three tectonic plates: the Eurasian, the Pacific and the Indian-Australian. It is surrounded by passive continental margins in the west and north and convergent margins in the south and east. Thick organic-rich sediments have accumulated in the SCS since the late Mesozoic (He, et al., 2006). These sediments are a continuing source to form gas hydrates at the site (Guo, et al., 2004). There are three areas in the SCS with potential gas hydrate deposits: northwestern continental shelf and slope, Dongsha Rise and Manila Trench in Bijianan Basin (Fig. 2) (McDonnell and Czarnecki, 2000, Wu, et al., 2005).

The QB is a potential gas hydrate-bearing basin on the northwestern continental shelf. It is an oil-bearing, fault-depression structural basin, in which organic-rich sediments of 5000 m in thickness have accumulated since the Cenozoic (He, et al., 2006). Seismic data show occurrences of high-pressure diapirs and gas plumes, faults, and gas springs, all of which indicate that the QB possesses suitable sources and tectonic conditions for gas hydrate formation (Chen, et al., 2004). High geothermal (4.6^{o}C/100m) and pressure (18-22 kPa/m) gradients and strong thermal fluids in the basin have accelerated maturation of hydrocarbons and their mobilization (Liu and Wang, 2004). Different stratigraphic traps provide good storage space (Zhu, et al., 2004). The presence of gas hydrates in the QB has been confirmed by geological and geophysical evidence (Chen, et al., 2004, Jiang, et al., 2005, Su, et al., 2005, Wu, et al., 2003)

The goals of this dissertation are to systematically investigate microbial abundance, diversity and metabolic functions in waters and sediments collected from Chaka Lake, Qinghai Lake and the QB with an integrated approach including culture-dependent and -independent means. The goals are:

1) to assess microbial abundance and diversity in the lake water column and the shallow sediments and to study the change of microbial community composition along a vertical salinity gradient in sediments (from nearly

freshwater at the bottom, ~9 m in depth to NaCl-saturated at the top) of Lake Chaka;

2) to evaluate the diversity and relative abundance of *Archaea* and some functional prokaryotes (specifically, ammonia-oxidizers) in the water column and anoxic sediments of Qinghai Lake;

3) to study microbial community composition in the sediments (with potential gas hydrate deposits) collected from the QB of the SCS.

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Figure captions

Fig. 1 Location and monsoon systems of the Tibetan Plateau

Fig. 2 Location of Lake Chaka, Qinghai Lake and South China Sea

Fig. 1

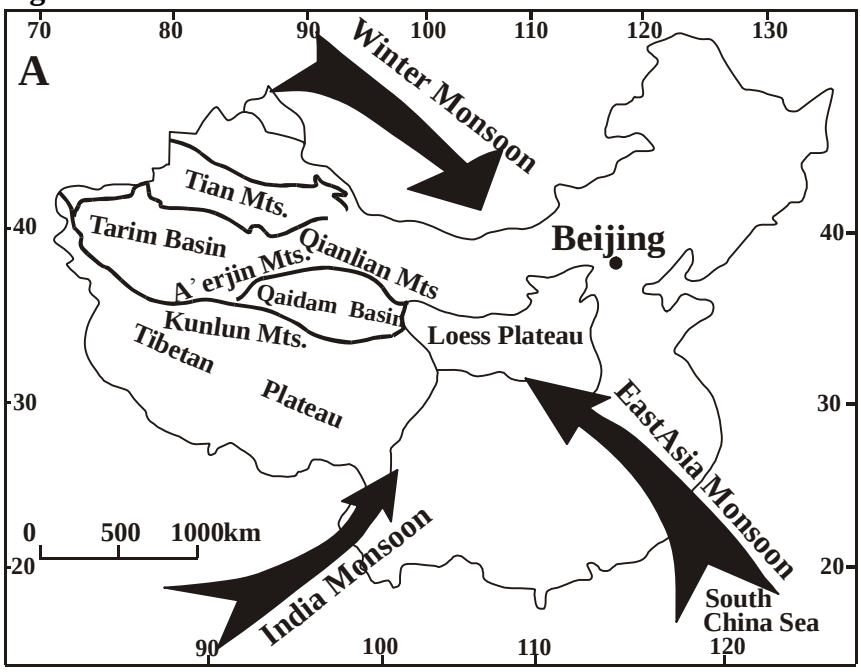
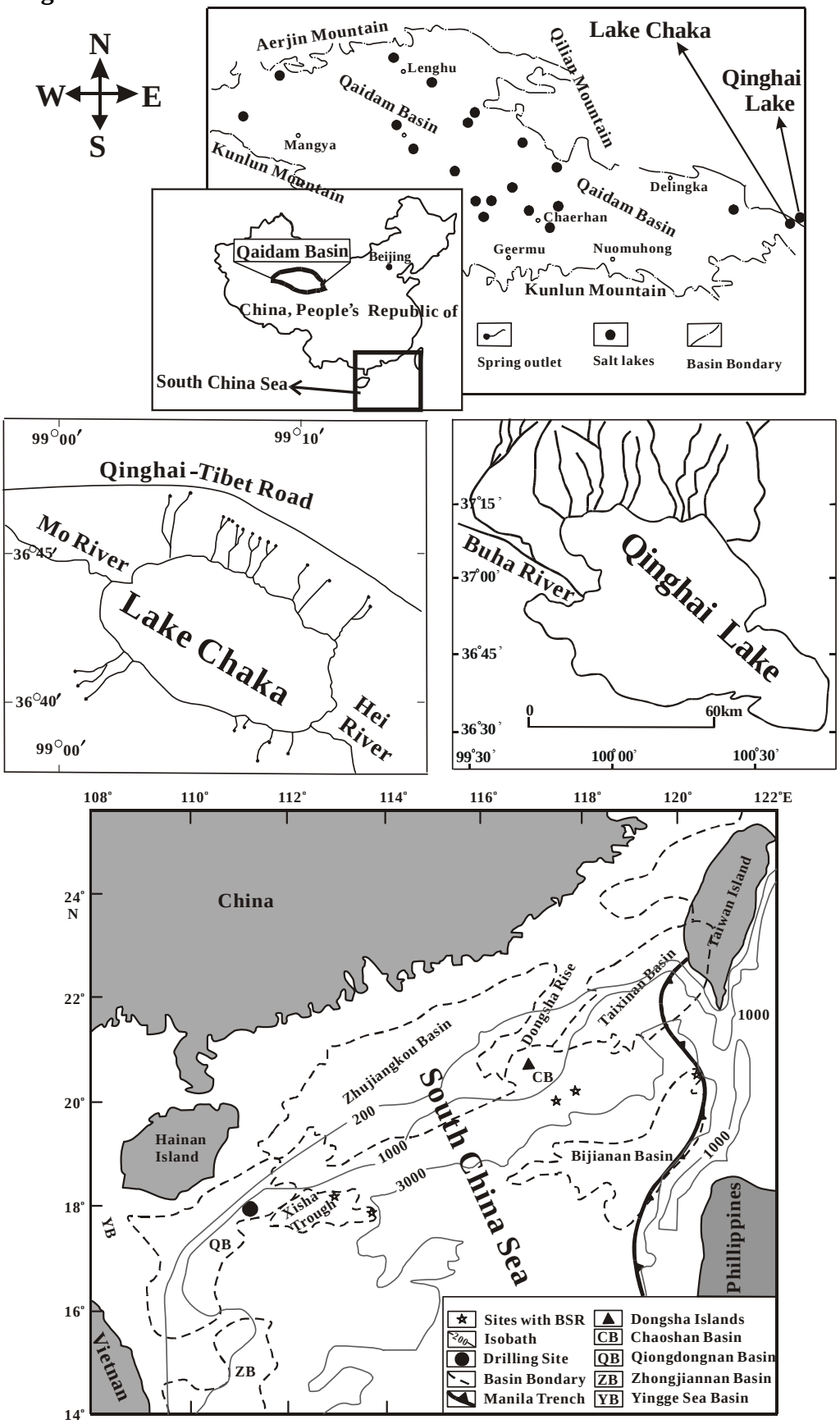


Fig. 2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Qaidam Basin
Mangya
Kunlun Mountain
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Lake Chaka
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Spring outlet
Salt lakes
Basin Bondary
99°00′
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Qinghai-Tibet Road
Mo River
36°45′
Lake Chaka
36°40′
Hei River
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Zhujiangkou Basin
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South China Sea
Hainan Island
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Bijianan Basin
1000
Xisha Trough
YB
QB
Phillippines
Vietnam
ZB
Sites with BSR
Isobath
Drilling Site
Basin Bondary
Manila Trench
Dongsha Islands
CB Chaoshan Basin
QB Qiongdongnan Basin
ZB Zhongjiannan Basin
YB Yingge Sea Basin

CHAPTER 2:

Microbial Diversity in Water and Sediment of Lake Chaka: An Inland Hypersaline Lake in Northwestern China

Running title: Microbial Diversity in Hypersaline Lake Chaka

Hongchen Jiang[1], Hailiang Dong[1*], Gengxin Zhang[1], Bingsong Yu[2], Leah R. Chapman[3], and Matthew W. Fields[3]

1: Department of Geology
Miami University
Oxford, OH 45056

2: Department of Geology
China University of Geosciences
Beijing, China, 100083

3: Department of Microbiology
Miami University
Oxford, OH 45056

*Corresponding author: Hailiang Dong
Department of Geology
Miami University
Oxford, OH 45056
Tel: 513-529-2517
Fax: 513-529-1542
Email: dongh@muohio.edu

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ABSTRACT

We employed culture-dependent and -independent techniques (SSU rRNA gene analysis) to study microbial diversity in water and sediment samples from Lake Chaka, a unique hypersaline lake (32.5% salinity) in Northwestern China. It is situated at 4000 m above sea level in a dry climate. The average water depth was 2 to 3 cm. Halophilic isolates were obtained from the lake water, and halotolerant isolates were obtained from the shallow sediment. The isolates exhibited resistence to UV and gamma radiation. Microbial abundance in the sediments ranged from 10^8 cells/g at the water-sediment interface to 10^7 cells/g at a sediment depth of 42 cm. A major change in the bacterial community composition was observed across the interface. In the lake water, clone sequences affiliated with the *Bacteroidetes* were the most abundant, whereas in the sediments, sequences related to low G+C Gram-positive bacteria were predominant. A similar change was also present in the archaeal community. While all archaeal clone sequences in the lake water sample belonged to the *Halobacteriales*, the majority of the sequences in the sediments were related to those previously obtained from methanogenic soils and sediments. The observed changes in the microbial community structure across the water-sediment interface were correlated with a decrease in salinity from the lake water (32.5%) to the sediments (approximately 4%). Across the interface, the redox state also changed from oxic to anoxic and may also have contributed to the observed shift in microbial community.

INTRODUCTION

Hypersaline lakes are considered extreme environments for microbial life (Oren, 1999) because of the effects on water activity and balance. Saline environments are globally distributed on Earth. Halophiles thrive in hypersaline niches and include prokaryotes and some eukaryotes (DasSarma and Arora, 2001). Among halophilic microorganisms are found a variety of heterotrophic and methanogenic archaea; photosynthetic, lithotrophic, and heterotrophic bacteria; and photosynthetic and heterotrophic eukaryotes. Previous studies have shown that the taxonomic diversity of microbial populations in terrestrial saline and hypersaline environments is low (DasSarma and Arora, 2001, Oren, 2001), and that, in general, microbial diversity decreases with increased salinity (Oren, 2002).

The study on microbial diversity in saline environments is important for two reasons. First, some of the earliest microbial life on Earth might have been halophilic because of high salt and organic compound concentrations in evaporitic environments, and thus research on microbial survivability and adaptation bears relevance to our understanding of the early evolution of life and the biosphere on Earth (Kunte, 2002). Understanding diversity within an environmental context is a necessary first step in studying survivability and adaptation of halophiles at different levels of tolerance. Second, because of the presence of hypersaline conditions on Mars (Catling, 1999, Moune, et al., 2003), studies of microbial diversity in terrestrial saline environments may shed light on extinct and/or extant life on Mars.

Microbial diversity in most hypersaline environments is often studied using culture-dependent and -independent methods (SSU rRNA gene analysis) (Oren, 2003). A variety of hapersaline environments have been surveyed for microbial diversity such as the Great Salt Lake in Utah, the Great Salt Plains of Oklahoma, the Dead Sea, the Mediterranean Sea, the Solar Lake in Sinai, Egypt, Antarctic hypersaline lakes, deep-sea brine sediments, and various salterns (evaporation ponds for salt-recovery) (Mancinelli, 2005, Oren, 2002, Ventosa, 2004). These previous studies have established that halophiles are distributed in both the *Archaea* and *Bacteria* domains. Within the domain *Archaea*, halophiles are classified in the *Halobacteriaceae*, the *Methanospirillaceae* and

the *Methanosarcinaceae*. All members of the family *Halobacteriaceae* are extreme halophiles (3 to 4 M salt). They are chemoheterotrophic and most members are aerobic. Halophiles are also widely spread within the domain *Bacteria*. Unlike archaea, most halophilic bacteria can only live at moderate salinity (up to 2.5 M salt). Bacterial halophlies vary widely in their physiological properties, including aerobic and anaerobic chemoheterotrophs, photoautotrophs, photoheterotrophs, and chemolithotrophs.

Despite these previous studies, our understanding of microbial diversity in hypersaline environments is still limited, especially in athalassohaline lakes (a saline lake not of marine origin but evolved from evaporation of fresh water) at high elevation. Lake Chaka in Northwestern China represents an ideal site for studying halophile diversity in such an environment. The lake is located on the Tibetan Plateau at an elevation of 4000 m above sea level, and possesses a salinity of 32.5% (or greater, depending on the season). The high salinity is developed via progressive evaporation of freshwater in the lake. The combination of high elevation (and thus high UV intensity) and salinity makes it an extreme environment. In addition, there exists a salinity gradient in the lake sediments, 32.5% or higher at the water-sediment interface to 0% at the 8-m depth (Jiang et al., unpublished data). This natural gradient is a result of progressive evaporation in the region over the past 50,000 years.

The goal of this research was to assess microbial diversity and abundance in lake water and shallow sediments (top 42-cm) in Lake Chaka and to correlate it with its environmental conditions. We integrated geochemical and microbiological approaches, including lake and pore water chemistry, sediment mineralogy and geochemistry, and culture-independent (SSU rRNA gene analysis) and -dependent microbiology. Bacterial halophiles were isolated under salinities that were similar to those measured in the lake water and sediment. We observed major differences in the microbial community between the lake water and the sediments, and these differences could be correlated with geochemical characteristics.

MATERIALS AND METHODS

Description of the study site. Lake Chaka (36º18´-36º45´N, 99º02´-99º12´E) (Fig.1) is a shallow salt lake in Northwestern China at an elevation of 3,214 meters above sea level. It possesses high salinity (32.5%). It is an elliptic and closed drainage basin, trending northwest-southeast in parallel to nearby mountains, and is located at the southern corner of Qaidam Basin, where a semiarid continental climate dominates. The Lake Chaka basin is approximately 80 km long and 30 km wide. Strong evaporation and little precipitation in this area (2264 mm evaporation versus 224 mm rainfall/year) have resulted in a nearly dry lake and high salinity (Liu, et al., 1996). The water depth and coverage of water in the lake vary seasonally. The area of liquid water in high-water period (summer) can reach 104 km^2 with an average water depth of 2 to 3 cm. The area of liquid water and water depth significantly decrease in the low-water period (winter). Average water temperature is 4.2^oC with -6 to -8^oC in winter and 6 to 20^oC in summer. Upper Pleistocene and Holocene rocks occur widely in the basin of Lake Chaka. The lake became progressively saline in the last 50,000 years. Liu et al. (Liu, et al., 2004) reported that the majority of water supply (80%) to the lake is from rivers and spring water in the surrounding area. The authors reported that the source waters contain high concentrations of cations and anions and concentrations as follows (mg/liter): Na^+ (145 to 343), K^+ (3 to 9), Mg^{2+} (26 to 72), Ca^{2+} (52 to 81), Cl^-(188 to 386), SO_4^{2-} (112 to 330), CO_3^{2-} (10 to 12), HCO_3^- (218 to 270).

Field measurements and sampling. Field measurements and sampling were conducted in August 2003. pH, temperature, and salinity were measured with pH and conductivity probes (water depth at the site of 2 to 3 cm). Field colorimetric Hach kits were used to measure soluble Fe (Fe^{2+}), sulfide, sulfate, phosphate, nitrite, and nitrate concentrations. Water samples were collected with 50-mL sterile centrifuge tubes. A sediment core of 42 cm by 8 cm in dimension (length by diameter) was collected using a gravity coring device. After collection, the samples were immediately stored in a refrigerator at 4ºC. Within two days, the samples were shipped clod (4ºC) to China University of Geosciences in Beijing and then shipped in a cooler (regular ice) to Miami University in Ohio. For the lake water sample, enrichments were set up and clone libraries were constructed. The sediment core was dissected into 2-cm-long sediment subsamples inside a Coy glove box filled with 95% N_2 and 5% H_2 (Coy Laboratory

Products, MI). The external layers of the sediment subsamples were removed using sterile tools. Five subsamples, designated as LCKS0 (the water-sediment interface), LCKS10 (10-12 cm depth), LCKS20 (20-22 cm depth), LCKS30 (30-32 cm depth), and LCKS40 (40-42 cm depth), were geochemically and microbiologically analyzed. The analyses included measurements of total organic carbon (TOC), mineralogy by X-ray diffraction (XRD), total microbial counts by acridine orange direct counting (AODC), and microbial diversity by SSU rRNA gene analysis. Phospholipid fatty acids (PLFA) analysis was performed for LCKS0, LCKS20 and LCKS40. Enrichments and isolations were performed for LCKS0. Samples were coded as follows, with LCKS20 as an example: LCKS, Lake Chaka sediment; 20, sediment sample depth in centimeters. Lake water samples were coded as LCKW.

Laboratory chemical analyses of water samples. Anion and cation compositions of the lake water sample were analyzed by high performance liquid chromatography (HPLC) and direct current plasma emission spectrometry (DCP). For determination of total organic carbon (TOC) content, the lake water sample was acidified to pH 3 to remove inorganic carbon, followed by analysis with an organic carbon analyzer (TOC-5000A, Shimadzu). Because of paucity of pore water in the sediments, "artificial pore water" was created and analyzed for acetate, lactate, formate, and sulfate concentrations. The "artificial pore water" was created by leaching 1 g of each sediment subsample with 50 ml of deionized water for 4 h, followed by centrifugation. The leaching step was repeated until all chemical species were leached. Subsequent analyses showed that one step was sufficient to completely leach acetate, lactate and formate, and but multiple steps (3-4 steps) were required to leach sulfate. For analyses, the supernatants from multiple steps were combined. Acetate, lactate, formate, and sulfate concentrations were measured using HPLC. An IonPac®AS11-HC column (4 by 250 mm) was used for acetate, lactate, and formate measurements, and an IonPac®AS14 column (4 by 250 mm) for sulfate. The concentrations were reported as micromolars per gram of wet sediment and were assumed to be proportional to those in natural pore water.

Sediment geochemistry. XRD was employed to analyze the five sediment subsamples (LCKS0, LCKS10, LCK20, LCKS30, and LCK40) for mineralogy following a previously used procedure. Concentrations of TOC, total nitrogen (TN), bioavailable

phosphorus, and soluble salt were determined in the Service Testing and Research laboratory of the Ohio State University. TOC content was analyzed with the dry combustion method, and total inorganic carbon was determined by U.S. Environmental Protection Agency method 9060A. These methods are available on-line at http://www.oardc.ohio-state.edu/starlab/references.htm. Bioavailable phosphorus was analyzed by following a previously published method (Kuo, 1996). The amount of soluble salts in the sediments was measured as the conductivity of the solution (mS/cm) when a certain amount of sediment (typically, 5 g) was mixed with an equal amount of water (5 g), following the methods described in (Rhoades, 1996).

Total microbial counts. AODC was performed for both the lake water and the sediments to determine the total microbial counts. For the lake water sample, 10 mL of water was stained and counted (Dong, et al., 2005). For the sediment samples, microbial cells were first detached from sediments (Bottomley, 1994) followed by staining and counting.

PLFA analyses of the sediment samples. Three sediment subsamples (LCKS0, LCKS20, and LCKS40) were chosen for PLFA analysis and shipped frozen (-80^{o}C) to Microbial Insights, Inc. (Rockford, TN). PLFAs were analyzed after extraction of the total lipid (White, et al., 1979) and separation of the polar lipids by column chromatography (Guckert, et al., 1985). The polar lipid fatty acids were derivatized to fatty acid methyl esters, which were quantified using gas chromatography (Ringelberg, et al., 1994). Fatty acid structures were verified by chromatography/mass spectrometry and equivalent chain length analysis.

Enrichment and isolation of microbes present in lake water. Enrichment experiments were performed for the lake water and one sediment sample (LCK0, the water-sediment interface sample) with a modified Kauri medium (Kauri, et al., 1990) for halophiles. Diluted modified R2A (DMR2A) was used for nitrate-reducers, and modified MB (MMB) medium (Difco) was used for general heterotrophs. The enrichment experiments were performed in 28-mL capacity Bulsch culture tubes incubated at 30ºC in a water bath with 60 rpm shaking. pH value for all three media was 7.0. Gradients of NaCl salt (5%, 10%, 15%, 20% and 25%) were used in multiple tubes to target halophiles with the modified Kauri medium. DMR2A was made as previously described except that

all final concentrations were diluted fivefold (Fries, et al., 1994). MMB medium contained the following, per liter: 0.5 g of sodium actetate, 0.5 g of yeast extract, 4.7 g of Middlebrook 7H9 (Difco), 0.5 g of Casamino acids, 0.5 g of sodium thiosulfate, 10 ml of mineral solution, and 2 g of NaCl. The mineral solution was the same as that used for DMR2A. Positive enrichments were transferred three times. The enrichment cultures were streaked onto agar plates consisting of the original enrichment media supplemented with 2% agar. The plating step was repeated three times to ensure purity. The colonies from the final set of agar plates were grown in liquid medium, preserved in 35% glycerol, and frozen at –80^{o}C for later analyses.

Physiological testing of halophilic isolates. The influence of temperature on growth was studied by incubation of inoculated medium (the modified Kauri medium) at temperatures between 10^{o}C and 5^{o}C with shaking (210 rpm) for 96 h. The influence of pH on growth was studied in the same medium, with the pH value varying from 4.9 to 9.0 (adjusted by HCl or NaOH). The culture tubes were incubated at 37oC (the optimum growth temperature for the isolates) with shaking (210 rpm). The cell growth was monitored with a spectrophotometer (600 nm). Experiments were performed to test for the NaCl and $MgCl_2$ requirement of the isolates by changing the NaCl and $MgCl_2$ concentrations in the Kauri medium (0-35% with 5% increments for the NaCl and $MgCl_2$ test). The culture tubes were incubated at 37^{o}C and pH 7.0 with shaking (210 rpm). The cell growth was monitored as above.

To test UV resistances of the halophilic isolates, two UV light sources with different wavelengths were used: 312 nm (UVB; EB28OC, 620 μm/cm^2 at a 6-in. distance; Spetronics Corp.) and 254 nm (UVC; UVG-11, 120 μm/cm^2 at a 3-in. distance; UVP Inc.). Cells (10^8 cells/ml) were irradiated at different doses of UV radiation using a previously described method (Baliga, et al., 2004). The viable cell numbers were determined by plate counts on agar plates (the Kauri growth medium supplied with 2% agar). The survival rate was calculated by dividing the number of the remaining cells by the initial number of cells. To test the resistance of the isolates to gamma radiation, cells were exposed to 0.0 to 7.0 kGy of gamma rays at room tempearture using a 3,600-Ci ^{60}Co source located at the Ohio State University Nuclear Reactor Laboratory at a dosage rate of 1.29 kGy/h. The number of viable cells was counted immediately postirradiation

with plate counts (the Kauri growth medium supplied with 2% agar). The survival rate was calculated in a similar manner.

PCR amplification and sequence determination of the isolates. For the isolates obtained from the DMR2A and MMB media, the following PCR amplification procedure was used. Cell lysates were made of isolated microorganisms for the PCR amplification of the SSU rRNA genes by boiling cells suspended in Tris-EDTA buffer for 5 min at 100°C. PCR reactions were treated with SeqMix (Q-Biogene, Irvine, CA) prior to sequencing reactions according to the manufacturer's instructions. The SSU rRNA gene was amplified with the universal primers FD1: 5' AGA GTT TGA TCC TGG CTC AG-3' and 1540R: 5'-GGA GGT GWT CCA RCC GC-3' as previously described (Yakimov, et al., 2001).

For the isolates obtained using the modified Kauri medium, the following procedure was used. Freshly grown isolates were suspended in boiling water for 10 min. The SSU rRNA gene was amplified with the bacterial forward primer Bac27F: 5'-AGA GTT TGG ATC MTG GCT CAG -3' and universal reverse primer Univ1492R: 5'-CGG TTA CCT TGT TAC GAC TT -3', or archaeal specific primers Arch21F (5'-TTC YGG TTG ATC CYG CCR GA-3'), 925R (5'-CCG TCA ATT CMT TTR AGT TT-3') and Univ1492. A typical PCR reaction (25 μL in volume) contained the following components: 10 mM Tris pH 8.3, 50 mM KCl, 1.5 nM $MgCl_2$, 200 μM each dNTP, 0.2 μM each primer, and 1.25 unit Taq DNA polymerase. The following standard conditions were used for bacterial 16S rRNA gene amplification: initial denaturation at 95°C for 5 min; 35 cycles of denaturation (30 s at 94°C), annealing (30 s at 55°C), and extension (2 min at 72°C); and a final extension at 72°C for 7 min. The standard conditions for amplification of archaeal 16S rRNA gene were: initial denaturation at 95°C for 5 min, 45 cycles of denaturing (30 s at 94°C), annealing (30 s at 54°C), and extension (2 min at 72°C); and a final extension at 72°C for 10 min. The PCR products were purified with GeneClean®Turbo kit (Qbiogene Inc., Irvine, CA) according to the manufacturer's suggested protocol.

For the sequence determination with ET Dye chemistry (Amersham Pharmacia Biotech Inc., Piscataway, NJ), primers FD1 and 529R (5′-CGC GGC TGC TGG CAC-3′)

and 1540R were used for the isolates obtained with DMR2A and MMB media. Primers Bac27F and Arch21F were used for the isolates obtained with the modified Kauri medium. All sequences were determined with an automatic 3100 DNA sequencer. The sequences were tested for chimeras by using the Ribosomal Database Project Chimera-Check program and were aligned with ClustalW. Phylogenetic analyses of partial 16S rRNA gene sequences were conducted using the MEGA (molecular evolutionary genetics analysis) program, version 2.1. Neighbor-joining phylogenies were constructed from dissimilatory distances and pair-wise comparisons with the Jukes-Cantor distance model.

Clone library construction for the lake water and sediment samples. Genomic DNA in the lake water sample and the sediment samples (0.5-0.7 gram) (LCKS0, LCKS10, LCKS20, LCKS30, and LCKS40) was extracted and purified with Ultra Clean Soil DNA Isolation Kit (MoBio Laboratories, Inc., Solana Beach, CA). Purified DNA from the samples was PCR-amplified according to the procedure of Failsafe Kit (Epicentre Communications Inc., Sausalito, CA). The PCR conditions were the same as those for the isolates (from the modified Kauri medium). Primer sequences for bacteria were Bac27F and Univ1492 and those for archaea were Arch21F and Arch958R (5'-YCC GGC GTT GAM TCC ATT T-3').

The PCR product was ligated into pGEM-T vector (Promega Inc., Madison, WI) and transformed into *Escherichia Coli* DH5α competent cells. The transformed cells were plated on Luria-Bertani (LB) plates containing 100 μg/mL of ampicillin, 80 μg/mL of X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) and 0.5 mM IPTG (isopropyl-b-D-thiogalactopyranoside) and incubated overnight at 37 ºC. Gene clone libraries of 16S rRNA were constructed, and 40-50 randomly chosen colonies per sample were analyzed for insert 16S rRNA gene sequences. Plasmid DNA containing inserts of 16S rRNA gene was prepared using QIAprep Spin Miniprep Kit (Qiagen). Sequencing reactions were carried out with primer Bac27F for bacteria and Arch21F for archaea with a DYEnamic ET terminator cycle sequencing ready reaction kit (Amersham Biosciences, Piscataway, NJ). The 16S rRNA gene sequence was determined with an ABI 3100 automated sequencer. Sequences were typically ~600-700 bp long. Phylogenetic analyses were carried out in the same manner as above.

Statistical analysis and sequence population diversity. We followed the approach of Humayoun et al. (Humayoun, et al., 2003) for these analyses. One major assumption was that sequences with similarities of greater than 97% were considered to represent the same phylotypes for the reasons stated in that study. 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. Rarefaction curves were constructed using software available on line at http://www.uga.edu/~strata/software.html.

LIBSHUFF (version 1.2) analysis was performed to compute the homologous and heterologous coverage within and between clonal libraries (Singleton, et al., 2001). The analysis estimates the similarity between clonal libraries from two different samples based upon evolutionary distances of all sequences. Thus, the sampled diversity of a community can be directly compared to another community. The predicted coverage of a sampled library is denoted by the homologous coverage, and the heterologous coverage is the observance of a similar sequence in a separate library. The values are reported over a sequence similarity range or evolutionary distance based upon a distance matrix. Analyses were performed accoding to specified directions given at the LIBSHUFF website (http:/www.arches.uga.edu/~whitman/libshuff.html).

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in the GenBank database under accession numbers DQ129871-DQ129877 and DQ247815-DQ247820 for the bacterial isolate sequences from the lake water, DQ395131 for the bacterial isolate sequence from the sediment (LCKS0), DQ129878-DQ129952 for the bacterial clone sequences, and DQ129953-DQ129989 for the archaeal clone sequences.

RESULTS

Lake water chemistry. Field measurements in August 2003 showed that salinity was 32.5%, with a pH of 7.4 and temperature of 16-17^{o}C. The colorimetric measurements indicated the following concentrations in lake water (μg/g): Fe^{2+}, 0.3; sulfide, 1; phosphate, 3; nitrite, 2. DCP analyses of lake water determined the concentrations of

major cations (mg/L) as: Li^+ (11), Na^+(73,311), NH_4^+ (25), Mg^{2+}(33,179), K^+ (4,957), Ca^{2+} (326), and Fe (2). HPLC analyses determined the concentrations of major anions (mg/L) as Cl^- (181,586), SO_4^{2-}(31,350), Br^-(72), F^-(292), HCO_3^- (170), NO_3^- (7), and $PO4_3^-$ (2).

Sediment properties and pore water geochemistry. Although a direct measurement of the redox state was not possible, visual observation indicated that the lake sediments were anaerobic. Gas bubbles were observed when sediments were disturbed, and an odor of hydrogen sulfide gas was detected. The sediments were dark. Thus, we inferred that the redox boundary was at the water-sediment interface (further confirmed below). XRD analysis of the sediment samples identified major minerals halite, quartz, kaolinite, and muscovite. Acetate, formate, and sulfate concentrations in the artificial pore water were 0.5 to 3.8, 0 to 1, and 70 to 200 μmol/g (wet sediment), respectively (Fig. 2A). Lactate was not detectable. TOC and total nitrogen content in the sediments were 0.8 to 1% and 0.11 to 0.17%, respectively (Fig. 2B). Bioavailable phosphorus was < 1 μg/g except for LCK0 (~1 μg/g). Soluble salt concentration in the sediments was in the range of 49 to 64 mS/cm (or ~3 to 4% assuming 1 mS/cm = 640 ppm).

AODC and PLFA data. Total cell counts in the lake water were 4.8×10^6 cells/ml. The total cell counts were higher in the sediments, ranging from 4.0×10^8 cells/g (dry weight) at the water-sediment interface to 4.2×10^7 cells/g at the 42-cm depth (Fig. 2C). Viable bacterial abundance in the sediments as determined by the total PLFA concentration (assuming a laboratory-determined conversion factor of 20,000 cells/pmole, (Chapelle, 2000)) was consistently lower than the AODC-determined cell biomass, ranging from 2.1×10^8 cells/gram of dry weight for LCKS0 to 1.7×10^7 cells/gram for LCKS40 (Fig. 2C). The difference between the AODC counts and PLFA biomass could be taken to indicate the archaeal abundance.

PLFA profiles for the sediment samples indicated that the proportion of terminally branched saturated fatty acids, indicative of *Firmicutes* or anaerobic Gram-positive bacteria, increased from approximately 22% in LCKS0 to 33% in LCKS40 (Table 1). The proportion of monoenoic fatty acids, indicative of Gram-negative bacteria, decreased from approximately 30% in LCKS0 to 20% in LCKS40. The proportion of the

characteristic biomarker for anaerobic metal reducers (branched monoenoic PLFA) increased from 0.8% in LCKS0 to 1.3% in LCKS40. Sulfate reducing biomarkers (mid-chain branched PLFA) remained the same at approximately 3 to 4% among the three samples. Eukaryotic biomarkers in LCKS0 and LCKS40 decreased from 6% in LCKS0 to 3% in LCKS40. Physiological status biomarkers indicated that the samples were undergoing a similar and moderate level of starvation. Sample LCKS40 showed a moderate level of microbial response to environmentally induced stress (i.e., *trans/cis* ratio of 0.57).

Isolate characteristics. Bacteria were isolated from both the lake water (*n*=10) and the sediment (*n*=8, from LCKS0) with three different media. Archaea were isolated from the lake water (*n*=3) with one medium (the modified Kauri medium). Bacterial isolates with significant sequence similarity to predominant clones were not obtained from the lake water sample. Archaeal isolates with moderate sequence similarity (~90%) to clones were obtained. All eight bacterial isolates from the sediment sample LCKS0 were similar to each other, and they were closely related (~97% similarity) to several clones from the same sample. Six bacterial isolates from the lake water and eight from the seidment were related to the genus *Halomonas* of the *Gammproteobacteria* group Itable 2). Two bacterial isolates from the water sample could be classified as *Firmicutes*, and another was classified as *Actinobacteria*. Three archaeal isolates from the water sample were closely affiliated with several species of the genus *Haloarcula* (Table 2).

Physiological testes were performed for some representative isolates. All water isolates tested were aerobic and exhibited an optimum growth temperature of 37^{o}C, pH of 7 to 8, and $MgCl_2$ tolerance of 2.5 to 5% (Fig. 3A). Whereas bacterium 10A exhibited the maximum growth rate at ~5% salinity, bacterium 25N and archaea 15A and 20A exhibited an optimal salinity of ~25%. Likewise, isolates E and W (representative of I, U, V, and G) could grow in the presence of up to 20% sodium chloride, but the maximal growth rate was observed in 5%. Growth was not observed when the sodium chloride concentration was above 25% (data not shown).

The isolates exhibited significant resitance to UV and gamma radiation (Fig. 3B), but the resistance levels were lower than that of *Halobacterium* strain NRC-1 (Baliga, et al., 2004, Kottemann, et al., 2005), an extremely halophilic archaeon. The bacterial

isolate 10A, which exhibited a low optimum salinity requirement (5%), showed a low resistance to UV and gamma radiation. One representative bacterial isolate from the sediment showed a slightly lower optimum growth temperature of 30^{o}C, optimum pH of 8, NaCl concentration of 5%, and $MgCl_2$ concentration of 7.5%. In contrast to the isolates from the lake water, the sediment isolate did not require any salt for growth.

Bacterial diversity. (i) Phototrophic bacteria. Three sequences from the LCKW library were related (99%) to phototrophic bacterium BN 9624 isolated from Abu Gabara Lake (Wadi Natrun, Egypt), which exhibits 36% salinity (Imhoff, et al., 1978).

(ii) *Alphaproteobacteria*. One sequence showed 94% similarity to *Alphaproteobacteria* strain ML6 (AJ315682) (Fig. 4A) from Mahoney Lake, South Central British Columbia (Yurkova, et al., 2002). Mahoney Lake is a meromictic saline lake (0.4 to 4%) with a surface pH of 9 and a pH of 8 near the chemocline. ML6 is an aerobic phototrophic bacterium that can use various organic carbon sources.

(iii) *Betaproteobacteria*. Nineteen sequences were affiliated with the *Betaproteobacteria* (Fig. 4A). One sequence (from LCKS0) was related to (98%) *Delftia acidovorans* strain B (AB074256), 7 (from LCKS10) related to (97%) *Petrobacter succinatimandens* (AY219713) and *Tepidiphilus margaritifer* (AJ504663), and 11 (from LCKS20) related to (97%) *Petrobacter succinatimandens* BON4 and strain HMD444 (AB015328). BON4 is a moderately thermophilic and nitrate-reducing bacterium that can grow optimally at pH 7.0 and 0.5% NaCl (tolerance up to 3% NaCl).

(iv) *Gammaproteobacteria*. Twenty-eight sequences were related to the *Gammaproteobacteria* (Fig. 4A). Many sequences in this group were related to several species of the genus *Halomonas*. *Halomonas*, a family of Gram-negative Proteobacteria, can tolerate or require a high salt concentration for growth. especially *H. salina*, *H. variabilis*, and *H. ventosae*. *Halomonas*, a family of Gram-negative *Proteobacteria*, can tolerate or require high concentration of salt for growth (Ventosa, et al., 1998).

Another group of clone sequences were related to (98-99%) *Stenotrophomonas* sp. An30 and 27 (AJ551168/5). An30 and 27 were obtained from deep-sea sediments in the west Pacific (GenBank description). One clone sequence (LCKS10-B8) was related to (97% similarity) bacterium HTB082 (AB010842) from deep-sea sediments from the Nankai Islands Iheya Ridge (1,050-m depth) (Takami, et al., 1999). HTB082 can grow

optimally at pH 7.6 and 3M (17.6%) NaCl. Two clone sequences were related to (96%) *Alcalilimnicola halodurans* (AJ404972) isolated from a water-covered site of Lake Natron, Tanzania. *Alcalilimnicola halodurans* can grow in the presence of 0-28% NaCl (w/v) with optimum growth at 3-8% NaCl (w/v) and pH above 8.5 (Yakimov, et al., 2001).

(v) *Epsilonproteobacteria*. Two sequences were grouped into the *Epsilonroteobacteria* (Fig. 4A) with 99% similarity to *Campylobacter* sp. NO3A (AY135396). NO3A is capable of oxidizing lactate with nitrate or nitrite as the electron acceptor.

(vi) Unclassified *Proteobacteria*. Three sequences (Fig. 4A) were related to an environmental clone (AY940550) recovered from Qinghai Lake, which is a saline (1.3% salinity) and alkaline (pH 9.4) lake in the same area as Lake Chaka (Dong, et al., 2005).

(vii) *Firmicutes* (Low G+C gram-positive bacteria). The low G+C Gram-positive clone sequences predominated the bacterial clone libraries (i.e. 71 out of 123 sequences) and were grouped into nine clusters (Fig. 4B). Sequences of 24 clones and 3 isolates formed Cluster 1. The majority of the sequences in that cluster were closely related to (98 to 99%) *Bacillus arseniciselenatis* strain E1H (AJ865469), a moderate halophile and alkaliphile from Mono Lake, Calif. E1H is a obligate anaerobe that is able to use Se(VI), As(V), Fe(III), nitrate, and fumarate as electron acceptors (Blum, 1998). Mono Lake is an alkaline (pH 9.8) and hypersaline (84 to 94 g/liter) soda lake. Four sequences in Cluster 1 were closely related to (98 to 99%) *Paraliobacillus ryukyuensis* (AB087828), a slightly halophilic, extremely halotolerant alkaliphilic anaerobe isolated from marine alga. It can tolerate NaCl concentration of 0 to 22% (optimum 0.75 to 3.0%) and grow at pH 5.5-9.5 (optimum 8.5) (Ishikawa, et al., 2002).

Ten clone sequences formed Cluster 2 (Fig. 4B), showing 99% similarity to an unidentified Hailaer soda lake bacterium F1 (AF275700) and *Alkalibacterium* sp. A-13 (AY347313) isolated from hypersaline Tanzania soda lakes (GenBank description). Three clone sequences formed Cluster 4 (Fig. 4B). These sequences were related to (94-95% similarity) *Halocella cellulosilytica* (X89072) and *Halanaerobium lacusrosei* (L39787), two isolates from hypersaline lake sediments or lagoons (Cayol, et al., 1995, Simankova, et al., 1993). *Halocella cellulosilytica* and *Halanaerobium lacusrosei* are

obligate anaerobes with optimal growth at pH 7.0 and NaCl concentrations of 15% and 20%, (w/v), respectively.

Eight clone sequences formed Cluster 5 (Fig. 4B). Five sequences were related to (99%) an uncultured low G+C Gram-positive bacterium (AJ495676) obtained from anoxic sediments underlying cyanobacterial mats of two hypersaline ponds in Mediterranean salterns (Moune, et al., 2003). These two hypersaline ponds have similar salinity to Lake Chaka (15-20% and 25-32% salinity, respectively). The rest of the sequences were related (90-99% similarity) to an uncultured low G+C Gram-positive bacterium (AF507875) from Mono Lake, CA.

In Cluster 6, one sequence was closely related to (98%) *Thermoanaerobacter ethanolicus* strain X513 (AF542520) isolated from the deep subsurface environments of the Piceance Basin, Colorado (Roh, et al., 2002). Strain X513 can use lactate, acetate, succinate, xylose, and glucose to reduce Fe(III) oxyhydroxide to form magnetite. Nine clone sequences formed Cluster 7 and they were related to (97%) *Alkaliphilus transvaalensis* (AB037677), which is extremely alkaliphilic (optimum pH of 10) and halotolerant (~4% sea salt) from mine water at 3.2 km below the land surface in an ultra-deep gold mine near Carletonville, South Africa (Takai, et al., 2001). Ten clone sequences and their relatives formed Cluster 9 (Fig. 4B). One of these relatives, *Anaerobranca californiensis* strain Paoha-1 (AY064218), was isolated from a hot spring in Mono Lake (Gorlenko, et al., 2004). It is an anaerobic, alkalithermophilic, fermentative bacterium, with an ability to reduce elemental sulfur, Fe(III), and Se(IV) in the presence of organic matter.

(viii) *Actinobacteria* (high G+C Gram-positive bacteria). Sequences of eight clones and one isolate were affiliated with the *Actinobacteria* group (Fig. 4B); the two closest relatives were *Nesterenkonia halotolerans* (AY226508) from hypersaline soil in Xinjiang Province, western China, and *Arthrobacter* sp. AS18 (AY371223) from lead zinc mine tailings in Huize county, Yunnan Province, China (Zhang, et al., 2004).

(ix) *Bacteroidetes*. Thirty-six clone sequences (34 from the LCKW and 2 from the LCKS0) were related to the *Bacteroidetes* group (Fig. 4B). They were 83-99% similar to cultivated and uncultivated *Bacteroidetes*, including *Salinibacter ruber* strain POLA 13 (AF323503), halophilic eubacterium EHB (AJ133744) and uncultured *Flavobacteriaceae*

bacterium (AF513959). POLA 13 was isolated from saltern crystallizing ponds in Mallorca, Balearic Islands, Spain, and can grow optimally in the presence of 150 to 300 g/liter total salt and at pH 6.5 to 8.0 (Anton, et al., 2002). The *Flavobacteriaceae* bacterium clone was obtained from hypersaline Lake Laysan and a brackish pond on Pearl and Hermes Atoll, Hawaiian islands (GenBank description).

Archaeal diversity in water and sediment. The majority of the archaeal sequences could be classified as the *Euryarchaeota* and *Crenarchaeota* (Fig. 5). The *Euryarchaeota* group consisted of two clusters. Eighty-nine clone sequences were grouped into a novel *Euryarchaeota* cluster within group III as defined by Jurgens et al. (Jurgens, et al., 2000), and the majority were closely related to (similarity 97 to 99%) an uncultivated archaeon (AY457656) from the Florida Everglades (Castro, et al., 2004) and an uncultivated archaeon (AJ310857) associated with methanogenic sediment in subtropic Lake Kinneret (Israel) (Nusslein, et al., 2001). Four clone sequences were related to an uncultivated archaeon (AY053471) associated with the Gulf of Mexico gas hydrates (Lanoil, et al., 2001).

Sequences of 3 archaeal isolates (from the lake water) and 26 clones (24, 1, and 1 from the lake water, LCKS0, and LCKS30, respectively) formed the *Halobacteriales* cluster in the *Euryarchaeota* group (Fig. 5). The isolates were phylogenetically related to (97 to 99%) several species of *Haloarcula. Haloarcula argentinensis* (D50849) is a halophilic archaeon isolated from the soils of the Argentine salt flats in Argentina (Ihara, et al., 1997) using a culture medium containing 16% NaCl. *Haloarcula marismortui* is another halophilic archaeon isolated from the Dead Sea. Its whole genome has been sequenced for further research (Baliga, et al., 2004). Most of the clone sequences from the lake water were related to clone sequences obtained along a transient soil salinity gradient at Salt Spring in British Columbia, Canada (Walsh, et al., 2005). Several sequences were related to (93%) *Halosimplex carlsbadense* (AB108676), which was isolated from salt crystals from the 250-million-year-old Salado formation in New Mexico (Vreeland, et al., 2002).

In the *Crenarchaeota* group, there were three small clusters. Four sequences formed the first and were closely related to (~98%) an uncultured archaeon (AY016470) recovered from coniferous forest and alpine tundra soils in the Front Range of Colorado

(GenBank Description). The second cluster contained two sequences related to uncultured archaea (U87519) retrieved from Lake Michigan sediment (MacGregor, et al., 1997). Three sequences formed the third. They were related to an uncultured archaeon (AF419646) from hydrothermal sediments in the Guaymas Basin, Gulf of California, Mexico. Three sequences were related to unclassified sequences (AJ578143 and AF119128) retrieved from methane seep areas (GenBank description) and deep-sea sediments from several stations in the Atlantic Ocean.

Distribution of bacterial and archaeal groups. The relative abundances of different phylogenetic groups in the bacterial clone libraries were calculated for all samples (Fig. 6). The phylogenetic compositions were significantly different between the lake water and the sediments. Whereas the clone library for the lake water was dominated by sequences affiliated with *Bacteroidetes* (63%), those of the sediment samples were dominated by sequences affiliated with *Firmicutes* (low G+C Gram-positive bacteria). Whereas the *Betaproteobacteria* were the second-most-abundant group in the LCKS10 and LCKS20 libraries, *Gammaproteobacteria* was the most abundant clones in the LCK30 library, followed by the low G+C Gram-positive group.

LIBSHUFF analysis was used to characterize the relationships between and among the different bacterial communities observed in the samples. The bacterial community in the water sample was markedly distinct from the communities in the sediment samples, and the communities in the sediment samples at 0-, 10- and 20-cm depth were the most similar (Fig. 7). The data suggested that the water sample was distinct from the sediments at any depth; that the interface, 10-, and 20-cm depths were more similar; and that the 30- and 40-cm depths were distinct from the other samples and each other. The results statistically significant ($p = 0.001$) with the number of clones analyzed; however, more clonal sequences need to be determined in order to elucidate possible relationships (e.g. succession) between the different depths and abiotic parameters.

The relative abundances of different phylogenetic groups in the archaeal clone libraries were also calculated for all the samples (Fig. 6). Again, there existed distinct difference in the phylogenetic compositions of the clone libraries between the lake water and the lake sediments. Whereas all the sequences in the library for the lake water were

affiliated with *Halobacteriales*, most sequences in the sediment libraries were related to clone sequences previously found in diverse environments (i.e., *Euryarchaeota* group III). Only a small percentage of sequences were related to the *Crenarchaeota* group.

Coverage of bacterial libraries. Coverage values and diversity index for the six bacterial clone libraries indicated that the bacterial sequence population from the interface sample was the most diverse and that from the lake water sample was the least diverse. There was a large range in the sequence coverage and diversity index. In contrast, the archaeal clone libraries showed a different trend. Although there was only one major phylogenetic group in the LCKW archaeal clone library, the diversity within that group was the highest, much greater than the inter-group and intra-group diversity of the other archaeal libraries (Table 3).

DISCUSSION

Microbial biomass. Comparisons between Lake Chaka and other hypersaline lakes indicated that the total abundance in the lake water was typical for such environments: total abundance was imilar to that in the Dead Sea (2×10^6 to 2×10^7 cells/ml) and lower than that in the Great Salt Lake, Utah ($\sim 7 \times 10^7$ cells/mL) (Oren, 1993). The small range in abundance in such diverse environments may suggest that salinity is the main factor in controlling microbial abundance. In general, abundance in unconsolidated sediments tends to be high, but in dry rock salt abundance can be low. Only the number of CFU has been reported in dry salt, ranging from 10 to 10^4 cells per g of dry salt (Kunte, 2002).

