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Distribution and Diversity of Aerobic Carbon Monoxide-Oxidizing Bacteria in Geothermal Springs of China, the Philippines, and the United States

JIAN YANG[1], ENMIN ZHOU[2], HONGCHEN JIANG[1]*, WENJUN LI[2]*, GENG WU[1], LIUQIN HUANG[1], BRIAN P. HEDLUND[3], and HAILIANG DONG[1,4]*

[1]*State Key Laboratory of Biogeology and Environmental Geology, China University of Geosciences, Wuhan, China*
[2]*State Key Laboratory of Biocontrol and Guangdong Key Laboratory of Plant Resources, College of Ecology and Evolution, Sun Yat-Sen University, Guangzhou, People's Republic of China*
[3]*School of Life Sciences, University of Nevada, Las Vegas, Las Vegas, Nevada, USA*
[4]*Department of Geology and Environmental Earth Science, Miami University, Oxford, Ohio, USA*

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Accumulating genomic evidence suggests that a variety of thermophilic bacteria contain *cox* operons and may be capable of aerobic carbon monoxide (CO) oxidation. However, little is known about the distribution and diversity of the *cox*-encoding (COXE) bacteria in natural geothermal environments. In this study, we examined *coxL* gene (encoding the large subunit of carbon monoxide dehydrogenase: CoxL) sequences retrieved from the sediments of 25 geothermal sites located in the Qinghai-Tibetan Plateau (QTP) and Yunnan Province (YP) of China, the Bacon-Manito Geothermal Production Field (BGPF) of the Philippines, and the Great Basin of the United States (USGB). Temperature and pH ranges of the studied hot springs were 22.1 to 90.8°C and 2.7 to 9.4, respectively. Phylogenetic analyses showed that most CoxL sequences were closely related to the classes *Actinobacteria*, *Deinococci*, *Ktedonobacteria*, *Thermomicrobia,* and *Clostridia*, and hot springs from different regions hosted different COXE communities. In addition, these hot springs harbored some COXE bacteria that were phylogenetically distinct from those inhabiting nongeothermal ecosystems. This study revealed no significant correlation between temperature or pH and the composition or diversity of COXE communities at the global scale. However, within a given region, temperature was correlated with the COXE bacterial community composition.

Keywords: *coxL* gene, *cox*-encoding (COXE), bacteria, diversity, hot springs

Introduction

Carbon monoxide (CO) is a trace gas in the Earth's atmosphere and is a common constituent of vapor emission from hydrothermal environments (Sokolova et al. 2009). CO can be used as electron donor and carbon source by aerobic CO-oxidizing bacteria (King and Weber 2007), which metabolize CO through carbon monoxide dehydrogenases (CODHs). CODHs are encoded by *cox* operons that have been identified in the genomes of numerous thermophilic prokaryotes (King 2013). To date, aerobic CO oxidation has been confirmed in several genera of moderately thermophilic bacteria, such as *Streptomyces* (*Actinobacteria*), *Thermomicrobium* and *Thermogemmatispora* (*Chloroflexi*), *Bacillus*, *Alicyclobacillus*, *Brevibacillus* and *Geobacillus* (*Firmicutes*), *Meiothermus* and *Thermus* (*Deinococcus-Thermus*), and *Pseudomonas* (*Proteobacteria*) (King 2013; King and Weber 2007). However, it is unknown whether all *cox* gene-carrying prokaryotes are capable of CO oxidation. For example, *Natronorubrum tibetense*, *Ktedonobacter racemifer*, and *Niastella koreensis* possess *cox* genes but fail to oxidize CO under experimental conditions (King 2013). Therefore, in the absence of evidence for CO oxidation activity, these *cox*-carrying prokaryotes can conservatively be considered *cox*-encoding (COXE) microorganisms.

Terrestrial hot springs host a variety of thermophilic microorganisms, which perform numerous redox processes including CO oxidation and thus play important roles in many geochemical cycles (Amend and Shock 2001; Shock et al. 2005). Over the last decade, many 16S rRNA gene-based phylogenetic studies have investigated microbial communities in terrestrial hot springs from a variety of locations, such as Tibet, China (Huang et al. 2011; Wang et al. 2013; Yim et al. 2006), Yunnan Province, China (Hou et al. 2013; Song et al. 2013), Thailand (Purcell et al. 2007), Yellowstone National

*Address correspondence to Hongchen Jiang, State Key Laboratory of Biogeology and Environmental Geology, China University of Geosciences, Wuhan 430074, China; Email: jiangh@cug.edu.cn; or to Hailiang Dong, Email: dongh@muohio.edu; or to Wenjun Li, Email: liwenjun3@mail.sysu.edu.cn

Park, USA (Kozubal et al. 2013; Meyer-Dombard et al. 2005; Miller et al. 2009), Great Basin, USA (Cole et al. 2013; Costa et al. 2009), the Philippines (Huang et al., 2013), and Canada and New Zealand (Sharp et al. 2014). All of these studies revealed the presence of sequences related to the above COXE-related genera, implying that COXE bacteria may inhabit terrestrial hot springs. However, to date, *Thermomicrobium roseum* is the only known aerobic CO-oxidizing bacterium that has been isolated from hot springs (Wu et al. 2009).

The *coxL* gene, encoding the large subunit of the CODH (CoxL) is highly conserved and has been used as a molecular biomarker for characterizing the distribution of COXE bacteria in natural environments (King 2003). *coxL* genes have been grouped into two divergent clusters, the form I or OMP group (based on the type enzymes from the genera of *Oligotropha*, *Mycobacterium*, and *Pseudomonas*) and the form II or BMS group (based on the type enzymes from the genera of *Bradyrhizobium*, *Mesorhizobium*, and *Sinorhizobium*) (King 2003; King and Weber 2007). However, some biochemical evidence suggests that the form II enzymes have a low affinity for CO and may not catalyze CO oxidation (King and Weber 2007; Lorite et al. 2000).

