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EXPLORING BIOTIC IRON TRANSFORMATION BY THE
HYPERTHERMOPHILIC ARCHAEON *PYROBACULUM*
ISLANDICUM

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This paper was prepared
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For Captain Thomas H. Lenagh

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ABSTRACT

Hyperthermophilic microorganisms, those that grow optimally above 80°C, have evolved unique methods of thriving in extreme environments. There are many diverse organisms that have the ability to reduce metals such as iron, yet little is known about how organisms, especially hyperthermophiles, utilize it. *Pyrobaculum islandicum* is a hyperthermophilic archaeon isolated from a continental hydrothermal system. *P. islandicum* grows optimally at 100°C, is an obligate anaerobe, and has the ability to reduce Fe(III) in an energy-generating metabolic process known as dissimilatory iron reduction. Unlike mesophilic model organisms (optimal growth between 20° and 50°C), *P. islandicum* does not have any known homologs of polyheme *c*-type cytochromes in its genome and requires direct attachment to iron for reduction to occur. This suggests that the metabolic pathway for dissimilatory iron reduction in *P. islandicum* is novel and warrants further investigation. In this study, *P. islandicum* was grown heterotrophically in Fe(III) media. We investigated iron transformation by determining growth rates of the organism, performing ferrozine assays to measure Fe(II) production, determining iron species by various spectroscopic methods, and visualizing iron-cell contact by electron microscopy. By studying iron reduction pathways in novel organisms, we will better understand the complexities of geothermal environments, a largely unstudied natural resource.

INTRODUCTION

Prior to the 1970s, environments that were considered too extreme to support life were ignored with respect to studying microorganisms. Studies over the last three decades have shown that these extreme environments host a vast number of microorganisms (Huber et al., 2000). With improvements in culture conditions and the breakdown of an anthropocentric view on the limits of life, extremophile research has shown that such environments not only contain, but also uniquely sustain a plethora of novel organisms. Extremophiles are classified depending on their optimal growth conditions; such as thermophiles (temperature between 50° and 80°C), hyperthermophiles (>80°C), psychrophiles (<20°C), acidophiles (pH<4), alkalophiles (pH>9), halophiles (salinity >4.0 M NaCl), and barophiles/piezophiles (pressure >500 atm) (Stetter, 1999).

Hyperthermophiles

The most extreme organisms that have been studied are the hyperthermophiles ('superheat-loving') that grow optimally at temperatures between 80° and 106°C (Table 1), temperatures at which

Table 1 –Basic features of hyperthermophiles (type species). *Pyrobaculum islandicum*, the organism used in this study, is enclosed in the box. Table adapted from Stetter (1999).

Species	Growth Conditions						Morphology
	Temperature (°C)			pH	ae, an	m, t	
	min	optimal	max				
<i>Thermotoga maritima</i>	55	80	90	5.5-9	an	m	Rods with sheath
<i>Aquifex pyrophilus</i>	67	85	95	5.4-7.5	ae	m	Rods
<i>Sulfolobus acidocaldarius</i>	60	75	85	1-5	ae	t	Lobed cocci
<i>Thermoproteus tenax</i>	70	88	97	2.5-6	an	t	Regular rods
<i>Pyrobaculum islandicum</i>	74	100	103	5-9	an	t	Regular rods
<i>Pyrobaculum aerophilum</i>	75	100	104	5.8-9	ae/an	m	Regular rods
<i>Desulfurococcus mobilis</i>	70	85	95	4.5-7	an	m	Cocci
<i>Sulfophobococcus zilligii</i>	70	85	95	6.5-8.5	an	t	Cocci
<i>Thermotoga maritimus</i>	75	88	98	5-7	an	m	Disks
<i>Igneococcus islandicus</i>	65	90	100	3.9-6.3	an	m	Irregular cocci
<i>Hyperthermus butylicus</i>	80	101	108	7	an	m	Lobed cocci
<i>Pyrolobus fumarii</i>	90	106	113	4.0-6.5	ae/an	m	Lobed cocci
<i>Pyrococcus furiosus</i>	70	100	105	5-9	an	m	Cocci
<i>Methanothermus sociabilis</i>	65	88	97	5.5-7.5	an	t	Rods in clusters
<i>Acidiamus infermus</i>	60	88	95	1.5-5	ae/an	t	Lobed cocci
<i>Stygiolobus azoricus</i>	57	80	89	1-5.5	an	t	Lobed cocci
<i>Ferroglobis placidus</i>	65	85	95	6-8.5	an	m	Irregular cocci
<i>Methanopyrus kandleri</i>	84	98	110	5.5-7	an	m	Rods in chains
<i>Thermococcus celer</i>	75	87	93	4-7	an	m	Cocci
<i>Aeropyrum pernix</i>	70	90	100	5-9	ae	m	Irregular cocci
<i>Mettalosphaera sedula</i>	50	75	80	1-4.5	ae	t	Cocci

ae— aerobic, an— anaerobic, m— marine, t— terrestrial

small biomolecules are known to rapidly degrade. Hyperthermophiles have extended the known upper temperature limit of life to 122°C. This has been achieved by a hyperthermophilic methanogen known as *Methanopyrus kandleri* (Takai et al., 2008). Some genera, such as *Pyrolobus*, that achieve optimal growth at 113°C, thrive at such high temperatures that they are capable of surviving autoclaving (Stetter, 1999). Due in part to comparisons of 16S rRNA sequences and the ability of hyperthermophiles to inhabit such a wide variety of ecosystems, it is thought that hyperthermophiles are perhaps the most primitive life forms on Earth (Huber et al., 2000).

Hyperthermophilic Environments

Hyperthermophiles are commonly found in hot terrestrial and submarine environments. There are, at present, about 90 species of hyperthermophilic archaea and bacteria that have been isolated from all over the world. Hyperthermophiles form communities in geothermally- and volcanically-heated aqueous environments at areas of sea-floor spreading, such as the Mid-Atlantic Ridge. These biotopes are essentially oxygen-free (anaerobic) due to the low solubility of O₂ at high temperatures and the presence of reducing gasses such as H₂S (Huber et

al., 2000). Natural terrestrial biotopes are mainly hot springs and solfataric fields, which generally have low salinities and a wide range of pH values. Solfataric fields are commonly found close to active volcanoes and consist of mud holes and surface waters heated by subsurface volcanic activity, such as those found at Yellowstone National Park. Chemical compositions of these fields vary from site to site, but many are rich in iron minerals such as ferric hydroxides (Stetter, 2006). Hyperthermophile communities are also found in submarine biotopes and, most impressively, in deep-sea black smoker vents where steep temperature gradients create areas within rock chimneys that support massive amounts of hyperthermophilic growth (Stetter, 2006).

Characteristics of Hyperthermophiles

Hyperthermophiles are small organisms (in the micrometer range) that are different from otherwise similar mesophilic organisms because all the cellular components of hyperthermophiles must be heat resistant (Stetter, 1999). Ester-linked lipids, which make up the cell membrane in mesophilic organisms, are known to increase in fluidity and permeability as temperature increases, allowing small molecules and ions to escape at high temperatures. Archaeal membranes contain ether-linked lipids that

are derived from diphytanyl-glycerol and show significant resistance and stability at high temperatures (Charlier and Droogmans, 2005). In order to protect the DNA double helix, hyperthermophiles possess an enzyme known as reverse gyrase, a type I DNA topoisomerase, that supercoils the DNA helix and is unique to hyperthermophiles (Confalonieri et al., 1993). Archaeal hyperthermophiles also possess histones, which are special proteins that bind to DNA to prevent melting by forming compact structures that seem to resemble eukaryotic nucleosomes (Musgrave et al., 1991). Because of the structural similarities between archaeal and eukaryal histone complexes, it has been suggested that eukaryal histones evolved from the histones found in archaea (Sandman and Reeve, 2000). The secondary structure of the DNA is further stabilized by having three hydrogen bonds between base pairs caused by the addition of GC base pairs (Charlier and Droogmans, 2005). Purified enzymes also show remarkable heat resistance *in vitro*. However, a complete understanding of the stabilizing ability of hyperthermophilic proteins will most likely have to wait until three-dimensional structural comparison and analysis are possible between hyperthermophilic proteins and homologous mesophilic

proteins. This suggests that the upper temperature limit of life is set by the stability of biomolecules at high temperatures (Stetter, 1999).

The ability to thrive at high temperatures is the distinctive physiological property of hyperthermophiles. Within these hot ecosystems, hyperthermophiles use an array of energy sources and utilize both aerobic and anaerobic respiration. Most hyperthermophiles are chemolithotrophic; that is, they use inorganic redox reactions as energy sources (Figure 1) and some are also capable of growing mixotrophically and/or heterotrophically (Stetter, 2006). During anaerobic respiration, hyperthermophiles are capable of nitrate-, sulfate-, sulfur-, carbon dioxide-, and ferric iron respirations. Heterotrophic hyperthermophiles use organic molecules whenever they are available in the environment and gain energy through aerobic respiration, the different types of anaerobic respiration, fermentation, or by using organic material as electron donors (Stetter, 1999).

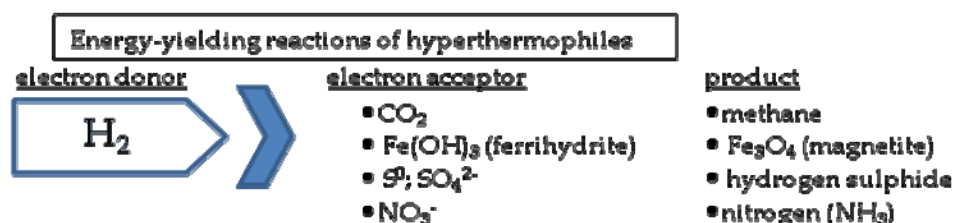


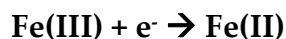
Figure 1—Simplified schematic of the energy-yielding reactions in chemolithotrophic hyperthermophiles. Figure adapted from Stetter (2006).

Microbial Metal Reduction

Microbial reduction and oxidation of metals have been identified as significant biogeochemical processes but the exact mechanisms by which they occur are still unknown (Weber et al., 2006). Microbial encounters with metals in the environment are unavoidable and include countless transition elements and metalloids including vanadium, chromium, manganese, iron, cobalt, nickel, copper, zinc, molybdenum, cadmium, lead, the precious metals gold and silver, and the metalloids arsenic, selenium, and antimony. These exist as cations, as oxyanions, in crystalline (mineral) form, or in an amorphous insoluble form. Archaea and bacteria often employ metal species that exist in more than one oxidation state (Cr, Mn, Fe, Co, Cu, As, and Se) as electron donors or acceptors in their metabolism. Only prokaryotes are capable of reducing

Mn(IV), Fe(III), and Co(III) on a large scale while conserving energy in the reaction (Ehrlich, 1997).

Thermophilic microorganisms have been shown to use many inorganic electron acceptors during metabolism; however the ability of hyperthermophiles to reduce metals has been demonstrated only in the last two decades (Slobodkin et al., 1999). Metal reduction can fulfill various functions in hyperthermophilic metabolism. These include energy generation (dissimilatory reduction), biosynthetic functions (assimilatory reduction), detoxification functions, or no definite function (nonspecific reduction) (Slobodkin, 2005). These processes are not mutually exclusive. For example, detoxification reactions can sometimes take place via dissimilatory reduction. Dissimilatory reduction involves far greater quantities of metals than any other reduction process. Many mineral compounds that contain metal used in reduction are insoluble or poorly-crystalline so organisms have adapted unique strategies of accessing these minerals (Slobodkin, 2005).

Insoluble Fe(III) Reduction

Equation 1

Fe(III) is the most abundant available terminal electron acceptor in many subsurface and hyperthermophilic environments (Luu and Ramsay, 2003). The ability of thermophiles to reduce trivalent iron was first described in 1976 for *Sulfolobus acidocaldarius* (Brock and Gustafson, 1976). Since then, a variety of microbes including thermophiles and hyperthermophiles, has been shown to use insoluble Fe(III) compounds as terminal electron acceptors in dissimilatory reduction during anaerobic growth (Figure 2). Prior to exploration of iron-reducing microorganisms, it was thought that Fe(III) reduction was entirely the result of abiotic processes such as changes in pH and/or redox potential (Luu and Ramsay, 2003). It is now understood that microbial respiration with Fe(III) oxides as terminal electron acceptors is a common process in anaerobic environments. Electrons from an organism's electron transport chain are deposited on an Fe(III) molecule, reducing it to Fe(II) (Luu and Ramsay, 2003).

One unique aspect of Fe(III) is that it is capable of forming different oxides and hydroxides, each with a distinctive crystal structure as seen in

Figure 3. These various crystal structures have different kinetic and thermodynamic properties, even when the chemical formulas are the same (Nealson and Saffarini, 1994). Particle size and surface area have been implicated in controlling the bioavailability of Fe(III) oxides for microbial reduction. The more crystalline an Fe(III) oxide, the less available it is to reduction by microorganisms (Roden and Zachara, 1996).

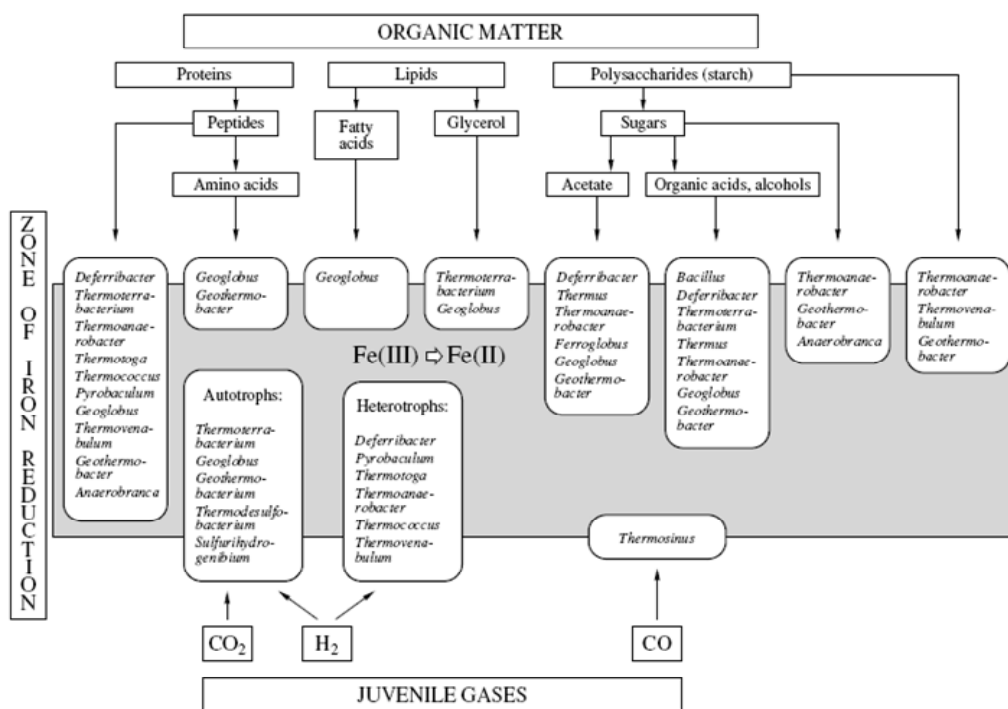


Figure 2—Utilization of organic and inorganic compounds as electron donors during the reduction of Fe(III) by thermophilic prokaryotes. Figure taken from Slobodkin (2005).

