RESEARCH ARTICLE

Microbial diversity in nonsulfur, sulfur and iron geothermal steam vents

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Abstract

Fumaroles, commonly called steam vents, are ubiquitous features of geothermal habitats. Recent studies have discovered microorganisms in condensed fumarole steam, but fumarole deposits have proven refractory to DNA isolation. In this study, we report the development of novel DNA isolation approaches for fumarole deposit microbial community analysis. Deposit samples were collected from steam vents and caves in Hawaii Volcanoes National Park, Yellowstone National Park and Lassen Volcanic National Park. Samples were analyzed by X-ray microanalysis and classified as nonsulfur, sulfur or iron-dominated steam deposits. We experienced considerable difficulty in obtaining high-yield, high-quality DNA for cloning: only half of all the samples ultimately yielded sequences. Analysis of archaeal 16S rRNA gene sequences showed that sulfur steam deposits were dominated by *Sulfolobus* and *Acidianus*, while nonsulfur deposits contained mainly unknown *Crenarchaeota*. Several of these novel *Crenarchaeota* lineages were related to chemoautotrophic ammonia oxidizers, indicating that fumaroles represent a putative habitat for ammonia-oxidizing Archaea. We also generated archaeal and bacterial enrichment cultures from the majority of the deposits and isolated members of the *Sulfolobales*. Our results provide the first evidence of Archaea in geothermal steam deposits and show that fumaroles harbor diverse and novel microbial lineages.

Introduction

Fumaroles occur when steam and volcanic gases escape through the Earth's crust as a result of magma degassing and/or geothermal heating of groundwater at a shallow depth (Ellis *et al.*, 2008; Costello *et al.*, 2009). As steam exits a vent or a cave, minerals are formed along the cooler walls and ceilings as 'steam deposits.' These types of steam environments are abundant in fumaroles and steam deposits are found in multiple oxidation states that represent potential nutrient availability for microorganisms. The high metal content, combined with the steam deposits exposed to constant high-temperature steam and gases (60–95 °C), make this environment interesting as both an extremophile habitat and a potential model for extraterrestrial life. However, the range of fumarole conditions extends from mild to extreme. Mild fumaroles have lower temperatures of 60–70 °C, pH 4–6, only a small metal content and lower steam flow. We sampled and analyzed chemically distinct fumaroles with cave orientations: the nonsulfur caves examined were both mild (~65 °C) and extreme (93 °C) in temperature, while both sulfur and iron cave/vents examined were extreme in temperature (> 82 °C). The metal content was low for the sulfur, but high for the iron cave/vents. Naturally, the more extreme conditions make diversity studies particularly challenging. Despite their ubiquity in geothermal environments, very few studies have explored the microbial diversity associated with fumaroles and most of these have focused on thermally heated soils surrounding vents (Mayhew *et al.*, 2007; Stott *et al.*, 2008; Costello *et al.*, 2009). So far, there have been no studies of Archaea and only one study of Bacteria in steam deposits (Mayhew *et al.*, 2007).

Archaea have been isolated, and DNA extracted, from fumarole aerosols (Ellis *et al.*, 2008), but despite considerable effort by a number of research groups, archaeal DNA has proven extremely difficult to obtain from steam deposits (Mayhew *et al.*, 2007). Previous studies have expended considerable effort extracting and PCR-amplifying DNA from steam (Ellis *et al.*, 2008). However, fumarole deposits

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are particularly recalcitrant to such procedures (Herrera & Cockell, 2007). Two factors appear to complicate DNA extraction from these deposits: acidity and substrate–DNA interactions (Henneberger *et al.*, 2006). Several studies have reported applying multiple alternative DNA extraction methods to isolate DNA from steam deposits, but with poor and inconsistent results (Henneberger *et al.*, 2006; Herrera & Cockell, 2007; Ikeda *et al.*, 2008). Indeed, several research groups ceased their attempts to extract archaeal DNA and moved on to extracting eukaryote and bacterial DNA because of the technical difficulties associated with DNA extractions (Ackerman *et al.*, 2007; Mayhew *et al.*, 2007). Nevertheless, Archaea are likely to persist in fumarole deposits as they are found living at extremely high temperatures, low pH and in chemically rich hot spring sediments (Brock & Mosser, 1975).

In this study, we collected steam deposits from fumaroles in three geothermal regions: Hawaii Volcanoes National Park (HAVO), Yellowstone National Park (YNP) and Lassen Volcanic National Park (LVNP). The deposits included three basic types commonly found around fumaroles: nonsulfur, sulfur and iron-type deposits. Over the course of the study, we developed novel environmental DNA recovery approaches to extract DNA from steam deposits and successfully PCR-amplified and cloned archaeal and bacterial 16S rRNA gene sequences. We also culture enriched Archaea and Bacteria from steam deposits in all three geothermal regions.

We sampled steam caves and vents where steam comes into direct and continuous contact with the cave ceiling or the vent surface, causing several types of steam-transported elements, molecules and gases to form deposits at the site of contact as a result of the following processes: (1) steam condenses to water and water evaporates, leaving behind salts of various types, nutrients such as sulfates, nitrates and other ions in the form of *evaporites*; (2) iron as Fe(II) oxidizes to Fe(III) and *precipitates* as iron oxides, hydroxides, oxyhydroxides or complex iron minerals; (3) volcanic gases such as H_2S and SO_2 rise and become less soluble in steam and deposit as solids known as *sublimates* as they contact cave or vent surfaces and are oxidized into sulfur *deposits*; (4) *particulates* carried by the flow of steam and gasses, passing over the cave/vent surface, are held by entrapment or interactions with the surface matrix. These may be represented by biofilms or minerals detached from subsurface steam passageway walls; (5) *Sediments* form as a result of erosion, transport and settling. While this process is commonly associated with flowing hot springs and bubbling pools, it does not take place in a fumarolic environment. Throughout this paper, we refer to the sampled material formed by fumaroles by a single term, steam deposits. We selected this term because it correctly refers to the first four processes described above, but it does not describe or include sediments or sedimentation.

Materials and methods

Study sites and sample collection

Steam deposit samples were collected from HAVO, YNP and LVNP between 2007 and 2009. An example of a nonsulfur Hawaiian fumarole is depicted in Fig. 1. Table 1 lists the geographical locations, global positioning system coordinates, temperature, pH and sample types with reference to the dominant chemistry for each site. Sites were chosen on the basis of both measured temperatures and visible differences in substrates to obtain selected chemical and physical sites. (The chemistry of the steam deposits was confirmed with X-ray microanalysis as well as wet chemical analysis and spectroscopy.) The final selection was also based on the physical features in which steam 'caves' were sampled. Figure 2 shows six of the steam vents sampled in the study that represent the various vent morphologies sampled. All the sites were essentially artesian in nature and were chosen because aerial contamination of the artesian steam sources was precluded by continuous steam flow through their lateral exit portals. To minimize contamination, only the ceilings of the caves were sampled unless it was clear that the floor and ceiling received continuous, positive flow and equal exposure

Fig. 1. Hawaii 1 nonsulfur cave, HAVO area. Arrow marks cave opening. The sample was taken from the cave ceiling at a distance of 1 m inside the cave.

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to the steam vapors. This occurred only in the Amphitheater Springs steam cave (Fig. 2c), which formed a lateral–conical ceiling. Steam deposits were also collected from cave-free steam vents (e.g. Fig. 2e) in which steam deposits were formed in the openings and around the entrance where oxygen and cooler temperatures are encountered.

