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Microbiology and geochemistry of Little Hot Creek, a hot spring environment in the Long Valley Caldera

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ABSTRACT

A culture-independent community census was combined with chemical and thermodynamic analyses of three springs located within the Long Valley Caldera, Little Hot Creek (LHC) 1, 3, and 4. All three springs were approximately 80 °C, circumneutral, apparently anaerobic and had similar water chemistries. 16S rRNA gene libraries constructed from DNA isolated from spring sediment revealed moderately diverse but highly novel microbial communities. Over half of the phylotypes could not be grouped into known taxonomic classes. Bacterial libraries from LHC1 and LHC3 were predominantly species within the phyla *Aquificae* and *Thermodesulfobacteria*, while those from LHC4 were dominated by candidate phyla, including OP1 and OP9. Archaeal libraries from LHC3 contained large numbers of *Archaeoglobales* and *Desulfurococcales*, while LHC1 and LHC4 were dominated by *Crenarchaeota* unaffiliated with known orders. The heterogeneity in microbial populations could not easily be attributed to measurable differences in water chemistry, but may be determined by availability of trace amounts of oxygen to the spring sediments. Thermodynamic modeling predicted the most favorable reactions to be sulfur and nitrate respirations, yielding 40–70 kJ mol^{-1} e^- transferred; however, levels of oxygen at or below our detection limit could result in aerobic respirations yielding up to 100 kJ mol^{-1} e^- transferred. Important electron donors are predicted to be H_2, H_2S, S^0, Fe^{2+} and CH_4, all of which yield similar energies when coupled to a given electron acceptor. The results indicate that springs associated with the Long Valley Caldera contain microbial populations that show some similarities both to springs in Yellowstone and springs in the Great Basin.

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INTRODUCTION

At temperatures above ~73 °C, the coupling of light energy to biomass production by photosynthesis is not known to occur (Brock, 1967a). Despite this, microbial growth is observed in hot springs at much higher temperatures (Davis, 1897; Setchell, 1903; Brock, 1967b). In these systems, primary production is limited to chemolithotrophy, where energy is derived from redox reactions involving inorganic substrates. Collectively, chemolithoautotrophic *Bacteria* and *Archaea* cultured from thermal environments have been shown to use a variety of electron donors such as H_2, NH_3, reduced metals, and reduced sulfur species and acceptors, such as O_2, NO_3^-, oxidized metals, CO_2, and various oxidized sulfur compounds (e.g. Huber *et al.*, 1998; Götz *et al.*, 2002; Kashefi *et al.*, 2002a,b; de la Torre *et al.*, 2008; Miroshnichenko *et al.*, 2009). The use of culture-independent methods for surveying natural microbial populations has confirmed that hot spring environments can contain diverse communities of both autotrophic and heterotrophic microorganisms (e.g. Barns *et al.*, 1994; Reysenbach *et al.*, 1994; Hugenholtz *et al.*, 1998; Blank *et al.*, 2002; Meyer-Dombard *et al.*, 2005). With a wide array of potential electron donors (e.g. H_2, CH_4, NH_4^+, H_2S, Fe^{2+} and other reduced metals) and acceptors (e.g. O_2, NO_3^-, SO_4^{-2}, CO_2, Fe^{3+}) often present in geothermal systems, the question arises: which

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redox couples and organisms are responsible for primary production in terrestrial hot springs above the temperature limit for photosynthesis?

This question can be addressed using a combination of geochemical and microbiological approaches. It has been proposed that the diversity of potential energy sources in thermal environments drives the diversity and composition of microbial communities, and that the most thermodynamically favorable reactions are preferentially utilized by microbes (Amend & Shock, 2001). Also, primary producers should be conspicuous members in autotrophic communities. Thus, comparison of 16S rRNA gene sequences obtained from culture-independent surveys to those of microbes with known metabolisms can identify potential autotrophs and the energy sources that they might be utilizing (Spear *et al.*, 2005). Several studies have used this approach in various Yellowstone National Park (YNP) hot spring environments (Inskeep *et al.*, 2005; Meyer-Dombard *et al.*, 2005; Spear *et al.*, 2005; Hall *et al.*, 2008). Spear *et al.* (2005) proposed that aerobic hydrogen oxidation was the dominant redox couple driving primary production in these systems, based on the ubiquity of H_2, the prevalence of aerobic, H_2-oxidizing members of the bacterial phylum *Aquificae*, and the higher predicted energy yield of aerobic oxidation of hydrogen compared to that of other electron donors. While *Aquificae* appear to be dominant members of many of these systems, the importance of H_2 in comparison to other electron donors such as formate and H_2S has been questioned (Windman *et al.*, 2007; D'Imperio *et al.*, 2008).

In contrast to hydrothermal systems in YNP, the microbiology of hot springs in the endorheic Great Basin region of the United States has received little attention. One study focused on arsenite oxidizing communities in the Alvord Desert of southern Oregon (Connon *et al.*, 2008), while others have concentrated on *Crenarchaeota* and associated ammonia oxidization genes and biomarkers in a variety of Great Basin hot springs (Pearson *et al.*, 2004, 2008; Huang *et al.*, 2007; Zhang *et al.*, 2008). Work by Costa *et al.* (2009) used a similar approach to that described above with respect to the geology and microbiology of four 75–80 °C, circumneutral springs within the Great Boiling Springs and Mud Hot Springs (GBS/MHS) area in northwestern Nevada. 16S rRNA gene libraries from sediments from these springs contained few or no phylotypes related to the *Aquificae*, suggesting that they play only a minor role in primary production. Thermodynamic modeling of potential chemolithotrophic reactions indicated that O_2 was the best electron acceptor; however, no single electron donor was dominant. Aerobic oxidation of several potential electron donors (CH_4, Fe^{2+}, sulfur and H_2S) yielded similar or slightly greater amounts of energy than reactions involving H_2.

The Long Valley Caldera, while located within the hydrologic Great Basin, is an actively volcanic, silicic caldera associated with the Mono-Inyo volcanic chain on the eastern flank of the Sierra Nevada. The region has been marked by periods of volcanic unrest since it was formed by the eruption of 600 km^3 of rhyolitic magma 730 ka (Sorey *et al.*, 1991). The area is underlain by a shallow (7–10 km) magmatic intrusion that is likely responsible for the geothermal activity and features of the area, including hot springs and fumaroles (Farrar *et al.*, 2003; Sorey *et al.*, 2003). Little Hot Creek (LHC), located on the eastern edge of the resurgent dome of the caldera, is a small group of circumneutral springs with source temperatures up to ~80 °C. LHC springs have thus far been the subject of only one microbiological study, which focused on phage community dynamics (Breitbart *et al.*, 2004). LHC provides a natural laboratory for comparing geothermal features of the Great Basin with those of YNP because of its unique location; it is within the hydrologic Great Basin and is also associated with a large, active, volcanic caldera. Here, we present a coordinated geochemical and culture-independent microbiological survey of three major springs in the LHC area, LHC1, LHC3 and LHC4. Physicochemical parameters of spring water are used to calculate the energy yields of various known and hypothetical chemolithotrophic reactions. In this context, we discuss the potential roles of the microbes identified in the springs and the chemolithotrophic metabolisms that might provide energy in these ecosystems.

