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# CHARACTERIZING FE(III) TRANSFORMATION IN A DEEP-SEA HYPERTHERMOPHILIC ARCHAEON

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This paper was prepared
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Professor Darby Dyar
for eight credits.

*For MEW, who kindled a spark that started a fire*

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I would like to express my utmost gratitude to James Holden and the Hyperthermophile Lab at UMass-Amherst, for welcoming me into their lab and sharing all of their resources with me. Everyone in the lab deserves my thanks for putting up with my presence for two years, but I would especially like to thank Jennifer Lin for her limitless patience and support. Jenn has educated me beyond measure and has taught me how to be a good scientist, in addition to being a great friend. Thank you to Marian Rice for allowing me to access the SEM, and for teaching me from scratch how to use the TEM. I also want to extend a special thank you to Jacqueline Knutson, who as a senior inspired my sophomore self to take over her role in this project and for giving me someone to look up to. The Massachusetts Space Grant Consortium deserves paramount appreciation for providing me with the financial resources to spend a summer in Amherst working on Sully.

My friends at Mount Holyoke have been a wonderful inspiration and support system for me over the last three years. Their achievements and accomplishments have encouraged me to keep going no matter what. Maddy, thank you for always bragging on me to strangers, and for at least pretending to be interested in my beloved organisms. I also want to thank my boyfriend, Carter, for accompanying me on all those journeys to the lab in the middle of the night to pull test tubes and never complaining about the alarm going off at 3am. Finally, thank you to my parents and sister for showing me unconditional love and support in all of my life pursuits.

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# ABSTRACT

Hyperthermophilic bacteria and archaea found in deep-sea hydrothermal ecosystems thrive in the chemical disequilibrium and high temperatures found in these environments, growing optimally between 80-110°C. These organisms flourish without the use of energy from the sun, instead utilizing the chemical-rich fluid released from hydrothermal venting structures for energy through chemosynthesis. The subject of this thesis is a recent archaea isolate from a venting structure in the Endeavour Segment of the Juan de Fuca Ridge in the Northeastern Pacific Ocean. The organism is officially known as Strain Su06, but nicknamed "Sully" after the hydrothermal structure from which it was extracted, as it is so newly discovered that it has yet to be given a scientific name or even be assigned a genus. It is a known iron-reducer, utilizing iron oxides for energy through the metabolic process of dissimilatory iron reduction, and is thought to belong to one of two hyperthermophilic archaeal genera; *Pyrodictium* or *Hyperthermus.* In this study, we investigated Sully by determining its growth rates under different conditions through ferrozine assays to measure Fe(II) production, and epifluorescence microscopy to determine cell concentration, in order to establish optimal growth temperature for the species. Cell characteristics and iron-cell contact were visualized through electron microscopy. The findings were then compared to species within the *Pyrodictium* and *Hyperthermus* genera in order to get closer to answering the question of which it belongs to.

The study of extremophilic archaea is fundamentally based in concepts of astrobiology. Because of their archaic nature and ability to exist at high temperatures, it is thought that similar organisms may have been spread throughout the solar system after heavy impacts or may be closely related to our last universal common ancestor. Additionally, the scientific investigation of iron-reducers' metabolic pathways is of interest in the study of biofuels. Gaining a further understanding of geothermal environments may lead to future sustainable energy resources.

# INTRODUCTION

The deep-sea ecosystem on Earth is a lush garden, rich in life that humans are just beginning to understand. Without the influence of the Sun, organisms dwelling thousands of feet below the ocean surface have evolved to use alternate sources of energy for survival. The existence of hydrothermal vents was predicted based on knowledge of tectonic activity, but this unique ecosystem was not discovered until 1977 when a group of marine geologists sought to study the East Pacific Rise (Campbell, 2000). Before this point, it was not known that life could flourish without sunlight. On a submersible dive, these scientists were astounded at the variety of charismatic life they found, from tubeworms and shrimp, to bivalves and crabs – all flourishing at the bottom of the ocean without any accessible solar energy (Lonsdale, 1977). Since this

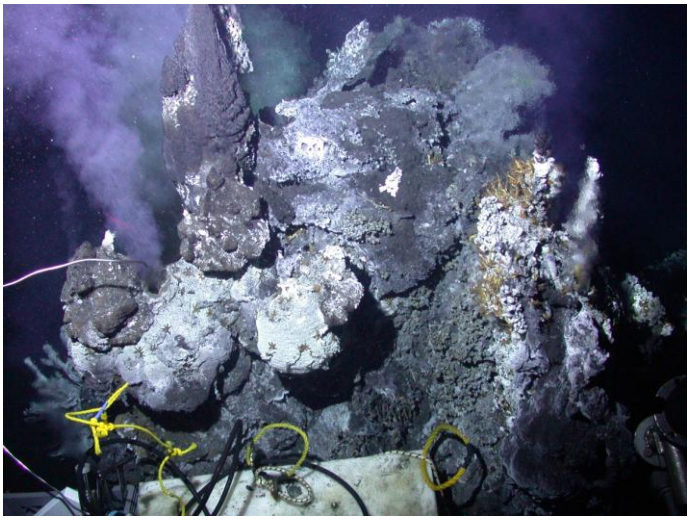

**Figure 1.** A black smoker chimney (Courtesy of Dr. James Holden).

groundbreaking discovery, scientists from around the world have been grappling to understand the complex ecosystem at deep sea ridges. Over 200 species of vent macrofauna and nearly 100 species of hyperthermophilic archaea and bacteria have been isolated and described since the late 1970s (Tunnicliffe, 1991; Huber et al. 2000).

These hydrothermal environments are found at sea-floor spreading centers, where seawater seeps into cracks in the crust and circulates beneath the sea floor. The water interacts with magmatic heat, as well as volatile compounds and heavy metals that have been leached from sub-surface, geothermally-heated rock. When the fluid is released (at temperatures of up to 400°C) through layers of the ocean crust and comes into contact with seawater, it is rapidly heated, causing the precipitation of sulfate minerals from the seawater. This temperature gradient and subsequent accumulation of precipitated metal sulfides results in the formation of the chimneys that characterize hydrothermal vents (Figure 1). This fluid, rich in compounds such as $H_2$, $H_2S$, $CO_2$, and iron-sulfides, nourishes the organisms that inhabit these environments surrounding the black smokers (Holden et al., 2011; Holden et al., 2012).

The chemical disequilibrium found in hydrothermal vent environments creates a habitat for a diverse array of life. One of the major groups of organisms is bacteria and archaea known as "extremophiles." The extremophiles found here are especially adapted to the sun-less, chemical-rich, and extremely hot environment. Their adaptation to extreme temperatures defines them as hyperthermophiles, organisms with optimal growth temperatures above 80°C. These environments are essentially anaerobic, meaning without oxygen, due to the low-solubility of oxygen at high temperatures and because of the presence of reducing gasses such as $H_2S$ (Huber et al., 2000). Due to these stipulations, these organisms have evolved with unique metabolic functions that are the focus of this study.

## *Project History and Goals*

As an Environmental Studies major and Astronomy minor, I never dreamt I could find a research project that encompassed nearly all of my academic interests. Jacqueline Knutson, Mount Holyoke Class of 2010, was my neighbor in our residence hall during my sophomore year, and one day when we were chatting in the hall way she told me about her

thesis project – studying deep-sea microorganisms that could have analogs on other worlds. Being fascinated by astrobiology and without a lab to call home, I immediately jumped at this opportunity and pestered Jacqueline about how I could get involved. I was in luck because she was looking for another MHC student to take over her research after she graduated. I started working at the Holden Hyperthermophile Lab in February 2010, and have since spent many hours and a summer researching the growth behavior of iron-reducing archaea alongside graduate students.

The study of extremophilic archaea is fundamentally based in concepts of astrobiology. Because of their archaic nature and ability to exist at high temperatures, it is thought that similar organisms may have been spread throughout the solar system after heavy impacts. Other theories involve extremophiles developing on other planets independently, such as below the ice on tectonically-active rocky planets and satellites. A key aspect of the research involves Mössbauer spectroscopy, which enables us to identify the chemical signatures of the organisms grown in the lab, such as Sully. These signatures can then be compared to spectroscopy of distant planets, telling us if similar

organisms could exist there. Furthermore, the scientific investigation of iron-reducers' metabolic pathways is of interest in the study of biofuels. Gaining a further understanding of geothermal environments may lead to future sustainable energy resources.

When I began this project, I sought to answer several research questions. The main focus of the project was characterizing Sully's growth parameters – What is the optimal growth range? Will it grow on different Fe(III) oxides? Will it grow without $H_2CO_2$ in the headspace? I also wanted to visualize the physical characteristics of Sully cells and how they interact with iron, utilizing both Scanning and Transmission Electron Microscopy. Lastly, I wanted to explore the genetic and physical characteristics of Sully in order to come closer to an understanding of its correct scientific classification, by analyzing whether it is a member of the *Pyrodictium* or *Hyperthermus* genus.

# BACKGROUND

## *Characterizing hyperthermophiles and their environment*

Hydrothermal vent environments are home to a variety of life forms. One of the major groups of organisms is hyperthermophilic bacteria and archaea, known as "extremophiles." Types of extremophiles are determined based on their optimal growth conditions, such as temperature, salinity, pH, and pressure. Examples include 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).

Because of their optimal growth temperature above 80°C, hyperthermophiles are found primarily in aqueous environments with geothermal and volcanic heating. They are specifically adapted to the hot, sunless, and chemical-rich hydrothermal ecosystem (Stetter, 1999). These environments are essentially anaerobic, meaning without oxygen, due to the low-solubility of oxygen at high temperatures and because of the

presence of reducing gasses such as $H_2S$ (Huber et al., 2000). As such, all cellular components of these microorganisms must be heat-resistant.

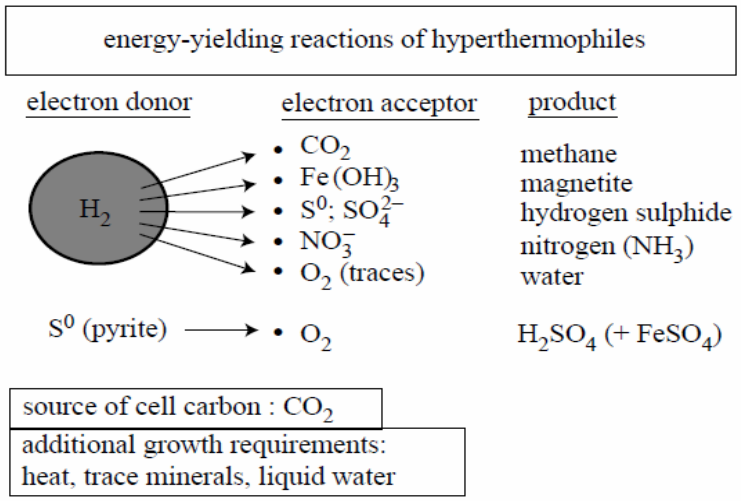


**Figure 2.** Diagram showing main energy-yielding reactions in chemolithoautotrophic hyperthermophiles (Stetter, 2006).

Most hyperthermophiles are anaerobes that require metalloenzymes with low reduction potentials to catalyze essential metabolic reactions (Holden et al., 2011). They are recognized as chemolithoautotrophs, and gain energy by utilizing inorganic redox reactions. In anaerobic respiration, hyperthermophiles are capable of nitrate-, sulfate-, sulfur-, carbon dioxide-, and ferric iron respiration (Knutson, 2010). Select hyperthermophiles use metals as terminal electron acceptors in a dissimilatory manner during respiration (Figure 2). In these hydrothermal environments, heavy metals that are inevitably encountered by hyperthermophiles include vanadium, chromium, manganese, iron,

cobalt, nickel, copper, zinc, molybdenum, cadmium, lead, the precious metals gold and silver, as well as the metalloids arsenic, selenium, and antimony. However, only prokaryotes are capable of reducing Mn(IV), Fe(III) and Co(III) on a large scale while still conserving energy in the reaction (Knutson, 2010). Organisms that utilize minerals require unique physiological mechanisms, because the reactants are insoluble (Holden et al., 2012).

Microorganisms that use metals as terminal electron acceptors have a significant effect on their geochemical environments. Microbial metal reduction may be utilized in removing metal and organic contaminants in various environments. Iron reducers like Sully have a particular influence because Fe(III) is one of the most abundant potential electron acceptors for organic matter decomposition in aquatic sediments and subsurface environments. Sully is a novel organism in its genus, because in most microorganisms that metabolize sugars or amino acids with the reduction of Fe(III), the primary products are typical fermentation acids, alcohols, and $H_2$. (Lovley, 1993). We don't yet know whether Sully can oxidize sugars and amino acids, but it grows autotrophically with CO2 in its headspace and uses Fe(III) as the sole electron acceptor.

Dissimilatory iron reduction (DIR) is a common characteristic among hyperthermophiles, and its significance in hyperthermophilic environemtns has only been acknowledged in the past decade (Kashefi et al., 2008). DIR requires higher quantities of metal than any other reduction reaction (Slobodkin, 2005). In cell respiration, electron transfer occurs in the cytoplasmic membrane towards an external terminal acceptor. An electrochemical proton gradient is generated across the membrane, which results in the formation of H+ dependent energy across the membrane. Currently, the physiological mechanisms for $O_2$, $NO_3^-$, and $SO_4^{2-}$ reduction are very well understood, but very little is known about how organisms (and hyperthermophiles in particular) utilize DIR (Holden et al., 2011; Kashefi et al., 2008). DIR generally involves three mechanisms: 1) direct cell-iron contact; 2) reduction and release of extracellular electron shuttles for non-enzymatic, extracellular iron reduction; and 3) the secretion of iron chelators that solubilize iron and return it to the cell for reduction (Holden et al., 2011). Figure 3 illustrates this interaction within the cell.