Table 1. Steam deposit sampling locations and physical conditions

Location	Latitude/longitude	Temperature (°C)*	pH	Type
HAVO[†]				
Hawaii 1 (H1)	ND	65.0	6.4	Nonsulfur cave
Hawaii 2 (H2)	ND	67.0 ± 0.6	6.9	Nonsulfur cave
Hawaii 3 (H3)	ND	82.0	3.1	Sulfur cave
Hawaii 4 (H4)	ND	82.0	3.1	Iron/sulfur cave
YNP[‡]				
Amphitheater Springs (AS)	44°48′1.4″N, 110°43′41.9″W	92.0 ± 0.4	4.8	Sulfur cave
Norris Geyser Basin (NGB1)	44°43′44.1″N, 110°42′19.0″W	61.0	4.5	Nonsulfur cave
Norris Geyser Basin (NGB2)	44°43′37.1″N, 110°42′12.6″W	93.0	5.5	Iron vent
Roaring Mountain (RM1)	44°46′45.9″N, 110°44′18.6″W	93.0 ± 0.1	4.8	Nonsulfur cave
Roaring Mountain (RM2)	44°46′46.2″N, 110°44′18.8″W	91.0 ± 0.8	4.8	Sulfur vent
LVNP[§]				
Sulphur Works (SW1)	40°26′58.50″N, 121°32′07.08″W	92.0 ± 0.1	5.0	Nonsulfur cave
Sulphur Works (SW2)	40°26′59.1″N, 121°32′06.18″W	93.0 ± 0.8	4.0	Sulfur vent
Sulphur Works (SW3)	40°26′59.88″N, 121°32′05.34″W	91.2 ± 1.0	4.2	Sulfur cave
Sulphur Works (SW4)	40°27′16.98″N, 121°32′7.26″W	90.0	3.0	Iron vent
Sulphur Works (SW5)	40°27′17.40″N, 121°32′07.92″W	84.0 ± 2.8	2.5	Iron cave

*Averages and SDs are reported for vents in which three or more measurements were taken at least 3 months apart between 2005 and 2009.
[†]Hawaii Volcanoes National Park, Hawaii.
[‡]Yellowstone National Park, Wyoming.
[§]Lassen Volcanic National Park, California.
ND, not determined.

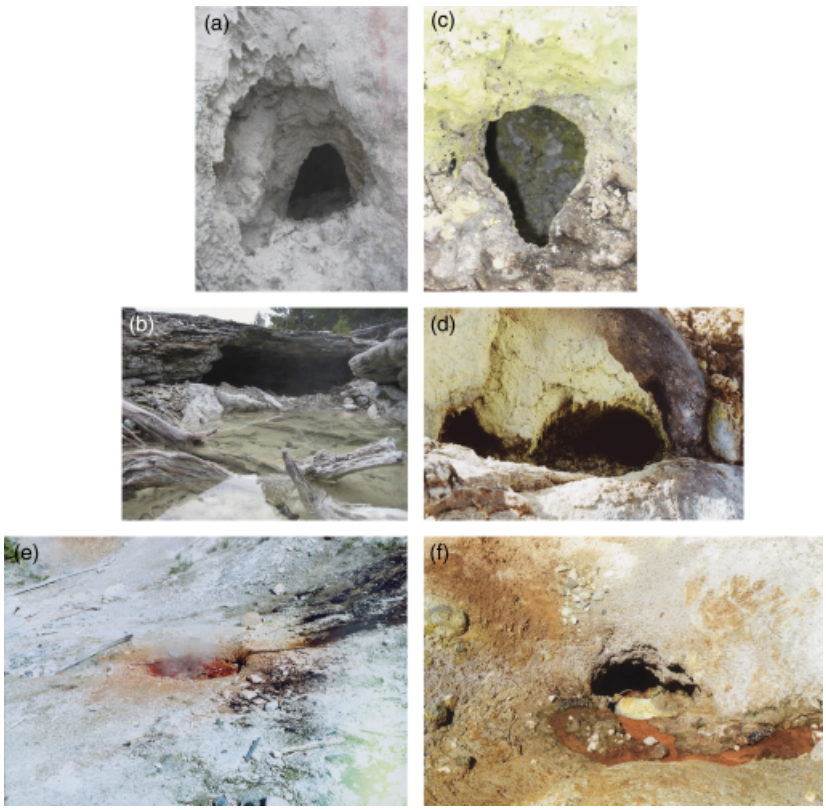

Fig. 2. Examples of vent and cave sample sites, ranging in size. (a) Roaring Mountain, YNP, nonsulfur steam cave, (b) Norris Geyser Basin, YNP, nonsulfur cave, (c) Amphitheater Springs, YNP, sulfur cave, (d) Sulphur Works, LVNP, sulfur cave, (e) Norris Geyser Basin, YNP, iron vent, (f) Sulphur Works, LVNP, iron cave.

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Approximately 1 cm^2 samples were collected from inner cave surfaces that were in direct and prolonged contact with rising steam. The samples were collected by scraping the surface using an extension pole holding a sterile 50-mL polypropylene screw-cap tube. Sampling depth was minimized to between 0.5 and 1 cm. Only the sterile lip of the sample collection tube was allowed to contact the surface inside of the steam cave. In some instances, a sterile spoon was used if the opening was too narrow to accommodate the 50-mL tube and holder or if samples were collected from cave-free steam vent deposits. Each spoon-collected sample was immediately transferred into a 50-mL sterile tube. The sample tube was capped following sample collection, chilled on ice and returned to the laboratory. Samples for cloning were frozen at –20 °C within 48–60 h of collection and samples for cultivation were left at room temperature. Inoculations into enrichment medium were completed within 5 days of sample collection.

Chemical analysis

In preparation for chemical analysis, samples of each steam deposit were extracted with water and separately with 0.1 N HCl. Extractions were prepared by placing a 1 : 5 (w/w) slurry of 2 g steam deposit in 10 mL Milli-Q ultrapure water (or 0.1 N HCl) in a sterile screw-cap 15-mL polypropylene tube. The tube was then shaken on a rotator (180 r.p.m.) for 60 min at 23 °C. The water or the HCl extract was filtered through a 0.22-μM filter (Millipore Corp., Billerica, MA) and analyzed. A Perkin Elmer Inductively Coupled Plasma Optical Emission Spectrometer (Optima DV 4300) was used to measure alkaline earth metals and selected elements as described previously (Mathur *et al.*, 2007). Nutrients were analyzed using standard methods of analysis scaled to 96-well flat-bottomed microtiter dishes (Mathur *et al.*, 2007). The conductivity and pH of the water extract were measured on Millipore filtered (0.2 μm) samples.

Energy-dispersive X-ray spectroscopy

For solid-state elemental analysis, samples of steam deposits were adhered to a 12-mm double-stick carbon conductive disc (Ted Pella, Redding, CA). Samples were analyzed at a working distance of 12 mm using a Hitachi S-2700 scanning electron microscope operated at 20 kV with an Oxford Instruments X-ray microanalyzer and INCA software (Oxfordshire, UK) to obtain solid surface information by energy-dispersive X-ray spectroscopy. A minimum of 1 00 000–3 00 000 X-ray counts was used to perform each analysis. Multiple scans were performed on each sample to obtain a representative X-ray spectrum from 0 to 20 keV.

Environmental DNA extraction and amplification

Before all DNA extraction attempts, samples were neutralized by adding 1 mL of 0.5 M Tris, pH 8.2, to approximately 0.75 g of each steam deposit sample (0.5–1.0 g of steam deposit was extracted for DNA in duplicate), followed by a wash of 10 mM Tris, pH 8.2 (Siering *et al.*, 2006). A number of DNA isolation approaches were attempted to isolate environmental DNA from the pH-neutralized steam deposit sample (0.75 g), which was added directly to the tubes. These methods included: (1) the UltraClean® Soil DNA Isolation kit (MoBio Laboratories Inc.), using the maximum yield protocol; (2) the PowerSoil® DNA Isolation kit and protocol (MoBio Laboratories Inc.); and (3) the NucleoSpin® Plant II kit and protocol (Macherey-Nagel GmbH & Co. KG, Germany), which included an extraction protocol for DNA from soil with cetyl trimethylammonium bromide as a detergent and polyvinylpyrrolidone for protection of the DNA in the extraction buffer. Following the failure to obtain sufficient DNA yields with any of the various kit protocols, we modified the methods by adding skim milk or other protein sources to the extraction protocol. Skim milk has been demonstrated to increase the DNA yields from volcanic soils (Ikeda *et al.*, 2008). To test the effect of complex proteins as DNA-binding competitors to increase yields from steam deposits, we followed the PowerSoil® DNA Isolation kit protocol while adding 50 mg of the following four dry substances to the bead-beating step: (1) Powdered nonfat skim milk (Carnation); (2) Casein hydrolysate (Difco); (3) Protein 1, Veggietones GMO-Free soya peptone; and (4) Protein 2, Veggietones vegetable peptone no. 1 (Oxoid Ltd, Basingstoke, UK). All four protein additives were UV-treated as follows: the protein powder was shaken on a sterile plastic Petri dish with the top removed in a biosafety cabinet. Then the plate was placed (cover-free) in an Ultralum MultiLinker UVC 515 cabinet and irradiated for 10 min (254 nm wavelength; 1.2×10^5 μJ cm^{-1}). The powder was then dispersed randomly around the dish and irradiated for another 10 min. The top of the Petri plate was replaced to ensure continued sterility and the protein powder was weighed on a UV-sterilized weighing paper using aseptic methods. To test for contamination of the protein additives, we performed negative extraction control experiments on each of the four additives by adding each one to a PowerSoil® bead-beating tube. A NanoDrop™ spectrophotometer was used to determine the yield and purity of DNA extractions for all the methods tested following the example of Urakawa *et al.* (2010).