Therefore, previous COXE bacterial diversity studies largely focused on the form I genes in a variety of environments, such as volcanic deposits (Dunfield and King 2004; King et al. 2008; Weber and King 2010), soils (Lynch et al. 2012), geothermally heated microbial biofilms (King 2013), and saline lakes (Yang et al. 2013b). However, little is known about the diversity and distribution of thermophilic COXE bacteria in terrestrial geothermal springs, despite their important roles in controlling CO flux in such environments (Sokolova et al. 2009).

The objectives of this study were: 1) to investigate the diversity and distribution of COXE bacteria in sediments and microbial mats collected from eighteen different hot springs on the Qinghai-Tibetan Plateau (QTP) and Yunnan Province of (YP) China, Bacon-Manito Geothermal Production Field (BGPF) of the Philippines, and the Great Basin of the United States (USGB) using *coxL* gene-based phylogenetic analysis; and 2) to assess the effect of temperature and pH on the composition or diversity of COXE bacteria.

Materials and Methods

Site Description and Sampling

The sampled hot springs in this study were distributed in four different areas (QTP, YP, BGPF, and USGB) (Table 1). Six hot springs were sampled in Naqu County of the QTP including four from Gulu (GL15, GL22, GL7, and GL20), one from Naqu (NQ1), and one from Guozu (GZ1) (Wang et al. 2013). Six hot springs were sampled from Tengchong County of Yunnan Province (YP) in southwestern China (Gumingquan: GmqP; Jiemeiquan: JmqL; Jinze: Jz; Shuirebaozha: SrbzD; Diretiyanqu-2: Drty-2; and Drty-3) (Briggs et al. 2013; Hou et al. 2013). Four hot springs were sampled in the BGPF of the Philippines: two from Balasbas Town (BAL-0 and BAL-1), one from Naghaso Town (NAG-7), and one from Balbagon

Table 1. Geographical locations, temperature, and pH of the investigated hot springs in this study

Area	Sample name	Spring name	GPS Location	Temperature (°C)	pH
QTP	GL15	Gulu spring No. 15	N30°52′34″ /E91°36′40″	80.0	9.1
	GL22	Gulu spring No. 22	N30°52′34″ /E91°36′34″	73.0	8.1
	GL7	Gulu spring No. 7	N30°52′31″ /E91°36′42″	66.0	8.8
	NQ1	Naqu spring No. 1	N31°38′45″ /E91°45′08″	54.0	7.4
	GL20	Gulu spring No. 20	N30°52′35″ /E91°36′34″	46.0	7.4
	GZ1	Guozu spring No. 1	N31°40′53″ /E91°51′21″	22.1	7.2
YP	JmqL	Jiemeiquan (Left)	N24°57′04″ /E98°26′10″	84.7	9.0
	Gmqp	Gumingquan (streamer pool)	N24°57′33″ /E98°26′11″	83.5	9.4
	Jz	Jinze	N25°26′29″ /E98°27′36″	80.7	7.0
	SrbzD	Shuirebaozha (downstream)	N24°57′01″ /E98°26′15″	72.1	8.3
	Drty-2	Diretiyanqu site 2	N24°57′14″ /E98°26′18″	66.0	2.8
	Drty-3	Diretiyanqu site 3	N24°57′14″ /E98°26′18″	53.0	2.7
BGPF	BAL-0	Balasbas spring No. 0	N13°06′36″ /E123°53′24″	90.8	4.3
	NAG-7	Naghaso spring No. 7	N13°07′48″ /E123°54′36″	64.1	3.7
	BAL-1	Balasbas spring No. 1	N13°06′36″ /E123°53′24″	60.5	5.2
	BAG-2	Balbagon spring No. 2	N13°07′12″ /E123°55′48″	59.9	6.6
USGB	GBS_A	Great Boiling Spring site A	N40°39′41″ /W119°21′58″	82.2	7.2
	GBS_B	Great Boiling Spring site B	N40°39′41″ /W119°21′58″	72.8	7.6
	GBS_C	Great Boiling Spring site C	N40°39′41″ /W119°21′58″	57.1	7.9
	GBS_D	Great Boiling Spring site D	N40°39′41″ /W119°21′58″	47.4	8.3
	SSW_F	Sandy's Spring West site F	N40°39′11″ /W119°22′30″	80.5	7.4
	SSW_G	Sandy's Spring West site G	N40°39′11″ /W119°22′30″	72.1	7.8
	SSW_H	Sandy's Spring West site H	N40°39′11″ /W119°22′30″	65.5	8.0
	SSW_I	Sandy's Spring West site I	N40°39′11″ /W119°22′30″	58.8	7.9
	SSW_J	Sandy's Spring West site J	N40°39′11″ /W119°22′30″	51.0	8.1

Town (BAG-2) (Huang et al. 2013). Four different locations in Great Boiling Spring (GBS) (GBS_A, GBS_B, GBS_C, and GBS_D) and five sites along the outflow channel of Sandy's Spring West (SSW) (SSW_F, SSW_G, SSW_H, SSW_I, and SSW_J) were sampled in the USGB (Cole et al. 2013; Costa et al. 2009). Sampling and field measurements were performed in Tibet, Tengchong, the Philippines, and the USGB in July 2010, June 2012, July 2011, and November 2011, respectively.

