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Halophilic *Archaea* determined from geothermal steam vent aerosols

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Summary

Hydrothermal vents, known as 'fumaroles', are ubiquitous features of geothermal areas. Although their geology has been extensively characterized, little is known about the subsurface microbial ecology of fumaroles largely because of the difficulty in collecting sufficient numbers of cells from boiling steam water for DNA extraction and culture isolation. Here we describe the first collection, molecular analysis and isolation of microbes from fumarole steam waters in Russia (Kamchatka) and the USA (Hawaii, New Mexico, California and Wyoming). Surprisingly, the steam vent waters from all the fumaroles contained halophilic *Archaea* closely related to the *Haloarcula* spp. found in non-geothermal salt mats, saline soils, brine pools and salt lakes around the world. Microscopic cell counting estimated the cell dispersal rate at approximately 1.6×10^9 cells year^{-1} from a single fumarole. We also managed to enrich microbes in high-salt media from every vent sample, and to isolate *Haloarcula* from a Yellowstone vent in a 20% salt medium after a month-long incubation, demonstrating both salt tolerance and viability of cells collected from high-temperature steam. Laboratory tests determined that microbes enriched in salt media survived temperatures greater than 75°C for between 5 and 30 min during the collection process. Hawaiian fumaroles proved to contain the greatest diversity of halophilic *Archaea* with four new lineages that may belong to uncultured haloarchaeal genera. This high diversity may have resulted from the leaching of salts and minerals through the highly porous volcanic rock, creating a chemically complex saline subsurface.

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Introduction

Fumaroles discharge steam at temperatures up to 180°C (Ferreira and Oskarsson, 1999), and the vapour formed proceeds directly into the air from the subsurface, often passing through a chimney where vapour and soil contact may occur (Brombach *et al*., 2000). The upper limit for microbial growth has been reported at 121°C (Kashefi and Lovley, 2003), and both archaeal and bacterial 16S rRNA gene sequences have been found at temperatures exceeding 300°C in deep sea hydrothermal vents (Atkinson *et al*., 2000; Takai *et al*., 2001; Robinson *et al*., 2005). Thus, in spite of the extreme heat of fumarole steam, the subsurface zone of vapour formation may harbour microorganisms able to disperse along with the steam water. Fumaroles are a ubiquitous feature of geothermal environments, and although the geology of fumaroles has been studied extensively, little is known about the microbiota of fumaroles. Numerous studies have explored the microbial diversity of geothermal springs and pools (Burton and Norris, 2000; Benlloch *et al*., 2001; Blank *et al*., 2002; Norris *et al*., 2002; Spear *et al*., 2005) but, to our knowledge, only one study has detected microbes in geothermal steam water and not from a fumarole (Bonheyo *et al*., 2005). The principle difficulty in studying the microbial constituents in geothermal steam has been the challenge of collecting sufficient quantities of steam water for analyses. For example, the recent effort by Bonheyo and colleagues (2005) to identify microbes in steam from flowing springs managed to collect only 20 cells in ~72 h.

In this article, we describe the successful deployment of a highly efficient and portable steam collector designed for sterile microbiological sampling of fumarole steam water (Fig. 1). Fumarole steam is extremely hot and very diffuse (see example site photo, Fig. 1C), making it challenging to collect in large enough quantities for microbiological analysis. Our device, which uses temperature differentials to condense steam into gamma-irradiated polypropylene centrifuge tubes (Fig. 1B), collected up to 4 ml min^{-1} of fumarole steam water from vents with steam as hot as 93.5°C. After collecting steam water, we used both culture-based and culture-independent methods to determine the identity and viability of cells in steam from thermal habitats in Russia (Kamchatka) and in the USA (Hawaii, New Mexico, California and Wyoming). In this

Fig. 1. Fumarole collection images.
A. Portable steam collector, side view; height = 15 cm, diameter = 10 cm.
B. Top view with cover off showing placement of orange-capped sterile 50 ml conical polypropylene tubes.
C. Photo of Roaring Mountain site showing example fumarole steam vents alongside the spring water channel.

study, we focused on identification and isolation of *Archaea*, a group including organisms that survive the highest known temperature extremes (Blöchl *et al*., 1997; Takai *et al*., 2001; Kashefi and Lovley, 2003). We also estimated cell concentrations and steam flow rates in order to gauge the dispersal potential of fumaroles.

Results and discussion

Sample collection

Steam samples were collected over a wide geographical area (Table 1). We evaluated the sample collector for latent heat release over a 5 min period in which an 8 ml

Table 1. Fumarole sampling locations and physical conditions.