Isolate characteristics. The isolates from the water sample were obligately halophilic. Although the water isolates exhibited a range of salinity requirements, in general, either the optimum or the upper salinity limit (25 to 30%) was consistent with that in the lake water (32%). Phenotypic traits are difficult to predict from phylogenetic relationships, but the physiological characteristics of the isolates were in general accordance with presumptive phylogenetic positions. One exception was the bacterial isolate LCKW-Isolate25N, which exhibited an optimum salinity of 25%, whereas its closest relative, *Arthrobacter* sp. strain AS18, was a freshwater bacterium (Zhang, et al., 2004). Nonetheless, the isolates and the predominant clones from the water sample were consistent in showing a halophilic nature. In contrast, the isolates from the sediment

showed a halotolerant nature with an optimum salinity of 5% or lower, consistent with the salinity in the sediment.

Bacterial diversity. Although the bacterial community was largely dominated by halophilic and halotolerant microorganisms in all the samples studied, there was a distinct difference in the composition of the community structure between the lake water and the sediments. Whereas the clone library for the lake water was dominated by sequences affiliated with *Bacteroidetes* group (mostly related to halophilic bacteria), those for the sediments were dominated by sequences related to low G+C Gram-positive bacteria. The water-sediment interface sample was most diverse and composed of mixture of phylotypes that were present in both the lake water and the sediments. The distinct change in the bacterial assemblage across the water-sediment interface was likely caused by difference in salinity and the redox state. Salinity in the lake water was 32.5%, and the precipitate on the lake surface was nearly pure salt. In the sediments, however, there were other minerals (quartz, calcite and feldspars) in addition to halite salt. The quantitative measurement of soluble salts indicated that there were only about 3 to 4% soluble salts.

Although a direct measurement of the redox state was not possible, we infer from our qualitative observations that it also contributed to the observed difference in microbial community between the lake water and the sediments for the following reasons. First, visual observation indicated that the redox boundary was at the water-sediment interface (i.e., dark sediments with sulfide odor). Second, the LCKW clone libraries (both bacterial and archaeal) were dominated by sequences related to aerobic halophiles (such as *Salinibacter ruber*, *Bacillus vedderi*, halophilic eubacterium EHB and *Halosimplex carlsbadense*). Multiple aerobic halophiles were isolated in this sample. The dominant sequences in the LCKS0 clone library, which was immediately below the interface, were related to anaerobic bacteria (i.e., *Bacillus arseniciselenatis*, *Halanaerobium lacusrosei*, *Alkaliphilus transvaalensis*, and *Anaerobranca californiensis*) and to environmental clones from anaerobic sediments. The isolate from LCK0 (LCK0-Isolate1) was closely related to a facultative bacterium *Halomonas hydrothermalis* isolated from deep-sea hydrothermal vent environments (Kaye, et al., 2004). Third, the highest diversity was observed for LCKS0, which showed a mixture of

phylotypes observed for LCKW and LCKS0, consistent with the notion that the water-sediment interface was the redox boundary.

In addition to halophiles in Lake Chaka, clone sequences related to iron-reducing bacteria (i.e., *Thermoanaerobacter ethanolicus*) were also present. A group of clone sequences related to an anaerobic Fe(III) and Se(IV) reducer *Anaerobranca californiensis* strain Paoha-1 (Gorlenko, et al., 2004) suggested that Fe(III) and Se(IV) reducing activity may be present in the lake. The presence of selenate and selenite reducers has been reported in saline lakes (Blum, et al., 2001) and alkaline environments, and these types of microorganisms may be important in such environments. The relatedness of a group of clone sequences to *Alkaliphilus transvaalensis* further suggests iron-reducing activity in Lake Chaka. The genus *Alkaliphilus* was recently established (Takai, et al., 2001) and the type species was isolated from an alkaline, deep subsurface habitat. It has an optimal pH of 10 and salt concentration of 0.5%. Other species of the genus *Alkaliphilus* has been shown to tolerate higher salt content (up to ~10% NaCl) and to reduce metals such as Fe(III), Co(III), and Cr(VI) (Ye, et al., 2004).

Archaeal diversity. All archaeal isolate and clone sequences in the lake water were affiliated with the *Halobacteriales*, a group of extremely halophilic, aerobic archaea that have a salinity tolerance of 3 to 4 M salt. Representatives include *Haloarcula* species, *Halosimplex carlsbadense*, and haloarchaeon strain Nh.2. In contrast, all the archaeal clone libraries for the sediment samples were dominated by sequences that were grouped to form a distinct cluster (the *Euryarchaeota* group III) (Fig. 5). The shift was most likely caused by a change in salinity and the redox state across the water-sediment interface. A salinity-caused shift in archaeal community composition has been previously observed in saline soils (Vreeland, et al., 2002). Clones among this group have been reported in a diverse range of environments, including marine sediments (Vetriani, et al., 1998) and the deep sea (Fuhrman and Davis, 1997). Most of the refence clone sequences in this group were obtained from methanogenic sediments, such as soils in the Florida Everglades (Castro, et al., 2004) and sediments in Lake Kinneret (Israel) (Nusslein, et al., 2001).

The facts that all the sequences in *Euryarchaeota* group III were closely related to environmental clones from freshwater lake in Finland (Fig. 5, VAL2 and VAL147) and that those clones belonged to *Thermoplasmales* (Jurgens, et al., 2000) suggest that this

cluster may belong to *Thermoplasmales*. Interestingly, sequences that belong to *Thermoplasmales* have been reported to be present in another hypersaline lake, Solar Lake, Sinai, Egypt (Cytryn, et al., 2000), and in saline soils (Walsh, et al., 2005). However, definitive identification must await acquisition of pure isolates and functional testing in future research.

In conclusion, we employed culture-dependent and –independent techniques to examine the difference in microbial diversity in the lake water, the water-sediment interface, and the sediments in a hypersaline lake in northwestern China. All these regions in the lake were inhabited by abundant microorganisms, which include representatives of *Bacteria* and *Archaea*. A significant difference in community structures was observed between the water, the water-sediment interface, and different depths of sediment. We attributed the observed differences to the high salinity in the water and the lower salinity in the sediments, and both from phylogenetic relationships and phenotypic characteristics of field isolates corroborated this idea. Differences in the redox state, i.e., oxidizing in the lake water and reducing in the sediment, most likely contributed to the observed differences as well.

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Table 1. PLFA composition pf samples from Chaka Lake

Sample Name	Biomass	% of total PLFA[a]						Physiological Status	
	Cells/g	TerBrSats	Monos	BrMonos	MidBrSats	Nsats	Polyenoics	Starved (cy/*cis*)[b]	Stress, *trans/cis*
LCK0	2.05E+08	22.1	29.7	0.8	3.6	37.4	5.9	0.47	0.17
LCK20	3.80E+07	22.5	28.1	0.7	3.1	43.1	2.5	0.47	0.29
LCK40	1.65E+07	32.5	19.2	1.3	3.7	41.0	2.9	0.43	0.57

[a] PLFA type: TerBrSats: Terminally branched saturated; Monos: Monoenoic; BrMonos: Branched monoenoic; MidBrDats: Mid-chain-branched saturated; Nsats: Normal saturated.

[b] cy, cyclopropyl.

Table 2. Microbial isolates obtained from the lake water and sediment samples

Isolate name and type	% Similarity to closest match	Closest match (GenBank accession no.)
Bacteria		
LCKW-IsolateE, I, V, and U	99	*Halomonas variabilis* (AY505527)
LCKW-Isolate 10A and 10N	99	*Halomonas salina* (AY505525)
LCKW- Isolate15N	98	Bacillus vedderi (Z48306)
LCKW- IsolateG	100	*Gracilibacillus* sp. strain BH 235 (AY762980)
LCKW- IsolateW	98	*Gracilibacillus* sp. strain BH 235 (AY762980)
LCKW- Isolate25N	100	*Arthrobacter* sp. strain AS18 (AY371223)
LCKS0-Isolate1[a]	99	*Halomonas hydrothermalis* (AF212218)
Archaea		
LCKW- Isolate15A	99.5	*Haloarcula marismortui* (X61689)
LCKW- Isolate20A	99.5	*Haloarcula marismortui* (X61689)
LCKW- Isolate20N	100	*Haloarcula argentinensis* (D50849)

Table 3. Coverage and diversity index for the clone libraries for the lake water and sediments from Chaka Lake

Sample (depth cm)	Bacteria		Archaea	
	% Coverage	N_t/N_{max}	% Coverage	N_t/N_{max}
LCKW (water)	98	2	66.7	4.5
LCKS0 (0-2)	76.4	8.5	86.4	1.3
LCKS10 (10-12)	79.2	4.8	85	1.2
LCKS20 (20-22)	80	3.9	85.7	1.2
LCKS30 (30-32)	82.6	5.8	70	1.7
LCKS40 (40-42)	81.3	2	89.5	1.1

FIGURES CAPTIONS

Figure 1. Location of Lake Chaka, northwestern China. Lake Chaka is a hypersaline lake on the southeastern corner of Qaidam Basin. The coring site is shown on the map.

Figure 2. A). Depth distributions of acetate, formate, and sulfate concentration in the Lake Chaka sediments as determined in the "artificial pore water" created by leaching the lake sediments with distilled water; B). Distribution of total nitrogen and organic carbon in the lake sediments; C). Microbial abundance distribution in the lake sediments as determined by AODC and PLFA analysis. Single samples were used, and so sample-to-sample variability assessment was not possible. Single samples were measured more than once, and analytical errors were smaller than the symbol sizes.

Figure 3. (A) Cell growth as a function of temperature, pH, and concentrations of NaCl and $MgCl_2$. LCKW-Isolate10A and LCKW-Isolate25N are bacterial isolates from the lake water. LCKW-Isolate15A and LCKW-Isolate20A are archaeal isolates from the lake water. LCK0-Isolate1 is a bacterial isolate from the sediment (LCKS0). (B) Survival rate of the bacterial and archaeal isolates in response to increasing UV and gamma radiation. The UV and gamma radiation resistance data for Halobacterium strain NRC-1 (Baliga, et al., 2004, Kottemann, et al., 2005) are plotted for comparison. *N/N0*, the number of cells remaining after irradiation/initial number of cells.

Figure 4 (A) Neighbor-joining tree (partial sequences, ~600 bp) showing the phylogenetic relationships of bacterial 16S rRNA gene sequences cloned from the Lake Chaka samples to closely related sequences from the GenBank. One representative clone type within each phylotype is shown, and the number of clones within each phylotype is shown at the end (after the GenBank accession number). If there is only one clone with a given phylotype, the number 1 is omitted. Clone sequences from this study are coded as follows for the example of LCKS30-B9: LCKS: Lake Chaka sediment; 30, sample depth in centimeters; B, bacterium; 9, clone number. The isolates were obtained from the lake water only.

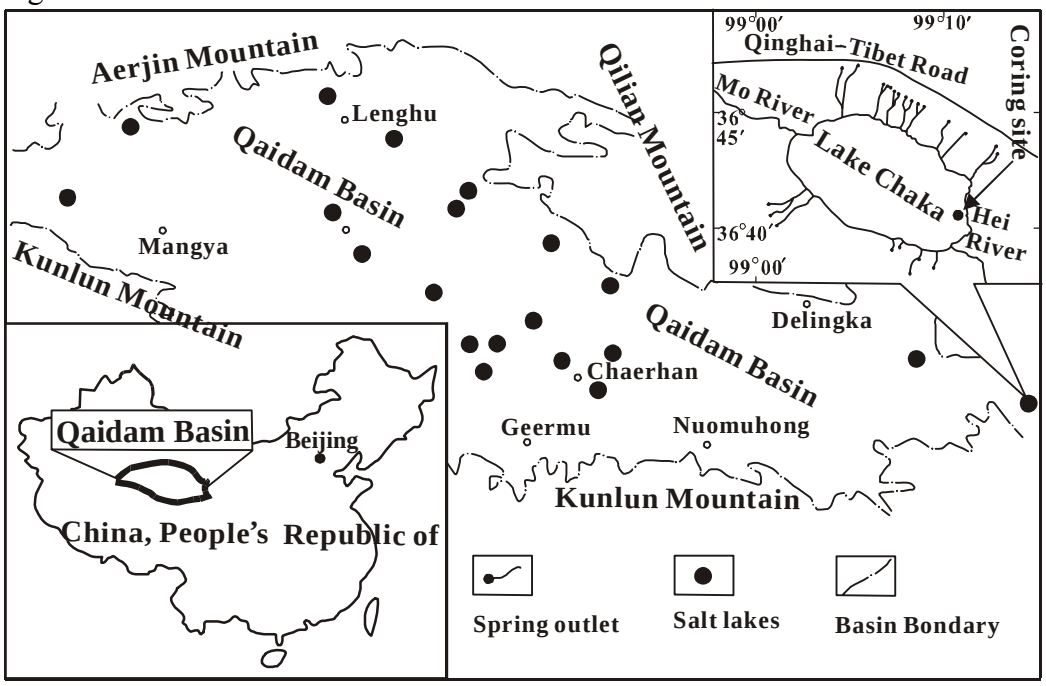
They are coded as follows: LCKW-isolate25N, Lake Chaka water, isolate25N. Scale bars indicate Jukes-Cantor distances. Bootstrap values of >50% (for 500 iterations) are shown. *Aquifex pyrophilus* is used as an outer group, and a single tree showing all bacterial sequences is created. Because of its large size of the tree, it is divided into two subtrees. Panel A is the first bacterial subtree showing the *Alpha-*, *Beta-*, *Gamma-*, *Epsilon-, and Delta-proteobacteria*. (B) This figure is the second subtree showing the *Firmicutes* (low G+C Gram-positive bacteria), *Bacteroidetes*, and *Actinobacteria*)

Figure 5. Neighbor-joining tree (partial sequences, ~600 bp) showing phylogenetic relationships of archaeal 16S rRNA gene sequences cloned from the Lake Chaka samples to closely related sequences from the GenBank database. The same algorithms as those for the bacterial tree (Figure 4) were used. *Aquifex pyrophilus* is used as an outer group. One representative clone type within each phylotype is shown and the number of clones within each phylotype is shown at the end (after the GenBank accession number).

Figure 6. Frequencies of phylotypes affiliated with the major phylogenetic groups in the bacterial and archaeal clone libraries for LCKW, LCKS0, LCKS10, LCKS10, LCKS20, LCKS30, and LCKS40. Alpha, Beta, Gamma, Delta, and Epsilon are *Alpha-, Beta-, Gamma-, Delta-*, and *Epsilon-proteobacteria*, respectively. High G+C: High G+C gram-positive.

Figure 7. Clustering of the differenct bacterial clone libraries based upon ΔCxy values deteremined from LIBSHUFF analysis. The tree was constructed with the unweghted-pair group method using average linkages in MEGA2. The parameter ΔCxy in the LIBSHUFF analysis represents the difference in coverage of the two sequence libraries (an increased ΔCxy represents greater dissimilatory between the given communities). The software for the analysis was used according to specified directions (http://whitman.myweb.uga.edu/libshuff.html).

Fig.1


Aerjin Mountain
Lenghu
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Qilian Mountain
Mangya
Kunlun Mountain
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99°10′
Qinghai–Tibet Road
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Mo River
36°45′
Lake Chaka
Hei River
36°40′
99°00′
Delingka
Qaidam Basin
Chaerhan
Geermu
Nuomuhong
Kunlun Mountain
Qaidam Basin
Beijing
China, People's Republic of
Spring outlet
Salt lakes
Basin Bondary

Fig.2

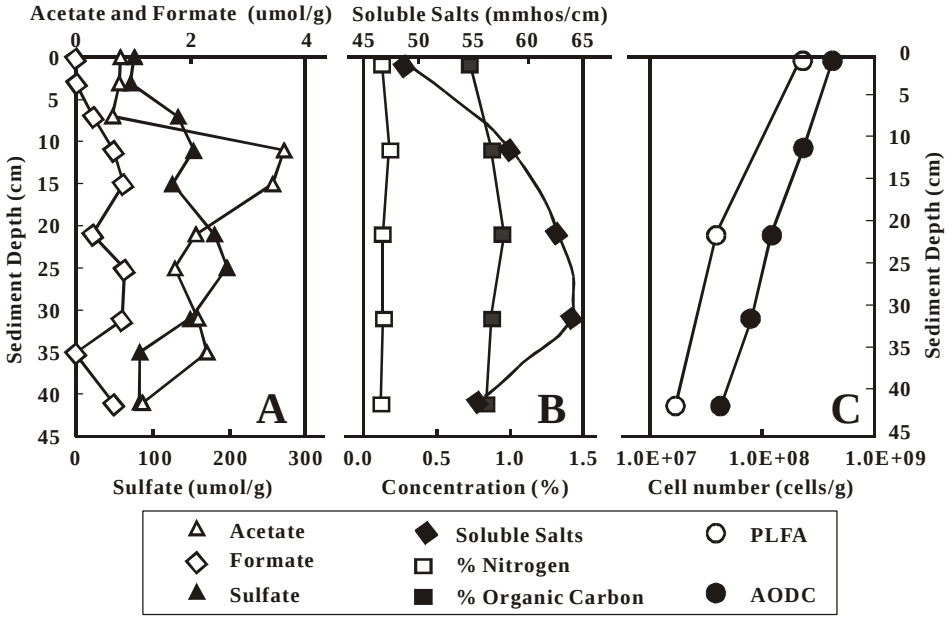

Acetate and Formate (umol/g)
Soluble Salts (mmhos/cm)
Sediment Depth (cm)
Sulfate (umol/g)
Concentration (%)
Cell number (cells/g)
A
B
C
Acetate
Formate
Sulfate
Soluble Salts
% Nitrogen
% Organic Carbon
PLFA
AODC

Fig. 3A

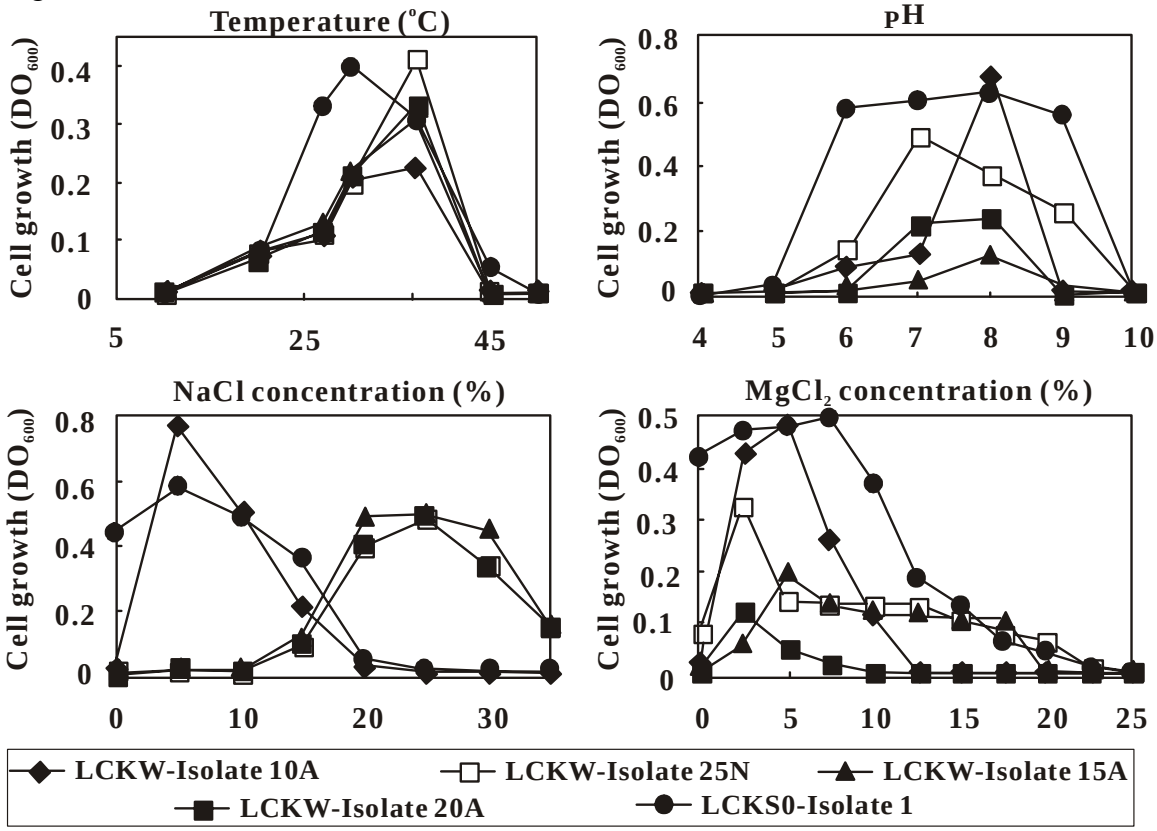

Temperature (°C)
pH
NaCl concentration (%)
MgCl2 concentration (%)
Cell growth (DO600)
LCKW-Isolate 10A
LCKW-Isolate 25N
LCKW-Isolate 15A
LCKW-Isolate 20A
LCKS0-Isolate 1

Fig. 3B

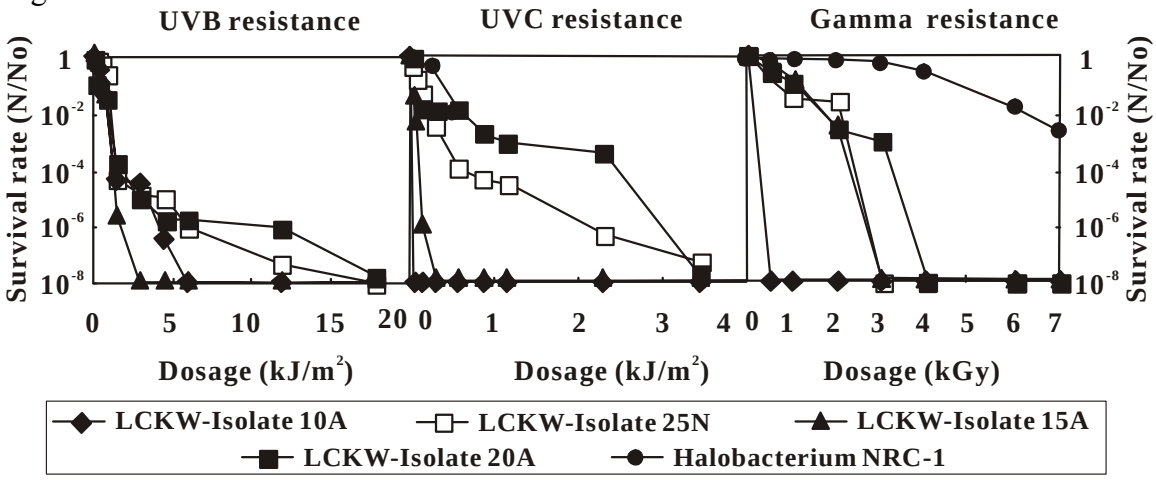
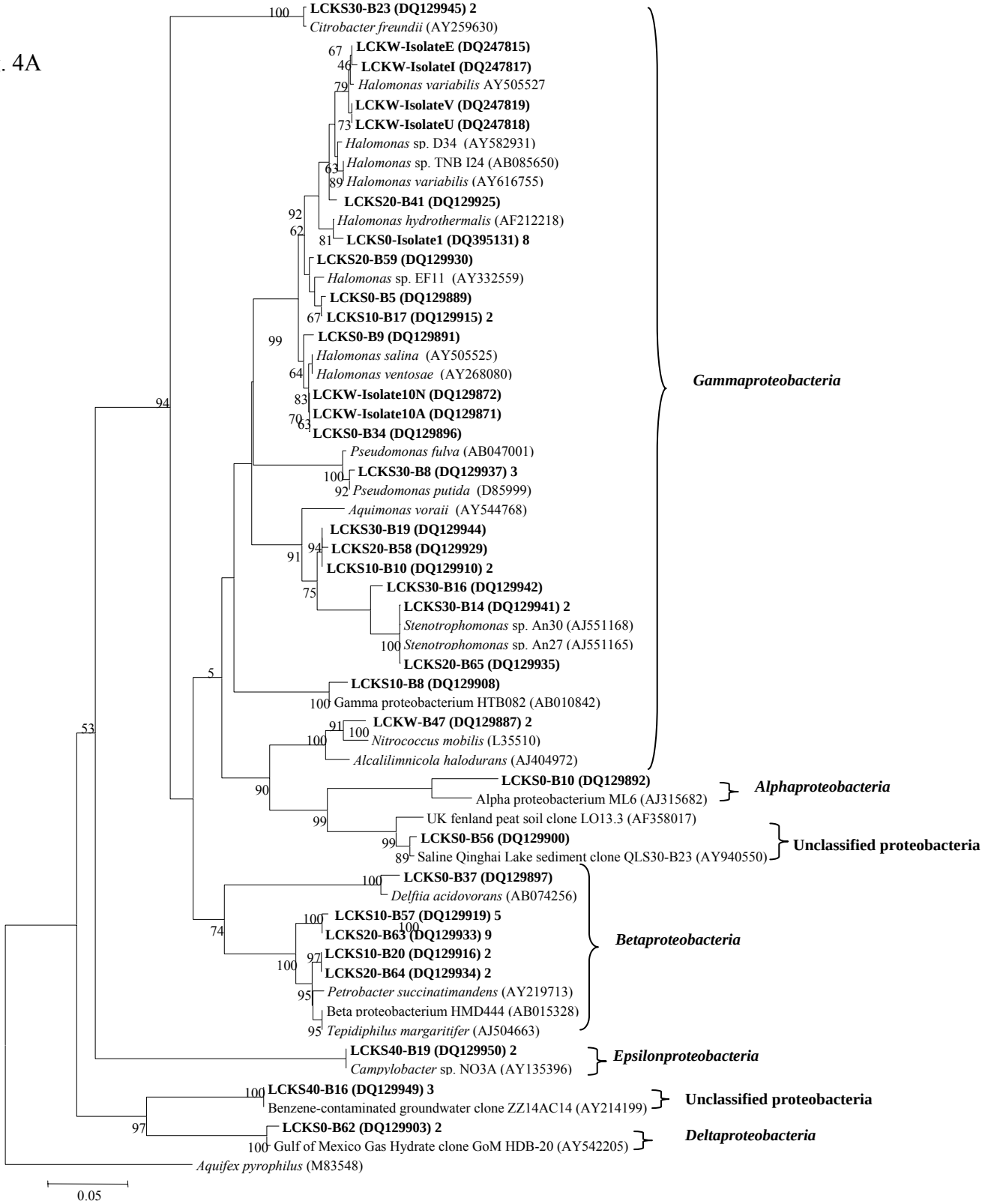

UVB resistance
UVC resistance
Gamma resistance
Survival rate (N/No)
Dosage (kJ/m2)
Dosage (kJ/m2)
Dosage (kGy)
LCKW-Isolate 10A
LCKW-Isolate 25N
LCKW-Isolate 15A
LCKW-Isolate 20A
Halobacterium NRC-1

Fig. 4A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LCKW-IsolateE (DQ247815)
LCKW-IsolateI (DQ247817)
Halomonas variabilis AY505527
LCKW-IsolateV (DQ247819)
LCKW-IsolateU (DQ247818)
Halomonas sp. D34 (AY582931)
Halomonas sp. TNB I24 (AB085650)
Halomonas variabilis (AY616755)
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Halomonas hydrothermalis (AF212218)
LCKS0-Isolate1 (DQ395131) 8
LCKS20-B59 (DQ129930)
Halomonas sp. EF11 (AY332559)
LCKS0-B5 (DQ129889)
LCKS10-B17 (DQ129915) 2
LCKS0-B9 (DQ129891)
Halomonas salina (AY505525)
Halomonas ventosae (AY268080)
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LCKS0-B34 (DQ129896)
Pseudomonas fulva (AB047001)
LCKS30-B8 (DQ129937) 3
Pseudomonas putida (D85999)
Aquimonas voraii (AY544768)
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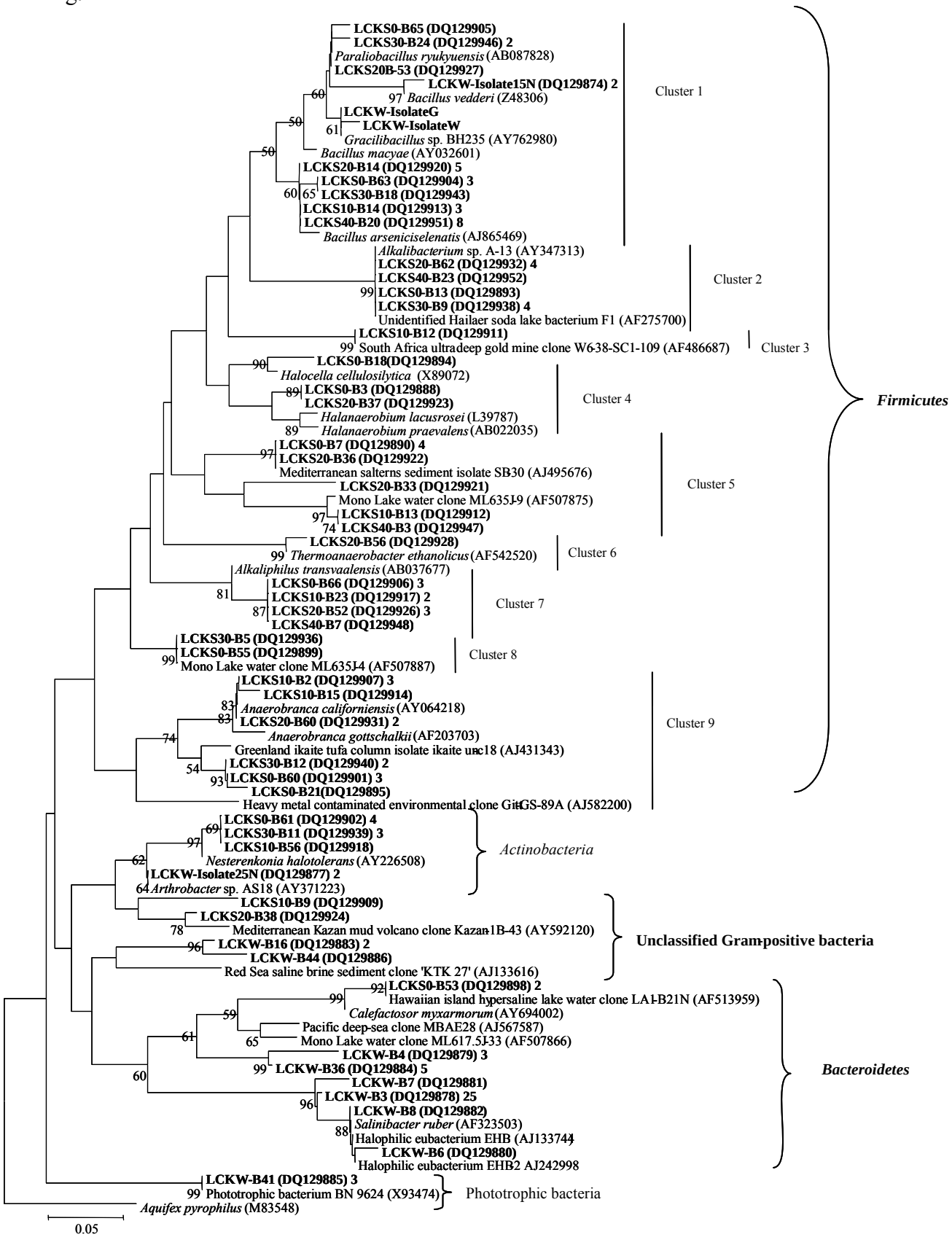
LCKS20-B58 (DQ129929)
LCKS10-B10 (DQ129910) 2
LCKS30-B16 (DQ129942)
LCKS30-B14 (DQ129941) 2
Stenotrophomonas sp. An30 (AJ551168)
Stenotrophomonas sp. An27 (AJ551165)
LCKS20-B65 (DQ129935)
LCKS10-B8 (DQ129908)
Gamma proteobacterium HTB082 (AB010842)
LCKW-B47 (DQ129887) 2
Nitrococcus mobilis (L35510)
Alcalilimnicola halodurans (AJ404972)
Gammaproteobacteria
LCKS0-B10 (DQ129892)
Alpha proteobacterium ML6 (AJ315682)
Alphaproteobacteria
UK fenland peat soil clone LO13.3 (AF358017)
LCKS0-B56 (DQ129900)
Saline Qinghai Lake sediment clone QLS30-B23 (AY940550)
Unclassified proteobacteria
LCKS0-B37 (DQ129897)
Delftia acidovorans (AB074256)
LCKS10-B57 (DQ129919) 5
LCKS20-B63 (DQ129933) 9
LCKS10-B20 (DQ129916) 2
LCKS20-B64 (DQ129934) 2
Petrobacter succinatimandens (AY219713)
Beta proteobacterium HMD444 (AB015328)
Tepidiphilus margaritifer (AJ504663)
Betaproteobacteria
LCKS40-B19 (DQ129950) 2
Campylobacter sp. NO3A (AY135396)
Epsilonproteobacteria
LCKS40-B16 (DQ129949) 3
Benzene-contaminated groundwater clone ZZ14AC14 (AY214199)
Unclassified proteobacteria
LCKS0-B62 (DQ129903) 2
Gulf of Mexico Gas Hydrate clone GoM HDB-20 (AY542205)
Deltaproteobacteria
Aquifex pyrophilus (M83548)
0.05

100

Fig. 4B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Bacillus vedderi (Z48306)
LCKW-IsolateG
LCKW-IsolateW
Gracilibacillus sp. BH235 (AY762980)
Bacillus macyae (AY032601)
LCKS20-B14 (DQ129920) 5
LCKS0-B63 (DQ129904) 3
LCKS30-B18 (DQ129943)
LCKS10-B14 (DQ129913) 3
LCKS40-B20 (DQ129951) 8
Bacillus arseniciselenatis (AJ865469)
Cluster 1
Alkalibacterium sp. A-13 (AY347313)
LCKS20-B62 (DQ129932) 4
LCKS40-B23 (DQ129952)
LCKS0-B13 (DQ129893)
LCKS30-B9 (DQ129938) 4
Unidentified Hailaer soda lake bacterium F1 (AF275700)
Cluster 2
LCKS10-B12 (DQ129911)
South Africa ultradeep gold mine clone W6-38-SC1-109 (AF486687)
Cluster 3
LCKS0-B18(DQ129894)
Halocella cellulosilytica (X89072)
LCKS0-B3 (DQ129888)
LCKS20-B37 (DQ129923)
Halanaerobium lacusrosei (L39787)
Halanaerobium praevalens (AB022035)
Cluster 4
LCKS0-B7 (DQ129890) 4
LCKS20-B36 (DQ129922)
Mediterranean salterns sediment isolate SB30 (AJ495676)
LCKS20-B33 (DQ129921)
Mono Lake water clone ML635J-9 (AF507875)
LCKS10-B13 (DQ129912)
LCKS40-B3 (DQ129947)
Cluster 5
LCKS20-B56 (DQ129928)
Thermoanaerobacter ethanolicus (AF542520)
Cluster 6
Alkaliphilus transvaalensis (AB037677)
LCKS0-B66 (DQ129906) 3
LCKS10-B23 (DQ129917) 2
LCKS20-B52 (DQ129926) 3
LCKS40-B7 (DQ129948)
Cluster 7
LCKS30-B5 (DQ129936)
LCKS0-B55 (DQ129899)
Mono Lake water clone ML635J-4 (AF507887)
Cluster 8
LCKS10-B2 (DQ129907) 3
LCKS10-B15 (DQ129914)
Anaerobranca californiensis (AY064218)
LCKS20-B60 (DQ129931) 2
Anaerobranca gottschalkii (AF203703)
Greenland ikaite tufa column isolate ikaite unc18 (AJ431343)
LCKS30-B12 (DQ129940) 2
LCKS0-B60 (DQ129901) 3
LCKS0-B21(DQ129895)
Heavy metal contaminated environmental clone GitC-GS-89A (AJ582200)
Cluster 9
Firmicutes
LCKS0-B61 (DQ129902) 4
LCKS30-B11 (DQ129939) 3
LCKS10-B56 (DQ129918)
Nesterenkonia halotolerans (AY226508)
LCKW-Isolate25N (DQ129877) 2
Arthrobacter sp. AS18 (AY371223)
Actinobacteria
LCKS10-B9 (DQ129909)
LCKS20-B38 (DQ129924)
Mediterranean Kazan mud volcano clone Kazan-1B-43 (AY592120)
LCKW-B16 (DQ129883) 2
LCKW-B44 (DQ129886)
Red Sea saline brine sediment clone 'KTK 27' (AJ133616)
Unclassified Gram-positive bacteria
LCKS0-B53 (DQ129898) 2
Hawaiian island hypersaline lake water clone LA1-B21N (AF513959)
Calefactosor myxarmorum (AY694002)
Pacific deep-sea clone MBAE28 (AJ567587)
Mono Lake water clone ML617.5J-33 (AF507866)
LCKW-B4 (DQ129879) 3
LCKW-B36 (DQ129884) 5
LCKW-B7 (DQ129881)
LCKW-B3 (DQ129878) 25
LCKW-B8 (DQ129882)
Salinibacter ruber (AF323503)
Halophilic eubacterium EHB (AJ133744)
LCKW-B6 (DQ129880)
Halophilic eubacterium EHB2 AJ242998
Bacteroidetes
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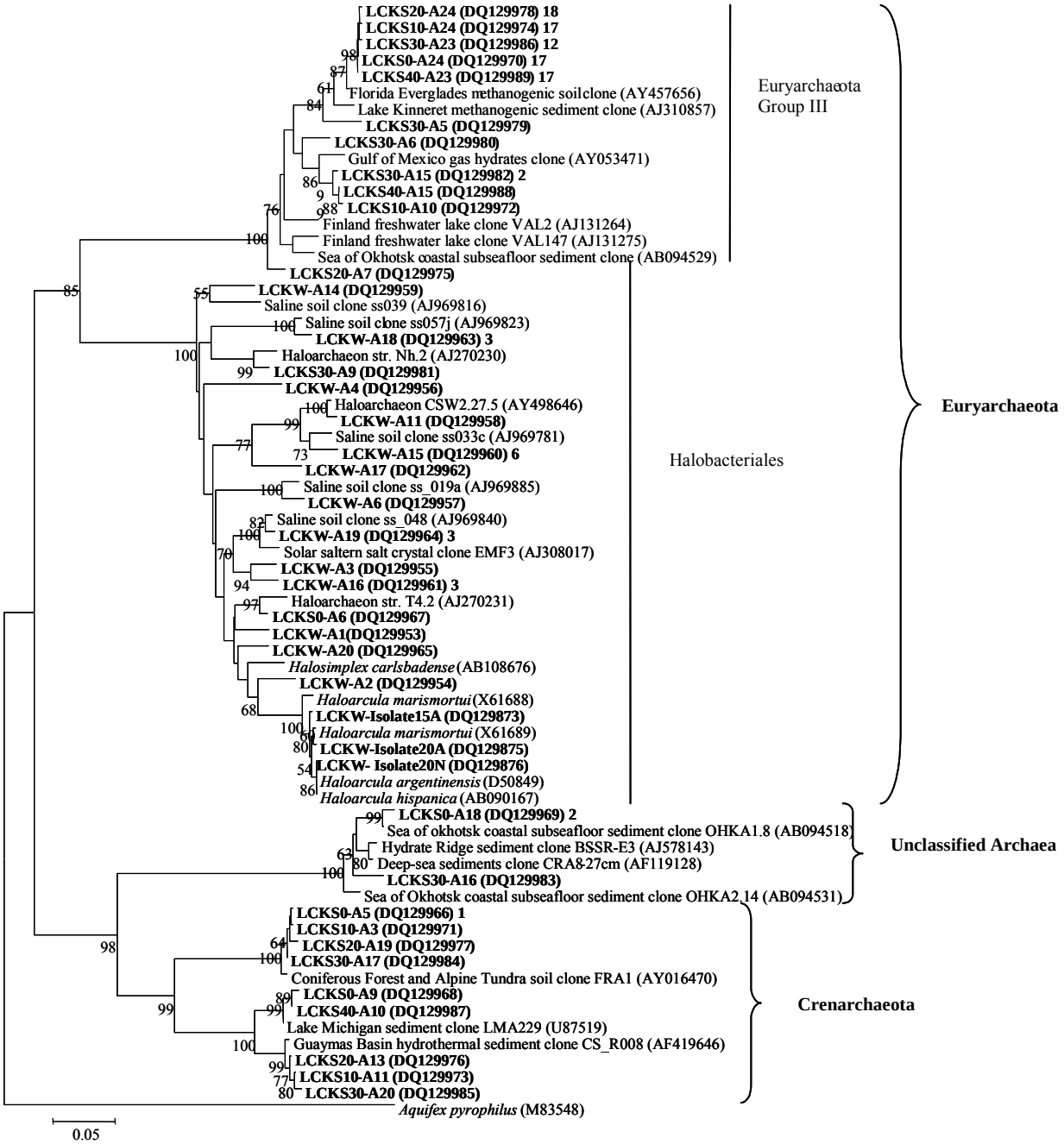
Phototrophic bacterium BN 9624 (X93474)
Phototrophic bacteria
Aquifex pyrophilus (M83548)
0.05

Fig. 5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LCKS40-A23 (DQ129989) 17
Florida Everglades methanogenic soil clone (AY457656)
Lake Kinneret methanogenic sediment clone (AJ310857)
LCKS30-A5 (DQ129979)
LCKS30-A6 (DQ129980)
Gulf of Mexico gas hydrates clone (AY053471)
LCKS30-A15 (DQ129982) 2
LCKS40-A15 (DQ129988)
LCKS10-A10 (DQ129972)
Finland freshwater lake clone VAL2 (AJ131264)
Finland freshwater lake clone VAL147 (AJ131275)
Sea of Okhotsk coastal subseafloor sediment clone (AB094529)
Euryarchaeota Group III
LCKS20-A7 (DQ129975)
LCKW-A14 (DQ129959)
Saline soil clone ss039 (AJ969816)
Saline soil clone ss057j (AJ969823)
LCKW-A18 (DQ129963) 3
Haloarchaeon str. Nh.2 (AJ270230)
LCKS30-A9 (DQ129981)
LCKW-A4 (DQ129956)
Haloarchaeon CSW2.27.5 (AY498646)
LCKW-A11 (DQ129958)
Saline soil clone ss033c (AJ969781)
LCKW-A15 (DQ129960) 6
LCKW-A17 (DQ129962)
Saline soil clone ss_019a (AJ969885)
LCKW-A6 (DQ129957)
Saline soil clone ss_048 (AJ969840)
LCKW-A19 (DQ129964) 3
Solar saltern salt crystal clone EMF3 (AJ308017)
LCKW-A3 (DQ129955)
LCKW-A16 (DQ129961) 3
Haloarchaeon str. T4.2 (AJ270231)
LCKS0-A6 (DQ129967)
LCKW-A1(DQ129953)
LCKW-A20 (DQ129965)
Halosimplex carlsbadense (AB108676)
LCKW-A2 (DQ129954)
Haloarcula marismortui (X61688)
LCKW-Isolate15A (DQ129873)
Haloarcula marismortui (X61689)
LCKW-Isolate20A (DQ129875)
LCKW- Isolate20N (DQ129876)
Haloarcula argentinensis (D50849)
Haloarcula hispanica (AB090167)
Halobacteriales
Euryarchaeota
LCKS0-A18 (DQ129969) 2
Sea of okhotsk coastal subseafloor sediment clone OHKA1.8 (AB094518)
Hydrate Ridge sediment clone BSSR-E3 (AJ578143)
Deep-sea sediments clone CRA8-27cm (AF119128)
LCKS30-A16 (DQ129983)
Sea of Okhotsk coastal subseafloor sediment clone OHKA2.14 (AB094531)
Unclassified Archaea
LCKS0-A5 (DQ129966) 1
LCKS10-A3 (DQ129971)
LCKS20-A19 (DQ129977)
LCKS30-A17 (DQ129984)
Coniferous Forest and Alpine Tundra soil clone FRA1 (AY016470)
LCKS0-A9 (DQ129968)
LCKS40-A10 (DQ129987)
Lake Michigan sediment clone LMA229 (U87519)
Guaymas Basin hydrothermal sediment clone CS_R008 (AF419646)
LCKS20-A13 (DQ129976)
LCKS10-A11 (DQ129973)
LCKS30-A20 (DQ129985)
Crenarchaeota
Aquifex pyrophilus (M83548)
0.05

Fig. 6

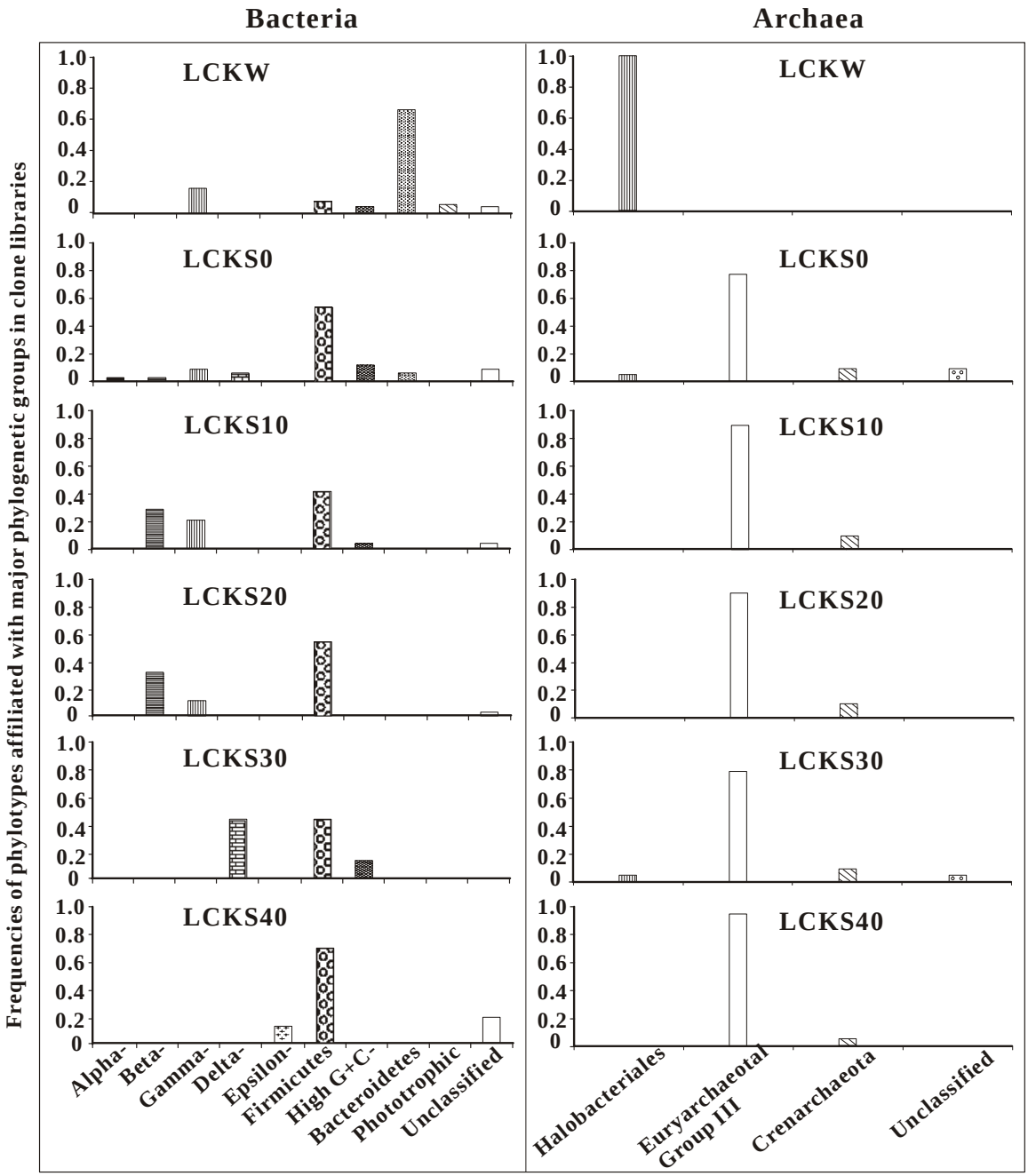
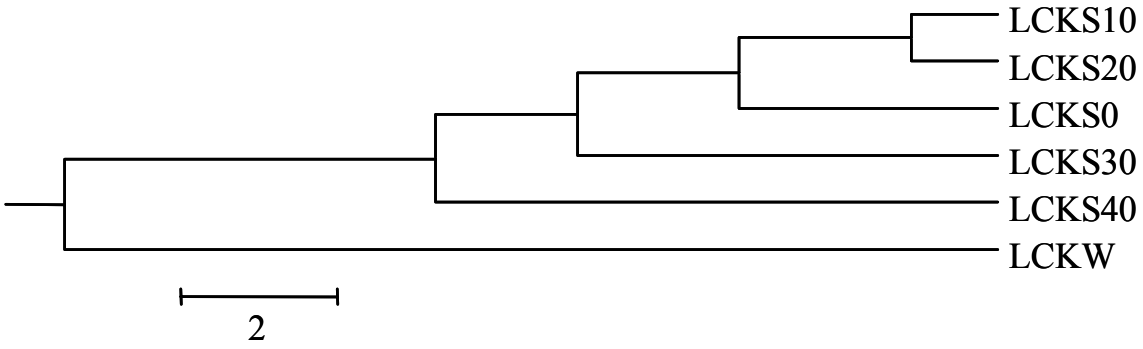

Bacteria
Archaea
Frequencies of phylotypes affiliated with major phylogenetic groups in clone libraries
LCKW
LCKS0
LCKS10
LCKS20
LCKS30
LCKS40
1.0
0.8
0.6
0.4
0.2
0
Alpha-
Beta-
Gamma-
Delta-
Epsilon-
Firmicutes
High G+C-
Bacteroidetes
Phototrophic
Unclassified
Halobacteriales
Euryarchaeotal Group III
Crenarchaeota
Unclassified

Fig. 7



CHAPTER 3:

Microbial Response to Salinity Change in Lake Chaka, a Hypersaline Lake on Tibetan Plateau

Running title: Microbial Response to Salinity Change in Saline Lake Chaka

Hongchen Jiang[1], Hailiang Dong[1,2*], Bingsong Yu[2,3], Xinqi Liu[4],Yiliang Li[5], Shanshan Ji[1], and Chuanlun L. Zhang[6]

1: Department of Geology
Miami University
Oxford, OH 45056

2: Geomicrobiology Laboratory
China University of Geosciences
Beijing, 100083, China

3: School of Earth Sciences
China University of Geosciences
Beijing, China, 100083

4: Nanjing Institute of Geography and Limnology
Chinese Academy of Sciences
Nanjing, China, 210008

5: Center for Environmental Biotechnology
University of Tennessee
10515 Research Drive, Suite 300
Knoxville, TN 37931

6: Savannah River Ecology Laboratory
University of Georgia
P.O. Box Drawer E
Aiken, SC 29802

*Corresponding author: Hailiang Dong
Department of Geology
Miami University
Oxford, OH 45056
Tel: 513-529-2517
Fax: 513-529-1542
Email: dongh@muohio.edu
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Summary

Previous investigations of the salinity effects on the microbial community composition have largely been limited to dynamic estuaries and coastal solar salterns. In this study, the effects of salinity and mineralogy on microbial community composition was studied by using a 900-cm sediment core collected from a stable, inland hypersaline lake, Lake Chaka, on the Tibetan Plateau, northwestern China. This core, spanning a time of 17,000 years, was unique in that it possessed an entire range of salinity from fresh-water clays and silty sands at the bottom to gypsum and glauberite in the middle, to halite at the top. Bacterial and archaeal communities were studied along the length of this core utilizing an integrated approach combining mineralogy and geochemistry, molecular microbiology (16S rRNA gene analysis and quantitative-PCR), cultivation, and lipid biomarker analyses. Systematic changes in microbial community composition were correlated with the salinity gradient, but not with mineralogy. Bacterial community was dominated by the *Firmicutes*-related environmental sequences and known species (including sulfate reducing bacteria) in the freshwater sediments at the bottom, but by halophilic and halotolerant *Betaproteobacteria* and *Bacteroidetes* in the hypersaline sediments at the top. Succession of proteobacterial groups along the salinity gradient, typically observed in free-living bacterial communities, was not observed in the sediment-associated community. Among *Archaea*, the *Crenarchaeota* were predominant in the bottom freshwater sediments, but the halophilic *Halobacteriales* of the *Euryarchaeota* was the most important group in the hypersaline sediments. Multiple isolates were obtained along the whole length of the core, and their salinity tolerance was consistent with the geochemical conditions. Iron-reducing bacteria were isolated in the freshwater sediments, which were capable of reducing structural Fe(III) in the Fe(III)-rich clay minerals predominant in the source sediment. These data have important implications for understanding how microorganisms respond to increased salinity in stable, inland water bodies.