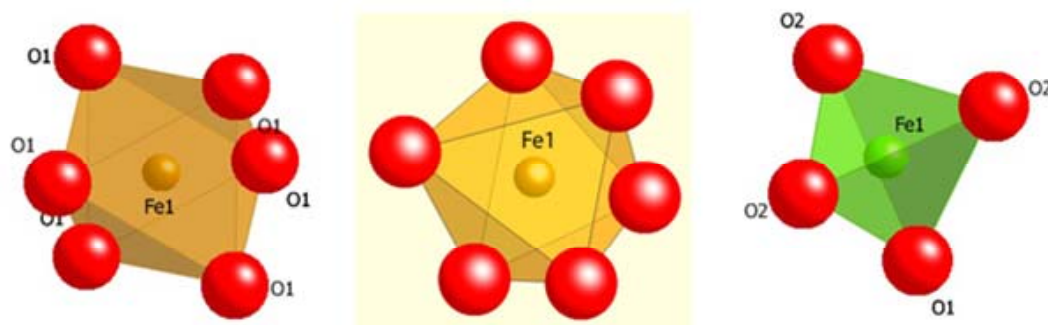


Figure 3—Polyhedra surrounding the iron atoms in the magnetite (Fe₃O₄), ferrihydrite (Fe(OH)₃), and maghemite (Fe₂O₃) crystal structures (from left to right). All of these are possible iron oxides/hydroxides that could be present in our experimental media.

There are three known methods of dissimilatory Fe(III) reduction: direct contact, production of an iron chelator, and the use of an electron shuttle (Figure 4). Thus far, the only successful studies of Fe(III) reduction pathways are from mesophilic bacteria, specifically from the genera *Geobacter* and *Shewanella*. These organisms contain multiple polyheme *c*-type cytochromes in their outer membranes and/or membrane-associated ferric reductases (Nevin and Lovely, 2002). Some archaea have polyheme cytochrome homologs in their genomes or have membrane-associated ferric reductases, although the pathways of these enzymes in archaea have yet to be successfully studied. Some archaea capable of dissimilatory Fe(III) reduction lack homologs for either of these pathways in their

genomes, giving rise to the notion that novel methods exist for dissimilatory Fe(III) reduction.

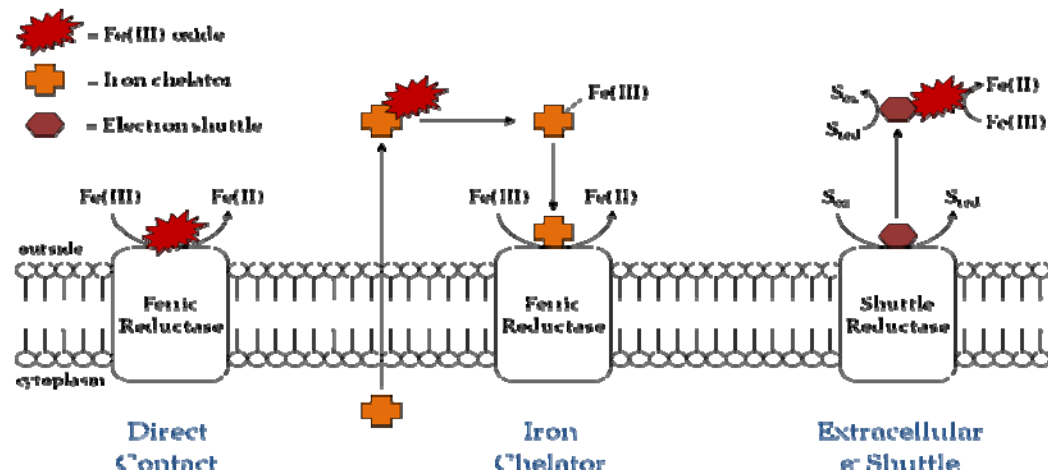


Figure 4—Methods of dissimilatory Fe(III) reduction (Holden)

Phylogenetic Relation of Hyperthermophiles

Based on the work of Carl Woese, small subunit ribosomal RNA was used as a basis to propose a new classification system of organisms that are contained, not in the traditional five kingdoms, but in three overarching domains: Archaea, Bacteria, and Eukaryota. This system was proposed to more accurately represent biodiversity, most notably in prokaryotes that were divided into separate domains (Woese et al., 1990).

Originally, the novel domain Archaea served as a category for microorganisms that did not neatly fit into the other domains; specifically

extremophiles. Today, these classifications rely heavily on differences found in the rRNA sequences of various species and are represented as a phylogenetic tree (Figure 5). Hyperthermophiles occupy the short, deep

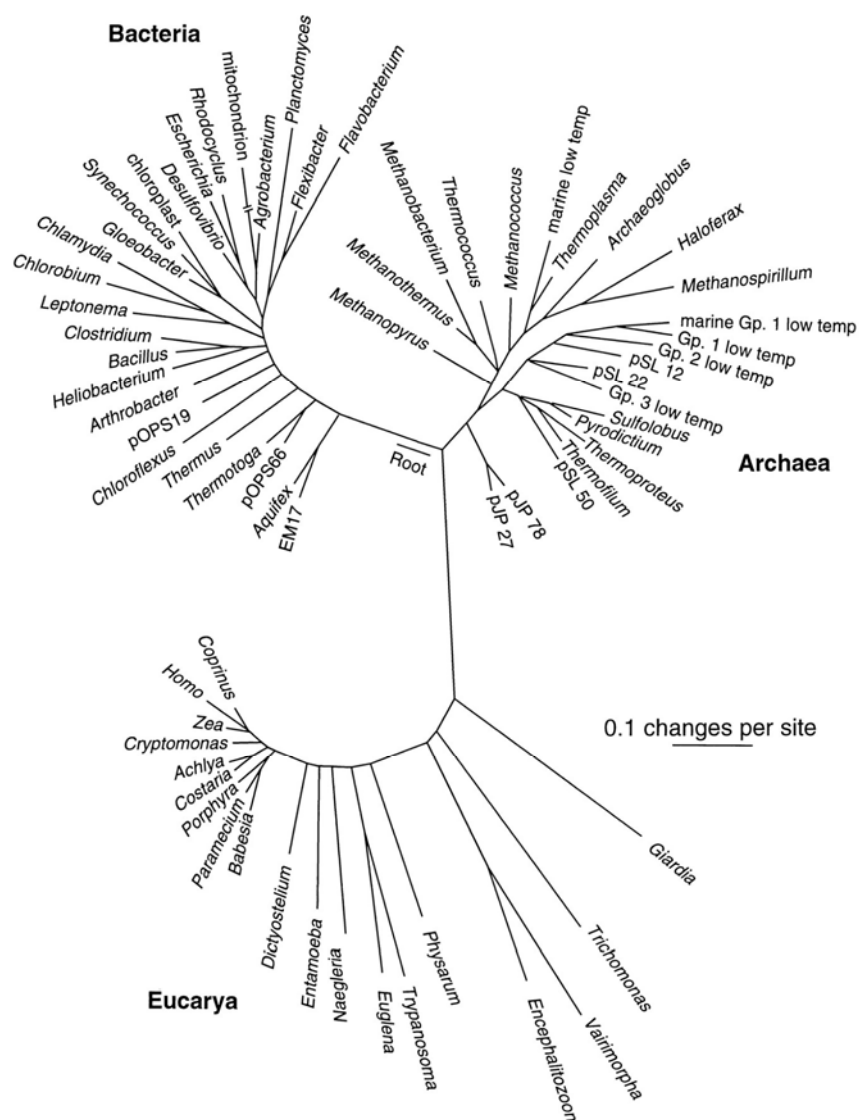


Figure 5—Universal phylogenetic tree with the domains Bacteria, Archaea, and Eucarya based on the 16S rRNA work of Woese et al. (1990). Figure taken from Pace (1997).

branches of the phylogenetic tree, signifying two things. First, these branches hold organisms that grow at the highest temperatures. Second, the shortness of these branches signifies that the organisms are slow to evolve and may be most closely related to the last common ancestor (Stetter, 1999; Vargas et al., 1998).

Currently there are around 90 known species of hyperthermophilic archaea and bacteria that have been isolated from various terrestrial and marine thermal systems throughout the world. Hyperthermophiles are generally divergent in both phylogeny and physiological properties and, at present, are grouped into 34 genera and 10 orders (Stetter, 2006).

The Hyperthermophilic Archaeon *Pyrobaculum islandicum*

Prior to isolation of *Pyrobaculum islandicum*, hyperthermophiles had exclusively been isolated from submarine hydrothermal systems. In 1987, Huber et al. described the isolation of seven strains of rod-shaped continental hyperthermophilic archaebacteria growing optimally at 100°C, obtained from a geothermal power plant and in solfatara fields in the Azores, Iceland, and Italy. All seven of the isolates were described as a new genus, *Pyrobaculum*, meaning “fire stick.” Within this genus, two new

species were noted, one of which was named *organotrophum*, which means “feeding on organic material,” and the other was named *islandicum*, meaning “Icelandic” as a description of its place of isolation (Huber et al., 1987).

Characteristics of *Pyrobaculum islandicum*

P. islandicum is a hyperthermophilic archaeon isolated from a geothermal power plant in Krafla, Iceland. Morphologically, *P. islandicum* appears as rod-shaped, nearly rectangular cells about 1.5 to 8 µm long and 0.5 µm wide with more than 80% of the cells being 2.5 µm in length. *P. islandicum* shows bipolar polytrichous flagellation with up to three flagella about 13 nm in diameter and up to 15 µm long (Figure 6). *P. islandicum* isolates do not contain muramic acid, an indication that their cell walls lack murein and they contain isopranyl ether lipids characteristic of archaea. Optimal growth is achieved at 100°C and at pH 6.8, but *P. islandicum* is capable of growing within a temperature range from 72° to 102°C and a pH range of 5 to 9 (Huber et al., 1987; Feinberg et al., 2008).

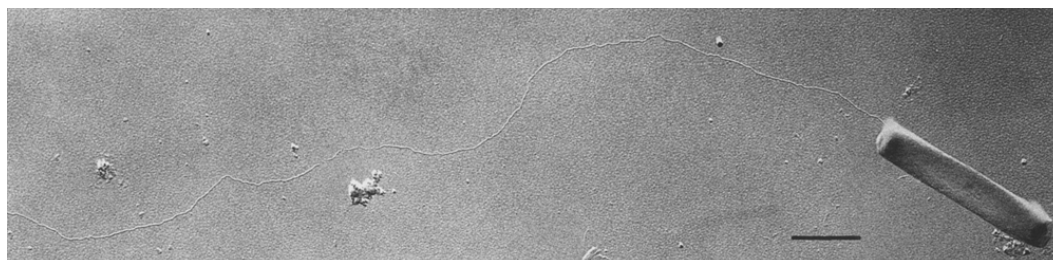


Figure 6—Electron micrograph of *Pyrobaculum islandicum* showing one polar flagellum. Bar: 1 μ m (Huber et al., 1987).

Metabolism of *Pyrobaculum islandicum*

P. islandicum is an obligate anaerobe that uses Fe(III), $\text{S}_2\text{O}_3^{2-}$, and S^0 as terminal electron acceptors during heterotrophic growth. It is also a facultative autotroph and grows mixotrophically and autotrophically with acetate and hydrogen (Hu and Holden, 2006). *P. islandicum* has also been shown to reduce U(VI), Tc(VII), Cr(VI), Co(III), and Mn(IV) with hydrogen as the electron donor (Kashefi and Lovley, 2000). In this study, *P. islandicum* is grown heterotrophically on Fe(III). Unlike other iron-reducing hyperthermophiles, *P. islandicum* uses both soluble Fe(III) citrate and insoluble Fe(III) oxide hydroxide as electron acceptors in dissimilatory iron reduction. *P. islandicum* requires direct contact with Fe(III) for growth and yields a magnetic Fe(II) end product (Feinberg et al., 2008). When grown heterotrophically in laboratory conditions, the organic compounds casein hydrolysate (Difco) and yeast extract (Difco)

are added, although the concentration necessary is remarkably low (0.001% yeast extract). This suggests that heterotrophic growth could occur naturally when such compounds were available in their environments, e.g., from decaying cell mass (Hu and Holden, 2006; Stetter 2006).

Fe(III) Reduction in *Pyrobaculum islandicum*

The mechanisms for Fe(III) reduction in *P. islandicum* are significantly different from the models for electron transport in the mesophilic bacteria *Geobacter* and *Shewanella* (Childers and Lovely, 2001). Table 2 outlines the differences between the different organisms. Childers and Lovely (2001) characterized a ferric reductase in *P. islandicum* that differs in location from the ferric reductases studied in mesophilic model organisms. Specifically, bacterial reductases are membrane-associated whereas activity in *P. islandicum* is localized in the cytoplasm. The most significant difference between *P. islandicum* and other well-studied dissimilatory Fe(III) reducers is the lack of polyheme *c*-type cytochromes. Although there is a presence of a *b*-type cytochrome, it has not been found to be critical for Fe(III) reduction activity (Childers and Lovely, 2001). This leads to the conclusion that phylogenetically diverse Fe(III) reducing

microbes utilize different methods for Fe(III) reduction. Studying Fe(III) reduction in organisms such as *P. islandicum* aids in our understanding of the various processes that occur in extreme or otherwise inaccessible environments.

Table 2—Fe(III) reduction comparison between the mesophilic model organisms *Geobacter* and *Shewanella* and the organisms used in this study, *Pyrobaculum*.

Characteristics	<i>Geobacter</i>	<i>Shewanella</i>	<i>Pyrobaculum</i>
Domain	Bacteria	Bacteria	Archaea
Growth conditions	Mesophile	Mesophile	Hyperthermophile
Cytochromes	Multiple polyheme c-type	Multiple polyheme c-type	Few monoheme c-type (hypothetical)
Conductive Pili	Yes	Sometimes	No
Requires Direct Contact with Fe(III)	Yes	No	Some species yes, others no
Ferric Reductase	Membrane Bound	Membrane Bound	Soluble (possibly)

Project Goal

The ways in which microorganisms use Fe(III) as a terminal electron acceptor and the mineral transformations that occur during this process have yet to be completely understood, especially in hyperthermophiles. Iron reduction by hyperthermophilic archaea, especially that of *P. islandicum*, represents a novel system for Fe(III) reduction that differs in many respects from the two most common

organismal models for microbial iron reduction studies, *Geobacter* and *Shewanella*. By determining the mechanisms behind iron reduction at high temperatures by hyperthermophilic archaea and gaining a fuller understanding of their interactions with metals in their environments, the processes that regulate the reactions at the cellular level can be modeled.

My project included measuring growth rates, using Mössbauer spectroscopy to characterize the species of Fe(III) used in media and the various species of Fe(III) and Fe(II) present during and after growth of *P. islandicum*, using colorimetric assays to assess and quantify Fe(II) production, and visualizing iron-cell contact by scanning electron microscopy. All of this was done to determine the metal transformations that occur during Fe(III) respiration by *P. islandicum* as well as the rates and constraints on these processes.

Understanding the ability of hyperthermophiles to reduce metals in the environment has implications in bioremediation of toxic metals and metalloids in environments hostile to mesophilic organisms. Geochemical evidence also points to Fe(III) reduction as one of the earliest forms of microbial metabolism. Studying this metabolic pathway will bring us closer to characterizing processes that occur in geothermal environments,

and help us better understand what life on early Earth may have been like. This information could also lead us to finding biosignatures in rock records. If these biosignatures are easily recognizable, they could lead us to finding evidence of life on other terrestrial bodies, where geothermal environments exist or may have existed; such as under the martian permafrost layer or on the moon Europa.

