

Diversity of Life at the Geothermal Subsurface—Surface Interface:
The Yellowstone Example
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John R. Spear and Norman R. Pace

Department of Molecular, Cellular, and Developmental Biology University of Colorado, Boulder Boulder, Colorado

Generally, studies of the terrestrial example of Yellowstone National Park indicate that the diversity of microbial life at the geothermal subsurface-surface interface is considerable. On the other hand, experiments in a subsurface well in Biscuit Basin suggest that the Yellowstone subsurface is highly reduced, with minimal in situ subsurface life at this location. The absence of life in the subsurface is likely due to low concentrations of available electron acceptors. Where subsurface thermal waters emerge to the surface, however, in the presence of oxidizing electron acceptors, microbial life blossoms on all growth surfaces at high temperatures. The geothermal subsurface-surface interface in the presence of both electron donors and acceptors, provides the key location for life to thrive and forms the cornerstone of the microbial ecosystem. Through molecular analyses, the identities of organisms present in a community can be determined by their phylogenetic types (phylotypes), their molecular signatures. Molecular sequences allow relationships to other life forms to be inferred. Comparisons of gene sequences of organisms and consideration of the geochemistry of a particular environment can help to explain how this geothermal system functions. Experimental results challenge some popular notions about the kinds of organisms that inhabit the geothermal realms and the energy sources that fuel them. In contrast to the popular notion that representatives of the phylogenetic domain Archaea dominate high-temperature ecosystems, members of the domain Bacteria are most abundant in the Yellowstone ecosystem. Moreover, while sulfur metabolism is generally proposed to be the primary energy source for life in this geothermal system, the main organisms identified by phylotype are related to organisms that utilize hydrogen, not sulfur, for energy. This implies that hydrogen is the main energy source that drives primary productivity in this and potentially other geothermal ecosystems. Primary dependence on hydrogen metabolism could be the common theme for high-temperature life in hydrothermal zones at mid-oceanic ridges, as well as for the earliest life on Earth and, potentially, for life on other planetary bodies.

INTRODUCTION

Recent molecular studies of environmental microbes without the traditional requirement for culture have provided a new perspective on the nature of life at high temperatures. Our knowledge of microbial diversity in general has been limited by the need to cultivate microbial organisms in order to study them. Yet, using standard techniques we do not cultivate >99.9% of the organisms seen microscopically in the environment [Amann, 1995]. Several molecular methods have been developed in studies of geothermal ecosystems that focus on the unique hotspots of Yellowstone National Park, Wyoming. Yellowstone's broad variety of geothermal springs with their associated microbiotic complexity are in many ways an approximate model of the microbial biology of Mid-ocean Ridge systems. As in Mid-ocean Ridge hydrothermal vents, Yellowstone geothermal hotspots present a variety of locations to observe different kinds of interfaces between the anoxic hydrothermal fluids and the oxic surface [Ball, 1998a, b]. Yellowstone also offers an accessible ecosystem, where the diversity of life is readily observable. The Yellowstone geothermal ecosystem, like the Mid-ocean Ridge, is potentially a modern-day analog of the oldest kind of ecosystem on Earth, where it is thought that life arose from hot, reduced environments about 4 billion years ago.

Over the past two decades, the techniques of molecular biology have been applied to problems in microbial ecology. It is now possible to identify and study microbiota in the environment without the traditional requirement for cultivation and the results have increased substantially our knowledge of the extent and nature of biological diversity. The development of "molecular phylogeny," the establishment of relationships of organisms through their DNA sequences, not their physiology as seen through culture studies, has given us a systematic view of biological diversity. The molecular techniques relate organisms objectively, subject to statistical analysis, by direct comparison of gene sequences. Biases in these molecular techniques, however, can cloud this objectiveness [Polz, 1998; Head, *et al.*, 1998]. Surmountable problems can be encountered at all stages of the molecular methodology, from DNA extraction efficiency to expressed primer bias in the polymerase chain reaction (PCR) to the cloning and sequencing of DNA. Specific gene sequences can be isolated directly from environmental DNA (a pool of genomic DNA composed of an unknown number of individuals) by cloning, and analyzed to determine the kinds of organisms, the "phylotypes," that constitute different ecosystems. The same sequences also can be used to design molecular tools with which to study morphology and quantitate organisms in the environment [DeLong, 1989]. From the molecular perspective we see that microbial life, in all three of the phylogenetic domains, Archaea, Bacteria,

and Eucarya, constitutes most of biological diversity. Even with the limited number of molecular studies that have been completed, thousands of new organisms have been detected. The use of molecular tools to identify organisms without culture is rapidly changing our view of the biological component of biogeochemistry. Knowledge of this biological component in turn can change our view of the metabolic basis that drives the microbiota that underlies the whole of any ecosystem.

PHYLOGENETIC PERSPECTIVE AND
MICROBIAL ECOLOGY*A Map of Life*

We can now define what we mean by "biological diversity" in terms of the relationships of gene sequences (representing organisms) compared. Comparisons of gene nucleotide sequences can be used to construct "maps" of biological diversity. The process is relatively simple. Gene sequences from different organisms are compared and the number of differences in the DNA sequences are considered to be some measure of "evolutionary distance" between the compared organisms. Just as maps can be made from distances between points, maps of evolution, "phylogenetic trees," can be made with the use of evolutionary distances between organisms. These maps can be made by comparisons of any gene sequence, but the small subunit ribosomal RNA gene sequences (SSU-rRNA), because of their ubiquity and high degree of conservation, have become the mostly widely used [Woese, 1987]. Before the application of molecular methods, only cultured organisms could be used as references for the paths of evolution. Because of the need for culture to examine the intricacies of microbial physiology in the laboratory, knowledge about the microbial world has been limited, and what we do know stems mostly from medically related organisms (pathogens). For example, 65% of published microbiology reports from 1991 to 1997 were on only eight genera of bacteria [Galvez, *et al.*, 1998]. We have little information about how similar or dissimilar, and what metabolisms are exhibited by uncultivated organisms in the environment.

