Hydrogen and Primary Productivity: Inference of Biogeochemistry from Phylogeny in a Geothermal Ecosystem

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ABSTRACT

The geochemical energy sources for microbial primary productivity in the >70°C geothermal springs of Yellowstone National Park have not been understood. Results from phylogenetic studies of geothermal communities indicate, unexpectedly, that hydrogen-metabolizing organisms, both known and novel, dominate these communities. Hydrogen (H_2) is the basis of diverse microbial metabolisms, yet little is known about how this important energy source functions in natural microbial communities. We measured source waters in the Yellowstone geothermal area by gas chromatography to survey the potential distribution of hydrogen concentrations in high-temperature waters (hot springs, streams, geothermal vents, and a well). The results indicate levels in excess of 300 nM H_2 in chemically diverse settings. Extensive, culture-independent molecular phylogenetic surveys of microbial communities from several locations with different chemistries also were conducted. These surveys were based on cloning and sequencing of 16S rRNA genes for phylogenetic analyses to determine the nature and quantities of microbial diversity that constitute these communities. Analyses of DNA sequences show a dominance of organisms of the phylogenetic kinds known to metabolize H_2 as an energy source. Sulfur metabolism is commonly invoked in geothermal bioenergetics, and organisms of the kinds that rely on sulfur metabolism occur in these settings but are not numerically prominent. Thermodynamic modeling of potential energy sources in these settings shows that a microaerophilic hydrogen-based metabolism is most energetically favorable, even in the presence of high concentrations of sulfide. Consequently, we propose that molecular hydrogen provides the main driving energy for primary productivity in this, and probably other, geothermal ecosystems.

Key Words

geothermal springs
hydrogen
phylogenetic survey
primary productivity

1.0 INTRODUCTION

Yellowstone National Park is a remarkable place to any visitor. The macro biota of flora and fauna are visible and ubiquitous. Even more amazing is the microbial world, with a wide diversity of life throughout Yellowstone's extreme temperature gradient. In recent years, several new developments in microbial ecology, many developed from studies of Yellowstone hot springs, have allowed us to view the microbial world in an entirely new fashion. Traditional, culture-based techniques have been supplemented by new molecular techniques that allow for a better assessment of the nature of microbial communities in Yellowstone hot springs. In turn, these techniques have allowed us to ask and answer new questions. Important questions, for example, relate to the kinds of energy metabolisms that fuel these microbial communities; how the local geochemistry influences the make-up of microbial communities and vice versa; and how primary productivity—the conversion of carbon dioxide into biomass—occurs at high temperature, where photosynthesis is absent.

These questions also are essential in the consideration of what sparked life on early Earth, or potentially elsewhere in the universe. For life to thrive, basic, energetically favorable metabolisms had to evolve based on the conditions that existed at the time. Early Earth's hot, reducing condition—with no oxygen and quantities of hydrogen—had to provide the energy needed for life to establish. Indeed, with new knowledge of volcanic activities spread across our solar system, the astrobiological implications for what kinds of chemistries and life are found in terrestrial hot springs has relevance to the search for life elsewhere. With a molecular approach, we examined several Yellowstone hot springs for the energetic basis that allows life to thrive above 70°C—the limit of photosynthesis, the mechanism responsible for most of Earth's current primary productivity. This chapter explains how microbial community analysis can point to new understandings of the chemistry that underpins an ecosystem. By a molecular analysis in the Yellowstone geothermal ecosystem, with interpretation likely to other geo-hydrothermal ecosystems, we find that hydrogen is probably the dominant energy source.

2.0 YELLOWSTONE MICROBIAL ECOLOGY

Yellowstone's geothermal springs, with their associated microbiotic complexity, are analogs of similar environments that occur worldwide. Yellowstone geothermal hot springs present the opportunity to observe different kinds of interfaces between anoxic hydrothermal fluids and the oxic surface (Ball et al. 1998a, 1998b). Yellowstone geothermal ecosystems, like those of the Mid-Ocean Ridge, are potential modern-day analogs of the oldest ecosystems on Earth, where primitive life may have arisen to form an ecosystem in high temperature environments.

Microbial communities associated with volcanic hot springs have attracted broad interest because of the unique thermophilic (heat-loving) properties of the organisms therein. Little attention has been given, however, to hot spring settings as whole microbial ecosystems. One fundamental consideration for any ecosystem is the energy budget—the contribution of different energy sources that fuel primary productivity. Most of Earth's biomass is the product of photosynthetic energy, but at temperatures higher than ~70°C photosynthesis is not known to occur. Nonetheless, robust microbial communities develop in some of the earth's hot springs to 110–120°C (Brock 1967, 1978; Jannasch 1985; Jannasch and Mottl 1985; Madigan et al. 2003). Consequently, high-temperature primary productivity must derive from chemolithotrophy—the production of biomass with the use of reduced inorganic energy sources such as sulfide, reduced metals, and molecular hydrogen (H_2)—or organic sources such as light hydrocarbons (methane and others). Many microorganisms with various chemolithotrophic metabolisms have been cultured from hot spring communities using hydrogen and sulfide as an electron donor, but the relative contributions of different potential energy sources to particular ecosystems have not been systematically addressed (Huber et al. 1989, 1992, 1995, 1998; Reysenbach et al. 2000a, 2000b; Norris et al. 2002).