MATERIALS AND METHODS

Pyrobaculum islandicum Growth Medium

P. islandicum medium was generally prepared just prior to inoculation. 100mL of medium contained the following:

Table 3—contents of *P. islandicum* growth medium

With Fe(III)	Per 100 ml
dH ₂ O	79 ml
10X <i>P. islandicum</i> salts	10 ml
DL Vitamins	1 ml
1000X <i>P. islandicum</i> elements	0.1 ml
10mM Na-Tungstate	10 µl
Yeast Extract	0.02 g
Tryptone	0.05 g
Iron Types: 1 of the following	
Fe Gel	10 ml
Fe Powder	35 mg
Phosphate Buffer	100 µl

When the pH of the medium was 6.8, the phosphate buffer was then added to maintain the pH. Then 10 ml of medium were aliquoted into Balch tubes and sealed with butyl rubber stoppers. These were placed on a vacuum manifold for 15 minutes and the headspace was flushed with argon gas. The tubes were placed upright in a 95°C incubator for 1 hour to be sterilized. After being removed from the oven, 0.1 ml of 50 mM

cysteine and 0.05 ml of 0.26 M FeCl_2 were added to each tube. After the addition of cysteine and FeCl_2 , the tubes were inoculated with inoculums from a previous culture.

Growth Curves

All of the tubes for each growth curve were inoculated with a 2.5% inoculum of a logarithmic-growth-phase culture that had been grown for >24 hours at 95°C on the same medium. The inoculated tubes were placed upright in an incubator at 95°C. Two tubes were removed from the incubator every 4 hours to determine the cell concentration at that time point. A third set of tubes was not inoculated and functioned as an abiotic control. One of these un-inoculated tubes was also pulled every 4 hours. Cell concentrations were calculated by cell counts performed after Acridine Orange staining. The specific growth rate (k) was determined by a best-fit curve through the logarithmic portion of the cell growth data.

Acridine Orange and Cell Counting

Acridine Orange (AO) is a fluorescent dye with an excitation wavelength of 490 nm and an emission wavelength of 526 nm. Cultures sat in a 1:1 solution of oxalate. After 1 hr, the culture/oxalate mixture was

pipetted onto 0.2 μm filters sitting under steel columns and two drops of AO were added. After 3 min, the filters were washed with oxalate and sterilized DI water. The columns were removed and the filters were placed onto slides that had been pre-treated with oil. Another drop of oil was added on top of the filter and a coverslip was placed on top of each slide. All slides were stored in the dark until they were used.

Using a high power mercury bulb, slides were viewed under fluorescent light and cells were counted in 15-25 different views at 100x and averaged for each slide.

Ferrozine Assay

A ferrozine assay is a colorimetric assay that measures the amount of Fe(II) in cultured cells. This was performed using an oxalate extraction technique, in which 0.1 ml of sample were added to 1.9 ml of degassed oxalate solution in a 5 ml serum bottle flushed with argon. After approximately 1 hr in this solution, 0.1 ml of the solution was added to 4.9 ml of ferrozine solution. The absorbance of the solution was read at 562 nm in a spectrophotometer. A standard curve was constructed by adding known concentrations (0 mM, 0.625 mM, 1.25 mM, 2.50 mM) of Fe(II) to 1.9 ml of degassed oxalate.

Scanning Electron Microscopy

Using a 1 ml syringe, 0.5 ml of suspension were taken from the anaerobic medium and, after carefully removing the needle, the syringe was attached to a Pop-Top™ Filter Holder that contained a 0.2 µm Nuclepore® polycarbonate membrane filter. The suspension was gently pushed through the filter until it was well-coated. The syringe was filled with 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 6.8 as a fixative. Then 3.5 ml were slowly and continuously pushed through the filter for 45 min. The filter was washed with 0.1 M phosphate buffer 3 times for 5 min each. The filter was then dehydrated in a graded series of ethanol. Each filter was immersed two times for 5 min in 30%, 50%, 70%, 90%, 95%, and three times in 100%. At 90%, the filters were removed from the syringes and placed in the holder for the critical point dryer. These were layered as follows: washer, filter with suspension, clean filter, metal screen, washer, etc. After the third immersion in 100% ethanol, the samples were placed in a Tousimis Samdri-780B critical point dryer and dried in liquid CO₂.

Using a Polaron 5100 Sputter Coater the samples were coated in gold for 75 s at a voltage of 2.5 kV and at a pressure of 0.07 mbar. Samples were viewed at Mount Holyoke College in an FEI Instruments Quanta 200 "environmental" scanning electron microscope and at the University of Massachusetts in a Carl Zeiss EVO 50 scanning electron microscope in high vacuum ($<10^{-4}$ torr) mode.

Fourier Transform Infrared (FTIR) Spectroscopy

Fourier Transform Infrared (FTIR) spectroscopy is an experimental technique for determining mineral identification. FTIR spectroscopy relies on detection of molecular vibrations (stretching and bending) between atoms in a crystal structure. This technique utilizes the spectral range from 1000 nm ($10,000\text{ cm}^{-1}$) up to 100,000 nm (100 cm^{-1}) in the far-infrared. IR spectra are acquired on a Fourier Transform Infrared (FTIR) spectrometer. These instruments measure two interferograms, one of light passing through the sample and the other without the sample. The light beam passes through both the sample and the reference, while the wavelength is changed. A computer analyzes the interferograms and determines what wavelengths were removed (i.e., absorbed) by the sample, and will show

up as absorption bands in the resulting spectrum.

For this project, samples of reacted and unreacted media were analyzed by FTIR at Stony Brook University at Laboratory for Infrared Spectroscopy (LIRS) facility, which includes a Thermo Fisher Nicolet 6700 FTIR spectrometer modified to also collect emissivity spectra in an environment purged of water vapor and CO₂. Measurements from 400-4000 cm⁻¹ were carried out using a KBr beamsplitter and a deuterated L-alanine doped triglycine sulfate (DLaTGS) detector with a KBr window. Data were matched to spectra of iron oxides in the collections of Dr. Glotch, as described in Glotch and Rossman (2009).

Mössbauer Spectroscopy of Samples

A thorough review of Mössbauer effect and spectroscopy can be found in the Appendix.

Variable temperature Mössbauer spectra were acquired on each sample under low He gas pressure at multiple temperatures ranging from 4-295K. A source of 100-70mCi ⁵⁷Co in Rh was used on a WEB Research Co. model W100 spectrometer equipped with a Janus closed-cycle He cooling system. Each sample was run for 2-24 hr and the results were

calibrated against an α -Fe foil. Typical count rates were between 500,000 and 900,000 non-resonant counts/hour.

Mössbauer Sample Preparation

P. islandicum growth medium, both prior to and post inoculation, was dried in a partially covered petri dish in the anaerobic hood for 3-7 days. The dried medium was removed from the hood and carefully scraped using a Scoopula™ into weigh paper. The sample amounts varied between 25-50 mg. Using an iron-free agate mortar and pestle (SPI Supplies), the samples were ground into a fine powder and mixed with sugar. Each sample was then loaded into 34 mm diameter plastic washers. The washer is sealed with an adhesive plastic cap on one side and thermal Kaptan tape on the other. The grinding of the sample and the addition of sugar assure random orientation in the sample and thin the sample to allow for the best data quality.

Mössbauer Fitting Procedures

The program used to fit paramagnetic spectra (unsplit spectra) is a subset of a number of programs developed by Eddy De Grave and Antoine van Alboom (Gent, Belgium) known as Mexfieldd.

“Mexfieldd uses Lorentzian line shapes to fit doublets with a fixed area ratio of 1:1 for the peaks. It solves the full Hamiltonian to determine single quadrupole splitting values (this is distinguished from other programs where distributions are found for one or more parameters). Other variables are isomer shift and width. Best fits are determined by minimizing the chi squared (χ^2) value. See Grant (1995) for a full description of how this is done. Comparison fits for some of our spectra were performed using Disd3e_dd, a program that uses velocity approximations instead of solving full Hamiltonians to obtain values for isomer shift and quadrupole splitting. It searches for a distribution of quadrupole splitting values, rather than the single value sought in Mexdisdd. Quadrupole splitting distributions provide a non-Lorentzian lineshape (the lineshape is a sum of Voight lines) that has proved more correct for fitting certain types of Mössbauer spectra (Rancourt 1994). Magnetically split spectra were fit using Mexdisdd, which solves the full Hamiltonian to obtain values for quadrupole splitting, isomer shift, and magnetic field, but provides a distribution of values for the magnetic field parameter, similar to the quadrupole splitting distributions mentioned above” (Sklute, 2006).

Most bulk iron oxides of interest in this project are magnetic with sextets in their spectra, particularly at low temperatures where magnetic ordering occurs. The Mössbauer parameters of bulk iron oxides are in Table 4. However, as described in the appendix, nanoparticles of iron oxides may instead have spectra that are doublets at room temperature because they behave as superparamagnets. It is possible that the iron oxides metabolized by *P. islandicum* might have extremely small grain sizes due to the small surface area available to the cell. If this is the case,

then the magnetite may have parameters typical of nanophase iron oxides as shown in Table 5.

Table 4—Selected iron oxide minerals, their formulas, and Mössbauer characteristics.

Mineral	Formula	Space	295K			4K		T _N , T _C (K)
		Group	B _{Hf}	δ	Δ	B _{Hf}	δ	
Goethite	α-FeOOH	Pnma	38.0	0.37	-0.26	50.6	-0.25	400
Akaganéite	β-FeOOH	I2/m	--	0.38	0.55	47.3	-0.81	299
				0.37	0.95	47.8	-0.24	
						48.9	-0.02	
Lepidocrocite	γ-FeOOH	Bbmm	--	0.37	0.53	45.8	0.02	77
Feroxyhite	δ'-FeOOH	P3 _{barm} 1	41.0	0.37	-0.06	53	~0.0	450
						52	~0.0	
Ferrihydrite	~Fe(OH) ₃	--	--	0.35	0.62	50	-0.07	115
			--	0.35	0.78	47	-0.02	25
Hematite	α-Fe ₂ O ₃	R3 _{bar} C	51.8	0.37	-0.20	53.5	-0.20	955
						or 54.2	0.41	
Magnetite	Fe ₃ O ₄	Fd3m	49.2	0.26	≤0.02	50.6	0.00	850
			46.1	0.67	≤0.02	36- 52	1.18--0.79	
Maghemite	γ-Fe ₂ O ₃	Fd3m	50.0	0.23	≤0.02	52.0	≤0.02	~950
		or P422	50.0	0.35	≤0.02	53.0	≤0.02	

Adapted from Murad and Cashion (2004). "B_{Hf} is the hyperfine splitting in T, and refers to the orientation of the nuclear magnetic moment (spin $I \geq \frac{1}{2}$) in an effective magnetic field that arises from magnetic ordering. δ is the shift up or down of nuclear levels that result from overlap of nuclear and s-electron charge distributions. Δ refers to the quadrupole splitting of (or quadrupole shift of already split) nuclear levels, and describes the orientation of the nuclear quadrupole moment (spin $I \geq 1$) in a distribution of electronic charge lacking spherical or cubic symmetry (electric field gradient ≠ 0). T_N and T_C are the temperatures at which each mineral will magnetically order (switching from a doublet to a sextet); T_N is the Néel temperature and T_C is the Curie temperature. The combination of these hyperfine interactions, which result from interactions between the nucleus and its surrounding electrons that lead to changes in the nuclear (and electronic) energy levels, provides information that enables characterization of the host material." See Dyar et al. (2006) for thorough discussion of these parameters (Sklute, 2006).

Table 5—Mössbauer parameters for nanophase magnetite as reported in the literature at room temperature.

Isomer Shift δ (mm/s)	Quadrupole Splitting Δ (mm/s)	Particle Size (nm)	Reference
0.34	0.78	--	Barkatt et al., (2009)
0.20	0.71	5	Woo et al., (2004)
0.05	0.70	11	
0.36	0.88	20	Fellenz et al., (2006)
0.20	0.77	--	Surowiec et al., (2008)
0.20	0.48	--	
0.38	0.75	6	Mikhaylova et al., (2004)
0.22	0.60	4	Goya et al., (2003)
0.22	0.14	--	Murad and Cashion, (2004)
0.27	0.58	10	Franger et al., (2007)*
0.22	0.74	80	Morales et al., (2010)*

*Mössbauer parameters estimated from plots in paper

RESULTS

Growth Rates and Conditions

We first measured the growth rates of *P. islandicum* and the respective production of Fe(II) using ferrihydrite, Fe(III) oxide, as an electron acceptor at 95°C. Both cell concentration and Fe(II) concentration increased over time (Table 6).

Table 6—*P. islandicum* cell concentration and Fe(II) concentration over time.

Time (hrs)	Number of cells per mL	Fe(II) Concentration (millimoles)
4	1.57E+06	3.7326
4	1.04E+06	5.961
8	3.15E+06	4.8468
8	2.25E+06	10.279
12	7.81E+06	7.5627
12	6.89E+06	2.6184
16	6.84E+06	12.646
16	1.29E+07	6.2396
20	9.67E+06	7.5627
20	1.11E+07	8.7465
24	1.33E+07	7.9805
24	1.33E+07	8.8162
32	1.65E+07	10.488
32	1.55E+07	10.557

P. islandicum reached stationary phase at approximately 22 hours of growth on ferrihydrite gel and death phase after the 32 hours. We used

the four time points to measure the cell concentration over time and found the doubling time to be 3.9 hours (Figure 7).

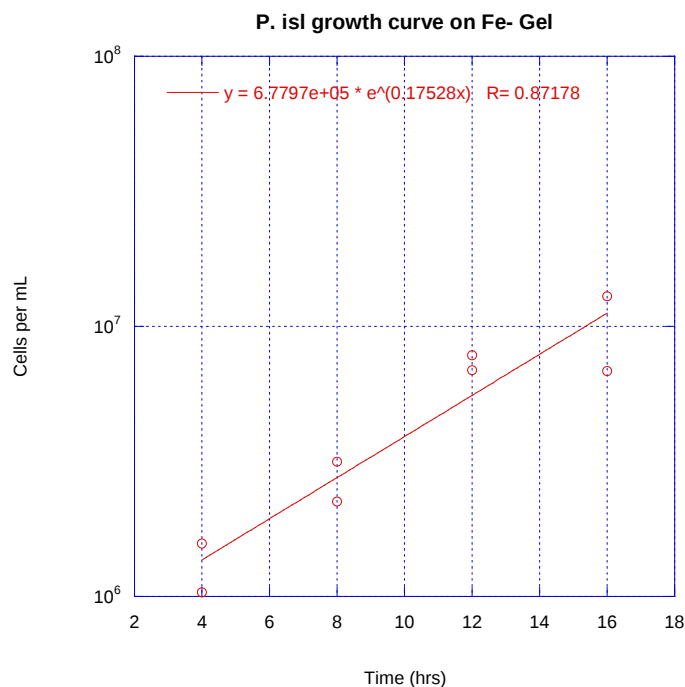


Figure 7—*P. islandicum* cell concentration over time.

The Fe(II) concentrations in the media were measured during growth at all time points and were found to increase with cell growth (Figure 8). Even after the cells have reached stationary phase (Figure 9), Fe(II) concentration continues to increase at the same rate as in exponential phase. It is possible that an abiotic reaction occurred and an uninoculated control should have been added. Additional experiments were designed to test this.

