Incidence and diversity of iron mineral-colonizing microorganisms in seven hydrothermal environments, Yellowstone National Park, USA

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Senior Integrative Exercise
9 March 2005

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Abstract

Terrestrial hydrothermal springs are geochemically diverse environments that host equally diverse lithotrophic microbial communities. Free energy calculations for redox disequilibria in Yellowstone National Park hot springs suggest that iron-bearing minerals should be rich sources of energy for microorganisms able to catalyze their transformation. Iron reducing and oxidizing microbes are widespread in hydrothermal environments and are important in global mineral cycling. Microorganisms in seven Yellowstone hot springs were baited with hematite, magnetite, and pyrite. Density and diversity of mineral-colonizing communities was assessed with fluorescence microscopy and 16S rDNA gene terminal restriction fragment length community profiling. Results indicate that microbes selectively colonize minerals in a pattern that is not predicted by either cell densities in the water column or cell densities on an inert surface. Mineral-specific colonization patterns suggest microbial use of iron minerals as metabolic substrates. Promising sites and techniques for further investigation are discussed.

Georef Keywords: Yellowstone National Park, iron minerals, biogeochemistry, hot springs, redox disequilibrium.
Introduction

Lithotrophic thermophiles, organisms that thrive at high temperatures (>45ºC) through the reduction and oxidation of inorganic materials, are an important link between geochemical and biological mineral cycles (Macalady and Banfield, 2003; Madigan et al., 2003). Occupying the shortest and deepest branches of the universal phylogenetic tree, lithotrophic thermophiles may be the extant organisms that bear closest resemblance to our last universal common ancestor (Vargas et al., 1998).

Terrestrial hydrothermal springs, such as Yellowstone National Park’s famous pools, geysers, and mudpots, are home to an astounding diversity of Bacteria and Archea as revealed by 16S rDNA-based phylogenetic surveys (Barns et al., 1996; Barns et al., 1993; Hugenholtz et al., 1998; Pace, 1997). However, thermophiles often defy standard laboratory culture and thus specific geochemical energy sources are known for only a handful of the organisms recognized through molecular phylogenetic studies (Pace, 1997; Shock et al., 2004a).

Beneath Yellowstone National Park (YNP), magmatically heated groundwater at equilibrium with rocks in the deep subsurface, convects upward and emerges as hot springs where faults cross topographically low areas (Fournier, 1989). Irregular mixing of high temperature fluids with shallow, dilute groundwater produces springs with diverse chemical compositions that support an equally diverse array of microbial metabolisms (Amend and Shock, 2001; Fournier, 1989; Reysenbach and Shock, 2002). Complex microbial communities are sustained by the constant influx of fluids bearing redox-active chemical species far from equilibrium at surface conditions. Exothermic reduction-oxidation reactions proceed toward equilibrium with a net release of energy but generally
equilibrate slowly due to kinetic barriers. Thermodynamically favored yet kinetically inhibited reactions are potential energy sources for lithotrophic microorganisms that overcome kinetic barriers through enzymatic catalysis and convert the released energy into a biologically useable form (Amend and Shock, 2001).

Everett Shock and colleagues (Arizona State University, Tempe AZ) have measured chemical compositions of dozens of YNP hot springs and calculated free energies for hundreds of redox disequilibria. Such calculations indicate which redox systems yield the most energy to microorganisms catalyzing the electron transfer and allow prediction of which metabolisms are most advantageous, and potentially most ecologically prevalent, in a given environment. These predictions can be used to guide microbiological investigations (Shock et al., 2004b). Interestingly, theoretical results indicate that many of the most energy-rich reactions in YNP hot springs involve oxidation or reduction of common iron-bearing minerals (Everett Shock, personal communication).

The present study seeks to employ these theoretical predictions in the search for microorganisms with specific metabolisms. I probed for thermophiles capable of metabolizing magnetite ($\text{Fe}_3\text{O}_4$), hematite ($\text{Fe}_2\text{O}_3$), and pyrite ($\text{FeS}_2$) through \textit{in situ} incubation and subsequent microscopic and molecular analysis of colonizing communities. This combination of theoretical prediction, \textit{in situ} incubation, and microscopic and molecular characterization has led to the successful isolation of novel Fe-oxidizing bacteria from a deep-sea hydrothermal area (Edwards et al., 2003a; Edwards et al., 2003b; McCollum and Shock, 1997).
Given the prevalence of dissimilatory iron reducing and oxidizing microorganisms in hydrothermal environments (Vargas et al., 1998) and the potential energy yields from iron minerals, microbes capable of deriving energy from such minerals should colonize the surfaces of introduced mineral samples with diversity and density dependent on the environment-specific energy potential. Here I report visual and molecular community characterization and cell count data demonstrating that mineral colonization is a selective process; surface communities vary in density and diversity between mineral substrates and incubation environments. While it is unknown whether the microbes observed on the surfaces of magnetite, hematite, and pyrite are using the minerals for energy metabolism, the selectivity of microbe-mineral interactions deserves further investigation as possible evidence for iron mineral metabolism in Yellowstone hot springs.