We propose that one way to gain insight into the relative contributions of potential energy sources available to microbial habitats is by assessment of the relative abundances of the organisms that comprise the communities. Microorgan-

isms that engage in primary productivity would presumably be the most abundant in any ecosystem, as is the case with large organisms in terrestrial ecosystems (Begon et al. 1996). If the relative abundances of particular physiological types reflect the relative amounts of different energy sources used for primary productivity, then a census of the physiologies that comprise a microbial community would correspond to a biological assessment of the energy demands of the particular ecosystem. Such a census cannot be achieved with culture-based methods because most naturally occurring microbes resist cultivation by standard techniques (Amann et al. 1995). With the advent of molecular techniques for the phylogenetic identification of organisms without the resort to culture, the relative abundances of microbial community constituents can now be determined.

In the most commonly employed molecular analysis of microbial community composition without culture, small subunit ribosomal RNA (SSU rRNA) genes are amplified by polymerase chain reaction (PCR) from natural community DNA using universally conserved sequences as priming sites, then cloned and sequenced for phylogenetic identifications. The collection of rRNA gene sequences approximates a census of the local phylogenetic types of organisms—the "phylotypes"—that comprise the community. If the phylotypes fall into relatedness groups with predictable forms of energy metabolism based on cultured representatives, the probable energy sources for the environmental organisms can be inferred.

Microbial communities associated with high-temperature hot springs in Yellowstone National Park and elsewhere have been analyzed using culture-independent methods. One unexpected finding of all studies has been the abundant occurrence of microorganisms of the Aquificales bacterial phylogenetic division (Reysenbach et al. 1994; Huber et al. 1998; Hugenholtz et al. 1998a; Skirnisdottir et al. 2000; Blank et al. 2002). Although sulfur metabolism is commonly cited as a main source of energy in these environments, all known representatives of Aquificales mainly utilize H_2 as an energy source. This dominance of Aquificales representatives suggests that H_2 may be the main energy source for primary productivity in these hot spring ecosystems.

Hydrogen had not been measured previously in the Yellowstone geothermal system, however, and only a few hot spring communities have been phylogenetically analyzed, with limited variation in the chemical composition of the settings. To further assess the energetic fuels that support Yellowstone hot spring communities, we have conducted extensive further phylogenetic analyses of Yellowstone communities in different chemical settings (Spear et al. 2005). In parallel, we determined the chemical compositions of the hot springs, including the first widespread measurements of molecular hydrogen in the Yellowstone hydrothermal system. We then used thermodynamic modeling based on the chemistries to evaluate the bioenergetic underpinnings of the hot spring communities.

3.0 MATERIALS AND METHODS

3.1 Sample Collection and DNA Extraction

The analysis of microbial communities using molecular phylogenetic methods begins with sampling and storage. It is critical that samples be frozen or otherwise preserved (e.g. formaldehyde, ethanol immersion) to prevent alterations in community structure due to deterioration and selective growth of some organisms. For our studies, samples for DNA extraction were collected from individual source springs by several methods, dependent on the nature of the spring. In some springs, sediments were collected and frozen in cryovials on liquid nitrogen in the field and stored at -80°C in the lab. In others, glass growth slides (plain microscope slides) were hung in a spring for periods of time ranging from days to months. Biofilms can then be scraped from the slides in the field, collected in cryovials, and frozen on liquid nitrogen. For this study, samples for analyses were collected in different seasons from 1999 to 2003.

Total community DNA can typically be obtained and prepared from ~1 g of the frozen samples as described (Dojka et al. 1998). Extraction of DNA from the thawed environmental sample is accomplished by some combination of physical and/or chemical disruption of the cells, such as mechanical beating with zirconium (glass) beads to disintegrate tough communities embedded in a soil or rock matrix. The homogenized sample is then usually subjected to extraction with phenol chloroform followed by precipi-

tation with alcohol in the presence of high salt. The DNA can be further purified to remove salts and any compounds that may inhibit PCR, such as humic compounds that commonly co-purify with DNA from, for example, soil (Barns et al. 1994). Extracted DNA is then run down a 1% agarose gel to check for both positive extraction and purity.

3.2 PCR Amplification of rDNA

This environmental DNA is used as a template for PCR amplification of SSU rRNA genes (16S for bacteria and archaea, 18S rRNA genes for many eucarya). The choice of PCR primer pairs determines the specificity of any search for diversity. If universally conserved primers are used, genes belonging to organisms from all three domains of life are amplified. Generally, researchers choose either universal primer pairs, or primer pairs tailored to amplify sequences of specific microbial domains, divisions, or taxa. For this study, initial PCR amplifications of environmental DNAs were done with the universal SSU rRNA degenerate primers 515F—5′GTGCCAGCMGCCGCGGTAA3′ and 1391R—5′GACGGGCGGTGWGTRCA3′ (Lane 1991). Full-length bacterial rRNA gene sequences were obtained with the PCR primers 8F (27F)—5′AGAGTTTGATCCTGGCTCAG3′ and 1492R—5′GGTTACCTTGTTACGACTT3′ (Lane 1991). Archaeal libraries were constructed from the use of the PCR priming pairs 4Fa—5′TCCGGTTGATCCTGCCRG3′ or 333Fa—5′TCCAGGCCCTACGGG with 1391R (Lane 1991).

