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Prokaryotic Community Analysis of a Hyperalkaline Spring in the Philippines Using 16S rRNA Gene Clone Library Construction

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The prokaryotic diversity associated with serpentinization-driven Manleluag Hyperalkaline (pH 11) Spring in Pangasinan, Philippines was investigated. DNA extracted directly from the sediment samples was used to construct clone libraries based on bacterial and archaeal 16S rRNA gene sequences. Phylogenetic analysis of 16S rRNA gene sequences from the clone library revealed that the clones were grouped into *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Cyanobacteria*, *Bacteroidetes*, and *Firmicutes*. Analysis of the archaeal 16S rRNA clones revealed the presence of sequences associated with members of *Euryarchaeota* and *Thaumarchaeota*. Most of the sequences from *Euryarchaeota* were related to *Methanobacteria* and *Methanomicrobia*. Some clones show little affiliation with known taxa and may represent novel sequences of organisms adapted to the hyperalkaline conditions. The populations found suggest the type of metabolisms that drive this specific environment, which include ammonia oxidation, and hydrogen-based and methanogenic metabolisms. This study represents the first analysis of prokaryotic diversity from community DNA of a hyperalkaline environment in the Philippines.

Key Words: 16S rRNA gene sequence, alkaliphiles, hyperalkaline spring, phylogenetic analysis, serpentinization

INTRODUCTION

Natural nonsaline alkaline environments are not common while saline alkaline soda lakes and soda deserts have been systematically studied. Nonsaline alkaline environments are much rarer and their microbial populations have not been well-documented. The genesis of nonsaline alkaline environments is related to a geochemical process known as serpentinization (Tiago et al. 2004). This process is exothermic and large quantities of hydrogen gas, methane and low-molecular weight organic compounds can emanate from these serpentinizing regions (Brazelton *et* al. 2011). Serpentinization is therefore a potential source of reducing power and organic carbon for organisms inhabiting the ultramafic subsurface (Brazelton et al. 2012).

A unique natural alkaline environment in the Philippines is the Manleluag Hyperalkaline Spring, Pangasinan which is characterized by hyperalkaline (pH 10-11.5) and highly

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reducing waters (- 200 to 300mV) with little dissolved oxygen present (<1 mg/L) (Vargas et al. 2009; Arcilla et al. 2007). The hyperalkaline water is effervescent with methane or hydrogen gas. The alkalinity of the spring is the result of the hydrolysis of Mg-rich rocks like gabbros while its highly reducing state is due to the oxidation of ion minerals like pyroxenes. Some of the reaction pathways are also strongly exothermic, frequently producing hydrothermal groundwaters that are often used as therapeutic springs in the Philippines.

Similar alkaline environments include the Ophiolitic complex of Semail in Oman (Bath et al. 1987), Cabeco de Vide aquifer of Portugal (Tiago et al. 2004), Maqarin site in Jordan (Pedersen et al. 2004), Del Puerto Ophiolite in California (Blank et al. 2009) and Tablelands Ophiolite in Canada (Brazelton et al. 2012). While microbial diversity surveys have been conducted in deep sea ultramafic hydrothermal systems, microbial diversity studies on terrestrial serpentinization-driven environments are rare (Tiago et al. 2012).

Due to their potential applications in biotechnological processes, there is renewed interest in alkaliphilic organisms. Many available enzymes do not withstand industrial reaction conditions. Thus, the characterization of microorganisms that are able to thrive in extreme environments has received a great deal of attention. Enzymes from microorganisms that can survive under extreme pH could be particularly useful for applications under highly alkaline reaction conditions.

To date, the microbial diversity of Manleluag Hyperalkaline Spring has not yet been explored. The study represents a unique opportunity to look for novel microorganisms suitable for scientific and economic exploitation through the phylogenetic analysis of bacterial and archaeal species as revealed by PCR- amplified 16S rRNA gene sequencing.

MATERIALS AND METHODS

Sample collection

Samples (MS1, MS2, MS3) of the upper sediment (10-20 cm) were collected from three different locations of the Manleluag Hyperalkaline Spring in Pangasinan, Philippines (15˚ 42' 12.71" N 120˚ 16' 56.96" E) using sterile scoopers and placed in sterile plastic bags. Samples were transported on ice and assayed immediately in the laboratory. The pH and temperature during the sampling were recorded. Composite sediment sample of MS1, MS2, and MS3 was analyzed at the Analytical Service Laboratory of the Agricultural Systems Cluster, University of the Philippines Los Baños, College, Laguna.

Extraction of Total Community DNA

The total community DNA was extracted from the composite sediment samples using PowerSoil®DNA Isolation Kit (MO BIO Laboratories, Inc., California, USA) following the manufacturer's instructions with modifications detailed below. For every replicate sample composed of 1 g sediment, three successive extractions were conducted and pooled following the protocol of Feinstein et al. (2009). Initial lysis, centrifugation and removal of supernatant containing crude DNA from the tube were followed by a second extraction involving the addition of new aliquots of bead solution and Solution C1 into the same tube. Subsequent centrifugation steps resulted in a second supernatant. New aliquots of bead solution and Solution C1 were again added to the same tube for a third extraction. Subsequent centrifugation steps resulted in a third supernatant. All supernatants obtained through successive extractions were combined for the final elution of DNA.