The GPS locations of all the selected hot springs were determined using a portable GPS unit (eTrex H, Garmin, USA). Water temperature and pH of hot springs were measured at the sampling locations using a portable meter (LaMotte, MD, USA). After field measurements, surface sediments (top ~1 cm) at each of the selected hot springs were collected into sterile 1.5-mL Eppendorf tubes and immediately frozen on dry ice. All sediment samples for DNA extraction were transported to the laboratory on dry ice and then stored in a -80°C freezer until further analysis.

DNA Extraction, PCR Amplification, and Phylogenetic Analysis

DNA extraction was performed on freshly thawed samples using FastDNA® SPIN Kit for Soil (Qbiogene, Inc. CA) according to the manufacturer's protocol. Bacterial *coxL* genes were amplified from the extracted DNA samples with the following OMP *coxL* gene-specific primer set: OMPf (5'-GGCGGCTT[C/T]GG[C/G]AA[C/G]AAGGT-3') and O/Br (5'-[C/T] TCGA[T/C]GATCATCGG[A/G]TTGA-3') (King 2003). All PCR reactions for the *coxL* genes were performed using the conditions as described previously (King 2003). PCR products (1,260 to 1,290 bp) were examined using gel electrophoresis on 1% agarose gel and appropriate bands were excised and purified with Agarose Gel DNA purification Kit (TaKaRa, Japan). *coxL* gene clone libraries (Table 2) were constructed by using the pGEM-T Easy (Promega, USA) cloning kit as previously described (Yang et al. 2013a). Twenty-five clones were randomly selected and screened for inserts by performing another round of PCR using the above primer set.

The positive PCR products were digested with restriction endonuclease *HaeIII* (TaKaRa, Dalian, China) for restriction fragment length polymorphism (RFLP) analysis. Digests were analyzed with electrophoresis on a 2% (w/v) agarose gel. Unique RFLP types were identified visually. Subsequently, rarefaction curves were constructed using aRarefactWin (www.uga.edu/strata/software/Software.html) (data not shown). RFLP screening was stopped when the rarefaction curves approached saturation (library coverage >80%, Table 2). One representative clone was selected for sequencing from each RFLP type. The *coxL* gene inserts were sequenced with an ABI 3100 automated sequencer using the reverse primer O/Br.

Table 2. Ecological estimates of the CoxL libraries from hot spring sediments in Qinghai-Tibetan Plateau (QTP) and Yunnan Province (YP) of China, Bacon-Manito Geothermal Production Field (BGPF) of the Philippines, and the Great Basin of the United States (USGB)

Area	Libraries	Clones	OTUs	Coverage(%)	Simpson	Shannon	Evenness	Chao 1
QTP	GL15	17	2	100	0.5	0.7	1.0	2.0
	GL22	24	2	100	0.4	0.6	0.9	2.0
	GL7	25	5	96	0.6	1.2	0.6	5.0
	NQ1	24	7	83	0.6	1.4	0.6	10.0
	GL20	21	3	100	0.6	1.0	0.9	3.0
	GZ1	14	5	86	0.6	1.3	0.7	5.3
YP	JmqL	12	2	92	0.2	0.3	0.7	2.0
	GmqP	23	5	91	0.6	1.2	0.7	5.5
	Jz	17	1	100	0.0	0.0	1.0	1.0
	SrbzD	21	4	95	0.5	1.0	0.6	4.0
	Drty-2	16	1	100	0.0	0.0	1.0	1.0
	Drty-3	11	3	91	0.5	0.9	0.8	3.0
BGPF	BAL-0	24	1	100	0.0	0.0	1.0	1.0
	NAG-7	24	2	100	0.3	0.5	0.8	2.0
	BAL-1	24	2	100	0.2	0.4	0.7	2.0
	BAG-2	25	2	96	0.1	0.2	0.6	2.0
USGB	GBS_A	25	2	96	0.1	0.2	0.6	2.0
	GBS_B	25	2	100	0.5	0.7	1.0	2.0
	GBS_C	25	3	92	0.2	0.3	0.5	4.0
	GBS_D	24	5	96	0.7	1.3	0.7	5.0
	SSW_F	25	3	96	0.5	0.8	0.8	3.0
	SSW_G	20	3	100	0.5	0.9	0.8	3.0
	SSW_H	20	2	100	0.2	0.3	0.7	2.0
	SSW_I	14	2	100	0.2	0.4	0.8	2.0
	SSW_J	17	2	100	0.4	0.6	0.9	2.0

For phylogenetic analysis, raw nucleotide sequences were checked and trimmed manually by using the BioEdit program (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The edited sequences were then translated into amino acid sequences. The resulting amino acid sequences were used to perform BLASTP (www.ncbi.nlm.nih.gov/blast/) against available CoxL sequences in the GenBank database and the closest references were chosen for constructing phylogenic trees. Sequences that were unrelated to CoxLs were removed from further analysis. Henceforward, all the analyses were conducted on the basis of the deduced amino acid sequences unless specified otherwise.

The CoxL sequences were clustered into operational taxonomic units (OTUs) using the nearest neighbor algorithm in DOTUR (Schloss and Handelsman 2005) at 90% identity cutoff, which corresponds to 97% level for the 16S rRNA gene (Weber and King 2010). One representative CoxL sequence was chosen from each OTU and was aligned with a reference set of CoxL sequences by using Clustal W implemented in the BioEdit program. Maximum-likelihood trees were constructed for the representative CoxL sequences and their references by using MEGA 5 with 1000 bootstrap replicates (Tamura et al. 2011). Putative *coxL* gene sequences determined in this study were deposited in the GenBank database under accession numbers: KC843664-KC843707 (QTP), KF697173-KF697188 (YP), KF524339-KF524350 (BGPF), and KF524264- KF524313 (USGB).