Introduction

Saline lakes are globally distributed on Earth (Oren, 2002) and constitute 45% of total inland water bodies (Wetzel, 2001). Hypersaline environments are considered extreme habitats for microbial life (Litchfield and Gillevet, 2002, Rothschild and Mancinelli, 2001). Salt-loving microorganisms thrive in these extreme habitats and are distributed among all three domains of life (DasSarma and Arora, 2001). Previous studies have shown that the taxonomic diversity of microbial populations in terrestrial saline and hypersaline environments is low (DasSarma and Arora, 2001, Oren, 2001), and that in general, microbial composition is primarily controlled by salinity (Jiang, et al., 2006, Oren, 2002). Major metabolic functions in hypersaline environments are limited due to energetic considerations (Oren, 2001, Oren, 2002).

Microbial research in saline environments is important for multiple reasons. First, biotechnological applications of halophilic microorganisms were recognized and employed even centuries ago, with a recently renewed interest (Oren, 2002). Potential exists to find novel applications with more in-depth research. Second, because of the presence of salt deposits and saline environments on Mars (Catling, 1999, Cooper and Mustard, 2002, Gendrin, et al., 2005, Langevin, et al., 2005, Mancinelli, et al., 2004, Squyres, et al., 2004), studies of microbial diversity in terrestrial saline environments may shed light on the forms of extinct and/or extant life on Mars. Third, primordial life on earth might have started in hypersaline environments (Dundas, 1998, Knauth, 1998), thus research on microbial survivability and adaptation in saline environments bears relevance to our understanding of the early evolution of the biosphere on Earth.

Previous investigations of the salinity effects on the microbial community composition have largely been limited to dynamic estuaries (Henriques, et al., 2006, Langenheder, et al., 2003, Zhang, et al., 2006) and coastal solar salterns (Casamayor, et al., 2002). These studies have definitively shown that salinity is a major factor in controlling microbial abundance, diversity, composition, and functions. However, because of the dynamic nature of these systems, it may be difficult to differentiate the two response strategies of microbial community to changing salinity: replacement of one taxa by another or slow adaptation of the same taxa (Wu, et al., 2006). Wu et al. (2006)

employed both culture-dependent and molecular methods to study the influence of salinity on the bacterioplankton community composition of 16 stagnant inland lakes located on the Qinghai-Xizang (Tibetan) Plateau. Those authors found that salinity, not altitude, is the dominant factor controlling the bacterioplankton community composition. However, Wu et al (2006) did not study how the diversity and composition of particle-attached microorganisms respond to increased salinity and alteration of mineralogy in lake sediments. This type of investigation is important because particle-attached and free-living bacterial community composition is fundamentally different (Acinas, et al., 1999, Crump, et al., 1999, DeLong, et al., 1993, Phillips, et al., 1999, Schweitzer, et al., 2001).

The Tibetan Plateau is the Earth's largest (2×10^6 km^2) and highest (average ~4500 masl, meters above sea level) plateau. The high elevation results from the collision between the Eurasian continent and the India sub-continent ~40-50 Ma ago (Kerr, 2006, Mulch and Chamberlain, 2006, Patriat and Achache, 1984). The tectonic uplifting of the Tibetan Plateau makes this location very unique with respect to its climatic systems: it lies at a critical and sensitive junction of three climatic systems: the East Asian Monsoon, the cold polar airflow from the Siberian high pressure, and the India monsoon. Dry climate dominates the region and there are several thousand lakes in this area with an increased geographic salinity gradient from south to north due to a gradually decreased annual precipitation (Yang, et al., 2003). The sediments in these saline lakes archive environmental changes and store abundant information about the history of climatic change, especially climatic variation, vegetation succession, glacial effect, and their connection with global change.

The main objective of this research was to study the change of microbial community composition along a vertical salinity gradient in sediments of Lake Chaka on the Tibetan Plateau (Fig. 1). Lake Chaka has the following unique characteristics: 1). There exists a complete range of salinity in the lake sediments from nearly freshwater (0.02%) at the depth of 9 m to hypersaline (32.5%) at the top as a result of progressive evaporation in the last 17, 000 years; 2). High elevation, dry climate, and relatively closed basins of the lake allow the development of stable microbial community and metabolic processes. This environmental stability may allow differentiation of the two strategies of microbial

response to changing salinity: replacement of one taxa by another or slow adaptation of the same taxa.

Lake Chaka is a hypersaline lake on the Northeastern Tibetan Plateau. The lake has become progressively saline in the last 17,000 years, as a result of evaporation in the region (Liu, et al., in submission). This progressive evaporation is recorded in lake sediments from freshwater clays and mud at 900-cm depth, sulfate minerals at 500-700 cm, to halite at 0-500 cm. In our previous study (Jiang, et al., 2006), we examined microbial diversity in lake water and shallow sediments (42 cm). In this research, we focused on how microbial diversity and composition changes along this salinity gradient in the sediments, by using a 900-cm long sediment core that covers the entire range of salinity. We employed an integrated approach, combining mineralogy and geochemistry, molecular microbiology (16S rRNA gene analysis and quantitative polymerase chain reaction), cultivation, and lipid biomarker analyses to achieve this objective. Our results show that the microbial community structure systematically changes along the salinity gradient and this change is consistent with geochemical conditions. These results have important implications for understanding how the microbial community adapts to changing environment (salinity and climate) in inland water bodies at high elevation.

Results

Sediment geochemistry

The 900-cm sediment core covered the entire range of salinity and a complete evaporative sequence of minerals, ranging from freshwater clays (illite, smectite, chlorite etc.) and fine sands at the bottom of the core, to evaporative gypsum and glauberite (Glauber's salts, $Na_2SO_4 \cdot 10H_2O$) in the middle, and halite at the top (Fig. 2A). The color of the core was white to light gray at the top (mostly halite salt grains), dark gray at ~500 cm and completely black at 570 cm, then changed to dark gray at ~700 cm. These colors might indicate an oxidizing environment near the top half of the core (< 570 cm) and a reducing (or microaerophilic) environment near the bottom half (570-900 cm). A direct measurement of the redox state of pore water was not possible because of its paucity.

Sulfate concentration was the highest at the 650-cm depth, consistent with the presence of sulfate minerals at this depth (Fig. 2A&B). The concentration of soluble salts increased from nearly zero at the bottom to near saturation at the 200-cm depth (~650 mS/cm, which is equivalent to ~40% salt, 1 mS/cm=640 ppm) followed by a decrease to 360 mS/cm at the top (Fig. 2B). From the bottom to the top of the core, pH decreased from alkaline (9.7) to nearly neutral (7.5), total nitrogen (TN) from 0.05 to <0.01%, and total organic carbon (TOC) from 2.0% to < 0.1% (Fig. 2C). The general decrease in the TN and TOC contents from the bottom to the top might reflect an overall decrease in primary productivity as salinity increased (Liu, et al., 2004). Titration measurement (total dissolution) indicated that total iron content in sample LCKS880 (the sediment sample where iron-reducing isolates were obtained) was 2.34% and 75% of that was present as Fe(II).

AODC and Q-PCR data

Total cell counts as determined by AODC decreased from 8×10^8 cells/g (dry weight, DW) at the bottom of the core to $<2 \times 10^8$ cells/g (DW) in the middle followed by an increase to 6×10^8 cells/g (DW) at the top (Fig. 2D). Q-PCR-determined cell abundance was at the same magnitude and displayed a similar pattern to the AODC-based cell counts. Overall, the bacterial abundance decreased with increasing salinity, but the archaeal abundance increased. In the bottom freshwater sediments, the number of *Archaea* was 2-3 times lower than that of *Bacteria*. In the high-salinity sediments near the top of the core, the number of Archaea was 2 times higher than *Bacteria*. Halophilic archaea were not detectable in the bottom freshwater sediments (LCKS880), increased to $\sim 1 \times 10^5$ cells/g in LCKS745, and became dominant in the salt layers ($\sim 10^8$ cells/g at 0-, 200- and 595-cm depths). In contrast, *Crenarchaeota* were predominant in the freshwater sediments (745- and 880-cm depths) but not detectable in the salt layers (Fig. 2D).

Phosphorlipid fatty acid (PLFA) analysis

Viable bacterial abundance in the core as determined by the total PLFA concentration (assuming a laboratory-determined conversion factor of 20000 cells/pmol (Chapelle, 2000) decreased from ~4×10^8 cells/g (DW) at the bottom to ~1×10^7 cells/g (DW) in the middle (halite layer, 200-415 cm) followed by an increase to 1×10^8 cells/g (DW) at the top. These PLFA-determined numbers of *Bacteria* were generally lower than the total number of *Bacteria* as determined by Q-PCR, suggesting either that a certain fraction of extracted DNA might be from non-viable *Bacteria* or that not all of the PLFA's were extracted due to sorption to sediment particles.

PLFA profiles (Fig. 3) in the sediment samples showed that the proportion of terminally branched saturated fatty acids, indicative of *Firmicutes*/anaerobic Gram-negative-like *Bacteria*, were present without any correlation with salinity. The proportion of monoenoic fatty acids, indicative of some *Proteobacteria*-like *Bacteria*, decreased from 32% in the salt layers (near the top) to 0 in the gypsum layer (635-645 cm) followed by an increase to 79% in the freshwater sediments. Mid-chain branched monoenoic components, diagnostic of sulfate reducing bacteria (SRB)-like microorganisms, were absent at the salt layers, but became detectable below 200 cm with a concentration of 4-54%. Polyenoics (polyunsaturated fatty acids, PUFAs), diagnostic of *Eukaryotes*, were present in almost all tested samples except for LCKS745. The abundance of PUFAs in the salt layers (0-645 cm) was higher than that in the freshwater sediments (745-880 cm), i.e., 5-23% vs. 0-4%.

Isolation of Bacteria and Archaea

Isolates were obtained from four of the five selected sediment subsamples (other than LCKS595). Failure to retrieve any isolates from LCKS595 may be due to the special mineral composition (gypsum) of this sample and/or the absence of aerobes and facultative anaerobes in the sample (only those media targeting aerobic/facultative organisms were used in our cultivation attempts.). Ninety isolates (Table 1) were obtained and were subjected to 16S rRNA gene analysis. Thirty-three of these isolates were *Bacteria* (Table 1), and 57 were *Archaea* (Table 1). The bacterial isolates were

distributed into three major groups: *Gammaproteobacteria*, *Actinobacteria*, and *Firmicutes* (Fig. 4).

In the *Gammaproteobacteria* group, all isolates belonged to three genera: *Halomonas*, *Pseudomonas*, and *Shewanella*. The 16S rRNA gene of more than a half (17 out 33) of the bacterial isolates from the sediments of different salinity (i.e., 4, 2 and 11 from LCKS000, LCKS200, and LCKS745, respectively) showed the best matches (99.4-99.7%) with several species of the genus *Halomonas*, i.e., *H. sulfidaeris*, *H. glaciei*, *H. boliviensis*, and *H. variabilis* (Fig. 4). *H. sulfidaeris* can tolerate up to 22% NaCl and perform anaerobic nitrate reduction (Kaye, et al., 2004). *H. glaciei* is capable of tolerating 12% NaCl (Castro, et al., 2004). *H. boliviensis* can grow in a medium with 0-25% NaCl. *H. variabilis*, a psychrotolerant species of *Halomonas*, was isolated at the sea ice-seawater interface from Terra Nova Bay Station, Ross Sea, Antarctica. A common feature of these *Halomonas* species/strains, including our isolates (LCKS000-Isolate25, LCKS200-Isolate9, and LCKS745-Isolate5, Fig. 5), is that they can tolerate a large range of NaCl salt.

Four isolates, two each from LCKS745 and LCKS880, were related (99.3-99.9%) to several species of *Pseudomonas* (Fig. 4). These *Pseudomonas* isolates were also related (~94%) to *Nitrococcus mobilis* isolated from Pacific Ocean surface water, which is a strict aerobe capable of oxidizing nitrite to nitrate and grows optimally in 70-100% seawater at pH 7.5-8.0 (Watson and Waterbury, 1971). These isolates showed an optimal salinity of 1.5% and maximal of 6% (LCKS745-Isolate34 and LCKS880-Isolate32, Fig. 5).

Four isolates from LCKS880 were closely related (99.2-99.7%) to *Shewanella putrefaciens* ACAM 576 and *Shewanella baltica* W145. ACAM 576 is capable of dissimilatory Fe (III) reduction with lactate or acetate as an electron donor (Bowman, et al., 1997). Our *Shewanella* isolate (LCKS880B-Isolate44) was capable of reducing ~30% Fe(III) in a nontronite sample (NAu-2). This isolate was also capable of reducing 64% and 42% Fe(III) in the source sediment (LCKS880) with and without AQDS, respectively (Fig. 6). It exhibited an optimal and maximal salinity of 1% and 6%, respectively.

The two bacterial isolates in the *Actinobacteria* (from LCKS745) were related (97.5-98.4%) to alkaliphilic *Micrococcus luteus* (Tiago, et al., 2004). In the *Firmicutes*, two

isolates (from LCKS745) were related (98.5%) to one bacterium isolated from a Hailaer soda lake (GenBank description). Three isolates (from LCKS000) were related (97.3%) to *Virgibacillus marismortui*. The strictly aerobic growth of *V. marismortui* occurs at 5-25% (w/v) total salts (optimal at 10%) and pH 6.0-9.0 (optimal at pH 7.5) (Arahal, et al., 1999).

All selected archaeal isolates (from LCKS000 and LCKS200 only) were grouped into the *Halobacteriales* group, most of which (49 out of 67) were phylogenetically related (99.0-99.7%) to *Halobacterium* sp. NRC-1 (AE005128) and *Halobacterium salinarum* (AJ496185). The others were related to *Halorubrum xinjiangense*. *H. salinarum* and *H. xinjiangense* can grow optimally at 15-17.5% and 18-21% NaCl, respectively (Feng, et al., 2004, Gruber, et al., 2004). Likewise, our halophilic archaeal isolates showed such NaCl requirements for optimal growth (LCKS000-Isolate39, LCKS000-Isolate10) (Fig. 5).

Bacterial 16S rRNA gene clone library analysis

Analysis of the 273 bacterial clones revealed that those clone sequences could be classified into: *Alpha-*, *Beta-*, *Delta-*, *Gamma-proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Verrucomicrobia*, and the KB1 group (Eder, et al., 1999) (Fig. 4).

Alphaproteobacteria. Two sequences from two sediment subsamples of the same salinity, LCKS595B19 and LCKS000B15, (Fig. 4), were related (99%) to *Rhodovibrio salinarum* (D14432) and *Rhodovibrio sodomensis* (AJ318524). Both *R. salinarum* and *R. sodomensis*, isolated from salterns, are purple non-sulfur phototrophic bacteria, with optimal growth conditions of 12-18% NaCl and pH 7.5-8.0 for the former and 9-12% NaCl and pH 6.8-7.0 for the latter (Hirschler-Rea, et al., 2003, Kawasaki, et al., 1993). The presence of phototrophic bacterial sequences at 595 cm may suggest preservation of ancient DNA in saline environments.

Betaproteobacteria. Thirty-five sequences (all from LCKS200B, the sample with the highest salinity) were affiliated with the *Betaproteobacteria* (Fig. 4). Four sequences

were related (98%) to Betaproteobacterium aquaspiB (AY322152). A cluster of 31 sequences was related (98-99%) to Arctic soil bacterium K2LI3 (DQ234463), Betaproteobacterium HTB091 (AB010869), *Janthinobacterium* sp. HHS7 (AJ846272), and Antarctic bacterium R-7614 (AJ440982). These close relatives were isolated from a diverse range of cold environments, such as Finnish Lapland forest soil (K2LI3) (Mannisto and Haggblom, 2006), deep-sea sediments from the Nankai Islands Iheya Ridge (1,050-m in depth) (Takami, et al., 1999), Himalayas glacier (HHS7) and Antarctic Dry Valleys (R7614) (Van Trappen, et al., 2002).

Gammaproteobacteria. Seventy clones were affiliated with the *Gammaproteobacteria* (Fig. 4). Forty clone sequences from the sediments of varying salinity formed a tight cluster and they were related (93-99.5%) to several species of the genus *Halomonas*: *H. sulfidaeris*, *H. glaciei*, *H. boliviensis*, *H. variabilis*. These *Halomonas* species can tolerate a large range of NaCl salt (Ventosa et al., 1998). The predominant group of our isolates belonged to this group, illustrating the consistency between the cultivated and molecular results.

Seven sequences of LCKS200 were related (99.5%) to *Psychrobacter okhotskensis* CMS32 (AJ748270), a psychrophilic and halotolerant (up to 12% NaCl) strain isolated from the Wright Valley region of Antarctica (Shivaji, et al., 2005). Four sequences from LCKS880 were related (99.4%) to *Shewanella putrefaciens* ACAM 576 and *Shewanella baltica* W145. Pure isolates of these Fe(III)-reducing bacteria were also obtained from this sediment subsample, again illustrating the consistency between our cultivation and molecular results. Thirteen clones (5, 3 and 5 from LCKS200B, LCKS745B and LCKS880B, respectively) were related (99%) to *Pseudomonas stutzeri*.

Deltaproteobacteria. Seventeen sequences, all from the sediments of the same salinity, i.e., LCKS000 and LCKS595B (23%), were affiliated with the *Deltaproteobacteria* (Fig. 4). Sixteen clones, 2 and 14 from LCKS000, and LCKS595B, respectively, were related (95-97%) to a sequence (AJ495688) retrieved from a hypersaline pond (25-32% salinity) in Mediterranean salterns. These clones were also remotely related (88-93%) to the members of the genera *Desulfonatronum* and *Desulfovibrio*. *D. cooperativum* and *D.*

thiodismutans were all alkaliphilic (optimum pH were 8.0-9.0 and 9.5, respectively) and obligately anaerobic sulfate-reducing bacteria (Pikuta, et al., 2003, Zhilina, et al., 2005). The NaCl concentration range for growth of these two bacteria was 0.1-8% and 1-7%, respectively. Three clones from LCKS595B were related to (99%) *Desulfosalina propionicus*, a sulfate-reducing bacterium isolated from the hypersaline sediments of the Great Salt Lake, Utah (GenBank Description).

Bacteroidetes. Forty-four sequences (40, and 4 from LCKS000B, and LCKS595B, respectively) formed the *Bacteroidetes* group (Fig. 4). Twelve clones were related (99%) to sequences retrieved from water of Lake Chaka (Jiang, et al., 2006) and hypersaline microbial mats of an Israel saltern. Thirty-two clones were 98% to 99.5% similar to *Salinibacter ruber* strain POLA 13 (AF323503) isolated from saltern ponds in Mallorca (Balearic Islands, Spain). POLA 13 can grow optimally in the presence of 150 to 300 g/liter total salt and at pH 6.5 to 8.0 (Anton, et al., 2000).

Firmicutes (low G+C Gram-positive bacteria). Eighty-two sequences were affiliated with the *Firmicutes* group (Fig. 4), with the majority from the freshwater sediments at 745 and 800 cm. Twenty-six sequences from these depths, i.e., 3 and 23 from LCKS745B and LCKS880B, respectively, were related (99.6%) to sulfate-reducing bacterium STP12 (AJ006607) and *Desulfosporosinus* sp. LauIII (AJ302078). STP12 is capable of using H_2, formate, butyrate, pyruvate and lactate as electron donors to reduce thiosulfate. LauIII is a lactate-utilizing sulfate reducer (Drake, et al., 2002). Our clone sequences were also related (91%) to *Desulfitobacterium metallireducens* (AF297871). This obligate anaerobe is capable of reducing Fe(III), Mn(IV), sulfur and thiosulfate with lactate, formate and other substrates as electron donors (Finneran, et al., 2002). Thirteen clones from LCKS745B showed 99% similarity to an unidentified Hailaer soda lake bacterium F27. Fifteen sequences (9 and 6 from LCKS000B and LCKS595B, respectively) were related to (99.5%) a halophilic bacterium, Isolate15N, isolated from Lake Chaka water, with an optimal salinity of ~20% NaCl (Jiang, et al., 2006). Eleven sequences (4, and 7 from LCKS745B, and LCKS880B, respectively) were related (98.6%) to *Alkaliphilus*

transvaalensis, a strictly anaerobic alkaliphile (pH range 8.5-12.5 with 10.0 for optimum) (Takai, et al., 2001).

Actinobacteria. Three clones from LCKS745B (Fig. 4), along with two isolates, formed the *Actinobacteria* group and were related to *Micrococcus luteus* with an optimal growth at pH 8.0-9.0 (Tiago, et al., 2004).

KB1 group. Seven clones from LCKS595B were affiliated with the KB1 group and they were related to one sequence retrieved from the brine-seawater interface of the Shaban Deep in Red Sea (Eder, et al., 2002). The sampling site where the reference clone sequence was obtained has a salinity of 24.2% and pH 6.1 (Eder, et al., 2002). The KB1 sequences were first identified in a sediment sample (salinity of pore water: 26%) of the Kebrit Deep in Red Sea (Eder, et al., 1999). The KB1 group members were inferred to be extreme halophiles and specifically adapted to brine bodies and sediments although none of them has been isolated yet (Eder, et al., 2001, Eder, et al., 2002).

Unclassified bacteria. Eleven sequences could not be classified (Fig. 4) and they were related to an environmental clone (AY940550) recovered from Qinghai Lake, which is a saline (1.3% salinity) and alkaline (pH 9.4) lake in the same area as Lake Chaka (Dong, et al., 2006).

Archaeal 16S rRNA gene clone library analysis

All 174 archaeal clones could be classified into the *Euryarchaeota* and *Crenarchaeota* (Fig. 7). The majority (~69%, 120 out of 174) of archaeal sequences fell within the *Euryarchaeota* group, including four groups: *Halobacteriales*, SA1 group (Eder, et al., 2002), Cluster 1, and Candidate division MSBL1 (van der Wielen, et al., 2005). The rest (~31%, 46 out of 174) belonged to the *Crenarchaeota*. The *Euryarchaeota* and *Crenarchaeota* phyla were clearly separated from each other according to salinity. The crenarchaeotal sequences were exclusively limited to freshwater sediments, i.e., at 745

and 880 cm depth. In contrast, the vast majority of the euryarchaeotal sequences (116 of 120) were found in saline and hypersaline sediments (i.e., depth of 595, 200 and 000 cm).

The Euryarchaeota. Two thirds of the euryarchaeotal sequences (80 sequences) from the sediments of the similar salinity, i.e., 42, 21, and 17 from LCKS000A, LCKS200A and LCKS595A, respectively, formed the *Halobacteriales* group. The majority of these sequences (49 out of 80) were nearly identical to *Halobacterium* sp. NRC-1 (AE005128) and *Halobacterium salinarum* (AJ496185). NRC-1 is an extremely halophilic archaeon and can tolerate extreme conditions such as UV and gamma radiation (Baliga, et al., 2004, Kottemann, et al., 2005). Fourteen sequences were closely related to (>99%) haloarchaeon CSW2.27.5 (AY498646), a member of the novel Antarctic Deep Lake (ADL) group isolated from an Australian crystallizer pond (pH 8.1, salt content, 33%) (Burns, et al., 2004).

In addition to close relatedness to NRC-1 and CSW2.27.5, there were several other clusters within the *Halobacteriales*. A group of clone sequences (9 and 3 from LCKS000 and LCKS595, respectively) were closely related to (>99%) *Halobacterium* sp. Ch12 and *Halobacterium* sp. AUS-1, two halophilic members within the *Halobacteriales* group. Twenty six sequences from the saline sediments (000, 200, 595 cm) were related to (92-99.9%) various archaeal clone sequences from Canada British Columbia Salt Spring (Walsh, et al., 2005) and clone sequences from Lake Chaka water (Jiang, et al., 2006).

The SA1 group contained about 30% of the *Euryarchaeota* sequences (16, 16, 2, and 2 from LCKS200A, LCKS595A, LCKS745A and LCKS880A, respectively), which were related to an archaeal clone (AJ347788) retrieved from the interface of hypersaline brine (salinity, 24.2%; pH 6.1) and seawater of the Shaban Deep, northern Red Sea (Eder, et al., 2002). The SA1 group branches at the family level, and has a large phylogenetic distance to the *Methanosarcinaceae* (20.7%), the *Methanomicrobiaceae* (21.3%), and the *Halobacteriaceae* (22.7%) (Eder, et al., 2002).

The *Crenarchaeota*. Fifty-four sequences (21 and 33 from freshwater sediments LCKS745A and LCKS880A, respectively) were clustered into the *Crenarchaeota* group. These sequences were not related to any cultivated *Crenarchaeota*, but to those recovered

from environmental clones. The largest cluster (26 sequences from LCKS880) were closely related to (>98%) methanogenic archaeon ET1-8 (Chin, et al., 1999). The second largest cluster (21 sequences from LCKS745) was related to (95-96%) clone sequences recovered from Korean Ganghwa Island tidal flat sediments (Kim, et al., 2005). A few sequences were also related to (96-99% similarity) those from diverse environments, such as Okhotsk Sea costal subseafloor sediments, petroleum-contaminated soils, Colorado Front Range soils, and Qinghai Lake sediments.

Distribution of bacterial and archaeal groups

The vertical profiles of archaeal and bacterial community structure were constructed based on the relative abundance of different phylogenetic groups in the clone libraries (Fig. 8). In the *Bacteria*, the *Firmicutes* sequences became less dominant with increasing salinity. For example, the *Firmicutes* sequences were predominant in the freshwater sediments at the bottom of the core (880 and 745 cm), but only constituted a small component in the saline sediments at 595 and 000 cm. These sequences were absent in the most saline sample (at 200 cm). *Bacteroidetes*-related sequences appeared only in the mid-salinity samples (LCKS000 and LCKS595).

In *Archaea*, the crenarchaeotal sequences were predominant in freshwater and low-salinity sediments near the bottom of the core, and were completely absent in the saline sediments near the top. In contrast, the euryarchaeotal sequences (mostly *Halobacteriales*) were only present in saline sediments, and were completely absent in the bottom two samples. The distinct difference in the microbial community between the freshwater sediments at the bottom and the saline sediments near the top of the studied sediment core was further confirmed by LIBSHUFF analysis (data not shown).

Coverage of 16S rRNA gene clone libraries

Coverage values, diversity index and rarefaction curves for the clone libraries all indicated that both the bacterial and archaeal communities from LCKS595 (at the

interface between low and high salinity) were the most diverse. The bacterial community from LCKS200 (with the highest salinity) and the archaeal community from LCKS745 (nearly freshwater sediment) were the least.

Discussion

Microbial biomass as a function of salinity

Total microbial cells as determined by AODC and Q-PCR and total bacterial cells as determined by PLFA and Q-PCR were consistent. These results were significant, considering the different nature of these techniques and assumptions involved in the conversions from gene copy numbers to cell numbers and from the amount of total PLFA to cell biomass. The lower bacterial cell numbers as determined by PLFA were consistent with the fact that this technique only measures viable bacterial cells, whereas Q-PCR measures all bacteria (both viable and non-viable).

Archaeal cells (specifically, halophilic archaea) were predominant in saline sediments, whereas bacterial cells and the *Crenarchaeota* in freshwater and low-salinity muds and silts. This systematic change in the relative proportion of *Archaea* vs. *Bacteria* with salinity was consistent with the fact that most obligate halophiles are *Archaea* (3 to 4 M salt). Unlike halophilic archaea, most halophilic bacteria can only live at a moderate salinity (up to 2.5 M salt concentration) (Ventosa, et al., 1998). Because of their different requirements for salt, halophilic bacteria and archaea tend to occupy different salinity niches with the former being dominant at low salinity and the latter being dominant at high salinity. Although this generalization has been made previously (Oren, 1993), our study has definitively demonstrated this.

Isolate characteristics at different salinities

Although only three different types of aerobic media were employed, we were able to isolate a large number of *Bacteria* and *Archaea*. Bacterial isolates included Fe(III)

reducers and *Pseudomonas*, and alkaliphiles in freshwater sediments at the bottom of the core, and halotolerant/halophilic organisms at the upper (saline) part of the core.

Fe-reducing isolates were nearly identical (99.3%) to *S. putrefaciens* and *S. baltica*, two known Fe(III)-reducing bacteria. The physiological test results indicated that these isolates were capable of reducing Fe(III) in a clay mineral, nontronite, showing a similar iron-reducing capacity to *S. putrefaciens* CN32 (Jaisi, et al., 2005) when the same nontronite mineral (NAu-2) served as electron acceptor. The possible Fe(III) source in the source sediments may be clay minerals (smectite and illite). Abundant organic carbon was available as possible electron donors (Fig. 2).

Acquisition of inferred alkaliphilic organisms (based on the 16S rRNA gene relatedness of isolate sequences to known alkaliphiles) at the bottom of the core, where the pH was alkaline, was not surprising. The close relatedness of 11 clone sequences to *Alkaliphilus transvaalensis*, a known alkaliphile isolated from a South African deep mine (Takai, et al., 2001), also suggests that there were abundant alkaliphiles in the bottom sediments of the core. However, more physiological testing of the acquired isolates and more cultivation work is necessary to further understand the metabolism of these organisms.

It was unexpected to obtain multiple isolates from different sediments of varying salinity that formed a tight cluster. All these isolates were closely related to various species of the genus *Halomonas* (Fig. 4). However, a close examination revealed that these *Halomonas* species have a wide range of salt tolerance (Ventosa et al., 1998). Thus, the range of salinity in the core sediments may still be within the tolerance level of the isolates.

The SA1/KB1 group at the low-high salinity boundary

Our study showed the presence of the deeply branching KB1 and SA1 groups in the lake sediments, suggesting that these groups may not be unique to the brine-seawater interface, as discussed by Eder et al. (2002). Interestingly, both KB1 and SA1 groups occurred at 595 cm, the depth at which salinity changed dramatically. The SA1 group also occurred at 200 cm, another depth where salinity changed. These data, along with

those by Eder et al. (2002), imply that these groups might be unique to those niches where sharp salinity gradient is present.

The relative importance of salinity and mineralogy in controlling bacterial diversity and composition

Between two potentially controlling factors of microbial community, salinity appeared to take the dominant role, and mineralogy the secondary. The dominant role of salinity was reflected in both microbial diversity and composition. For example, the sample with the highest salinity (LCKS200) exhibited the lowest diversity, as consistent with a previous study (Benlloch, et al., 2002) and the general ecological principles that more extreme environments decrease diversity (Frontier, 1985, Hacine, et al., 2004). The sediment sample at the interface between high and low salinity (LCKS595) showed higher microbial diversity than the sediment samples above and below (diversity indices and rarefaction analyses, data not shown). This enhanced microbial diversity, activity and biogeochemical cycling at the interface between low (such as seawater) and high (such as anoxic brine) salinity has been observed in previous studies (Daffonchio, et al., 2006, Parkes, et al., 2005) and has been ascribed to the presence of chemical gradients, such as electron acceptors and donors. Indeed, our geochemical data clearly show gradients of TOC, TN, pH, and sulfate concentrations at or near 595 cm (Fig. 2).

The salinity effect was also clearly reflected in the composition of the bacterial community. This composition between the salt layers at the top (0, 200 and 595 cm) and the silty sandy layers at the bottom (745 and 800 cm) was distinctly different. For example, the *Firmicutes* group (freshwater and halotolerant organisms) was predominant in the low-salinity sediments (745 and 880 cm), but this group was either minor or absent in high-salinity sediments (0, 200 and 595 cm). Instead, the *Betaproteobacteria* and the *Bacteroidetes* became important with increased salinity. The predominance of the *Firmicutes*-related sequences in low-salinity sediments and their absence in high-salinity sediments are consistent with previous studies. For example, the *Firmicutes* are absent in a number of hypersaline environments (Benlloch, et al., 2002, Demergasso, et al., 2004),

but abundant in low-salinity environments such as deep-sea sediments (Li, et al., 1999) and meromictic lakes of marine salinity from Eastern Antarctic (Bowman, et al., 2000).

It is interesting to note that sequences related to sulfate-reducing bacteria (SRB) were retrieved from most samples (such as LCKS880B37 and LCKS595B18) except for the highest-salinity sample LCKS200 (~42% salinity). These data imply that SRB may be present in sediments of up to 23% salinity (LCKS595), as consistent with the suggestion based on bioenergeric considerations (Oren, 2001, Oren, 2002).

Relative to the dramatic effects of salinity on both bacterial diversity and composition, mineralogy appeared to play a minor role. For example, the two samples with a similar salinity but distinct mineralogy (0 and 595 cm) had a qualitatively similar composition of both bacterial and archaeal community.

Lack of succession of sediment-associated Proteobacteria as a function of salinity

Wu et al. (2006) showed that with increasing salinity, the relative abundance of the *Betaproteobacteria* decreases, whereas that of the *Alphaproteobacteria* and the *Gammaproteobacteria* increases. This observation is consistent with many other studies on inland waters (Bockelmann, et al., 2000, Brummer, et al., 2000, Glockner, et al., 1999), dynamic saline systems such as estuaries (Bouvier and del Giorgio, 2002, Cottrell and Kirchman, 2003, Crump, et al., 1999, del Giorgio and Bouvier, 2002, Henriques, et al., 2006, Kirchman, et al., 2005, Zhang, et al., 2006) and costal solar salterns (Benlloch, et al., 2002). For example, Henriques et al. (Henriques, et al., 2006) examined successions of multiple groups of bacteria and reported dominance of the *Alphaproteobacteria* and *Gammaproteobacteria* in the marine-brackish section of the Ria de Aveiro estuary (Portugal) and the *Betaproteobacteria*, the *Deltaproteobacteria* and the *Epsilonproteobacteria* in the freshwater section of the estuary. The reasons for such succession are suggested to be related to cell inactivation/death due to dynamic hydrological conditions such as mixing of riverine and estuarine waters (Bouvier and del Giorgio, 2002), but existence of such successions in stable water bodies appear to suggest that dynamic mixing may not be a necessary condition (Wu, et al., 2006).

However, the *Proteobacteria* groups in our study did not show such successions: the *Betaproteobacteria* sequences were not even retrieved from the lowest salinity sediment (880-cm depth), but they were abundant in the salt layer (200-cm depth) with the highest salinity. In addition, the relative abundance of the *Alphaproteobacteria* and the *Gammaproteobacteria* did not show any systematic change along the salinity gradient. Other studies have also shown different succession patterns with salinity. For example, Langenheder et al. (Langenheder, et al., 2003) showed that the *Alpha-* and *Beta-proteobacteria*, and *Gammaproteobacteria* were more abundant under freshwater conditions. Bernhard et al. (Bernhard, et al., 2005) analyzed bacterioplankton community structure in Tillamook Bay, Oregon and its tributaries, to evaluate phylogenetic variability and its relationship to changes in environmental conditions along an esturine gradient. The authors observed that the *Gammaproteobacteria* and the *Betaproteobacteria* and members of the *Bacteroidetes* dominated in freshwater samples, while the *Alphaproteobacteria*, *Cyanobacteria* and *Chloroplast* genes dominated in marine samples. It appears that when bacteria are attached to solid particles, salinity-related successions are generally not present (Selje and Simon, 2003). Indeed, multiple studies have reported that particle-attached and free-living bacterial community composition are fundamentally different (Acinas, et al., 1999, Crump, et al., 1999, DeLong, et al., 1993, Phillips, et al., 1999, Schweitzer, et al., 2001).

Change of archaeal composition with salinity

Our data definitively demonstrated that with increased salinity the relative abundance of the *Crenarchaeota* decreased, but that of the *Euryarchaeota* increased. These data are in agreement with the distribution of the *Crenarchaeota* in freshwater and slightly saline environments. The *Crenarchaeota* are generally absent in high salinity environments (Benlloch, et al., 2002, Cytryn, et al., 2000, Demergasso, et al., 2004, Maturrano, et al., 2006) with the exception of Walsh et al. (Walsh, et al., 2005) who detected the presence of crenarchaeotal sequences in a soil of 13% salinity.

Some *Crenarchaeota* sequences detected in this study were related to clones retrieved from methane/organic-rich or putative methane hydrate-bearing deep-sea

sediments. Interestingly, Dong et al. (2006) made similar observations in sediments of Qinghai Lake, a lake approximately 150 km to the east of Lake Chaka. It may be tantalizing to speculate that these Tibetan lakes might harbor methane-oxidizing archaea. Definitive evidence would have to come from geochemical as well as microbial data (such as negative $\delta^{13}C$ values).

Response strategies of microbial community to increased salinity

Two possible strategies for microbial community to respond to increased salinity are replacement of taxa or gradual adaptation of the same taxa (Wu et al., 2006). Our data support a combination of both mechanisms. The taxa replacement mechanism appears to operate at the phylum level, as changes of major phylum/groups were observed with increased salinity. However, at the microdiversity (species-level), the gradual evolution and adaptation appears to take place. For example, multiple clone and isolate sequences from both low and high salinity samples (LCKS880B, LCKS745B, LCKS595B, LCKS000B) clustered together and were closely related to the genus *Halomonas* (Fig. 4). Many species within this genus have a wide range of salinity tolerance. Thus, the same species, such as *H. boliviensis*, a known organism with salinity tolerance of 0-25% NaCl (Quillaguaman, et al., 2004), may gradually increase its salt tolerance with increased salinity. This slow adaptation may be accomplished via either de novo synthesis or uptake of glycine betaine and/or ectoine from the natural environment (Ventosa, 2004). Considering the fact that the age span of the 900-cm sediment core is 17,000 years (Liu, et al., in submission), this adaptation may have taken place over this time frame.

Experimental procedures

Study site

Lake Chaka (36°38'-36°45'N, 99°02'-99°12'E, 3200 masl) is a hypersaline lake on the NE Tibetan Plateau, approximately 150 km west of Qinghai Lake (Fig. 1). Upper Pleistocene and Holocene rocks occur widely in the basin of Lake Chaka. The lake became progressively saline in the last 17,000 years (Liu et al., in submission). The Lake Chaka region, isolated from oceanic air masses, is characterized by a highly continental climate. Local meteorological data in the region indicate that the mean annual temperature is 3.5 °C, with a low mean monthly temperature of -12.4°C in January and a high mean monthly temperature of 14.4°C in July. The mean annual precipitation (197.6 mm) is far less than the mean annual evaporation (2074.1 mm).

The lake has no outlet but is fed by fresh water from Mo River at its northwestern margin, Hei River at its southeastern margin, and springs on its northeastern and southwestern bank (Fig.1). It covers an area of 105 km^2 with a catchment area of about 11,600 km^2. The depth of lake water varies largely from 50-60 cm in the rainy season (June-July) to just 1 cm in the dry season (January – March). Like many other salt lakes in the Qaidam Basin, the salinity of the Chaka lake water at present is between 31.7 to 34.7%. The lake water chemistry is dominated by ions Na^+, Mg^{2+}, Cl^-, and SO_4^{2-} but also contains smaller amounts of Ca^{2+}, K^+, CO_3^{2-} and HCO_3^- (Liu et al., 2004) The lake water is currently saturated with respect to halite.

Sampling

In October 2004, a 900-cm long sediment core was drilled from the southeastern part of the lake (Fig. 1) at a water depth of 2 cm using a hydraulic pressure-driven coring device made in China (HT150C, chamber length 1 m, inner diameter 7 cm). PVC pipes were used to retrieve the sediment core. The interior and exterior surfaces of the PVC pipes were alcohol sterilized. The stratigraphic sequences recovered from the core generally consist of halite from 0 to 546 cm, a mixture of soluble and sparingly soluble sulfate salts

with dark siliciclastics from 546 to 693 cm, and dark siliciclastic layer from 693 to 900 cm. This site was selected because a complete evaporative mineral sequence could be obtained. After collection, the whole core was cut into 100-cm segments and they were temporarily stored at 4^{o}C for 2 days. The core segments were then brought to Miami University, Ohio, USA (in less than 1 day) in thermally insulated boxes at 4^{o}C. Upon arrival, the core segments were immediately dissected into 5-cm- subsamples inside a Coy glove box filled with 97% N_2 and 3% H_2 (Coy Laboratory Products, Grass Lake, MI). The subsamples were designated using a combination of the lake name and depth in centimeters, i.e., LCKS200 (Lake Chaka Sediment from 200-205 cm in depth). Each sediment subsample was homogenized and then split into three aliquots: one for geochemistry (frozen at -80^{o}C), one for cultivation (refrigerated at 4^{o}C) and the other for DNA extraction (frozen at -80^{o}C).

Sediment geochemistry

Seventeen subsamples (LCKS000, LCKS140, LCKS200, LCKS285, LCKS415, LCKS490, LCKS595, LCKS630, LCKS640, LCKS645, LCKS650, LCKS660, LCKS670, LCKS745, LCKS815, LCKS815, and LCKS880) were analyzed for soluble salts, pH, sulfate, total organic carbon, and total nitrogen. Due to the dryness of the core, it was not possible to obtain pore water chemistry data. Instead, total soluble salts, total soluble sulfate, pH, total organic carbon (TOC) and total nitrogen contents of selected sediment subsamples were measured in the Service Testing and Research Laboratory of the Ohio State University, USA. These methods are available on-line at http://www.oardc.ohio-state.edu/starlab/references.asp. X-ray diffraction (XRD) analysis was carried out on the sediment subsamples as previously described (Jiang, et al., accepted). Because iron-reducing isolates were obtained from LCKS880 and they were used to reduce Fe(III) in this sediment sample, total iron and ferrous iron concentrations in this sediment sample were determined following a previously published method (Andrade, et al., 2002).

Total microbial counts

Acridine orange direct count (AODC) was performed on selected sediment subsamples (13 subsamples, LCKS000, LCKS140, LCKS200, LCKS285, LCKS415, LCKS490, LCKS595, LCKS630, LCKS640, LCKS645, LCKS745, LCKS815, and LCKS880) according to the procedure described previously (Jiang, et al., 2006).

Isolation of microorganisms

Five subsamples (salt grains: LCKS000, LCKS200 and LCKS595; muddy silty sand: LCKS745 and LCKS880) were chosen for cultivation. The salt grains and muddy silty sands were dissolved or dispersed in 20% and 2% (w/v) sterilized NaCl brine, respectively, for three hours before plating onto petri dishes with nutrient media (Vreeland and Powers, 1999). Three media (targeting aerobic/facultative heterotrophs) with a variable NaCl concentration were used (Table 1): seawater basal salts with 0.1% yeast extract (SS) (Rodriguez-Valera, et al., 1985), MS salts with 0.1% yeast extract (Maturrano, et al., 2006) and MR2A medium (Litchfield, et al., 1999). The petri dishes were incubated at 30^{o}C for 1 to 8 weeks. Based on the color, size, and shape (Table 1), round colonies were picked, and were directly subjected to 16S rRNA gene analysis following the procedures by Jiang et al. (2006).

Physiological characterization of selected isolates

Physiological tests (salinity tolerance) for selected isolates were performed according to Jiang et al. (2006). Microbial reduction of Fe(III) in NAu-2 (an iron-rich smectite clay from the Clay Minerals Society) and the source sediment (from which the isolates were obtained) using iron-reducing isolates (*Shewanella* sp., see the result section) were performed following the procedures described previously (Jaisi, et al., 2005). Briefly, both the source sediment (freeze dried) and NAu-2 were suspended in bicarbonate buffer (2.5 g $NaHCO_3$ and 0.1 g KCl per 1 L DI water) to reach a final concentration of 5 mg L^{-1} in the test tubes, which were then purged with N_2:CO_2 gas mix (80:20) and sealed with

butyl rubber stoppers. Before inoculation, 100 μM AQDS (9, 10-anthraquinone-2, 6-disulfonic acid) was added in selected treatments and 100 mM Na lactate was added in all test tubes (including controls) to serve as electron donor. Freshly washed iron-reducing cells in bicarbonate buffer (same as above) were added by using sterile syringes to reach a final concentration of 10^7 cells/mL. Duplicate was run on all tests.

PLFA analyses of the sediment samples

Lipid analyses of thirteen selected sediment subsamples along the length of the core, covering the entire range of salinity, were performed at the Savannah River Ecology Laboratory of the University of Georgia. Briefly, lyophilized sediment samples were extracted with a single-phase organic solvent system that was composed of chloroform, methanol, and aqueous phosphate buffer (50 mM, pH 7.4) in a ratio of 1:2:0.8 (v:v:v) (White, et al., 1979). After overnight extraction, equal volumes of chloroform and nanopure water were added to the extractant, resulting in a two-phase system. The lower organic (lipid-containing) phase was collected and fractionated on a silicic acid column into neutral lipids, glycolipids, and polar lipids. The polar lipids were subjected to a mild alkaline methanolysis to produce fatty acid methyl esters (FAMEs), which were analyzed by gas chromatography/mass spectrometry (GC/MS) according to a previously published procedure (Zhang, et al., 2004).