After determining the contamination-free DNA extraction method with the highest yield, UltraClean, 11.96 ± 2.1 ng μL^{-1} and PowerSoil/Protein 1, 11.28 ± 4.6 ng μL^{-1}, we carried out PCR amplification of archaeal and bacterial 16S rRNA gene sequences from the community DNA. The primers used

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to amplify archaeal 16S rRNA gene were 21Fa (5′-TTCCGGTTGATCCYGCCGGA-3′) and 915Ra (5′-GTGCTGCCCCGCCAATTCCT-3′). The bacterial primers used were 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 'universal' 1492R (5′-GGYTACCTTGTTACGACTT-3′). The PCR conditions were as follows: an initial denaturing step of 95 °C for 10 min, followed by 29 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 90 s. There was a final extension of 72 °C for 20 min with storage at 4 °C until removal from the thermocycler. The resulting PCR products were run on a 1% agarose gel (150 V for 1 h). Negative controls were also screened for contaminants. PCR samples that displayed positive bands at the appropriate marker (~900 bp for Archaea and ~1500 bp for Bacteria) were cleaned using an UltraClean™ PCR Clean-up Kit (MoBio Laboratories Inc.) as per the manufacturer's protocol and quantified using a NanoDrop™ spectrophotometer.

Clone library and phylogenetic analysis

Purified PCR products were cloned using a TOPO-TA Cloning Kit® (Invitrogen) following the manufacturer's instructions. Thirty positive (white) colonies were picked from each clone library and screened via PCR for inserts of the correct size using M13 primers (~900 bp for Archaea and ~1500 bp for Bacteria). M13 PCR products were then run on a 1% agarose gel (150 V for 1 h) and the PCR products of positive clones were cleaned for sequencing using an UltraClean™ PCR Clean-up Kit (MoBio Laboratories Inc.). Purified DNA (approximately 7–17 ng μL^{-1}) was then sent to the San Diego State University MicroChemical Core Facility for sequencing with both forward and reverse M13 primers.

Sequence chromatograms were quality checked and the ends were trimmed manually to lengths somewhere between 500 and 600 nucleotides. The complementary strands were then edited and aligned using the automated base-calling and alignment programs PHRED and PHRAP (http://www.phrap.com) and submitted to NCBI BLAST through the XPLORSEQ program (Frank, 2008). The final edited and aligned archaeal and bacterial sequences were identified taxonomically using the naïve Bayesian rRNA Classifier (Wang *et al.*, 2007) in the Ribosomal Database Project (RDP) version 10 (Cole *et al.*, 2005). Because all the archaeal sequences appeared to belong to the *Crenarchaeota*, to determine the relationship of our cloned archaeal sequences with cultured organisms, we downloaded all the available full-length cultured *Crenarchaeota* sequences from the RDP 10 database so that they could be included in a multiple sequence alignment for phylogenetic analysis. We also downloaded a set of uncultured *Crenarchaeota* that matched our sequences based on the RDP classifier analysis. Cloned steam deposit sequences and the RDP 10 downloaded sequences were aligned using GREENGENES (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) and imported into the ARB small-subunit rRNA sequence database of 16S rRNA gene sequences for Archaea (Ludwig *et al.*, 2004). A NEXUS-formatted text file containing the multiple sequence alignment of our unknown archaeal sequences, as well as the cultured and uncultured *Crenarchaeota* sequences, was exported from ARB for phylogenetic analysis.

Phylogenetic analysis was performed using the PAUP* program (Swofford, 2003). Both maximum parsimony (MP) and a neighbor-joining (NJ) analysis were performed to find the best tree and statistical support was determined using bootstrap resampling under MP and NJ criteria. We searched for the shortest MP tree using a random addition sequence heuristic search strategy with 100 replicates. The maximum number of trees kept during each search was limited to 1000. For the MP bootstrap analysis, 100 bootstrap replicates were performed and each replicate consisted of a heuristic search with 10 random addition sequence heuristic search replicates. We also performed an NJ analysis and an NJ bootstrap analysis with 1000 replicates.

Steam deposit culture

Medium components, including nutrients, pH, temperature and duration of incubation, were selected to optimize microorganism growth (Ellis *et al.*, 2008). Given the shallow sampling depth and the Eh measurements of condensed steam, which were highly positive in the three vents tested (Table 2), we used aerobic conditions for the initial culturing attempts. Cultures were established with the addition of environmental steam deposits into 10 mL of Brock's medium, which included 100 μL of a 100× stock (7.5 mM) iron citrate, 0.1% yeast extract (Difco, BD), prepared as a 10% solution. Inoculations consisted of the addition of 1–5% steam deposit (0.1–0.5 g) per 10 mL medium for each vial (Brock, 1978). The vials were incubated at 55 and 70 °C. Although these temperature conditions are low relative to the steam temperature, we have used these temperatures in the past to obtain cultures from condensed steam (Ellis *et al.*, 2008). The cultures were checked for evidence of growth every day over the course of several months. Subcultures were also set up using the same conditions after growth was evident.

Archaeal colonies were grown on Gelrite overlays (0.4%/0.8%) prepared by pouring 0.4% Gelrite medium over the surface of a 0.8% Gelrite medium plate already inoculated. The colonies were always found growing intermixed with Bacteria. The same medium conditions (as described previously) were used to prepare the solid medium Brock plates, except that the 0.4%/0.8% solid medium also contained 0.2% glucose, 0.1% dextran T10 and 0.1% tryptone. To determine the identity of the isolates, DNA was extracted from selected colonies or liquid cultures using the lysozyme extraction protocol. The enzymatic lysis buffer [20 mM Tris,

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Table 2. Steam deposit chemistry based on ICP and nutrient analysis

Analyte (mg L^{-1})	HI1	HI2	HI3	NGB1	NGB2	RM1	RM2	AS	SW2	SW5
Na	3.454	3.463	3.360	3.361	9.070	2.421	2.356	3.517	2.356	3.453
Ca	2.285	3.592	4.205	2.069	17.558	0.596	0.762	2.147	1.240	2.870
Al	1.024	1.082	4.052	1.106	26.467	4.432	3.877	1.184	8.431	2.006
Fe (total)	0.975	0.998	2.755	1.062	37.986	3.084	0.609	1.067	1.674	0.997
Si	18.579	29.222	21.326	41.999	13.579	19.311	9.433	18.536	9.733	25.706
B	0.697	0.700	0.705	0.783	20.067	2.332	1.597	0.806	1.036	0.760
K	2.864	2.519	2.510	2.635	0.393	0.371	0.377	2.680	0.367	3.401
Mg	0.119	1.434	1.892	0.00	4.528	0.0245	0.122	0.0223	0.256	0.945
Zn	0.0147	0.0195	0.0315	0.000446	0.156	0.0598	0.0170	0.0328	0.0619	0.0362
Mn	0.00604	0.00646	0.0532	0.00209	0.666	0.00	0.000748	0.00625	0.0157	0.0256
Mo	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Se	0.0289	0.0220	0.0242	0.0273	0.0104	0.0171	0.0241	0.0230	0.0191	0.0251
Ni	0.00	0.00	0.00	0.00	0.141	0.0173	0.587	1.726	0.0416	0.00
Pb	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cr	0.00	0.00	0.00	0.00	0.0150	0.00	0.00	0.00	0.00	0.00
Cd	0.00164	0.00215	0.00199	0.00178	0.00724	0.0229	0.00701	0.00171	0.008739	0.00236
Cu	0.00	0.00	0.809	0.00	0.108	0.498	0.00	0.00	0.000994	0.00
Hg	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
As (total)	0.0242	0.0266	0.0435	0.0126	0.0629	0.0109	0.0126	0.0210	0.0194	0.0209
S	0.0312	0.00	14.474	0.00	154.914	50.763	230.530	6.629	250.428	17.833
Sr	0.00379	0.00858	0.0148	0.00317	0.0879	0.00459	0.00588	0.00514	0.0832	0.0387
NH_4, N-NH_4 (μM)	19.550	79.023	3.650	54.652	16.702	18.391	25.941	1.504	66.573	5.780
PO_4, P-PO_4 (μM)	0.00	27.372	0.00	49.650	28.108	5.593	25.171	0.00	74.493	0.00
SO_4 (μM)	0.00	0.00	185.00	0.00	820.0	355.0	1243.6	0.00	175.0	222.0
Conductivity (μS $cm^{-1)}$	13.9	29.3	257.0	45.8	1399.0	1420.0	91.5	239.0	8970.0	516.0
Eh (mV) Steam	ND	ND	ND	ND	ND	+185.0	+368.0	ND	+272.0	ND