Methods

**Thermodynamic calculations**

Gibbs free energy for coupled redox reactions is determined by comparing the equilibrium state to the environmental (disequilibrium) state. Overall Gibbs free energy for a reaction \((r)\) is given by:

\[
\Delta_r G = \Delta_r G^\circ + RT \ln Q_r
\]  

(1)

Where \(T\) is temperature and \(R\) is the gas constant. Standard (equilibrium) Gibbs energy is given by:
\[ \Delta G^\circ = -RT \ln K \]  

Where \( K \) is the equilibrium constant. \( Q_r \), the activity product, is defined as:

\[ Q_r = \prod a_i^{v_i} \]

Where \( a_i \) is the activity of the \( i \)th reaction constituent in the environment in question and \( v_i \) is the reaction coefficient. Activities were calculated by the procedure described by Amend and Shock (2001) from measurements of pH, temperature, major and minor gas concentrations, and magmatic gas levels made by GEOPIG in 1999-2001 (Shock et al., 2004a). Equilibrium constants come from a customized database developed by Everett Shock and colleagues. Free energy data was kindly provided by Melanie Holland (Arizona State University, Tempe AZ).

**Yellowstone National Park field sites**

Seven persistent hot springs with diverse geochemistries (see appendix) were selected for field incubations: Figure 8 Pool, Obsidian Pool, and OB1-heim Pool in the Greater Obsidian Pool Area (GOPA) near Mud Volcano; Lobster Claw Spring and Sylvan Spring in the Gibbon Geyser Basin; and Bison Pool and Boulder Spring in sentinel meadows near the Lower Geyser Basin area (Figures 1 & 2). The location of the incubation in each pool was chosen to coincide with GEOPIG (Group Exploring Organic Processes in Geochemistry, Arizona State University) chemical sampling sites.
Figure 1. Map of Yellowstone National Park, USA. Towns, major geyser basins, and water bodies are indicated. Study areas are marked with unofficial names of hot springs used for field incubations.

Figure 2 (Next Page). Yellowstone National Park field incubation sites. Sylvan Springs Area near Gibbon Geyser Basin: A) Sylvan Springs proper; B) Lobster Claw Spring. Greater Obsidian Pool Area near Mud Volcano: C) OB1-heim Pool; D) Figure 8 Pool; E) Figure 8 Pool close-up; F) Obsidian Pool proper. Sentinel Meadows in the Lower Geyser Basin: G) Bison Pool; H) Boulder Spring; I) Close-up of Boulder Spring. Arrows indicate the location of field incubations.
**Preparation of minerals**

Characteristics of minerals used in field studies are listed in table 1. Mineral slabs were prepared to bait microorganisms for microscope studies and glass slides were used as inert control surfaces. 2-cm square slabs of magnetite and hematite and 1cm square slabs of pyrite for field incubation were cut to 2-4 mm thickness and polished to 10 microns. Magnetite, hematite, and pyrite were crushed with a hammer and sieved. The 7.93 mm to 1.68 mm fraction was used to bait microorganisms for DNA extraction. Minerals and glass slides were sonicated, soaked in ethanol, and rinsed repeatedly in deionized water. All materials used in field incubations were autoclaved prior to introduction into the environment.

**Flow basket incubations**

Mineral slabs, chips, and glass slides were placed in four-inch tetrahedral polyetheretherketone (PEEK) mesh flow baskets designed specially for this study. Baskets were suspended in hot springs such that contents were completely submerged but not touching sediments (Figure 3).

After 8 to 11 days incubation, baskets were removed from the springs. Mineral slabs were fixed in 4% paraformaldehyde in 1% phosphate buffered saline solution (PBS: 0.137 M NaCl, 0.005 mol/L Na₂HPO₄, 0.003 mol/L KCl, 0.001 mol/L KH₂PO₄, pH 7.3) for 2 hours (Edwards et al., 2003a). Following fixation, slabs were transferred to 50 mL Falcon tubes containing 1:1 PBS buffer/Ethanol solution and frozen until analysis. Mineral chips were removed from baskets and transferred into 50 mL Falcon tubes containing 2x buffer
<table>
<thead>
<tr>
<th>Supplier</th>
<th>Source</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnetite</td>
<td>Arizona Mining and Minerals Museum</td>
<td>Arizona Granular massive compact, anhedral crystals, fine grained with silicate inclusions.</td>
</tr>
<tr>
<td>Hematite</td>
<td>Arizona Mining and Minerals Museum</td>
<td>Arizona Massive, course grained, containing both red, earthy material and metallic tabular crystals.</td>
</tr>
<tr>
<td>Pyrite</td>
<td>Dakota Matrix Minerals Company (Thomas Loomis)</td>
<td>Colorado Aggregates of striated euhedral, cubic crystals with sparse silicate inclusions.</td>
</tr>
</tbody>
</table>

Table 1. Sources, suppliers, and descriptions of minerals used in field incubations.