The concentration and purity of the extracted total community DNA was estimated by using NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Delaware, USA). The quality of the DNA was evaluated by measurement of the A_{260}/ A_{280} and the A_{260}/A_{230} ratios.

PCR Amplification of 16S rRNA Gene for Clone Library Construction

PCR amplification of nearly full length bacterial 16S rDNA was performed with the Bacteria–specific primer 27F(AGAGTTTGATCCTGGCTCAG) paired with universal primer 1492R (GGGTTACCTTGTTACGACTT) (Lane 1991). Amplification was carried out in a 25 µL reaction mixture with the following components: 1X PCR buffer (5 PRIME, Maryland, USA), 0.2M each dNTP, 1µM primer, 1mM $MgCl_2$, 1.25 U *Taq* DNA polymerase (5 PRIME, Maryland, USA), 0.8 % Dimethyl sulfoxide (DMSO), 0.4 µg/µL bovine serum albumin (BSA) and 3µL DNA template. PCR was performed using the Thermo Electron Px2 Thermal Cycle under the following conditions: one cycle of initial denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min and 30 s; and one cycle of final extension 72°C for 10 min with a holding temperature of 4°C.

Archaeal 16S rDNA was amplified with Archaea–specific primer pair 21F (TTCCGGTTGATCCYGCCGGA) and 958R (YCCGGCGTTGAMTCCAATT) (Delong 1992). Amplification was carried out in a 25 µL reaction mixture with the following components: 5X 2G Robust Hotstart Buffer (Kapa Biosystems, Massachusetts, USA), 0.2M each dNTP, 1 µM each primer, KAPA 2G Robust Taq polymerase (Kapa Biosystems, Massachusetts, USA) and 3 µL DNA template. PCR was performed using G-Storm™

GS1 (G-STORM, UK) under the following conditions: one cycle of initial denaturation at 95°C for 2 min; 30 cycles of denaturation at 94° C for 20 s, annealing at 53° C for 30 s and extension at 72°C for 4 min and one cycle of final extension 72°C for 10 min with a holding temperature of 4°C.

Construction of 16S rRNA Gene Clone Libraries and Preparation of Plasmid DNA from the Clones

The PCR products generated from total community DNA were cloned into a pGEMT easy vector (Promega, USA) and transformed into *Escherichia coli* JM109 (Promega, USA) following the manufacturer's instructions. Positive clones were picked by blue/ white selection. Recombinant plasmids from the libraries were extracted using alkaline lysis plasmid miniprep as described by Sambrook et al. (1989). Extracted plasmids with the correct insert were double digested using the following pairs of restriction enzymes: *Eco*RI- *Hind*III for archaeal clones and *Eco*RI – *Dra* I for bacterial clones in 20 µL reaction volume. After digestion, 8 ul of the digestion product was run in 1.5% agarose gel at 50V. The gel was stained with ethidium bromide and visualized using the BioRad Gel Documentation System (BioRad USA). Restriction enzyme digestion patterns of each library were grouped visually and representative clones were selected for sequencing.

Sequencing and Phylogenetic Analysis of 16S rRNA Gene Data

Recombinant plasmids from representative clones from each restriction pattern were submitted to Macrogen, Inc., Seoul, South Korea for Sanger sequencing using T7 primer. Analysis of DNA sequences and homology searches were completed using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov) against a nucleotide sequence database (blastn). The 16S rDNA sequences were placed within tentative taxa by determining the taxonomic class of the closest relative in GenBank of sequences that formed a phylogenetic clade. Multiple sequence alignments of 16S rDNA sequences were performed using ClustalW (Larkin et al. 2007). Evolutionary distances were calculated according to Kimura two-parameter correction method (Kimura 1980) and phylogenetic trees were constructed using neighbor-joining method (Saitou and Nei 1987) through MEGA 5 software (Tamura et al. 2011). Statistical significance of the tree branches was evaluated by bootstrap analysis based on 1000 bootstrap replicates. All sequences generated by this study have been deposited in the GenBank database (accession numbers KJ947121- KJ947155).