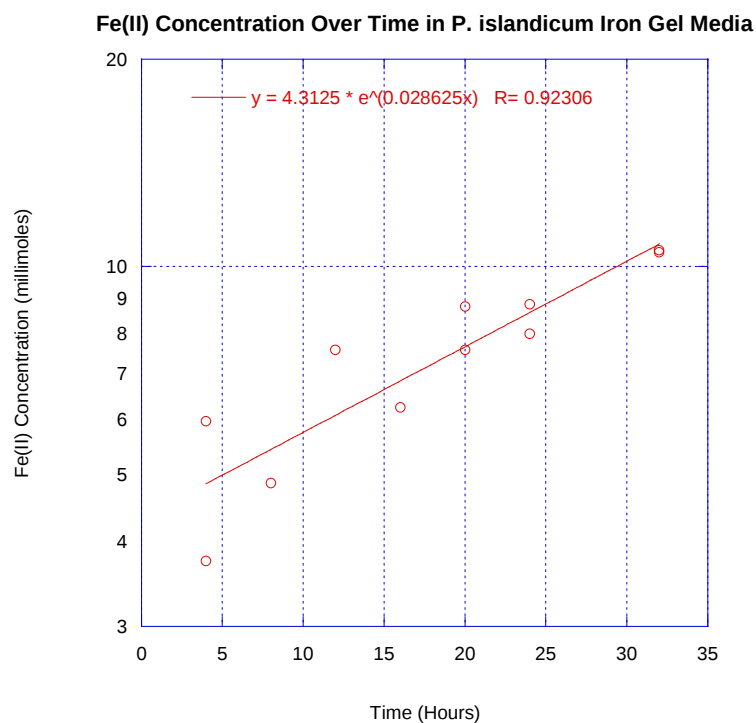


Figure 8—Fe(II) concentration in the media during *P. islandicum* growth.

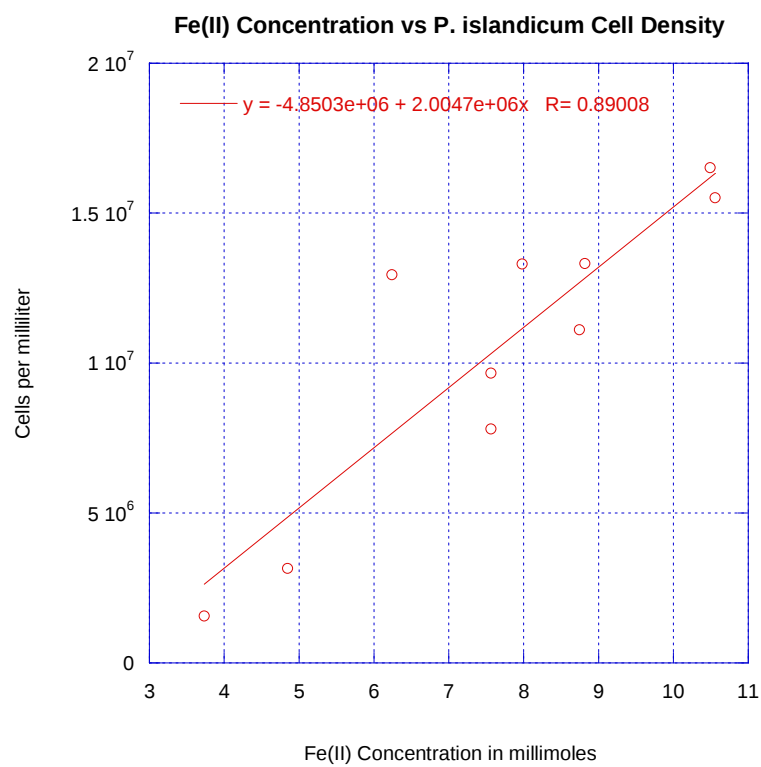


Figure 9—Fe(II) production as related to the cell concentration of *P. islandicum*.

In order to optimize Fe(II) production and cell growth, the amount of FeCl₂ was varied to determine the concentration of FeCl₂ necessary for cell growth and whether or not this is necessary in the media. We also used a third set of tubes; these were not inoculated and were used as a control for abiotic Fe(II) production. Cells had a faster doubling time and showed more growth when no FeCl₂ was added (Table 7 and Figure 10).

Table 7—Summary of data with varying concentrations of FeCl₂.

FeCl ₂	doubling time(hrs)	specific growth rate k =	highest cell concentration (cells mL ⁻¹)	highest Fe(II) concentration (mM)	Δ Fe(II) Concentration (mM) (growth – control)
2x	3.155	0.219	2.4100E+07	17.06	7.8
1x	2.55	0.271	4.2086E+07	12.5	5.55
0.5x	2.00	0.346	3.3772E+07	12.06	8.08
None	1.87	0.379	6.7254E+07	9.29	6.69

The greatest optimization of growth occurred when FeCl₂ was not added. Figure 11 shows the growth of *P. islandicum* over time with no FeCl₂. In Figure 12, the concentration of Fe(II) is shown over time in both the inoculated and the uninoculated control media and Figure 13 gives the Fe(II) production in relation to cell concentration.

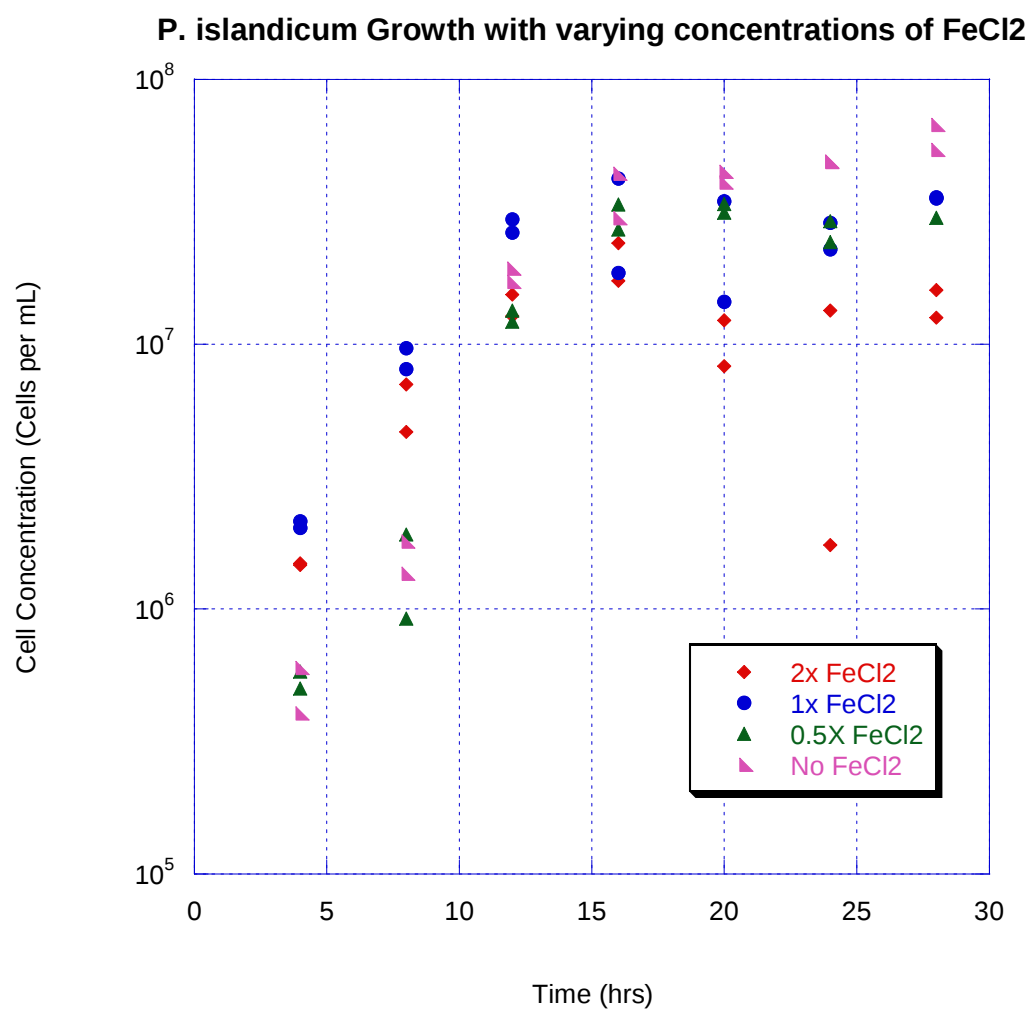


Figure 10—The growth of *P. islandicum* with varying concentrations of FeCl₂ added to the media.

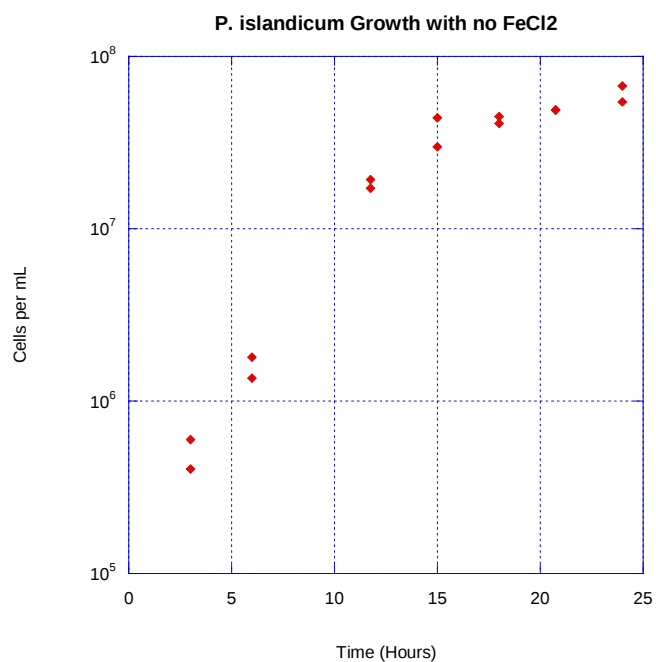


Figure 11—Growth of *P. islandicum* with no FeCl₂.

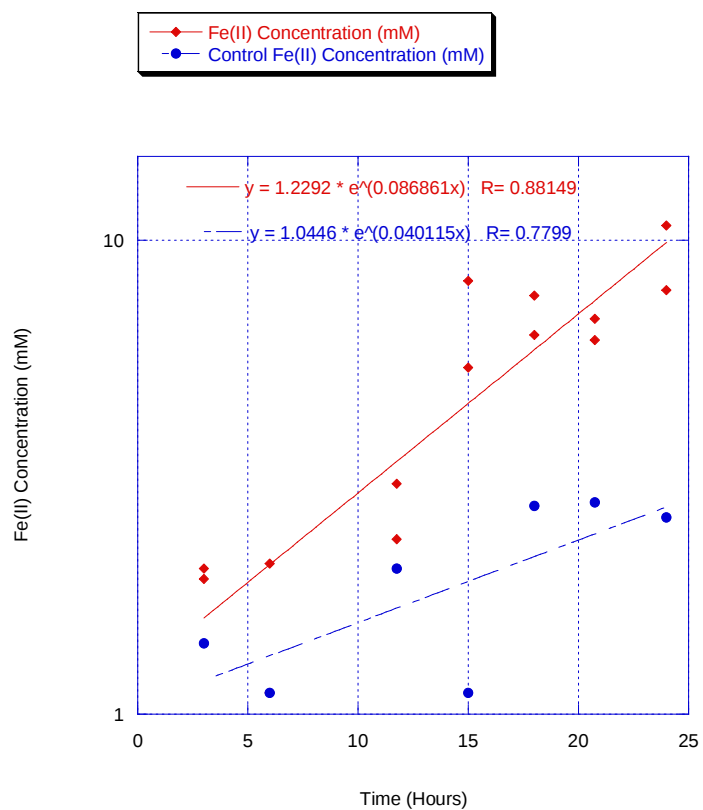


Figure 12—Fe(II) concentration over time in the inoculated (red) and uninoculated (blue) media with no FeCl₂.

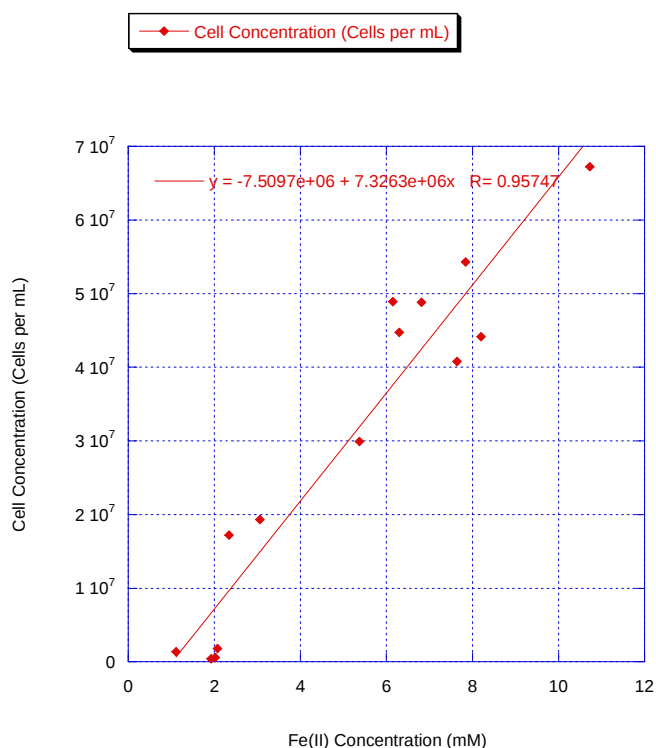


Figure 13—*P. islandicum* cell concentration in relation to Fe(II) concentration. As the cell concentration increases the concentration of Fe(II) increases.

P. islandicum was grown in media without FeCl₂ again. The tubes were left on the vacuum manifold for an hour rather than the typical 15 minutes (Table 8), in an attempt to remove the maximum amount of oxygen to prevent re-oxidation of Fe(II) produced. Figure 14 shows the growth of *P. islandicum* over time. In Figure 15 the concentration of Fe(II) is shown over time in both the inoculated and the uninoculated control media. Figure 16 gives the Fe(II) production in relation to cell concentration. As was the case in Figure 12, Figure 16 shows that there is

some abiotic Fe(II) production but it is lower than biotically mediated

Fe(II) production.

Table 8—Summary of growth curve with no FeCl₂ and left to vacuum for 1 hour.

time (hrs)	cell concentration (Cells mL ⁻¹)	ferrozine λ 562nm	Fe(II) concentration (mM)	Ferrozine λ 562nm of control	Fe(II) concentration (mM) control	Δ Fe(II) concentration (mM) (growth – control)
2.5	6.31E+05	0.009	0.71	0.014	0.944	-0.234
2.5	4.69E+05	0.009	0.71			
5.5	1.52E+06	0.042	2.28	0.005	0.52	1.165
5.5	1.28E+06	0.017	1.09			
8.5	3.77E+06	0.023	1.37	0.015	0.99	0.455
8.5	3.59E+06	0.026	1.52			
11.5	8.35E+06	0.052	2.76	0.006	0.56	1.7
11.5	2.75E+06	0.031	1.76			
17.5	1.68E+07	0.064	3.33	0.036	1.99	0.815
17.5	1.66E+07	0.042	2.28			
23.5	1.09E+07	0.066	3.43	0.071	3.66	0.365
23.5	1.74E+07	0.091	4.62			
27	1.75E+07	0.101	5.1	0.015	0.99	3.915
27	1.57E+07	0.093	4.71			

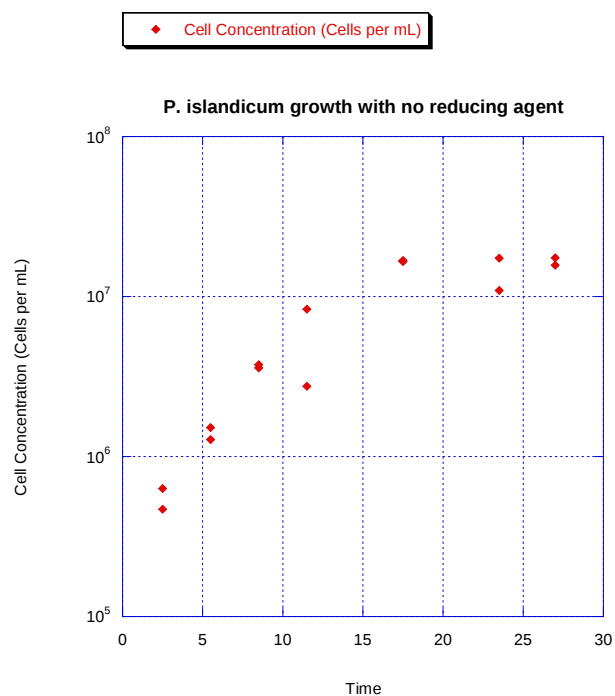


Figure 14—Growth of *P. islandicum* with no FeCl₂ added to the media and vacuumed for 1 hour.