Statistical Analysis

Library coverage was calculated using the equation $C = 1 - (n1/N)$, where n1 represents the number of OTUs that occurred only once in the clone library and N indicates the total number of clones analyzed in the library (Jiang et al. 2006). Diversity indices (Shannon (H), Simpson ($1\text{-}D$), Buzas and Gibson's Evenness (e^H/S), and *Chao1*) were calculated by the PAST software package (http://folk.uio.no/ohammer/past/) (Hammer et al. 2001).

The Shannon index was calculated following the equation

$$H = -\sum_i \frac{n_i}{n} In \frac{n_i}{n}$$

where n_i is the number of clones in OTU_i and n is the total number of clones. The Simpson index was calculated using $1 - D$, where D is equal to

$$\sum_i (\frac{n_i}{n})^2$$

.

Buzas and Gibson's evenness is expressed as e^H/S, where H is Shannon index and S is the total number of OTUs. *Chao1* is an estimate of total species richness, and it was calculated using the equation: $Chao1 = S + F_1(F_1 - 1) / (2 (F_2 + 1))$, where F_1 is the OTU number of singleton sequences and F_2 is the OTU number of doubleton sequences. Principal coordinate analysis (PCoA) was conducted using UniFrac (http://unifrac.colorado.edu/) (Lozupone et al. 2007). Cluster analysis was performed to test for significant similarity in CoxL composition among different samples at the OTU level based on Bray–Curtis indices.

Mantel tests were performed to estimate the correlation between biotic and environmental similarity matrices by using the PAST software package. The biotic and environmental distance matrices were constructed according to a previous study (Yang et al. 2013b). Briefly, the biotic matrices based on CoxL composition were constructed using Bray–Curtis distances (defined as follows: Bray–Curtis distance $= 1 - d$, where d refers to the Bray-Curtis similarity index). The abiotic matrices of environmental variables (temperature and pH were the only parameters measured at all sites studied here) were constructed on the basis of the Euclidean distances.

To compare the COXE bacterial diversity retrieved in the geothermal features in this study with that described in non-geothermal environments, *coxL* gene sequences were collected from the following published studies: Hawaiian volcanic deposits (Weber and King 2010), Japanese volcanic deposits (King et al. 2008), and Qinghai-Tibetan lakes (Yang et al. 2013b). In order to avoid any bias resulting from different primers, only *coxL* gene sequences derived from the same primer set (OMPf and O/Br) and the same PCR protocol were included in this analysis. Finally, all the *coxL* gene sequences were translated into amino acid sequences and then were subjected to OTU identification using nearest neighbor algorithm in DOTUR at 90% cutoff (Schloss and Handelsman 2005). The diagrams of OTU presence/absence were then plotted by using R v3.1.0 in the ggplot2 package (R Core Team 2014).

Results

Geochemistry of the Sampling Sites

The geochemistry of the springs sampled in this study has been described previously (Costa et al. 2009; Hou et al 2013; Huang et al 2011; Huang et al. 2013). Temperatures measured at the sampling sites were 22.1−80.0°C (QTP), 53.0–84.7°C (YP), 59.9–90.8°C (BGPF), and 47.4–82.2°C (USGB) (Table 1). The pH of the hot springs was different among the sites: the QTP and USGB hot springs were slightly alkaline with a narrow range (7.2–9.1); the BGPF hot springs were acidic, with a pH range of 3.7 to 6.6; and the YP hot springs had a wide range of pH (2.7–9.4) (Table 1).

Phylogenetic Analyses and Clustering of Putative CoxL Sequences

All samples were tested positive for *coxL* gene PCR. A total of 517 *coxL* gene clones were derived from the libraries from QTP, YP, BGPF, and USGB samples (125, 100, 97, and 195 clones, respectively). These clones were grouped into 24, 16, 7, and 24 OTUs for the QTP, YP, BGPF, and USGB samples, respectively (Table 2). The coverage values were 83–100% for the constructed clone libraries (Table 2). Hot spring sediments from different regions hosted distinct COXE bacterial populations

(Figures 1 and 2). With a few exceptions, samples from the same region were grouped together (Figure 2 and Figure 3).

For example, the CoxL sequences from the QTP samples were closely related to those found in *Actinobacteria* (~53% of total QTP clones), *Alphaproteobacteria* (~14%), and *Betaproteobacteria* (~11%). The YP samples mainly consisted of CoxL sequences related to those in *Alphaproteobacteria* (56%) and *Deinococci* (25%). The CoxL sequences in the BGPF samples were closely related to those in *Deltaproteobacteria* (~25%), *Ktedonobacteria* (~42%), and *Thermomicrobia* (~25%). CoxL sequences related to those found in *Clostridia* and *Deltaproteobacteria* were abundant in clone libraries from the USGB samples, accounting for ~28% and ~17% of the total USGB clones, respectively (Figures 1 and 2).