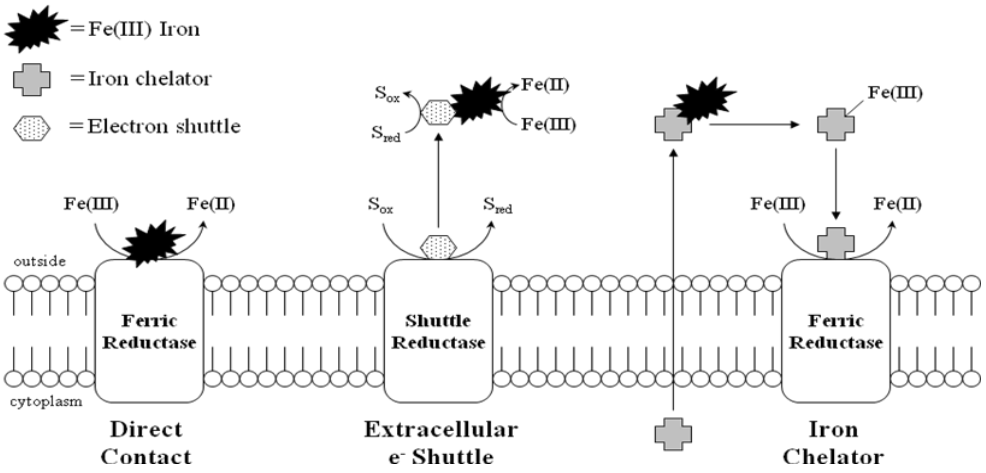


**Figure 3.** Schematic demonstrating reactions that take place during dissimilatory iron reduction (DIR) (Holden et al., 2011).

## *Phylogeny of Hyperthermophiles*

Life first emerged on Earth between 3.5 and 3.9 Gyr ago, during the Early Archaean eon. At some point after its emergence, the universal phylogenic tree of life split into three domains: the Archaea, the Bacteria, and the Eukarya. The earliest branching point is represented by the split between the arm of the bacteria, and that shared by the archaea and eukarya. As seen in Figure 4, hyperthermophilic species form a cluster around the root of the phylogenic tree (Stetter, 2006). Molecular phylogeny allows us to establish genetic relationships amongst organisms, and to determine the evolutionary progression of metabolic and microbial diversity (Amend and Shock, 2001). Currently, roughly 90 species of

hyperthermophilic bacteria and archaea have been isolated, and are very divergent in terms of their phylogeny and physiological characteristics: they comprise 34 genera and 10 orders (Stetter, 2006). The position of these organisms in the phylogenic tree provides strong evidence for a hyperthermophilic archaeon as a last universal common ancestor (LUCA) or as a member of the more inclusive term, the last common community (LCC) (Stetter, 2006). Combined evidence, primarily determined from isotopic data, places the advent of the LCC earlier than 3.5 Gya (Line, 2002). Further evidence from geological and microbiological research supports the theory that electron transport to Fe(III) may have been the first form of microbial respiration in the early stages of the evolution of life (Kashefi and Lovley, 2003). DIR is also hypothesized to have existed in Precambrian marine environments through Fe redox cycling, which potentially would leave behind geological and isotopic signatures (Percak-Dennet et al., 2011).

In the history of Earth, the period of late heavy bombardment (LHB) was strong enough in magnitude to evaporate all of the water on the planet. This catastrophic epoch and boiling of the oceans may have been responsible for selecting hyperthermophilic biota (and, in the

process, destroying most others) (Line, 2002). Some hypothesize that two branches may have survived, which today can be traced back to the deepest branches in Bacteria and Archaea, as illustrated in Figure 4.

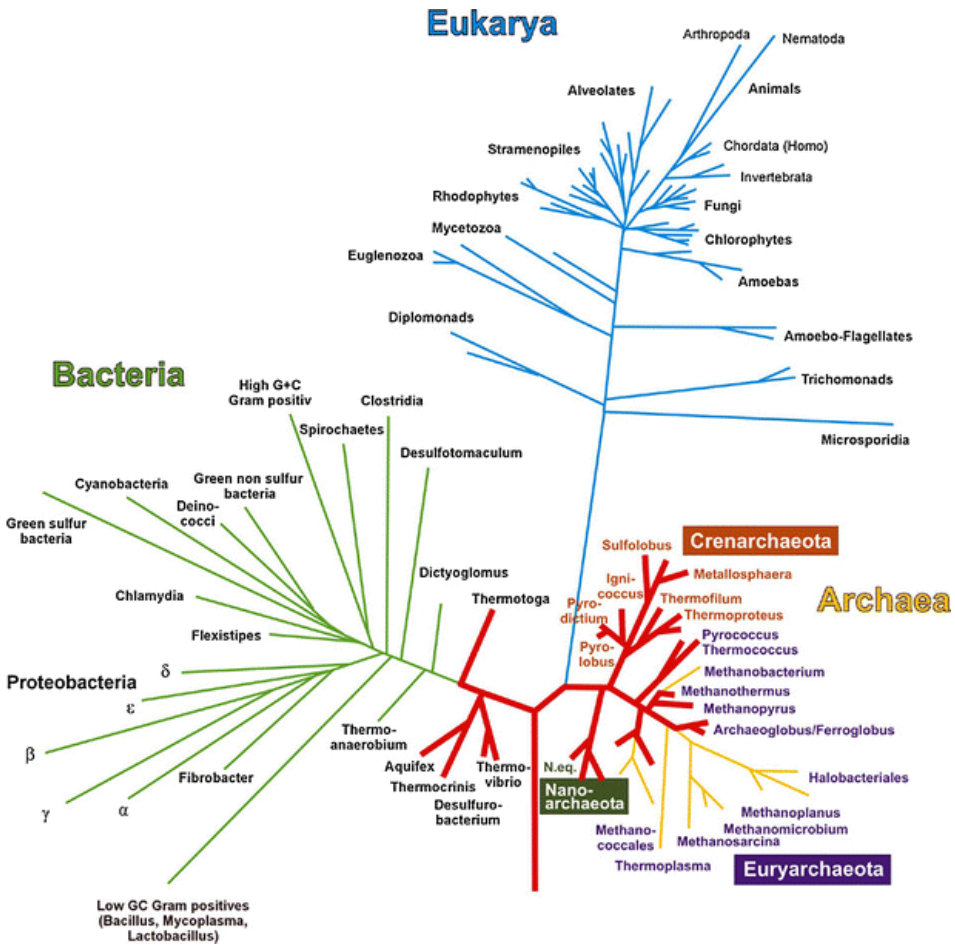


**Figure 4**. Phylogenic tree based on ribosomal RNA. Thick red lineage lines represent hyperthermophiles (Stetter, 2006).

Furthermore, in the absence of ozone on early Earth, the surface was likely saturated in high levels of UV radiation for more than two Gya, resulting in strong natural selection for hyperthermophiles in a deep-sea

hydrothermal habitat safe from radiation. Another theory posits that the LHB may have delivered living cells to Earth, which perhaps were thermophiles (Line, 2002).

Potential early chemosynthesis in organisms also favors a hyperthermophilic LCC. The hydrothermal habitat favors the abiotic synthesis of ammonia, as well as the formation of key elements of the citric acid cycle from CO and $H_2S$ in the presence of metal catalysts (Line, 2002). $FeS_2$ and $H_2S$, two compounds found in abundance in hydrothermal environments, are thought to be likely candidates as a first energy source. Further evidence, provided predominantly by extant species within the bacteria and archaea arms of the phylogenic tree, suggests that the LCC was anywhere from thermophilic to extremely thermophilic and was capable of nitrogen-fixation (Line, 2002). The presence of elements such as molybdenum, iron and sulfur in the nitrogen-fixing complexes suggest a hydrothermal legacy, and a history of anoxic conditions as the nitrogen-fixing enzyme cannot function in the presence of oxygen (Line, 2002). Scientists have made claims that "all basic types of bioenergetics processes probably existed 3.5 billion years ago and the biogeochemical cycling of carbon, nitrogen and sulfur was established as we know it today" (Line,

2002). It is believed that the "canonical" genetic code was already highly evolved before the LCC split off from the phylogenic root (Line, 2002).

## *"Sully"*

**Taxonomy:**

- **Domain:** *Archaea*
- **Kingdom/Phylum:** *Crenarchaeota*
- **Class:** *Thermoprotei*
- **Order**: *Desulfurococcales*
- **Family:** *Pyrodictiaceae*
- **Genus:** *Pyrodictium or Hyperthermus*

**Full length 16S rRNA sequence for Strain Su06:**

GGGCTCGTTATGTTGTTTATATAGCATGGGAGTCGTGCGCCCCCGGATGCGGGGGCGC
GGCGGACGGCTGAGTAACACGTGGCCAACCTACCCTCGGGACGGGGATAACCCCGG
GAAACTGGGGCTAATCCCCGATAGGCGAGGGGGCCTGGAACGGGTCCCTCGCCGAA
AGGGCGCGCGGAGCTTCCCCGCGCGCCGCCCGAGGATGGGGCTGCGGCCCATCAGGT
AGTTGGCGGGGTAACGGCCCGCCAAGCCGATAACGGGTAGGGGCCGTGGGAGCGGG
AGCCCCCAGATGGGCACTGAGACAAGGGCCCAGGCCCTACGGGGCGCACCAGGCGC
GAAACCTCCGCAATGCGGGCAACCGTGACGGGGTCACCCCGAGTGCCGCCGATAAG
GCGGCTGTTCCCCGCTGTAGGAAGGCGGGGGAGTAAGCGGGGGGCAAGTCTGGTGTC
AGCCGCCGCGGTAATACCAGCCCCGCGAGCGGTCGGGATGATTACTGGGCCTAAAGC
GCCCGTAGCCGGCCCGGTAAGTCCCCCCCTAAAGCCCCGGGCTCAACCCGGGGAGTG
GGGGGGATACTGCCGGGCTAGGGGGCGGGAGAGGCCGAGGGTACTCCCGGGGTAGG
GGCGAAATCCGATAATCCCGGGAGGACCACCAGTGGCGAAGGCGCTCGGCTGGAAC
GCGCCCGACGGTGAGGGGCGAAAGCCGGGGGAGCAAACCGGATTAGATACCCGGGT
AGTCCCGGCTGTAAACGATGCGGGCTAGGTGTTGGGCGGGCTTGGAGCCCGCCCAGT
GCCGCAGGGAAGCCGTTAAGCCCGCCGCCTGGGGAGTACGGCCGCAAGGCTGAAAC
TTAAAGGAATTGGCGGGGGAGCACCACAAGGGGTGGAGCCTGCGGCTTAATTGGAGT
CAACGCCGGGAATCTTACCGGGGGCGACAGCAGGATGACGGCCAGGCTGACGACCT
TGCCCGACGCGCTGAGAGGAGGTGCATGGCCGTCGCCAGCTCGTGCCGTGAGGTGTC
CGGTTAAGTCCGGCAACGAGCGAGACCCCCACCCCTAGTTGCTACCCCGGGGGCCAC
CCCGGGGGCACACTAGGGGGACTGCCGCCGTCCAAGGCGGAGGAAGGAGGGGGCCA
CGGCAGGTCAGCATGCCCCGAATCCCCCGGGCTGCACGCGGGCTACAATGGCGGGGA
CAGCGGGATCCGACCCCGAAAGGGGGAGGCAATCCCTTAAACCCCGCCGCAGTTGG
GATCGAGGGCTGCAACTCGCCCTCGTTGAACGCGGAATCCCTAGTAACCGCGCGTTA
GCATCCGCGCCGGAATCATTCCATATAA