Location	Latitude/longitude	Temperature (°C)	pH	Type
Sulphur Springs, Valles Caldera National Reserve, New Mexico	53°54′26″ N 106°36′59″ W	89	2.0	Sulfur
Sulphur Works, Lassen Volcanic National Park, California	40°26′60″ N 121°32′02″ W	93	3.2	Sulfur
Roaring Mountain, sulfur vent, Yellowstone National Park (YNP), Wyoming	44°46′47″ N 110°44′15″ W	87	3.01	Sulfur
Roaring Mountain, origin water sample, YNP[a]	44°46′46″ N 110°44′19″ W	93.5	0.87	Non-sulfur
Amphitheater Springs, YNP sulfur vent	44°48′04″ N 110°43′44″ W	76	3.2	Sulfur
Scott's Vent, Mutnovsky Volcano, Kamchatka, Russia	52°28′02″ N 158°09′34″E	94	3.9	Sulfur
Rick's Vent, Hawaii	N/A	82	2.7	Sulfur

a. Water sampled at the origin of the southern effluent of Roaring Mountain.
N/A, not available.

sample was condensed from steam as a control. Under ideal conditions we calculated that a mean value of 2.51 kJ g^{-1} was required to condense control water vapour samples. Given that the change of phase from vapour to liquid (i.e. the latent heat of vaporization-condensation of water) is 2.26 kJ g^{-1} at 100°C, the condenser is reasonably effective and on average requires only an additional ~251 J release per gram control vapour sample collected.

Figure 2 presents the collection rate of several sulfur and non-sulfur vent samples in the field. At Amphitheater Springs sulfur vent, samples collected on two different days showed different collection rates, likely due to a change in the volume of fumarolic steam. In contrast, there was little variability in collecting control samples on two different days. The results showed that steam sample collection in the field is effective despite day-to-day variability and multiple samples can be collected within a 3 h period.

Our portable steam collector proved extremely effective for capturing significant numbers of microbes from high temperature acidic fumarole steam waters in Kamchatka, Hawaii, New Mexico, California and Wyoming (Figs 3 and 4; Table 1). With these collections we were able to estimate cell concentrations in fumarole steam water, extract DNA for polymerase chain reaction (PCR) cloning methods, and isolate *Archaea* from the enrichments and subcultures. DNA-4′, 6′-diamidino-2-phenylindole (DAPI) staining and phase-contrast microscopy showed that steam from all vents contained cells (Figs 3 and 4). Direct

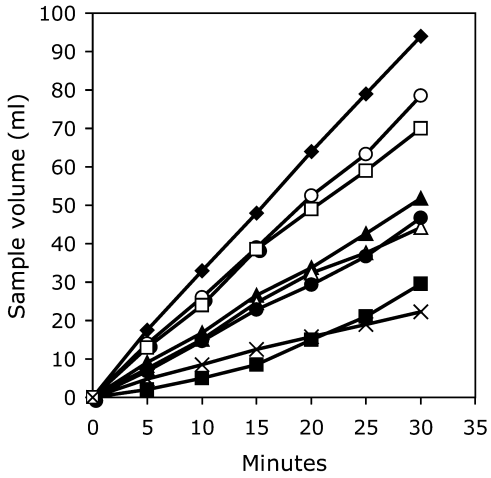

Fig. 2. Collection rates at steam vents. Sulphur Works, Lassen Volcanic National Park, sulfur vent (◆); Roaring Mountain, Yellowstone National Park, sulfur vent (■), non-sulfur vent (□); Amphitheater Springs, sulfur vent 5/19/2006 (●), 5/20/2006 (○); controls collected on two consecutive days (▲ and △); Hawaii, sulfur vent (×).

