

FLEXIBILITY IN THE MINERAL DEPENDENT METABOLISM OF A  
THERMOACIDOPHILIC CRENARCHAEOTE

by

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DEDICATION

For my wife Ivonne who has been the pillar of support and love in my life and for my daughters Martina and Trinidad who are the ones that inspire my life every day.

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## ABSTRACT

This dissertation focuses on understanding the metabolic flexibility of a thermoacidophilic crenarchaeote, the mechanisms underlying its physiology, and the consequences for its ecology. *Acidianus* strain DS80 was isolated from an acidic spring in Yellowstone National Park (YNP) and displays versatility in energy metabolism, using soluble and insoluble substrates during chemolithoautotrophic, chemoheterotrophic, and/or chemolithoheterotrophic growth, and is widely distributed among YNP springs. This flexibility suggests that strain DS80 is of utility as a model thermoacidophile that allows investigation towards how metabolically flexible microorganisms select among available substrates and how these traits influence their natural distributions. Moreover, this plasticity allows investigation of the agreement between thermodynamic metabolic predictions and physiological measurements. Here, I showed that strain DS80 prefers growth with redox couples that provide less energy ( $\text{H}_2/\text{S}^\circ$ ) when compared to other redox couples ( $\text{H}_2/\text{Fe}^{3+}$  or  $\text{S}^\circ/\text{Fe}^{3+}$ ). I present a bioenergetic-physiological argument for this preference, suggesting that the preferential use of substrates in metabolically versatile strains, such as strain DS80, is dictated by differences in the energy demand of electron transfer reactions rather than the energy supply. These observations may help explain why thermodynamic approaches alone are often not enough to accurately predict the distribution and activity of microorganisms in environments. I then showed that genome-guided predictions of energy and carbon metabolism of organisms may not agree with physiological observations in the laboratory, and by extension, the environment. Using a suite of physiological experiments, I provide evidence that the availability of electron acceptors influences the spectrum of potential electron donors and carbon sources that can sustain growth. Similarly, the availability of  $\text{H}_2$  enables the use of organic carbon sources in DS80 cells respiring  $\text{S}^\circ$ , thereby expanding the ecological niche of this organism by allowing them to compete for a wider array of substrates that are available in dynamic environments. Finally, I showed that strain DS80 can use several minerals for chemolithotrophy and that the use of specific metabolisms dictates the requirement for direct access to these minerals. Taken together, the results shown here provide novel insight into the extent and mechanisms of metabolic flexibility of chemolithotrophs and the consequences for their ecology.

## CHAPTER ONE

## GENERAL INTRODUCTION

Microbial communities that inhabit hydrothermal environments exhibit limited biodiversity making them highly amenable to study. As such, these environments and the communities they support have served as model systems for addressing key questions in astrobiology and earth sciences, including those that seek to understand the factors that control the distribution of microorganisms and their activities in natural systems and those that aim to study the physicochemical conditions where life could be detected (i.e., habitability) (Shock & Holland, 2007). Several key aspects make hot spring environments attractive for astrobiological study including their extremes of pH, temperature, and chemical compositions (oftentimes in synchrony in what can be termed polyextremophilic conditions) which present unique opportunities to study the factors that limit the distribution of life on Earth. Moreover, the organisms that are detected in hot spring environments are often deeply rooted evolutionarily (Pace, 1997; Schwartzman & Lineweaver, 2004) and thus provide contemporary analogs for studies of the biochemical processes that supported early forms of life on Earth. This is particularly true for organisms that inhabit high-temperature ( $>73^{\circ}\text{C}$ ) hot spring transects, since this exceeds the upper temperature limit for photosynthesis (Brock, 1967; Boyd, *et al.*, 2010a; Cox *et al.*, 2011; Boyd *et al.*, 2012; Hamilton *et al.*, 2012). Microorganisms in these high-temperature environments are supported by chemical energy in the form of soluble and mineral based substrates (Amend & Shock, 2001; Spear, *et al.*, 2005a; Shock *et al.*, 2010;

Colman *et al.*, 2016). Thus, the metabolism of these organisms is likely similar to that which was present on early Earth prior to the emergence of anoxygenic photosynthesis ~ 3.2 Ga (Des Marais, 2000).

Hydrothermal environments are common both in terrestrial and marine settings. In terrestrial continental settings, hydrothermal environments are often localized at plate boundaries, both divergent (i.e., Iceland) and convergent (e.g., Kamchatka, Russia; El Tatio, Chile). However, hydrothermal environments can also be localized away from these plate boundaries at hot-spots [e.g., Yellowstone National Park (YNP), USA] (Smith & Braile, 1994; Christiansen *et al.*, 2002). The largest concentration of hot springs on Earth is in YNP and the springs and their microbial inhabitants have been subjected to study since the late 19<sup>th</sup> century (Davis, 1897). Springs in YNP range in temperature from ambient to 93°C (boiling at the elevation of YNP) and in pH from <1 to >10 (Amenabar *et al.*, 2015). To this end, YNP and the springs that it hosts is a natural laboratory for the study of fundamental questions in astrobiology and earth sciences. This thesis is organized into 5 integrated chapters.

In the first chapter, I will first introduce the geological setting and geophysical characteristics of YNP hydrothermal systems, describing the factors that influence the compositional variability of YNP hot springs. I will focus my work primarily on one type of YNP hot springs, termed Acid-Sulfate-Chloride springs, since these are the focus of my thesis. In this chapter, I will emphasize how microorganisms and their activities influence their geochemical environment and how this, in turn, influences the distribution and activity of microorganisms. I will focus on the availability of nutrients that support

microbial growth, specifically mineral forms of nutrients and how microbes cope with the low solubility of these minerals.

In chapter 2, I present a characterization of a sulfur-reducing crenarchaeote that I isolated from an Acid-Sulfate-Chloride spring. This organism exhibits extreme versatility in its metabolism, which raises intriguing questions as to how this organism “chooses” among available substrates. In this work, I present a bioenergetic-physiological argument for substrate preference in this organism. The results of this study offer new insights into the role of metabolic efficiency in the choice of substrates and help to explain why microorganisms and their activities often deviate from those predicted when using bioenergetic data alone.

In chapter 3, I characterized the extent of metabolic flexibility in this crenarchaeote using genomic and physiological approaches. The results indicate that electron acceptor availability influences the spectrum of potential electron donors and carbon sources that can sustain growth in this taxon. In addition, I showed that the availability of hydrogen ( $H_2$ ) enables the use of organic carbon sources in cells respiring elemental sulfur ( $S^0$ ), thereby expanding the ecological niche of this organism by allowing them to compete for a wider array of the spectrum of substrates available in dynamic environments, such as hot springs.

In chapter 4, I present results of a detailed study aimed at examining how this crenarchaeote accesses mineral substrates to support metabolism despite their low solubility. I focus on the use of redox active minerals that are commonly encountered in Acid-Sulfate-Chloride springs including sulfur and iron (hydr)oxides minerals. The



results presented in this chapter indicate that the requirement for cells to access minerals is dependent on the mode of metabolism that the cell employs, in particular, if the mineral is being used as an oxidant or a reductant (or both through what can be termed as a disproportionation reaction). Moreover, the data suggest that the activity of the organism indirectly expands its own metabolic niche, by catalyzing reactions whose products drive abiotic reactions and increase availability of the minerals that it is metabolizing. Finally, chapter 5 is a general conclusion for material presented in chapters 2 to 4, alongside a section describing future directions for this research.

### Hydrothermal Ecosystems in YNP

Hydrothermal environments such as hot springs, fumaroles, or geysers are dynamic surface features that form as the result of the interaction between water, heat, and rocks (Heasler *et al.*, 2009). Oxygen-rich surface water (meteoric water) infiltrates the geothermal system in a recharge area. This water then descends into the subsurface through fractures and/or pores in rocks where it is heated and injected with volcanic gases (Heasler *et al.*, 2009). This hot hydrothermal fluid then flows upwards due to density differences and ultimately emerges on the surface at the discharge area to form different types of hydrothermal features (Heasler *et al.*, 2009; Amenabar *et al.*, 2015).

The chemical composition of hydrothermal fluids is dependent on processes that take place deep in the subsurface as well as those that take place near the surface of the hydrothermal features (Nordstrom *et al.*, 2005). Upon reaching shallower depths, decreasing pressure causes rising hydrothermal fluids to undergo boiling and phase

separation, resulting in the partitioning and concentration of chemical species between the vapor phase and the liquid phase (White *et al.*, 1971; Truesdell & Fournier, 1976; Fournier, 1989; Nordstrom *et al.*, 2009). Systems receiving substantial deep liquid phase input tend to be alkaline and rich in sodium ( $\text{Na}^+$ ) and chloride ( $\text{Cl}^-$ ) due to interaction with deep magmatic brines and water rock interactions leading to the formation of alkaline hot springs or geysers. On the other hand, hydrothermal systems that receive substantial vapor phase input tend to be acidic due to near surface oxidation of sulfide, which ultimately yields sulfuric acid (Fournier, 1989; Xu *et al.*, 1998; Nordstrom *et al.*, 2005). Through this mechanism, acidic hot springs and fumaroles are formed, and in rare occasions acidic geysers. Phase separation of hydrothermal fluids at shallow depths results in the formation of hot springs of circumneutral pH that can be found within a few meters of acidic pH hot springs (Nordstrom *et al.*, 2005). A plot of the frequency of YNP hot springs as a function of their pH revealed a bimodal distribution of hot springs types that are centered on sulfuric acid (vapor phase influenced) and bicarbonate (liquid phase influenced) buffered springs (Amenabar *et al.*, 2015) (Figure 1.1a).

In addition to chemical variation induced by subsurface boiling and phase separation, chemical variability of hydrothermal fluids among hot springs is largely attributable to compositional differences between the underlying bedrock and to the interaction of hydrothermal fluids with these rocks. Leaching of minerals from the bedrock changes the composition of both the rocks and the hydrothermal water as it flows through the subsurface (Fournier, 1989), which is reflected by the conductivities of the hot springs (Figure 1.1b-c). Protons generated through the oxidation of sulfide contribute

to the leaching of different minerals from subsurface bedrock. Protons along with soluble products of water-rock interactions results in the high conductivities of hydrothermal fluids associated with low pH springs in YNP, when compared to alkaline systems (Figure 1.1c). As it is observed in panel b from Figure 1.1, little correlation exists between hot spring conductivity and temperature suggesting that temperature likely plays a secondary role on defining the ionic strength of hydrothermal fluids in comparison to pH. These subsurface processes results in hydrothermal fluid compositions that are extraordinarily diverse when compared to other natural surface waters (Amenabar *et al.*, 2015).

#### Types of Thermal Features Present in YNP

The geological setting and geophysical characteristics of the Yellowstone Plateau volcanic field make YNP the largest and most diverse thermal field on Earth, including more than 14,000 geothermal features in the form of geysers, mud pots, fumaroles, frying pans, and hot springs (Fournier, 1989; Hurwitz & Lowenstern, 2014). Based on their chemical composition, YNP hot springs can be classified as one of three primary groups: Alkaline-Siliceous Springs (AS), Sulfidic-Carbonate-rich Springs (SC) and Acid-Sulfate-Chloride Springs (ASC) (Breckenridge & Hinckley, 1978; White *et al.*, 1988; Kruse, 1997; Inskeep & McDermott, 2005; Hurwitz & Lowenstern, 2014). AS springs are typical thermal features present in Upper, Middle, Lower, and West Thumb Geyser Basins in YNP and are thought to be the liquid component (i.e., end member) that remains following phase separation (Fournier, 1989; Hurwitz *et al.*, 2007; Hurwitz &

Lowenstern, 2014). These springs are characterized by having fluids with pH values that range from 7.6 to 9.1, and fluids with water chemistries dominated by  $\text{Na}^+$ ,  $\text{Cl}^-$  and dissolved silica ( $\text{SiO}_2$ ) (Fournier, 1989).

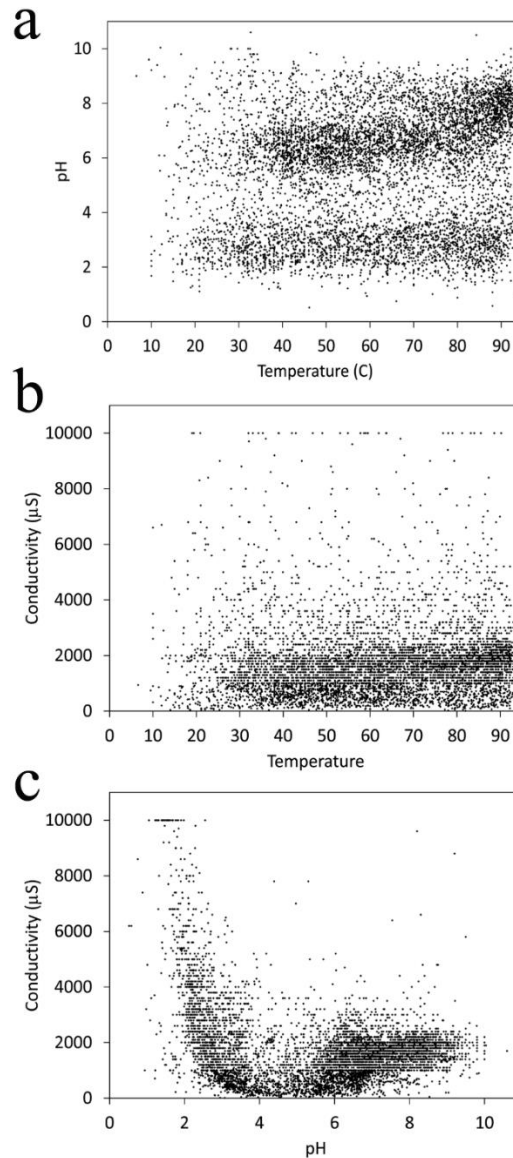


Figure 1.1. pH, temperature and conductivity measurements plotted against each other for ~7700 hot springs in Yellowstone National Park. Data was compiled from the Yellowstone Research Coordination Network website (<http://www.rcn.montana.edu/>). Figure adapted from Amenabar et al., (Amenabar *et al.*, 2015).

SC springs, such as those found at Mammoth hot springs, harbor fluids that are rich in dissolved inorganic carbon (DIC) generated through interactions between hydrothermal fluids and sedimentary rocks (Fournier, 1989; Kharaka *et al.*, 2000; Nordstrom *et al.*, 2005; Hurwitz & Lowenstern, 2014). In addition to dissolution of sedimentary carbonate minerals, a mantle origin has been suggested for the high concentrations of DIC in the Mammoth area (Kharaka *et al.*, 2000). Upon discharge to the surface, fluids saturated in DIC exsolve carbon dioxide ( $\text{CO}_2$ ) as the solution cools, resulting in an increase in the pH of these waters and leading to the precipitation of carbonate minerals (Sorey & Colvard, 1997; Fouke *et al.*, 2000; Nordstrom *et al.*, 2005). The hydrothermal fluids associated with these types of springs are dominated by calcium ( $\text{Ca}^{2+}$ ), magnesium ( $\text{Mg}^{2+}$ ), sulfate ( $\text{SO}_4^{2-}$ ), and DIC (Fournier, 1989; Nordstrom *et al.*, 2005; Hurwitz & Lowenstern, 2014). In addition, these hot springs also contain elevated concentrations of sulfide ( $\text{HS}^-$ ). However, is not yet known if this  $\text{HS}^-$  is derived from abiotic or biotic reactions (Fouke *et al.*, 2000).

The last group of YNP hot springs, ASC springs, are the result of the interaction between meteoric water and  $\text{H}_2\text{S}$ -enriched vapor produced through subsurface boiling and phase separation (Fournier, 1989; Nordstrom *et al.*, 2005). These hot springs can be found across YNP but are especially concentrated in the Norris Geyser Basin (NGB) (Inskeep & McDermott, 2005). ASC springs are vapor-dominated systems characterized by acidic thermal fluids with elevated  $\text{SO}_4^{2-}$  and  $\text{Cl}^-$  concentrations (Fournier, 1989; Inskeep & McDermott, 2005; Meyer-Dombard *et al.*, 2005; Nordstrom *et al.*, 2005; Hurwitz & Lowenstern, 2014). As mentioned in the previous section, the low pH of this

type of springs likely results from the oxidation of reduced sulfur species ( $\text{H}_2\text{S}$  and  $\text{S}^\circ$ ) near the surface to yield sulfuric acid. The acidic thermal water then mixes with  $\text{Cl}^-$ -rich subsurface waters to produce the chemical signature of ASC spring (Inskeep & McDermott, 2005; Nordstrom *et al.*, 2005). A set of abiotic and biotic reactions shapes the chemical composition of ASC springs, which are going to be described in the next section.

### Geomicrobiology of ASC Hot Springs

It has been widely recognized that pH, temperature, and water chemistry shapes the distribution and composition of microbial communities in hydrothermal areas (Boyd, *et al.*, 2010a; Hamilton *et al.*, 2011; Boyd *et al.*, 2012; Hamilton *et al.*, 2012; Alsop *et al.*, 2014). The upper temperature limit for photosynthetic life is  $\sim 73^\circ\text{C}$ , therefore microbial life in high temperature hot springs ( $>73^\circ\text{C}$ ) is supported by chemical energy (chemosynthetic communities) (Brock, 1967; Boyd, *et al.*, 2010a; Cox *et al.*, 2011; Boyd *et al.*, 2012; Hamilton *et al.*, 2012) supplied primarily by volcanic degassing (volatiles) or water rock interactions (solutes) (Amend & Shock, 2001; Spear, *et al.*, 2005a; Spear, *et al.* 2005b).

Source waters from ASC springs located in NGB contain concentrations of dissolved gases such as  $\text{H}_2$ ,  $\text{H}_2\text{S}$ ,  $\text{CO}_2$ , and methane ( $\text{CH}_4$ ) that are oversaturated with respect to earth surface atmospheric conditions (Inskeep & McDermott, 2005). In addition to these dissolved gases, ASC thermal waters typically contain micromolar concentrations of dissolved ferrous iron [ $\text{Fe(II)}$ ] and arsenite [ $\text{As(III)}$ ] (Inskeep &

McDermott, 2005). Therefore, ASC spring waters contain a suite of reduced compounds that may support microbial respiration and growth (Langner *et al.*, 2001; Inskeep *et al.*, 2004; Inskeep *et al.*, 2005; Inskeep & McDermott, 2005; Nordstrom *et al.*, 2005).

ASC springs often have defined outflow channels with steep physical and chemical gradients. As the source waters flow downstream along the outflow channel, the temperature decreases and the amount of dissolved oxygen ( $O_2$ ) increases gradually as the fluid equilibrates with atmospheric  $O_2$  (Langner *et al.*, 2001; Inskeep & McDermott, 2005). Because of the positive redox potential of  $O_2$ , microorganisms with aerobic respiratory capabilities present in the outflow channel should preferentially choose dissolved  $O_2$  as the oxidant to support metabolism, particularly in cooler transects of these springs (Amend & Shock, 2001). Oftentimes, the microbially-mediated oxidation of  $H_2S$ , dissolved  $Fe(II)$ , and dissolved  $As(III)$  with  $O_2$  will result in the formation of  $S^0$  and mineral oxide mats. The concentrations of other dissolved gases including  $H_2$ ,  $H_2S$ , and  $CO_2$  also often decrease along the outflow channel due to off-gassing and microbial activities resulting in different chemical gradients of these dissolved gases (Langner *et al.*, 2001; Lindsay *et al.*, in preparation). The combination of biotic and abiotic chemical transformations along the outflow channels including degassing, mineral precipitation, and oxidation-reduction reactions results in different chemical zones in acidic springs that correspond to specific transitions in the microbial taxa and processes that are present. For simplicity, these are separated as the sulfur depositional zone and iron/arsenic depositional zone, described below.

### Sulfur Depositional Zone

Located immediately downstream of the source in many (but not all) acidic hot springs is the sulfur zone, where  $\text{H}_2\text{S}$  oxidation and concomitant  $\text{S}^\circ$  precipitation lead to an accumulation of  $\text{S}^\circ$  solid phase of characteristic yellow color (Xu *et al.*, 1998; Langner *et al.*, 2001; Inskeep & McDermott, 2005; Nordstrom *et al.*, 2005). This is due to the low solubility and slow abiotic reactivity of  $\text{S}^\circ$  in water below  $100^\circ\text{C}$  (Kamyshtny, 2009). The yellow color is due to an allotrope (a solid phase of the pure element which differs by its crystal structure) of  $\text{S}^\circ$ ; pure  $\text{S}^\circ$  is white (Steudel & Eckert, 2003). In this zone of  $\text{S}^\circ$  deposition,  $\text{H}_2\text{S}$  concentrations range from greater than  $100\ \mu\text{M}$  at the spring source to  $2\text{--}5\ \mu\text{M}$  within several meters down gradient (Langner *et al.*, 2001). The deposition of  $\text{S}^\circ$  around the source and the outflow channel of ASC springs provides a solid phase that may serve as a surface for microbial colonization (Boyd *et al.*, 2009). In addition,  $\text{S}^\circ$  may serve as an energy source or terminal electron acceptor capable of supporting the growth of chemotrophic microorganisms (Muyzer & Stams, 2008).

Indeed, diverse thermoacidophilic microorganisms have been shown to use  $\text{S}^\circ$  to grow, either as an electron donor (sulfur oxidation) or as an electron acceptor (sulfur reduction), including thermophiles which have been shown to dominate hot spring microbial communities (Seeger *et al.*, 1986; Friedrich *et al.*, 2001; Yoshida *et al.*, 2006; Boyd *et al.*, 2007; Muyzer & Stams, 2008; Boyd *et al.*, 2009; Boyd & Druschel, 2013; Giaveno *et al.*, 2013; Urschel *et al.*, 2016). The low solubility of elemental sulfur in pure water ( $478\ \text{nM}$  at  $80^\circ\text{C}$ ) (Kamyshtny, 2009) and evidence from cultivation studies indicating that the  $\text{S}^\circ$ -reducing thermoacidophilic crenarchaeote *Acidilobus*



*sulfurireducens* does not associate with bulk  $S^{\circ}$  (Boyd *et al.*, 2007) suggests that microorganisms in acidic environments might be reducing a soluble form of  $S^{\circ}$  (Boyd & Druschel, 2013).  $S^{\circ}$ -reducing thermoacidophiles increase the solubility of  $S^{\circ}$  by driving production of  $H_2S$ , which reacts with bulk  $S^{\circ}$  to form polysulfides ( $S_x^{2-}$ , primarily  $S_5^{2-}$  at acidic pH) (Giggenbach, 1972). However, in acidic solutions,  $S_x^{2-}$  is unstable and disproportionates according to the reaction:  $2 S_5^{2-} + 4 H^+ \rightarrow \alpha-S_8^{\circ} + 2 H_2S$ , ( $\alpha$  denotes a polymorph of  $S^{\circ}$ ) to yield sulfide and soluble nanoparticulate  $S^{\circ}$ , which serves as the electron acceptor supporting  $S^{\circ}$ -dependent cells (Boyd & Druschel, 2013). On the other hand,  $S^{\circ}$ -oxidizing thermoacidophiles must deal with the low solubility of sulfur by other mechanisms than that described above. This is because without production of  $H_2S$  through  $S^{\circ}$  reduction, cells cannot increase  $S^{\circ}$  solubility. This suggest that  $S^{\circ}$  oxidizers might require direct contact to the solid mineral to use it as energy source, which has been shown for several  $S^{\circ}$  oxidizing *Sulfolobus* spp. (Brock *et al.*, 1972).

In addition to  $S^{\circ}$  oxidation or reduction,  $S^{\circ}$  can simultaneously serve as an electron donor and acceptor in a process called sulfur disproportionation (Muyzer & Stams, 2008). Sulfur disproportionation can be described as an inorganic fermentation in which sulfur compounds with an intermediate oxidation state such as  $S^{\circ}$ , sulfite ( $SO_3^{2-}$ ), or thiosulfate ( $S_2O_3^{2-}$ ) serve as both electron donor and electron acceptor in a metabolic, energy-generating process (Bak & Cypionka, 1987; Thamdrup *et al.*, 1993). However, because of the instability of  $SO_3^{2-}$  and  $S_2O_3^{2-}$  in acidic conditions (Nordstrom *et al.*, 2005), the disproportionation of these two sulfur compounds are likely of only minor importance in acidic hot springs. Despite the abundance of  $S^{\circ}$  in thermal environments,

only one thermophile with the ability to disproportionate  $S^0$  has been described: *Thermosulfurimonas dismutans* (Slobodkin *et al.*, 2012). Similar to all previous microorganisms reported to disproportionate  $S^0$ , *T. dismutans* is a bacterium, suggesting this process might be restricted to this domain (Bak & Cypionka, 1987; Bak & Pfennig, 1987; Thamdrup *et al.*, 1993; Lovley & Phillips, 1994; Janssen *et al.*, 1996; Finster *et al.*, 1998; Pikuta *et al.*, 2003; Sorokin *et al.*, 2008; Sorokin *et al.*, 2011; Slobodkin *et al.*, 2012). In addition to  $S^0$ , the other two most common oxidation states of sulfur compounds present in the spring environment are sulfide ( $S^{-2}$ ) and sulfate ( $S^{+6}$ ) (Amend *et al.*, 2004). Both compounds can be used by microorganisms either as an electron donor or as electron acceptor, respectively (Castro *et al.*, 2000; Fishbain *et al.*, 2003; Amend *et al.*, 2004; Koschorreck, 2008; D'Imperio *et al.*, 2008).

#### Iron/Arsenic Depositional Zone

Adjacent and downstream from the sulfur depositional zone, As(III) and Fe(II) are oxidized, resulting in brown colored mineral mats comprised of amorphous hydrous ferric oxide (HFO) solid phase that contain sorbed As(V) (Langner *et al.*, 2001; Inskeep *et al.*, 2004). This zone of As(III) and Fe(II) oxidation coincides with a decline in  $H_2S$  due to degassing and oxidation to  $S^0$  (Inskeep & McDermott, 2005). The mechanisms of formation of this As(V)-HFO solid phase are thought to be biological (Langner *et al.*, 2001; Inskeep & McDermott, 2005). Moreover, the dynamics between Fe(II)-oxidizing and Fe(III)-reducing populations have been hypothesized to play a key role in defining HFO mat thickness (Inskeep & McDermott, 2005).

The oxidation of As(III) commences when the concentration of H<sub>2</sub>S drops below 5  $\mu$ M (Langner *et al.*, 2001; Inskeep & McDermott, 2005). The extent of As(III) oxidation increases down the outflow channel, as evinced by the accumulation of the HFO solid phase. This As(V)-HFO solid phase can potentially serve as an electron acceptor capable of supporting Fe(III)-reducing microorganisms. Thermophiles capable of reducing iron oxides have been shown to inhabit hot spring microbial communities (Lovley *et al.*, 2004; Slobodkin, 2005; Kashefi *et al.*, 2008; Slobodkina *et al.*, 2012; Fortney *et al.*, 2016); however, iron reduction in the HFO depositional zone from ASC springs has been subject to far less study (Kozubal *et al.*, 2012).

Iron bioavailability is low in most environments not necessarily because of low total iron content but due to the low solubility and the slow dissolution kinetics of iron-bearing minerals (Kraemer, 2004). The solubility of iron, however, is strongly influenced by pH whereby iron is highly insoluble at neutral to alkaline pH and more soluble as the pH decreases (Kraemer, 2004). Several mechanisms are utilized by microorganisms to respire solid phase iron oxides (Hernandez & Newman, 2001; Weber *et al.*, 2006), including the use of chelators or siderophores that solubilize insoluble minerals facilitating their transport into the cell (Gralnick & Newman, 2007). Other mechanisms involve extracellular electron transport to the surface of the solid mineral via outer membrane c-type cytochromes that can interact directly with the solid surface to carry out respiration (Richardson, 2000; Beliaev *et al.*, 2001; Magnuson *et al.*, 2001; Myers & Myers, 2001). Other mechanisms involve soluble electron shuttles such as flavins and quinones (Newman & Kolter, 2000; von Canstein *et al.*, 2008). The third mechanism

involves the use of an extracellular component such as pili as nanowires that could transfer the electrons from the bacterium to the solid phase (Reguera *et al.*, 2005; Gorby *et al.*, 2006). Under acidic conditions that characterize ASC springs, the mechanism of accessing iron oxides as electron acceptors is not known since acidophilic iron reducers have yet to be described in any detail. Iron oxide reducing thermoacidophilic microorganisms [e.g., *Acidicaldus* sp. (Kozubal *et al.*, 2012)] might reduce solubilized Fe(III) ions or ion complexes generated during acid leaching of the solid phase as described in chapter 4.

### Geomicrobiology of “Dragon Spring”

The core of this thesis is based on a thermoacidophilic creanarchaeote belonging to the *Acidianus* genus isolated from “Dragon Spring”, an ASC spring located in the Hundred Springs Plain area of NGB in YNP (44°43'55" N, 110°42'39" W, YNP thermal inventory ID NHSP106). Detailed reports describing the aqueous- and solid- phase chemistry of “Dragon Spring” have been published previously (Langner *et al.*, 2001; Inskeep & McDermott, 2005) and thus will be just briefly reported here. “Dragon Spring” is classified as an ASC spring because of its distinctive fluid chemical signatures dominated by millimolar levels of Na<sup>+</sup>, Cl<sup>-</sup>, and SO<sub>4</sub><sup>2-</sup> with a fluid pH of 3.1 (Langner *et al.*, 2001; Inskeep & McDermott, 2005; Ball *et al.*, 2010). This thermal feature presents characteristic depositional zones of S<sup>0</sup> and iron oxides that exhibit a distinctive sequence represented by unique microbial-mineral mat colors and morphologies (Figure 1.2) (Langner *et al.*, 2001; Inskeep & McDermott, 2005). The source of “Dragon Spring”

contains a suite of reduced inorganic compounds and dissolved gases including As(III), Fe(II), H<sub>2</sub>S, H<sub>2</sub>, and CH<sub>4</sub>, that may serve as potential energy sources for chemolithotrophic microorganisms (Langner *et al.*, 2001; Inskeep & McDermott, 2005). In addition to these compounds, “Dragon Spring” also contains significant concentrations of DIC (4.4 mM) and dissolved organic carbon (DOC) (80 µM) (Langner *et al.*, 2001; Inskeep & McDermott, 2005) that may support autotrophic (primary productivity) and heterotrophic populations, respectively. Like other ASC springs, “Dragon Spring” exhibits a defined outflow channel that has been previously characterized (transect along the outflow channel from spring source to 5.6 m) with respect to pH, temperature, DIC, DOC, total Fe, Fe(II), H<sub>2</sub>S, SO<sub>4</sub><sup>2-</sup>, As(V), and total As (Langner *et al.*, 2001). The concentration of H<sub>2</sub>S and DIC, as well as the temperature of the hydrothermal fluids, decrease as the source waters flow downstream along the outflow channel. On the other hand, the concentration of total As remained constant at 33 µM along the outflow channel; however, the ratio of As(V):As(total) increased, indicating As(III) oxidation along the channel (Langner *et al.*, 2001). There was also a correlation between the oxidation of As(III) and a decline in the concentration of H<sub>2</sub>S. The concentration of total Fe, and Fe(II) remained constant along the outflow channel at 60 µM with the majority of the dissolved total Fe represented by Fe(II). The concentrations of SO<sub>4</sub><sup>2-</sup> and DOC increased slightly and the pH decreased slightly along the outflow channel (Langner *et al.*, 2001), which may be due to evaporative concentration. These physical and chemical changes along the outflow channel result in gradients of dissolved compounds that often

correlate to biological zones of characteristic colors, as was described in the previous sections, and microbial community compositions.



Figure 1.2. “Dragon Spring” located in NGB, YNP, showing the transition of the S<sup>0</sup> depositional zone and iron/arsenic depositional zone. Photograph taken September 2016. Photo credit: M. Amenabar.

Like the geochemistry of “Dragon Spring”, the microbiology of this thermal feature has also been previously characterized (Jackson *et al.*, 2001; Langner *et al.*, 2001; Boyd *et al.*, 2007; D'Imperio *et al.*, 2008; Boyd *et al.*, 2009; Colman *et al.*, 2016). Different microbes potentially involved in the sulfur and iron cycles in this hot spring have been described. Based on clone libraries of 16S rRNA gene sequences from both Bacteria and Archaea, Jackson *et al.*, (Jackson *et al.*, 2001) showed the presence of two dominant bacterial group in the sulfur and iron/arsenic depositional zones, comprised by *Hydrogenobaculum*- and *Desulfurella*-like organisms. Physiological analysis of cultivated members from the *Hydrogenobaculum* genus (Shima & Suzuki, 1993; Jackson *et al.*, 2001; Eder & Huber, 2002; Donahoe-Christiansen *et al.*, 2004; Macur *et al.*, 2004;

D'Imperio *et al.*, 2008) suggest that members detected in “Dragon Spring” are likely involved in primary productivity using energy derived from the oxidation of  $H_2$ ,  $H_2S$ ,  $S^0$ , and/or As(III) coupled to the reduction of  $O_2$ . The presence of  $CO_2$  (4.4 mM),  $H_2$  (60 nM),  $H_2S$  (60  $\mu M$ ), As(III) (33  $\mu M$ ), and  $S^0$  in source fluids from “Dragon Spring” (Langner *et al.*, 2001; Inskeep & McDermott, 2005) suggest this to be an environment favorable for the growth of these *Hydrogenobaculum*-like microorganisms. In support of this, D'Imperio *et al.*, (D'Imperio *et al.*, 2008) isolated several members of the *Hydrogenobaculum* genus from the sulfur depositional zone of “Dragon Spring” that can couple the oxidation of  $H_2$  or  $H_2S$  to  $CO_2$  fixation. In addition, Boyd *et al.*, (Boyd *et al.*, 2009) showed  $CO_2$  fixation by microbial communities associated with  $S^0$  flocs in “Dragon Spring”. The most abundant 16S rRNA sequences detected were related to *Hydrogenobaculum* strains previously isolated from this spring (D'Imperio *et al.*, 2008), supporting the role of these microbes in primary productivity in the environment. Similarly, Donahoe-Christiansen *et al.*, (Donahoe-Christiansen *et al.*, 2004) successfully isolated a *Hydrogenobaculum* strain (H55) from the iron/arsenic depositional zone of “Dragon Spring” that fix  $CO_2$  using  $H_2$  as energy source; however, in contrast to several *Hydrogenobaculum* strains isolated from the sulfur depositional zone (D'Imperio *et al.*, 2008), strain H55 can oxidize As(III) to As(V) and was unable to grow chemolithotrophically with  $H_2S$ . The iron/arsenic HFO mats commence when the concentration of  $H_2S$  drops below 5  $\mu M$  (Inskeep & McDermott, 2005), likely due to the activity of the microorganisms involved in As(III) oxidation, such as strain H55, being inhibited by  $H_2S$  (Langner *et al.*, 2001; Inskeep & McDermott, 2005).

The other dominant bacterial group present in “Dragon Spring” S<sup>0</sup> depositional mats is related to *Desulfurella* sp. Previous reports have shown that representative members of this genus can grow chemolithotrophically with H<sub>2</sub> as energy source and S<sup>0</sup> as electron acceptor or chemoorganotrophically using different organic carbon sources (Bonchosmolovskaya *et al.*, 1990; Miroshnichenko *et al.*, 1994; Miroshnichenko *et al.*, 1998). The presence of H<sub>2</sub> (60 nM), DOC (80 µM), CO<sub>2</sub> (4.4 mM), and S<sup>0</sup> in “Dragon Spring” (Langner *et al.*, 2001; Inskeep & McDermott, 2005) suggest this to be an environment favorable for *Desulfurella*-like organisms. In addition to these two dominant bacterial groups, other bacterial sequences present in the iron/arsenic depositional zones are related to *Meiothermus* sp. and *Acidimicrobium* sp. Physiological analyses of cultivated members from these genera suggest that organisms related to *Meiothermus* sp. and *Acidimicrobium* sp. are putatively involved in aerobic oxidation of organic carbon or Fe(II), respectively (Clark & Norris, 1996; Nobre *et al.*, 1999). The presence of DOC (80 µM) and Fe(II) (60 µM), in “Dragon Spring” (Langner *et al.*, 2001; Inskeep & McDermott, 2005) would presumably support the growth of these *Meiothermus*- and *Acidimicrobium*-like microorganisms.

“Dragon Spring” also harbors a diverse group of archaeal populations related to uncultured microorganisms (Langner *et al.*, 2001; Inskeep & McDermott, 2005)(Takacs-Vesbach *et al.*, 2013). However, because the closest relatives of these sequences have not been cultivated it is hard to infer functions to these groups. Moreover, a few members from the domain Archaea have been isolated from “Dragon Spring”. Boyd *et al.*, (Boyd *et al.*, 2007) isolated and characterized two S<sup>0</sup>-reducing thermoacidophilic crenarchaeotes



from the sulfur depositional zone that are capable of using organic substrates as energy and carbon source. Sequence analyses of the 16S rRNA genes indicated that these isolates represented novel species from the Acidilobales order and were designated as *Caldisphaera draconis* sp. nov. and *Acidilobus sulfurireducens* sp. nov (Boyd *et al.*, 2007).

Interestingly, the majority of work conducted on “Dragon Spring” cited above was conducted prior to 2009 when the temperature of the source of the spring was ~73°C and the pH was relatively constant at 3.0. Since then, the temperature has steadily risen and the pH has become slightly more acidic, which might favor a more Sulfolobales (e.g., *Sulfolobus*, *Acidianus*) dominated ecosystem (Colman *et al.*, in review). Indeed, *Acidianus* sp. DS80 was isolated from “Dragon Spring” in 2012 when the temperature was ~80°C (described in chapters 2 and 3). Other *Acidianus* strains described in this thesis (described in chapter 4) were isolated from high-temperature acidic springs from geographically distinct locations in YNP. These include a S<sup>o</sup>-rich spring in the Crater Hills area informally called “Alice Spring” [80.7°C, pH 2.32; YNP thermal inventory ID CHA043 (Inskeep, *et al.*, 2013a; Inskeep, *et al.*, 2013b)], a S<sup>o</sup>-rich spring in Nymph lake area informally called “NL\_2” [80.0°C, pH 2.50; YNP thermal inventory ID NMC051 (Inskeep, *et al.*, 2013a; Inskeep, *et al.*, 2013b)], a S<sup>o</sup>-rich spring in NGB (88.6°C, pH 2.66; 44°43'45.25"N; 110°42'39.16"W, YNP thermal ID not available), and a S<sup>o</sup>-rich spring in NGB (89.7°C, pH 2.79; YNP thermal inventory ID NHSP116). In contrast to “Dragon Spring”, aqueous- and solid-phase chemistry and microbiology of these springs are not necessarily available, except where described below.

“Alice Spring” and “NL\_2” are turbulent pools that contain suspended minerals including  $S^{\circ}$  and  $SiO_2$  and low levels of  $H_2S$  ( $<5\mu M$ ) (Inskeep, *et al.*, 2013a; Inskeep, *et al.*, 2013b). Both springs contain a suite of different dissolved gases including  $H_2S$ ,  $H_2$ , and  $CH_4$ , that may serve as potential energy sources for chemolithotrophs. In addition to these compounds, “Alice Spring” and “NL\_2” contain abundant DIC (1.4 mM and 0.6 mM, respectively) that might support primary producers (Inskeep, *et al.*, 2013a; Inskeep, *et al.*, 2013b).

The microbiology of “Alice Spring” and “NL\_2” has been previously studied by metagenomics approaches (Inskeep *et al.*, 2010; Inskeep, *et al.*, 2013a; Inskeep, *et al.*, 2013b). Analysis of the individual sequences retrieved from these springs revealed that they were dominated by members of the order Sulfolobales. “Alice Spring” and “NL\_2” contained two and one major Sulfolobales types, respectively, which are related to *Sulfolobus* sp. and *Stygiolobus* sp. Based on metabolic genes identified in the assembled metagenomes, these archaeal populations are likely involved in the oxidation of  $H_2$ ,  $S_2O_3^{2-}$ ,  $H_2S$  and/or  $S^{\circ}$  as energy sources and fixing  $CO_2$ . Additionally, these populations are putative facultative anaerobes able to respire  $S^{\circ}$  or  $O_2$ . (Inskeep, *et al.*, 2013a). The physicochemical conditions present both in “Alice Spring” and “NL\_2”, including acidic pH, high temperatures, and variable concentrations of  $H_2$ ,  $H_2S$ ,  $S^{\circ}$ , and DIC suggest these to be suitable environments for the growth of these Sulfolobales populations.

*Acidianus* sp. as a Model for the Study of Thermoacidophiles

Chapter 2, 3 and 4 are based on the metabolic characteristics of a thermoacidophile that belongs to the *Acidianus* genus. This genus belongs to the Sulfolobales order within the crenarchaeal branch of Archaea (Huber & Prangishvili, 2006). Cells are irregular cocci and exhibit cell diameters ranging from about 0.5 to 2.0  $\mu\text{m}$  (Huber & Stetter, 2015; Amenabar *et al.*, 2017). The *Acidianus* genus is composed of physiologically versatile thermoacidophiles that can grow as facultative anaerobes with the ability to oxidize or reduce  $\text{S}^0$  depending on  $\text{O}_2$  availability. Under aerobic conditions, they can grow lithotrophically using  $\text{S}^0$  or  $\text{H}_2$  as an electron source. Under anaerobic conditions, cells can respire  $\text{S}^0$  using  $\text{H}_2$  as electron donor. Some species of this genus can also respire Fe(III) with  $\text{H}_2$  or  $\text{S}^0$  (Yoshida *et al.*, 2006; Giaveno *et al.*, 2013). Therefore, in contrast to the related *Sulfolobus* genus (Huber & Prangishvili, 2006), growth occurs under anaerobic conditions via reduction of  $\text{S}^0$ . All members of this genus are extreme thermophiles to hyperthermophiles with optimal growth temperatures between 70°C and 90°C and optimal growth pH between 0.8 to 3.0 (Giaveno *et al.*, 2013). *Acidianus* species are capable of autotrophic and facultative heterotrophic growth (Huber & Stetter, 2015). Under autotrophic conditions they generate energy by oxidation of  $\text{H}_2$ ,  $\text{S}^0$ , Fe(II), sulfidic ores or tetrathionate, fixing  $\text{CO}_2$  as a carbon source. Alternatively, heterotrophic growth occurs by oxidation of organic substrates (Huber & Stetter, 2015).

Some representative species of this genus include *A. ambivalens* (Fuchs *et al.*, 1996), *A. manzaensis* (Yoshida *et al.*, 2006), *A. infernus* (Seegerer *et al.*, 1986), *A. brierleyi* (Seegerer *et al.*, 1986), *A. sulfidivorans* (Plumb *et al.*, 2007), *A. tengchongensis* (He *et al.*,

2004), and *A. copahuensis* (Giaveno *et al.*, 2013). *Acidianus* species have been isolated mainly from terrestrial thermal sites but also from geothermally heated acidic marine environments at the beach of Vulcano Island, Italy (Seegerer *et al.*, 1986). The capacity to inhabit both terrestrial and marine hydrothermal systems reflects the broader metabolic flexibility of this genus.

### Metabolic Flexibility of Microbes Present in Terrestrial Hydrothermal Systems

In addition to the previously described chemical variability present in YNP hot springs, hydrothermal environments are dynamic ecosystems that vary chemically or physically both spatially and temporally (Fournier *et al.*, 2002; Hurwitz & Lowenstern, 2014). Microbes inhabiting these environments cope with these fluctuating conditions by broadening their metabolic capabilities (Kassen, 2002). This strategy allows cells to acclimate to spatio-temporal changes in the availability of nutrients, providing a competitive advantage to metabolically-flexible microbes.

Different forms of metabolic flexibility have been described including facultative autotrophy, where chemosynthetic communities may alternate between autotrophic and heterotrophic metabolisms depending on substrate availability (Siebers *et al.*, 2004; Huber & Prangishvili, 2006; Urschel *et al.*, 2016), mixotrophy where both inorganic and organic carbon compounds could be used simultaneously (Jochimsen *et al.*, 1997; Eiler, 2006; Ramos-Vera *et al.*, 2010), and heterotrophy where microbes can use an array of organic carbon sources (Prokofeva *et al.*, 2014; Schut *et al.*, 2014).

Genome sequencing efforts continue to reveal the prevalence of flexibility in the energy metabolism of microorganisms, particularly in the ability of strains to use a variety of inorganic and organic electron donors and a variety of electron acceptors, (Grzymiski *et al.*, 2008; Swan *et al.*, 2011; Sheik *et al.*, 2014). Moreover, the advent of metagenomics has provided new insight into the extent of the metabolic potential of microbial communities, suggesting that metabolic flexibility is widespread among microbes (Walsh *et al.*, 2009; Swan *et al.*, 2011; Inskeep, *et al.*, 2013a; Anantharaman *et al.*, 2016). However, how metabolically flexible microorganisms select among available and utilizable substrates in the environment is not well understood.

#### Prediction of Microbial Substrate Preference

The availability of nutrients in natural environments is variable, both in space and time (Fournier *et al.*, 2002; Hurwitz & Lowenstern, 2014). This is particularly true in ASC springs which have been shown to vary considerably in their geochemical characteristics on a seasonal basis (Fournier *et al.*, 2002), which is likely attributable to the variability in the supply of meteoric water feeding these systems. How this variability shapes the distribution and activity of microorganisms in the environment has been of great interest to geomicrobiologists (Amend & Shock, 2001; Meyer-Dombard *et al.*, 2005). One of the most successful approaches toward addressing this fundamental question has been the application of thermodynamic calculations to geochemical data, which makes it possible to predict the variety and abundance of chemical energy

available for the maintenance and/or growth of microbial populations in an environment (Amend *et al.*, 2003; Shock *et al.*, 2010).

Thermodynamic-based approaches rank suites of redox reactions according to the amount of energy that can be dissipated into a redox tower for use in visualize their tendency to either donate or accept electrons (Weber *et al.*, 2006; Hansel *et al.*, 2015; Madsen, 2011). This redox tower predicts that electron donor/acceptor pairs that maximize the amount of energy dissipated should be used first in a given environment, thereby creating trophic structure and affecting the distribution of microorganisms and their metabolic activities (Lovley *et al.*, 1994). Similarly, microbial metabolisms that utilize redox couples that yield more energy should dominate in mixed cultures and presumably also in the environment (Cordruwisch *et al.*, 1988; Hansel *et al.*, 2015). By extension, a metabolically flexible organism should prefer electron donor/acceptor pairs that maximize the amount of energy when multiple redox pairs are available.

Such thermodynamic-based approaches have been widely used to rationalize the observed distribution of microbial populations and their activities in a variety of environments (Lovley & Klug, 1983; Nealson & Stahl, 1997; Spear, *et al.*, 2005a) and have been widely applied to hot spring communities (Amend & Shock, 2001; Amend *et al.*, 2003; Meyer-Dombard *et al.*, 2005; Shock *et al.*, 2010). However, these predictions sometimes fail to accurately describe the distribution of organisms and metabolic processes in natural environments (Canfield & Des Marais, 1991; D'Hondt *et al.*, 2004; Boyd, *et al.*, 2010b) and engineered systems (Marounnek *et al.*, 1989), suggesting that other factors must also be considered. For example, how variable is the cost associated

with synthesizing the machinery that allows for the use of various redox couples and how efficient are cells at capturing released energy and converting this to biomass synthesis. Variations in these key parameters may impart deviations from predictions based on thermodynamic data from geochemical measurements in the environment, when they are considered alone.

Therefore, microorganism inhabiting dynamic environments such as hot springs, have to deal with the changing concentration of substrates both in space and time and with the low solubilities of some compounds, such as  $S^{\circ}$  or Fe(III). In the following chapters, I present work aimed at addressing the following research questions: 1) What dictates preference for substrates that support microbial energy metabolism, 2) What controls the extent to which a strain exhibits flexibility with respect to its energy metabolism, and 3) How do microbes access substrates to support their energy metabolism?

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CHAPTER TWO

MICROBIAL SUBSTRATE PREFERENCE DICTATED  
BY ENERGY DEMAND RATHER THAN SUPPLY

Contribution of Authors and Co-Authors

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## Microbial substrate preference dictated by energy demand rather than supply

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Growth substrates that maximize energy yield are widely thought to be utilized preferentially by microorganisms. However, observed distributions of microorganisms and their activities often deviate from predictions based solely on thermodynamic considerations of substrate energy supply. Here we present observations of the bioenergetics and growth yields of a metabolically flexible, thermophilic strain of the archaeon *Acidianus* when grown autotrophically on minimal medium with hydrogen ( $\text{H}_2$ ) or elemental sulfur ( $\text{S}^\circ$ ) as an electron donor, and  $\text{S}^\circ$  or ferric iron ( $\text{Fe}^{3+}$ ) as an electron acceptor. Thermodynamic calculations indicate that  $\text{S}^\circ/\text{Fe}^{3+}$  and  $\text{H}_2/\text{Fe}^{3+}$  yield three- and fourfold more energy per mole of electrons transferred, respectively, than the  $\text{H}_2/\text{S}^\circ$  couple. However, biomass yields in *Acidianus* cultures provided with  $\text{H}_2/\text{S}^\circ$  were eightfold greater than when provided  $\text{S}^\circ/\text{Fe}^{3+}$  or  $\text{H}_2/\text{Fe}^{3+}$ , indicating that the  $\text{H}_2/\text{S}^\circ$  redox couple is preferred. Indeed, cells provided with all three growth substrates ( $\text{H}_2$ ,  $\text{Fe}^{3+}$  and  $\text{S}^\circ$ ) grew preferentially by reduction of  $\text{S}^\circ$  with  $\text{H}_2$ . We conclude that substrate preference is dictated by differences in the energy demand of electron transfer reactions in *Acidianus* when grown with different substrates, rather than substrate energy supply.

Microorganisms and their activities drive global biogeochemical cycles and interconnect ecosystem processes affecting plant, animal and human health <sup>1, 2</sup>. Consequently, a central goal in microbial ecology is to predict the distributions and



activities of microorganisms in natural environments<sup>3</sup>. The application of thermodynamic calculations to geochemical data makes it possible to predict and infer the variety and abundance of chemical energy available to microbial populations<sup>4,5</sup>. Such approaches have been widely used to rationalize the observed distribution of microbial populations and their activities in a variety of environments<sup>6,7,8</sup>.

Thermodynamic-based approaches rank assemblages of redox reactions according to the amount of energy that can be dissipated, with the distribution of microorganisms and their activities presumed to follow this sequence of reactions due to competition for energy-rich substrates<sup>6,7,9,10</sup>. However, thermodynamic-based predictions often fall short of accurately describing the distribution of microorganisms and metabolic processes in natural<sup>11,12,13,14</sup> and engineered systems<sup>15</sup>. To provide insight into why these predictions often fail to play out in natural systems and to improve our ability to accurately predict patterns of substrate usage and their effects on biogeochemical cycles, we aimed to quantify the bioenergetics and biomass yields of a metabolically versatile organism. To limit the influence of confounding variables in our determination of the efficiency of biomass synthesis, we sought to identify an autotrophic strain with the ability to grow on defined mineral medium with minimal biosynthetic deficiencies (minimal auxotrophy) and that exhibits versatility in its energy metabolism.

The thermoacidophilic strain *Acidianus* DS80, within the crenarchaeal order Sulfolobales, was isolated under autotrophic conditions on defined mineral medium (supplemented with vitamins) from ‘Dragon Spring’ (pH 3.1, 78 °C), Yellowstone National Park, Wyoming, USA. Strain DS80 displays versatility in lithotrophic energy

metabolism involving hydrogen ( $H_2$ ) or elemental sulfur ( $S^\circ$ ) as electron donors and  $S^\circ$  or ferric iron ( $Fe^{3+}$ ) as electron acceptors (**Supp. Fig. 1**) when grown at pH 3.0 and 80 °C. Moreover, the ability of strain DS80 to couple the iron, sulfur and hydrogen cycles in overlapping ways, in addition to the broader metabolic flexibility and global distribution of *Acidianus* in thermal environments<sup>16, 17, 18</sup>, makes strain DS80 a good model system to examine factors that influence substrate preference.

**Growth yields and substrate transformation kinetics.** Generation times ( $T_n$ ) of autotrophically grown DS80 cells provided with the redox couples  $H_2/S^\circ$ ,  $S^\circ/Fe^{3+}$ , or  $H_2/Fe^{3+}$  were statistically indistinguishable (**Table 1**), indicating that measurements of  $T_n$  are not sensitive to processes that dictate substrate preference. The amount of  $CO_2$  assimilated per cell (**Table 1, Supp. Table 1-2**) when cultures were provided with  $H_2/S^\circ$  or  $S^\circ/Fe^{3+}$  was also statistically indistinguishable ( $P = 0.59$ ). This is consistent with similar cellular morphologies (**Fig. 1; Supp. Fig. 2**) and cell sizes ( $1038 \pm 68$  nm and  $1128 \pm 38$  nm in diameter, respectively) when grown under these conditions (**Table 1**). In contrast, the amount of carbon assimilated from  $CO_2$  per cell when grown with  $H_2/Fe^{3+}$  was twofold lower than when cells were grown with  $H_2/S^\circ$  or  $S^\circ/Fe^{3+}$  (**Table 1**), which was reflected by the smaller size ( $826 \pm 55$  nm diameter) and the twofold smaller calculated volume of cells grown with  $H_2/Fe^{3+}$ . Since the amount of  $CO_2$  assimilated per unit cell volume (**Table 1**) was not significantly different ( $P > 0.33$  for all pairwise comparisons) among cells grown under these three conditions, these observations suggest that the differences in the electron transfer pathways attending utilization of the substrates involved in the cells' energy metabolism and their thermodynamic efficiencies (energy

conserved as chemical bonds or ionic gradients as opposed to energy lost as heat)<sup>19</sup> lead to an observed phenotype at the morphological level.

To further explore this possibility, measured rates of substrate transformation were used to calculate the amount of energy released during log phase growth assuming stoichiometric conversion of reactants to products as depicted by reactions listed in **Table 1**. The amount of CO<sub>2</sub> assimilated per  $\mu\text{J}$  of energy released in log phase cells grown with H<sub>2</sub>/S<sup>°</sup> was about eightfold greater than in cells grown with S<sup>°</sup>/Fe<sup>3+</sup> or H<sub>2</sub>/Fe<sup>3+</sup> (**Table 1**). This indicates that the electron transfer pathways used to conserve energy in cells growing with H<sub>2</sub>/S<sup>°</sup> are more efficient than those operating in cells grown under iron reducing conditions, allowing for greater biomass synthesis. Furthermore, a plot of the change in the overall Gibbs free energy per mole of electron transferred as a function of culture incubation time (**Fig. 2a**) revealed different slopes among the three growth conditions. The growth condition with the shallowest slope was H<sub>2</sub>/S<sup>°</sup> which is consistent with a lower energy demand for biomass synthesis in cells respiring S<sup>°</sup> than those respiring Fe<sup>3+</sup> (**Fig. 2b**).

The energy demand for biomass synthesis in cells respiring S<sup>°</sup> is probably lower than that reported here. This is due to the observation that S<sup>°</sup> reducing thermoacidophilic Archaea reduce nanoparticulate S<sup>°</sup> that is generated when hydrogen sulfide (H<sub>2</sub>S; from respiration of S<sup>°</sup>) reacts with bulk S<sup>°</sup> to form polysulfides (S<sub>x</sub><sup>2-</sup>)<sup>20</sup>. Under acidic conditions (pH <6.0), S<sub>x</sub><sup>2-</sup> rapidly disproportionate to yield H<sub>2</sub>S and nanoparticulate S<sup>°</sup>, the latter of which is thought to be the electron acceptor in thermoacidophiles reducing S<sup>°</sup><sup>20</sup>. However, the involvement of other intermediates in the reduction of S<sup>°</sup> by

thermoacidophiles cannot be excluded. The activity of nanoparticulate  $S^\circ$  in previous growth experiments <sup>20</sup> and in the growth experiments reported here is not known, but is likely to be far lower than the activity of 1.0 specified for bulk  $S^\circ$  in thermodynamic calculations considering previously determined low concentrations of  $S^\circ$  (478 nM at 80°C in equilibrium with excess  $S^\circ$  solid phase) in abiotic aqueous solutions under controlled experimental conditions <sup>21</sup>. Furthermore, in the case of  $Fe^{3+}$  reduction, the chemical equilibrium models that were used took aqueous  $Fe^{3+}$  complexes into account and thus thermodynamic calculations were based on the concentration of free  $Fe^{3+}$  only, which was quite low (0.19 mM) when compared to total  $Fe^{3+}$  (17.2 mM). Taken together, these observations suggest that cells grown in the presence of  $H_2/S^\circ/Fe^{3+}$  will preferentially utilize the  $H_2/S^\circ$  couple, despite this couple being energetically less favorable per mol  $e^-$  transferred than the  $H_2/Fe^{3+}$  and  $S^\circ/Fe^{3+}$  couples (**Fig. 2**).

**Preferential use of redox couples.** We tested the hypothesis that DS80 will preferentially utilize the  $H_2/S^\circ$  couple by growing cells in the presence of all three growth substrates ( $H_2/S^\circ/Fe^{3+}$ ), simultaneously. Concentrations of soluble  $Fe^{3+}$ ,  $Fe^{2+}$ ,  $SO_4^{2-}$ , and  $H_2S$  were determined to track the use of available electron donors and acceptors (**Supp. Table 4**). In addition, the number of cells produced, the amount of  $CO_2$  assimilated per cell, and cell size were quantified. Gibbs free energies for each of the three individual redox couples ( $H_2/S^\circ$ ,  $S^\circ/Fe^{3+}$ ,  $H_2/Fe^{3+}$ ) at the start of the competition experiment varied by < 1% of the Gibbs free energies computed at the onset of growth experiments prepared with only a single possible redox couple. During growth in the presence of all three growth substrates the concentration of  $SO_4^{2-}$  in biological controls never exceeded

that of abiological controls. Since oxidation products of  $S^{\circ}$  (that is, thiosulfate and sulfite) are unstable in aqueous solutions with  $pH < 4.0$ , in particular in the presence of  $Fe^{3+}$  <sup>22</sup>, and ultimately degrade to  $SO_4^{2-}$  in acid solutions <sup>23</sup>, this observation indicates that cells were not oxidizing  $S^{\circ}$  and thus not growing with the  $S^{\circ}/Fe^{3+}$  couple (that is, oxidizing  $S^{\circ}$ ).

Within the first day of growth the concentration of  $Fe^{2+}$  increased with a near stoichiometric decrease in the concentration of soluble  $Fe^{3+}$  (**Fig. 3a**). This differed from cells grown with  $H_2/Fe^{3+}$  only, where detectable  $Fe^{2+}$  was not observed until 6 days of growth (**Supp. Fig. 1**). This suggests that the  $Fe^{2+}$  produced in the  $H_2/S^{\circ}/Fe^{3+}$  cultivation was due to abiological reduction of  $Fe^{3+}$  by  $H_2S$  produced from the biological reduction of  $S^{\circ}$ . Indeed, following depletion of soluble  $Fe^{3+}$  after 48 hrs, the concentration of  $H_2S$  increased rapidly as did concentrations of cells and amounts of  $CO_2$  assimilated, consistent with growth with the  $H_2/S^{\circ}$  couple. These observations are independent of whether cells used as inoculum were grown with  $H_2/S^{\circ}$ ,  $S^{\circ}/Fe^{3+}$  or  $H_2/Fe^{3+}$  (**Supp. Fig. 3**) indicating that preference for  $H_2/S^{\circ}$  is not due to a “priming effect” or a difference in the need to acclimate to new cultivation conditions. Further evidence that DS80 cells prefer to utilize  $H_2/S^{\circ}$  when provided with  $H_2/S^{\circ}/Fe^{3+}$  comes from the size of cells in cultures, which were statistically indistinguishable ( $P = 0.47$ ) from those provided with  $H_2/S^{\circ}$  but which were significantly larger ( $P < 0.01$ ) than cells grown with  $H_2/Fe^{3+}$ . Likewise, the amount of  $CO_2$  assimilated per cell in cultures provided with  $H_2/S^{\circ}/Fe^{3+}$  was statistically indistinguishable ( $P = 0.62$ ) from measurements made on cells provided with  $H_2/S^{\circ}$  and the lag phase corresponded with cultures grown with  $H_2/S^{\circ}$  (**Table 1**).