Figure 3. Field incubations in Yellowstone National Park’s Boulder Spring. Arrow: PEEK mesh flow baskets containing glass slides, mineral slabs, and mineral chips.
A (200 mM Tris [pH 8], 50 mM EDTA, 200 mM NaCl, 2 mM sodium citrate, 10 mM CaCl$_2$) and frozen until analysis (Hugenholtz et al., 1998).

**DAPI Staining and Cell Counts**

Magnetite, hematite, and pyrite slabs and glass slides from each site were stained with DAPI (4’,6-diamidino-2-phenylindole) to visually characterize microbial communities. Polished surfaces of preserved slabs were covered with filtered 20 mg/L DAPI solution and stained in the dark for 10 min. Following staining, slabs were rinsed with PBS and viewed with a Carl Zeiss Axioplan 2 light microscope under illumination with an EXFO X-Cite 120 fluorescence light source. Florescence micrographs were captured with a Carl Zeiss AxioCam HRc color camera and Carl Zeiss AxioVision 4.0 imaging software.

Cell counts were used as a proxy for biomass density in hot spring water and on mineral surfaces. Hot spring water samples collected by GEOPIG (summer 2004) were vacuum filtered through a 0.1 μm Osmonics Poretics polycarbonate filter. Filters, and mineral surfaces, and glass slides were DAPI stained and viewed as described above. Cell densities were calculated from visual counts of cells in 25-40 fields.

**DNA extraction**

Total community DNA was extracted from approximately 10g of mineral chips per experimental group following the MoBio Ultraclean Soil DNA MegaPrep kit protocol. DNA was concentrated using an ethanol precipitation method: 10% volume sodium acetate and 200% volume cold 100% ethanol was added to DNA solution in Tris
methylamine/HCl. DNA was precipitated overnight at –20°C and pelleted by centrifugation at 13,000g for 10 min. Pellet was washed 70% ethanol and, dried, and resuspended in 10µl TE (10 mM Tris HCl, 1 mM EDTA, pH 8).

**PCR amplification of 16S rDNA**

Community 16S rDNA was amplified by polymerase chain reaction with bacteria-specific forward primer 8F (5’-AGAGTTTGATCCTGGCTCAG-3’), universal reverse primer 1492R (5’-GGYTACCTTGTTAGGACTT-3’) for routine amplifications, and universal reverse primer 1492R-G-FAM (5’-/56-FAM/GGYTACCTTGTTAGGAC-TT-3’) for fluorescent tagging (Operon Technologies, Alameda, CA). Each 25µl reaction contained 100ng DNA template, 120 ng of each primer, 2.5 µl 10x HotMaster Taq buffer with 25 mM Mg⁡²⁺, 2.5 µl 2 mM dNTP mixture, 1 U HotMaster Taq polymerase (Eppendorf, Westbury, NY). All reaction mixtures were incubated in a thermal cycler (BIO-RAD DNA Engine PTC-200 Peltier Thermal Cycler) with the following program: 2 minutes initial denaturation at 94°C, then 35 cycles of 45 seconds denaturation at 94°C, 20 seconds annealing at 54°C, and 90 seconds extension at 65°C, 5 minutes final extension at 65°C, and at 4°C storage. Successful amplification was confirmed with agarose gel electrophoresis (1% agarose) of PCR products and SYBR green staining (Molecular Probes, Eugene, OR).

**T-RFLP community profiling**

Terminal restriction fragment length polymorphism (T-RFLP) analysis was used to create molecular profiles of mineral-colonizing bacterial communities. Although T-
RFLP does not yield any phylogenetic or physiological data about specific members of the community, it produces unique, reproducible community fingerprints at higher sensitivity and a lower cost than competing techniques (Moeseneder et al., 1999; Osborn et al., 2000). This technique exploits the natural variation in 16S rDNA gene sequence between taxa. 16S sequences are amplified in a PCR reaction in which one of two primers is fluorescently tagged. PCR products are digested with a restriction enzyme and the length of tagged fragment is measured with an automated DNA sequencer (Liu et al., 1997). Because of variation in sequence, the restriction enzyme creates terminal fragments with lengths that vary between bacterial groups (henceforth referred to as operational taxonomic units (OTUs)).

T-RFLP was performed at the Nevada Genomics Center (University of Nevada, Reno). PCR products were purified with a Qiagen MinElute filter plate on the Qiagen BioRobot 3000, dried and resuspended in 5µl water, then digested with HhaI restriction enzyme. Restriction fragments were mixed with GeneScan 500LIZ size standard and analyzed by capillary electrophoresis on the ABI Prism 3730 DNA analyzer. Fragment lengths were calculated by comparison to the size standard with the GeneMapper program.