DNA isolation, PCR amplification and phylogenetic analyses

Genomic DNA of the five selected sediment samples (the same as those used for cultivation) was extracted as previously described (Jiang, et al., 2006). Total community DNA was amplified using 16S rRNA gene primer sets, Bac27F/Univ1492R and Arch21F/Univ1492R (Table 2) for *Bacteria* and *Archaea*, respectively. PCR conditions were established and PCR products were purified according to Jiang et al. (2006). The PCR reactions were run for 25 cycles. Ten clone libraries (five samples for *Bacteria* and *Archaea*, respectively) were constructed and plasmid DNA of randomly selected clones was extracted. The 16S rRNA gene sequencing reaction was carried out using primer

Bac27F for *Bacteria* and Arch21F for *Archaea* with the BigDye Terminator version 3.1 chemistry (Applied Biosystems, Foster City, CA, USA). The 16S rRNA gene sequence was determined with an ABI 3730 automated sequencer. Sequences were typically ~700 bp long. Clone sequences were manually checked for chimeras using Ribosomal Database Project II (http://wdcm.nig.ac.jp/RDP/html/index.html) and identified chimeric sequences were removed. Phylogenetic and statistical (rarefaction curve, diversity indices, and LIBSHUFF) analyses were carried out in the same manner as previously described (Jiang, et al., 2006). Briefly, neighbor-joining phylogenies were constructed from dissimilar distance and pairwise comparisons with the Jukes-Cantor distance model using the MEGA (molecular evolutionary genetics analysis) program, version 3.1. Rarefaction and LIBSHUFF analyses were performed using software available online at http://www.uga.edu/~strata/software/Software.html and http://whitman.myweb.uga.edu/libshuff.html, respectively.

Quantitative polymerase chain reaction (Q-PCR)

The same five sediment subsamples as those used for cultivation and clone library construction (see above) were used to determine the abundance of certain groups of microorganisms along the salinity gradient. 16S rRNA gene primers (Table 2) specific for *Archaea* (Arch349F/Arch806R), *Bacteria* (Bac331F/Bac797R), halophilic *Archaea* (HK1F/H589R), and *Crenarchaeota* (669F/886R) were employed for quantification of total archaeal, bacterial, halophilic archaeal and crenarchaeotal 16S rRNA gene copy numbers, respectively. Amplification conditions were 50^{o}C for 2 min, and then 95^{o}C for 15 min, followed by 45 cycles (15s at 94^{o}C, 30s for annealing at the temperatures shown in Table 2, and 30s at 72^{o}C) in a reaction volume of 25 µl, containing 12.5 µl of SYBR-Green Master Mix (QIAGEN, Valencia, CA) and 5 pmol of each primer. PCR products from the same environmental samples using *Archaea*-(21F/1492R), *Bacteria*-(27F/1492R), halophilic *Archaea*-(HK1F/H589R) and *Crenarchaeota* (133F/1492R)-specific primers were used as standards, which, with serial dilutions, were used to yield standard curves for each group of organisms. All quantitative PCR reactions for the standard curves were performed in duplicate. The amplification yielded reliable

exponential patterns with template amounts in the range of 10^4 to 10^8 16S rRNA gene copies. The data were used to create standard curves correlating the C_T values with 16S rRNA gene copy numbers. Linear plots between the Ct value (not shown) and log(copy numbers/reaction) were obtained with correlation coefficients of R^2=0.993, 0.965, 0993 and 0.991 for *Archaea*, *Bacteria*, halophilic *Archaea* and *Crenarchaeota*, respectively. For all samples, 16S rRNA gene copy numbers were converted to cell numbers by a conversion factor of 3.6 (Klappenbach, et al., 2001).

Nucleotide sequence accession numbers

The sequences determined in this study have been deposited in the GenBank database under accession numbers EF201669- EF201714 for the archaeal clone sequences, and EF201715- EF201773 for the bacterial clone sequences, and EF201774- EF201785 for isolate sequences.

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Table 1. Distribution of cultured bacterial and archaeal isolates from Lake Chaka sediments

Depth (cm)	Media (20% salinity)			Media (2% salinity)		
	SS[a]	MS[b]	MR2A[c]	SS	MS	MR2A
0-5	2 bacteria 10 archaea	3 bacteria 17 archaea	2 bacteria 5 archaea			
200-205	2 bacteria 7 archaea	8 archaea	10 archaea			
595-600	No growth	No growth	No growth	No growth	No growth	No growth
745-750				4 bacteria	11 bacteria	2 bacteria
880-805				No growth	3 bacteria	4 bacteria

a: Rodriguez-Valera and colleagues (1985).
b: Maturrano and colleagues (2006).
c: Litchfield and colleagues (1999).
The media salinity was designed to approximately match the measured values in the respective samples. Blank cells in the table represent gaps (unnecessary trials) in the experimental design.

Table 2. Primers used in this study

Primers	Primer sequence (5'→3')	Annealing temp. (^{o}C)	Target group	References
Arch21F	TTC YGG TTG ATC CYG CCR GA	54*	Domain *Archaea* (16S rRNA gene)	(DeLong, et al., 1994)).
Bac27F	AGA GTT TGG ATC MTG GCT CAG	55*	Domain *Bacteria* (16S rRNA gene)	(Lane, 1991).
133F	TGT TGA CTA CGT GTT ACT GAG	52*	Crenarchaeota (Q-PCR)	(Simon, et al., 2000)).
Univ1492R	CGG TTA CCT TGT TAC GAC TT		Universal (16S rRNA gene)	(Lane, 1991).
Arch349F Arch806R	GYG CAS CAG KCG MGA AW GGA CTA CVS GGG TAT CTA AT	50	Domain *Archaea* (Q-PCR)	(Takai and Horikoshi, 2000).
HK1F H589R	ATT CCG GTT GAT CCT GCC GG AGC TAC GGA CGC TTT AGG C	56	Halophilic *Archaea*(Q-PCR)	(Litchfield and Gillevet, 2002).
669F 886R	CGA CGG TGA GGG ATG AAA G CCA GGC GGC AAA CTT AAC	55	Crenarchaeota (Q-PCR)	(Simon, et al., 2005).
Bac331F Bac797R	TCC TAC GGG AGG CAG CAG T GGA CTA CCA GGG TCT AAT CCT GTT	54	Domain *Bacteria* (Q-PCR)	(Nadkarni, et al., 2002).

* When the primer works with Univ1492R

Figure captions

Fig. 1. A geographic map showing lakes on the Tibetan Plateau and the location of Lake Chaka in Northwestern China. The drilling site in the lake is shown.

Fig. 2. (A) Lithology of the 900-cm sediment core showing a progressive change of evaporative minerals from the bottom to the top. (B) Depth distributions of soluble salts, sulfate concentration and pH along the length of the core. (C) Depth distribution of total nitrogen and organic carbon content along the core. (D) Microbial abundance distribution along the core as determined by AODC, PLFA and Q-PCR. Single samples were measured multiple times, and analytical errors were smaller than the symbol sizes (*the *Crenarchaeota* in LCKS000, LCKS200 and LCKS595 were not detectable; **Halophilic archaeal abundance in LCKS745 was 9.92×10^4 cells g^{-1}. Halophilic *Archaea* in LCKS880 were not detected).

Fig. 3. Depth profile of PLFA composition for 10 selected sediment subsamples. TerBrStas: terminally branched saturated fatty acids; Monos: monounsaturated fatty acids; MidBrSats: mid-chain branched saturated fatty acids; Nsat: normal straight chain saturated fatty acids; Polyenoics: polyunsaturated fatty acids.

Fig. 4. Neighbor-joining tree (partial sequences, ~700 bp) showing the phylogenetic relationships of bacterial 16S rRNA gene sequences cloned from the sediment subsamples of the 900-cm sediment core to closely related sequences from the GenBank database. One representative clone type within each phylotype is shown, and the number of clones within each phylotype is shown at the end (after the GenBank accession number). If there is only one clone sequence within a given phylotype, the number "1" is omitted. Clone sequences from this study are coded as follows for the example of LCKS595B19: LCKS, Lake Chaka sediment; 595, sample depth in centimeters; B, bacterium; 19, clone number. The isolate sequences are coded as described in the text: LCKS000B-Isolate25, Lake Chaka sediments from 000 cm depth, bacterial isolate 25. Scale bars indicate the Jukes-Cantor distances. Bootstrap values of >50% (for 500 iterations) are shown. *Aquifex pyrophilus* is used as the outgroup.

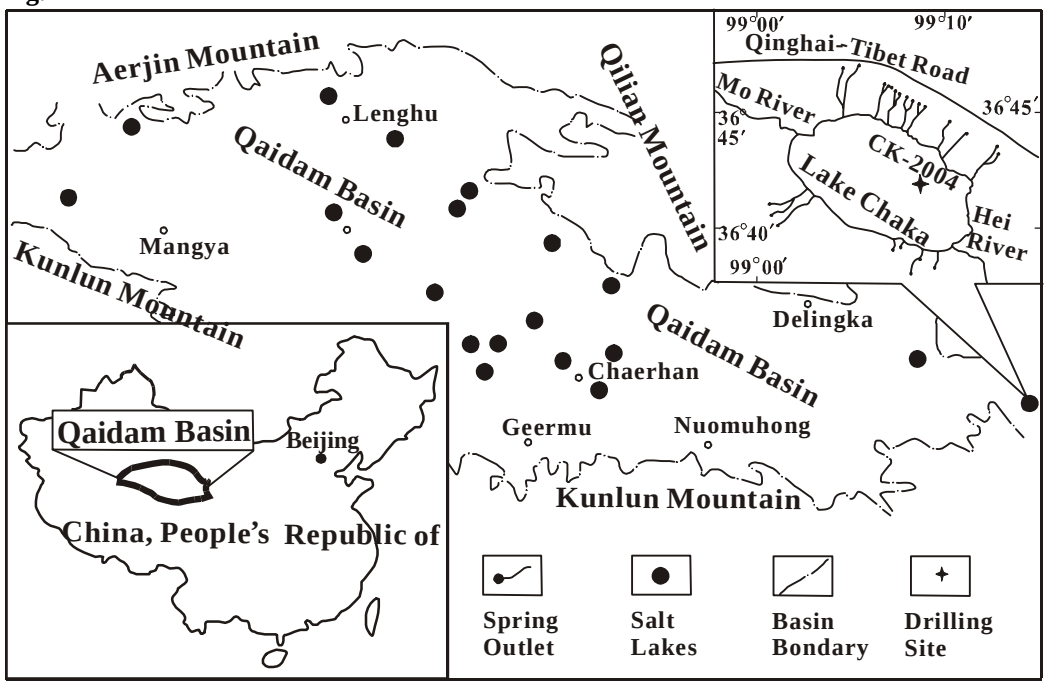
Fig. 5. NaCl requirements of representative isolates from the core. The histogram shown for each isolate indicates the range of NaCl concentration within which visible growth occurred. The dot on each bar indicates the optimum NaCl concentration to support growth.

Fig. 6. Time-course production of Fe(II) from microbial reduction of Fe(III) in NAu-2 (A) and the source sediment (LCKS880) (B), respectively, by an isolate (LCKS880-Isolate44). The Fe(II) concentration was measured by Ferrozine assay.

Fig. 7. Neighbor-joining tree (partial sequences, ~700 bp) showing the phylogenetic relationships of archaeal 16S rRNA gene sequences cloned from the sediment subsamples of the 900-cm core to closely related sequences from the GenBank database. The same algorithms as those for the bacterial tree (Fig. 5) were used. *Aquifex pyrophilus* is used as an outer group. One representative clone type within each phylotype is shown, and the number of clones within each phylotype is shown at the end (after the GenBank accession number).

Fig. 8. Schematic figure of the sediment core and frequencies of phylotypes affiliated with major phylogenetic groups in the bacterial and archaeal clone libraries for LCKS000, LCKS200, LCKS595, LCKS745, and LCKS880. *α, β, γ,* and *δ*: *Alpha-, Beta-, Gamma-,* and *Delta-proteobacteria*; F: *Firmicutes*; B: *Bacteroidetes*; V: *Verrucomicrobia*; KB1: Kebrit Deep (Red Sea) brine-seawater interface group; UCB: unclassified bacteria; H: *Halobacteriales*; SA1: SA1 Group of the *Euryarchaeota*; CdM: Candidate division MSBL1 of the *Euryarchaeota*; C1: Cluster1 of the *Euryarchaeota*; C: *Crenarchaeota*.

Fig. 1



✦ **Drilling site**

Fig. 2

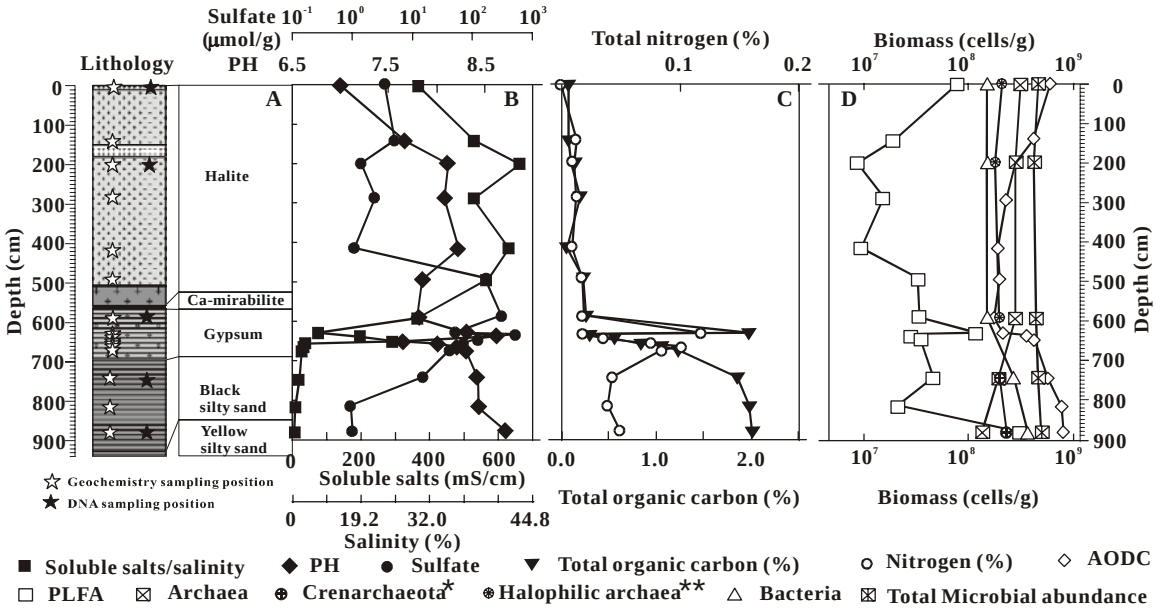


Fig. 3

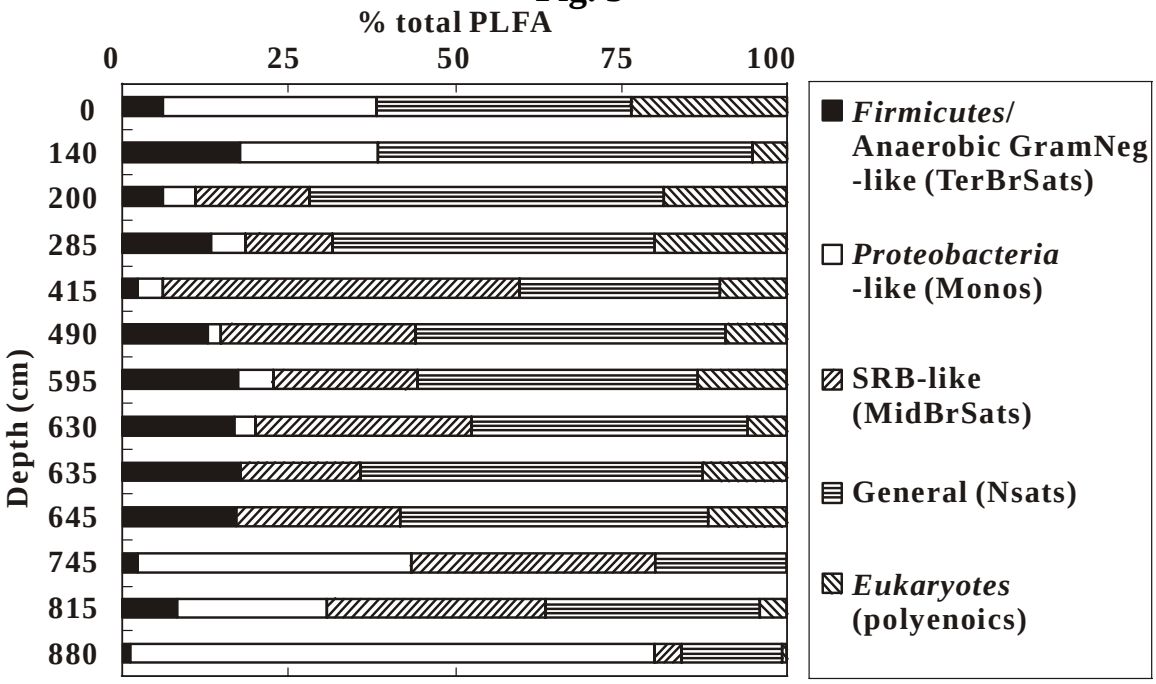
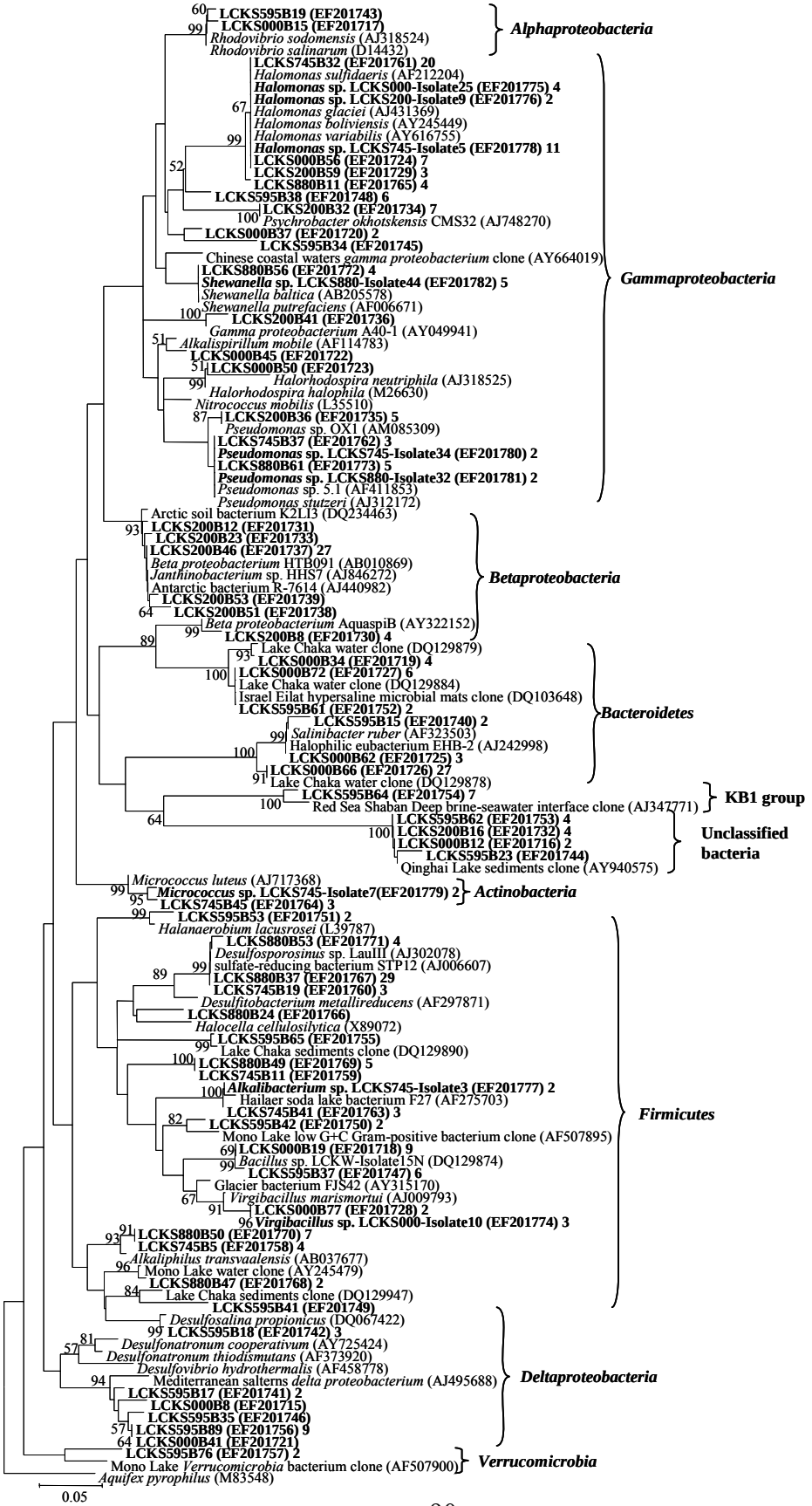

% total PLFA
0
25
50
75
100
Depth (cm)
0
140
200
285
415
490
595
630
635
645
745
815
880
Firmicutes/ Anaerobic GramNeg -like (TerBrSats)
Proteobacteria -like (Monos)
SRB-like (MidBrSats)
General (Nsats)
Eukaryotes (polyenoics)

Fig. 4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Rhodovibrio salinarum (D14432)
Alphaproteobacteria
LCKS745B32 (EF201761) 20
Halomonas sulfidaeris (AF212204)
Halomonas sp. LCKS000-Isolate25 (EF201775) 4
Halomonas sp. LCKS200-Isolate9 (EF201776) 2
Halomonas glaciei (AJ431369)
Halomonas boliviensis (AY245449)
Halomonas variabilis (AY616755)
Halomonas sp. LCKS745-Isolate5 (EF201778) 11
LCKS000B56 (EF201724) 7
LCKS200B59 (EF201729) 3
LCKS880B11 (EF201765) 4
LCKS595B38 (EF201748) 6
LCKS200B32 (EF201734) 7
Psychrobacter okhotskensis CMS32 (AJ748270)
LCKS000B37 (EF201720) 2
LCKS595B34 (EF201745)
Chinese coastal waters gamma proteobacterium clone (AY664019)
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Shewanella sp. LCKS880-Isolate44 (EF201782) 5
Shewanella baltica (AB205578)
Shewanella putrefaciens (AF006671)
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Gamma proteobacterium A40-1 (AY049941)
Alkalispirillum mobile (AF114783)
LCKS000B45 (EF201722)
LCKS000B50 (EF201723)
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Halorhodospira halophila (M26630)
Nitrococcus mobilis (L35510)
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LCKS880B61 (EF201773) 5
Pseudomonas sp. LCKS880-Isolate32 (EF201781) 2
Pseudomonas sp. 5.1 (AF411853)
Pseudomonas stutzeri (AJ312172)
Gammaproteobacteria
Arctic soil bacterium K2L13 (DQ234463)
LCKS200B12 (EF201731)
LCKS200B23 (EF201733)
LCKS200B46 (EF201737) 27
Beta proteobacterium HTB091 (AB010869)
Janthinobacterium sp. HHS7 (AJ846272)
Antarctic bacterium R-7614 (AJ440982)
LCKS200B53 (EF201739)
LCKS200B51 (EF201738)
Beta proteobacterium AquaspiB (AY322152)
Betaproteobacteria
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Lake Chaka water clone (DQ129879)
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LCKS000B72 (EF201727) 6
Lake Chaka water clone (DQ129884)
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LCKS595B15 (EF201740) 2
Salinibacter ruber (AF323503)
Halophilic eubacterium EHB-2 (AJ242998)
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LCKS000B66 (EF201726) 27
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Bacteroidetes
LCKS595B64 (EF201754) 7
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KB1 group
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LCKS200B16 (EF201732) 4
LCKS000B12 (EF201716) 2
LCKS595B23 (EF201744)
Qinghai Lake sediments clone (AY940575)
Unclassified bacteria
Micrococcus luteus (AJ717368)
Micrococcus sp. LCKS745-Isolate7(EF201779) 2
Actinobacteria
LCKS745B45 (EF201764) 3
LCKS595B53 (EF201751) 2
Halanaerobium lacusrosei (L39787)
LCKS880B53 (EF201771) 4
Desulfosporosinus sp. LauIII (AJ302078)
sulfate-reducing bacterium STP12 (AJ006607)
LCKS880B37 (EF201767) 29
LCKS745B19 (EF201760) 3
Desulfitobacterium metallireducens (AF297871)
LCKS880B24 (EF201766)
Halocella cellulosilytica (X89072)
LCKS595B65 (EF201755)
Lake Chaka sediments clone (DQ129890)
LCKS880B49 (EF201769) 5
LCKS745B11 (EF201759)
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Hailaer soda lake bacterium F27 (AF275703)
LCKS745B41 (EF201763) 3
LCKS595B42 (EF201750) 2
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Bacillus sp. LCKW-Isolate15N (DQ129874)
LCKS595B37 (EF201747) 6
Glacier bacterium FJS42 (AY315170)
Virgibacillus marismortui (AJ009793)
LCKS000B77 (EF201728) 2
Virgibacillus sp. LCKS000-Isolate10 (EF201774) 3
LCKS880B50 (EF201770) 7
LCKS745B5 (EF201758) 4
Alkaliphilus transvaalensis (AB037677)
Mono Lake water clone (AY245479)
LCKS880B47 (EF201768) 2
Lake Chaka sediments clone (DQ129947)
LCKS595B41 (EF201749)
Firmicutes
Desulfosalina propionicus (DQ067422)
LCKS595B18 (EF201742) 3
Desulfonatronum cooperativum (AY725424)
Desulfonatronum thiodismutans (AF373920)
Desulfovibrio hydrothermalis (AF458778)
Mediterranean salterns delta proteobacterium (AJ495688)
LCKS595B17 (EF201741) 2
LCKS000B8 (EF201715)
LCKS595B35 (EF201746)
LCKS595B89 (EF201756) 9
LCKS000B41 (EF201721)
Deltaproteobacteria
LCKS595B76 (EF201757) 2
Mono Lake Verrucomicrobia bacterium clone (AF507900)
Verrucomicrobia
Aquifex pyrophilus (M83548)
0.05

Fig. 5

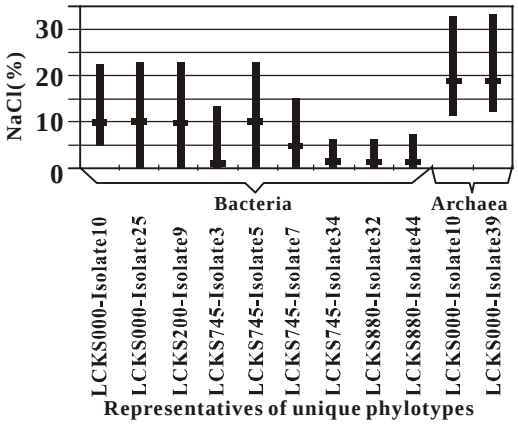
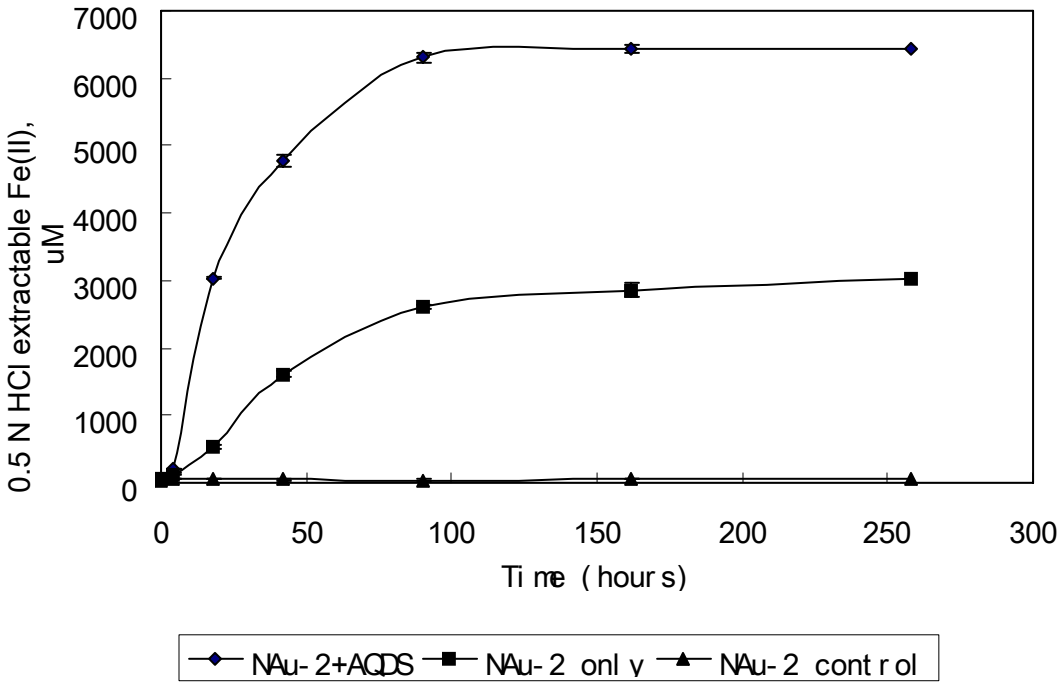

NaCl(%)
30
20
10
0
Bacteria
Archaea
LCKS000-Isolate10
LCKS000-Isolate25
LCKS200-Isolate9
LCKS745-Isolate3
LCKS745-Isolate5
LCKS745-Isolate7
LCKS745-Isolate34
LCKS880-Isolate32
LCKS880-Isolate44
LCKS000-Isolate10
LCKS000-Isolate39
Representatives of unique phylotypes

Fig. 6 A


7000
6000
5000
4000
3000
2000
1000
0
0.5 N HCl extractable Fe(II), uM
0
50
100
150
200
250
300
Time (hours)
NAu-2+AQDS
NAu-2 only
NAu-2 control

B

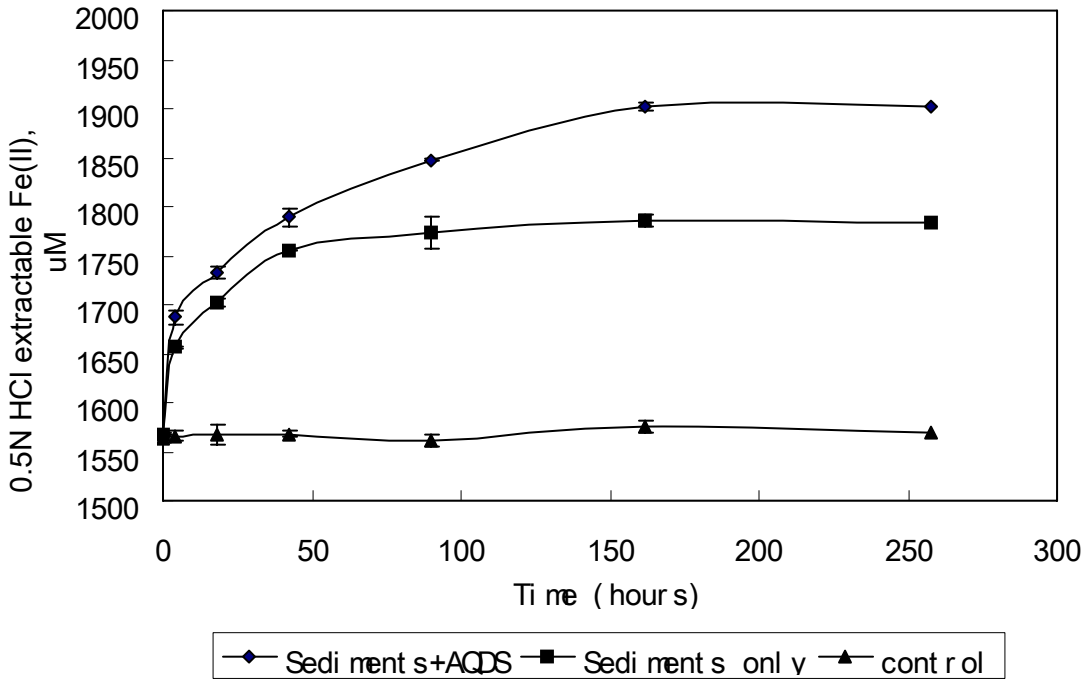
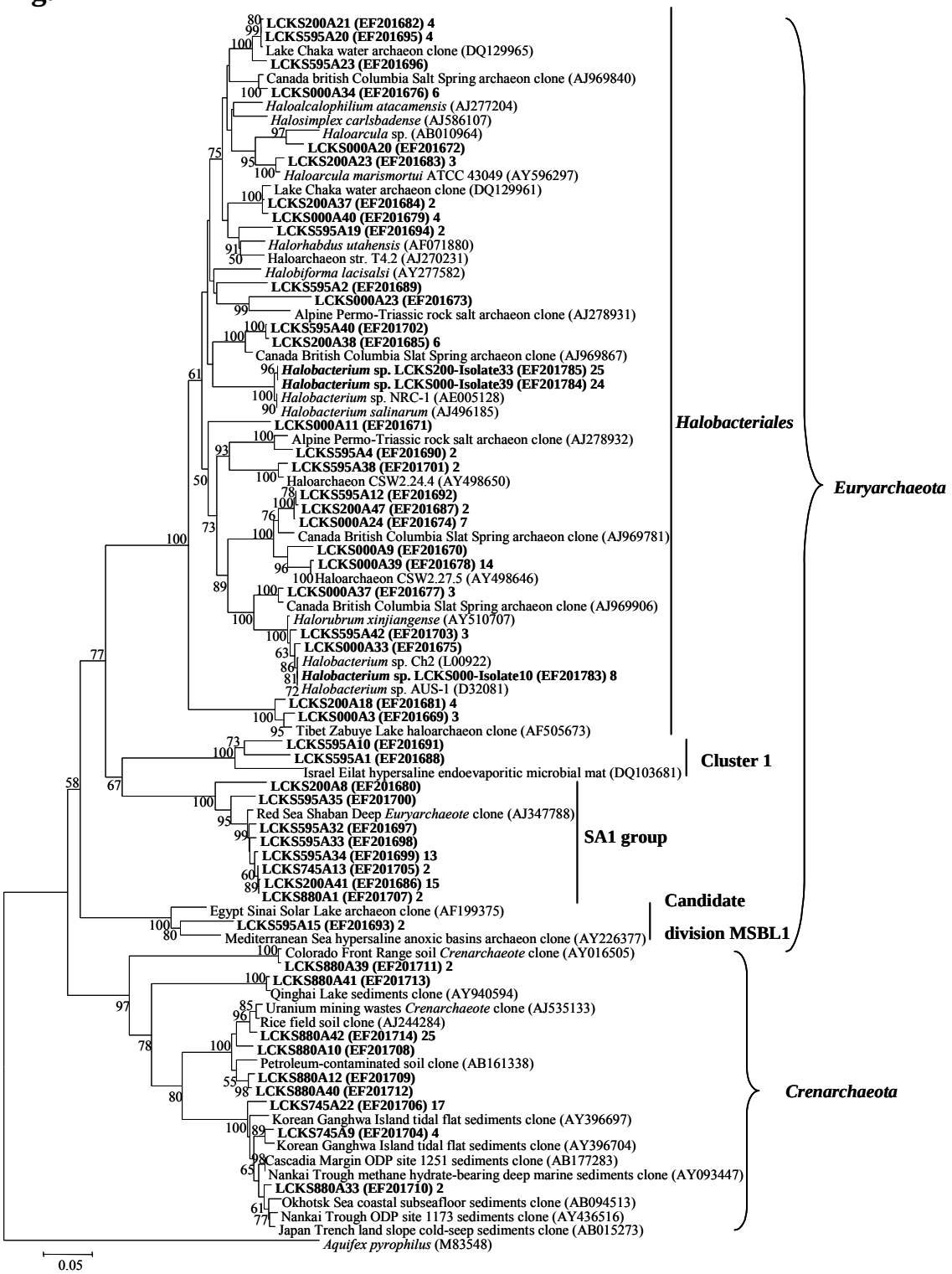

2000
1950
1900
1850
1800
1750
1700
1650
1600
1550
1500
0.5N HCl extractable Fe(II), uM
0
50
100
150
200
250
300
Time (hours)
Sediments+AQDS
Sediments only
control

Fig. 7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Lake Chaka water archaeon clone (DQ129965)
LCKS595A23 (EF201696)
Canada british Columbia Salt Spring archaeon clone (AJ969840)
LCKS000A34 (EF201676) 6
Haloalcalophilium atacamensis (AJ277204)
Halosimplex carlsbadense (AJ586107)
Haloarcula sp. (AB010964)
LCKS000A20 (EF201672)
LCKS200A23 (EF201683) 3
Haloarcula marismortui ATCC 43049 (AY596297)
Lake Chaka water archaeon clone (DQ129961)
LCKS200A37 (EF201684) 2
LCKS000A40 (EF201679) 4
LCKS595A19 (EF201694) 2
Halorhabdus utahensis (AF071880)
Haloarchaeon str. T4.2 (AJ270231)
Halobiforma lacisalsi (AY277582)
LCKS595A2 (EF201689)
LCKS000A23 (EF201673)
Alpine Permo-Triassic rock salt archaeon clone (AJ278931)
LCKS595A40 (EF201702)
LCKS200A38 (EF201685) 6
Canada British Columbia Slat Spring archaeon clone (AJ969867)
Halobacterium sp. LCKS200-Isolate33 (EF201785) 25
Halobacterium sp. LCKS000-Isolate39 (EF201784) 24
Halobacterium sp. NRC-1 (AE005128)
Halobacterium salinarum (AJ496185)
LCKS000A11 (EF201671)
Alpine Permo-Triassic rock salt archaeon clone (AJ278932)
LCKS595A4 (EF201690) 2
LCKS595A38 (EF201701) 2
Haloarchaeon CSW2.24.4 (AY498650)
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LCKS200A47 (EF201687) 2
LCKS000A24 (EF201674) 7
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LCKS000A9 (EF201670)
LCKS000A39 (EF201678) 14
Haloarchaeon CSW2.27.5 (AY498646)
LCKS000A37 (EF201677) 3
Canada British Columbia Slat Spring archaeon clone (AJ969906)
Halorubrum xinjiangense (AY510707)
LCKS595A42 (EF201703) 3
LCKS000A33 (EF201675)
Halobacterium sp. Ch2 (L00922)
Halobacterium sp. LCKS000-Isolate10 (EF201783) 8
Halobacterium sp. AUS-1 (D32081)
LCKS200A18 (EF201681) 4
LCKS000A3 (EF201669) 3
Tibet Zabuye Lake haloarchaeon clone (AF505673)
LCKS595A10 (EF201691)
LCKS595A1 (EF201688)
Israel Eilat hypersaline endoevaporitic microbial mat (DQ103681)
LCKS200A8 (EF201680)
LCKS595A35 (EF201700)
Red Sea Shaban Deep Euryarchaeote clone (AJ347788)
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LCKS595A33 (EF201698)
LCKS595A34 (EF201699) 13
LCKS745A13 (EF201705) 2
LCKS200A41 (EF201686) 15
LCKS880A1 (EF201707) 2
Egypt Sinai Solar Lake archaeon clone (AF199375)
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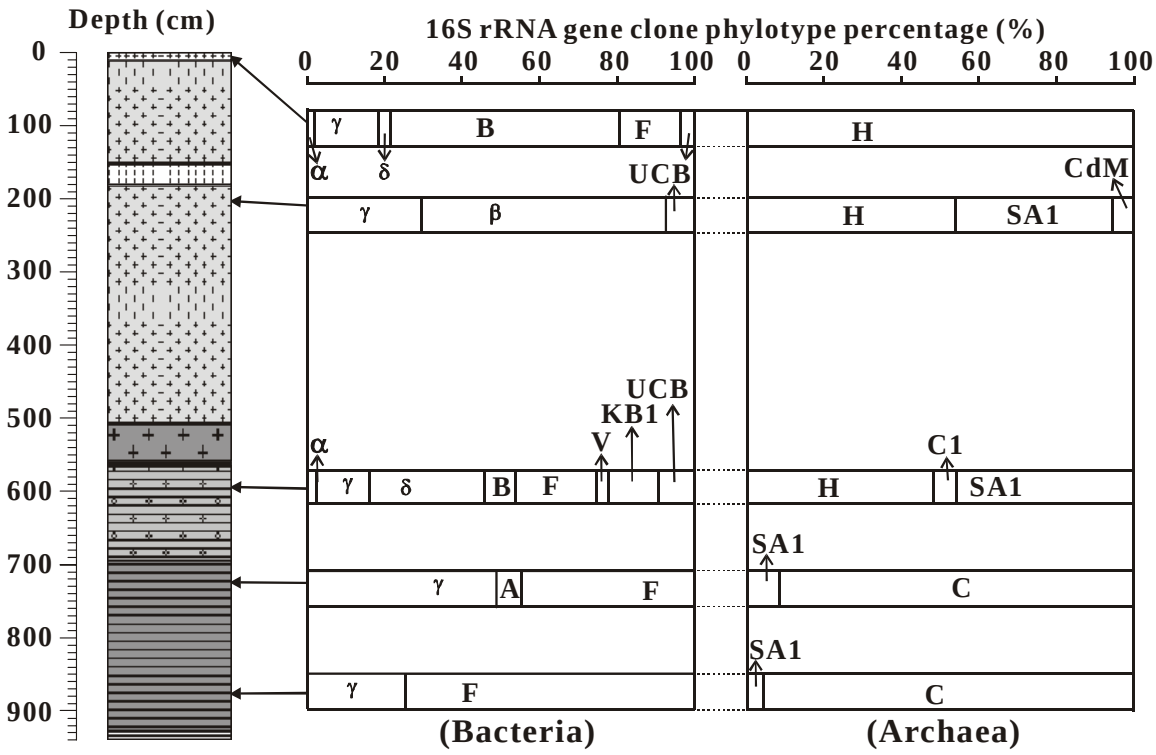
Mediterranean Sea hypersaline anoxic basins archaeon clone (AY226377)
Colorado Front Range soil Crenarchaeote clone (AY016505)
LCKS880A39 (EF201711) 2
LCKS880A41 (EF201713)
Qinghai Lake sediments clone (AY940594)
Uranium mining wastes Crenarchaeote clone (AJ535133)
Rice field soil clone (AJ244284)
LCKS880A42 (EF201714) 25
LCKS880A10 (EF201708)
Petroleum-contaminated soil clone (AB161338)
LCKS880A12 (EF201709)
LCKS880A40 (EF201712)
LCKS745A22 (EF201706) 17
Korean Ganghwa Island tidal flat sediments clone (AY396697)
LCKS745A9 (EF201704) 4
Korean Ganghwa Island tidal flat sediments clone (AY396704)
Cascadia Margin ODP site 1251 sediments clone (AB177283)
Nankai Trough methane hydrate-bearing deep marine sediments clone (AY093447)
LCKS880A33 (EF201710) 2
Okhotsk Sea coastal subseafloor sediments clone (AB094513)
Nankai Trough ODP site 1173 sediments clone (AY436516)
Japan Trench land slope cold-seep sediments clone (AB015273)
Aquifex pyrophilus (M83548)
0.05
Halobacteriales
Cluster 1
SA1 group
Candidate division MSBL1
Euryarchaeota
Crenarchaeota

Fig. 8


Depth (cm)
16S rRNA gene clone phylotype percentage (%)
0
100
200
300
400
500
600
700
800
900
0 20 40 60 80 100
0 20 40 60 80 100
γ
B
F
α
δ
UCB
γ
β
UCB
KB1
V
α
γ
δ
B
F
γ
A
F
γ
F
H
CdM
H
SA1
C1
H
SA1
SA1
C
SA1
C
(Bacteria)
(Archaea)

CHAPTER 4:

Diversity and Abundance of Ammonia-Oxidizing Archaea and Bacteria in the Sediments of Qinghai Lake, Northwestern China

Running title: Diversity and Abundance of AOA and AOB in Lake sediments

Hongchen Jiang[1], Hailiang Dong[1,2*], Bingsong Yu[2,3] and Ji Shen[4]

1: Department of Geology
Miami University
Oxford, OH 45056

2: Geomicrobiology Laboratory
China University of Geosciences
Beijing, 100083, China

3: School of Earth Sciences
China University of Geosciences
Beijing, China, 100083

4: Key Laboratory of Lake Sediment and Environment
Nanjing Institute of Geography and Limnology, Chinese Academy of Sciences,
Nanjing, Jiangsu 210008, China

*Corresponding author: Hailiang Dong
Department of Geology
Miami University
Oxford, OH 45056
Tel: 513-529-2517
Fax: 513-529-1542
Email: dongh@muohio.edu

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Abstract

Ammonia oxidation, mostly mediated by ammonia oxidizers, is a key step in global nitrogen cycling. In this study, the abundance and diversity of *amoA* genes in anoxic lake sediments were evaluated on a 5-m sediment core collected from a saline lake, Qinghai Lake, on the Tibetan Plateau. A highly integrated approach was employed including geochemistry, functional gene (*amoA*) analysis and quantitative PCR (Q-PCR). Clone sequences affiliated with anammox bacteria were not retrieved. The presence of ammonia-oxidizing archaea (AOA) and putative aerobic ammonia-oxidizing bacteria (AOB) was confirmed in anoxic saline lake sediments with PCR and subsequent clone sequencing. Both AOB and AOA appeared to respond differently to increasing salinity: AOB were more sensitive to salinity change than AOA. The AOB community was composed of *Nitrosomonas*- and *Nitrosospira*-like sequences, and the AOA community was predominated by sequences related to those previously retrieved from soils and sediments. The AOB *amoA* genes were more abundant than AOA *amoA* genes, which is opposite to the previous findings in soils and oceans. Both AOA and AOB may play a key role in denitrification in anoxic saline sediments of Qinghai Lake. These data have important implications for better understanding of the potential role of ammonia oxidizers operate in anaerobic saline lake sediments.

Keywords: amoA, ammonium-oxidation, Archaea, Bacteria, Qinghai Lake.

1. Introduction

Ammonia oxidation, through either nitrification (**$NH_3 \rightarrow NO_2^- \rightarrow NO_3^-$**) or anammox ($NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$) is an important part of the biological nitrogen cycle globally. This process is mediated by either ammonia-oxidizing bacteria (AOB) or ammonia-oxidizing archaea (AOA). Most of the latter belong to non-thermophilic Crenarchaeota [see review by Francis et al. (2007) and refs therein], and they occur abundantly in soils (Leininger, et al., 2006), marine water columns and sediments (Francis, et al., 2005, Wuchter, et al., 2006), estuary sediments (Beman and Francis, 2006), thermal springs (Zhang et al. unpublished results and (Weidler, et al., 2007)) and wastewater treatment plant bioreactors (Park, et al., 2006).

AOB comprise three groups: obligate aerobes as monophyletic groups within the *Beta-* and *Gamm-proteobacteria* and anaerobes (anammox bacteria) within the order *Planctomycetales*. Since the first anammox bacterium was discovered by Strous et al. (1999), molecular techniques, e.g. 16S rRNA gene analysis, fluorescence in situ hibridization (FISH), etc., have been performed to describe the distribution and diversity of anammox bacteria in a variety of environments [see reviews by Francis et al. (2007) and Dalsgaard et al. (2005), and refs therein]. Anammox bacteria have been found to occur widely in marine environments (Schmid, et al., 2007). Some factors, such as sediment mineralization rates, nitrate availability and organic concentration, may the control anammox process within sediments (Dalsgaard, et al., 2005). However, no molecular work involving anammox and AOA has been performed so far on anoxic saline lake sediments.

Aerobic AOB have long received attention and have been widely surveyed with 16S rRNA gene and *amoA* gene analyses [see review by (Kowalchuk and Stephen, 2001)]. Aerobic ammonia oxidation is predominantly carried out by the genus *Nitrosomonas* and *Nitrosospira* within the *Betaproteobacteria* and the genus *Nitrosococcus* within the *Gammproteobacteria* (such as *Nitrosococcus oceanus*, the only known widely-distributed marine AOB species within the *Gammproteobacteria*, can oxidize nitrite to nitrate). Under anoxic conditions, however, in addition to their nitrification metabolic pathway, some *Nitrosomonas* strains are able to reduce nitrite or nitrogen dioxide with hydrogen, ammonium, or simple organic compounds as the electron donors (Abeliovich and

Vonhak, 1992, Bock, et al., 1995, Schmidt and Bock, 1997). Energy provided in this pathway is sufficient to allow survival under anerobic conditions, although it is not thought to support growth (Kowalchuk and Stephen, 2001). In certain settings, AOB have been shown to contribute minimally to autotrophic ammonia oxidation (Jordan, et al., 2005). Instead, for example, ammonia-oxidizing archaea (AOA) are more abundant than AOB in soils and oceans (Leininger, et al., 2006, Wuchter, et al., 2006), but it is not yet known if this observation is true in freshwater and saline lake environments.