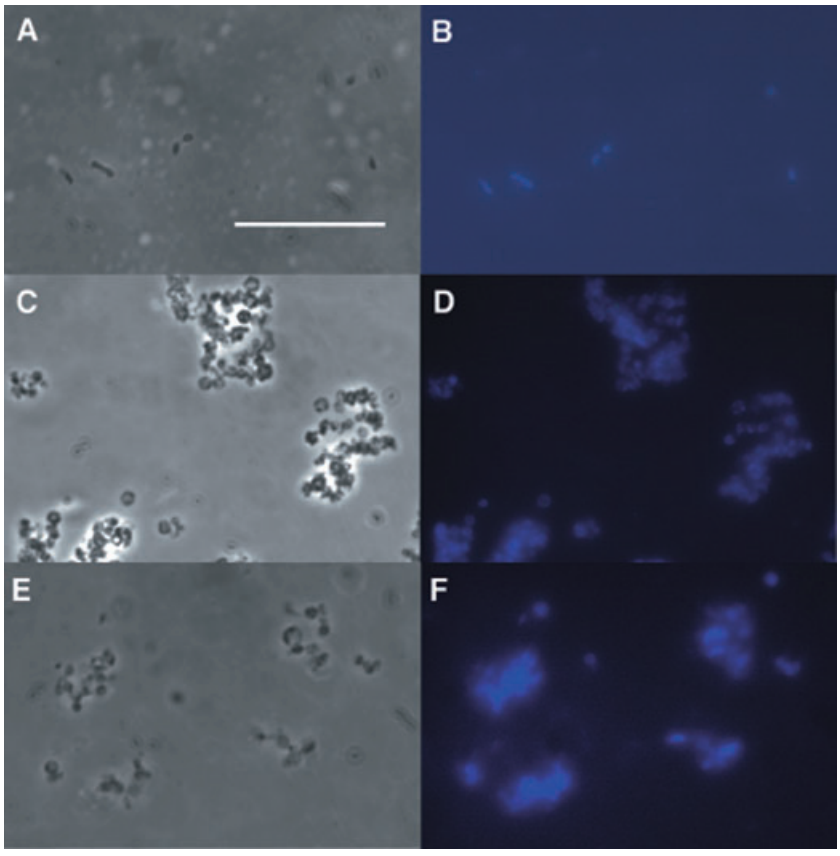

Fig. 3. DAPI and phase-contrast microscopy showing vent microorganisms. Cells visualized by phase contrast and by DAPI fluorescence.
A. Amphitheater vent phase contrast.
B. Amphitheater vent DAPI.
C. Amphitheater vent enrichment phase contrast.
D. Amphitheater vent enrichment DAPI.
E. Amphitheater vent subculture phase contrast.
F. Amphitheater vent subculture DAPI.
Bar (A)–(F), 20 μm.

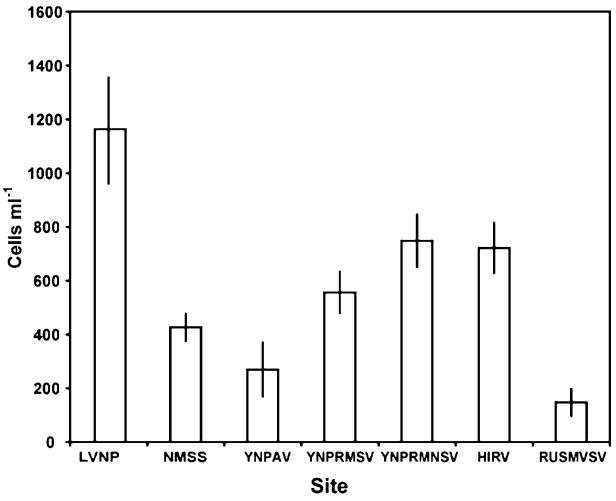

Fig. 4. Environmental vent cell counts using DAPI. Intact cellular estimates were collected by counting at least 500 cells or 100 fields. LVNP, Lassen Volcanic National Park Sulphur Works fumarole; NMSS, New Mexico Sulphur Springs fumarole; YNPAV, Yellowstone National Park Amphitheater Springs vent; YNPRMSV, Yellowstone National Park Roaring Mountain sulphur vent; YNPRMNSV, Yellowstone National Park Roaring Mountain non-sulphur vent; HIRV, Hawaii Rick's vent; RUSMVSV, Russia Mutnovsky Volcano Scott's vent. Error bars represent 95% confidence intervals constructed using standard error and *t*-values (0.05) based on $n > 100$. A correction factor of 284 was then applied to account for sample volume and area counted.

microscopic analysis with DAPI confirmed that several morphological types were present in Hawaii samples (data not shown), which contained the greatest diversity of phylotypes of any of the fumarole samples (Fig. 5). Cell counts made on fumarole samples found considerable variability in cell concentrations ranging from a low of 150 cells ml^{-1} in Kamchatka, Russia Mutnovsky Volcano Scott's vent to a high of 1100 cells ml^{-1} in Lassen Volcanic National Park (Fig. 4). In order to establish the cell dispersal potential of a typical fumarole, we estimated the steam water flow rate for the Amphitheater Springs (AS) fumarole. At this fumarole, we calculated the steam collection rate at 2.11 ± 0.57 ml min^{-1}. Given that the collector had an efficiency rate of $13.8 \pm 0.53\%$ (lab tests; data not shown), we estimated that AS fumarole had a flow rate of 15.3 ml min^{-1}. Assuming a constant rate of steam production, we estimated the cell dispersal rate at approximately 3×10^3 cells min^{-1} and 1.6×10^9 cells $year^{-1}$ from this single fumarole. Our method could not determine how far the cells might travel, which will be a subject for further study.