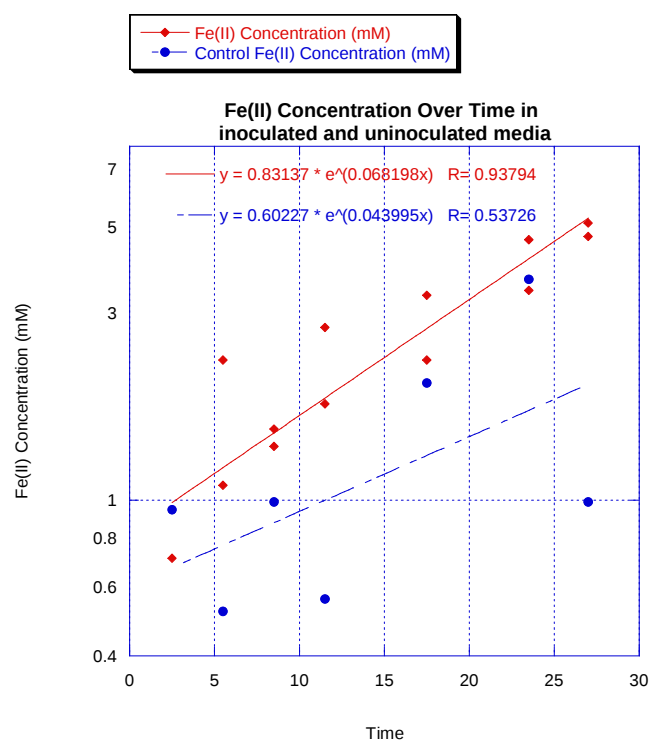


Figure 15-- Fe(II) concentration over time in the inoculated (red) and uninoculated (blue) media with no FeCl₂ and vacuumed for 1 hour.

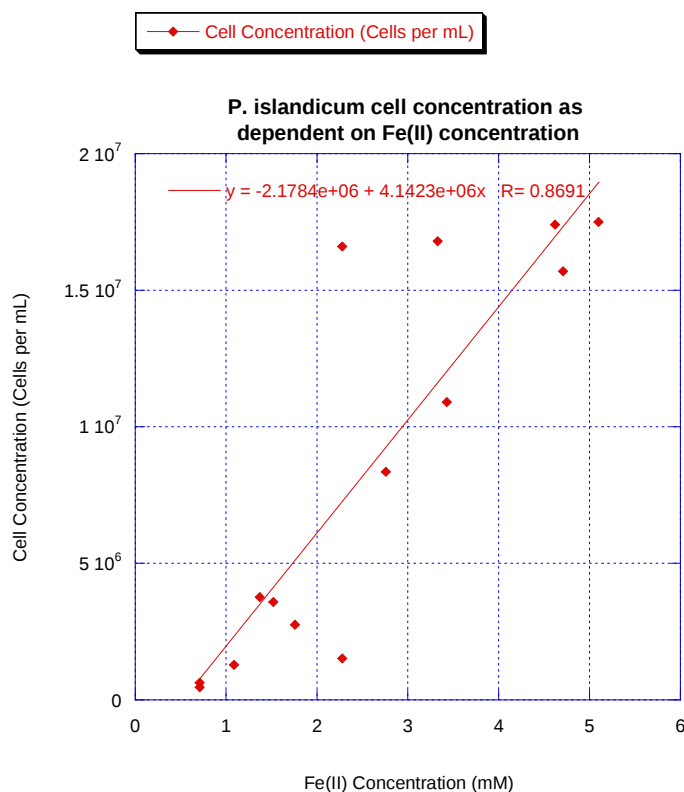


Figure 16—*P. islandicum* cell concentration in relation to Fe(II) concentration. As the cell concentration increases the concentration of Fe(II) increases.

Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) was used to visualize the cellular association with iron. We examined cells from two different time points from our growth curve: one from the tubes pulled during exponential phase and the other from tubes in stationary phase. Table 9 outlines basic image observations. For cells in the tubes pulled during exponential phase, the average length was 2.9 μ m and the average width

was $0.47\mu\text{m}$. The cells showed regular rectangular morphology, had smooth edges, and had small particles of iron on their surfaces (Figure 17 and Figure 18). The average length for cells in stationary phase was $7\mu\text{m}$ and the average width was $0.99\mu\text{m}$. All of the cells imaged in stationary phase were longer and appeared to be degrading based on their irregular edges and flaky surface area as well as the lack of definitive iron particles along their surfaces (Figure 17 and Figure 19).

Table 9—SEM image details

Figure	Phase	Length (μm)	Width (μm)	Original Mag (X)	Scale bar
17A, 18	exponential	3.4	0.55	59,270	200nm
17B	exponential	2.9	0.45	50,000	200nm
17C	exponential	2.4	0.40	60,000	200nm
17D, 19	stationary	7.5	1.0	20,000	$1\mu\text{m}$
17E	stationary	7.5	1.3	30,000	$1\mu\text{m}$
17F	stationary	6.0	0.67	30,000	$1\mu\text{m}$

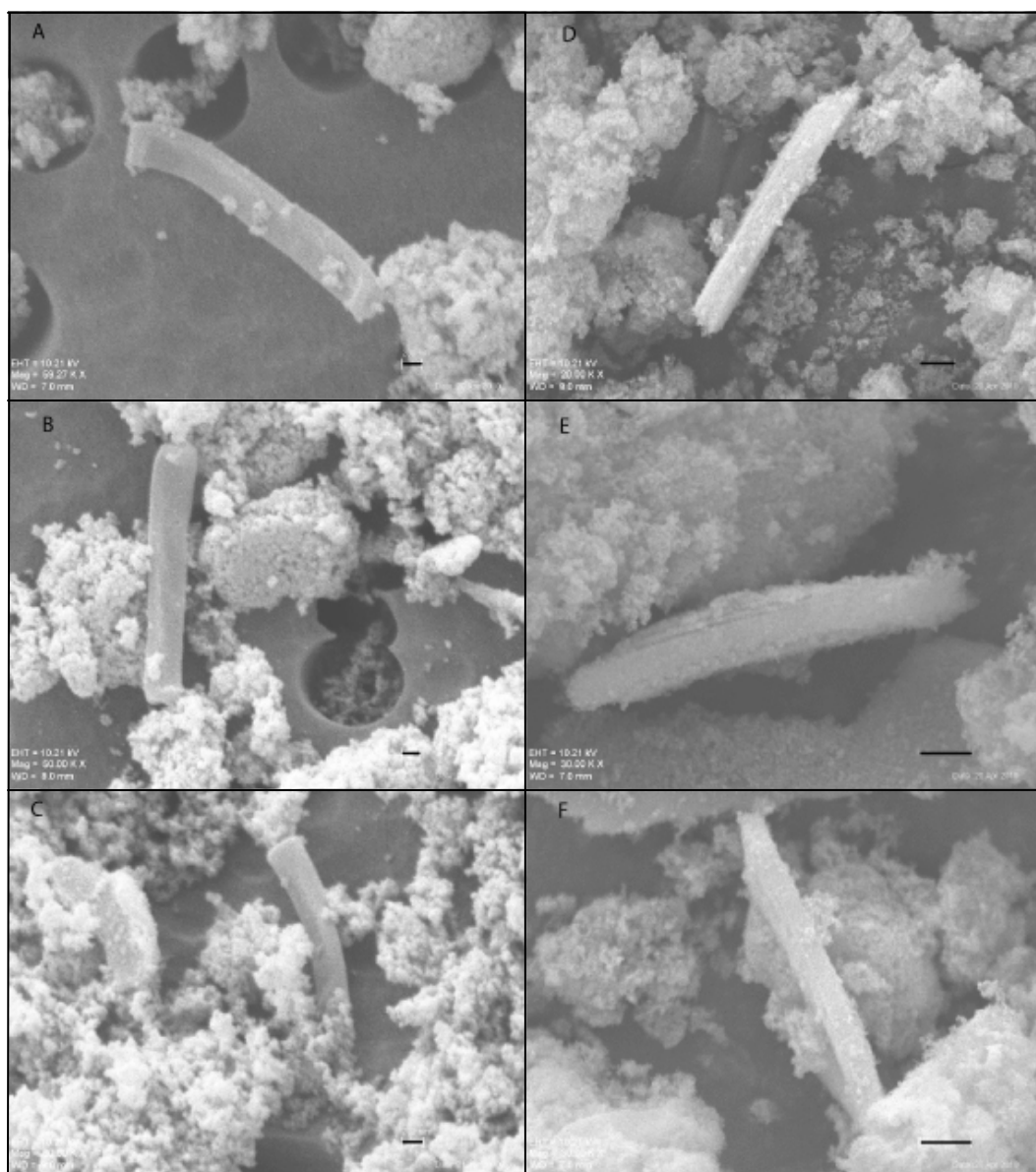


Figure 17—SEM micrographs of *P. islandicum*. A, B, and C were cells growing in exponential phase. These cells showed regular morphology, were between 2-3 μ m long, and had small iron particles on their surface (bar=200nm). The cells in D, E, and F were in stationary phase. These cells were elongated and appeared to have signs of degradation (bar=1 μ m).

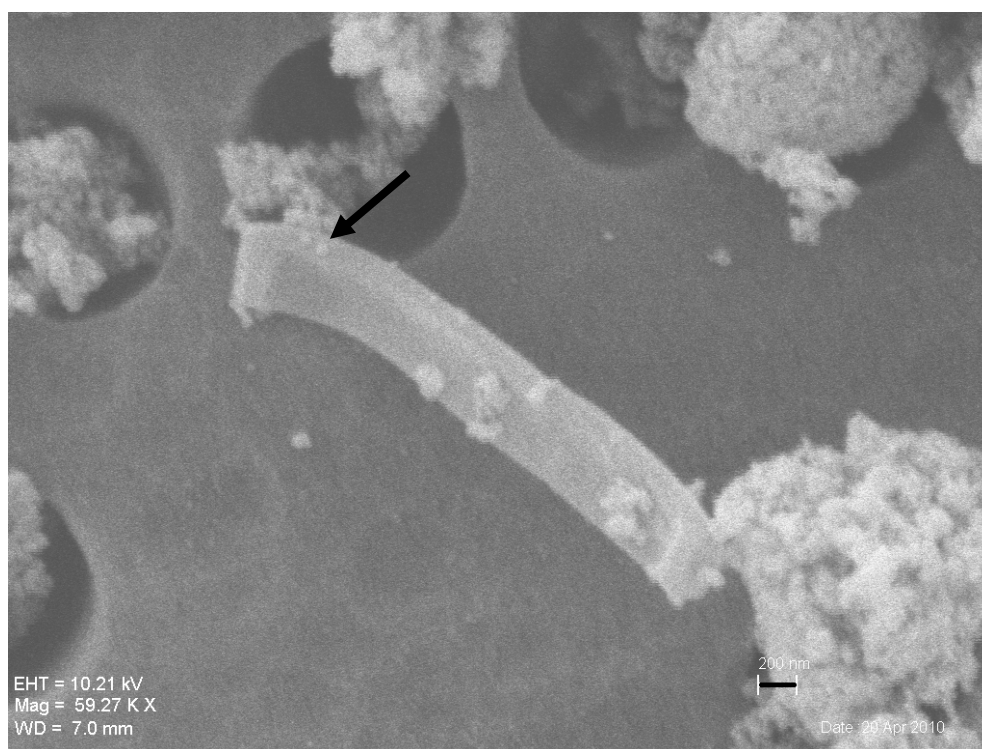


Figure 18—Micrograph of *P. islandicum* in exponential phase. Iron particles are clearly visible on the surface of the cell. Nanophase iron is indicated by the arrow (bar=200nm).

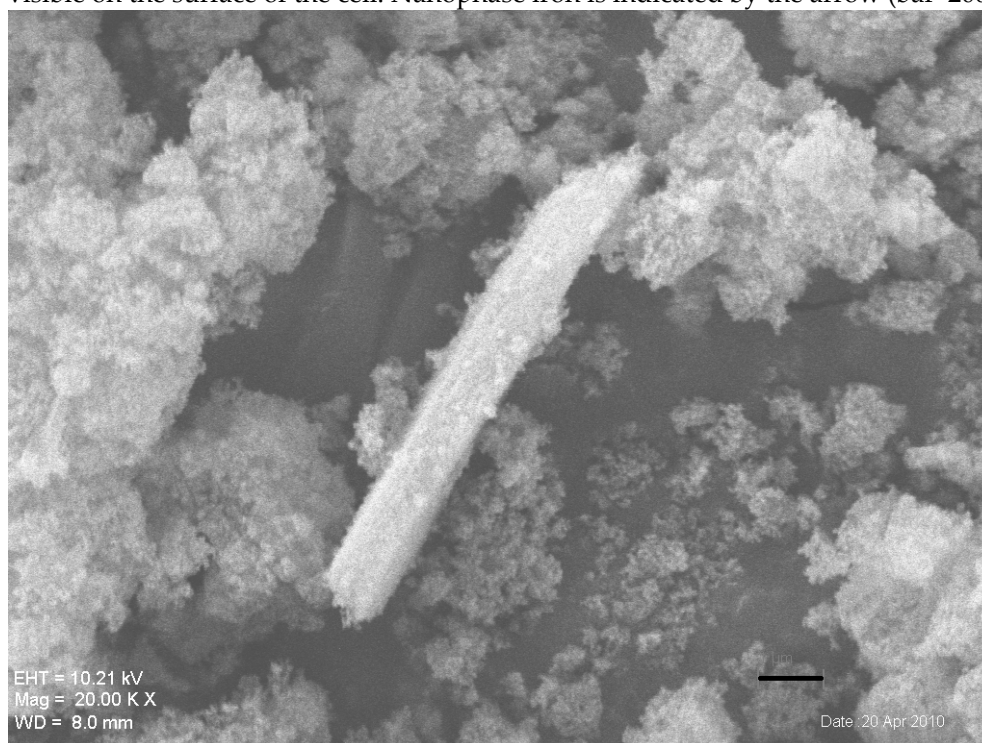


Figure 19—Micrograph of *P. islandicum* in stationary phase. Elongation, irregular edges and surface area are indications of cellular distress (bar=1μm).

Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectra were collected by Dr. Timothy Glotch at Stony Brook State University of New York. Figure 20 compares unreacted and reacted Fe to three lab standards.