To maximize the representation of environmental rDNA sequences amplified in PCR, an annealing temperature gradient was used to find an optimal annealing temperature—one that gives a strong PCR product visualized with gel electrophoresis—which was ~55°C for most primer pair combinations. All PCR reactions using these primer pairs were incubated in an Eppendorf gradient thermocycler at 94°C for 2 min, followed by 29 cycles at 94°C for 30 sec, 55.5°C for 1 min, and 72°C for 1.5 min, followed by a 72°C step for 12 minutes. Each 25-µL reaction contained 30 mM Tris (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.05% BSA (Sigma), 1M betaine, 0.2 mM of each dNTP, 50 ng of each oligonucleotide primer, 0.5 units of Taq Polymerase, and approximately 600 ng of environmental DNA template.

3.3 Cloning and Sequence Analysis

PCR reactions were analyzed on a 1% agarose gel to check for amplification. Positive PCR reactions were gel-purified using a Qiagen Gel Extraction Kit (Qiagen Inc.) as per kit instructions. Before cloning, 30 µL of gel-purified PCR product was incubated with 2 µL of dNTPs for 20 min at 72°C in 1X buffer with 1.5 mM $MgCl_2$. Reactions were cloned with the TOPO TA Cloning Kit for Sequencing (Invitrogen) as per kit instructions. Unique recombinant clones were determined using the restriction fragment length polymorphism (RFLP) analysis (Dojka et al. 1998). PCR products were cut with *HinP1* and *Msp1* restriction endonucleases for a minimum of three hours, moist, at 37°C. Sequencing reactions were prepared with the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences) and sequenced on a MegaBACE™ 1000 DNA Sequencing System (Amersham Biosciences) in 96-well format. Unique sequences were assembled and put through the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al. 1997) at the NCBI BLAST website (**http://www.ncbi.nlm.nih.gov/blast**) to determine approximate phylogenetic position, using XplorSeq 2.0, an in-house software package developed by Dr. Daniel Frank.

3.4 Phylogenetic Analysis

Chimeric sequences were identified using secondary-structure analyses and the CHECK_CHIMERA software program of the Ribosomal Database Project (Maidak et al. 2001). Sequences that showed ≥99% identity to common contaminants of rRNA-based molecular surveys were excluded from further analyses (Tanner et al. 1998). The remaining environmental rRNA gene sequences were then aligned to other known SSU rRNA sequences using the ARB database of more than 25,000 SSU rRNAs in the ARB software package (**http://arb-home.de**) followed by phylogenetic analysis with version 4.0b8 of PAUP* (Swofford 2001). Sequence alignments used in phylogenetic analysis were minimized/masked using the Lane mask that cuts off hypervariable regions of the SSU rRNA alignment from the analysis. A total of 1334 homologous nucleotide positions were included in the alignments for phylogenetic analyses (Lane 1991). Phylogenetic

relationships were tested using reference taxa from the currently described taxa in the literature. Heuristic searches were performed using evolutionary distance, maximum-parsimony (nreps = 100 or 200) and maximum-likelihood (nreps = 1 or 10) analyses on the alignments to find the best tree possible as previously described (Barns et al. 1994). Distance and maximum-parsimony analyses were conducted by PAUP*.

3.5 Molecular Methodology Notes

The molecular assessment of microbial communities is susceptible to errors at several steps in the process, which can result in skewed community descriptions. For instance, DNA from different organisms may be differentially extracted. Microbes in spore stages or ensheathed in polysaccharides may not be extracted as readily as free-living vegetative cells. PCR primer bias is always a concern and steps can be taken to reduce this source of bias (Polz and Cavanaugh 1998). The choice of primer pairs, the reaction conditions (Mg^{2+} concentration, annealing temperature), and additives (acetimide, betaine, bovine serum albumen) all may influence the composition of the reaction products. Steps to reduce bias prior to cloning include varying the reaction conditions in multiple replicate reactions and keeping the PCR cycle-number low. PCR products generated under different conditions can be pooled prior to cloning. The type of cloning vector may select particular products preferentially, and the use of several different vectors is advisable. Despite these potential sources of error, there is generally good agreement between the distribution of organisms in clone libraries and their abundance in the environment where it is possible to assess them (Pace 1997). In any case, analysis of phylotype libraries remains far and away the most comprehensive method available for community analysis.

For applied examples of these molecular methodologies in various environments consider the following references (Barton et al. 2001; Blank et al. 2002; Dawson and Pace 2002; de la Torre et al. 2003; DeLong 1992; Dojka et al. 1998; Frank et al. 2003; Hugenholtz and Pace 1996; Hugenholtz et al. 1997, 1998a, 1998b; Pace 1997, 1999, 2001; Reysenbach and Cady 2001; Reysenbach et al. 1992, 1994, 1998, 2000a, 2000b; Spear et al 2002, 2003, 2005; Ward et al. 1990a, 1990b, 1997).