RESULTS AND DISCUSSION

Physicochemical Characteristics of Manleluag Hyperalkaline Spring

The measured temperature of the site was 35°C. The sediment was alkaline with a pH of 11.5 (Table 1). The sediment from the spring was classified as sandy loam and the clay, sand and silt content were found to be 3, 77, and 20%, respectively, with a relatively low amount (1.31%) of organic matter (Table 1). Some elements detected from the sediment include sodium, calcium and chlorine, while potassium, magnesium, iron, copper and manganese were not detected. The chemical composition data were similar with that of Cabeco De Vide in Portugal, an alkaline groundwater generated by active serpentinization, dominated by ions such as hydroxide, chloride, sodium, and calcium (Tiago et al. 2004). Similar geochemical characteristics have also been reported for hydrothermal carbonate vents of the Lost City near the Mid-atlantic Ridge (Kelley et al. 2005), alkaline borehole, South Africa (Moser et al. 2005), and the Samail ophiolite in Oman (Nicolas et al. 2000).

Table 1. Physicochemical characteristics of sediment sample obtained from Manleluag Hyperalkaline Spring, Pangasinan.

PARAMETERS	SEDIMENT
Temperature (°C)	35
pH	11.5
Textural classes (%)	
Clay	3
Sand	77
Silt	20
Organic matter (%)	1.31
Na (ppm)	29
Ca (ppm)	5
Cl (ppm)	83
K (ppm)	ND
Mg (ppm)	ND
Fe (ppm)	ND
Cu (ppm)	ND
Mn (ppm)	ND

ND - not detected

Construction of 16S rDNA Clone Libraries

Two 16S rRNA gene libraries resulted from the clones of archaeal and bacterial PCR products amplified both from the DNA obtained from the sediment of Manleluag Hyperalkaline Spring. Amplicons obtained from three separate amplifications were pooled prior to cloning. Pooling the PCR products will minimize PCR drift, a result of random events occurring in the early cycles of the reaction. In this case, separate PCR experiments in general do not produce biases towards the same group; thus, the bias will not be repeatable (Wagner et al. 1994).

Analysis of Bacterial 16S rRNA Gene Clone Library

Two 16S rRNA gene libraries resulted from the clones of archaeal and bacterial PCR products amplified both from the DNA obtained from the sediment of Manleluag Hyperalkaline Spring. A total of 122 clones were produced from PCR products generated using bacterial primer pair 27F- 1492R. The 16S rRNA gene inserts of the bacterial clones resulted in eight different groups based on the double restriction digestion patterns. Based on restriction digest patterns, three representatives from each unique group, except for the groups with only one or two representatives in the bacterial clone library, were selected, resulting in 18 bacterial clones selected for sequencing. Analysis of the bacterial clone library revealed that the 16S rDNA sequence types clustered with members of *Proteobacteria, Cyanobacteria, Firmicutes* and *Bacteroidetes* (Figure 1). Some of the sequences could not be assigned to any described taxon. Among the sequenced representative clones from the different restriction patterns, 72% were highly similar to sequences of known phylogenetic groups while 28% were described as unclassified environmental sequences (Table 2).

Sequence types related to the Alphaproteobacteria. BLAST analysis showed that clone SB25, SB27, and SB28 had highest hits (96% similarity) corresponding to *Rhodobacter* sp., originally isolated from a hydrothermal vent in slow/ultraslow-spreading Indian ridge (NCBI, FJ997595). In Manleluag Hyperalkaline Spring, the abundance of H_2 and organic compounds as electron donors and presence of CO_2 (Vargas et al. 2009) could support the growth of this group of organisms. Moreover, members of this group are mesophilic and moderately halophilic (Horikoshi 2011) allowing them to survive in environments like the Manleluag Hyperalkaline Spring with moderate temperature and with the presence of elements such as Na^+ and Cl^-.

Sequence types related to the Betaproteobacteria. Clones SB67, SB74 and SB79 shared 99% similarity and high bootstrap support (99%) with uncultured *Burkholderiales* bacterium (NCBI, EU449563) within the class *Betaproteobacteria* (Fig. 1) detected as a significant component of soil microbial communities (Ceja-Navarro et al. 2010) and low salinity aquatic ecosystems. The presence of *Betaproteobacteria* in this study is consistent with the results reported by Moser et al. (2005) from an alkaline environment.

Sequence types related to the Gammaproteobacteria. Clones SB65 and SB26 had the highest BLAST similarity with *Pseudomonas* sp. (99%) with 100% bootstrap support. The clones also clustered with members of the *Gammaproteobacteria* such as representatives from genera *Methylomicrobium* and *Halomonas* (Fig. 1). Genus *Pseudomonas* has been isolated from Mono Lake (California, USA) a saline- alkaline (pH 10) environment but it is not yet reported in serpentinization-driven environments.

Sequence types related to the Cyanobacteria. Clone SB7 grouped with members of the photosynthetic phylum *Cyanobacteria*, and corresponded to the 16S rDNA of an uncultured cyanobacterium clone (NCBI, FJ902618) with 94% similarity. The cyanobacteria in this serpentinizing habitat could be similar to the types of microorganisms linked to biological precipitation of carbonates. It has been reported that the potential sources of inorganic carbon in serpentinizing systems like Manleluag Hyperalkaline Spring include dissolved carbon dioxide in circulating groundwater, magmatic gases, and carbonate minerals (Abrajano et al. 1988). Thus, the presence of cyanobacteria-related sequences in the study site demonstrates the ability of this group of organisms to thrive in hyperalkaline pH environment driven by serpentinization.