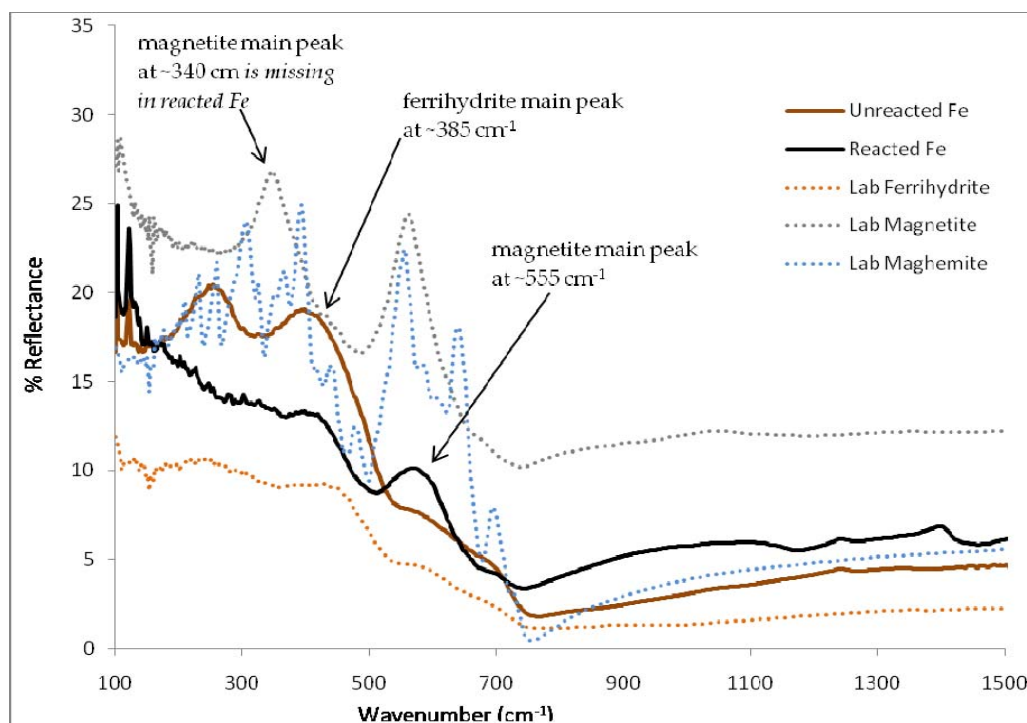


Figure 20—FTIR analysis of the unreacted Fe(III) gel used in *P. islandicum* medium (solid brown line) and the reacted, magnetic Fe (solid black line). These were compared to three lab Fe oxides. The main peak of the unreacted medium was found at 385cm⁻¹ which corresponds to ferrihydrite. The reacted medium showed a peak at 555cm⁻¹ which corresponds with magnetite, but was missing the second magnetite main peak at 340cm⁻¹.

Figure 21 shows a direct comparison of the unreacted Fe and the lab ferrihydrite and the spectra are quite similar, confirming the Mössbauer measurement (discussed later) that identified the Fe oxide in

the starting media to be ferrihydrite. The main peak is at 385cm^{-1} . The unit, cm^{-1} , is a reciprocal of wavelength and is proportional to the energy of the light. Figure 22 directly compares reacted magnetic Fe to lab magnetite. A peak exists at 555cm^{-1} but a second peak at 340cm^{-1} is missing in the reacted medium. This suggests that the reacted sample is an intermediate between ferrihydrite and magnetite, but it strongly supports the hypothesis that reduction is occurring.

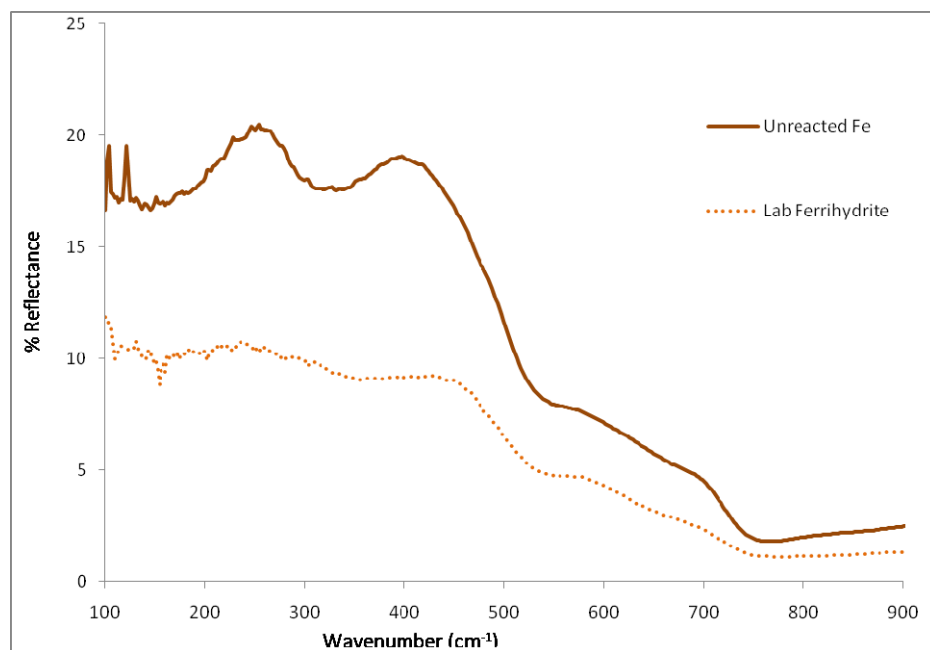


Figure 21 —FTIR spectrum directly comparing unreacted Fe to Lab ferrihydrite

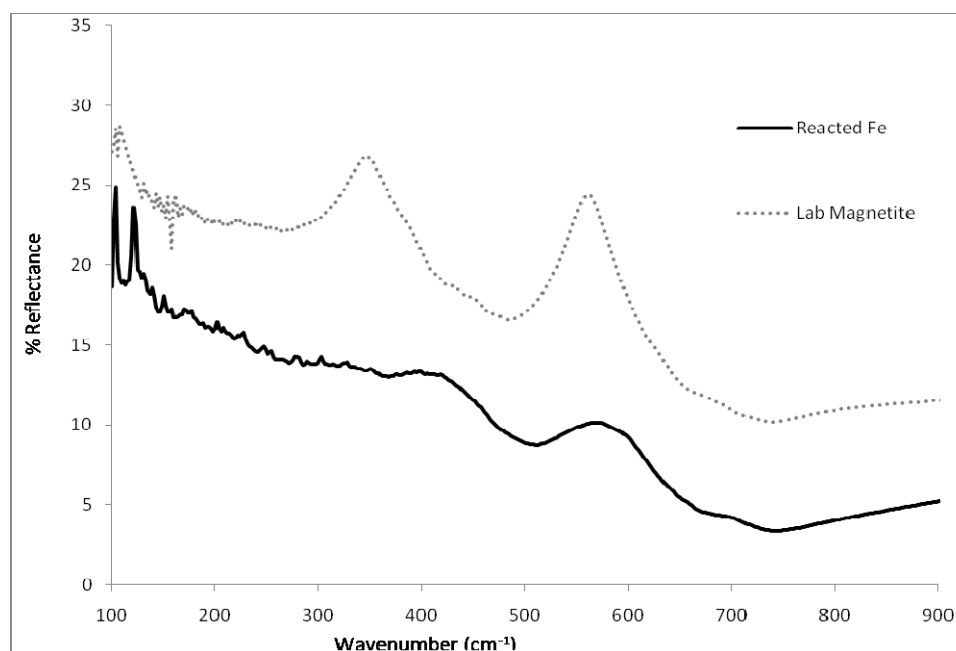


Figure 22—FTIR spectrum directly comparing reacted, magnetic Fe to Lab magnetite

Mössbauer Spectroscopy

The Mössbauer data presented here are part of a preliminary study of the transformations of the iron during *P. islandicum* growth. Spectra were acquired at a range of temperatures because different iron species magnetically order at different temperatures. Magnetic ordering is indicated in Mössbauer spectra by the presence of a sextet rather than a doublet. At 4K, both the unreacted Fe gel and the reacted medium are sextets (Figure 23). Magnetite, the most likely end product in spent *P. islandicum* media, magnetically orders at higher temperatures than ferrihydrite if it is present in large grain sized (i.e., >1000nm). Our 295K

spectra of the unreacted and reacted media show no evidence of the characteristic sextet from bulk magnetite. However, the reacted media at 295K does show both broadening of the central doublet and the onset of magnetic ordering. Closer examination of the reacted medium shows that splitting occurs near 175K while the unreacted medium has yet to split even at 150K (Figure 24). This may represent a contribution from nanophase magnetite.

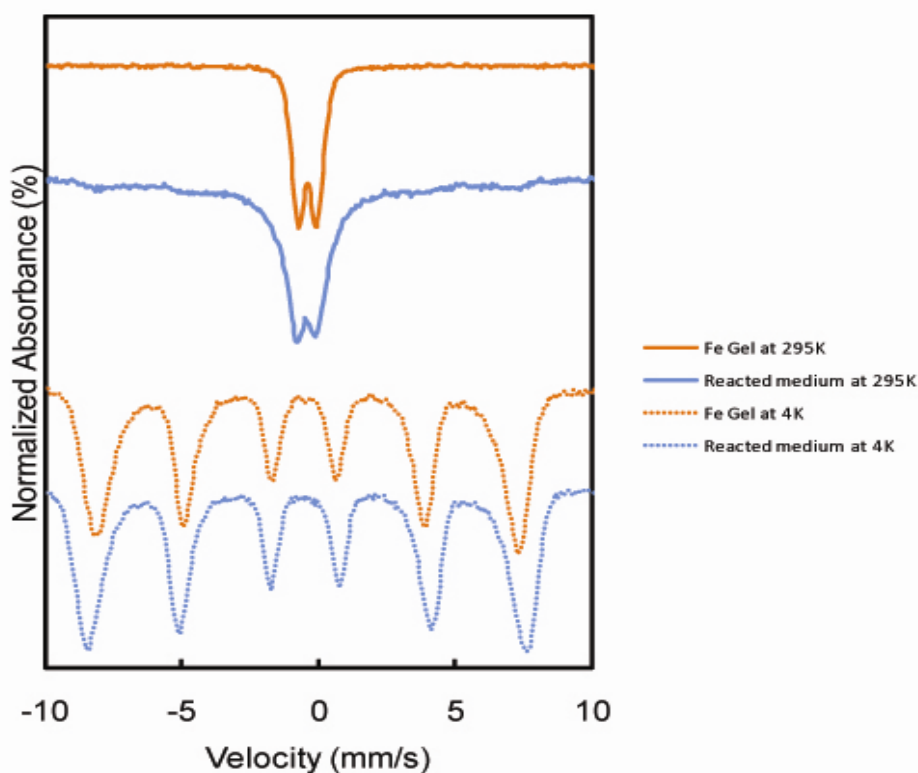


Figure 23—Overlaid Mössbauer spectra of unreacted Fe gel (orange) and reacted medium (blue) at room temperature and low temperature. Magnetic ordering occurs at low temperature in both species which is shown by the sextets at 4K. Broadening at room temperature in the reacted medium may be due to particle size or the existence of another phase of Fe.

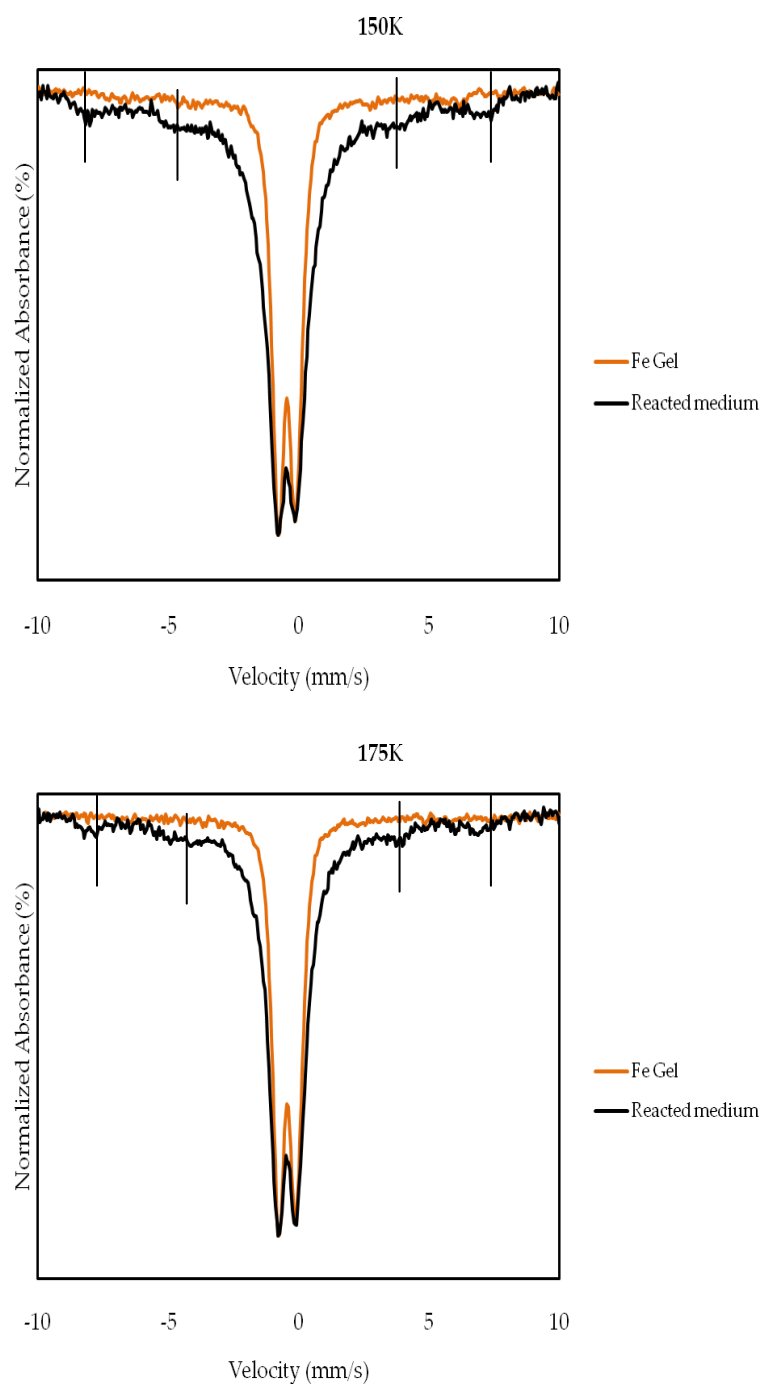


Figure 24—Spectra of Fe gel and reacted medium at 150K and 175K. Splitting appears to occur at 175K for the reacted medium and is more apparent at 150K. The starts of the sextets are marked by the lines.

DISCUSSION

Results of this work lay the foundation for future studies of *P. islandicum* in several ways. We have started optimizing the conditions for growth and iron reduction by varying the contents of the medium and have clues as to the importance of both the type and the size of the Fe(III) oxide used in the starting media. Using SEM images, the differences between cells growing in exponential phase and cells in stationary phase were examined. We used two spectroscopic methods to identify the minerals present in the unreacted and reacted media, and interpret these as evidence of bioreduction. These themes are discussed in more detail in the following sections, along with suggestions for future work on this topic.

Growth Rates and Conditions

Experimental data from growth curves shows that the overall average doubling time for *Pyrobaculum islandicum* is 2.7 hours. Without FeCl₂, the average doubling time is reduced nearly 50%. Also, with the subtraction of a FeCl₂, cells also grew to higher concentrations. This

suggests that the addition of FeCl_2 may be inhibitory and that it is unnecessary. This was contrary to our starting assumption that increases in FeCl_2 would aid in starting Fe(III) reduction and therefore cells would not only grow to higher concentrations but the doubling time would also be lower. When higher concentrations of FeCl_2 are added to the media, the opposite effect occurs. When too much FeCl_2 is added to the media there appears to be abiotic reduction of Fe(III) , which may account for the low cell concentration. Future work will thus likely proceed without the use of FeCl_2 .

Because abiotic reduction of Fe(III) can occur, the results from our first growth curve were viewed with some skepticism. We noticed that Fe(II) production continues to occur after stationary phase has been reached and cells are no longer growing. This seemed to be a product of a secondary abiotic reaction, and so we added uninoculated controls in all subsequent growth curves. Although there is some abiotic reduction of Fe(III) it does not look to be substantial enough to change the fact that the bulk of the reduction is mediated by *P. islandicum*.