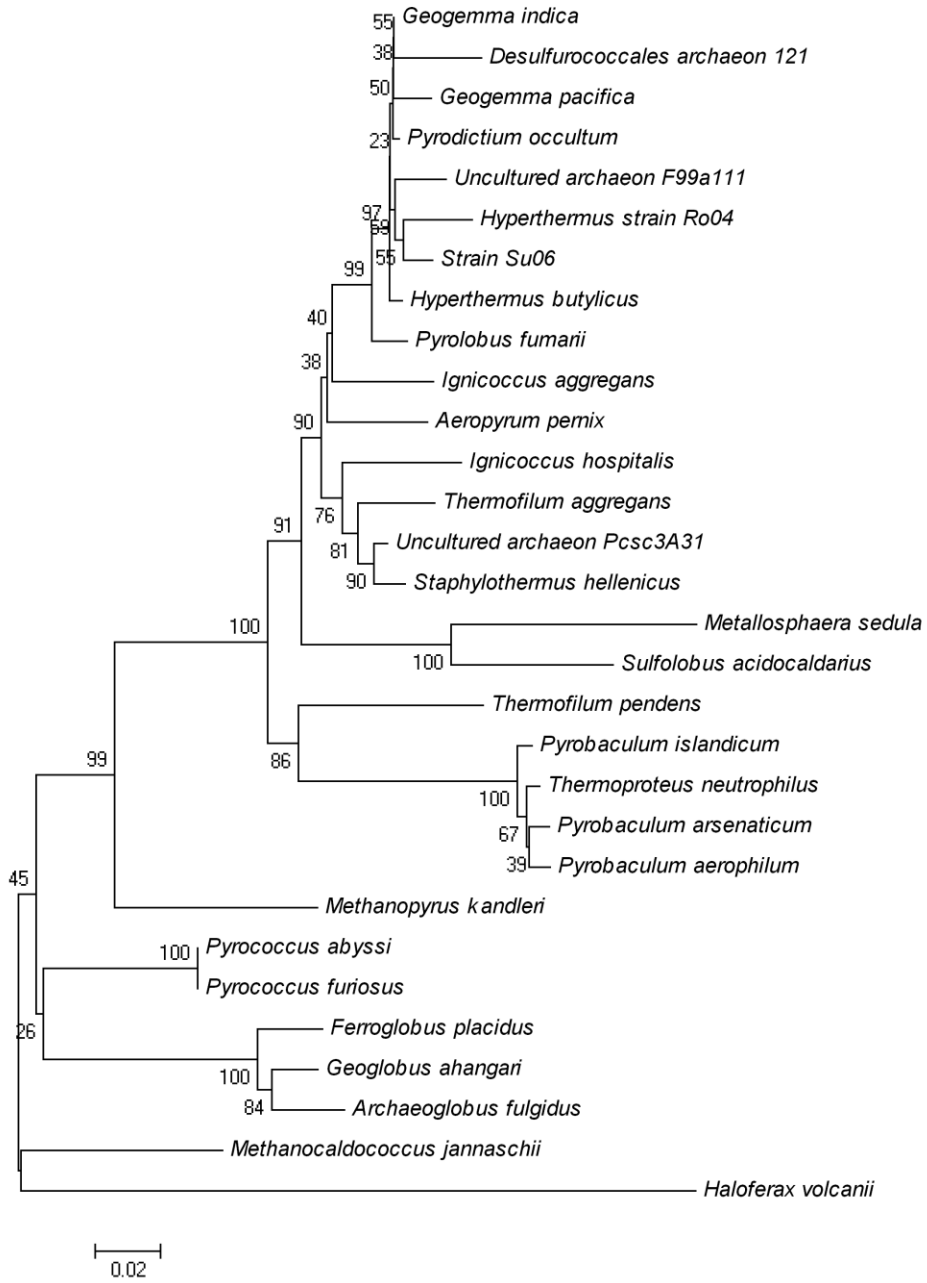


**Figure 5.** Phylogenetic tree showing hyperthermophilic archaea species. Sully is represented as "Strain Su06." Numbers indicate bootstrap values, or the percentage of reliability between each branch. For this tree, because there are so few sequences (because of few strains and few pure cultures), we determine that bootstrap values of 50% or higher indicate that that section of the tree is correct. Used with permission from Jennifer Lin, UMass Holden Hyperthermophile Lab.

Su06, nicknamed "Sully" after the hydrothermal structure from which it was isolated, is an organism that has been recently discovered. It was one of several isolates from an individual venting structure of the same name from the Juan de Fuca Ridge in 2006. Due to its very recent discovery, little is known about Sully, and the organism does not yet have a scientific name. This project is focused on the chemosynthetic nature of Sully, particularly its metabolic interactions involving iron reduction. Sully is an obligate anaerobe, is especially tolerant of high temperatures, and is capable of reducing Fe(III) using the energy-generating metabolic process of dissimilatory iron reduction (DIR) (Figure 3), which requires direct attachment to iron in order for reduction to occur (Holden et al., 2011). At present, it is undetermined whether Sully belongs to the genus *Pyrodictium* or *Hyperthermus*. New 16S rRNA data suggests that it may be the third *Hyperthermus* species being studied in our lab, in addition to *Hyperthermus butylicus* and Roane (another hyperthermophile from a volcanic structure of the same name in the Endeavour Segment; named *Hyperthermus hephaistosi* in Figure 5).

*Discovery of the genus* Pyrodictium

The genus *Pyrodictium* was first discovered in 1983 by Karl Stetter and his colleagues, when six new species were isolated from a submarine solfataric field off of Vulcano, Italy (Stetter et al., 1983). They found that the optimum temperature for their coccoid isolates was approximately 105°C, and that their growth was stimulated by $H_2$ gas (Stetter et al., 1983). The researchers also found that the organisms were capable of fermenting carbohydrates, proteins, cell homogenates, acetate and formate. Isovalerate, isobutyrate, butanol and $CO_2$, and in the presence of sulfur, $H_2S$ was formed. The discoverers coined the genus after the extreme temperatures in their environment, literally meaning "fire-loving network" – they found these coccoid cells appeared to be entrapped in fiber networks (Stetter et al., 1983) (Figure 7).

*Discovery of the genus* Hyperthermus

The genus *Hyperthermus*, belonging to the family *Pyrodictiaceae* along with the *Pyrodictium* genus, was first described by Zillig et al. in 1990. Zillig et al. (1990) note that *Hyperthermus* is a novel genus within a major branch of archaea, and is distinctly different from other related

genera such as *Pyrodictium*. As of this writing, the only known member of the genus is *H. butylicus*, which was isolated from a submarine solfataric source at temperatures up to 112°C off the coast of the island Sao Miguel, Azores, near the mid-Atlantic ridge in the northeastern Atlantic ocean. *H. butylicus* is known to grow up to 107°C, and gains energy by reducing sulfur and fermenting peptides. Additionally, Zillig et al. (1990) found that *H. butylicus* was the first archaeon for which fermentation products were identified. The genome of *H. butylicus* was first mapped by Brugger et al. (2007), who also confirmed that sulfur reduction to $H_2S$ is an important energy-generating process for the organism. *H. butylicus* is one of the many organisms being explored in the Holden lab along with Sully and Roane, the latter of which was confirmed to also possess fermentative growth properties.

*Sully Sample Collection Site*

The Juan de Fuca Ridge is an area of seafloor spreading located in the Northeastern Pacific Ocean, off of the coasts of British Columbia, Canada and Washington State, USA where the Juan de Fuca and Pacific plates diverge. The Endeavour Segment is one of the best-studied deep-

sea hydrothermal systems, and has numerous massive sulfide chimneys that are currently forming (Ver Eecke et al., 2009). This remarkable system is home to expansive hydrothermal fields, as well as many smaller fields and diffuse-flow sites, all of which are situated above magma chambers. More than 800 individual active and extinct edifices have been documented in the central area of the ridge, with some chimneys reaching 45m tall and 50m wide. This extraordinary ridge site is the only one in the world where seismic activity has been directly correlated to heat flux at the individual vent field scale. The faults within the rift zone are used by both magmas and upwelling hydrothermal fluids, where there is significant chemical disequilibrium. The greatest heterogeneity is found near the base of the western axial valley wall, where a myriad of various basalt types occur just meters apart. This environmental diversity creates a prime location for the establishment of a rich hydrothermal ecosystem (Kelley et al., 2012).

Microbial life in the Endeavour system is diverse and highly influenced by sediments. Fe(III) is one of the most naturally abundant terminal electron acceptors in subsurface environments. Previously it was believed that Fe(III) reduction to Fe(II) was an abiotic process, resulting

from changes in pH and/or redox potential (Luu and Ramsay, 2003). The abundance of Fe(III) in hot sediments surrounding hydrothermal vents likely led to Fe(III) reduction as an important process in these environments (Kashefi and Lovley, 2003). Previous analyses of organisms inhabiting the hydrothermal zone found that the exterior of the structure is a mixed community of bacteria and archaea, while the interior is dominated by archaea. The Endeavour system has high numbers of organisms under the order *Desulfurococcales*, which include hyperthermophiles that reduce Fe(III) oxides. Microbes within the order *Thermococcales* were found in lesser numbers, with few to no *Methanococcales*, or methanogens. The low concentrations of $H_2$ at this particular venting site may explain the dominance of Fe(III) oxide reducers and lack of methanogens. The microbial community is mainly fueled by sulfur oxidation coupled to $CO_2$ fixation, as well as $N_2$ fixation, indicating that nitrogen reduction is also occurring in Endeavour's subseafloor. Previous studies have shown that community diversity decreases with decreasing reactivity of the sulfide component and increasing presence of alteration products, and that weathered minerals are predominantly colonized by Fe-oxidizing organisms. These data

demonstrate that mineral-oxidizing microbes have a significant effect on the weathering of seafloor sulfide deposits (Kelley et al., 2012).

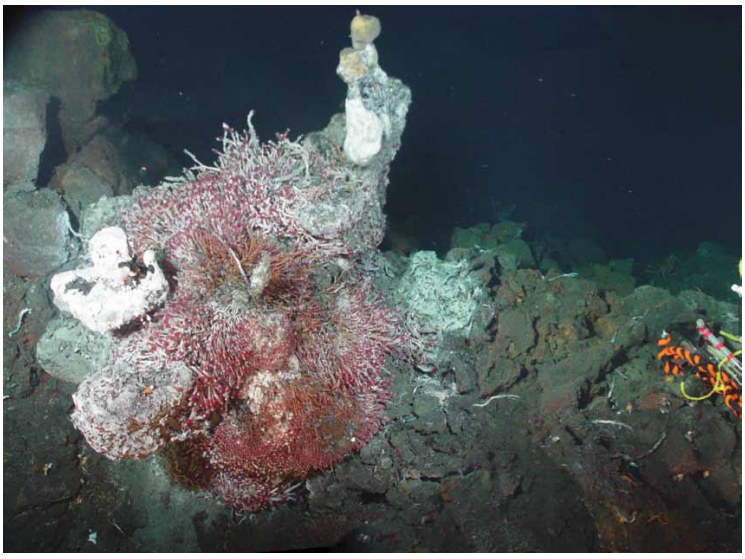

**Figure 6.** Hydrothermal structure known as Sully showing multiple black smoker orifices. Main Endeavour Field, Endeavour Segment of the Juan de Fuca Ridge (Kelley et al., 2012).

Sully, the specific edifice from where the original Su06 samples were collected, is shown in Figure 6. Sully, found in the Bastille Complex in the southern region of Endeavour, is an intensely dynamic hydrothermal structure, with many fluctuations occurring in its venting temperature and chemical composition in the past few decades. A 1999 diking event, where melt pulse and seismic activity increased vent fluid temperatures, chimney growth, volatile concentrations, and flocculent output affected hydrothermal venting sites within the region. Since 2000, Sully has shown a significant decrease in venting temperature -- In 2000, it was venting fluids of up to 380°C, and was down to 230°C when it was

last sampled in 2008. Furthermore, $CO_2$ concentrations at Sully are approximately half of those prior to the 1999 diking event. Current hydrogen concentrations at Sully are 10 times less than they were in 1995, and $CH_4$ dropped dramatically in half after the 1999 event, and continuously rose and fell until 2004, when it began to steadily increase. High $CH_4$ concentrations are associated with elevated ammonia levels, which may indicate the decomposition of the organic material in sediment buried within the volcanic edifice. In Sully, the dramatic decline in $CH_4$ levels may point to increased thermogenesis rates of the buried organic carbon induced by the intruded dike (Kelley et al., 2012).

## *Imaging Sully*

*Pyrodictium abyssi*, a potential close relative of Sully that was extracted from the Mid-Atlantic ridge at a depth of 3600 meters was studied by Rieger et al. in 1995. By utilizing both scanning and transmission electron microscopy, Rieger et al. found that *P. abyssi* cells grow in flake formations in the lab, and upon closer examination discovered that the cells are connected through a network extracellular tubules (Figure 7). The colonies of cells, appearing as flakes in lab culture medium, were found to be 1-10mm in size, and exhibited vigorous growth in high temperatures (Rieger et al., 1995).

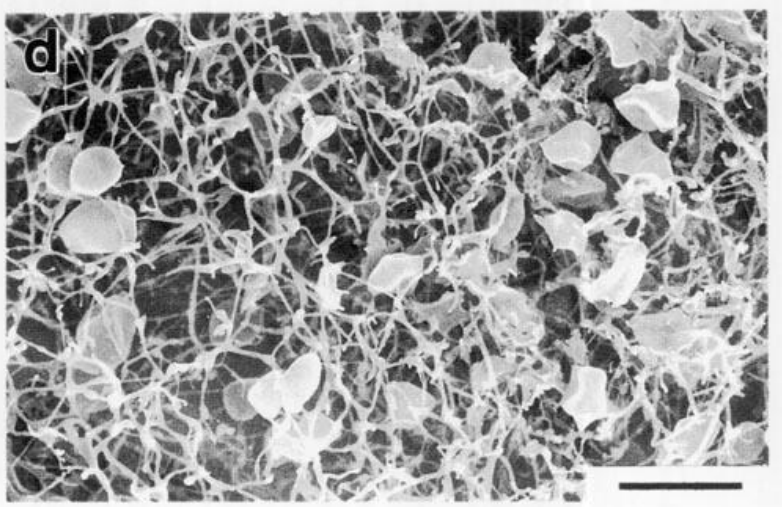


**Figure 7**. Scanning Electron Micrograph of a well-preserved, three-dimensional network structure of *Pyrodictium* cells, consisting of cells and tubules. Image shows irregular, non-spherical shape of cells. Scale bar 3μm (from Rieger et al., 1995).