3.6 Hydrogen and Water Chemistry

For this study we measured the distribution of aqueous H_2 concentrations in Yellowstone waters (hot springs, streams, geothermal vents, and a well; Spear 2002) with a modified bubble-stripping method (Chapelle et al. 1997). In this method, water is pumped through a chamber that contains an introduced atmospheric gas bubble. The gases dissolved in the water equilibrate with the gas bubble, and the bubble is removed for analysis. Source waters were pumped through insulated H_2-impermeable tubings for 20 minutes at a flow rate of 200 mL/min with a 12-V powered Masterflex peristaltic pump (Cole-Parmer) through a 250 mL glass bottle bubble stripping device (Chapelle et al. 1997). A 20 mL atmospheric air bubble was introduced into the bottle after filling completely with the source water to be measured. The temperature of the bubble was measured by a thermistor attached to a digital thermometer (Cole-Parmer). Bubbles were collected with an airtight syringe and transferred to nitrogen charged H_2-impermeable glass septum vials and sent to Microseeps Inc. (**http://www.microseeps.com**) for analysis of H_2, CH_4, CO_2, and light hydrocarbons on a Trace Analytical RGA3 reduction gas analyzer (Chapelle et al. 1997).

Henry's Law describes the ratio of the aqueous-phase concentration of a gas to its equilibrium partial pressure in the gas phase alone. The temperature effect on Henry's constant is relatively small for most gases. However, for hydrogen, Henry's constant decreases by 28% from 0°C to 100°C. To adjust for equilibrium partial pressure at elevated temperature, and thus consider actual aqueous H_2 concentrations in hot springs, we used Henry's Law:

$$C_W = C_G H_C \quad (1)$$

where C_W is the concentration of the gas in the water, C_G is the concentration (partial pressure) of the gas in the bubble, and H_C is Henry's constant for that gas, which is the solubility of a gas at a given temperature. Henry's constant is typically measured, but can also be estimated. There were no values of H_C available for H_2 at the high temperature of the hot springs, so we estimated H_C with Ostwald's expression:

$$H_C = P_{H2}/RTC_W \quad (2)$$

where P_{H2} is 1.22 x 10^{-5} atm, R is the gas constant (0.0821 L atm mol^{-1} K^{-1}), T is the measured bubble temperature in Kelvin, and C_W is 1x10^{-8} mol/L. Values reported in this study reflect this temperature correction.

Sulfide measurements were conducted at each location with a CHEMetrics colorimetric assay (CHEMetrics Inc.). Samples for water chemistry were collected by pumping water out of each spring, filtered by syringe through a 0.2 µm filter, and acidified with ultra pure nitric acid (Sigma). Samples were held at 4°C until analysis. Anions, cations, and elemental analyses were conducted in the Laboratory for Environmental and Geological Studies of the Geology Department at CU-Boulder on a Dionex Series 4500I Ion Chromataograph, ARL 3410+ Inductively Coupled Plasma Atomic Emission Spectrometer, and a Varian Inductively Coupled Plasma Mass Spectrometer as per standard methods.

3.7 Thermodynamic Modeling

The amounts of chemical energy available from lithoautotrophic reactions were quantified using the equation:

$$\Delta G_R = \Delta G_R^\circ + RT \ln Q^\circ \quad (3)$$

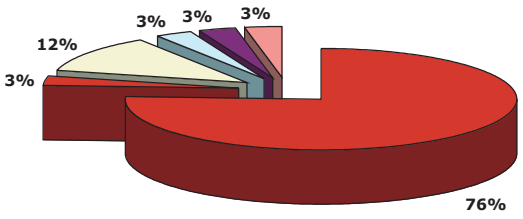
where ΔG_R is the Gibbs energy of the metabolic reaction, ΔG_R° is the standard Gibbs energy of the reaction, and Q is the activity quotient of compounds involved in the reaction. Values of ΔG_R° were calculated with the computer program SUPCRT92 and thermodynamic data contained therein (Johnson et al. 1992). Values of Q were determined with the measured composition of hot spring fluids. Because these are dilute solutions, activity coefficients were assumed equal to one for all dissolved compounds. Distributions of dissolved CO_2 and sulfide were calculated from the measured concentrations of these compounds, together with appropriate dissociation constants and the measured pH, assuming the species were in equilibrium.

4.0 RESULTS AND DISCUSSION

4.1 Microbial Diversity of Yellowstone Hot Springs

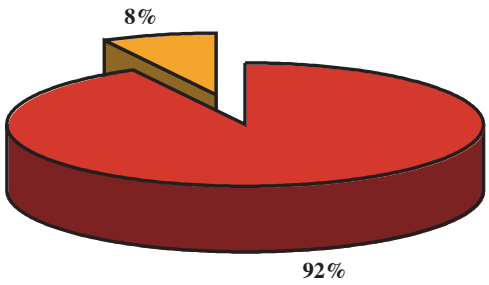
Despite the often daunting complexity and diversity of microbial communities, their composition presumably reflects the energy sources available. In the case of the Yellowstone hot springs, at temperatures >70°C, above the photosynthetic limit, the composition of the microbial communities led to a fundamental insight about the principal driver of hot spring ecosystems at the anoxic-oxic boundary. Popular attention had focused on the oxidation of sulfur compounds (e.g. sulfide) as a main source of energy for these communities. Therefore it was a surprise when a few phylogenetic analyses of microbes in Yellowstone and other hot springs above 70°C indicated that molecular hydrogen, not sulfur, may be the primary source of energy in these hot spring environments. The importance of hydrogen in this ecosystem follows from observations that the dominant masses of organisms in >70°C Yellowstone hot spring communities are representatives of the bacterial divisions Aquificales and Thermotogales (Reysenbach et al. 1994; Hugenholtz et al. 1998a, 1998b; Blank et al. 2002). Cultivated representatives of Aquificales, e.g., *Thermocrinis ruber,* isolated from Octopus Spring mainly thrive by microaerophilic oxidation of H_2 at high temperatures (Huber et al. 1995, 1998). In Octopus Spring, >80% of the rRNA gene sequences obtained were associated phylogenetically with Aquificales. In Obsidian Pool, 30% of the gene sequences were representatives of this phylogeny. Most of the cloned rRNA gene sequences from other sites examined also grouped phylogenetically with Aquificales.