Results

Free energies for reactions involving magnetite, hematite, and pyrite are shown in Figure 4. Reactions are grouped according to mineral substrate and ordered according to free energy in Obsidian Pool, the site with the most complete data set. The same horizontal scale is used across groups to allow direct comparison of free energies.
Magnetite Oxidation Reactions:

\[
\begin{align*}
2\text{FeO}_4\text{s, }0.5\text{O}_2\text{aq }3\text{H}_2\text{O} & \rightarrow 6\text{FeOOH}_4\text{s, }1\text{NO}_2\text{[2]} \\
2\text{FeO}_4\text{s, }0.5\text{O}_2\text{aq }3\text{FeO}_3\text{s, }2\text{NO}_2\text{[2]}
\end{align*}
\]

Magnetite Reduction Reactions:

\[
\begin{align*}
\text{Fe}_2\text{O}_4\text{s, }1\text{CO}_3\text{g, }1\text{H}_2\text{O} & \rightarrow 1\text{HCO}_3\text{g, }3\text{FeO}_3\text{s, }2\text{H}_2\text{O}\text{[2]}
\end{align*}
\]
Hematite Reduction Reactions:

\[ \begin{align*}
1\text{Fe}_2\text{O}_3 + 1\text{CO}_2 + 3\text{H}_2\text{O} &\rightarrow 2\text{Fe}_2\text{O}_3 + 1\text{H}_2\text{O} + 2\text{CO}_2 \\
1\text{Fe}_2\text{O}_3 + 1\text{CO} + 4\text{H}_2\text{O} &\rightarrow 2\text{Fe}_2\text{O}_3 + 1\text{H}_2\text{O} + 4\text{H}_2\text{O} \\
3\text{Fe}_2\text{O}_3 + 1\text{CO}_2 + 3\text{H}_2\text{O} &\rightarrow 2\text{Fe}_2\text{O}_3 + 1\text{H}_2\text{O} + 3\text{CO}_2 \\
1\text{Fe}_2\text{O}_3 + 1\text{H}_2\text{O} &\rightarrow 1\text{Fe}_2\text{O}_3 + 1\text{H}_2\text{O} \\
4\text{Fe}_2\text{O}_3 + 1\text{CO}_2 + 8\text{H}_2\text{O} &\rightarrow 4\text{Fe}_2\text{O}_3 + 1\text{H}_2\text{O} + 8\text{H}_2\text{O} \\
3\text{Fe}_2\text{O}_3 + 1\text{H}_2\text{O} &\rightarrow 1\text{Fe}_2\text{O}_3 + 3\text{H}_2\text{O} \\
4\text{Fe}_2\text{O}_3 + 1\text{CO}_2 + 8\text{H}_2\text{O} &\rightarrow 4\text{Fe}_2\text{O}_3 + 1\text{H}_2\text{O} + 8\text{H}_2\text{O} \\
3\text{Fe}_2\text{O}_3 + 1\text{H}_2\text{O} &\rightarrow 1\text{Fe}_2\text{O}_3 + 3\text{H}_2\text{O} \\
12\text{Fe}_2\text{O}_3 + 1\text{CH}_4 + 12\text{H}_2\text{O} &\rightarrow 12\text{Fe}_2\text{O}_3 + 1\text{H}_2\text{O} + 12\text{H}_2\text{O} \\
8\text{Fe}_2\text{O}_3 + 1\text{CO}_2 + 4\text{H}_2\text{O} &\rightarrow 8\text{Fe}_2\text{O}_3 + 1\text{H}_2\text{O} + 4\text{H}_2\text{O} \\
11\text{Fe}_2\text{O}_3 + 1\text{H}_2\text{O} &\rightarrow 11\text{Fe}_2\text{O}_3 + 1\text{H}_2\text{O} \\
\end{align*} \]
Pyrite Oxidation Reactions:

4FeS₂ + 7O₂ + 14H₂O → 4FeSO₄ + 8SO₄²⁻ + 27H⁺

Pyrite Reduction Reactions:

FeS₂ + 2H₂O → Fe₂⁺ + 2H₂S
Figure 4. Average overall Gibbs free energy in calories per mole electrons transferred for coupled redox reactions involving magnetite (A & B), hematite (C), and pyrite (D & E) in Lobster Claw, Figure 8, OB1-heim, Sylvan, Obsidian, and Bison springs. More exothermic conditions plot towards the right. Reactions are listed on the y-axis and ordered according to increasing free energy in Obsidian Pool. The number of electrons transferred is given in brackets after each reaction. Bars are the total range of free energy values for each reaction based on multiple samples, and as such reflect geochemical variability of the spring.
Magnetite, which contains both \( \text{Fe}^{3+} \) and \( \text{Fe}^{2+} \), can serve as either an electron acceptor or donor in Fe reduction or oxidation, respectively (Figures 4a & b). Oxidation results in the transformation of magnetite to hematite, goethite (FeOOH), or pyrite when coupled to the reduction of \( \text{HCO}_3^- \) (aq), CO (g), \( \text{CO}_2 \) (g), O\(_2\) (aq), \( \text{NO}_2^- \) (aq), \( \text{NO}_3^- \) (aq), \( \text{SO}_4^- \) (aq), or S (s). Reduction of magnetite results in dissolution by conversion of \( \text{Fe}^{3+} \) to \( \text{Fe}^{2+} \) and the oxidation of \( \text{H}_2\text{S} \) (aq), CO (g), \( \text{NH}_4 \) (aq), H\(_2\) (g), \( \text{CH}_4 \) (g), \( \text{NO}_2^- \) (aq), or FeS\(_2\) (s). Magnetite reduction reactions show greater free energy variability between pools than oxidation reactions. Reduction is much more favorable in acidic environments than in neutral and basic environments. Hematite, with only \( \text{Fe}^{3+} \), can only serve as an electron acceptor (Figure 4c). Reduction of hematite, coupled to the oxidation of the species mentioned above, yields considerably less energy than the reduction of magnetite and shows a similar environmental variation. \( \text{Fe}^{2+} \) in pyrite can be oxidized to \( \text{Fe}^{3+} \), and the sulfur in pyrite can be reduced (Figures 4d & e) though pyrite reduction is an unfavorable process in the environments considered. Many pyrite oxidation reactions are highly energetic with a bias towards acidic sites.

Visual descriptions of microbial communities colonizing experimental surfaces are summarized in Table 2. Observed morphotypes were classified as cocci (small, spherical cells), rods (elongated, cylindrical cells), or chains (multiple cells linked together end to end). Rods conveniently divided into two groups: short rods with an aspect ratio of ~ 3:1, and long rods with an aspect ratio of ~ 10:1. Chains were difficult to classify as they are found in a variety of different lengths, are notoriously hard to focus, and the edges of individual DAPI stained cells are often difficult to discern. Chains that
Table 2. Morphotype diversity observed by fluorescence microscopy on the surfaces of magnetite, hematite, pyrite, and glass slides incubated in seven Yellowstone hot springs. Relative numerical importance of each morphotype is represented by font size.

<table>
<thead>
<tr>
<th></th>
<th>Boulder</th>
<th>Bison</th>
<th>Obsidian</th>
<th>Sylvan</th>
<th>OB1-heim</th>
<th>Figure 8</th>
<th>Lobster Claw</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Magnetite</strong></td>
<td>Cocci</td>
<td>Long rods</td>
<td>Cocci</td>
<td>Long chains</td>
<td>Cocci</td>
<td>Short rods</td>
<td>Cocci</td>
</tr>
<tr>
<td></td>
<td>Long rods</td>
<td>Short rods</td>
<td>Short rods</td>
<td>Short chains</td>
<td>Short rods</td>
<td>Short chains</td>
<td>Short chains- rods</td>
</tr>
<tr>
<td></td>
<td>Cocci</td>
<td>Chains</td>
<td>Cocci</td>
<td>Long chains</td>
<td>Cocci</td>
<td>Short chains</td>
<td>Rods</td>
</tr>
<tr>
<td><strong>Hematite</strong></td>
<td>Cocci</td>
<td>Long rods</td>
<td>Cocci</td>
<td>Long chains</td>
<td>Cocci</td>
<td>Short rods</td>
<td>Cocci</td>
</tr>
<tr>
<td></td>
<td>Long rods</td>
<td>Short rods</td>
<td>Short rods</td>
<td>Short chains</td>
<td>Short rods</td>
<td>Short chains</td>
<td>Rods</td>
</tr>
<tr>
<td></td>
<td>Chains of rods</td>
<td>Chains</td>
<td>Cocci</td>
<td>Long chains</td>
<td>Short rods</td>
<td>Short chains- rods</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Long rods</td>
<td>Cocci</td>
<td>Short chains</td>
<td>Short chains</td>
<td>Short chains</td>
<td>Cocci</td>
<td>Rods</td>
</tr>
<tr>
<td><strong>Pyrite</strong></td>
<td>Chains of rods</td>
<td>Long rods</td>
<td>Short rods</td>
<td>Cocci</td>
<td>Short rods</td>
<td>Short chains- rods</td>
<td>Cocci</td>
</tr>
<tr>
<td></td>
<td>Long rods</td>
<td>Short rods</td>
<td>Short chains</td>
<td>Cocci</td>
<td>Short rods</td>
<td>Short chains</td>
<td>Rods</td>
</tr>
<tr>
<td></td>
<td>Long rods</td>
<td>Cocci</td>
<td>Short chains</td>
<td>Cocci</td>
<td>Short rods</td>
<td>Cocci</td>
<td>Rods</td>
</tr>
<tr>
<td><strong>Glass Slide</strong></td>
<td>Long rods</td>
<td>Long rods</td>
<td>Cocci</td>
<td>Short rods</td>
<td>Cocci</td>
<td>Short chains- rods</td>
<td>Cocci</td>
</tr>
<tr>
<td></td>
<td>Long rods</td>
<td>Short rods</td>
<td>Cocci</td>
<td>Long chains</td>
<td>Cocci</td>
<td>Short chains</td>
<td>Rods</td>
</tr>
</tbody>
</table>