On the *Hyperthermus* side, in 1990 Zillig et al. imaged the hyperthermophile *H. butylicus*. Electron micrographs of the species (which

were also imaged in the TEM using a negative staining technique) are comparable to those taken of Sully cells. Flagella (denoted as pili in the subtext of the image in its original publication) are visible in Figure 8a, a "duplex form" of a cell is seen in 8b as in images of Sully. Figure 8d shows a cell with a "tail," likely made of cytoplasm, as seen in a few Sully pictures. Additionally, the *H. butylicus* cells do not grow in networks like *Pyrodictium* species do, thus giving evidence that Sully is likely a member of the *Hyperthermus* genus.

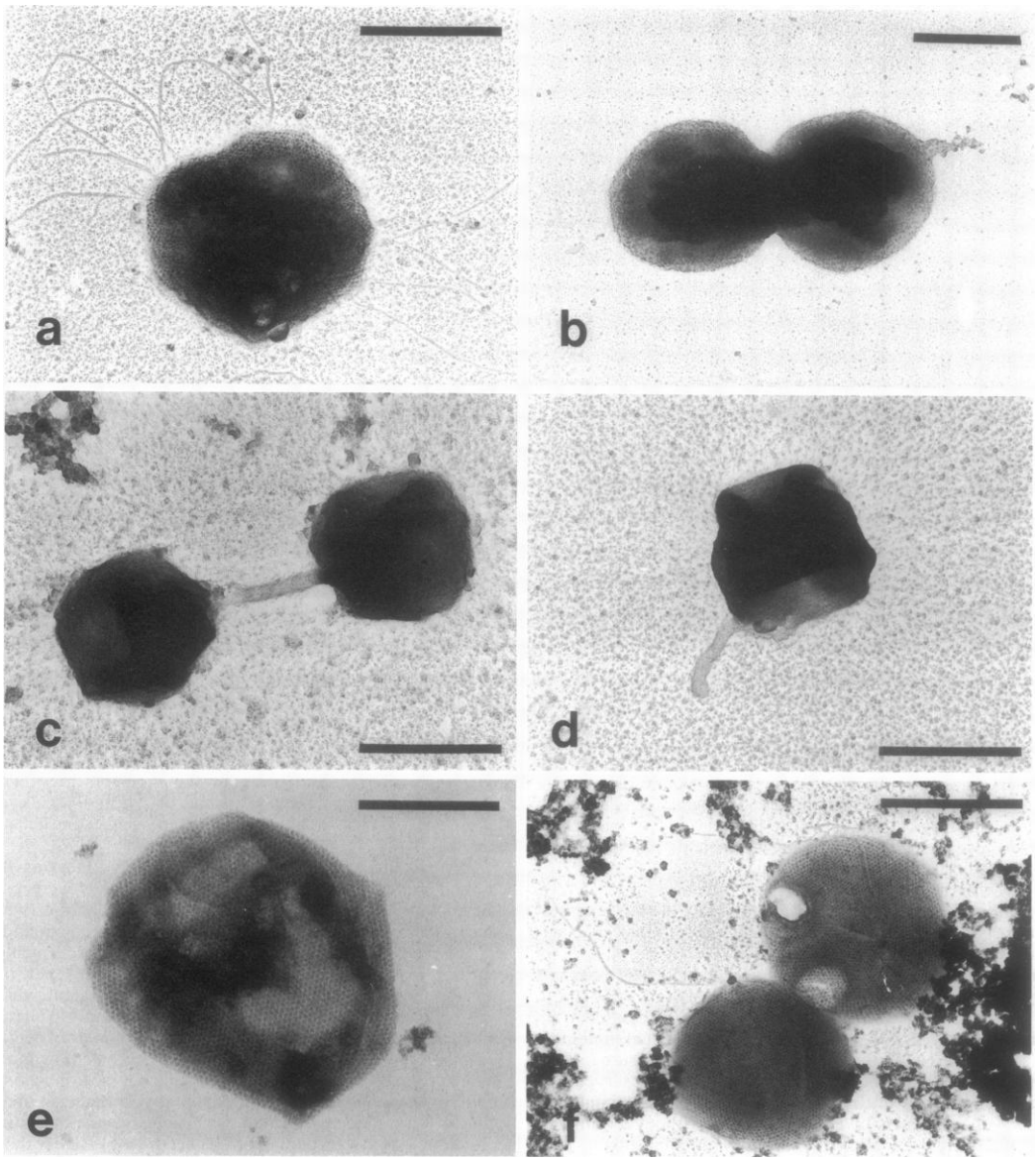


**Figure 8.** Electron micrographs of *H. butylicus*: a, Single cell with pili; b, duplex form; c, cells connected by a string of cytoplasm; d, cell with a tail; e, ghost exhibiting an S layer; f, duplex form exhibiting an S layer and vacuoles. Scale bar = 1.0μm (from Zillig et al., 1990).

# METHODS

The experimental design for testing the growth conditions for Sully was based on trying to determine the upper and lower limits for ideal temperatures at which the cells would flourish. A normal experiment consisted of running two different temperature experiments in two separate ovens at the same time. Two replicate test tubes were taken out of each oven at each of the six time points. The time points ranged from 4-7 hours. For a normal experiment, two 50mL bottles of startup media and 24 10mL test tubes were prepared (Table 1). The 24 test tubes were divided in half for the two growth curves, so that 12 would be controlled under one temperature and 12 under another. Both the startup and test tube media were incubated at 95°C for one hour before inoculation to control for any bacterial contamination of non-hyperthermophiles in the media.

After completion of the time points, the samples were analyzed utilizing a ferrozine assay to determine Fe(II) concentration and acridine orange staining for counting cells with epifluorescence microscopy using a mercury bulb. Using these methods, the doubling time at each

temperature was calculated based on the regression line for the data from cell concentration over time. Fe (II) production (mM) was also calculated over time and compared to cell concentration to determine a linear growth relationship for the various temperatures. All data analysis was done primarily in Kaleidagraph and Microsoft Excel secondarily.

### *Sully Growth Medium Preparation*

Startup media was usually prepared in batches of 350mL, with a pH of 6.8 (Table 1). Fe Gel (poorly crystalline Fe(III)oxide that is mostly made up of ferrihydrite) was added to all media used for experiments in this thesis. 50mL of media was allocated to two serum bottles each and 240 mL divided among 24 Balch test tubes, all sealed with butyl rubber stoppers. The bottles were vacuumed for an hour on a glass manifold, and then the headspace in each was flushed with $H_2CO_2$ gas. The bottles were incubated at 95°C for one hour for sterilization purposes, at room temperature for half an hour, and then inoculated with 0.5mL of 50mM cysteine, and 0.3mL of 250mM $FeCl_2$. Each bottle was also inoculated with 0.5mL Su06 startup culture from a previous experiment that had been grown under logarithmic growth phase conditions. The serum bottles

were then over-pressurized for 10 seconds each with $H_2CO_2$ gas, and incubated at 85°C for 24 hours. The test tubes were vacuumed for 10 minutes, flushed with $H_2CO_2$ gas, and incubated at 95°C for one hour. Then each tube was inoculated with 0.25mL startup culture, 0.1mL of 50mM cysteine and 0.05mL 250mM $FeCl_2$. After being inoculated, each Su06 tube was over-pressurized with $H_2CO_2$ gas for 10 seconds. The Su06 test tubes were then divided equally into two incubators with different controlled temperatures, depending on the experiment. Two Su06 test tubes were pulled from each incubator every 4-7 hours over the course of 24-42 hours, depending on the time-point length being controlled in that particular experiment.

**Table 1.** Components of a 350mL batch of Sully growth medium.

| Ingredients | Per 350 mL |
|---|---|
| dH20 | 270mL |
| Fe Gel | 35mL |
| 10x Marine Salts | 35mL |
| DL Vitamins | 3.5mL |
| Trace Elements (Wolf's Mineral Solution) | 3.5mL |
| Sodium bicarbonate | 0.875g |
| Yeast Extract | 0.07g |

### *Ferrozine Assay*

To measure the Fe(II) oxide concentration within each test tube and rate of Fe(II) production over time, a ferrozine assay in the spectrophotometer was performed on a sample from each test tube. In a normal experiment, 27 5mL bottles were prepped with 1.9mL oxalate inside the anaerobic chamber filled with argon. Here, oxalate is used to dissolve Fe(II) oxide so that it can be measured in solution. 0.1mL of media from each of the 24 test tubes was injected into 24 of the oxalate bottles. 0, 50 and 100μL of ferrozine standard were injected into the remaining 3 bottles to use as standards. These standards allowed for the calculation of a standard curve. After allowing the bottles to incubate at room temperature for one hour, 4.9mL of ferrozine solution was put into small glass test tubes, and 0.1mL of the oxalate-media solution was injected into the tubes from each 5mL bottle. After measuring each ferrozine standard, the contents of the small test tubes were poured into cuvettes and measured at 562nm in a spectrophotometer.

### *Epifluorescence Microscopy*

In order to obtain a cell count sample for each time point, cell samples from each test tube in the experiment were stained using the acridine orange (AO) staining protocol. AO is a fluorescent dye, with an excitation wavelength of 490nm and an emission wavelength of 526nm (Knutson, 2010). The dye stains DNA within the cells, but can also have nonspecific binding to debris within the cultures. Exactly 0.25mL of 37% formaldehyde was placed in 24 eppendorfs, and 0.5mL of culture was injected into each eppendorf. 3mL of oxalate was put into plastic screw-top test tubes in order to dissolve iron. The contents of each eppendorf were then pipetted into each plastic test tube, and were incubated at room temperature for an hour. The cultures were then pipetted onto 0.2μm filters within steel columns on top of a vacuum. Two drops of AO were added to each filter, and after 3 minutes the filters were washed with oxalate and DI water, vacuuming in between each wash. The columns were then removed, and the filters placed onto glass slides that had been pre-treated with oil. Another drop of oil was added to the filter, and a coverslip was applied to each. All slides were stored in the dark until they were examined under the microscope. After completing the staining

protocol, the Su06 cells were stained and viewed under the microscope using the mercury bulb and oil-lens at 100x magnification. The cells within 10 viewing planes were counted for each of the 24 Su06 samples and then averaged.

### *Scanning Electron Microscopy (SEM)*

Samples were viewed at Mount Holyoke College in an FEI Instruments Quanta 200 environmental scanning electron microscope in high vacuum mode. In high-vacuum mode, the pressure of the sample chamber is reduced to $<10^{4}$ torr. The absence of gas molecules prevents reactions with the electrons in the beam, and thus clearer, more complete images of the sample can be taken. Soft, biological samples and medium-hard minerals and metals cannot be viewed under this pressure and require a lower voltage. As a result, low-vacuum SEM provides only surface details of the sample. In order to view a liquid medium such as that of the Sully culture under such enormous pressure, the samples must be dried, mounted and sputter-coated in gold prior to imaging in the SEM.

Exactly 0.5mL of Sully culture was extracted from an anaerobic test tube and dissolved in 0.5mL of oxalate. Two samples were taken: one

from the top to obtain images with as little as iron as possible, and one from the bottom of the eppendorf where some insoluble iron remained. A 1mL syringe was used to extract each sample, and then attached to a Pop-Top™ Filter Holder that contained a 0.2μm Nuclepore® polycarbonate membrane filter. The Sully culture was pressed through the filter until it was well-coated. A syringe was filled with 2.5% gluteraldehyde in 0.1M phosphate buffer (pH 6.8) as a fixative. Exactly 1mL of this fixative was pushed slowly and continuously through the filter for 50 minutes, with approximately 0.1mL of fixative pushed through every five minutes. Each filter was then dehydrated in a graded series of ethanol. The filter was immersed two times with 1mL of ethanol for five minutes each in 30%, 50%, 70%, 90%, and 95% ethanol, and three times in 100% ethanol. After the 70% ethanol wash, the filters were removed from the syringes and placed in the holder for the critical point dryer which was then immersed in 90%, 95% and 100% ethanol. These were layered as follows: washer, filter with culture 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 $CO_2$ (Knutson, 2010).

After the critical point drying process was complete (approximately one hour), a Polaron 5100 Sputter Coater was used to coat the samples in gold, for 75 seconds at a voltage of 2.5kV and at a pressure of 0.07mbar. Once the mounted samples were coated in gold, they were placed in the sample chamber of the SEM, and the chamber was vacuumed to $<10^4$ torr. The image was adjusted according to variables including voltage, electron beam spot size, magnification, working distance, convergence angle (depth of field), current (brightness), and contrast in order to get the highest quality images possible.

### *Transmission Electron Microscopy (TEM)*

Samples were viewed at Mount Holyoke College on a Philips CM100 Transmission Electron Microscope at 80kV. Formvar Carbon support film 200 mesh grids (stabilized with silicone monoxide) were used to mount the sample, and a negative stain technique was used to prepare the Sully cultures for the TEM. In advance of the negative staining procedure, the carbon grids (if they had been used previously) were exposed to ultra-violet light for three hours, four-six inches away from the light source, in order to sterilize them. Brand-new carbon grids did not need to be exposed to UV light. We experimented with two different

preparation methods, one using oxalate to dissolve iron in the culture and one without oxalate that retained iron in the sample. For those samples that were dissolved in oxalate prior to sample preparation, Sully cultures were dissolved in a 1:1 oxalate culture in eppendorfs. Two samples were taken from the eppendorf: one from the top to obtain images with as little as iron as possible, and one from the bottom where some insoluble iron remained.