METHODS

Sample locations and collection

Samples were obtained from LHC hot springs LHC1 at GPS location N37°41.436′ W118°50.664′, LHC3 at N37°41.456′ W118°50.639′ and LHC4 at N37°41.436′ W118°50.653′ (Datum: WGS84) in the Long Valley Caldera, located near Mammoth Lakes, CA, USA. Water temperature, conductivity and pH were measured in the field using a LaMotte 5 Series hand-held meter (LaMotte, Chestertown, MD, USA). Alkalinity, ammonia, nitrate, nitrite, silica, sulfide and oxygen were measured in the field by titration or by colorimetric assays using LaMotte reagents (LaMotte). Sulfide and oxygen were measured with hot samples taken directly from the spring. Sulfide was diluted with ~25 °C double-distilled H_2O (1:2) and titrated using the Pomeroy methylene blue method. Oxygen was measured using the azide-modified Winkler method. Other field measurements were made with freshly cooled samples. Alkalinity was determined by titration with sulfuric acid to pH 4.5. Silica was titrated using the heteropoly blue method. Ammonia was determined by Nesslerization. The sum of nitrate and nitrite was determined by diazotization of nitrite following cadmium reduction. Nitrite was determined by diazotization without the reduction step. Samples for major ions were filtered through a well-rinsed 0.2-μm hydrophilic polyether-sulfone filter (Pall Scientific, East Hills, NY, USA) in the field, transported on ice, and quantified at Arizona State University (ASU) by ion chromatography (anions: IonPac AS11

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Analytical and IonPac AG11 Guard columns; cations: IonPac CS12A Analytical and IonPac SG11 Guard columns; conductivity detection; Dionex, Sunnyvale, CA, USA). Samples for trace elements were filtered using the same filters, acidified with nitric acid and analyzed at ASU by high-resolution inductively coupled plasma mass spectrometry (ICP-MS; Element II, Thermo Scientific, Waltham, MA, US). Samples for hydrogen and oxygen isotopes were sealed in 40 mL amber vials (Scientific Products LLC, Miami, OH, USA) with no headspace and analyzed at ASU by high-temperature pyrolysis (TC/EA) mass spectrometry (Thermo Scientific MAT253). Samples for dissolved gas analysis were collected using a gas stripping technique described by Spear *et al.* (2005) and analyzed by gas chromatography by Microseeps (Pittsburgh, PA, USA). Sediment samples for DNA extraction were collected aseptically from the top 1 cm of submerged sediment at each site, frozen immediately on dry ice and stored in a −80 °C freezer after transport to the laboratory. Samples for mineralogical analysis by X-ray diffraction (XRD) at UNLV were collected from the same fraction of sediment.

Mineralogy

The XRD analyses were made on clay fractions separated by centrifugation and sedimentation following rinsing with distilled water to achieve dispersion. Pastes of K- and Mg-saturated clays (<2 μm) were smeared on glass slides. The K-saturated sample slides were examined by XRD at 25 °C and after heating at 350 and 550 °C for 2 h. The Mg-saturated samples were analyzed at 25 °C and after being dried for 2 h at 65 °C in a desiccator containing a pool of ethylene glycol. The desiccator vent was closed upon removal from the oven and the slides were stored in the desiccator at least 12 h prior to XRD analysis. All samples were examined by XRD (CuKα radiation) using a PANalytical X'PERT Pro diffractometer (PANalytical, Almelo, the Netherlands), equipped with an X'Celerator detector (PANalytical).

16S rRNA gene amplification and sequencing

DNA was extracted from 0.5 g of frozen sediment using the FastDNA® SPIN for Soil Kit (Q-BIOgene, Irvine, CA, USA), eluted in 50 μL of DES (Q-BIOgene) and stored at −20 °C until use. Due to low amounts of biomass in the samples and high background absorbance in the extracts, the amount of DNA could not be accurately quantified. 16S rRNA genes were amplified from DNA extracted from the three springs with primers specific for *Bacteria* or *Archaea* using polymerase chain reaction (PCR). For bacterial 16S rRNA genes, forward primer 8bF (5′-GRG TTT GAT CCT GGC TCA G, where R is an A or G; Burggraf *et al.*, 1992) was used in combination with one of two reverse primers, 1406uR (5′-ACG GGC GGT GTG TRC AA; Lane, 1991) or 1512uR (5′-ACG GHT ACC TTG TTA CGA CTT, where H is an A, C or T; Lane, 1991). For archaeal rRNA 16S genes, forward primer 8aF (5′-YCY GGT TGA TCC TGC C, where Y is a C or T; Burggraf *et al.*, 1991) was used in combination with either 1406uR or 1512uR. The primer names are the same as those used in Eder *et al.*, 1999. The same PCR conditions were used for all primer sets: 96 °C for 4 min; 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 90 s; final extension at 72 °C for 5 min. Reaction mixtures (25 μL) contained 200 nM of each primer, 200 μM each of dATP, dGTP, dTTP and dCTP (Promega, Madison, WI, USA), 0.65 units of Go*Taq* polymerase (Promega), 5 μL of 5x GoTaq buffer (Promega), and 1 μL of template DNA. 16S rRNA gene libraries were constructed with product from each PCR using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Libraries constructed using PCR product obtained with primer sets 8bF/1406uR, 8bF/1512uR, 8aF/1406uR and 8aF/1512uR were named L1, L4, L2 and L5, respectively. Frozen glycerol stocks from each library were sent to the Nevada Genomics Center for plasmid isolation and sequencing using a Prism 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) with the applicable forward PCR primer (8aF or 8bF).

Phylogenetic analyses

Sequences from the bacterial (L1 and L4) and archaeal (L2 and L5) libraries were pooled and operational taxonomic units (OTUs) were assigned at the 3% sequence divergence level by DOTUR, using the nearest neighbor algorithm (Schloss & Handelsman, 2005). The highest quality representative of each OTU was selected based on phred score and compared to the NCBI database using BLASTN (Altschul *et al.*, 1997) to identify the closest relatives of each OTU at the phylum level (phylogenetic binning). The same representative was used for complete sequencing with vector primers (M13F and M13R) by High-Throughput Sequencing (Seattle, WA, USA) or Functional Biosciences (Madison, WI, USA). Complete 16S rRNA gene sequences were assembled using EMBOSS (Rice *et al.*, 2000). Sequences were checked for chimeric artifacts using Bellerophon (Huber *et al.*, 2004) and Mallard (Ashelford *et al.*, 2006), and verified using Pintail (Ashelford *et al.*, 2005). Phylogenetic relationships were inferred based on a comparison of maximum-likelihood analyses optimized using heuristic search, neighbor-joining calculation, and maximum-parsimony calculation in ARB (Ludwig *et al.*, 2004). Sequence data were submitted to GenBank under accession numbers EU924220–EU924261.

Thermodynamic modeling

Chemical data were used to calculate the Gibbs free energy available from potential metabolic reactions using the formula $\Delta G_r = \Delta G^\circ_r + RT\ln(Q)$ (Amend & Shock, 2001). In this expression R is the gas constant, T is the temperature (K), ΔG°_r is the standard Gibbs free energy at *in situ* temperature

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and pressure, and Q is the activity product, defined as the product of the activities of reaction products divided by the activities of the reactants, each raised to the power of their stoichiometric coefficient. Values for ΔG°_{r} were calculated using SUPCRT92 (Johnson *et al.*, 1992), using data and parameters from Helgeson *et al.* (1978), Shock *et al.* (1989, 1997), Shock & Helgeson (1990) and Shock (2009). Activities were calculated using EQ3/6 (Wolery, 1992). Results are expressed as the chemical affinity (A) of the reaction, where A is quantitatively equal to $-\Delta G_r$.

RESULTS AND DISCUSSION

The objective of this study was to characterize the microbial communities present in three caldera-associated Great Basin hot spring habitats and to determine how the microbial diversity may be related to the geochemistry of the environment. To this end, the microbial and geochemical datasets were used in conjunction with thermodynamic modeling to form hypotheses regarding organisms and processes that may be important in the ecology of the hot springs.