To assess whether  $\text{Fe}^{3+}$  reduction observed early in cultures provided with  $\text{H}_2/\text{S}^\circ/\text{Fe}^{3+}$  was due to abiological reduction by  $\text{H}_2\text{S}$ , sterile medium containing  $\text{H}_2/\text{S}^\circ/\text{Fe}^{3+}$  was titrated with  $\text{H}_2\text{S}$  (**Fig. 3b**) at rates that corresponded with those determined in cultures grown with  $\text{H}_2/\text{S}^\circ$  only (**Supp. Fig. 1**). Production of  $\text{Fe}^{2+}$  and consumption of soluble  $\text{Fe}^{3+}$  were observed at similar rates and over similar time frames in these abiotic experiments (**Fig. 3b**) when compared to biotic assays (**Fig. 3a**). The amount of soluble  $\text{Fe}^{2+}$  detected could not account for the amount of  $\text{Fe}^{3+}$  reduction that had taken place. Evidence that this was due to formation of insoluble iron sulfides comes from depletion in the concentration of soluble  $\text{Fe}^{2+}$  in both abiotic and biotic assays (**Fig. 3**) concomitant with the formation of black precipitates in cultures (**Supp. Fig. 4**), which are likely iron mono- or di-sulfides <sup>24</sup>.

**Energy demand dictates substrate preference.** The results of these experiments raise questions about why the energy demands differ in cells supported by these reactions, and where and how electron transfer takes place in cells that grow preferentially with  $\text{H}_2/\text{S}^\circ$  over  $\text{S}^\circ/\text{Fe}^{3+}$  or  $\text{H}_2/\text{Fe}^{3+}$ . Respiration of  $\text{S}^\circ$  with  $\text{H}_2$  in *Acidianus* is mediated by a short electron transfer chain comprising a membrane-associated [NiFe]-hydrogenase and a membrane-associated sulfur reductase complex, linked by a quinone cycle, with S reduction thought to take place on the outer side of the membrane <sup>25</sup>. Respiration of  $\text{Fe}^{3+}$  with  $\text{H}_2$  or  $\text{S}^\circ$  in crenarchaeotes, including *Acidianus*, is not well understood but in DS80 likely involves the same [NiFe]-hydrogenase, since there is a single hydrogenase encoded in its genome, and a sulfur-oxygenase reductase (SOR) complex <sup>26</sup> which is also encoded in the draft genome (IMG submission identifier 92883;

estimated to be 99% complete; data not shown). Despite the absence of a characterized  $\text{Fe}^{3+}$  reduction pathway, DS80 cells grown with  $\text{Fe}^{3+}$  as the electron acceptor had numerous hair-like protrusions extending from the plasma membrane (**Fig. 1; Supp. Fig. 5**) that resembled “nanowires”<sup>27, 28</sup>. Such structures were observed when cells were grown with “soluble”  $\text{Fe}^{3+}$  [i.e.,  $\text{Fe}_2(\text{SO}_4)_3$ ] (**Figs. 1e & 1f**) or insoluble ferric oxyhydroxides such as ferrihydrite or hematite (**Supp. Fig. 6**) but not with  $\text{S}^\circ$  as oxidant (**Fig. 1d**), suggesting that this is a generalized physiological response to growth via  $\text{Fe}^{3+}$  reduction. Intriguingly, these structures were often observed in association with mineral precipitates of unknown composition in  $\text{Fe}^{3+}$  grown cultures (**Figs. 1; Supp. Figs. 5a & 5b**). Such structures were not observed when cells were provided with  $\text{H}_2/\text{S}^\circ/\text{Fe}^{3+}$  (data not shown), which is consistent with these cells growing preferentially via the  $\text{H}_2/\text{S}^\circ$  redox couple under this condition.

C-type multiheme cytochromes (cMHC) have been suggested to be involved in iron reduction in both Bacteria<sup>29, 30</sup> and Archaea<sup>31, 32</sup>, and have been suggested to be the electrically conductive component of nanowires in *Shewanella*<sup>33</sup>. However, many iron reducers, including a number of archaea from the Sulfolobales and Thermoproteales<sup>31, 34</sup>, lack detectable c-type cytochromes<sup>35</sup> or the genes encoding for c-type cytochromes. Consistent with this observation, BLASTp searches failed to detect conventional (CXXCH)<sup>36, 37</sup> and unconventional heme binding motifs (CXXXH and CXXXXH)<sup>37</sup> in the DS80 and the closely related *Acidianus copahuensis* genome<sup>16</sup>, suggesting that they also do not encode for cMHCs. Collectively, these observations point to a different

mechanism of  $\text{Fe}^{3+}$  reduction in members of the Sulfolobales that is independent of c-type cytochromes.

Although the physical properties conferring conductivity to microbial nanowires has been controversial, it has been recently suggested that overlap of the pi-pi orbitals of aromatic amino acids in the structural pilin protein PilA in *Geobacter* imparts metallic-like conductivity to the structures, independent of the presence of cMHC<sup>38,39</sup>. The primary structural components of nanowires (type IV pili) include an assembly ATPase, a transmembrane protein, a pilin protein, and a prepilin peptidase<sup>40,41</sup>. BLASTp analyses of proteins encoded in the DS80 genome revealed the presence of the ATPase, a transmembrane protein, and a prepilin peptidase in an apparent operon while screens for proteins with class III signal peptides thought to be specific for type IV pili using the FlaFind (ver. 1.2) server<sup>42,43</sup> revealed several type IV pilin-like proteins in the DS80 genome. Pilin proteins that have been shown to be involved in electron transfer are enriched in aromatic amino acids, with the more conductive pilins averaging an aromatic density (number of aromatic rings in amino acids divided by the total number of amino acids) of 0.15<sup>44</sup>. Intriguingly, one of the pilin proteins encoded in the DS80 genome is predicted to have an aromatic density of 0.12, which may point to its potential involvement in  $\text{Fe}^{3+}$  reduction.

While the role of pili-like structures in  $\text{Fe}^{3+}$  reduction in strain DS80 is not clear, their synthesis would involve an additional energetic cost to cells grown with  $\text{Fe}^{3+}$  but not with  $\text{S}^0$ . In addition, if these structures were involved in  $\text{Fe}^{3+}$  reduction, it is possible that energy is lost as heat during successive electron transfers along the array of electrically



conductive components (e.g., aromatic amino acids) leading to a lower thermodynamic efficiency per electron transported. Energy loss through either of these mechanisms would represent additional energetic costs to cells, which could otherwise contribute to biomass synthesis and more efficient cellular yields. The longer lag times associated with growth via  $\text{Fe}^{3+}$  reduction when compared to  $\text{S}^{\circ}$  reduction (**Table 1; Supp. Fig. 1**) may point to the influence of these mechanisms on the inefficiency of converting energy captured to biomass synthesis. Alternatively, the short lag associated with the  $\text{H}_2/\text{S}^{\circ}$  versus the  $\text{H}_2/\text{Fe}^{3+}$  and  $\text{S}^{\circ}/\text{Fe}^{3+}$  couples may suggest that the biochemical machinery necessary to process the  $\text{H}_2/\text{S}^{\circ}$  couple is the energetically less costly to assemble when compared to the machinery necessary to process the other two redox couples.

Collectively, these observations indicate that the preferential use of electron donors and acceptors in metabolically versatile strains, such as DS80, cannot be understood in terms of energetic supplies based on thermodynamic analysis or on measurements of  $T_n$  alone. Rather, these data show that combining thermodynamic calculations with measurements of biomass yields provides a metric to describe the physiology of cells in a way that more accurately captures the physiological nuances experienced by cells and that dictate differences in energy demands attending substrate utilization. We suggest that a combined approach, as outlined above, could be used to develop more accurate models for describing the distribution of microorganisms and their activities in natural and engineered environments.

## METHODS

**Sample collection and enrichment of *Acidianus* strain DS80.** Samples for enrichment and isolation of a chemolithotrophic strain capable of metabolizing substrates that can be easily tracked were collected from the source of ‘Dragon Spring’, an acidic hot spring (78°C; pH 3.1; determined with a YSI combination pH and temperature probe) located in the Hundred Springs Plain area of Norris Geyser Basin in YNP (44°43’55” N, 110°42’39” W). The source of ‘Dragon Spring’ has clear depositional zones of sulfur and iron oxides and samples were collected from this area (see images in <sup>45, 46</sup>). Detailed reports describing the aqueous- and solid-phase chemistry of ‘Dragon Spring’ have been published previously <sup>45, 46</sup> and thus only basic chemical measurements will be reported here. Ferrous iron ( $\text{Fe}^{2+}$ ) and total sulfide ( $\text{S}^{2-}_{\text{total}}$ ) were quantified using Hach ferrozine pillows and Hach sulfide reagents 1 and 2, respectively, and a Hach DR/890 spectrophotometer (Hach Company, Loveland, CO). The concentration of  $\text{Fe}^{2+}$  in source water was 55  $\mu\text{M}$  and the concentration of  $\text{S}^{2-}_{\text{total}}$  was 46  $\mu\text{M}$ .

Flocculent elemental sulfur ( $\text{S}^0$ ) precipitate and spring water were sampled aseptically using a sterile syringe and this precipitate slurry was immediately injected into a sterile,  $\text{N}_2$ -purged serum bottle. The serum bottle was placed in an insulated thermos bottle containing spring water to keep the samples at ambient spring temperature during the 120-min. transit to the lab where they were immediately used to inoculate enrichments. Roughly 250  $\mu\text{L}$  (~100 mg of dry solids) of the  $\text{S}^0$  precipitate slurry was used to inoculate a base salts medium containing  $\text{NH}_4\text{Cl}$  (0.33 g  $\text{L}^{-1}$ ),  $\text{KCl}$  (0.33 g  $\text{L}^{-1}$ ),  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (0.33 g  $\text{L}^{-1}$ ),  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (0.33 g  $\text{L}^{-1}$ ), and  $\text{KH}_2\text{PO}_4$  (0.33 g  $\text{L}^{-1}$ ) <sup>47</sup>. The

pH of the base salts medium was adjusted to 3.0 with concentrated hydrochloric acid. Eighteen mL of base salts medium were dispensed into 70 mL serum bottles and bottles and their contents were subjected to autoclave sterilization. Following autoclave sterilization, filter-sterilized Wolfe's vitamins ( $1 \text{ mL L}^{-1}$  final concentration), filter sterilized SL-10 trace metals ( $1 \text{ mL L}^{-1}$  final concentration), and  $\text{S}^{\circ}$  (sulfur precipitated powder, EMD Millipore, USA;  $5 \text{ g L}^{-1}$  final concentration; sterilized by baking at  $100^{\circ}\text{C}$  for 24 h) were added, and the bottles and contents were deoxygenated by purging with sterile nitrogen gas passed over heated ( $210^{\circ}\text{C}$ ) and hydrogen reduced copper shavings. The serum bottles were sealed with butyl rubber stoppers and heated to  $80^{\circ}\text{C}$  prior to the replacement of the headspace with  $\text{H}_2$  and  $\text{CO}_2$  (80%:20%) or  $\text{N}_2$  and  $\text{CO}_2$  (80%:20%). The serum bottles were then inoculated with the sulfur precipitates slurry recovered from the source of 'Dragon Spring' and were incubated at  $80^{\circ}\text{C}$ .

Progress of enrichments was monitored by filtering cells onto  $0.22 \text{ }\mu\text{m}$  black polycarbonate filters (Millipore, Billerica, MA), staining cells with SYBR Gold Nucleic Acid Gel Stain (Life Technologies, Inc., Grand Island, NY) at a final concentration of 1/4000 (vol/vol), followed by epifluorescence microscopy using an Axioskop 2 Plus microscope (Zeiss, Thornwood, NY). Following 4 rounds of dilution to extinction, a single morphotype was observed, which was designated as strain DS80. The purity of the culture was further confirmed by PCR. Briefly, PCR amplification of 16S rRNA gene was performed according to previously described protocols<sup>48, 49</sup> using archaeal primers 344F (5'-ACGGGGYGCAGCAGGCGCGA-3') and 915R (5'-GTGCTCCCCGCCAATTCCT-3') and bacterial primers 1100F (5'-

YAACGAGCGCAACCC-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3').

Bacterial 16S rRNA gene amplicons were not detected. Archaeal 16S rRNA gene amplicons were purified, cloned, sequenced, assembled, and analyzed using previously published protocols <sup>47</sup>. A single 16S rRNA gene phylotype was recovered and this was 100% identical to the 16S rRNA gene from *Acidianus hospitalis*. Nucleotide sequence of the 16S rRNA gene of strain DS80 have been deposited in the GenBank database under the accession number KX608545. Genomic DNA was subjected to Illumina MiSeq sequencing, assembly, and annotation as described previously <sup>50</sup>. The assembled genome (IMG submission identifier 92883), which is estimated to be 99% complete (data not shown).

**Physiological characterization.** The temperature range for growth of strain DS80 was tested between 40 to 100°C, at pH 3.0. The pH range for growth was tested between 1.0 and 6.0, at 80°C. Optimal growth was observed at 80°C and pH 3.0 and thus all experiments were conducted under these conditions. The ability of strain DS80 to utilize H<sub>2</sub> or S<sup>0</sup> as electron donors and Fe<sup>3+</sup> or S<sup>0</sup> as electron acceptors was tested in anoxic base salts medium amended with trace elements and vitamins as previously described <sup>47</sup>. Medium was supplemented with S<sup>0</sup> (5 g L<sup>-1</sup>) or Fe<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub> (3.7 g liter<sup>-1</sup>). Fe<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub> was prepared at a pH 1.9 (fully dissolved) and was added to medium with a pH of 7.5 (final pH when mixed was 3.0). A headspace gas mixture of 80%:20% N<sub>2</sub>-CO<sub>2</sub> was used when S<sup>0</sup> was the electron donor and Fe<sup>3+</sup> the electron acceptor. A headspace gas mixture of 80%:20% H<sub>2</sub>-CO<sub>2</sub> was used when S<sup>0</sup> or Fe<sup>3+</sup> were supplied as the electron acceptor. All

cultures were incubated at 80°C. Cultures were prepared in triplicate for both biological and abiological (un-inoculated) assays as described above.

**Preferential substrate use determination.** Cells of strain DS80 were grown in the presence of  $H_2$ ,  $S^\circ$ , and  $Fe_2(SO_4)_3 \cdot H_2O$  to test for the preferential use of  $H_2/S^\circ$ ,  $H_2/Fe^{3+}$ ,  $S^\circ/Fe^{3+}$  as a redox couple. Anoxic base salts medium amended with trace elements and vitamins was used, as previously described <sup>47</sup>. Medium was supplemented with  $S^\circ$  (5 g L<sup>-1</sup>) and  $Fe_2(SO_4)_3 \cdot H_2O$  (3.7 g liter<sup>-1</sup>), using a headspace gas mixture of 80%:20%  $H_2$ - $CO_2$ . Cultures grown with  $H_2/S^\circ$ ,  $S^\circ/Fe^{3+}$  or  $H_2/Fe^{3+}$  were separately used as inoculum to rule out any “priming effect” or carryover effect from previous growth experiments. Abiotic experiments were performed by titration of sterile medium containing  $H_2/S^\circ/Fe^{3+}$  with  $Na_2S$  at rates that corresponded with those determined in cultures grown with  $H_2/S^\circ$ . Cultures were prepared in triplicate for both biological and abiological (un-inoculated) assays and incubated at 80°C as described above.

**Evaluation of growth and activity.** Growth of strain DS80 with different electron donors and acceptors was quantified in terms of cell density, dissolved inorganic carbon (DIC) assimilation (described below), sulfur or iron reduction activity, or sulfur oxidation activity. Cell density was determined by staining with SYBR Gold and enumeration via epifluorescence microscopy, as described above. A subsample (0.9 mL) of culture was briefly centrifuged (14,000 x *g*, 1 min.) to remove particulate iron before activity measurements. Dissolved sulfide concentrations were determined with the methylene blue reduction method <sup>51</sup>. Total sulfide production (dissolved and gaseous) was calculated using standard gas-phase equilibrium calculations as described previously

<sup>47</sup>. The concentration of total and ferrous iron was determined using the Ferrozine method; the concentration of ferric iron was calculated as the difference between total (obtained after reduction of all iron with hydroxylamine hydrochloride) and ferrous iron measurements <sup>52</sup>. The concentration of sulfate was determined after precipitation with barium chloride, as previously described <sup>53</sup>. The addition of  $\text{Fe}_2(\text{SO}_4)_3 \cdot \text{H}_2\text{O}$  was considered in the measurement of sulfate production.

**Carbon assimilation.** The amount of  $\text{CO}_2$  assimilated into cellular biomass in cultures of DS80 was determined using a radioisotope tracer method as described previously <sup>50</sup>. Briefly, a 160 mL serum bottle containing 55 mL of base salts medium (described above) with a 20%  $\text{CO}_2$  (V/V) headspace (balance was  $\text{H}_2$  or  $\text{N}_2$ , depending on experimental condition) was amended with  $^{14}\text{C}$ -labeled sodium bicarbonate (American Radiolabeled Chemical) to a final concentration of 27.5  $\mu\text{Ci}$ . Given that the  $\text{pK}_a$  for bicarbonate/ $\text{CO}_{2(\text{aq})}$  at  $80^\circ\text{C}$  is  $\sim 6.4$  <sup>54</sup>, we assumed that the added bicarbonate completely dissociated to  $\text{CO}_2$  when added to medium (pH 3.0) and equilibrated equally among the unlabeled DIC pools. The amount of  $^{14}\text{C}$  assimilated into cellular biomass was quantified by removal of 2 mL subsamples of culture from each serum bottle (followed by replacement of the gas phase) with sterile gas. Concentrated hydrochloric acid was added to subsamples to achieve a final pH of less than 2.0 in order to volatilize unreacted DIC. Following 2 hrs of equilibration and degassing, subsamples were filtered onto 0.22  $\mu\text{m}$  white polycarbonate membranes, washed with sterile base salts medium, and dried at  $80^\circ\text{C}$  for 24 hrs. Dried filters were transferred to 20 mL liquid scintillation vials containing 10 mL CytoScint ES<sup>TM</sup> liquid scintillation fluid (MP Biomedicals, USA) and

radioactivity associated with filtered biomass was measured on a Beckman LS 6500 liquid scintillation counter (LSC) (Beckman Coulter, Inc., Indianapolis, IN). Rates of CO<sub>2</sub> assimilation were calculated from LSC counts using previously described methods<sup>50</sup>. The amount of C assimilated per cell (fmol/cell) during autotrophic growth was calculated by dividing the total amount of C assimilated from CO<sub>2</sub> during exponential growth by the number of new cells produced over this time.

**Energetics.** The total amount of energy that can be obtained by an organism catalyzing a redox reaction in a non-standard state displaced from equilibrium is given by equation 1:

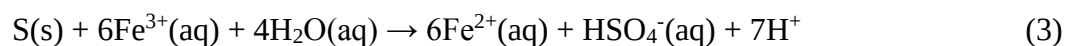
$$\Delta G = \Delta G^\circ + 2.303 RT \log Q$$

(1)

where  $\Delta G$  represents the Gibbs free energy of reaction,  $\Delta G^\circ$  represents the standard Gibbs free energy of reaction (J mol<sup>-1</sup>),  $R$  designates the ideal gas constant (~8.314 J mol<sup>-1</sup> K<sup>-1</sup>),  $T$  is the temperature in Kelvin (K), and  $Q$  is the activity product. The activity ( $\alpha$ ) of each reactant and product can be calculated from its aqueous concentration and the ionic strength of the aqueous solution in which the reaction proceeds using the extended Debye-Hückel equation<sup>55</sup>.

As was previously described by Urschel et al.<sup>50</sup>, the activities of reactant and product at each time point during growth of strain DS80 on H<sub>2</sub>/S<sup>0</sup>, S<sup>0</sup>/Fe<sup>3+</sup> and H<sub>2</sub>/Fe<sup>3+</sup> were calculated based on the ionic strength and composition of the base salts medium, as well as the measured or estimated concentration of each reactant and product, using the freeware chemical equilibrium modeling application Visual MINTEQ 3.0. Since we did

not quantify differences in H<sub>2</sub> concentration during the growth of strain DS80, we assumed a constant aqueous H<sub>2</sub> concentration equal to the saturation concentration of H<sub>2</sub> in H<sub>2</sub>O at 80°C and 1 atmosphere of partial pressure (~768 μM). Reactant and product activities were then used to calculate logQ based on the following chemical reactions (number in parenthesis indicates equation number):



ΔG° of the different reactions was calculated using the SUPCRT online program provided by the “Group Exploring Organic Processes in Geochemistry” from Arizona State University.

Calculated log Q and ΔG° values at each time point during exponential phase were then used to calculate ΔG with equation (1) above, and the resulting ΔG value was divided by the total number of electrons transferred per mol substrate transformed to determine the energy available per mol electron transferred kJ (mol e<sup>-</sup>)<sup>-1</sup> at each time point during exponential phase. Because of the differences in the stoichiometry of the chemical reactions 2, 3 and 4, the biomass yield per μJ of energy was calculated in order to be able to directly compare the efficiency of coupling energy conservation to biomass synthesis under these three growth conditions. The difference in the amount of C assimilated between two sampling points during exponential growth was then divided by the difference in total energy dissipated (**Fig. 2, Supp. Tables 1-3**) between these time points to compute the amount of C assimilated per unit of energy dissipated (fmol CO<sub>2</sub>



$\mu\text{J}^{-1}$ ). These calculations were conducted using measurements made during exponential phase growth (**Supp. Fig. 1**).

**Transmission electron microscopy.** Cells were fixed on ice with 2% paraformaldehyde and 1.25% glutaraldehyde and washed with Hepes buffer as previously described<sup>56</sup>. Cells were negatively stained with sodium phosphotungstate and imaged on a Leo912AB transmission electron microscope (TEM) equipped with a 2K X 2K CCD camera, operated at 100-kV accelerating voltage. The diameters of negative stained cells were measured from the TEM micrographs. Cells from all growth conditions were prepared for TEM analysis using the same protocol in order to account for modification of cell size associated with the preparation technique.

**Field emission scanning electron microscopy.** Cells were fixed with 2% paraformaldehyde and 1.25% glutaraldehyde overnight at room temperature. Cells were washed four times with distilled water. Washed cells were subjected to a series of dehydration steps with 25% ethanol for 20 min, 50% ethanol for 20 min, 75% ethanol for 20 min, 95% ethanol for 30 min. and three final dehydrations using 100% ethanol for 60 min each. Dehydrated cells were then subjected to critical point drying using a Tousimis Samdri®-795 critical point dryer. Dried cells were coated with iridium and imaged on a Zeiss SUPRA 55VP Field Emission Scanning Electron Microscope.

**Data Availability.** The data that support the findings of this study are available from the corresponding author on request. The draft genome sequence obtained for strain DS80 has been deposited in the Joint Genome Institute IMG database, IMG submission identifier 92883, genome ID 2690315630.

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**Competing Financial Interests** The authors declare no competing financial interests.

**Table 1:** Generation times ( $T_n$ ), lag phase times, yields of carbon assimilated per cell, measured cell diameters, yields of carbon assimilated per unit cell volume, and yields of carbon assimilated per microjoule of energy released in cultures of *Acidianus* sp. DS80 grown at 80 °C in base salts medium (pH 3.0) with CO<sub>2</sub> as sole carbon source with the electron donors and acceptors specified.

| Redox reaction                                                                                                                                                                                              | $T_n$<br>(hrs) | Lag phase<br>(hrs) | fmol CO <sub>2</sub><br>/cell | Cell diameter<br>(nm) | fmol CO <sub>2</sub><br>/m <sup>3</sup> <sup>b</sup> | fmol CO <sub>2</sub><br>/μJ |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------|--------------------|-------------------------------|-----------------------|------------------------------------------------------|-----------------------------|
| H <sub>2(g)</sub> + S <sup>0</sup> <sub>(s)</sub> → H <sub>2</sub> S <sub>(aq)</sub>                                                                                                                        | 35 ± 17        | 24                 | 3.00 ± 0.34                   | 1038 ± 68             | 4.41 ± 3.24                                          | 267.4 ± 36.2                |
| S <sup>0</sup> <sub>(s)</sub> + 6Fe <sup>3+</sup> <sub>(aq)</sub> + 4H <sub>2</sub> O <sub>(aq)</sub> → 6Fe <sup>2+</sup> <sub>(aq)</sub> + HSO <sub>4</sub> <sup>-</sup> <sub>(aq)</sub> + 7H <sup>+</sup> | 33 ± 19        | 96                 | 3.44 ± 1.90                   | 1128 ± 38             | 4.39 ± 2.92                                          | 31.7 ± 1.7                  |
| H <sub>2(g)</sub> + 2Fe <sup>3+</sup> <sub>(aq)</sub> → 2Fe <sup>2+</sup> <sub>(aq)</sub> + 2H <sup>+</sup>                                                                                                 | 27 ± 4         | 120                | 1.64 ± 0.23                   | 826 ± 55              | 4.85 ± 2.87                                          | 34.2 ± 5.0                  |
| Competition Experiment (H <sub>2</sub> / S <sup>0</sup> / Fe <sup>3+</sup> ) <sup>a</sup>                                                                                                                   | 31 ± 2         | 24                 | 3.32 ± 0.25                   | 1017 ± 29             | 5.87 ± 1.83                                          | N/D                         |

The average and standard deviation of three replicate cultures are presented. In the case of cell diameter determinations, the average and standard deviation of measurements of > 10 cells, based on TEM, are presented. <sup>a</sup>Redox reaction is not defined since multiple potential electron donors and acceptors are present in the cultivation medium. <sup>b</sup>Calculated assuming a spherical (coccioid) morphology (**Fig. 1**). Abbreviations: N/D, not determined.

**Supplemental Table 1.** Number of cells, amount of carbon assimilated, measured analyte ( $\text{H}_2\text{S}$ ,  $\text{Fe}^{3+}$ ,  $\text{Fe}^{2+}$ , and  $\text{SO}_4^{2-}$ ) where applicable, and calculated amounts of energy available per mol electron transferred during the specified times in cultures of *Acidianus* sp. DS80 grown at  $80^\circ\text{C}$  in base salts medium (pH 3.0) with  $\text{CO}_2$  as the carbon source,  $\text{H}_2$  as electron donor, and  $\text{S}^\circ$  as electron acceptor.

| Substrate $\text{H}_2$ / $\text{S}^\circ$ ( $\text{e}^-$ donor/acceptor) |                    |                         |                             |                         |                         |                           |                                                 |
|--------------------------------------------------------------------------|--------------------|-------------------------|-----------------------------|-------------------------|-------------------------|---------------------------|-------------------------------------------------|
| Time                                                                     | Cells              | $\text{CO}_2$<br>(fmol) | $\text{H}_2\text{S}$<br>(M) | $\text{Fe}^{3+}$<br>(M) | $\text{Fe}^{2+}$<br>(M) | $\text{SO}_4^{2-}$<br>(M) | $\Delta G$<br>(kJ (mol $\text{e}^-$ ) $^{-1}$ ) |
| 0                                                                        | $2.66 \times 10^5$ | $4.07 \times 10^6$      | $2.95 \times 10^{-5}$       | N/A                     | N/A                     | N/A                       | -28.20                                          |
| 24                                                                       | $2.66 \times 10^5$ | $4.10 \times 10^6$      | $2.95 \times 10^{-5}$       | N/A                     | N/A                     | N/A                       | -28.20                                          |
| 48                                                                       | $1.10 \times 10^6$ | $4.74 \times 10^6$      | $5.61 \times 10^{-5}$       | N/A                     | N/A                     | N/A                       | -27.25                                          |
| 72                                                                       | $2.76 \times 10^6$ | $6.15 \times 10^6$      | $1.18 \times 10^{-4}$       | N/A                     | N/A                     | N/A                       | -26.15                                          |
| 96                                                                       | $4.56 \times 10^6$ | $1.14 \times 10^7$      | $2.57 \times 10^{-4}$       | N/A                     | N/A                     | N/A                       | -25.01                                          |
| 120                                                                      | $5.73 \times 10^6$ | $1.49 \times 10^7$      | $4.62 \times 10^{-4}$       | N/A                     | N/A                     | N/A                       | -24.15                                          |
| 144                                                                      | $7.59 \times 10^6$ | $2.09 \times 10^7$      | $7.35 \times 10^{-4}$       | N/A                     | N/A                     | N/A                       | -23.47                                          |
| 168                                                                      | $8.07 \times 10^6$ | $2.07 \times 10^7$      | $9.46 \times 10^{-4}$       | N/A                     | N/A                     | N/A                       | -23.10                                          |
| 192                                                                      | $7.70 \times 10^6$ | $2.04 \times 10^7$      | $1.08 \times 10^{-3}$       | N/A                     | N/A                     | N/A                       | -22.90                                          |

Abbreviations: N/A, Not Applicable.

**Supplemental Table 2.** Number of cells, amount of carbon assimilated, measured analyte ( $\text{H}_2\text{S}$ ,  $\text{Fe}^{3+}$ ,  $\text{Fe}^{2+}$ , and  $\text{SO}_4^{2-}$ ) where applicable, and calculated amounts of energy available per mol electron transferred during the specified times in cultures of *Acidianus* sp. DS80 grown at  $80^\circ\text{C}$  in base salts medium (pH 3.0) with  $\text{CO}_2$  as the carbon source,  $\text{S}^\circ$  as the electron donor, and  $\text{Fe}^{3+}$  as the electron acceptor.

| Substrate $\text{S}^\circ / \text{Fe}^{3+}$ ( $\text{e}^-$ donor/acceptor) |                    |                         |                             |                         |                         |                                        |                                                             |
|----------------------------------------------------------------------------|--------------------|-------------------------|-----------------------------|-------------------------|-------------------------|----------------------------------------|-------------------------------------------------------------|
| Time                                                                       | Cells              | $\text{CO}_2$<br>(fmol) | $\text{H}_2\text{S}$<br>(M) | $\text{Fe}^{3+}$<br>(M) | $\text{Fe}^{2+}$<br>(M) | $\text{SO}_4^{2-}$<br>(M) <sup>1</sup> | $\Delta\text{G}$<br>(kJ (mol $\text{e}^-$ ) <sup>-1</sup> ) |
| 0                                                                          | $2.04 \times 10^5$ | $4.21 \times 10^6$      | N/A                         | $9.25 \times 10^{-3}$   | B.D.                    | 0.00                                   | -41.83                                                      |
| 24                                                                         | $2.04 \times 10^5$ | $4.33 \times 10^6$      | N/A                         | $9.24 \times 10^{-3}$   | $1.00 \times 10^{-5}$   | $1.67 \times 10^{-6}$                  | -82.95                                                      |
| 48                                                                         | $2.10 \times 10^5$ | $4.32 \times 10^6$      | N/A                         | $9.22 \times 10^{-3}$   | $2.88 \times 10^{-5}$   | $4.81 \times 10^{-6}$                  | -79.84                                                      |
| 72                                                                         | $2.46 \times 10^5$ | $4.21 \times 10^6$      | N/A                         | $9.18 \times 10^{-3}$   | $7.48 \times 10^{-5}$   | $1.25 \times 10^{-5}$                  | -77.02                                                      |
| 96                                                                         | $2.57 \times 10^6$ | $6.67 \times 10^6$      | N/A                         | $8.36 \times 10^{-3}$   | $8.96 \times 10^{-4}$   | $1.49 \times 10^{-4}$                  | -69.46                                                      |
| 120                                                                        | $4.62 \times 10^6$ | $1.29 \times 10^7$      | N/A                         | $7.01 \times 10^{-3}$   | $2.24 \times 10^{-3}$   | $3.73 \times 10^{-4}$                  | -66.24                                                      |
| 144                                                                        | $6.14 \times 10^6$ | $1.98 \times 10^7$      | N/A                         | $5.57 \times 10^{-3}$   | $3.68 \times 10^{-3}$   | $6.14 \times 10^{-4}$                  | -64.06                                                      |
| 168                                                                        | $6.87 \times 10^6$ | $2.49 \times 10^7$      | N/A                         | $4.45 \times 10^{-3}$   | $4.81 \times 10^{-3}$   | $8.01 \times 10^{-4}$                  | -63.53                                                      |
| 192                                                                        | $8.09 \times 10^6$ | $2.73 \times 10^7$      | N/A                         | $2.55 \times 10^{-3}$   | $6.70 \times 10^{-3}$   | $1.12 \times 10^{-3}$                  | -59.88                                                      |
| 216                                                                        | $1.01 \times 10^7$ | $3.47 \times 10^7$      | N/A                         | $1.85 \times 10^{-3}$   | $7.40 \times 10^{-3}$   | $1.23 \times 10^{-3}$                  | -58.61                                                      |
| 240                                                                        | $1.03 \times 10^7$ | $3.10 \times 10^7$      | N/A                         | $1.11 \times 10^{-3}$   | $8.14 \times 10^{-3}$   | $1.36 \times 10^{-3}$                  | -56.81                                                      |

<sup>1</sup>Calculated by subtracting  $\text{SO}_4^{2-}$  in cultures from  $\text{SO}_4^{2-}$  in abiotic controls.

Abbreviations: N/A, Not Applicable; B.D., Below Detection

Detection limits for  $\text{Fe}^{2+}$  and  $\text{SO}_4^{2-}$  are 0.3 and 5.0  $\mu\text{M}$ , respectively.

**Supplemental Table 3.** Number of cells, amount of carbon assimilated, measured analyte ( $\text{H}_2\text{S}$ ,  $\text{Fe}^{3+}$ ,  $\text{Fe}^{2+}$ , and  $\text{SO}_4^{2-}$ ) where applicable, and calculated amounts of energy available per mol electron transferred during the specified times in cultures of *Acidianus* sp. DS80 grown at  $80^\circ\text{C}$  in base salts medium (pH 3.0) with  $\text{CO}_2$  as the carbon source,  $\text{H}_2$  as the electron donor, and  $\text{Fe}^{3+}$  as the electron acceptor.

| Substrate $\text{H}_2$ / $\text{Fe}^{3+}$ ( $\text{e}^-$ donor/acceptor) |                    |                         |                             |                         |                         |                                        |                                                             |
|--------------------------------------------------------------------------|--------------------|-------------------------|-----------------------------|-------------------------|-------------------------|----------------------------------------|-------------------------------------------------------------|
| Time                                                                     | Cells              | $\text{CO}_2$<br>(fmol) | $\text{H}_2\text{S}$<br>(M) | $\text{Fe}^{3+}$<br>(M) | $\text{Fe}^{2+}$<br>(M) | $\text{SO}_4^{2-}$<br>(M) <sup>1</sup> | $\Delta\text{G}$ (kJ<br>(mol $\text{e}^-$ ) <sup>-1</sup> ) |
| 0                                                                        | $1.85 \times 10^5$ | $4.28 \times 10^6$      | N/A                         | $9.25 \times 10^{-3}$   | B.D.                    | B/D                                    | -68.56                                                      |
| 24                                                                       | $1.95 \times 10^5$ | $4.33 \times 10^6$      | N/A                         | $9.25 \times 10^{-3}$   | $3.17 \times 10^{-6}$   | B/D                                    | -110.41                                                     |
| 48                                                                       | $2.08 \times 10^5$ | $4.21 \times 10^6$      | N/A                         | $9.24 \times 10^{-3}$   | $7.50 \times 10^{-6}$   | B/D                                    | -107.88                                                     |
| 72                                                                       | $2.08 \times 10^5$ | $4.17 \times 10^6$      | N/A                         | $9.24 \times 10^{-3}$   | $1.60 \times 10^{-5}$   | B/D                                    | -105.65                                                     |
| 96                                                                       | $3.17 \times 10^5$ | $4.19 \times 10^6$      | N/A                         | $4.60 \times 10^{-3}$   | $3.63 \times 10^{-5}$   | B/D                                    | -101.15                                                     |
| 120                                                                      | $8.17 \times 10^5$ | $4.57 \times 10^6$      | N/A                         | $9.10 \times 10^{-3}$   | $1.56 \times 10^{-4}$   | B/D                                    | -98.92                                                      |
| 144                                                                      | $1.22 \times 10^6$ | $5.21 \times 10^6$      | N/A                         | $8.84 \times 10^{-3}$   | $4.09 \times 10^{-4}$   | B/D                                    | -96.01                                                      |
| 168                                                                      | $2.33 \times 10^6$ | $7.35 \times 10^6$      | N/A                         | $8.40 \times 10^{-3}$   | $8.58 \times 10^{-4}$   | B/D                                    | -93.70                                                      |
| 192                                                                      | $4.05 \times 10^6$ | $8.82 \times 10^6$      | N/A                         | $7.82 \times 10^{-3}$   | $1.43 \times 10^{-3}$   | B/D                                    | -92.00                                                      |
| 216                                                                      | $8.59 \times 10^6$ | $1.55 \times 10^7$      | N/A                         | $6.63 \times 10^{-3}$   | $2.62 \times 10^{-3}$   | B/D                                    | -89.75                                                      |
| 240                                                                      | $1.39 \times 10^7$ | $2.47 \times 10^7$      | N/A                         | $4.73 \times 10^{-3}$   | $4.52 \times 10^{-3}$   | B/D                                    | -87.16                                                      |
| 264                                                                      | $1.40 \times 10^7$ | $2.82 \times 10^7$      | N/A                         | $4.19 \times 10^{-3}$   | $5.06 \times 10^{-3}$   | B/D                                    | -86.47                                                      |

<sup>1</sup>Calculated by subtracting  $\text{SO}_4^{2-}$  in cultures from  $\text{SO}_4^{2-}$  in abiotic controls.

Abbreviations: N/A, Not Applicable. B.D., Below Detection

Detection limits for  $\text{Fe}^{2+}$  and  $\text{SO}_4^{2-}$  are 0.3 and 5.0  $\mu\text{M}$  respectively.

**Supplemental Table 4.** Number of cells, amount of carbon assimilated, and measured analyte ( $\text{H}_2\text{S}$ ,  $\text{Fe}^{3+}$ ,  $\text{Fe}^{2+}$ , and  $\text{SO}_4^{2-}$ ) where applicable during the specified times in cultures of *Acidianus* sp. DS80 grown at 80°C in base salts medium (pH 3.0) with  $\text{CO}_2$  as sole carbon source.

| Substrate $\text{H}_2/\text{S}^\circ/\text{Fe}^{3+}$ |                    |                      |                          |                      |                      |                                     |
|------------------------------------------------------|--------------------|----------------------|--------------------------|----------------------|----------------------|-------------------------------------|
| Time                                                 | Cells              | $\text{CO}_2$ (fmol) | $\text{H}_2\text{S}$ (M) | $\text{Fe}^{3+}$ (M) | $\text{Fe}^{2+}$ (M) | $\text{SO}_4^{2-}$ (M) <sup>1</sup> |
| 0                                                    | $2.57 \times 10^5$ | 4.07E+06             | 6.72E-08                 | 8.83E-03             | 2.08E-05             | B/D                                 |
| 24                                                   | 2.57E+05           | 4.07E+06             | 6.72E-08                 | 8.18E-03             | 4.76E-04             | B/D                                 |
| 48                                                   | 6.83E+05           | 4.56E+06             | 1.88E-05                 | 7.54E-03             | 1.04E-03             | B/D                                 |
| 72                                                   | 2.27E+06           | 5.97E+06             | 1.33E-04                 | 4.06E-03             | 2.76E-03             | B/D                                 |
| 96                                                   | 3.44E+06           | 7.19E+06             | 3.35E-04                 | 2.37E-03             | 2.28E-03             | B/D                                 |
| 120                                                  | 5.82E+06           | 1.43E+07             | 5.85E-04                 | 1.26E-04             | 4.78E-04             | B/D                                 |
| 144                                                  | 7.05E+06           | 1.73E+06             | 1.27E-03                 | 4.48E-06             | 3.72E-05             | B/D                                 |
| 168                                                  | 7.52E+06           | 2.01E+06             | 1.41E-03                 | 6.98E-06             | 1.31E-05             | B/D                                 |

<sup>1</sup>Calculated by subtracting  $\text{SO}_4^{2-}$  in cultures from  $\text{SO}_4^{2-}$  in abiotic controls.

Abbreviations: B.D., Below Detection

Detection limits for  $\text{SO}_4^{2-}$  is 5  $\mu\text{M}$  respectively.

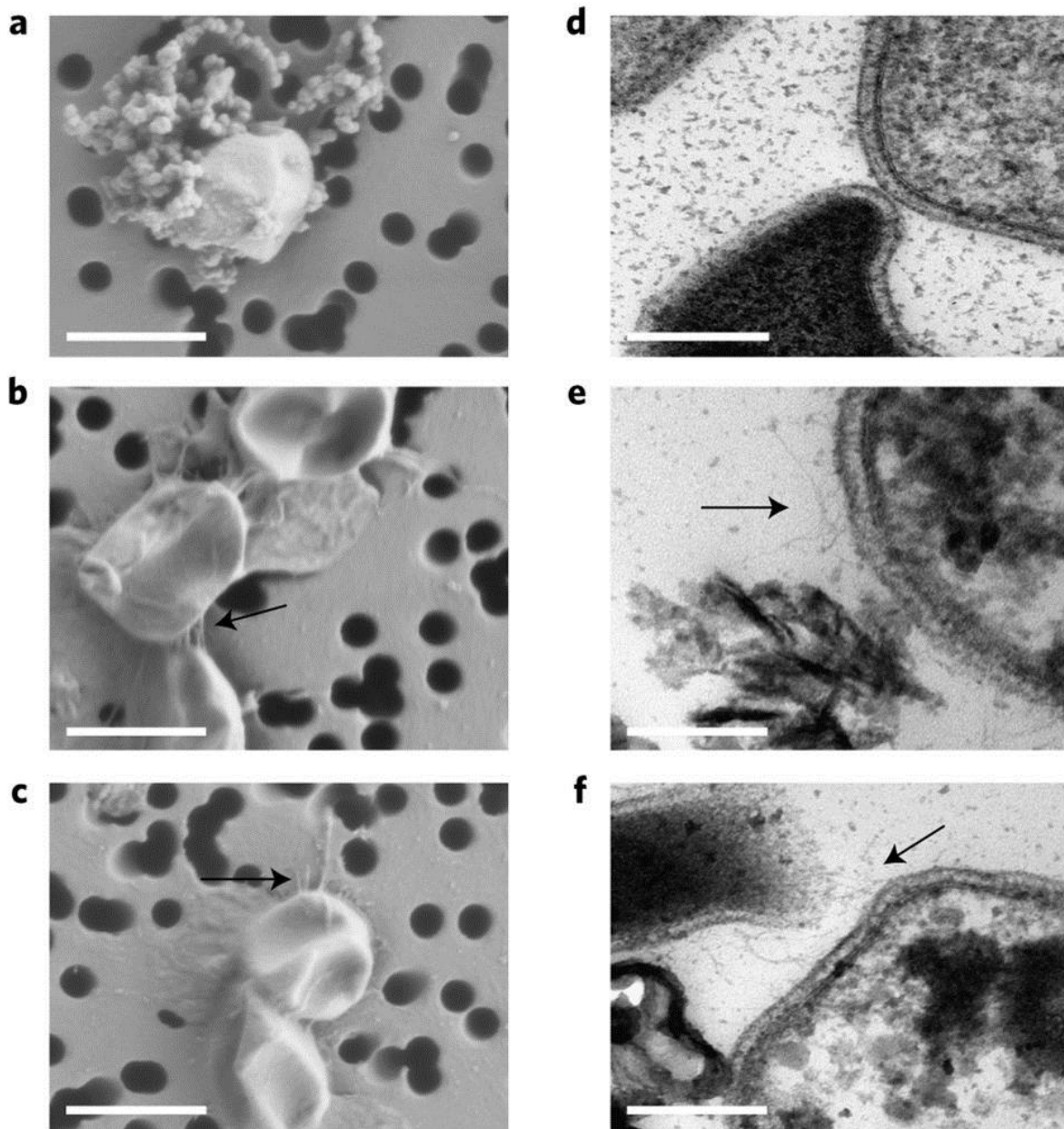


**Figure 1. Electron micrographs of DS80 cells grown with different electron donor/acceptor pairs reveal differences in morphology.** **a–c**, Field-emission scanning electron micrographs (FE-SEMs) of  $\text{H}_2/\text{S}^\circ$  (a),  $\text{S}^\circ/\text{Fe}^{3+}$  (b) and  $\text{H}_2/\text{Fe}^{3+}$  (c) grown cells with arrows denoting pili-like structures where present. Globules outside cells in panel **a** correspond to nanoparticles of  $\text{S}^\circ$  (ref. 20). FE-SEM scale bars represent 1,000 nm. **d–f**, Thin-section transmission electron micrographs (TEMs) of cells grown with  $\text{H}_2/\text{S}^\circ$  (d),  $\text{S}^\circ/\text{Fe}^{3+}$  (e) and  $\text{H}_2/\text{Fe}^{3+}$  (f), with arrows denoting pilin-like structures where present. Insoluble precipitates of unknown composition are visible in **e** and **f**. TEM scale bars represent 200 nm.



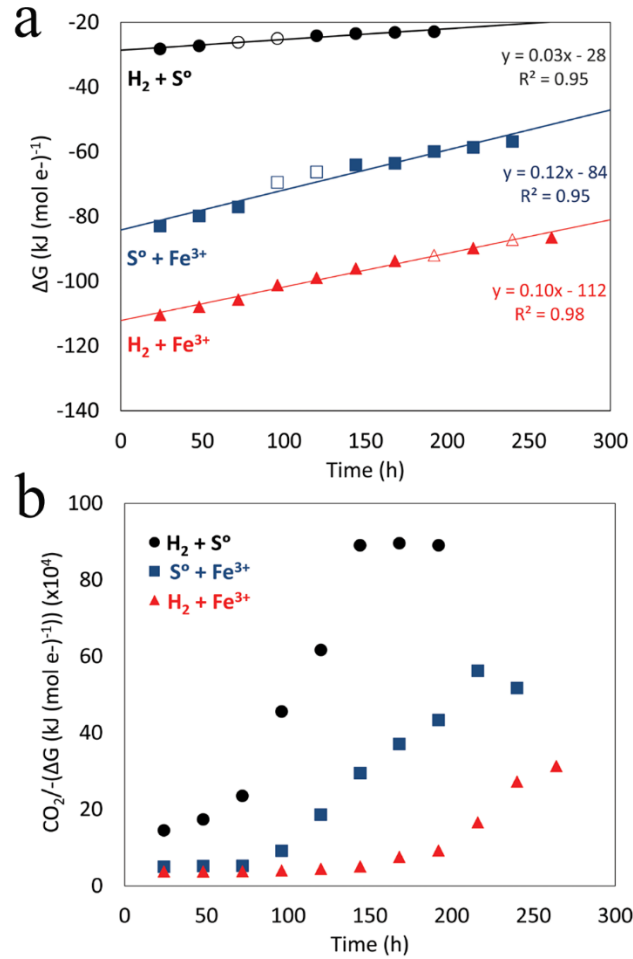


**Figure 2. Bioenergetics and biomass yields per kilojoule of energy dissipated in DS80 cultures provided with  $H_2/S^0$ ,  $S^0/Fe^{3+}$  or  $H_2/Fe^{3+}$ .** **a**, Available Gibbs free energy ( $\Delta G$ ) per mole of electrons transferred in growth experiments. Trends in the data are illustrated with the least-squares best-fit regressions. The standard deviations of the slope of the regression for  $H_2/S^0$ ,  $S^0/Fe^{3+}$  and  $H_2/Fe^{3+}$  grown cultures are 0.002, 0.010 and 0.004, respectively. Open symbols represent logarithmic growth and are the points where generation times and growth yields were calculated. **b**, Carbon assimilated (fmol) divided by the negative value of ( $\Delta G$ ) per mole of electrons transferred in cultures of DS80 provided with  $H_2/S^0$ ,  $S^0/Fe^{3+}$  and  $H_2/Fe^{3+}$  as a function of incubation time.

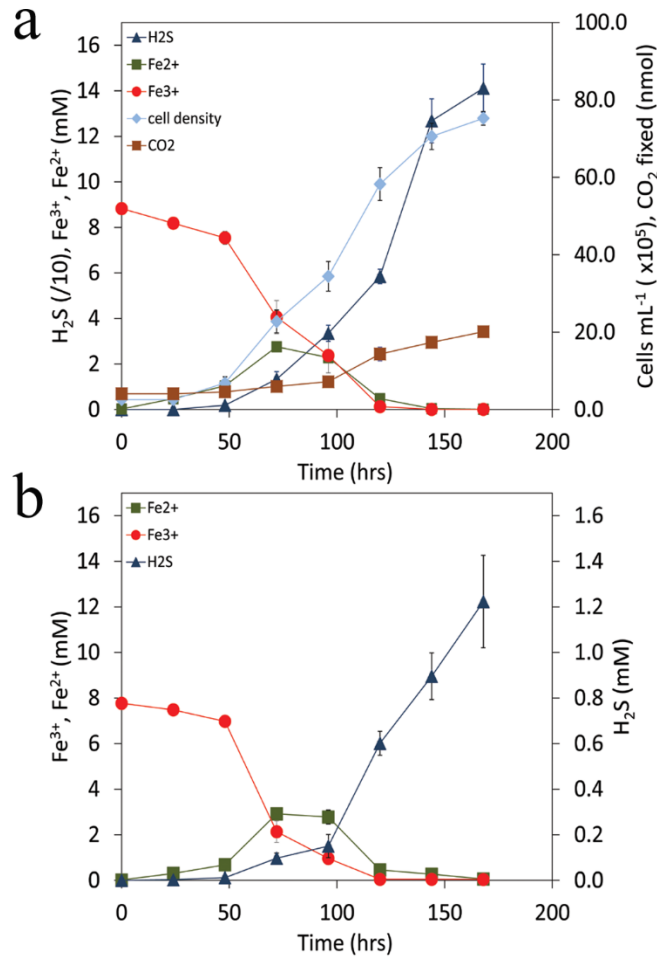
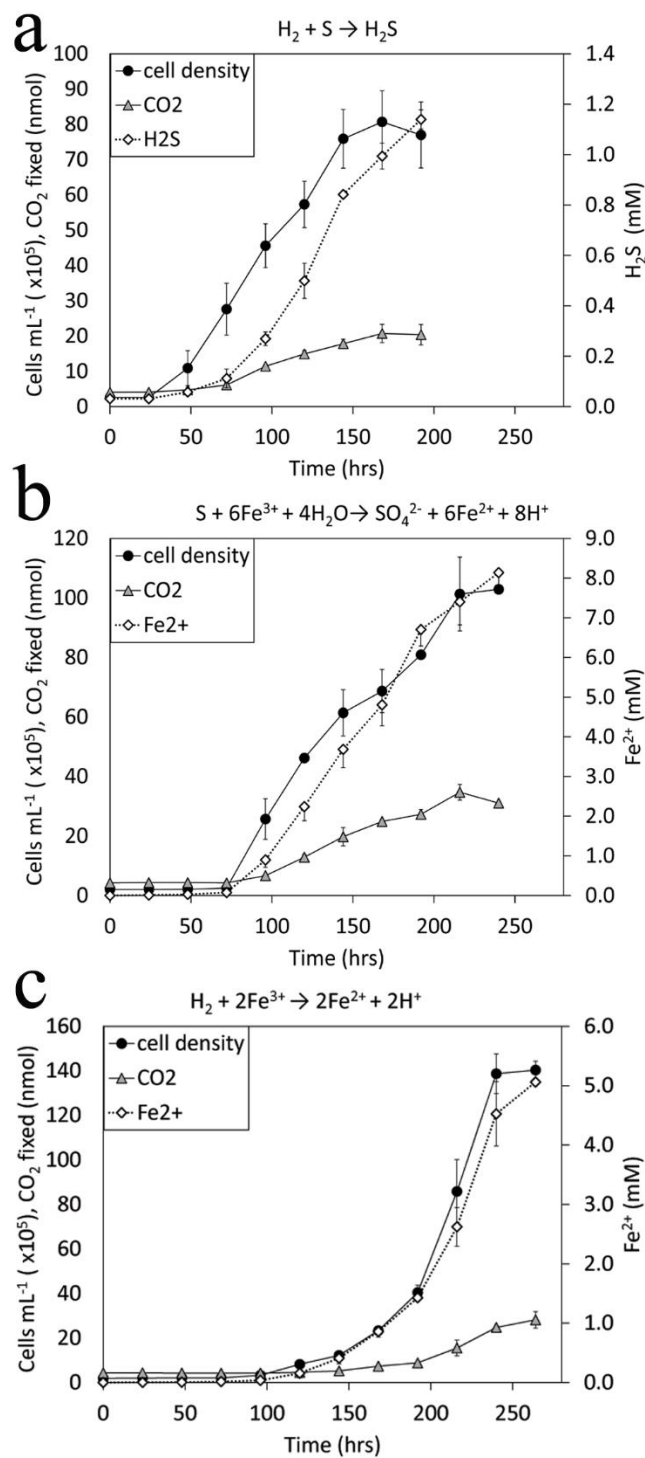
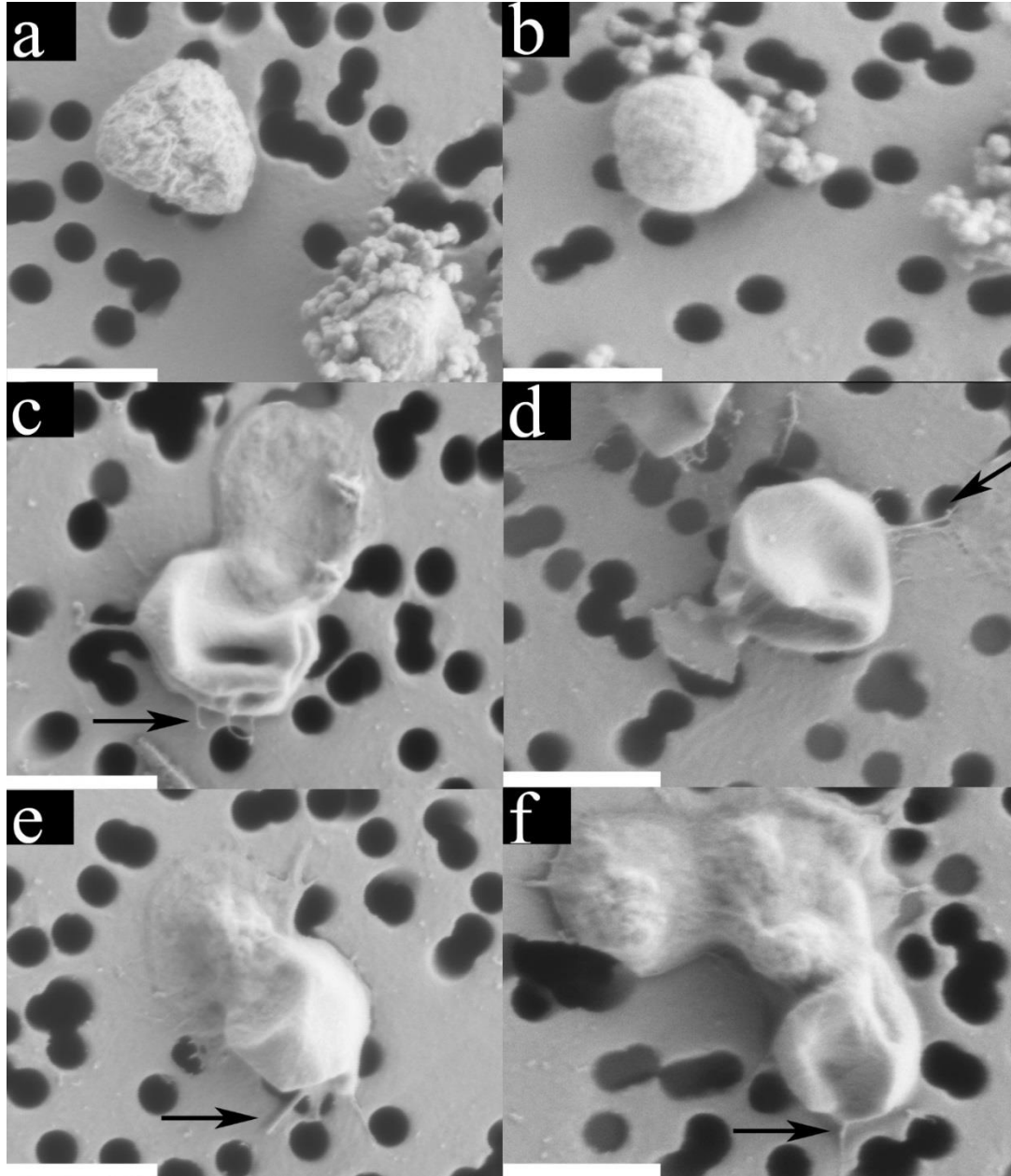


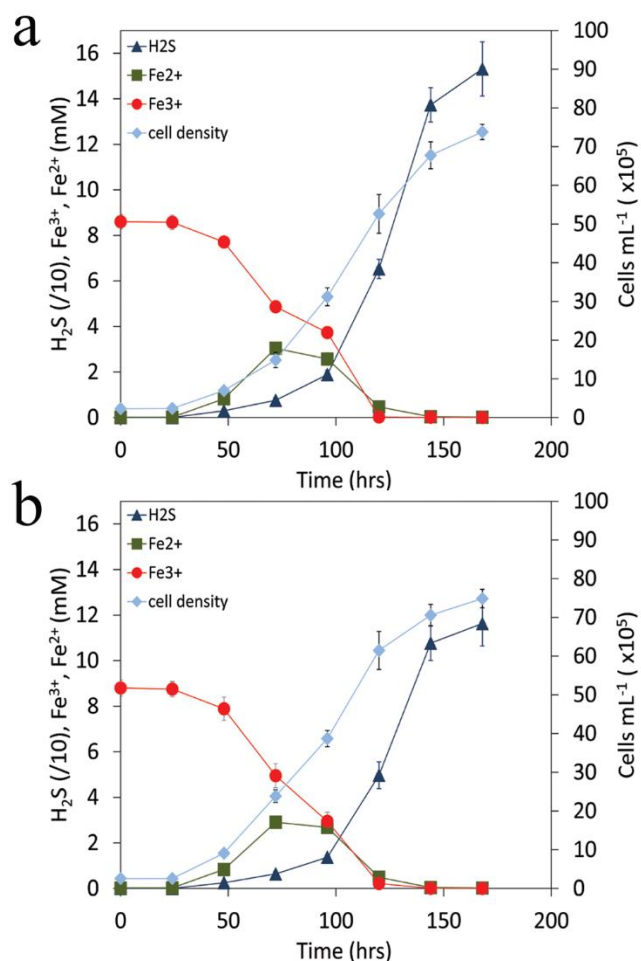
**Figure 3. Growth and/or substrate transformation activities in biotic and abiotic experiments provided with  $\text{H}_2/\text{S}^\circ/\text{Fe}^{3+}$ .** **a**,  $\text{H}_2/\text{S}^\circ$  grown DS80 cells provided with  $\text{H}_2/\text{S}^\circ/\text{Fe}^{3+}$  as potential electron donors and acceptors and  $\text{CO}_2$  as the sole carbon source.  $\text{S}^\circ/\text{Fe}^{3+}$  and  $\text{H}_2/\text{Fe}^{3+}$  grown cells (Supplementary Fig. 3) exhibited indistinguishable growth kinetics and activities when they were used as inoculum. Error bars reflect the standard deviation of measurements made on three replicate cultures. **b**, Titration of anoxic and sterile cultivation medium containing  $\text{H}_2/\text{S}^\circ/\text{Fe}^{3+}$  with  $\text{Na}_2\text{S}$  reveals a similar activity profile when compared to the biological assays. Error bars reflect the standard deviation of measurements made on three replicate assays. Depletion in the concentration of soluble  $\text{Fe}^{2+}$  in both abiotic and biotic assays was due to formation of insoluble iron sulfides (Supplementary Fig. 4).