Sequence types related to the Firmicutes. Clones SB94, SB93 and SB91 were found to be affiliated with members of the phylum *Firmicutes*, also called the low G+C Gram-positive Bacteria. The clones formed a common lineage with uncultured *Firmicutes* bacteria detected in sulfur-containing freshwater (NCBI, AB476673) and from subterranean high pH groundwater (NCBI, AM778006) associated with serpentinization (Tiago and Verissimo, 2012) (Fig. 1). The presence of *Firmicutes* in the bacterial community is consistent with the physiological adaptations of this group to high pH environments (Moser et al. 2005).

Sequence types related to the Bacteroidetes. Clone SB48 showed highest similarity (99%) to uncultured *Bacteroidetes* bacterium (NCBI, JF727707) from a saline-alkaline soil (Fig. 1). Sequences related to members of *Bacteroidetes* were detected in Cabeco de Vide aquifer in Portugal, a serpentinization-driven subterrestrial alkaline aquifer (Tiago and Verissimo 2012). This phylum has moderately and extremely halophilic members (Horikoshi 2011) that can possibly grow in Manleluag Hyperalkaline Spring due to the presence of Na^+ and Cl^-.

Unclassified environmental sequences. Clones SB80, SB99, and SB73 were found to be related to 16S rRNA genes of uncultured bacterium clone (NCBI, AM778016) recovered from subterrestrial high pH groundwater associated with serpentinization (Tiago and Verissimo 2012). Clones SB76 and SB106 were related to the 16S rDNA sequence of an uncultured bacterium clone from anaerobic digesters for animal waste treatment (NCBI, GQ139068). Based on phylogenetic analysis, all these unclassified clones formed a cluster with some uncultured members of the phylum *Bacteroidetes* (Fig. 1).