To expand the survey of the organisms that comprise the Yellowstone hot spring communities, we undertook a molecular phylogenetic analysis of several Yellowstone hot springs that had not been characterized (Spear et al. 2005). We looked at hot springs with a spectrum of sulfide concentrations—from very high, >200 µM (Washburn Spring area) to very low, near zero (a West Thumb area spring). We also conducted analyses of hot springs with high (>300 nM) and low (<15 nM) H_2 concentrations. **Figure 1 (next page)** shows results from two locations with associated phylogenetic and relevant chemical analyses. Lower Washburn Spring, **Figure 1A**, is a location with some of the highest measured concentrations of sulfide in the Park. **Figure 1B** shows an unnamed pool in the West Thumb Region that has no detectable aqueous sulfide, and approximately the same H_2 concentration as Lower Washburn Spring. Clone libraries from both pools are dominated by phylotypes of the kinds that thrive on



- Aquificales; *Hydrogenothermus*
- Aquificales; *Hydrogenobacter; T.ruber* sp.
- Thermodesulfobacteria; *Geothermobacterium*
- Thermotogales; EM-3
- OD-1
- Delta-Proteobacteria; *Syntrophobacter*

Figure 1A. Lower Washburn Spring, Canyon Region. Pool is: 86°C; pH = 6.2; 19 nM in H2; 167 μM in H2S. Pie chart results are from an examination of 192 clones of PCR priming pair 8F and 1492R.



Aquificales; EM-17 Thermotogales; EM-3

Figure 1B. Unnamed Pool, West Thumb Region. Pool is: 88ºC; pH = 5.5; 16 nM in H_2 ; non-detectable in H_2S; 16.8 μM in arsenic. Pie chart results are from an examination of 96 clones of PCR priming pair 8F and 1492R.

the oxidation of molecular hydrogen. Thus, hydrogen as a dominant energy source is indicated by the kinds of microbiota detected (Spear et al. 2005).

The total phylogenetic distribution of rRNA genes amplified with the use of universal PCR primers from five hot springs in Yellowstone are shown in **Figure 2A**. The figure provides some perspective on the overall microbial organismic composition of the Yellowstone geothermal ecosystem. Each of the hot spring communities was dominated by bacteria, which typically comprised >95% of the rRNA genes obtained from community DNA. Archaea are popularly considered to be particularly conspicuous in geothermal and other "extreme" environments, but these and all previous rRNA gene surveys indicate that such organisms are, in fact, generally not more abundant than bacteria (Hugenholtz et al. 1998b). Most of the archaeal sequences encountered were related to environmental crenarchaeote sequences previously observed in Obsidian Pool (Barns et al. 1994, 1996a, 1996b), none with a specific relationship, (i.e., >97% similarity) to a cultured organism (Spear et al. 2005, **Figure 2B**).

4.2 Hydrogen in Yellowstone Hot Springs

Molecular hydrogen is a highly versatile energy source. It is the most abundant element in the universe and also has the highest electron donation potential. Indeed, earliest life likely utilized H_2 as a primary energy source (Morita 1999). Organisms in general that can gain energy by the use of hydrogen as an electron donor include photolithoautotrophs, photolithoheterotrophs, chemolithoautotrophs, and chemolithoheterotrophs. The electron acceptors for metabolism can be oxygen, nitrate, sulfate, and carbon dioxide. In addition, various elements such as sulfur, manganese, and iron, can all serve as electron acceptors in hydrogen oxidation. Hydrogen, however, is not the sole electron donor in the Yellowstone geothermal ecosystem. Hydrogen sulfide, popularly considered the dominant electron donor, is certainly present in several hot springs, and sulfur-oxidizing organisms are readily cultured from hot springs. Nonetheless, in clone libraries from >70°C hot springs, rRNA sequences from organisms of the kinds that metabolize hydrogen sulfide, thiosulfate, or elemental sulfur are not numerically conspicuous and, when found, are spring-dependent, i.e., geochemically dependent (Skirnisdottir et al. 2001; Norris et al. 2002; **Figure 1A-B**). In the Yellowstone geothermal waters above 70°C that we tested, the springs with the most microbial biomass have relatively low sulfide concentrations. Conversely, hot springs with abundant sulfide tend to have relatively low biomass (Barns et al. 1994, 1996a, 1996b; Hugenholtz et al. 1998a, 1998b).