Perspective on the nature of microbial diversity changed in the late 1970s with the development of molecular phylogeny. From this phylogenetic perspective has come the recognition that there are three distinct relatedness groups, the domains of life, as shown in Figure 1 [after Pace, 1997]. This sequence-based definition of biological diversity replaces the traditionally taught view of biology as composed of only five kingdoms. Figure 1 is a map of evolutionary relationships between representative SSU-rRNA sequences. The diagram shows that all life on Earth is

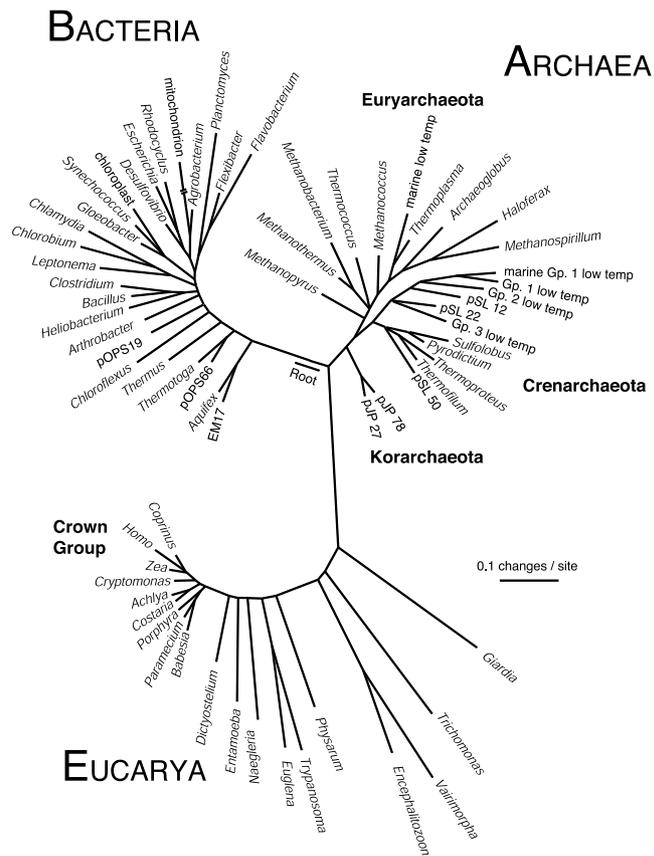


Figure 1. A universal phylogenetic tree based on small subunit ribosomal RNA (SSU-rRNA) sequences. This map was constructed with 64 SSU-rRNA sequences representing several divisions within each domain. “Kingdom” level branches of the Archaea are represented with their name for each branch within the domain. The “crown” group of Eucarya is comprised of animals (represented by *Homo*), plants (*Zea* (maize)), and fungi (*Coprinus*). The scale bar represents 0.1 changes per nucleotide. After Pace, 1997.

related. This map is not a measure of time, rather it is a measure of evolutionary distance. Lines in the tree are from single sequences but represent members of relatedness groups, e.g. *Homo* for the animals kingdom; *Zea* (maize) for the plants kingdom, etc. Line-lengths are proportional to the evolutionary distance, the change in the SSU-rRNA gene that separates the organisms represented. A similar relationship map is obtained by comparative phylogenetic analysis of other genes involved in the central information pathway. All cells contain a core suite of genes necessary for life—replication and evolution, which are required for vertical inheritance in a line of descent. Comparisons of some metabolic genes can yield results that are inconsistent with Figure 1, however, there is no consistent alternative. These incongruities are due to, and part of, the extensive evidence for inter- and intradomain lateral gene transfers [Woese, 2000].

Molecular Methodology

The molecular perspective on organismal relationships is not only intellectually satisfying, but also provides a basis for the study of natural microbial communities without culture. With this perspective, organisms can be described as sequences instead of physiological properties. Figure 2 describes the basic molecular methods that have been refined over the past decade to study microbes in the environment without culture. First, genomic DNA, representative of the resident organisms from a particular environment, is obtained by grinding and chemical extraction. The polymerase chain reaction (PCR) then is used to amplify the SSU-rRNA genes (rDNA) present in the environmental DNA. The SSU-rDNA is however, a mixture of the rRNA genes for the entire community. Cloning of the PCR product from environmental DNA is carried out to separate the multitude of different rRNA genes. At least two restriction enzymes are used to cut the cloned PCR product into a variety of sizes, each specific to the DNA sequence of the individual organisms. The SSU-rDNA sequence is different in different organisms, so will be enzymatically cut at particular locations unique to the

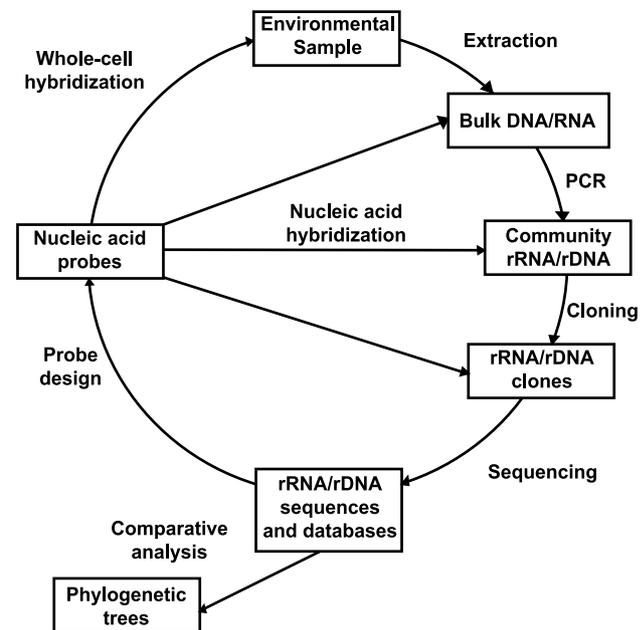


Figure 2. A flowchart for molecular phylogenetic analyses of communities. The environmental sample can be a soil, sediment, water, microbial mat, tissue (plant or animal), or other sample. Extraction, PCR, cloning, sequencing, probe design and hybridization steps all follow with different buffers, cycling times for PCR, and protocols possible for each step. For review of an environmental application and methods followed see Dojka, *et al.*[2000]. Arrows indicate the interconnected utility of the process for which all or part can be adjusted for the particular application.