Site description and mineralogy

The LHC site consists of several spring sources of varying temperature and discharge rates that eventually converge to form LHC, which flows eastward to the Owens River. The springs sampled (LHC1, 3 and 4) represent three of the hottest sources with the greatest water discharge in the immediate area (Fig. 1). LHC1 and LHC4 emerge from the ground at a slight incline and have no appreciable source pools, while LHC3 has a small conical source pool ~1 m in diameter (Fig. 1). Water (from the sediment/water interface) and sediment (top ~1 cm) samples were taken from the spring sources, as close as possible to the center of the source. LHC1 was sampled at the deepest point in the outflow channel at the source (~10 cm below the air/water interface), while LHC3 and LHC4 were sampled ~25 cm below the air/water interface. The spring designated as LHC1 is named 'LHC-1' by the US Geological Survey (USGS) (http://lvo.wr.usgs.gov/images/lhc_map.gif), and LHC4 in this study corresponds to 'LHC site 3' in Breitbart *et al.* (2004). Although no measurements of spring discharge rates were made in this study, LHC4 was estimated to have a surface flow rate of 0.2 m s^{-1} by Breitbart *et al.* (2004). Additionally, the USGS measured flow rates monthly from 1987 to 1995 that ranged from 3.4 to 3.8 L s^{-1} at a flume several meters downstream from the LHC1 source (http://lvo.wr.usgs.gov/lhc_main.htm).

Sediment in LHC1 and LHC4 sources was black in color, whereas LHC3 contained a mix of black and gray sediment. There were no obvious streamers, mats or other biomass present at the spring sources at the time of sampling, however, small amounts of streamer growth have been periodically observed 1–2 m downstream of LHC1 at a constriction in the outflow. The mineralogical composition of the total clay (<2 μm) fraction of the LHC1 and LHC4 sediment samples was similar but distinct. Both samples consisted primarily of smectite, with lesser amounts of illite, kaolinite, and carbonate-bearing apatite. LHC1 also contained clinoptilolite and a trace amount of quartz and plagioclase feldspar. The LHC3 sediment consisted of illite and calcite with lesser amounts of smectite or other expansive mineral. The smectite or expansive mineral present was interpreted to be poorly crystalline and/or very small particle size based on the broad, low intensity peaks observed in the diffractograms.

Water chemistry of LHC Springs

Over 80 different chemical measurements were made in each of the three springs including pH, conductivity, dissolved gases, major ions, and trace elements. Selected data are shown

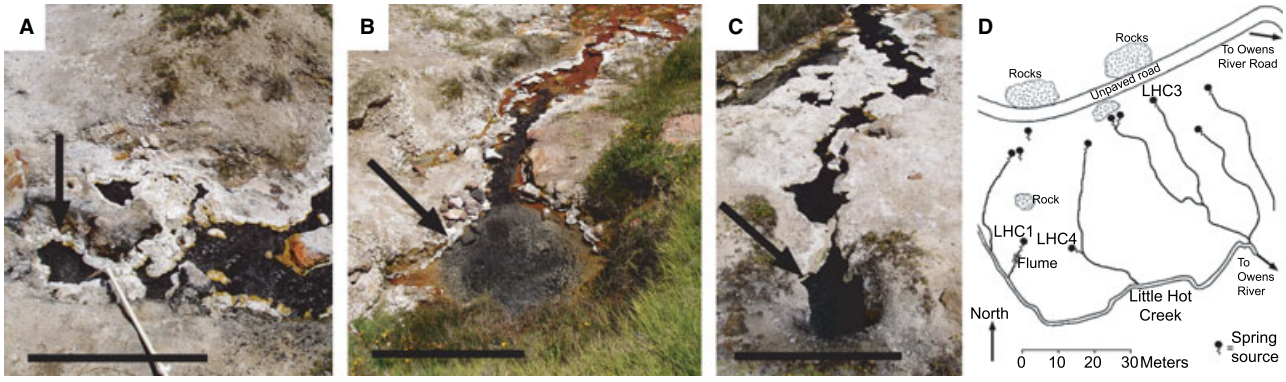

Fig. 1 Spring sources of LHC1, 3 and 4. Digital photographs of springs LHC1 (A), LHC3 (B) and LHC4 (C), with the source of each spring indicated with a black arrow. Sediment samples were taken near the center of each indicated source. Photographs were taken on a separate trip to the LHC springs in June, 2008. The white PVC tube in the LHC1 source (A) contains a hose connected to a peristaltic pump leading to the gas stripping apparatus (not shown). The black bar in the lower left corner of each figure indicates ~1 m in scale. (D) The relative locations of LHC1, 3 and 4 are shown within the LHC spring system, adapted from an image available on the USGS website (http://lvo.wr.usgs.gov/images/lhc_map.gif)

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in Table 1, and additional data (full ICP-MS, H and O isotopes) are provided in Table S1. LHC1, 3, and 4 were all close to 80 °C with near-neutral pH and high alkalinity. The high alkalinity of the hot springs is due to neutralization of carbonic acid from the large amounts of magmatic CO_2 that are found in soil and water throughout the Long Valley area (Farrar *et al.*, 1995). Geothermal hot springs are often categorized based on their chemical properties. A 2005 study in YNP by Meyer-Dombard *et al.* (2005) grouped three YNP systems (Bison Pool, Obsidian Pool and Sylvan Spring) based on geochemical measurements and designated them as vapor-dominated (acid-sulfate), water-dominated (alkaline-chloride and carbonate), or a mix of these two systems. The relatively high water discharge, circumneutral pH, and high chloride concentration as well as most major cations in LHC springs are indicative of water-dominated systems, whereas high levels of sulfide and $\sum NH_3$ are characteristic of vapor-dominated systems in YNP (Fournier, 1989; Nordstrom *et al.*, 2005). In general, the chemistry of the LHC springs resembles that of Obsidian Pool (Shock *et al.*, 2005), where main differences include apparent anoxia in the LHC system in addition to higher alkalinity, conductivity, sulfide concentration, and several solutes (Na^+, Cl^-, NH_4^+, and SO_4^{2-}). Obsidian Pool also had characteristics of both the water- and vapor-dominated systems, and was interpreted as a dilute acid-sulfate fluid by Meyer-Dombard *et al.* (2005). It is likely that the LHC springs represent a mix of these two systems as well.

Table 1 Spring temperature and selected chemistry*

	LHC1	LHC3	LHC4
Field measurements			
Water temperature (°C)†	82.5 ± 0.1	79.0 ± 0.1	78.7 ± 0.1
Conductivity (mS cm^{-1})†	2.68 ± 0.05	2.38 ± 0.05	2.13 ± 0.04
pH†	6.75 ± 0.01	6.97 ± 0.01	6.85 ± 0.01
Alkalinity (ppm $CaCO_3$)‡	530	510	530
Dissolved silica (mM)‡	1.1	1.3	1.2
Total sulfide (μM)‡	20.9	16.4	16.4
Total ammonia (μM)‡	198	63.7	68.2
Nitrite (μM)§	b.d.	b.d.	b.d.
Oxygen (μM)§	b.d.	b.d.	b.d.
Ion chromatography			
Cations¶			
Sodium (mM)	17.3	16.5	16.0
Calcium (μM)	549	530	740
Lithium (μM)	372	350	341
Potassium (μM)	673	644	628
Anions¶			
Chloride (mM)	5.24	5.06	4.88
Sulfate (mM)	1.00	0.958	0.933
Nitrate (μM)	2.42	2.44	2.46
Bromide (μM)	1.55	1.21	1.30
Gases‡			
Hydrogen (nM)	14.0	8.8	4.9
Methane (nM)	588	119	306
Ethane (nM)	2.5	5.0	1.03
Ethylene (nM)	1.75	2.57	1.39
ICP-MS¶			
Total As (μM)	9.8	9.75	9.79
Total Fe (μM)	1.56	2.08	2.18

*All three springs were sampled on June 29, 2006.
†Error is based on the manufacturer's specification (LaMotte).
‡Measured only once during this sampling trip.
§Oxygen and nitrite were below detection (b.d.) limits of 3 and 0.1 μM, respectively.
¶Analytical error associated with duplicate measurements of a single sample was <10%.