The chemical properties of the steam samples are compared in Table 2. Fumarole steam had low salinity especially when compared with spring water samples from Roaring Mountain (Yellowstone National Park Roaring Mountain spring source water), which has analytes in higher concentrations (Table 2). Polymerase chain reaction amplifications using 'universal' archaeal primers of GenomiPhi-treated DNA extractions produced bands from five of the seven fumarole steam samples and one spring water sample (Table 1). None of the GenomiPhi-treated negative DNA extraction controls (for outside air contamination or reagent contamination) produced PCR bands with the archaeal primers even when we extended the reactions to 35 cycles to increase detection of contaminants. These controls indicated that any *Archaea* identified either by cloning or by culturing were collected from geothermal steam and not from aerial or other possible environmental sources. Restriction fragment length polymorphism (RFLP) analysis of the PCR clone libraries uncovered very little sequence diversity in the libraries: two to four different sequences per 96-well plate. We sequenced approximately 900 bp of each unique PCR clone in each plate. Phylogenetic analyses of these sequences determined that the fumarole steam samples contained microbes related to cultured and uncultured halophilic *Archaea* (Fig. 5). These related *Archaea* had been obtained from a diverse array of saline environments, including a brine pool (Egypt) (Oren *et al*., 1999), salt lakes (Turkey and India), saline soils (Argentina) (Ihara *et al*., 1997), a subglacial hypersaline lake (Antarctica) (Bowman *et al*., 2000) and water from the Dead Sea (Israel) (Arahal *et al*., 1996). There was robust statistical support (high posterior probability and bootstrap support) for the inclusion of Yellowstone National Park and Russia fumarole rRNA gene sequences within the genus *Haloarcula* (Fig. 5). These sequences fell into two well-supported monophyletic clusters, suggesting that they may represent new *Haloarcula* spp. Maximum parsimony (MP) and neighbour-joining (NJ) bootstrap support values for the relationships amongst *Haloarcula* spp. were low, but there was high support for the monophyly of the genus and for the inclusion of six clone sequences within this group. However, given the propensity for *Haloarcula* to maintain two distinct rRNA gene sequences it would be difficult to predict how many new species we might have discovered.

Based on our determination of haloarchaeal-related sequences in steam vent water, we attempted to enrich for halophilic microbes from the collected water. We managed to generate successful enrichment cultures using vent water samples in media with 12.5–20% salt concentration at temperatures of 37°C and 51°C (data not shown). From the Amphitheater Springs vent, we further subcultured a *Haloarcula* sp. (Fig. 3), although we have yet to produce a completely pure culture of the organism. Given that the highest enrichment growth temperature we achieved was 51°C, we do not have evidence that the vent-associated microbes grow well at extremely high temperatures. However, lab temperature measurements of steam water made during and after collection