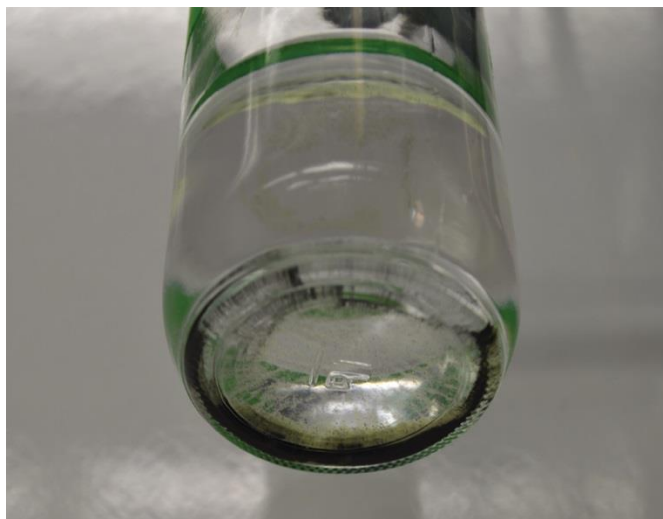
**Supplemental Figure 1.** Growth kinetics and substrate transformation activities of cultures of DS80 grown with  $\text{H}_2/\text{S}^\circ$  (**a**),  $\text{S}^\circ/\text{Fe}^{3+}$  (as ferric sulfate) (**b**), or  $\text{H}_2/\text{Fe}^{3+}$  (as ferric sulfate) (**c**). All cultures were provided with  $\text{CO}_2$  as a carbon source. Error bars reflect the standard deviation of measurements made on three separate cultures.



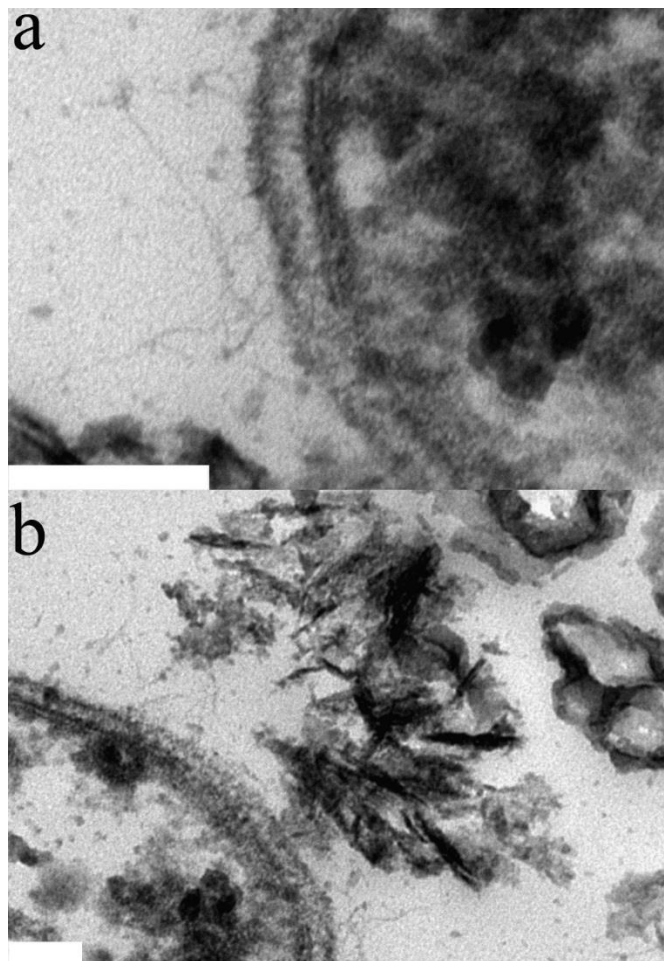
**Supplemental Figure 2.** Field emission scanning electron micrographs (FE SEMs) of cells grown with  $H_2/S^\circ$  (**a,b**),  $S^\circ/Fe^{3+}$  (**c,d**), and  $H_2/Fe^{3+}$  (**e,f**) with arrows denoting pilin-like structures where present. Scale bars represent 1000 nm.



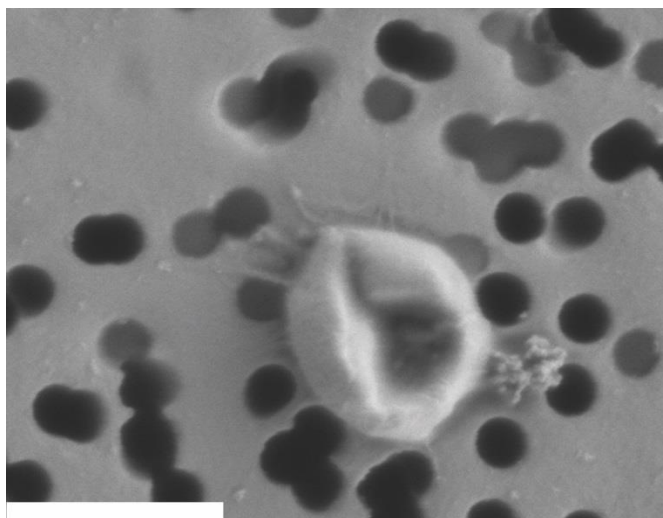
**Supplemental Figure 3.** Growth kinetics and substrate transformation activities of cultures of DS80 grown with  $\text{H}_2/\text{S}^\circ/\text{Fe}^{3+}$  (as ferric sulfate). The inoculum used in experiments presented in the “a” panel was grown with  $\text{S}^\circ/\text{Fe}^{3+}$  (as ferric sulfate) whereas the inoculum used in experiments presented in the “b” panel was grown with  $\text{H}_2/\text{Fe}^{3+}$  (as ferric sulfate). All cultures were provided with  $\text{CO}_2$  as a carbon source. Error bars reflect the standard deviation of measurements made on three separate cultures.



**Supplemental Figure 4.** Insoluble, black iron sulfide precipitates produced during microbial growth in medium containing  $\text{H}_2/\text{S}^\circ/\text{Fe}^{3+}$ .



**Supplemental Figure 5.** Thin section transmission electron micrographs (TEMs) of cells of strain DS80. Scale bars represent 100 nm. Both images show the presence of a precipitate of unknown composition.



**Supplemental Figure 6.** Field emission scanning electron micrograph (FE SEM) of cells grown with  $H_2$ /ferrihydrite. Scale bar represent 1000 nm.



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CHAPTER THREE

EXPANDING ANAEROBIC HETEROTROPHIC

METABOLISM WITH HYDROGEN

Contribution of Authors and Co-Authors

Manuscript in Chapter 3

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## Expanding Anaerobic Heterotrophic Metabolism with Hydrogen

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**Originality-Significance Statement:** Here we show that electron acceptor availability limits the spectrum of electron donors and carbon sources that can sustain growth in a thermoacidophilic archaeon. The addition of molecular hydrogen to growth assays relieves this limitation, allowing cells to use a range of organic substrates as carbon sources under anaerobic conditions through what can be described as mixotrophic or chemolithoheterotrophic metabolism. In this role, hydrogen expands the ecological niche of these cells, allowing them to compete in environments that are dynamic, such as hot springs.



## SUMMARY

The thermoacidophilic and facultatively autotrophic *Acidianus* strain DS80 displays versatility in its energy metabolism and can grow autotrophically and heterotrophically with elemental sulfur ( $S^{\circ}$ ), ferric iron [Fe(III)], or oxygen ( $O_2$ ) as electron acceptors. Here we show that autotrophic and heterotrophic growth with  $S^{\circ}$  as the electron acceptor is obligately dependent on  $H_2$  as electron donor; organic substrates such as acetate can only serve as a carbon source. In contrast, organic substrates such as acetate can serve as electron donor and carbon source for Fe(III) or  $O_2$  grown cells. In  $S^{\circ}$  or Fe(III) grown cells, the amount of  $CO_2$  assimilated is incompletely suppressed by acetate. In Fe(III) grown cells, the amount of acetate oxidized is incompletely suppressed by  $H_2$ . The addition of  $CO_2$  incompletely suppresses acetate assimilation and influences growth kinetics in  $H_2$ /Fe(III) grown cells but has no effect on  $H_2$ / $S^{\circ}$  grown cells. These results indicate that electron acceptor availability influences the spectrum of potential electron donors and carbon sources used by this facultatively mixotrophic strain. Moreover, the availability of  $H_2$  enables the use of organic carbon sources in cells respiring  $S^{\circ}$ , thereby expanding the ecological niche of this organism by allowing them to compete for a wider array of substrates.

## INTRODUCTION

Hydrothermal environments can be dynamic and can vary chemically or physically on time scales that range from seconds (e.g., earthquakes), to days (e.g., temperature variations, diurnal cycling), to seasons (e.g., precipitation) (Hurwitz and Lowenstern, 2014). A common adaptive strategy for microorganisms that inhabit dynamic environments, such as hot springs, is to harbor flexibility in their energy metabolism (generalist strategy). Generalist strategies contribute to a broadening of ecological niche space, both spatially and temporally, and therefore offer a competitive advantage in environments where nutrient flux is dynamic (Kassen, 2002). However, a generalist strategy can also lead to a competitive disadvantage in environments where nutrient availability is not dynamic or where a specialist can outcompete a generalist for available resources.

Many forms of microbial generalists have been described, and these often refer to flexibility in the mechanisms used to acquire a specific element such as carbon or nitrogen, the source of energy used to power metabolism, or in the overall mode of energy metabolism (Kassen, 2002). In the case of carbon, for example, microbial generalists include facultative autotrophs that are capable of switching between autotrophic and heterotrophic growth contingent on the availability of suitable organic substrates (Siebers et al., 2004; Huber and Prangishvili, 2006; Urschel et al., 2016), mixotrophs that utilize both inorganic and organic carbon compounds simultaneously (Jochimsen et al., 1997; Eiler, 2006; Ramos-Vera et al., 2010; Carere et al., in press) and

metabolically diverse heterotrophs that can utilize an array of organic carbon sources (Prokofeva et al., 2014; Schut et al., 2014).

Among characterized thermophilic organisms, crenarchaeotes may exhibit the most generalist lifestyles as they relate to carbon and energy metabolism. For example, the crenarchaeal orders Sulfolobales and Desulfurococcales harbor representatives that range from being strict aerobes to facultative or obligate anaerobes and from obligate autotrophs to facultative autotrophs (Huber and Prangishvili, 2006; Huber and Stetter, 2006). Common among all known members of the Sulfolobales and to some members of the Desulfurococcales is the ability to utilize elemental sulfur ( $S^{\circ}$ ) as an electron donor and/or acceptor, regardless of their sensitivity to oxygen and their mode of carbon metabolism. Despite exhibiting an overall generalist lifestyle, however, several  $S^{\circ}$  metabolizing members of the Sulfolobales and Desulfurococcales exhibit characteristics that are more reflective of specialization. For example, the putatively mixotrophic strains *Acidianus ambivalens* [Sulfolobales; (Zillig et al., 1986; Laska et al., 2003)] and *Stetteria hydrogenophila* [Desulfurococcales; (Jochimsen et al., 1997)] were shown to be incapable of heterotrophic growth via reduction of  $S^{\circ}$ ; but exhibited autotrophic growth via reduction of  $S^{\circ}$  that was obligately dependent on hydrogen ( $H_2$ ) as the electron donor. Potential reasons for the obligate requirement of  $H_2$  during  $S^{\circ}$  reduction in these strains were not further explored, but could provide important new insight into the limitations of flexibility in energy metabolism as a generalist strategy as well as the ecological and evolutionary drivers of adopting this metabolic lifestyle.

We previously isolated an *Acidianus* strain (strain DS80; order Sulfolobales) from a sulfur-rich, acidic hot spring in Yellowstone National Park (YNP), Wyoming, U.S.A (Amenabar et al., 2017). Like other *Acidianus* strains (Seegerer et al., 1986; Zillig et al., 1986; Plumb et al., 2007; Giaveno et al., 2013), strain DS80 was shown to display versatility in its energy metabolism involving hydrogen ( $H_2$ ) or elemental sulfur ( $S^\circ$ ) as electron donors and  $S^\circ$  or ferric iron ( $Fe^{3+}$ ) as electron acceptors (Amenabar et al., 2017). The strain also was shown to be capable of aerobic autotrophic growth with  $S^\circ$  serving as electron donor and aerobic heterotrophic growth using a variety of organic substrates. Under anaerobic chemolithotrophic growth conditions, experiments showed that this strain preferentially utilizes the  $H_2/S^\circ$  redox couple rather than the  $H_2/Fe^{3+}$  or  $S^\circ/Fe^{3+}$  couples, despite the lower energy yield associated with the former (Amenabar et al., 2017). We suggested that this preference is due to more thermodynamically efficient coupling of electron transfer reactions in an evolutionarily optimized membrane-bound [NiFe]-hydrogenase and membrane-associated sulfur reductase (Amenabar et al., 2017), which co-purify as a complex in *A. ambivalens* (Laska et al., 2003). Electron transfer between the [NiFe]-hydrogenase and the sulfur reductase in *A. ambivalens* is mediated by sulfolobusquinone; it is unclear if *S. hydrogenophila* has a similar complex due to the lack of a publicly available genome for this strain. Nonetheless, it is possible that evolutionary optimization of efficient electron transfer reactions between  $H_2$  and  $S^\circ$  in this complex lead to specificity in the source of reductant (via reduced quinol) that can be used to reduce  $S^\circ$  via a sulfur reductase complex. If true, then electrons derived from organic carbon during heterotrophic metabolism may not be capable of being transmitted

through electron transport chains involving  $S^{\circ}$  as the terminal oxidant, perhaps explaining the obligate requirement for  $H_2$  as an electron donor during  $S^{\circ}$  dependent growth in *A. ambivalens* and *S. hydrogenophila*.

In the present study, we aimed to further define the role of  $H_2$  in the metabolism of *Acidianus* strain DS80 when grown with various electron acceptors [ $Fe(III)$ ,  $O_2$ , and  $S^{\circ}$ ] under autotrophic, mixotrophic, and heterotrophic growth conditions. The genome sequence of DS80 was reconstructed and interrogated with respect to genes encoding for proteins that putatively allow for the use of specific electron donors, electron acceptors, and carbon sources during growth. Like other *Acidianus* strains and members of the Crenarchaeota in general, genomic signatures consistent with flexibility in energy and carbon metabolism were observed. Physiological analyses were used to substantiate predictions of substrates capable of supporting growth. Results are discussed considering the role of  $H_2$  in allowing for heterotrophic growth, with an emphasis placed on biochemical mechanisms potentially explaining observed patterns of substrate usage in this and other thermophilic strains.

## MATERIALS AND METHODS

**Culture Conditions.** *Acidianus* strain DS80, previously isolated from “Dragon Spring”, Yellowstone National Park, Wyoming (Amenabar et al., 2017), was cultivated in anoxic base salts medium containing  $NH_4Cl$  ( $0.33\text{ g L}^{-1}$ ),  $KCl$  ( $0.33\text{ g L}^{-1}$ ),  $CaCl_2 \cdot 2H_2O$  ( $0.33\text{ g L}^{-1}$ ),  $MgCl_2 \cdot 6H_2O$  ( $0.33\text{ g L}^{-1}$ ), and  $KH_2PO_4$  ( $0.33\text{ g L}^{-1}$ ). The pH of the medium was adjusted to 3.0 with concentrated hydrochloric acid. Briefly, 55 mL of medium was

dispensed into 160 mL serum bottles and was subjected to autoclave sterilization. Following autoclave sterilization and while still hot ( $\sim 90^{\circ}\text{C}$ ), filter-sterilized Wolfe's vitamins ( $1\text{ mL L}^{-1}$  final concentration), filter sterilized SL-10 trace metals ( $1\text{ mL L}^{-1}$  final concentration), and elemental sulfur ( $\text{S}^{\circ}$ ;  $5\text{ g L}^{-1}$  final concentration; sterilized by baking at  $100^{\circ}\text{C}$  for 24 h) or filter sterilized ferric iron in the form of  $\text{Fe}_2(\text{SO}_4)_3 \cdot \text{H}_2\text{O}$  ( $3.7\text{ g L}^{-1}$ ) were added. The bottles and their contents were deoxygenated by purging with sterile nitrogen ( $\text{N}_2$ ) gas passed over heated ( $210^{\circ}\text{C}$ ) and hydrogen ( $\text{H}_2$ ) reduced copper shavings. The serum bottles were sealed with butyl rubber stoppers and heated to  $80^{\circ}\text{C}$  prior to the replacement of the headspace with 80%:20%  $\text{H}_2$ : $\text{CO}_2$ , unless otherwise specified. All growth experiments were conducted at  $80^{\circ}\text{C}$  in medium with a pH of 3.0.

**Isolation of Genomic DNA and Sequencing.** Biomass for DNA extraction was concentrated via centrifugation ( $14,000 \times g$ , 20 min.,  $4^{\circ}\text{C}$ ) from a single 55 mL autotrophically grown culture provided with  $\text{H}_2$  and  $\text{S}^{\circ}$  as electron donor and acceptor, respectively. The cell pellet was re-suspended in 1 mL of sterile base salts medium and carbon disulfide ( $\text{CS}_2$ ) was added to solubilize and partition  $\text{S}^{\circ}$  into the organic phase to improve the recovery of DNA, as has been described previously (Boyd et al., 2007).  $\text{CS}_2$  was added to a final concentration of 10% vol./vol., and following phase separation, the aqueous phase was collected and concentrated by centrifugation ( $14,000 \times g$ , 20 min,  $4^{\circ}\text{C}$ ). Genomic DNA was extracted from the cell pellet using a modified phenol-chloroform extraction procedure. Briefly, cells were first re-suspended in 150 mM Tris-HCl buffer (pH 7.5) containing 15 mM EDTA (pH 8.0) and then were subjected to three cycles of sonication (1 min. each) on ice. Lysozyme was added to the extract to a final

concentration of  $1 \text{ mg mL}^{-1}$  and the extract was incubated at  $37^{\circ}\text{C}$  for 30 min. Following incubation, proteinase K ( $200 \text{ } \mu\text{g mL}^{-1}$  final concentration) and sodium dodecyl sulfate (1% final concentration) were added to the extract and the extract was incubated at  $50^{\circ}\text{C}$  for 30 min. Cell debris was removed by centrifugation ( $9,000 \times g$  for 20 min.) and the resultant clarified phase was extracted with two volumes of phenol/chloroform and subjected to centrifugation ( $14,000 \times g$ , 10 min.,  $4^{\circ}\text{C}$ ). The soluble phase was again collected and extracted with two volumes of chloroform/isoamyl alcohol (24:1) and centrifuged ( $14,000 \times g$ , 10 min.,  $4^{\circ}\text{C}$ ). Following centrifugation, the soluble phase was collected and DNA was precipitated from this fraction by adding two volumes of 100% ethanol, followed by incubation at  $-20^{\circ}\text{C}$  for 12 hr. and centrifugation ( $14,000 \times g$ , 10 min.,  $4^{\circ}\text{C}$ ) (Sambrook and Russell, 2001). The pellet was collected, dried in a laminar flow hood, and re-suspended in  $50 \text{ } \mu\text{L}$  of molecular grade water. The concentration of DNA in the extract was determined using a Qubit 2.0 Fluorimeter (Invitrogen, Carlsbad, CA, USA) and a Qubit dsDNA HS Assay kit (Molecular Probes, Eugene, OR, USA).

Total genomic DNA was sequenced at the Genomics Core Facility at the University of Wisconsin-Madison using the Illumina MiSeq platform and the 2 x 300 bp v3 sequencing kit. Paired-end DNA fragments were prepared according to the manufacturer's protocol. Illumina adapters were removed and reads with a length of less than 50 bp were removed using Trimmomatic (version 0.33) (Bolger et al., 2014). FastQC (version 0.11.4) (Andrews, 2010) was used to quality filter remaining reads to only include bases with a 26 Phred average value, or higher. Quality filtered reads were assembled using SPAdes (version 3.8.1) (Bankevich et al., 2012; Nurk et al., 2013) with

k-mer lengths ranging from 37 to 127. The genome assembly with contigs exhibiting the highest N50 length (k-mer of 121 bp) was used for further analysis. Contiguator (Galardini et al., 2011) was used to identify contigs with high similarity alignments to *Acidianus hospitalis* strain W1 with the following parameters: e-value of  $1e^{-20}$ , contig threshold of 1000 bp, contig coverage threshold of 20%, hit length threshold of 1100 bp, and gap size for overlapping contigs of 100 bp. CheckM (Parks et al., 2015) was used to estimate genome completeness based on the presence of 217 housekeeping genes common to Thermoprotei. Average Amino Acid Identity (AAI) and Average Nucleotide Identity (ANI) were used to assess the genomic relatedness between strain DS80 and its closest relative with an available genome, *A. hospitalis* W1, using available online tools (Goris et al., 2007; Rodriguez-R and Konstantinidis, 2014, 2016). Lastly, the extent of genomic synteny between strain DS80 and *A. hospitalis* W1 was analyzed using Circos software (Krzywinski et al., 2009).

Phylogenomic analyses were conducted by surveying the DS80 assembly for conserved single copy archaeal-specific phylogenetic marker genes (n=104) using the Amphora2 software package (Wu and Scott, 2012). Publicly available phylogenetic reference genomes were downloaded and analyzed as above to represent the diversity of available Sulfolobales genomes. Only reference genomes with >50% of the phylogenetic marker genes present in the assembly were included. Phylogenetic analysis was conducted using a concatenated alignment of the phylogenetic marker protein encoding genes, with individual alignment of each of the 104 amino acid sequences using Clustal Omega v.1.2 (Sievers et al., 2011). The concatenated amino acid alignment was subjected



to Maximum Likelihood (ML) phylogenetic reconstruction using RAxML v. 8.2.4 (Stamatakis, 2014) specifying the LG protein substitution model (PROTCATLG) with 100 ML bootstraps. *Pyrobaculum islandicum* (order: Thermoproteales) was used as an outgroup to root the Sulfolobales. The partial genome sequence of strain DS80 has been deposited in the Joint Genome Institute IMG database, IMG submission identifier 92883, genome ID 2690315630.

**Physiological Characterization.** Carbon sources that support growth of strain DS80 when S<sup>0</sup> or Fe(III) served as terminal electron acceptor were evaluated in anoxic base salts medium, prepared as described above. Putative carbon sources capable of supporting growth were identified based on analysis of the DS80 genome (described below). Glucose, fructose, galactose, mannose, lactose, maltose, sucrose, yeast extract, peptone, and casamino acids were added to anoxic base salts medium to achieve a final concentration of 0.1% (wt./vol.). Sodium formate and sodium acetate were added to base salts medium to achieve a final concentration of 200  $\mu$ M, since previous studies have shown these organic acids to be toxic to acidophiles at concentrations higher than 200  $\mu$ M (Urschel et al., 2016). All carbon sources were tested for their ability to serve as a carbon source (100% H<sub>2</sub> headspace) or as a carbon and electron donor (100% N<sub>2</sub> headspace). The ability of strain DS80 to grow with carbon monoxide (CO) as a carbon source (90%:10% H<sub>2</sub>:CO headspace) or as a carbon and electron donor (90%:10% N<sub>2</sub>:CO) was also examined. The ability of these different carbon sources to support mixotrophic growth, with respect to carbon metabolism, was evaluated as described above using a 80%:20% N<sub>2</sub>:CO<sub>2</sub> headspace. The ability of strain DS80 to grow

heterotrophically with these different carbon sources when O<sub>2</sub> served as terminal electron acceptor (serum bottle headspace was air) was also evaluated in cultures grown on a rotary shaker (100 rpm) at 80°C.

Growth and activity of strain DS80 with different carbon sources in the presence of various electron donors and acceptors was quantified in terms of cell density and production of total sulfide (S<sup>2-</sup>; proxy for S<sup>0</sup> reduction) or ferrous iron [Fe<sup>2+</sup>; proxy for Fe(III) reduction], as previously described (Amenabar et al., 2017). The results presented represent the average and standard deviation of triplicate measurements conducted on triplicate cultures.

**Carbon Assimilation and Mineralization.** The amount of acetate assimilated into cellular biomass or mineralized and released as CO<sub>2</sub> was determined in cultures provided with S<sup>0</sup> or Fe(III) as terminal electron acceptor, in the presence or absence of H<sub>2</sub>. Briefly, 160 mL serum bottles containing 55 mL of base salts medium (described above) and S<sup>0</sup> or Fe(III) with a 100% H<sub>2</sub> or 100% N<sub>2</sub> headspace was amended with acetate to a final concentration of 100 µM [45:1 ratio of unlabeled acetate to 1-[<sup>14</sup>C] sodium acetate (CH<sub>3</sub><sup>14</sup>COONa)]. Each assay received a total of 5 µCi of 1-[<sup>14</sup>C]-acetate. At each time point, a 2 mL subsample of culture and a 1 mL subsample of gas was removed from each vial for use in determining the amount of 1-[<sup>14</sup>C]-acetate assimilated into biomass or mineralized as CO<sub>2</sub>, using scintillation counting as previously described (Urschel et al., 2016). Three mL of sterile N<sub>2</sub> or H<sub>2</sub> (depending on the cultivation condition) was added to each vial at each sampling interval to maintain atmospheric pressure in the vials. Conversion of the assimilation or mineralization of 1-[<sup>14</sup>C] labeled

acetate to the assimilation or mineralization of total acetate (unlabeled plus labeled) was performed as previously described (Urschel et al., 2016).

#### **Influence of Acetate on the Assimilation of Dissolved Inorganic Carbon**

**(DIC).** The amount of DIC assimilated into cellular biomass was determined in cultures of DS80 provided with  $S^{\circ}$  or Fe(III) as terminal electron acceptor in the presence of increasing concentrations of acetate. Briefly, 160 mL serum bottles containing 55 mL of base salts medium and  $S^{\circ}$  or Fe(III) with a 80%:20%  $H_2$ : $CO_2$  headspace (described above) were amended with a total of 5  $\mu$ Ci of [ $^{14}C$ ]-bicarbonate, followed by the addition of 0, 50, 100 or 200  $\mu$ M [ $^{12}C$ ]-acetate. Cultures were inoculated and the influence of acetate on the assimilation of the amount of  $^{14}CO_2$  assimilated into biomass was determined as previously described (Urschel et al., 2016).

**Influence of DIC on the Assimilation and Mineralization of Acetate.** The amount of acetate assimilated into cellular biomass was determined in cultures provided with  $S^{\circ}$  or Fe(III) as terminal electron acceptor in the presence or absence of added  $CO_2$ . Briefly, 160 mL serum bottles containing 55 mL of base salts medium and  $S^{\circ}$  or Fe(III) with a 80%:20%  $H_2$ : $CO_2$  headspace (described above) was amended with a total of 5  $\mu$ Ci of 1- $^{14}C$ -acetate, followed by the addition of [ $^{12}C$ ]-acetate to achieve a final concentration of 100  $\mu$ M. Cultures were inoculated and the influence of  $CO_2$  on the assimilation/mineralization of 1- $^{14}C$  acetate was determined as previously described (Urschel et al., 2016).

## RESULTS

**DS80 Genomic Properties and Phylogeny.** Assembly of the DS80 genome resulted in 133 non-overlapping contigs after curation. Of the 2604 genes encoded on these contigs, 2570 (98.6%) encoded for proteins. The DS80 genome is estimated to be 99% complete based on the presence of 216 of 217 conserved, taxonomic marker genes and has a predicted genome size of 2.19 Mbp with an average GC content of 34.4% (**Supp. Fig. 1**). These values are similar to those obtained from the genome of *A. hospitalis* W1, which is ~ 2.13 Mbp with an average GC content of 34.2% (You et al., 2011) but are slightly lower than those obtained from the genome of *A. copahuensis*, which is 2.45 Mbp with an average GC content of 35.6% (Urbieta et al., 2014). A genome sequence of *A. ambivalens* is not publicly available, precluding a detailed genomic comparison between DS80 and this strain.

Phylogenomic reconstruction of a concatenation of 104 single copy housekeeping marker genes (**Supp. Fig. 2**) indicates a close evolutionary relationship between strain DS80 and *A. hospitalis* W1. Consistent with this analysis, the AAI between the DS80 genome and *A. hospitalis* W1 was 97.6%, while the ANI of 8257 genome fragments in the DS80 genome when compared to those from *A. hospitalis* W1 was 98.5% (**Supp. Figs. 3 and 4, respectively**), both of which correspond to intraspecific levels of homology (Konstantinidis and Tiedje, 2005; Konstantinidis et al., 2006). Of the 133 contigs in the DS80 genome, 117 aligned with *A. hospitalis* W1 at 70% nucleic acid identities or higher. The remaining 16 contigs were found to be more similar to non-*Acidianus* genomes within the Sulfolobales order (**Supp. Fig. 5**).

The 2570 proteins encoded in the DS80 genome were subjected to BLASTp analysis (threshold of 30% sequence identities) against the proteins identified in *A. hospitalis* W1 (n=2389), and vice versa, to identify differences in the proteins encoded in their respective genomes. A total of 97 proteins from *A. hospitalis* W1 were not identified in the genome of strain DS80 (**Supplementary File 1**). It is possible that these genes are encoded in un-sequenced portions of the DS80 genome. However, this scenario is not likely considering the high completeness estimate for DS80 which takes into account numerous conserved markers that are distributed throughout the genome (i.e., they are not necessarily co-localized). A total of 146 proteins encoded in the DS80 genome were not identified in the complete *A. hospitalis* W1 genome (**Supplementary File 2**). Foremost among these were genes encoding for an uptake [NiFe]-hydrogenase and a sulfur reductase complex (described below). Consistent with the ability of DS80 to conserve energy with the  $H_2/S^\circ$  redox couple (Amenabar et al., 2017), these proteins have previously been shown to purify as a complex in cells of *A. ambivalens* grown with  $H_2/S^\circ$  (Laska et al., 2003). Thus, despite the close phylogenomic relationship between *A. hospitalis* and DS80 (**Supp. Fig. 2**), key differences exist that likely drove their divergence and influence their respective ecologies.

**Genomic Prediction of Electron Donor Usage.** The partial genome of DS80 encodes proteins with homology to those that allow for use of a variety of single carbon compounds as putative electron donors or carbon sources (**Fig. 1**). For example, a complement of genes encoding the majority (15 out of 16) of the enzymes involved in the

hydroxypropionate-hydroxybutyrate pathway of CO<sub>2</sub> fixation, first described in *Metallosphaera sedula* (Berg et al., 2007), was identified in the DS80 genome. This is consistent with the apparent universal distribution of this CO<sub>2</sub> assimilation pathway among members of the Sulfolobales with available genome sequences (Berg et al., 2010) and with the ability of strain DS80 to grow with CO<sub>2</sub> as the sole carbon source (Amenabar et al., 2017). A full suite of proteins encoding a Ni-dependent carbon monoxide dehydrogenase (CODH) and an “H”-type formate dehydrogenase (FDH-H) were identified, which exhibit 99% and 98% sequence identities to homologs in *A. hospitalis*, respectively. As mentioned above, the genome also encoded for homologs of a membrane bound [NiFe]-hydrogenase (Hyn) organized in a gene cluster containing 12 open reading frames (9964 bp in length) and the gene synteny was identical to that reported for *A. ambivalens* (Laska et al., 2003) (**Supp. Fig. 6**). Homologs of Hyn were not detected in the genome of *A. hospitalis*. Phylogenetic analyses of the large subunit (HynL) of the DS80 Hyn indicates that it is a Group 1g [NiFe]-hydrogenases (membrane bound H<sub>2</sub> uptake hydrogenase) (Greening et al., 2016) and the small subunit (HynS) encodes for a N-terminal twin-arginine motif (RRxFxK), indicating that it is likely transported to the outer side of the plasma membrane by the twin-arginine translocation (TAT) pathway in a manner similar to other Group 1 hydrogenases (Vignais et al., 2001). However, unlike most Group 1 [NiFe]-hydrogenase gene clusters which encode for a cytochrome *c* subunit and function to couple oxidation of H<sub>2</sub> to the reduction of cytochrome *c* (Vignais et al., 2001), Group 1g [NiFe]-hydrogenase gene clusters,

including that from DS80 and *A. ambivalens* (Laska et al., 2003), do not encode for a cytochrome *c* subunit.

The DS80 genome encodes several other enzymes putatively involved in sulfur metabolism, including a cytoplasmic sulfur oxygenase reductase (SOR) that has been shown or hypothesized to function in  $S^0$  oxidation in other members of the *Acidianus* genus such as *A. brierleyi* (Emmel et al., 1986), *A. ambivalens* (Kletzin, 1989), and *A. tengchongensis* (He et al., 2000) (**Fig. 1**). In addition to inorganic electron donors and carbon sources, the genome of DS80 encoded for numerous sugar transporters and glycosidases suggesting an ability to utilize carbohydrates as carbon sources. Moreover, homologs of genes predicted to encode amino acid and oligopeptide transporters, as well as genes encoding proteolytic enzymes including endopeptidases, aminopeptidases, and carboxypeptidases were identified in DS80 genome, suggesting an ability to hydrolyze peptides and oxidize amino acids. Genes encoding a complete suite of homologs of the tricarboxylic acid (TCA) cycle, gluconeogenesis pathway, and the non-phosphorylative Entner-Doudoroff (ED) pathway (**Fig. 1. and Supp. Fig. 7**) were also detected in the DS80 genome, suggesting an ability for this strain to utilize organic substrates during heterotrophic metabolism, similar to what has been predicted for other *Acidianus* strains based on comparative genomic analyses (You et al., 2011; Urbietta et al., 2014). The presence of a gluconeogenesis pathway would also allow strain DS80 to assimilate organic carbon when the TCA cycle is not operational. Such a situation may occur when organic carbon substrates are used only as a source of carbon while other electron donors, such as  $H_2$ , serve as electron donor.

**Genomic Prediction of Electron Acceptor Usage.** The DS80 genome encodes for homologs of a multimeric sulfur reductase (Sre) complex that has been shown to be involved in  $S^{\circ}$  reduction in *A. ambivalens* (Laska et al., 2003; Veith et al., 2011). The Sre complex in DS80 consists of homologs of *sreABCDE* that are syntenous with those encoded in the genome of *A. ambivalens* and are between 79 and 95% homologous at a protein level (**Supp. Fig. 6**). Similar to SreA from *A. ambivalens*, SreA in the DS80 genome is predicted to encode a twin-arginine motif, suggesting export of the alpha subunit of Sre by the twin-arginine translocation (TAT) pathway. Respiration of  $S^{\circ}$  with  $H_2$  in hyperthermophiles, including *Acidianus ambivalens* (Laska et al., 2003) and *Thermoproteus tenax* (Siebers et al., 2011), is thought to be mediated by a short electron transfer chain comprising a membrane-associated Hyn and a membrane-associated Sre complex linked by a quinone cycle (Laska et al., 2003). Due to similarity in the synteny of genes and homology of Sre and Hyn proteins encoded in the DS80 genome and those in *A. ambivalens* (Laska et al., 2003), it is possible, if not likely that the Sre and Hyn function as a complex in DS80 cells.

Despite a demonstrated ability to reduce soluble and mineral forms of Fe(III) (Amenabar et al., 2017), homologs of enzymes implicated in extracellular Fe(III) reduction including porin cytochrome trans-outer membrane protein complexes (Liu et al., 2014) and multi-heme *c* type cytochrome membrane protein complexes (Shi et al., 2012) were not identified in the DS80 genome. The presence of several oxidases including a terminal quinol oxidase encoded in the DS80 genome is consistent with the



ability of strain DS80 to grow aerobically with O<sub>2</sub> as terminal electron acceptor (Amenabar et al., 2017), similar to what has been predicted in *A. hospitalis* (You et al., 2011). The presence of genes allowing for aerobic respiration is consistent with numerous other members of the *Acidianus* genus, which have been shown to be facultative anaerobes (Huber and Stetter, 2015).

**Physiological Characterization of *Acidianus* Strain DS80.** To test genomic predictions of the ability of DS80 to utilize a wide variety of substrates to support growth, cultures were provided with a range of inorganic (CO<sub>2</sub>, CO) and organic compounds (formate, acetate, glucose, fructose, galactose, mannose, lactose, maltose, sucrose, yeast extract, peptone and casamino acids) as energy and/or carbon sources with S<sup>0</sup>, Fe(III), and O<sub>2</sub> as electron acceptors and activities and the production of cells were monitored (**Table 1**). Cells were able to couple oxidation of a number of inorganic (i.e., CO<sub>2</sub>) and organic carbon sources (i.e., formate acetate, glucose, galactose, mannose, lactose, maltose, yeast extract, peptone, and casamino acids) to reduction of Fe(III) to sustain growth, regardless of whether H<sub>2</sub> was provided to cultures. This indicates that these substrates can be used as both a carbon source and as an electron donor. In contrast, when S<sup>0</sup> served as electron acceptor, these inorganic and organic carbon sources could only support growth if H<sub>2</sub> was also provided in what can be described as chemolithoheterotrophic growth. This indicates that CO, formate, acetate, glucose, galactose, mannose, lactose, maltose, yeast extract, peptone, and casamino acids can only serve as a carbon source and not as a reductant during growth with S<sup>0</sup> as electron acceptor. The addition of CO<sub>2</sub> to cultures provided with these carbon sources did not

allow for growth under  $S^{\circ}$  reducing conditions in the absence of  $H_2$  (data not shown), indicating that the lack of growth was not due to cells being obligate mixotrophs. All of the aforementioned inorganic and organic substrates could support growth under aerobic conditions and growth under these conditions was not dependent on the presence of  $H_2$  (**Table 1**).

**Carbon Assimilation and Mineralization.** Growth experiments indicating that DS80 could not couple heterotrophic metabolism with  $S^{\circ}$  reduction but could couple heterotrophic metabolism to  $Fe(III)$  and  $O_2$  reduction prompted additional experiments to determine the ability of strain DS80 to assimilate and/or oxidize (mineralize) acetate with different electron acceptors. In cultures provided with  $S^{\circ}$  as the terminal electron acceptor,  $H_2$  as electron donor, and acetate as a carbon source, the density of cells, the amount of acetate assimilated, and the concentration of total sulfide increased concurrently (**Fig. 2A**).  $^{14}CO_2$  was not detected above background levels in the headspace of cultures confirming that acetate could only serve as a carbon source and not an electron donor when  $S^{\circ}$  served as electron acceptor. Indeed, no evidence for acetate oxidation/mineralization or assimilation was observed in cultures provided with  $S^{\circ}$  as the terminal electron acceptor and acetate as a carbon and electron source (data not shown), consistent with growth assays indicating that this cultivation condition did not support growth.

In cultures provided with  $Fe(III)$  as electron acceptor,  $H_2$  as electron donor, and acetate as a carbon source, the cell density, the amount of acetate assimilated, and the concentration of total  $Fe^{2+}$  increased concurrently (**Fig. 2B**).  $^{14}CO_2$  was detected in the

headspace indicating that acetate was also being oxidized/mineralized under this condition. However, cultures provided with Fe(III) as the terminal electron acceptor and acetate as a carbon and electron source (no added H<sub>2</sub>) had a significantly greater amount of <sup>14</sup>CO<sub>2</sub> headspace (**Fig. 2C**). This indicates that H<sub>2</sub> can serve as an additional source of reductant in Fe(III) reducing cells grown with acetate.

The cell yield per mol of Fe(III) reduced when grown with H<sub>2</sub> as electron source and acetate as carbon/electron source was 1.6-fold lower (1.1 versus 1.8 cells per mol Fe(III) reduced, respectively) than when cells were grown with acetate as the sole carbon and electron source. This suggests that cells were more efficient at coupling energy metabolism with biomass synthesis when acetate served as both the electron and carbon source. The amount of C assimilated per cell produced in log phase cultures when grown with acetate/Fe(III) provided with H<sub>2</sub> was not significantly different. These data suggest that electrons recovered from H<sub>2</sub> are involved in less efficient respiratory chains in DS80 than electrons from acetate under Fe(III) reducing conditions. The production of <sup>14</sup>CO<sub>2</sub> in the headspace of acetate-amended cultures provided with Fe(III)/H<sub>2</sub> (**Fig. 2B**) suggests that this strain may be growing mixotrophically with respect to its energy metabolism when it is provided with acetate. It has been previously shown that cells growing mixotrophically have to invest in the synthesis and maintenance of the proteins involved in the types of metabolisms that they might be using (Eiler, 2006), in this case chemolithotrophy and chemoheterotrophy. Energetic costs associated with the synthesis and maintenance of these proteins may lower the overall metabolic efficiency of the cells, thereby leading to no difference in the growth rates of cells.

**Influence of Acetate on DIC Assimilation.** To explore the effects of acetate on DIC assimilation, DS80 cells were provided with  $\text{H}_2/\text{S}^\circ/\text{CO}_2$  or  $\text{H}_2/\text{Fe(III)}/\text{CO}_2$  and these cultures were amended with increasing concentrations of acetate (up to 200  $\mu\text{M}$ ). The amount of DIC assimilated into biomass decreased systematically with increasing concentrations of acetate, relative to unamended controls, in cultures provided with  $\text{H}_2/\text{S}^\circ$  (**Fig. 3A**). However, the magnitude of the suppression of DIC was not proportional to the amount of acetate provided, in particular when cells were provided with acetate at a concentration of 100  $\mu\text{M}$  or more. This indicates that acetate was not limiting activity and that cells were assimilating DIC in the presence of acetate. Moreover, despite evidence indicating that acetate suppresses DIC assimilation in cells provided with  $\text{H}_2/\text{S}^\circ$  (**Fig. 3A**), amendment of cultures with acetate had no effect on the production of cells (**Fig. 3B**). Together, these results indicate that cells provided with  $\text{H}_2/\text{S}^\circ$  are simultaneously using DIC and acetate to meet their carbon demands in what can be termed mixotrophy with respect to their carbon metabolism.

Similar to cells provided with  $\text{H}_2/\text{S}^\circ/\text{CO}_2$ , rates of DIC assimilation in cells provided with  $\text{H}_2/\text{Fe(III)}/\text{CO}_2$  decreased systematically with increasing concentrations of added acetate (**Fig. 3C**). The magnitude of the suppression of DIC was also not proportional to the amount of acetate provided and amendment with acetate also did not influence the production of cells (**Fig. 3D**). Together, these results indicate that cells provided with  $\text{H}_2/\text{Fe(III)}$  are simultaneously using DIC and acetate to meet their carbon

demands in what can be described as mixotrophy with respect to both their carbon and their energy metabolism.

**Influence of DIC on Acetate Assimilation/Mineralization.** The addition of CO<sub>2</sub> did not substantially affect the growth kinetics of cells provided with H<sub>2</sub>/S<sup>0</sup>/acetate (**Fig. 4A**), when compared to those not provided with CO<sub>2</sub> (**Fig. 2A**). In contrast, the addition of CO<sub>2</sub> to cultures provided with H<sub>2</sub>/Fe(III)/acetate (**Fig. 4B**) resulted in a 411% increase in the number of cells produced at stationary phase ( $9.72 \times 10^6$  cells mL<sup>-1</sup> versus  $2.36 \times 10^6$  cells mL<sup>-1</sup>), when compared to cells provided with only H<sub>2</sub>/Fe(III)/acetate (**Fig. 2B**). At stationary phase, the ratio of acetate mineralized to that assimilated was 1:4 for H<sub>2</sub>/Fe(III)/acetate grown cells in the absence of CO<sub>2</sub> (**Fig. 2B**) versus 3:4 for cells grown in the presence of CO<sub>2</sub> (**Fig. 4B**), likely due to a decreased reliance on acetate as a source of carbon (i.e., cells are fixing CO<sub>2</sub>). Consistent with this interpretation, H<sub>2</sub>/Fe(III)/acetate grown cells provided with CO<sub>2</sub> assimilated 0.7 fmol of acetate per cell at stationary phase when compared to 3.7 fmol of acetate per cell when H<sub>2</sub>/Fe(III)/acetate grown cells were not provided with CO<sub>2</sub>. These observations confirm that strain DS80 is mixotrophic with respect to both its carbon and energy metabolism, when Fe(III) is provided as an electron acceptor.

## DISCUSSION

Genomic and physiological characterization indicate that *Acidianus* strain DS80 exhibits flexibility in its metabolism at the level of electron donors, electron acceptors, and carbon sources capable of supporting metabolism. However, flexibility in the energy

and carbon metabolism of DS80 was shown to have its limitations, and in particular, was dependent on the electron acceptor that was provided during growth. For example, DS80 was capable of growing mixotrophically with respect to its carbon metabolism (simultaneous assimilation of CO<sub>2</sub> and organic carbon) and energy metabolism (simultaneous oxidation of H<sub>2</sub> and acetate) when Fe(III) served as electron acceptor; mixotrophic growth was not rigorously examined when O<sub>2</sub> served as electron acceptor. In contrast to growth with Fe(III) as electron acceptor, cells provided with S<sup>°</sup> as electron acceptor could grow mixotrophically with respect to their carbon metabolism (assimilation of CO<sub>2</sub> and acetate) and this activity was obligately dependent on H<sub>2</sub> as an electron donor. This observation is similar to those made for *S. hydrogenophila* and to *A. ambivalens*, both of which were shown to be obligately dependent on H<sub>2</sub> as electron donor during S<sup>°</sup> dependent growth (Zillig et al., 1986; Jochimsen et al., 1997). However, unlike strain *Acidianus* strain DS80 and *A. ambivalens* (Zillig et al., 1986), both of which can be described as facultative mixotrophs with respect to their carbon metabolism during S<sup>°</sup> dependent growth with H<sub>2</sub>, *S. hydrogenophila* is dependent on trace yeast extract for growth and is an obligate mixotroph. Thus, despite genomic data indicating an apparent ability to utilize a range of organic substrates as electron donor and carbon source when Fe(III) or O<sub>2</sub> served as electron acceptor, these substrates could only serve as a carbon source and not an electron donor when S<sup>°</sup> served as electron acceptor. The dependence on H<sub>2</sub> as an electron donor during heterotrophic growth with S<sup>°</sup> as electron acceptor can be described as chemolithoheterotrophic growth, which has been described (albeit not

necessarily defined as such) in sulfur oxidizing Bacteria (King, 2007; Ghosh and Dam, 2009) and sulfur reducing Archaea (Jochimsen et al., 1997).

Several possible explanations exist for why members of the genus *Acidianus* may not be able to respire  $S^{\circ}$  using the organic compounds tested here. The first involves the cellular localization of the reductase complex, which may constrain delivery of electrons to the complex due to specificity of the Sre for electron carriers. Respiration of  $S^{\circ}$  with  $H_2$  in hyperthermophiles, including *Thermoproteus tenax* (Siebers et al., 2011), *Acidianus ambivalens* (Laska et al., 2003), and presumably DS80 based on genomic inference, is mediated by a short electron transfer chain comprising a membrane-associated [NiFe]-hydrogenase and a membrane-associated  $S^{\circ}$  reductase complex (Sre1) linked by a quinone cycle (Laska et al., 2003) (**Fig. 5A**). Respiration of  $S^{\circ}$  in these strains is similar to the membrane-bound respiratory system found in mesophilic  $S^{\circ}$ /polysulfide reducing bacteria such as *Wolinella succinogenes* (Hedderich et al., 1998). The Sre1 operon in *T. tenax*, *A. ambivalens*, and *Acidianus* strain DS80 encodes for five subunits (ABCDE), with the alpha subunit (SreA) encoding for a twin arginine motif indicating that it is localized on the external side of the plasma membrane. However, the genome of *T. tenax* also encodes for a second Sre operon (Sre2) that includes only three subunits (ABC); none of the subunits encode for twin-arginine motifs indicating a cytoplasmic orientation of this membrane bound complex (Siebers et al., 2011) (**Fig. 5B**), which may influence the source of reductants that can be used to reduce this complex.

Unlike *A. ambivalens* and strain DS80, *T. tenax* can respire  $S^{\circ}$  using organic compounds. In *T. tenax*, organic compounds are completely oxidized to  $CO_2$  via the oxidative TCA cycle. During organotrophic growth, *T. tenax* conserves energy via a membrane-bound electron transport chain with  $S^{\circ}$  as the terminal electron acceptor (Siebers et al., 2004; Siebers et al., 2011). *Thermoproteus* strain CP80 also exhibited the ability to couple organic acid oxidation to the reduction of  $S^{\circ}$  and the ability to grow autotrophically with  $H_2$  and  $S^{\circ}$  (Urschel et al., 2016); the genome of CP80 was found to encode for both cytoplasmic (Sre2) and extracellular oriented (Sre1) Sre complexes. We suggest that differences in the orientation of the Sre complex (extracellular versus cytoplasmic) allows for use of electron donors present in the cytosol by Sre2, including NAD(P)H produced from the TCA cycle, which allows for electrons derived from oxidation of organic carbon to be coupled to the reduction of  $S^{\circ}$  (**Fig. 5B**).

A second possible explanation for the difference in the ability to respire  $S^{\circ}$  with organic electron donors in the Thermoproteales (i.e., *Thermoproteus* spp.) but not the Sulfolabales (i.e., *Acidianus* spp.) is in the types of co-factors involved in reducing  $S^{\circ}$ . For example, it has been suggested that succinate dehydrogenase (complex II) in *T. tenax* (Siebers et al., 2004; Siebers et al., 2011) might be involved in the reduction of  $S^{\circ}$  when organic compounds serve as electron donor. Succinate dehydrogenase is a membrane-bound component of the TCA cycle and can provide an entry point for electrons derived from the oxidation of succinate into the membrane quinone pool, which can then be used to reduce  $S^{\circ}$  via the membrane bound Sre (Siebers et al., 2004; Siebers et al., 2011) (**Fig. 5C**). Like *T. tenax*, members of the Sulfolobales, including DS80 and *A. ambivalens*, also



encode for succinate dehydrogenase (Lemos et al., 2001; Schafer et al., 2002). However, as isolated succinate dehydrogenase from *A. ambivalens* was shown to contain bound caldariellaquinone (Lemos et al., 2001; Schafer et al., 2002), suggesting that electrons from succinate oxidation are transferred to this specific quinone pool. Biochemical studies indicate that caldariellaquinone cannot be used to reduce membrane-bound Sre in *A. ambivalens* (Laska et al., 2003). Rather, the specific quinone that is used to reduce Sre in *A. ambivalens* is sulfolobusquinone, which differs from caldariellaquinone in that it lacks a mercaptan side chain (Collins and Langworthy, 1983; Thurl et al., 1986; Elling et al., 2016).

While caldariellaquinone is produced by *A. ambivalens*, it is usually only found in the membranes of aerobically grown cells and not  $S^{\circ}$  grown cells (Laska et al., 2003). Likewise, caldirellaquinone is the predominant quinone in aerobically grown *Sulfolobus solfataricus* cells; sulfolobusquinone is detected in greater amounts in cells grown under progressive  $O_2$  limited conditions (Nicolaus et al., 1992). Moreover,  $H_2/S^{\circ}$ /acetate grown DS80 cells appear to not operate the TCA cycle as evidenced by the lack of  $CO_2$  produced over the growth period (**Fig. 2A**); the C1 (carboxy position) label of acetate should be released as  $CO_2$  by the second turn of the TCA cycle. Thus, differences in the specificity of the membrane bound Sre complex for sulfolobusquinone versus caldariellaquinone may be another reason why DS80, and *Acidianus* strains in general, cannot link organic carbon oxidation via the TCA cycle to the reduction of  $S^{\circ}$ . Further evidence that this phenomenon is due to the specificity of Sre for electron donors comes from the observation that electrons from  $H_2$  generated via the sole hydrogenase encoded

in the genome can be used to reduce Fe(III), albeit with a low efficiency of energy conservation as indicated by a 1.6-fold reduction in cell yields when H<sub>2</sub> is provided to acetate grown Fe reducing cells.

The ability of strain DS80 to grow with organic substrates as sole electron donors respiring Fe(III) and with O<sub>2</sub> (**Table 1**) but not with other oxidants is a potentially widespread phenomenon among hyperthermophiles. For example, *Ferroglobus placidus* cannot utilize acetate as the sole electron donor when either nitrate or thiosulfate was provided as the electron acceptor (Hafenbradl et al., 1996). However, acetate was shown to be capable of supporting growth when Fe(III) was provided as the electron acceptor (Tor et al., 2001). These observations are consistent with those from DS80 and support the notion that the metabolic versatility of hyperthermophilic microorganisms may be expanded when Fe(III) is provided (Vargas et al., 1998). These observations also suggest the possibility that the mechanisms for iron reduction are not necessarily specific, which may allow for co-factors or oxidoreductase complexes and their associated co-factors with potentials negative enough to non-specifically reduce Fe(III). The results shown here indicate that providing H<sub>2</sub> as electron donor can also allow for use of alternative electron acceptors (i.e., S<sup>0</sup>) when organic carbon sources are available to these cells, thereby allowing for expansion of the ecological niches of these cells. It is not known if addition of H<sub>2</sub> would allow for organotrophic growth in nitrate or thiosulfate grown *F. placidus* cells.

The higher amount of acetate mineralized by Fe(III) reducing DS80 cells when acetate served as electron donor when compared to H<sub>2</sub>/acetate as electron donors suggests that the addition of H<sub>2</sub> decreases the flux of low potential electrons derived from acetate into electron transport chains and a potential diversion of acetate away from oxidation via the TCA cycle and toward gluconeogenesis. Indeed, observed growth yields of DS80 with Fe(III) when acetate was used as carbon and energy source were higher than those when both H<sub>2</sub> and acetate were present. Here, less efficient electron transfers involving the H<sub>2</sub>/Fe(III) redox couple, additional energetic costs associated with reduction of acetate during gluconeogenesis pathways, and additional energetic costs associated with synthesis of the enzymes necessary to metabolize H<sub>2</sub> and to perform gluconeogenesis could influence the overall efficiency of coupling energy metabolism to biomass synthesis. Such observations beg the question as to why DS80 cells would metabolize H<sub>2</sub> when acetate (or potentially other organic substrates) are present. One possible explanation is that DS80 evolved in a H<sub>2</sub> rich environment where constitutive expression of hydrogenase enzymes or H<sub>2</sub> induced regulation of these enzymes is ecologically advantageous. Such environments may include hot springs, where H<sub>2</sub> concentrations and flux are high (Spear et al., 2005; Windman et al., 2007; Bergfeld et al., 2011). Further experimentation is needed to verify whether Hyn is constitutively expressed in DS80.

Many of these same H<sub>2</sub> rich acidic and high temperature hot spring environments where *Acidianus* strains have been detected are hypoxic, have abundant S<sup>0</sup> precipitates (Inskeep and McDermott, 2005; Boyd and Druschel, 2013; Colman et al., 2016), and tend to have low concentrations of Fe(III), either dissolved or as a solid phase. This is due to

constraints imposed by temperature on the populations involved in oxidizing Fe(II) (Langner et al., 2001; Kozubal et al., 2008) and to the low rates of abiotic Fe(II) oxidation at acidic pH (Nordstrom and Southam, 1997). Even in a metabolic generalist such as *Acidianus* strain DS80, such conditions may select for optimization of enzymatic machinery capable of coupling  $H_2$  and  $S^\circ$  for energy metabolism, leading to specificity of the redox coupling between these complexes via sulfolobusquinone. The availability of organic substrates is temporally variable in hot spring environments (Schubotz et al., 2013; Schubotz et al., 2015) and would represent a selective pressure to adapt mechanisms (i.e., gluconeogenesis) that allow for use of organic substrates as carbon sources when available all the while maintaining efficient  $H_2/S^\circ$  based autotrophic energy metabolism. Indeed,  $S^\circ$  reducing cells provided with organic compounds and  $H_2$  assimilated the organic compounds into biomass but did not oxidize them.

Intriguingly, a key difference between *Acidianus* strain DS80 and W1, despite being nearly identical from a phylogenetic perspective (**Supp. Fig. 2**), is that the former encodes for Sre and Hyn while the latter does not. Acquisition of these key traits in strain DS80 (or loss of these traits in strain W1) would allow for DS80 to outcompete its ancestor in environments rich in  $H_2$  and  $S^\circ$  and could have led to their ecological divergence. To this end, we suggest that  $H_2$  expands the carbon metabolism of strain DS80 when  $S^\circ$  serves as electron acceptor. This facultatively chemolithoheterotrophic form of metabolism, where an inorganic compound serves as an energy source and an organic compound serves as the carbon source, could expand the ecological niche of thermoacidophiles in hot spring environments and allow them to compete for a wider

array of electron donors, carbon sources, and electron acceptors whose availability varies temporally. Based on work presented here, and the work of others (Jochimsen et al., 1997; King, 2007; Ghosh and Dam, 2009), we suggest that chemolithoheterotrophic metabolism is likely much more common in nature than is currently recognized.

In summary, the data presented here suggest that the use of electron donors and carbon sources can be influenced by the types of electron acceptors available (e.g., Fe(III) versus  $S^0$  reduction) and the presence or absence of  $H_2$  (i.e.,  $S^0$  reduction). These data also clearly indicate that predictions of the metabolism of a cell based on the presence or absence of genes or geochemical/thermodynamic data indicating energetic favorability of a given redox reaction may not play out in natural systems. As an example, the presence of acetate kinase and sulfur reductase in the genome of DS80, and other Sulfolobales, may lead one to believe this organism can grow via coupling of acetate oxidation to  $S^0$  reduction. Likewise, the presence of acetate and  $S^0$  in an acidic hot spring, combined with the general thermodynamic favorability of the reduction of  $S^0$  by full oxidation of acetate (Amend and Shock, 2001), might lead one to predict this metabolism could take place. We suggest the need to more completely characterize the physiology of microbial cells and to integrate this with genomic and thermodynamic data to more accurately predict the distribution of microorganisms and their metabolic activities in natural systems.

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**Table 1.** Growth of *Acidianus* strain DS80 in base salts medium (80°C; pH 3.0) with specified substrates as carbon sources when S°, Fe(III), or O<sub>2</sub> served as electron acceptor. The ability for each carbon source to support growth was tested in the presence (+) and absence (-) of H<sub>2</sub>.

| Carbon source   | S°                 |                    | Fe(III)            |                    | O <sub>2</sub>     |
|-----------------|--------------------|--------------------|--------------------|--------------------|--------------------|
|                 | H <sub>2</sub> (+) | H <sub>2</sub> (-) | H <sub>2</sub> (+) | H <sub>2</sub> (-) | H <sub>2</sub> (-) |
| CO <sub>2</sub> | +                  | N/D                | +                  | N/D                | N/D                |
| CO              | +                  | -                  | +                  | +                  | N/D                |
| Formate         | +                  | -                  | +                  | +                  | +                  |
| Acetate         | +                  | -                  | +                  | +                  | +                  |
| Glucose         | +                  | -                  | +                  | +                  | +                  |
| Fructose        | -                  | -                  | -                  | -                  | -                  |
| Galactose       | +                  | -                  | +                  | +                  | +                  |
| Mannose         | +                  | -                  | +                  | +                  | +                  |
| Lactose         | +                  | -                  | +                  | +                  | +                  |
| Maltose         | +                  | -                  | +                  | +                  | +                  |
| Sucrose         | -                  | -                  | -                  | -                  | -                  |
| Yeast extract   | +                  | -                  | +                  | +                  | +                  |
| Peptone         | +                  | -                  | +                  | +                  | +                  |
| Casamino acids  | +                  | -                  | +                  | +                  | +                  |

+ (growth); - (no growth); N/D not determined.