Qinghai Lake (36°32'-37°15'N, 99°36'-100°47'E), the largest saline (12.5 practical salinity unit, psu) and alkaline (pH 9.3) lake on the Tibetan Plateau in NW China, represents a pristine environment, where perturbations from human activities have been minimal. This lake provides an opportunity to perform a focused study of natural (not human-altered) ammonia oxidation process and to fill in the current knowledge gap (i.e., lack of specific studies of anammox and AOA processes in saline lake sediments) and to answer the above question (i.e., which, between AOA and AOB, predominate the other). Previous molecular work (16S rRNA gene analysis and FISH) performed in Qinghai Lake showed that the *Alpha*-, *Beta*- and *Gamma*-*Proteobacteria* and the *Cytophaga*/*Flavobacterium*/*Bacteroidetes*(CFB) are dominant in the water column (Dong, et al., 2006, Wu, et al., 2006) and at the water-sediment interface (Dong, et al., 2006), and that low G+C Gram-positive bacteria are the predominant group in the top 40 cm anoxic sediments. Clone sequences affiliated with *Planctomycetes* (that may contain anammox bacteria) were retrieved only from subsamples at the water-sediment interface and 20 cm depth (Dong, et al., 2006).

The main objective of this research was to study the diversity of AOB and AOA and their relative abundance in the anoxic Qinghai Lake sediments. We employed an integrated approach including geochemistry, quantitative polymerase chain reaction (Q-PCR), and 16S rRNA gene/functional gene (*amoA*) analyses. Our results suggested 1) anammox is not important in anoxic Qinghai Lake sediments, 2) AOA do not outnumber AOB under anaerobic conditions in Qinghai Lake, and 3) the putative aerobic AOB may be able to survive in anoxic sediments by switching to low-level state (resting cells) or to the denitrification pathway with the reduction of nitrite or nitrogen dioxide. These results

have important implications for understanding global nitrogen cycling in lake ecosystems.

2. Materials and Methods

2.1. Description of the study site.

Qinghai Lake is a perennial lake located in a structural intermontane depression at the northeastern corner of the Qinghai-Tibetan Plateau (Fig. 1). The lake has an area of 4300 km^2 and lies within a catchment of limestones, sandstones, and shales (Henderson, et al., 2003). The average water depth is 19.2 m, and the maximum is 28.7 m. The altitude of the lake is 3196 m above sea level (masl), and the surrounding mountains rise to above 5200 masl (Dong, et al., 2006). The evaporation of the lake (~1400 mm/year) is in excess of mean annual precipitation (~400 mm/ year), resulting in the development of a saline lake with salinity of 12.5 g/L (Dong, et al., 2006, Shen, et al., 2005).

Recent seismic survey suggests that Qinghai Lake is separated into two subbasins by a normal faulting horst in the middle of the lake (An, 2003). The northern subbasin is more dynamic than the southern one because of input of a major river in the northwest and strong northwest wind. So the entire water column in the northern subbasin is well mixed in summer. In comparison, the southern subbasin is relatively quiet, within which the depositional environment is stable and water stratification occurs seasonally (CAS, 1979). Dissolved oxygen showed a slight gradient from 6 ppm at the surface to 3 ppm at the bottom in the water column in the eastern depression of the southern subbasin with a relatively long water column (~23 m) (Dong, et al., 2006), within which we selected our sampling site at approximately 15 km north of a fishing station called Erlangjian for easy access (labeled QH-2005 on Fig. 1) and a stable depositional environment.

2.2. Sediment Core Collection and Processing.

A 5-meter sediment core was collected in 2005 using a platform drilling rig (Shen et al., 2005) (Fig. 1). The core was retrieved in three segments (~2, 2, and 1 m) and they were cased inside polyvinyl chloride tubings that had been sterilized with alcohol. Upon retrival, the core segments were immediately capped and frozen in dry ice. In less than 1 week, the core segments were taken to China University of Geosciences in Beijing, where they were stored in a -80^{o}C freezer. Within 1 month, the core segments were

shipped to Miami University. Four subsamples of this sediment core were selected for geochemistry and microbiology work: QLS1: 30-45 cm, QLS2: 345-355 cm, QLS3: 399-415 cm, and QLS4: 458-470 cm. These subsamples represent different geochemical and climatic conditions (Shen, et al., 2005). Through multi-proxy analyses of a parallel sediment core, Shen et al. (2005) concluded that the Qinghai Lake area underwent an abrupt increase in temperature and precipitation during the period of 10.8-8.5 cal. kyr BP (corresponding to 500-399 cm in depth of the sediment core), and after that, Qinghai Lake generally underwent a dry and cold period until present. The sediment core was dissected inside a glove box (Coy Laboratory Products, Ann Arbor, MI), and the external layers were carefully removed using sterile tools to avoid possible contamination.

2.3. Geochemical Analyses.

Sediment pore water samples were collected following centrifugation of ~60 grams of each sediment subsample (at 5000 g for 10 minutes). The volume of pore water from each sediment subsample was ~11-23 mL. A strong odor of hydrogen sulfide was evident from pore water. pH measurements were made on pore water with a pH meter immediately after collection. For measurements of anions and cations, aliquots of pore water were fixed with HCl solution (0.1N HCl final concentration). The diluted pore water samples were analyzed by high performance liquid chromatography (HPLC) for anions (IonPac®AS14 column 4×250mm) and direct current plasma emission spectrometry (DCP) for cations. Major anions and cations were combined to calculate the salinity (psu). Sediment mineralogy was determined by X-ray diffraction according to Jiang et al. (Jiang, et al., in press).

2.4. DNA isolation, PCR amplification and phylogenetic analyses.

Genomic DNA was extracted from 0.5 g of the four selected sediment subsamples using a previously described method (Dong, et al., 2006, Jiang, et al., 2006). The *amoA* gene was amplified using AOB and AOA specific primer sets (Table 1). The 16S rRNA gene of *Planctomycetales* and anammox were amplified using specific forward primers coupled with universal reverse primer 1392R (Table 1). PCR conditions were the same as those described in Rotthauwe et al. (1997), Francis et al. (2005), Neef et al. (1998) and Schmid et al. (2003) for AOB- and AOA *amoA* gene, *Planctomycetales*- and anammox-16S rRNA gene, respectively. The PCR reactions were run for 25 cycles in triplicates.

The 16S rRNA gene of *Planctomycetales* and anammox could not be amplified. Successful triplicate PCR products of the same sample were pooled and purified according to the procedures described previously (Jiang, et al., 2006). Plasmids DNA of randomly selected clones were extracted as previously described (Jiang, et al., 2006). The AOB and AOA *amoA* gene fragments of selected clones were sequenced by using *AmoA*-1F and *amoA*F, respectively (Table 1), with the BigDye Terminator version 3.1 chemistry (Applied Biosystems, Foster City, CA, USA). The *amoA* gene sequences were determined with an ABI 3730 automated sequencer. Nucleotide sequences were assembled and edited by using Sequencer v.4.1 (GeneCodes, Ann Arbor, MI). Obtained clone sequences were manually checked for chimeras using Ribosomal Database Project II (http://wdcm.nig.ac.jp/RDP/html/index.html) and identified chimeric sequences were removed. From eight clone libraries (four each for AOB and AOA, respectively), a total of 89 bacterial (~490-bp) and 139 archaeal (~635-bp) *amoA* sequences were subjected to phylogenetic and LIBSHUFF analyses according to Jiang et al. (2006). Briefly, neighbor-joining phylogenies were constructed from dissimilar distance and pairwise comparisons with the Jukes-Cantor distance model using the MEGA (molecular evolutionary genetics analysis) program, version 3.1. Rarefaction and LIBSHUFF analyses were performed using software available online at http://www.uga.edu/~strata/software/Software.html and http://whitman.myweb.uga.edu/libshuff.html, respectively. Operational taxonomic units (OTUs) assignment and rarefaction analyses were performed using DOTUR (Schloss and Handelsman, 2005).

2.5. Quantitative polymerase chain reaction (Q-PCR).

The same primer sets (AmoA-1F/AmoA-2R and Arch-amoAF/Arch-amoAR) were employed for quantification of total bacterial and archaeal *amoA* gene copy numbers, respectively. Amplification conditions were 50^{o}C for 2 min, and then 95^{o}C for 15 min, followed by 45 cycles (15 s at 94^{o}C, 30 s for annealing at 53^{o}C and 50^{o}C for bacterial and archaeal *amoA* gene, respectively, and 30 s at 72^{o}C) in a reaction volume of 25 µl, containing 12.5 µl of SYBR-Green Master Mix (QIAGEN, Valencia, CA) and 5 pmol of each primer. Purified plasmid DNA of Clone QH3amoAB18 and QH3amoAA7 (obtained in this study) was used as standard AOB and AOA functional gene, respectively, which, with serial dilutions, were used to yield two standard curves (one for AOB and the other

for AOA). All quantitative PCR reactions for the standard curves were performed in duplicate. The amplification yielded reliable exponential patterns (bacterial and archaeal *amoA* gene) with a template amount in the range of 10^4 to 10^8 *amoA* gene copies. The data were used to create standard curves correlating the C_T values with *amoA* gene copy numbers. Linear plots between the Ct value (not shown) and log(copy numbers/reaction) for AOB and AOA *amoA* genes were obtained with correlation coefficients of R^2=0.993 and 0.984, respectively.

2.6. Nucleotide Sequence Accession Numbers.

The sequences determined in this study are being deposited in the GenBank database and accession numbers will be available at the revision stage.

3. Results

3.1. Sediment Properties.

X-ray diffraction results indicated that the sediment samples were dominated by quartz, calcite, illite, kaolinite, albite, microcline, pyrite, dolomite, palygorskite, and smythite in decreasing order of abundance. Pore water chemistry analysis (Fig. 2) showed that salinity decreased from 11.3 psu at the top (0-10cm) to 3-4 psu at the bottom of the sediment core (Fig. 2A). The sulfate concentration displayed a dramatic decrease with depth, from 14.5 mM at the top of the sediment core to a negligible level at the bottom (Fig. 2B). Both the strong hydrogen sulfide odor and the decrease/disappearance of sulfate are indicative of sulfate reduction under anaerobic condition. Ammonium concentration was highest near the top (~5.6 mM), but low (less than 2 mM) deeper within the core (Fig. 2C). The nitrate concentration was less than 1 mM (average) throughout the core (Fig. 2D). pH did not show any significant variation throughout the length of the sediment core with an average value of ~9.4 (Fig. 2E).

3.2. Relative Abundance of AOA and AOB.

Both AOA and AOB were detected in the four selected sediment subsamples. The number of AOB *amoA* gene copies showed about one order of magnitude higher than that of AOA and decreased from 1.77×10^7 copies/g in QLS1 (higher salinity) to $6\text{-}8\times10^6$ copies/g in the three bottom samples (lower salinity). The number of AOA *amoA* gene copies was about 10^6 copies/g and did not show any apparent change throughout the length of the core (Table 2).

3.3. Diversity of AOB.

A total of 89 AOB *amoA* gene clone sequences were subjected to sequence similarity analysis. These sequences belonged to one monophyletic lineage (*Betaproteobacteria*) and could be classified into two clusters: Group1 within *Nitrosospira*-like sequences and Group2 within *Nitrosomonas*-like sequences (Fig. 3). None of these clones showed close identity to any known AOB within *Nitrosospira* and *Nitrosomonas* genus. All sequences (16) from QLS1 (higher salinity) and three sequences from QLS2 and QLS3 (2 and 1, respectively) formed Group1, which were closely related to (98-99% identity) clone sequences from estuary water bodies (Bernhard, et al., 2005). In contrast, Group2 contained clone sequences only from the low-salinity sediment samples (QLS2, QLS3 and QLS4) and these sequences were only remotely related (~81-83% identity) to *Nitrosomonas eutropha* (U51630).

3.4. Diversity of AOA.

A total of 139 AOA *amoA* gene clone sequences could be grouped into two clades: soil/sediment clade and hot spring clade (Fig. 4). The majority (92%) of the AOA *amoA* gene clone sequences fell into the soil/sediment clade and most of them showed close identity (95-99%) to clones retrieved from soil (Leininger, et al., 2006), estuary sediment (Beman and Francis, 2006), and marine water and sediment (Francis, et al., 2005). A small fraction of clone sequences (approximately 8%) are related to *amoA* gene clone sequences revovered from thermal springs [Zhang et al. unpublished results and (Weidler, et al., 2007)].

3.5. Statistical analysis of amoA gene clone libraries.

Both OTUs calculations (Table 3) and rarefaction analyses (Fig. 5) indicated the AOA diversity was greater than AOB in the selected sediment subsamples. *amoA* genes (both AOB and AOA) in the low-salinity samples (QLS2, QLS3 and QLS4) are more diverse than the high salinity sample (QLS1) (Fig. 5). LIBSHUFF analysis showed a clear difference for both AOB and AOA communities between high-salinity (QLS1) and low-salinity samples (QLS2, QLS3 and QLS4) (Fig. 6).

4. Discussion

4.1. AOB and AOA abundance.

This study is the first to apply Q-PCR analysis to quantify the relative abundance of AOA and AOB *amoA* genes in anoxic saline lake sediments. One important observation in this study was the predominance of AOB *amoA* genes over AOA by up to one order of magnitude (Table 3), which is in contrast to previous observations that AOA are more abundant than AOB in soils (Leininger, et al., 2006) and ocean waters (Wuchter, et al., 2006). Our results indicated that AOB may out-compete AOA in anoxic saline lake sediments. With increasing salinity, AOA *amoA* gene abundance did not show an apparent change, but the abundance of AOB *amoA* gene increased up to a half of magnitude from a lower salinity at the bottom to a higher salinity at the top, which suggests that AOB are more sensitive to salinity change (albeit small) than AOA.

4.2. AOA and AOB diversity.

The inability to amplify anammox and *Planctomycetales* might indicate either the absence or low abundance (minority) of this group in the anoxic Qinghai Lake sediments. Dalsgaard et al. (2005) claimed that nitrate availability might regulate anammox in anaerobic marine sediments. Thus, the absence and/or minority of anammox in the Qinghai Lake sediments may be due to the low concentration of nitrate (Fig. 2D).

The exclusive presence of *Betaproteobacteria* AOB sequences is in accordance with one observation that all cultured AOB isolates from non-marine ecosystems belong to the monophyletic *Betaproteobacteria* (Kowalchuk and Stephen, 2001). However, further examination of the AOB commnity in the Qinghai Lake sediments revealed that its composition shifted with salinity: *Nitrosospira*-like sequences were only predominant at the top (higher salinity) but absent at the bottom (lower salinity), where *Nitrosomonas*-like sequences dominated instead. Although pH was thought to be a factor controlling the relative abundance of *Nitrosospira* and *Nitrosomonas* in the terrestrial environments (Kowalchuk and Stephen, 2001), we cannot ascribe the observed shift of the AOB composition to the pH effect, because pH was approximately constant throughout the sediment core. Thus, it may be the salinity difference, albeit small, that caused the change in the AOB community composition between the top and bottom samples of the core. This observation is consistent with that of Bernhard et al. (2005), who found a strong link between salinity and AOB community composition in Plum Island Sound estuary in north-eastern Massachusetts, where *Nitrosospira*-like sequences were predominant at

high-salinity sites and both *Nitrosomonas*-like and *Nitrosospira*-like sequences coexisted at mid- and low-salinity sites. This indicates that *Nitrosospira*-like AOB might be able to adapt to a wider range of salinity than *Nitrosomonas*-like ones.

Originally, the *Betaproteobacteria* AOB were claimed to be obligate aerobes (Watson, et al., 1989). Subsequently, Diab et al. (1992) proposed that AOB could survive anoxic conditions by switching their metabolism either to a low-rate state (resting cells) or to an opposite one (nitrite denitrification). The denitrification gene *nirK*, encoding for the nitrite reductase has been identified in the genome of *Nitrosomonas europaea* (Chain, et al., 2003). Under anoxic conditions, some *Nitrosomonas* strains are either capable of oxidizing ammonium with nitrogen dioxide or nitrogen tetroxide (Schmidt and Bock, 1997) or denitrifying with nitrite as as the electron acceptor and hydrogen or simple organic compounds, such as pyruvate, as the electron donor (Abeliovich and Vonhak, 1992, Bock, et al., 1995). However, nitrogen oxide is not readily available in natural environments under anoxic condtions (Schmidt, et al., 2002). So the NO_2-dependent ammonium oxidation metobalism cannot explain the presence of *Nitrosomonas*-like AOB in the anoxic Qinghai Lake sediments. In contrast, the availability of nitrite in the anoxic sediment is likely. Nitrite production rate in anoxic marine/estuary sediments has been found several times higher than, or at least similar to, its consumption rate (Dalsgaard and Thamdrup, 2002, Rysgaard, et al., 2004, Thamdrup and Dalsgaard, 2002). Thus, in addtion to the possiblity to switch their metablism to a low-rate state, AOB in the anoxic Qinghai Lake sediments may reduce nitrite with hydrogen or simple organic compounds as electron donors. So the aquisistion of AOB sequences from the anaerobic Qinghai Lake sediments might be suggestive of denitrification instead of anaerobic oxidation of ammonium.

The majority of the AOA *amoA* gene clone sequences fell into the soil/sediment clade (Fig. 5), suggesting a low temperature origin, as consistent with recent discoveries that ammonia oxidation is carried out by nonthermophilic Crenarchaeota (Francis, et al., 2005, Nicol and Schleper, 2006). So far, however, the only cultured AOA strain, *Nitrosopumilus maritimus*, is only capable of aerobic ammonia oxidation (Konneke, et al., 2005). AOA *amoA* gene clone sequences retrieved in the present study showed less than 85% identity to *Nitrosopumilus maritimus* (Fig. 4). There might be unknown

metabolic pathways, through which not-yet-cultured AOA could survive either by oxidizing ammonia or switching their growth state under anoxic conditions perhaps similar to their AOB counterparts.

In conclusion, molecular work performed in this study indicates that anammox is not important in anoxic saline lake sediments. AOA may not out-compete AOB under anaerobic conditions. The putative aerobic AOB can survive in the anoxic sediments, within which they may have switched to low-level state (resting cells) or to the denitrification pathway by reducing nitrite or nitrogen dioxide with hydrogen, ammonium, or simple organic compounds as the electron donors. AOB and AOA responded differently to increasing salinity: AOB were more sensitive to salinity change than AOA. Within AOB, the genus *Nitrosomonas* and *Nitrosospira* showed different responses to salinity change: *Nitrosospira*-like AOB may be able to tolerate a wider range of salinity than *Nitrosomonas*-like AOB.

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Table 1. Primers used in this study

Primers	Primer sequence (5'→3')	Annealing temp. (°C)	Target group	References
AmoA-1F AmoA-2R	GGG GTT TCT ACT GGT GGT CCC CTC KGS AAA GCC TTC TTC	53	AOB (*amoA* gene)	(Rotthauwe, et al., 1997)
Arch-amoAF Arch-amoAR	STAATGGTCTGGCTTAGACG GCGGCCATCCATCTGTATGT	50	AOA (*amoA* gene)	(Francis, et al., 2005)
Amx-368	CCT TTC GGG CAT TGC GAA	48-58*	Anammox (16S rRNA gene)	(Schmid, et al., 2003)
PLA46	GAC TTG CAT GCC TAA TCC	48-58*	*Planctomycetales* (16S rRNA gene)	(Neef, et al., 1998)
Universal 1392R	ACG GGC GGT GTG TAC		Universal (16S rRNA gene)	(Lane, 1991)

*Thermal-gradient PCR was performed with Amx-368-1392R and PLA46-1392R primer sets.

Table 2. Quantitative analyses of AOB and AOA by Q-PCR.

Sample	amoA gene copies	
	AOA	AOB
QLS1	1.15E+06	1.77E+07
QLS2	6.79E+05	7.91E+06
QLS3	1.52E+06	6.17E+06
QLS4	3.10E+06	8.31E+06

Table 3. Phylogenetic affiliations of AOB and AOA *amoA* gene clones retrieved from the Qinghai Lake sediments.

No. of clones	AOB				AOA			
	QLS1	QLS2	QLS3	QLS4	QLS1	QLS2	QLS3	QLS4
Total clones sequenced	16	19	24	30	24	23	45	47
No. of OTUs*	1	3	4	3	6	8	12	12
No. of unique OTUs	0	0	2	0	1	1	2	3

*Determined by DOTUR with a cutoff value of 3%.

FIGURE CAPTIONS

Fig. 1. A geographic map showing lakes on the Tibetan Plateau and the location of Qinghai Lake, NW China, and the drilling site in the lake (The symbol sizes are not proportional to the sizes of the lakes).

Fig. 2. Depth distribution of certain geochemical parameters in the pore water of the Qinghai Lake sediments.

Fig. 3. Neighbor-joining tree (partial sequences, ~490 bp) showing the phylogenetic relationships of bacterial *amoA* gene sequences cloned from four sediment subsamples of the 5-m core 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. 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 QLS1amoAB18: bacterial *amoA* clone number 18 from QLS1. Scale bars indicate the Jukes-Cantor distances. Bootstrap values of >50% (for 1000 iterations) are shown.

Fig. 4. Neighbor-joining tree (partial sequences, ~635 bp) showing the phylogenetic relationships of archaeal archaeal *amoA* gene sequences cloned from four sediment subsamples of the 5-m core to closely related sequences from the GenBank database. The same algorithms as those for the bacterial tree (Fig. 4) were used. One representative clone type within each OTU is shown, and the number of clones within each OTU is shown at the end.

Fig. 5. Rarefaction curves showing the relative richness of archaeal and bacterial *amoA* gene clone libraries for the four subsamples studied. OTUs were defined based on a 3% cutoff.

Fig. 6. Clustering of the different AOB (A) and AOA (B) amoA gene clone libraries based on ΔCxy values determined from the LIBSHUFF analysis. The tree was constructed with the unweighted-pair group method using average linkages in MEGA3.1. The parameter ΔCxy in the LIBSHUFF analysis represents the difference in coverage of any two clone libraries (the larger ΔCxy, the greater dissimilarity between the given clone libraries). The software for the analysis was available at http://whitman.myweb.uga.edu/libshuff.html.

Fig. 1

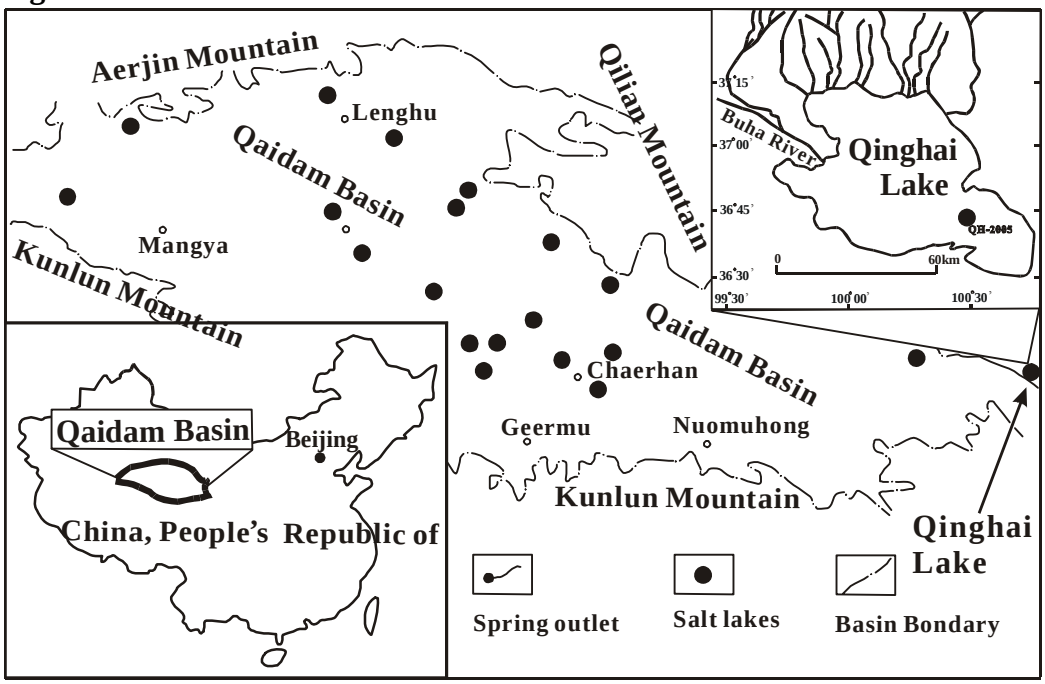

Aerjin Mountain
Lenghu
Qilian Mountain
Qaidam Basin
Mangya
Kunlun Mountain
Qaidam Basin
Chaerhan
Qaidam Basin
Beijing
Geermu
Nuomuhong
China, People's Republic of
Kunlun Mountain
Qinghai Lake
Spring outlet
Salt lakes
Basin Bondary
Buha River
Qinghai Lake
QH-2005
0
60km
37°15'
37°00'
36°45'
36°30'
99°30'
100°00'
100°30'

Fig. 2

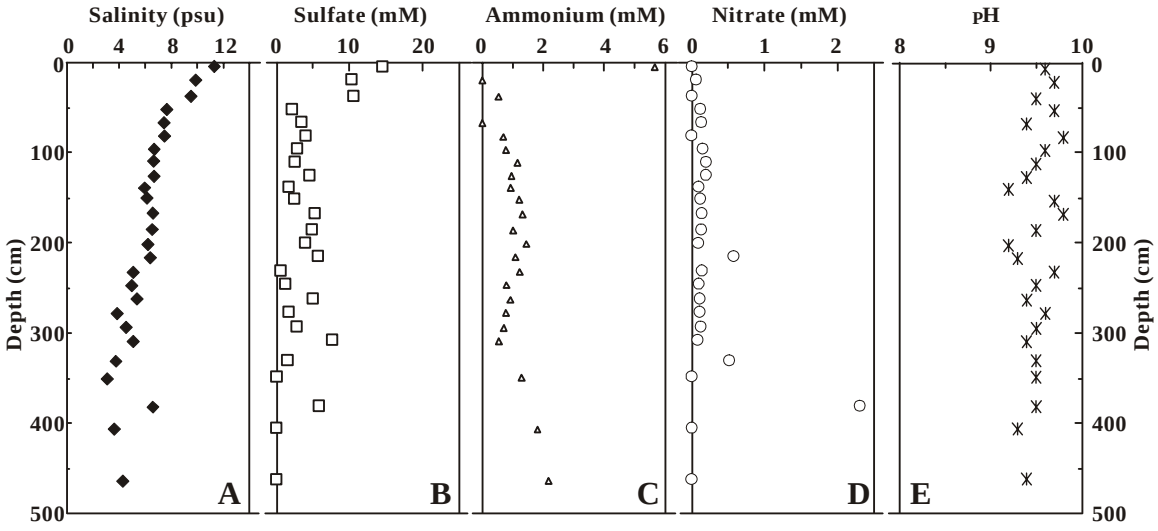

Salinity (psu)
Sulfate (mM)
Ammonium (mM)
Nitrate (mM)
pH
Depth (cm)
A
B
C
D
E

Fig. 3

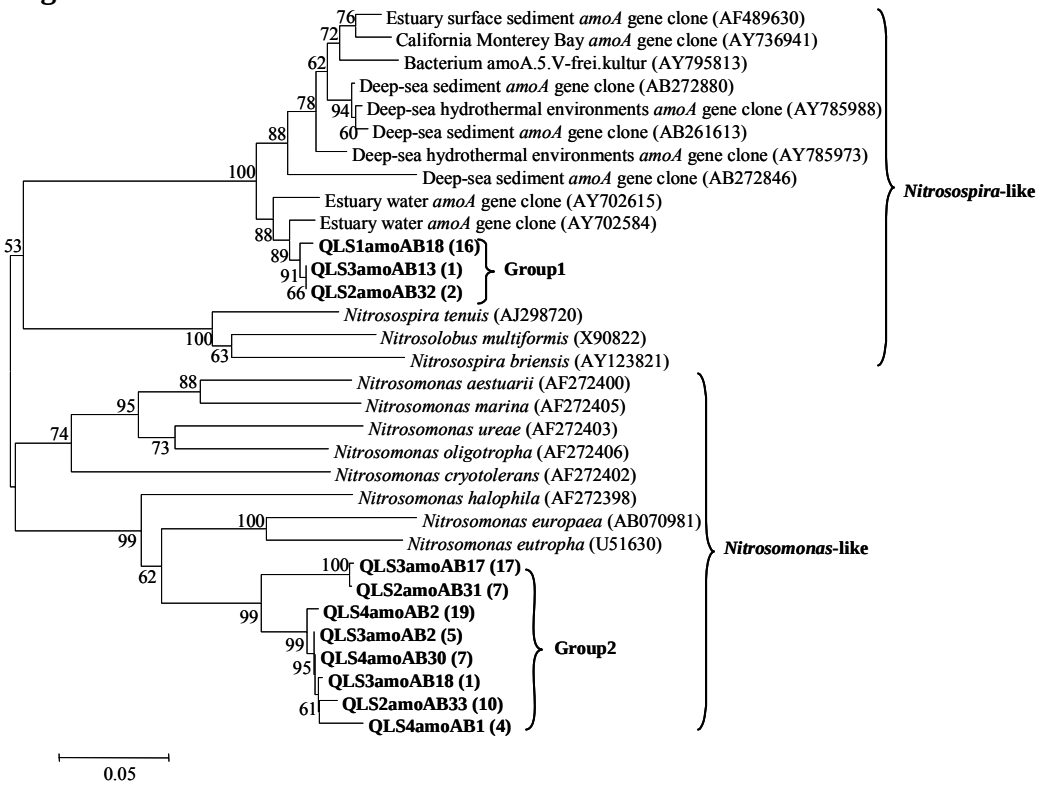
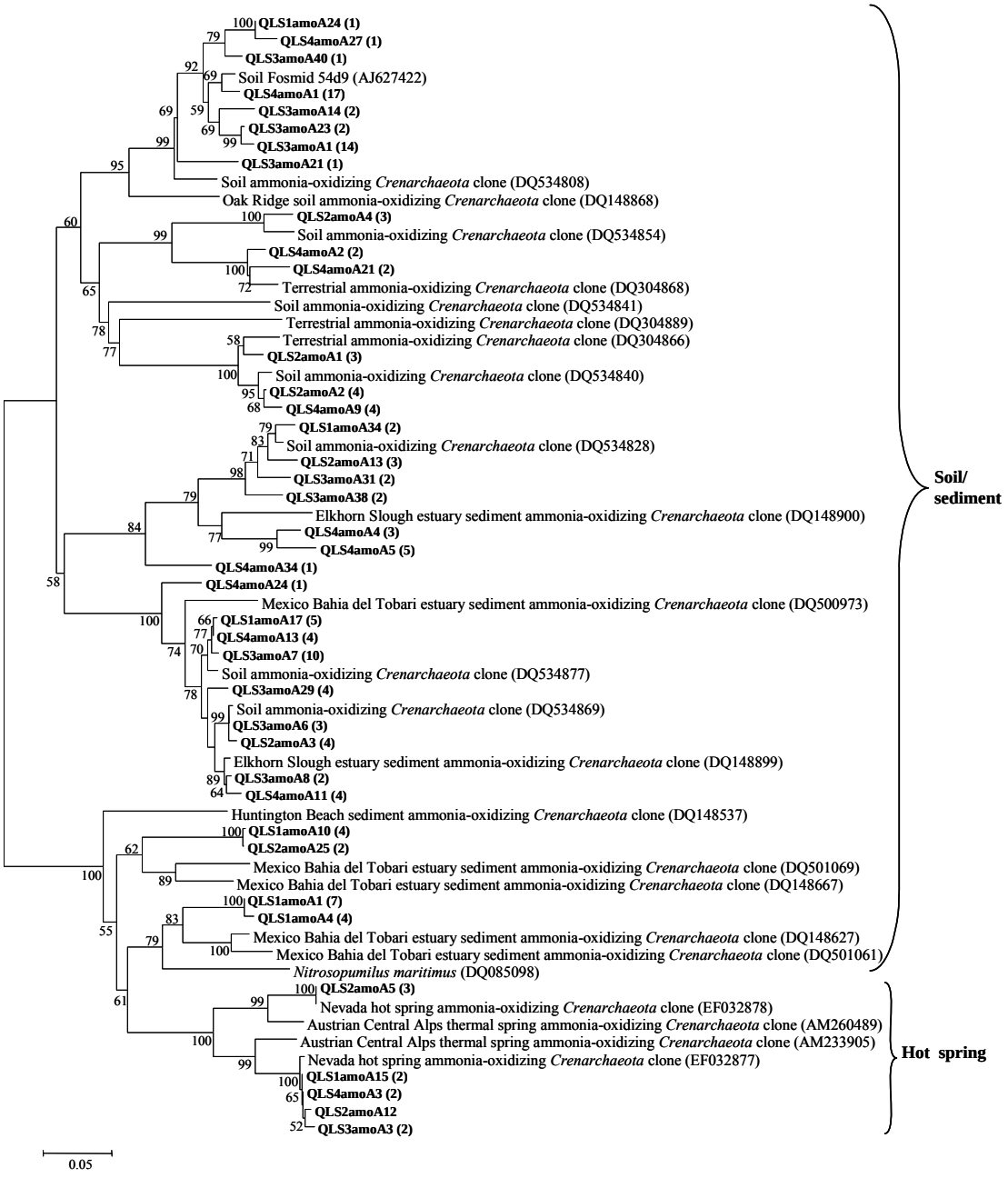

Estuary surface sediment *amoA* gene clone (AF489630)
California Monterey Bay *amoA* gene clone (AY736941)
Bacterium amoA.5.V-frei.kultur (AY795813)
Deep-sea sediment *amoA* gene clone (AB272880)
Deep-sea hydrothermal environments *amoA* gene clone (AY785988)
Deep-sea sediment *amoA* gene clone (AB261613)
Deep-sea hydrothermal environments *amoA* gene clone (AY785973)
Deep-sea sediment *amoA* gene clone (AB272846)
Estuary water *amoA* gene clone (AY702615)
Estuary water *amoA* gene clone (AY702584)
QLS1amoAB18 (16)
QLS3amoAB13 (1)
QLS2amoAB32 (2)
Group1
Nitrosospira tenuis (AJ298720)
Nitrosolobus multiformis (X90822)
Nitrosospira briensis (AY123821)
Nitrosospira-like
Nitrosomonas aestuarii (AF272400)
Nitrosomonas marina (AF272405)
Nitrosomonas ureae (AF272403)
Nitrosomonas oligotropha (AF272406)
Nitrosomonas cryotolerans (AF272402)
Nitrosomonas halophila (AF272398)
Nitrosomonas europaea (AB070981)
Nitrosomonas eutropha (U51630)
QLS3amoAB17 (17)
QLS2amoAB31 (7)
QLS4amoAB2 (19)
QLS3amoAB2 (5)
QLS4amoAB30 (7)
QLS3amoAB18 (1)
QLS2amoAB33 (10)
QLS4amoAB1 (4)
Group2
Nitrosomonas-like
0.05

Fig. 4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QLS4amoA27 (1)
QLS3amoA40 (1)
Soil Fosmid 54d9 (AJ627422)
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QLS3amoA14 (2)
QLS3amoA23 (2)
QLS3amoA1 (14)
QLS3amoA21 (1)
Soil ammonia-oxidizing Crenarchaeota clone (DQ534808)
Oak Ridge soil ammonia-oxidizing Crenarchaeota clone (DQ148868)
QLS2amoA4 (3)
Soil ammonia-oxidizing Crenarchaeota clone (DQ534854)
QLS4amoA2 (2)
QLS4amoA21 (2)
Terrestrial ammonia-oxidizing Crenarchaeota clone (DQ304868)
Soil ammonia-oxidizing Crenarchaeota clone (DQ534841)
Terrestrial ammonia-oxidizing Crenarchaeota clone (DQ304889)
Terrestrial ammonia-oxidizing Crenarchaeota clone (DQ304866)
QLS2amoA1 (3)
Soil ammonia-oxidizing Crenarchaeota clone (DQ534840)
QLS2amoA2 (4)
QLS4amoA9 (4)
QLS1amoA34 (2)
Soil ammonia-oxidizing Crenarchaeota clone (DQ534828)
QLS2amoA13 (3)
QLS3amoA31 (2)
QLS3amoA38 (2)
Elkhorn Slough estuary sediment ammonia-oxidizing Crenarchaeota clone (DQ148900)
QLS4amoA4 (3)
QLS4amoA5 (5)
QLS4amoA34 (1)
QLS4amoA24 (1)
Mexico Bahia del Tobari estuary sediment ammonia-oxidizing Crenarchaeota clone (DQ500973)
QLS1amoA17 (5)
QLS4amoA13 (4)
QLS3amoA7 (10)
Soil ammonia-oxidizing Crenarchaeota clone (DQ534877)
QLS3amoA29 (4)
Soil ammonia-oxidizing Crenarchaeota clone (DQ534869)
QLS3amoA6 (3)
QLS2amoA3 (4)
Elkhorn Slough estuary sediment ammonia-oxidizing Crenarchaeota clone (DQ148899)
QLS3amoA8 (2)
QLS4amoA11 (4)
Huntington Beach sediment ammonia-oxidizing Crenarchaeota clone (DQ148537)
QLS1amoA10 (4)
QLS2amoA25 (2)
Mexico Bahia del Tobari estuary sediment ammonia-oxidizing Crenarchaeota clone (DQ501069)
Mexico Bahia del Tobari estuary sediment ammonia-oxidizing Crenarchaeota clone (DQ148667)
QLS1amoA1 (7)
QLS1amoA4 (4)
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Mexico Bahia del Tobari estuary sediment ammonia-oxidizing Crenarchaeota clone (DQ501061)
Nitrosopumilus maritimus (DQ085098)
QLS2amoA5 (3)
Nevada hot spring ammonia-oxidizing Crenarchaeota clone (EF032878)
Austrian Central Alps thermal spring ammonia-oxidizing Crenarchaeota clone (AM260489)
Austrian Central Alps thermal spring ammonia-oxidizing Crenarchaeota clone (AM233905)
Nevada hot spring ammonia-oxidizing Crenarchaeota clone (EF032877)
QLS1amoA15 (2)
QLS4amoA3 (2)
QLS2amoA12
QLS3amoA3 (2)
Soil/ sediment
Hot spring
0.05

Fig. 5

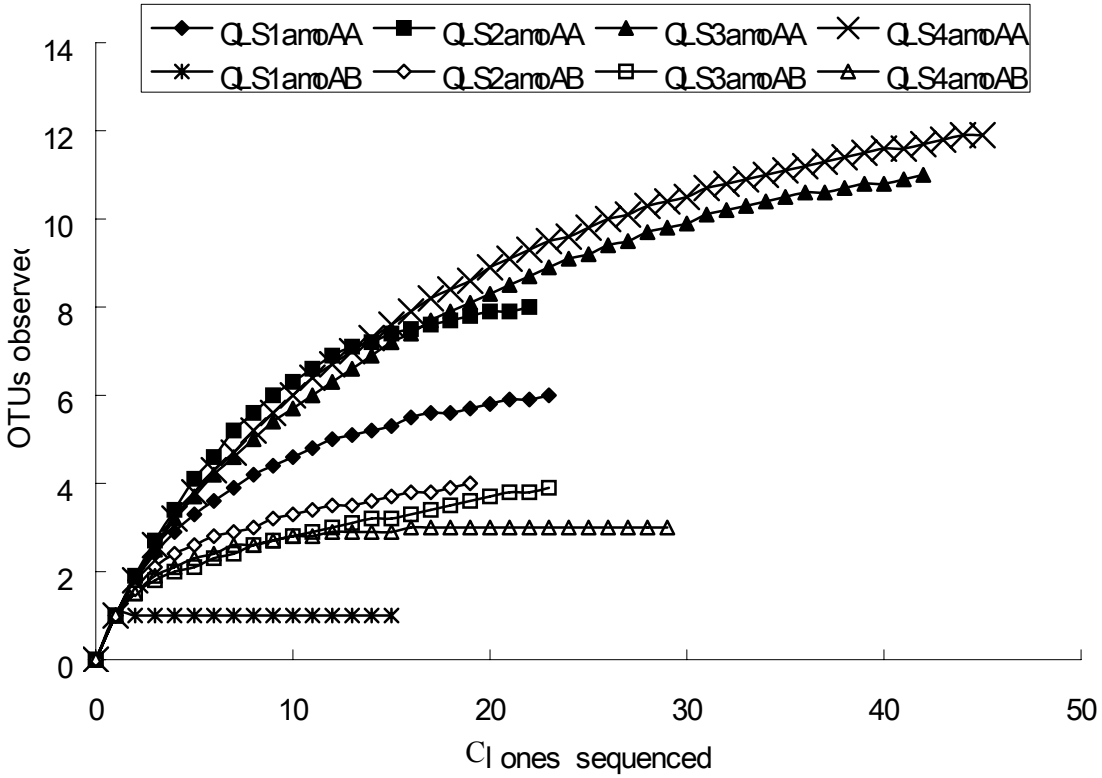
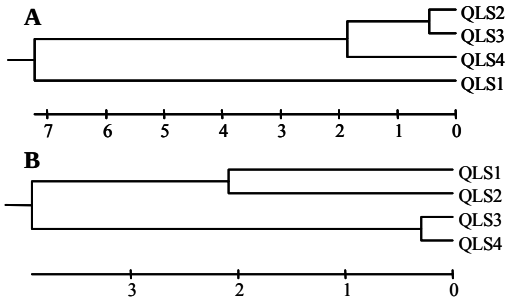

QLS1amoAA
QLS2amoAA
QLS3amoAA
QLS4amoAA
QLS1amoAB
QLS2amoAB
QLS3amoAB
QLS4amoAB
OTUs observed
Clones sequenced
0
2
4
6
8
10
12
14
0
10
20
30
40
50

Fig. 6


A
QLS2
QLS3
QLS4
QLS1
7
6
5
4
3
2
1
0
B
QLS1
QLS2
QLS3
QLS4
3
2
1
0

CHAPTER 5:

Dominance of Putative Marine Benthic Archaea in Sediments from Qinghai Lake, Northwestern China

Hongchen Jiang[1], Hailiang Dong[1,2*], Bingsong Yu[3,2], Qi Ye[4], Ji Shen[5], Harry Rowe[6], and Chuanlun Zhang[4]

1: Department of Geology
Miami University
Oxford, OH 45056

2: Geomicrobiology Laboratory
China University of Geosciences
Beijing, 100083, China

3: School of Earth Science
China University of Geosciences
Beijing, China, 100083

4: Savannah River Ecology Laboratory
University of Georgia
P.O. Box Drawer E
Aiken, SC 29802

5: Key Laboratory of Lake Sediment and Environment, Chinese Academy of Sciences;
Nanjing Institute of Geography and Limnology, Chinese Academy of Sciences,
Nanjing, Jiangsu 210008, China

6: Earth and Environmental Sciences
University of Kentucky
Lexington, KY 40506

*Corresponding author: Hailiang Dong
Department of Geology
Miami University
Oxford, OH 45056
Tel: 513-529-2517
Fax: 513-529-1542
Email: dongh@muohio.edu

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Abstract

Recent studies have revealed important and versatile roles that Archaea play in a wide variety of environments on Earth. In this study, we investigated the abundance and diversity of archaeal community in a 5-m sediment core collected from Qinghai Lake on the Tibetan Plateau. An integrated approach was employed including geochemistry, quantitative polymerase chain reaction (Q-PCR) and 16S rRNA gene analysis. Here, we show that *Archaea* dominated the prokaryotic community in the lake sediments. Members of putative marine benthic groups (Marine Benthic Group-B, C & D) and Miscellaneous Crenarchaeotic Group were dominant, many of which were previously reported to be present in deep seas only. These results suggest that these groups are not limited to marine sediments. Despite their ubiquitous presence in aquatic environments, metabolic functions of these important groups largely remain unknown. Although many of these groups (such as MBG-B and D) have typically been found in methane-hydrate deposits in marine environments, our carbon isotopic and molecular data do not support such an origin in the Qinghai Lake sediments. Instead, a large percentage of these groups appear to be ammonia-oxidizing archaea.

Introduction

Archaea, one of the three domains of life on Earth, is composed of two major phyla: the Euryarchaeota and the Crenarchaeota (Woese, et al., 1990). Archaea are widely distributed in various extreme environments of both terrestrial and marine ecosystems. The cultured Crenarchaeota are composed of four orders: *Caldisphaerales*, *Desulfurococcales*, *Sulfolobales*, and *Thermoproteales* (Chaban, et al., 2006). Most of pure cultures in this phylum are thermophilic, with the exception of an ammonia-oxidizing archaeon (Konneke, et al., 2005). The cultivated members of the *Euryarchaeota* include halophiles, thermophiles, methanogens, and sulfate reducers (Chaban, et al., 2006). However, genomic studies indicate that the well-known archaeal cultures mentioned above represent only a very small fraction of total archaeal organisms present in nature, and our knowledge of archaeal diversity, metabolic pathways, and ecology properties is rather limited [See reviews by Schleper et al. (2005) and Chaban et al. (2006)].

Certain groups of uncultured Archaea were thought to be confined to specific geographical locations. For example, members of Marine Group (MG) -I, -II, -III & -IV were originally phylogeneticly recognized in sea water [DeLong et al. (1992, 1994), Fuhrman et al. (1992, 1997), Lopez-Garcia et al. (2001)]. Marine Benthic Group (MBG)-A, -B, -C, -D & -E were found in a wide range of marine sediments, surficial as well as subsurface, and in hydrothermal vents (Vetriani, et al., 1999). MG-I and MBG-A, -B & -C belong to the Crenarchaeota, and MG-II, -III & -IV and MBG-D & -E belong to the Euryarchaeota (Lopez-Garcia, et al., 2001; Vetriani, et al., 1999). Multiple researchers confirmed the presence of these groups in marine environments as summarized by Teske (Teske, 2006), but they have never been found in inland lakes. In the present study, however, the phylogenetic analysis of the archaeal 16S rRNA gene sequences retrieved from sediments in Qinghai Lake (the largest inland lake in China) may change our viewpoint about the distribution patterns of these groups.

Qinghai Lake ($36^{o}32'$-$37^{o}15'$N, $99^{o}36'$-$100^{o}47'$E), the largest saline (12.5 practical salinity unit, psu) and alkaline (pH 9.3) lake on the Tibetan Plateau in NW China, represents a pristine environment, where perturbations from human activities have been minimal (Dong, et al., 2006). Previous 16S rRNA gene analysis of shallow lake

sediments (top 40 cm) showed the predominance of some aforementioned marine groups in the archaeal community (Dong, et al., 2006). In that study, for example, the *Crenarchaeota* Cluster II, and Cluster III correspond to MCG, MBG-C and MBG-B, respectively, the *Euryarchaeota* Cluster I & II correspond to MBG-D. However, the identity of these putative marine groups was not clearly recognized at that time.

The main goal of this research was to examine the abundance and diversity of Archaea in the Qinghai Lake sediments. We employed an integrated approach including geochemistry, quantitative polymerase chain reaction (Q-PCR), and 16S rRNA gene analyses. Our result indicated that Archaea dominate the prokaryotic community in the sediments and that the marine benthic groups (MBG groups) may not be specific to marine environments, instead, members of these groups can be abundantly present in lake ecosystems.

Results

Geochemistry of Sediments. X-ray diffraction results indicated that the sediment samples were dominated by quartz, calcite, illite, kaolinite, albite, microcline, pyrite, dolomite, palygorskite, and smythite in decreasing order of abundance. Pore water chemistry analysis (Fig. 2) showed that salinity decreased from 11.3 psu at the top (0-10 cm) to 3-4 psu at the bottom of the sediment core (Fig. 2A). The sulfate concentration displayed a dramatic decrease with depth, from 14.5 mM at the top of the sediment core to a negligible level at the bottom (Fig. 2B). Both the strong hydrogen sulfide odor and the decrease/disappearance of sulfate were indicative of sulfate reduction under anaerobic condition. pH did not show any significant variation throughout the length of the sediment core with an average value of ~9.4 (Fig. 2C), which was the same as that in lake water. The $\delta^{13}C$ and $\delta^{18}O$ values of the selected sediment subsamples (Table 1) were 0.68 to 3.11‰ and -1.31 to 1.81 ‰, respectively.

Cell Abundance and Quantification of 16S rRNA genes. Total prokaryotic cells as determined by Q-PCR (sum of bacterial and archaeal biomass) were about 10^8-10^7 cells/g (dry weight) throughout the 5-m long sediment core, decreasing from ~6.7×10^8 cells/g at the top of the core to ~6.7×10^7 cells/g at the bottom (Table 2). Archaeal cells were dominant (51-86% of total prokaryotic cells) over bacteria throughout the core

(Table 2). The relative abundance of crenarchaeotal cells was 20-35% of the total prokaryotic cells (Table 2).