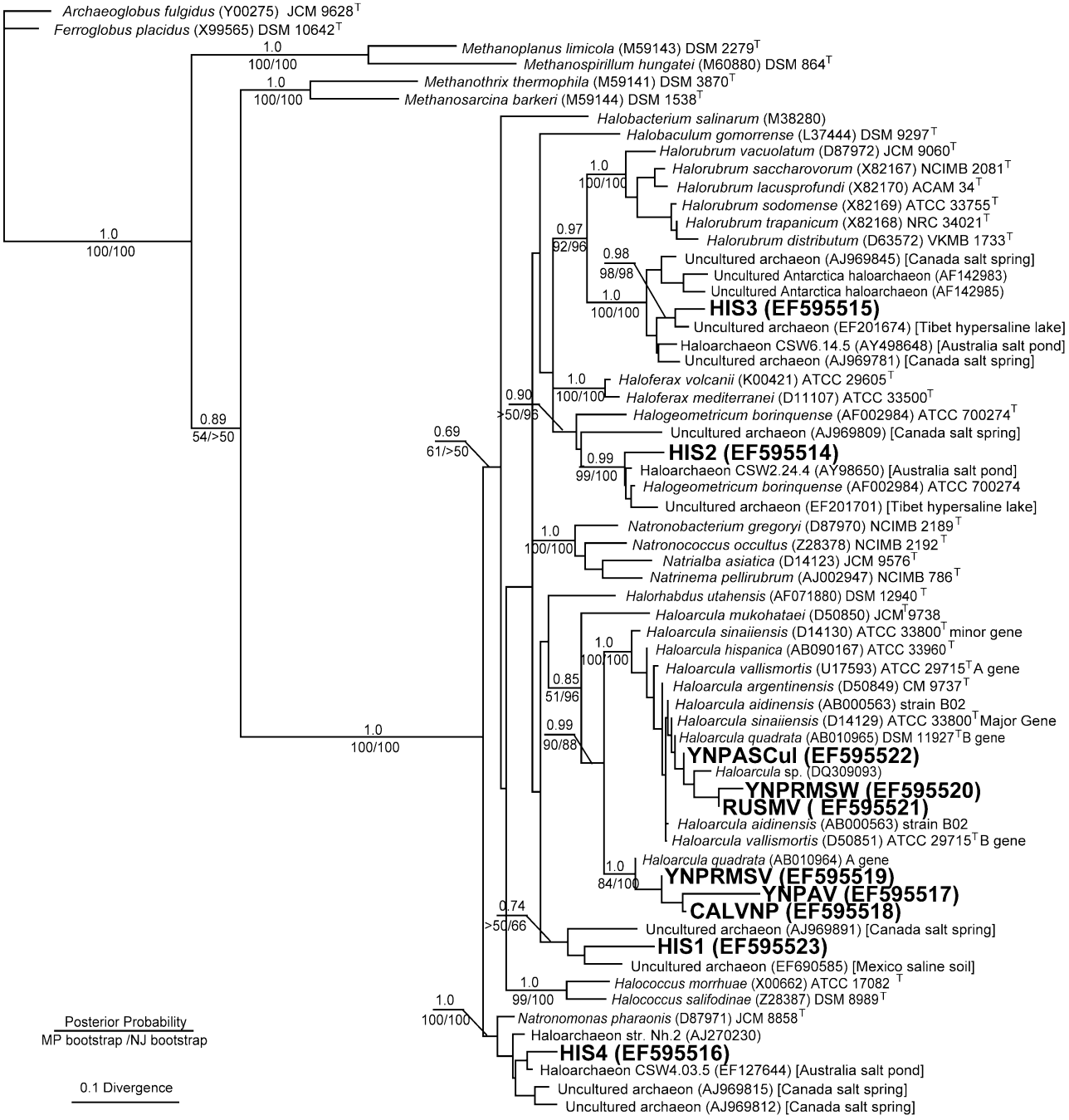

Fig. 5. Phylogenetic analysis of cloned rRNA gene sequences isolated from fumarole steam water. The analysis included both cultured and uncultured rRNA gene sequences of halophilic *Archaea* obtained from highly saline environments around the world. The presented phylogenetic tree was based on a Bayesian analysis. Organism names are followed by the GenBank accession number in parentheses and the culture collection identification number (e.g. ATCC 33800), if from a typed strain. Several *Haloarcula* spp. contain two distinct rRNA gene sequences (indicated as A/B, major/minor; Oren *et al*., 1999). The numbers above the branches indicate Bayesian posterior probability values, while the numbers below the branches indicate maximum parsimony (MP) and neighbour-joining (NJ) bootstrap values respectively. To reduce clutter, support values are shown at select, strongly supported nodes important for understanding the relationships of the clone sequences to cultured and uncultured haloarchaea. Codes: HIS, Hawaii fumarole; YNPASCul, Yellowstone National Park Amphitheater Springs fumarole culture; YNPRMSW, Yellowstone National Park Roaring Mountain spring source water; RUSMV, Russia Mutnovsky Volcano fumarole; YNPRMSV, Yellowstone National Park Roaring Mountain fumarole; YNPAV, Yellowstone National Park Amphitheater Springs vent; CALVNP, California Lassen Volcanic National Park fumarole. Sequences of clones and one culture in this tree are represented by GenBank Accession No. EF595514–EF595523.

Table 2. Steam vent and hot spring chemistry.

Analyte (mg l^{-1})	SSNM	LVNPSW	YNPRMSV	YNPRMNSV	YNPRMSW	YNPASV	HISV	MVKR
Na	0.868	1.70	0.828	0.666	5.84	0.634	1.57	1.43
Ca	0.756	0.876	0.00	0.00	2.65	0.00	0.789	0.452
Al	0.00	0.307	0.00	0.00	14.8	0.0389	0.281	0.423
Fe (total)	0.00	0.433	0.00	0.00	5.63	0.283	0.335	0.163
Si	0.744	0.342	0.225	0.0676	382	0.207	0.482	8.20
B	0.00	0.441	0.00	0.00	0.00	0.00	0.505	0.369
K	0.674	1.75	0.601	0.00	20.5	0.557	1.22	0.631
Mg	0.246	0.0432	0.266	0.00	0.327	0.00	0.0451	0.330
Zn	0.0424	0.0115	0.276	0.127	0.0286	0.316	0.0231	1.09
Mn	0.00	0.0187	0.00	0.00	0.0589	0.00	0.0189	0.00
Mo	0.0269	0.00	0.0272	0.0270	0.0277	0.0265	0.00	0.00
Se	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.0184
Ni	0.00	0.00289	0.0110	0.00	0.00	0.0272	0.00573	0.0122
Pb	0.000163	0.00	0.00568	0.00165	0.00127	0.00435	0.00	0.00
Cr	0.00	0.00612	0.0179	0.00535	0.000150	0.0362	0.00557	0.00
Cd	0.00	0.0138	0.00	0.00	0.00	0.00	0.0137	0.0401
Cu	0.00	0.00	0.00	0.00	0.00	0.172	0.00	0.00
Hg	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.0183
AS (total)	0.0140	0.00	0.00	0.000590	0.134	0.0112	0.00	0.0128
S	0.480	1.82	0.166	5.180	128	9.77	0.146	1.09
Sr	0.0381	0.00	0.0325	0.0312	0.0426	0.0320	0.00	0.00435
NO_2/NO_3, $N\text{-}NO_3$	0.0342	0.0440	0.0421	0.0202	0.794	0.0420	0.0300	0.0486
NH_4, $N\text{-}NH_4$	0.407	0.230	0.013	0.011	1.79	0.473	0.244	0.529
PO_4, $P\text{-}PO_4$	0.288	0.0560	0.0607	0.0565	0.00517	0.245	0.0910	0.00
SO_4	100	25.0	75.0	75.0	250	80.0	30.0	7.50
Conductivity ($\mu S\ cm^{-1}$)	46.8	139	91.5	47.6	3040	336	8.60	22.3