Potential electron donors for chemolithotrophy detected in the springs included hydrogen, sulfide, methane and ammonium (Table 1). Hydrogen and methane were present at the low end of the range of values measured in a variety of YNP hot springs (Spear *et al.*, 2005) and in the GBS/MHS system (Costa *et al.*, 2009). Levels of ammonium were also similar to those in GBS/MHS springs, while sulfide was considerably higher (Costa *et al.*, 2009). Nitrate and sulfate were the most abundant potential electron acceptors detected in all three springs. Nitrate was not detected in the field using a colormetric assay with a theoretical detection limit of 1 μM (LaMotte) but was detected by ion chromatography. Dissolved oxygen was not detected in any of the springs by the azide modification of the Winker method, which has a detection limit of ~3 μM (LaMotte). Because sulfide is known to cause negative interference with this method at a stoichiometry of approximately 2:1 to O_2 (Ingvorsen & Jørgensen, 1979), the practical detection limit for O_2 thus ranged from 11 to 13 μM, based on the measured sulfide levels (Table 1). The inability to detect O_2 may have reflected true anoxia, sulfide interference or rapid microbial utilization of available O_2 in the water. It is therefore not clear to what extent O_2 is available as a terminal electron acceptor in these springs.

Overview of 16S rRNA gene libraries

Polymerase chain reaction of bacterial and archaeal 16S rRNA genes using two different primer sets targeting each domain yielded product from DNA from all three springs. Sequences from the two bacterial (L1 and L4) and archaeal libraries (L2 and L5) from each spring were combined, chimeric or poor quality sequences were removed, and the remaining sequences were grouped into OTUs, or phylotypes, at the species level (>97% identity). After dereplication of identical OTUs between the springs, the libraries in total contained 20 archaeal phylotypes, representing 204 sequences, and 21 bacterial phylotypes, representing 201 sequences. Rarefaction curves and Chao1 estimators calculated by DOTUR (Chao, 1984; Schloss & Handelsman, 2005) for the individual libraries indicated that most clone libraries were sampled nearly to saturation, except for LHC1 L2 and L4 and LHC4 L2 and L4 (Table S2). The rarefaction data also indicated that libraries constructed from PCR product using different reverse primers did not always sample the environments equally. This

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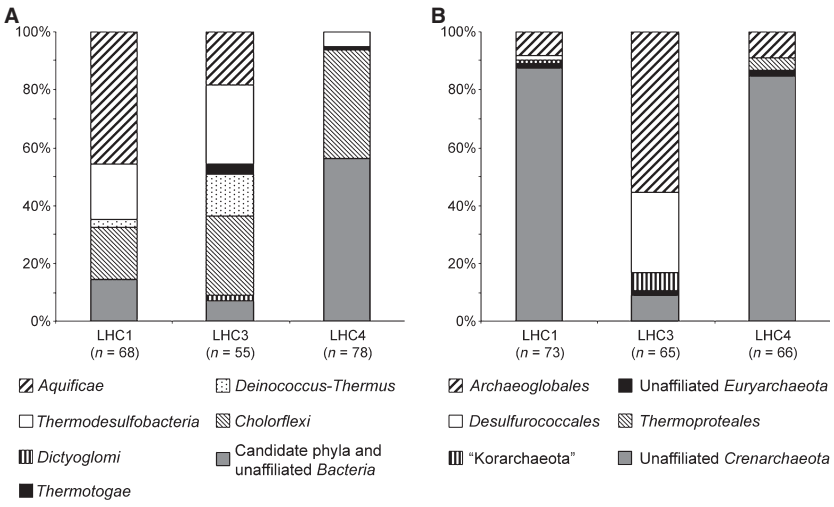

Fig. 2 Phylogenetic binning of sequences in 16S rRNA clone libraries. Sequences in the LHC1, LHC3, and LHC4 16S rRNA clone libraries constructed using PCR primers specific for *Bacteria* (A) or *Archaea* (B) were grouped based on their relationship to known phyla or orders and are displayed as a percentage of the total number of sequences (*n*) in each library.

exemplified the utility of using multiple primer pairs to mitigate primer bias and to achieve more comprehensive sampling across an environment.

Phylogenetic binning based on closest relatives in the NCBI database revealed a moderate degree of phylum-level diversity. The percent abundance of 16S rRNA gene sequences representing various phyla (and orders for *Archaea*) and their distribution in each clone library is shown in Fig. 2A (*Bacteria*) and B (*Archaea*). OTUs were binned in a given phylum if their representative sequence had ≥85% identity to sequences in accepted phyla, or if they were consistently monophyletic with accepted phyla based on phylogenetic analyses (Figs 3 and 4; Hugenholtz *et al*., 1998). A list of OTUs and their closest BLASTN hits and percent identity to 16S rRNA genes of cultured and uncultured organisms is shown in Table S3. Over 70% of the sequences in bacterial libraries (representing 13 OTUs) could be grouped within six formally named bacterial phyla: *Aquificae*, *Thermodesulfobacteria*, *Thermus–Deinococcus*, *Thermotogae*, *Chloroflexi* and *Dictyoglomi*. Of the remaining sequences, 58 (seven OTUs) grouped within the candidate phyla OP1, OP9, OPS8, and OP11/SR1. An additional sequence could not be affiliated with known or predicted phyla; it was <85% identical to 16S rRNA genes of known or candidate phyla and did not consistently group with known or candidate phyla in phylogenetic analyses. For the archaeal libraries, 72.5% of the sequences fell within the phylum *Crenarchaeota*. The majority of these (126 sequences, 15 OTUs) were not affiliated with known archaeal orders, while 19 sequences (one OTU) grouped with the order *Desulfurococcales* and three sequences (one OTU) belonged to the *Thermoproteales*. Of the remaining sequences, 48 belonged to the order *Archaeoglobales* within the phylum *Euryarchaeota*, three were *Euryarchaeota* unaffiliated with known classes, and five grouped closely with members of the candidate phylum 'Korarchaeota', each represented by one OTU.

Analysis of bacterial libraries

The inferred phylogenies of the 21 bacterial species-level phylotypes identified in the springs, along with the percentage of sequences among *Bacteria* from each spring, are shown in Fig. 3. The results were somewhat variable between the three springs, despite their similar chemistries. Bacterial libraries from LHC1 and 3 showed broadly similar distributions at the phylum level, while LHC4 appeared to be less diverse. Sequences grouping within the phylum *Aquificae* were unequally distributed within the three springs, being dominant in LHC1 (45.6% of the library), present in LHC3 (18.2%), but not detected in LHC4. In LHC1, all but one of these sequences were represented by a single OTU with ~95% identity to *Thermocrinis ruber*. *Aquificae* in LHC3 grouped into two OTUs distinct from those in LHC1, each representing a similar number of 16S rRNA sequences related to either *T. ruber* or *Sulfurihydrogenibium* spp. (represented as *Sulfurihydrogenibium azorense* in Fig. 3). *Thermocrinis ruber* is a microaerophilic chemoautolithotroph that can use H_2, sulfur and thiosulfate as electron donors, but can also use formate and formamide as sole carbon and electron sources (Huber *et al*., 1998). *Sulfurihydrogenibium* spp. are also facultative chemoautolithotrophs, but collectively can use a wider array of electron donors (reduced Fe and As, in addition to sulfur and thiosulfate) and terminal electron acceptors (including nitrate and oxidized Fe, As and Se compounds) (Nakagawa *et al*., 2005). A single sequence from LHC1 was 99% identical to the 16S rRNA gene of *Hydrogenobacter subterraneus*, which requires reduced sulfur compounds for growth, cannot use H_2 and is unusual among the *Aquificae* in that it is an obligate heterotroph (Takai *et al*., 2001). *Aquificae* are often abundant in 16S rRNA gene libraries from circumneutral, as well as some acidic, springs in YNP ranging in temperature from 56 to 92 °C, and are believed to be important primary producers (Reysenbach *et al*., 1994; Meyer-Dombard *et al*.,