ND, not determined.

pH 8, 2 mM EDTA, 1.2% P40 detergent, nanowater (filtered with a 0.22-μm Millipore filter) and 20 mg mL^{-1} lysozyme) was added to a pellet of cells, swirled and incubated at 37 °C for 30 min. Proteinase K and buffer AL (lysis buffer, Qiagen) were added and incubated at 70 °C for 10 min to lyse the cells. Lysozyme was excluded when DNA was extracted from archaeal colonies. DNA was then purified using the Qiagen DNeasy Blood and Tissue Kit. Purified DNA was quantified on the NanoDrop™ spectrophotometer. The purified DNA was amplified by PCR using archaeal specific primers (21Fa and 915Ra), or bacterial-specific primers (27F and 'universal' 1492R), using the same conditions as described previously. Amplified PCR products were then cleaned using ExoSAP-IT® (USB) and purified DNA was sequenced at the San Diego State University MicroChemical Core Facility.

Results

Chemical analysis

Table 2 summarizes the analysis of steam deposits for alkaline earth metals, trace elements and selected nutrients. Nonsulfur samples contained the highest concentration of silica. This included the Sulphur Works nonsulfur cave at LVNP (data not shown). X-ray data characterized the dominant chemistry of steam deposit sample sites as nonsulfur, sulfur, iron or iron over sulfur as described previously for flowing spring sediments (Mathur *et al.*, 2007). Figure 3 shows the representative X-ray microanalysis spectra of nonsulfur, sulfur, iron and iron over sulfur steam deposit samples. The nonsulfur cave/vent samples had the highest peak containing silica, except for Hawaii 1 nonsulfur cave, which had the highest peaks at iron and oxygen. Nonsulfur samples also contained oxygen, carbon, aluminum, calcium and magnesium at relatively lower levels. The sulfur spectrum showed that the samples were composed mainly of sulfur. The sulfur cave/vent samples also contained oxygen, silica and titanium. The iron cave/vent samples had the highest peak at iron, except for Norris iron vent, which had its highest peak at silica. The iron samples also contained peaks at oxygen, aluminum, carbon, potassium, titanium, sulfur and phosphorous. The iron over sulfur cave sample had the highest peak at sulfur, with peaks also for silica, oxygen, iron, aluminum, carbon and titanium. A principal component analysis was performed to examine whether there was agreement between the deposit site chemistry (Table 2) and the mineralogy types, nonsulfur, sulfur and iron. When all the chemical components and sampling sites were considered, the chemistry data did not strongly cluster the environmental types. Clustering occurred when the

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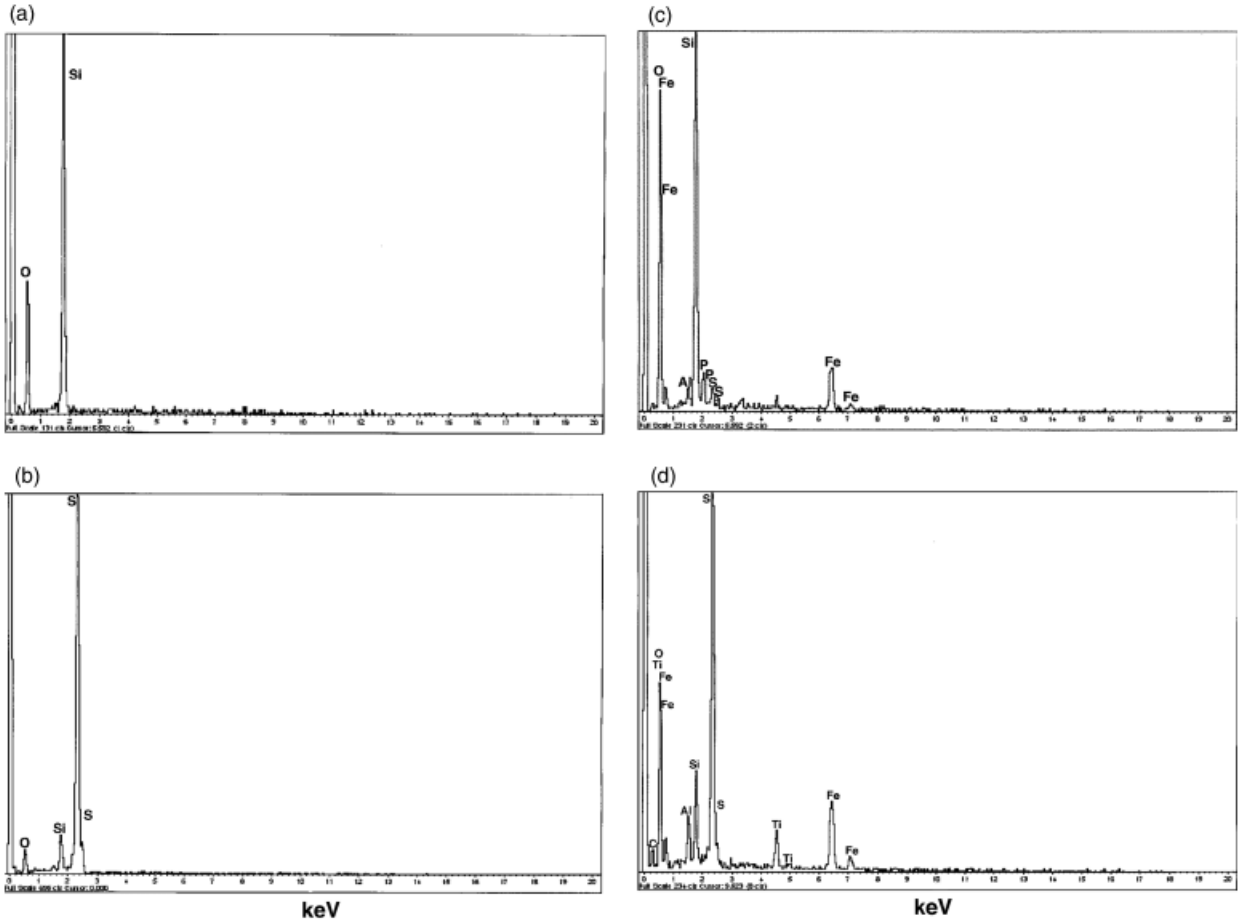

Fig. 3. Comparison of spectra (nonsulfur, sulfur, iron and iron over sulfur) from Norris Geyser Basin, Sulfur Works and Hawaii. (a) Spectrum of white–gray–greenish steam deposits sampled from Norris Geyser Basin nonsulfur cave; oxygen and silica are prominent, (b) Spectrum of yellow steam deposits sampled from Sulphur Works sulfur cave; sulfur is the highest peak, (c) Spectrum of reddish-brown steam deposits sampled from Sulphur Works iron cave; iron is prominent, (d) Spectrum of yellowish-brown steam deposits sampled from Hawaii 4 iron over sulfur cave; sulfur and iron were the main elements.

analysis was limited to defining features and nutrients of the deposit site mineralogy (Supporting Information, Fig. 4S). With the limited analysis, 69% of the variance in the five chemical variables was explained by two factors: one primarily driven by S, SO_4 and Fe and the other by pH and NH_4. Overall, we found the chemistry of each site to be somewhat unique, with large variations within each defined mineralogy steam deposit type.