SSNM = Sulphur Springs, Valles Caldera National Reserve, New Mexico, sulfur vent; LVNPSW = Lassen Volcanic National Park, California, Sulphur Works, sulfur vent; YNPRMSV = Yellowstone National Park, Wyoming (YNP) Roaring Mountain, sulfur vent; YNPRMNSV = YNP Roaring Mountain, non-sulfur vent; YNPRMSW = YNP Roaring Mountain, flowing spring water sampled at origin; YNPASV = YNP Amphitheater Springs, sulfur vent; HISV = Rick's vent, Hawaii sulfur vent; MVKR Scott's vent, Mutnovsky volcano, Kamchatka, Russia, sulfur vent.

determined that microbes collected in our tubes were exposed to water temperatures exceeding 75°C for a minimum of 5 min and probably a good deal longer. The temperature of the water during the collection phase, lasting between 10 and 30 min (Fig. 2), was only a few degrees cooler after condensation than the temperature of the fumarole steam as it exited the vent, and after collection the water in the conical tubes remained above 75°C for 5 min and more than 50°C for 15 min. This observation, combined with our enrichment culture success, shows that the halophiles aerosolized in fumarole steam survive high temperature exposure for significant periods of time, even though they may not grow optimally at extreme temperatures. These results provide further support for the hypothesis that steam aerosolization provides a potential means for halophile dispersal from fumaroles. In November 2007, researchers reported finding *Sulfolobus* viruses in the air column above geothermal pools. Their results suggest that these viruses may disperse among pools via steam droplets, indicating that steam may provide a general means of dispersal in geothermal ecosystems (Snyder *et al.*, 2007).

Interestingly, the Hawaii steam samples contained the greatest diversity of halophilic *Archaea*. We discovered four distinct, and phylogenetically diverse, lineages that were all at least 10% divergent from any cultured sequences (Fig. 5). There was strong support for the clustering of all of the Hawaii clones within the halophilic *Archaea*. The inclusion of haloarchaeal sequences from uncultured and untyped organisms from other studies suggested that the Hawaii sequences may belong to new genera (Fig. 5). One of the more interesting findings was the relationship of the HIS3 sequence to uncultured sequences determined from hypersaline lakes in Tibet and Antarctica (Fig. 5).

Salinity appeared to be the most significant commonality among the environments of the reference sequences we included in the tree, although we cannot exclude the possibility that these environments do not share other features of equal importance. This suggests that the underground fumarole habitats were highly saline. Subsequent to the identification of halophilic rRNA gene sequences, we were able to isolate and subculture *Haloarcula* sp. from the AS fumarole (Fig. 3C and D). The organism appeared in culture after nearly complete evaporation of the initial culture enrichment. More recently, we managed to re-isolate organisms from this same fumarole in media specifically designed to isolate *Haloarcula* (20.6% salt concentration) after incubation at 51°C for 29 days (Oren *et al.*, 1999).

One potential source of these halophilic *Archaea* in fumaroles may be the highly saline zone of vapour