Aqueous 1-2% uranyl acetate (a form of uranium which has been depleted of radioactivity but is still harmful when in contact with skin) was used as the negative staining medium. A droplet of the Sully culture was extracted from the anaerobic test tube and dissolved in a 1:1 oxalate culture in an eppendorf. Two samples were taken: one from the top of the eppendorf to obtain images with as little as iron as possible, and one from the bottom where some insoluble iron remained. A droplet of each sample type was placed on a piece of Parafilm® M laboratory sealing film, and a carbon grid was floated on top of the droplet for one minute. Using fine tweezers, the carbon grid was removed from the Sully droplet at an angle (so as to keep the coating of the culture intact), and then blotted gently with the edges of a small piece of filter paper. The carbon grid was then

transferred to a drop of uranyl acetate for one minute, blotted again, and left in a petri dish to dry with the sample coating facing upwards. After drying for 2-3 minutes, the samples were mounted inside the sample holder of the TEM.

### *16S rRNA Genetic Analysis*

Sully was grown in 50 ml of medium and diluted 1:1 (vol/vol) with a marine TPE buffer, which included Tris pH 7.0 (100mM), EDTA (10mM), and sodium phosphate (300mM) to prevent osmosis. The Fe(III) oxide in the medium was dissolved using 1:1 (vol/vol) filter-sterilized oxalate solution. The cells were spun at 4000 rpm for 1 h, and genomic DNA was extracted using a Power Soil DNA Isolation kit (MoBio). The entire 16S rRNA gene sequence was amplified using archaeal primers 4aF (5'-TCC GGT TGA TCC TGC CRG-3') and 1391R (5'-GAC GGG CRG TGW GTR CA-3'). PCR mixtures were adapted from those described previously. Each 30 μl reaction included 3 μl of 10× KlenTaq Mutant Reaction Buffer (DNA Polymerase Technology), 2.5 mM of dNTP, 10 μM of each primer, and 0.1 μl of Omni KlenTaq (DNA Polymerase Technology). The PCR started with an initial denaturation step at 96°C for 5 min, followed by 30

cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min 30 sec, and a final extension at 72°C for 6 min. PCR products were run and excised from a gel using a QIAquick gel extraction kit (Qiagen). Sequencing was performed at the University of Massachusetts-Amherst Sequencing Facility. The resulting sequences were aligned using ClustalW in BioEdit, and compared to sequences in GenBank using BLAST. Aligned sequences were imported into Mega 4.0 and plotted into a phylogenetic tree. Bootstrap analysis was performed in Mega 4.0 with 1000 replicates (Adapted from Jennifer Linn's laboratory methods, used with permission)

# RESULTS

## *Growth Rates and Conditions*

Sully has a particularly narrow range of growth conditions, and does not react well to altered environmental variables. Temperature was essentially the only variable that could be controlled in experiments to get any sort of comparable growth rates. For example, Sully would not grow on any other Fe(III) oxide besides ferrihydrite (Table 2), or without the presence of an Fe-Gel and an $FeCl_2$ inoculum in the growth culture.

After a given growth curve conducted at an experimental temperature was complete, we measured the growth rates of Sully by calculating cell concentration (which yielded doubling time in hours) and Fe(II) production using ferrihydrite, an Fe(III) oxide, as an electron acceptor. In general, the rate of cell doubling and Fe(II) production increased over time in most experiments, but their rates were dependent on how optimal that particular temperature is for Sully. Here we examine and discuss the indicators of growth optimality.

*Calculation of Doubling Time*

In order to determine the optimal growth temperature for Sully, we calculate the rate at which the cell concentration is doubled. Doubling time is calculated based on cell concentration data gathered from the epifluorescent microcopy counting technique. These values are then plotted against their incubation time, and the data are fit with an exponential trendline (Figure 9). In Figure 9, the last two data points from the final time point are excluded from the trendline, because at this point the cells have clearly stopped growing, have entered stationary phase, and will subsequently enter death phase. From the trendline equation, doubling time is calculated by taking the natural log of 2 and dividing by the exponential coefficient. For instance, doubling time for the cell concentration data displayed in Figure 9 would be calculated with the following equation:

Line equation: y = 3.0408e+06*e^(0.16389x)

[ln(2)]/0.16389 = **4.23 hours = doubling time for Sully at 85°C.**

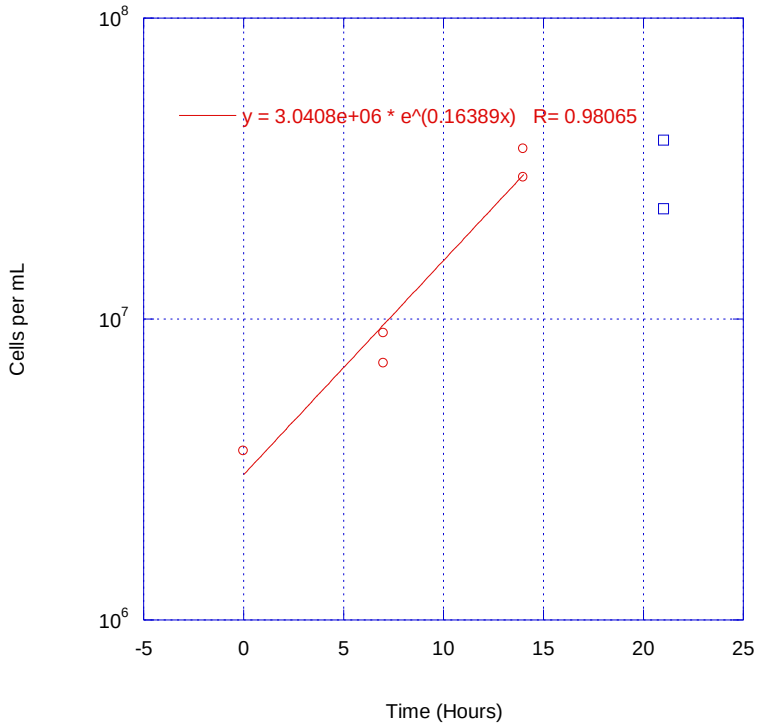


**Figure 9**. Example of Sully cell concentration data plotted against incubation time for one experimental growth curve grown at 85°C. Red trendline shows line of best fit for the data. Equation of the line and R value are displayed.

*Fe(II) Rate of Production*

In addition to cell concentration (Figure 9), Fe(II) production was also measured, which determines the rate at which Sully reduces Fe(III) to Fe(II). Figure 10 shows the exponential increase of Fe(II) over time in a growth curve for Sully at 85°C. More Fe(II) is being produced the longer the culture is incubated at this temperature. The last two data points are eliminated from the trendline calculation because the cell has entered

stationary or death phase and is no longer producing Fe(II) at an exponential rate.

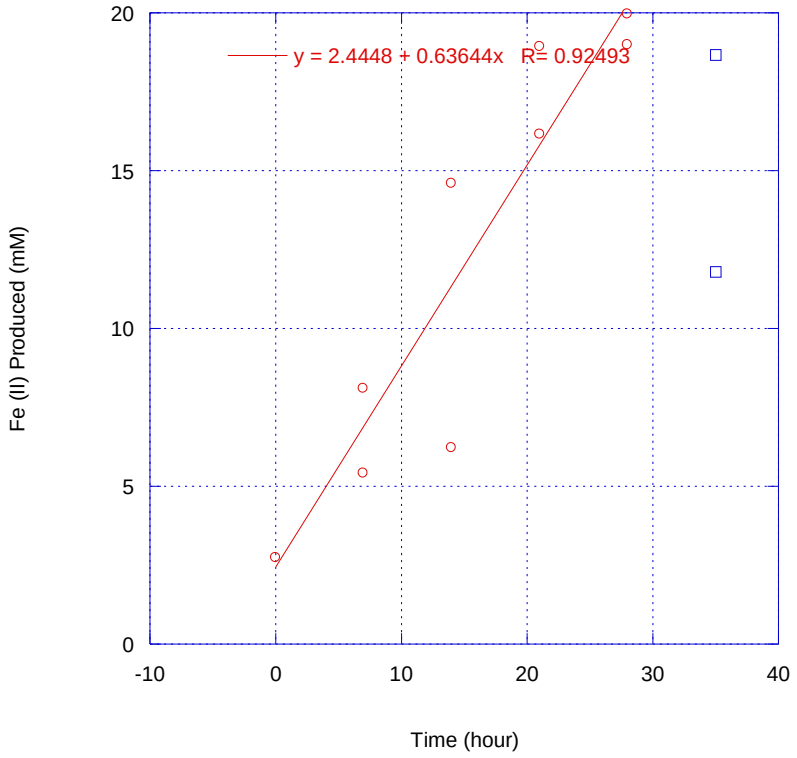


**Figure 10.** Example of Sully Fe(II) production (mM) data plotted against time for one experimental growth curve grown at 85°C. Red trendline shows line of best fit for the data. Equation of the line and R value are displayed.

*Doubling Time Data*

All doubling time data from each growth curve were collected and plotted against the experimental growth temperature. Overall trends are visible: Sully grows optimally between 78°C and 92°C. However, there is some variation within this optimal range. Figure 11 shows a consolidated

version of Sully growth curve data, with outliers (unexpected data points at that temperature) removed. A clear trend is visible – doubling times between 78°C and 92°C all fall below 10 hours. Figure 12 analyzes this optimal range in-depth, and it is clear that the 87°C time point is somewhat of an outlier as compared to the rest of the more uniform points in the range.

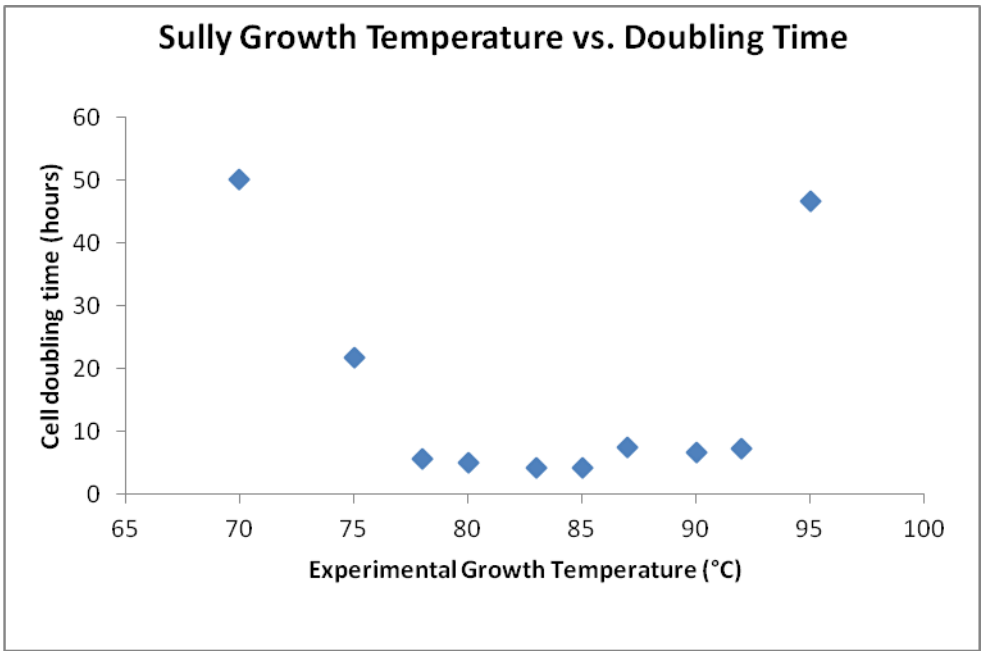


**Figure 11**. Consolidated version of all growth data – excludes all outlier points which skew the overall temperature range for optimal growth. A more defined U-shape is visible, with lower doubling times indicating faster cell growth and more optimal temperatures.

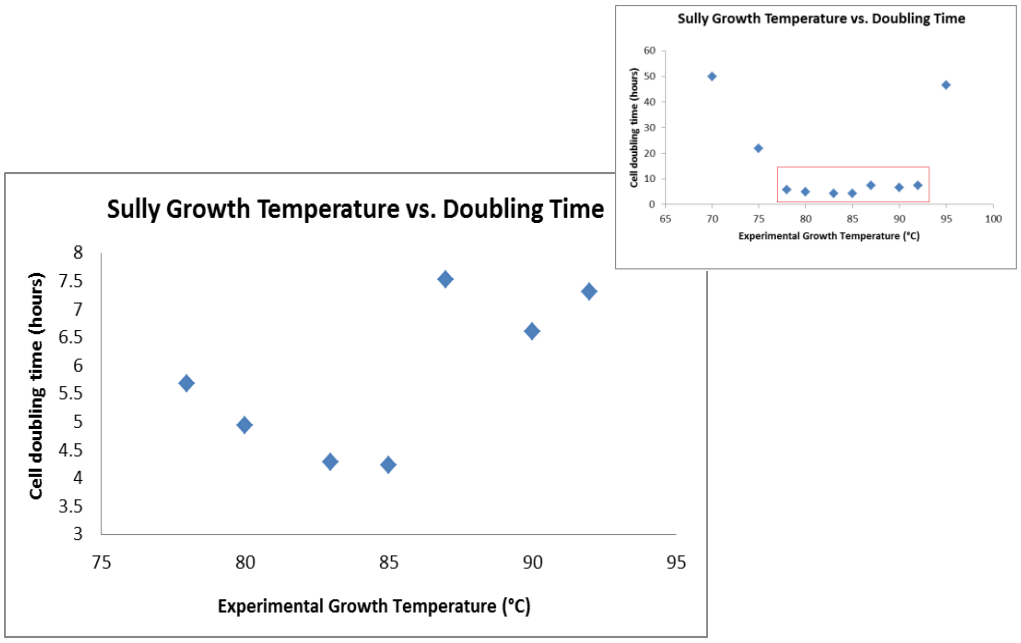


**Figure 12.** Data for temperatures between 78°C and 92°C, the ideal temperature range as defined in the previous graphs. When zoomed in on this portion of the data, the point at 87°C appears to be an outlier and skews the curve of optimal growth temperature.