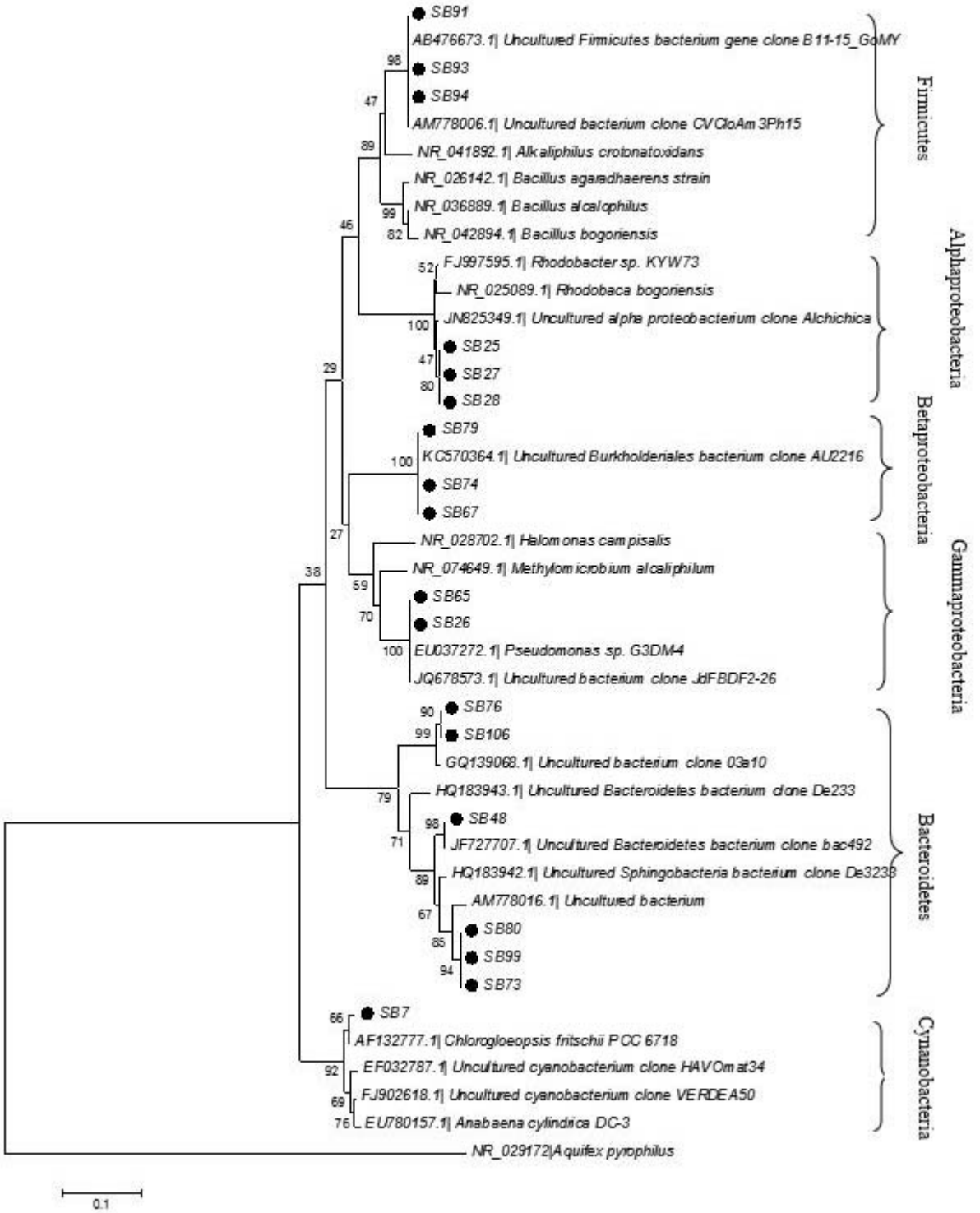

Figure 1. Phylogenetic tree showing the relationship among bacterial 16S rDNA sequences from Manleluag Hyperalkaline Spring in Pangasinan, Philippines with reference sequences obtained through BLAST analysis. The sequence alignment was performed using the CLUSTALW program and the tree was constructed using Neighbor joining with Kimura 2 parameter distances in MEGA 5 software. Bootstrap values (1000 replicates) are shown at the nodes. Sequences in shaded circles are obtained from the present study. *Aquifex pyrophilus* was used as an outgroup.

Table 2. Bacterial 16S rDNA sequences and their nearest phylogenetic affiliations.

CLONE NO.	ACCESSION NO. OF NEAREST NEIGHBOR	% SIMILARITY	NEAREST PHYLOGENETIC NEIGHBOR	PHYLOGENETIC GROUP
SB25	FJ997595	96%	*Rhodobacter* sp.	α-proteobacteria
SB27	FJ997595	96%	*Rhodobacter* sp.	α-proteobacteria
SB28	FJ997595	96%	*Rhodobacter* sp.	α-proteobacteria
SB7	FJ902618	94%	Uncultured cyanobacterium	Cyanobacteria
SB67	EU449563	99%	Uncultured Burkholderiales bacterium	β- proteobacteria
SB74	EU449563	99%	Uncultured Burkholderiales bacterium	β- proteobacteria
SB79	EU449563	99%	Uncultured Burkholderiales bacterium	β- proteobacteria
SB80	AM778016	91%	Uncultured bacterium clone CVCloAm3Ph5	unclassified
SB99	AB546008	91%	Uncultured bacterium gene for 16S rRNA	unclassified
SB73	JQ738993	92%	Uncultured bacterium clone SA_178	unclassified
SB65	EU037272	99%	*Pseudomonas* sp. G3DM-4	γ-proteobacteria
SB26	DQ205301	99%	*Pseudomonas* sp. HI-B17	γ-proteobacteria
SB48	JF727707	99%	Uncultured Bacteroidetes bacterium	Bacteroidetes
SB94	AB476673	99%	Uncultured Firmicutes bacterium clone: B11-15_GoMY	Firmicutes
SB93	AM778006	99%	Uncultured bacteriumclone CVCloAm3Ph15	Firmicutes
SB91	AM778006	99%	Uncultured bacteriumclone CVCloAm3Ph15	Firmicutes
SB76	GQ139068	96%	Uncultured bacterium clone 03a10	Unclassified
SB106	GQ139068	96%	Uncultured bacterium clone 03a10	Unclassified

Analysis of Archaeal 16S rRNA Gene Clone Library

The cloning of the PCR products generated from archaeal primer pair 21F – 958R produced 120 clones. Double enzyme restriction digestion revealed seven different digestion patterns. For sequencing, three representative clones were chosen from each unique restriction pattern, resulting in 21 clones selected for sequencing. Approximately 43% of sequenced clones were related to sequences from unclassified environmental clones and approximately 57% of the sequenced clones had relatively high levels of similarity (≥96%) with their closest counterparts in the database, mostly belonging to uncultured members of the phylum *Thaumarchaeota* and *Euryarchaeota* (Table 3).

Sequence types related to the Euryarchaeota. Euryarchaeota- related clones were mainly associated with methanogens as revealed by BLAST analysis (Table 3) specifically within the orders *Methanobacteriales*, *Methanomicrobiales,* and *Methanosarcinales*.