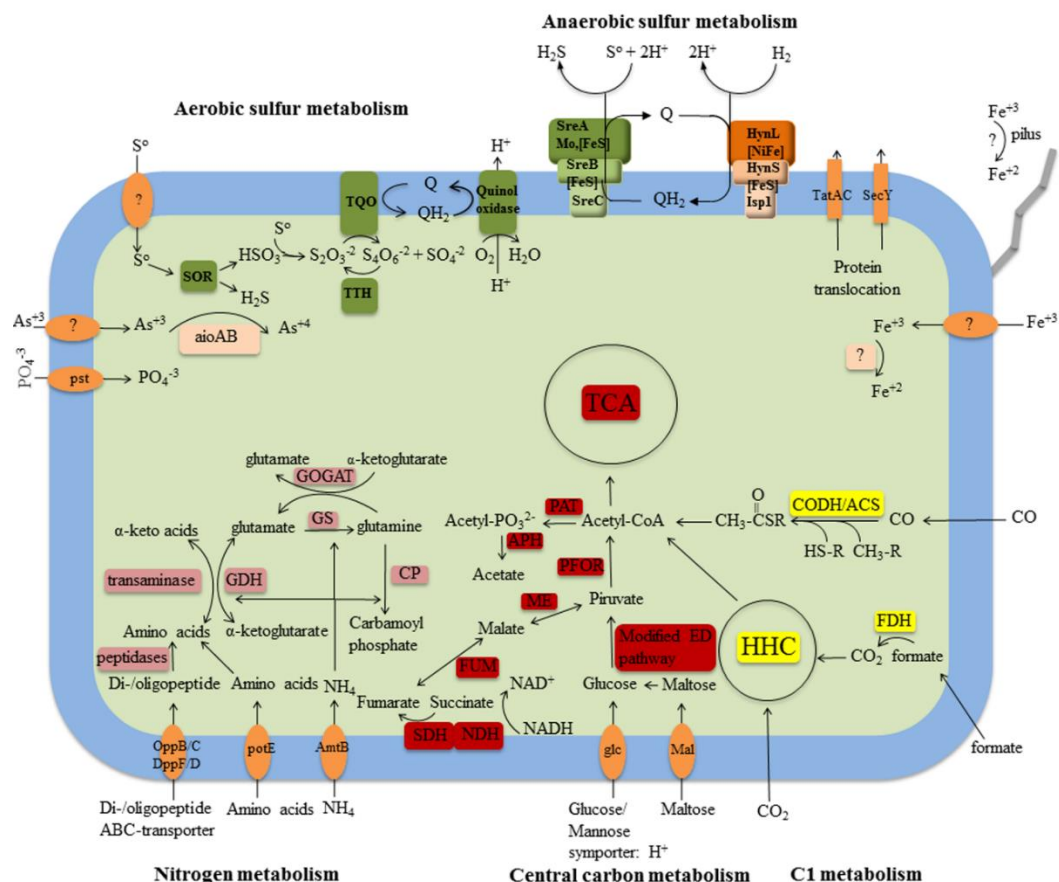


**Figure 1.** Diagram of metabolic capabilities of *Acidianus* strain DS80. Abbreviations: SOR, sulfur oxygenase reductase; TQO, thiosulfate quinone oxidoreductase; TTH, tetrathionate hydrolase; Q, quinone; CODH/ACS carbon monoxide dehydrogenase/acetyl-CoA synthase; FDH, formate dehydrogenase; HHC, hydroxypropionate-hydroxybutyrate cycle; PAT, phosphate acetyltransferase; APH, acylphosphate phosphohydrolase; PFOR pyruvate:ferredoxin oxidoreductase; ME, malic enzyme; FUM, fumarate hydratase; SDH, succinate dehydrogenase; NDH, NADH dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase; GDH, glutamate dehydrogenase; CP, carbamoyl phosphate synthetase. Extracellular electron transport via pili has been hypothesized to be involved in iron reduction in *Acidianus* strain DS80 previously (Amenabar et al., 2017).



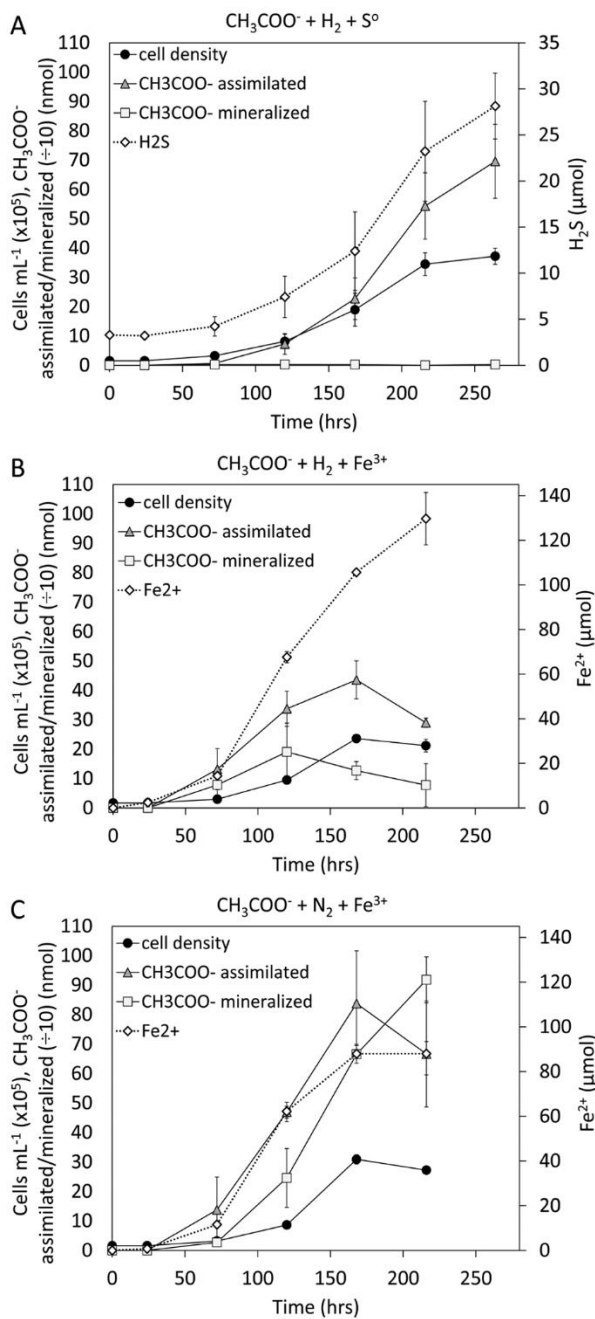


**Figure 2.** Growth and substrate transformation activities of cultures of *Acidianus* strain DS80 provided with H<sub>2</sub>, acetate and S<sup>0</sup> (A), H<sub>2</sub>, acetate and Fe<sup>3+</sup> (provided as Fe<sub>3</sub>SO<sub>4</sub>) (B) or with acetate and Fe<sup>3+</sup> (provided as Fe<sub>3</sub>SO<sub>4</sub>) (C). No increase in cell density, acetate mineralization/assimilation activity, or S<sup>0</sup> reduction activity was observed when cultures were provided with only S<sup>0</sup> and acetate (data not shown). Error bars reflect the standard deviation of measurements made on three replicate cultures.

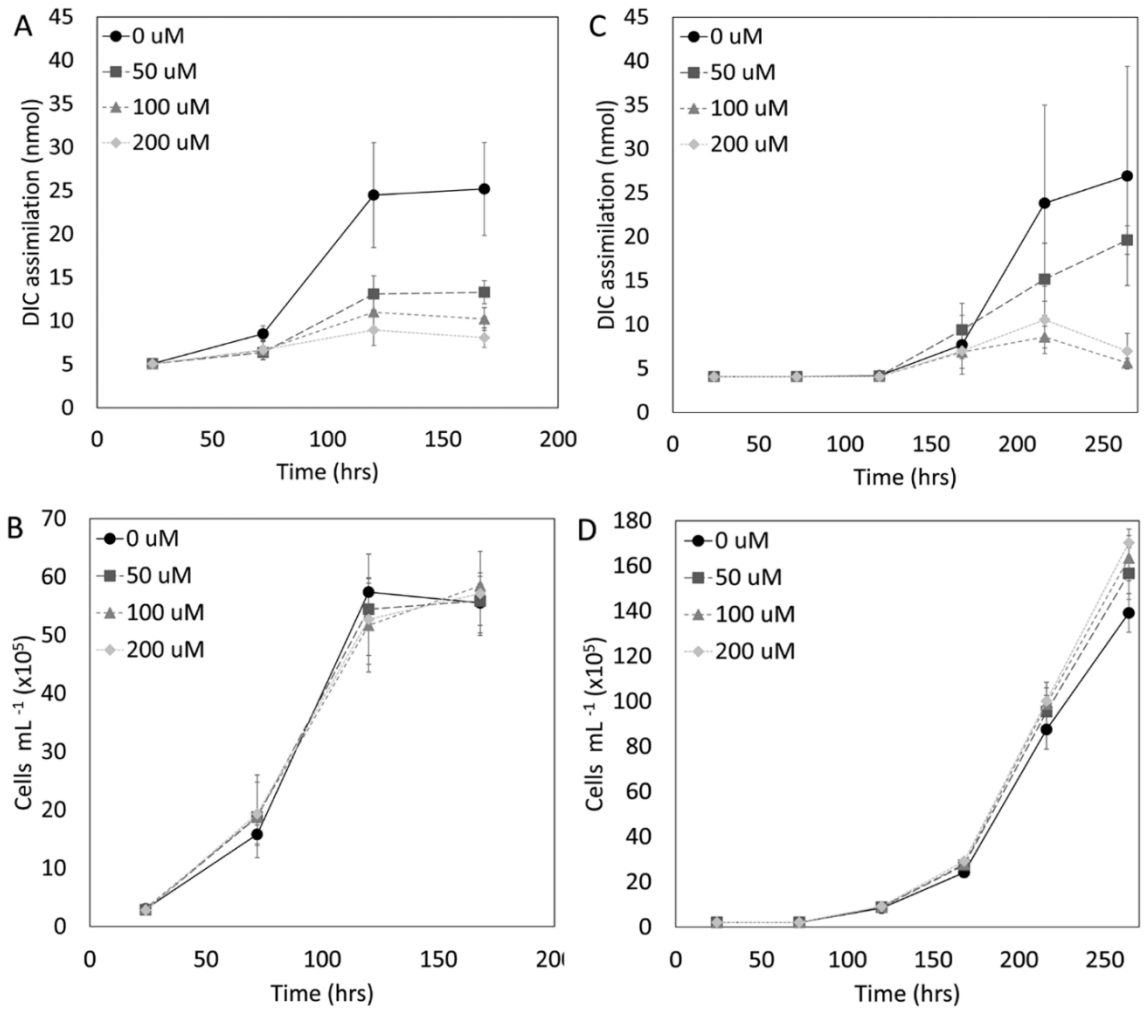


**Figure 3.** DIC assimilation and the number of cells produced in cultures of *Acidianus* strain DS80 provided with  $\text{H}_2$ ,  $\text{S}^\circ$ , and  $\text{CO}_2$  (**3A** and **3B**, respectively) or  $\text{H}_2$ ,  $\text{Fe}^{3+}$  [provided as  $\text{Fe}_2(\text{SO}_4)$ ], and  $\text{CO}_2$  (**3C** and **3D**, respectively) amended with 0, 50, 100 or 200  $\mu\text{M}$  acetate. Error bars reflect the standard deviation of measurements made on three separate cultures.

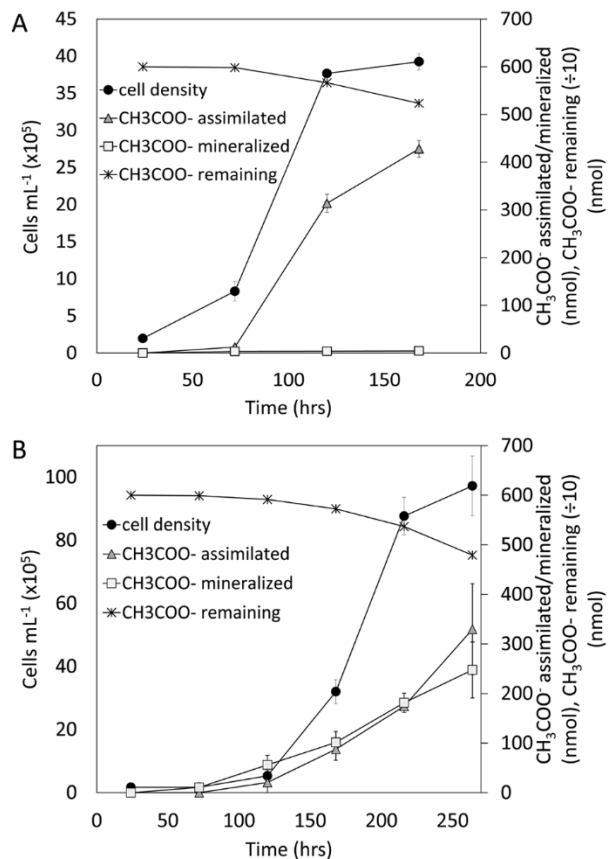


**Figure 4.** Acetate assimilation and mineralization in the presence of CO<sub>2</sub> (headspace of 20%/80% vol./vol. CO<sub>2</sub>/H<sub>2</sub>) in cultures of *Acidianus* strain DS80 provided with H<sub>2</sub>, S<sup>0</sup>, CO<sub>2</sub>, and 100 μM total acetate (**A**) or with H<sub>2</sub>, Fe<sup>3+</sup> (provided as Fe<sub>3</sub>SO<sub>4</sub>), CO<sub>2</sub>, and 100 μM acetate (**B**). Error bars reflect the standard deviation of measurements made on three separate cultures. The concentration of acetate remaining in culture medium was calculated by mass balance.

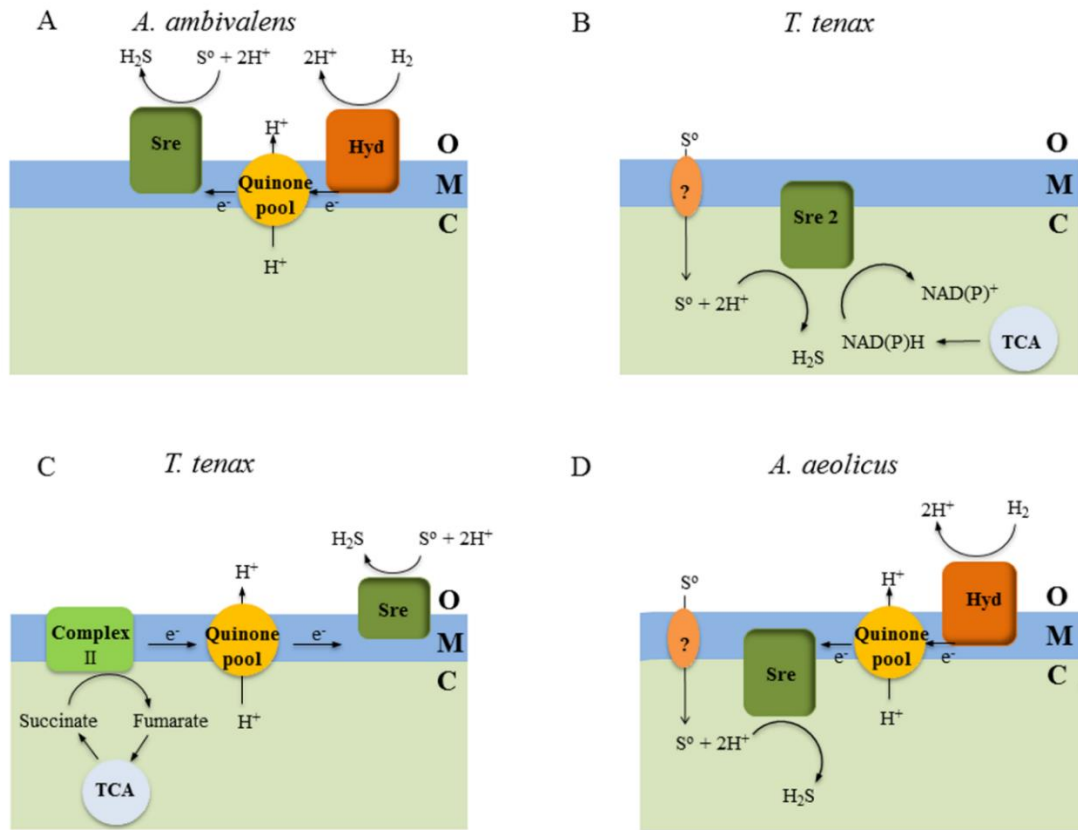
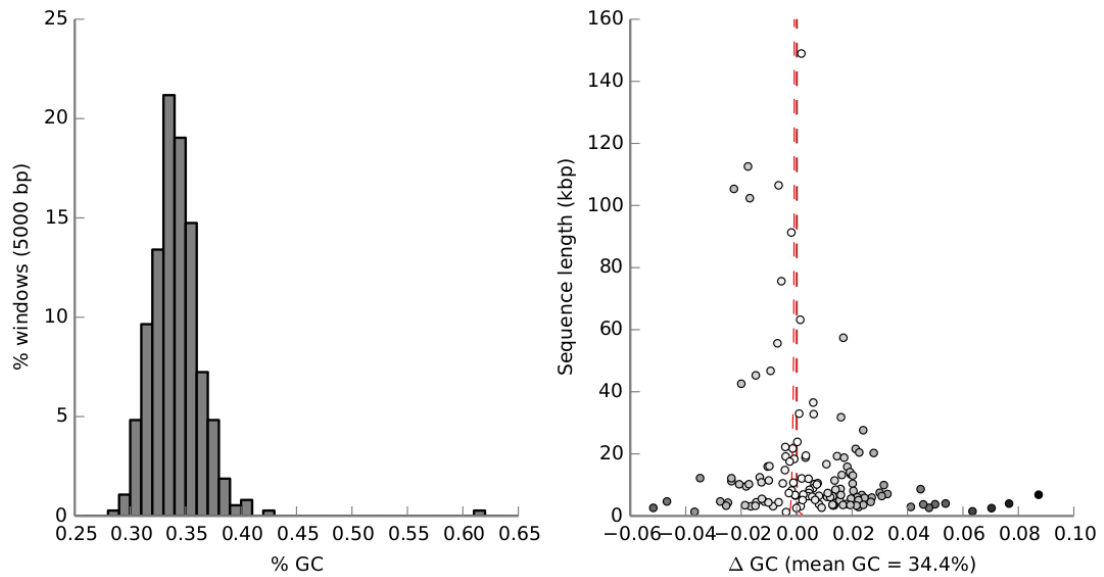
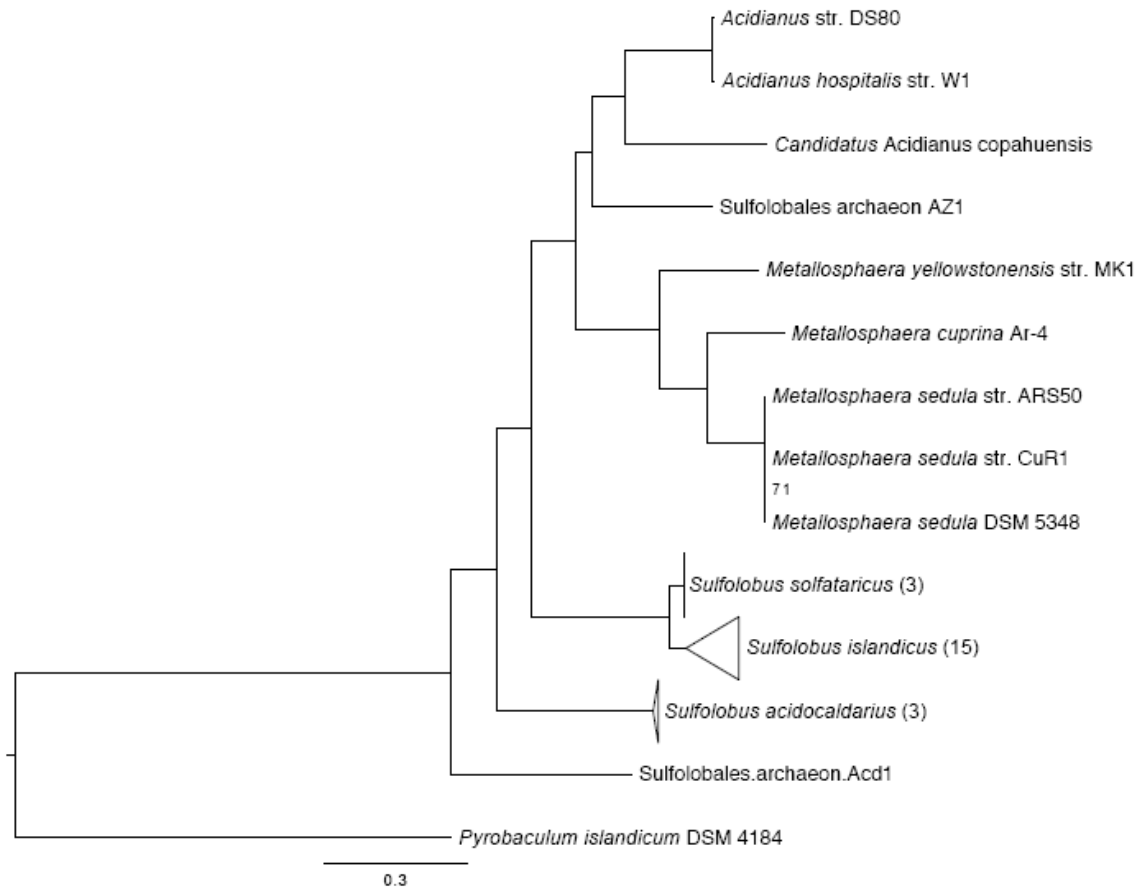


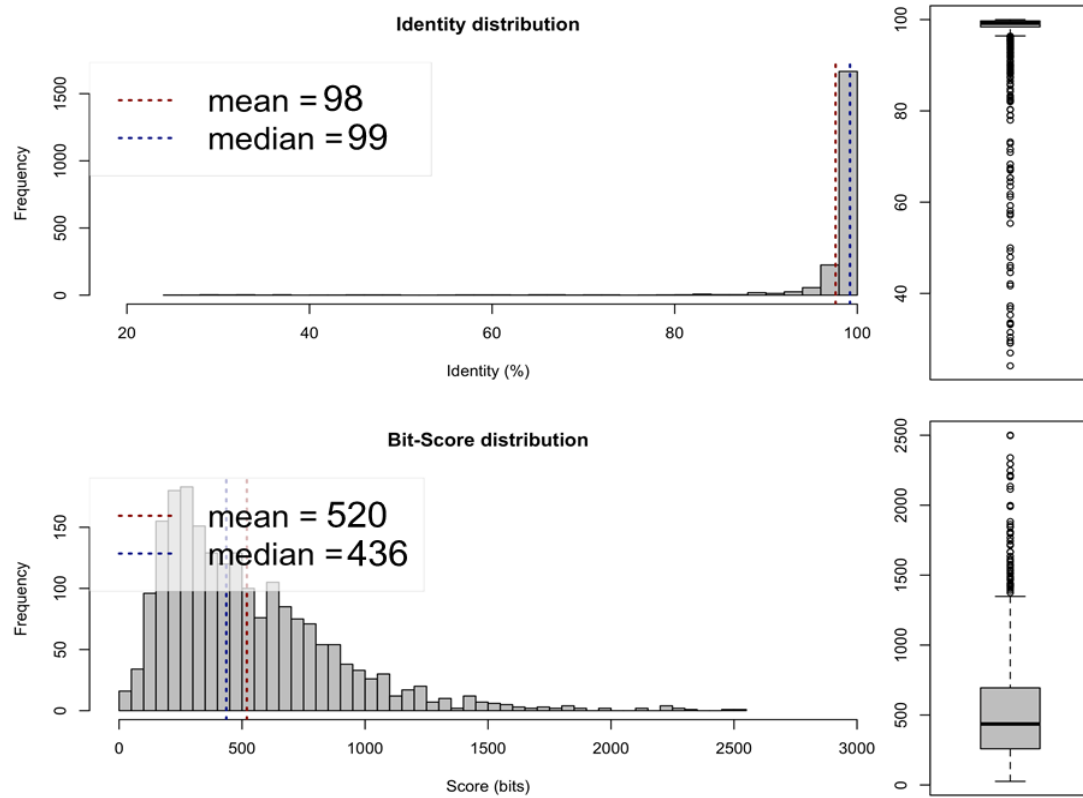
**Figure 5.** Diagrams of proposed pathways for  $S^0$  reduction with  $H_2$  or organic carbon substrates as sources of reductant. Abbreviations: Sre, sulfur reductase; Hyd, group 1 [NiFe]-hydrogenase; TCA, tricarboxylic acid cycle; O, outside of cell; M, membrane; C, cytosol.



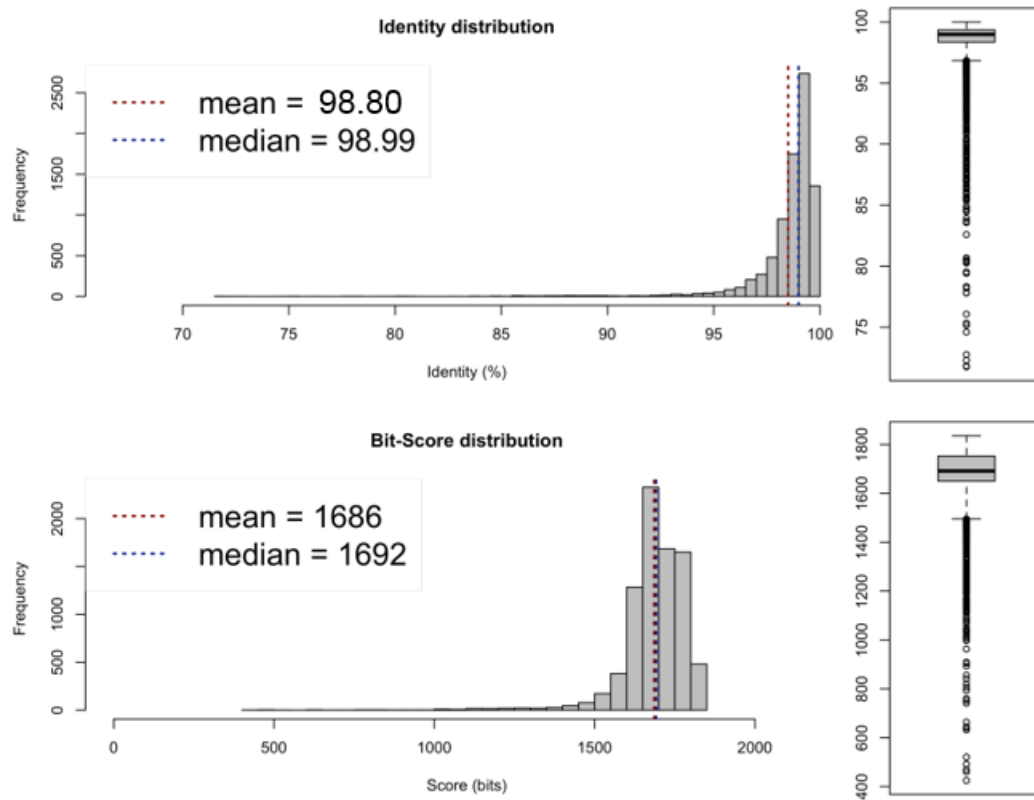
**Supplementary Figure 1.** Quality assessment via GC content of the *Acidianus* strain DS80 genome assembly. Left panel shows average GC content of contigs calculated over a non-overlapping sliding window of 5000 base pairs. The right panel shows the size of each genomic contig as a function of its deviation from the mean GC of the entire assembly. The red lines denote the expected deviation from the mean using a 95% confidence interval.



**Supplementary Figure 2.** Phylogenetic placement of *Acidianus* strain DS80 within the *Sulfolobales* order. The Maximum Likelihood tree was constructed using a concatenation of 104 phylogenetic marker genes for a total of 30 archaeal genomes. All nodes shown exhibited bootstraps of 100% except for one node where the bootstrap value is indicated. Scale bar shows expected number of substitutions / site. *Pyrobaculum islandicum* was specified as an outgroup for the *Sulfolobales* genomes.

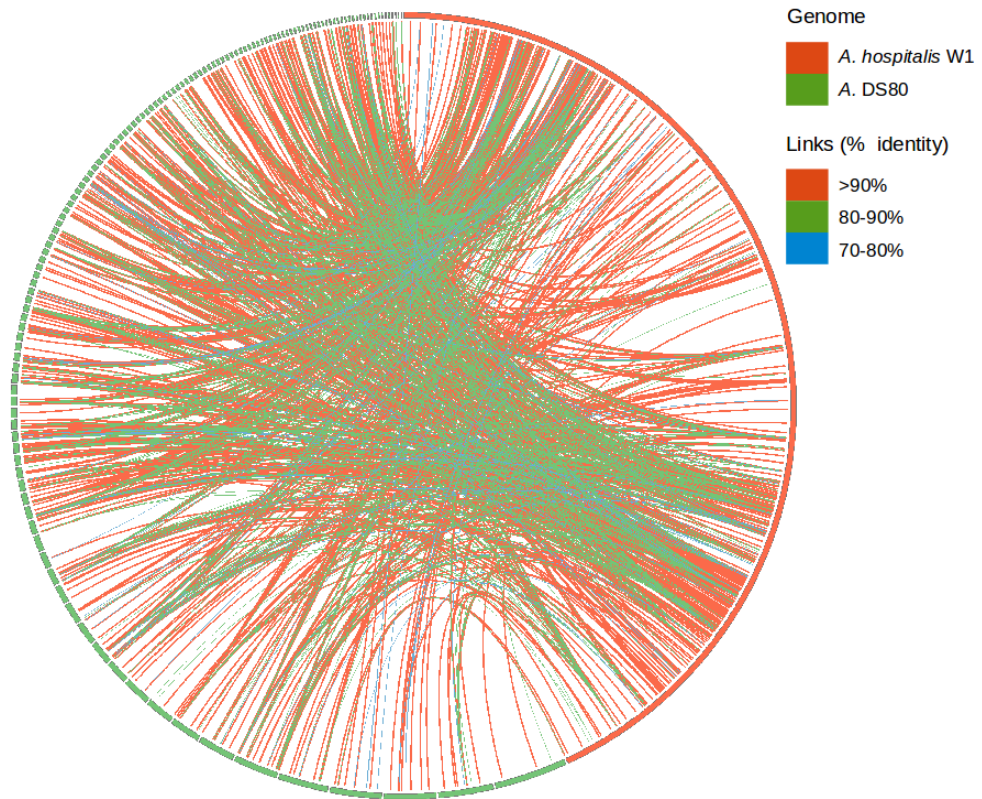


**Supplementary Figure 3.** Average Amino Acid Identity (AAI) of proteins encoded in the *Acidianus* strain DS80 genome when compared to the genome of *Acidianus hospitalis* W1. The top panel represents the percent identity while the bottom panel represents the bit score distribution.

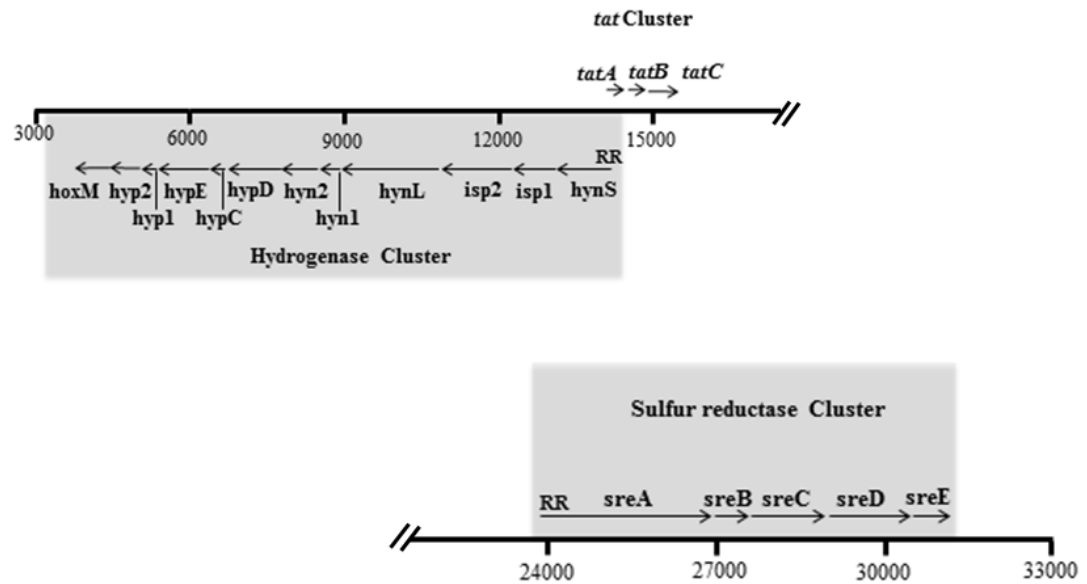


**Supplementary Figure 4.** Average Nucleotide Identity (ANI) of the genome of *Acidianus* strain DS80 when compared to the genome of *Acidianus hospitalis* W1. The top panel represents the percent identity while the bottom panel represents the bit score distribution.

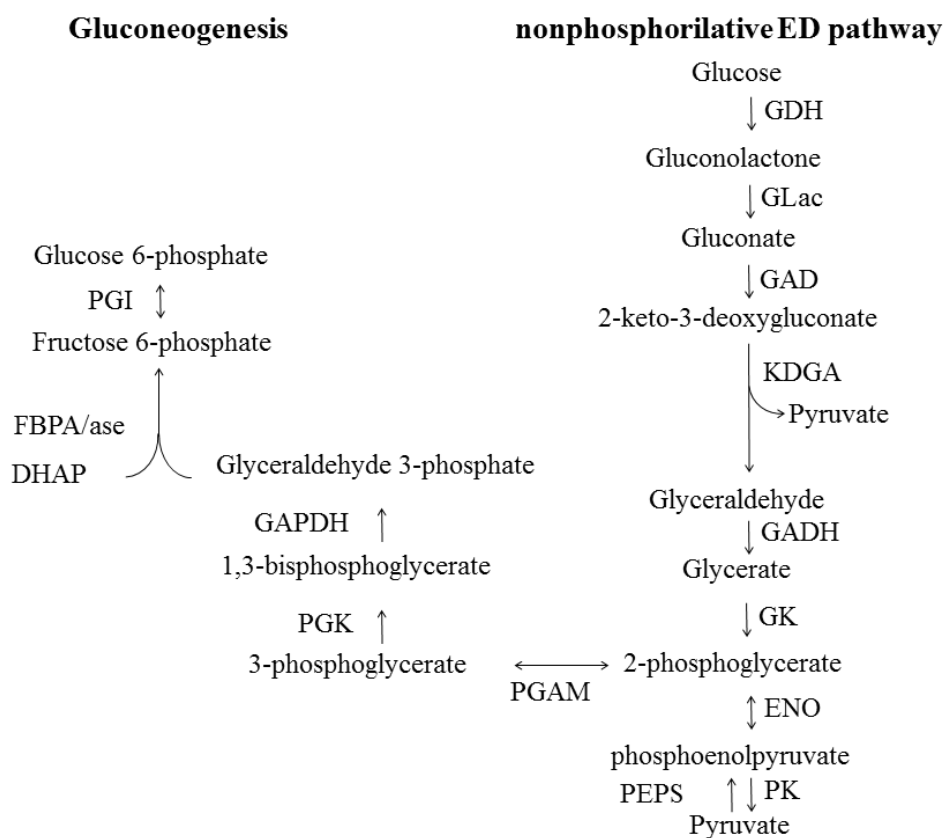




**Supplementary Figure 5.** Complete genome mapping between the *Acidianus hospitalis* W1 genome and 133 contigs generated from the sequencing of the genome of *Acidianus* strain DS80. Here, the orange strip (right) represents the genome from *A. hospitalis* W1 while the green strip (left) represents the 133 contigs. Colored links represents regions of high similarity as obtained from BLASTn. The red links represents the regions that were >90% identical, green links represent regions that were between 80-90% identical, and blue links represent regions that were between 70-80% identical.



**Supplementary Figure 6.** Gene clusters encoding the [NiFe]-hydrogenase (*hyn*) and the sulfur reductase complex (*sre*) in *Acidianus* strain DS80. The ruler demarcates base pair positions, arrows indicate ORFs, RR indicate a conserved twin-arginine motif, the *tat* cluster encodes proteins required for the twin-arginine protein translocation pathway (TAT).



**Supplementary Figure 7.** Gluconeogenesis and glucose degradation via a nonphosphorylative Entner-Doudoroff (ED) pathway. GDH: glucose dehydrogenase, Glac: gluconolactonase, GAD: gluconate dehydratase, KDGA: 2-keto-3-deoxy-gluconate aldolase, GADH: glyceraldehyde dehydrogenase, GK: glycerate kinase, ENO: enolase, PK: pyruvate kinase, PEPS: phosphoenolpyruvate synthetase, PGAM: phosphoglycerate mutase, PGK: phosphoglycerate kinase, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, DHAP: dihydroxyacetone phosphate, FBPA/ase: fructose bisphosphate aldolase/phosphatase, PGI: glucose-6-phosphate isomerase.

**SUPPLEMENTARY FILE 1**

>AEE92940.1 conserved hypothetical protein [Acidianus hospitalis W1]  
MSELDLFLNRKKSIEKKEEKSTAENVEKSEERIEKAQEEKKEENSTVESEKEEETL  
KVEGKKDEKREKTVENTPESTKNNIEDKGKKEDSEEDSIESIMKKFLEKDPKIGIW  
SYPAYLVLQYLYNTKPGFKMSRVAKEALEYGLKHMYPDLFSKAEEKISQLKQR

>AEE93052.1 hypothetical protein Ahos\_0159 [Acidianus hospitalis W1]  
MKSKATLLALLILLMPGIFYSDALYYHGESQFCTSKLIKIYTFSYHLPNANVRS  
SVNSGNNVSIKICEYNPPPPHSQKLINVTIKICLPKSSKVFLIPIIYNISKNGPKLSYC  
CISLTFTSLPNTYKACLVTAKAPQNKTFYFVTECNYYFVNVCWTFCGPPPTAW  
ANILLMFIVQEGGANYVYFWSINITLSPAHPPPPGPPPPPPPPSPYCYIVEYPNLCP  
LKVAQIIFSLYKL

>AEE93063.1 conserved hypothetical protein [Acidianus hospitalis W1]  
MGVSLFLIILLIVVVSIIYHNL TQVSQYSILYISNFSKIEDIIALNASTLFIVGSKTCGNI  
SHGVAGYYYLNDNEFKQVNL SKLFCCGAIYSVAYNGSALMLAGACKISGVLHP  
AIALISYNKVTNLTSYIPMGYYPGRALAVAWFNKTWFIGGNFLFNLDQHYVSVM  
FLIGLKGNSTSNLTLNISNILNQLPEGCILSLSVGHNCILIGGKFVIYLVLFKFNSTSI  
YEINMTKSIFKYPGFIISLSQFNSSTWVVGGEYFPVNSTICHPLYLVLYNSLNSCINYI  
KLNYTIGFVTSVSSYNGNIVVSLRVPFKTPQGVGGTVILEGKINALKQTFDEVNI  
SINQLLSLNNRIIGGGYKVTGNADEGILIFLNEN

>AEE93064.1 binding-protein-dependent transport, inner membrane component  
[Acidianus hospitalis W1]  
MASSNTVQTSRPFIRIVIRRLIARLVLLFGVINFNFFIFQVIPQLVGLNPLEFYVPPSF  
KTSLNRNAIIEGLEKSYGLNQPICIRYIDYIKDLFTFNFGESLYYQEPILKIEQNFP  
VTAEIVIPSLIITTIMAIFFGIYSATKEGKVS DHVISNAAIITYFIPAFWLGFIIWYYLT  
IQYNLFPSSYTFAIIESRKDPLAIYTVLIPPILT SFTSFGVRTILMRNNSIDVFNQDF  
VTILKAKGVPRSRILFRHVFKN SFLPVFTRVGIDFAFLLSGVVFCDFVNISGLGTL  
LVTAAENFDIFLLEGDFYIISLFAIVVLTLMDFIYILIDPRVKLT

>AEE93068.1 hypothetical protein Ahos\_0176 [Acidianus hospitalis W1]  
MSEEVSEIVSKSEKILSFFISVISIIISFYLGIFSYYLLILLFLSVSVFIFRYNLLIKLILS  
KNDKISII PRPSLEKRRALQNLIISSLIFSPFLLIYIFPSILWITFTIAIVTSWPFSIIAL  
LVIYILEKRRGVKIYKYVIFDERLDEINIKEYGIIAYRQDSL RKQ

>AEE93090.1 ribosomal protein L13ae [Acidianus hospitalis W1]  
MNPPTPMIKRPNYSFEYPHERKSTREGRGFS LGELKAANLTPEKARKLGLRV DK  
RRKSVHQENV DALKKYLDELNKEKSTQSKT

>AEE93100.1 hypothetical protein Ahos\_0208 [Acidianus hospitalis W1]  
MSIIYAYNAYCYHIEQQYEPISSLNAARMISESNCSNGIKLTATFAVLNNGSCY  
LIMCVINV GDRVVNYTFTINLYQLSGNYCELGQKLVYFSIYNNSSEFDLFPIIPGGP  
AIFQNTTIHIYPGERLVYYYEIKGYYQSLFQCKFVQFPNGYTIKVYDVFNQQVT  
LHVQLR

>AEE93102.1 VapC-type toxin [Acidianus hospitalis W1]  
MKILIDTSFILPALGIDVGESVLNII EFDNHEIYYTELSLLEAMWMIRRLKEGNN  
VDFKIVRTGLKSVFKTYHLLKIPISAYINAVNDMRHSDLIDMIIYYTAKAYNLKFL  
SLDKKLKELDKENIVIDKL

>AEE93123.1 SirA family protein [Acidianus hospitalis W1]

MIKRIYKRLDYSGVNSCDSAVGEILGYLDTLKDKEAIEIVLDADWKLKDLQDFIS  
KTKYKILDVKKDDGKYIVTVGEE

>AEE93127.1 Molybdopterin-Binding domain of MopB superfamily [Acidianus hospitalis W1]

MLKLNRRDFLKLSGITALGASLAYLSSKGSFINKLFTESQTNQETALEDQDVIAFT  
TCYMCLGRCTMQYQITANNLIRYAGGDVVGRVNNGATCPIGPTAAMHYLSPAR  
MKYPLLRPPTAERGTGQFIRISWEDLYDILLNGDNAAFLQERGWKYGFGLKQIY  
QCCPEQFVMVTGRDQYNPTENRYFAAMFGTPNEYEHGGFCAANVAIGAVYVA  
GSTYWEYGYFDDHHAKVLILAGATQDHFPRTGFRRRITMVKERGGTIVLFQPDQR  
PNLGPISNIWVPIRPGYDGLLISSIIHELLRLHKQGMQQNPPMPYIDEEYLKWYSN  
APWLVITNPDGTIDPPTASNVGLFLRVTNKNGEWTPAVMGSDGNIYAFTDVPWQ  
NNVEPMLEYEGDVTVPVAPGSTQTITVHVKSFAKLLEDQFLVDDYAPENVADKV  
GYPADQITALAQLLGDTAMRSPVIEVPGGWVDYLGRTHNEYIGRPVATYIMRGI  
SAHTNGFTTTRLFMLEALLGAIDTPGGYRAKYPPRPIPDGDFWPSYEATPDPR  
ARITQADVQSGEIPATGPNGESYVGTGGININERVYNDGTVVLSANKVHYP  
VITSTNPFVYTPDQLVIDENGRPLLIDRGYSWEFPAQHRSYNITMWDAGFQFP  
YPVKFLLWHITNPYWDNSYDIDKDMRLALMKNPDGTYVVPFIGIVDTFYGNSVP  
FVDIVLPDTTFLERYGEHSTLDRPITTPDIVADNIEHPILPPLFDSRPFSDVEIELGYL  
LGLSAFVNPDGSPKYPNGFGDFLWKWQISPGTGMLIAGRGADGTECKGSPNPN  
QVKLYTYPGKVPAYQYGNQNIYPGLQEFPVTNGELPSFVPGKTQKIGHAEGEYE  
LPLNIRYLRHVNMYYLQWAHSVGLIAYVKPIITQIYSEPIARFRLAAYGMWKG  
NAYFYMYLKTGNQQYLQLAQQNANVPNDTYGQYIAQIKKIAFWPIPKWYEPL  
SWTPDGANPQEYALLTEHRHVTPWFYHHWENHNPNWLRPLLRYAPVFMSTEDC  
KKYGITDFDWVVFQSWTGETARLMVVCDETTTRPGVVWYWKARNIRPGVLAIPP  
NSPEATISSMFNDVYSIVMPPSMGGASPTDYNLNLKAALIGTQGNPALLNFDPH  
TGKTSWGLRLKIVAVLGKGNYSYGLSDPYSLAVASAPFNPPPNLPYLQYNA  
YKTPDEMGLNWAYVNQTYQWQQQKQVIRPLSQSSSSS

>AEE93128.1 Integral transmembrane protein NrfD [Acidianus hospitalis W1]

MGLTPPPPPFQYPVSWGPDMPDHQAIWGAKVALAYFFIGLGSMLAAITAANDV  
RVSVPATGGVTESIRDIAFKFSVGVASLIALILGLGFLVLDAGKPSTAWQIITYGL  
QYGRWESWMFLGTIFLIVLFILDIAYSAILWGNMRPNFLSMLFNKISSWYVVKGK  
MYSMRYFLIGIMIIFGILGASYGGVLFSTQTNIGLWRNPTIILLFPVSGLSAAATGL  
LLTLIKDPSIRDNTNLLWSKIDLYAEASEATLTALFLYIGYISTYASESVYQVLFG  
RYSIYFWVLVVTLGIAIPMLLELALHKVNPRYHKEYIPIVTVLVLIGAASLRYVVI  
ASAYFYPPPLSPFSPTQMFPWGTYWGE

>AEE93129.1 hypothetical protein Ahos\_0237 [Acidianus hospitalis W1]

MSSFNTKFFNFENKNNTVIELSKYACSEIFNEKEVEILCKIIEPKSLYKLYIDTNIP  
IATLWRIVNRLRENGYLVHEKRGVYKVTYKGALLIYLVGCNKFELSLKYISELY  
NLCINKVKNILNYIIEVSNNKYSICVLEFDDIIKIIPFVLFDA LNGLYIPSDTIEFVLKA  
LNGHPSVISDEGCHIPEITNEGEKILLGKCKNNGFVSNHKCPHVRKILDKKFSKEF  
LILL

>AEE93177.1 hypothetical protein Ahos\_0286 [Acidianus hospitalis W1]

MKHRIFTYIIFMILLASLSSTLMLAQSQGYKVNINVSSSGKIEVCLYSPVTSGEVG  
DAIIPISHHGFLIQKTTIEQGLCFIKVDKIEYNTQPEFMRLPLILLVAIIVVFVEKGLK  
VI

>AEE93198.1 hypothetical protein Ahos\_0307 [Acidianus hospitalis W1]

MMNKVIEDFLKADEIARKIYYHKFKKYVGLIYLLAGAYAPLTEAVILILKVSSLST  
MSYILTGMFIAVLIPIFFINDRLLRLHGIISKMNKIYKRSIYDQIYDIVMKILFVLVI  
LGGILYHFCNFSSLVAFWILELWLLYTFHFLKFKMRVEDWVAMISPIGLILQAFTG  
SLMIIFVIGWITAGIISLVRAYGKF

>AEE93207.1 oxidoreductase domain protein [Acidianus hospitalis W1]

MKVAVVGCNGWGRVHLHALRKIGTEYYVFSRDEEKAKECMKKYEAKAYFTKY  
EDVLRSDTDIIDLVVSHDKHFEMGIEAMKAGKHLMLEKPIARTIEEAKGLINFSRE  
KGVKFMVLEQFYFDSTVRKAKELLPNLGKLSLILVRSTHFNQPKEWRAVKEKM  
GGGALIDGGVHFIDTLLNLGGEYEEIKSFCRKYFSNMEGEDTTVASFKFTGGHLG  
LLIYSWSIKNPPKVPFAFEIYGEKGS LVEDPNSRVPGKPYGDIIFNGERIEVEKVDPI  
EVEIREFLKAVENNTVEVPMKPEIALRDLEAVLKIYDECNP

>AEE93210.1 D-galactarate dehydratase/altronate hydrolase domain protein [Acidianus hospitalis W1]

MAVTIKGYIRENGAVGIRNHVLILPLDDLNSNSAAVGVSKLIRGTTVIPHPYGRQLQF  
GRDLDLFFHILAGTGANPNVAGVIIIIGIEENWANKVADEIAKTGKPVEVFPIEGYG  
DLRTIERASRKALEMVQDASEKQRTEVDISSIVMSIKCGESDTSGLASNPAGV  
VVD RMVDQGATVMFGETSEL TGAEDIVADKMATPELREKFMKIYKEYVDIIQRE  
GVDLLGSQPTQGNIKGGLSTIEEKALGNIQKLGHRKVNCVLDYLDPLNKS KDGG  
TLCFVNTSSAGAEAVTLFAAKGSVIHLFTTGQGNVVGHPPIPVKITANPKTASQM  
SEHIDVDVSDLLDLKISLEEAGERIYQYMLRVMNGRLTRAEVLQHDEFSPIKLYIS  
A

>AEE93211.1 mandelate racemase/muconate lactonizing family protein [Acidianus hospitalis W1]

MYVKLSDKESYGWGESLIAGSGIIGAYSSIIRELIKPLIEEKYNIRSPFEFEEVFEKI  
MFSAGNCGIVTGAISAVEMAMWGLKARKMKIPLYELFGGKIKDYVKVYGSFPR  
FSKIDDLIKAVEFTVNKGFDFIKLHQPPSTTLEAVKAVRERFKEIKIAVDLNSPFDL  
SEAKNFADKIARYEVEWIEEPLWPPNDYFALEKLTKVSPVPIAAGENEYTLYGFR  
KLLESGVSYIQPDVAKIGGISKFLKVLDLASSYGVKVAPHDRPDSSIVSLIYTLNL  
ALTRNEIKIVEYPISDLPKDLFNEPTFEKGYVRPPENIEINNSILNKYSYINRIRVLH  
FSDLNDKLIKYG

>AEE93212.1 transcriptional regulator, GntR family [Acidianus hospitalis W1]

MVKIISMSNLAEKAYHEILNAIVTGQYKPGQRLTEDQLCKDLNMSRTPIREALR  
MLLSDGVVKKDKTSYSVVYISAEEARMLYELRIPLEGHA AKLATMRANEEDIK  
LGDILQRIKEEMEKENPDPLTLAELNGSFHEEVALLSKNKYLYNCLHDIRVKLKI  
VRTSLFTSYERRKDEYNEHYSVFLAIKEKDPEKA EKIMIEHEKKVLEFLEKRVFLF  
MK

>AEE93218.1 conserved hypothetical protein [Acidianus hospitalis W1]

MVLYSWKDEIKAGVPLGGIGTGKLEINN RGKLVNITVFGNLSPIKSVRGFHIFISP  
DEGDPFFLEKDLRIRNMREVDELYYEGVYPFCYLTGKKGEIKVTLEAFSPPIPRNI  
KDSNIPAVGLSIKVS GSKKGTIYLSFPNFVGSTSIGRINRKVENGIIFTNIRTNEYDP

RRGEVGISSYPTLIISQYNINVDAREAFKTFIYKDLYEDDNVWRNINKVKKSDEHE  
 VVGFWDDPAGIIASNYEENKEIRFVIAWYSRAKSYQYPYGFYYHNRFSGVLEVA  
 QYFMKNYEELRKRTIEWKERIMNINLPDWLKDIAINSSYILTSSTWLDEKGRFGV  
 LEAPEVFPVVGTIAGLCYEGALPVLLLFPCLEKEFIKELANVIREDDGYVPHDLGIW  
 SFDSPIEGTTAPPRWKDLNPTFILLVYRYFKFTGDVDLRETYPKMLKAYEWMLT  
 RSIEAEGSGDTAFDVLPIKGKNPMLLTLFIASALALRETKKVLNEKDESTDLSKLR  
 EMLNSLYNGKYFIAWEGQEGIFMAQLLGEWWTELLGLENVTDEEKISSALRYML  
 EVNGKASEYCTPNLVKENGEVVKISPQAYSSWPRLVFAMGWIGSKRDKRWFEIV  
 KKEWDNIIKKGVVWNQPSRVNSINGNPEPENYLDHYIGNPSIWSFIVKEFI

>AEE93219.1 Probable cationic amino acid permease [Acidianus hospitalis W1]

MGKDLNKGSIISLIGLTFITIAGVLPPIAIEVAAFISDAGGAIIWPVILGYLLFLAVS  
 LPILEYTRLVSFSGGYGLAEIGFGKTVGKFTALCNYLFYNSWQMANAFFIGWLI  
 VDTLYILYGIMLPCYDWIIISILTLFITFLITIQHPRNLSRILIIAILSSLILVIASALYVI  
 LKSPYNSLYLNPSSSPSGFTGIALATAVVGFTYFTGYASPLFYSEEGIESRKNVW  
 KAIYLGLTISAITIALVAYSEVISVPSSNLQVIGSSSMPQLVSWINYLPPIFLMLNIII  
 AIIISLIAFGGSGAQARLLWAMSRDKFIKSEWINRLNKNVPRNAALLNLIFALSI  
 VLIASLLMMHYGYNPNTVETAFYVAGTLSTILWYFHHFIPEMGYSLCKNTRN  
 LNFL

>AEE93220.1 conserved hypothetical protein [Acidianus hospitalis W1]

MILKGEELGFSEIFFGIGRGVHTRSSIEALANAKELLKIANKLGYYSFVDINPSILEE  
 LGASPRNLSIFKEAGFSAVRVDCGFSVDDILKIRDIGIELNAYTFPEDKIEYLLRNI  
 DPEKVKATHNYYPVIGSGISIKELIEKSKPFVKSGIPVGAFFVSVPSVRSDTTVEELR  
 DKNPRESARILFSTGVISRVLIGDAHPTDEEMIDLSKVL

>AEE93223.1 conserved hypothetical protein [Acidianus hospitalis W1]

MKLANNFGIPIGSLGTGKIDFFNDLTIGNATIMNNWSNPLRVIRGFHIVDLSTSMF  
 LQGNPARNSEIKFEVKIPKSIEANALFPEVEYEIKNPDFTIKVYSPFVPNDLKDSSL  
 PLIVFNIKKGKGIISFPNLTGSRRWGRVNYKIEGKVNGVLMTNLKAQSDPAYGE  
 IFLGCQGCHSYVGYRYWIPALKEGMTEVDVSIFNLDKLKEGEGINRYIIPYAREEI  
 GGIVWKEIDGEENFYLTWFFNGRPGYYPYGHYYENWFQSAVDVAEYALKRKP  
 VELDNTQDWINDAVRNSMYVLTYSWLTGDGR LAVYEDPQISLLMNTIGGMTW  
 DLSFALLEYFPELVKKMDEHFGNFIIDGEVPHDLGEESIENPIYGASYPYSWNDL  
 GPTWILMIYRDYKFTNDLSFLKRNYNKMKEVIDWLIKDEEDNDGIPDSKGGFDN  
 SYDGTMYGTSSYIGSLFLCALKAFFIESSKILSYDYSKYEEILNKAKSSLES LWNG  
 RYFINWKYKDQKNESCLNSQLLGEFWCNLLGLGNVIDEDKIKTALKYIYEHNGK  
 ASKYCLVNSVNPDGSIDESTDQMKSCWPRISFAIASHMIMKGMINEGIEIARKEW  
 ETISSRYPNQPSKINAFNGEHFGLPYYIGSLSIYLVKYAIKNTPHR

>AEE93226.1 Alpha-mannosidase [Acidianus hospitalis W1]

MRNDAEVLRSISYILANSFYDIKYFKWSKENENEFYVDINNNNGYFMIVINYEGS  
 ALVKVNSQPYYALDGYHNTFPLPEGKIRVNALFSPYKAFGQKVEINPGTPVIFRR  
 NEDAYKLWAYLNATLQLAKNSQDYLREKLLKVLTTETLSLVPFVSVSRDQLLLAS  
 KYWNDFP SHLLEFSQEMKYEFHEGNYKEALTYLKEKLGELETFGKAGELIGF  
 AHAHMDTAWLWNFDETRRKVARTFSTVLNLSKYDFQYIQSMALYYEWIKED  
 YPELFNKIKEKVKEGWILGAGWVEFDANLPSGESIARQLLYSQEFYLSNFGKIA  
 EILWLPDTFGFSAQLPQIMKLSGIKYFATHKVFWNDTNKFPYSVFNWIGIDGTSIP

SIAFGNGKGGYNSEFTIDSVMSQWNNWKDKDQPMLYSYGYGDGGGGPTEDML  
 NFAEVINELPSLPKVCLTGGIPEYKPENSWSEDELYVETHRGVYTSHSKMKYLHRR  
 AECTLRDAEIWSTIAGRYDKEIVNLWKILLKDEFHDLVLPGSAINVEYKIVYPELEG  
 IISKANKIIDESIKAIAGEGDRLMFFNSLSWDREDVAVVNEELPNSQKVDESYLVI  
 VKVPSVGYSEYKEVKYSPVKVDGLLMENDYLKVCLDENGSLISIFDKEENREIIK  
 SPSKLIFYENIPGWADAWDIEKSYKDTYFEVKAYKYEIKERGPIRACIRFYKFRN  
 SEITQDVCLNANSRRIDFKTRTKIPDRELLLKAWYYFDLNSTEAVYEIPFGVIKRR  
 TTTSNSWELAKFEVPIKWMDIYEDDYGVAVLNDGKYGIAVERSNVGLAKTPI  
 YPDYETDSEENVFTYSIYPHKGNWISAEVYKRAYELNYPPIRATKGKGGEKSFVSV  
 NPRNIILEALKPSEDGNGIILRLYNVENNRGIGEIKLWKSPNTAYKTNILENEKLED  
 VNRNKDEISFNRYEIIISIKIE

>AEE93230.1 conserved hypothetical protein [Acidianus hospitalis W1]

MDKLMLFTIAFLLLGIFMSFFTFSSNSIVYCNHFKVSLSDRGGISIPYKGSTGDEV  
 VLLGYSNSTIKVLELNPPGGAILLGSFKGNFSKTFTALTFCITFEGCNCPASVSAK  
 IIVYNTSFSPIGYTIAGIFIFLGLIFLGYKALKSLRNKVNRSKS

>AEE93233.1 ABC-type dipeptide/oligopeptide/nickel transport system, permease,  
 DppB [Acidianus hospitalis W1]

MNKVWLIRRIAMSFITILVTIIISWILIEYSPESPANAILSNIRANLAANPKFVKIYES  
 EVQYYESLIPHGNPVIEALTYIANVLRGNLGLDVITEIPVTKLIAQALPWTIFVST  
 SLIISFFLGIRIGQKMGYKRGTKIDSALMSTFTVMKSIPIYISGALLLYILGYNLHWF  
 PTGGAYCPCVPMGLTFIGCVLYHSALPILTLTLANLATWALHMRANTIYTLGEDY  
 VTFAEIRGVKDKTIETKYVGRNALLPLYTSLIIGIGFSFGGSVFIEEIFSYPGVGHL  
 YNAITNNDYTLEMGIIFIIITAVVFGVLIADLTYSIDPRVRYE

>AEE93234.1 conserved hypothetical protein [Acidianus hospitalis W1]

MKSVGILSQAWRREKINELKEIAEKAGKMGYNEIWSGVDPNYIEGIKEISAVA  
 EKYNYMYFFIDINPDIMRGFGASPTNLKVFKDLKIGGIRLDYGFSDTIKMANNDLGL  
 KIELNASIFPLDKLDYVVKNVKDIENTLKASHDFYPIKYTGLSLESAIAKSKEFKER  
 GIPVAVFVAPPREIEGRNTIETLRGKSIGKAASILFNSRYIDRVILGHPPTDEELKE  
 LIEAKEYTTIRVLVYPGLTEEEKKVFTVFSDVRVKEYTIGLSKMMENNIKPRNIVR  
 RFRGAVTVMNCRKNVVEVWIFKKEVEADSRFNVIGEVYYDDMDLLDYIGENMN  
 VKLVPIYMKQS

>AEE93235.1 cellobiose binding protein [Acidianus hospitalis W1]