formation in the subsurface (Brombach *et al*., 2000). The soil zone that is in contact with the emerging vapour may also be a source of halophiles (Oren, 2002). Compared with the other fumaroles sampled, the porous volcanic Hawaiian rocks appear to provide a substantially different habitat for microbial growth, which may explain the relatively high diversity of 16S rRNA gene sequences in the Hawaiian fumarole steam. All of the waters that eventually form the fumarolic steam from the active Hawaii volcano are meteoric: rainwater passes through the ground until it reaches a level where it is heated by contacting rocks that convect heat from the hot magma below. The heated water rises through small passageways and fissures to the surface where it escapes into the air as steam. This steam consists of nearly pure water, as seen by a much lower conductivity (8.6 μS cm^{-1}) when compared with the other fumarole steam waters (139.3 μS cm^{-1} Lassen; 336 μS cm^{-1} Amphitheater Springs vent). We suspect that the percolation of rainwater through porous rocks from above may leach out sodium and chloride containing compounds and that in a transition zone where the water evaporates, salt is deposited. The presence of sulfur in the Hawaii collection site indicates that deep faults and fractures extend to the magma and allow volcanic gases with hydrogen sulfide to reach the surface. These fractures could also reach brackish waters in Hawaii where salinity is higher and could contribute to subsurface salt deposits and biofilms that would contact steam vapours. The increased deposits of salts and other elements left behind may provide an explanation for the greater diversity of halophilic *Archaea* in the Hawaiian subsurface. Recently, one of us (R.W. Bizzoco) discovered microbes in salt deposits collected from a steaming lava cave not far from the fumarole collection site. Further studies will ascertain whether these types of deposits may contain microbes similar to the ones found in fumarole steam.

Because this was the first study of microbes in fumarole steam, our main goal was to identify the types of microorganisms found in fumarole steam water and determine their potential viability. Although we only detected a relatively limited diversity of microbes in any of the habitats, we expect that further studies will uncover much greater diversity, particularly of halophilic bacteria. Even after concentration, the low cell numbers in the steam water samples required us to take the unusual step of GenomiPhi amplification to generate enough material for 16S PCR amplification. The combination proved effective and the GenomiPhi negative controls were also negative in subsequent 16S PCR amplification. However, the extra GenomiPhi amplification step may have added another level of bias to the process in addition to any bias of the 'universal' PCR amplification. Thus, we cannot say with any confidence that the sequences we determined from the aerosols were the dominant organisms in the environment and we make no claims to that effect.

Another unknown aspect of the study was the diversity and prevalence of bacteria in the fumarole steam aerosol samples. The salt-media enrichment cultures and microscopy work revealed an abundance of bacterial cells, in addition to archaeal cells, in all the steam water samples. We have isolated bacteria on solid media after enrichment in 20% salt liquid media from Hawaiian fumaroles. Based on 16S rRNA gene sequence analysis, two isolates recovered were related to *Geobacillus* spp., two were *Bacillus* spp. and four others were related to uncultured bacteria. Future studies will involve a more complete assessment of uncultured diversity including exploration of the diversity of bacteria in fumaroles, determination of the ratio of bacteria to archaea and culturing halophilic bacteria from the steam vent samples. We will also continue our ongoing efforts to culture halophilic *Archaea* from fumarole steam, especially from Hawaii where molecular DNA analysis demonstrated the highest diversity.

Experimental procedures

Study sites and steam collection

Table 1 lists the geographical locale of each fumarole studied, and reports the corresponding temperature, pH and Global Positioning System coordinates. Our portable steam sampler (PSS) is a self-contained unit that includes a condenser, collector and handle for remote attachment (Fig. 1A and B). Prior to deployment, the PSS was cleaned with Sparkleen® laboratory detergent (Fisher Scientific) and 10% bleach, then rinsed, autoclaved and stored in a sterile commercial autoclave bag. The sample collection device was held above the vent surface in the flow of the steam without contacting any surfaces. After 40 ml was condensed in gamma-irradiated sterile 50 ml tubes, the screw cap sample tube was closed and the sample chilled for transport to the laboratory. The sampling device uses temperature differentials to condense hot steam water inside the device, which drips down inside and collects in the conical tubes. The temperature of the water samples was measured in a controlled laboratory setting during and immediately after collection by condensing sterile water from a boiling hotpot and recording the sample temperature at 0 min, 5 min, 10 min and 15 min after collection. To control for contamination a field sample of vapours from sterile water was collected alongside each of the fumaroles. This served as the negative environmental control to check for potential air contamination of the steam waters during collection. We screened the environmental control using both microscopy (DAPI staining and cell counts) and PCR methods (see below).

Environmental DNA extraction and amplification

The pH of each sample was adjusted to between 7 and 8 aseptically using 0.5 N sodium hydroxide. The DNA was then extracted from 5 to 10 ml of environmental sample and