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organism. The fragments are then separated electrophoretically and the different banding patterns, restriction fragment length polymorphisms (RFLPs), are compared. The results provide a rough assessment of the extent of diversity in an environment; high community diversity is represented by multiple RFLP types, and lower community diversity represented by fewer or often repeated RFLP types.

The heart of the molecular methodology lies in the determination of the nucleotide sequences of the SSU-rDNA that are used to compare organisms. At this stage of the technology, sequences are determined by the use of automated DNA sequencers. The DNA sequences can be compared to other SSU-rRNA sequences in the public data bases, followed by thorough phylogenetic analyses with statistical applications in various software programs such as ARB (<http://www.mikro.biologie.tu-muenchen.de>) and PAUP (Sinauer Associates, Sunderland, MA). A second important component of the molecular technology is to characterize cellular morphology and distribution in the natural environment for cells that are likely difficult to culture. For this, unique regions in SSU-rRNA sequences can be targeted with fluorescently tagged oligonucleotide (nucleic acid) probes, which can bind, in a hybridization step, to samples from the original environment. This process is known as fluorescence in situ hybridization (FISH). FISH can identify specific organisms in a mixture of different organisms from a particular environment. Fluorescent illumination of particular species among all species present can indicate cell quantity, morphology and growth preferences of the organisms identified by the probes.

BACTERIA IN YELLOWSTONE HOTSPRINGS

The Bacteria

Figure 3 is a diagram of the currently known groups of the phylogenetic domain Bacteria. The main relatedness groups are termed "divisions." For most of the history of microbiology, the primary focus was on representatives of the domain Bacteria, and only a small representation of total bacterial diversity could be examined because of the reliance on culture techniques. These culture-dependent studies of environmental microbiota have tended to indicate that representatives of some bacterial divisions are cosmopolitan in the environment, *Proteobacteria* and *Cytophagales* for example, whereas other divisions are found in only certain ecological niches such as a particular hot spring in Yellowstone [Hugenholtz, 1998a]. Only four of the 36 bacterial divisions represented have been the source of 90% of all cultivated bacteria characterized by SSU-rRNA sequences [Hugenholtz, 1998b]. When Carl Woese first described the 16S rRNA gene-based phylogenetic tree of Bacteria in his

landmark 1987 study, only 12 divisions of bacteria were known (Figure 3 inset) [Woese, 1987]. New bacterial divisions are still being discovered and an unknown number will be found in the coming years. Currently, more than one-third of the bacterial divisions have no cultured representatives and are known only by "phylotype," their SSU-rRNA sequence identification (hollow wedges, Figure 3). Another one-third of the bacterial divisions have only a few representatives that comprise the group (thin, filled wedges). Taken together, the emerging perspective indicates that the microbial world is vast and little-known.

Bacteria and Yellowstone Hotsprings

Microbes have long been studied in Yellowstone National Park, mainly beginning with the efforts of Tom Brock and students [Brock, 1978]. Because of their novelty, much attention has been focused on high-temperature archaea. Consequently, the popular perception has arisen that "extreme" high temperature environments such as Yellowstone are the particular province of archaeal species. However, sequences and direct assays indicate instead that representatives of Bacteria, in fact, constitute the bulk of the biomass in the Yellowstone high-temperature communities. This has been shown in Obsidian Pool, Octopus Spring, Calcite Spring, and in other settings [Hugenholtz, 1998a; Ward, 1998; Reysenbach, 1994; Blank, 2001]. For instance, Hugenholtz [1998a] showed with membrane hybridization that archaeal members of the communities examined did not dominate and tended toward rarity. This study additionally screened 122 cloned rDNA sequences from Obsidian Pool and found 38 unique sequences from 12 novel bacterial divisions (termed "OP" in Figure 3) [Hugenholtz, 1998a]. These new division-level relatedness groups are known only through their sequences, so are termed "candidate" divisions. Obsidian Pool, in the Mud Volcano region of Yellowstone, has a temperature that ranges between 75-95°C and contains high concentrations of reduced iron, sulfur species, and high hydrogen [Spear, 2001a]. Representatives of these 12 candidate OP divisions have since been found in a number of other environments including the human ear, hypersaline microbial mats and in a hydrocarbon polluted aquifer, showing their wide occurrence [Frank, 2002; Spear, unpublished; Dojka, 1998]. Other sequences from Obsidian Pool fell into the recognized taxonomic divisions of *Aquificales*, *Thermotogales*, *Thermodesulfobacterium*, *Thermus-Deinococcus*, the green non-sulfur group (GNS), and sulfate-reducing bacteria, a group within the (-) *Proteobacteria*. Lithotrophic metabolic capabilities of several of these groups, i.e. energy production from inorganic chemicals such as hydrogen, reduced sulfur or reduced iron, indicate that these processes are main metabolic