Developing the PowerSoil/Protein 1 method

Our goal was to determine archaeal diversity in fumarole deposits. This required the recovery of amplifiable DNA and so far this had failed for Archaea (Mayhew *et al.*, 2007). Sulfur steam deposits represent a challenging habitat, due to potential low pH DNA damage, but this is not a problem with near-neutral pH nonsulfur fumaroles. We focused on problems with chemical extractions because in sulfur steam caves, like in flowing springs, most organisms attach to surfaces of sulfur crystals or in nonsulfur caves to silica grains. Microscopic 4′-6-diamidino-2-phenylindole-DNA examination of steam cave sulfur deposits revealed microcolonies on crystal surfaces. Thus, focusing on problems with chemical rather than physical approaches seemed to offer the means for the recovery of high-yield and -quality DNA. Problems with physically inaccessible-occluded endolithic sites were not pursued further. In such samples, evaporation, concentration, and silica deposition entrap organisms, but steam flow in caves/vents is continuous, so significant evaporation is unlikely. In flowing sulfur springs, we successfully isolated DNA using the lysozyme method, but this method failed with sulfur steam cave deposits because the DNA did not amplify. Thus, we looked for and found another method: UltraClean DNA kits. To improve DNA quality, we changed to PowerSoil and examined the effect of Protein 1 and Protein 2. The method succeeded

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when we obtained DNA amplicons for cloning and returned clean sequences from Archaea in fumarole deposits. Our approach is presented in the section below.

Environmental DNA extraction

Various extraction methods were attempted for environmental DNA isolation from the steam deposits following pH neutralization to protect DNA from acid-induced depurination and degradation (Jordan, 1960). Table 1 and Figure S1 show the DNA yields of the various extraction methods attempted. The two methods with the greatest consistent yields were the UltraClean® Soil DNA Isolation kit and the PowerSoil® DNA Isolation kit with the addition of Protein 1 (Table S1; Fig. S1). Further analysis of the extraction results showed that, although the yields between these two methods were similar, the PowerSoil/Protein 1 extraction method produced purer DNA. The 260 : 280 ratio for the PowerSoil/Protein 1 method was consistently closer to 1.8 than the Ultraclean method alone and the 260 : 230 ratio was consistently higher as well (Table S1; Fig. S2). Fig. S3 shows representative examples of typical UV spectra for each of five different extraction methods. The addition of skim milk to the extractions, following Takada-Hoshino & Matsumoto (2004), did improve the yields slightly (Table S1; Fig. S1). However, unlike Takada-Hoshino & Matsumoto (2004), PCR tests of the negative extraction controls of the skim milk samples were positive for bacterial DNA. Ikeda *et al.* (2008) performed an extensive study on the usage of skim milk in environmental DNA extractions and also demonstrated that there was thermophilic bacterial DNA contamination in skim milk (Ikeda *et al.*, 2008). Adding casein to the bead-beating protocol yielded approximately 2–10 ng μL^{-1} of DNA. A final comparison was made between Protein 1 and Protein 2 (data not shown). The control experiments resulted in no evidence of archaeal or bacterial DNA contamination from either of the proteins after amplification of the negative extraction controls. In the end, Protein 1 yielded the most DNA with the greatest purity of all the methods and was determined to be the best yield-boosting additive when used with the PowerSoil™ DNA Isolation Kit. Using this method, DNA was successfully extracted and PCR amplified from sites in HAVO, YNP and LVNP. Hawaii 1 and 2 nonsulfur caves were positive for Archaea and Bacteria DNA, Hawaii 3 sulfur cave was positive for Archaea and Hawaii 4 iron over sulfur cave was positive for Archaea. YNP Norris nonsulfur cave was positive for Archaea and Bacteria, while LVNP Sulfur Works iron cave was positive for Bacteria.

Clone library and phylogenetic analysis

Positive PCR products were selected for TOPO-TA® cloning (Invitrogen) and the M13 PCR products were sequenced.

Table 3. Archaeal cloned sequences top matches using NCBI BLAST

Sample site	# Clones	%ID	Species	Accession #
H1	4	99	Uncultured archaeon	DQ791908
	1	95	Uncultured archaeon gene	AB213053
H2	1	99	Uncultured archaeon	DQ791908
H3	12	98	*Sulfolobus* sp.	AY907890
	6	97–98	*Sulfolobus islandicus*	CP001401
H4	35	96–99	*Sulfolobus* sp.	AY907890
	3	98	*Sulfolobus islandicus*	CP001401
	1	98	*Sulfolobus islandicus*	CP001399
	1	99	Uncultured archaeon	DQ179001
NGB1	7	95	Uncultured thermal soil archaeon	AF391991
	1	96	Uncultured archaeon	AY882838
	1	99	Uncultured archaeon	DQ179001

Archaeal 16S rRNA gene sequences were successfully determined from nonsulfur, sulfur and iron over sulfur sample sites. The sulfur steam deposits were dominated by *Sulfolobus* (Table 3), while the nonsulfur steam deposits had diverse uncultured members of *Crenarchaeota*. Figure 4 shows the phylogenetic relationships of these non-*Sulfolobus* uncultured *Crenarchaeota* sequences. Archaeal clone sequences have been deposited in GenBank as accession numbers GU221912–GU221926. A diverse array of bacterial 16S rRNA gene sequences was also obtained from nonsulfur and iron sample sites (Table 4). Only a fraction of these sequences closely matched the existing sequences in GenBank. Table 4 summarizes the Bayesian classification, as well as the top NCBI BLAST hits and top known hits. Bacterial clone sequences have been deposited in GenBank as accession numbers GU292497–GU292532.

Scope of the analysis

Our goal was to find evidence of Archaea in fumarole deposits. We expected that with higher temperatures, archaeal diversity would be low and this is precisely what we found. At the highest temperature sites that returned archaeal sequences, H3 sulfur and H4 iron over sulfur, diversity was the lowest. When a successful PCR product and cloning of a deposit site was accomplished, we prepared a clone library, sequenced 30 clones from each site and analyzed the returned sequences. The diversity of most sites was low as reported for the number of clones returned (Table 3). In the libraries, we saw only one to three novel clones. These sequences were repeated several times within the library, indicating that we achieved coverage of the clone library. This does not fully eliminate the possible bias that might arise as a result of PCR primers. As samples were processed, failure was seen at different steps in the procedure. With nonsulfur samples, two were excluded from

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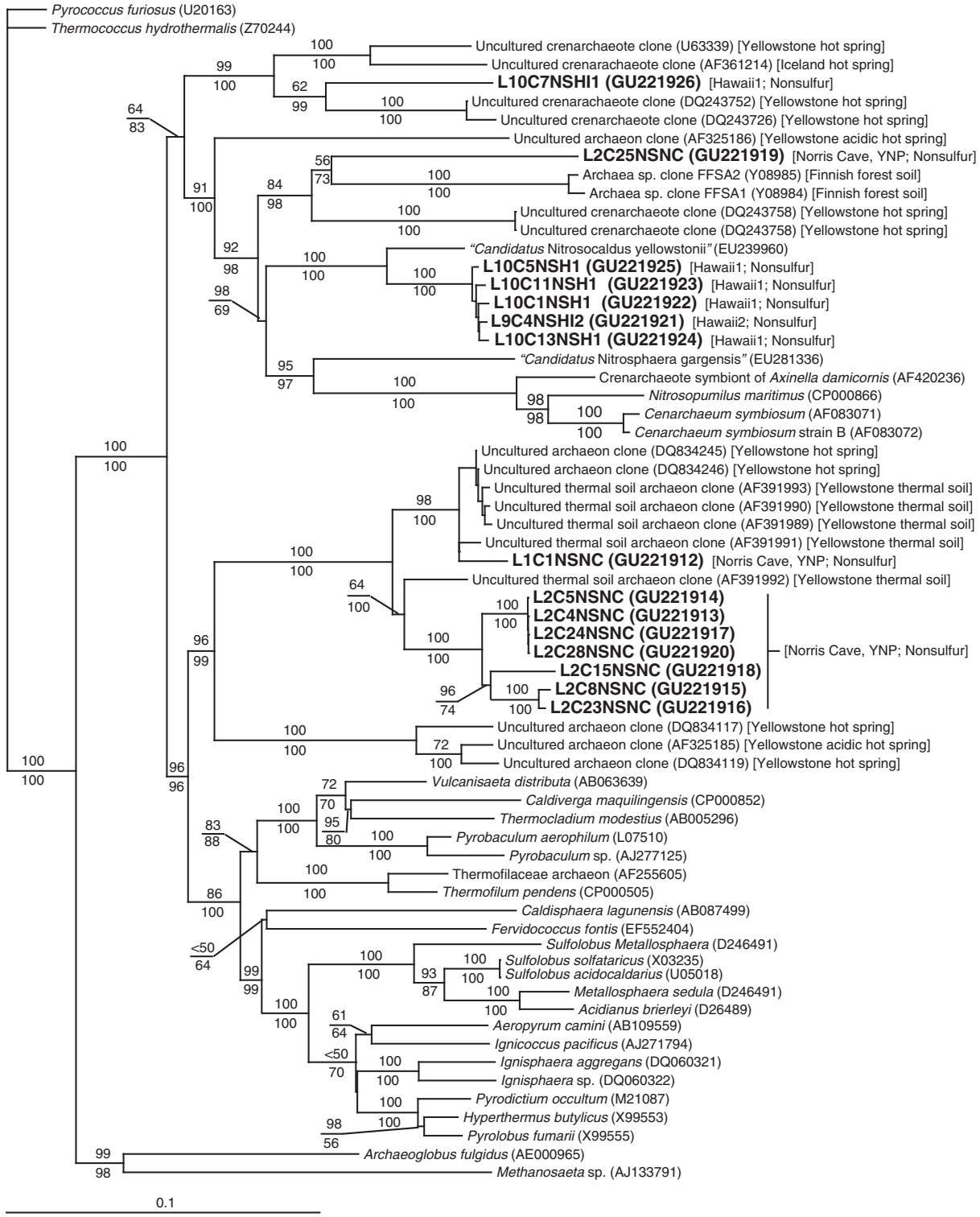