MSNQKLLKYISIVVLSLIIPAAASSALSLAATSSCPTLYLAYVTSSPYQSLSTFNPN  
 LFDGGLGGDFYGLIFGYTITLNVSNNEVIPCLISNWTFSNWEQVWQNETVNVTI  
 TLRHSGWANGQPVTAYDILATCLILDMYSAPPYPNYTIVNNYTHIIEPPGRMSPV  
 LLSHTLICSIGLGEVALIMSYQQYKPLIQQIESDWTQLQHNNVTLIKEFRDKIHSYV  
 PPGPISANYNGPYYVASITPSEIVLEKNPYYFAASNVKFNKIIVYQYQSTSDFYAA  
 LFKGQISLDLPSVSVCPKVLSELPSCYKVISIPNPGGWALYFNFKNPWLMVQV  
 RQAIAYVLNRTSIALAGGYPKFTPVKVPNGIPCCFSYLKQFITPAVSNLNPYCTNL  
 SKAKQLLESVGFTCKNGQWYTPNGTPFTLTILEPFTLSPCVNMMNVIKDELTA  
 FIPTTYVCTNVKVNHEHYDTGTGYDLVFQHWGGFCIGTVDWYLETQYLNIGIP  
 LNVTQWDGIVTLPNGTQVNVVKLYGETVSPNSTSQLIAANDEMAYALNYYLPA  
 LPLVFQSCQIVVNTKQLNVPPSNSWFWQEYFYGIAGTAIYQLGFTDGFIPVIVTT  
 TPPTTTTPPTTTVTSTLPVVDIAVGIVVVIIVAAVVIIMRRR



>AEE93236.1 conserved hypothetical protein [Acidianus hospitalis W1]

MRKNFLIASWLFGLGNSFSSPIIGLYIYINSSINYMLKFLLISSIFILLGYILVGYLTT  
FWKEALTYYYKLGILLFISFYILMFILNVKSYRFISFLGILYGLAQGFYWSGWDIIFY  
NIPHKLKFFNKSAYLGFFTNLVSPAVYGSILTIFHNNGYGILFLLTALTLLLSAILA  
ESVKVSSFKFNIRRSISVFNENKTYRYTMTALAIVSGVNYILTNVNTIIHYKIATDY  
LNFIIISYSMSVITLISIIYVIRDKLSGILKPYNVVLSSSLLGISSVSIIVGFPLFYLVF  
SLTSPLIYPIIDVYNWNNMDRRFLTEYLVNRQIFLNSGRILSSLSEVILSQISGYEILP  
LIPLIIIASIIFVRYNSKEKIQQDVL

>AEE93237.1 bile acid beta-glucosidase [Acidianus hospitalis W1]

MKYSFTYNISSGIPLGGIGTGSVEIRADGRLYEWTIFNNGGYAERQDLRFTYYLDE  
QDSFFAVRQKGIVRVLQAFDYNFGGSPYILPWLRPVERVEFNGEPPIAYLEFVDKI  
SVNMKAFFSPIPTDLKNSSLPSAIFTLESNEESDFMFGIKNPFNEGNVDFKDNLTIL  
SGEVEPNDRYQGNLCIKILAEYPFARVIERHPSEEFQLWHEFRENGRLENKLGK  
GNFALIGGKGRKVTFILSWYFPNHLLADGRRIGHYYENFFSNCLDVAKYVESNLT  
YLESKTTAFHDLLYNVKGIDSWIADLVGSQTLTLIKSTWLAKDGGFGIWEGYFNT  
ADKRKTANQYPYTDGPLHTALNTIDVLTYSLPTILNLFPELAEKIILQFKDKLIEEN  
TPEHVVSLSFEENREKFLEKLSKDPSPPTDGEKLYSTINEIVKETGKDPKGRVPH  
FFTDNLRVDEYHRVDLNPEFILMTYLIAKTTGDLNLFKEIFPKMKEALESTMKTQ  
TYDGLIYHTLPAGLEWLRYVNNKLNLPBGDNNASILGHNLIPLSMQTFDDWSM  
IGITSFTSILWISSIQAVNDACSNLKINCSYDYESLVKKLIDYLVNGEYFDLWYDP  
KSKMRDKACNASQILGHWYSTLLGLRFLDDSLVKTTLSIVKYNLKEEEGLLNG  
AYPNGYRPLKRNYQNQLNLPATTQIDTPWVGVEFYVASHLIYEKLRDEGEKILRN  
IYERYKLAGNFWNHLEWGSYHLRPLDSLMVIHAYEGLKYDGITKTLTIDPAVEE  
LSWIIFLPTGWGKIEISKQKVSIMFHGVLEINKLKAEPKKVLLNGKEANLPIELK  
EGDSLILY

>AEE93238.1 Alpha-galactosidase [Acidianus hospitalis W1]

MSIKMRKISLEKVDDIVSPTLSFSDGKYLEYRVHNSFTILNFDEKIGIKSSLQFSY  
AEIGIEVPKKGFLALTISPVDYSNGYKYYNELYTYGRTDAVKPGNLSYPVFREFK  
NFDDINKVGPCWSYSYVEGELVYPTIAVLFFKEEGISNNYVTYLVNSQEGVTSSIRE  
NKLIIEGKYTPNKVFWALFIGRSEDPYESIRAAAFKEMSKCDNVKLREEKLKPSILG  
KLGWCSWNAFLTNISESKVLDVIKILDRGIKLSYVLIDDGWQKLENKVMASIDP  
DEVKFPGGFRRTVNVLKKGIEKVGLWHTINIYWNGYNEKVKEELGDGERTNG  
GYQIPHQLDRVLKVYYNFHKRVKDNGFSFVKVDNQWVIRKYSKPDEIEKAVQL  
SASLNGLDVMNCMSMVPECYTNYFLSNIMRTSNDYIPMWKEDAKLHLLFNAYN  
SLFFSNIAYPDYDMFVSYYDDYALPHLIFRIFSGGPVYITDKDPSRTNVELLRKVM  
EDKVLTVDFPGLVTKDILFVNPLREEKLLKLASKSNGIPVAVVNINSMRIKDKIR  
AEDFPYPLNGDLMYYMVIRGEHGYLKDLELELDEMEAEILVISSKGSPIGLKEYL  
LPPATIKDGRTLASGTLVFLDEEIKEIKVNKGYYQVNLPP

>AEE93239.1 CcmE superfamily domain protein [Acidianus hospitalis W1]

MRTLLKIGLIFIILSFVVGVGGAFFTLRTISSFFVIPTKVTVITIPSDSNFTIHYVNNG  
SAICLYYTNTSCVKILGIPSSATKIENNINGKTWSRSFIFLPTVKEGNITIVNPNPFP  
VKVITYKLSYIPPSYANPFRPFLDLLSSITLIVAGTLLIVGLILILGVVLKS

>AEE93249.1 CRISPR-associated protein, Cas4 [Acidianus hospitalis W1]

MISGTTVKHFAYCPQIVRLEAMGFTEAMREGLEVDKEKTINSLYGVLKPL  
NIVAKPIFRYNDLVGSPDYVLFSSNYVTPLDVKQGKERIDHVMQILFYLYIMEMK  
GFNVKEGLLYYVQQGRLKKVRYSFERNHVTRIIQEIRNAMKKGKVKVTQPVSKC  
KNCGFFHWCRPKIEGSIKAVIE

>AEE93250.1 CRISPR-associated protein, Csx1 [Acidianus hospitalis W1]

MKILFAPIGDPSGYVNVEYLVNGEGPYNTNASFIAISQALKIDKLVVYAGLSLCDT  
NCNDYKCKKDSVTQKVAQKLGNEFDLLIAPNIYGTRFIQKDRKNTLYFNFIYFNS  
LKILEDNKPDEIYIDITHGINYMPLLATDAIRLATYTYIVENNKNNGVNLTIYNSEPV  
TKGNQGPYAIDDIYSEKITVRQALSSILSPFLSSESKNVIKNKVFKVIRNKANSENV  
NTSSCNADFIYHIVNALSAGIFLYLILKKDEINSCLNVVENILGKLDFNNFAVQLNF  
ENGKVIYDENIPIEISYIHSLLKVSNHIAQGSNKISEIRRMAENYSTSDSVKYVILNE  
ISRIEEKNEIPNSPTLYAKIRGTDSPKICLPEKRNLIHGGFEENVTFWKCAGEDIC  
VSYTDGKCDCIENVEKHLG

>AEE93251.1 hypothetical protein Ahos\_0360 [Acidianus hospitalis W1]

MNYVELLNNLLEHAGIEADISPFYISLIFLHELTHHVIDWRTSSGTFSYLQEDERL  
CEYTAFTLTQAILGNLLRNQAFQTFVHEDHIHIFEGMANEAIIGFPVPGFFIPLNMD  
LPPQHNQVQELLSLIYYWNRDKDPLYKPLVLAWVNEMDLVRFLSQNLTVGLDN  
AWNKKGLPSVYTKVRFV

>AEE93262.1 hypothetical protein Ahos\_0371 [Acidianus hospitalis W1]

MEFIITCSNGLTYSTVYLYIKNNKDVIRRYIPQDSVQNVNVLIDFVRNNSFLAHG  
GFILPSTPDVIENIYGRLLDDTSLKLNDFLDKINKIKLSKE

>AEE93305.1 amidohydrolase [Acidianus hospitalis W1]

MDIEKLLKKDVLEIEDKIIERRKIHENPELSYKEYNTAKLVAETLKSGLIEVKVGV  
GLPTAVLGILKTSKPGKVVALRADMDALPVEEMTDLPFKSKIKGVMHACGHDT  
HVAMLLGGAMLLAKNIDMLSGEVRFIFQPAEEDGGLGGAKPMIDAGVMDGVD  
YVFGLHISSAYPAGVFATRKGPLMATPDAFKITVHGKGGHGSAPHETIDPIYISLLI  
ANAIYGITARQIDPVQPFIISITSIHSGTKDNIIPDDAVMEGTIRSLDENVRKKALDY  
MERIVSSICGIYGAECKVEFMKDVYPITVNDPETTEEVMRILNNISKVEETQPILGA  
EDFSRFLQKAKGTFFLGTRNEKLGCIYPNHSSKFCVDESVLKLGALAHAAALSIE  
FTNKKE

>AEE93306.1 Amidohydrolase 3 [Acidianus hospitalis W1]

MYNEWLPVAFSLEGYPKRRGNAPIKEMMKFNVNVALGHDCIMDPWYPLGTGN  
MLQVLFMAIHMDQLTGVEELLNSINLITFNSAKAWRVKNYGIKEGNDANLLITN  
ADDVIDLFRFMEPPKFVVIKKGKIIAKDGKYVLFNGKWEEVKRKP

>AEE93307.1 hypothetical protein Ahos\_0419 [Acidianus hospitalis W1]

MEDDLTQKLISLVEEYKEVNYKAQEIFKELKLLMRKGAINPWRVNMIDSEGKFID  
EYLNEIEKELEIEKEIRLKKK

>AEE93311.1 hypothetical protein Ahos\_0423 [Acidianus hospitalis W1]

MGVFPGIYLPFPFFAYYLSISLISYTLWGFYFYSSISILSSQYKILSTRSSHNLQIM  
YLSKDSITILILNFIIAFNNLQTYL

>AEE93347.1 amidohydrolase [Acidianus hospitalis W1]

MNIEEIAEKLLKDESEIENDVITIRILHENPELPFQETNTSRLEEKLRSLGIQTRRL  
STTVIGLIDSGKPGKTVLRVQISALPITEKTNLQFSSKSSGIMHACGH DANVAML  
LGAAQLLVKNKDLLSGKVKLIFQAGEEEDLGAKEVISNHELDDVDYVFGLVHVP

FIPSGFFATRKGALMPSSSNFKIRVKGLGGHVSSPHSTLDPFISAQIVNLLDGLTSR  
IVNPLDGFTLSITSIHSGTKSNIIPDEAVMEGTIRGFDVFTIEKVKSKIKSLVDSLCK  
SFNADCEVVFSNDNCPPLINYPEITSRAMDILNNLRPIVEIEPVMLADDFSRYLQL  
KPGCYIFIGTRNLEKGCYIPHTSPMFKLDEDILKYGSAALALLAISFSKEESIFK

>AEE93422.1 conserved hypothetical protein [Acidianus hospitalis W1]

MLRLIGGA AVAVIAPKGSELF SRTYKDADYFGLSSQRKEITS LLEGLNMEPNKRF  
NALHGYNRLMFYDPVLNSTIDVFLDEFVMCHKLILKDRLKIYYPSIPPSDLLLT  
MQIINLTENDKKDIAALLYDFEIGDKDEDKTLDGNYIAKLLSEDWGFYKTFTINH  
DRMKEYLKGNTYAPNILPKLDKLEMIESHPSLKWKMRAKIGERVKWYEEPEE  
VNTNFQGAGSGI

>AEE93873.1 hypothetical protein Ahos\_0987 [Acidianus hospitalis W1]

MRLIIAAMSRKRRLGIEGKNYHIFHYCFIFIIFLFHFTFFKDISYCLVVC SYISLGKF  
GSVALQLPIRSCFTSTLFLQLLRGSLFPFSLLYNTWTVRLQYWGSTRTLYFHPLPI  
VVRGNRLGFL

>AEE94069.1 DNA polymerase B1 [Acidianus hospitalis W1]

MLKEYFFHLYNVAKQITLFDSEIKQETHSEGKGV EKEEEVQQLEVENKRGQVEW  
IKEAKEGRTY YLLGVDYDGGKKGKAVMKLYDPETQEAAILYDNTGHKSYFLVDL  
DPEKVRKIAKIIRDPSFDHLEVVT KIDPYTWKPITLT KIVVKDPLAVRRMRNYAPK  
AYEAHIKYFN NYIYDMQLIPGMPYKVNKGRLEAIIPKVDTEEVT KAFQDSDEVTK  
EMALQWAPLFESEIPSIKRVAVDIEVFTPMKGRVPDPYKAEFP IISISLAGSDGLKK  
VLVIEREDVGEGNTNNEGISVEKFKTEREMLSRFFEIVKTYPLLLTFNGDDFDVPY  
IYFRSLKLGF FPEEIPFDIGSRET KFLPGFHIDLYRFFFNKAVRNYAFEGKYSEYNL  
DAVASALLGMSKV KVDLSLIDLDISKLIQYNFRDSEITLKL TTFNNDLTWKLIVLF  
ARISKL GIEELTRTEISAWVKNLYYWEHRKRNWLIPLKEEILQRSSTLKTSAVIK  
KGYKGAVVIDPPVG VFFNVTVLDFASLYPSIIRTWNLSYETVDIENCKNIVDVKD  
ETGQVLH HVCMDRPGITAVITGLLRDFRVKIYKKKSKQKDLDPERKMMYEVVQ  
RGMKVFINATYGVFGAESFPLYAPAVAESVTALGRYVITSTVKKAQEVGTRVLY  
GDTDSLFLYQPTQEQLNTIHKWVKENFKLDLELDKSYRFVAFSGLKKNYFGVFTD  
STFDIKGMLAKKRNTPEFLKEAFKEVKDLMGTINSPSDLEKVKKEVAEKVKSVY  
DRLKNKEYNLDELAFRVMLS KDLD SYTKNTPQHVKAA MQLRALGIQVLPRDMI  
LFVKVKSKEGVKPVQLAKLTEIDL DKYYDAVKSTFEQLL KSLGISWDEIASTISID  
SFFKF

>AEE94103.1 UBA/THIF-type NAD/FAD binding protein [Acidianus hospitalis W1]

MDRFSRQLLT LGIDVQEKIMSTKVLVAGCGALGSSIAELLVRLGVKELKIVDADV  
VELSNLHRTHL FTEKDLMKPKVLACKEFLEKINSEVKIEPIFDIIDETNAEDLVKD  
VDVVF DGLDNIN YRLILNDACVKYEKPLIHAGVS GEYGN AKLVIPGKTSCLACFL  
QPSDSRNACDIIGTTTVVPN FLASIQVQLFINYL RGYSEDELVIADLKDRLDKIK  
MKRNPQCEACSLHEYKYLRHMDINCGLTRA EKAEGDRIFSSNGVELYKYNGGYI  
ICYNQKCFKKRIA

>AEE94104.1 conserved hypothetical protein [Acidianus hospitalis W1]

MSIISLRDKYMVDISYKSAFKARLLGNASSLLIPGWAGQELARAAIYNLEKKNLIE  
SLSLSIAEAPFDVISGAILFIILPIKFYYIEFLYVLVALGNIVGWSIGIAYVYSTAGR  
HVETEKRLMKLIGMEKYYFLLLQ GKDSIRNSIGNKRFIMYMALSFLGYLVLSAGL

YPLIPNYFYDIVVTMAYFAATLFFPIPGASGVSELALAIVLPSSYVFDIIIILEYVSYAL  
GFIFLREISFSELKKDIDKIKKDGELYKRADS

>AEE94118.1 conserved hypothetical protein [Acidianus hospitalis W1]

MRIKENANMGVETSSSLRYLGGIIGTLLEAVITLDCMQENCVKEGLKRYNSMESF  
QRYEVYPAISAGMRVLKDASSPERIFRQGIVVKTTDTGDWIFYIGGISQYWTSDQ  
LIYQGGSQASSQGKLNRIIDDFVNKGGLGVVPLYKERAPSVWYNPVLFKDCQ  
GSFGIFWNYLGEFQAGVLSIFSNAFNILRYTEDLIKAGKASLAYSSYGNYLSRA  
AENDVMRPASDNPYVYLALGTNPLVAKSHGLQIYPSFTFDTVTSDVSSCCENIMP  
EPYCCSYFLKYVRFNDIDIGAPVYATLPCGTSCSTFGLAGLIMGISSMTVNNVQLI  
YLTIAQPPSDFTTSAIIEWSKTMGFYDSLNLKFEAGKRFKKAITDLSTAFPEFIATA  
AALTVDWLESYEEGLKRAEVKARELNELWLLTNWLVSRCQQFPIIREVYIL

>AEE94317.1 hypothetical protein Ahos\_1434 [Acidianus hospitalis W1]

MWKKKFSLLFHVQVVEVTKEDEIIQAVKEDEDLSPYDALHYVISRRYSNSQCGSR  
LQRKDRPLFFLFIKIKTRAKSKIYRIINISNSSLLKNYRPRKTPYYVNIHRKNEKLA  
LNLVTVQEYCTNPPGKNTPEGACDYNGEVSTPEPITLGSLLLTGEAESGVSSPAP  
NSG

>AEE94430.1 conserved Sulfolobus plasmid protein [Acidianus hospitalis W1]

MERELTQKQKILLVLAKRGSLTLEELERYTKIPRNSLLKNLSELKKEGKITRGWL  
HIAGRKYRKYSIKTSILKELGIE

>AEE94434.1 conserved Acidianus plasmid protein [Acidianus hospitalis W1]

MSSKIPIKSKYSWNELNRAKEAITFFEAVIQPSLERDGISTTVIESMKISFMRDVLNI  
ISQKYAQTHAPLDVTEAVKIIDDELPKLLKESSPGLDVNGILNMWSLVKKILSGGE  
TENEQKVGGNNSTTTTTTNKK

>AEE94435.1 conserved Acidianus plasmid protein [Acidianus hospitalis W1]

MNDKETVKLLQYHHLLYKNLYSNNFSETARLVIDEHLGSFKPNKVQIILQFFRNL  
KNGIIPAIARDLQGLTAILLIKDEGYMTKLKKTKFADYLIAKLQDSQAFTITRSD  
ENKKVTIFDDEKEFLDEFKEKHGLDNDQAFYELLSTTFAFSIWRTLINLSKERV  
MTELNECFGVLQQFALLLGILKVSQCKAKVYVEFFPYFFKNEFMEMIEDVI

>AEE94441.1 sulphite exporter TauE/Safe [Acidianus hospitalis W1]

MIPIWLLIIIGIAVGALTGITGSSGVLIIVPALSYLGLSFQDAIGSSLLVDVITTL SVI  
FVYFRHGNVDLKLISLILGLGAVMGAQVGSIAIAFVVPDRALESVFFVFTAYMAYV  
SFKRSRNPKNLKNLRTASYVIAPTLAFLIGTVTGTLGASGGIMFIAVMMLLSFI  
DVKRMIGTATLAMLFSAISGATAYAISGRSDIISVIIIGLTALVSGYYFARLANKM  
RPSYIYMFLGVSFVVTSVSELFKVI

>AEE94442.1 conserved hypothetical protein [Acidianus hospitalis W1]

MLRIHTKKGDKLFLYVQIILWLIYVMIDLGLFTNKLLLFATIALALSVVLIALVLTS  
YSYLNVRITPERVEVGKWKIPLDQVREIRVLRNNGPTADLIIVYEGGERIIEVK  
NWKVLETFQRYKENIV

>AEE94459.1 hypothetical protein Ahos\_1576 [Acidianus hospitalis W1]

MKLKFSYFGILSMKAKTIAVALAGILTGLLWIIPSFFISMITMLYILGRAKLFFLP  
II SYIISESVTLLLFMILYKFVSRTYAIAFLVTASIISFIAPFLAFLVSILIGES

>AEE94479.1 hypothetical protein Ahos\_1598 [Acidianus hospitalis W1]

MLGKFDSKIVGLGESDEVYDLPFYWRIKYVKGKLSEIRFPHSLFDGILFFIDSRTS  
FNHVSIELARISKPKTKLYLILLDKMALEEKEAYVKLLSDKFELIGDEKNILVLYN  
KKQKNKRFBVNTIYVYHPNLAKDGITAYAKHLIYRLKKRI

>AEE94481.1 hypothetical protein Ahos\_1601 [Acidianus hospitalis W1]

MAFPTRAGGALSTSLSSLSNSSLVQYGQPHRDPLQLVHDPQRETLPNPHRNLYLEY  
LGEVLLSSSRALLSISAVSFIARPPQLPQGAQSPRDWINISRYGLALVSFSDTDVG  
SIKPIL

>AEE94489.1 VapC-type toxin [Acidianus hospitalis W1]

MVYADTDFFLALLKEKDWLKEKALKLLEEYRGNIKTSLTTFIELMLLSKRYGLD  
PVRVTLSVMELTRYFDEKVLKASVLISQGMGVFDAFHAAAFSEEEIISSDHVYEEF  
GFRRIKLDDP

>AEE94491.1 conserved hypothetical protein [Acidianus hospitalis W1]

MGLNLILSYIDLDVDDVYVSSLLKIMIEDKRLREHFELLSRLSSLTPRQQLRVL  
DYILYISNKNEMGLTSLKIQCSPREVVYSITLLSLNPRIVSLYEALKICDKKLEILNK  
FTNNLEDIILNPKLIDFLREKINGEGFDKAHLELLILLEFALKLRNEGYHVKLNDYF  
GDLLLDTNASFEEQMKIRDQIIEEYKRKYNVDSAEVGDVFGMVDSVDKIKEYM  
RKILDVGGGRKQCLLVA

>AEE94509.1 conserved hypothetical protein [Acidianus hospitalis W1]

MDDLRAKNVITFTDVGECTNCGVYGPYEFVFWSSHTRRVYGFCSLKCFFREWL  
TTRAIYPKR

>AEE94529.1 Radical SAM domain protein [Acidianus hospitalis W1]

MLSKYNIFLDDYKIMFNTLTGYAIRLTDEEIEKLKKGEVPEELKDIEEGFSATFDE  
FLQKFKEVLEPTLVLTYRCNFDCVYCFQKAFRNNSSVSDKVVRGFIKYVRTHA  
NGRKVRVTYFGGEPLLELRRIKEISTQLSDLKYSFSIVTNGSLLTRHVFDELKSLG  
LTHVQITLDGPREVHDKRRYFVGKGSYDIILKNLKEIQDEVNVVLRVNIDVNNL  
DSFRDLLRDLKQNGITKVRLDPLVHDNVFRNEYWDYTFPKDEEGEILVKLWET  
AKDEGFEPQDVFRGLGLCAHFEDEDIVDVPFGNIYPCWAFTGNPLYVKGYLTED  
GDVVIYEKLSGERALNLWKKCKDCPYMPLCLGGCRFFSVLNRKGYDGIDCRKK  
SYEAIMKLLKYFV

>AEE94543.1 zinc finger C2H2-type domain protein [Acidianus hospitalis W1]

MAKRVLKCELCGQVFASNSLYYQHKVLQHSDYKPIVKEDGYECPICHEKRKRL  
EPMLTHMGLQHLINNPRIEIVQ

>AEE94585.1 conserved hypothetical protein [Acidianus hospitalis W1]

MDCCFNYIGFVRREDNSASRNSVVRIVIKEEYVEGLKGVEEFSHLIIYHLHLANL  
NKGLKRVRGGVEVGVFATRSQHRPNPIGISVVELIKREGNTLYVKGINAFDGT  
PVLDIKPYDEWDSILNPKVPEWHKIEMRSRITDSFYFL

>AEE94608.1 hypothetical protein Ahos\_1731 [Acidianus hospitalis W1]

MEQQQGEYVIWKDYPLSSYRRNALIGTSIIGAIFLLTVILAVLGIHIAVGVTYILT  
RRANEYVVTNKRAMHIKFGKVLKQVDLSTPGLVVSTVNPRYYSSRVAGQHVVE  
DVVFLVNGVEVLRFNKTSKGDELIAKLHSMGFQ

>AEE94613.1 allantoin permease family protein [Acidianus hospitalis W1]

MSVKPRSILTLWFSSNTAVAKYAIGLIPATLGLSPTLTIISLTGLNFLASIIILGIATSM  
GPRFNKEQIRISQDVFNNGWKAFSFLNFLNTLGWFIVNVTLLGGFALSVLTTNNVAL  
GGLIIVVIDVSVVIFGSSFIHKFESYVSLLLVALGIFISAEILTVPMKIPEGGSFLSFW

VVFLTSFGAILSWSPLYASDYSKGLNLDFFKSLFYTSLGVTVPSPVWLSYLGYSAL  
AIGKSSPIADVGLGVLGKYFLAGVVMVLGIVSADALNLYTNTVALTSIVNIKREIA  
TIIAGVLGFIAFYFIYKNFLDFLINFLSSSLGYWISPWIGVLIAYLIKRDWKRAWVS  
FSLSVIILPFMNLRQYGIPYEGFISSMMGGIDVSQIVMFFVSILFYLM

>AEE94614.1 VapC-type toxin [Acidianus hospitalis W1]

MKGEFLLDSSALYPLLNYADKVDVSKIYILSLTFYEVGNAIWKEFYIHKRIKDPFT  
LSLLFQKFLHKLKVLSDPPANEVMKVAVDKGLTFYDASYVYSAEANSITLVSED  
KELIKKANAIPLKEFIRL

>AEE94617.1 CRISPR-associated protein, Cas6 [Acidianus hospitalis W1]

MLALVKTTYNTVPLTDVVLPSPSSKVLKYILSGKLFPSLANLVKSRDKQKPFIS  
NLGYGDVRLISDGSEVIKINANSRLKATLSFPFLDGIQNEITEGVYETPYGKFSLL  
DSIEIVDIKSLKNVNNYENANIYVKFLTPTLLSSKILLPSSLASKYKQVNSGFSLLP  
SIGLIIAYAYRNYYAILGNTNGEEYASRAFKLGVLINAFKIVGFNLRPKTVIIGRD  
SKKRLRETRGTIGWIEFDVVHDKFKRLAIEYLLIASYLGLGRGRGIGLGEIKFELK  
RRKD

>AEE94618.1 CRISPR associated protein, CsaX [Acidianus hospitalis W1]

MRIPLYNIFGDSYVKQVAIEAGKYEILRNAIEIDNSLKEVLKIAAGDIKSREEKIK  
NKIVNILPLSGNDKRAWEKILKCYNFGNIATLSEVLYKFSPEAEKCNVDTAPSFV  
KPEFYEARIPGIPGGSKSKMSVDGSYLVAAGWVLTRLGKVKVNEGNWLGV  
QLFTTTKSNLYKILENIKYIPGIKPETAFAIWIADKIINVNLNVYTLKVYLISDAVG  
QSPTTIEEGFVLDLNLKLLMNKEMINDDIVYIASDALNIDSNTRNYSAKIINLVYEVI  
SGSKRIEDLLYFANRDLIMISESNDERIPLLKRISRYANYLSNVTTFS

>AEE94619.1 CRISPR-associated HD domain protein [Acidianus hospitalis W1]

MVYLSKPCAFIKQTLFDHSMGSYAVLSKFINYTYAVISRRLKSVGIIQLKPEEVK  
RLIEISVKLHDIGKAAEYYQNQFDENCLSNKKEPSFIFHEIGSSFFYYSNYTNDN  
KVKRLLALAALNHLNAIRGLADFKPNQLPKGAKEEMLKIEKYGKVLLSKLGFNF  
EVRDYTTDDYIKMMNDFINRPKDEDLKLYKLHNLFLAPIIIGDNLDSDHYARSISER  
RRFIKLLLEVELNEDSII

>AEE94621.1 CRISPR-associated protein, Cas5 [Acidianus hospitalis W1]

MIYSKVFLKLHWGFSVSVPSVSKSRPSVYLPPPTTLIGALSYGKYRGYDTVLVNR  
RPGSPAYNLLNVKAAASFSDFRGTYIEDIVRNVLILYFQRSGRKTDPKYRYGVIPS  
GKVYSPNGKLNVVYVTDSIDRSELERLSWSITRIGCKECLVSVEDVELGEAKKVS  
GKVRTRYYPDNVKIIDGKVEYVEFWNEKGFLWGSESDRIRYALPIITYPLMSVE  
VEVEAKEAYEVGGEYVVF

>AEE94622.1 CRISPR-associated protein, Csa2 [Acidianus hospitalis W1]

MISGSARFLINVESLNGVESVGNLTKHRTAPVVVKTTSTGYLIRYVPVISGESLAHA  
YQASLVDIKSMNLPVGLYSSQYEFIKYSSDEVLEKEEGISAPSSSNDVRRFEVEVL  
LKDIVSDVGGFMYAGKYPVRRTSRIKFGYMIPALTGEELPAQLEAQFHVRYSSK  
VEERQAIFNVEVSSALYTLTFLSLDDDLIAPSTIGNEVEGEEEGQKTDRVKA  
KSLYSILTGNFGGKRSRFLPSMKLMSMVVTVTDFPFIEPGHTDDYIKVSVERLN  
KAKSIFNSKNVEVFTINNENIEVPSNVKTLSSAEDLIDELIKSKK

>AEE94623.1 CRISPR-associated protein, Csa5 [Acidianus hospitalis W1]

MDEEKIEGIVRRRIANLFATTYLYSESSMLVDRFANALSKEAVAKVLYDAQRIVQ  
MGLDRDEIKSDIVKIKEKDYPAIVITKEKEKSVQIIGYLPTDQDVEDFLSLIEKDVY  
YARKAGALAMSIANRTKLGGSQ

>AEE94628.1 CRISPR-associated protein, Cas4 [Acidianus hospitalis W1]

MASVTDLKDFMLCPVIPWIRKKLGWREPITEGQRIAKNVKPRELANDLPDPKYY  
EVYMRDKSTGLSGIIDVLARDIVAEIKAFNRRFYGHFRIQLLSYAYLAERNGFRA  
KDAILILGREKRLKIEVRKEHIEYVEKITNKLVESLEEDSPP

>AEE94629.1 CRISPR-associated DNA-binding protein, Csa3 [Acidianus hospitalis W1]

MKSYFVTLGFNESYLLRLLNETSAQKEDKLLIVVPSPIVEGTKAAIENLRIQTLRL  
RYPNPEVIEIKISTFDDILSQLLDNLLPLQEPIISDLTMGMRLMGALILAILVSRKKF  
TIYLRDENGGSRVVSNSSDINALFKEYSSEEFKMLYNRFKNNTISTFELSKILGKS  
EKTIQNKISELKKLGILTQKGKDRTVELTSLGLNVIKLIKNSRLYIENKIQ

>AEE94632.1 hypothetical protein Ahos\_1755 [Acidianus hospitalis W1]

MNEIVVHTHIIGQKHMFKITEFINTLSRKYHFNYDLYLHPITPSRDYVRTYSIPYE  
HFSKEGKWIIEEELLNVFEKELARIKKENNVNKNDF

>AEE94635.1 DEAD-like helicase superfamily [Acidianus hospitalis W1]

MSEFIHPALDKEYNINGEVSSSLVTETGNSIPYGVLLADETGLGKTVIISLYLLYLK  
IRGLANNVLIASPKSITYQWRKELLDKASLKFKVINSGRDFEKINNNKFNIIVSIDL  
LKTTKGIQFIDKLNNYQIDVAVIDEAHHVLSANDTLRRRMIIKVREKSKSLLLATA  
TPFRGVKEEEFIMHLLGQEYIFIGRIKENIEDTDGNPIFKPRKSITVKIELNFRWNFI  
YDHLNKMIDRLNTENIIKLILKKRLASSVYSFLITYNHIIDSKILLQTK

>AEE94637.1 conserved hypothetical protein [Acidianus hospitalis W1]

MNLLTVKKYLFKQSLTKFQEIQLKYINSILQDILVPILCDYKNIDIISQITDIESFYF  
GFKQNPSSISEKLALPLYISNRFFIASSGPRFGSIYEDLIRSFLNFGYQVDERVNIF  
KYTLFSEYYQKKYSKQKVIDFIARKDNKLYLIEQRTSEHSGGRTGQESLLDKFK  
VFLNWIIEKSADPLINKGLREIYLIIFISYSQKHEILTEQNVNRGRINSLEIYIVENLG  
DYFTRLTNKGFKTECIDLASCLEKYRRLIFYTNQIKIEFRIMLGEEFYKEILGEKYS  
SLKNKILNEELGDDLWVIYSILPYELRLYYEEGFMWSNRIYEMLSRYNNIITSAS  
NEDDIINKIASKIIDQKDLRLLETNNLSKQYEYKTLCAASLILYALKCPSRLGI

>AEE94639.1 Cytosine methylase [Acidianus hospitalis W1]

MQTSIDDFLNNKYRPVLFRLDIVKLRTNYATHDLYPYPAKFIPNVVRYFIEAYTKP  
GETLFDPFAGSGTVAIEAEITGRNYILWDLNPIIEILVKAETWKDEISEKIFEIDFNY  
NKSFIPKWKNLGYWYPKEFLEQLSKLWAYYHDHPNPLVAIPLFKITKYFSYAELE  
FPKLYKSKFAIERVNGLLSNWKEIMRDMYWKEVERVIRKVKEFQSLCKCVSSG  
KVYGGVDVLSESPNVDAVITSPPYLQAQYIRTFKIELYWLGYDDNKIRELSKK  
EIPYNKPPNVEIRSKTFKEYLEKIEEFGNKKLVDIYITYFRSLAYFFGKVNARTLGI  
FVGVPKVRRLRPIDEILKEHLESLGWKHEVTFIDKIVSRRIKEVNKNPATKLEDE  
RTPTEHLLIMRKN

>AEE94640.1 conserved hypothetical protein [Acidianus hospitalis W1]

MYVLFKGTLTNFLSLDDYKTRKSKLIQKYPQGSFIWGFNRSSKLLLENQVRAFLY  
LNKSESRLKGGIVLEGEIIDIAELSEKYWPEGEWKYYVTLKIIYIPKSVLSTTDTR  
WKIIDLDKLKEIGVKILPGIQKIDNELGRRIEKLLGEIDAN

>AEE94641.1 conserved hypothetical protein [Acidianus hospitalis W1]

MYDKLSTSEIILLTLREKGGKVDIDKLQEIIEFYNSKCTSEICEKIDPNDKKLIANS  
LYYLTIVGLVTLEKEKIDSKEVYEIIKKYKDYLFSPVVLYYPYLGRRFVLPVLP  
KFTRRDIKNLILFLEGLKENEPSLKIENDIEKLKMLMKTRTFVKLTGDGEEIASKL  
LTEYKVKV

>AEE94650.1 conserved pro-fuselloviral protein [Acidianus hospitalis W1]

MRKSLSLLGLGLLILFLEIPISAIHAGATVPLPLGTDPNWVIEFYPCSYPSGVSPSN  
YSVTVNFTIPGFTHTSGCYLAFVLTTWVEASEYTGIVYSGTVYALQLGVVVFVYS  
SNTYVINEQVWTCNGNEIYHNYTLVTTTIHPNVETLITLLYRYDNGYVATGILQE  
CTSPITLIDVHYPTSYRYGCTIFYVINTYGFTDPS

>AEE94651.1 conserved pro-fuselloviral protein [Acidianus hospitalis W1]

MEFLSLLIFVLGVTVAILTIVLSPDFAKAKPNKRNLIIFLSIILVGIGLWLFHIDHYT  
YICSSAPQNVNCSVPSLGTVYAPSKVIPPPSYASLWPVFLASGIGLMGFSVLIQIVR  
SKK

>AEE94652.1 conserved hypothetical protein [Acidianus hospitalis W1]

MYDGILIFVIDAFLCAYKGSSTPVYTVKGIPVASGVYLQGEIKYLDVLEKLNNN  
VLAQFIVFFSNGTVWCKKICKCWVYGCSPAYSAYSIIEAPSPETGVYIELPIVEGG  
MINFEFAYCVNGNQYAGPGTQNCFCCTIANIISLERTSCNKATVFLCNGWQRKCS  
GWDYLYKFVYPCGISTYWL

>AEE94673.1 hypothetical protein Ahos\_1797 [Acidianus hospitalis W1]

MVNSEMLKRIASNLLSIIFVSMVAFMSIHSYATNEGRSIIHVIDGEKPIYNARLQI  
YVFTPLGLVYLTTLFTNRCGNAILQYSSLLSYVGTAINFKSIGIQIMGTYNSTTKV  
LHYNVKSCIIPIAEIVHPNATKIITLNLHYKKVIKKAHDNNQNIHKIIPMSNCPPI  
PSPGPYYEWWVLVKQQSINNVPPIFAWGKQCTPGQSIIFLCSSSSATVCTWAAGS  
ISICGAPISFKIVGKSAQVCSTLLHMPYLVRVCNGTGYPGCMLYANATIGLAEFQ  
LYYVYPCSGYAYPCNGYFNETYLEHFDTNLLYSLPSTARYPNYPWIYQELEPILS  
TYVKSVPYIQSAKIYYMQRIFFFSQNYSRVCNVYLPLGLAIPVGDIIVSLFVPEIPI  
ISPDILATLIIGITATQNNQYIRLVCLDLLSLKCKSYCYVDIEVGYIPQEYYNNGFN  
TVFPFYFVKIISPESMCVSTNILTPTKPCTPPGIYVPPGGNLRFTLNITLGGTDILAP  
HGTICVMVIPVGQNPTWTHRYSVDGNTLTICVPSGVLFYGTNWTVTIYYRTSN  
GTIIYKCSCTWIFYAYS

>AEE94682.1 conserved hypothetical protein [Acidianus hospitalis W1]

MDCCFNYIGFVRREDNSASRKSIVKIVIKEEYVEGLKGVEEFSHLIIHYHLHLASSN  
KGLKRVRGGVEVGVFATRSHRPNPIGISVVELVKREGNALYVKGINAFDGPV  
LDIKPYDEWDSIPNPRVPEWHKMEMKSRVTD

>AEE94683.1 hypothetical protein Ahos\_1808 [Acidianus hospitalis W1]

MDASLIKLLSKESKMKQEEIEERLSSASKAIQYVISFNYYEKEYTESVTLSGNYKFIR  
LKPFNRLSFKKVFLSTSSKILEGQVKMNAEGKGEVKIKDLGNFQNEIETFNKGK  
KEMEVELEFKVIFTNELLKDIKKYLEIQEKVYGTRRLFSINFNVMLSPILRYRQS  
LSFPRLEKAMAIEAKYLSKKGFPEKFKVLDLFLKVNKGLTEFLIDFPDFGYIGIIEI  
E

>AEE94687.1 exodeoxyribonuclease III Xth [Acidianus hospitalis W1]

MKIVTWNVNGLKAITRKGSLEVLKYDVVMLQEIRTSDDLPLDLLFSGLAIESFSA  
KKKGYSVMTLTRYKPINVIKGLNVEEFDEEGRVLTLEFEKLFLINSYFPRAGDE  
LKRLDFKIKFDKTVVEEFMMKLRERKPVIIICGDFNAVDRDKDSSFWDEREPAITPQ



EREWLNHVINDLGFIDAYKLVNPNKNEFTWRSYRFKWKAMRIDYCLVSSELKNE  
IKNCEVLKIEGSDHYPLLELNIESP

>AEE94719.1 pyrimidine dimer DNA glycosylase [Acidianus hospitalis W1]

MRLWSIHPKYLDAHGLLGLWREGLLAQKVLLGETRGYKNHPQLIRFKRTSDPVL  
YIGTYLYYVYLEGVRRGYSFNKEKIVKYDLTLRMPVTEGQINYEFRHLEKLR  
NPKMYAELLQVKITEVNPIFFVIKGDVEEWEKVAFFNTQNAI

>AEE94722.1 conserved hypothetical protein [Acidianus hospitalis W1]

MILKPKISWFCLNRLKTPYVYWKSVIVILENPSKTLVVDVSEDQLSTYRPPKEIINF  
KFFYKIGKIDDENIKYIGCIAQELQKNMKAIPQPKA

>AEE94724.1 hypothetical protein Ahos\_1851 [Acidianus hospitalis W1]

MLSNNRIDMVWKEKDEVKKSVELTDVGKEVGKSSLSAAAGITKDGIEIGKEL  
TKSSIELAKSTEREIKDTTKVAVDSTKELIEDLTDGVKDAMKGPIREMKNLGDGD  
QGKQENK

>AEE94762.1 major facilitator superfamily MFS\_1 [Acidianus hospitalis W1]

MQRIKIFAYSWIITFLQLLFRSAWGVISVPVAMQFHLSVQIGLVLTsfyVGYVISS  
TPWGLFIDLHGKVAVLISLVSSIVILLIFLPSYDIIIFSyllSGLIVSALFPSSVKI  
VSVKLSPLYFYLGMLESSAPFAVLLGLISGLLYNYWRIFYLLSVSLLSVFFITLS  
MKDDYVSGKRRETKVLFTRFILLSILVRSGEMWVSWGTTAWLFPFLVLYFHFCN  
SSLIFLLFSLGLVSSTVVSSKLPLGIGKKVQISLILYVLFLLFLTRVSWLSFFL  
GIFSFSFRSPTDTLIVKFAGKENSSSSIGLANTISQIGSLLAPISIGFAVKFSPLLGI  
FTLSAGALLSLAVSFLI

>AEE94763.1 hypothetical protein Ahos\_1890 [Acidianus hospitalis W1]

MRFLGIDTSGDHDISSPYVIGYAISSDESSSIEKSNIGRAISEARAILNKNPYVFHAYH  
DDKKVREVLLDSLIIKHGKGGVIIIILNRKFVNSFFFPLSKLIDKCSYAVYDNPFDH  
VLSKTVKKSTKVTSSKSLTPLYLPLQVADYFHSIWWEIFSHGLKDFNQFEKILSIT  
DELVVIGLKNSTLVKT

>AEE94768.1 conserved hypothetical protein [Acidianus hospitalis W1]

MKEVQLNVKHEVEVLELFSDPDFALPRIIPGYESHTGKDTFQVIGVLGLYPYIM  
EGRVFKGSVIKYVFRMIDGLKGAGNIEISSIENSIFLVDYEGDSKSMFESSFEKGI  
KKLSKSINEDIRLERIKRI

>AEE94785.1 conserved TM helix repeat-containing protein [Acidianus hospitalis W1]

MLPLVIYSISAAISNLETDFINAIPSILFIITVIIGYIIAKLVSDVVARVLHNFSASYPE  
LRIGTGLIAGTVEALIIILALIAFSLNLGPASVYVDYIARYLPYLAGAILLLTLGT  
SLVNLFTDFANRQIGQTDPFANVIIQVLRGLYAVIITVAAELAIFYWIPSINSYLFY  
GIIIGSIIILFSFTITGKAIDEISKAHPETASVLGYARLVLYAVFILIAIGHIVQPFGNVT  
AIIQTFAWGLAIAFAIIVIPLVYALAKKIIQ

>AEE94818.1 PaREP1 domain-containing protein [Acidianus hospitalis W1]

MQVEIPDVIYAFITESLGKDASDVLVDAFLSTLDKGNRLKLYVKLSEYERKEID  
GEACWKALSIVFKAIAEIEGMEISSYQDYSLADYLSFKLNTEIHKYFLNAEKL  
HAEYHPRPQDKDSLVRMDHCKKLIEVGKEYLRKSKSLPEEI

>AEE94880.1 conserved hypothetical protein [Acidianus hospitalis W1]

MLMTLLPSSIGGLMGLWEEQAVSLSFKGLELSDESSLSSSGGVSDYPDCPPYERC  
SPELMVGTMSPLGGNPRPSRAGRSQLLTALKDSIYRQL

>AEE94960.1 Dinitrogenase iron-molybdenum cofactor biosynthesis protein [Acidianus hospitalis W1]

MRIAIPVTKGRVDGPGE GEEVLIYEIDGDEVKLVEKYENPALKATAARGAHMLK  
SALDKGVNAVIVAEIGPPGVRLKKGKAKIFLAEGLTVEEAELEKLKRGELQETDKP  
THDEHHHGF

>AEE95052.1 ribonuclease HII [Acidianus hospitalis W1]

MVIAGVIIDEKIEGMLKEVGVKDSKILSREKREKLFEIIEFAEAYIVNKAYPNEID  
SSNLNELTYSKVIQIIYASLPLHPKIVTIDKVGNE DIVIMKIRELGLKDNVVFNADV  
KYIEASAASIIAKVTRDRIIDSLKKIYGDFGSGYPSDPKTRKWIIEIYEKDKNNPPPII  
RRTWKTLTKIAPNYYISKG

>AEE95067.1 ABC-type Na<sup>+</sup> efflux pump permease [Acidianus hospitalis W1]

MKDLIKKEWTDVKRDRKLLGSILLPLILLPLIGVILFAAIVSQPPVVDIINENYDNI  
KYVKELCSYIQGNNGGIVYINSTQPADVEVFPSCFYENISDINRTAIVYISYVISSRS  
SALQLVENGLYNILYNTSIQRIKYLENMSHTEVSPSIIRDPLEVTLLYKLPTGEQAS  
YQQSQFSQLARIVAIILFPAATPVIFFVSDSIMGEKERKTLESLLASPISSRAFI FSKL  
IISIILGLISSIGDLIGLVAFSLFAPYIIGESITFSATFAILVIIVYLAMILLTASMSIIVLL  
LLGGSSRN VQIINFITSF GMIA SFSSLFLNFGDLSYPLSLILGIPYVQLVASIMFYVF  
GLIEESIFSISITLLASIFLLLLASRFLDSERLLLK

**SUPPLEMENTARY FILE 2**

>PROKKA\_00243 hydrogenase 2 maturation endopeptidase

MSVKIIGLGNRLYGDDAVGSLTAACMEELGLPAFDAGANGFQALSAIENGDIVFF  
IDIVQMDEEEGIFKVDLDKADFVEITDPHRLTPLQVLSLSARSNNKPKEAYIVGIK  
PEYIDWPGISDAAIKRLEKVLQKFKKFISTYGIDVDIDKVIQCVKSKSKEPW

>PROKKA\_00246 Hydrogenase expression/formation protein HypE

MSYNKIQISHGNGGKETQQLLQRLFFSRLPLELKRVKGGVGIDYPDDGAVLQNN  
LVVATDSHTVNPYFFPGGDIGYLAVSGTLNDVIMMGAKPIAMLDIVVSEGFID  
DLEKITGSMIELLKKNYNIPLVGGDFKVIETAMKNSIIINTVGLGVVETGSPIVDAI  
KPGDKIIVTGPVGVHGA VIAAMQYNISTNLKSDVRPLFELLEVF SKFKGYVHAAR  
DPTRGGLAATLNEWAQLTGNVIVIEEAKVPVLEDVKAITEVTGLDPLNLASEGV  
GVLAVDSNVEDDVLETLSLGFEPASIGYVVEPKSEKGIVVAKTAVGGAKILEMP  
TGDIVPRIC

>PROKKA\_00248 Hydrogenase expression/formation protein HypD

MELPKQIDALFRENGELAKSITEQIHKLAPIVSKEIGREKIKIMNFCGSHEWVTTH  
YGLRALMPETVELIPGPGCPVCVTPSSDIENVIKLAMEGYTVYTFGDVFKLP TVK  
HYKKDEVGSLAAARALGADVRIVYSFVDAIEDAKKSGKPSVFFGIGFETTTPSYA  
VLFEKEKVPKNLLFYSSLKLTAPAAEFVRLHKSGLVPVSGVIGPGHVSSIIGAI  
SWDFFPREYKIPAVVTGFEPIDVLGVLKILQDLHSGDITYTNLEYKRVVKYQGN  
VFAQVEINKVFKPVD AIWRGIGTIPKSGLDLREEYSMYNAREQLGIKEKPWDYDL  
PPGCRCNEVTLGIA YPTDCPMFMKACTPARPWGPCMVSMEGACAVWARFGSTE  
RITKIVSEVK

>PROKKA\_00251 Uptake hydrogenase large subunit

MSNPLTMKIDPITRVAGHLGLTATVDPNTGQVINNQAYTYVTMFRGFVFLRGR  
QPPDAVHITSRSCGVCGAAHANASVRANDMALGAVPYPLGQALRNLAYAMTD  
MIYDHSIILNVLEGPDFS AEVMQQYYPSCWQAAQQTQAEYS DIHGFSTIADIMTA  
LNPFTGWLWQKSVKYQIIAREAGVLIYGRHSHPPMLIPGGIGTDLSVGESLFTQY  
MYRLTKLTAWTKVVIAAWMDLANFLINNC DYETQGLTYAKPTYISSYGYESPVL  
YSNLGDNYQDIYKNYDSLANTADEGPQTVFRATIVRNGELLSKSFIDLNVAQLEF  
VNSSFYHDWAKITSPFTETDPVGNTLAWGLQSDGVNLYMYHPWNKTTIPNPM  
APNFM DKYSWDAEPRLVWKDGTMWAYETGPWARLHAVAHYHPNSPIVKNGQI  
TIELPTISELPSDLPSGAGPAWTVEWLPPDESTTLSRILGRAVDMAAAIFTAWDNL  
EYALEVYMKNQSSPQTSRPWKQPSFSLGVGQYEVP RGTVRHWMVVKDYTIANY  
QYHAPTTANVSPRDNNCNGPWCINGQAIGAFEMS VINTKIMEEVPPDQWVG YDF  
LRAIRSFDPCLVCAAHFEIKGKNKKLEHIITPVCNS

>PROKKA\_00252 succinate dehydrogenase/fumarate reductase iron-sulfur subunit

MAVTEQEAINLEALKRAIDDVFY SQIDSTILYYLQSCVNCKACEAACPFPTPSLK  
YSPVNKA EIARQLYRPRFTVWGKTIGRIIGGSKKYL SLEEAETMADYVWHCTNC  
GACMFVCPMAIDSGALIDLLRQVTFKAGMTPKIYTDIEQLEVSGKYWDIPVFRDA  
WNNLISKMKEAVGKDIPLDKKNSEVLFYASIYEAMVYPDVLIKAAKILDMLGKD  
WTFMSKPLGIRPPVGLVIGDKEGALDVMKR VYAYFTEMSPKYVMLTAGGFEYP  
ALRYTMHEVLKVRPKYEVVHVTELLAKWYEEKEFEIEPIDEVITWHDPCQLGRR  
GGVFEAPRVL MKALSKNFKELPNHGVNSFCCSRGGGGCGLSSMVENMAKAIGIP

ISEKDKKFLDSTLEPLIKAGKIKVDEINKVKASEVLTGCPVCIESIGFSIDYYHANA  
KVNHIIELLADRIKVIKK

>PROKKA\_00253 hypothetical protein

MQIPYATYPVGNSLYPLALDLFVPTVFFFFLGASYRVFRYLVITYQKPFIPYPSSEL  
RQVGSGHKIAALVNTFANSSRVGIKRPVTTGVGLLFHIALVLIIFLLAQHMIFWA  
YYIPPYSALFPLAIPESSEDGLLALTTPNHASTTLFVNDIWGPLTVILNGQFLTFFLI  
IFLGVYLGHKLLLAVEGLSLRAGDWWFFVLLYIDILLGFFATAHIPNNVYWYDN  
LLGAHILVAEIIIATLPFTRGFHMFIFYLGKVREWFYFMVYRRGEK

>PROKKA\_00254 Periplasmic [NiFe] hydrogenase small subunit precursor

MSFKISRDFLKLALTATMLTAPEWDKAVAKAVDMIKNGDVNIVWFEAQACEG  
NTTAIIQATDPSVAEVLFGASPLVGP GSVKISFWPSIMPQQGEQAVQILEDIIAGNY  
NPYVLVIEGSFPDENTAQQYNPNPNPGYFGMLGPKTLNQWVAELLPNNAVAVLTV  
GNCASYGGLIADKVYQPPPKFITPSWSPSTGAIGFFDDPLRGYKGLLSRFYEDNY  
LNAQAGAAPFYTFVKNPNAYLDITSPSPASVKPAIAVPGCPANGNGIMRTLNLV  
LWAGGLAPLPELDQYWRPLYFFKYTVHEQCPRAAWYAAGDFRTQPGQPTAACL  
FEVGCKGPVSNCPWNKYGWVGGIGGPTRTGAVCIGCTMPGFTDLYEPFYKPLQ  
VPVPSSTLTAGLIAAGGIAIGAAASIAEKKMVQKGLRGS

>PROKKA\_00263 hypothetical protein

MDWKVFLIKNSINLSEIESKKIVPLNIKAIMKTNGIGTKGFTFAGTSFPLFHYYLP  
KRTLYPNKKRSAPKIAFRIYFLLYIIPYIYIVKYVPFFSRG

>PROKKA\_00272 Polysulfide reductase chain A precursor

MESEKSKNGLRISRDFLKVSAITAAAAAGYAASKNFVFDIFSPVDEEQLQPG  
YQYSYAPNMCGICSSVCDILVNVEQNGNYIRAREIDGNPLSTLNYGKVCARGRS  
GTLITYNKDRLKKPLIRTGPKGTWAFREAEWDEAIQYILQKFQELNVQPWEIVLS  
GGAIPCGNYKPEFIPFTFAAQIPSMAASPMQACLFGEHLGINLTIGTFDIHGDELAD  
DFEHSSLIVVWGNNGNPAGVFNKGSFRFGKGLANGAFVIVLDPRQSEAASKADL  
WIPVKPGSDLAIAMGLIKYLIDNNYDDYFTRYHTNAPFLVYQENG VYVPYTAN  
YEDGTVQAFYVYDEISQIVQVPPYTNTNMYSVNGQTIKPALRVQNLTTPDGKQ  
LITVFQALEQAVSQFTIDYVAQIADV DKS LIEEFYFRVGTMRPIDIASGQKGQEGS  
YTTMWRKAIGIIMALTGNIDVRGGWIYSGDHREGAIQLFQTYTSMVNSGQVKPG  
ILIQRQPVLMSVPVLNLP GQMMQYVATALAYCNPSFWQHGHPAVWCAYNSTLT  
SQGLKPAAAFSLFPDTGVYEATQGQVKWNGQPYNVKVVL TCCVNPAKNMQES  
SWKQILQNTFVIMVDIFPTDTALYADVILPDATYIEREEPITGRGATPD SGYRRRW  
QAIPRVYPYTIPELDFVLLSYKLGFFEQYVTWMANALGLPADQLVQAMQNEM  
MPFLQYLEKYGTYP RWGEFVGKAFADVQSQIIAQQLNTTPDDVLNKLRTGCVIT  
VNTFEDYVQNNERIPWNIPAGLPTGRIEISTILYYYVIQNFGYDPTWDPILAYVPP  
NWNAGYAVTPGQFVQPQPPYNDPTFKPTPEMFYISFKVPPIAYTFTTNVSTLEAI  
TSNSYHTNIYQFAWINKETAQSLGINEGDWIAIISKLTGAKLIVRAHLTEWIRPDTI  
GVPEPWGQSNPALTYSTRSLKNFGNRAITHLWPQSYDPLTGHRMIQQFTVIVRK  
ATPDEISEYTQLAQVQTQETIPSQENIPGNYTVPENYTISGNYTT

>PROKKA\_00274 Polysulphide reductase, NrfD

MNIKLWTWTIVFILIGGGIMFYGMSYNPLGWAQGFVSFTEPNDEPIPWGLLVIGYI  
FFGVIGTG VSTYNSLYELFNKNHKERNPFDKIKLRNEWLALAVLIPGWIMVFAST  
YKP AEALFIYLSFRSTSRIAWNGVLYALVGIGIIAEILALIGEKIKEEGKRGILDRFL

AWLSSKTTFEIPGMLKLVVDIDALIVGYAILAELILDANLGSVFGYLSTWVYEFGP  
FMSILLVVASFYSGIAMISFITPLYSWLRRSTTVTPPTVQPVATHISGSGSVDPEED  
NTIFKVLARDGLLATIGIGFLILWWVWLTATNQETLPWAELLINGSWSLIFWIGLV  
LIGIIPVLYSIA YKKESKGWLFASSIFVLLGWFLITLADVIPAISWYYSVSPVSP  
PGSTWAYELRSNFNGVAQYTQLPFAVSSYDLVWFAGSVLFLLG VYTLGVLLLPL  
EEEEKPKHVWIFK

>PROKKA\_00284 cobalt-precorrin-6Y C(5)-methyltransferase

MRKIYAVGISPSPGLISCKA VELIGKAPVVLIYDTPFPELEEIIKNKRVIKLPKPGFK  
DPNVIEENIRTLKSIDADWGVFLEIGDPSIRNPLFYHILGGSKD FEIEVIPGISSVTAV  
FSRLGIHVRHFCMLGSEEDLLNNLIDKCDLFVIVNIHKEHAEIFKKLKEHG YDVT  
FIENCCNEKEKITHEYDGETYWIIAIAKKDLPERML

>PROKKA\_00287 hypothetical protein

MRKDILIAVIISLALAIILSLPDILEPVARSVLTYLG VYWAFVFIIGVIHGLKPDE  
HTWPITVSYGLMQSSIRRALLSTAVFAGALTIVWASLSALTSEVL PFFSLHDFDPY  
VDMVVGVTMISVALFILSVGKKGKVESADYRVIWVHGLAAAFSGDFIIVLVLTTL  
LIPVIPSNLGFMIGLLFGIGSFISQSVVVVFVYKGV LKVS KDFTIMEKAGKLALLM  
LGVFMIFLG VYCLLSL

>PROKKA\_00293 Periplasmic binding protein

MKRRDFIIAITTLALASIAGGVIFYDNEGNKYSGEVKIIDSMGR TVYVPKEINKVV  
ALPGTTLGMVIYAGGLDKISGIEQIELRNMWGQDCWLA YHDKFKGLPIVGQGGP  
NATPDPSAILTAKPQVIHEDQLYAQVMDPNQLQEETKIPVIVVYTFSPKRIGELGPN  
TFKSSMSLLGELLGTTDRTNELNEYVNSLVNDLNSRTSNISYRPSVYVGGLPYKA  
GSEGFLGTDVDFEVLNLINTKSVVDNLGYSPGFYNIDFSYLLQTQPEFVFIDEGNL  
ETVISEFSQNK

>PROKKA\_00294 putative siderophore transport system permease protein YfhA

MLKTEIILKHRKKLIFILVFLLIITFFAYLNLGYYHTTLSQVLDYLTNKIPYDSPLSA  
VLSRLRLRALVAIFAGALMGVGGAVLRGTMRNPLASPFTLGIPQASALGVALVLI  
ISSNLSFFLLQSPFILPLFAFLMALLQVFFIILLSRALGMSSGALILSA

>PROKKA\_00304 Transposase DDE domain protein

MRNYVEKQGMEVIEKLLERARKISLEV LKGVKEVDLSIDWTTKTWY GKPVKGL  
GSSEKGSSWNYATATTKYKGVLLLA FIPQVNDMTKDEIVKALVEQV VAMGFK  
VRLITLDAGFYTIDVLNFISQFKYIIA VPVGDKVYEEFDGDYTTNSKRHKKDEQ  
VKFRLLVYSKKKVRKRKTLVYFARATNLDLPKGEVLKLYNKVRGP IETS YRNI  
KAFLPFTSSTKFVFRTLIFVLAVAFYSLYTVFKGEV GREQFRVLLILLFSDDL FYLK  
DFLFKSIEPLINNIDLFSRR