What is the source of the hydrogen in hot springs? Apps and van de Kamp (1993) have reviewed the nature of hydrogen and

Figure 2A. Pooled Universal Libraries

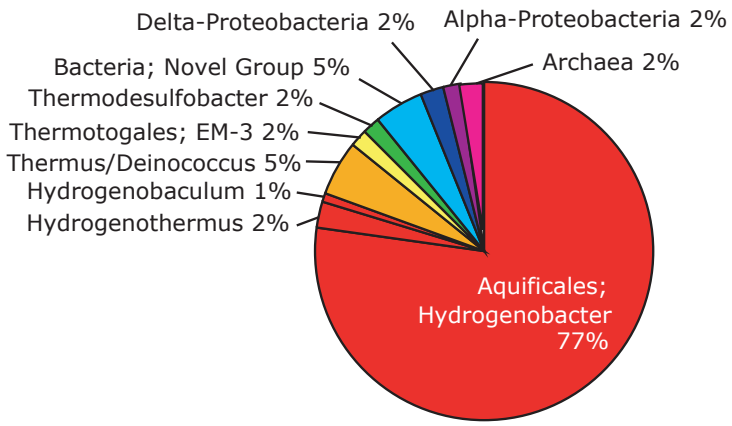


Figure 2B. Pooled Archaeal Libraries

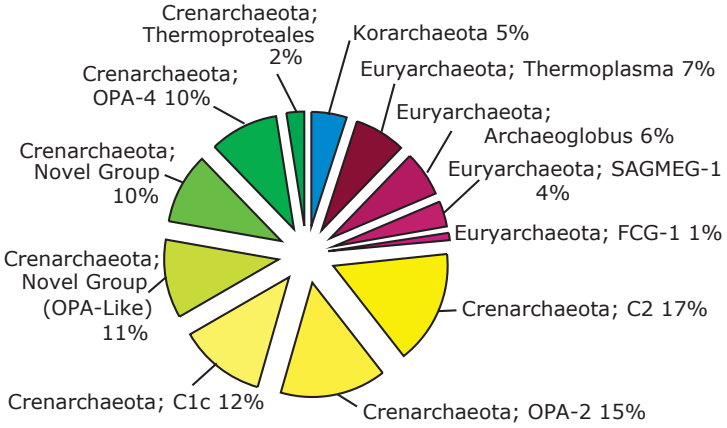


Figure 2. Cumulative rRNA gene analyses. A. Distribution of sequences by phylogenetic group as identified with ARB and PAUP. Universal PCR Primers (515F and 1391R) were used with environmental DNA templates from five hot springs and resultant sequences were compiled for the assemblage. 5% of the sequences are from one potentially new candidate bacterial division encountered in this study. **B.** Distribution of archaeal sequences in three hot springs with two archaeal-specific PCR primer pairs. The majority, 77% of the sequences, are identified as crenarchaeotes. 18% fall within Euryarchaeota, and 5% within Korarchaeota. Abbreviations: OPA-2, OPA-4 and OPA-like represent environmental DNA sequences from a previous study of Obsidian Pool (Barns et al. 1996); FCG-1 represent sequences from marine/hydrothermal vent benthic archaea; and SEGMEG-1 represent sequences from deep South African mines.)From Spear et al. 2005.)

Table 1. Elemental analyses of total dissolved ions in several Yellowstone hot springs.

Constituent (mg/L)	Obsidian Pool	Obsidian Pool Prime	Queens Laundry	Green Pool, South of QL	West Thumb Pool
pH	6.5	5.7	8.0	7.6	7.3
Temperature (°C)	80	74	89	70	89
Hydrogen (nM)	133	325	28	7	15
Fluoride	5.2	18.0	26.0	15.7	13.6
Chloride	25.0	305.0	239.4	101.9	152.7
Bromide	0.03	0.75	0.64	0.29	0.33
Phosphate	0.0	0.0	0.1	0.03	0.14
Sulfate	149.2	231.0	19.0	9.5	23.7
Silica	96.0	166.0	142.6	102.8	107.9
Iron	0.11	0.26	DL	0.15	DL
Magnesium	9.14	1.12	0.004	0.04	0.03
Calcium	21.17	6.58	0.50	0.66	0.40
Sodium	56.2	272,0	277.2	155.8	239.8
Potassium	21.7	34.3	12.7	18.8	11.2
Elements (ppb):					
Lithium	199.4	1170.7	1996.0	650.3	1383.9
Aluminum	349.0	206.3	282.0	28.0	66.4
Chrome	9.8	20.6	30.8	23.4	40.9
Manganese	427.0	50.1	0.87	32.7	13.2
Nickel	3.5	1.3	0.3	0.3	0.2
Zinc	2.3	2.2	DL	4.6	0.7
Arsenic	DL	526.4	1312.9	585.0	1264.9
Strontium	159.6	38.4	1.0	0.2	1.0
Cadmium	DL	0.2	0.2	DL	0.4
Barium	96.8	24.2	3.9	2.6	3.4
Titanium	0.04	0.4	0.2	0.1	0.2