Fig. 4. Phylogenetic analysis of cloned archaeal rRNA gene sequences isolated from fumarole steam deposits. The analysis included both cultured and uncultured 16S rRNA gene sequences of Archaea obtained from the phylum *Crenarchaeota* (RDP version 10 database). Organism names and clone identifiers are followed by the GenBank accession number in parentheses. The numbers above the branches indicate MP, while the numbers below the branches indicate NJ bootstrap values. Codes: H1, Hawaii 1; H2, Hawaii 2; NC, Norris Cave, YNP; NS, nonsulfur cave. Clone names in bold print precede GenBank accession numbers; the geographic location of the sample site and the dominant chemistry are indicated in parentheses following accession numbers.

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Table 4. Identification of bacterial sequences from two Hawaiian steam vent deposit communities using RDP classification and BLAST

Clone ID	RDP classification	Prob %	Top BLAST	Accession #	% ID	Taxon affiliation	Accession #	%ID
Hawaii 1 (H1)								
L11C1HI1NSC	*Bacillales*	97	*Bacillales* bacterium	DQ999995	97	*Alicyclobacillus pohliae*	AJ607431	95
L11C10HI1NSC	Bacteria	100	Uncultured bacterium	DQ791434	99	*Thermoanaerobacter tengcongensis*	AE008691	86
L11C11HI1NSC	Bacteria	100	Uncultured bacterium	DQ791411	94	*Sphaerobacter thermophilus*	CP001824	88
L11C12HI1NSC	*Bacillales*	99	*Alicyclobacillaceae* bacterium	AB362268	95	*Alicyclobacillus pohliae*	AJ607429	93
L11C13HI1NSC	Bacteria	99	Uncultured bacterium	EF444701	99	Green nonsulfur bacterium*	AF445735	85
L11C18HI1NSC	*Gemmata*	99	Uncultured bacterium	FJ207091	99	*Gemmata* sp.	AM902601	94
L11C20HI1NSC	*Bacillales*	97	*Alicyclobacillaceae* bacterium	AB362268	94	*Alicyclobacillus pohliae*	AJ607429	93
L11C21HI1NSC	*Bacillales*	97	*Bacillales* bacterium	DQ999995	96	*Alicyclobacillus pohliae*	AJ607431	94
L11C25HI1NSC	Bacteria	100	Uncultured bacterium	DQ791411	100	*Thermaerobacter subterraneus*	EU214630	89
L11C27HI1NSC	Bacteria	100	Uncultured bacterium	DQ791402	98	*Chloroflexi* bacterium*	AB433073	86
L11C28HI1NSC	*Bacillales*	94	*Alicyclobacillaceae* bacterium	AB362268	94	*Alicyclobacillus pohliae*	AJ607429	93
L11C30HI1NSC	Bacteria	99	Uncultured bacterium clone	DQ791434	98	*Thermoanaerobacter tengcongensis*	AE008691	87
Hawaii 2 (H2)								
L5C1HI2NSC	*Sphingomonas*	100	Uncultured bacterium	GQ128420	99	*Sphingomonas* sp.	FJ192268	99
L5C21HI2NSC	*Sphingomonas*	100	Uncultured bacterium	GQ128420	99	*Sphingomonas* sp.	FJ192268	99
L5C23HI2NSC	Bacteria	100	Uncultured bacterium	EU490269	98	*Chloroflexi* bacterium*	GQ143781	90
L5C4HI2NSC	Bacteria	100	Uncultured bacterium	EU490281	96	*Chloroflexi* bacterium*	GQ143781	91
L5C5HI2NSC	Bacteria	100	Uncultured bacterium	EU490269	98	*Chloroflexi* bacterium*	GQ143781	90
L5C6HI2NSC	*Rhodothermus*	99	Uncultured *Rhodothermus* sp.	EU249949	99	*Rhodothermus marinus*	EU214605	95
L5C7HI2NSC	Bacteria	100	Uncultured bacterium	EU490269	98	*Chloroflexi* bacterium*	GQ143781	90
L6C14HI2NSC	*Bacillales*	97	*Bacillales* bacterium	DQ999995	96	*Bacillus cellulosilyticus*	AB043852	94
L6C16HI2NSC	Bacteria	96	Uncultured bacterium	FN545885	95	*Marinithermus hydrothermalis*	NR_028639	83
Norris Geyser Basin (NGB1)								
L3C2NNSC	Bacteria	99	Uncultured bacterium	GU113055	95	*Chloroflexi**	DQ450731	87
L3C7NNSC	*Proteobacteria*	92	Uncultured thermal soil bacterium	AF391977	99	*Thialkalivibrio thiocyanodenitrificans*	AY360060	95
L3C11NNSC	*Bangiophyceae*	100	*Cyanidioschyzon merolae*	AY286123	100	N/A	–	–
L3C15NNSC	*Meiothermus*	92	Uncultured bacterium	AY191889	99	*Meiothermus timidus*	AJ871170	91
Sulphur Works (SW5)								
L4C3SWFeC	*Bacillales*	95	Uncultured bacterium	EU419142	99	*Bacillus badius*	AB098575	87
L4C5SWFeC	*Leptospirillum*	100	*Leptospirillum*	FJ228330	98	*Leptospirillum ferriphilum*	EF025341	93
L4C8SWFeC	Bacteria	99	Uncultured bacterium	AY935137	95	*Alicyclobacillus hesperidum*	AB059679	86
L4C13SWFeC	*Actinobacteria*	92	Uncultured bacterium	DQ906083	91	*Ferrimicrobium acidiphilum*	AF251436	89
L4C15SWFeC	*Actinobacteria*	100	Uncultured *Ferrimicrobium*	EU383022	99	*Ferrimicrobium acidiphilum*	AF251436	99
L4C17SWFeC	*Gammaproteobacteria*	100	Uncultured bacterium	EF446180	95	*Acidithiobacillus thiooxidans*	AY830900	94
L4C18SWFeC	*Alicyclobacillus*	100	*Alicyclobacillus disulfidooxidans*	AB089843	99	N/A	–	–
L4C22SWFeC	*Acidimicrobium*	100	Uncultured bacterium	EU419133	100	*Acidimicrobium ferrooxidans*	CP001631	99
L4C23SWFeC	*Deltaproteobacteria*	98	Uncultured bacterium	EF409834	98	*Alkalilimnicola ehrlichii*	CP000453	94
L4C27lSWFeC	*Alicyclobacillus*	100	*Alicyclobacillus disulfidooxidans*	AB089843	99	N/A	–	–
L4C30SWFeC	*Leptospirillum*	100	Uncultured *Leptospirillum*	EF612426	99	*Leptospirillum ferriphilum*	GU168001	99

If the top BLAST hit was unknown, the nearest formally described taxon affiliation was also included.

*Top 100 BLAST hits did not included formally labeled taxon.

N/A, not applicable.