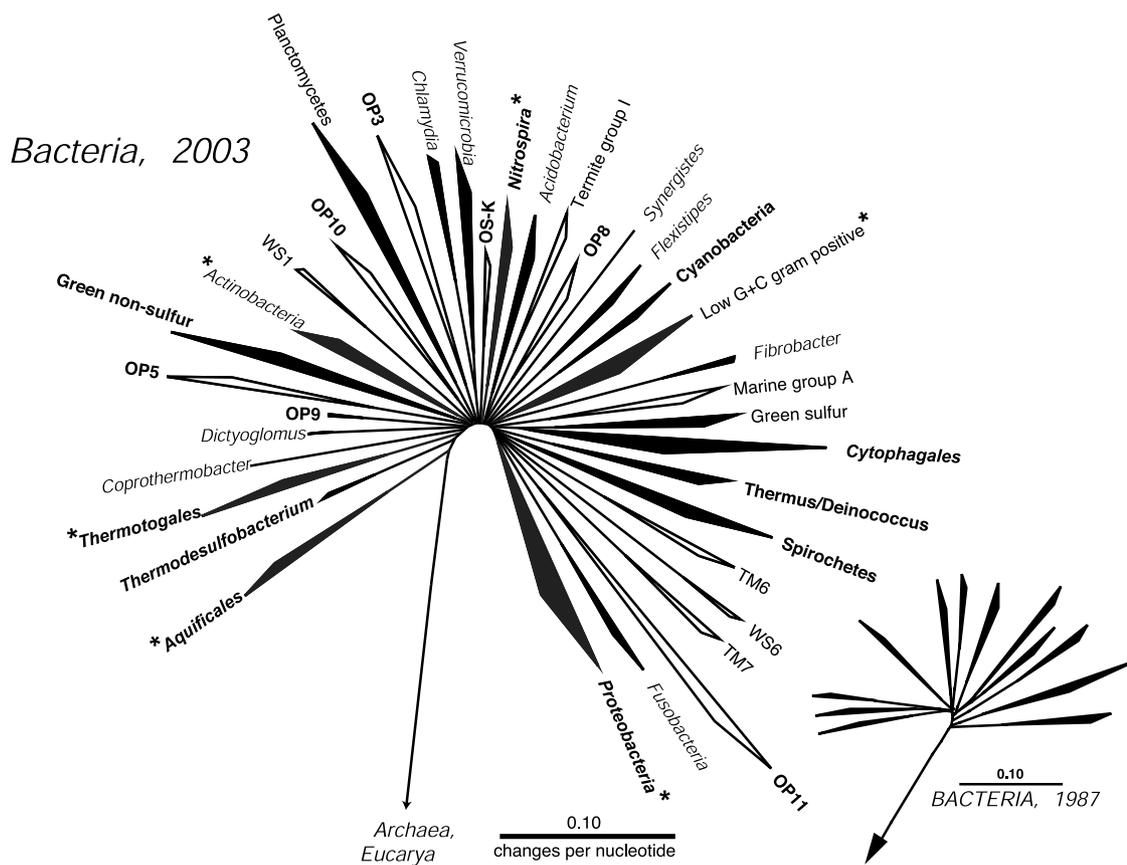


Figure 3. A radiation diagram for Bacteria based on SSU-rRNA sequences. Division-level groupings of at least two SSU-rRNA sequences are depicted as wedges. The depth of wedges represent the branching depth of the representatives selected for each particular division. Divisions filled with black represent divisions with at least one cultivated representative, white wedges are represented only by their environmental sequence-phylogeny, retrieved from environmental samples. Divisions indicated with an asterisk possess known hydrogen-oxidizers. Bold-face division titles are those divisions that have demonstrated representation within Yellowstone National Park hot spring surveys. The width of the wedges are proportional to the number of known cultivars (filled) or phylotypes (white). The figure also describes the impact that uncultured Yellowstone microbes have had for the delineation of the bacterial domain. Organisms detected at Yellowstone through the application of the described molecular methods have resulted in the identification of several novel bacterial divisions indicated by the “OP” groups [Hugenholtz, 1998a] and the “OS-K” group [Ward, 1998]. Inset diagram, lower right, shows that in 1987 there were only 12 divisions known [Woese, 1987]. Today, there are approximately 50, 36 represented here. The scale bar indicates 0.1 changes per nucleotide in the SSU-rRNA gene sequence. The diagram is adapted from Hugenholtz [1998b].

themes in this hot spring. About 30% of the rRNA gene sequences obtained from Obsidian Pool were associated phylogenetically with *Aquificales*, while about 12% were associated with sulfate-reducing bacteria [Hugenholtz, 1998a]. Taken together with a low total organic carbon content of 3 mg/L, indicates that sulfate reduction with hydrogen as an electron donor could be an important metabolic theme for primary production in this spring [Hugenholtz, 1998a].

Current Studies

Molecular surveys underway in other Yellowstone hot springs reveal that several of these candidate OP divisions

are widely represented around the Park. Approximately 10% of the sequences obtained from both Octopus Spring and Queens Laundry in the Lower Geyser Basin, pools that are 75-95°C, comprise members of candidate divisions OP 9 and 10 respectively. Work is ongoing to ascertain the relation between these phylogenetic sequences and their metabolic activity, which must be correlated with in situ geochemistry. One extremely important indication from the sequences is that hydrogen-metabolism may play a key role in these ecosystems. This is because most of the identifiable phylotypes are closely related to organisms known to derive their energy from hydrogen metabolism. Hydrogen concentrations have not been systematically measured in

Yellowstone geothermal waters. Prompted by the microbiological results, we currently are surveying the distribution of hydrogen in several geothermal settings. We have found bulk aqueous phase H_2 concentrations to range from a low of 4 nM (probably background in the measurements) in Yellowstone Lake, to 30 nM in Queens Laundry, to a high of 320 nM in a pool adjoining Obsidian Pool [Spear, 2002a]. This continuous supply of strong electron donation potential, food, probably provides the main energy basis for diverse microbiota in these hot springs. For comparison, the H_2 formation rate in the central region of a termite hindgut has been measured by microelectrode to be ca. 200 nmol (g termite \times h)⁻¹ and can accumulate to as high as 40 (M within the center of the hindgut [Ebert, 1997].