methane from subsurface environments and list several potential processes for the generation of H_2. Although subject to debate, Stevens and McKinley proposed that water reacts with iron-rich Columbia River basalt (CRB) to produce molecular hydrogen at concentrations as high as 60 μM (Stevens and McKinley 1995, 1996; Lovley and Chapelle 1996; Madsen 1996; Anderson et al. 1998). Later work in the CRB has showed that hydrogen production can average 5 nmol of H_2 $(m^2$ of basalt$)^{-1}day^{-1}$—sufficient to support observed concentrations of methane produced by microbes in the CRB (Stevens and McKinley 2000). In the case of Obsidian Pool, the sediment is rich in reduced iron (at >15 g/kg) that could react with the water to produce abiogenic hydrogen (Hugenholtz et al. 1998b). In the case of geothermal waters with low iron content such as Octopus Spring, the specific chemical source of H_2 is in the subsurface and not known. Regardless of source, and in the presence of a strong metabolic electron acceptor like oxygen, bulk aqueous phase H_2 is likely to serve as the main electron donor for naturally occurring microbial communities in geothermal springs >70°C.

Hydrogen concentrations had not been measured extensively in Yellowstone hot springs primarily because hydrogen concentrations in water are difficult to assess. Hydrogen has a solubility of 1.69 mL/100 mL of water

at 27°C, can react with several metals, and can molecularly diffuse through many materials. In ambient seawater, hydrogen concentrations range from 0.2-1.5 nM at the surface to 0.2-0.4 nM in deep water (Winn et al. 1995). Measurements of hydrogen in Yellowstone hot springs indicate, however, the general presence of hydrogen in relatively high concentrations, from 10 to >300 nM (Spear et al. 2005). Along with hydrogen, anion, cation, and elemental analyses are shown for a few Yellowstone hot springs in **Table 1**. For life to utilize H_2, others have shown with both *in situ* sediments and cultured mesophilic, hydrogenotrophic methanogens, that approximately 4 nM concentrations of H_2 are sufficient to sustain growth (Lovley and Klug 1982; Lovley et al. 1982; Lovley 1985). Rates of growth per mole of H_2 have been shown for some anaerobes to be −5 to −6 kJ/mol H_2 for cultivated homoacetogens and −9 to −12 kJ/mol H_2 for methanogens (Conrad and Wetter 1990). Zinder (1993) notes that methanogens, and possibly most organisms, are energetically adapted to utilize the low nM levels of H_2 in natural habitats.

4.3 Thermodynamic Modeling

We used thermodynamic models to test the efficacy of the potential energy sources available to the microbial communities that we analyzed both phylogenetically and chemically (Spear et al. 2005). Our thermodynamic calculations are based on the concentrations of dissolved constituents in the hot springs and the calculated free energies of reaction (kJ/mole electron or oxygen). Models show that dissolved sulfide, methane, and H_2 are the principal potential energy sources available to these communities through the processes of sulfide oxidation, methanotrophy, hydrogen oxidation, methanogenesis, and sulfate reduction. Based on the chemical compositions of the hot springs, relative potential energy yields of the available different chemical energy sources can be estimated. Based on complete oxidation of the available reduced compounds in the hydrothermal fluids, sulfide, where available, would constitute the largest source of chemical energy, followed by methane (where available) and hydrogen (data not shown). Although this might seem inconsistent at first with the apparent dominance of putative hydrogen-metabolizing

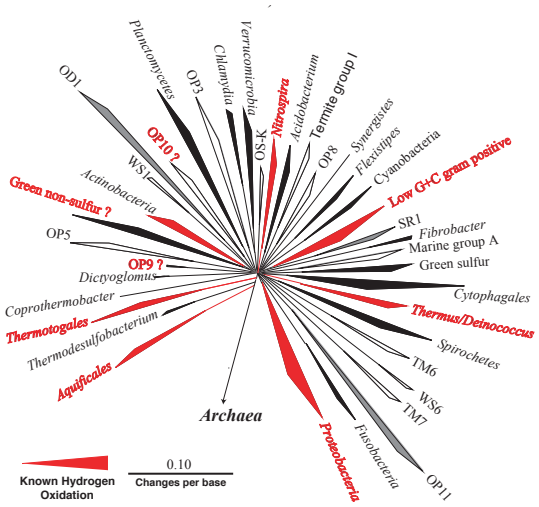


Figure 3. A graphical representation of the domain *Bacteria*. Thirty-eight bacterial divisions are represented of the at least 50 known. Width of wedges represent how many known sequences there are per division. Filled wedges have at least one known cultivar with known kinds of metabolisms/chemistries. Hollow wedges are known only by their phylotype, or environmental DNA sequence. Wedges in red depict bacterial divisions with known capability to oxidize molecular hydrogen.

organisms, the most abundant potential energy source is not necessarily preferentially utilized.

Energetic comparisons for the complete oxidation of reduced compounds presume that sufficient O_2 is available to consume all H_2S, H_2, and CH_4 introduced into a hot spring. However, hot springs typically are low in O_2 concentration due to the highly reduced nature of the spring source waters and the poor solubility of atmospheric O_2 in hot water (Ball et al. 1998a, 1998b). Diffusion of atmospheric O_2 into a hot spring, therefore, is slow and unlikely to saturate all reductants simultaneously. Competition for O_2 would limit consumption of reduced compounds, particularly in those parts of the springs that are in close proximity to the source of the reduced compounds. Consequently, in the case of hot springs it is more appropriate to compare the amount of metabolic energy available from oxygen-consuming reactions on the basis of the energy that can be obtained per mole of O_2. In all hot springs ana-

lyzed, more energy is available from each mole of O_2 for oxidation of H_2 than for oxidation of sulfide or methane (Spear et al. 2005). Thus hydrogen oxidizers are expected to out-compete sulfide oxidizers and methanotrophs for available O_2 under oxygen limiting conditions.