In addition, one CoxL OTU was affiliated with the form II/BMS clade and ten CoxL OTUs formed three clades that

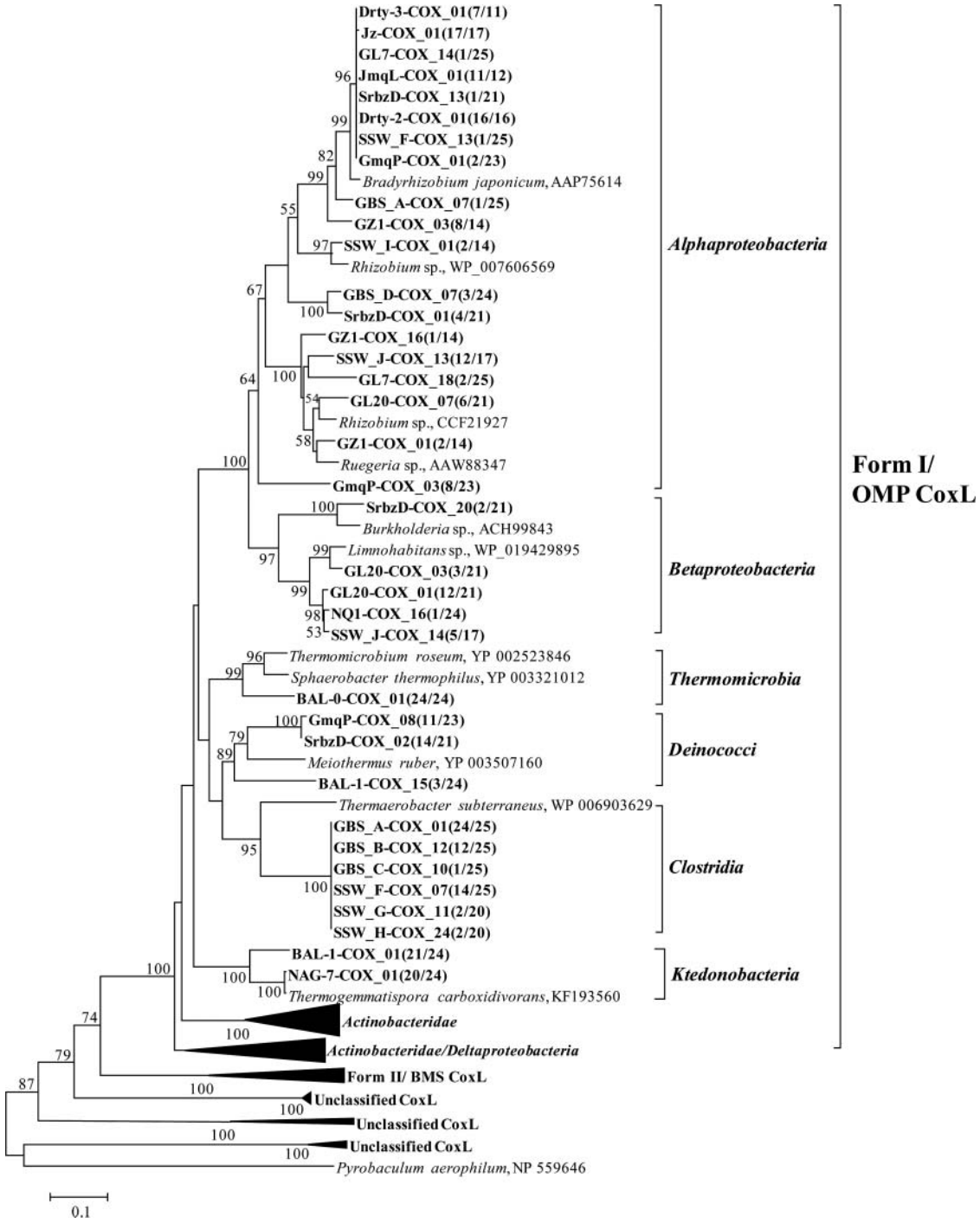

Fig. 1. Continued

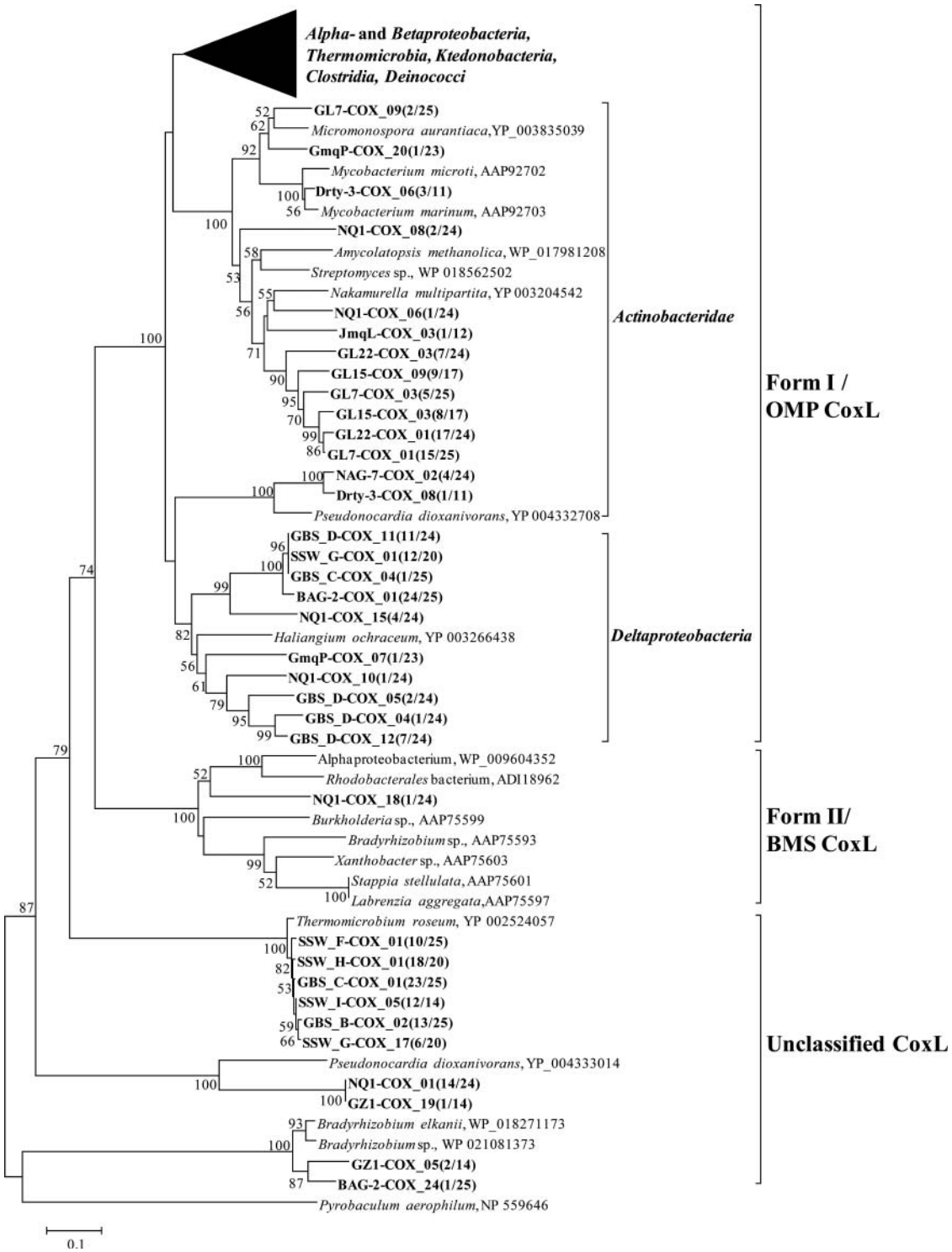

Fig. 1. Maximum-likelihood tree showing the phylogenetic relationships between the putative CoxL sequences obtained in this study and closely related sequences from the GenBank database. The sequences from this study are colored for different regions: QTP (green), YP (blue), BGPF (red) and USGB (orange). Sequences are coded as follows for the example of GZ1-COX_01 (2/14): CoxL sequences of clone No. 01 from hot spring sediment GZ1, and this sequence represent 2 clones in the 14-clone library. The scale bar indicates the expected number of changes per homologous position. Bootstrap values > 50% are shown (1000 replicates). The putative CoxL sequence from *Pyrobaculum aerophilum* was used as the outgroup.