In these molecular studies we find there are no distinctions between the ecological boundaries of bacterial and archaeal habitats and that representatives of the domain Bacteria tend to dominate the Yellowstone high-temperature communities. By dominance we mean that colonization of growth surfaces suspended in hot springs (glass slides; glass wool; cotton fiber) tends to result almost exclusively in bacterial, not archaeal, biofilms (unpublished). Hugenholtz [1998a] used membrane hybridization to show that in a typical hot spring community, community rDNAs were composed primarily of bacterial representatives. These bacteria were then shown to comprise the majority of the microbial diversity within at least one hot spring, Obsidian Pool [Hugenholtz, 1998a]. We do not know which microbial members of the three domains of life comprise the majority of the biomass in this ecosystem. These results are directly relevant to the kinds of life that might be found at the geothermal subsurface-surface interface and around the hydrothermal zones of the world.

ARCHAEA IN YELLOWSTONE HOTSPRINGS

The Archaea

So far, there are three known main relatedness groups or “kingdoms” within the domain Archaea: Euryarchaeota, Crenarchaeota, and Korarchaeota (Figure 1) [Woese, 1990; Barns, 1996]. The split between these relatedness groups of Archaea is the deepest intradomain branch in the three-domain tree. Once thought to be confined to “extreme” locations and environments, archaea are as ubiquitous as bacteria. The misperception of archaeal dominance in extreme environments was due to the fact that most cultivated representatives of archaea derived from such environments [Dawson, 2000]. Archaea are profoundly different from both Bacteria and Eucarya, not only in phylogenetic assessments, but also in several fundamental properties. For instance, archaea produce membranes made of ether-linked lipids instead of ester-linked lipids used by both bacteria and

eucaryotes. In many ways archaea seem similar to eucaryotes or bacteria. For example, the transcription mechanism of archaea is similar to that of eucaryotes and distinct from bacteria with regard to genetic signals and protein factors. On the other hand, in many metabolic enzymes archaea resemble bacteria more than eucaryotes. Further, the deep division between Euryarchaeota and Crenarchaeota is reflected in profound biochemical difference. Euryarchaeota, for instance, possess histones associated with the chromosome as do the Eucarya. The Crenarchaeota, however do not produce histones; the mechanisms that fold chromosomal DNA in these organisms is still unknown.

The cultured Crenarchaeota are relatively homogeneous in their physiological properties, mainly thermophiles that use hydrogen as an energy source and some form of sulfur as an electron acceptor. On the other hand, cultured examples of Euryarchaeota exhibit considerable physiological diversity that consists of methanogens, extreme halophiles, and thermophiles. The molecular studies in many environments show, however, that the cultured kinds of Crenarchaeota comprise only one relatedness group, distinct from several other thermophilic and low-temperature, non-cultured relatedness groups that have been discovered in surveys of marine and terrestrial microbiota. Indeed, SSU-rRNA gene sequences of uncultured organisms make-up the majority of the known crenarchaeal phylogenetic diversity (Figure 4) [Dawson, 2000; DeLong, 1992]. The main diversity of Crenarchaeota are not thermophiles in “extreme” environments after all, but instead are low-temperature organisms of currently unknown physiology. Low-temperature archaea are probably ubiquitous throughout the environment [DeLong, 1992; DeLong, 1994; Hershberger, 1996; Dawson, 2000; Karner, 2001]. In the deep sea biome, low-temperature crenarchaeota are thought to comprise 20-39% of the biomass [Karner, et al., 2001]. This is an amazing estimate considering that the metabolic traits of these organisms and their functions in the ecosystem remain a complete mystery.

Many novel thermophilic lineages, some that relate to low-temperature lineages discovered in molecular surveys, have been encountered in several geothermal environments. Figure 4 describes the phylogeny of Crenarchaeota based on the approximately 300 rRNA gene sequences currently available [Dawson, 2000; Hugenholtz, 2002]. The figure illustrates that the great majority of crenarchaeal diversity is represented by non-cultured organisms (hollow wedges). Figure 4 also shows that some low-temperature lineages appear to have arisen from high-temperature lines. This is indicated by the fact that the low-temperature sequences group with thermophiles in phylogenetic trees, but exhibit long line segments in the trees. Presuming that modern organisms represented by short branches are more similar to the

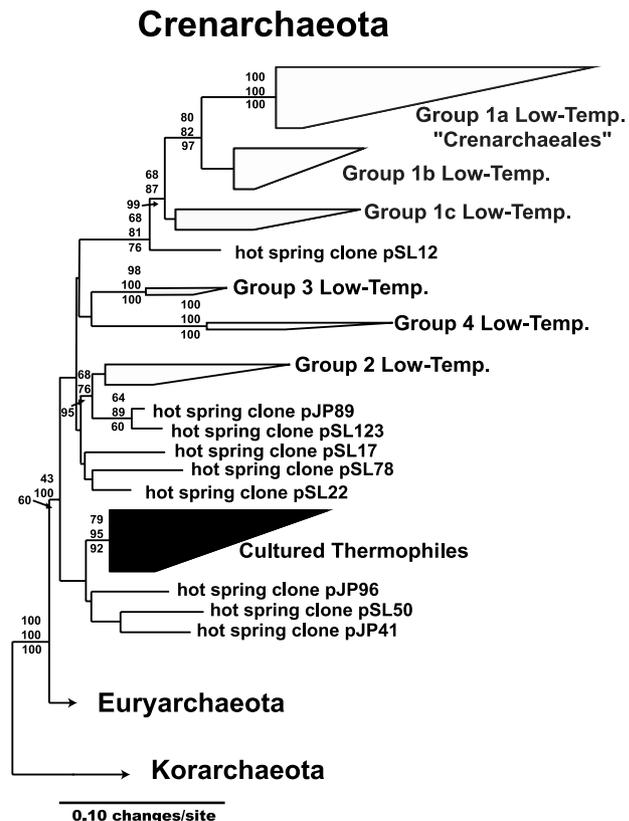


Figure 4. Phylogenetic representation of the Crenarchaeota. This figure is based on multiple bootstrapped analyses with three different phylogenetic methods. The size of the wedges is proportional to the number of rRNA sequences in that particular division. The one division filled with black has several cultivated representatives, the white wedges are represented only by their environmental sequence-phylogroup, retrieved from environmental samples. The scale bar indicates 0.1 changes per nucleotide in the SSU-rRNA gene sequence. After Dawson, 2000.

ancestral organisms than those represented by long branches, then thermophilic ancestry is indicated [Dawson, 2000].