Sequences related to Methanobacteriales. Clones SA65, SA40, and SA70 exhibited high sequence similarity (98% - 99% similarity) to uncultured taxa (NCBI, AY695843 and DQ230925) and with 99% bootstrap support (Figure 2). Members of the order *Methanobacteriales* are distinguished from other methanogens by their limited range of catabolic substrates. They are generally hydrogenotrophic, using H_2 to reduce CO_2 to methane, although some members can use formate, CO or secondary alcohols as electron donors for CO_2 reduction. Moser et al. (2005) studied the warm, alkaline meteoric water emanating from a borehole intersecting quartzite-hosted fractures near a mining region in South Africa and

Table 3.Archaeal 16S rDNA sequenced clones and their nearest phylogenetic affiliations

CLONE NO.	ACCESSION NUMBER OF NEAREST NEIGHBOR	% SIMILARITY	NEAREST PHYLOGENETIC NEIGHBOR	PHYLOGENETIC GROUP
SA83	AB236067	99%	Uncultured *Methanomicrobia* archaeonclone:SP-ProM-B	Euryarchaeota
SA1	AB236067	99%	Uncultured *Methanomicrobia* archaeonclone:SP-ProM-B	Euryarchaeota
SA2	AB236067	98%	Uncultured *Methanomicrobia* archaeonclone:SP-ProM-B	Euryarchaeota
SA65	AY695843	99%	*Methanobacteriium* sp. SA-12	Euryarchaeota
SA40	DQ230925	98%	Uncultured *Methanobacterium* sp. clone uNO27FW100501SAB122	Euryarchaeota
SA70	AY695843	99%	Uncultured *Methanobacteria* sp. SA-12	Euryarchaeota
SA15	AB355126	99%	Uncultured archaeon gene clone Hados.Sedi.Arch.5	unclassified
SA32	AB355126	99%	Uncultured archaeon gene clone Hados.Sedi.Arch.5	unclassified
SA39	AB355126	99%	Uncultured archaeon gene clone Hados.Sedi.Arch.5	unclassified
SA41	JQ668660	96%	Uncultured archaeon clone OTU-G1-5 16S	unclassified
SA66	JQ668660.	96%	Uncultured archaeon clone OTU-G1-5 16S	unclassified
SA67	JQ668660.	96%	Uncultured archaeon clone OTU-G1-5 16S	unclassified
SA82	HQ678247	99%	Ammonia oxidizing archaeon clone FQ-LF-Archaea-OTU3-S1-R4	unclassified
SA84	HQ678247	99%	Ammonia oxidizing archaeon clone FQ-LF-Archaea-OTU3-S1-R4	unclassified
SA85	HQ678247	99%	Ammonia oxidizing archaeon clone FQ-LF-Archaea-OTU3-S1-R4	unclassified
SA117	AB161339.1	99%	Uncultured archaeon gene for 16S rRNA, partial sequence,clone:ASC40	unclassified
SA118	JN562349	99%	Uncultured archaeon clone SEAA1BE121	unclassified
SA119	JN562349	99%	Uncultured archaeon clone SEAA1BE121	unclassified
SA68	AJ133791	99%	*Methanosaeta* sp Clone A1	Euryarchaeota
SA74	EF420177	99%	Uncultured Methanosaetaceae archaeon clone 18-2B	Euryarchaeota
SA94	AJ133791	99%	*Methanosaeta* sp. Clone A1	Euryarchaeota

reported the presence of sequences related to the genus *Methanobacterium* whose presence possibly could be linked to the abundance of H_2. The high levels of H_2 in Manleluag Hyperalkaline Spring could possibly support members of genus *Methanobacterium.* The characteristics of the study site such as having moderate temperature, presence of hydrogen as product of serpentinization, presence of Na^+ and Cl^-, and alkaline environment allow the growth of this group of organisms.

Sequences related to the Methanomicrobiales. Clones SA83, SA1, and SA2 were found to be closely affiliated to the 16S rRNA gene sequence of an uncultured archeon (AB236067) having 98% - 99% similarity and supported