purified with a Soil Extraction Kit (MoBio) using the maximum yield protocol as per the manufacturer's directions. A minimum of 5 ng μl^{-1} of environmental genomic DNA was extracted for whole genome amplification. To produce sufficient genomic DNA for molecular analysis, the environmental genomic DNA was amplified using a GenomiPhi kit (GE Healthcare) following the manufacturer's instructions. Whole genome amplifications of environmental samples were performed at 37°C for 18 h. This produced between 1.5 and 3.5 μg of total environmental DNA. The GenomiPhi product was then purified using a DNeasy Tissue Extraction Kit (Qiagen) following the manufacturer's instructions. The cleaned DNA was eluted with 100 μl of purified water (Sigma). DNA was amplified using archaeal-specific primers: 21F (5′-TTCCGGTTGATC CYGCCGGA-3′) (DeLong, 1992) and 915R (5′-GTGCTGCCCCGCCAATTCCT-3′) (Stahl and Amann, 1991) with broad specificity for *Euryarchaeota* and *Crenarchaeota*. We limited PCR cycles to a maximum of 25 to reduce the potential for amplification of contaminants. Polymerase chain reaction was carried out in a total reaction volume of 50 μl including 1 μl of sample (15–35 ng) DNA as template, each deoxynucleoside triphosphate at 200 μM, 1.5 mM $MgCl_2$, 5 μl 10× buffer (10× concentration: 500 mM 1 M KCl, 100 mM Tris-HCl pH 8.4, 1% gelatin), each primer at 0.4 μM, 4 μl of bovine serum albumin (10 mg ml^{-1}), and 0.5 μl of 1 unit μl^{-1} REDTAQ DNA polymerase (Sigma-Aldrich). The PCR conditions included an initial denaturing step of 95°C for 10 min, followed by 25 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 3 min. The PCR then underwent a final elongation step at 72°C for 20 min and the product was stored at room temperature (22°C) for 1 h. We also screened the negative environmental extractions and negative reagent controls using PCR with 35 cycles to screen for contaminants. The resulting PCR products were gel-purified using a Gel Extraction Kit (MoBio) following the manufacturer's instructions.

Clone library construction

Gel-extracted PCR products were cloned using a TOPO-TA cloning kit and following the manufacturer's instructions (Invitrogen). For every vent analysed, 96 (white) colonies were inoculated into LB-glycerol (15%), grown overnight at 37°C and 1 μl of the culture was screened directly (no DNA extraction) via PCR with M13 primers to check for inserts. RFLP analysis was used to identify unique clones from each library for sequencing following the procedures outlined in Kelley and colleagues (2004). The PCR products of clones with unique banding patterns were cleaned for sequencing using ExoSap (USB) using the manufacturer's instructions and the cleaned products were sent to the San Diego State University MicroChemical Core Facility for sequencing. Polymerase chain reaction products were sequenced in both directions with the 21F and 915R primers generating approximately 900 bp for each clone sequenced. Sequences were deposited in GenBank as Accession No. EF595514–EF595523.

Sequence and phylogenetic analyses

Near relatives were identified using BLAST. A Fasta file of the cleaned sequences was then aligned using the green genes website (http://greengenes.lbl.gov/cgi-bin/nph-index-cgi), and the aligned sequences were imported into the ARB bacterial and archaeal database of 16S rRNA gene sequences (Ludwig *et al*., 2004). Phylogenetic analyses were performed using PAUP* program (Swofford, 2002) and MrBayes (Huelsenbeck and Ronquist, 2001). The program Mr MODELTEST2 (Posada and Crandall, 1998) was used to select the optimal substitution model for Bayesian analyses. Bayesian analyses were performed using the General Time Reversible (GTR) model (Yang, 1994). This model incorporated a gamma-distributed among-site substitution rate heterogeneity and a fraction of sites constrained to be invariable (GTR+I+G). Bayesian analyses were performed with four independent Markov chains run for 3 000 000 MCMC generations. Trees were sampled every 200 generations with a burn-in of 2000 trees. The best MP tree, or set of trees, was found through a random addition sequence heuristic search strategy with 100 replicates. The maximum number of trees kept during each search was capped at 1000. For the MP bootstrap analyses, we performed MP searches on 100 bootstrap replicated data sets using the same heuristic search strategy except with 10, rather than 100, search replicates. We also performed a NJ bootstrap analysis with 1000 replicates.

Steam culture and enrichment

Media components, including nutrients and conditions, pH, temperature and duration of incubation, were selected to optimize enrichments and subculture of steam microorganisms. Enrichment cultures were established using the environmental steam vapours that had been condensed into vent water. For each steam sample collected, we attempted isolations using four different enrichment culture conditions. The first included adding 0.1 ml of 10% yeast extract (pH 5.8) made up in Brock's medium (Brock *et al*., 1972) to 10 ml of the condensed water sample, yielding a final concentration of 0.1% yeast extract. (For the other three culture conditions, we added 1 ml of condensed steam water to 10 ml of Brock's medium adjusted to pH 3, 4 and 5 respectively.) Duplicate samples of all four enrichments were incubated at both 55°C and 70°C. The enrichment cultures were checked for evidence of growth every 2–3 days over the course of several months. Once we achieved growth in the AS fumarole, we set up subcultures using halophilic specific medium and incubation conditions as described by Oren and colleagues (1999), which were also checked every few days for growth (Oren *et al*., 1999).

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