The relatedness group of Korarchaeota is represented by only a few environmental rRNA gene sequences, all from hot springs such as where they were first discovered, in Yellowstone's Obsidian Pool [Barns, 1994; Barns, 1996]. This group is significant in that its branch point from the other lines of Archaea is deep in the overall map of life (Figure 1). This group branches more deeply in evolution than even the Euryarchaeota/Crenarchaeota separation and thus is a candidate for a third "kingdom" of Archaea [Dawson, 2000]. This conclusion needs to be confirmed, however, by analyses of further sequences or other study. While no representatives of the Korarchaeota have been brought into pure culture, organisms represented by the rRNA gene sequences pJP27 and pJP78 have been cultured

in community, probed by FISH, and shown to be rod-shaped with strong affinity for growth surfaces [Burggraf, 1997; Huber, 1995]. The mixed culture was developed in high hydrogen concentrations, so these representatives of Korarchaeota are likely to be chemolithotrophs, or at least sustained by or dependent upon lithotrophic (e.g. hydrogen) primary production. The deeply divergent nature and short lengths of the Korarchaeota lines in phylogenetic trees indicate that such organisms are the most closely related of any currently sequenced phylotypes to the universal ancestor of all life [Woese, 1998]. Where detected, korarchaeotes are abundant, and probably exist globally in chemical settings similar to Obsidian Pool.

Archaea and Yellowstone Hot Springs

Although archaea do not dominate the high-temperature Yellowstone ecosystem, they certainly are present in abundance and exhibit broad diversity. Novel organisms identified by SSU-rRNA gene sequences from Obsidian Pool provided the first indication that the extent of diversity within the Crenarchaeota kingdom was not well represented by cultured organisms. *Barns and coworkers* [1996] identified many new environmental sequences that expanded dramatically the known diversity of Crenarchaeota. Indeed, most of the crenarchaeal sequences obtained from Obsidian Pool branched more deeply from the crenarchaeal line of descent than most of the cultured species. Included were two sequences that grouped with SBAR5, a crenarchaeal sequence from pelagic marine picoplankton. The specific affiliation of these two clone sequences, together with their nested position within other thermophilic lineages of crenarchaeotes, also suggests that the low-temperature marine crenarchaeota are descendants of ancestral thermophiles (Figure 4) [Barns, 1996].

Only a few euryarchaeal sequences have been obtained from Obsidian Pool or other Yellowstone hot springs. Cultured representatives of thermophilic euryarchaeota are, however, well known. Organisms such as *Thermococcus*, *Thermoplasma* and *Methanothermus* are model laboratory organisms, but they have not yet been detected in environmental surveys of Yellowstone hot springs. The few representatives of euryarchaeota identified in Obsidian Pool were most similar to the thermophilic sulfate-reducing marine organism *Archaeoglobus fulgidus* [Barns, 1996]. As with the crenarchaeal kingdom, the deepest branchings and shortest line segments in the euryarchaeal kingdom are represented by thermophilic organisms. The uniform branching of thermophilic lineages from the base of the archaeal domain indicates that the last common ancestor for Archaea was thermophilic. This is consistent with theories that postulate a high-temperature origin of life.

Current Studies

In summary, much remains to be done in order to document the prevalence and kinds of archaea that occur in Yellowstone's geothermal springs. Indeed, the surface of this fascinating ecosystem has only been examined lightly for the extent of archaeal diversity. Efforts are underway in this and other laboratories to expand the survey of Archaea. In turn, this will provide insight into the extent of diversity that we can expect to find at mid-ocean ridges and other high-temperature microbial ecosystems.

EUCARYA IN YELLOWSTONE HOTSPRINGS

The eucaryotes in our common experience are mainly large organisms such as plants, animals and fungi. As seen in Figure 1, however, most of eucaryal diversity in fact is comprised of microbial organisms. Large, complex organisms such as plants, animals and fungi, constitute only three of the 30-40 recognized eucaryotic kingdoms. The actual extent of the diversity of microbial eucaryotes remains unknown, and what is known of diverse eucaryotes comes primarily from the analysis of a relatively few cultured organisms. As with most representatives of Bacteria and Archaea, most microbial eucaryotes probably are difficult to culture. Historically, descriptive efforts have focused on morphologically conspicuous aerobic eucaryotes. These surely do not represent most of the naturally occurring diversity of eucaryotes. The natural environment, therefore, is a rich source of novel eucaryotic diversity, both in oxic and anoxic environments. Indeed, molecular studies such as those that revealed novel representatives of Bacteria and Archaea also have identified novel eucaryal species deeply divergent from organisms known from culture [Dawson, 2002]. In terms of evolution, eucaryotes have been considered "younger," or more recently derived than the bacterial and archaeal domains, however, analyses of SSU-rRNA and other gene sequences indicate that the domain Eucarya is as old as the domain Archaea [Dawson, 2002; Woese, 2002]. The most deeply divergent lineages of Eucarya are anaerobic or aerotolerant organisms; this is consistent with the idea that life arose on an anoxic early Earth.