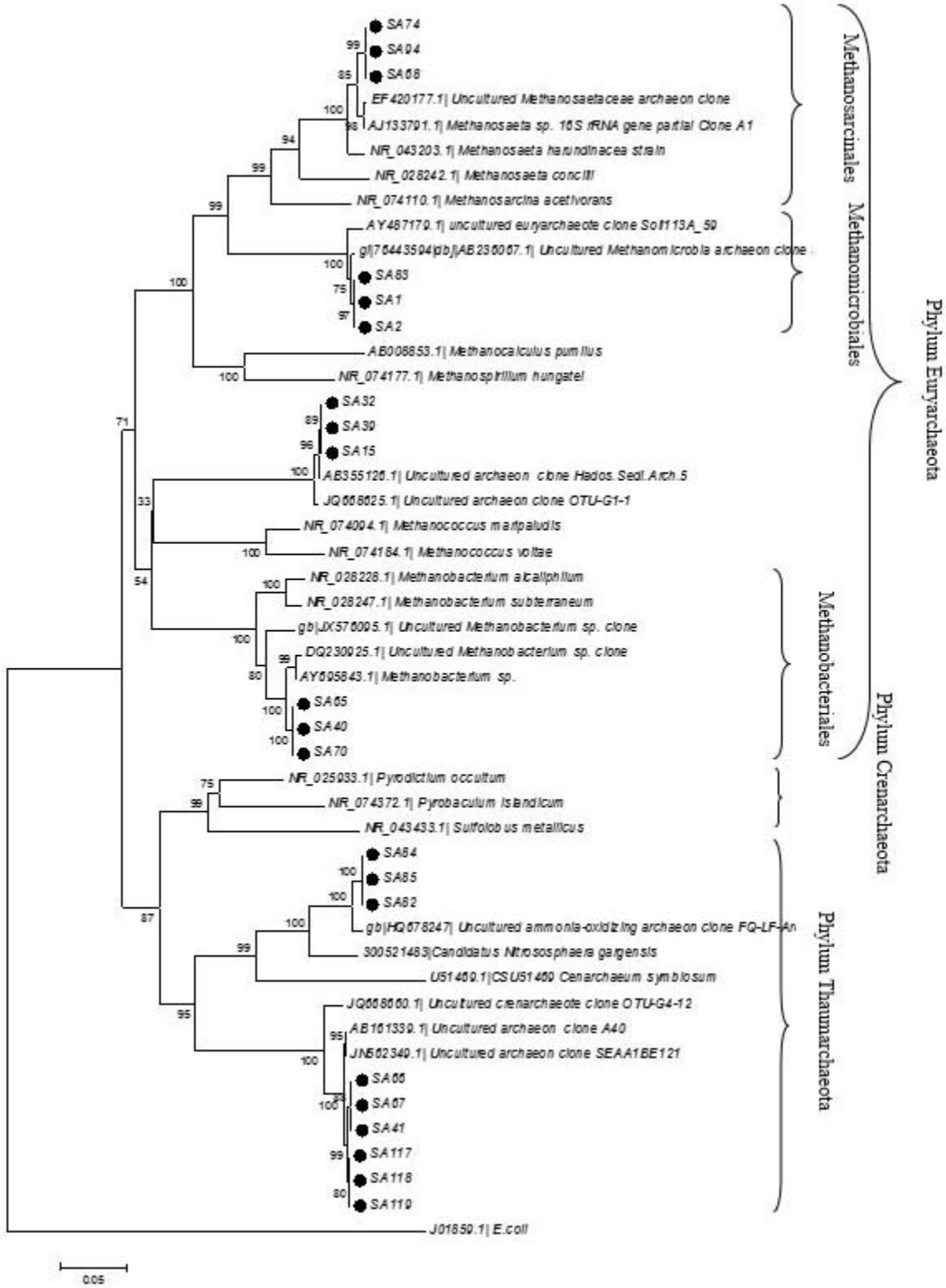

Figure 2. Phylogenetic tree showing the relationship among archaeal 16S rDNA sequences from Manleluag Hyperalkaline Spring in Pangasinan, Philippines with reference sequences obtained through BLAST analysis. The sequences alignment was performed using the CLUSTAL W program and the tree was constructed using Neighbor joining with Kimura 2 parameter distances in MEGA 5 software. Bootstrap values (1000 replicates) are shown at the nodes. Sequences in shaded circles are obtained from the present study. *Escherichia coli* was used as an outgroup.

by bootstrap value of 100 (Fig. 2). *Methanomicrobiales* can use H_2 and CO_2 as substrates for methanogenesis although many species can also utilize formate and alcohols (Garcia et al. 2006). The Manleluag Hyperalkaline Spring appears to be limited in electron acceptors like oxygen because of its highly reducing nature (Vargas et al. 2009). However, CO_2 and H_2 are present which favor the metabolism of this group of organisms.

Sequences related to the Methanosarcinales. Clones SA68, SA74, and SA94 had the highest similarity (99%) to *Methanosaeta* sp. clones (NCBI, AJ133791 and EF420177). The phylogenetic placement of these sequences clustering with *Methanosaeta harundinacea* (NCBI, NR_043203) strain was supported by very high bootstrap support of 100% (Fig. 2) and hence these clones most likely belong to family *Methanosaetaceae* of the order *Methanosarcinales*. The family *Methanosaetaceae* contains a single aceticlastic genus *Methanosaeta* that all grow by the aceticlastic reaction and acetate (from the fermentation of hydrolysed organic matter) serves as the only substrate for methanogenesis (Boone et al. 1993). Analysis of the sediment sample revealed the presence of low amounts of organic matter, possibly contributed by the surrounding vegetation.

The relatively low amount of organic matter may be due to its conversion to simpler substrates that can be utilized by methanogens. The aceticlastic genus *Methanosaeta* has also been detected in the Lost City hydrothermal field (Brazelton et al. 2011). The 60 to 75°C water venting from the Lost City carbonate towers is similar to Manleluag Hyperalkaline Spring in that it has a high pH (9 to 11), high concentrations of abiogenic CH_4, and elevated H_2 concentrations produced through serpentinization, although the Manleluag site has a lower temperature of 35°C. The presence of Methanosarcinales-related sequences is consistent with anaerobic microbial metabolisms linked to serpentinization processes that produce high amounts of hydrogen, methane and low molecular weight organic compounds (McCollom and Seewald, 2001).