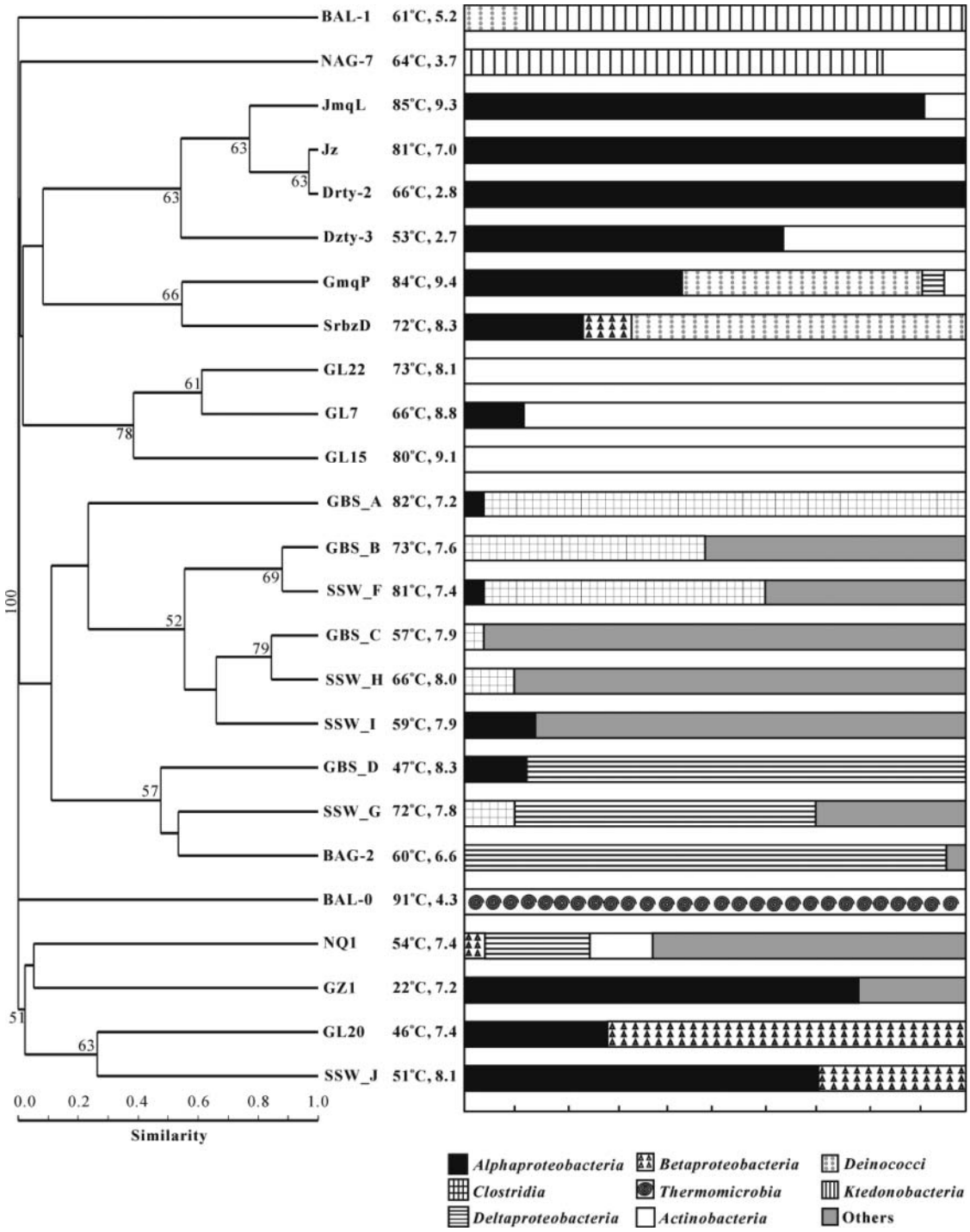

Fig. 2. Cluster analysis of the COXE bacterial community compositions in the investigated hot spring sediments based on Bray-Curtis similarity. The topology was constructed with the pair group algorithm using the PAST software package. Bootstrap values > 50% are shown (1000 replicates). The right panel represents community structures, which shows the frequencies of clones affiliated with major phylogenetic classes.

were related to, but distinct from form I and form II CoxL enzymes (i.e. unclassified clades of CoxL; Figure 1B). The retrieval of the form II CoxL genes was possibly due to the primer set used in this study, which targets amino acid motifs that are conserved in both form I and form II *coxL* genes (Dunfield and King 2004). These motifs may also be conserved in related molybdenum hydroxylases that may or may not have significant CO-oxidation activity (e.g. the unclassified clades of CoxL). Many known carboxydotrophs and carboxydovores encode multiple molybdenum hydroxylase enzymes whose functions are not completely understood (King 2013).

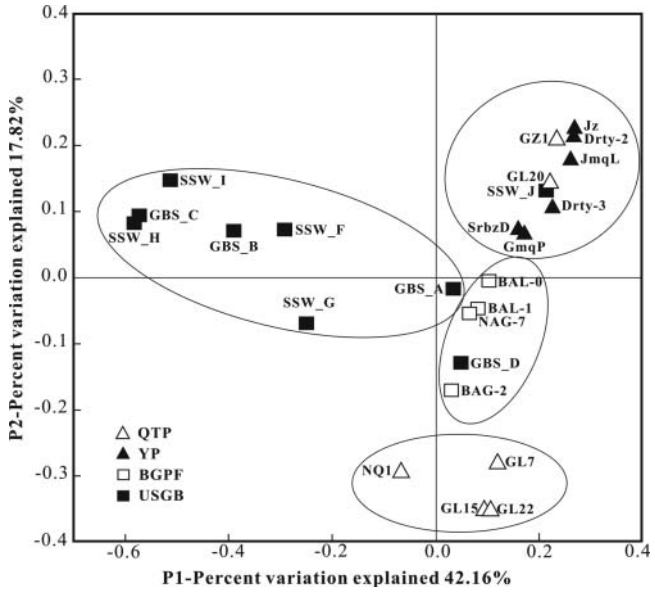

Fig. 3. Ordination diagrams plotting the distribution of sedimentary COXE bacterial assemblages based on abundance-weighted UniFrac PCoA analysis. P1: principal coordinate 1, P2: principal coordinate 2.

Correlation of CoxL Richness, Diversity, and Community Composition with Temperature and pH

No significant correlation was observed between CoxL richness and diversity and temperature or pH ($P > 0.05$, data not shown). For example, high CoxL richness was observed in mid temperature range for the QTP samples. In the USGB springs, CoxL richness decreased slightly with increasing temperature in the GBS samples, while the opposite trend was observed in the SSW samples. Mantel tests showed that COXE bacterial community composition was not significantly ($P < 0.05$) correlated with temperature or pH at the global scale. However, within a given region, temperature did affect the distribution of COXE bacterial community composition. For example, Mantel tests indicated that COXE bacterial communities were significantly correlated with temperature in the QTP ($r = 0.539$, $P = 0.017$) and USGB ($r = 0.448$, $P = 0.004$) springs.