There have been limited surveys for microbial eucaryotes in Yellowstone's geothermal waters, and these have only been conducted with culture techniques or by direct observations. Molecular studies of Yellowstone hot springs have not yet detected eucaryotes at the highest temperatures, approaching the boiling point of water. Indeed, there are no known hyperthermophilic (growth $>80^{\circ}\text{C}$) eucaryotes, and it is generally believed that the upper temperature limit for eucaryotes is at the low-end of the thermophilic range, at 60°C for some protists, algae, and fungi [Rothschild and Mancinelli, 2001].

To date, there has been no systematic study or survey of microbial eucaryotes in the low thermophilic (growth $45\text{-}60^{\circ}\text{C}$) temperature ranges at Yellowstone.

METABOLIC BASIS OF THE YELLOWSTONE
HIGH-TEMPERATURE ECOSYSTEM

Hot spring microbial communities popularly are considered dependent on sulfur metabolism to derive energy for growth. Phylogenetic analyses of microbes in Yellowstone and other hot springs indicate, however, that molecular hydrogen, not sulfur, is the primary source of energy in these hot spring environments. This conclusion follows from the observations that the dominant masses of organisms in hot spring communities are representatives of the bacterial divisions *Aquificales* and *Thermotogales* [Hugenholtz, 1996, 1998a,b]. These kinds of organisms are known to derive energy for primary productivity from hydrogen metabolism. For instance, about 30% of the rRNA gene sequences obtained from Obsidian Pool were associated phylogenetically with *Aquificales*. Cloned rRNA gene sequences from Octopus Spring, hydrocarbon-containing Calcite Spring, and other sites also grouped phylogenetically with *Aquificales* [Reysenbach, 1994 and 2000]. Cultivated representatives of *Aquificales*, for example, *Thermocrinis ruber*, isolated from Octopus Spring, all thrive by microaerophilic oxidation of H_2 at high temperatures [Huber, 1998].

Molecular hydrogen is a highly versatile energy source. In general, organisms that can use hydrogen as an electron donor include photolithoautotrophs, photolithoheterotrophs, chemolithoautotrophs, and chemolithoheterotrophs. The electron acceptor for metabolism at the geothermal subsurface interface is probably mainly oxygen, although sulfur and sometimes elemental sulfur also can serve as electron-acceptors in hydrogen-oxidation. Organisms of the kind that metabolize hydrogen sulfide are not numerically conspicuous, however. Indeed, Yellowstone geothermal waters with the most microbial biomass have relatively low sulfide concentrations. Conversely, hot springs with abundant sulfide tend to have relatively low biomass [Barns, 1995; Hugenholtz, 1998a; Spear, unpublished].

The nature of the microbial constituents of Yellowstone hot spring communities predicts the availability of hydrogen in these geothermal waters, but a comprehensive survey for the occurrence of hydrogen has not been conducted. Ongoing measurements of hydrogen in Yellowstone hot springs indicate, however, the general presence of hydrogen in relatively high concentrations, from 10 to >300 nM [Spear, 2002a]. Thus, these results are consistent with the suggestion that molecular hydrogen, not sulfur, is the driving energy source for most microbial life at the geothermal surface-subsurface interface.

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. Hydrogen has been found in most submarine hydrothermal fluids, at concentrations that range from 96 (M to 1 mM [Seyfried and Mottl, 1995; Winn, *et al.*, 1995; Morita, 2000]. These hydrogen concentrations however, are from high temperature (300–450°C) vent fluids. Once mixed with seawater, buoyant plumes then spread from the vent outlet, and hydrogen concentrations drop at least one-thousand fold, to nM concentrations, similar in nature to those of Yellowstone. Ambient seawater hydrogen concentration spans from 0.2 to 1.5 nM at the surface to 0.2 to 0.4 nM in deep-water [Winn, 1995]. Thus vent zones with diluted, circulating vent fluids, provide a significant electron donation potential that can provide hydrogen, fuel for life. It seems likely, due to the reactive nature of hydrogen, that microbiota will make full use of this important energy source by rapidly oxidizing the hydrogen. This is likely to occur in Yellowstone and in the mixed and diluted buoyant plumes that arise from the hydrothermal fluid emanation of mid-ocean ridges.

What is the source of the hydrogen? Apps and van de Kamp have reviewed the nature of hydrogen and methane from subsurface environments and list several different processes for the generation of H₂ [1993]. Stevens and McKinley [1995; with debate by Madsen, 1996 and Lovley, 1996; followed by Stevens and McKinley, 1996; and Anderson, 1998] found that water reacts with iron-rich Columbia River basalt (CRB) to produce molecular hydrogen at concentrations as high as 60 (M. Later work in the CRB has shown that hydrogen production can average 5 nmol of H₂ (m² of basalt)⁻¹ day⁻¹ [Stevens and McKinley, 2000]. This supports observed concentrations of methane produced microbially in the CRB [Stevens and McKinley, 2000] and suggests that nM concentrations of hydrogen can provide the reduction potential necessary for life. 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, 1998a]. Though subject to debate in terms of the quantities (refs. noted above), the occurrence of any bulk aqueous phase H₂ provides reason to believe that H₂ could serve as a primary electron donor for naturally occurring microbial communities. Potentially relevant for H₂-generation both at Yellowstone and mid-ocean ridges is the reaction between dissolved gases in the system, C-H-O-S in magmas, particularly those with basaltic affinities [Apps and van de Kamp, 1993]. Gold 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, 1992]. Gold maintains that H₂ and CH₄ should be chemically stable in Earth’s upper mantle, and that migration into the crust occurs continuously. In addition,

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 [Hoehler, 1998]. There are, of course, many potential biogenic sources of H₂ in both oxic and anoxic environments, that are utilized by hydrogen-metabolizers (for review see [Nandi and Sengupta, 1998]).