Due to the strictly anaerobic nature of *P. islandicum* as reported in Huber et al. (1987), and the possibility that the iron was being re-oxidized after reduction, we attempted to evacuate as much oxygen as possible from the tubes in our final growth curve. This was achieved by leaving the tubes on the vacuum manifold for an hour. Typically these tubes are only vacuumed for 15 minutes prior to being flushed with argon gas. There was not, however, any significant difference in growth after 1 hour on the manifold. It does not appear that evacuating the tubes for a longer amount of time makes a difference, so, in the interest of expediting experimental time, tubes will continued to be vacuumed for 15 minutes only as this seems to be sufficient for oxygen removal.

Visualizing Iron Reduction

In all cases, the brown, paramagnetic, insoluble Fe(III) oxide was converted to a black, ferromagnetic precipitate (e.g., that responds to an external magnetic source). This corresponds well with a study conducted by Kashefi and Lovley (2000) in which the reduction of Fe(III) oxides and other metals was studied. They reported this black medium to be magnetite on the basis of XRD data, which also indicated that the magnetite produced was ultrafine-grained.

The presence of ultrafine-grained (superparamagnetic) magnetite has implications for how we visualize iron-cell contact as well as for the interpretation of Mössbauer results. SEM was used for this purpose rather than light microscopy because it produces high resolution images, has greater depth of field, and shows fine surface detail.

Comparing our micrographs from exponential phase to the micrograph in Figure 6, the morphologies and sizes of these cells are quite similar (Huber et al., 1987). Unexpectedly, we found that cells from stationary phase showed signs of cellular distress. They were elongated, with irregular edges and surface, and did not appear to have iron particles on their surface. Based on the amount of iron surrounding these cells, Fe(III) does not appear to be limiting growth at this stage.

Visualizing surface detail was most important because *P. islandicum* requires direct contact with iron for reduction to occur. The question remains; what are the mechanisms behind which this takes place? Although there are iron particles visible on the surface of the cell in Figure 18, there is some indication that there are even smaller iron particles that are being utilized by *P. islandicum*. On the top, left side of the cell in Figure

18, two very small particles can be seen. These particles are, to the best approximation, about 50nm. If particle size is highly important for cellular access to Fe(III), then it is possible that lack of surface area on Fe(III) grains is the limiting factor in *P. islandicum* growth. If this is the case, then increasing the surface area of the ferrihydrite in the starting media should promote increased Fe(III) reduction.

To address this possibility, dried Fe gel has been micronized (bulk particle size $<1\mu\text{m}$) to use in media for future work. Preliminary work has shown that cells are capable of growing on this micronized media but the resultant reacted media does not respond to an external magnetic source. This suggests that the smaller particles and their increased surface area are being used for Fe(III) reduction but the reduced particles may be superparamagnetic. We have started low-temperature Mössbauer studies of these samples in the reacted media to characterize them at a temperature range at which they become ferromagnetic and therefore have more diagnostic Mössbauer parameters (Table 4).

Fe(III) Reduction and Spectroscopic Fe-Species Identification

It has been well established that insoluble Fe(III) oxide can be used in reduction processes by *Pyrobaculum islandicum* (Kashefi and Lovley, 2000; Childers and Lovley, 2001; Feinberg et al., 2008). However, the exact species of Fe(III) oxide in the starting media had yet to be identified in the Holden laboratory. Using two different spectroscopic methods, Fourier Transform Infrared (FTIR) spectroscopy and Mössbauer spectroscopy, ferrihydrite was identified in the unreacted media and both ferrihydrite and magnetite were found in the reacted media.

The broadening of the central doublet (Figure 23) in the reacted media as well as the onset of magnetic ordering show a contribution from nanophase magnetite that is mostly a doublet but may be just large enough to begin to show ordering at 295K. At 4K, the Mössbauer spectra of both unreacted and reacted media show complete magnetic ordering. Modeling of the overlapping sextets from nanophase magnetite and magnetically-ordered ferrihydrite will be needed to distinguish the relative contributions from each phase. Because both ferrihydrite and magnetite order magnetically somewhere between 295K and 4K, it should also be possible to find an intermediate temperature at which their relative contributions can be better resolved. Further experimentation is needed to

do this. From the Mössbauer work done in this study we can conclude that the spectra at least show a difference in state of iron in the reacted versus the unreacted media, and that these differences are consistent with the co-existence of ferrihydrite and magnetite in the reacted medium.

Future Directions

Once we have identified the temperature where the various co-existing iron species can be resolved, it may be possible to track the changes in iron over the entire growth of *P. islandicum* so that the changes in mineral composition and oxidation state can be followed throughout growth. Understanding the transformation of iron will give us insights into the mineralogical changes that occur in geothermal environments and how hyperthermophilic organisms and dissimilatory iron reducers fit into the primary production scheme in such environments. The use of different Fe(III) oxides, such as akaganéite, goethite, and hematite, in the starting material should also be investigated because these are also available in hyperthermophilic environments (Stetter, 1999). Other iron minerals such as Fe(III) sulfates and Fe(II) sulfides should also be investigated and can be characterized using both Mössbauer and FTIR.

The next step in our Mössbauer investigations should be to directly compare spectra from *P. islandicum* reacted medium to bulk and nanophase magnetite lab standards over the entire temperature range from 4-295K to see how closely these spectra match up. Using FTIR spectroscopy on these samples can confirm any findings. Therefore the compositions, Fe valence state, and crystal structures of iron-containing minerals will be characterized before and after transformation. It is quite possible that magnetite is not the only iron end-product after transformation and that soluble Fe(II) may also be a possible end-product. This has been the case in preliminary growth studies on micronized ferrihydrite and these samples need to be studied further to determine the type of iron present.

Once we have determined the Fe(III) particle size that is optimal for *P. islandicum* growth, it will be important to visualize this interaction. In June of 2010, the University of Massachusetts at Amherst will have available an extreme high-resolution (XHR) SEM, the FEI Magellan™ 400L system. Using this system, particles <1nm can be imaged in high resolution. This platform will give us the opportunity to view the cell-iron interface at magnification and resolution that traditional SEMs are unable

to reach. It may be possible to view a physical characteristic (e.g., direct contact between cell and iron particle if one exists) that lends insight into the reduction mechanism.

We hope eventually to couple our iron reduction studies with geochemical studies to show if these organisms leave a biosignature that can be detected in naturally-occurring samples using spectroscopic techniques. For example, in remote-sensed spectra of planetary surfaces it may be possible to identify the unique spectral signature of our ferrihydrite-magnetite intermediate (Figure 20), if this phase is solely formed by bioreduction.

We also wish to determine if the ferric reductase activity of *P. islandicum* is the primary dissimilatory iron reduction mechanism for this organism and how this and other dissimilatory iron reduction mechanisms are used to generate ATP and conserve energy for the cells. Improved understanding of the physiology of hyperthermophilic Fe(III) oxide reduction by archaea will provide insights into metabolic processes occurring in geothermal environments and deep within the Earth's crust where physiology, growth rates and constraints, diversity, and distribution patterns of the organisms there have yet to be understood.

The high abundance of Fe(II) oxide and the lack of other terminal electron acceptors make dissimilatory iron reduction an important process within subsurface and geothermal environments. Not only does this have implications for biogeochemical studies on Earth, but these results can be used to explore other terrestrial bodies within our solar system by translating spectroscopic techniques to instrumentation used on exploratory spacecrafts.

APPENDIX

Mössbauer Effect and Spectroscopy

One tool in understanding microbial iron transformation is Mössbauer spectroscopy, or resonant γ -ray emission/absorption spectroscopy. In the study of solids, Mössbauer spectroscopy reveals the interaction of electromagnetic radiation in the gamma radiation range (10^4 to 10^5 eV) in atomic nuclei, more specifically the emission of a γ -ray from the excited state of a nucleus and the resonant absorption of the γ -ray by the ground state of an identical nucleus that lose no energy to recoil. The Mössbauer effect was first identified by then-graduate student Rudolf L. Mössbauer in Heidelberg, Germany in 1957.

Resonant Absorption

Mössbauer spectroscopy distinguishes the oxidation states of Fe^{2+} and Fe^{3+} on the basis of nuclear transitions. The process of resonant absorption is well understood and highly utilized in many other forms of spectroscopy. As an example, photons of specific energies can excite an electron from one energy level into another. When the atom returns to its

ground state, the emitted photon gives the precise energy absorbed. The Mössbauer effect relies on this principle, but the process is occurring in the nucleus of an atom. A γ -ray excites transitions between nuclear energy levels such that the γ -ray emitted from the source nucleus is absorbed and remitted by the absorber nucleus (Figure 1A). The γ -ray used for the Mössbauer effect comes from the decay of ^{57}Co into ^{57}Fe (Figure 2A).

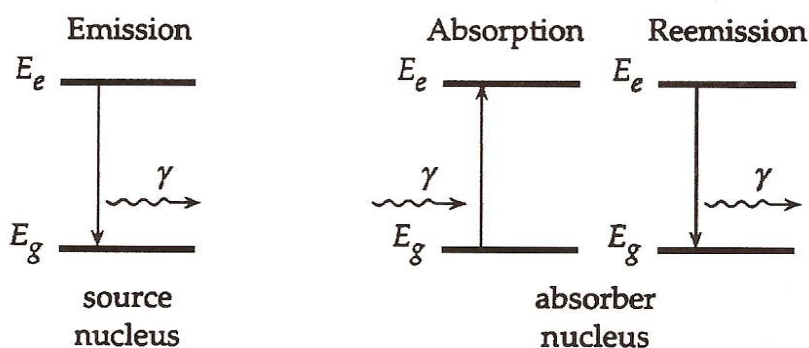


Figure 1A—Schematic representation of nuclear resonance fluorescence (Grant, 1995)

The use of ^{57}Fe as a nuclide source is particularly useful in the investigation of minerals because it optimizes the conditions for recoil-free emission and absorption of γ -rays (Dyar et al., 2006). The energetic processes relevant in the investigation of minerals of ^{57}Fe are shown in Figure 3A. ^{57}Co has a half-life of 270 days. It decays primarily to the 136.3-keV level of ^{57}Fe , an excited state for ^{57}Fe . Return to the ground state

occurs two different ways. The γ -ray can return directly to ground and emit a 136.3-keV gamma photon, a process that occurs approximately 9% of the time. Otherwise, it drops to the 14.4-keV state before dropping to its ground state. Of these 14.4-keV transitions approximately 11% result in the emission of a γ -ray, which is abundant enough for Mössbauer experiments to be performed. The other 89% of de-excitation events are noted in Figure 3A but are not relevant to this particular study (Sklute, 2006).

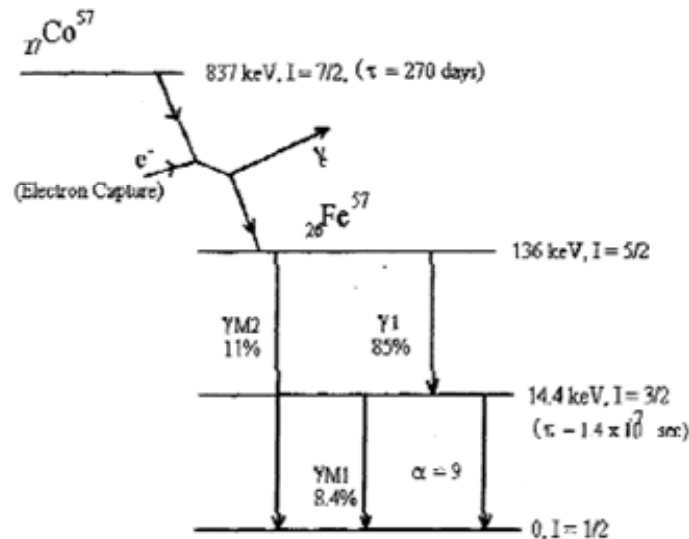


Figure 2A—Graphic representation of the energetic properties of ^{57}Fe . The decay of ^{57}Co causes emission of three different energies of γ -rays. Mössbauer spectroscopy uses the 14.4keV transition. The energy given is in percent of decays (Emerson, 2008).

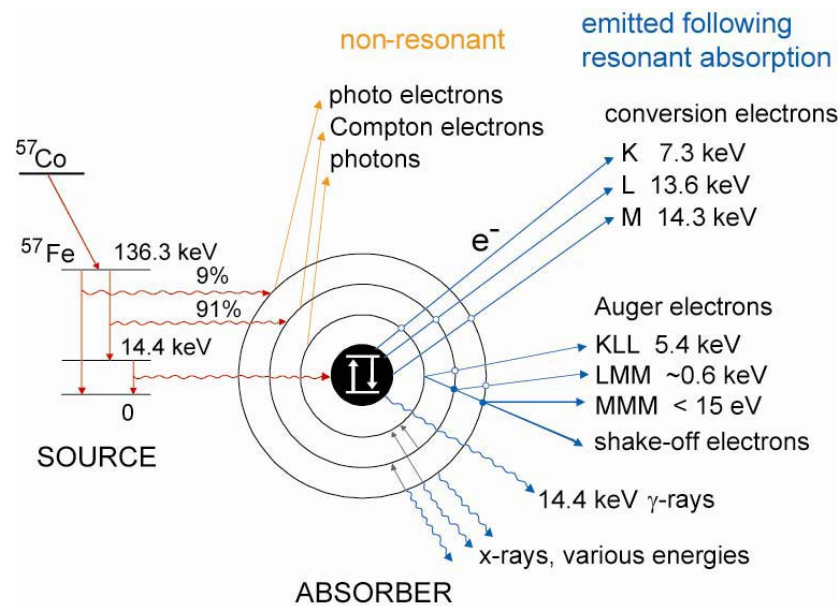


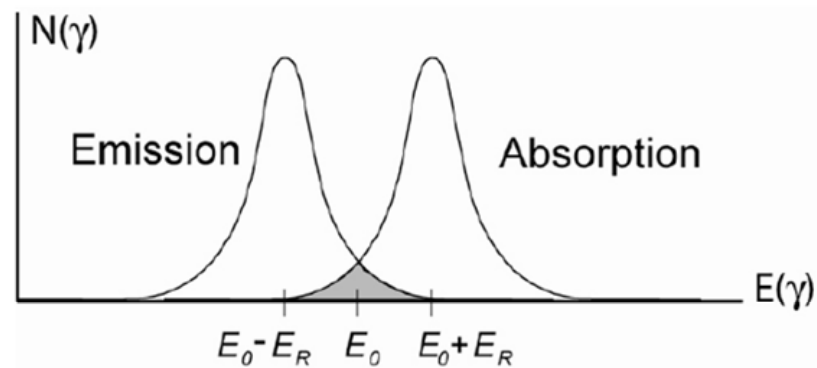
Figure 3A—The nuclear decay for ^{57}Co to ^{57}Fe and the various emission processes that can follow resonant absorption of a γ photon (Dyar et al., 2006)

The Lorentzian distributions for the energies of the emitting and absorbing atoms are shown in Figure 4A. Overlap of these two distributions is necessary for resonant absorption to occur, however slight the overlap between the two distributions may be. The decrease in the intensity of γ -rays is represented by the amount of overlap between the Lorentzian distributions for the source and the absorber (sample).

Momentum and Recoil Free Fraction

The γ -rays from the nucleus of an atom result in a specific energy and emission at a certain momentum. Based on conservation of momentum and the assumption that the emitting atom is originally at

rest, both the emitting atom and absorbing atom would experience recoil. The energy is then shared between the nuclear recoil and the emitted γ -ray (Dyar et al., 2006). By eliminating recoil, photon emission and absorption can occur at the same energy. The γ -rays emitted from a source that can be absorbed without recoil are the recoil-free fraction. The Mössbauer effect can then be achieved in a solid matrix. To obtain a large recoil-free fraction, the Mössbauer nuclide must be locked into a crystal or metallic matrix such that momentum recoil on emission or absorption of a γ -ray is taken up by the solid as a whole and thus is not reemitted.