were clearly identifiable as long (>5 cells) or short (=< 5 cells), or clearly composed of
rods or cocci are tabulated as such. Relative importance of each morphotype is indicated
by the font size. Examples of the morphotypes listed in Table 2 are shown in Figure 5.

Morphotype data show variation both between pools and between surfaces
incubated in the same pool. With the exception of OB1-heim and Figure 8, in every pool
there are one or two morphotypes with a limited distribution: in Boulder, chains are
restricted to hematite and pyrite, in Bison cocci are restricted to magnetite and glass and
in Sylvan to magnetite and pyrite, Lobster Claw’s rods are restricted to hematite and
pyrite, while in Obsidian cocci and chains are notably absent from pyrite.

Cell counts for whole water samples varied by three orders of magnitude between
pools (Figure 6a). Densities ranged from $2.6 \times 10^4 \pm 1.40 \times 10^4$ cells/ml in Boulder to
$3.85 \times 10^6 \pm 1.06 \times 10^6$ in Sylvan. Obsidian, Sylvan, OB1-heim, and Figure 8 had
densities on the order of $10^6$, while Bison and Lobster Claw densities were on the order
of $10^5$ cells/ml. Significantly higher densities were observed in subneutral springs than in
acidic or alkaline springs.

For cell counting and molecular profiling I focused on the differences between
hematite and magnetite. Surface colonization varied several orders of magnitude both
between pools and between different surfaces incubated in the same pool (figure 6b).
Densities ranged from Lobster Claw magnetite with $1.43 \times 10^3 \pm 5.12 \times 10^2$ cells/ml, to
Bison hematite with $1.25 \times 10^5 \pm 3.45 \times 10^4$ cells/ml. Pools can be divided roughly into three
groups. OB1-heim and Boulder show a similar pattern of very sparse colonization on
minerals and densities an order of magnitude higher on glass. Sylvan and Lobster Claw
Figure 5. Examples of morphotypes used to qualitatively characterize biodiversity on mineral slabs and glass slides. A) Abundant cocci colonizing a hematite surface incubated in Lobster Claw Spring. B) Long rods colonizing a glass slide incubated in Bison Pool. C) A pyrite surface incubated in Figure 8 Pool showing a diverse array of morphotypes. Short rods are indicated with the white arrow, a long chain with the yellow arrow, and a short chain with the green arrow.
Figure 6. Microbial cell densities: A) suspended in the water column, and B) colonizing the surfaces of hematite, magnetite, and glass slides. Bars are one standard deviation. Fine sediment accumulation made cell counting impossible on Obsidian surfaces and Sylvan glass slide.
Microbe-mineral interactions influence weathering and mineral cycling and provide an important link between geology and the biosphere (Fenchel et al., 1988; Lovely, 2000). Free energy calculations of redox disequilibria indicate that reduction and oxidation of magnetite, hematite, and pyrite coupled with electron donors and acceptors present in Yellowstone hot springs are can proceed to equilibrium with a net release of energy via numerous pathways (Figure 4). Variation along the x-axis is a product of the disparity in chemical composition between pools.

A total of fifteen 16S terminal fragment alleles, corresponding to fifteen OTUs, were distributed over seven hot springs (Table 4). Several OTUs (numbers 1, 2, 3, 4, 6, 8, 12, 13, 14) were confined to one mineral in one pool. OTUs 5, 10, & 15 were widespread, appearing in extractions from both minerals and multiple pools. OTU9 and OTU11 were isolated from both minerals, but were confined to Lobster Claw and Figure 8 respectively. T-RFLP data demonstrate variability in biodiversity of colonizing communities: maximum biodiversity was observed on Figure 8 magnetite, hosting 5 distinct OTUs. The majority of surfaces however were dominated by 1-3 OTUs (table 3).

**Discussion**

Microbe-mineral interactions influence weathering and mineral cycling and provide an important link between geology and the biosphere (Fenchel et al., 1988; Lovely, 2000). Free energy calculations of redox disequilibria indicate that reduction and oxidation of magnetite, hematite, and pyrite coupled with electron donors and acceptors present in Yellowstone hot springs are can proceed to equilibrium with a net release of energy via numerous pathways (Figure 4). Variation along the x-axis is a product of the disparity in chemical composition between pools.