>PROKKA\_00307 Hydrogenase-4 component B

MNVLIISLIIVPIIANAFFIKGIKYSTIASGIAEVLLS LLLLNKIPIVCYFYVTSFTWYFI  
IMVSSIYLLSALFSINY LKGERTKISERTYFLLLNFFASSMFFALVINNLGLMWVGI  
EATTISTILLVITEGTEIAMEVGWRYTHIVSAGVTFAFISIILVYYCTHSLVVS YLVM  
NHYSSTLRIASAIALVGFGTKVGIFPVNTWLPDAHSEAPAPISAMFSGVLLPVAL  
YVLYQFYKICSIFTLYSWIAVISIVIASISMASQVFFKRLFAYSTIENMNLALLGLAS  
GQILGAVILLISHAFGKAGAFYSSGLLIKTYESKRIEYGLWKNTFIPYSLLSSLA  
VTGTTPPGTFIGEFLLSTLIEKCAIEFIIVLIALSTAFISVNYHVS KM TFKGERDSL N  
EDKSLGIISLISAIISLGIGIFIIAWWSL

>PROKKA\_00308 Hydrogenase-4 component G

MRRKIGKLGKFCLYNDGYVECEEEIEKRTPAYGSFDFVYGPSAGGLMESVRL  
DIITFGEYIRNVIVNPYYKSRTIKIKGKGINDALLYIERINAPFTASHTIAFLMAIEK  
ALGIEVPYELLLDRIAEIELERIRNNLLVIERLTESAGFQVPMNSLLWLVEKVNRII  
GKYFGHRYFFGVNYYGGINVEEGKIKDLEIIEKEFNDLFNSLIESRIFIDRLQGNGII  
KDEESIGPVARAANLEYDARNEEIGLPYKDLEFKISTYDSADAFGRFIVRGKEIFE  
SLNMLSRINLKKIEYKGLAEGKAIGRVESPSGDLAYYVEVSNEGKISEIYLLSPS  
SVNIKLFSSKSMVKNIFTDLPFNWESFGIWISEIGVKFE

>PROKKA\_00310 Hydrogenase-4 component B

MLQLLILFLLLVSIIVISLFNRKIGYSLLGIASALIIYQGLHCIFFLIAGIVWLLSSAFSI  
FYDNYGKWLSPLFITSILGMYIVLISNNYLEFLAGWEIMSIPAYAIIGLNKKIDYPA  
FTFMAFGELSTVLILSAFIYSYSITDNIYFTELSSPLPFIISTFGFIVKMGIFPFLVME  
WLPIAHGSAPANSSAILSATMTLMGVYGIKIASLTQRTIVTQDFGFILLGMGSFSV  
FFGALYAYVSEHVKGLLAFSTIENNGSILTIGIGVYLISQGVLSQFALDVTLVFALA  
HSIAKTGLFFISGSVEGESLTYIKIAKSLRGKIGGILLSSMSGLLPNIGGVAAWGL  
LESFLMLAYVEHSVLSIPIISGSIVAMGEGLATGALIKFISYTQIFKLGKTEGSVKV  
SIVLLVGIIVLLGVSYSIFFRGFTAGVPCLGMLEGLLISSYGEPPGGISPLYVLLLL  
FIISLVTFGIFGKPKIRISETWNNGEKIEDQFSSFALANNIRMMLSKLLRTKILENEV  
VFSADIFWEAMYSIAKAYKKFSRILSTSVMNSSLSSYYIFYMILAFIITLLAMII

>PROKKA\_00311 Formate hydrogenlyase subunit 4

MNEIIETHIQVLAVILLSPLYAGILDKLKANVSTRRGQSIFQPYDIFKLLKKESV  
SINASAVFIYSPYVVFYSIYVLISFVIPVYVYPQPIIFTPTVDFLGGALLFSLAAFLKIISA  
MDSGSNFVALGTSRVISFNFLGEATLITVFFAVALITGTNNPYVELKFAENPVYYL  
ALDHVFASVAFFMLWLFETGKLPVLESSGLAEMGMIDDALTYEYSGKLLALLKW  
GSYMKQYLLGVSLLNVFILPWGLQTGILGAIEDLGIMFLKWFLIFIAVVIDTSLA  
KLRLYKVQDFLAVAFVISILSLIFSVIEYD

>PROKKA\_00315 hypothetical protein

MLRVISISVFFVAVILSLFAPLLASSNYTINYSGRYEVHYLVTTYFRAYGNNTTIKVS  
GHNVSSENFIVCNKSLATVASSINPNITSLFNLHIYNSYYYVLSENNSRICIKFYF  
TNSSTMLIPYYLPINISSGNYSIVILNENITSGKVVKYNGTSIVYCKGDILNITFTAL  
CLNPFHYYYIHGYYAYNKSNNILQSYENIFSGKVNNTRGKAPLTFVEILQLQRTY  
EAPVSHTSNIFTYIEIGGIVIIILVLSVLIKKKKFK

>PROKKA\_00319 Dipeptide transport system permease protein DppB

MGLASYLAKRAIERLILLIFTAFVWMLVLGIPQLVGINPAYHFINPSEFLHSKNPE  
LAEKLAIESLDRDLGLNYPLEVQFFIYFIHMLTLNLGVCNVNHQSVAVCLLCALPF  
TVILTIPPVLFQTLASIYLGSIKRAIKRNTKTDVAIMSYFIVDYNLPFVVSFLWFLF  
AVVLKIYPISVYNEVHDWTNIVTDLKVFWLPWIILTFIYGFSTRGILMRNAMVEN  
LDSDFIKYERLAGLREKIVRAHARRVSIIPVIVRTAIDLAFASGDFFIEVYFGIPGL  
GYKLYYAALGDEIEIVLGSTFVLTLYAIIILYFVDLVEFIIDPRARRSVK

>PROKKA\_00320 Bacterial extracellular solute-binding proteins, family 5 Middle

MKSKSMYYKILLGIIVLLGGMIVSEAAGVLVAPSALKQHVPVPAIEYCKFGPYLG  
SIVFLWNYTDCKQIYACFKCFGNIQLINAVPASQFLSSCIETLEKLATVSVYDEVT  
YSFIFIQVNFKYYPENNVYFRWAIASLINPACVESQILCNGLGKGVPSGLYICPAVFP  
CLASNTTLISELYSIYNAHESYCPKRACQYLKDAGLVYNPSLGEWTPNGTPVVI

PVYIQEDFYNCMAWINMLESNACKIHFRKNLDIIEYQPYCLETDLVSLKYGLINF  
 GYLSYTAIILDDYYSLGPLAPVEEFNCYVNSTVNAQLSAAAYTKDPTFSQALVNLA  
 NALVDLQWDEPWILGWTTCTIPAIINNYGYVASPDEGYTFTYNDIHEGNTITGR  
 LVEAIGTCKFKAPNMDILYCNFYSSSEIWDQAVPEPLTEPPSEPTPLGLQPFVANY  
 KIIPIHNVTINGHKKIINGTEIHYCFVHNATWINGMPLTALDYNFSIWYFDMPGYCPN  
 YNPLGLNLNHVYIGNYFGIPVYVNYSEIPSYAMKIVYGSMPSLVYSCVPSNNTYE  
 IKLYFNSTAACLLSSSNEYIVPPCIYLNKPCQYNPDYPSFVCLAYKGELPGGFPW  
 YIYEWNYSIGAIVKPFVGNFLWNPKACTYNVTAGSVASITTNITQIIASPYIVVPLN  
 GTVIKTAPKPCCPITNATGYAEVWAYGSTTPIEKLTLTHVAGDEYKVEVPTSSLTP  
 NEFYVVFINATYYAPITVMGHTMMMPHYAYRWYAVYPYSPTATTHVSNVSKLI  
 SCASHLTSYTPPTSSVTVNSTLLTYTPPSVPSVSDAVLAMIGITVVLALIGVAILIR  
 FK

>PROKKA\_00321 hypothetical protein

MRSNQVGIIIFIILIVVAAIGIYLNSEISALSSSYKSLASKYNALKSEFYTMNSSYT  
 NLKANYTELSNNYNTLKYYFTTLLGYYESLNESLNLEDGYATAYQVLEYLASSN  
 PKEIANMFCPKVTGFISVGKINGSFSGVVNVSKMFSQVFAYPIVRAFLCCGVVYN  
 SSSHCLILSALVKYCNVNSTGGTTFVYVLYHMTLSNPSMFTWKISSINVYNYFNEI  
 QYQMALDGLTYIHAICSKDTPVISELGIGQFPSYVFFCSNLPLAGNYTVPQLNSLL  
 KNVTPFNIRIDYYNFTAVGNSLSGVIYAYVNMEYNGHTFCGELKITEHAEIQSNG  
 LPEIYQISFCKM

>PROKKA\_00327 hypothetical protein

MATEFIEVVANGLSTLSDPLGILMEIIGVVWTYVLVCIVILIFSFLDICLRNLIIFENK  
 ILCCIYPQYLHIIS

>PROKKA\_00405 hypothetical protein

MIECNFDDTDILKNILHDNSTADEIMNFLDTPLTRYKDYFECRKNNENILRLIKIASD  
 IQQSLNLRFNIAHILLCFIADKTREKKISSPTPREDELERPLTEDEIENIKDNLMEV  
 CNFLKAALTLEFIISCSNGLTYSTVYKE

>PROKKA\_00421 hypothetical protein

MDNATVDCASAEMEHFKEPSELETYESGELIIFSNNKFLCYAGVIITEA

>PROKKA\_00424 Prolyl oligopeptidase family protein

MENDTRTPLVPVLRVYVQELQKGGKTFEFHVIPNLGHAIYKIDDAIDLLLPAIFLK  
 KLFG

>PROKKA\_00443 Heme-binding protein A precursor

MFTAVFQELVEFNGSNYHEVVPVIAENYTTPNYQNYFYLRPCVHFADGVQVN  
 ASTVWFSLYRTILMGQGPVANYIGLLFNSTVYAVSDYAIWGVVYAIENATGL  
 HTVGNYNLTAHVLAAYILSHFNANNATIQKIMSYPNQAVVVKGPYEVEINTLEPY  
 KFFLYDIASWWGAIVNPVCVDMHGGVVPNSPNSCLDEHGMNGTGPPYVIVKVCP  
 GFSTIELEANPNYWAVGKSVSAVAEPAHIKYIIINYGLSHNDRVEDFVKNQAQISY  
 ISIPYLSQILGTSPYNEIPMNASFRNFGAEPGVLYISLNTQKFPTNITDFRLAIEHAIN  
 YSTLLSIFSNGKPLAAEFVGPISPQFPGYNPGNLSVYCYNPSLAMHYLNEAGY  
 EGHFYVVLPNGSTIGDTSQTQLPSLSLYALAPVNELEQDELEIITHDLSQIGISTTV  
 YYVLPSTVDGWTTVNGTPVMVNLGWVPDWPDPVFQQLMPLTGVPYGGISGNL  
 AWVDNPELQSMYNTLPFITNTTLQEEMVAKAYSIIYNCAPYVWLPVPDITYFFVQ  
 PYVHGFVYNPNYVGYFYNNMYYNGTYTYTT

>PROKKA\_00633 hypothetical protein

MSESSSIILQSAYGSTSISDFASTIGGIIGAASAIMQSVENAECEANVDALKYCDSY  
KQMATSNTYYSILPAMIVPARMSALPLTLTIMDFVRHGILVKTSYKD VYVYIGGI  
SKDWRGKVSRLTVYQGGASFTFSGDLDTLKS LTVENFPIAMIPLHGSTKSKFVNP  
PCPKCRLTKKQADINAMTKVITEQVGGSFSDFLMLAKGLKSASPVTVAIIPNYV  
PYILKYIYEDMLGYNQIPIFKSSDGRYYIRGAIHNELKAISSRFPVFNFAVSFGLGS  
LSQYEGLYGVTIFGNGLYSSLC SVTNPILNNVTGNLRLALMGETPLIFGTPIFDDG  
VCTTYCTGNYNLEGFLSPSSPASAVGSVAGTILQLSLVTPPPNSYSIQDLEAYAQ  
WLGIVENFRKLLSVVIKVS AVVSALVSVYNMEE EVAEDIINSLDWLGKLFENDV  
DTIARKIYDEVNSGNHDESSHTDDEGRELHNAYMCARFGICG

>PROKKA\_00634 hypothetical protein

MESYVDKDGYLHIEKFKN SKVHIWDFGDFIIVSPHDNLNEIRGSENTDQLELLV  
NLA FVYMDKDQINDICEINKLHTGEHMAGYCDRECIPPEWFRFLQYAEVRIKTH  
NPRQIYEILHGLVSLLLLKVY GKCEGHEVSCEKAIEILKSALTSEDKNFRAEVWEN  
LELERLEDVDIKKFLGQEINAYYELLYDPEIRERVWKEEVREKSY YLDNLILNKL  
FGQKICTSLNSLHILADKILKDKLPELLTSNDIFIRINSWIVFGEESPPSIDKKYFLEL  
LSTEDLHYRLLLWEESISLLGKGLLTINDLAERKKYLIDL VKNIKGNEKYKSENLA  
NLLKKMGIINEAELT

>PROKKA\_00781 hypothetical protein

MLTEENLTQICVIPILGRSGLSWMNPTYYKIANKV TYEESVNQMILPSAMPQLLT  
YQPALNYLAVAREKDFYKSDTSTYISLSSPDDLLEISGRFPYPYPAYYSSGVNY  
YGDYPSQLFLGYLRVVISNFP RYSPFYTRNGLLYPKVYNHNVSSDFMLNNMSIG  
APVYLSKYNPNGKLCTDYGV LGLVSAITDFYADIGGNIVTDRSLLSQLPPDDFV  
AGYYILTLVKVPEPSNINQYIKWLKQENAVNIVLEGIRNAKNAISSLISSGVLAGIA  
FSAVMAVDEWESEEQIIRDAKTYVDKLNKIYNEALNYATSCIENSPNLPRNAKIIY  
IQQVQQYLSNLMYEINAEQEEENILSWLENEIYNYLSNQGFTPACDPHEEE

>PROKKA\_00926 hypothetical protein

MKSRDILLLLLIINSIIVYCFTKNILYSTIDIFLAIGIYFANYIISRKFADQ

>PROKKA\_00927 hypothetical protein

MGGRQKTTLFMTKEIKKEKRGTATGQQTSSK

>PROKKA\_00929 hypothetical protein

MKTLIVFLNKIDINKILYLQDKKDIYILNEILHIPISFYNWENNCYEEKILDYVSK  
KLDNLSFENIFLLTTLKLCNKITQKQGKIEIINVDDENMMRKLIAST

>PROKKA\_01048 Sporulation initiation inhibitor protein Soj

MNGKVIAIHNFKGGVGKTTLTAVLGMGLAVNYRVLLIDFDPQMSLTQIFVKEER  
RKEILQQSIRPESDRSAYALIREISDVIVENFIHVSKTSKGEVKVSLDIIPGSYVSTF  
HAMFKGYFPMFDEFVKKELNFKREKYDFILIDTSPSDVVTIKPVLRASDYLIPE  
DGTIEAFNAMLIFLKEALPKYIWSSFDNPRVLG SVLTKVRRNSVKLLFEHNKLLL  
NEISENTEAMKHIYDPPYFGANDDHPEDFILSSNKEYLSDLIWRNESIPPIGE VFDK  
LFLVSEKLQPDLYSYFYKV FYRLPSEVVRRVREW

>PROKKA\_01050 phenylalanyl-tRNA synthetase subunit alpha

MGKISTDKYIFLT PRAYIVIYLLKVGKAKASEIAESTQIPYQTVIQNIRWLLAEGYII  
KEQKDEEIYVLT DKGRHLATTELEKIRKLIEVVQ

>PROKKA\_01051 MarR family protein



MKENYRVLSETEQYILLYLISIKEGVAVKEIYEEMKLSPNAVTQAVNKLLNEGLL  
 TEKREEVFPRRLIYLSEKGRKIAQLLLQMQEIIKVNQ  
 >PROKKA\_01053 hypothetical protein  
 MSTNIQPFDTISTPLGNCYVYTIHSSFINRLGSFDCGEKEQNEFIRRLAVGHYLAGI  
 NHTFLLCNDLAGFVSFSSYSYAFAEGFIGKIREVMKEEFNRRGLPHEFNISFKK  
 LPVILLGQLGIDKRYQGKGVGSVFNFIIPYSLGYCINNSCIGILVYTKTAKEFYE  
 KLGFQLIYEDKSGGSNYFYSLYKNVLEVERYKAFLA  
 >PROKKA\_01055 hypothetical protein  
 MKGLIAGLIIVVILAIVYYLYTEGYFYFYSVDINGVYVTIDSNFLGIKNSLHVSYSNT  
 TISAHGDQDITLKIYLYNNNPLLSVKVTNVSVSHPTLISVTPVPSEISPHNNETLCI  
 VIKTPMCNYAGAVDIIYATEG  
 >PROKKA\_01112 Anaerobic glycerol-3-phosphate dehydrogenase subunit C  
 MIEEYKYIINKEIKVYDVMEYLLKLSREGKIKLPRKVNMTIFYHTPCHLKYLKIGL  
 PGVTIMRSLGAKVEIADKGCSDGIDGGWGLRNYDKAKIIGKKMMEAFSQSNAEVF  
 ATECPLAGLQIFKASGKRPLHPIQVLKEALSSG  
 >PROKKA\_01225 hypothetical protein  
 MDKIKVFIDSKNPGGFSWIVPLPDKTLVGSLSYSNPKLFLPVVDKRLIEIHGGSIPR  
 VKPIKPQSSIIIVFGDRTGLIKTFTGGGIFGIAELISSNNYTKTFSELSKEIRKQYYLTT  
 FAEKSWRIWLSLARIYKDKTIKAEKEFDHSMMLFSH  
 >PROKKA\_01255 hypothetical protein  
 MFIELYNPLIDVSRSAFISDLASIDKVILQLKMAKR  
 >PROKKA\_01297 hypothetical protein  
 MNKKERRVAVLVKGKEITAICVFRGQFLEHLFLGKSREEVLSQFNNSSVSKEITST  
 SPSNDLEEICRFIVEKISQKINKVNS  
 >PROKKA\_01486 hypothetical protein  
 MIKYFLNGNQVTLLLEDNSSLFIMDLQIKLDSNVTLTASKIQFVDVNIPFKYGNIL  
 KKGICKINDKLFICYAIAELKGSISEYDENKVKEVYKEVVEVLNNVI  
 >PROKKA\_01499 hypothetical protein  
 MFIVPFIFLIYGILSPIYFAILKGKLSNEKAFLFTWTLSPFLISYVYNCIFIFYILVIS  
 NFIFLYVVLNDKLRKYLWNGVFLFLVLAFLIEFIYKIF  
 >PROKKA\_01651 hypothetical protein  
 MARYLHYISEGEIRDKWYAKIVEDLTRMGIDNVSIPELIVRGNEKADLVITYICKN  
 DYCYPFLFEFKNELNKSHERICYQARNYASIINPYYTICVDLTKSLYYLIIYHGSN  
 AILNMMFQSYDELFNYFFSIFLPRSLSVPLNFTIPKNIVINKLFASPTYERFLRAEFV  
 RILDKNNFCAISECSSINSQGSNIVKPDLAIDSPCNNEKLIDCEYPFLVLEFKSQY  
 DITAVSQVRKYKDSLFPYYYGEVYGVGSVVKIEINNSKNQQIFNGSIRIGNPYLND  
 LNILGVISRLPKVSAHQGPLNGRYTIDELVKMGAKISRRNPAFNVRIAIQGFSFLFL  
 DRGGVKYPNTDYTHIDCPAKLTRLVFLILAVLYNGANFKTED  
 >PROKKA\_01653 hypothetical protein  
 MVSITTVLLPTITSLIIFIITTLFTLFYREILYSILNKISELSKIPDTYLTLYNIDLQRNE  
 LANLLFTLRGIRDSYLAHINEYELSSKHIIIPSDIKNYALEMDNKCRKHILKVLES  
 AIKKIFNPFQEEVRSRSVLLQKINYLIASLEIDKSFINFPSYTSEKYKQTFIVNSMAF  
 SILSAALISLGIIVQLFINNLIYFLILYLLIYLYTSFMITMYYYARSYNVDIEESSTNK

LYRLLKLLYCFPLISFLLAFVTLLVFYIVFFIIMIHVNIHNILLYLWAWITIFSIRSFK  
EIKQNIDNMRERLKRFEIYKEYFRKTS

>PROKKA\_01661 hypothetical protein

MIPKYPEHLTQESRLEVVKSYILHELASLTRDKTPDIINIDELVKKCREHKKGIEED  
CKEIYDLFYGLSLMFKRKFAKEITAGSCTWKEDSVKLKDLIISTEIEGFPGYKYK  
DIEEIVKNEEIKRKICEEGENYGKRRGKLAEDPIIVVKEGNKYRILDGNGRAFYKL  
SKEKCDINKEINAYIGECNGVHNICIPFGIPHFIEGYLGLLIQRLD

>PROKKA\_01662 hypothetical protein

MKFTEKAKIEIIFGDGHFDRQIIKHAIEKNKDKYCQILLFIPESGRKGKGRRTINTGID  
GVLKFIKEDVTRFSLGYIIFIDKEHCSSDCENDVNKLANKYGISISATQKIAEDLDI  
YKLTCSIGNKKFFMYFIFLGFYCCIEDFILIKICKSNTSKFTGEGCCKKYKEIFKSV  
QKEMKESCIEKTYDELFAIARVLEEFTTIKM

>PROKKA\_01665 hypothetical protein

MVTPGLPHQNNIQQIGYKLLSMLNFQGEKVDEVAKTLSACLWNDSVENKSGAY  
DVSPQTVRNYVEKQGMEVIEKLLERARKISLEV LKGVKEVDLSIDWTTKTWYGK  
PVKGLGSSEKGSWNYATTKYKGKVLLAFIPQVNGMTKEEIVKILVEQVAMG  
FKVRLITLDAGFYTIDVLNFIQFKYIIA VPVG DVKVFEEDGDYTTNSKRHRDE  
QVKFRLLVYSKEKVRRKKKSLVYFARATNLDLPKREVL DLYNKVVRGP IETSYRN  
IKAFLPFTSSTKFVFRTLIFVLAIMLYSLYTIFKGEVGREEFRLLLILFPDLFNPENF  
TFNVIKTLIYTIDFLRR

>PROKKA\_01673 hypothetical protein

MIIGKNEDVTTSNLYIEREKKILKILENKNNAKKVIDIYRGCAIKYSDFISIYPYIKKY  
NVIVYFLYLLSKVKGQYKNANPNNYVYFYKKVIDTISDPREYLNNDINNINITLS  
RNVYNYLEKYDIINNIIKYESIMP

>PROKKA\_01674 N-glycosyltransferase

MASICVYDTVFNNVNTVEESVKS VWSPDYDIVVDNYSKDGTWEKLQELKKEY  
NLKLLRLKSSRGKGRAYALEQCPENSITAYFDLDTYYNQNFHNTI

>PROKKA\_01711 hypothetical protein

MQVYSVMNDQMCLGNTINSQSYEIIIDNDSGIFIKFSPLTFSVETHEAIVSIYRDKD  
GRIYAIDCNAST

>PROKKA\_01735 hypothetical protein

MNEYVVRIVSKDHRRIGEINIRYTANDDDDESIRIAKSVCEKLGGEIEEVIIGKV  
MGTITGAGSSLSKSDDDKFLGAITGLIVGALVGHIDSAITKVIYSKKYPCIKPRPF  
EDIVNYINIDRLEEIVNER

>PROKKA\_01736 PIN domain protein

MNVDEIINLNCNSQC VIFDTSVIIDYTRILLRRNYERKRNILSRIFNQCSRKAITTLIYE  
EILAGSDEDVQLQQFLSDFMILPFDIDEAIFASRLEKQIRNKGLKPKGENWRIDLFI  
SAFAYTQRCYILTKDEDFTKMLNCEKLDEVEYYVCRS

>PROKKA\_01737 PD-(D/E)XK nuclease superfamily protein

MVVKEIIHKHLSYTSIPEFFDGEYWPSQIWYCLRKQYYSRISPIPM SLESKKFTTLG  
TIIHEFIAETLKKEESVRVESEVPIRIPHPNRHDVVISGRADDIIIIVTVGKSRYVVEV  
KTIDDLEGKRSHLPKKEHIAQLNVYLRAYPN SKGIILYVDRGSFEMEEFEISFNP  
LFNETIKRVEMLHDYLIKRELPPPEAKENQDMKWQCDFCEYRAKCEKQISSNNN  
K

>PROKKA\_01789 hypothetical protein

MMSKRNKGEFPMKGRVSSLVKHEGIIIFDLSLKGFYNWCKNRLKYLGFNPFIT  
PYKYDHQIMIYARLIQGYIITTDKDFLKCERAILKVDKYEKMYVKMLKELHEKL  
S

>PROKKA\_01797 hypothetical protein

MKSLILYISFLSLSSIISKSFPLLISFCRLSIINLTYSKGCLVIAKLTNSLITYSKIIGFP  
NSPLEILFIKRNALSWFISFLLRSEYINEVSRIIIATQLLLVIFGNPYSDKDFLH

>PROKKA\_01810 hypothetical protein

MIHLCGTIEGIKYKPKFLLPVLQTYHDLSEGIKHSSSFIYKRSGNEFAVSWWVSPK  
RTRSPYPYARVYNTLQFSKGKIVTIIPIMKDEGVDGDRDFIQWDTVALMSLLGVYV  
IIGYYVKASKNPKYKNKVTSQEFDEYEYLEKKFDELSNYRSDALHWNMNELSNLK  
QIGEKALSYKRISSETKVTFHDLASAHKRIEKVMSDVEAFKNFSRTLKKAQYR  
ESITRQPKERTYGNKGIIIDIKNYLGGFYHFTVDEVFFNSKKNKVCLIEAKNTKNSA  
LPSEDDIKDGLLKMILYTNLKDLYYISEKQEKIKVNDFTPMLRLTTEKEVNMSNK  
DYTVLKSLLLEEAKENHFEILFNNKKVNNFINNKPELIDFIC

>PROKKA\_01811 DNA adenine methyltransferase YhdJ

MMRVIFGDSRNMKEVEDNSVGLVLTSPPYNAPFDFPDLFPSYADYLSLLNGVG  
KEIFRVLEEGRVAVFVTADVRIHGELYPIVADLIKIMQSLGFKYQERIIWKKPEGYI  
RISRRSGVLIQHPYPLYYPDNVYEDIVVFKKPGKFIPRNKEESKIDVNFQREKW  
YLSVWEITNVLNNKYKYTAPFPEELARRIITLYSYVGDTVLDPFAGTGTTLKV  
ANELKRNAIGYEIDLELKDIIERIGVNTLFGKPQIEIHEREDAKRLRTKLREKIEEK  
LQNK

>PROKKA\_01812 hypothetical protein

MSFNIPNYPLTKVYKFCSYILSCRREPYSELEEQCEGEDGFDEAPYVLEGGGLTTA  
PVDGMGSRTRGMRTD

>PROKKA\_01815 hypothetical protein

MRKSLSLGLGILVFLMELVMPLSLVKASSGAIPICSVGCDNKTCNYQGYAFSF  
CGTNCOPYVKQVSMCLRIPSSLPSLLVKCDNYTNVYGVWVGLSPYKACYNAGQL  
FVQAVVSFIVKKSGDFIQLIVFYIKHGQEPVALYGKCFPLSKFAGTVMYVLMSSY  
YYSNCFVCFKDSNGCKLTYDAFPCYKSALIICEAPSHGNVVICGEQRYLLWYPL  
PDDSFNVGFAVEGSGNVRYACVYTFRYATRAPMLLCELHNGHYVFAWKSNC  
NSYSAKAYTSCICSFICGIIHLGYTECPPTCSIL

>PROKKA\_01818 hypothetical protein

MSLIQKLWFRVLSYVGIFFLSWFLDIGLFIVLYEKIFYPGFNTLLYTDFSASVYALL  
LSFYFLKKKYFFSLIVPTLNFFIGLVFLAFEALVLIGNS

>PROKKA\_01892 hypothetical protein

MPISVQILISNYIQYKMNVDQFISSVGYILNPITTYCYVYVQGIVTLGLMKGLIINIVA  
DAIFLLLFYIAFRSIQIKP

>PROKKA\_01904 hypothetical protein

MKNNVKIKMIGTSIVLLIILMSTMSSFMKIKYVTAASCCGAYKCPTISCKNVKIICS  
LKWFCYPCPKNCPRITVRCRVIIYEACGLSTISGKKYLPFVVVLGSLAKCCWYE  
YDSGCNGLNSKYSTTMTSSIDLIKYPACTCILCEKPTGFWAKCITGAISTTSQIGIT  
YCGISAQASVSITNYVYRLYGEQTSCTSPKYCQQWRWSLGVCGTNPAAHAWEW

WAAATLIITPPLNGNELAPASKLCIKSSVLGNFWNSDWWLEFTVLIPPPCLYQHH  
ETSRTHIC

>PROKKA\_02030 hypothetical protein

MNYIEFAKNLERLRLGVFTTNDAIRIVGKPKNYVNLFLHRLYKKGLIIRISKSRYA  
LAGAGKLELAYALAENGYMSTLSALFYHNLINQDPNSVDIVNTSLET

>PROKKA\_02105 hypothetical protein

MAKKITKKIEDATKDVAKEAEKAGEKVKEGAERVIPKKGKKKQE

>PROKKA\_02163 Oligopeptide transport system permease protein OppB

MKIGILLFSIILLSSGIIISAVSTITKQCELVNYGYGSVANPPQYWREELWSVLNKG  
MPSAYIVLNGTRIVNLGNVSSVQLNSSYFYVYVIKGYVQPYAYLSPIGILLIFLGT  
ATGFRGMILLIQERALGELSKGYAVGGSIYKYVIKRLASFVISMIVSSVVIILEAI  
HGVCITKSIFELITFSMGYSRHYGISVTSLLTSLAFTSLLTGIAFALTVYLSPLFVLM  
KAILGSSVINSLLRRWKYIGNALASWVIAIGLIYLFHVYLVNVLPIGSPKGHLIYYLI  
LPLVSLFFPFIFIGIFANRILLSVSKVPQEFIAKGLSRDVLVFRHILGNLTVVTLSISA  
AFVEMLIAEFLVEAIFAWPGLGYLIRIGVDYDDFKIVEGVLMYSSIVIFSNIADVI  
YGIIDPRVTR

>PROKKA\_02164 Oligopeptide transport system permease protein OppC

MIKVYVILLSLIIGLSLFFTFYELPQGEPLHCPCLSYPLGTyingDNMVMNNAEAI  
NTLIFGVVAGIVETFIADVYGSVSGLMGSKIRIIMVRISDSINTVPRILLLSIALIYG  
VPTGTSLKANFFITAIIVGLTGWANYSRQVSEYLFSSSTLSKIVPKTPFSLLFFSN  
KEEIYNLSKKFIIPAMIDGISTYTAMGVIAGVGDPRFPTLTLLTVASRYLPYWWL  
YLPALFRAIVIVLLYLISDNLK

>PROKKA\_02165 Oligopeptide transport ATP-binding protein OppD

MLEYVNIYGDILNDFSMKVESNLGILGQRDSGKEEIIFYTLLLKKPKKGKILLNGN  
EIKEDNVNDIRWREISAVFYNPYSMFNPIYNIASHFAEIVMSHDIGDYNFAVDIAK  
EFIKILGLDSSVLEKFSYQLTPLEAKKVTLALATFLEPKYVLIDDIEFGINDNGRAV  
VINSIIDLESIVSSKFIVLDNDPAVISRVSEFVVLYKKGKIIERGSNVSEVYHPYTL  
LLRGEISTNYLGKGCPSYSDNCRYSSLKCKEKEPEEIKVGNNYVKCLLYSWLK

>PROKKA\_02302 RNA-binding protein YhbY

MISEKIKKAKAEHPTIRIGKNGLTEGLINEIKRQLKDNEVVKIRIGIKDQDRREIAK  
KVAELTNAKLVEVRGYTFILMKNE

>PROKKA\_02306 hypothetical protein

MAEKKTEINSFSDLIKALFKDDNWYLGGFIILFIIFDLIAYK

>PROKKA\_02346 hypothetical protein

MLDLSDVFKEISGLEIYNKTYVFEGDSYVSLLRKLESIKDIEKVEICSSDYVYLK  
NNIKVNITKISEYFQSMKDTLKYLIKLSKIDVRERITIDSYILIKRLKNLISYFGN  
LCICNSSLIIRGEIIVEVPYKLLKGLKMLLDDNNIPNGYYIVKTDKFDINKLKEIK  
YDYVYVKITRKAIVDYFVGKDYLELREIVIPNTQ

>PROKKA\_02379 hypothetical protein

MEILIPALIGGFITTAFTYLLRKPSFADTLIFYAIYAAYYMGLILYFNMIPLAIV  
TASLIIAVLAWKFLKKINIF

>PROKKA\_02426 hypothetical protein

MVSKLKINLSLSVIMGFISIFILVTLCTFYLNSTIIPCKSEIPIGVGFAVLLITGFFIY  
KFRKGLF

>PROKKA\_02429 Aspartate-proton symporter

MADKVGINIKDNPFRKEAGIFSLIAFSLGNIIGSGILILPAVTASLAGPLSPFVWLI  
GGILLLPVVIVYSKLARIFPFVVGSKIRFINVTHGKVMASVVGWLYLIGTLLTIPIEAE  
ATANYLSYVFPSIMYRGHPHTIIGYFIESIIVIIIYLLVYLGIRGLSISVNTITYAKLGILI  
VYVLAVGYLDFHLSNFSMKFSPSFSNFLYAIALTMFAYGGFRSAMVYAGESKSD  
TGKAIMIAFVLSMIIYTLVPLVFIASTSVLSSGWSSLSKMSAPLAESAIIAGVPAL  
GALFILDGVISPSGASLIGAGDLIRYSYALAKAGSFPKVLGKVDVKRGIPILPVILF  
GAFSLLALFMLSTFEESIGYLVATRILAYSTGPVSLFILSEKKSDKVLSFIAFLVTGL  
IFSFGVFPKTLFGTLVILLTILAMIPFSRNYIPALWYLGFSGIVTLITYFTLPTLFFLPI  
IILASTAFFYLATKTAKGDGENDL

>PROKKA\_02435 hypothetical protein

MENAYKILTEGVIMTRKWFLAFLILIMVVATIPLSLYYYHAHSVNNAPLINSILKL  
SGSDLPLILVTYTHNHIRETVTVFQNGEYYCIFVSFTNVGNKAIVLHVTNMFKPNST  
IGIFGVKILGQPNTFYLPSSSLTFGVEILDLENNKSYSPPCILIHTTLPKFFCGFPV  
MISLENFTFYPGETIWCLERLCYAYLICACSHLVTLKLGACSHLVTLKPGKYEVI  
VSSWLTNCTIRIPICVNNIIGFYLINQNGKMYAILPPGVCKIEICNIQINFTRVCNIE  
YEIDNAQQLTSRFPCLTNCGHFIPLNSKIWVNGQEYNVTIYSTSHLPSIC

>PROKKA\_02442 hypothetical protein

MYFVDKIKRMVKENGFMVMIVGRQGVGKTTYAMRTLYHYYKDWDKVLESM  
TFDPIELLRKRKHVDAILIDDAGVHLSKYDWRDKNDFAKFFNLIRDVTNFVIFTTP  
NEGDILKNVRDKLSYLA VIHGHVRPRKGKKIRDGQYQYLGDEYWADGGYATIIY  
KRKLVINSTTNYKPDYRYVEVMHFDMFYKIPDDVRQRYAEMRAKVTQALIDE  
LTGKVTAKKYDELIERIKAKAKLYYDKAHD SYKMKVDGDISLPKDIAESIFSSPK  
NGVNDNPKNASNSEN

>PROKKA\_02443 hypothetical protein

MSVICPICGNPTTIKEVKLDKDDIEKAIKEERTPLDFGMRIYNSADDValseikRVF  
GGFIGKDLFDMIMDNFNEIKRLAYEHQNDVLRKFADDHTYVIHVYYVLEVFRYI  
ILSPAIMDFLQNKVKGENDDVKEAILKLLMLANPLLLYIMSENSKKGK

>PROKKA\_02445 hypothetical protein

MTNLMKADWDLIEKMIDKMLS DHMRTWDTYNYFVINDDTVLVKAYVGDRLTF  
TIKAKLVGETLEVVEVS

>PROKKA\_02446 hypothetical protein

MIVTIPYRFIEDHAWCEKDFGEYLPYADDAQYEFTVDEVEFDYADLKDIVEAYT  
DDIIDILLDDEQLRNALLKKMRSRKIDVSTIIDAKDEKNAER

>PROKKA\_02452 putative MFS-type transporter EfpA

MNELSKSVLFSSLPSSFISLEATMILIVIPLIAKDFSITYIKASTLLSIYIVTEVLLSIPF  
SLLAEKVGVKMVII LGTMIMALSSFLIIFSNSFLLILILRIFQGVGASMVLLTSLSYA  
SLIGSDEERGKMIGLNHTIVSLGYVFGLPLGGIIAEINWKYLFATS SVLSFISLFLILS  
LNEVHAKSGLGINIIYTSLIFDGII LGLYPLFLLLSLVGLVMTLWKIKLGKVFYLT  
SLSGFLHSITRNMIAAFFVFYLYSLHLPALIIGSLVLLYPLSFTFVSFYAGKLHDKY  
KLRVASFGFASMALSSLSFLNVVLGEFLLGASSGIATTSNTAYTMSSVRNKDRI  
VASGIRSVQGVVSNITIGLIIASYFRFTLTPYVETTVLLNLASLLILGIVVFFQANT

>PROKKA\_02454 hypothetical protein

MIDPLVCDAFKRLKDGRLLMMEVDGVKIFFKEEITPGIICDLTLRSTNYNLICKIEY  
DVRKEKVVMMVSCNGFKGDKVKAKIVESMRRAGMLYSSWGGEMF

>PROKKA\_02455 hypothetical protein

MLLKIKLKGKTFFKSQVKGSLSFLKNVLLVRNQNRVLFVDYLDDKVNLLGGYR  
VPFFLEGQLYFYEVIEVP EEYVPYLPICAKAVEEKLLPLYRNKRLSCSEDLTVVIE

>PROKKA\_02456 hypothetical protein

MDIRTMNKVVLISSAVVYSVNIILSVALYSLLSYVGLPVSSVVERSILVSVPLMSA  
LFNAVILTMIVMSLKLA EYYGIAKSVLAIGIYSGYILAFSPPTYLILFMGSIMALNL  
VQILLYYYAKLQKLMFG

>PROKKA\_02463 hypothetical protein

MPKVLFFYFFGSMLMDLVKRRKTYL FKARVKALLWVISKLPEIYRKRKNEIFINEE  
ELIRRLIVKHHSKI

>PROKKA\_02464 Glycosyl transferase family 2

MVTLIIIVNVKGDNL PKLLESKRSSYNDFELIIVDDEQISGPFKLIKIEKDLGP  
AYCRNFASYAKGEYLLFLDNDELLPDTIFKAVEFIKQNQNTIVQLKLVYPNGLI  
DSCGGVLDELGYPIELGRGDKSAENC FEIREILYAKSASLLVSKKIFDELKGFDE  
YFY

>PROKKA\_02474 CRISPR-associated protein (Cas\_Cmr3)

MTFIMIKPLKPV SFRWGGEFSPTLSGPMNKGISEPLPLPSTIAGFLYSLATGGGKRE  
NLEKLSLENESVKVKLWGPILYHRGKYAHSYPSKLIELDNWELKLNEDGKVEE  
VEATNLLGVINKIGIMRFDNKT VIESMLYTQELIKIEGGIIVEVEQDVKEGYT M  
GGESSIVKVEPWKFTPPEEGDCGLVISPIILSPTEE VVSVD DFYDVEIEGCGKLGD K  
IVEGIPVKIGLIGLFNIAYNVRRPIYPAILPGSVLKC NKRG NVGKFTSWGSILPVN  
CRKSN

>PROKKA\_02475 CRISPR-associated protein

MSEEIFKIKTLALLHDP PWKPSITSSIRGVKGGKVLEEIVGEFNLSYYNKSKDVH  
ENEGLTFAKLLGIKDDEIEKYMEIVHKADRFSASLERYAVEQGVGSVVVQEWKY  
NIFNINHRIP LKKEIDKECIENYVRWIVNNTRNLGWREKYNTLYALIEVAWYHFC  
PGYYPLADTRVGTYSIFDHL YATASMVNWF IKGDL SGYLVKIDLP AIQLIISKARK  
AWDFWGGSYLLSWITYATVEPLIQKYGV DIVLSPFMGLNPFFVSRLNLPKKIKDE  
NTYWEFNDLMFEPTQPVM PATVLMALPEYENLKDLYEKAWEKIVNKNLNLGIDK  
RLLSYPITPIRIKIVDLRELSKV KLYDFS KFM YLLKLVNEEEKVVKVFYGITANS  
FANKFTIEKYEKKELYNLCTSCGILPAVIENDRDLEE GEKLCQYCYV KRKLHKYL  
EKEYKMQLIPSTIDIANIYNWRDILEKEEEIQGAEEFAKDLRLPESLRSLDTPTRRV  
MFYYYTTPPKEDRLMLKYLKSGSLKTEEIYYAIVKGDGDFIGKKLWRGIVK GK  
SFEDYVKVVS RVPVKDIEEME KEMKKVFNVRTSNGEIQIPVTPDY LITLSRALMV  
ISLKDAKT VSEDGFLIYAGGDDVAFLAPISELSALKLLKNTRLNYWGADKLEDLN  
LESSEGAIGFLKLGNMIFDAPIAYGRSYGVYITHYKDPFFYSWEIATQLEEMKDKI  
EGKDV TIILRGRGELNFEDSAILKNSELEEVEKLYCEIKGKKELSKSFIKDV LGDEI  
VMRCANNRNSLLNSALKYYYVERNKLKKNVQYTPPQNINVIKAVDYFDDNYKR

>PROKKA\_02477 CRISPR-associated protein (Cas\_Cmr5)

MDYIDFVIEY GKKLEKKREECKNIDAFIRRAEDFPSLVVQEGLVPAMTFYYSKAE  
KVINIENADCEELTNEGKGYSVYLSFLIDVLRNFTNLKCTSQLDCIREVRQGEVV  
VTRKILPILVEMKKVSNIVG

>PROKKA\_02478 hypothetical protein

MIGLSDIISIADKLVQQYLVFDGEFEAFTPWWGGNEFGYTFYPQPHYPEAKEIIGK  
TKWTIRTL CGHVPRELGFSEGEKAAASQVKVIVEHLNEG NMYFTSVPKIKCSRK  
DYIRRLNAMREAGYQLLFKGYRGKNPLVNEQLKYKEYYELYKLLTIPRFYLKA  
MGSSEM QPNQVMQELLRSQPMRPLRIRIKVYSKNKEISKLFVSAMITTLRLLGI  
GSAANRGFGRFKLVKVNYAIEGIGEDIK CERIHDEQFPSFPSEQDIREINPVCASIG  
GRKYTADTVEGVLSAIGIATLKSTWKAYCGNLKSGREYHTWVLGLPREVAGK  
GYVGEDRRQYRRQSYIVISPTVDNGVLLLPFFANDYHPIFHQDHRKKEVTDIMRV  
TPTCKADQSSSSVRDHVNTALDWLEVLLSCGR

>PROKKA\_02479 RAMP superfamily protein

MKGIPFVVYAITSLHVGMRSSGVVDLP IQRDPQGFPIIFSSTFKGALKQYCGTVY  
NAINDNGRIDCSKANVCCCLFGGEEETDITSIISLSDLYPIAIPVPSLDKGYVYLTSR  
YLINTVVDLFAVNVEEGVKLFSSTVSESKEQQQNNTQQNETSSIVSESKVTIGTD  
IEVPLVKVSSNLLNILSSLGSI AKKLNEGIAIEEDDSKAVLEIERGIIKYTRNRINLNT  
KTVVAGSLWTEEYLP HGSIFAGIMFLNTPRRNKYCNDKICDDQCASQKVNEFIKN  
KEFYLVN VGGKESIGKGVVKVRILG

>PROKKA\_02480 hypothetical protein

MTFQMKRYVERFGDY YGVTVYRLYPDQLMQEEYLGIGSTSYRKRLAELLRKEIS  
WLSYQSKTLTSQELLSPRIKYRDTKYKLSL PARIFDDLICKVRCKYSIDKIKASS  
DPRMEYERKEGEITLGDRKRELYFYAGDWIKIVGIPDSIINNLF EFTISRQSVKH  
ILGRAVIYAYMCMKENEYCSTMIVPTTPGEEIGYLVIPNSKFLEYLSDEL RKTIEEE  
VEGKRIPFCKSCIYRNICPYS

>PROKKA\_02481 CRISPR-associated endonuclease Cas1

MLLIVKNATIKKSGSDLIISWDNSNKQVSTLDLEMVVIVGNNVNISSEVINFLSSL  
NIPILIHGKKVDTVLVTPFINTIAEIRRKFYTLDEGSQILLAKRFIEGKIRGMINVAK  
YFSYIDKLQVNEIDVRIRAKDKKSLILEEAELSKEAWEELKKFMPSFQGRKPRGE  
DPINRAIDYAYSIIYSLSTHALIAAGLDPYSGLLHVNQGRPSLVYDFSEMFKPVAI  
HTVIAVSRRSKLSLDSEGYLKKEGLELITKHLYSILYKRKRRTIRGEFYRKANDLK  
KFILDAVDFQPFYKPLP

>PROKKA\_02482 hypothetical protein

MPLIRLKLKISPFITTDFTGKFVKTLLINTNPELEKIFEDKKYPSPKPLRITPLLEDK  
KEEPVYPKV VITRFGEVAGKPKNSPNPISISGLYHVYIGYEKSLEPEINLTITKLLG  
GVELKYGNDVRVKLIEYEKVEQEVPREFNSVKVYFITPAIFVDPFVKVSQTDKDR  
AKRFVPFPPLIFSVNVYDLFKETYKRNIIRLSYSLIESHTILNTVTKVWYYYD GKW  
LPGVIGYAKFFLRKGIHKNAKEAVKKILENAIEMGVGSGRAAGFGFVKMKFDTT  
S

>PROKKA\_02483 hypothetical protein

MTSIVFNIGFSSEYVIKALTYRGVQGINKIFLITALPKDELSKKRNEDA ITTILNYLN  
VVGIKNVKVLSDVNTTDFDKILLQISTELGKIDDDIEIYLIGGM RILLLSLYYIAQI  
MSKIKKIKAI AFDESMQNSYELLLTIPKIPATSSQIELLKALT KRQSITEVSRKLEKS  
ESTVLKQIESLGELVDCEKSGKTRECEITNIGKVVLNLIQSGA

>PROKKA\_02484 hypothetical protein

MSDQTDEKLIFLVRESLNSISLRQGVKEAKVKSPQSYAFHTLSVATLSLDICRAIY  
NSSDLGKSQLEKLSENYNLPFEELCFYGGFLHDWNKLEGNEEAAKDIIKKLGLPE

EFLYGIPTMAEGHLPDNLHMPVWVSIKLADMILLISDIGSVKDVIFYFANSDAYKN  
 AVEALKNYNLELNYISSTFRLFTLIASKDLLDKIFSKEAGYFPLMSYSDGLVFLRR  
 KNSQQILLSKVLDLLNKEIFSSSSQGIEEKVNEIKKCITNKEDLFRQMNIDVKSVIY  
 DEKGKVRQLNAFLPSKVCKPFEDVVGNDNKSIEVAKRIIEELRGDVPFGLVLY  
 FVNKFSKGDEDYIRRGGLGINEKSLSYLENITDVQEKIDKILELIEKKYPATQGVDK  
 TLLYYIKHSFSGNVLDLPSAVESPKDYCVVCGMPIYSDNPVRFVQYASELRGK  
 AEIWIPREKGLEEIDRVRRDDWKVCPICIYEANLMRDRITPPYFIVTFYPGIPISLLNII  
 DFDQSQGIRYYVDEGETYLNAFEKLGGKLMPTKKALPAYFSSKVIHKASEVTNF  
 NLSTRLSKSDVNKLLPYTPMLSIIFLTSPVLISSDIHEIPIIHERIVSITSTYNYNFMKS  
 LDSNLTTLYSIFAYSADAMKKICRSDLDNCLGYLTEESDLYSSVDPALGVLSI  
 GMGVGTPIENDEKFFSTFLPISGFLKLVAGMVSKMGESIKSSIFSIAVVLKDIKSQ  
 KVSKEYDVTGFLRDGIDMFFKTSSVVKDKEDRVGISVNASISSLENKYSLLDDSHRA  
 QAYSALQNIFDTLYRIEEEADRSLAISIANTLNWLIIAYKLVSQGDKS

>PROKKA\_02485 hypothetical protein

MNGMEVVSKILGKEKLDQFFADLSKPNKENAVPRGRVANVFLVILAEGELVIRH  
 EGGEDVTLATLDGEKYPMILHEKLISAWRREMLEQLRHDYDNNKEEINKIRGKS  
 DEWHCSLRPTAAAGRREERIGGLCRECPNCMIFGYAVGEEKEYNLKSRIEGDVY  
 LSTLPERKSVVIRTFNAVDEPTHTTFIQSEGARTGALFRLSLIKVGTPTFVGKIAMK  
 DLSPAEFALSLYTLVNTTRIGGIKSDFGKIRIIIPAIALCDHEVSSGYEIFEEVKEIDN  
 ASEIVRKINAHVKAFENECQVFTSNDFSTVITDIVSKNKHDIKEAWNGLNFKKS  
 IEEFIKSK

>PROKKA\_02486 hypothetical protein

MKLYKANFYLQGVLIFFSTEVRPMISAGNSMGSYITPSPHVHNYPVMYGMLGKSA  
 EAYFVFPQLQYLDYPERRRGRAKTSKGLQYTSIRKLLLEDFFKKGKDSFYTFPLLPVK  
 FSVSSFLLSAESWSYALLTRSPTKNVFPRITSYSAFMPSQFTTYVVTQGNVKLPE  
 WIRIGKKRWGIFKVEYEEVKINKVEKKKNELTSIPINYKDAEFFGFKISTFSKVLET  
 PNVEEGIIGWAVLEECEVINDKICLPIRWR

>PROKKA\_02487 DEAD/DEAH box helicase

MSVSFTIKEAYIETGNISINGIRLRRFQEELFDSLKGYSRILLRAPTGSGKTFTLLLG  
 AIKSYLEKNLYPVVGIYPSRALVYDQARSIQETLQRMGFKEEEKNTFVGRNLING  
 EDKGEISIKINILTSEVKEIPREFQFPTSPSIVFTVPEYPYMFITGMNKQSIASQVLEA  
 SMKYSFDEAINALAVRRSDVRDLLNYFSVFFNGYWFIDEFHLYSGISRNSMLTLV  
 EMFEKFNSVIKSNKTIVFSSATPVPITIEKVIEVKTSKEGSKIRGRRTKIVFHLVGKNP  
 QENLVNYVPQEYKNGKTAIILDRVYYVAELCNKVNDAAVVGIDKSFGHCRKV  
 NKDLDDKENFIIGNQAISFGIDLDELGFIIHAHDAETLIQRLGRFGRHGDSEVHIFIE  
 AKDKVVRELKNLVNKEITYDDFLKLVDKIYEKRVDDKLDKIFFSKIRHDLVIRTF  
 SLIYAISSQGEQVYNLVKNWYPQKVDELKIRPSYDDYFYVFAYRPGNLTGKWCDG  
 GSDDLFLSIRNFKYREDGCFTNETLRETPIIPRKLDKRVKCSFMQFKEFNENLRPR  
 LVLRKQNYLSILMGGMKEFNDSYVVLLTEECVNWKNFSEMAKLVSTYESAPIYK  
 DEDMKTVIGLALFI

>PROKKA\_02490 hypothetical protein

MLTQIILILMEYINLSRPFYSRETVAYALANYVSGLSSWRAVLPHSTLLYYYRRLS  
 YVRYAVPLSGVYAIDETQVRLINGQYYYVWVIARDVKTKGIPFFMVTSVRSGVHV  
 LVVMANMKRAEEVARGIFKVRVDKVIYLLHDGATAYNAFSWLNVEHEKVTFNE



RDYAEQGFRLKHLASIDFHFPWNSNKFTITRWLSTFFLIYNILYTPTYLLDKGV  
IINVNISNE

>PROKKA\_02492 hypothetical protein

MELVRKRQQTLEELAKPLAFLVAKVEGIYRNSEELNNRNFTLRNFFYNIDDPQK  
YGIDNEFIYPLIVERRSNIIDYNKVVREILLNNFLKLEIYDCGNYSVFTKQFDPSLKC  
NYRILKC

>PROKKA\_02493 hypothetical protein

MAVKIGRSTLPSFVVSLSVLVVLTIYTLTLLSYSRLMGFKDYFSQMSLDPYAFPLF  
SVLTALSFTLIYLTCKNKSVIFLTIPLIAFILARFYLPWFSPQYGSEFYDAGGFLAR  
TEYVLYTGHTTPIYPLDQIVRQEPGFWEANAMLIDIMNGIPTSPATAFILAFVLYY  
NVVEVLVYTPIVLYVFRKLGLDIYKSFGLIIFGFEFSPNLHYVDQTYATPLYWLL  
LLLVLVSVKEGGLKHSVPIAVLSSGIILSQLGVAFSFFGLLAIALYYLRKNRTPLY  
YTALFGIGWISYLNILAMVYGGYVVLGYDVVTFYINPSSAITVVSQDLRPLPIW  
AEIVFYEAIIYMAITLTPALLFFLLSRKSDKKEEYLMMLAVLILITSLIVGPVATKLG  
AGYITRIPQQMLPFMAMAFAEISFSRKLKPLFATMVIILVLIGSLYYYEGRNFEAT  
LNNNYFEFLYTYAVSKPPSFLTAYCHLSINATLTGYSISGLIQSYYYEYGNFSIIQ  
EIVYNTSLRDGIIMAPGIVIGIS

>PROKKA\_02494 Alpha-D-kanosaminyltransferase

MRIALIHHSVCGYGGMEVVSQRLYYYFTKRGHEVTLFSTSACQGMNVRQVKVL  
KIKPDIQIPLEKVNVDYEVVHALSFLSYFNISVSSMLKGKKVVSFLAAKTLRTHPN  
LFYRLVGPIYEDYIIKKGLKLANAVTVKNLGDKKVLEKFNANPFLIPDGLDDVYF  
KPLNEDFRQEVLRFNLEEGEFALYVGRIHKLKGVEVFIKALQLSNFKGVIVGSG  
GKVEGTNVVYAGSLDDLKIALIDSSCCVVIPSLSDFVEGFSAIAASEAWSRRKPVI  
ASSVGALKYRVKEGVNGFLFKPGDYKALAKILPKAKDFKVKIPELVLPWETVV  
SMYEDVYKKTLEDNFEK

>PROKKA\_02496 hypothetical protein

MKGKWATFIPTFVALMLRLYPYLYTREPFTTISWDNLYLSNMLLSGKVPPLSSLLI  
HPTLGSEFFSAIFSKVTSIYPISFEPLIIPAVGSISVLIYLMVRNFGVKVAFLSSTFLA  
LFFGEALIDASVKNGVYGEPLFLLAIWLMMRKSGLKEYFLLFLISLSLIPVYFYF  
TGFLFLIALFFTLYDRDWKKFISVSLPLLILFPHLSTIIRPIYLLALGVYIAFYFLTIFL  
LKFYVKGYSVISGLIVVLVILSLFVKLPYATVFPLYFLPFAVPYIVGFYYINEKKLTY  
VNLFLSSLLVLLFSVLIKTLSFYIAYRVMDYISIPLAMLFGLGGRVTKLIPFILLI  
LATSNFFAIFATVNLQLPYTGGITEVYTQPEFFAVSFSCNNVLAEVSTCVKFQFLF  
QYFGQSVDRYSAICYLIGRAPPTLFLLTPLSLSQGYVLNYNENLPNPVPANYEEK  
LNNVSLIFNDGAVQLYMG

>PROKKA\_02498 hypothetical protein

MIEDEVLGVIMAIIVVAAVVAVTLAFFNPGNNYTAIGLLGINGKIGNYPSHLVVN  
QSVTLYLFYINHEGKAMYYIIEKLGNYTTVLNSTYPGYNLPVLNVYQVIVGND  
ENATLPVVIKIPYPVIDGKVFFILYYYNGSEVYSGEWLQLILNVTE

>PROKKA\_02499 hypothetical protein

MVTANLERDRIRSAVIKYKGLTLGEIVERVSKELNVKDYEIAKVIYKMVEDGEIR  
LVDPNEGFLNFLLYHSIWFFLVLVIFGTIISVFLNFLPLRYFFGIIFVMYLPGATLTE  
LVYPPKKEDLSQLGRLAMGIGLSLALDPVGLFLNYGPGIFVNTSLVSLTLLTLAFS  
LGALFRKYSNYRLLKEVEKVEK

>PROKKA\_02500 hypothetical protein

MRSELGISLGIFGTLLSLSSFFILRDNTLTALGIGIVIIGLTLISIRDEGDISMIIEGGLA  
NLELLEDLDVVSQKGYYFPNGNKNVNVYIALNGKLSFPEPQGIITTQDGSSVLILHP  
PIYVIKDLNKSLSLISEYVIERSLAEDVKVIKNGNVYALEVKGSKVYTPGRVKLV  
MGSCVSSIVASIIALKEGKPCIIKEEKGDDKRFTALIEVLP

>PROKKA\_02502 hypothetical protein

MRKDALLSLSMLLIILSLAVITVYQQPVAVKEIHLPNVVVRQINQIVSTYYQYVIN  
NISVENFTLALQMVSKAPSQYSELNSNLSALIFYLQSLNSTSLSLKEYYLLKTNE  
SLSQLKNLVGFYSLNVTLTPIYSLLNKIYVGILQQESELIKTEIQLSVTNSTVPGGNI  
TITGKLLTYNGTPLPFYTVYITLLNKQYSVITNSIGSFELNLTLPQIYVREIEVTAYF  
LPTNVYAGSLTTAKVFLNYYQPEIQASLNQTRGFEGEKLTLNLFELKTISTDNTIVIS  
IFNSSYTITDVEPNVTYSTIVTLPNITGPQNITVTSLPQGNIPASVKLTVNVTYFSA  
NISFKSPGFVIAGLPANIHGCTTPKFNGEVLVMIGNNKYYTHVTDGQFSVSVVIPP  
TLNLGNTPISVSALPPYSGTTVKSVFVINVLDFVPLGVVGYLAYYALSSRTVEVK  
REEKKKEELGERKKFIFTDPIALAYYQALTAIEKITNEEMRDYYTVREYLNLVRG  
KINENKYNAFSRLTFLFELRVYGNVYTLDLAEVNELLKVIMS

>PROKKA\_02503 hypothetical protein

MKLILISSLLSILVVLTHIYVTPSTTPFSVFSNSPLGLSSAERYFTPSNNVVIIVQPQQ  
NVTQLVKKILINNGTVILVGDNSVINNTLKKFGAYIGSCIIRDNVFNAGNDSEIEVF  
YKGNLAVFFDSHPINVTPLVESSPYSYTITQKGPFTVVGMIKVHNMGRIVVISSP  
YVLTNYYIKENLNFLYAIIGKSAYIYPNVFSPLDYVKALLNQLSYYSYIVLLLSLAI  
FRLKFKRKNEQSKTMLKILLSLHPDWDENVLRRIYDDRGE

>PROKKA\_02504 ATPase family associated with various cellular activities (AAA)