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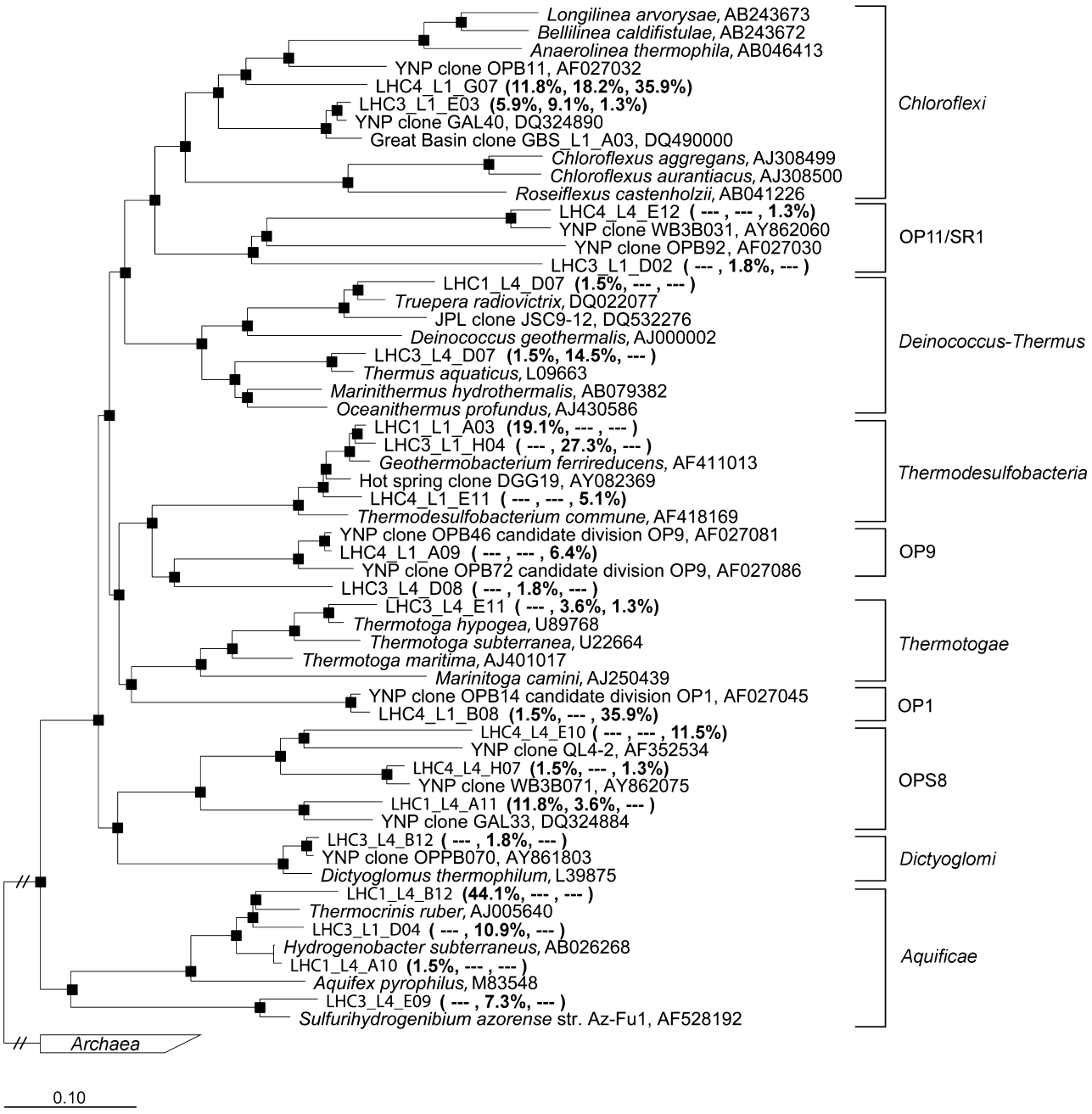

Fig. 3 Inferred phylogeny of *Bacteria* in LHC springs. Phylogenetic relationships were inferred based on a distance matrix using the maximum likelihood, maximum parsimony, and neighbor-joining software supplied with ARB. The resulting phylogeny of the maximum likelihood method is shown. Black squares at nodes indicate branching pattern supported in three separate analyses (maximum likelihood, neighbor joining, and maximum parsimony). Sequences identified in this study have the notation LHC, followed by the spring number, the library number and the clone name. This is followed in parentheses by three values identifying the number of sequences represented by the phylotype in springs LHC1, 3 and 4, respectively, expressed as a percentage of the total number of sequences in the library from that spring; dashes denote that the phylotype was not present in a given spring. The names of sequences of cultured and uncultured bacteria identified in other studies are followed by their accession number. The scale bar indicates the number of changes per nucleotide position.

2005; Spear *et al.*, 2005; Boyd *et al.*, 2009). Among Great Basin hot springs, *Aquificae* were abundant in Alvord Hot Springs (Connon *et al.*, 2008), but were only minor constituents of sediment communities in the GBS/MHS area (Costa *et al.*, 2009).

Sequences grouping within the phylum *Thermodesulfobacteria* were detected in all three springs; however, each spring contained a unique phylotype (Fig. 3). In general, *Thermodesulfobacteria* are thermophilic chemolithoautotrophs and heterotrophs capable of using oxidized sulfur and iron

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compounds as terminal electron acceptors. Sequences in LHC1 and LHC3 were closely related (98 and 97% identity, respectively) to the 16S rRNA gene of *Geothermobacterium ferrireducens*, a chemolithoautotroph that obtains energy by reducing ferric iron to magnetite by using H_2 as an electron donor (Kashefi *et al.*, 2002a). The phylotype in LHC4 was 95% identical to the recently isolated *Caldimicrobium rimae*, a facultative chemolithoautotroph that couples sulfur or thiosulfate reduction to oxidation of H_2 (Miroshnichenko *et al.*, 2009).

Only two phylotypes were present in libraries from all three springs, LHC4_L1_G07 and LHC3_L1_E03 (Fig. 3). These were moderately abundant in all three springs, collectively representing 17–37% of the total bacterial sequences (Fig. 2A). They both branch deeply within the *Chloroflexi*, a metabolically diverse phylum containing both phototrophs and chemoheterotrophs. The more abundant phylotype, LHC4_L1_G07, branches deeply within the 'Anaerolineae', while LHC3_L1_E03 does not reliably affiliate with named classes within the *Chloroflexi*. The 'Anaerolineae' encompass mesophilic to moderately thermophilic, filamentous, anaerobic heterotrophs (Sekiguchi *et al.*, 2003; Yamada *et al.*, 2007). Sequences with >98% identity to these two LHC phylotypes were abundant in hot springs in the GBS/MHS system (Costa *et al.*, 2009). Apart from this, closely related sequences (>90% identity) have only been found in two YNP springs, where they were minor constituents of 16S rRNA gene libraries (Blank *et al.*, 2002; Ackerman, 2006). Other OTUs grouping with known phyla included close relatives of the heterotrophic *Thermus aquaticus*, *Truepera radiovictrix*, *Thermotoga hypogea* and *Dictyoglomus thermophilum*. With the exception of the *T. aquaticus* relative, LHC3_L4_D07 (14.5% of LHC3 library), these were represented by only one or two sequences in the libraries, if they were detected at all.

The LHC4 clone libraries contain the largest relative abundance of unaffiliated bacteria, with about 44% of the clones showing no close relationship to any of the cultivated phyla. Of these, 64% comprise a single OTU with 99% sequence identity to members of the candidate division OP1 (OPB14; Fig. 3), which was first identified in Obsidian Pool in YNP (Hugenholtz *et al.*, 1998). A single member of the same OTU was also found in LHC1. The remaining unaffiliated bacterial clones from LHC4 are mainly members of the candidate divisions OP9, OPS8, and OP11/SR1.