*Comparison of Growth Rate and Fe(II) Production*

Figure 13 shows a comparison between Sully's growth rate and Fe(II) production per cell per second at each experimental temperature. According to the graph, the optimal growth for Sully appears to be between 80°C and 85°C. The outlier at 87°C, as discussed above, is depicted here and appears to skew the shape of the curve. Ideally, the 87 point would fall somewhere between 0.1 and 0.15 $k^{-1}$. The graph depicts an inverse relationship between growth rate and Fe(II) production. Here,

Fe(II) production is measured per cell per second, which is different compared to the Fe(II) production data from each individual growth curve, as depicted in Figure 10. Because there are more cells present in the cultures at the optimal growth rate, there is less Fe(II) production per cell. When there are fewer cells, the Fe(II) production rate is higher per cell. From these data we can conclude that Sully is producing less Fe(II) per cell per second when it is growing at a more optimal temperatures.

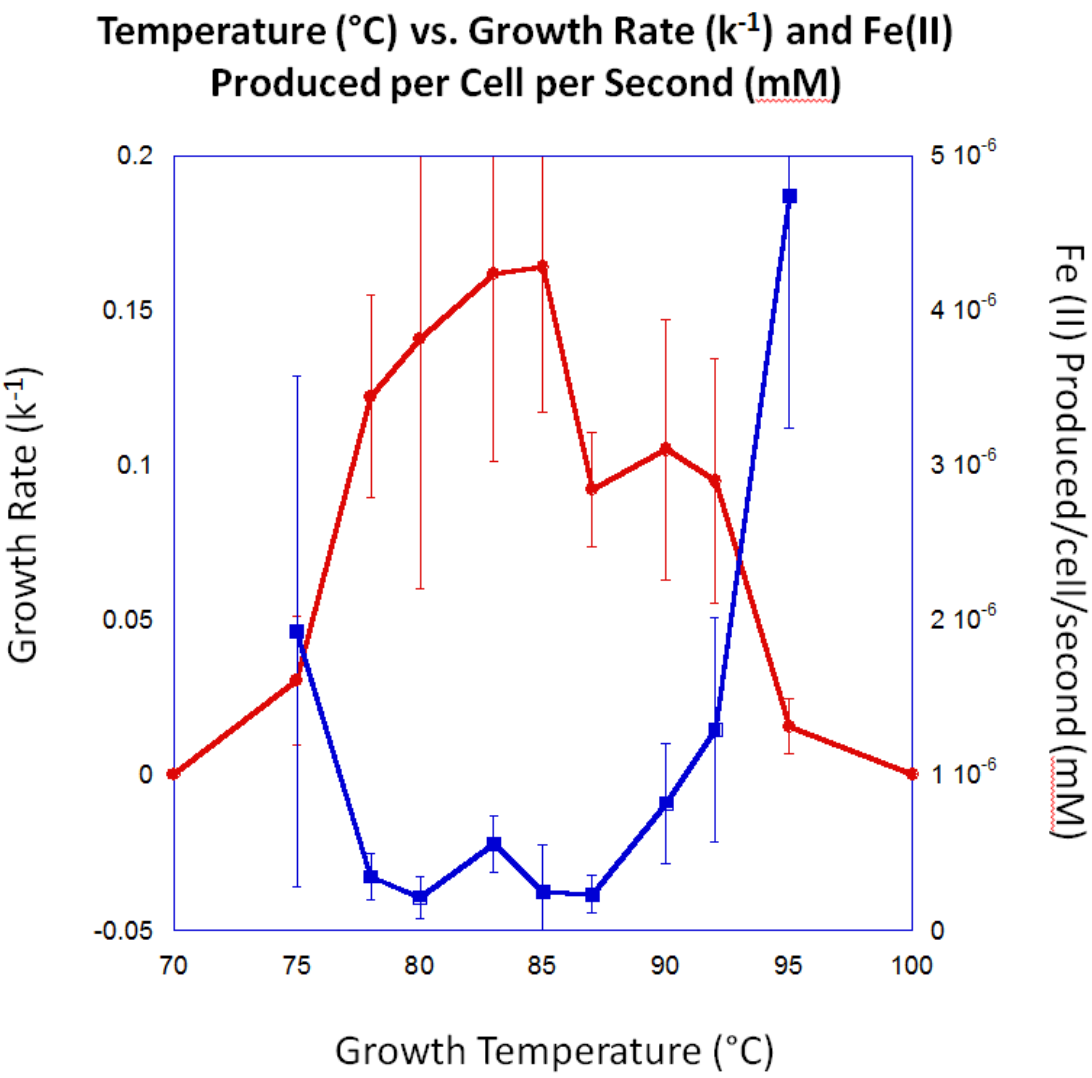


**Figure 13.** Growth rate ($k^{-1}$) is shown in red; Fe(II) production (mM) is shown in blue. Red point determines the growth rate at that temperature, and each blue point determines the Fe(II) produced per cell per second at that specific temperature.

## *Growth of Strain Su06 on different types of Fe(III)oxide*

**Table 2.** Results of experimental growth of Sully on various types of Fe(III) oxides. From the table it is clear that Sully did not grow on any other Fe(III) oxides.

| **Fe(III)Oxide (10mM)** | **Transfer 1** | **Transfer 2** | **Transfer 3** |
|---|---|---|---|
| Lepidocrocite | x | x | x |
| Goethite | x | x | x |
| Hematite | x | x | x |
| Maghemite | x | x | x |

After another experiment in our lab was conducted on Roane (a likely close relative of Sully) to determine what other types of Fe(III) oxides it could grow on, we determined it would be interesting to do the same for Sully. However, over the course of working with this organism, we have found that it has very narrow growth conditions, and as such it did not grow at all on any other experimental types of Fe(III) oxides, including Lepidocrocite, Goethite, Hematite, and Maghemite through three transfers. (Table 2). As of this writing, Sully is known to be able to grow on ferrihydrite alone.

## *Scanning and Transmission Electron Microscopy*

From the images of Sully taken in the electron microscopes, we can ascertain several characteristics about the cell. First of all, we can tell that Sully is a coccoid that is about 1.0m in diameter. We also know that Sully has only one membrane, so when a cell was imaged that had two visible membranes (Figure 19a), it was clearly not a Sully cell. Flagella, a motility adaptation in microorganisms, are visibly connected to the cells in Figures 16a, 16b, 16d 16e, 17a, and 17c. Some cells that were imaged look healthy (appear darker, thicker), while others appear transparent and/or fuzzy. These cells are expericing lysing, a process in which the cell membrane begins to deteriorate in stationary/death phase, and begin to lose their structure. The images also show clear interaction with iron particles, although the images do not tell us which DIR processes Sully uses. However, the images do speak to the fact that iron particles appear to be directly attached to the cell. This interaction is more visible in the TEM images, while the SEM images also show some iron particles on the surface of the Sully cells. Figures 16b and 16d show invagination by iron particles, in which they are embedded in the cell membrane. Furthermore, Figure 16f is an image showing Sully with an appendage, similar to one

demonstrated by *H. butylicus* in Figure 8d. Overall, the images of the Sully cells are more similar in appearance to those of Hyperthermus species Roane (*H. hephaistosi*) and *H. butylicus* (Figures 8 and 14). Lastly, some contamination by flagellated bacterial rods was visible in the SEM images, possible causes and actions taken are outlined in the discussion section.

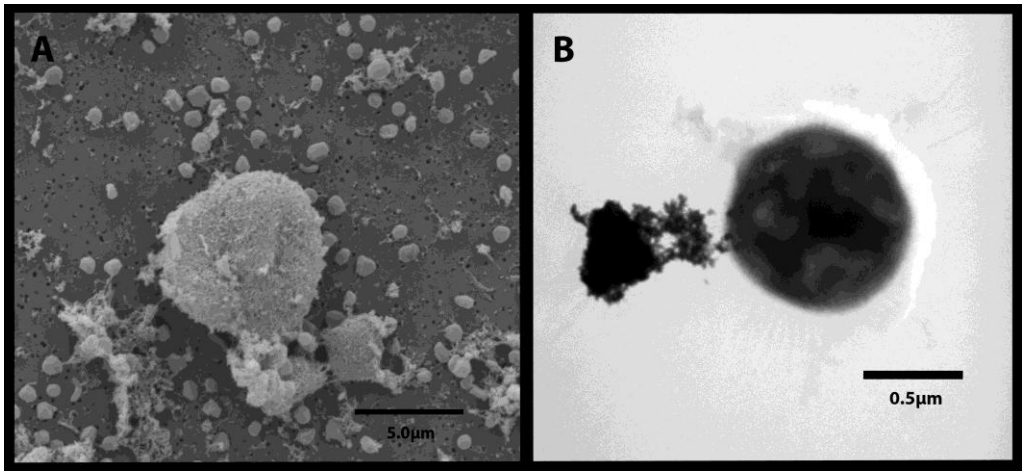


**Figure 14.** Electron micrographs of Roane (*H. hephaistosi*). Image A (scanning electron micrograph) depicts a large mass (potentially a cell, but not Roane) surrounded by smaller Roane cells. Image B (transmission electron micrograph) shows a close-up of a Roane cell interacting with iron particles.

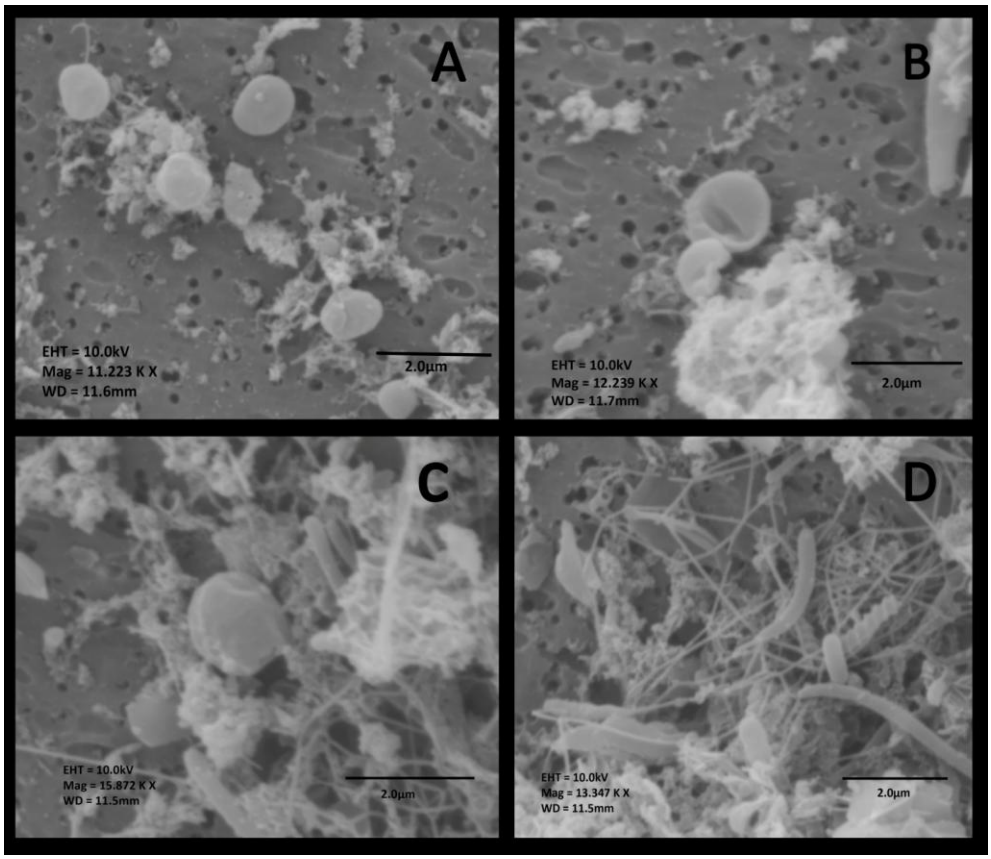


**Figure 15**. Image A shows a cluster of potential cells that all seem to be interacting with iron clumps but not with each other; some iron-cell contact is visible and cells look healthy. Image B shows a cell near a clump of iron, which may have a deteriorated cell clinging to it. Image C shows a likely cell with visible surface detail and possible iron attachment. Tubule-like structures are visible, which may be remnants of a former "network," but also may be caused by contaminating flagellated bacterial rods. Image D shows cluster of cells and contaminating rods in a network of tubules or flagella. Undetermined whether these are caused by the contaminating bacteria or the Sully cells. There appears to be two types of contamination, based on the presence of a corkscrew shaped cell in addition to the smooth rods.