MMIEVKNDYIKRILDGLDSLIIGQEELKTVLILSLLTEGNLLIEGPVGVGKTVTAK  
VFSMLIGGEFKRIQMTSDLLPSDILGTPFYNIKDGSWSIKKGPIFANVVFIDELNRA  
PARTQSALLQAMQEKEVTIENTIFKLPSPFMVIATQVPYAEGTYTLPHYVEIDRSY  
GIEVDLPSKEDEIKILDVSYLTDNPDVKPVITPEKALELTKVVKEVKVSDEVKEYI  
VSLISSLREDKDLMYKLSVRASISLYRASKAIAFLDSRNFPVDDVKFVFPYVVYH  
RIVLKPEAIEKNKKEKIKAVLESVKVVK

>PROKKA\_02505 hypothetical protein

MITDKGFKFLIILISSFLSVLTPTITLVAITLVSILLLDYLSLVRKKVLNLKFKAEEVI  
AGERLSFDLPPLGEFVNQKFVKFENKGSKEAIIIDTRFFGKYVLKPKVVLKSFLG  
LFSVVKDSGIEIDLTIPRFLREVILALGELYGIGGLGYKLSRPGLEYVESREYIE  
GDPLNRIDFNATAKTGTLHIKYFLDEGSLGEAIIIFDIRSPGRYTADYLASKFLSAIL  
NSDRSIVLITDGVKTYRYEGNRDYILKIALYWVFRIALDKGEEIYEIVPPKIVSELE  
KVLKVNRRNGTTESPYKLSYEMREVIVIAHPLYDTVKLLRFLADAKSRGIKVKVI  
YPESPWLDSEDLERAYIEYQSFRKVTKVIEDLVEG

>PROKKA\_02509 dTDP-4-dehydrorhamnose 3,5-epimerase

MPFEFEELGLGVLLIKPRVFPDNRGYFKEIFKESDFKKMGIPTPLQVNVFSKYGV  
VRGLHYQLPPKEQGKIVTVIKGKILDVAVDIRKGSSFGKYVSAELSEDNHMYMLWI  
PPGFAHGFQALESSTIVYFITHNEYSPPHERCNVYSMIDWPIKDIISEKDSECPPLE  
KAEVFDESH

>PROKKA\_02512 GalNAc(5)-diNAcBac-PP-undecaprenol beta-1,3-glucosyltransferase

MIDNDTVVLLLFSSQYANVLSIVIPTSNAEDTIGELLESIKAQDFHDDYEVIIDNGS  
 KDKTEEIVKKYMNILPIKYFKYKEKLGVANSSKNAGINKASREFILFLDHDVTLPP  
 GTLFQLSTILNKENYNIVFQLKLVYPNGLIDSCGGLIDELGYPRELRKGEPAENQC  
 IDVTDILYAKGSAMLISKLLDELKGFDPNYFYEYDDTDICFRAIKRGYKVKFLPI  
 SIFHHEHGALPKDLRSREIRLTIFYLESRRLYFLLKNFSRGYLFRKMPKVLFFYFLGS  
 MLMDLVKRRKTYLKFARVKALLWVISKLPFIYKKRKNEIFINEEELIRRGLIVKH  
 QLKR

>PROKKA\_02513 hypothetical protein

MSNYIKKAKIAIKSRKLLKNWTISILKYAFKNPEILLCKDNSVIKVSDEFANILS  
 LYYEGKIINCSNNSIGFYVNGNTYWIPIVNEILIASGEFDSILKALYYNWRYNSKY  
 WEKGNIKFKHIAEIIATFEDEEYGYVDVKNKSIADIGAFVGDTAIYFAIKGAKKV  
 ISIEHPGAYEELVENIRINGLEGKIVPLNIAVGDKGYIVISNVETKQAPVTLFKES  
 EGNIGIKVKMETLNNVIKMYNLETDILKMDCEGCEYNLILNDYEAVSKFDQLAFE  
 YHAYNTGMSIYKLIQLLERYNCKFVDEHIYRKNDPNWDKSKIGMLYCVKRK

>PROKKA\_02515 D-inositol 3-phosphate glycosyltransferase

MKILFALISTGLAGGVRYIFEVANGLKDKGHDVKIVALAGDHSWFKNLRAEVIY  
 KPLNLMFSSIVYAFYKFYRYVTLRSAKRNPYDTFLLISSKLGVRPDIIIELAEIIRS  
 FDSDVTIATYYLTAFSVWFSQKNPFYLMQDFPELVEGNEGKLGGLNMFYHSLKL  
 PFSFITVSSYAKQLIVENNPATAKVIAHPGVDLNIFRPRRTNSQNQKKRVMVILRG  
 SRVKGDEVALEVLKNVNKKVPIHVVFVGGKSLVRHYSKTIGLDFEYSVFSNVSD  
 EVLAELYSSSDVFLHTSYAESFGLPPEAMACGTPVMTDNKGSRDY AIDGFNA  
 LISQPGDVKSLSDNLLKVIQDDKLRRERMIENGLETAKKFTWAKTVENFEKALKEI  
 N

>PROKKA\_02518 hypothetical protein

MGLDTALLYLSLEHNNLLHIPEKLTIRVGTGISTYSKVIDYSKFLENKNKLVCFSS  
 NRQLEDFRYLGSLITSCKQCKKAIQRSILFLELYLSAENEVFKCSYKAQLSSLNSL  
 FFSSIRYYFDGTISVKDLYNVAKGVLAQLILGRKKVSEMRSKRDFESFQNIKT

>PROKKA\_02519 Glycosyl transferases group 1

MKTISVILFDMSTLGGIQLMTVDTLCLLAKNGYNVTLVTMSLSERVLTSLRNCEG  
 NIKIKLLPKISNVTFQMIYSTFYKKDESFSINMHGDIQPIASDIVYFHQFNTDYRIRS  
 SSLKRIILLPQYSIRKRFIEELRRRGSLVLVNSSWTQAEAKYFWNLDAKILYPPVH  
 TEKFRKIDNTNRSDVVLTSRFSRDRRLDNVLNVAKELKKTRFIIAGYLQDPEYFL  
 ELKMKKSENIELYPNVNEGTKLRLLSVSKVYFNPTPYIEGFGISVIEGMSAGLIPV  
 TRNKGGVIDFVPKEYMFNEINEALSKIDYGIKNWNFYSMRMMKELADRFSLENY  
 EKNLLDIVSKYL

>PROKKA\_02524 N,N'-diacetyl bacillosaminyl-diphospho-undecaprenol alpha-1,3-N-  
 acetylgalactosaminyltransferase

MASELGKICHEVIPIPFKNEGFKSYLRDKREFILHVGTRGEKNVPVSVEAVRLLRE  
 RGFNVNLVIVGPAAHYWKEKFKYDWIIPKVVVDNEELKELYARAIALILPSTFEGF  
 PYTTLEASASGTPMVGSNCIPEEALIDGYNGFRINGINPKNYAEKLEMILSNRELW  
 EKISSNGKELVKNYDYISISKRYLELASE

>PROKKA\_02525 Glycosyl transferase family 2

MCKLAVLITSYNRSELFKKTLKSWLDREEVDTLIIKADSSKREEYEGYKEALEEIK  
GKSVIYEISDKRSGPTTAKNRVLEMALQNDCRFMIYADDDHYIPTESDVKGSLKI  
LLNGSIGLNPFSR

>PROKKA\_02526 Undecaprenyl-phosphate mannosyltransferase

MGLEEVVTFLIPTLNERDGIGLVIDEIKSLGFNKIIVVDGNSTDGTAEIAKEKGAK  
VIYQQGKGKALAIMTGLKHVDTPYVAVIDGDYTYNPADAIKALKMMEDYDEVI  
VVRGNRKNIPLLNRFGNWWITKMFNLLFGTRLKDVLSGFYVLRNEVKGVNFEA  
KGFSIEVAISAYLASQSKRIGEVIQDYRARKGESKLKKIQGLKILFDTILLA AKFNP  
VFLIFVVGSVLLLPAILSYVALDYLAHYHIFHFLAFIGFSLFNVGLISLLSVLAL  
YLKRMEYRLNEKIEGLKRDV

>PROKKA\_02533 NurA domain protein

MEFGEAIKSLFSLSSIDQPYLGVSPTTSFEDSTPVEVEEELGVCDSEEFSLDSSS  
RMINVRGANVYFASLYANLVKDHIMIPQVDVPFIAIKASDEVKRLIEGDQMLSK  
IVAINNVNGEPYSPDYKDDNIDELRISLENYAIGKSNYVTIVDGPPIPGPYIQMVG  
EPYKSAFITLANQRKKDRLIGIVKRLNYSRKLRRSGIHKDPSLMNATDDVIVEYLG  
RGKEFYTTPIKKEIDVGNSHYTRYMVYVKVREGVFRVESLSKDLLCKGVYTAR  
KYSSYRGIPEFIEVADRISKRLSASAFIFAFNIARVTTGVNYEDWENLRTAELDAG  
E

>PROKKA\_02534 hypothetical protein

MDKFAKILDKINYLEVKFTYNGCLVEVDDKNYLDLAIDLVEKYNPLFFSLRYGR  
VKVLISSTEIETNVDLFNALDDIVKRIRNVNNFYLEIEKYGERERRIIDKVNSVKG  
KQLLTYLGIKKNYVEVGASRIIGEISRTSELQRLELPPLISEPPRLDSGVGFEEVIKL  
NQNVRFYNCDDVEVIEDAYGEIDCNEISPRVLKGIPLSYVHAGKKGTCLISQSKTL  
VVSPTSLLNEEALAEFNELPYFTITVIVDPSRDLKEVFKKMEEINPGKSTETYVY  
KDRIYMYTSNDRKQLTLNKVKITNH

>PROKKA\_02535 hypothetical protein

MIGRSDERDHFVIFVRLIPSRSKISKRSPIKEVKLKKPTKLLLTQGDLLIKLLKEGINF  
ETLKENAFKSLLSIKYFTEHYPLFRDWSVIDDFKEELNNEIDLITVINALNKPASV  
DFLNTHSHFLNIQGGIYLSPKLGETDGIFRRQGHRRYIVRQEHVIGIYLLKKGGKI  
RLIHMGEYPHREISITAEDEVYQPFEARLESQVDIQDHNVIIR

>PROKKA\_02537 hypothetical protein

MSSFSPSSPSFVDSWIISRLKKENSVS LTIMAEDYAAERGISKRSARRILERHLKDL  
VENGIVERLPTYPVSFKLKKLP IQYLLPQTKNFRLNV SFDLSLNNHNSKTLGNSA  
NLKQKLQELRKIGLRVITLANAFRQKGLLTAQHKVSIRPGTREHSDIAFLYNENIK  
EWNRSVLVFENDDGEFMKMNVTRFNDQKKA FMNLIKSNKALDNAFKKYRDA  
IMITLTIPHIFPLVIPVKEGKRIVSFIPLQDSILTQLKKKMTDWIRKMWKGRKIETFT  
AYEYHGDYVLHVHVLIFGIPYLIDWNRKYGKKKEDALTYVRSYRSYNIPLRSD  
LETKMKEGKLEPKDKTRISKYIFTALLDMWIMDILTDFGSALHMNLLQAYLDYK  
KKEKIQGPINEIHRKNGKWKGKPPKDAIEEYSSGAAYRKVISPKQYVLKYVTM  
AYAIAQGGNVEEKDQAKVYGYWLF GKRFNSYSPSLLPKKKEVKIPYWHFVGVF  
NRLNLPDFVMNNLILDLT

>PROKKA\_02538 hypothetical protein

MLDITQLLIPGVKLQKLTRHINNKGREYEYSQYFIYIPRDFEAYVKNKEWLVTIIV  
DGKEFPIGIKSPSRHNNYYIITLPNDLEYWYWERKLYQKVDVLLQRP

>PROKKA\_02539 hypothetical protein

MKIITQVKAKVTNGDFKSLTINGIYIAIDRHNAYLNDIEIPKKIAEYVIDHVKADSI  
PELHLNYVEYEANLTLEIEDYELTIRQRGKELLTITFRGNMALLLTPKHSDKVRN  
ADKLRRILKREVMAYA

>PROKKA\_02550 hypothetical protein

MKVDFS DY EK VYNELGGIPGWLTYFGFRYYEIREFDKAMNETLNYAKRLIISEFE  
HFLLDKMI AKERYYTIMRSTANCNNWSGIRNELEAKEGIKISDSELSNYLNHLLD  
SSWLTKVDDRYCPSEPLIADSSLPQSGRPKITSLKINL

>PROKKA\_02555 Haloacetate dehalogenase H-2

MLYAFDVYGT LFDVNSIVDEIKDIELVKEWRRKQLEYTWLLTIMGRYESFWEITR  
KALIYTMKKFNV SIDVDKAMKTWLNLT PYEDVVEALSKINARKVTLTNGDEWM  
IRELLRNSNLLGYFDEIITAEKVKKYKPAKDVYKLVEGAIFISSNPWDIAGARNAG  
LQAIYVNR YGYNLEEIDIKDKIKIKSLKELVK

>PROKKA\_02559 hypothetical protein

MDDNNKENIRPGNIISSII GGILGGIVMVIVLMIGAMTSLPPTAFPMVMGMALG  
ASINIAIIVGIIAHFVVSIVIGVIFVAIVSYIRPLNLKSSKKALGLGLDAGLTVYLVAF  
IPVAMFMFAPVMIHMMGSSAATILPDVLGIAVIGHILYGLILGISAFKIGVRLS

>PROKKA\_02561 hypothetical protein

MVRSARSGVHVLVILAASMEELAGKYFKRVDEVVYDGASIYNAFTWLVNVNHRK  
VTFGKR DYAEQGFRSVKHRLSPMDKHFPWNSNAKTIERWFATYFLIYNILYCLV  
YLLDKGVIINVNISNE

>PROKKA\_02564 hypothetical protein

MNLHQA YLDYKRKEKIQGPINEIHRIDGKWKKGKPPKDSVIEHSSGACYRKVISP  
KQYVLKYVTKMAY AIAQGGNVEEKDQAKVFGYWLF GKRFNSYSPSLLPKKEL  
KIPYWHFVG VFNQLDIPDYVTDNLILDLT

>PROKKA\_02565 hypothetical protein

MIIGKSPLSYFVWKYMSEYVCNPLYPYPLFYDAKGDLLTSRLAYIGYLRKLQVK  
RNEVRIA VYPDYVLP HKARVNLSPLKSIEFVVP IHSLENDMVIVEEFG

>PROKKA\_02567 hypothetical protein

MSWDKSVKESIKWHDNMEVEKEYEYTVGPAGEEFFKGLKQGKIIGSKCSKCGKIY  
IPARTYCENC FVKINNYVEIKKEEAYVD TYTHIYKDDEGRQLDKPIYIAMIRFPNTV  
GGLLCYAEGNVKVGGKAKITSFDWPLRVTV

>PROKKA\_02570 acetyl-CoA acetyltransferase

MNQIAIIGTGWYGFKPSTPEVS FREMVFEASTRAYQDAGNINPRNDVDSFISCQE  
DFWEGISISDEFAPDQLGGALRPTMTVAGDSLQGLAHAFMQINSGIADIVAIEAHS  
KASDILTYSEIVKLSMDPTYVRSINPQNFHFIAGLDSVKFMERKKISREDLAMVVE  
KNKNSGLSSPRASYASKISAQDV LNKEFVVYPLTELDIAPFVDGAIVIVVSTEEIA  
RKLKKDDYVIIKGISYATDSANLETSELGKANYMRIASDLAYKMANVTSPRRYF  
DAVFVDDRYSYKELQHLEGLRISDDPSKDLNEGNFSPQGEIPVNPLGGHLAKGVP  
LEASGFSLLLD AIDYIKQGKIEKALVG SWRGLPTFTGSVVVVEKP

>PROKKA\_02571 Acyl-CoA dehydrogenase

MSESLIIQSLREFLRREVEGVSAKIDKEDSYPRELVRKL GELGFLIPLYSNLSHYD  
MLKILEEIAKVSGSLALIVDAQGE LAGEMRLYGNETQKKNFLEPMSKGEIISFA  
LTEPSGGSDVGS MKTYAEKKGSWIVNGHKMWITQGIYADIFITVAKTGN SRKDL

SVFIIPRGNCVETSKIEVMGNRGTGTAEVIFHECEVPEDYVIGEVNKGWEMINSVL  
 EVGRLAISGIAIGLAEKALEEAFQWANSREAFGNKLYNFQGLRWYFADSIKINS  
 LKALAKEVSRKFDDGSNDKGVYVSMLKLLSAEIAQEVVDISLQILGGLGYAKGT  
 SVERIYRDVRLMRIGEGSDEIQRHIISKYSEKYGIPILD

>PROKKA\_02572 Glycosyl transferases group 1

MRILISPPMRFGKEFLGGMRRRTLEVYSRIGEEVYLCVDERTLPQVDPEISPLLSKF  
 HLVSSSSTDKKTYLKGFNCLKYARKVDVLISYSEYSLSVYYTWLLSLFSRKPFIV  
 VVHHVTEEIRGSKFLRTVIARSKGIICLDNPEVYEELKSLFPNKVILTSTNGVNVED  
 YYASEEKICDGI FVGNYGERKGSRYLFQIWDKVNEKIDAKLCIIGKGWSEIPKNSV  
 YYGFTEREKIDLLAKSKVFVFPSTYEGFALAVAEALSAYLPVVTWDLKWSERY  
 PVAIRVTYPDISSFAEEVVKLLKDEKLRKSIGEKSRFAKALNWEKAAEIEKKAIIY  
 KTIESK

>PROKKA\_02580 Methyltransferase domain protein

MRFIEQINYSLFENFVLEQYNTEIQGDVVDIGANIGDSTIYFALKGASHVYA FEPL  
 PSVYKVALENIKLNGLENKITLINA AVGSKEGKVKVPSNINTEESGGFYIKNQGD  
 VEVPRFSLNRIMTMTRNPYLLKMDCEGCEADVIFSSSELD FNKIFVESHQGITKIPH  
 KKLIKRLLEEQRKQCQERMKIDDNTKLFYCIKLN

>PROKKA\_02585 hypothetical protein

MPRPEDLPSSRRDVAESLGDALRECDVDYYILVGNKISPLITMSLDYTLAFAWA  
 FIDRYNKAIGE VNRI LNIA GRGGVYEAEIFYGLGLASIIANAAGLGKVVKPSDAD  
 TALHIASFTIRLVASPDLIKSVLGALEPLYGKAPHRYLELLALASN MENLDFITVR  
 YTFDKLNEILDNYGDVVRGYAWSLVDAIRAYAHLLRRYFYHFNSEEVGYMVGR  
 VVDLLNELGRFKSSLGLIAWTEALDPALSLEDVRRLMEEKLGIDALVKASEVLEK  
 LNDMRKR VQELMRDEEFMSYIESWSVKANEKAVKIGILEAASLLKYALAIYRLE  
 NNELDEARDLFNKAAEYREIGEYENYLVL RDWALRVEAIKGS LVGEKLVNEFR  
 QLYEETFSKEHFKPTAKYLSNASRTLGN YLVSLALTGDHETINKLLEEHLRVLNA  
 DGHVSVLTRLMLNALLGPRGGLSGELKGKLSVNPEELIDAFGSHMYSKSLPALR  
 VALNIAKPEDGIKLCEEFNDEVCIDSVLAVKGN SVAVKQLRERVINAFNAFNNSL  
 KGFGFDVESLINKLRGMVSGLDDRS LVQLIAPSTSMARLALMLHALISGNKELAK  
 ALALDGAISSAISSTSKLLGRLFLEVYKECCDLGKDEFRLAIARLFFLHT

>PROKKA\_02586 hypothetical protein

MINGQVPIENVTINGQTY YVIDADKVN PADIASCWRWVNNFVTAINTPGAYAA  
 LLSGDSPVFTWENLTGT VAYETYRNGIRGLGWGIVLTQWNYR

>PROKKA\_02587 hypothetical protein

MPPDQQHNQNY YQYGVFWLYNWFQAENNNMNIFFLMNENICNYS LDLEQYIW  
 DSNCWLFGCSPFSAY AIPPNP PASTNSGEPIFAQSYIIAYNTAFNQETSGYNLNYQ  
 KALNNYNKFTYSDVNGNFVPRTFGYKEEYNRLSQRFYTN YFKMMQVYM

>PROKKA\_02588 hypothetical protein

MSSGAENEEQVFLPITNSIPSVNNNQITIGSSVSIEYNQSGQHVSQIDDKGLHNILV  
 LTGYAIDENTGELIPTFDPCDYVKGILISGKILEGNPFKIIKIIGIPSNKLYIIRKKDVH  
 GNITFSFPIKINQLSTGT YQVDLG NKVTSFESLNGDVATKIVNEVLAKIYKLSEKQ  
 KKNLYTGVKEIFNYYSIQS

>PROKKA\_02591 RAMP superfamily protein

MEKLLLSLKLRALYPLVGGYNRHSINEFYEENVVRPTEIKGLWRWWNRVLFNTVS  
 YVKEGKLYTYDSIDRLFEDVFGSENKKS AVRLEVITDGGSDNHFELSNVELDKLI  
 DCLKASREEKVNLD FRDNTLIVEIEGST EIPISFKSNLDIDKIKDIVYKNKLLSFELL  
 GFKSIKINTKISDKEVIKEVLRDLITNYLEYFNIKQEVTF TLNIYLDKSREHKQNFE  
 DKLKFALYSLLVFILLGGIGRKT SRGFGSL SIVYAKCYDGVCS DLESYINEILKVE  
 NEKELKDKLVQLIHKIEYNVLFPG EKHRLEKVDFSNNMVYFYFNLG SFLEHTLFV  
 NEADPNKVKCILD TVSEAVIIKERKCIQLIQNYLAFLV LFCGNRANKKSVINSTQY  
 IYDIDDY LKSLVCNSFDSASWFAKYSRIFDCKKRRPSALRFKILSINNGKTFL LTYL  
 LIPSYIEYIKNNPVKVNYNKICKDLKTLADCVSENVKSCQFNIR

>PROKKA\_02596 hypothetical protein

MRIMFLDEHVDNNSTLIGGIYLD MDEIRLIIDAINSAWDKLGTEWDFHYVKDKKI  
 VRNEMPVSEIFLNSLHSYFERRHTLIVVHKDVEALKKHNDEFYPTVIFNLYSFPVF  
 LSKSDYALIDKNPQVTVMKNIIEKIGYPKIFYDVDMLVNSMDIGTKNCILKIVKNII  
 RSKKGIAIQSLDRAYNLRKEEAIKRV MKWGLFCADYVVSSMDKEWRKVDIYGR  
 EI

>PROKKA\_02597 cytosine permease

MEEDEIKRKVIEASSGIPELGKIGREIETIGVLPVPDSIRKISSLDVFIFWAMASASA  
 ATPLAGYLLYGVGIPSFLIIVSLATHI GLIPAGLFSEIGRQKPIVALIQARGTFGYFPA  
 NALSLLYTFVNMGWFGNLAVGA EILSSVSGISFAIWSVILGIIQIALVIFGAKWLN  
 YFYKFTSPLLIISYGVLA FYLFYCYHPALSTMMNPSIPINWGLDINLILTF SILSWA  
 YKITTITRFAKPYDRPSISYFLSPSLGIMLPVFLMGLLGYISQAATGNWNLA AVYK  
 PGDPIWVLVA AVGASIAIIHTNSMNLYP AVADLLVSMETFMKRMKTYRLSQPLS  
 AIVLGSVSII LAILGILNYVENFLLL VGDILLPFTFILIIDWYTGLRNSDILSFYYNNG  
 LKVKALIALAIGFLLNYYNIYGIIGY YFPYQVLGSLIAAGIYYIEKKI

>PROKKA\_02598 (R)-stereoselective amidase

MVKIAMIQMGGVESKEANIKKALDYAKSAIKDGAELIVYNELFTTQYFAATEDP  
 KFFDLAEPDDGPTVRTFAEFSKQYKVGMVITFFFEEDKKIRGIYYDTSVFIKDG NV  
 LGKYRKTHIPQVP GYYEKFYFKPGKDYPVFD FGEYKVGGVICYDRHFPEGVRILT  
 LKGADIVTIPTTTNFYPETWELELRAHAAFN TIYVVG VNRTPEVFQGREIDYFGKS  
 LVADPMGRILKEMDSQEGYEIV

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CHAPTER FOUR

FLEXIBILITY IN MINERAL DEPENDENT ENERGY  
METABOLISM BROADENS THE ECOLOGICAL  
NICHE OF A THERMOACIDOPHILE

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## Flexibility in Mineral-Dependent Energy Metabolism Broadens the Ecological Niche of a Thermoacidophile

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**ABSTRACT**

The thermoacidophile *Acidianus* was shown to be widely distributed in Yellowstone National Park hot springs that spanned large gradients in pH (1.60 to 4.84), temperature (42 to 90°C), and mineralogy. To characterize the potential role of flexibility in mineral-dependent energy metabolism in broadening the ecological niche space of this strain, we characterized the spectrum of minerals capable of supporting metabolism. *Acidianus* strain DS80 displayed versatility in mineral-dependent metabolism and used elemental sulfur ( $S^0$ ) and a variety of iron (hydr)oxides, consistent with the presence of these minerals in the hot springs where DS80 was detected. Strain DS80 reduced, oxidized, and disproportionated  $S^0$ . Cells growing via  $S^0$  reduction or disproportionation did not require direct access to the mineral to reduce it whereas cells growing via  $S^0$  oxidation did require direct access to the mineral, observations that are attributable to the role of  $H_2S$  in solubilizing bulk  $S^0$ . Moreover, cells growing via iron (hydr)oxide reduction did not require access to the mineral and higher rates of reduction were observed with poorly crystalline iron oxides, suggesting that cells were reducing  $Fe^{3+}$  ions that were being leached by the growth medium. Growth via disproportionation of  $S^0$  occurred near thermodynamic equilibrium [ $-8.2 \text{ kJ (mol e}^{-})^{-1}$  transferred] and was highly sensitive to product formation. These results highlight the versatility in the mineral-dependent metabolism of this strain and potentially explain its widespread ecological distribution. Moreover, these results underscore the importance of abiotic reactions and their feedbacks with biotic reactions in increasing the availability of mineral-based substrates capable of supporting growth.

## INTRODUCTION

Microbial life in high temperature hot spring environments ( $>73^{\circ}\text{C}$ ) is supported by chemical energy (Boyd et al., 2012; Boyd et al., 2010; Cox et al., 2011; Hamilton et al., 2012) supplied by volcanic degassing (volatiles) or water rock interactions (solutes) (Amend and Shock, 2001; Spear et al., 2005). The amount of and variability in the composition of chemical sources of energy is dependent on processes that take place deep in the subsurface as well as those that take place near the surface (Nordstrom et al., 2005). The primary subsurface process that influences the geochemical composition of hydrothermal fluids is subsurface boiling and separation of fluid into a vapor phase and a liquid phase (Fournier, 1989; Nordstrom et al., 2009; Truesdell and Fournier, 1976; White et al., 1971). Partitioning of hydrogen sulfide ( $\text{H}_2\text{S}$ ) into the vapor phase, interaction of this  $\text{H}_2\text{S}$  enriched vapor with infiltrating near surface, oxygen ( $\text{O}_2$ ) rich meteoric fluids, and oxidation of  $\text{H}_2\text{S}$  to sulfuric acid is thought to drive the development of acidic spring waters (Nordstrom et al., 2009). In turn, these acidic waters are more effective in leaching minerals from subsurface bedrock than alkaline liquid phase influenced waters, which can lead to diverse geochemical compositions (Amenabar et al., 2015; Fournier, 1989).

Variation in the availability of electron donors and acceptors, both in soluble and insoluble (mineral) forms, drives variation in the taxonomic and functional composition of chemotrophic hot spring communities (Alsop et al., 2014; Colman et al., 2016; Inskeep et al., 2013; Inskeep et al., 2010). Redox active minerals that can support microbial metabolism in hot spring environments include elemental sulfur ( $\text{S}^{\circ}$ ) (Boyd and

Druschel, 2013; Boyd et al., 2007b; Colman et al., 2016; Inskeep and McDermott, 2005) that results from the incomplete near surface oxidation of  $\text{H}_2\text{S}$  (Colman et al., in review; Inskeep and McDermott, 2005; Langner et al., 2001; Nordstrom et al., 2005; Xu et al., 1998).  $\text{S}^\circ$  is generally more common in acidic hot springs (Boyd et al., 2007b; Inskeep et al., 2013), since these springs are influenced to a greater degree by vapor phase input, which is enriched in  $\text{H}_2\text{S}$  relative to liquid phase input (Nordstrom et al., 2009).

Numerous reports have demonstrated the role of  $\text{S}^\circ$  as an electron donor (Amenabar et al., 2017; Friedrich et al., 2001; Huber and Prangishvili, 2006) and as an electron acceptor (Amenabar et al., 2017; Boyd and Druschel, 2013; Boyd et al., 2007b; Boyd et al., 2009; Huber and Prangishvili, 2006) in supporting microbial metabolism in hot spring environments. Far less is known of the role of  $\text{S}^\circ$  when it serves as both electron donor and as an electron acceptor in a process termed  $\text{S}^\circ$  disproportionation (Finstter et al., 1998; Janssen et al., 1996; Lovley and Phillips, 1994; Muyzer and Stams, 2008; Slobodkin et al., 2012; Thamdrup et al., 1993). To our knowledge, only one thermophilic bacterium with the ability to disproportionate  $\text{S}^\circ$  has been described to date (Slobodkin et al., 2012). All other reported organisms capable of growth via  $\text{S}^\circ$  disproportionation are neutrophiles within the bacterial domain (Finstter et al., 1998; Janssen et al., 1996; Lovley and Phillips, 1994; Muyzer and Stams, 2008; Slobodkin et al., 2012; Thamdrup et al., 1993).

In addition to  $\text{S}^\circ$ , common redox active minerals capable of supporting microbial metabolism in hot springs include iron (hydr)oxides (Fortney et al., 2016; Inskeep and McDermott, 2005; Kashefi et al., 2008; Langner et al., 2001; Lovley et al., 2004;

Slobodkin, 2005; Slobodkina et al., 2012), which result from the near surface oxidation of  $\text{Fe}^{2+}$  with oxygen (Inskeep et al., 2004; Kozubal et al., 2008).  $\text{Fe}^{2+}$  is more soluble in acid (Johnson et al., 2012) and is enriched in the source waters of many acidic hot springs (**Supp. Fig. 1**) (Ball et al., 2010). As such, many acidic springs have iron (hydr)oxide depositional zones (Beam et al., 2016; Inskeep and McDermott, 2005; Inskeep et al., 2004; Kozubal et al., 2008; Langner et al., 2001). Despite these observations, only a few thermoacidophiles have been shown to couple  $\text{Fe}^{3+}$  reduction to growth in acidic high-temperature conditions (Amenabar et al., 2017; Giaveno et al., 2013; Yoshida et al., 2006).

We previously isolated a thermoacidophilic crenarchaeote belonging to the *Acidianus* genus, designated strain DS80, from an acidic hot spring known as “Dragon Spring” in the Norris Geyser Basin (NGB) in Yellowstone National Park (YNP) (Amenabar et al., 2017). Our prior characterization indicates that strain DS80 displays versatility in lithotrophic energy metabolism involving hydrogen ( $\text{H}_2$ ) or  $\text{S}^\circ$  as electron donors and  $\text{S}^\circ$  or soluble/mineral forms of ferric iron ( $\text{Fe}^{3+}$ ) as electron acceptors (Amenabar et al., in review; Amenabar et al., 2017). Aside from  $\text{Fe}^{3+}$  and  $\text{S}^\circ$ , the spectrum of mineral-based substrates that can support growth of DS80 is not known. Moreover, despite the ability of strain DS80 to respire these solid minerals, questions remain as to how these cells couple redox transformation of multiple solid phase minerals when they serve as obligate electron donor and acceptor (e.g., coupling  $\text{S}^\circ$  oxidation to iron (hydr)oxide reduction).

In this study, we determined the distribution of strain DS80 across pH and temperature space in 73 hot springs in YNP. The widespread distribution of this strain in hot spring sediments that contained combinations of S° and iron (hydr)oxides prompted additional physiological studies aimed at further characterizing the flexibility in the mineral-dependent energy metabolism of strain DS80. In specific, we aimed to characterize *i*) the role of strain DS80 in the formation of solid phase minerals (e.g., through oxidation of Fe<sup>2+</sup> or Mn<sup>2+</sup>), *ii*) to determine the spectrum of solid phase minerals commonly detected in hot spring environments (S°, iron (hydr)oxides) in supporting growth, and *iii*) the mechanisms involved in accessing mineral substrates during metabolism. Results suggest that flexibility in the mineral-dependent metabolism of strain DS80, combined with unique mechanisms of acquiring these mineral substrates for use in energy metabolism, enables a widespread distribution of this strain in low pH hot spring habitats.

## **MATERIALS AND METHODS**

**DNA extraction and PCR amplification of sulfur oxygenase reductase (*sor*) gene.** Sediments from 73 thermal features in YNP were collected aseptically as previously described (Hamilton et al., 2011). Sampling sites were chosen to include a wide range of temperature and pH combinations available in the thermal features of YNP as well as to sample geographically distinct areas of the Park. The pH and temperature of each thermal feature were measured on-site with a YSI pH100CC-01 pH meter and a YSI EC300 conductivity meter (YSI, Inc., USA), respectively. Genomic DNA was extracted

from approximately 100 mg of sediment using the FastDNA Spin Kit for soils (MP Biomedicals, Santa Ana, CA). Genomic DNA from five isolates (see below for description) was also extracted using this kit. The concentration of DNA in the extract was determined using a Qubit 2.0 Fluorimeter (Invitrogen, Carlsbad, CA, USA) and a Qubit dsDNA HS Assay kit (Molecular Probes, Eugene, OR, USA).

DNA extracts were subjected to PCR amplification of archaeal and bacterial 16S rRNA genes to ensure that the DNA was suitable for PCR (i.e., of sufficient quality). PCR amplification of 16S rRNA genes was performed according to previously described protocols (Boyd et al., 2007a; Hamilton et al., 2013) using archaeal primers 344F (5'-ACGGGGYGCAGCAGGCGCGA-3') and 915R (5'-GTGCTCCCCCGCCAATTCCT-3') and bacterial primers 1100F (5'-YAACGAGCGCAACCC-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). An annealing temperature of 55°C or 61°C was used for bacterial or archaeal primers, respectively. Bacterial 16S rRNA gene amplicons were not detected in any of the DNA extracts from isolated cultivars. Archaeal 16S rRNA gene amplicons from each isolate were purified, cloned, sequenced, assembled, and analyzed using previously published methods (Boyd et al., 2007a).

Purified DNA that was determined to be “PCR-amplifiable” was subjected to PCR amplification of the *sor* gene. Primers *sorF* (5'-GGACCTTGGGAGCCAATTTA-3') and *sorR* (5'-CTCCAGAATGTTCTTCATCA-3') were designed to target positions 10 to 588 in the *sor* gene from *Acidianus* strain DS80 (IMG gene ID 2690452492, genome ID 2690315630) as well as other *sor* homologs from *Acidianus* spp. available in the NCBI database. These primers were specifically designed to not

amplify the *sor* fragment from closely related Sulfolobales strains (i.e., *Sulfolobus solfataricus*). PCR amplification of *sor* was optimized using genomic DNA from *Acidianus* sp. DS80 as a positive control and genomic DNA from *S. solfataricus* (kindly provided by Mark Young) as a negative control. PCR cycling conditions included an initial 4 min denaturation step (94°C) followed by 30 rounds of denaturation (94°C, 1 min), annealing (50°C; 1 min), and extension (72°C, 1 min), with a final extension step at 72°C. PCR reactions included ~ 0.5 ng of DNA, 1.5 mM MgCl<sub>2</sub> (Invitrogen), 200 µM of each deoxynucleotide triphosphate (Sigma-Aldrich, USA), 0.5 µM of each forward and reverse primer, 0.4 µg µL<sup>-1</sup> molecular-grade bovine serum albumin (Roche, USA), and 0.25 U Taq DNA Polymerase (Invitrogen) in 1X PCR buffer (Invitrogen) (50 µL final volume). PCR amplification was verified by electrophoresis in a 1.5% agarose gel.

**Culture conditions.** *Acidianus* strain DS80, previously isolated from “Dragon spring”, YNP, Wyoming (NHSP 106; YNP thermal inventory ID) (Amenabar et al., 2017), was cultivated in anoxic base salts mineral medium as previously described (Amenabar et al., 2017). The pH of the medium was adjusted to 3.0 with concentrated hydrochloric acid. Briefly, 55 mL of medium was dispensed into 160 mL serum bottles and was subjected to autoclave sterilization. For growth via S<sup>0</sup> disproportionation, 18 mL of media was used instead of 55 mL to increase the headspace/liquid volume ratio, allowing for greater partitioning of H<sub>2</sub>S into the gas phase [pK<sub>a</sub> of H<sub>2</sub>S<sub>g</sub>/HS<sup>-</sup> is ~6.6 at 80°C (Amend and Shock, 2001)] thereby maintaining a more favorable environment (thermodynamically) as described in more detail in the discussion. Following autoclave sterilization and while still hot (~90°C), filter sterilized (0.22 µm) Wolfe’s vitamins (1



mL L<sup>-1</sup> final concentration) and filter sterilized (0.22 µm) SL-10 trace metals (1 mL L<sup>-1</sup> final concentration) was added. S<sup>o</sup> (sterilized by baking dry at 100°C for 24 h) was added at a final concentration of 5 g L<sup>-1</sup>. Fe<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub> • H<sub>2</sub>O (sterilized by 0.22 µm filtration) was added at a final concentration of 3.7 g L<sup>-1</sup> while ferrihydrite [prepared aseptically using sterilized reagents according to previously described procedures (Lovley and Phillips, 1987)] was added at a final concentration of 1 g L<sup>-1</sup>. Briefly, ferrihydrite was formed by neutralizing a filter sterilized (0.22 µm) 0.4 M solution of FeCl<sub>3</sub> to a pH of 7.0 with filter sterilized (0.22 µm) NaOH followed by washing with filter sterilized (0.22 µm) deionized water. The suspension of synthetically produced ferrihydrite was deoxygenated by stirring under sterile N<sub>2</sub> passed over heated (210°C) and hydrogen (H<sub>2</sub>) reduced copper shavings and repeated flushing of the headspace in a sealed serum bottle.

Following addition of nutrient amendments, the bottles and their contents were deoxygenated by purging with O<sub>2</sub>-free, sterile nitrogen (N<sub>2</sub>), as described above. The serum bottles were sealed with butyl rubber stoppers and heated to 80°C prior to the replacement of the headspace. A headspace gas mixture of 80%:20% N<sub>2</sub>:CO<sub>2</sub> was used when S<sup>o</sup> was supplied as the electron donor and Fe<sup>3+</sup> the electron acceptor or when S<sup>o</sup> was supplied as electron donor and acceptor (disproportionation). A headspace gas mixture of 80%:20% H<sub>2</sub>:CO<sub>2</sub> was used when S<sup>o</sup> or Fe<sup>3+</sup> (soluble and mineral) were supplied as the electron acceptor. All cultures were grown autotrophically in medium with a pH of 3.0 and were incubated at 80°C. Unless otherwise stated, all growth experiments were performed in triplicate, and a single uninoculated control was included for use in monitoring abiotic chemical reactions.

The ability of strain DS80 to oxidize  $\text{MnCl}_2$  and  $\text{FeCl}_2$  was tested under aerobic autotrophic growth conditions. Following autoclave sterilization, base salts mineral medium was supplemented with filter sterilized ( $0.22\ \mu\text{m}$ ) Wolfe's vitamins ( $1\ \text{mL L}^{-1}$  final concentration) and filter sterilized ( $0.22\ \mu\text{m}$ ) SL-10 trace metals ( $1\ \text{mL L}^{-1}$  final concentration).  $\text{MnCl}_2$  and  $\text{FeCl}_2$  were added from filter sterilized ( $0.22\ \mu\text{m}$ ) solutions to a final concentration of  $0.25\ \text{g L}^{-1}$  and  $3\ \text{g L}^{-1}$ , respectively.

**Mineral surface requirements.** The requirement for physical contact with minerals when they serve as electron donor or acceptor, or both electron donor and acceptor (disproportionation conditions), was evaluated by sequestering minerals in dialysis tubing with varying pore size diameters, as previously described (Boyd and Druschel, 2013). Four different growth conditions were examined: 1)  $\text{H}_2$  as electron donor and  $\text{S}^\circ$  as electron acceptor, 2)  $\text{S}^\circ$  as electron donor and ferric sulfate as electron acceptor, 3)  $\text{H}_2$  as electron donor and ferrihydrite as electron acceptor, and 4)  $\text{S}^\circ$  as electron donor and acceptor (disproportionation).  $\text{S}^\circ$  was sequestered in dialysis tubing in conditions 1, 2, and 4 while ferrihydrite was sequestered in dialysis tubing in condition 3.  $\text{S}^\circ$  or ferrihydrite were added to dialysis membranes (Spectrum Laboratories, Gardena, CA) with pore sizes of 6-8 kDa or 12-14 kDa, followed by closure with dialysis clips. Prior to use, all dialysis membranes were incubated at  $80^\circ\text{C}$  in nanopure deionized water for 4 hours to remove preservatives and this was repeated a total of 3 times, with replacement of deionized water each of the times. Experiments where direct contact was allowed between cells and minerals (when  $\text{S}^\circ$  or ferrihydrite was not sequestered) were

performed in the presence of dialysis membranes (12-14 kDa) to account for the potential interactions between cells, membranes, and/or minerals.

**Characterization of activity with solid phase iron minerals.** The ability for strain DS80 to reduce various soluble and mineral forms of ferric iron was examined using  $S^0$  (5 g L<sup>-1</sup>) as an electron donor with an 80%:20% N<sub>2</sub>-CO<sub>2</sub> headspace in anoxic base salts medium. Sterilized medium was prepared as described above and was supplemented with Fe<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub> • H<sub>2</sub>O (3.7 g L<sup>-1</sup>), ferrihydrite (1.0 g L<sup>-1</sup>), goethite (1.0 g L<sup>-1</sup>), or hematite (1.0 g L<sup>-1</sup>). Commercially available goethite ( $\alpha$ -FeOOH) and hematite ( $\alpha$ -Fe<sub>2</sub>O<sub>3</sub>) were used in these experiments.  $\alpha$ -FeOOH and  $\alpha$ -Fe<sub>2</sub>O<sub>3</sub> were sterilized by baking at 100°C for 24 h. Synthetic ferrihydrite was produced as described above. Cell production was only monitored at the end of these growth assays.

**Evaluation of growth and activity.** Growth and/or activity of strain DS80 with different electron donors and acceptors was quantified in terms of cell density and production of total sulfide (S<sup>2-</sup>; proxy for  $S^0$  reduction), production of sulfate (SO<sub>4</sub><sup>2-</sup>; proxy for  $S^0$  oxidation), production of ferrous iron (Fe<sup>2+</sup>; proxy for Fe<sup>3+</sup> reduction) or production of ferric iron (Fe<sup>3+</sup>; proxy for Fe<sup>2+</sup> oxidation) using methods previously described (Amenabar et al., 2017).

## RESULTS

**Distribution of *Acidianus* spp. in YNP hot springs.** Primers specific for the amplification of *sor* from *Acidianus* spp. were used to determine the distribution of these genes in sediments sampled from 73 hot springs that spanned a pH gradient from 1.60 to

9.27 and a temperature range from 21.1°C to 90.1°C as a proxy for the distribution of strain DS80 and closely related *Acidianus* strains. *sor* amplicons were detected in DNA extracts from 18 of the 73 samples, all of which had acidic pH (<4.9) and elevated temperatures (>42°C) (**Fig. 1**). The 18 samples were collected from hot springs from geographically distinct locations in YNP including the One Hundred Springs Plain area of NGB, Crater Hills (CH), Geyser Creek, the Obsidian Pool area, the Sylvan Spring area, and the Nymph Lake (NL) area. These sediments visibly contained varying combinations of S° and iron (hydr)oxides. This suggested the potential use of these minerals by *Acidianus* spp. Moreover, these results suggest that *Acidianus* spp. have a broad ecological niche that spans a pH gradient of 1.60 to 4.84, a temperature gradient of 42.3°C to 89.7°C, and a gradient in the availability of redox active mineral substrates.

To confirm that *Acidianus* spp. were present and viable in these springs, dilution to extinction serial enrichments were conducted using anoxic mineral salts growth medium amended with H<sub>2</sub> as electron donor and S° as electron acceptor with CO<sub>2</sub> as a carbon source. Positive enrichments were obtained from all 5 of the springs where enrichments were performed. These include a S° rich spring in CH informally called “Alice Spring” [80.7°C, pH 2.32; YNP thermal inventory ID CHA043 (Hochstein et al., 2016)], a S° rich spring in NL informally called “NL\_2” [80.0°C, pH 2.50; YNP thermal inventory ID NMC051 (Inskeep et al., 2013)], a S° rich spring in NGB (88.6°C, pH 2.66; 44°43'45.25"N; 110°42'39.16"W, YNP thermal ID not available), anhydrous ferric oxide and S° rich spring in NGB informally called “Dragon Spring” [80.0°C, pH 3.09; YNP thermal inventory ID NHSP 106 (Langner et al., 2001)], and a S° rich spring in NGB

(89.7°C, pH 2.79; YNP thermal inventory ID NHSP116). Images of these springs are presented in **Supp. Fig. 4**. Serial passage of these enrichments, in combination with dilution to extinction, resulted in cultures with a single morphotype. Sequencing of 12 archaeal clones from each of the five cultures yielded a single 16S rRNA gene phylotype. 16S rRNA gene sequences from the 5 cultures ranged from being 99% to 100% identical to the 16S rRNA gene from *Acidianus hospitalis* (accession number: CP002535). We used the culture isolated from “Dragon Spring” (NHSP 106), designated as strain DS80 (Amenabar et al., in review; Amenabar et al., 2017) and that has a 16S rRNA gene (accession number: KX608545) that is 100% identical to that from *A. hospitalis*, in the physiological studies described below.

#### **Role of DS80 in the potential formation of different solid phase minerals.**

Analysis of the DS80 genome revealed the presence of genes putatively involved in the transport of divalent metal cations [i.e., homologs of MntH (Makui et al., 2000)] that may allow for transport of  $\text{Mn}^{2+}$  or  $\text{Fe}^{2+}$  for use as electron donors (Amenabar et al., in review). These observations prompted experiments aimed at determining the ability for strain DS80 to oxidize these metals, and potentially contribute to the formation of  $\text{MnO}_2$  or iron (hydr)oxides in hot spring environments. Under aerobic conditions, strain DS80 was unable to couple growth to the oxidation of  $\text{Fe}^{2+}$  or  $\text{Mn}^{2+}$  (data not shown), suggesting it is not involved in the formation of iron (hydr)oxides or  $\text{MnO}_2$  in hot spring environments.

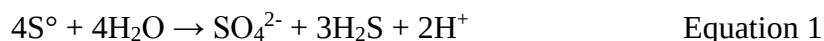
**Surface requirement for mineral dependent growth.** The ability for “insoluble” minerals such as  $S^0$  and iron (hydr)oxides to support growth prompted an investigation into the requirement for access to mineral surfaces by strain DS80 when these are supplied as sole electron donors or acceptors, or both.  $H_2/S^0$  grown DS80 cells grew and reduced  $S^0$  when it was sequestered in dialysis tubing with different pore sizes indicating that physical contact with bulk solid-phase  $S^0$  was not necessary (**Fig. 2**). Moreover, rates of growth and activity were dependent on the dialysis membrane pore size, with higher rates detected when  $S^0$  was sequestered in dialysis membranes with larger pores. This indicates that the sulfur compound that is serving as an electron acceptor exhibits a distribution of particle sizes

Specific growth yields, or the number of cells produced per mol of product produced, when strain DS80 was cultivated with direct access to  $S^0$  or when  $S^0$  was sequestered in dialysis membranes were not significantly different (**Table 1**). This indicates that sequestration of  $S^0$  in dialysis membranes did not impact the functioning of cellular metabolism. Generation times, calculated during the steepest period of growth, were  $25.7 \pm 5.7$  hrs when cells were provided access to  $S^0$  whereas they were  $40.4 \pm 2.2$  hrs and  $178.9 \pm 77.3$  hrs when  $S^0$  was sequestered in dialysis tubing with pore sizes of 12 to 14 kDa and 6 to 8 kDa, respectively. It is not clear that cells grown with  $S^0$  sequestered in dialysis membranes with the restricted pore size of 6 to 8 kDa entered log phase growth, which may indicate cell death is nearly balancing cell production in these cultures. Moreover, the final cell densities decreased by 41% and 68% when  $S^0$  was sequestered in dialysis tubing with pore sizes of 12 to 14 kDa and 6 to 8 kDa,

respectively, when compared to cultures grown with  $S^{\circ}$  in the bulk medium. The decrease in growth rate and cell production when  $S^{\circ}$  is sequestered in dialysis membranes with smaller pore sizes indicates that the sulfur compound that is serving as an electron acceptor is limiting growth.

Growth was not observed when cells were grown with  $S^{\circ}$  as electron donor and  $Fe^{3+}$  (as ferric sulfate) as the electron acceptor when  $S^{\circ}$  was sequestered in dialysis tubing (**Fig. 3**). Thus, in contrast to cells reducing  $S^{\circ}$ , cells oxidizing  $S^{\circ}$  require direct physical contact with the mineral to catalyze its oxidation. This suggests that the soluble form of  $S^{\circ}$  that serves as electron acceptor in cultures reducing  $S^{\circ}$  is either not present, is not in high enough concentration to support growth in cultures oxidizing  $S^{\circ}$ , or is unable to serve as electron donor for these cells.

Cells of strain DS80 were capable of  $S^{\circ}$  disproportionation under autotrophic growth conditions and this reaction supported cell growth. The cell density, the amount of total sulfide and the amount of sulfate increased concurrently in cultures of strain DS80 grown with  $S^{\circ}$  as the sole electron donor and acceptor (**Fig. 4**).  $S^{\circ}$  was disproportionated to hydrogen sulfide ( $H_2S$ ;  $4.95 \pm 1.12 \mu\text{mol}$  at the end of exponential phase) and sulfate ( $SO_4^{2-}$ ;  $1.36 \pm 0.10 \mu\text{mol}$  at the end of exponential phase) at a stoichiometry close to 3 mols sulfide ( $3.67 \pm 1.05$ ) per mol of sulfate produced, which agrees with the predicted stoichiometry of  $S^{\circ}$  disproportionation to these products:



Cells growing via  $S^{\circ}$  disproportionation, like those growing via  $S^{\circ}$  respiration, did not require direct physical contact with the mineral to catalyze its simultaneous oxidation

to  $\text{SO}_4^{2-}$  and reduction to  $\text{H}_2\text{S}$  (data not shown). This suggests that the soluble form of  $\text{S}^\circ$  that serves as electron acceptor in cultures reducing  $\text{S}^\circ$  is also capable of supporting growth under disproportionation conditions.

The requirement for access to solid phase  $\text{Fe}^{3+}$  as an electron acceptor was also evaluated in cultures of DS80. Cells were capable of growth with ferrihydrite sequestered in dialysis membranes (**Fig. 5**), indicating that DS80 cells do not require direct access to this solid phase mineral to use it as electron acceptor. However, differences in growth kinetics and  $\text{Fe}^{3+}$  reduction activity were observed when cells were allowed direct contact with ferrihydrite when compared to those when ferrihydrite was sequestered in dialysis membranes. While the specific growth yield, or the amount of iron reduced per cell produced, did not vary significantly under these conditions (**Table 1**), cells provided with direct access to ferrihydrite exhibited log phase growth. In contrast, cultures grown with ferrihydrite sequestered in dialysis membranes did not appear to enter a log phase of growth which may be due to oxidant ( $\text{Fe}^{3+}$ ) limitation. Like cells growing with sequestered  $\text{S}^\circ$ , this may indicate that cell death is nearly balancing cell production in cells growing with sequestered ferrihydrite.

The generation time (calculated during the steepest period of growth) of cultures provided direct access to ferrihydrite was  $74.8 \pm 2.8$  hrs whereas generation times of cultures grown with ferrihydrite sequestered in dialysis tubing with a pore size of 12 to 14 kDa or 6 to 8 kDa were  $156.8 \pm 37.8$  hrs and  $121.2 \pm 57.7$  hrs, respectively, the latter of which were statistically indistinguishable ( $P = 0.32$ ). The observation that rates of iron reduction and generation times of cultures provided with ferrihydrite sequestered in



dialysis tubing with a pore size of 12 to 14 kDa or 6 to 8 kDa were not significantly different, indicates that the soluble form of  $\text{Fe}^{3+}$  that is being reduced does not exhibit a size distribution over this size range. This could include  $\text{Fe}^{3+}$  ions or soluble  $\text{Fe}^{3+}$  containing complexes (see Amenabar et al., 2017 for a discussion). Alternatively, this could indicate that a soluble electron shuttle is being used to reduce  $\text{Fe}^{3+}$ , although this shuttle would necessarily be limited in size to  $< 6$  kDa. Regardless, these observations suggest that strain DS80 is reducing the solid phase ferrihydrite, but it can be sustained by reduction of acid solubilized ferric iron. Moreover, the rate of dissolution may be what is limiting activity and growth. Alternatively, these data may indicate that the metabolic costs of synthesizing compounds or complexes associated with extracellular electron transport leads to a reduction in growth rate and activity.

**Effect of iron source on rates of reduction.** Observations indicating that DS80 cells are possibly reducing a soluble form of  $\text{Fe}^{3+}$  prompted additional experimentation aimed at characterizing the effect of  $\text{Fe}^{3+}$  mineral solubility on the rate of  $\text{Fe}^{3+}$  reduction. Cells of strain DS80 utilized a variety of  $\text{Fe}^{3+}$  sources as electron acceptors, including ferric sulfate [ $\text{Fe}_2(\text{SO}_4)_3$ ], amorphous ferrihydrite ( $\text{Fe}_2\text{O}_3 \bullet 0.5\text{H}_2\text{O}$ ), goethite ( $\alpha\text{-FeOOH}$ ), and hematite ( $\alpha\text{-Fe}_2\text{O}_3$ ) (**Fig. 6**). Rates of  $\text{Fe}^{3+}$  reduction when autotrophically grown cells were supplied with  $\text{Fe}_2(\text{SO}_4)_3$ ,  $\text{Fe}_2\text{O}_3 \bullet 0.5\text{H}_2\text{O}$ ,  $\alpha\text{-FeOOH}$ , and  $\alpha\text{-Fe}_2\text{O}_3$  as electron acceptor and  $\text{S}^\circ$  as electron donor were  $2532 \pm 450 \text{ nmol hr}^{-1}$ ,  $238 \pm 1 \text{ nmol hr}^{-1}$ ,  $93 \pm 4 \text{ nmol hr}^{-1}$ , and  $39 \pm 2 \text{ nmol hr}^{-1}$ , respectively. These rates of  $\text{Fe}^{3+}$  reduction were broadly consistent with the equilibrium solubility of the different iron sources tested:  $\text{Fe}_2(\text{SO}_4)_3 > \text{Fe}_2\text{O}_3 \bullet 0.5\text{H}_2\text{O}$  ( $K_s = 3.55$ )  $> \alpha\text{-FeOOH}$  ( $K_s = 0.36$ )  $> \alpha\text{-Fe}_2\text{O}_3$  ( $K_s = -0.53$ )

(Kraemer, 2004). In all growth conditions, cell concentrations at the end of the growth experiment significantly exceeded those at the start of the experiments (data not shown), indicating that these redox reactions could support cellular growth.

## DISCUSSION

The detection of *Acidianus* spp. *sor* genes and the cultivation of *Acidianus* strains with 16S rRNA genes identical to DS80 across wide gradients in pH, temperature, and mineralogical composition motivated physiological studies aimed at determining whether the spectrum of minerals that have been detected in hot springs could support growth of this strain. Moreover, a prior report that showed the ability of strain DS80 to couple redox transformation of two solid phase minerals [oxidation of  $S^{\circ}$  via reduction of ferrihydrite (Amenabar et al., 2017)] prompted additional experiments aimed at understanding the requirement for access to mineral surfaces in order for those minerals to support metabolism.

Strain DS80 was capable of growth with mineral substrates including  $S^{\circ}$  and a variety of iron (hydr)oxides, consistent with the presence of many of these minerals in the hot springs where DS80 and closely related strains were isolated from [e.g., “Dragon spring” (Inskeep and McDermott, 2005; Langner et al., 2001); “NL\_2” and “CH\_1” (Inskeep et al., 2013)]. Previous studies have shown members of the Sulfolobales are capable of using a variety of minerals to support metabolism; however, most of these studies focused on the role of strains in the dissolution of sulfidic ore minerals (pyrite, sphalerite, and chalcopyrite) or the oxidation of  $S^{\circ}$  (Huber and Prangishvili, 2006). Just a

few thermoacidophiles, including strain DS80, have been shown to reduce  $\text{Fe}^{3+}$  (Amenabar et al., 2017; Giaveno et al., 2013; Yoshida et al., 2006). However, only *Acidianus manzaensis* and strain DS80 have been shown to reduce iron minerals (Amenabar et al., 2017; Yoshida et al., 2006).

Strain DS80 was shown to couple  $\text{S}^0$  disproportionation to growth, which is the first report of the occurrence of this metabolic process in an archaeon and is the first report of the occurrence of this process in acidic conditions. To this end, strain DS80 appears to be evolutionarily optimized in its ability to use  $\text{S}^0$  to support metabolism, with a demonstrated ability to reduce, oxidize, and disproportionate this mineral. This observation, combined with the ability of this strain to use several iron (hydr)oxides minerals, potentially explains the observed widespread distribution of this strain and closely related strains across numerous springs with varying assemblages of minerals. Arguably, the diverse assemblage of minerals capable of supporting growth of this strain is comparable to that of *Geobacter* spp. and *Shewanella* spp., the bacterial models for understanding the use of minerals in microbial metabolism (Fredrickson et al., 2008; Lovley et al., 2004; Nealson and Scott, 2006; Shi et al., 2007; Shi et al., 2009).

The demonstrated use of multiple minerals to support metabolism of DS80 prompted experiments aimed at understanding mechanisms for how these cells access minerals during growth. Dialysis membranes were used to limit the access of cells to the surface of  $\text{S}^0$  and iron minerals during growth. Like the thermoacidophile *A. sulfurireducens* (Boyd and Druschel, 2013), strain DS80 did not require direct access to  $\text{S}^0$  to use it as an electron acceptor (**Fig. 2**). Boyd and Druschel suggested that *A.*

*sulfurireducens* was reducing a more soluble form of  $S^{\circ}$  (nanoparticulate  $S^{\circ}$ ) that existed in a range of particle sizes (Boyd and Druschel, 2013). This nanoparticulate  $S^{\circ}$  was shown to be formed through a series of biological/abiological feedbacks, whereby  $H_2S$  formed through the reduction of  $S^{\circ}$  generated soluble linear chains of  $S_x^{2-}$  (3-6 S atoms) through nucleophilic attack on bulk  $S^{\circ}$  rings. The formed  $S_x^{2-}$  rapidly disproportionates under acidic conditions, yielding soluble  $S_8$  molecular rings (denoted as  $S^{\circ}$  here) that rapidly aggregate or coarsen, reaching average diameters of 400 nm within several minutes of  $S_x^{2-}$  disproportionation (Boyd and Druschel, 2013; Garcia and Druschel, 2014). Thus, the rates of  $S_x^{2-}$  production and disproportionation, the former of which is dependent on the rate of  $H_2S$  produced by biological activity, and the kinetics of coarsening are likely to generate a distribution of soluble  $S^{\circ}$  particle sizes available for use in metabolism. Consistent with this notion, growth experiments with strain DS80 reported here and those reported previously for *A. sulfurireducens* (Boyd and Druschel, 2013) showed that the electron acceptor (i.e., nanoparticulate  $S^{\circ}$ ) that supported growth exhibited an apparent size dependence, with higher rates of reduction and shorter generation times observed when  $S^{\circ}$  was sequestered in dialysis membranes with larger pore sizes. These results suggest that a similar mechanism involving biological and abiological feedbacks is likely supporting  $S^{\circ}$  dependent growth in strain DS80.