Unclassified archaeal sequences. Four different groups of restriction patterns were considered unclassified environmental archaeal clones based on BLAST analysis. The first group composed of clones SA15, SA32, and SA39 were found to be related (99% similarity) to the 16S rDNA sequence of uncultured archaeon clone (NCBI, AB355126) obtained from Manzala Lake in Egypt with neutral to alkaline pH (Elsaied and Maruyama 2008). In the second group, clone SA117 was found to be affiliated with uncultured archaeon clone (NCBI, AB161339) recovered from petroleum-contaminated soil (Kasai et al. 2005). Meanwhile, clones SA18 and SA119 shared 99% similarity with uncultured archaeon clone (NCBI, JN562349) recovered from a constructed wetland. The third group comprising clones SA41, SA66, and SA67 had 96% similarity to an uncultured archeaon clone (NCBI, JQ668660) from anaerobic digestion of sewage. Based on phylogenetic analysis, clones SA41, SA66, SA67, SA117, SA118, and SA119 clustered with uncultured Crenarchaeote clone (NCBI, JQ668660) (Fig. 2). In the last group, clones SA82, SA84 and SA85 showed 99% similarity to an ammonia-oxidizing clone (NCBI, HQ678247) from agricultural soils.

It is important to note that the clones SA82, SA84, SA85, SA41, SA66, SA67, SA117, SA118, and SA119 clustered together with *Candidatus Nitrososphaera gargensis* and *Cenarcheum symbiosum*, both members of the newly proposed phylum *Thaumarchaeota.*

It was apparent in the phylogenetic tree (Fig. 2) that the cluster formed was phylogenetically distinct from the members of thermophilic Crenarchaeota. Thus the clones mentioned could be members of the phylum *Thaumarchaeota* which were previously considered within the mesophilic *Crenarchaeota.* The temperature (35 °C) of the study site makes it a likely habitat that could harbor mesophilic Crenarchaeota. The clustering of some clones to members of phylum *Thaumarchaeota* suggest the presence of putative ammonia-oxidizing Archaea within the hyperalkaline spring indicating that these organisms are ecologically relevant members of this community (Buckley et al. 1998).

The hydration of peridotites through serpentinization in the Zambales Ophiolite, which includes Manleluag Hyperalkaline Spring, contributes to the gas compositional signatures at the site (Abrajano et al. 1990). This process creates strongly reducing conditions and produces fluids that are highly enriched in molecular hydrogen and methane (McCollom and Seewald 2013). The presence of this energy source in many serpentinizing environments is reflected in the microbial composition obtained through genomic and cultivation methods (Perner et al. 2010; Brazelton et al. 2012). The impact of serpentinization upon fluid chemistry imposes a unique set of conditions upon the organisms and biological activities operative in such ecosystem. Serpentinizing environments are typically rich in electron donors but the availability of terminal acceptors is frequently limited. In addition, the microbial communities in serpentinizing ecosystems face physiological challenges in terms of high pH and low concentrations of dissolved inorganic carbon (Schrenk et al. 2013). A common observation in the most extreme alkaline fluids at many sites includes low cell abundances and low taxonomic diversity (Schrenk et al. 2004; Tiago et al. 2004). For both bacterial and archaeal library, most cloned sequences have low sequence identity to previously identified sequences and may represent new taxa that have

not been previously cultured. Most of the prokaryotic populations detected in the Manleluag Hyperalkaline Spring had highest phylogenetic similarities with uncultured environmental sequences from diverse types of environments. Some of these source environments have neutral pH while some have alkaline pH with no serpentinization activity. The same observation was reported by Tiago and Verissimo (2012) in a study involving a subterranian high pH groundwater associated with serpentinization.

Relying on 16S rDNA sequences to ascribe function assumes that phylogenetically related populations have conserved functional properties and that function of novel organisms can be inferred by comparison with species that have been previously cultivated and characterized (Gray and Head 2001). In addition, rDNA clone libraries may not represent a complete picture of the microbial community (Janssen 2006). The limitations of these assumptions have been recognized (Janssen 2006) and a cautious approach is therefore indicated. Nevertheless, this study represents the first DNA-based analysis of this unique environment, and the results provide baseline information on the dominant bacterial and archaeal populations in Manleluag Hyperalkaline Spring.

CONCLUSION

The strategy of total community DNA extraction from the sediment and sequence analysis of cloned 16S rRNA genes enabled the detection of archaeal and bacterial sequence types from the extreme environment of Manleluag Hyperalkaline Spring. Although the populations revealed in this study may not represent the totality of the prokaryotic community in this spring, the results suggest the type of metabolisms that drive this specific environment which include H_2 and methane metabolisms. Further studies on the microbial community of the Manleluag Hyperalkaline Spring may help to understand the role of these organisms in this serpentinization-driven system and reveal novel microbes with unique properties that may have important industrial applications.

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