4A—Lorentzian distributions of the emitting and absorbing nucleus. The degree of overlap indicates the percent of resonant γ -ray emission and re-absorption events. Overlap is exaggerated in this figure (Blackwell, 2000).

Mössbauer Spectrometer and γ -Ray Detection

The most common experimental set-up for measurement of the Mössbauer effect, and the one used in this study, is transmission

spectroscopy. The configuration for a Mössbauer experiment is shown in Figure 5A.

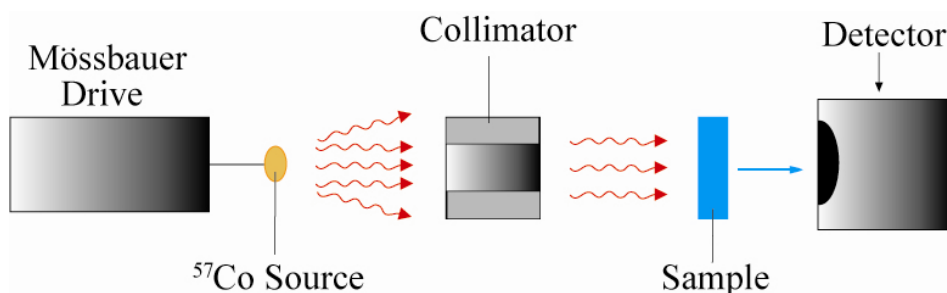


Figure 5A—Diagram of transmission Mössbauer spectroscopy (Dyar et al., 2006)

In Mössbauer transmission spectroscopy, gamma rays from a radioactive source, such as ^{57}Co , are passed through a collimator and then through a sample. The decrease in the intensity of the beam of γ -rays is detected. The detector counts the number of γ photons that pass through the sample. In order to obtain a spectrum, a range of transmission energies is created by adding a slight Doppler velocity to the source. The source is oscillating, modifying the γ -ray emission so that γ -rays with multiple energies can be observed. The resultant spectrum is a graph of transmission versus Doppler velocity. The acquisition of a Mössbauer spectrum from this added Doppler shift is shown in Figure 6A. The absorber interacts with γ photons with energies that vary with the amount

of Doppler shift. Therefore, the final curve produced (at the bottom of Figure 6A) is the proportion of the change in the amount of overlap between the source and the absorber.

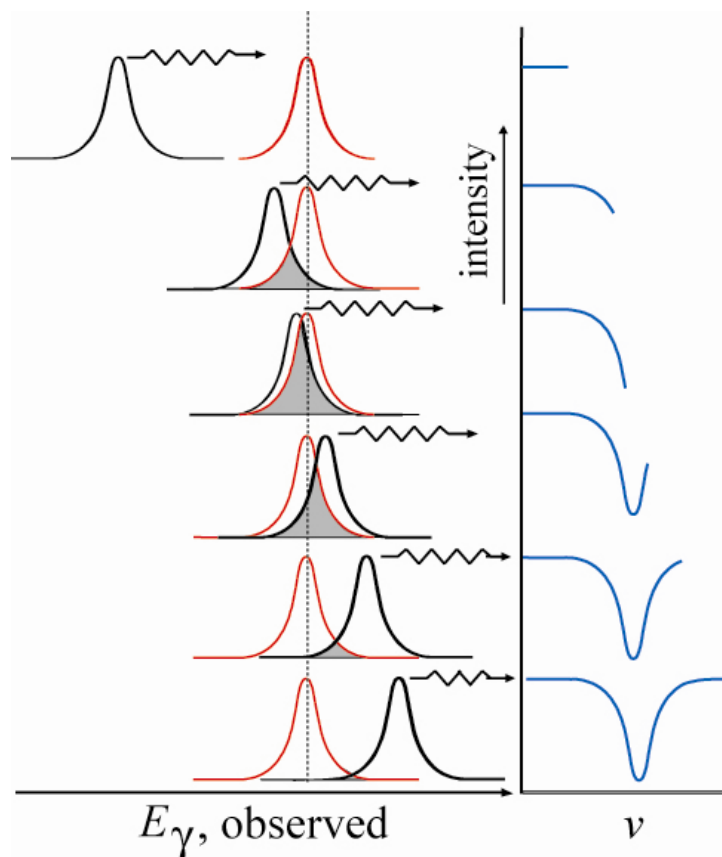


Figure 6A—Schematic of acquisition of Mössbauer spectrum. The source is moved by a Doppler velocity, creating an overlap of the source and absorber energy distributions. When there is no overlap, the Mössbauer spectrum shows 100% transmission (0% absorption). As more overlap is created by the Doppler velocity, fewer gamma rays get through to the detector, creating a drop in absorption that forms the peak in the spectrum (Sklute, 2006).

Mössbauer Parameters

Isomer Shift

If both the emitting and absorbing atoms are experiencing the same local environments, the source peak would be at zero velocity. However, this assumption does not hold true and would not be experimentally interesting. Because the local electronic environments of the two atoms differ, a spectrum of an absorber shows a shift of the spectrum away from zero by an amount that is characteristic of the environment of the iron absorber, known as the isomer shift. Isomer shift is recorded in mm/s, and is denoted by IS or δ .

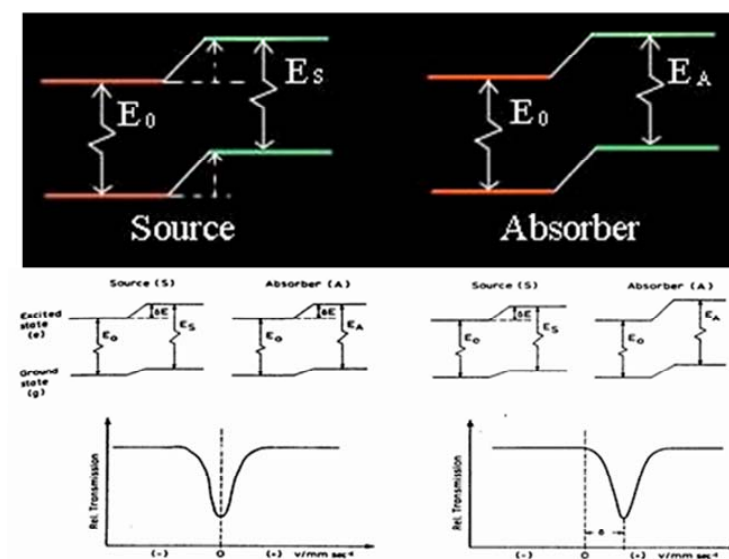


Figure 7A—The isomer shift is a shift in Mössbauer energy levels based on the different electronic environments of the emitting and absorbing nucleus (Sklute, 2006).

Isomer shift arises because of the atomic interactions between the charge density surrounding the nucleus and the energy levels of the nucleus itself. The interaction does not cause a split in nuclear energy levels, but a shift in energy from the ground to excited state transition. The result is an isomer shift that is relative to a standard, usually an α -Fe standard (Figure 7A). In a Mössbauer spectrum, isomer shift is observed when the Doppler velocity modifies the source's γ -ray energy to account for the difference between source and absorber. Because of the dynamics of the electronic environment, isomer shift is sensitive to differences in oxidation state, spin state, covalency and coordination. Figure 8A shows the variation of isomer shift with respect to oxidation and spin state for Fe^{3+} and Fe^{2+} . Fe^{3+} generally has a lower isomer shift than that of Fe^{2+} .

Quadrupole Splitting

The characteristic doublets found in Mössbauer spectra are directly related to the quadrupole moment and splitting interactions. The quadrupole moment is the result of asymmetry in the nucleus, or a non-spherical electronic charge density. The nucleus is then capable of interacting with the electric field gradient (EFG) created by other charges

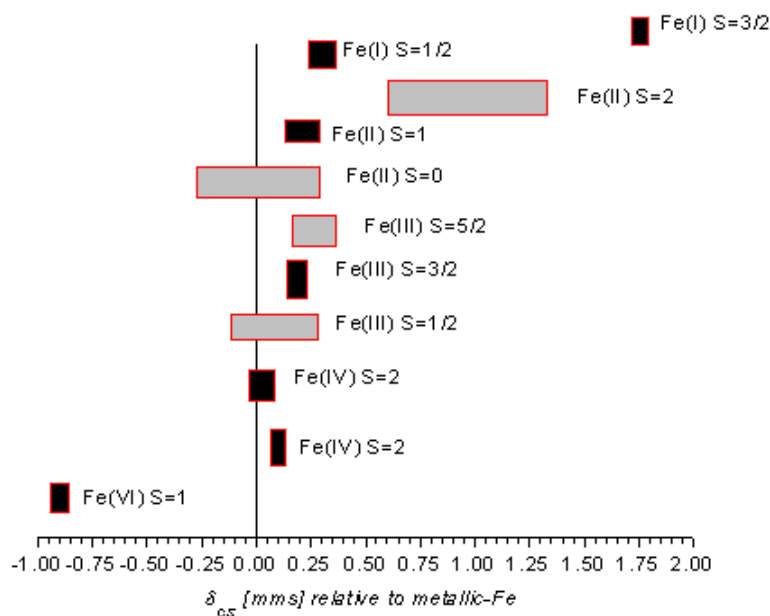


Figure 8A—Isomer shift ranges with respect to spin and oxidation state (Greenwood, 1971).

in the crystal. The nuclear levels lose degeneracy, giving the atom two different energy levels, causing the nuclear energy levels to split. The quadrupole split (QS, Δ , or ΔE_Q) is the distance between the two peaks, or doublet, that results in the spectrum and measures the energy difference between the two split states (Figure 9A). Quadrupole splitting is also sensitive to the coordination and oxidation states of the atom because changes in the electronic shape changes the electronic field.

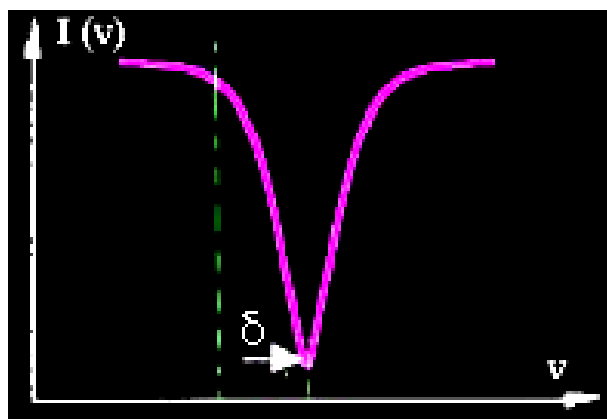


Figure 9A—Quadrupole splitting results from the change in the shape of a nucleus causing the nuclear energy level to split. The resulting Mössbauer spectrum has two peaks, known as a doublet. The distance between these two peaks is the quadrupole split, measuring the energy difference between the two split states (Sklute, 2006).

Magnetic Hyperfine Interactions

Based on the nuclear electronic environment, it is possible for the nuclear energy levels to be further split apart. There is a magnetic moment associated with ^{57}Fe nucleus and this is capable of interacting with an external magnetic field that is either applied or arises naturally from the structure of the iron-bearing mineral. This results in a loss of degeneracy of the nuclear levels that causes the appearance of six peaks to appear in a spectrum (sextet) rather than a doublet (Figure 10A). This is known as magnetic hyperfine (Zeeman) interaction. In an ideal sample, the ratio of areas of the sextet peaks is 3:2:1:1:2:3 and the widths of the peaks are

assumed to be the same. The IS of a sextet is between the centermost peaks.

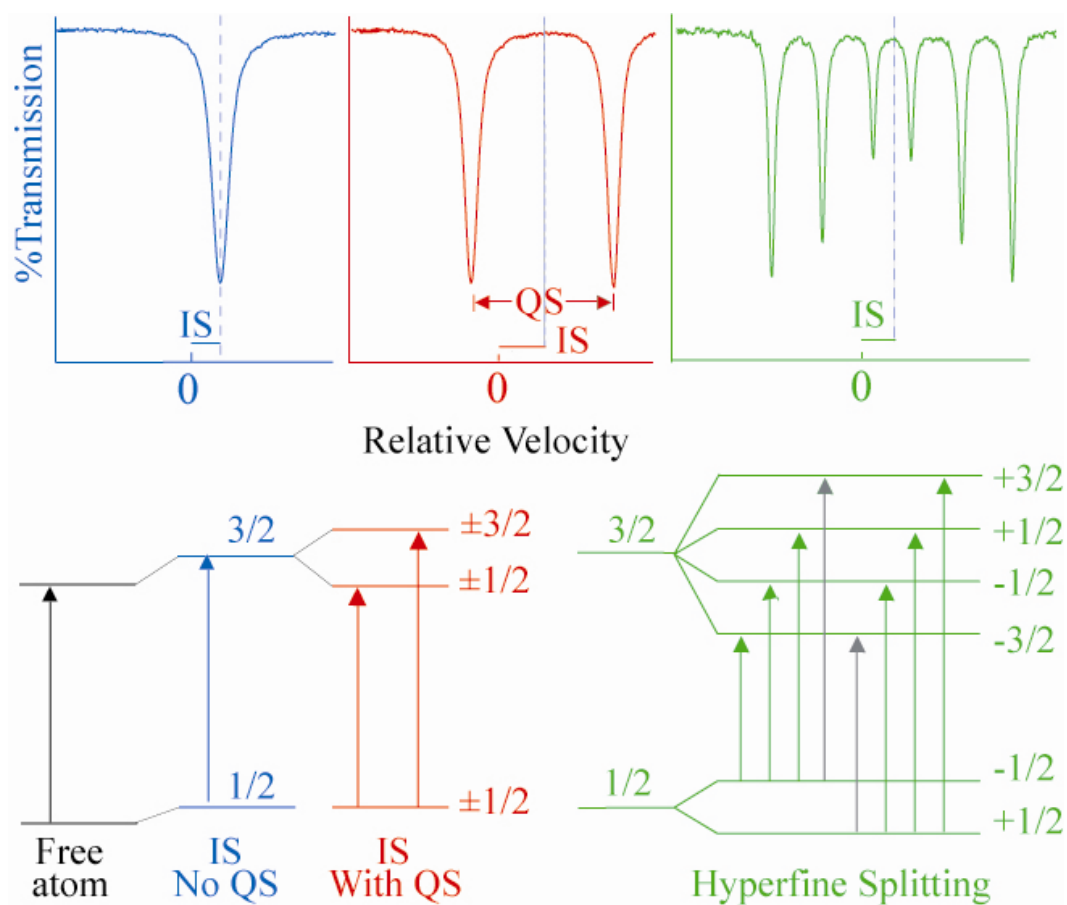


Figure 10A—The different nuclear energy levels when the source and absorber atoms are in different local environments (Dyar et al., 2006).

Superparamagnetism

In some special cases, a sample may appear as a doublet even when the temperature is dropped to a level where magnetic ordering would otherwise occur. This happens when the material is composed of

nanoparticles, or crystallites which are generally between 1-10nm but can be as large as 20nm depending on the material. When a sample exists in nanoparticle form, even when the temperature is dropped low enough that magnetic ordering would occur, the direction of magnetization can be changed for each nanoparticle. Therefore the magnetic field averages to zero. In this way, a ferromagnetic material would behave in a paramagnetic way. Normally, coupling forces in ferromagnetic minerals cause alignment of neighboring atoms which results in an internal magnetic field. This alignment makes it possible to distinguish ferromagnetic samples from paramagnetic samples.

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