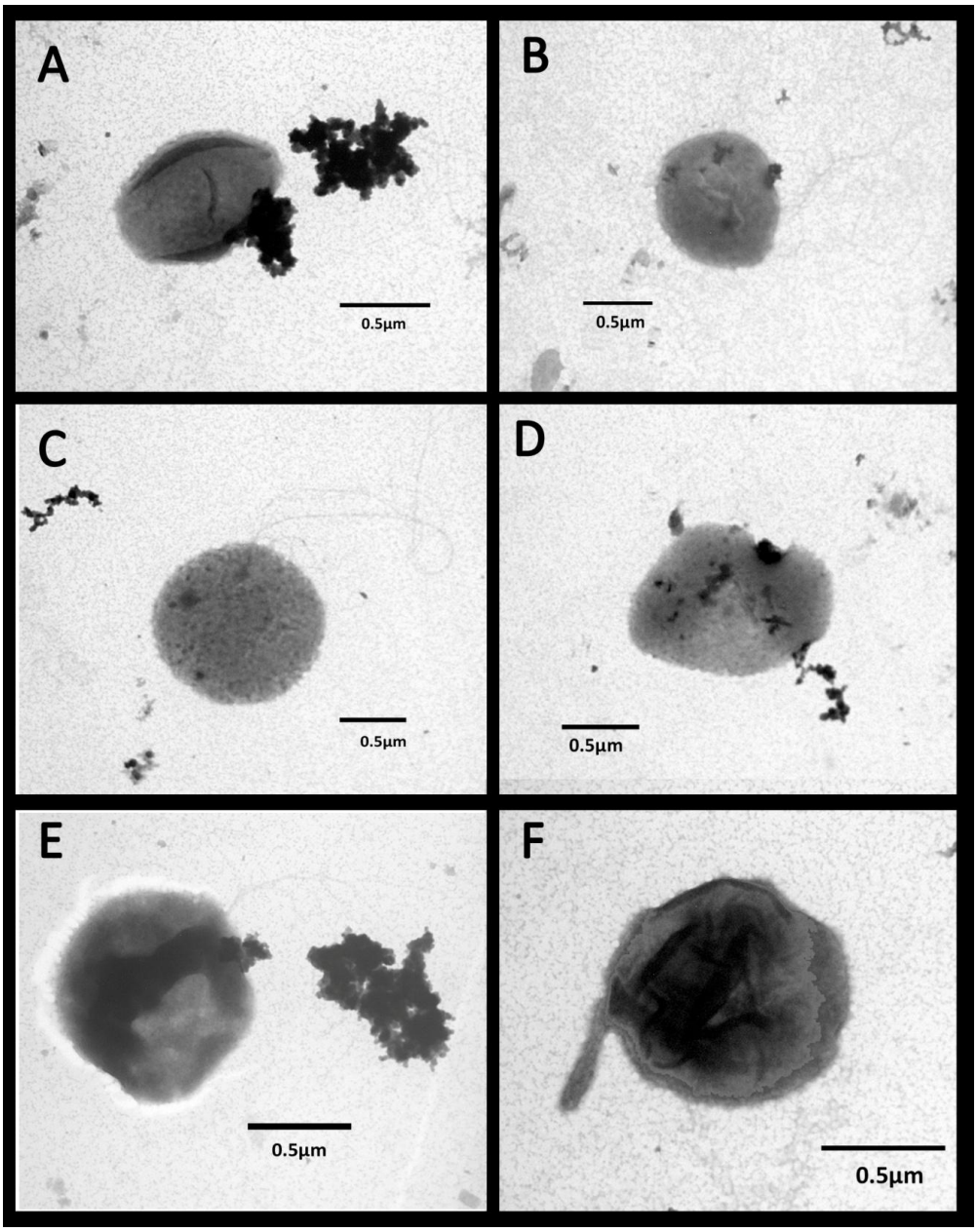


**Figure 16**. Cells A and B appear to have been growing in exponential phase; iron attachment and flagellation are visible, and cell walls appear intact. Cells C and D show fuzzy edges, indicating that they may be in distress and entering stationary phase. Cell C still has its flagella intact, while Cell D shows membrane invagination by iron particles. Cell E shows a clear connection between the iron clump and iron on the cell, as well as flagellation. However, it appears to also be fuzzy and losing its membrane. Cell F shows surficial cell detail and a possible appendage.

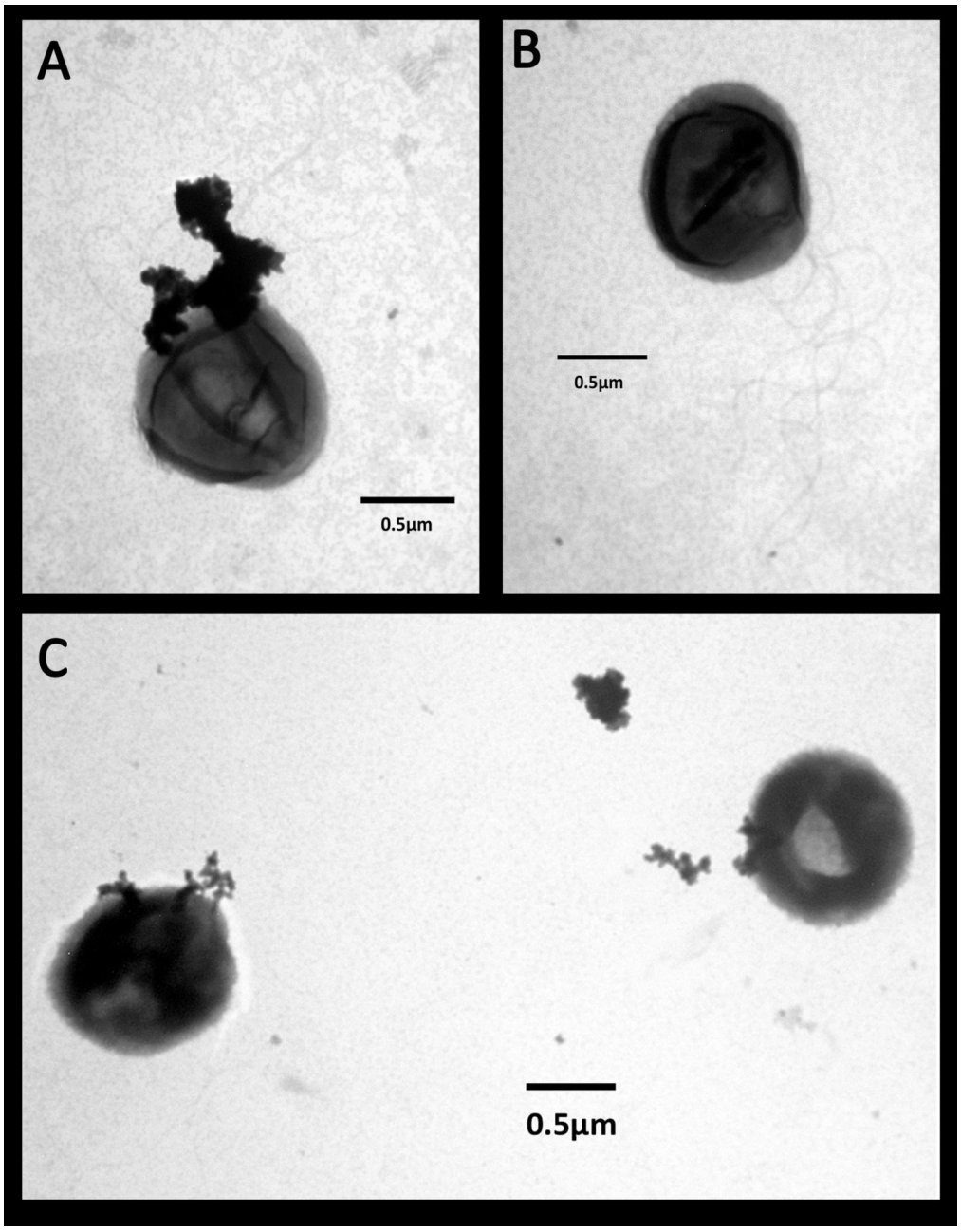


**Figure 17**. Cell A shows clear iron attachment, surficial cell detail, and flagellation. Cell B also has visibly distinctive flagella; both cells A and B look healthy. The cells in Image C appear to have fuzzy edges; despite showing iron attachment, they may be entering stationary phase.

# DISCUSSION

## *EM Preparation Techniques*

Most EM images were taken from samples that were taken at the fourth or fifth time point, so as to capture as many cells as possible. Images were taken from samples grown at various temperatures. Temperature was not an important control in visualizing iron contact with the cell. However, in the future it may be interesting to look at how cells from time points that are determined to be in stationary phase differ from those growing in exponential phase.

We found that the oxalate iron dissolution process prior to sample preparation for the TEM may be too stressful for the cells, as it appears to damage their structure and integrity. TEM images in Figures 16 and 17 in the results section were taken from samples that were not dissolved in oxalate prior to mounting. Figure 18 shows cells that were dissolved in oxalate prior to imaging. They appear to be warped and in distress as compared to their counterparts that were not dissolved in oxalate. We can conclude that this warping is not from conditions within the test tube,

because no similarly shaped cells were found in any of the TEM images that had not used oxalate to dissolve iron in the sample. For the SEM images, the oxalate technique did not appear to cause any damage to the cells.

Furthermore, Figure 19 shows two cells that were originally thought to be Sully, but were determined to be too large and must be a different type of cell. Cell A (TEM) is more than 20μm in diameter, and the ridges along its perimeter indicate that it has two membranes – Sully is known to only have one. Cell B (SEM) is smaller, and appears to be interacting with what looks like iron, but is also too big to be considered a Sully cell. This cell and others like it were imaged in the samples, and were similar in size and appearance to human red blood platelets.

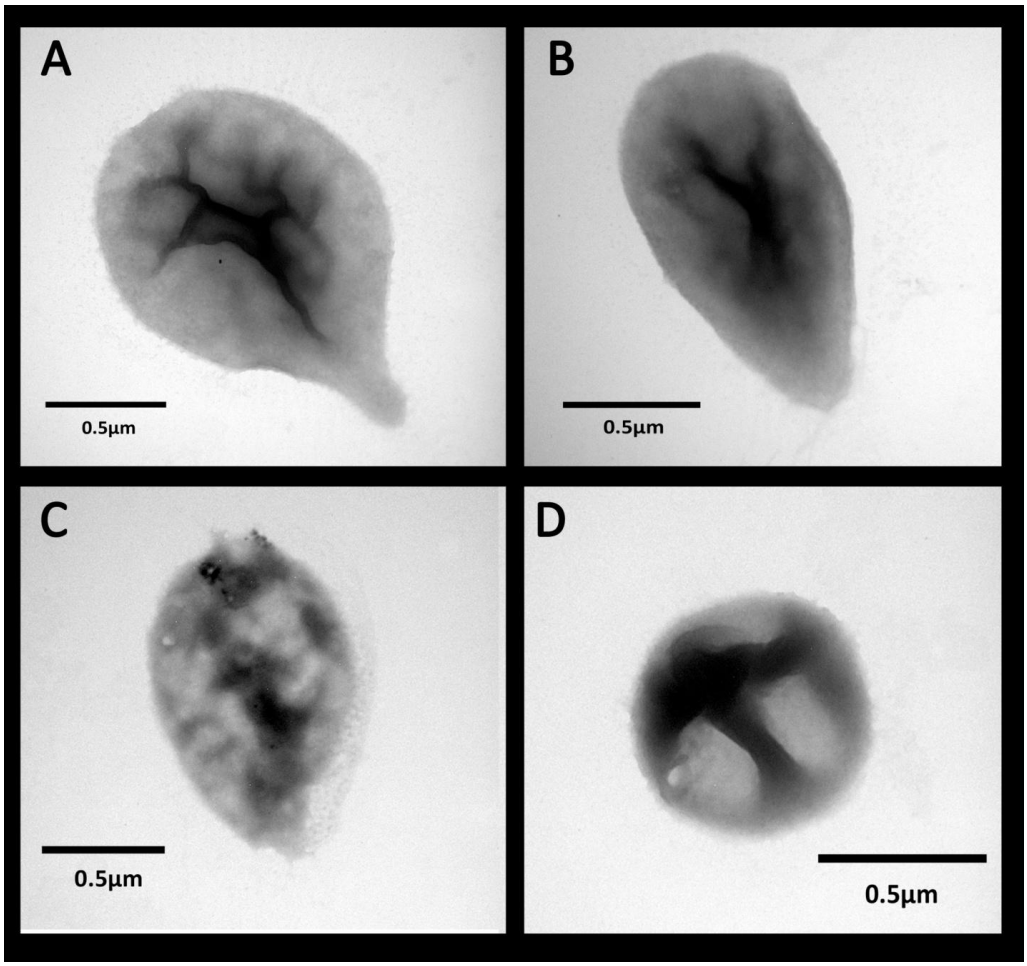


**Figure 18.** Cell A shows surface detail of the cell as well as warping/stretching that may be due to distressing oxalate technique to dissolve iron in the culture. Cell B shows this effect as well; cell is particularly elongated but still shows remnants of flagella. Cell C is a "ghost cell" – its membrane is being destroyed because it is dying, a process known as lysing. Cell D also shows fuzzy edges, a sign of distress and lysing - likely is in stationary phase. Cell C and Cell D may have experienced lysing naturally, or it may be due to the destructive oxalate technique.