Use of Fe(III), including Fe(III) in common minerals, as a terminal electron acceptor in energy metabolism is a widespread and highly conserved feature among thermophilic microorganisms.

...
Table 3. Suspended cell densities, surface colonization densities, and operational taxonomic units (OTUs) detected by T-RFLP analysis for seven Yellowstone hot springs. (N/A= data not available; --- = not detected).

<table>
<thead>
<tr>
<th></th>
<th>Temp. (°C)</th>
<th>pH</th>
<th>H₂O cell density (cells/ml)</th>
<th>Surface Colonization Density (cells/mm²)</th>
<th>OTUs Detected</th>
</tr>
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<td>5.65</td>
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<td>N/A</td>
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<td>5.02</td>
<td>1.56*10⁶</td>
<td>3.21*10⁴</td>
<td>9.82*10¹</td>
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Table 4. Terminal fragment lengths in base pairs (bp) of the 15 unique alleles detected from Hha1 cut 16S rDNA genes isolated from mineral colonizing bacterial communities. Each allele represents an operational taxonomic unit (OTU).

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<td>5</td>
<td>67</td>
<td>10</td>
<td>280</td>
<td>15</td>
<td>404</td>
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(Bridge and Johnson, 1998; Johnson and McGinness, 1991; Kashefi et al., 2002; Kashefi and Lovely, 2000; Vargas et al., 1998). Fe(II) mineral oxidizing bacteria, although not as well characterized, are also recognized in a variety of hydrothermal habitats (Bridge and Johnson, 1998; Brock et al., 1976; Edwards et al., 2004; Edwards et al., 2003a). The energy metabolism of a solid requires specialized electron transfer machinery such as excreted chelating agents or membrane-bound electron transfer proteins (Newman, 2001). While dissimilatory iron-metabolizing organisms have been isolated in Yellowstone it is unknown whether they have the requisite biochemical capacity to take advantage of iron in specific mineral forms.

A series of questions must be answered positively in order to demonstrate that microorganisms are using a solid substrate in energy metabolism: 1) Are microorganisms colonizing the surface in question? 2) Is colonization surface-specific? 3) Is colonization influenced by the metabolic characteristics of the substrate? 4) Are surface microorganisms influencing the rate of mineral transformation? 5) Are microorganisms conserving energy from the reaction of the mineral’s transformation? The present study provides some answers to the first three questions, and suggests strategies for answering the final two.

Are microorganisms colonizing the surfaces in question?

For all hydrothermal environments studied, the answer in emphatically ‘yes’. Bacteria frequently adhere to solid surfaces via secreted extracellular polymers, the association affording protection, access to nutrients, and/or a favorable geochemical microenvironment (Marshall, 1999). It is unlikely in these turbulent environments that
the high cell densities observed on some surfaces resulted from planktonic organisms settling out of suspension. Cell densities in the water column appear to vary with pH, with subneutral springs supporting densities one to two orders of magnitude higher than acidic and alkaline springs (Figure 6a). Surface cell densities do not follow this pattern and show little correspondence to suspended cell densities (Figure 6b). Bison and Lobster Claw stand out as environments with high mineral colonization densities that are not predicted by the low concentrations of cells in the water column. In contrast, OB1-heim surface have disproportionately low densities for the concentration of suspended cells.

*Is colonization surface-specific?*

While colonization is necessary for energy metabolism of a mineral substrate colonization doesn’t necessarily imply metabolic use (Crundwell, 2003). As observed on glass slides microbes will adhere to any solid substrate (figure 6b). However, if microbial colonization were indiscriminate with regards to the substrate we would expect to see identical cell densities on all surfaces within the same pool. In fact, we see surface-specific colonization in every environment studied- variations of two orders of magnitude between magnetite, hematite, and glass slides were observed in some pools. Glass slide density is fairly consistent between pools suggesting that the controls on inert surface colonization differ little between pools.

Surface-specific colonization patterns were also observed in community biodiversity. Distinct assemblages of OTUs were detected on magnetite and hematite in every environment studied. Distinctions between hematite and magnetite-colonizing communities are also apparent from morphotype descriptions. The lack of correlation
between OTU and morphotype number has several causes: DAPI stains the DNA of both Bacteria and Archea, while T-RFLP analysis was limited to Bacteria by use of a domain-specific PCR primer; many taxa have identical morphologies and can assume different morphologies depending on environmental conditions (Zinder and Dworkin, 2001). This underscores the need for DNA-based biodiversity measurements to complement microscopic observations.

*Is colonization influenced by the metabolic characteristics of the substrate?*

Microbes are known to selectively colonize the surfaces of minerals that contain essential nutrients or metabolic substrates (Rogers and Bennett, 2004). Initial attachment of suspended cells is a random process, however, bacteria attached to surfaces grow and reproduce in place (Marshall, 1999); organisms that find themselves in a favorable environment will thrive and proliferate. High densities would therefore be expected on minerals serving as energy sources.