Microbial mats are another ecosystem where hydrogen-metabolism is emerging as a conspicuous theme, and some results may be applicable to 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 orientation are prevalent throughout examined mats. The specific microbes involved and their supportive energy metabolisms 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 influences individual microbial metabolisms and system-level biogeochemistry [Hoehler *et al.* 2001; Ward, *et al.*, 1998]. While in this case the hydrogen is a result of primary production by photosynthetic organisms, this strong electron donor is then available for hydrogen-based metabolisms of associated organisms, supported only indirectly by photosynthesis. This hydrogen-metabolism, in turn, defines the thermodynamics of the mat microenvironment and determines the organisms that can thrive in the community, for instance, anaerobic sulfate-reducing bacteria, archaeal methanogens, and bacterial *Green non-Sulfur* and *Nitrospira* members. It is likely that many of the same kinds of hydrogen-dependent organisms will be encountered in hydrothermal environments, where the source of hydrogen is, however abiogenic rather than biogenic.

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. Laboratory experimentation with hydrogen-metabolizing organisms is technically a challenge and, therefore, has received relatively little attention. Only six of the 36 phylogenetic divisions of Bacteria have at least one representative shown to engage in hydrogen-metabolism (asterisks in Figure 3), but it seems likely that hydrogen-metabolism is far more widespread. In Obsidian Pool, where the bulk of the biomass appears to be composed of known hydrogen-oxidizers, other organisms (for instance, representatives of the *Green non-Sulfur* division) also are abundant [Hugenholtz, 1998a]. Are such organisms engaged in hydrogen oxidation?

ENERGY GRADIENTS

The fluids of the Yellowstone geothermal system are comprised of both heated meteoric rainwaters and hot groundwater from deep sources. Energy gradients for microbiological growth are provided, on the one hand, by the meteoric waters that contain organic compounds, and, on the other hand, by the hot, reduced groundwaters that likely contain considerable electron donation potential. This potential is probably primarily in the form of hydrogen or reduced compounds such as hydrogen sulfide, but the groundwaters probably have little electron acceptance potential. The terminal electron acceptance potential for the Yellowstone system is provided by atmospheric oxygen, ecosystem sulfates and ecosystem carbonates. Thus, all of the unique microbial life so far described in geothermal settings occurs in places where the unique and reduced subsurface waters mix with these electron acceptors. Complex microbial communities occur as lush biofilms on surfaces that are pH, Eh, chemical, and temperature-dependent. The results of microbial processes at the oxic-anoxic boundary are observable in thermal features named for their microbially provided color, such as Grand Prismatic Spring and Fountain Paint Pots. Rich, complex microbial life is ubiquitous at all of these subsurface-surface interfaces. This complexity of life has been seen in the microbial mats of Octopus Spring, the "slimy" black sediments of Obsidian Pool, and in the geothermal silica sinter of a number of Yellowstone hot springs [Ward, 1998; Hugenholtz, 1998a; Blank, 2002].

LIFE IN THE YELLOWSTONE SUBSURFACE

There has been considerable speculation about the potential occurrence of a substantial, subcrustal biomass on Earth [Gold, 1992; Whitman, 1998]. It is possible that the Yellowstone subsurface contains an active microbial ecosystem, but we think it unlikely. We have looked, using in situ growth surfaces (contact slides), for microbial life in one subsurface location in the Park, namely in the groundwater zone that underlies Biscuit Basin [Spear, 2001b]. There, Well Y-7, drilled by the U.S. Geological Survey in 1968, penetrates 75 m into the subsurface. This well is filled with water of temperatures that range from 48°C at the surface to 140°C at the bottom; these temperatures are seasonally dependent with significant variation. We found a few microbial cells at the surface of the well, evidently not growing, and some cells as far down as about 30 m. We tried to encourage growth of in situ microbiota on these growth surfaces in submerged growth chambers that contained a time-released growth medium [Spear, et al., 2002b]. We did not observe colonization on the glass slides that were suspended

at various depths and temperatures (48 to 140°C) down the length of the well from one to six months [Spear, 2002b]. In contrast, growth surfaces in a control chamber suspended at an interface zone in Octopus Spring were heavily colonized. Limited searches for microbial life in five subsurface geothermal-energy wells in Iceland similarly found few organisms in geothermal waters (five wells screened, 70-130°C range), and not in significant concentrations [Marteinsson, 2001]. We believe that the subsurface, at least at the Well Y-7 location, is too reduced and/or too hot (i.e. above 120°C) for microbial life. It seems likely that a significant amount of subsurface microbial life in any environment-geothermal, hydrothermal, rock, or mineralized soil-can only occur at interfaces, where a reduced zone meets an oxidized zone and there is a significant flux, a mix of geochemical compounds, to allow for the reactions necessary to maintain life. This implies that an indigenous, widespread, subsurface, microbial biosphere is likely to be rare or non-existent in all geo- and hydrothermal zones.

CONCLUSION

Molecular phylogenetic analyses and perspective applied to even a few Yellowstone geothermal communities have contributed significantly to our current view of microbial diversity and the kinds of organisms to be expected in other geothermal settings. Results focus perspective on the chemical basis of high-temperature ecosystems. Particularly conspicuous is the abundance of organisms in the Yellowstone geothermal ecosystem that for energy probably depend on the most fundamental and abundant element, hydrogen. A hot, anoxic, hydrogen-rich Earth, provided the conditions necessary for the emergence of a universal ancestor for all life [Woese, 1998]. The fact that life can be based energetically on the most abundant element in the universe, indicates the potential for life elsewhere than Earth. Hydrogen metabolism is likely to be a dominant theme in many places on our own Earth; the hot springs of geothermal areas and the mid-ocean ridges; the deep subsurface; the hydrosphere; and within our own bodies.

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Department of Molecular, Cellular and Developmental Biology,
347 UCB, Boulder, Colorado, 80309 USA