Phylogenetic Diversity. Approximately 42-55 clones were sequenced for each sediment subsample and a total of 334 clone sequences were subjected to the phylogenetic analysis (Table 3). These numbers of clones represented 86.0-93.2% of the coverage of each clone library (Table 3). All clone sequences retrieved in this study were classified into three euryarchaeotal (Fig. 3A) and six crenarchaeotal groups (Fig. 3B), respectively. These groups include *Methanomicrobia*, *Methanosarcinales*, and Marine Benthic Group-D in the *Euryarchaeota* (Fig. 3A), and MBG-C, Miscellaneous Crenarchaeotic Group (MCG) (Inagaki, et al., 2003), MG-I, "SCA clone" group (Bintrim, et al., 1997), and MBG-B (synonymous with the deep sea archaeal group, DSAG) (Takai, et al., 2001) in the *Crenarchaeota*. About 74% (247 out of 334) of all clone sequences retrieved in this study were affiliated with putative marine groups MBG-B, -C, -D, and MG-I. Our clone sequences showed close identities (~96-99.9%) to those retrieved in various methane/organic-rich or methane hydrate-bearing marine sediments, including the Black Sea (Knittel, et al., 2005), the Peru Margins (Inagaki, et al., 2006), the Guaymas Basin (Teske, et al., 2002), the Alaska Skan Bay (Kendall, et al., 2007), the Gulf of Mexico (Lanoil, et al., 2001; Lloyd, et al., 2006; Pi, et al., in revision), South China Sea (Jiang, et al., 2007), Nankai Trough (Newberry, et al., 2004; Reed, et al., 2002), the Cascadia Margins (Inagaki, et al., 2006; Lanoil, et al., 2005), and the Sea of Okhotsk (Inagaki, et al., 2003).

Both the total and the unique number of OTUs did not show any apparent variations with depth, which suggested that at the selected OUT cut-off value (3%) the archaeal diversity did not change with depth (Table 3). However, the analysis of major group affiliation showed that the two bottom subsamples (QLS399 and QLS458) were more diverse than the five top subsamples (from QLS0 to QLS345) (Table 3). In the top five samples, all sequences belonged to three groups: MBG-B, MBG-D, and either MBG-C (QLS0, QLS30, and QLS345) or MCG (QLS118 amd QLS240). In the two bottom samples, the archaeal clone libraries were composed of seven and six groups, respectively (Table 3). In QLS0, QLS30, QLS118, and QLS345, sequences affiliated with MBG-D and MBG-B were predominant (95%, 98, 90, and 98%, respectively) with a few

sequences within either MBG-C or MCG. In QLS240, the MCG was the dominant followed by MBG-D and MBG-B. The two bottom subsamples possessed very different community compositions. The dominant groups in QLS399 and QLS458 were *Methanomicrobia* (77%) and MBG-C, respectively. Consistent with analysis, the LIBSHUFF results (not shown) clearly showed that there was a distinct difference in the archaeal community composition between the two bottom subsamples and the five top subsamples (Table 3).

Discussion

Archaea abundance. The Q-PCR analysis demonstrated that Archaea dominated (51-86% throughout the 5-m-long core) the prokaryotic community in the Qinghai Lake sediment. Such a percentage of Archaea was lower than the highest record (98%) reported for sulfate-methane transition zones in ODP sites of the Peru Margins (Biddle, et al., 2006). To our best knowledge, however, no such information in other inland lake sediments was available for comparison.

Possible reasons for the presence of marine groups in inland lake sediments. To our best knowledge, this study is the first to retrieve so many putative marine benthic groups in sediments from an inland lake. The putative marine archaeal groups, MG-I, -II & -III, IV and Marine Benthic Group-A, -B, -C, -D & -E, were phylogeneticly recognized in marine environments (DeLong, 1992; DeLong, et al., 1994; Fuhrman and Davis, 1997; Fuhrman, et al., 1992; Lopez-Garcia, et al., 2001; Vetriani, et al., 1999). The presence of these groups in diverse marine environments was subsequently confirmed by a number of studies, including cold seeps at the Hydrate Ridge and the Black Sea (Knittel, et al., 2005), hydrothermal vent chimneies (Takai and Horikoshi, 1999; Takai, et al., 2001), methane-rich, organic-rich or hydrate-bearing marine sediments in coastal subseafloor or near continental margins (Biddle, et al., 2006; Dhillon, et al., 2005; Inagaki, et al., 2006; Inagaki, et al., 2003; Lanoil, et al., 2005; Lloyd, et al., 2006; Newberry, et al., 2004; Parkes, et al., 2005; Sorensen, et al., 2004; Sorensen and Teske, 2006). Given the fact that past studies have shown their exclusive presence in marine environments, it is surprising to observe their predominance in lake sediments. Because Qinghai Lake is far from any marine influence, it is reasonable to

assume that the microorganisms from which the DNA was derived are indigenous to the lake. Because these groups have never been detected in terrestrial environments, any contamination from terrestrial sources (such as inflow rivers and dust particles) would not explain their presence.

Given the fact that biogeographical control appears to be insignificant, it is a logical next step to look for any environmental controls. However, it is not an easy task to determine the relative importance of environmental factors in controlling the distribution of these various archaeal groups. Among the main possible environmental parameters, pH, salinity, and water chemistry might be the important ones. pH is alkaline for both Qinghai Lake and the oceans, although the pH in the Qinghai lake sediment is higher than typical marine pH by one unit. Both Qinghai Lake and the oceans are saline, although the salinity of seawater is twice the value of that for Qinghai Lake water. Water chemistry of both environments is dominated by Na and Cl (as opposed to soda lakes), although their specific concentrations and other ions differ. So the similarity in geochemistry between Qinghai Lake and oceans might explain the presence of marine benthic groups of Archaea in both environments. Some sequences from MBG-D, MBG-B, and MBG-C groups have been previously retrieved from lakes (such as Lake Dagow, Lake Kinneret and Lake Chaka) and they were closely related to those from Qinghai Lake (Fig. 3A & B). The pH of Lake Kinneret water varies from ~9.5 on the surface to ~7.5 at the bottom depending on different locations and depths (http://www.ocean.org.il/Eng/KineretDataCenter/ph.asp) and its lake sediments are also alkaline (Avnimelech, 1983; Stiller and Magaritz, 1974). Its salinity is 0.5-0.6 psu and its water is also Na- and Cl-type (Kolodny, et al., 1999). Lake Chaka water is hypersaline, but the sediments have a range of salinity and pH values (Jiang, et al., 2007). Interestingly, those sequences from Lake Chaka sediments that belong to MBG-B and MBG-C groups (i.e., DQ129969 in the MBG-B group and EF201704 in the MBG-C group of Fig. 3B) were retrieved from those depth intervals in a sediment core where pore water was slightly saline and alkaline (Jiang, et al., 2007). Again, these conditions are broadly similar to those of Qinghai Lake. Lake Chaka also has Na- and Cl- type water chemistry. The corresponding information about Lake Dagow is not available. In summary, all these geographical locations described above have similar water chemistry

to oceans: alkaline, saline and Na-Cl-type water chemistry. These limited data appear to suggest that saline and alkaline conditions favor the presence and/or dominance of the marine benthic groups (MBG-B, C, and D, and MG-1), regardless of the specific environment (lake or ocean). However, unlike abundant presence of these marine sequences in Qinghai Lake, only a few marine sequences were retrieved from other lakes previously (Lake Dagow, Lake Kinneret, and Lake Chaka). Thus, in addition to the saline and alkaline conditions, other factors might have played a role in controlling the distribution and relative abundance of the putative marine benthic groups of Archaea.

Presence of MBG-B and MCG. Inagaki et al. (2006) discussed the relationship between the distribution patterns of archaeal and bacterial communities in deep-sea sediments and total organic carbon/methane hydrates. In hydrate-free, organic-poor sediments on the Pacific Ocean Margin, archaeal community is dominated with Marine Crenarchaeotic Group I (MCG-I) and Marine Benthic Group A. In hydrate-free but organic-rich sediments, the community is dominated with Miscellaneous Crenarchaeotic Group (MCG) and South African Gold Mine Euryarchaeotic Group (SAGMEG). In contrast, in hydrate-containing sediments, the MBG-B (DSAG) is the predominant group in archaeal community. Based on the different biogeographical distribution of MBG-B, Inagaki et al. (2006) speculated that the uncultivated MBG-B group might play a role in sulfate reduction and methane oxidation. However, this proposed speculation has not been confirmed because none of the MBG-B group members has been cultured and their metabolic pathways are therefore not known. On one hand, this group has been retrieved in organic/methane-rich or hydrate-bearing sediments, such as the Cascadia Margins (Lanoil, et al., 2005), Nankai Trough (Newberry, et al., 2004), the Guaymas Basin (Teske, et al., 2002) and the Gulf of Mexico (Lloyd, et al., 2006; Martinez, et al., 2006), supporting the Inahaki et al. speculation. On the other hand, this group is absent in other well-known hydrate-bearing sediments of the Gulf of Mexico (Lanoil, et al., 2001; Mills, et al., 2003; Mills, et al., 2005; Mills, et al., 2004; Reed, et al., 2006), suggesting that the presence of MBG-B members may not be specifically linked to sulfate reduction or methane oxidation.

In our study, the MBG-B group was detected in hydrate-free Qinghai Lake sediments, further suggesting that this group is not specific to gas hydrates. Furthermore,

MCG group were co-present with the MBG-B group. These two groups have been found to be co-present in some organic/methane-rich or hydrate-bearing marine sediments, such as the Peru Margins (Biddle, et al., 2006; Inagaki, et al., 2006; Parkes, et al., 2005; Sorensen, et al., 2004; Sorensen and Teske, 2006), the Cascadia Margins (Inagaki, et al., 2006), the Sea of Okhotsk (Inagaki, et al., 2003), Nankai Trough (Reed, et al., 2002), Mediterranean (Coolen, et al., 2002) and the Gulf of Mexico (Pi, et al., in revision). These studies effectively show that it is difficult to make any specific links between the presence or absence of these groups and geochemical conditions (presence or absence of gas hydrates, organic-rich or poor sediments etc.).

Absence of Methane Oxidation. To further test if the detected clone sequences were derived from methanogens or methanotrophs, attempts were made to amplify the methyl coenzyme M reductase alpha-subunit (mcrA) gene. Except for QLS399, this gene was not PCR-amplified in any other samples, suggesting that MBG-B and MCG were not methanogens. This was confirmed from the carbon isotope data. The $\delta^{13}C$ values of carbonates were much higher than those typically found in putative methane-derived carbonates (the normal range: -61‰ to -35‰ PDB) (Jorgensen, 1992; Suess and Whiticar, 1989; Whiticar and Faber, 1986). The $\delta^{13}C$ values of carbonates in the Qinghai lake sediments are within the normal range for freshwater carbonates (Clark and Fritz, 2000). Recent studies have consistently shown depleted ^{13}C values for authigenic carbonates related to methane oxidation (Canet, et al., 2003; Lein, et al., 2002; Martin, et al., 2007; Mazzini, et al., 2004; Mazzini, et al., 2006; Muralidhar, et al., 2006). The $\delta^{18}O$ values are lower than those (~3 ‰ to ~4.5‰, or higher) for putative methane-derived carbonates (Jorgensen, 1992; Suess and Whiticar, 1989; Whiticar and Faber, 1986).

Speculation of functions of the marine groups within the Crenarchaeota. Whereas it is premature to speculate the specific functions of MBG-B, MBG-C, MCG, SCA clones, and Marine Group I of the Crenarchaeota, it is attempting to speculate that they might be ammonia oxidizers. Our separate study (Jiang, et al., 2007, in revision, previous chapter) previous chapter determined the abundance of ammonia-oxidizing archaea with Q-PCR in these same samples. When the AOA gene abundances were compared with those for crenarchaeota, it became clear that nearly 30-100% of all

crenarchaeota are AOA. Definitive confirmation will have to await other complementary approaches, such as metagenomic and fosmid library approaches.

Conclusion

Molecular work performed in this study indicated that Archaea dominated the prokaryotic community in Qinghai Lake sediments, in which members of putative marine benthic groups were predominant. The dominance of so-called marine benthic groups in the Qinghai Lake sediments suggest that geographical isolation does not control their distribution patterns. Instead, environmental conditions may be more important. Although these groups are commonly associated with methane-rich gas hydrates, they are not methanogens. Our data appear to suggest that they may be ammonia oxidizers.

Materials and Methods

Description of the study site. Qinghai Lake is a perennial lake located in a structural intermontane depression at the northeastern corner of the Qinghai-Tibetan Plateau (Fig. 1). The lake has an area of 4300 km^2 and lies within a catchment of limestones, sandstones, and shales (Henderson, et al., 2003). The average water depth is 19.2 m, and the maximum is 28.7 m. The altitude of the lake is 3196 m above sea level (masl), and the surrounding mountains rise to above 5200 masl (Dong, et al., 2006). The evaporation of the lake (~1400 mm/year) is in excess of mean annual precipitation (~400 mm/ year), resulting in the development of a saline lake with salinity of 12.5 psu (Dong, et al., 2006; Shen, et al., 2005).

Recent seismic survey suggests that Qinghai Lake is separated into two subbasins by a normal faulting horst in the middle of the lake (An, 2003). The northern subbasin is more dynamic than the southern one because of a major river input in the northwest and strong northwest wind. So the entire water column in the northern subbasin is well mixed in summer. In comparison, the southern subbasin is relatively quiet, within which the depositional environment is stable and water stratification occurs seasonally (CAS, 1979). Dissolved oxygen typically shows a slight gradient from 6 ppm at the surface to 3 ppm at the bottom in the water column in the eastern depression of the southern subbasin (~23 m water depth) (Dong, et al., 2006), within which we selected our sampling site at

approximately 15 km north of a fishing station called Erlangjian for easy access (labeled QH-2005 on Fig. 1) and a stable depositional environment.

Sediment core collection and processing. A 5-meter sediment core was collected in 2005 using a platform drilling rig. The core was retrieved in three segments (~2, 2, and 1 m) and they were cased inside polyvinyl chloride tubings that had been sterilized with alcohol. Upon retrieval, the core segments were immediately capped and frozen in dry ice. In less than 1 week, the core segments were taken to China University of Geosciences in Beijing, where they were stored in a -80^{o}C freezer. Within 1 month, the core segments were shipped to Miami University, Oxford, OH, USA. Seven subsamples of this sediment core were selected for geochemistry and microbiology work: QLS0: 0-10 cm; QLS30: 30-45 cm; QLS118: 118-133 cm; QLS240: 240-255 cm; QLS345: 345-355 cm; QLS399: 399-415 cm; and QLS458: 458-470 cm. The sediment core was dissected inside a glove box (Coy Laboratory Products, Grass Lake, MI), and the external layers were carefully removed using sterile tools to avoid possible contamination.

Geochemical analyses. Sediment mineralogy was determined by X-ray diffraction according to Jiang et al. (2007). Sediment pore water samples were collected following centrifugation of ~60 grams of each sediment subsample (at 5000 g for 10 minutes). The volume of pore water from each sediment subsample was ~11-23 mL. A strong odor of hydrogen sulfide was evident from pore water. pH measurements were made on pore water with a pH meter immediately after collection. For measurements of anions and cations, aliquots of pore water were fixed with HCl solution (0.1N HCl final concentration). The diluted pore water samples were analyzed by high performance liquid chromatography (HPLC) for anions (IonPac®AS14 column 4×250mm) and direct current plasma emission spectrometry (DCP) for cations. Major anions and cations were combined to calculate the salinity (psu). Carbon isotope analysis on both hypochlorite-treated (Formolo, et al., 2004) and untreated sediment subsamples was performed at the University of Kentucky according to the method described by Seltzer et al. (Seltzer, et al., 2002).

DNA isolation, PCR amplification and phylogenetic analyses. Genomic DNA was extracted from 0.5 g of seven selected sediment subsamples using a previously described method (Dong, et al., 2006; Jiang, et al., 2006). The 16S rRNA gene was

amplified using Arch21F (5'-TTC YGG TTG ATC CYG CCR GA-3') (DeLong, et al., 1994) and Univ1492R (5'-CGG TTA CCT TGT TAC GAC TT-3') (Lane, 1991). PCR conditions were the same as those described in Jiang et al. (Jiang, et al., 2006). Because of the possible presence of the mcrA gene (specific to methanogens) in the samples, it was amplified with ME1 (5'-GCM ATG CAR ATH GGW ATG TC-3') and ME2 (5'-TCA TKG CRT AGT TDG GRT AGT-3') according to Hales et al. (Hales, et al., 1996). The PCR reactions were run for 35 cycles in triplicates. The mcrA gene was successfully amplified in only one sample (QLS399), and thus no down-stream work was performed mcrA gene. Successful 16S rRNA gene PCR products were pooled and purified according to our previously described procedure (Jiang, et al., 2006). Clone libraries were constructed and plasmid DNA of randomly selected clones were extracted as previously described (Jiang, et al., 2006). Selected clones were sequenced by using Arch21F with the BigDye Terminator version 3.1chemistry (Applied Biosystems, Foster City, CA, USA). The 16S rRNA gene sequence was determined with an ABI 3730 automated sequencer. Nucleotide sequences were assembled and edited by using Sequencer v.4.1 (GeneCodes, Ann Arbor, MI). Obtained clone sequences were manually checked for chimeras using Ribosomal Database Project II (http://wdcm.nig.ac.jp/RDP/html/index.html) and identified chimeric sequences were removed. Briefly, neighbor-joining phylogenetic trees were constructed from dissimilar distance and pairwise comparisons with the Jukes-Cantor distance model using the MEGA (molecular evolutionary genetics analysis) program, version 3.1. Rarefaction and LIBSHUFF analyses were performed using software available online at http://www.uga.edu/~strata/software/Software.html and http://whitman.myweb.uga.edu/libshuff.html, respectively. Operational taxonomic units (OTUs) assignment and rarefaction analyses were performed using DOTUR (Schloss and Handelsman, 2005). The cut-ff value for defining OTUs was 3% (Humayoun, et al., 2003).

Quantitative polymerase chain reaction (Q-PCR). Q-PCR was carried out using SYBR-Green Master Mix (QIAGEN, Valencia, CA). Primers specific for *Archaea*, *Bacteria*, and *Crenarchaeota* were employed for quantification of total archaeal, bacterial, and crenarchaeotal 16S rRNA genes, respectively, according to procedures

described previously (Jiang, et al., 2007). Purified PCR products of 16S rRNA gene of *Sulfolobus acidocaldarius* DSM639 and *Shewanella putrefaciens* CN32 was used as standard archaeal and bacterial 16S rRNA gene, respectively, which, with serial dilutions, was used to yield standard curves (one each for total Archaea, Crenarchaeota and Bacteria). All quantitative PCR reactions for the standard curves were performed in duplicate. The amplification yielded reliable exponential patterns (Archaea, Crenarchaeota and Bacteria) with template amounts in the range of 10^4 to 10^8 16S rRNA gene copies. The data were used to create standard curves correlating the C_T values with 16S rRNA gene copy numbers. Linear plots between the log (gene copy number) and Ct value (not shown) for Archaea, Crenarchaeota and Bacteria were obtained with correlation coefficients of R^2=0.986, and 0.990, 0.991, respectively. All Q-PCRs were performed using a Rotor-Gene ^{TM}3000 real time thermal cycler (Corbett Life Science, Sydney, Australia). Melting curve analysis was performed to determine the melting point of the amplification products and to assess reaction specificity. After the Q-PCR reaction was complete, temperature ramped from 72 ^{o}C to 95 ^{o}C, rising by 0.2 degree each step, waiting for 45 s on first step, and then 5 s for each step afterwards. The melting curve of each run had only one peak. For all samples, 16S rRNA gene copy numbers were converted to cell numbers by a conversion factor of 3.6 (Klappenbach, et al., 2001).

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in the GenBank database under accession numbers EU109972–EU110051.

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Table 1. Carbon and oxygen isotopic compositions in the Qinghai Lake sediments.

Sample	Untreated		Hypochlorite-treated	
	$\delta^{13}C$	$\delta^{18}O$	$\delta^{13}C$	$\delta^{18}O$
QH0-10	1.21	0.02	1.16	-0.27
QH10-30			1.80	0.40
QH30-45	1.75	0.60		
QH74-89			1.53	0.66
QH118-133			3.11	1.81
QH240-255			1.36	-0.50
QH302-317			1.11	-0.45
QH345-355	0.77	0.22	1.13	-0.55
QH399-410	1.59	-0.24	1.93	0.84
QH458-470	0.68	-1.31	1.05	-1.26

Table 2. Quantitative analyses of cell abundance determined by Q-PCR.

sample	Total cells/g	Bacteria		Archaea		Crenarchaeota	
		cells/g	A*	cells/g	B*	cells/g	C*
QH0-10	6.69E+08	3.23E+08	48.4%	3.45E+08	51.6%	1.36E+08	20.4%
QH30-45	4.77E+08	1.68E+08	35.3%	3.08E+08	64.7%	1.74E+08	26.2%
QH118-133	1.80E+08	3.02E+07	16.8%	1.50E+08	83.2%	2.66E+07	14.8%
QH240-255	1.38E+08	1.88E+07	13.6%	1.19E+08	86.4%	3.78E+07	27.3%
QH345-355	8.52E+07	2.02E+07	23.7%	6.50E+07	76.3%	3.00E+06	35.2%
QH399-410	1.04E+08	4.53E+07	43.6%	5.87E+07	56.4%	2.67E+06	25.7%
QH458-470	6.07E+07	1.27E+07	18.9%	5.44E+07	81.1%	2.00E+06	29.8%

*A, B, and C are ratios of bacteria, archaea, and crenarchaeota, respectively, to total cells.

Table 3. Phylogenetic affiliations and compositions of archaeal 16S rRNA gene clones retrieved from the Qinghai Lake sediments.

	No. of clones						
Clone library	QLS0	QLS30	QLS118	QLS240	QLS345	QLS399	QLS458
Depth (cm)	0-10	30-45	118-133	240-255	345-355	399-410	458-470
Total clones sequenced	42	44	50	55	48	47	48
No. of OTUs*	10	8	17	10	10	13	12
No. of unique OTUs	4	3	7	5	6	9	6
Coverage (%)	90.5	93.2	86.0	90.9	87.5	80.9	87.5
Euryarchaeota							
Methanomicrobia						36 (77%)**	1 (2%)
Methanosarcinales						1 (2%)	
MBG-D	19 (45%)	36 (82%)	37 (74%)	20 (36%)	28 (58%)	3 (6%)	3 (6%)
Crenarchaeota							
MBG-C	2 (5%)	1 (2%)			1 (2%)	1 (2%)	26 (54%)
MCG			5 (10%)	32 (58%)		1 (2%)	8 (16%)
Marine Group I						2 (4%)	
"SCA clones"							3 (6%)
MBG-B	21 (50%)	7 (16%)	8 (16%)	3 (6%)	19 (40%)	3 (6%)	7 (15%)

*Determined by DOTUR with a cutoff value of 3%; **clone library composition.

Figure Captions

Fig. 1. A geographic map showing lakes on the Tibetan Plateau and the location of Qinghai Lake, NW China, and the drilling site in the lake (The symbol sizes are not proportional to the sizes of the lakes).

Fig. 2. Depth distribution of certain geochemical parameters in the pore water of the Qinghai Lake sediments.

Fig. 3. (A) Neighbor-joining tree (partial sequences, ~700 bp) showing the phylogenetic relationships of archaeal 16S rRNA gene sequences cloned from seven sediment subsamples of the 5-m core 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. 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 QLS399-A46: archaeal 16S rRNA gene clone number 46 from QLS399. Scale bars indicate the Jukes-Cantor distances. Bootstrap values of >50% (for 1000 iterations) are shown. Aquifex pyrophilius is used as the outgroup. Panel A is the first archaeal subtree showing all euryarchaeotal sequences retrieved in this study. (B) This figure is the second archaeal subtree showing all crenarchaeotal sequences.

Fig. 1

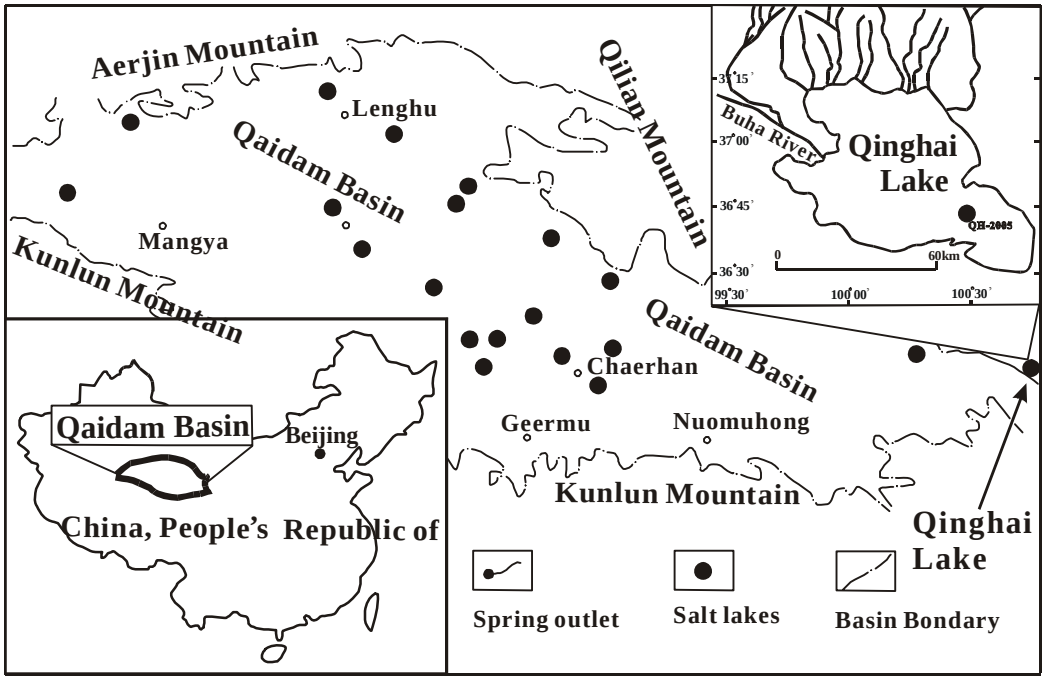

Aerjin Mountain
Lenghu
Qilian Mountain
Qaidam Basin
Mangya
Kunlun Mountain
Buha River
Qinghai Lake
QH-2005
0
60km
35°15'
37°00'
36°45'
36°30'
99°30'
100°00'
100°30'
Chaerhan
Qaidam Basin
Geermu
Nuomuhong
Kunlun Mountain
Qinghai Lake
Qaidam Basin
Beijing
China, People's Republic of
Spring outlet
Salt lakes
Basin Bondary

Fig. 2

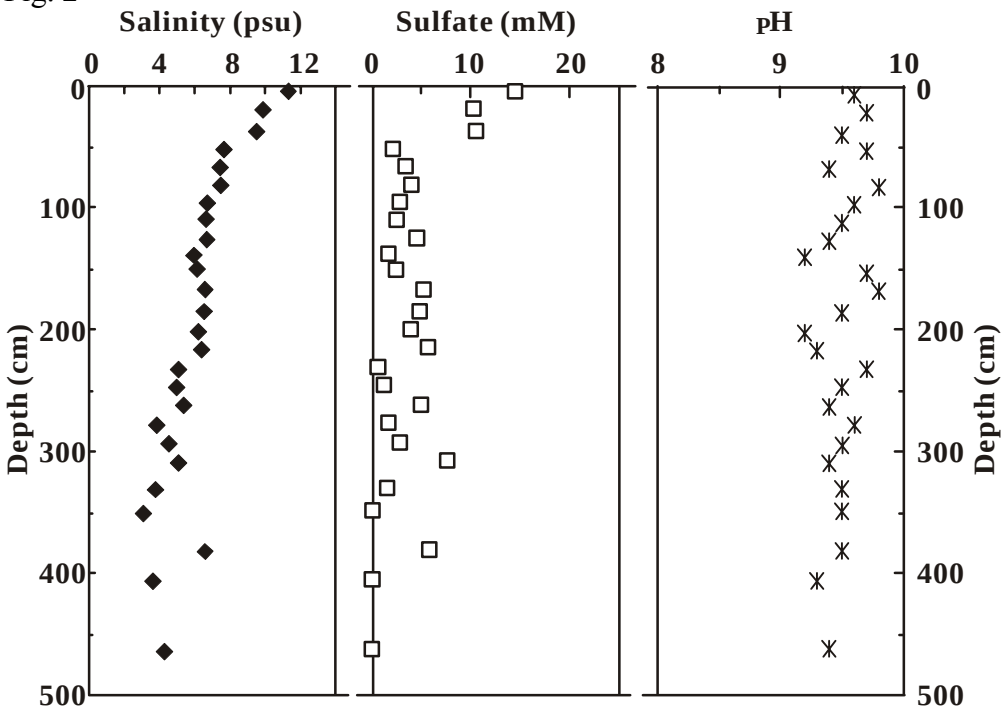
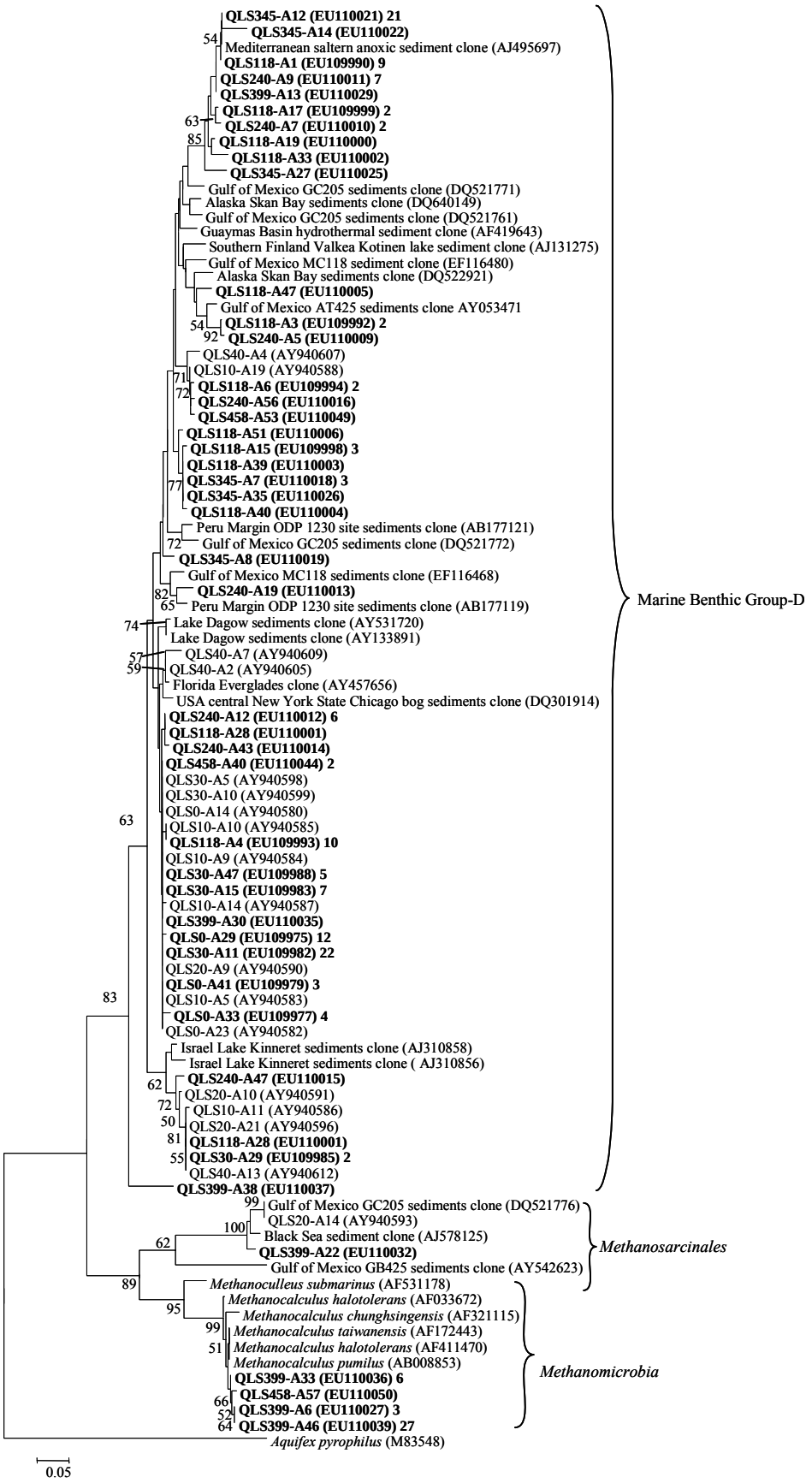

Salinity (psu)
Sulfate (mM)
pH
0
4
8
12
0
10
20
8
9
10
0
100
200
300
400
500
Depth (cm)
Depth (cm)

Fig. 3A


QLS345-A12 (EU110021) 21
QLS345-A14 (EU110022)
Mediterranean saltern anoxic sediment clone (AJ495697)
QLS118-A1 (EU109990) 9
QLS240-A9 (EU110011) 7
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Southern Finland Valkea Kotinen lake sediment clone (AJ131275)
Gulf of Mexico MC118 sediment clone (EF116480)
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Methanocalculus chunghsingensis (AF321115)
Methanocalculus taiwanensis (AF172443)
Methanocalculus halotolerans (AF411470)
Methanocalculus pumilus (AB008853)
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QLS458-A57 (EU110050)
QLS399-A6 (EU110027) 3
QLS399-A46 (EU110039) 27
Aquifex pyrophilus (M83548)
Marine Benthic Group-D
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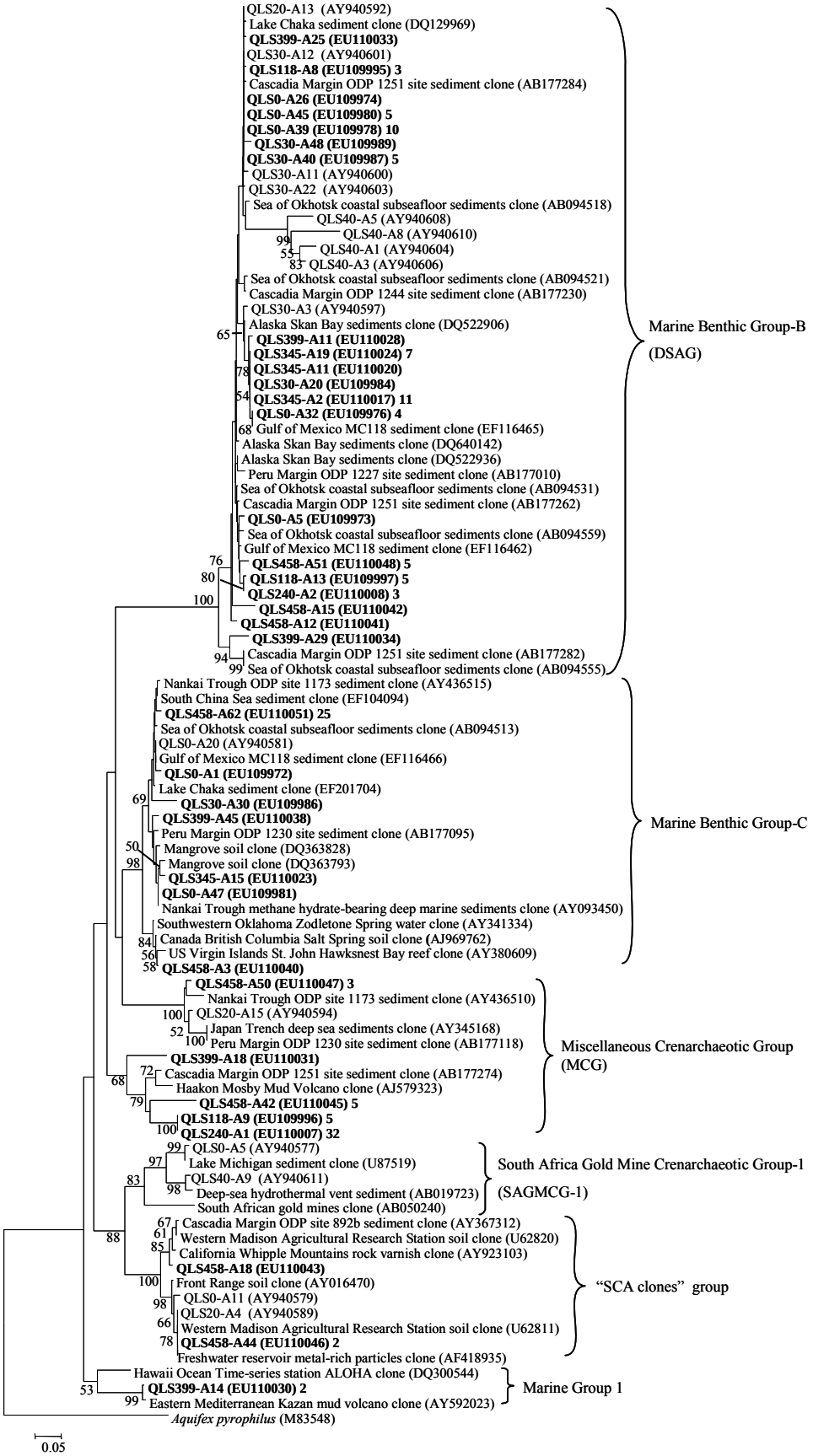
Methanomicrobia
0.05

Fig. 3B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Sea of Okhotsk coastal subseafloor sediments clone (AB094531)
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Marine Benthic Group-B
(DSAG)
Nankai Trough ODP site 1173 sediment clone (AY436515)
South China Sea sediment clone (EF104094)
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Sea of Okhotsk coastal subseafloor sediments clone (AB094513)
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Lake Chaka sediment clone (EF201704)
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US Virgin Islands St. John Hawksnest Bay reef clone (AY380609)
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Japan Trench deep sea sediments clone (AY345168)
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Cascadia Margin ODP 1251 site sediment clone (AB177274)
Haakon Mosby Mud Volcano clone (AJ579323)
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QLS240-A1 (EU110007) 32
Miscellaneous Crenarchaeotic Group
(MCG)
QLS0-A5 (AY940577)
Lake Michigan sediment clone (U87519)
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Deep-sea hydrothermal vent sediment (AB019723)
South African gold mines clone (AB050240)
South Africa Gold Mine Crenarchaeotic Group-1
(SAGMCG-1)
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California Whipple Mountains rock varnish clone (AY923103)
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Front Range soil clone (AY016470)
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Freshwater reservoir metal-rich particles clone (AF418935)
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Eastern Mediterranean Kazan mud volcano clone (AY592023)
Marine Group 1
Aquifex pyrophilus (M83548)
0.05

CHAPTER 6:

Microbial Diversity in the Deep Marine Sediments from the Qiongdongnan Basin in South China Sea

Running title: Microbial Diversity in South China Sea Sediments

Hongchen Jiang[1], Hailiang Dong[1,2*], Shanshan Ji[1], Ying Ye[3], and Nengyou Wu[4]

1. Department of Geology
Miami University
Oxford, OH 45056

2: Geomicrobiology Laboratory
China University of Geosciences
Beijing, 100083, China

3. Department of Earth Science
Zhejiang University
38 Zheda Road
Hangzhou, Zhejiang Province, China 310027

4. Guangzhou Marine Geological Survey, CGS
477 Huanshi Dong Road
Guangzhou, Guangdong Province, China 510075

*Corresponding author: Hailiang Dong
Department of Geology
Miami University
Oxford, OH 45056
Tel: 1-513-529-2517
Fax: 1-513-529-1542
Email: dongh@muohio.edu

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ABSTRACT

The continental shelf and slope in the northern South China Sea is well known for its prospect of oil/gas/gas-hydrate resources. To study microbial communities and their roles in carbon cycling, a 4.9-m sediment core was collected from the Qiongdongnan Basin on the continental slope of the South China Sea during our cruise HY4-2005-5 in 2005. Geochemical, mineralogical, and molecular phylogenetic analyses were carried out. Sulfate concentration in pore water decreased with depth. Abundant authigenic carbonates and pyrite were observed in the sediments. The bacterial community was dominated by aerobic and facultative organisms. Bacterial clone sequences belonged to the *Gamma*-, *Alpha*-, *Deltaproteobacteria* and *Firmicutes* group, and they were related to Fe(III) and/or Mn(IV) reducers, sulfate reducers, aromatic hydrocarbon degraders, thiosulfate/sulfite oxidizers, and denitrifiers. Archaeal clone sequences exhibited greater overall diversity than the bacterial clones with most sequences related to Deep-Sea Archaeal Group (DSAG), Miscellaneous Crenarchaeotic Group (MCG), and Uncultured Euryarchaeotic Clusters (UECs). Archaeal sequences related to *Methanosarcinales*, South African Gold Mine Euryarchaeotic Group (SAGMEG), Marine Benthic Group-D (MBG-D) were also present. Most of these groups are commonly present in deep-sea sediments, particularly in methane/organic-rich or putative methane hydrate-bearing sediments.

Keywords: 16S rRNA gene analysis, Archaea, Bacteria, diversity, South China Sea.

INTRODUCTION

Methane, a greenhouse gas and the main component of natural gas, is mainly stored as a free compound in permafrost regions or as hydrates in continental slope sediments (Kvenvolden, 1988, Milkov, 2004). Gas hydrates are crystalline solids of gas and water molecules under high pressure. The estimated total biomass of methane carbon in gas hydrates is more than 10 trillion tons, twice the amount of all known coal, oil, and other fossil fuels. Communities of microbes and macrofauna flourish in these environments and often mediate methane production and consumption (Strous and Jetten, 2004).

In the past several years, there has been an increased interest in gas hydrate research, primarily due to the potential economic and environmental significance of such deposits. Ample evidence has now demonstrated that sulfate-reducing bacteria and anaerobic methane-oxidizing archaea (ANME) form a stable consortium (Boetius, et al., 2000, Orphan, et al., 2001) in many gas hydrate deposits, such as surface-breaching gas hydrate mounds in the Gulf of Mexico (Lanoil, et al., 2001, Mills, et al., 2003, Mills, et al., 2005) and methane hydrate-bearing marine sediments in the Nankai Trough (Reed, et al., 2002). However, this syntrophic consortium is not universal in all gas hydrate deposits. The ANME may be absent or less dominant in certain hydrate environments. For example, Inagaki et al. (2003) showed that only a few sequences of anaerobic methane oxidizers were present in the coastal subseafloor sediments of the Sea of Okhotsk, but instead the Deep-Sea Archaeal Group (DSAG) was predominant. The DSAG, none of which has been cultivated yet, was first found at hydrothermal vents (Takai and Horikoshi, 1999) and deep-sea sediments (Vetriani, et al., 1999), and subsequently retrieved from many benthic marine sediments (Sorensen and Teske, 2006). Miscellaneous Crenarchaeotic Group (MCG), ubiquitous in both terrestrialenvironments and marine sediments (Sorensen and Teske 2006), often co-occur with the DSAG in organic-rich and/or hydrate-bearing deep-sea sediments (Inagaki, et al., 2006). None of the MCG has been cultivated either.

Inagaki et al. (2006) found distinct distribution patterns of archaeal and bacterial communities in deep-sea sediments with and without methane hydrates. In hydrate-free, organic-poor sediments on the Pacific Ocean Margin, archaeal community is dominated

with Marine Crenarchaeotic Group I (MCG-I) and Marine Benthic Group A. In hydrate-free but organic-rich sediments, the community is dominated with Miscellaneous Crenarchaeotic Group (MCG) and South African Gold Mine Euryarchaeotic Group (SAGMEG). In contrast, in hydrate-containing sediments, the DSAG is the predominant group in archaeal community. The authors also found distinct distribution pattern in bacterial community. For example, the uncultured JS1 is the predominant group in hydrate-bearing sediments, but the *Chloroflexi* group is dominant in hydrate-free sediments.

The goal of this research was to study microbial community composition in the South China Sea (SCS) sediments, a site with potential gas hydrate deposits. One sediment core was collected from the Qiongdongnan Basin of the South China Sea. Geochemical, mineralogical, and phylogenetic analyses (16S rRNA gene analysis) were carried out, and the relationship between geochemical environments and microbial diversity/inferred physiological functions were discussed.

MATERIALS AND METHODS

Introduction to the South China Sea.

The South China Sea is a site with potential gas hydrate deposits (Guo, et al., 2004, McDonnell and Czarnecki, 2000). The SCS is located at the junction of three tectonic plates: the Eurasian, the Pacific and the Indian-Australian. It is surrounded by passive continental margins in the west and north and convergent margins in the south and east. Thick organic-rich sediments have accumulated in the SCS since the late Mesozoic (He, et al., 2006). These sediments are a continuing source to form gas hydrates at the site (Guo, et al., 2004). There are three areas in the SCS with potential gas hydrate deposits: northwestern continental shelf and slope, Dongsha Rise and Manila Trench in Bijianan Basin (Fig. 1) (McDonnell and Czarnecki, 2000, Wu, et al., 2005).

The Qiongdongnan Basin (Fig. 1) is one of potential gas hydrate-bearing basins on the northwestern continental shelf. It is an oil-bearing, fault-depression structural basin, in which organic-rich sediments of 5000 m in thickness have accumulated since the Cenozoic (He, et al., 2006). Seismic data show occurrences of high-pressure diapirs and

gas plumes, faults, and gas springs, all of which indicate that the Qiongdongnan Basin possesses suitable sources and tectonic conditions for gas hydrate formation (Chen, et al., 2004). High geothermal (4.6^{o}C/100m) and pressure (18-22 kPa/m) gradients and strong thermal fluids in the basin have accelerated maturation of hydrocarbons and their mobilization (Liu and Wang, 2004). Different stratigraphic traps provide good storage space (Zhu, et al., 2004). The presence of gas hydrates in the Qiongdongnan Basin has been confirmed by geological and geophysical evidence (Chen, et al., 2004, Jiang, et al., 2005, Su, et al., 2005, Wu, et al., 2003).

Sample Collection

In September 2005, a 4.9-m sediment core was collected using a gravity piston corer from the continental slope of the Qiongdongnan Basin during our cruise HY4-2005-5 (Fig. 1). The site (111^{o}4$^{'}$E, 18^{o}2$^{'}$N) is located at about 150 km southeast of Sanya, Hainan Province, China. The sampling site has a water depth of 1508 m. This site was selected because of relatively shallow water depth and soft sediments (so that gravity piston coring was possible). After collection, the sediment core was stored onboard at 4^{o}C (approximate in-situ temperature at the water-sediment interface) for 3 weeks. After the ship came back onshore, the core was dissected into 5-cm sediment subsamples in a flow-through (N_2) anaerobic chamber at Guangzhou Marine Geological Survey, China. The external layers of each subsample were removed using sterile tools to avoid any possible contamination. Selected subsamples throughout the length of the core (17 in total) were shipped frozen to Miami University for geochemical and microbiological analyses.

Geochemical Analyses

Sediment pore water samples were collected following centrifugation of ~50 g of the sediment subsamples (5000 x g for 10 minutes). The volume of pore water from each sediment subsample was ~13-20 mL. A strong odor of hydrogen sulfide was evident from pore water. pH measurements were made on pore water with a pH meter immediately after collection. For measurements of cations, aliquots of pore water were fixed in HCl solution (0.1N HCl final concentration). Samples for anion analysis were not fixed. The diluted pore water samples were analyzed by high performance liquid chromatography

(HPLC) for anions (IonPac®AS14 column 4×250mm) and direct current plasma emission spectrometry (DCP) for cations. Major anions and cations were combined to calculate total dissolved salt (TDS). The total organic carbon (TOC) content of two sediment subsamples (one was from the top and the other from the bottom) were determined in Service Testing and Research Laboratory of the Ohio State University, USA. The method is available on-line at http://www.oardc.ohio-state.edu/starlab/references.asp. X-ray diffraction (XRD) was employed to analyze mineralogy in the sediments. Samples were X-rayed with a Siemens D500 X-ray diffraction system using Cu radiation with a monochromator, 0.02 two-theta steps from 2 to 40 degrees, and a count time of 2 seconds per step. Mineral identification was made with a combination of search-match software and manual search. A Zeiss Supra35 VP-FEG scanning electron microscope (SEM) equipped with EDAX Energy Dispersive X-ray microanalysis system was used to identify minerals and to image their morphologies. Selected sediment subsamples for SEM observations were vacuum-dried and gold-coated.

Total Microbial Counts

Microbial cell abundance in the sediments was measured by acridine orange direct count (AODC) following the procedure described in Jiang et al. (Jiang, et al., 2006).

DNA Extraction, PCR Amplification, and Cloning of the Sediment Sample

Three sediment subsamples (top, middle and bottom of the 4.9-m core) were initially selected to determine any possible change of microbial diversity with depth. The procedures for genomic DNA isolation, PCR amplification and cloning of the 16S rRNA gene were similar to our previously published protocol (Jiang et al. 2006). The bacterial forward primer was Bac27F: 5'-AGA GTT TGG ATC MTG GCT CAG -3', the archaeal forward primer was Arch21F (5'-TTC YGG TTG ATC CYG CCR GA-3') and the universal reverse primer was Univ1492R: 5'-CGG TTA CCT TGT TAC GAC TT -3'. The PCR reactions were limited to 25 cycles in order to lower bias (Acinas, et al., 2005). Triplicate PCR products were pooled to obtain sufficient quantities for subsequent sequencing.