In OB1-heim and Boulder springs, where the glass slide showed the highest colonization density (Figure 6b), microbial density is clearly not correlated with energy richness of the substrate. Why glass should be a more favorable surface in these environments is unknown. Boulder surfaces do show notable differences in community composition (Tables 2 & 3), but densities are low enough that this could result from chance colonization by suspended cells.

Based on free energy predictions magnetite should be the most favorable substrate in every environment, and since it presents energy sources for both iron oxidizers and reducers, should host a higher diversity of colonizers. Two springs, Sylvan and Figure 8,
showed the highest cell density on magnetite. Both T-RFLP data and morphotypes confirm that Figure 8 has high community diversity, and a single taxon, OTU8, is unique to Figure 8 magnetite. Bison has lower overall diversity, but two taxa, OTU3 and OTU6, are present only on Bison magnetite.

Hematite hosted the highest density in two springs, Sylvan and Lobster Claw. The reduction of hematite is most favorable in acidic environments (Figure 4c). Lobster Claw is therefore the spring most likely to host hematite metabolizing organisms. Magnetite is still however, a much richer energy source in both environments, so higher cell densities and Lobster Claw’s increased biodiversity on hematite remain a mystery. It is possible that the crystal structure of hematite make Fe$^{3+}$ more accessible to biological molecules or that hematite presents a higher concentration of Fe$^{3+}$ to dissimilatory iron reducers.

*Are surface microorganisms influencing the rate of mineral transformation?*

Microbes can facilitate mineral transformations either indirectly, by changing local environmental parameters such as pH and ionic strength, or directly, by dispatching a biological oxidant or reductant to garner energy from the reaction of a mineral’s transformation (Crundwell, 2003). A useful method for assessing the impact of biological activity on mineral transformation rate is sealed bottle incubations. A known quantity of mineral in a sealed vessel is innoculated with either live or poisoned hot spring water and incubated at environmental temperatures. Chemical compositions are measure before, during, and after the incubation. Many possible magnetite and pyrite oxidation reactions involve the transformation of magnetite into another insoluble phase (Figure 4a), therefore scanning electron microscopy and XRD analysis of minerals following
incubation should be used to identify solid products of microbe-mediated transformation (Dong et al., 2000; Edwards et al., 2003a).

*Are microorganisms conserving energy from the reaction of the mineral’s transformation?*

Although high densities of microorganisms adhering to some minerals suggests that they are deriving physiological benefit from the association, it is impossible to determine by observation alone whether this is because of the mineral’s metabolic energy yield or other properties. Since the biological activities can passively enhance mineral transformation by changing microenvironmental chemistry (Crundwell, 2003), laboratory culture under controlled conditions is essential for determining whether candidate organisms can support growth and proliferation by catalyzing mineral transformations. Eventual laboratory culture of candidate organisms will also be essential for determining which pathways shown in Figure 4 are being employed by microorganisms and the relationship between pathways employed and native geochemical environment.

**Conclusions and Future Directions**

Pursuing the rate measurement and culturing studies just described is warranted based on the results of the microbial “fishing expedition” reported here. Mineral-specific densities and community compositions suggest that iron mineral metabolizing bacteria are active in Yellowstone hydrothermal environments. T-RFLP data indicate that they are potentially taxonomically diverse and variable in their distribution: some groups (ex. OTU #s 5, 10, 15) are widespread among pools while many have a more restricted
distribution. Based on dense mineral-specific colonization and presence of multiple unique OTUs I suggest Bison and Lobster Claw, two springs with distinct geochemistries (see appendix), as promising sites for further investigation and possible isolation of novel mineral metabolizing microbes. Cell counts and T-RFLP analyses for pyrite, and T-RFLP analysis of colonizing communities on an inert surface should complement the current study.

“Baiting” is a useful tool for applying theoretical predictions to the study of natural microbial communities. The broad application of predictive microbiology techniques will link the study of geochemical and biological mineral cycling and enhance our understanding of hydrothermal systems as complex ecosystems.
Acknowledgements

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References


Kashefi, K., and Lovely, D. R., 2000, Reduction of Fe(III), Mn(IV), and Toxic Metals at 100°C by Pyrobaculum islandicum: Applied and Environmental Microbiology, v. 66, p. 1050-1056.


Gradient Gel Electrophoresis: Applied and Environmental Microbiology, v. 65, no. 8, p. 3518-3525.


Appendix

Figure 1. Chemical characteristics of hot springs used for \textit{in situ} incubations. Measurements were made in the field and in subsequent laboratory analyses by GEOPIG in 2003. Alkalinity is reported as mg/kg CaCO$_3$. All other concentrations are reported in ppm.

<table>
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<th></th>
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<th>Conductivity (µS)</th>
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<th>NO$_2^-$</th>
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