Analysis of archaeal libraries

The inferred phylogenies of the 20 archaeal species-level phylotypes, along with the percentage of sequences among *Archaea* from each spring, are shown in Fig. 4. LHC3 archaeal libraries were dominated by members of the phylum *Euryarchaeota*. A single phylotype that groups with the order *Archaeoglobales* dominated LHC3 and was present but less abundant in LHC1 and 4 (Figs 2B and 4). The LHC sequences, which appear to represent a new genus or family (Fig. 4), were between 91% and 93% identical to 16S rRNA genes from members of the genera *Archaeoglobus, Geoglobus* and *Ferroglobus*. These genera represent anaerobic chemolithoautotrophs and heterotrophs, which collectively can use NO_3^-, Fe^{3+} and oxidized sulfur compounds as terminal electron acceptors (Kashefi *et al.*, 2002b; Hartzell & Reed, 2006). H_2 is usually the exclusive inorganic electron donor, although *Ferroglobus* spp. can additionally use H_2S and Fe^{2+} (Hafenbradl *et al.*, 1996). The next most abundant OTU in the LHC3 archaeal libraries is a member of the *Desulfurococcales* with the most closely related cultivated lineage being *Thermosphaera aggregans* (Fig. 4). As most members of the *Archaeoglobales* and *Desulfurococcales* are anaerobes, their prevalence in LHC3 suggests an anaerobic environment.

Libraries from LHC1 and LHC4 are both dominated by uncultivated members of the *Crenarchaeota*, comprising a total of 14 phylotypes, and host only small percentages of *Euryarchaeota*, in contrast to LHC3 in the spring ecosystem. Three of these phylotypes dominated libraries from LHC1 and 4 (LHC3_L2_E01, LHC3_L2_F12 and LHC3_L5_F12; Fig. 4), and were present in LHC3 as well. Their predominance suggests that they represent important members of the microbial communities in LHC springs. The divergence of these phylotypes from cultivated *Archaea*, however, makes it difficult to predict their metabolism and possible role in these ecosystems. Similar to the GBS/MHS springs, no sequences in the archaeal libraries grouped with 'Great Basin hot spring crenarchaeota cluster I' sequences, previously identified by Huang *et al.* (2007) from relatively cooler springs (49–67 °C). Also, no relatives of the recently cultivated, aerobic, ammonia oxidizing crenarchaeon '*Candidatus* Nitrosocaldus yellowstonii' were detected. These were abundant in GBS/MHS springs with a higher residence time but absent in libraries from Sandy's Spring West, which has a high flow rate and low oxygen levels, qualitatively similar to the LHC springs (Costa *et al.*, 2009). Libraries from springs LHC1 and 3 also contained a phylotype with 98% identity to the 16S rRNA gene of '*Candidatus* Korarchaeum cryptofilum', a heterotrophic anaerobe that represents a third phylum within the *Archaea* (Elkins *et al.*, 2008).

Similar to the bacterial libraries, there was a lack of continuity among the archaeal populations of the three springs, in spite of the likelihood of a common source for geothermal fluid in all three springs (Sorey *et al.*, 1991). Although LHC1 and 3 exhibited similar bacterial libraries, the LHC3 archaeal library was unique. The mineralogical component of LHC3 sediment also was distinct, where illite was the major clay mineral rather than smectite. Past studies of microbial interactions with iron-containing minerals indicate that mesophilic, iron-oxidizing *Bacteria* are capable of respiring iron-containing clay minerals such as smectite (Kostka *et al.*, 1996, 1999). More recently, it was observed that *Bacteria* may play a role in

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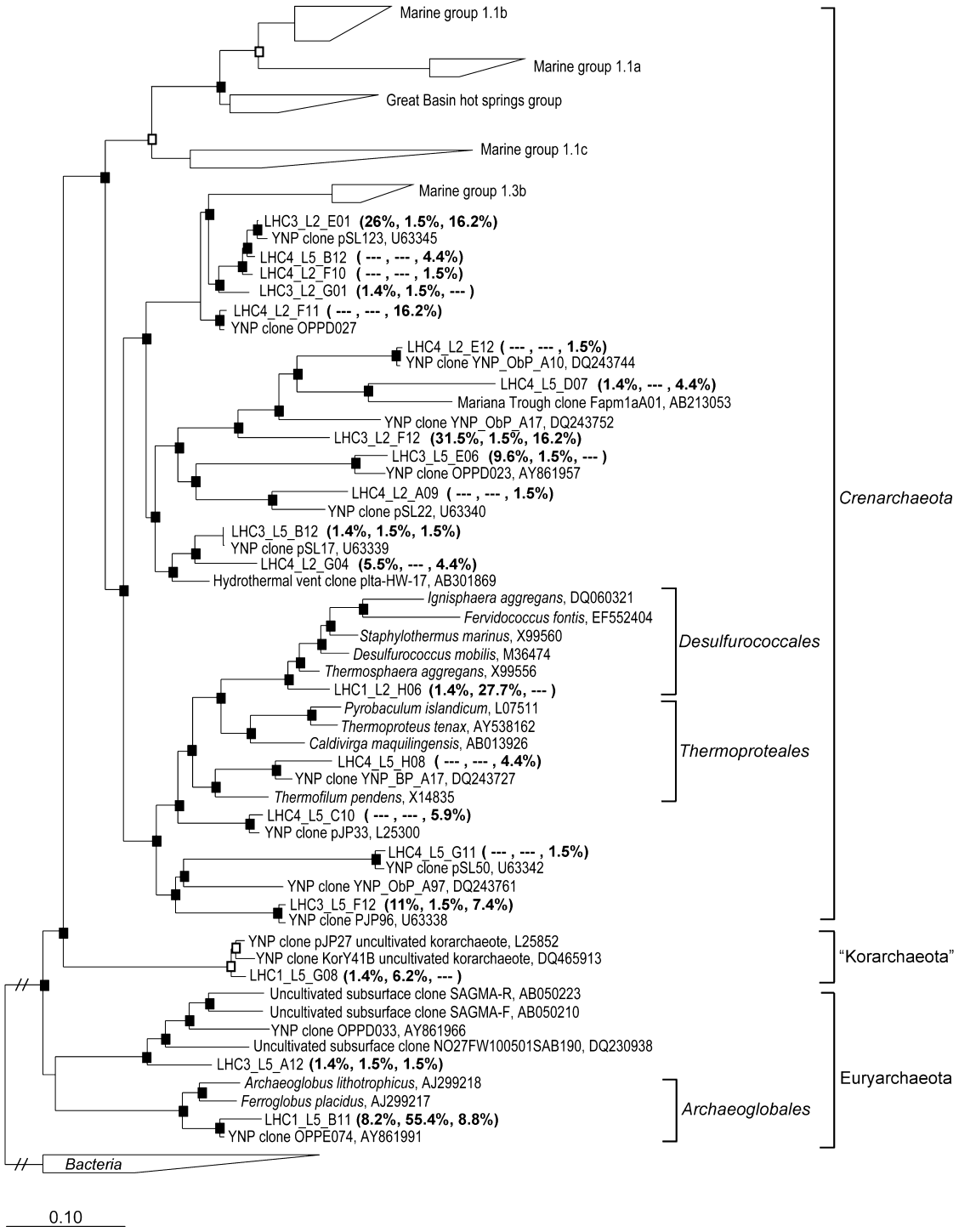

Fig. 4 Inferred phylogeny of *Archaea* in LHC springs. Phylogenetic relationships were inferred based on a distance matrix using the maximum likelihood, maximum parsimony, and neighbor-joining software supplied with ARB. The resulting phylogeny of the maximum likelihood method is shown. Black squares, branching pattern at this node was supported in three separate analyses (maximum likelihood, neighbor joining, and maximum parsimony). Open squares, branching pattern at this node was supported in two of three analyses. The branching pattern at unmarked nodes was only supported in maximum likelihood analysis. Sequences identified in this study have the notation LHC, followed by the spring number, the library number and the clone designation. This is followed in parentheses by three values identifying the number of sequences represented by the phylotype in springs LHC1, 3 and 4, respectively, expressed as a percentage of the total number of sequences in the library from that spring; dashes denote that the phylotype was not present in a given spring. The names of sequences of cultured and uncultured *Archaea* identified in other studies are followed by their accession number. The scale bar indicates the number of changes per nucleotide position.