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analysis: RM1 and SW1. At RM1, DNA yield and quality were only fair and at SW1 the yield was high, but quality was low. No amplification was seen. Out of a total of 27 different trials from sulfur steam deposits, 13 amplified and returned cloned sequences and all were from Hawaii. With sulfur samples from Yellowstone RM2, AS, Lassen SW2, SW3, the DNA yield was always adequate, low to moderate in quality, but no amplification occurred in any samples. Iron steam deposit DNA at Lassen cave was high yield, high quality and returned only bacterial clones; no archaeal PCR bands were seen, but archaeal cultures were grown. At the SW4 iron vent, low-yield, low-quality DNA did not amplify. Norris iron vent NGB2 DNA was high yield low quality and DNA did not amplify. As can be seen, what began as a 3-site, 14-sample analysis had decreased, allowing us to focus on the samples that yielded PCR-amplified DNA and clones.

Table 5. Success (+) and failures (−) with cloning and enrichment culturing of Archaea and Bacteria from various steam deposits

	Clones		Cultures	
Sample site	Archaea	Bacteria	Archaea	Bacteria
H1	+	+	+	+
H2	+	+	−	+
H3	+	−	−	+
H4	+	−	−	−
NGB1	+	+	+	+
RM1	−	−	−	+
RM2	−	−	+	+
AS	−	−	+	+
NBG2	−	−	+	+
SW1	−	−	+	+
SW2	−	−	+	+
SW3	−	−	+	+
SW4	−	−	+	+
SW5	−	+	+	+

Steam deposit cultures

We were able to culture microorganisms from most of the sample sites (Table 5). Archaea and Bacteria grew in a liquid medium with an optimum pH for microbial growth at pH 4.5, the pH of most of the steam samples. All the isolates, both bacterial and archaeal, were obtained via liquid culturing at 55 °C, although we have recently managed to generate positive enrichment cultures at 85 °C (data not shown). Typically, we detected evidence of growth in our enrichment cultures between 5 and 12 days of incubation. Bacteria were isolated on a solid medium using 1.5% agar at 55 °C and we often observed visible colonies within a week of inoculation. Archaea grew on Gelrite overlays at 55 °C usually within a week of inoculation and were always found growing intermixed with Bacteria. Archaea and Bacteria were enriched from all sample sites in YNP and LVNP, except for the Roaring Mountain nonsulfur cave, where only Bacteria were grown and isolated. Archaea were isolated from Hawaii 1 nonsulfur cave. Bacterial isolations at Hawaii sample sites were from Hawaii 1 and Hawaii 2 nonsulfur caves and Hawaii 3 sulfur cave. Figure 5 illustrates examples of the high concentration of cells found in the cultures. DNA was extracted from the archaeal enrichment cultures, and we were able to obtain PCR products in most cases. However, for unknown reasons, only a few 16S rRNA gene sequences were of high quality or pure 16S rRNA gene sequences from a single organism and the BLAST matches of these sequences are reported in Table 6.

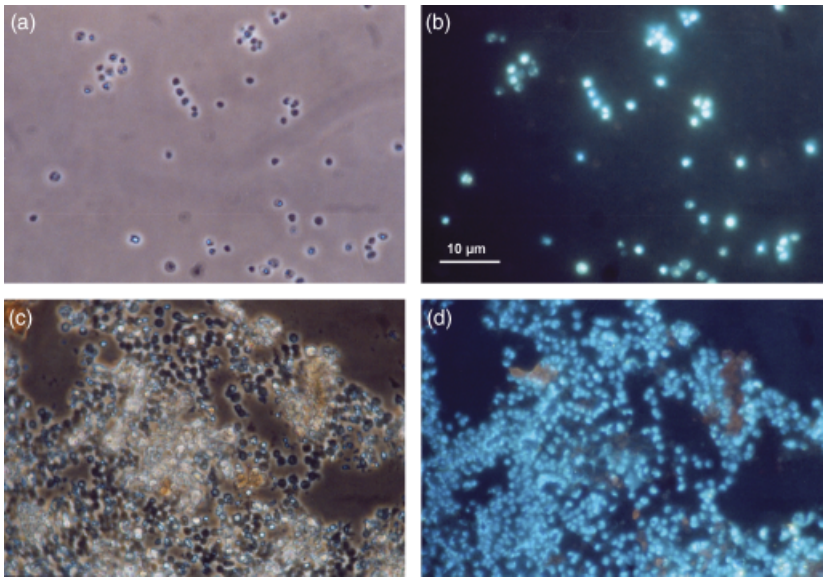

Fig. 5. Phase-contrast and 4′-6-diamidino-2-phenylindole (DAPI) microscopy showing microorganisms enriched from steam deposits. Cells visualized by phase contrast are phase dense and appear dark and those by DAPI fluorescence are bright blue. (a) Amphitheater Springs sulfur vent steam deposit culture, phase contrast. (b) Amphitheater sulfur vent steam deposit culture, DAPI. (c) Amphitheater Springs sulfur cave steam deposit culture, phase contrast exhibiting bright yellow sulfur crystals with dark microorganisms. (d) Amphitheater Springs sulfur cave DAPI steam deposit culture, showing sulfur crystals with microorganisms.

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Table 6. Culture isolated archaeal sequences top matches using NCBI BLAST

Sample sites	%ID	Species ID	Accession #
AS	97	*Acidianus brierleyi*	FJ154515
	83	Uncultured *Sulfolobus* sp.	DQ832062
	87	*Sulfolobus metallicus*	EU419200
RM2	98	*Acidianus brierleyi*	FJ154515
SW2	93	Uncultured *Sulfolobales*	DQ924847
SW3	97	Uncultured *Sulfolobus* sp.	DQ832062

Discussion

Our culture-independent molecular analysis and culturing approaches both confirmed that Archaea commonly inhabit fumarole deposits. We successfully isolated environmental DNA from steam deposits utilizing a novel DNA extraction approach and, together with PCR-based cloning methods, determined archaeal 16S rRNA gene sequences from multiple steam deposits (Table 5). Our improved DNA extraction method, which included pH neutralization and the addition of protein to the isolation procedure, resulted in the highest DNA recovery (Table S1; Fig. S1) and purity (Figs S2 and S3) of the many methods we attempted, and may be of general utility for recalcitrant environmental samples. Moreover, the protein utilized in our method was produced without the use of animal products, which helped avoid bacterial DNA contamination. Although the DNA extractions yielded DNA for many steam deposit samples, we were still not able to obtain archaeal or bacterial PCR products for more than half the steam deposits we investigated, despite the fact that we were able to culture Archaea from many of these same steam deposits (Table 6). Clearly, more work needs to be carried out to enhance DNA extraction methods with these steam deposit types, although our improvements represent an important breakthrough in this regard.

We investigated three fumarole types, nonsulfur, sulfur and iron, but ultimately, only the nonsulfur steam deposit sites and two Hawaii sulfur sites, H3 and H4 iron over sulfur, yielded DNA that could be amplified, cloned and sequenced. No other sulfur or iron deposit sites produced DNA that could be sequenced, even with sufficient yields of high-quality DNA. The decrease in the sample pool size during this study indicates the difficulty we had in obtaining PCR-amplifiable DNA for cloning, especially with the low pH of sulfur steam sites. Despite being unable to obtain sequences from every steam deposit sample, we learned that Archaea and Bacteria were widely distributed in both mild and extreme fumarole deposit sites of the three chemical types and many could be cultured. We found that no universal method for DNA extraction exists and in particular that low-pH sulfur steam deposits were extremely challenging sites to investigate for diversity. We may be able to overcome the obstacles to cloning in these habitats with improved sample collection methods, using Tris buffer and dry ice freezing at the time of sample collection to better preserve samples for more efficient DNA recovery.