Sequence and Phylogenetic Analyses

Six clone libraries (three for bacteria and the other three for archaea) were constructed. A sufficient number of bacterial and archaeal clones were randomly picked for sequencing analysis until rarefaction curves were fully saturated. The 16S rRNA gene sequencing reaction was carried out using primer Bac27F for *Bacteria* and Arch21F for *Archaea*. The 16S rRNA gene sequence was determined with an ABI 3730 automated sequencer with the BigDye Terminator version 3.1 (Applied Biosystems, Foster City, CA). Sequences were typically ~700 bp long. Phylogenetic analyses were carried out in the same manner as described in Jiang et al. (2006). Rarefaction curves, operational taxonomic units (OTUs) (see below) and phylogenetic analyses on the bacterial and archaeal clone libraries (three each) indicated that there was no systematic difference in microbial community among the three selected subsamples. Thus, the three clone libraries for *Bacteria* and *Archaea*, respectively, were combined. The combined clone libraries were designated as SCS-QBSB (South China Sea Qingdongnan Basin sediments bacteria) and SCS-QBSA (South China Sea Qingdongnan Basin sediments archaea), respectively.

Statistical Analysis of Clone Libraries

For this analysis, one major assumption was that sequences with sequence identity of greater than 97% were considered to represent the same OTUs for the reasons stated in a previous study (Humayoun, et al., 2003). Coverage (C) was calculated as: $C = 1-(n_1/N)$, where n_1 is the number of OTUs that occurred only once in the clone library and N is the total number of clones analyzed (Mullins, et al., 1995). Chao-1 and ACE (abundance-based coverage estimators) nonparametric analyses of the clone libraries were performed and rarefaction curves constructed utilizing DOTUR (distance based OUT richness determination) (Schloss and Handelsman, 2005).

Nucleotide Sequence Accession Numbers

The unique sequences determined in this study have been deposited in the GenBank database under accession numbers EF104065-EF104084 for the bacterial clone sequences, and EF104085-EF104115 for the archaeal clone sequences.

RESULTS

Pore Water Chemistry

The total dissolved salt and pH did not show any significant variations throughout the entire length of the sediment core (Fig. 2A&B) with an average value of ~38 g/L and ~7.8, respectively. The sulfate concentration showed an approximate decreasing trend from ~27 mM at the top to ~21 mM at the bottom (Fig. 2C). The concentrations of cations and other anions of pore water (Table 1) as measured by DCP and HPLC analyses were similar to seawater and did not show any trend with sediment depth.

Sediment Properties

X-ray diffraction identified the major minerals in the sediments as quartz, albite, illite, and kaolinite with a small amount of pyrite, barite, calcite, aragonite, magnesite, gypsum, and anhydrite. SEM observations revealed a large number of authigenic minerals such as clay minerals, pyrite, barite, carbonate, gypsum and anhydrite etc. (Fig. 3). Euhedral (octahedral) and framboidal pyrite were abundant (Fig. 3E-H). The TOC content in the sediments increased from 0.84% at the top to 1.2% at the bottom (Fig. 2C). Total cell counts in the selected samples were ~10^7 cells/g (Fig. 2D).

Bacterial Clone Library Analysis

A total of 195 16S rRNA gene clone sequences (95 and 100 for bacteria and archaea, respectively) were subjected to sequence similarity analysis. The bacterial clone sequences could be classified into four lineages: *Alpha*-, *Gamma*-, and *Delta-proteobacteria*, and *Firmicutes* (Fig.4).

(i) *Alphaproteobacteria*. Twelve clones (13% of the bacterial library, Fig. 4&6) were affiliated with the *Alphaproteobacteria* group (Fig. 4). Three sequences were related (94%) to *Kordiimonas gwangyangensis* (AY682384) isolated from marine sediments in -Gwangyang Bay of the South Sea, Republic of Korea. *K. gwangyangensis*, strictly

aerobic, is able to degrade high-molecular-mass polycyclic aromatic hydrocarbons (Kwon, et al., 2005). Five sequences were related (93-99%) to Slope strain DI4 (AF254104), isolated from continental slope sediments off the coast of New England. DI4 is an acid-producing thiosulfate oxidizer (Teske, et al., 2000). Four sequences clustered with *Sulfitobacter* strains, members of marine alphaproteobacteria (Gonzalez and Moran, 1997). Because most *Sulfitobacter* species can oxidize sulfite to sulfate (Gonzalez and Moran, 1997), it is possible that the *Sulfitobacter*-related sequences retrieved in this study may have originated from sulfite oxidizers.

(ii) *Gammaproteobacteria*. Most (82%, 78 out of 95, Fig. 4 and 6) sequences in the bacterial library fell into the *Gammaproteobacteria* group. Fourteen sequences were related (97-99%) to one clone sequence (DQ462284) retrieved from South China Sea sediments (GenBank description) and also related (94-97%) to another clone sequence (AY375069) recovered from Western Pacific Warm Pool sediments (Zeng, et al., 2005). Two sequences were related (99%) to *Pseudomonas stutzeri* (DQ289057), a denitrifying member in the *Gammaproteobacteria* isolated from deep subsurface oil-bearing rocks (GenBank description). Some strains of *P. stutzeri* are capable of decomposing complex aromatic hydrocarbons (Grimberg, et al., 1996). Six clones were related (90-99%) to *Alcanivorax jadensis* (AJ001150) isolated from the coast intertidal sediment in German North Sea (Bruns and Berthe-Corti, 1999, Fernandez-Martinez, et al., 2003). *A. jadensis* is a moderately halophilic, hydrocarbon-degrading bacterium, and able to grow facultatively and reduce nitrate. Four sequences were related (99%) to one clone retrieved from German Wadden Sea water, which has a salinity of 2.6% and a pH of 7.9 (Stevens, et al., 2005). These conditions are similar to those of the SCS-QBS pore water (Fig.2). Five clones showed a high similarity (99%) to *Shewanella kaireitica* (AB094598). *S. kaireitica* was isolated from Japan Suruga Bay deep-sea sediments and could reduce sulfate and nitrate to H_2S and N_2, respectively (Miyazaki, et al., 2006). Thirty-two sequences were related (98-99%) to *Marinobacter* sp. MED91 (AY136115) isolated from the Eastern Mediterranean Sea. Most members of the genus *Marinobacter* can degrade certain components of petroleum (Gonzalez and Whitman, 2004). Fifteen clones were closely related (99%) to various species of *Halomonas*, among which *H. meridiana*

(AF212217), a facultative aerobe, was isolated from low-temperature hydrothermal fluid at 2580 m depth on the Southern East Pacific Rise (Kaye, et al., 2004).

(iii) *Deltaproteobacteria*. Two (Fig. 4) sequences were related (97%) to one *Pelobacter* clone (AJ271656) retrieved from Black Sea shelf sediments where manganese and sulfate reduction prevailed, and this clone was thought to be retrieved from organisms mediating Fe(III) reduction (Thamdrup, et al., 2000).

(iv) *Firmicutes*. Three clones were grouped into *Firmicutes*. One cultivated reference in this group, *Clostridium caminithermale* (AF458779) was a slightly halophilic and moderately thermophilic bacterium isolated from an Atlantic deep-sea hydrothermal chimney. *C. caminithermale* is strictly anaerobic and cannot use elemental sulfur, sulfate, thiosulfate, sulfite, nitrate or nitrite as electron acceptors (Brisbarre, et al., 2003).

Archaeal Clone Library Analysis

Although the majority of bacterial clones were mostly related to cultured isolates, very few archaeal clones exhibited such relatedness. The archaeal clone sequences could be grouped into two phyla: *Euryarchaeota* and *Crenarchaeota* (Fig.5).

i) *Euryarchaeota*. Forty-six archaeal clones clustered into the *Euryarchaeota*, which consisted of seven subgroups: Marine Benthic Group-D (MBG-D) (Vetriani, et al., 1999), Terrestrial Miscellaneous *Euryarchaeotic* Group (TMEG) (Takai, et al., 2001), *Methanosarcinales*, South African Gold Mine *Euryarchaeotic* Group (SAGMEG) (Takai, et al., 2001), and three uncultured *Euryarchaeotic* Clusters (UEC). Six sequences were grouped into the MBG-D and they were related to those retrieved from hydrate-bearing sediments from the costal subseafloor of the Sea of Okhotsk (Inagaki, et al., 2003), and organic-rich but hydrate-free sediments from site 1227 of the Peru Margin (Inagaki, et al., 2006). Three sequences were grouped into the TMEG and related to one clone (DQ301986) recovered from the sediments of Peru Margin site 1227 (Sorensen and Teske, 2006). Three sequences were grouped into *Methanosarcinales* and related to one clone (AY177814) retrieved from Antarctic coastal marine sediments (Purdy, et al., 2003). These three sequences were also related to two methanogens: *Methanococcoides burtonii* and *Methanococcoides alaskense* isolated from water samples of Antarctic Ace Lake and Alaska Skan Bay, respectively (Franzmann, et al., 1992, Singh, et al., 2005). *M.*

burtonii can use tri-, di- and mono-methylamine and methanol as substrates for growth and methanogenesis (Franzmann, et al., 1992). In comparison, *M. alaskense* can only metabolize trimethylamine as sole organic substrate for methanogenesis (Singh, et al., 2005). One common property of these two methanogens is that neither of them can use acetate, dimethylsulfide, formate and H_2:CO_2 as substrates (Franzmann, et al., 1992, Singh, et al., 2005).

Five clones formed the SAGMEG and grouped with those retrieved from the deep marine sediments of the Okhotsk subseafloor (AB094535) and ODP Margin site 1227 (AB177002). SAGMEG clone sequences were first found in South African gold mines and later in deep-sea and hydrothermal environments (Sorensen and Teske, 2006).

Twenty-nine clone sequences formed three UECs (29%, Fig. 5&6). Two clones were grouped into the UEC-1 and were related (98%) to one sequence (DQ363844) recovered from Hainan Island (China) mangrove soil (GenBank description). Twenty clones formed the UEC-2 and they were related to one sequence (AY396650) retrieved from Korean Ganghwa Island tidal flat sediments (Kim, et al., 2005). A group of seven clones in the UEC-2 were related to one sequence (AY592049) retrieved from anaerobic methane-oxidizing microbial community of mud volcano in Kazan, eastern Mediterranean (GenBank description). Seven clones formed the UEC-3 and were related to those retrieved from Eastern Mediterranean deep-sea sediments (AY627512, GenBank description) and North-east Pacific Ocean Baby Bare Seamount crustal fluids (AY704379).

ii) *Crenarchaeota*. In the *Crenarchaeota* phyla were two subgroups: DSAG and MCG. Twenty-seven clones (27%, Fig. 5&6) were grouped to form the DSAG, most of which were closely related (~99%) to sequences retrieved from Nankai Trough methane seeps (Nunoura, et al., 2006) and methane hydrate-bearing deep marine sediments from Nankai Trough (Reed, et al., 2002), Hydrate Ridge, Cascadia Margin off Oregon (Knittel, et al., 2005), and several ODP Peru Margin sites (Inagaki, et al., 2006).

Twenty-seven archaeal clones (27%, Fig. 5&6) were grouped into the MCG and they were related (93-99%) to some sequences recovered from subseafloor sediments and hydrothermal fluids (Okhotsk subseafloor sediments, ODP Peru Margin site 1227

sediments, ODP Nankai Trough site 1173 sediments, North-east Pacific Ocean Baby Bare Seamount crustal fluids, and Eastern Mediterranean deep-sea sediments).

Coverage, richness and sequence diversity of the libraries. The coverage values were 97% and 99% for the bacterial and archaeal library, respectively. Both the coverage values and richness analysis (chao-1 and ACE nonparametric estimators and rarefaction curves) (Fig. 7) indicated that the number of OTUs in this study nearly reached saturation. The diversity indices (N_t/N_{max}) for the bacterial and archaeal libraries were 2.97 and 4.35, respectively, which together with richness analyses (Fig. 7) - indicated the archaeal community was more diverse than the bacteria.

DISCUSSION

Microbial Abundance

Microbial cell abundance in the SCS sediments was similar to those from certain ocean margin, gas hydrate-free/bearing sediments (Inagaki, et al., 2006, Inagaki, et al., 2003, Reed, et al., 2002, Schippers, et al., 2005), but about 2 orders of magnitude lower than that in gas hydrate-bearing sediments in the Gulf of Mexico (Lanoil, et al., 2001). Parkes et al. (Parkes, et al., 2000) concluded that the microbial abundance in marine sediments is related to TOC concentration. The TOC concentration in the SCS sediments was within the range of 1-10% (dry weight) for ocean margin gas hydrate-free/bearing sediments (Schippers, et al., 2005), but much lower than that in the Gulf of Mexico sediments (>3.6%, dry weight) (Sassen, et al., 1994).

Bacterial Diversity

Three prominent features were observed in the bacterial diversity: 1) very low diversity; 2) relatedness of most (more than 70%) clone sequences to cultured organisms; 3) predominance (82%) of the *Gammaproteobacteria* group. The lower bacterial diversity in the studied SCS sediments than its archaeal counterpart (Fig. 6) was consistent with the trend observed for gas-hydrate bearing sediments in the Sea of Okhotsk (Inagaki, et al., 2003), but opposite to the trend observed for the Gulf of Mexico (Mills, et al., 2003) and the Nankai Trough (Reed, et al., 2002). Most bacterial sequences were related to the genera *Halomonas*, *Marinobacter*, *Pseudomonas*, *Shewanella*,

Pelobacter, *Clostridium*, and *Sulfitobacter*. These genera have been isolated in volcanic ash layers within costal subseafloor sediments from the Sea of Okhotsk (Inagaki, et al., 2003) and in methane hydrate sediments (ODP Peru Margin site 1230) (Biddle, et al., 2005, D'Hondt, et al., 2004). Physiological functions of these relatives include Fe(III) and Mn(IV) reduction (*Pelobacter*), sulfate reduction (H_2S production) (*S. kaireitica*), decomposition of complex aromatic hydrocarbons and denitrification (*P. stutzeri*), hydrocarbon degradation (*A. jadensis*) and thiosulfate/sulfite oxidation (*Sulfitobacter* spp.). Although caution must be exercised to infer any physiological functions from phylogeny (based on sequence relatedness to known cultures), these sequence data appear to suggest that sulfate and iron reduction coupled with oxidation of hydrocarbons may be an important process in the SCS sediments. These inferred metabolic processes are consistent with decreased sulfate concentration in pore water with depth (Fig. 2) and precipitation of pyrite (Fig. 3). Oxidation of hydrocarbons (possibly coupled with sulfate reduction) may have resulted in increased alkalinity and thus carbonate precipitation (Fig. 3).

The rate of sulfate decrease with depth (~12μM cm^{-1}) was much lower than that in methane/organic-rich or putative hydrate-bearing marine sediments, such as methane-rich sediments of the upwelling area off Namibia (~25-70 μM cm^{-1}) (Niewohner, et al., 1998), surface sediments above gas hydrate at Hydrate Ridge, NE Pacific Ocean (~2600 μM cm^{-1}) (Treude, et al., 2003), organic-rich sediments of Gulf of Mexico cold seeps (~250 μM cm^{-1}) (Joye, et al., 2004), and methane-rich sediments of the Chilean upwelling region (~70-170 μM cm^{-1}) (Treude, et al., 2005). So sulfate reduction at our study site may not be as active as in other systems, which might indicate that the fluxes of methane or petroleum was absent or minor, or microorganisms mediating sulfate reduction were not physiologically active.

The relative abundance (82%) of the *Gammaproteobacteria* in the SCS sediments was much higher than any other methane/organic-rich or putative hydrate-bearing marine sediments. To our knowledge, our study is the second to show this dominance in marine sediments. Such a high dominance of the *Gammaproteobacteria* has only been observed once, in the volvanic ash layers of the coastal subseafloor sediments from the Sea of

Okhotsk (Inagaki, et al., 2003). The reason for this dominance may be ascribed to their opportunistic life strategies of the *Gammaproteobacteria* (Pinhassi and Berman, 2003).

The number of *Firmicutes*-related gene sequences was very low. The *Firmicutes* group has been claimed to be widespread in marine environments (Hugenholtz, et al., 1998), but may be absent in gas hydrate-bearing sediments. *Firmicutes*-related gene sequences were not recovered from gas hydrate sediments from the Cascadia Margin (Bidle, et al., 1999, Inagaki, et al., 2006, Marchesi, et al., 2001), the Sea of Okhotsk (Inagaki, et al., 2003) and the Nankai Trough (Kormas, et al., 2003, Newberry, et al., 2004, Reed, et al., 2002). However, *Firmicutes*-related gene sequences were recovered in Gulf of Mexico gas hydrate sediments (Lanoil, et al., 2001, Mills, et al., 2003, Mills, et al., 2005). These data observations suggest that *Firmicutes* cannot specifically be related to gas hydrates. Other detected phylotypes (such as *Pseudomonas*, *Marinobacter*, *Shewanella*, and *Pelobacter*) are all commonly present in marine sediments, but their relationship to gas hydrates is not clear.

Archaeal Diversity

In comparison to the bacterial clone library, archaeal sequences in the studied SCS sediments exhibited a higher diversity. Clone sequences were clustered into nine phylogenetic lineages including seven groups of *Euryarchaeaota* (i.e., MBG-D, TMEG, *Methanosarcinales*, SAGMEG and three UECs) and two groups of *Crenarchaeota* (i.e., DSAG and MCG). Most of these groups have been detected in various marine sediments (Inagaki, et al., 2006, Sorensen and Teske, 2006), including the TMEG and SAGMEG that were first detected in terrestrial environments (Grobkopf et al., 1998; Takai et al., 2001). In particular, the presence of the DSAG and MCG members in the SCS sediments was consistent with previous studies on hydrate-bearing and/or organic-rich marine sediments (Inagaki, et al., 2006, Sorensen and Teske, 2006).

Another important observation in this study was lack of ANME sequences in the SCS sediments. The syntrophic consortia of SRB and anaerobic methane oxidizers is typically present in gas hydrate communities (Boetius, et al., 2000, Orphan, et al., 2001). However, our clone libraries did not show any sequences related to ANME (Fig. 5&6). Instead, *Methanosarcinales*-related sequences were retrieved. The methanogenic

Methanosarcinales have been reported to predominate over ANME in the Gulf of Mexico gas hydrate sediments (Lanoil, et al., 2001). Thus, it may be possible that anaerobic methane oxidation is carried out by *Methanosarcinales* via a process called reverse methanogenesis (Valentine and Reeburgh, 2000). Several studies have indicated that *Methanosarcinales* may be capable of anaerobic methane oxidation at gas hydrate sites (Hinrichs, et al., 1999, Orcutt, et al., 2005, Pimenov, et al., 1997). Of course, other groups, such as the DSAG, might be capable of anaerobic methane oxidation, but their functions are yet to be discovered.

In summary, the bacterial community in the SCS sediments was predominated by *Gammaproteobacteria*, but the archaeal community was highly diverse and consisted of 7 groups within the *Euryarchaeota* and 2 groups within the *Crenarchaeota*. The major groups within both communities were consistent with those previously detected in various marine environments, including methane/organic-rich and/or putative gas hydrate-bearing marine sediments. Combined geochemical and microbial data suggest that the SCS sediments may be undergoing a coupled process of hydrocarbon degradation and sulfate/ iron reduction. Presence or absence of gas hydrates in the SCS sediments could not be determined with certainty without further evidence (such as isotopic data).

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Table 1 pore water chemistry

Concentration (g/L)												TDS
Na^+	NH_4^+	K^+	Mg^{2+}	Ca^{2+}	Fe (mg/L)	F^-	Cl^-	Br^-	NO_3^-	PO_4^{3-}	SO_4^{2-}	(g/L)
11.7-12.7	<0.06	0.54-0.68	1.34-1.52	0.36-0.53	9.0-53.2	0.003-0.005	20.0-21.8	0.07-0.08	BD	BD	2-2.6	36.3-39.2

*BD: below detection limit

FIGURE CAPTIONS

Figure 1. A location map of the Qiongdongnan Basin, South China Sea. The drilling site is labeled on the map.

Figure 2. Depth profile of pH (A), total dissolved salt (B) and sulfate (open square) & total organic carbon (open circle) concentration (C) in pore water, and microbial abundance in the sediments as determined by AODC (D).

Figure 3. SEM images for bacterial cell (A), clay minerals (B), and authegenic minerals (C-H): C, Aragonite (typical acicular structure, see arrow); D, Calcite; E-H, framboidal pyrites. Energy dispersive X-ray spectra (C, D, G) showing the chemical composition of aragonite, calcite, and pyrite, respectively.

Figure 4. Neighbor-joining tree (partial sequences, ~700 bp) showing phylogenetic relationships of bacterial 16S rRNA gene sequences retrieved from the SCS Qiongdongnan Basin sediment sample to closely related sequences from GenBank. One representative clone type within each phylotype is shown and the number of clones within each phylotype is shown at the end (after the GenBank accession number). If there is only one clone sequence in a given phylotype, the number "1" is not shown. Clone sequences from this study are coded as follows, with SCS-QBS-B44 as an example: SCS: South China Sea sediment; QBS: Qiongdongnan Basin sediment, B, bacteria; 44, clone number. So it reads bacterial clone 44 from the South China Sea Qiongdongnan Basin sediment. Scale bars indicate Jukes-Cantor distances. Bootstrap values of >50% (for 500 iterations) are shown. *Aquifex pyrophilus* is used as an outgroup.

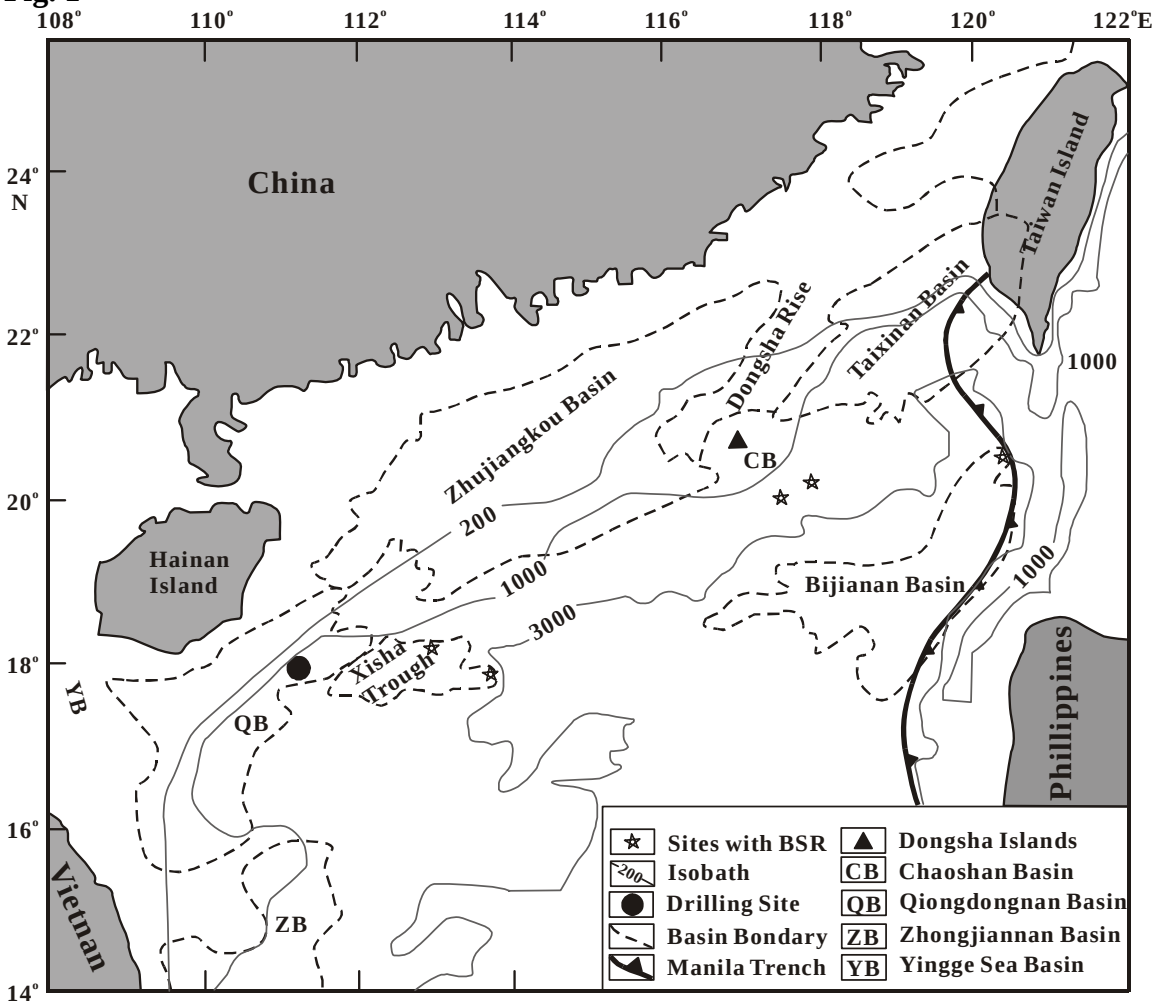
Figure 5. Neighbor-joining tree (partial sequences, ~700 bp) showing phylogenetic relationships of archaeal 16S rRNA gene sequences cloned from the SCS

sediment sample to closely related sequences from GenBank. The same algorithms as those for the bacterial tree were used. *Aquifex pyrophilus* is used as an outgroup. One representative clone type within each phylotype is shown and the number of clones within each phylotype is shown at the end (after the GenBank accession number). If there is only one clone sequence in a given phylotype, the number "1" is not shown.

Figure 6. Frequencies of OTUs affiliated with major phylogenetic groups in the bacterial and archaeal clone libraries for the South China Sea sediments. α, γ, and δ: *Alpha-, Beta-*, and *Gammaproteobacteria*; F, *Firmicutes*; M, *Methanosarcinales*; UEC, Uncultured Euryarchaeotic Clusters; SAGMEG, South African Gold Mine Euryarchaeotic Group; DSAG, Deep-Sea Archaeal Group; MCG, Miscellaneous Crenarchaeotic Group; MBG-D: Marine Benthic Group -D.

Figure 7. Observed and estimated OTU richness of archaeal and bacterial 16S rRNA genes retrieved from the sediments of the Qiongdongnan Basin in the South China Sea by Chao-1 (A), ACE (B) and rarefaction curves (C) analyses. Error bars are 95% confidence intervals given by DOTUR.

Fig. 1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112°
114°
116°
118°
120°
122°E
24°
N
22°
20°
18°
16°
14°
China
Taiwan Island
Dongsha Rise
Taixinan Basin
1000
Zhujiangkou Basin
CB
200
1000
Hainan
Island
Bijianan Basin
1000
3000
Xisha
Trough
YB
QB
Phillippines
Vietnan
ZB
Sites with BSR
Isobath
200
Drilling Site
Basin Bondary
Manila Trench
Dongsha Islands
CB Chaoshan Basin
QB Qiongdongnan Basin
ZB Zhongjiannan Basin
YB Yingge Sea Basin

Fig. 2

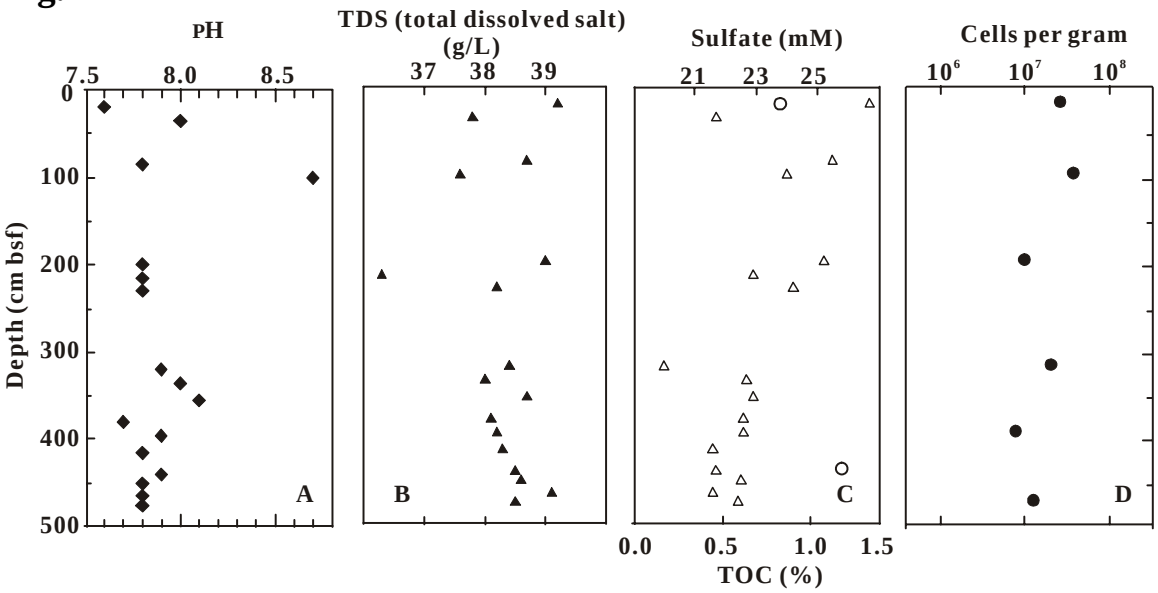
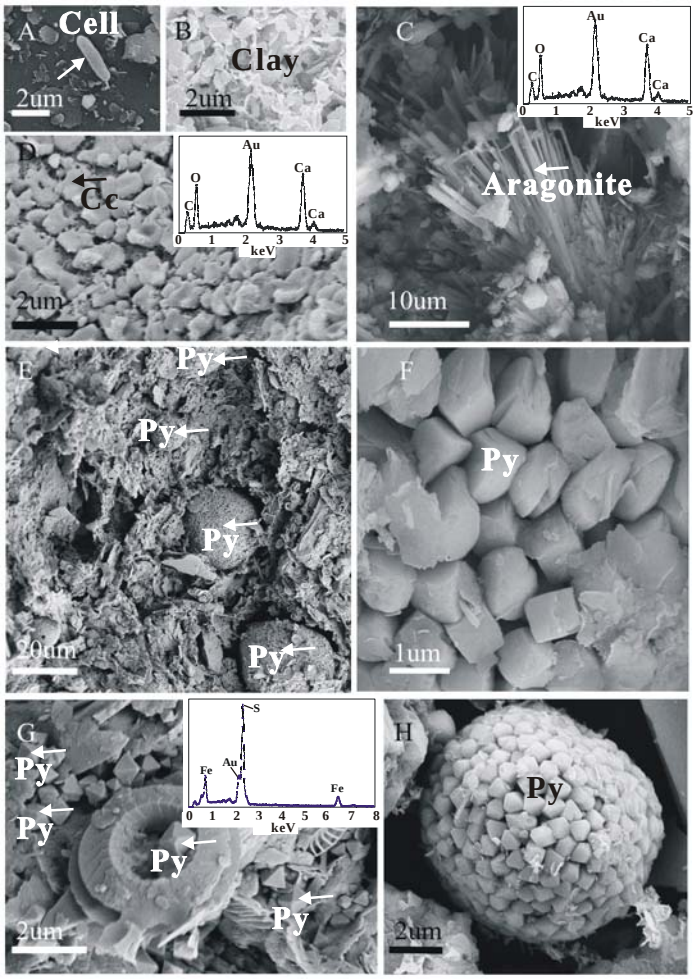

pH
7.5
8.0
8.5
TDS (total dissolved salt)
(g/L)
37
38
39
Sulfate (mM)
21
23
25
Cells per gram
10^6
10^7
10^8
Depth (cm bsf)
0
100
200
300
400
500
A
B
C
D
0.0
0.5
1.0
1.5
TOC (%)

Figure 3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Clay
2um
C
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Ca
Ca
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keV
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Cc
Au
O
Ca
Ca
0 1 2 3 4 5
keV
2um
E
Py
Py
Py
Py
20um
F
Py
1um
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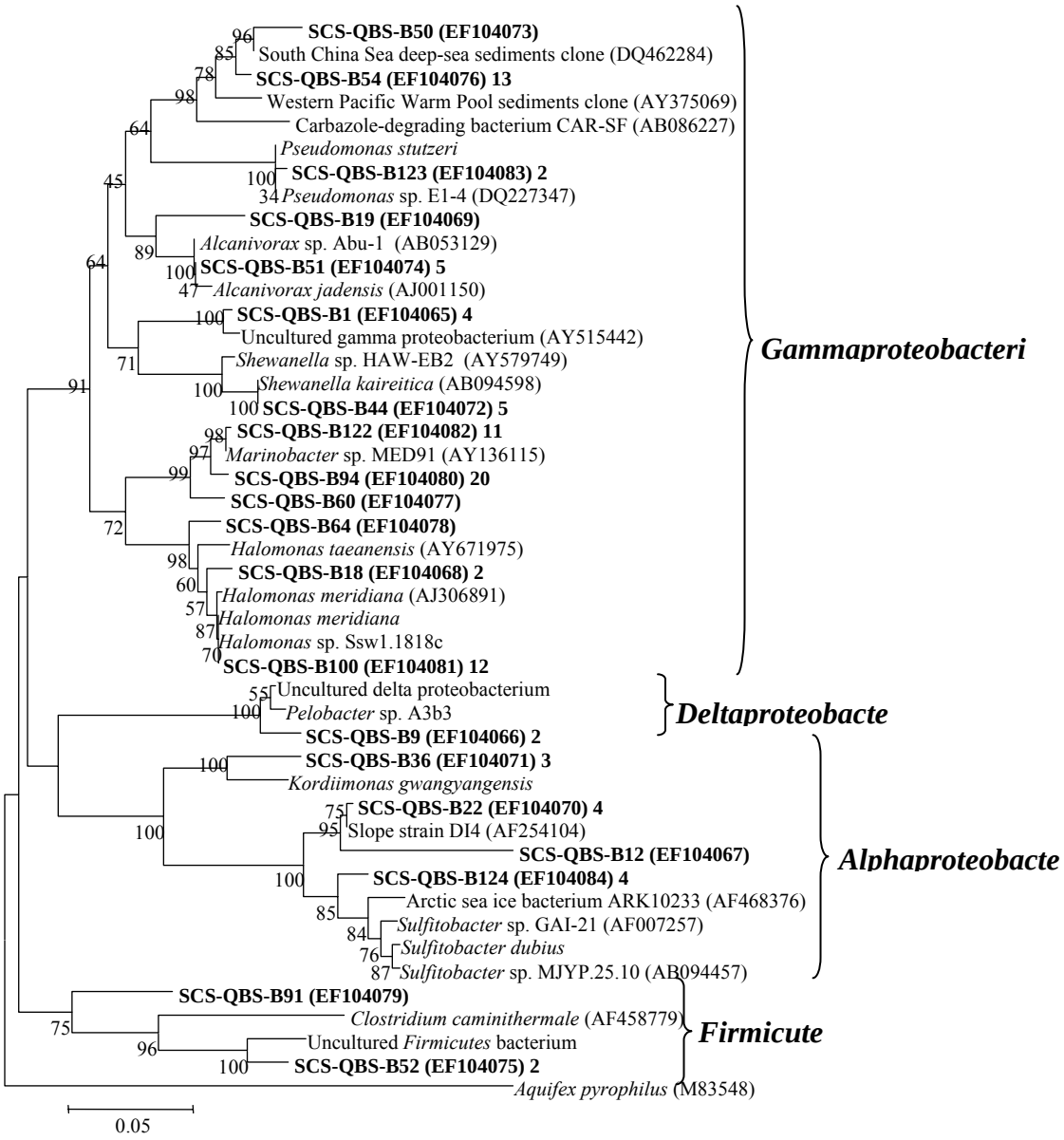
keV
2um
H
Py
2um

Fig. 4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SCS-QBS-B19 (EF104069)
Alcanivorax sp. Abu-1 (AB053129)
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Alcanivorax jadensis (AJ001150)
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Uncultured gamma proteobacterium (AY515442)
Shewanella sp. HAW-EB2 (AY579749)
Shewanella kaireitica (AB094598)
SCS-QBS-B44 (EF104072) 5
SCS-QBS-B122 (EF104082) 11
Marinobacter sp. MED91 (AY136115)
SCS-QBS-B94 (EF104080) 20
SCS-QBS-B60 (EF104077)
SCS-QBS-B64 (EF104078)
Halomonas taeanensis (AY671975)
SCS-QBS-B18 (EF104068) 2
Halomonas meridiana (AJ306891)
Halomonas meridiana
Halomonas sp. Ssw1.1818c
SCS-QBS-B100 (EF104081) 12
Gammaproteobacteri
Uncultured delta proteobacterium
Pelobacter sp. A3b3
Deltaproteobacte
SCS-QBS-B9 (EF104066) 2
SCS-QBS-B36 (EF104071) 3
Kordiimonas gwangyangensis
SCS-QBS-B22 (EF104070) 4
Slope strain DI4 (AF254104)
SCS-QBS-B12 (EF104067)
SCS-QBS-B124 (EF104084) 4
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Sulfitobacter sp. GAI-21 (AF007257)
Sulfitobacter dubius
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Clostridium caminithermale (AF458779)
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Aquifex pyrophilus (M83548)
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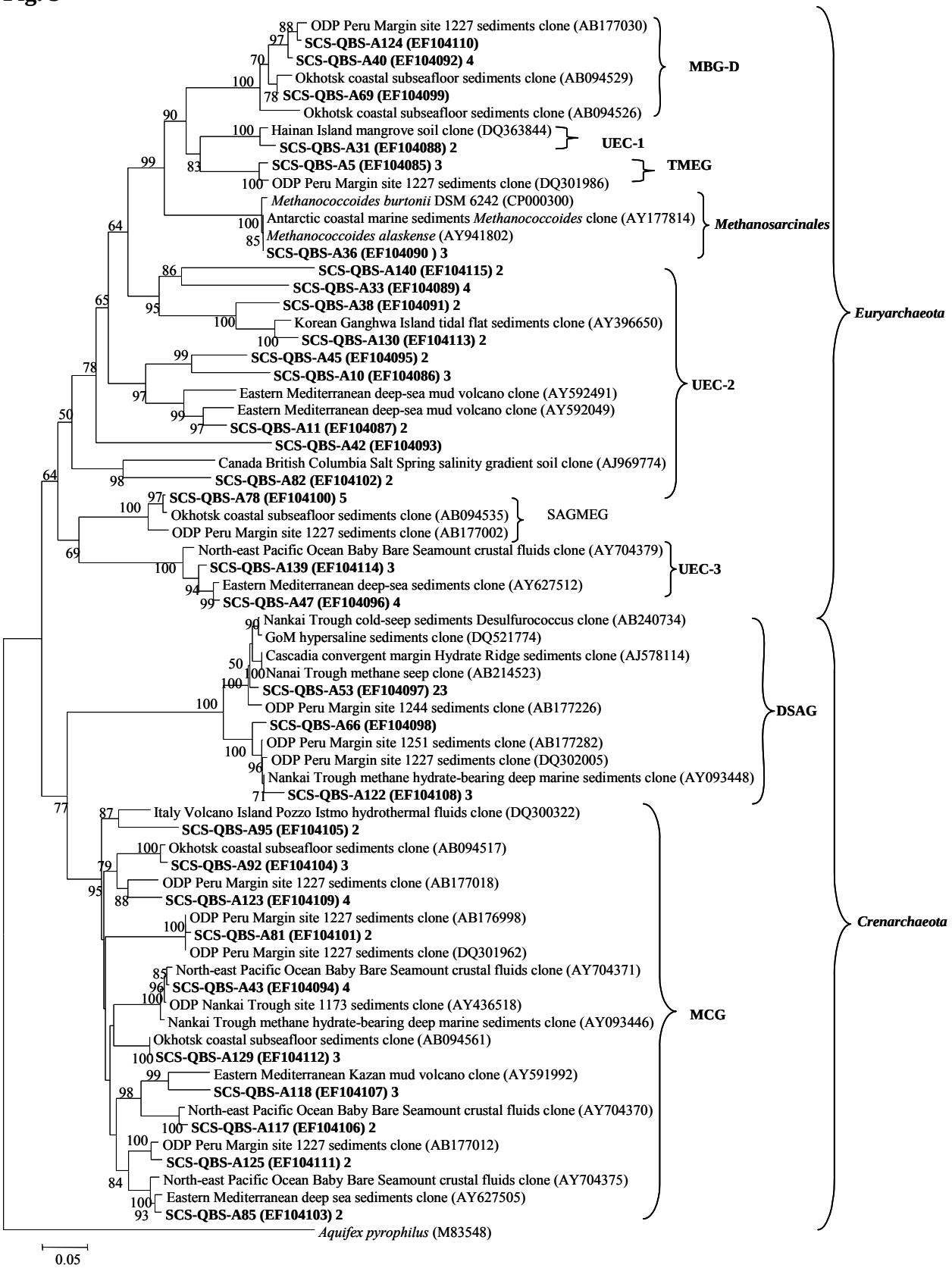
Fig. 5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Okhotsk coastal subseafloor sediments clone (AB094526)
MBG-D
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UEC-1
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TMEG
Methanococcoides burtonii DSM 6242 (CP000300)
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Methanococcoides alaskense (AY941802)
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Eastern Mediterranean deep-sea mud volcano clone (AY592049)
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UEC-2
SCS-QBS-A78 (EF104100) 5
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SAGMEG
North-east Pacific Ocean Baby Bare Seamount crustal fluids clone (AY704379)
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Eastern Mediterranean deep-sea sediments clone (AY627512)
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UEC-3
Euryarchaeota
Nankai Trough cold-seep sediments Desulfurococcus clone (AB240734)
GoM hypersaline sediments clone (DQ521774)
Cascadia convergent margin Hydrate Ridge sediments clone (AJ578114)
Nanai Trough methane seep clone (AB214523)
SCS-QBS-A53 (EF104097) 23
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SCS-QBS-A66 (EF104098)
ODP Peru Margin site 1251 sediments clone (AB177282)
ODP Peru Margin site 1227 sediments clone (DQ302005)
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DSAG
Italy Volcano Island Pozzo Istmo hydrothermal fluids clone (DQ300322)
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Okhotsk coastal subseafloor sediments clone (AB094517)
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Fig. 6

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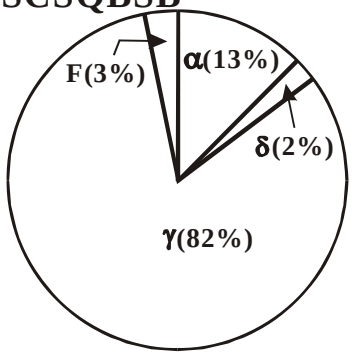

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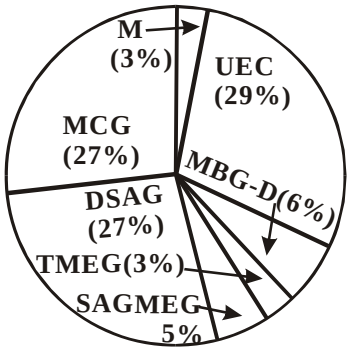


Fig. 7A
(chao-1)

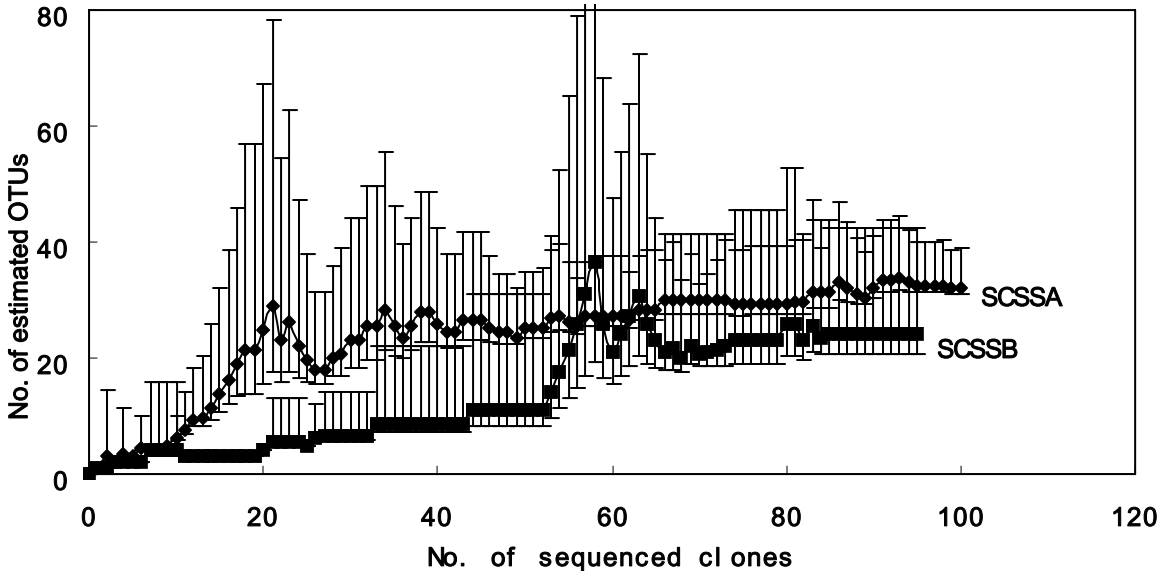


Fig. 7B
(ACE)

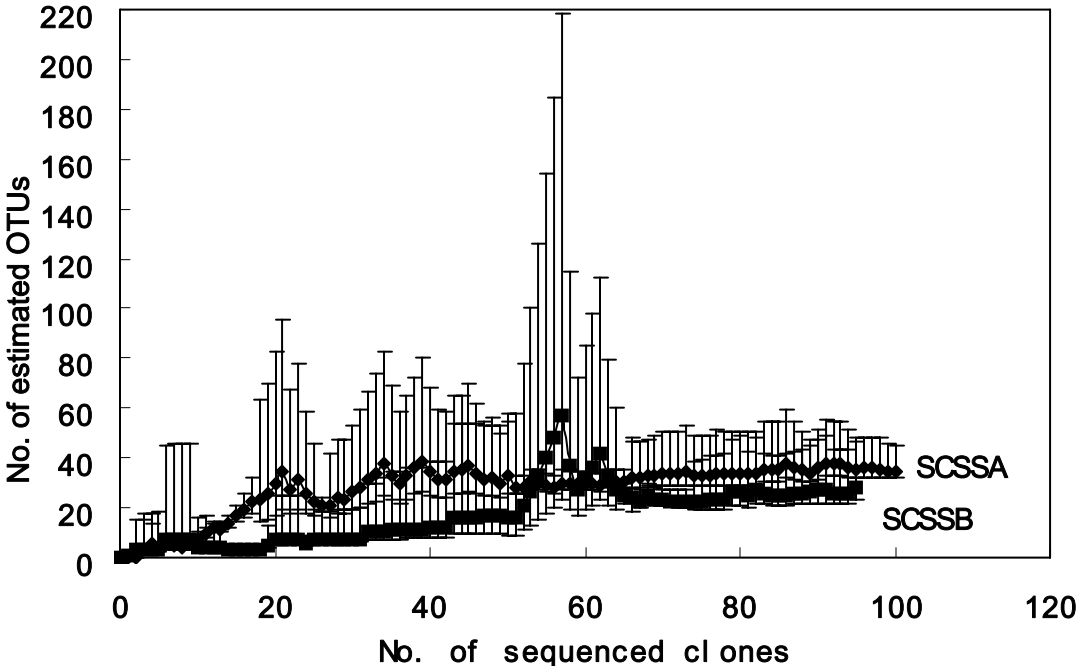
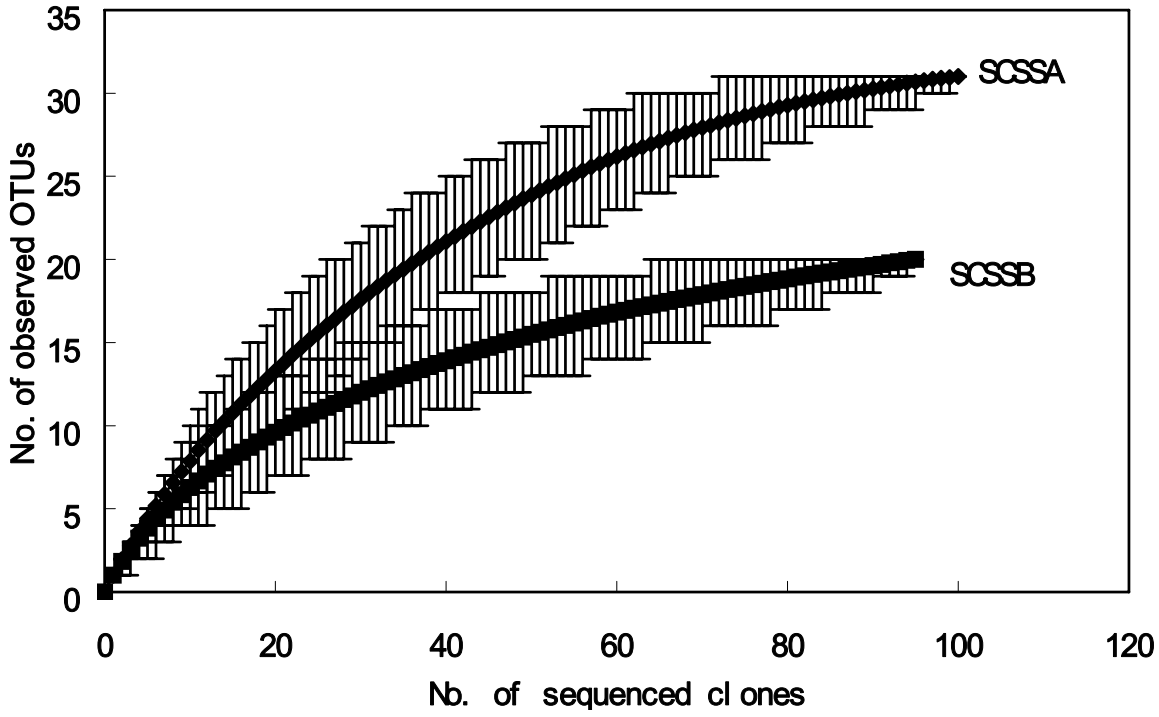


Fig. 7C
(Rarefaction curve)



CHAPTER 7:

SUMMARY

This study is the first comprehensive investigation into microbial communities in sediments of athalassohaline lakes on the Tibetan Plateau and conclusively demonstrates that the salinity plays a key role in controlling microbial communities (e.g. microbial abundance, composition and structure) in Lake Chaka. With the integration of culture-dependent and -independent techniques, microbial communities in the water and sediments of Lake Chaka were explored in depth. Because of their different requirements for salt, halophilic bacteria and archaea tend to occupy different salinity niches with the former being dominant at low salinity and the latter being dominant at high salinity. Archaeal cells (specifically, halophilic archaea) were predominant in saline sediments, whereas bacterial cells and the *Crenarchaeota* in freshwater and low-salinity muds and silts. Between two potentially controlling factors of sediment-associated microbial community, salinity appeared to predominate over mineralogy. Previously observed succession of *Proteobacteria* subgroups as a function of salinity observed in water columns (both oceans and lakes) does not seem to be applicable to sediment-associated microbial community.