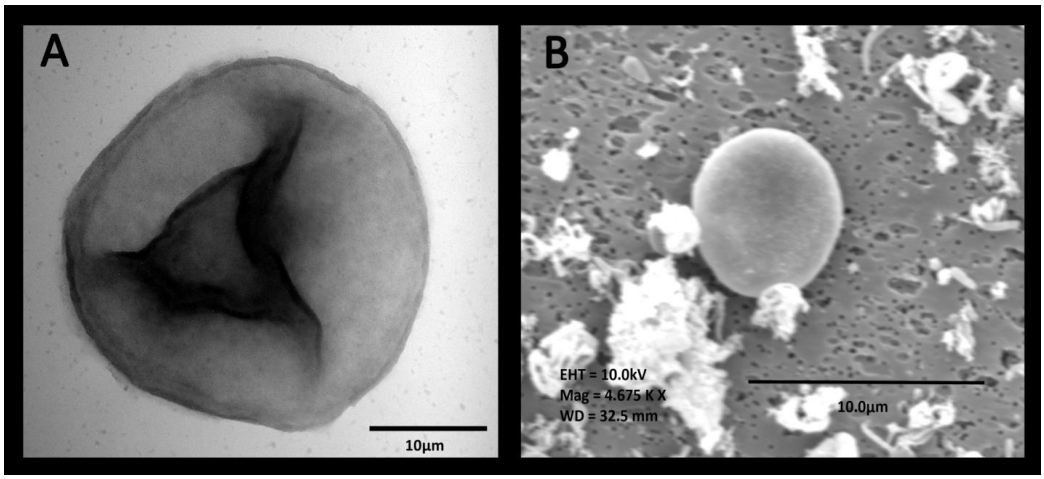


**Figure 19.** Two cells which are round in shape, but are not the right size to be a likely Sully cell. Cell A was taken in the TEM and Cell B was taken in the SEM.

## *Sources of Error – Contamination Problems*

Towards the end of the timeline for my experiments, my colleague Jenn and I noticed that some of my samples showed evidence of bacterial contamination (Figures 20, 15c, 15d). It is difficult to discern bacterial contamination from the regular debris found in the samples. However, once we spotted flagellated rod-shaped cells, we knew that the samples were probably contaminated. I went back through all of the 50mL startup cultures from the past year, AO-stained all of them, and checked for contamination. Every single startup culture showed some bacterial contamination, although the rods differed in size, shape and flagellation.

It is likely that the bacterial rod cells only started growing once the tubes were out of the oven, because they cannot withstand such high temperatures. It is possible that the bacteria grew on the remaining yeast in the cultures that had not been used by the cells during their exponential phase after the tubes had been removed from the high-temperature ovens. Thus, the Sully cultures that grew at those temperatures produce valid cell concentration and Fe(II) production data because they are largely unaffected by the contamination.

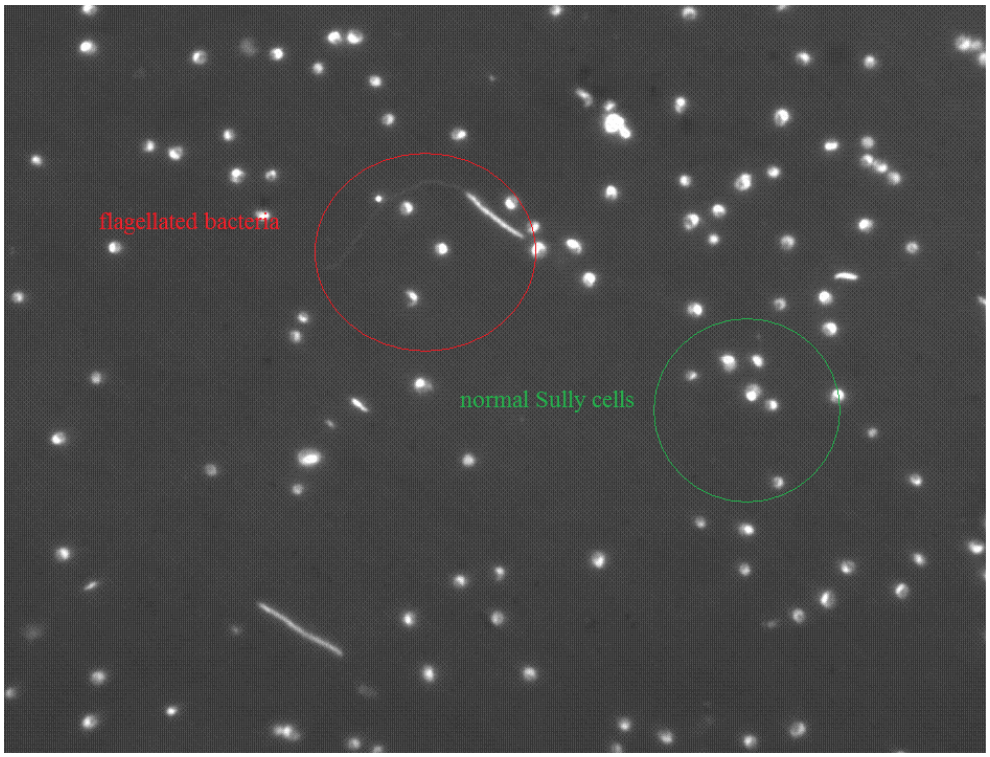


**Figure 20**. Image from fluorescent microscopy feed depicting normal Sully cells and evidence of bacterial contamination.

*Comparison of Staining Methods*

In the midst of trying to solve the mystery of what was causing contamination in the Sully samples, Jenn and I realized that her AO staining process had yielded clean, pure samples free of bacterial contamination, while most of the cultures I stained appeared to be contaminated under the microscope. Even though we followed the protocol for staining exactly the same, her samples were clearly not contaminated, and mine clearly were. We spent one afternoon staining the same Sully cells from the same cultures independently, to determine whether the contamination was coming from our own actions or if it had grown inside the tubes. We each stained two of the same Sully samples, and as predicted, found that Jenn's cells were not contaminated, and mine were. Thus, we were able to determine that the contamination was coming from the staining process, and not from inside the tubes themselves, although we still could not figure out what we were doing differently. However, what is important is that we can be sure that the contamination did not grow along with the Sully cells, and that it could not be present within the culture test tubes themselves.

## *Astrobiological Applications*

*Mars*

In considering possible locations of extraterrestrial life, Mars is consistently believed to be the best candidate for the development of life on another major planet elsewhere in the solar system. With its mean distance of 1.52 astronomical units from the sun, it is located in the outer periphery of the habitable zone that encircles the sun. There is growing substantial evidence that early Mars and Earth harbored similar physical and chemical surface properties. According to geological erosional features captured by Mars orbiters and landers, in the time preceding 3.5 Ga Mars had a wetter and warmer climate, including liquid water on the surface. Around this same time, Earth was already experiencing the early stages of the emergence of biological life forms. Microbial prokaryotes dominated the Earth's biosphere during its first 2 Ga of history before unicellular eukaryotes emerged (Horneck, 2000).

Considering the prospect of development of life on Mars can be difficult to imagine. The Martian surface as we understand it seems contrary to what we know about the conditions needed for life – it is cold,

dry, rocky, and harsh. However, as we have learned more about terrestrial biology, we have begun to understand that life can develop in the seemingly most unlikely places, such at the bottom of the ocean and in the polar ice caps. Continued research on the geological history of Mars has suggested that possible "oases" for the development of life could have existed on early Mars. Some of these may include volcanic regions, in rocks, areas of extreme UV radiation, and deep-sea hydrothermal vents (Rothschild, 1990). We can compare terrestrial analogues of extremophile communities, and postulate potential niches for Mars, and in particular those which include sub-surface regions rich in heavy metals and sulfide minerals, where chemosynthetic organisms thrive (Horneck, 2000).

The existence of extremophiles on Earth has instigated speculation about whether microbial life could weather harsh extraterrestrial climates, and if they can be used as an analogue for understanding the emergence of life in these climates (Olsson-Frances and Cockell, 2010). Hydrothermal systems are considered to be the most favorable environment for the origin of life on Earth, which makes them a strong candidate for the emergence of life on Mars, as well. In considering the Last Universal Common Ancestor (LUCA) of all modern life forms, hyperthermophilic

archaea are believed to be closest genetically to LUCA (Shock, 1996). Oxidizing and reducing (redox) reactions between water and rock establish the potential for organic biogenesis in and around hydrothermal systems (Shock, 1996). The disequilibrium within these systems is essential to the synthesis of organic compounds, and likely the emergence of life (Pope et al., 2006). All life requires a source of energy for respiration and reproduction, which in hydrothermal systems is produced in the form of thermodynamic disequilibria between chemical species in close proximity, which is seen in redox reactions (Westall et al., 2011).

Impact hydrothermal systems, which result from impact cratering proximal to an aqueous environment, may provide further answers in the search for understanding of Mars' biological history. Our understanding of the emergence of early life forms in terrestrial analogues of these systems has piqued interest in many to explore the habitability of these impact hydrothermal environments (Pope et al., 2006). On Earth, hydrothermal mineralization has been found in most impact craters, through the combination of water and the high-energy force that is characteristic of impacts. Research on the Sudbury impact crater found extensive subaqueous hydrothermal systems, which were comparable to

large volcanic systems (Pope et al., 2006). A focused fluid flow from a melting ice sheet fed an extensive network of hydrothermal vents (<200° C) on the crater floor, which researchers determined to be analogous to deep-sea hydrothermal vents (Pope et al., 2006). These hydrothermal vents continue to seep fluid even after being covered by volcanic and melt sheet fluid, which we understand were both active processes on Mars (Pope et al., 2006).

Extrapolations by several different researchers have concluded that impact hydrothermal systems could have remained on the Martian surface anywhere from 8,000 to 4 billion years ago (Pope et al., 2006). According to Pope et al. (2006), large hydrothermal systems, such as those found in the Sudbury crater in Canada, are probably a very rare occurrence on Mars. The cratering that did happen would have occurred during the Noachian period (>3.5 Ga), and thus that is the only epoch which would have experienced impact hydrothermal activity. Therefore, the Noachian epoch is probably the best candidate for when life could have existed on Mars. During this time period, the surface is believed to have been much different than it is today. It was characterized by liquid water, availability of energy and elements essential to life, and a thick

atmosphere comprised of $CO_2$; likely very similar to the surface of the Earth during that time (Olsson-Francis and Cockell, 2010). Mars did not have a global ocean as the Earth did earlier on, but there is evidence for a large ocean in the Martian Northern Hemisphere during the Noachian epoch (Westall et al., 2011).

In the end of the Noachian and in to the Hesperian, Mars lost most of its protective atmosphere, and thus liquid water was no longer stable on the surface, transforming the climate into the cold and dry one we are familiar with now. While this likely destroyed putative life on the surface, organisms that retreated below the surface may have survived (Olsson-Francis and Cockell, 2010). The gradual decreasing pressure and temperature may have pushed these emergent organisms into "protective oases," where they could persist today. Some of these life-forms may have retreated into rocks, or the polar ice caps, where evidence of their existence would likely be found today if we were to explore the Martian surface. According to Shock (1996), "the single most important parameter for modeling the geochemical emergence of life on the early Earth or Mars is the composition of the rock which hosts the hydrothermal system." Impact hydrothermal systems result in crystallization of carbonates and

silicates, which can then fossilize and preserve microbial life forms, as well as their chemical signatures (Pope et al., 2006). Horneck (2000) agrees that the fossilization of dead microorganisms in sedimentary rock on Earth (which first appeared 3.8 Ga) can give us morphological clues about the emergence of life on Mars. Because of the analogues between the early habitable environments on Earth and Mars, archaean sediments and the fossil biota they contain would be relevant to the understanding of Noachian habitats, and potentially, Noachian life forms (Westall et al., 2011).

*Europa*

This rocky, icy moon of Jupiter is one of the most favorable sites in the search for extraterrestrial life. Europa is believed to have an oxygen atmosphere, as well as liquid water ocean beneath the thick layer of ice on its surface. Even if they receive no energy from the sun, subsurface bodies of water may still be able to support lithoautotrophic biological processes (Cavicchioli, 2002). Volcanic activity on Europa may be possible, due to its proximity to Jupiter and consequent tidal interactions that cause warming in the core. These characteristics could facilitate the

development of hydrothermal vents below the icy surface (Chela-Flores, 1998).

Recently, scientists were able to drill through the ice in the Antarctic Lake Vostok, which could hold answers to mysteries about the development of life in extreme conditions. This ancient lake, which is over 1000m deep in some places, is predicted to hold life that may have originated 30 million years ago, and that microorganisms in the ice could live by growing in liquid veins that surround ice crystals. Furthermore, cores taken from the upper ice mass have revealed viable yeasts and actinomycetes, unicellular algae, diatoms, and spore-forming bacteria, all thousands of year sold (Cavicchioli, 2002). These organisms could serve as analogs for conditions in the subsurface permafrost on Europa, and it is believed that similar ancient and possibly extant life forms may be discovered if we were to drill through the ice, which is not beyond current human technological capabilities.

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