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the transformation of smectite to illite (Zhang *et al.*, 2008). Finally, it was recently shown that members of the *Archaeoglobale*s and *Desulfurococcales* are capable of respiring iron bound in smectite, and that they may be involved in the transformation of smectite to illite at high temperature (Kashefi *et al.*, 2008). Thus, the abundance of illite in LHC3 may be explained by the predominance of members of the *Archaeoglobales* and *Desulfurococcales.*

Thermodynamics of chemolithotrophy in LHC

Thermodynamic modeling of 75 known and theoretical chemolithotrophic reactions (Table S4) provided a measure of the potential Gibbs free energy available to organisms from various potential metabolisms in the hot springs. The results for selected reactions, grouped by their terminal electron acceptor, are shown in Fig. 5. In general, the results are similar to those obtained in studies applying a similar approach to several YNP and GBS/MHS springs (Inskeep *et al.*, 2005; Shock *et al.*, 2005, 2009; Hall *et al.*, 2008; Costa *et al.*, 2009). Because of their similar chemistries, energy yields for a given reaction were essentially the same in the three springs, usually not varying by more than ~2 kJ mol^{-1} e^- transferred. Considering reactions for which all reactants and products were detected in the springs, dissimilatory nitrate reduction to ammonia (Reactions 9–15, Fig. 5) and sulfur respirations

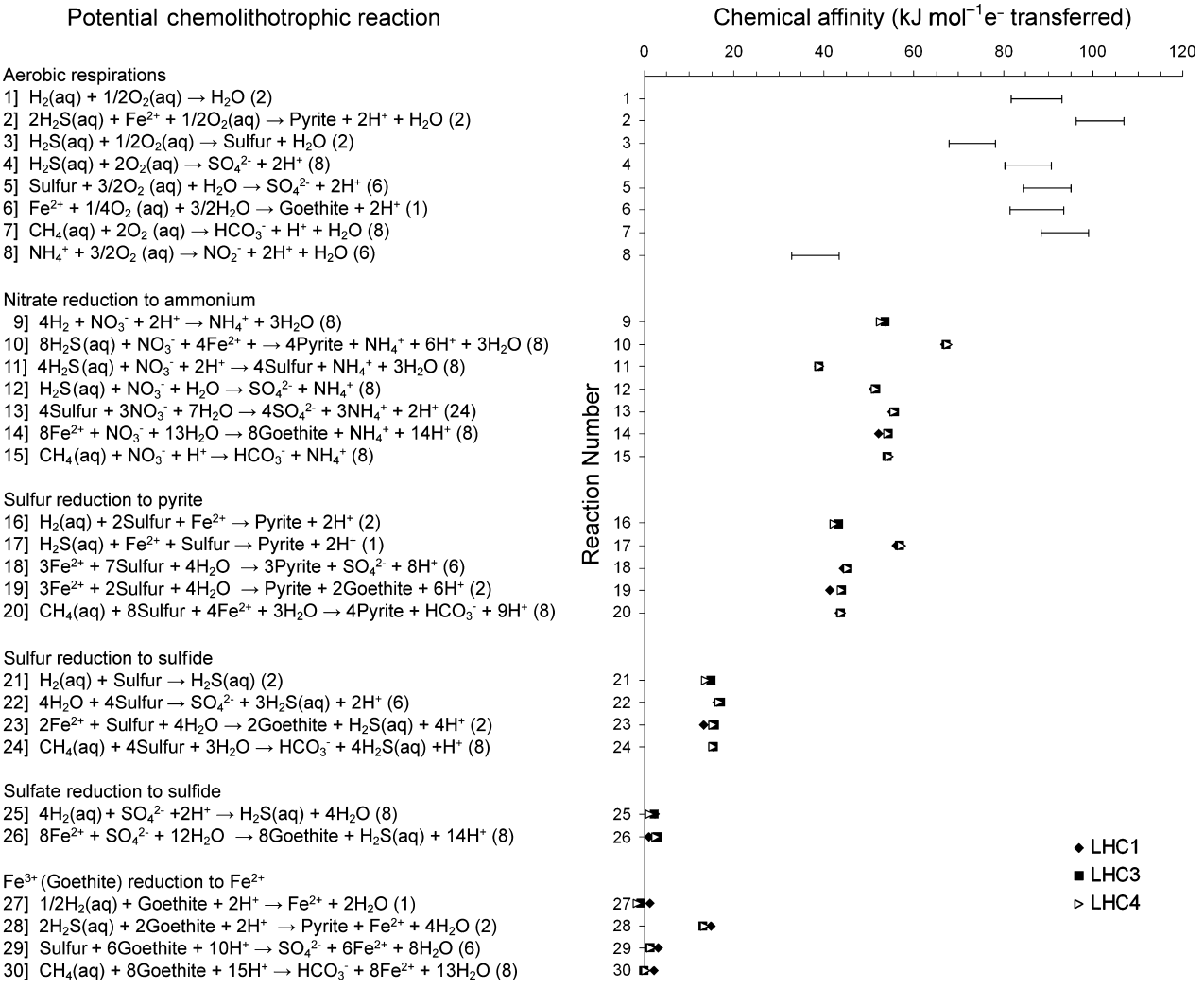

Fig. 5 Energy yields for known and hypothetical chemolithotrophic reactions. The chemical affinities, expressed as kJ mol^{-1} e^- transferred, are plotted as points on the x-axis for selected reactions calculated using measured spring chemistry for LHC1, 3 and 4 (Amend & Shock, 2001). The reactions are grouped by terminal electron acceptor. The number in parentheses after each reaction indicates the number of electrons transferred. For reactions 1–8, bars represent the range of affinities, lowest to highest in all three springs collectively, for aerobic respirations calculated using two hypothetical oxygen concentrations (10–12 M and 10–6 M). To calculate the chemical affinity of aerobic ammonium oxidation (reaction 8), 100 nM nitrite was assumed. Reactions involving ferric and ferrous iron are represented by goethite (FeOOH) and Fe^{2+}, respectively. Very similar chemical affinities (usually within 2 kJ mol^{-1} e^-) were obtained when Fe^{2+} was replaced with magnetite as a reactant or when goethite was replaced with magnetite or hematite as a product in these equations. This situation is the same when $HCO3^-$ is replaced by CO2 in the equations (Table S4).

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(Reactions 16–26) coupled to the oxidation of H_2, H_2S, sulfur, Fe^{2+} and CH_4 were predicted to yield the most energy. Although nitrite was not detected in the spring water, a lower bound for chemical affinities of nitrate reduction to nitrite was estimated using a hypothetical concentration of nitrite at the level of detection (0.1 μM). This resulted in chemical affinities ~10 kJ mol^{-1} e^- higher than the corresponding reaction involving nitrate reduction to ammonium (Reactions 9–15, Fig. 5), indicating that nitrate reduction in general is favorable in the LHC springs. When using a given terminal electron acceptor, no electron donor was obviously dominant in terms of the energy yield, in contrast to some YNP springs where H_2 oxidation was predicted to be the most energetically favorable (Spear *et al.*, 2005).

Calculation of the energy available from metabolisms attributed to characterized organisms revealed that many either do not yield the ~10 kJ mol^{-1} necessary to support microbial growth, or yield only nominally more energy (~15 kJ mol^{-1}) than the minimum requirement (Schink, 1997). An example of this would be sulfate reduction, which is known to be carried out by members of the *Thermodesulfobacteria* and *Archaeoglobales*. Although 16S rRNA gene sequences related to these groups were present in all three springs, sulfate reduction coupled to H_2 or Fe^{2+} oxidation yields very little energy (Reactions 25 and 26, Fig. 5). Ferric iron reduction carried out by *Geothermobacterium ferrireducens*, close relatives of which were found in LHC1 and LHC3, is another example of a reaction with a low energy yield. In pure culture *G. ferrireducens* reduces poorly crystalline Fe^{3+} [represented as the hydroxide mineral goethite (FeOOH) in our equations] to magnetite using H_2 as an electron donor (Kashefi *et al.*, 2002a). This reaction had a negative chemical affinity (Table S4), and a similar reaction yielding Fe^{2+} was essentially at equilibrium in the three springs (Reaction 27, Fig. 5). This suggests that the relatives of *G. ferrireducens* in LHC may derive energy using alternative redox couples, or that they may inhabit microenvironments within the spring sediment where conditions make goethite or amorphous ferric hydroxide reduction by H_2 more favorable. Several other reactions representing types of known microbial metabolisms, such as methanogenesis and sulfate reduction to sulfur, yielded negative chemical affinities and were thus not predicted to support life in the springs (Table S4). It is possible that these reactions were more favorable in conditions present in the spring sediment, illustrating a limitation on predicting thermodynamics in spring sediment using bulk spring water chemistry.