Discussion

Composition of COXE Bacteria in Hot Springs

COXE bacterial populations in the studied hot springs were compositionally different from those in nonthermal environments (e.g., saline lake sediments, volcanic deposits) (Figure 1 and Figure 4). For example, *Actinobacteria*-, *Alphaproteobacteria*-, and *Betaproteobacteria*-like CoxL sequences were dominant in saline lake sediments (Yang et al. 2013b) and Hawaiian/Japanese volcanic deposits (King et al. 2008; Weber and King 2010). *Ktedonobacteria*-like CoxL sequences were predominant in the Bare volcanic deposits of Kilauea Volcano, Hawaii (Weber and King 2010). Although these CoxL groups were present and even abundant in some of the studied hot springs, additional groups were also abundant, including *Deinococci*, *Thermomicrobia*, and *Clostridia* (Figure 1 and Figure 2). These results suggest that temperature may be a key factor selecting certain thermophilic COXE bacteria in geothermal systems, which are totally different from those in nonthermal environments (Cole et al. 2013; Hou et al. 2013).

Although many *Actinobacteria*-, *Alphaproteobacteria*-, and *Betaproteobacteria*-like CoxL sequences were also detected in the present study, they were compositionally different from those in nonthermal environments that have been surveyed with the use of the same primers as used in this study (Figure 4). *Actinobacteria* and *Proteobacteria* are physiologically diverse and are thus widely distributed in diverse environments (e.g., soils, hot springs, lakes, oceans) (Hedlund et al. 2012; Jiang et al. 2012; Nakagawa et al. 2005; Spain et al. 2009; Stackebrandt and Embley 2000). Therefore, it is not surprising to observe the *Proteobacteria*- and *Actinobacteria*-like CoxL sequences in the investigated hot spring samples.