Members of the *Crenarchaeota* appeared to dominate the archaeal diversity in fumarole steam deposits. Both culture and culture-independent approaches found *Sulfolobus* species in sulfur and mixed iron/sulfur steam deposits (Tables 3 and 6). Culture approaches also detected species of *Acidianus* alongside *Sulfolobus* in the Yellowstone steam vent (Table 6). The presence of *Sulfolobales* makes sense in these environments as we would expect to find microorganisms that metabolically depend on sulfur as an electron donor. On the other hand, the nonsulfur steam deposits (Hawaii 1, Hawaii 2 and Norris Cave, YNP) all yielded sequences that matched uncultured *Crenarchaeota* in other geothermal settings (Table 3). A phylogenetic analysis of these sequences with *Crenarchaeota* representing all major cultured lineages, as well as uncultured *Crenarchaeota* sequences obtained from GenBank, found that our sequences clustered strongly with a diverse array of uncultured *Crenarchaeota* (Fig. 4). Based on the relative sequence divergences, our data indicated that we identified members of five distinct and previously uncultured genera. Most of the sequences formed strongly supported (98–100% bootstrap values) monophyletic groups with uncultured Archaea identified previously from geothermal soils or hot springs (Fig. 4). Interestingly, some of the *Crenarchaeota* clustered with the so-called 'Group I' *Crenarchaeota* and were closely related to a recently cultured ammonia oxidizer, '*Candidatus* Nitrosocaldus yellowstonii' (Könneke *et al.*, 2005; de la Torre *et al.*, 2008). The isolation of an ammonia-oxidizing autotrophic marine crenarchaeon revealed the first instance of nitrification in the Domain *Archaea* (Könneke *et al.*, 2005). Marine ammonia-oxidizing archaea (AOA) appear to be widespread and important members of the world's oceans (Urakawa *et al.*, 2010). The phylogeny of AOA crenarchaea has been the subject of a recent proposal for a third phylum within the Archaea: *Crenarchaeota, Euryarchaeota* and newly proposed *Thaumarchaeota* for marine planktonic *Crenarchaea* and closely related organisms (Brochier-Armanet *et al.* 2008). Clones from Hawaii nonsulfur steam deposits branched monophyletically were closely related to AOA. In near-neutral/basic pH systems, ammonia partitions as a vapor (Jones, 1963; Nordstrom *et al.*, 2005) rising with the steam column and concentrates at the steam deposit site similar to the way H2S concentrates in acidic systems. If these Hawaii organisms can be demonstrated by enrichment culture, *amo*A gene sequences or other methods to be AOA then this represents a new AOA habitat. It would also suggest a parallelism between ammonia concentration and oxidation in neutral/basic systems and H_2S concentration and oxidation in acidic habitats. The number of published

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uncultured *Crenarchaeota* sequences continues to grow exponentially (Hershberger *et al.*, 1996; Robertson *et al.*, 2005; Robertson *et al.*, 2009) and a phylogenetic analysis incorporating many more sequences would be necessary to solidify the broader relationships among these groups. Nevertheless, our analysis uncovered many novel archaeal lineages and nonsulfur steam deposits appear to be rich sources of novel thermophilic Archaea.

Microscopic investigation found that enrichment cultures grew Archaea-like cells from the majority of the steam deposit samples (Fig. 5). Based on visual identification by microscopy, 10 of the cultures had spherical Archaea-like cells and all except one of the enrichment cultures had rod-shaped Bacteria-like cells (Table 5). Despite the clear evidence of the Archaea-like cells, and the confirmation of Archaea from the clone libraries, we only obtained six pure archaeal isolates from four different sulfur-type vents. All of these isolates were related to cultured and uncultured *Sulfolobus* or *Acidianus*, although they appeared to be distantly related to cultured organisms based on the BLAST identity values (Table 6). Both *Sulfolobus* and *Acidianus* are able to grow aerobically, oxidizing sulfur or iron. Anaerobically, *Acidianus* carries out Fe(III) reduction and sulfur reduction, utilizing H_2 as an electron donor (Segerer *et al.*, 1986; Yoshida *et al.*, 2006), or with one species unable to use H_2, H_2S serves as an electron donor with Fe(III) reduction (Plumb *et al.* 2007). The ability of *Acidianus* to change its mode of metabolism from aerobic to anaerobic in response to its environment likely explains the recurrent appearance of this organism in cultures isolated from high-temperature steam deposits present in cave and vent steam deposits.

Crenarchaeota were enriched from the nonsulfur vents, but we were unable to isolate organisms in pure culture. The Archaea from in these enrichments always grew with Bacteria on the Gelrite overlays, and we were not able to separate the archaeal isolates from bacterial isolates on the Gelrite. With the exception of one set of sequences that were closely related to a cultured autotroph high-temperature ammonia oxidizer (*Candidatus* Nitrosocaldus yellowstonii), the Archaea found in the nonsulfur vents were highly divergent from any well-characterized organisms, making physiological inferences uncertain. The fact that silica and oxygen were the main components in the nonsulfur deposits (Fig. 3) leaves open the question of how these organisms derive their energy. Interestingly, ammonia was one of the most abundant nutrients in the steam vent deposits, especially in the nonsulfur vents (Table 2), suggesting that ammonia oxidation may also be important in these habitats. Futher work, including attempts to cultivate ammonia oxidizers, is needed to determine whether these vent deposit Archaea are autotrophic or whether they are obligate symbionts as members of the complex bacterial communities in these steam deposits. These enrichment cultures may also be a ready source of interesting new hyperthermophilic archaeal viruses (Snyder *et al.*, 2003).

Our culture-based studies showed that organisms clearly survive at the measured steam deposit temperatures. It is generally difficult to grow novel thermophilic organisms from environmental samples and the likelihood for success was low because we used only a single pH and a single temperature. In addition, the laboratory culture incubation temperature was much lower than what we measured in the steam vents. In spite of using a single pH and a lower incubation temperature, the enrichment cultures grew well (Table 5). Furthermore, the isolated microorganisms made a great deal of sense, given the habitats (e.g. *Sulfolobales* in sulfur steam deposits; Table 6). The fact that we obtained cultures at a lower temperature supports the idea that a range of temperatures may exist for these organisms, and it is possible that optimum temperature for growth may not coincide with the environmental temperature. In a previous study, we were able to grow halophiles captured in hot steam ($> 85\,^{\circ}C$), at relatively low temperatures ($55\,^{\circ}C$), but not at higher temperatures (Ellis *et al.* 2008). Nevertheless, the steam deposit environments have rich microbial communities that are continually bathed in very hot steam and many of the organisms present likely grow optimally at these temperatures (e.g. *Acidianus* relatives). Thus, we are continuing our efforts to culture organisms from these steam cave/vent deposits at a hotter temperature to better approximate the actual conditions.

Although the main focus of our study was on the presence and diversity of Archaea in steam vent deposits, we also report the discovery of bacterial 16S rRNA gene sequences from four of the HAVO steam vent deposit communities. Overall, the Bacteria of these communities appeared to be more diverse than the Archaea and many of the 16S rRNA gene sequences we determined are from organisms that had no cultured representatives (Table 4). The nonsulfur caves contained nonphotosynthetic Bacteria (*Planctomycetes*) similar to those found in the Red Layer Microbial Observatory (Boomer *et al.*, 2009), YNP and Chloroflexi (green nonsulfur Bacteria) and were similar in composition to Hawaiian volcanic soils (Gomez-Alvarez *et al.*, 2007). Our studies of the bacterial communities are quite preliminary at this point, but certainly confirm our ability to derive bacterial sequences from these environments, confirming the results of Mayhew *et al.* (2007). Further study of these unknown steam deposit organisms will provide a better understanding of the bacterial communities in fumarole deposits.

The diversity of steam deposits raises the fundamental question of whether the organisms detected in this study arose from the subsurface or by some other means such as aerial movement (Bonheyo *et al.*, 2005; Snyder *et al.*, 2007). The steam vents we studied were similar in many respects to

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flowing hot springs, with a continual discharge from the subsurface along an aquifer or a silica-lined exit channel. Also, like flowing springs, steam vent water carries both Archaea and Bacteria to the surface, where they can be detected in condensed samples (Ellis *et al.*, 2008). At this point, our results on cloning and culturing of steam deposits (Tables 3, 4, and 6) now suggest that the organisms are dislodged from volcanic sites or biofilms below the surface as steam/water contacts the organisms and are conveyed by the continuous steam column to the deposit site. However, direct evidence for this is presently lacking, leaving this a fertile area for future study.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. DNA yield ($ng\,\mu L^{-1}$) from various environmental DNA extraction methods.
Fig. S2. Quality of DNA comparison between the UltraClean™ soil DNA isolation kit and the PowerSoil™ DNA isolation kit with the addition of protein 1, showing the PowerSoil™ DNA isolation kit producing higher quality DNA.
Fig. S3. Representative UV spectra of environmental DNA samples extracted using five extraction methods.
Fig. S4. Principal component analysis.
Table S1. Comparative summary of environmental DNA extraction methods.

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