Thermodynamic calculations based on hypothetical O_2 concentrations at, and many of orders of magnitude below, the method detection limit (~10 μM) predicted highly energetic aerobic respirations coupled oxidation of to CH_4, H_2, sulfur, Fe^{2+}, NH_4^+, and H_2S (Fig. 5, Reactions 1–8). The prevalence of sequences in LHC1 and 3 related to *Thermocrinis* spp., which are obligate microaerophiles, suggests that O_2 is indeed present and utilized by microbes in these springs. The highest enzymatic affinities for O_2 measured for cytochrome oxidases involved in energy conservation by aerobic respiration in mesophilic bacteria range from 3 to 8 nM (Contreras *et al.*, 1999). If respiratory enzymes in thermophiles have similarly high affinities, then O_2 could serve as a terminal electron acceptor even at nanomolar concentrations, approximately three orders of magnitude below the level of detection.

Changing theoretical O_2 concentrations from 10^{-6} to 10^{-12} M in the thermodynamic models resulted in a drop in chemical affinity of only ~10–12 kJ mol^{-1} e^-, which is still well above the corresponding reaction using the 'next best' electron acceptor, nitrate. This highlights the relative insensitivity of the chemical affinity of many of these reactions to measureable levels of reactants or products, other than $[H^+]$, as previously noted (Inskeep *et al.*, 2005). As an example, for aerobic hydrogen oxidation to have an equivalent chemical affinity to the same reaction with nitrate as electron acceptor, thereby making the two electron acceptors 'competitive' strictly in thermodynamic terms, it would require unreasonably low theoretical O_2 concentrations (~1 molecule L^{-1}; 10^{-24} M). These levels are obviously far below what could efficiently be utilized by biological systems. Thus, in contrast to the thermodynamic limitations on metabolisms such as sulfate reduction discussed above, aerobic respiration in the LHC system may be kinetically limited, for example by affinity (K_m) of respiratory enzymes for O_2 or by rates of O_2 transport to the sediment via diffusion or turbulence (Inskeep *et al.*, 2005). In this light, the variable presence of *Aquificales* sequences in the three springs might be due to heterogeneity of O_2 penetration within the springs or to the depth below the air–water interface at which the sediment sample was taken, rather than to some measured aspect of spring chemistry. For example, LHC3 and 4 spring sources were considerably deeper (~30–60 cm total, sampled at ~25 cm depth) than the source of LHC1 (~10 cm), where *Aquificales* represented about half of the bacterial 16S rRNA gene library.

CONCLUSION

This work describes the first combined geochemical and microbiological characterization of three circumneutral, ~80 °C springs in the LHC area of the Long Valley Caldera. Synthesis of the chemical measurements, microbial censes and thermodynamic modeling presented here allows some general conclusions to be drawn regarding chemolithotrophy in LHC. The temperature and chemistry of spring waters from LHC1, 3 and 4 were all very similar, and the three springs all shared populations of anaerobic (*Thermodesulfobacteria*, *Archaeoglobales*) and phylogenetically novel *Bacteria* and *Archaea*. It is well known that the abundance of sequences in 16S rRNA gene libraries may not reflect the abundance or importance of their host organisms in the environment (Reysenbach *et al.*, 1992; von Wintzingerode *et al.*, 1997).

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Because sediment was sampled from only one site from each spring, the differences in 16S rRNA gene libraries may also be the result of heterogeneity of microbial populations within the spring. Notwithstanding these limitations, we speculate that the varying abundance of close relatives of aerobic *Aquificales* (*Thermocrinis* spp.) and anaerobic *Archaeoglobales* and *Desulfurococcales* may be due to differing levels of anoxia in the three springs. In LHC1, the abundance of *Thermocrinis* spp. suggests a microaerobic environment where primary production is coupled to oxidation of H_2 or reduced sulfur compounds. In contrast, the minor presence of *Thermocrinis* spp. in LHC3, coupled with an increased abundance of anaerobic *Archaeoglobales* and *Desulfurococcales*, suggests a microaerobic to anaerobic environment. The prevalence of *Thermodesulfobacteria* and *Archaeoglobales* in LHC3 implicate H_2 as an important electron donor for chemolithotrophy. In addition to trace amounts of oxygen, sulfur and nitrate may be preferred electron acceptors, while sulfate and ferric iron reduction are predicted to be thermodynamically limited. The absence of *Aquificales* in LHC4, which is otherwise similar to LHC1 in terms of archaeal and bacterial 16S rRNA gene libraries, suggests a strictly anaerobic environment. The prevalence of novel *Bacteria* and *Archaea* in this spring make extrapolation of phylogenetic data to predict important chemolithotrophic metabolisms particularly difficult. Because thermodynamic models indicate that O_2 levels far below the detection limit would still allow for highly energetic aerobic respirations, the variable abundance of *Thermocrinis* spp. in the springs may be determined by kinetic limitations on oxygen transport from the atmosphere to the spring sediment.

The LHC springs, in general, displayed similarities to circumneutral springs in both YNP and the Great Basin in terms of their chemistry and microbiology. The energy yields of various chemolithotrophic reactions predicted for these springs were broadly similar to those obtained for YNP and other Great Basin hot springs (Inskeep *et al.*, 2005; Meyer-Dombard *et al.*, 2005; Shock *et al.*, 2005, 2009; Hall *et al.*, 2008; Costa *et al.*, 2009). The abundance of *Aquificales* in LHC1 and of novel *Crenarchaeota* in LHC1 and LHC4 are reminiscent of springs in YNP (Meyer-Dombard *et al.*, 2005; Spear *et al.*, 2005). The prevalence of deeply divergent members of the phylum *Chloroflexi* in all springs is characteristic of the GBS/MHS area in the Great Basin (Costa *et al.*, 2009). When combined with ongoing, parallel studies of other Great Basin hot spring systems, the data presented here will facilitate a thorough comparison of Great Basin and YNP hot springs in terms of their geochemistry, microbiology, and biogeography.

The chemical data and thermodynamic predictions described here will be used to inform attempts at culturing microorganisms from these springs. The abundance of novel bacterial clones in LHC4 and archaeal clones in LHC1 and 4 suggest that a substantial portion of the microbial population of LHC springs is not well understood. Understanding the nature of this so-called biological 'dark matter' (Marcy *et al.*, 2007) and its role in hot spring ecology is a major goal of current and future cultivation and cultivation-independent studies.

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SUPPORTING INFORMATION

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

Table S1 Additional spring water chemistry not shown in Table 1.

Table S2 Selected output from DOTUR (Schloss & Handelsman, 2005) for individual bacterial (L1 and L4) and archaeal (L2 and L5) 16S rRNA gene libraries from springs LHC1, 3 and 4.

Table S3 Top BLASTN hits of LHC OTU sequence representatives to full-length or near-full-length (covering >80% of the sequence) 16S rRNA gene sequences from cultured and uncultured organisms in the NCBI database.

Table S4 Chemical affinity (numerically equivalent to the ΔGr of the reaction) of potential metabolic reactions in LHC1, 3, and 4, normalized to the number of electrons transferred per reaction (indicated in parentheses after each reaction).

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