The second important contribution of this dissertation study is that the community of ammonia-oxidizing archaea (AOA) (along with ammonia-oxidizing bacteria, AOB) was investigated for the first time in lake environments. Both AOA and AOB are present and active in Qinghai Lake, but not in the Chaka Lake (high salinity). In oxic water column, AOA are more abundant than AOB, but this trend is reversed in anoxic sediments. AOB are aerobic organisms, and their survival in anoxic sediments may indicate that they may have switched to low-level state (resting cells) or to the denitrification pathway by reducing nitrite or nitrogen dioxide with hydrogen, ammonium, or simple organic compounds as the electron donors. In addition, AOB appear to be more sensitive to salinity change than AOA.

The third significant contribution of this dissertation study is the finding of abundant presence of putative marine benthic groups of archaea in the inland Qinghai Lake. These groups were previously thought to be present in deep-sea sediments. However, our molecular work indicates that *Archaea* dominate the prokaryotic community in the Qinghai Lake sediments, within which members of putative marine benthic groups are abundantly present. The presence and dominance of so-called marine benthic groups in the Qinghai Lake sediments suggested that inland lakes may have some microbial groups in common with those in the oceans. These results suggest that environmental conditions (not geographical isolation) may control the microbial distribution patterns in aquatic environments. The metabolic functions of these Archaea are largely unknown. Although the 16S rRNA sequences of these groups are closely related to those recovered from methane-rich environments such as gas hydrates, our carbon isotope data do not support their functions as methanogens. Out limited molecular data (quantitative PCR) suggest that they may be ammonia oxidizers. If verified by further molecular work such as metagenomics and fosmid library, this would extend the environmental niches from soils/costal waters to deep sea and lakes.

The fourth contribution of this study is that the microbial community in the potential methane-hydrate bearing zones of the SCS was systematically characterized by phylogenetic analyses for the first time. The major groups within both *Archaea* and *Bacteria* in collected sediment samples were consistent with those previously detected in various marine environments, including methane/organic-rich and/or putative gas hydrate-bearing marine sediments. However, presence or absence of gas hydrates in the SCS sediments could not be determined with certainty from the view of microbial community

In spite of many achievements of this research, a few more questions remain open and warrant future research:

1) The relationship between environmental conditions and microbial abundance/diversity needs to be expanded to include more lakes from the region. In this research, only two lakes representing contrast geochemical conditions were studied. The Tibetan Plateau has thousands of lakes with multiple

environmental gradients (Zheng, 1995) and they are ideal candidates to study how microbial and geological processes are coupled.

2) This research reveals that ammonia-oxidation by Archaea and Bacteria is an important process. However, the relationship between geochemical conditions and microbial functions can be further studied. For example, we do not know how the abundance and diversity of AOA and AOB change in response to changes in salinity, pH, ammonia concentration, and other variables. What are nitrification rates and how AOA and AOB contribute to the overall nitrification activity in lakes of different environmental conditions?
3) Because of the presence of some prokaryotic groups (e.g. the putative marine benthic groups as discussed in Chapter 5) commonly present in both oceans and inland lakes, I infer that some other prokaryotes abundant in marine environments might be present in inland ecosystems as well, such as aerobic anoxygenic phototrophic bacteria (AAPB). AAPB were recently reported to be abundant in marine plankton. So far, however, there has been no molecular work characterizing the AAPB community in inland lake ecosytems. Is it possible for AAPB to be abundantly present in inland lake (such as Qinghai Lake) water columns? If yes, any difference in community composition and structure from marine environments?
4) Functional gene (encoding photosynthesis centers) sequences originated from aerobic anoxygenic phototrophic bacteria were primarily retrieved from both water column and sediments (not shown in this study). The AAPB-originated DNA in the sediments was of fossil origin since these bacteria require light for growth, which is absent in the dark sediments. This indicates that low temperature and anoxic conditions in the Qinghai Lake sediments, and adsorption to sediment particles may have effectively protected fossil DNA from degradation. Fossil DNA of obligate phototrophic algae as well as their traditional lipid derivatives have been studied for implications in many fields including paleoecology, archaeology, and paleontology. However, no study so far has been performed on fossil DNA derived from obligate phototrophic bacteria. Thus, such AAPB-originated fossil DNA may be another biomarker to

characterize the phototrophic bacterial community, water temperature and other paleoecological parameters (Holocene and older) of Qinghai Lake, and possibly of other lakes on the Tibetan Plateau.

5) Some relics (fragments) of algae (coccolithophorids) were observed under SEM in sediments from both Qinghai Lake and the South China Sea (not shown in this study). These algae are also obligate phototrophs. Fossil DNA of such algae and their characteristic lipid molecules (e.g. alkenones and alkenoates, specific biomarkers of haptophytes) have been employed in other environments (e.g. Holocene sediments of the saline, meromictic Mahoney Lake and eastern Mediterranean) by Coolen and his colleagues (Coolen and Overmann, 1998; Coolen et al., 2004), successfully disclosing the microbial diversity and water geochemistry (temperature, salinity, etc.) evolution in the Holocene water columns. So it is worthwhile to evaluate such haptophyte fossil DNA and their characteristic lipids in both Qinghai Lake and the South China Sea.

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APPENDIX:

Archaeal lipids and 16S rRNA genes characterizing gas hydrate-impacted sediments in the Gulf of Mexico

Running title: Archaea associated with gas hydrates in the Gulf of Mexico

Yundan Pi,[1†‡] Qi Ye,[2†] Hongchen Jiang,[3†] Ann Pearson,[4] Shuguang Li,[1] John Noakes,[5] Randy Culp,[5] Hailiang Dong,[3] Chuanlun Zhang[2*]

1: School of Earth and Space Sciences
the University of Sciences and Technology of China, China

2: Department of Marine Sciences and Savannah River Ecology Laboratory
University of Georgia, USA

3: Department of Geology
Miami University, USA

4:Department of Earth and Planetary Sciences
Harvard University, USA

5:Center for Applied Isotope Studies
University of Georgia, USA

[†]These authors contributed equally.
[‡] Current address: Department of Earth and Planetary Sciences, Harvard University, USA

* Corresponding Author:
Chuanlun Zhang,
Tel: 803-725-5299,
Fax: 803-725-3309,
E-mail: Zhang@srel.edu

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Abstract

The abundance and community structure of archaea may be significantly altered in marine sediments when impacted by gas hydrates or cold seeps. We studied the intact lipids and the phylogenetic compositions of archaea from marine sediments adjacent to or within a region of methane seeps and hydrate mounds in the Mississippi Canyon Block 118 in the Gulf of Mexico. Our primary goal was to use lipid biomarkers to examine changes in community structure of archaea in normal marine versus hydrate-impacted sediments. Glycerol dialkyl glycerol tetraethers (GDGTs) showed distinct patterns between hydrate and non-hydrate samples, suggesting variation in archaeal communities caused by the presence or absence of gas hydrates. In particular, GDGT-1 to GDGT-3 having one to three cyclopentyl rings, respectively, were significantly enhanced in the hydrate or methane-rich samples. Deviation of lipid profiles from normal marine sediments was verified and 16S rRNA genes were sequenced to provide a phylogenetic explanation of the archaeal populations possibly causing the deviation. Clone libraries from the hydrate-associated samples showed the predominance of ANME-1 subgroups, which are known to produce tetraether lipids and may be responsible for the enhanced archaeal lipids in the hydrate samples. Our study demonstrated the consistency between phenotypic and phylogenetic properties of archaea, which responded to the impact of gas hydrates in the marine environment.

Introduction

The domain *Archaea* contains the major phyla *Crenarchaeota* and *Euryarchaeota*. *Crenarchaeota* inhabit not only extreme environments such as sulfidic, hot, salty or anoxic waters, but also are widespread in other locations such as lakes, open seas and sediments (*e.g.*, DeLong, 1992; DeLong *et al.*, 1994; Fuhrman *et al.*, 1992; Hershberger, 1996; MacGregor *et al.*, 1997; Pearson *et al.*, 2004; Schleper *et al.*, 2005; Dong *et al.*, 2006; Zhang *et al.*, 2006). These non-thermophilic crenarchaeota may account for one third of the planktonic prokaryotes in the open ocean (*e.g.*, Karner *et al.*, 2001). Mounting evidence indicates that many non-thermophilic crenarchaeota use ammonia as a major energy source for autotrophic growth (Francis *et al.*, 2005; Könneke *et al.*, 2005; Treusch

et al., 2005; Leininger *et al.*, 2006; Nicol & Schleper, 2006; Wuchter *et al.*, 2006). Other molecular and geochemical studies show that nonthermophilic crenarchaeota can take up amino acids and organic carbon, as well as inorganic carbon, indicating some capacity for heterotrophy or mixotrophy (Ouverney & Fuhrman, 1999, 2000; Herndl *et al.*, 2005; Ingalls *et al.*, 2006; Teira *et al.*, 2006).

Euryarchaeota include important groups of microorganisms that mediate methane production (methanogens) and methane oxidation (ANME groups). In particular, anaerobic oxidation of methane (AOM) consumes about 90% of methane produced in marine sediments (Valentine & Reeburgh, 2000) and plays a critical role in controlling flux of methane into the atmosphere. While numerous species of methanogens have been identified (*e.g.*, Boone & Castenholz, 2001), none of the ANME groups has been brought into pure culture. However, geochemical and genomic evidence indicates that some, if not all, of the ANME groups oxidize methane using reverse methanogenesis working in syntrophy with sulfate-reducing bacteria (*e.g.*, Hoehler *et al.*, 1994; Boetius *et al.*, 2000; Orphan *et al.*, 2001a,b; Michaelis *et al.*, 2002; Valentine, 2002; Hallam *et al.*, 2004; Nauhaus *et al.*, 2005).

Crenarchaeota and *Euryarchaeota* also have distinct lipids. Glycerol dialkyl glycerol tetraethers (GDGTs) are the core lipids of *Crenarchaeota* (Koga *et al.*, 1993). *Euryarchaeota* (*e.g.*, methanogens and halophiles), on the other hand, commonly are characterized by isoprenoidal diethers such as archaeol and *sn*-2-hydroxyarchaeol (Koga *et al.*, 1998; Hinrichs *et al.*, 1999; Pancost & Damsté, 2003). However, some of the *Euryarchaeota* such as *Thermococcus* and *Thermoplasmatales* also contain tetraethers as core lipids (Uda *et al.*, 2001; Macalady *et al.*, 2004).

Substantial evidence indicates that the GDGTs found in normal marine sediments predominantly originate from planktonic and/or benthic crenarchaeota (*e.g.*, DeLong *et al.*, 1998; Biddle *et al.*, 2006). The GDGTs from cold areas such as the Antarctic almost exclusively are composed of only crenarchaeol and GDGT-0; whereas in warmer areas such as the Arabian Sea, the GDGT assemblages also contain appreciable amounts of GDGT-1, GDGT-2, GDGT-3 and crenarchaeol-isomer (Schouten *et al.*, 2002). On the other hand, available information from gas hydrate or cold hydrocarbon seep

environments indicates that the ANME-1 group (but not ANME-2) may also produce GDGT-0 to GDGT-3 as core lipids (Aloisi *et al.*, 2002; Blumenberg *et al.*, 2004).

The Gulf of Mexico is an informative place to study biological effects on the carbon cycle, because dynamic microbial communities exist in association with gas hydrates or cold seeps/mud volcanoes in the marine sediments (Lanoil *et al.*, 2001; Mills *et al.*, 2003, 2005; Zhang *et al.*, 2002, 2003, 2005; Joye *et al.*, 2004; Orcutt *et al.*, 2004, 2005; Zhang & Lanoil, 2004; Lloyd *et al.*, 2006; Martinez *et al.*, 2006; Reed *et al.*; 2006; Yan *et al.*, 2006). One unique feature of the Gulf of Mexico in comparison with other hydrate locations is that gas hydrates formed there contain methane not only from microbial methanogenesis but also from thermogenic break down of petroleum hydrocarbons in the deep subsurface (Sassen et al., 2001, 2004). The abundance of archaea is enhanced significantly in the hydrate environment and these organisms may play an important role governing exchange of carbon between sediments and the deep ocean (Zhang *et al.*, 2003; Pancost *et al.*, 2005). Here we present an integration of lipid profiles and DNA sequence data to achieve a better understanding of archaeal community biogeochemistry, in association with gas hydrate-impacted sediments in the Gulf of Mexico.

Materials and methods

Sample collection and description

Gravity cores of 3-m or 10-m length (7 cm diameter) were collected in the Mississippi Canyon (MC) Block 118 in May 2005, onboard the R/V Pelican. MC 118 comprises a portion of the continental slope with water depths ranging from 800 to 990 m (Fig. 1) (Woolsey *et al.*, 2005). About 12 cores were taken near or on top of an elevated sedimentary topography within MC 118 (approximately 1 km^2 in area; Fig. 1), which is manifested in a mosaic of methane seeps, hydrate mounds, communities of macrofauna, *Beggiatoa* mats, and mounds of authigenic carbonate mounds (Sassen et al., 2006). All samples for this study were from the 10-m long cores (cores 1, 8 and 9). Core 1 was in normal marine sediment and cores 8 and 9 were on top of the "hydrate mound" (Fig. 1). A core (NBP) from the normal marine sediment at Grand Canyon Block 233 (Li *et al.*, 2007) was also included in this study.

Samples were examined visually onboard for evidence of gas hydrates before being sub-sampled for analyses of lipids and DNA. The lithology was then characterized in detail in the laboratory at the University of Southern Mississippi. In general, all cores were dominated by mud (particle size < 0.063 mm) and contained less than 10% sand (particle size ranging from 2-0.063 mm) in most intervals (Brunner, personal communication). In core 1, the mud was olive or dark olive in color. A thin oxidized layer (~5 cm) occurred around 300 cm and 400 cm. In core 9, the mud was gray and contained carbonate nodules or pyrite shells in the top 150 cm section. Gas expansion and oil staining were noticeable in core 9 but absent in core 1, suggesting possible dispersion of hydrate in core 9. Lithological description was not available for core 8 but the lithology in this core may be similar to that in core 9 because they are both on top of the elevated topography (Fig. 1).

About 50 grams of sediments were collected at different depth intervals (5-486 cm below sediment surface). The samples were immediately stored at -20°C onboard and stored at -80°C in the laboratory until analysis.

Porewater sulfate and sulfide

Porewater chemistry was performed on core 1 and core 9. The frozen samples were thawed to room temperature in an anaerobic chamber to minimize the oxidation of reduced species such as hydrogen sulfide. Porewater was collected by centrifugation (5000 x *g*) using 10-15 grams of wet sample and immediately analyzed for sulfate and hydrogen sulfide using Hach kits (Zhang et al., 2007) according to manufacturer's instructions (Hach Company, Loveland, CO).

Lipid analysis

About five grams of a lyophilized sample were used for extraction of total lipids using a single-phase organic solvent system comprised of chloroform, methanol, and aqueous 50-mM phosphate buffer (pH 7.4) in the ratio of 1:2:0.8 (v:v:v; Zhang *et al.*, 2006). After about 12 hours, equal volumes of chloroform and nano-pure water were added, forming a two-phase system. The organic phase was collected and reduced in volume under pure N_2 gas.

Total lipids were transesterified in 2 ml of methanol and hydrochloric acid (95:5; v/v) in a heating block at 70°C for two hours to hydrolyze polar side-chains of GDGTs. After cooling to room temperature, 1 ml of solvent-extracted nano-pure water and 2 ml of CH_2Cl_2 were added. The transesterified lipids were passed through a C-18 solid phase extraction (SPE) column. The GDGT fraction was eluted with 1:3 ethyl acetate:hexane and was dissolved in 1.4% isopropanol in hexane.

GDGTs were identified using an Agilent 1100 series high performance liquid chromatograph (HPLC) with atmospheric pressure chemical ionization-MS using a Zorbax NH_2 column (2.1 x 150 mm, 5 μm particle size) and/or a Prevail CN column (2.1 x 150 mm, 3 μm particle size) at 30°C (Hopmans *et al.*, 2004; Pearson *et al.*, 2004). Conditions for atmospheric pressure chemical ionization-MS were nebulizer pressure of 60 lb/in^2, drying gas flow of 6.0 liters/min and 350°C, vaporizer temperature of 375°C, voltage of 3 kV, and corona of 5 μA. Spectra were scanned over the *m/z* range from 1,250 to 1,350.

Archaeal 16S rRNA gene analysis

Three archaeal clone libraries were constructed using samples from MC 118 core 9 (GoMT: 5-15 cm; GoMM: 15-30 cm; GoMD: 30-35 cm) to provide phylogenetic information for the unusual GDGT profiles detected in this core. Genomic DNA was extracted from 7.5-g frozen sediment using the Ultraclean Mega Prep Soil DNA kit (MoBio Laboratories, Inc, Solana Beach, CA). The precipitated DNA was purified by gel electrophoresis plus mini-column preparation (Wizard DNA Clean-Up System, Promega, Madison, WI). Archaeal 16S rRNA gene fragments (approximately 930 bp) were amplified in a Perkin-Elmer 9700 Thermal Cycler with the primer pair Arch-21F (5'-TTC CGG TTG ATC CYG CCG GA-3') and Arch-958R (5'-YCC GGC GTT GAM TCC AAT T-3') (DeLong, 1992) according to the procedure of the FailSafe PCR system reaction mix G (Epicenter Biotechnologies, Madison, WI). To minimize sample biases and to obtain enough amplicon for cloning, the number of cycles in a PCR reaction was limited to 30 and three replicated amplifications were carried out for each sample. The combined PCR products were purified from a low-melting point agarose gel (1.0%) using the QIA Quick Gel Extraction Kit (QIAGEN, Inc., Valencia, CA). The purified DNA was ligated

with pCR 2.1vector and competent *Escherichia coli* cells were transformed according to manufacturer's instructions (Topo-TA cloning kit, Invitrogen, Carlsbad, CA). Forty to 50 colonies from each sample were randomly chosen for sequencing. Plasmid DNA containing inserts were prepared using a QIAprep Spin miniprep kit (QIAGEN, Valencia, CA). Sequencing reactions were carried out using ABI BigDye Terminatior v.3.1 (Applied Biosystems. Foster City, CA) and sequenced on an ABI 3100 automated sequencer. Clone sequences were manually checked for chimeras using Ribosomal Database Project II (http://wdcm.nig.ac.jp/RDP/html/index.html) and identified chimeric sequences were removed. Phylogenetic analysis was done according to Jiang *et al.* (2006). Sequences with identities of greater than 97% were considered to represent the same operational taxonomic unit (OTU) (Humayoun *et al.*, 2003). Coverage (C) was calculated as: $C = 1-(n_1/N)$, 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 (Mullins *et al.*, 1995). Rarefaction analyses were performed using DOTUR (Schloss & Handelsman, 2005). The program LIBSHUFF (http://whitman.myweb.uga.edu/libshuff.html) was performed to examine the difference in archaeal community structure between different layers of the sediments.

Nucleotide sequence accession numbers

The unique sequences determined in this study have been deposited in the GenBank database under accession numbers EF116459- EF116485.

Results

Concentrations of porewater sulfate and hydrogen sulfide

In core 1, sulfate concentrations ranged from 24.6 mM to 35 mM. In core 9, sulfate concentrations were below 0.3 mM. In contrast, sulfide concentrations were less than 2.5 μM in core 1 but 27.8 to 979.4 μM in core 9 (Table 1). However, it was possible that pore-water sulfide had undergone partial sulfide oxidation during exposure of sediment core on board the ship and during laboratory storage because of its sensitivity to oxygen in contact with the sample. Thus, measurements of sulfide may be regarded as potential

underestimates of actual concentrations. Nevertheless, the results clearly indicated that extensive sulfate reduction occurred in core 9. This is consistent with the presence of carbonate nodules or pyrite shells observed in core 9 but not in core 1. The abundances of carbonate and pyrite minerals are both enhanced during sulfate reduction coupled to the oxidation of methane and/or other hydrocarbons (Formolo et al., 2004).

Archaeal lipids

Twenty-four samples were analyzed for GDGTs. Representative GDGT profiles from GC 233 NBP and MC 118 Cores 1, 8, and 9 are shown in Fig. 2. Archaeal lipids from GC 233 NBP and MC 118 core 1 are characterized by high relative abundance of crenarchaeol (peak I) and GDGT-0 (peak II) (profiles a and b, Fig. 2), which are typical of normal marine sediments (*e.g.*, Schouten et al., 2002). Archaeal lipids from the hydrate-affected samples, however, are characterized by relative decreases in crenarchaeol and GDGT-0 and increases in GDGT-1 and GDGT-2 (peaks III and IV); GDGT-3 (peak V) increases to a lesser extent in hydrate-affected samples (Table 2; profiles c and d, Fig. 2).

In all three horizons from GC233 NBP, the crenarchaeol accounted for 42-43% and the GDGT-0 30-33% of total GDGTs (Table 2). The remaining GDGTs accounted for less than 12% for each compound and decreased in abundance in the order of GDGT-2 (10-11%), GDGT-1 (8-10%), the crenarchaeol isomer (4-5%), and GDGT-3 (1-2%) (Table 2). GDGTs from MC 118 core 1 had similar distributions to those from GC 233 NBP. In particular, no significant differences were observed in the relative abundances of crenarchaeol, GDGT-0, GDGT-1 and the crenarchaeol isomer between the two locations in the 0-30 depth range (Table 2).

In contrast, GDGTs from MC 118 cores 8 and 9 had distinct patterns with depth. In core 8, crenarchaeol and GDGT-0 were relatively less abundant below 100 cm (10-17% and 26%, respectively) than above 30 cm (28-43% and 30-34%, respectively; Table 2); while GDGT-1 and GDGT-2 were significantly enriched below 100 cm (20-23% and 31-33%, respectively) compared to shallower depths (10-12% and 11-21%, respectively). Similarly, GDGT-3 was also more abundant in the lower (below 100 cm) section (5-7%) than in the upper (above 30 cm) section (3%). Overall, in core 8, crenarchaeol and its

isomer decreased with depth; whereas, GDGT-1, GDGT-2, and GDGT-3 increased gradually with depth (Table 2).

Core 9 showed a nearly opposite pattern of distributional change in GDGT composition as a function of depth. A boundary appeared to exist between 35 and 100 cm, where below 100 cm, the GDGT profiles were similar to those in GC 233 NBP and MC 118 core 1 (34-43% and 26-36%, for crenarchaeol and GDGT-0, respectively); whereas above 35 cm, crenarchaeol and GDGT-0 were relatively much less abundant (13-18% and 14-16%). The crenarchaeol isomer also is slightly less abundant in the upper (above 35 cm) section (Table 2). The most abundant GDGTs in the shallow horizons, interestingly, are GDGT-2 and GDGT-1 (39-46% and 16-18%; Table 2).

Diversity of archaeal 16S rRNA genes

A total of 42-44 clones were sequenced from each of the top three intervals (5-15 cm, 15-30 cm, 30-35 cm) of core 9 in order to provide phylogenetic information about the archaeal communities that may be responsible for the unusual profiles of GDGTs in this core. The number of clones represented 82.5-95.0% of coverage of each clone library (Table 3) and along with rarefaction analysis (data not shown) indicated that sequences in each library reached or was close to saturation. Statistical analysis of the three 16S rRNA archaeal libraries using the LIBSHUFF program showed the archaeal community structure in the bottom sample (30-35 cm) was significantly different ($P \leq 0.005$) from that in the top (5-15 cm) or the middle (15-30 cm) sample. The community structure, however, is similar between the top and middle samples ($P > 0.025$).

The overall archaeal 16S rRNA gene sequences were dominated by *Euryarchaeota*, which in turn were dominated by the ANME-1a subgroup (61-71% of the total clones at each depth; Table 3). The 15-30 cm interval also included a couple of representatives from the ANME-2a subgroup and the terrestrial miscellaneous euryarchaeotal group (TMEG); the 5-15 cm and 30-35 cm intervals had a few representatives of the ANME2-c subgroup (Table 3). On the other hand, a large proportion (23-24%) of *Thermoplasmatales* was found in the 5-15 cm and the 15-30 cm intervals but not in the 30-35 cm interval (Table 3).

The number of crenarchaeotal 16S rRNA gene sequences was limited at each interval analyzed (Table 3). The 5-15 cm interval had five clones (11%) belonging to the marine benthic group B (MBG-B); while the 30-35 cm interval had 3 clones (7%) belonging to the miscellaneous crenarchaeotal group (MCG) and 4 clones (9%) belonging to MBG-B. No crenarchaeota were detected in the 15-30 cm interval.

Figures 3 shows the phylogenetic relationships of these euryarchaeotal and crenarchaeotal sequences. All euryarchaeotal sequences were closely related (~96-99%) to clones retrieved from methane hydrate-bearing or methane-rich habitats, including the Black Sea, California continental margin, Eel River, Cascadia Margin, Hydrate Ridge, Guaymas Basin, Gulf of Mexico, Nankai Trough, Peru Margin, and R/V Cape Hatteras Blake Ridge (Fig. 3). Within crenarchaeota, the MCG sequences were closely related to those from Okhotsk coastal subseafloor sediment and the Gulf of Mexico, and the MBG-B sequences were closely related to those from Peru Margin sediments, Cascadia Margin sediments associated with gas hydrates, or other locations in the Gulf of Mexico. However, none of our crenarchaeotal sequences was closely related to marine group I of the planktonic crenarchaeota (*e.g.*, DeLong, 1998), suggesting the 16S rRNA gene sequence data reflect sampling of the *in-situ* sedimentary community.

Discussion

Archaeal lipids associated with gas-hydrate hosting sediments

The GDGT profiles from the Gulf of Mexico cores GC 233 NBP and MC 118 core 1 (Fig. 2a,b and Table 2) are similar to those observed in other warmer areas such as the Arabian Sea and Cariaco Basin (*e.g.*, Schouten *et al.*, 2002; Zhang *et al.*, 2006). These observations are consistent with the generally-accepted interpretation that GDGTs in normal marine sediments mostly are contributed by the settling and sedimentation of planktonic crenarchaeota from the water column (Schouten et al., 2000). Although separated by over 300 km in distance, the similarity in GDGT profiles between GC 233 NBP and MC 118 core 1 suggests that the planktonic crenarchaeota in this region are homogeneously distributed in the upper water column.

In contrast, the unusually high relative abundances of GDGT-1, GDGT-2, and GDGT-3 in MC 118 cores 8 and 9 on top of the hydrate mound suggest significantly enhanced biomass of methane-oxidizing archaea (Table 2). Qualitatively, clone libraries of archaeal 16S rRNA genes suggest that euryarchaeaota responsible for these enhanced concentrations of GDGT-2 may be dominated by the ANME-1 group (Fig. 3; Blumenberg *et al.*, 2004). Some of the anomalous GDGTs also may come from the *Thermopasmatales,* particularly in the 5-15 cm and 15-30 cm intervals in which sequences of this group were found (Fig. 3); however, the physiology of the non-thermophilic *Thermoplasmatales* is unknown and caution needs to be excised when evaluating the contribution of these organisms to GDGTs in the marine environments. On the other hand, the enhanced biomass of the ANME-1 methane-oxidizing archaea can be reasonably linked to syntrophic sulfate-reducing bacteria. This AOM process commonly is observed near gas hydrates or in cold seep environments (*e.g.*, Boetius *et al.*, 2000; Boetius & Suess, 2004; Orphan *et al.*, 2002; Orcutt *et al.*, 2004; Lloyd *et al.*, 2006). Indeed, sulfate in core 9 was significantly depleted (Table 1); whereas sulfate in core 1 (Table 1) and GC 233 NBP (Formolo *et al.*, unpublished data) showed no appreciable decrease with depth. Other methane-oxidizing archaea, in particular the identified ANME-2 group (Fig. 3), also may have contributed to the observed sulfate reduction in core 9. It must be noted that sulfate reduction can also be coupled to oxidation of petroleum hydrocarbons and the process of AOM can sometimes contribute only a small fraction of the total observed sulfate reduction (Formolo et al., 2004; Orcutt et al., 2005; Lloyd et al., 2006). Thus, a precise correlation cannot be made between sulfate reduction rates, methane oxidation, and quantification of GDGTs putatively associated only with ANME-1 archaea.

Impact of gas hydrates on phylogenetic distributions of archaea in marine sediments

Extensive research has demonstrated that normal marine sediments are dominated by non-thermophilic crenarchaeota and non-ANME groups of euryarchaeota whereas shallow sediments affected by cold seeps and gas hydrates are dominated by ANME-groups of euryarchaeota (*e.g.*, Inagaki *et al*., 2003; Lanoil *et al.*, 2005; Wang *et al.*, 2005; Biddle *et al.*, 2006; Inagaki *et al.*, 2006; Sorensen & Teske, 2006; Webster *et al.*, 2006;

Kendall *et al.*, 2007). In particular, marine benthic group B and *Thermoplasmatales* appear to be cosmopolitan in normal marine sediments (Orphan *et al.*, 2001a; Teske, 2006). In our samples, crenarchaeota dominate the normal marine sediments in the Gulf of Mexico as observed using denaturing gradient gel electropherisis (Huang et al., manuscript in preparation).

AOM communities are ubiquitous in sediments of cold seeps and gas hydrates, although the relative distribution of ANME-1 and ANME-2 varies between locations. For example, ANME-1 is predominant in the Black Sea, whereas ANME-2 is predominant at Hydrate Ridge (Knittel *et al.*, 2005; Nauhaus *et al.*, 2005). In the Gulf of Mexico, both ANME-1 and ANME-2 are conspicuous (Fig. 3). In GC 185 and GC 234 (Mills *et al.*, 2003, 2005) both ANME-1 and ANME-2 appeared to be widely distributed (Table 4). However, in GB 425 (Lanoil *et al.*, 2005) and the Florida Escarpment (Reed *et al.*, 2006), ANME-2 is predominant; whereas, in GC 205 (Lloyd *et al.*, 2006) and MC 118 (this study), ANME-1 is predominant (Table 4). In particular, Lloyd *et al.* (2006) observed that ANME-1 at GC 205 was represented by a single sub-group (ANME-1b).

A number of factors have been proposed to affect the distribution of ANME-1 and ANME-2, which include concentration of methane (Blumenberg *et al.*, 2004, 2005; Girguis *et al.*, 2005), sensitivity to oxygen (Knittel *et al.*, 2005), salinity (Lloyd *et al.*, 2006), and temperature (Nauhaus *et al.*, 2005). For example, ANME-2 is less sensitive to oxygen and thus predominant in the Hydrate Ridge sediments are overlain by oxic seawater, while ANME-1 is more sensitive to oxygen and thus predominant in the anoxic Black Sea (Knittel *et al.*, 2005). The difference, however, also could be explained by temperature-dependent variations in rates of sulfate reduction in association with AOM. In the Black Sea, sulfate reduction rates increase with increasing temperature, whereas in Hydrate Ridge, sulfate reduction rates decrease with increasing temperature (see Figure 1 of Nauhaus et al., 2005). It must be noted, however, rates of sulfate reduction were overall 10-30 times higher in the Black Sea than in the Hydrate Ridge (Nauhaus et al., 2005); thus the effect of temperature on rate of sulfate reduction must be viewed in the context of local habitat. It is possible that both oxygen and temperature affect the distribution of ANME groups. The effect of methane is best demonstrated in well-controlled microcosms, which show that ANME-1 out-competes ANME-2 under high

methane-flow conditions (Girguis *et al.*, 2005). In natural environments, however, methane flux may vary considerably spatially and temporally and both ANME-1 and ANME-2 may be able to live on a wide range of methane concentrations (Lloyd *et al.*, 2006). An effect of salinity also has been proposed, based on the observation that high salinity (2,200 mM) is associated with the ANME-1b subgroup at GC 205, where all other ANME subgroups did not occur, suggesting that ANME-1b may have adapted to this extreme environment (Lloyd *et al.*, 2006).

Samples from core 9 at MC 118 represent normal salinity and the bottom water temperature is about 4.6-5.7ºC (Higley, Sassen, Sleeper, personal communications). Methane concentration in the pore-water of core 9 was not measured thus the effect of methane on ANME group distribution cannot be evaluated. While salinity does not seem to be a major factor affecting the community structure of methane-oxidizing archaea at MC 118 (see Lloyd *et al.*, 2006), the lower-end of temperature range (4.6ºC) at this site is closer to that observed in the Black Sea and it is tempting to suggest that the enhancement of ANME-1 group is related to the cold temperature. It is also intriguing to observe that GC 205 and MC 118 have similar water depths (876 m and 890 m, respectively) and similar water temperature (5.8ºC at GC 205; Lloyd *et al.*, 2006) and both sites are dominated by ANME-1 (Table 3), thus further suggesting the role of temperature in affecting ANME-1. Other unknown variables may also affect the distribution of ANME groups associated with gas hydrates.

In summary, glycerol dialkyl glycerol tetraethers showed distinct patterns between non-hydrate and hydrate-hosting cores at MC 118. Non-hydrate samples were dominated by GDGT-0 and crenarchaeol, which normally accounted for greater than 70% of total GDGTs. Methane-influenced samples had significantly higher ratios of GDGT-1, GDGT-2 and GDGT-3, with correspondingly decreasing relative amounts of crenarchaeol. Cloning of the archaeal 16S rRNA genes from the hydrate samples indicated the predominance of ANME-1 subgroups, which may have contributed to the enhanced production of GDGT-1 to GDGT-3. Our results demonstrated the effectiveness of integrating lipid biomarkers and molecular DNA in characterizing archaeal community structures impacted by gas hydrate-hosting or methane-rich sediments.

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Table 1. Pore-water chemistry of sulfate and hydrogen sulfide for core 1 and core 9 at MC 118.

Sample	$SO_4^{=}$ (mM)	$\sum H_2S$ (μM)
Core 1_5-10 cm	25.0	0.6
Core 1_15-20 cm	26.0	0.6
Core 1_25-30 cm	35.0	0.9
Core 1_100-105 cm	28.6	0.6
Core 1_184-189 cm	30.4	2.2
Core 1_200-205 cm	24.6	0.2
Core 9_15-30 cm	0.2	979.4
Core 9_167-182 cm	0.1	461.8
Core 9_476-486 cm	0.0	27.8

Table 2. Relative abundance of glycerol dialkyl glycerol tetraethers (GDGTs) in sediment cores collected from GC 233 and MC 118 in the Gulf of Mexico.

	Relative abundance (%)[†]					
Depth (cm)	GDGT-0	GDGT-1	GDGT-2	GDGT-3	Crenarchaeol	Cren-isomer
GC 233 NBP						
0~4	33	10	10	1	43	4
12~14	31	10	11	1	42	5
24~26	33	8	10	2	42	5
MC 118 core 1						
5~10	31	10	8	3	43	4
15~20	35	9	10	2	42	3
25~30	33	9	10	3	41	4
100~105	30	8	10	2	45	5
105~110	37	9	7	2	44	2
184~189	39	8	13	5	33	2
200~205	35	7	7	3	45	4
205~210	35	8	9	ND[*]	44	4
MC 118 core 8						
5~15	30	10	11	3	43	3
20~30	34	12	21	3	28	2
105~115	26	20	31	5	17	2
130~138	26	23	33	7	10	1
MC 118 core 9						
5~15	16	16	39	9	18	2
15~30	16	18	39	10	13	3
30~35	15	17	46	8	13	2
100~110	36	10	11	3	38	2
167~182	33	11	15	3	34	4
200~210	31	7	10	4	42	6
310~320	34	10	9	NA	43	5
476~486	30	12	8	3	43	4
500~510	27	9	18	5	39	3

[†]The standard error of the relative abundance is ~ ± 2%.
[*]not detected.

Table 3: Phylogenetic affiliations of archaeal 16S rRNA genes retrieved from MC 118 core 9 in the Gulf of Mexico.

	Number of clones/OTUs		
	GoMT (5-15 cm)	GoMM (15-30 cm)	GoMD (30-35 cm)
Total clones sequenced	44	42	43
Euryarchaeota			
ANME-1a	27	30	29
ANME-2a		1	
ANME-2c	2		7
TMEG		1	
Thermoplasmatales	10	10	
Crenarchaeota			
MCG			3
MBG-B	5		4
Number of OTUs*	11	9	8
Number of unique OTUs†	7	4	3
Coverage (%)	82.5	90.4	95.0

*Determined by DOTUR with a cutoff value of 3%.

Table 4: Distribution of different phylogenetic groups (in percentage of total clones) in archaeal clone libraries from different locations in the Gulf of Mexico.

Sample	[a]GC185+GC234	[b]GC234	[c]FE*	[d]GC205	[e]GB425	[f]AT425	[g]MC118
Location	27°46'N, 27°44'N 91°30'W, 91°13'W	27°44'N 91°13'W	26°01.8'N 84°54.9'W	27.°43.0'N 90°32.0'W	27°33'N 92°32'W	27°34.1'N 88°29.7'W	28°51.5' 88°29.5'
Water depth	~650 m	575 m	3,288 m	876 m	600 m	~1295 m	890 m
Total clones	125	100	68	310	134	93	129
Euryarchaeota							
ANME-1	20	58	6	59	0	17	67
ANME-2a	2	0	34	0	41	3	1
ANME-2b	0	0	22	0	39	0	0
ANME-2c	30	13	34	0	0	0	7
ANME-2d	2	2	0	0	0	0	0
Methanomicrobiales	14	13	0	4	0	0	0
Methanosarcinales	15	8	0	9	0	78	0
Thermoplasmatales	6	1	0	20	6	0	16
TMEG	0	0	0	0	0	0	1
Unclassified Euryarch.	0	3	0	0	4	1	0
Crenarchaeota	10	2	3	8	10	0	9

Note: a, Mills et al., 2003; b, Mills et al., 2005; c, Reed et al., 2006; d, Lloyd et al., 2006; e, Martinez et al., 2006; f, Lanoil et al., 2001; g, this work.
*FE = Florida Escarpment.

Figure captions:

Fig. 1. Map of site MC 118 in the Gulf of Mexico, modified from Woolsey *et al.* (2005). Colors of the background were obtained using multibeam image by C&C Technologies; the yellow and brown colors indicate shallower water and the light green, light blue, and deep blue indicate deeper water (see insert).

Fig. 2. HPLC-MS chromatograms of intact GDGTs from Gulf of Mexico sediments. A) surface sediment from GC 233 (NBP), B) surface sediment from MC 118 core 1, C) sediment from about 1 m depth in MC 118 core 8, and D) shallow sediment from MC 118 core 9. Shown GDGT series are: I, crenarchaeol and the regio-isomer of crenarchaeol VI, II = GDGT-0, III = GDGT-1, IV = GDGT-2, V = GDGT-3.

Figure 3. Neighbor-joining tree (partial sequences, ~700 bp) showing phylogenetic relationships of eurarychaeota and crenarchaeota using archaeal 16S rRNA gene sequences retrieved from core 9 of the GoM sediment to closely related sequences from the GenBank. One representative clone type within each phylotype is shown and the number of clones within each phylotype is shown at the end (after the GenBank accession number). Number is not shown if there is only one clone within a given phylotype. Clone sequences from this study are coded as follows, with GoMD22 as an example: GoM: Gulf of Mexico; D, bottom; 22, clone number. T stands for top and M for middle in GoMT and GoMM, respectively. Scale bars indicate Jukes-Cantor distances. Bootstrap values of >50% (for 500 iterations) are shown. *Aquifex pyrophilus* is used as the outgroup.

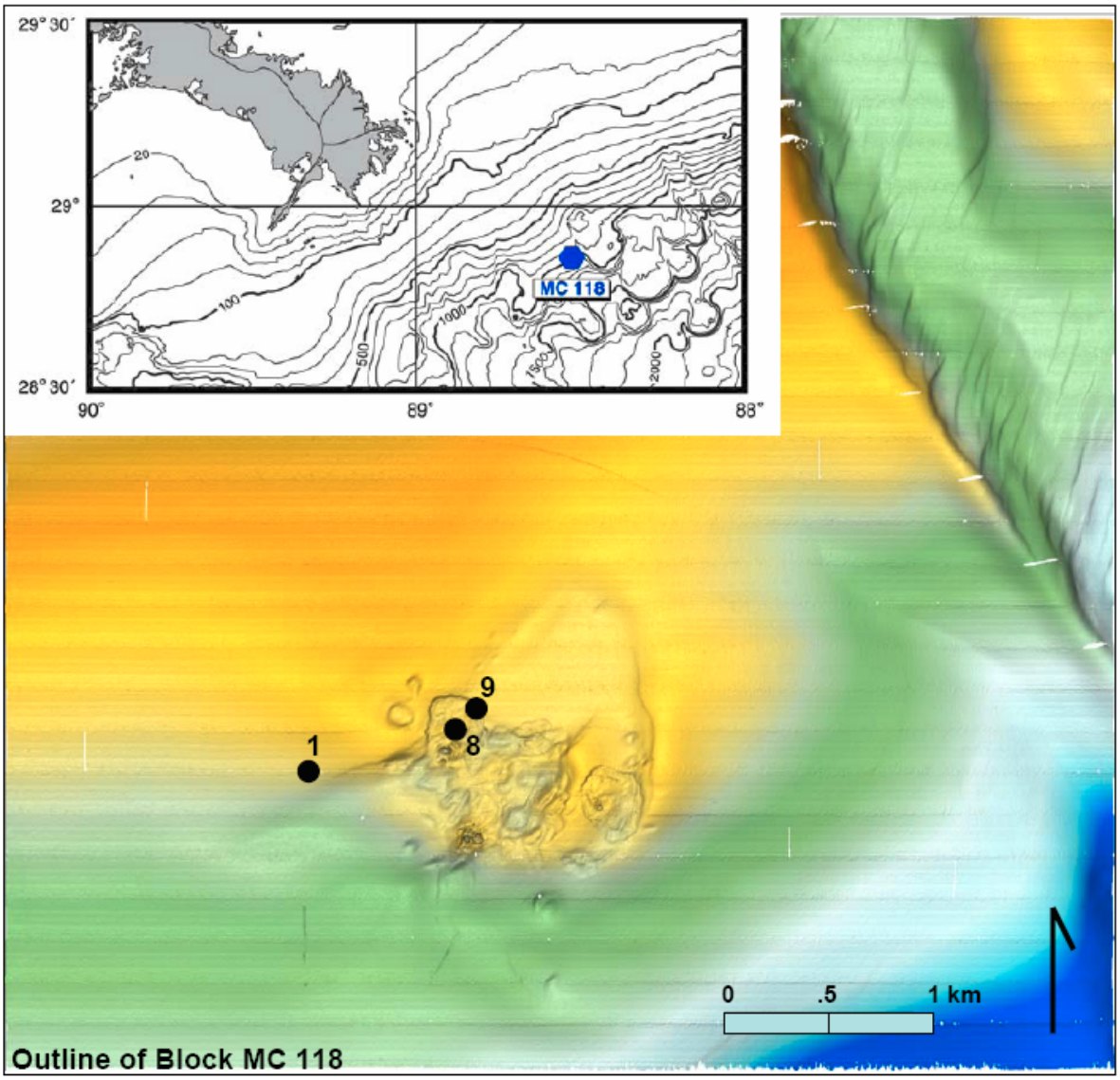

Fig. 1. Pi et al., 2007

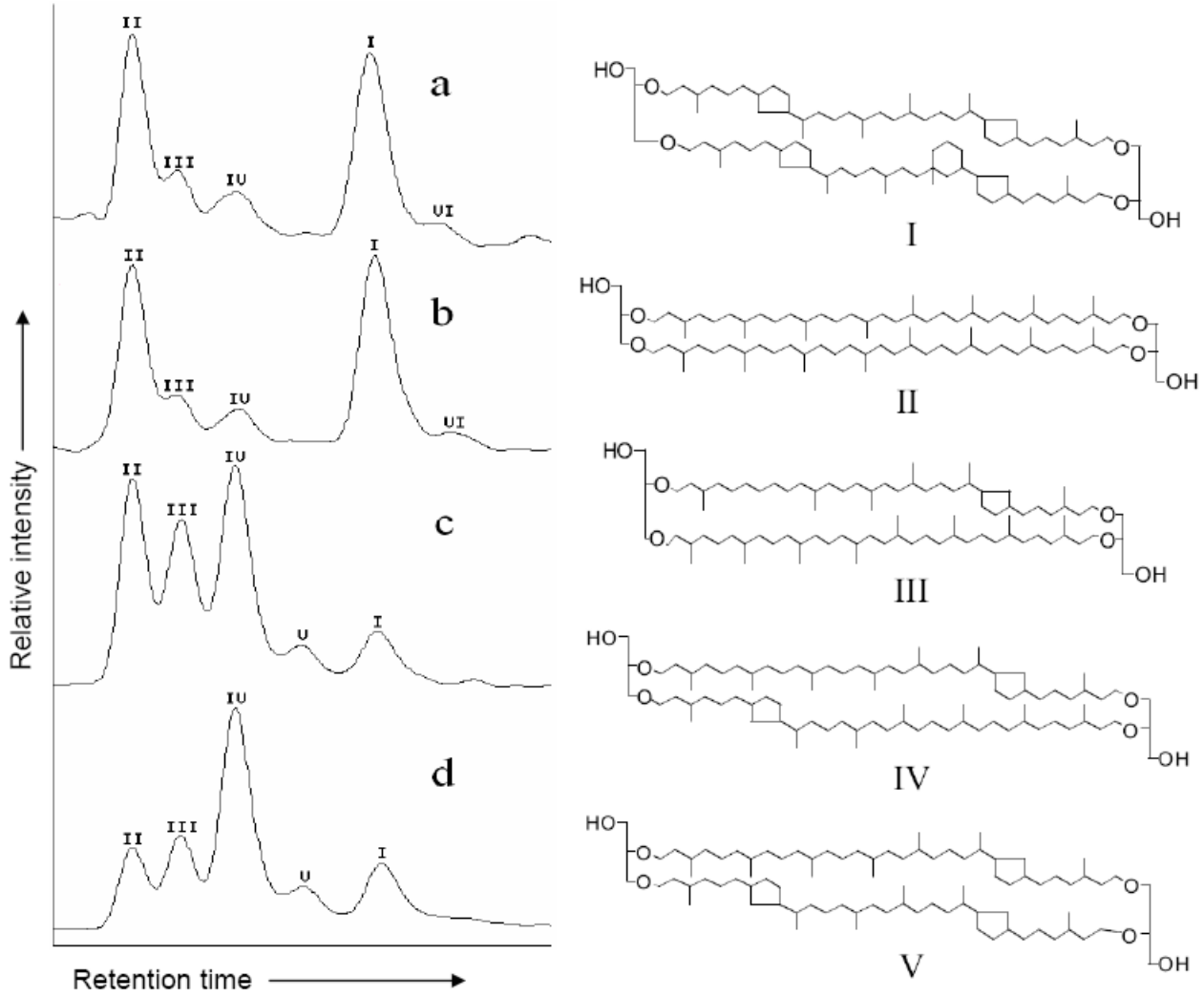

Fig. 2. Pi et al., 2007

Figure 3. Pi et al., 2007