In addition, COXE bacterial communities were compositionally different in these hot springs among different regions. For example, the *Ktedonobacteria*-like and *Clostridia*-like CoxLs were only found in the BGPF and USGB samples, respectively; the *Actinobacteria*-like and *Deinococci*-like CoxLs were only dominant in the QTP and YP samples, respectively (Figures 1 and 2). This may result from different water chemistry in the studied hot

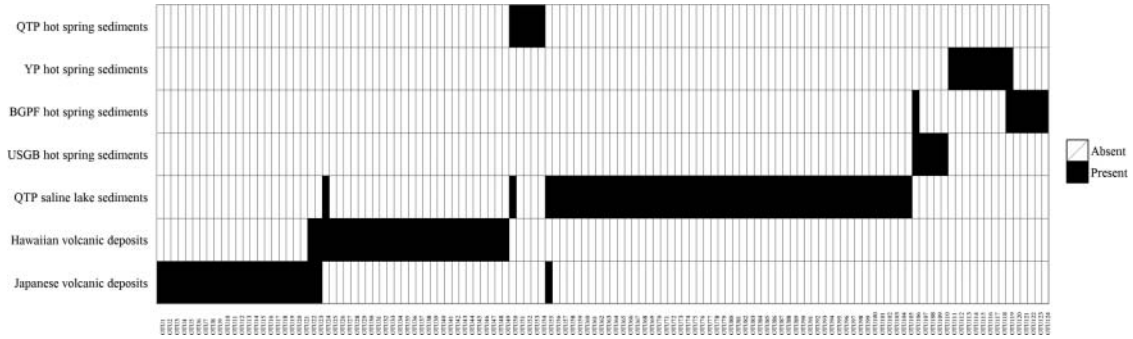

Fig. 4. Diagram showing the presence (black) or absence (blank) of CoxL OTUs from hot springs in this study, Hawaiian volcanic deposits (Weber and King 2010), Japanese volcanic deposits (King et al. 2008), and Qinghai-Tibetan lakes (Yang et al. 2013b). X-axis indicates OTUs, with each of the grids representing one OTU.

springs. However, the underlying reason still awaits further investigation.

Effects of Temperature and pH on COXE Bacterial Distribution in Hot Springs

Our study of COXE bacterial communities failed to reveal any clear relationships between diversity or richness and temperature or pH, despite clear relationships between the diversity of the entire microbial community and temperature in the U.S. Great Basin (Cole et al. 2013) and Tengchong hot springs (Wang et al. 2013). Correlations between microbial diversity and temperature have been shown in a number of studies of terrestrial geothermal systems (Boyd et al. 2012; Cole et al. 2013; Everroad et al. 2012; Meyer et al. 2013; Meyer-Dombard et al. 2011; Miller et al. 2009; Wang et al. 2013), including one intercontinental study covering a wide range of pH values (Sharp et al. 2014).

It is unclear why this study of COXE bacteria failed to uncover such relationship. It is possible that such a correlation may be obscured by the recovered mesophilic CoxL sequences that could have derived from surrounding soils (e.g., *Rhizobium* sp., *Bradyrhizobium* sp., *Burkholderia* sp.). A recent study observed a high number of putative soil contaminants in geothermal springs during the times of high precipitation (Briggs et al. 2014). The effect of such soil contamination could be particularly important for high-temperature springs because these soil-derived COXE bacteria could dilute indigenous hot spring COXE bacteria, especially if COXE bacterial abundance in high-temperature springs is low.

Within some regions, temperature appeared to play an important role in shaping COXE bacterial community composition. For example, for the QTP samples, high temperature samples (GL22, GL7 and GL15, >66°C) were dominated by *Actinobacteria*-like CoxL sequences, while low temperature samples (NQ1, GL20 and GZ1) were dominated by proteobacterial CoxL sequences. Likewise, for the USGB samples, *Clostridia*-like CoxL sequences were dominant in the high temperature samples (GBS_A, GBS_B and SSW_F, >73°C), and unclassified CoxL sequences were dominant in the samples with moderate temperatures (GBS_C, SSW_H and SSW_I, 57-66°C), whereas proteobacterial CoxL sequences were dominant in the low temperature samples (GBS_D and SSW_J). This was also demonstrated by the Mantel test.

The temperature response of COXE bacterial community structure was consistent with our previous investigations for bacteria in the same regions (Cole et al. 2013; Wang et al. 2013). In contrast, our results showed that temperature was not correlated with the COXE community structure in the YP and BGPF springs (data not shown). Many previous studies have shown that temperature affects hot spring microbial communities at a local scale when other conditions are similar (Boyd et al. 2012; Cole et al. 2013; Everroad et al. 2012; Meyer et al. 2013; Meyer-Dombard et al. 2011; Miller et al. 2009; Sharp et al. 2014; Wang et al. 2013).

The different response pattern of COX bacteria to temperature in different geothermal regions could be ascribed to other environmental conditions that may be important to COX bacteria. It is possible that temperature was important in shaping COX bacterial community structure in the QTP and USGB because these samples had similar pH (i.e., neutral to slightly alkaline with a small pH range). In contrast, the YNP and BGPF samples have large ranges of both temperature and pH (from acidic to neutral or alkaline) (Figure 2 and Table 1). In this case, the temperature effect may be obscured by the pH effect. Therefore, when multiple environmental conditions vary at the same time, it is difficult to isolate the effect of any single variable.

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