The occurrence of H_2 at relatively high concentrations in high temperature Yellowstone hot springs supports the indication from the molecular phylogenetic work that molecular hydrogen is the driving energy source for most microbial life in these ecosystems. Hydrogen is ubiquitous in anoxic environments and is likely to be a utilizable energy source by many if not most microbes. Yet, remarkably little is known about the distribution of hydrogen metabolism among all microbiota. **Figure 3** is a phylogenetic diagram of known bacterial diversity. As shown, only a handful of the fifty or more known phylogenetic divisions of *Bacteria* are known to metabolize hydrogen. However, it is likely that representatives of most or all of these groups are capable of hydrogen metabolism. Laboratory experimentation with hydrogen-metabolizing organisms is technically a challenge and, therefore, has received relatively little attention. In Obsidian Pool, where the bulk of the biomass (dominant rRNA genes) appears to be composed of known hydrogen oxidizers, other organisms such as representatives of the Green nonsulfur bacterial division also are abundant (Hugenholtz et al. 1998b). The question is, are such organisms engaged in hydrogen oxidation?

4.4 Hydrogen in Other Environments

Gold (1992) has speculated about a "deep hot biosphere" on Earth, suggesting that hydrogen and/or light hydrocarbons could serve as a source of energy in the subsurface. Gold maintains that H_2 and CH_4 should be chemically stable in the earth's upper mantle, and that migration in the crust occurs continuously. In addition, it is known that thermodynamic control of hydrogen concentrations is exerted in anoxic sediments by pH, temperature, and the individual and combined effects of various terminal electron acceptors such as nitrate, sulfate, carbon dioxide, iron, and manganese. These can have order-of-magnitude effects on hydrogen concentrations in environmental settings, including the whole of the earth's crust (Hoehler et al. 1998). There are, of course, many potential biogenic sources of H_2 in both oxic and anoxic environments that can be utilized by hydrogen metabolizers (for review see Nandi and Sengupta 1998).

Microbial mats are another ecosystem in which hydrogen metabolism is emerging as a conspicuous theme, and some results may be applicable to geo-hydrothermal settings. Microbial mats are finely layered, highly structured, complex ecosystems that occur globally. They accommodate a wide range of physiological types of organisms from oxygenic photosynthesizers to obligate anaerobes, all in close proximity. Complex community structure and spatial distribution are prevalent throughout examined mats. The specific microbes involved in the mats and the energy metabolisms that support them are relatively little understood and probably vary according to the local geochemical setting. Even in the case of photosynthetic microbial mats, hydrogen produced by metabolic processes that accompanies primary productivity influences individual microbial metabolisms and system-level biogeochemistry (Ward et al. 1998; Hoehler et al. 2001). Metabolic hydrogen is then available for hydrogen-based metabolisms of associated organisms that are supported only indirectly by photosynthesis. This hydrogen metabolism, in turn, strongly influences the thermodynamics of the mat microenvironment and plays a major role to determine the kinds of organisms that can thrive in the community; for instance, anaerobic sulfate-reducing bacteria, archaeal methanogens, and bacterial Green nonsulfur and Nitrospira members.

Other contributions in this symposium also demonstrate the importance of H_2 in varied Yellowstone geochemical settings. These include acid-sulfate springs in the Norris Geyser Basin (Inskeep and McDermott, this volume) and alkaline siliceous springs in the Calcite Springs area (Reysenbach et al., this volume).

5.0 CONCLUSIONS

This study of Yellowstone hot springs is a clear case in which inferences from phylogeny can drive hypotheses about the importance of specific sources of energy in an ecosystem (Spear et al. 2005). We used three lines of evidence to infer the main energetic basis for life in high

temperature geothermal springs. The thermodynamic modeling links the phylogenetic and geochemical results to suggest that H_2 is a dominant energetic theme in hot springs above the temperature limit for photosynthesis. In the phylogenetic surveys we conducted of five high-temperature Yellowstone hot springs, in both low and high-sulfide settings, the high number of Aquificales representatives was informative because all cultured members of the group have a very restricted physiology. Eventually entire environmental genomes will be available, and the presence or absence of metabolic genes will inform the physiological capacity of an entire community. As microbial ecology progresses, new revelations will emerge about the nature of life and the chemistries that fuel life.

ACKNOWLEDGMENTS

We thank the Yellowstone Center for Resources for years of continued access into the Yellowstone geothermal ecosystem. Support for this work has come from an NSF LExEn grant to N.P., an NSF Microbial Biology Postdoctoral Fellowship to J.S., and an Agouron Institute Postdoctoral Fellowship to J.S. Thanks to Montana State University and the Thermal Biology Institute for hosting the Geothermal Biology and Geochemistry in Yellowstone *workshop.*

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