The requirement for direct access to  $S^{\circ}$  differed depending on whether the cells were using the mineral as an oxidant, a reductant, or both as a reductant and oxidant. Unlike DS80 cells reducing  $S^{\circ}$ , cells that were oxidizing  $S^{\circ}$  required direct access to the mineral. The requirement for direct contact to  $S^{\circ}$  to oxidize this mineral is consistent with

microscopic observations of cultures of other  $S^{\circ}$  oxidizing crenarchaeotes such as *Sulfolobus* spp. that were shown to be attached to  $S^{\circ}$  crystals in both lab cultures and in various hot spring environments (Brock et al., 1972). The results of the dialysis membrane experiments in cultures oxidizing  $S^{\circ}$ , which show no growth or activity when  $S^{\circ}$  is sequestered, also further substantiate the proposed mechanism for how cells access  $S^{\circ}$  under reducing conditions since the  $H_2S$  needed to initiate the series of abiotic reactions that solubilize  $S^{\circ}$  was incapable of being produced (Boyd and Druschel, 2013). Moreover, the soluble form of  $S^{\circ}$  that supported growth during  $S^{\circ}$  reduction exhibited an apparent size distribution, with membranes that had larger diameter pores supporting more activity and shorter generation times (**Fig. 2**), consistent with coarsening of formed nanoparticulate  $S^{\circ}$ . DS80 cells also did not require direct access to  $S^{\circ}$  to grow via  $S^{\circ}$  disproportionation (data not shown), suggesting that the  $H_2S$  formed through this process functioned to solubilize S through a mechanism like what is described above.

The lack of detection of  $SO_4^{2-}$  in cells growing with  $H_2/S^{\circ}$  (data not shown) and the stoichiometry of the products formed during growth with  $S^{\circ}/Fe^{3+}$  (to control for potential abiotic oxidation of  $H_2S$  by  $Fe^{3+}$  and to discount that cells were growing via disproportionation) suggests that the presence of  $H_2$  or  $Fe^{3+}$  favors growth via reduction or oxidation of  $S^{\circ}$  over disproportionation, respectively. This is likely a consequence of the energetic yields of these reactions, where the standard Gibbs free energy change ( $\Delta G^{\circ}$ ) at  $80^{\circ}C$  for the  $H_2/S^{\circ}$  and  $S^{\circ}/Fe^{3+}$  redox couples are  $-47.57 \text{ kJ mol}^{-1}$  and  $-298.76 \text{ kJ mol}^{-1}$ , respectively, in comparison to the thermodynamically unfavorable  $\Delta G^{\circ}$  of  $121.71 \text{ kJ mol}^{-1}$  for the  $S^{\circ}$  disproportionation condition [ $\Delta G^{\circ}$  calculations performed as

previously described (Amenabar et al., 2017)]. Consistent with the thermodynamic constraints for the  $S^0$  disproportionation growth condition, initial attempts to cultivate strain DS80 through disproportionation conditions failed. To produce and maintain favorable thermodynamics in bacterial cultures growing via  $S^0$  disproportionation at circumneutral to alkaline pH, other researchers have added sulfide scavenging compounds (either catalyze oxidation or precipitation of sulfide) such as ferric iron minerals (Finster et al., 1998; Janssen et al., 1996; Slobodkin et al., 2012; Thamdrup et al., 1993). However, for reasons mentioned above, adding ferric iron to cultures of DS80 growing via the disproportionation condition promotes oxidation of  $S^0$ . To overcome this limitation, we maximized the headspace to liquid volume ratio in our cultures (while still allowing for enough volume for sampling) to keep aqueous sulfide concentrations as low as possible. In doing so, we maintained favorable thermodynamics for the reaction over the course of a cultivation cycle (**Fig. 7**) and were able to demonstrate this unique biological activity. To our knowledge, this is the first report of a microbial strain that can reduce, oxidize, and disproportionate  $S^0$ .

The  $\Delta G$  of the  $S^0$  disproportionation reaction that sustained activity and cell production in cultures of DS80 ranged from  $-73.4 \text{ kJ mol}^{-1}$  to  $-32.9 \text{ kJ mol}^{-1}$  [ $-18.3$  to  $-8.2 \text{ kJ (mol e}^{-})^{-1}$  transferred respectively] (**Fig. 7**), which is close to thermodynamic equilibrium. Moreover, this range of values is similar than those that have been measured during log phase growth of  $-30 \text{ kJ mol}^{-1}$  (Frederiksen and Finster, 2004) but higher than the estimated value of  $-92 \text{ kJ mol}^{-1}$  (Thamdrup et al., 1993) for two neutrophilic bacterial cultures.

The  $\Delta G$  for cultures of DS80 during the growth cycle is close to the minimum Gibbs energy yield of  $-20 \text{ kJ mol}^{-1}$  suggested to be required to sustain microbial life (Schink, 1997) a value which is based on the energetics associated with formation of 1/3 of a mol of ATP from ADP and  $P_i$ . Growth of strain DS80, would be expected to be more favorable in a natural, open system where products were exsolved or consumed by another member of the community.

DS80 cells grew via reduction of iron minerals without direct access to the surface (**Fig. 5**), albeit with slower rates and slower generation times than when provided with access to the surface. This indicates that cells can reduce solid phase iron minerals, but are capable of simultaneous reduction of  $\text{Fe}^{3+}$  ions or complexes by acid dissolution of the mineral. These  $\text{Fe}^{3+}$  ions or complexes are free to diffuse across the dialysis membrane, allowing for iron reduction activity without direct access to the mineral. In contrast to  $S^\circ$ , growth assays indicated that the soluble form of iron that supports iron reduction does not exhibit a size distribution. Consistent with the notion of cells reducing a solubilized form of Fe and not the bulk mineral, cells were not obligately associated with the solid phase iron (hydr)oxides when they were given access to the mineral (data not shown). Moreover, the rate of reduction of a variety of iron minerals broadly corresponds to their solubility (Kraemer, 2004), with higher rates of reduction with poorly crystalline ferrihydrite than with crystalline hematite. It is difficult to deconvolute whether this is due to an increased ease of depositing electrons into poorly crystalline mineral lattices, as has been shown for a *Shewanella alga* strain (Roden and Zachara, 1996), or if this is due to increased reduction of soluble  $\text{Fe}^{3+}$  ions due to differences in

mineral solubility. Both considerations are likely important in natural hot spring systems, since both  $\text{Fe}^{3+}$  ions [**Supp. Fig. 1**; e.g., (Ball et al., 2010)] and a variety of Fe (hydr)oxides have been detected in acidic spring precipitates [e.g., (Inskeep et al., 2004; Kozubal et al., 2008; Livo et al., 2007)].

## CONCLUSIONS

Evidence presented here indicates that strain DS80 is capable of using the spectrum of redox active minerals that are commonly identified in acidic hot springs for growth and metabolism. These observations which may help to explain the apparent widespread distribution of this and related strains across broad temperature and pH gradients in hot springs. Other *Acidianus* strains may be equally as flexible in their mineral dependent metabolism, which help to explain the distribution of this genus in geochemically diverse thermal environments outside of Yellowstone, including both terrestrial and marine thermal sites (Giaveno et al., 2013; He et al., 2004; Plumb et al., 2007; Segerer et al., 1986; Yoshida et al., 2006).

The demonstrated ability of DS80 to grow via transformation of minerals suggests that it is likely influencing the chemical composition of the environment. As an example, there is no direct evidence that  $\text{Fe}^{3+}$  reduction is occurring in the iron oxide depositional zone from “Dragon Spring”; however, the limited accumulation of iron oxide (<1 cm depth in many regions) suggests that  $\text{Fe}^{3+}$  reduction must be an important component of the Fe cycle in these microbial mats (Inskeep and McDermott, 2005). The ability of strain DS80 to reduce iron minerals either with  $\text{H}_2$  or  $\text{S}^\circ$  at conditions mimicking those of the



spring environment may explain the limited accumulation of iron oxide observed in “Dragon Spring”. The same could be true for  $S^0$ , where the authors have observed rain events where the entirety of the  $S^0$  mats present in “Dragon Spring” were flushed out of the system, only to reappear the next day. This suggests that the rate of  $S^0$  precipitation, which must be high, is balanced by the rate of utilization by strains such as DS80 or other  $S^0$  reducing populations identified in this spring (Boyd et al., 2007b) or by natural flushing of the flocculant mineral.

Strain DS80 was shown to reduce, oxidize, and disproportionate  $S^0$ , indicating that the strain is evolutionary optimized to grow in sulfur rich hot spring habitats where the availability of electron donors (e.g.,  $H_2$ ) and electron acceptors (e.g., ferric iron) are likely to vary, both spatially and temporally (Fournier et al., 2002). Growth of strain DS80 was observed under conditions approaching thermodynamic equilibrium [-18.3 to -8.2 kJ (mol  $e^-$ )<sup>-1</sup> transferred] and this value was heavily dependent on the concentration of dissolved sulfide. This underscores the importance of sulfide scavenging mechanisms, either biological or abiological, in natural environments or on sulfide exsolving from the system in maintaining the feasibility of this process. This is the first report of the occurrence of  $S^0$  disproportionation in an acidophile and in an archaeon, thereby broadening the taxonomic and ecological space for where this process is of putative importance. We suggest that strain DS80 is a useful model microorganism for the study of the physiology and biochemistry of transformations of  $S^0$ , including the little studied process of  $S^0$  disproportionation.

Different minerals and different modes of metabolism imparted different requirements for surface access during growth. Cells reducing iron minerals did not need access to those minerals, presumably due to leaching of  $\text{Fe}^{3+}$  ions by the acidity of the growth medium, although growth and iron reduction activities were higher when direct access to the mineral was provided. Cells oxidizing  $\text{S}^{\circ}$  required direct access to the mineral whereas those reducing or disproportionating the mineral did not. A series of abiotic and biotic reaction feedbacks involving the biological production of  $\text{H}_2\text{S}$ , the formation of unstable  $\text{S}_x^{2-}$ , and the coarsening of nanoparticulate  $\text{S}^{\circ}$  are implicated in explaining these differences. Importantly, the interplay between the kinetics of these reactions likely influence the availability of  $\text{S}^{\circ}$  for reduction; more work is needed to identify the rate limiting step in this proposed feedback mechanism. We suggest a similar interplay of biotic- and abiotic-catalyzed processes are taking place in hot spring environments and together increase the availability of minerals capable of supporting microbial metabolism and growth.

## ACKNOWLEDGEMENTS

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**Table 1.** Growth yields of strain DS80 in base salts medium (80°C; pH 3.0). Cells were grown autotrophically with H<sub>2</sub> or S<sup>0</sup> as electron donor and S<sup>0</sup> or ferrihydrite as electron acceptors or with S<sup>0</sup> serving as both electron donor and acceptor (S<sup>0</sup> disproportionation). S<sup>0</sup> and ferrihydrite were sequestered in dialysis membranes of different pore sizes (pore sizes of 12 to 14 kDa and 6 to 8 kDa) to prevent physical contact with the bulk mineral. Values reported represent the average and standard deviation of triplicate measurements.

| Growth Condition                       | Direct or indirect access to mineral | Growth Yield (Cells/pmol product) <sup>a</sup> |
|----------------------------------------|--------------------------------------|------------------------------------------------|
| S <sup>0</sup> + H <sub>2</sub>        | Control                              | 12.0 ± 1.0                                     |
|                                        | 12-14 kDa                            | 12.4 ± 0.4                                     |
|                                        | 6-8 kDa                              | 15.0 ± 3.7                                     |
| S <sup>0</sup> + Fe <sup>3+</sup>      | Control                              | 7.3 ± 0.8                                      |
|                                        | 12-14 kDa                            | N.G.                                           |
|                                        | 6-8 kDa                              | N.G.                                           |
| S <sup>0</sup><br>(disproportionation) | Control                              | 14.0 ± 4.1                                     |
|                                        | 12-14 kDa                            | N.D.                                           |
|                                        | 6-8 kDa                              | N.D.                                           |
| H <sub>2</sub> + Fe <sup>3+</sup>      | Control                              | 3.4 ± 1.3                                      |
|                                        | 12-14 kDa                            | 2.7 ± 0.5                                      |
|                                        | 6-8 kDa                              | 3.9 ± 0.5                                      |

<sup>a</sup>Growth yields are reported as per pmol H<sub>2</sub>S (S<sup>0</sup> + H<sub>2</sub> growth condition), per pmol of Fe<sup>2+</sup> (S<sup>0</sup> + Fe<sup>3+</sup> and H<sub>2</sub> + Fe<sup>3+</sup> growth conditions), and per pmol of H<sub>2</sub>S (S<sup>0</sup> disproportionation growth condition). Abbreviations: N.G., No growth; N.D., not determined.

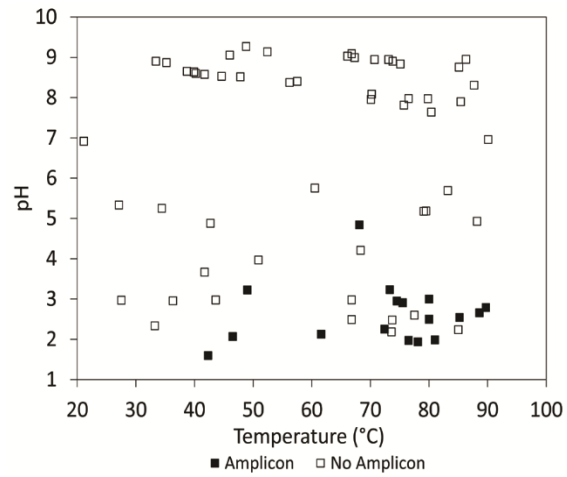


**Figure 1.** The presence (black rectangles) or absence (open rectangles) of *Acidianus* spp. *sor* amplicons in DNA extracts from sediments collected from 73 hot springs in Yellowstone National Park plotted as a function of the pH and temperature of those springs.

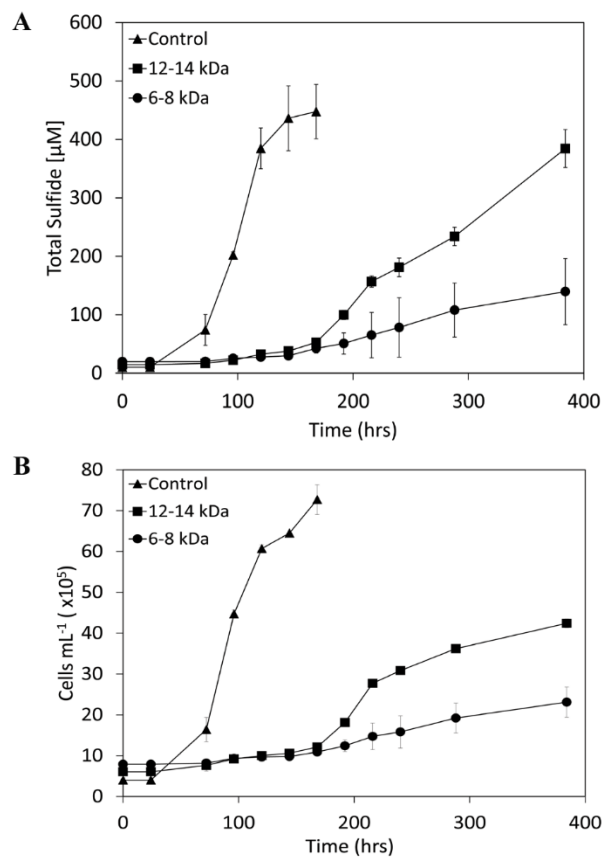


**Figure 2.** Total sulfide produced (**A**) and cell concentrations (**B**) in cultures of strain DS80 grown autotrophically with  $\text{H}_2$  as electron donor and  $\text{S}^\circ$  as electron acceptor.  $\text{S}^\circ$  was provided in the bulk medium (control) or was sequestered in dialysis membranes (pore sizes of 12 to 14 kDa and 6 to 8 kDa) to prevent physical contact with the bulk mineral.

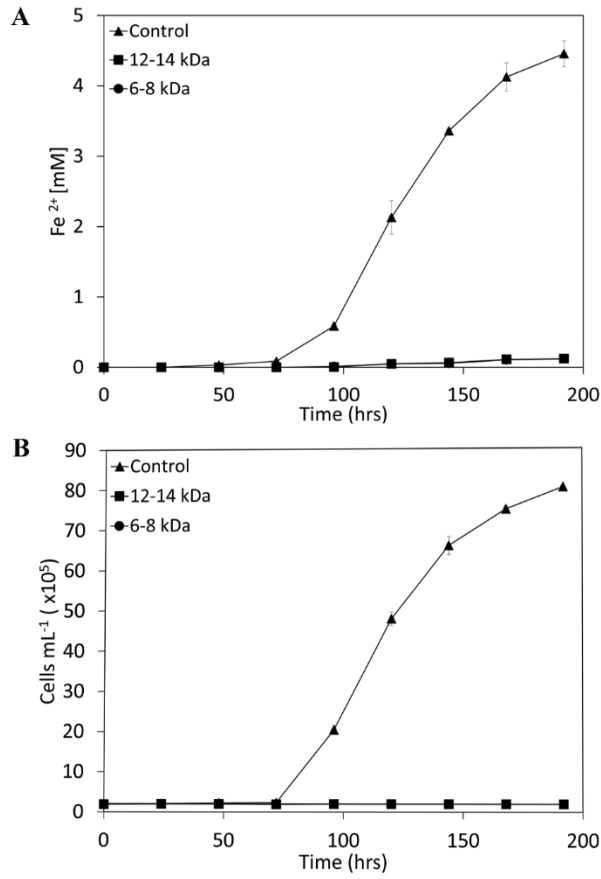


**Figure 3.**  $\text{Fe}^{2+}$  concentrations (**A**) and cell concentrations (**B**) in cultures of strain DS80 cultivated autotrophically with  $\text{S}^{\circ}$  as electron donor and  $\text{Fe}^{3+}$  (provided as ferric sulfate) as electron acceptor.  $\text{S}^{\circ}$  was provided in the bulk medium (control) or was sequestered in dialysis membranes (pore sizes of 12 to 14 kDa and 6 to 8 kDa) to prevent physical contact with the bulk mineral.

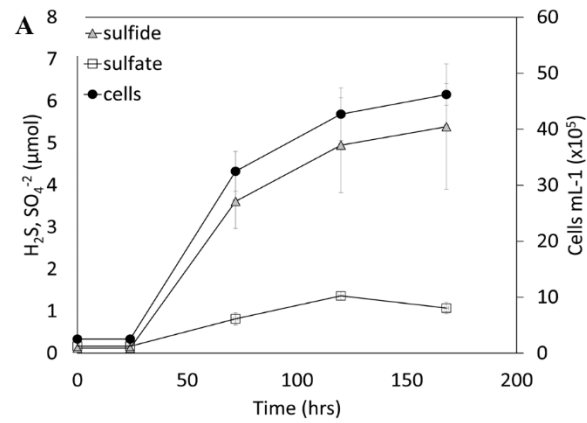


**Figure 4.** Autotrophic growth of strain DS80 with  $\text{S}^0$  provided as electron donor and electron acceptor ( $\text{S}^0$  disproportionation).

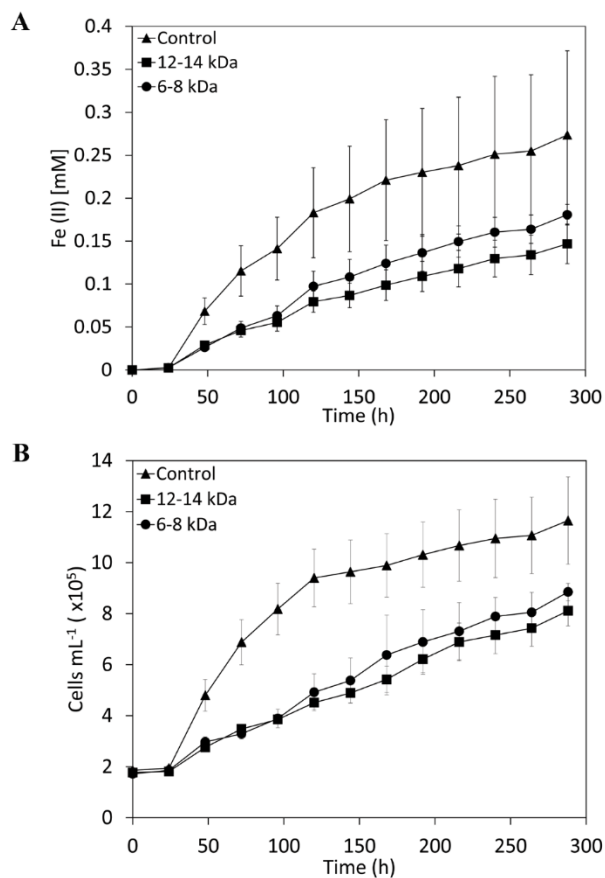


**Figure 5.**  $\text{Fe}^{2+}$  concentrations (**A**) and cell concentrations (**B**) in autotrophic cultures of strain DS80 grown with  $\text{H}_2$  as electron donor and ferrihydrite as electron acceptor. Ferrihydrite was provided in the bulk medium (control) or was sequestered in dialysis membranes (pore sizes of 12 to 14 kDa and 6 to 8 kDa) to prevent physical contact with the bulk mineral.



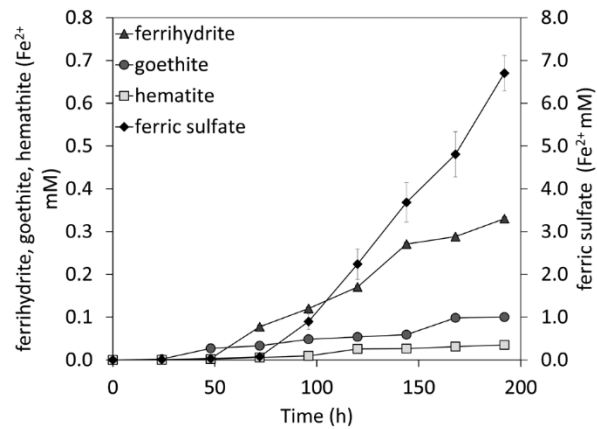


**Figure 6.** Fe<sup>2+</sup> production in cultures of strain DS80 grown autotrophically with S<sup>0</sup> as electron donor and ferrihydrite, goethite, hematite, or ferric sulfate as electron acceptor.

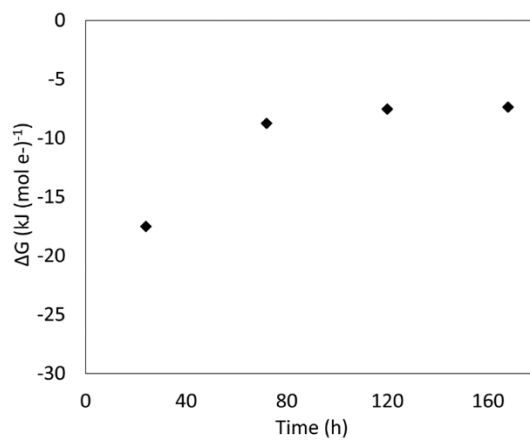
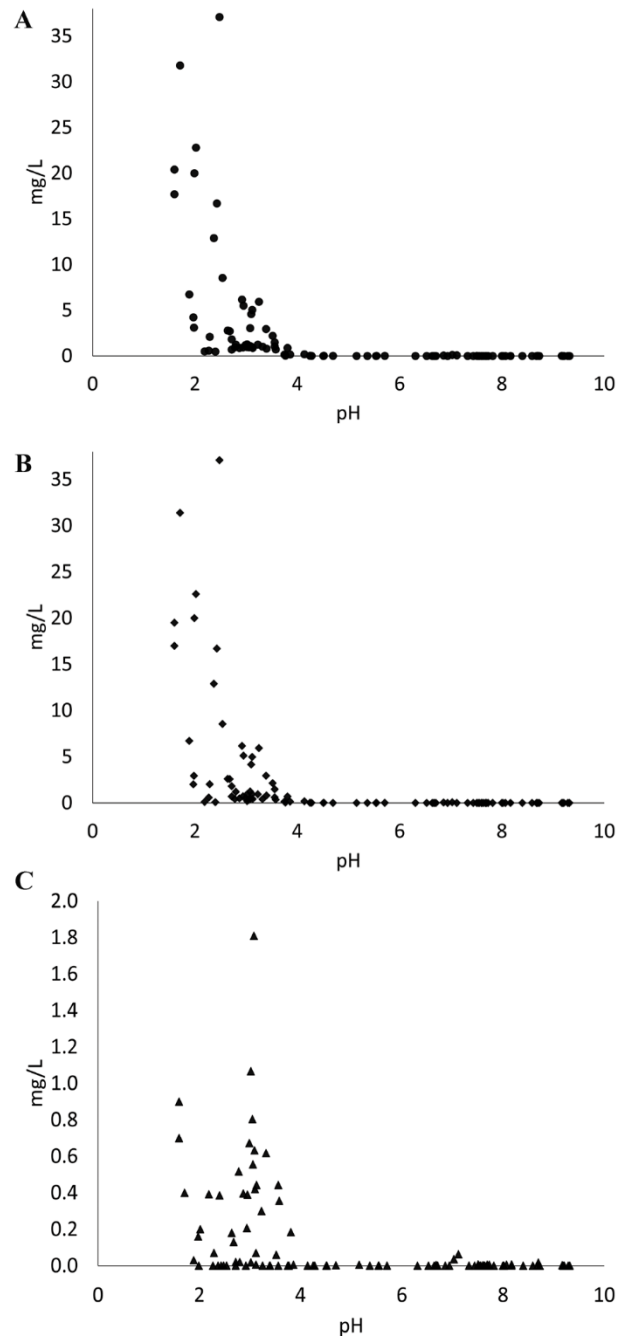
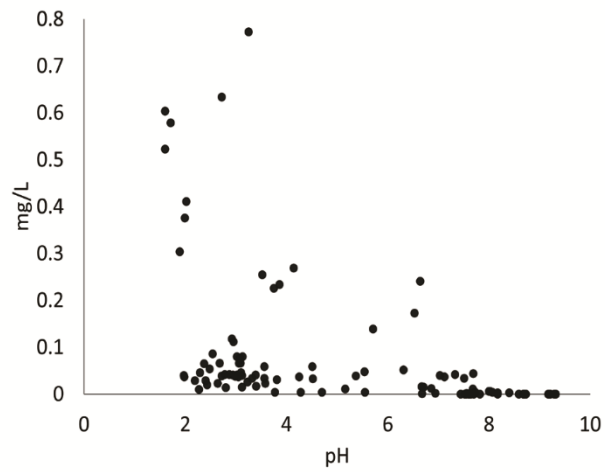


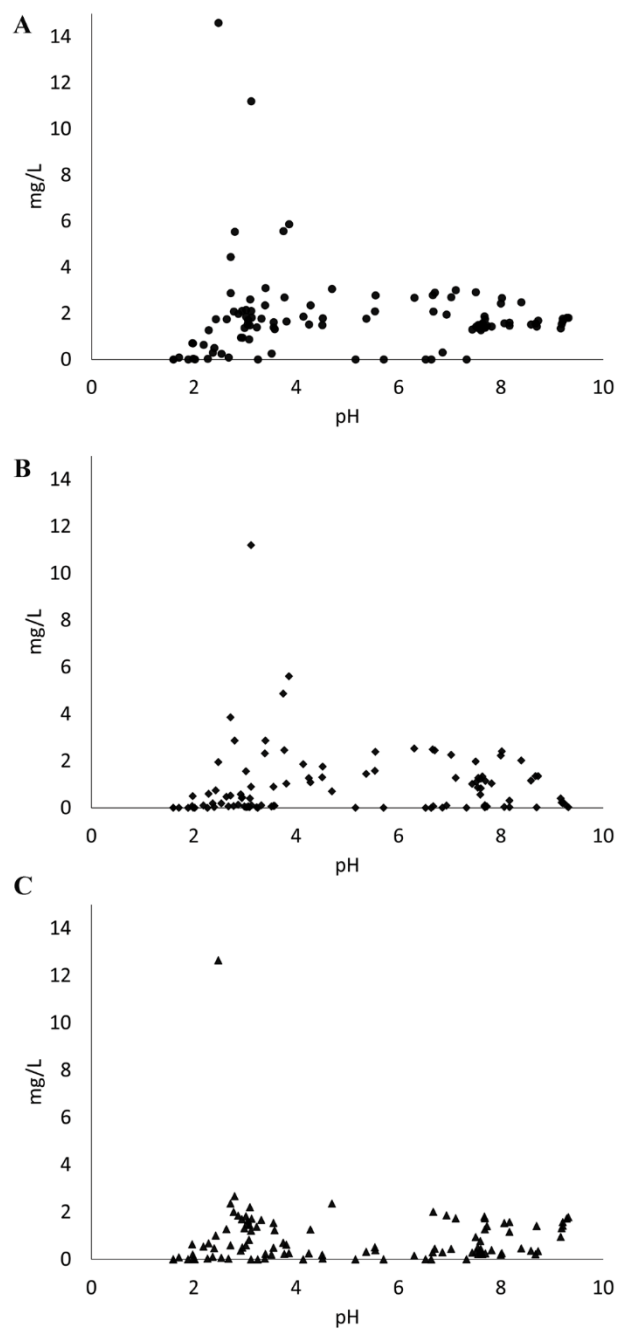
**Figure 7.** Available Gibbs free energy ( $\Delta G$ ) per mole of electrons transferred in DS80 cultures grown autotrophically with  $S^{\circ}$  as electron donor and acceptor ( $S^{\circ}$  disproportionation).



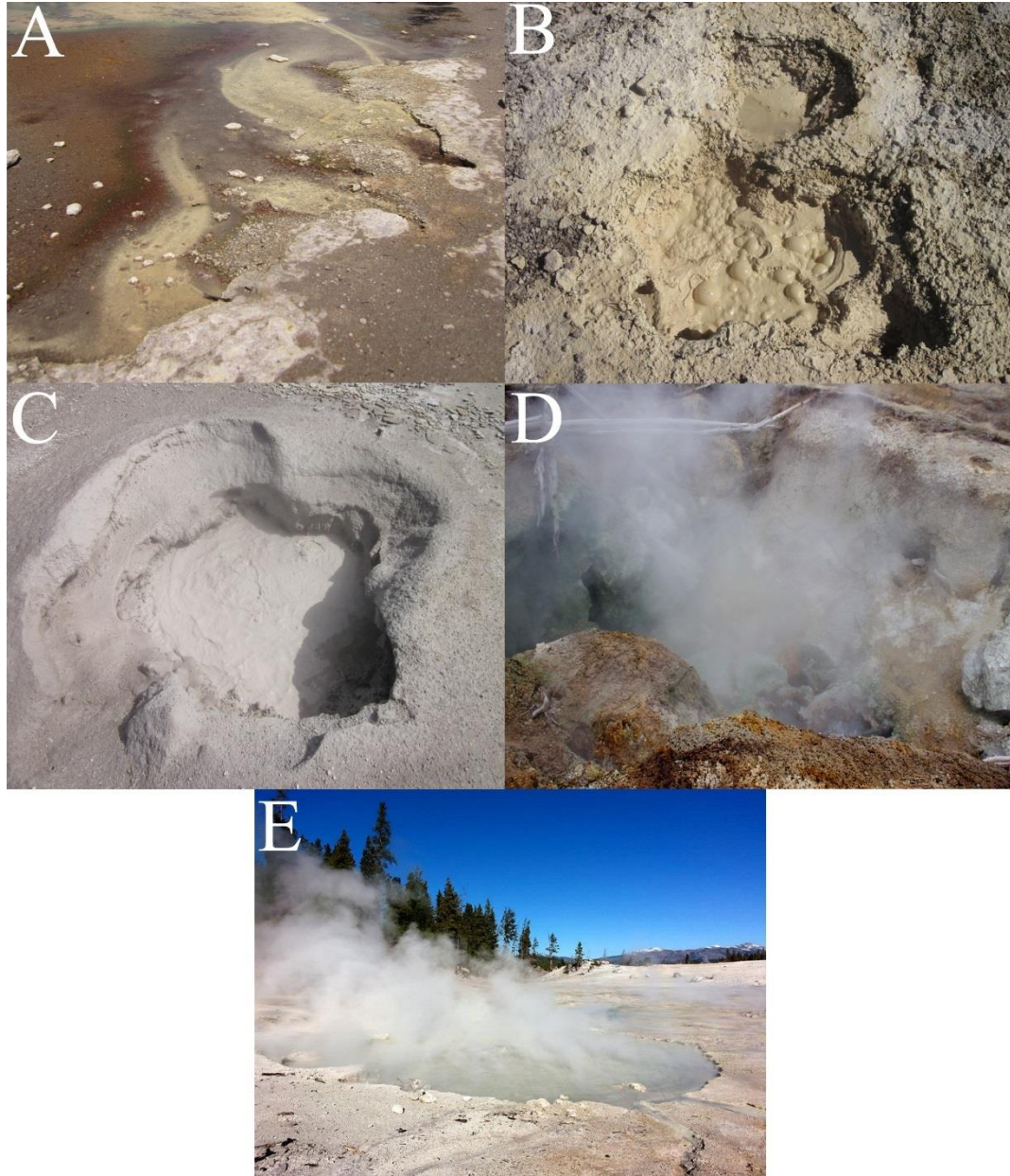
**Supplementary Figure 1.** Concentrations of dissolved total iron (A), iron (II) (B), and iron (III) (C) plotted as a function of hot spring pH in 103 features in Yellowstone National Park. Data was compiled from water chemistry analyses performed by the United States Geological Survey (Ball et al., 2010).



**Supplementary Figure 2.** Concentrations of dissolved total manganese plotted as a function of hot spring pH in 103 features in Yellowstone National Park. Data was compiled from water chemistry analyses performed by the United States Geological Survey (Ball et al., 2010).



**Supplementary Figure 3.** Concentrations of dissolved total arsenic (A), arsenic (III) (B), and arsenic (V) (C) plotted as a function of hot spring pH in 103 thermal features in Yellowstone National Park. Data was compiled from water chemistry analyses performed by the United States Geological Survey (Ball et al., 2010).



**Supplementary Figure 4.** Images of the acidic hot springs in Yellowstone National Park where *Acidianus* strains were isolated. (A) “Dragon spring” (YNP thermal ID NHSP 106) in the Norris Geyser Basin has a temperature of 80.0°C and pH of 3.09; (B) Unnamed spring (YNP thermal ID NHSP116) in the Norris Geyser Basin has a temperature of 89.7°C and pH of 2.79; (C) Unnamed spring (44°43'45.25"N; 110°42'39.16"W, YNP thermal ID not available) in the Norris Geyser Basin has a temperature of 88.6°C and pH of 2.66; (D) “NL\_2” (YNP thermal ID NMC051) in the Nymph Lake thermal area has a temperature of 80.0°C and pH of 2.50; (E) “Alice spring” (YNP thermal ID CHA043) in Crater Hills has a temperature of 80.7°C and pH of 2.32. Photo credits: Eric Boyd (A), Maximiliano Amenabar (B, C), Melody Lindsay (D, E).

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## CHAPTER FIVE

## CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

This dissertation focused on a sulfur-reducing crenarchaeote, named *Acidianus* strain DS80, isolated from an Acid-Sulfate-Chloride spring in Yellowstone National Park (YNP). The research presented in chapter 2 aimed to better define how metabolically flexible microbes select among available substrates and how this could be applied to better understand why the observed distribution of microorganisms and their activities often deviate from thermodynamic predictions using geochemical data obtained from natural environments. To accomplish this goal, we characterized the bioenergetics and growth yields of the metabolically flexible strain DS80 when grown autotrophically. Strain DS80 displayed versatility in its energy metabolism involving hydrogen ( $H_2$ ) or elemental sulfur ( $S^\circ$ ) as electron donors and  $S^\circ$  or ferric iron ( $Fe^{3+}$ ) as electron acceptors. The ability of strain DS80 to use these substrates in an overlapping way makes strain DS80 a good model system to examine factors that influence substrate preference.

A central tenet in microbiology is that growth substrates that maximize energy yield should be preferentially utilized by microorganisms (Froelich et al., 1979; Lovley and Klug, 1983; Nealson and Stahl, 1997; Roden and Wetzel, 2003). Thermodynamic calculations indicated that the redox couples  $S^\circ/Fe^{3+}$  and  $H_2/Fe^{3+}$  yield three- and four-fold more energy per mol of electron transferred, respectively, than the  $H_2/S^\circ$  redox couple. To test if strain DS80 used substrates based only on energy supply, we compared the generation times ( $T_n$ ) and growth yield of strain DS80 when grown with the redox

couples  $\text{H}_2/\text{S}^\circ$ ,  $\text{S}^\circ/\text{Fe}^{3+}$ , or  $\text{H}_2/\text{Fe}^{3+}$ . Growth experiment revealed that  $T_n$  were statistically indistinguishable between the three growth conditions indicating that measurements of  $T_n$  are not sensitive to processes that dictate substrate preference. However, biomass yields in *Acidianus* cultures provided with  $\text{H}_2/\text{S}^\circ$  were eight-fold greater than when cultures were provided with  $\text{S}^\circ/\text{Fe}^{3+}$  or  $\text{H}_2/\text{Fe}^{3+}$ . In addition, cells growing with the  $\text{H}_2/\text{S}^\circ$  redox couple required less energy to grow than cells respiring  $\text{Fe}^{3+}$ . Moreover, cells respiring iron, but not  $\text{S}^\circ$ , produced protrusions outside the membrane that resembled “nanowires” (Reguera et al., 2005; Gorby et al., 2006) which may represent an additional energetic cost to the iron reducing cells, an observation that might explain the lower amount of energy required for biomass synthesis in cells respiring  $\text{S}^\circ$ . These observations indicated that the electron transfer pathways used to conserve energy in cells growing with  $\text{H}_2/\text{S}^\circ$  are more efficient than those operating in cells respiring  $\text{Fe}^{3+}$  allowing for increased biomass synthesis. Indeed, cells of strain DS80 grown in the presence of all three growth substrates ( $\text{H}_2/\text{S}^\circ/\text{Fe}^{3+}$ ), simultaneously, preferred to use the  $\text{H}_2/\text{S}^\circ$  redox couple over the other redox couples.

Based on these observations we concluded that the preferential use of electron donors and acceptors in metabolically versatile strains, such as strain DS80 and probably other microorganisms as well, is dictated by difference in the energy demand of electron transfer reactions rather than the energy supply. This has significant implications in astrobiology and earth sciences, as it indicates that thermodynamic calculations of available energy alone are not enough to predict the distribution of microorganisms and their activities in natural systems. Furthermore, these observations may help explain



deviations from a standard stratification of metabolisms based on thermodynamic data in the environment (Canfield and Des Marais, 1991; D'Hondt et al., 2004; Boyd et al., 2010; Hansel et al., 2015). Results presented in chapter 2 suggested that combining thermodynamic calculations with measurements of biomass yields could be used to develop more accurate models for describing the distribution of microorganisms and their activities in the environment.

The research presented in chapter 3 sought to characterize the extent of the metabolic flexibility of strain DS80 using genomic and physiological approaches. Predictions from genomic data suggested the ability of strain DS80 to use several electron donors, electron acceptors, and carbon sources to support growth. Paramount among this work was the effort to identify the extent to which genomic predictions of substrate usage play out in cultures, as these approaches have been widely used to predict the metabolic potential of microorganisms. Moreover, metagenomic approaches have propelled these types of studies and have been used to develop new approaches to cultivate novel microbes from the environment based on their metabolic potential (Walsh et al., 2009; Swan et al., 2011; Inskeep et al., 2013; Anantharaman et al., 2016). However, we showed that predictions of substrate combinations capable of supporting the energy metabolism of cells from genomic data do not always support growth. For example, similar to other *Acidianus* strains and members of the Crenarchaeota in general (Huber and Prangishvili, 2006), strain DS80 showed genomic signatures consistent with the potential to respire  $S^{\circ}$  and to use organic substrates as energy and carbon sources. However, physiological studies showed that strain DS80 cannot reduce  $S^{\circ}$  using organic

substrates as their sole energy and carbon source. These results were unexpected considering that these carbon sources supported aerobic growth and  $\text{Fe}^{3+}$  respiration. Similar observations have been reported in other hyperthermophiles where the ability to use organic substrates depends on the presence of  $\text{Fe}^{3+}$  as the electron acceptor (Tor et al., 2001). The ability of strain DS80 to grow with organic substrates as sole electron donors respiring  $\text{Fe}^{3+}$  or  $\text{O}_2$  but not with other oxidants is a potentially widespread phenomenon among hyperthermophiles and supports the notion that the metabolic flexibility of hyperthermophilic microorganisms may be expanded when  $\text{Fe}^{3+}$  is provided (Vargas et al., 1998). Together, these observations indicated that the availability of electron acceptors influences the spectrum of potential electron donors and carbon sources that can sustain growth and also suggested that the mechanisms for iron reduction are not necessarily specific to the source of reductant.

Intriguingly, our data showed that the availability of  $\text{H}_2$  as an electron donor enables the use of organic substrates as carbon sources in cells respiring  $\text{S}^\circ$ , thereby expanding the carbon metabolism of strain DS80 when  $\text{S}^\circ$  serves as electron acceptor. The observation that autotrophic and heterotrophic growth of cells of strain DS80 respiring  $\text{S}^\circ$  is obligately dependent on  $\text{H}_2$  as an electron donor suggest that this mechanism of  $\text{S}^\circ$  reduction is highly specific to  $\text{H}_2$ , likely due to an optimization in the efficiency of electron transfer reactions between complexes involved catalyzing  $\text{H}_2$  oxidation and  $\text{S}^\circ$  reduction. These observations, in addition to the likely non-specific mechanism of  $\text{Fe}^{3+}$  reduction, also help to explain the better growth yields of cells respiring  $\text{S}^\circ$  when compared to cells respiring  $\text{Fe}^{3+}$ , as reported in chapter 2.

Growth where an inorganic compound is used as an energy source and an organic substrate is used as a carbon source could be described as a chemolithoheterotrophic metabolism. This mixed growth phenotype led us to reconsider the approaches that microbiologists normally use to characterize the physiology of microorganisms. This is because microbiologists typically test the ability of several carbon sources to support microbial growth, rather than test multiple substrates simultaneously as individual carbon or electron sources. Therefore, by testing a matrix of electron donors/carbon sources, the true ecological niche of an organism, with respect to its carbon and energy metabolism, will be better characterized. Together the results presented in chapter 2 and 3, indicated that predictions of the energy and carbon metabolism of a cell based on thermodynamic calculations from geochemical data, or based on the presence or absence of genes in the genome, may not play out in natural or laboratory environments.

The metabolic flexibility displayed by strain DS80, in particular the observation that  $H_2$  allows for the use of organic carbon sources, suggests that the ecological niche of this thermoacidophile is broader than previously considered, thereby allowing cells to compete for a wider array of substrates in dynamic environments, such as hot springs (Fournier et al., 2002). In chapter 4 we determined the distribution of strain DS80 across 73 hot spring environments using culture-dependent and culture-independent approaches. Results from these studies revealed that strain DS80 was widely distributed in YNP hot springs. These hot springs visibly contained varying combinations of  $S^0$  and iron (hydr)oxides suggesting the potential for these substrates to support microbial growth.

We further characterized the metabolic flexibility of strain DS80, this time focusing on the strains ability to use minerals in its energy metabolism.

Strain DS80 displayed versatility in mineral-dependent metabolism and was able to use  $S^0$  and several iron (hydr)oxides in its energy metabolism, potentially explaining the widespread ecological distribution of this and related *Acidianus* strains (Huber and Stetter, 2015). In addition, strain DS80 was capable of coupling cell production to the disproportionation of  $S^0$  to  $H_2S$  and  $SO_4^{2-}$ . It is important to note that  $S^0$  disproportionation is a thermodynamically unfavorable reaction (Bak and Cypionka, 1987) and how this explains our initial failure to grow the strain via this reaction. However, this limitation can be overcome if sulfide is removed from the culture, making the reaction thermodynamically more favorable. Previous researchers have used ferric iron minerals as sulfide scavenging agents to increase the thermodynamic favorability for bacterial growth (Thamdrup et al., 1993; Janssen et al., 1996; Finster et al., 1998; Slobodkin et al., 2012). However, the ability of strain DS80 to reduce ferric iron (as was mentioned in chapter 2, 3 and 4) prevents the use of these compounds to remove sulfide in cultures of DS80. We devised another mechanism to decrease the concentration of aqueous sulfide under acidic conditions: maximizing the headspace to liquid volume ratio to increase partitioning of aqueous sulfide to the gas phase. By applying this approach, we were able to generate thermodynamically favorable conditions that allowed for growth under this condition. This is the first report of  $S^0$  disproportionation in an archaeon and was the first report of the occurrence of this process in an acidophile. In

addition, this is the first report of a strain that can reduce, oxidize and disproportionate  $S^{\circ}$ .

These observations prompted additional experiments aimed at examining how this thermoacidophile accesses these mineral substrates to support metabolism despite their low solubility. We evaluated the surface requirements to  $S^{\circ}$  and iron minerals by sequestering these minerals in dialysis membranes of different pore sizes during growth. The results presented in this chapter indicate that the requirement for cells to access bulk minerals is dependent on the mode of metabolism that the cell employs, in particular whether the mineral is being used as an oxidant or a reductant. Cells reducing iron minerals did not required direct access to those minerals, presumably due to the leaching of  $Fe^{3+}$  ions by the acidity of the growth medium, although growth and iron reduction activities were higher when direct access to the mineral was provided. On the other hand, cells growing by  $S^{\circ}$  reduction or disproportionation did not need access to the mineral to reduce it whereas cells growing via  $S^{\circ}$  oxidation did require direct access to the mineral, observations that were attributable to the role of  $H_2S$  in solubilizing bulk  $S^{\circ}$  (Boyd and Druschel, 2013). These results suggested that both abiotic and biotic reactions are key to explaining the ability of thermoacidophiles to use solid minerals under acidic conditions.

In summary, the metabolic flexibility displayed by strain DS80 when using both soluble and insoluble substrates, its ability to use these substrates during chemolithoautotrophic, chemoheterotrophic, or chemolithoheterotrophic growth, and the widespread distribution of this genus across geochemical gradients underscores its utility

in studies of the biogeochemical cycling of minerals in hydrothermal environments.

Future investigations will focus on the following subjects:

#### Role of Pili-Like Structures in $\text{Fe}^{3+}$ Reduction in Strain DS80

Cells of strain DS80 grown with  $\text{Fe}^{3+}$  as the electron acceptor had numerous hair-like protrusions extending from the plasma membrane that resembled “nanowires” (Reguera et al., 2005; Gorby et al., 2006). Although these structures were not observed in cells reducing  $\text{S}^{\circ}$  we do not know if these structures are directly involved in the reduction of  $\text{Fe}^{3+}$ . Future work, including the full detection of the genes involved in the production of these pili-like structures and the knockout deletion of these genes, will be performed to evaluate if these mutations prevent the ability of DS80 mutants to reduce iron. Additionally, future experiments could include studies aimed at determining if these structures are electrically conductive or not and to identify what components of the structures confer conductance.

#### Transcriptomic Studies of Different Growth Conditions

Little is known about the mechanism of sulfur and iron reduction and sulfur disproportionation in thermoacidophiles. The availability of DS80 genome and the ability of this strain to couple growth via reduction of  $\text{S}^{\circ}$  and  $\text{Fe}^{3+}$  and via  $\text{S}^{\circ}$  disproportionation make this a suitable strain for transcriptomics aimed at identifying the genes involved in these different metabolic pathways. In addition, transcriptomics studies may shed light on the pathways of electron transfer under various growth conditions, which could be used to guide further studies aimed at identifying metabolic costs associated with synthesis of

these structures using thermodynamic approaches (Dick and Shock, 2011). Such data could help in identifying why cells prefer to reduce  $S^{\circ}$  over  $Fe^{3+}$  when grown autotrophically with  $H_2$ , despite  $Fe^{3+}$  having a more positive redox potential. Moreover, these studies would shed light on the enzymes involved in  $S^{\circ}$  disproportionation in a thermoacidophile and will likely allow us to identify some marker genes to study the distribution of this metabolism in the environment.

### Probing Mechanisms of Dissimilatory Iron Reduction in Acidophiles

The current proposed model of dissimilatory iron (hydr)oxides reduction (DIR) at neutral conditions suggest that the iron isotope fractionations signatures observed during DIR is driven by electron exchange between  $Fe(II)_{aq}$  and reactive component of the iron oxide at the mineral surface (Crosby et al., 2005; Crosby et al., 2007). However at low pH the amount of sorbed  $Fe(II)$  on to  $Fe$ -(hydr)oxides in abiotic experiments is negligible (Reddy et al., 2015) and therefore without sorbed  $Fe(II)$ , no electron exchange with surface  $Fe(III)$  should occur. To test if DIR under acidic conditions produces an isotopic fractionation, we will examine the degree of iron isotope fractionation in cultures of DS80 grown with two solid phase iron minerals under acidic conditions: goethite and ferrihydrite. The results of these experiments will provide new insights into the mechanism used by thermoacidophiles, such as DS80, to respire solid iron minerals and will potentially reveal novel mechanisms involving biology and iron isotopic fractionation at low pH.

### Surface Requirement During Mineral Reduction

Our preliminary data (data not shown) indicate that strain DS80 can use manganese oxides and arsenic sulfides (orpiment and realgar) to growth. Future work, including the characterization of DS80 growing with these minerals and the requirements for surface association will be performed. These studies will provide new insight into the involvement of abiotic processes (e.g., abiotic oxidation of arsenic sulfides and solubilization of arsenic) in supporting growth under these conditions. A set of dialysis experiments will be prepared as described in Chapter 4 to sequester these minerals and prevent direct access to the mineral surface. In addition, field emission electron microscopy, will be used to observe cells grown with direct access to the minerals to identify the extent to which cells attach to surfaces.



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## APPENDICES

APPENDIX A

METABOLIC AND TAXONOMIC DIVERSIFICATION IN CONTINENTAL  
MAGMATIC HYDROTHERMAL SYSTEMS

Contribution of Authors and Co-Authors

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Metabolic and taxonomic diversification in continental magmatic hydrothermal systems

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## 1. INTRODUCTION.

Hydrothermal systems integrate geological processes from the deep crust to the Earth's surface yielding an extensive array of spring types with an extraordinary diversity of geochemical compositions. Such geochemical diversity selects for unique metabolic properties expressed through novel enzymes and functional characteristics that are tailored to the specific conditions of their local environment. This dynamic interaction between geochemical variation and biology has played out over evolutionary time to engender tightly coupled and efficient biogeochemical cycles. The timescales by which these evolutionary events took place, however, are typically inaccessible for direct observation which impedes experimentation aimed at understanding the causative principles of linked biological and geological change unless alternative approaches are used. A successful approach commonly used in geological studies is to use comparative analysis of spatial variations to test ideas about temporal changes that occur over inaccessible (i.e., geological) timescales. The same approach can be taken to examine the links between biology and environment to reconstruct the sequence of evolutionary events that resulted in the diversity of organisms that inhabit modern day hydrothermal environments and the mechanisms by which this occurred. By combining molecular biological and geochemical analyses within robust phylogenetic frame works through approaches commonly referred to as phylogenetic ecology <sup>1,2</sup>, it is now possible to take advantage of variation within the present – the distribution of biodiversity and metabolic

strategies across geochemical gradients – to recognize the extent of diversity and the reasons that it exists.

The distribution of organisms and their metabolic functions in modern environments is rooted in the selection for physiological adaptations that allowed variant populations to radiate into new ecological niches. Such radiation events are recorded in extant organismal distribution patterns (e.g., habitat range), as well as in the genetic record of the organisms as they are distributed along environmental gradients<sup>3,4</sup>. These patterns are the result of vertical descent, which is a primary means by which organisms inherit their metabolic or physiological potential from their ancestors, although lateral gene transfer, gene loss, and gene fusions are also likely to influence extant distribution patterns. Overall, these phenomena manifest as a positive relationship between the physiological and ecological relatedness of organisms and their evolutionary relatedness<sup>1,2,5,6</sup>. Because of the correlation between the phylogenetic relatedness of microbial taxa and their overall ecological similarity, an analysis of the overall phylogenetic relatedness and structure of communities of interacting organisms can be used to investigate the contemporary ecological processes that structure their composition<sup>1</sup>. The aforementioned attributes of biological systems offer ‘a window into the past’ as extant patterns in the distribution of species or metabolic function can be used to infer historical constraints imposed by environment on the diversification of species and or metabolic function<sup>7-13</sup>.

The majority of phylogenetic studies conducted on early life have used gene or protein sequence data obtained from culture collections, which can make it difficult to place results in an ecological context. For example, phylogenetic reconstructions of contemporary genes or proteins indicates that the first forms of life (**Figure 1**)<sup>14</sup> and many of the physiological functions that sustained it [e.g., hydrogen oxidation<sup>15</sup>] may have a hydrothermal heritage. Additional evidence in support of thermophilic character for early forms of life comes from an analysis of resurrected proteins that are likely to share attributes of the Last Universal Common Ancestor of Archaea and Bacteria. Biochemical characterization of these proteins indicate that they function more efficiently at elevated temperatures when compared to more mesophilic conditions<sup>16</sup>. This is consistent with the elevated temperatures predicted for near surface environments in Archaean oceans based on silicon and oxygen isotopic data (55°C to 85°C)<sup>17-19</sup>. However, more recent studies based on phosphate isotopic data suggest that Archaean oceans were clement (<40°C)<sup>20</sup>. While this seems contradictory to the aforementioned phylogenetic and molecular reconstruction data, it is consistent with other phylogenetic studies of ribosomal RNAs suggest that LUCA was a mesophilic organism which then evolved to form the thermophilic ancestor of *Bacteria* and *Archaea*<sup>21,22</sup>. Clearly, there is much still to be learned about the nature of early forms of life on Earth and the characteristics of the environment that drove its emergence and early evolution. The variation in the geochemical composition of present day hydrothermal environments is likely to encompass much of the geochemical compositional space that was present on early Earth<sup>23</sup>, at the time when key metabolic processes (e.g., methanogenesis, iron

reduction, sulfur metabolism, and photosynthesis) that sustain populations inhabiting these systems are thought to have evolved. The geochemical variation present in modern hydrothermal environments provides an ideal field laboratory for examining the natural distribution of taxa and their metabolic functionalities and to define the range of geochemical conditions tolerated at the taxonomic and metabolic levels (i.e., habitat range or zone of habitability)<sup>24,25</sup>. Approaches that integrate molecular biological and geochemical data collected simultaneously within an evolutionary framework have potential to provide new insights into the factors that drove the diversification of metabolic processes that sustain life in hydrothermal environments. The studies reviewed here are aimed at providing a better understanding of the interplay between the geological processes that fuel hydrothermal systems, the characteristics of the modern-day inhabitants of these systems, and the role that geological processes have had in shaping the diversification of ancestral populations that inhabited hydrothermal systems in Earth's past. In doing so, this chapter will bring together topics on geology, geochemistry, microbial physiology, and phylogenetics in continental magmatic systems to explore the taxonomic and metabolic evolution of microorganisms in the context of the geochemically variable hydrothermal environments that they occupy.

## **2. GEOLOGICAL PROCESSES THAT DRIVE GEOCHEMICAL VARIATION IN CONTINENTAL HYDROTHERMAL SYSTEMS.**

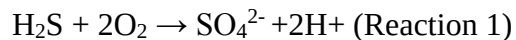
Hydrothermal systems form as the result of interaction of magmatic fluid with a heat source. Three key components are required for the formation of a hydrothermal system:

a source of water, a source of heat, and permeability of the rock strata overlying the heat source <sup>26</sup>. Although water sources, heat sources, and bedrock permeability may vary across hydrothermal systems, they all include the following general features: 1) A recharge area where water enters the system from the surface, 2) a subsurface network allowing this water to descend, come into contact with the heat source, and leach minerals from surrounding rock to form hydrothermal fluids, and 3) a discharge area where newly-formed hydrothermal fluids driven to the surface by heat-induced pressure or density changes emerge on the surface to form hydrothermal features such as hot springs, geysers, fumaroles or mudpots. Magmatic gases such as hydrogen, carbon dioxide, methane, and hydrogen sulfide play an important role in the chemistry of hydrothermal fluids in both marine and continental systems. Since many of these gases are the driving force behind chemotrophic microbial metabolisms <sup>25,27,28</sup> and thus their consideration is essential to understanding the distribution and metabolic diversity of microbial life inhabiting these systems. Moreover, abiotic synthesis reactions, as recently reviewed by McCollum and Seewald <sup>29</sup>, are capable of producing a variety of reduced hydrocarbons (e.g., formate, carbon monoxide, methane) that likely fuel chemotrophic populations in hydrothermal ecosystems.

Hydrothermal systems are typically classified according to the heat source which drives them. Those systems driven by heat from volcanic activity are termed “magmatic” [e.g., Yellowstone National Park (YNP), USA; Kamchatka, Russia; Tengchong, China], while those systems driven by the natural temperature increase that occurs with increasing depth (i.e. heat emanating from the Earth’s mantle, radioactive heat, etc.) are termed

“non-magmatic” (e.g., hot springs in the Great Basin, USA; Hot Springs National Park, USA).

Continental magmatic hydrothermal systems are recharged by precipitation which descends through cracks and fissures into the deep subsurface (**Figure 2**), where it mixes with and is heated by ion-rich magmatic fluids to temperatures as high as 500°C<sup>30-33</sup>. Rising hydrothermal fluids can undergo boiling and phase separation, resulting in the partitioning and concentration of chemical species between the vapor phase (such as ammonia, hydrogen sulfide, and metal cations) while non-gaseous ions (e.g., chloride) remain in the liquid phase<sup>34-37</sup>. Systems receiving substantial deep liquid phase input tend to be alkaline and rich in sodium and chloride due to interaction with deep magmatic brines. Vapor continues to rise and spread through rock crevices, forming a vapor-filled subsurface area termed the “vapor zone”. Some vapor cools and condenses at the surface, where it again mixes with the liquid phase hydrothermal fluids in hot springs, or forms mud pots. Remaining vapor rises via rock fissures and is ejected at the surface through fumaroles. Hydrothermal systems (e.g., fumaroles or hot springs) that receive substantial vapor phase input tend to be acidic due to near surface oxidation of sulfide and the generation of protons (i.e., acidity) according to reaction 1:



The partitioning of sulfide in the vapor phase and its near surface oxidation result in sulfuric acid buffered systems ( $\text{pK}_a \sim 2.0$  at 20°C and 3.0 at 100°C) whereas systems

receiving low sulfide tend to be buffered by bicarbonate ( $pK_a \sim 6.4$  at  $20^\circ\text{C}$  and  $6.6$  at  $100^\circ\text{C}$ )<sup>38</sup>. The bimodal distribution of these hot spring types can be delineated by plotting the frequency of hot springs as a function of their pH, as has been done for globally distributed hot springs<sup>39</sup> as well as in a focused study of Yellowstone National Park (YNP), Wyoming<sup>40</sup>. In **Figure 3A**, the pH and temperature combinations for 7700 hot springs in YNP are plotted revealing two clusters of spring types that span the gradient of spring temperature. Mixing of hydrothermal fluids with near surface meteoric fluid yields the variations in spring pH that differ from this bimodal distribution. This phenomenon can be illustrated by the plot in **Figure 3C**, whereby springs with pH that lie between sulfuric acid and bicarbonate buffered springs have lower conductivity (total dissolved ions), indicative of mixing and dilution of highly conductive acidic spring water with lower conductive and near neutral meteoric water.

The protons generated through the oxidation of sulfide contribute to the weathering of subsurface bedrock (e.g., rhyolite) resulting in the high conductivities associated with the low pH springs in YNP, when compared to alkaline systems. The fact that little correlation exists between hot spring conductivity and temperature (**Figure 3B**) suggests that temperature plays a secondary role to pH in dictating the ionic strength of hydrothermal fluids. The differential weathering of subsurface bedrock by vapor- and liquid-phase dominated hydrothermal systems has important ramifications for the type of hot springs formed. Vapor phase dominated systems tend to be limited in liquid and form fumaroles (steam vents), mudpots (dissolved clay or volcanic ash), and hot springs (upon mixing with meteoric fluid) whereas liquid phase dominated systems often form hot

springs or geysers. Geysers can also form from acidic waters; although, this is a rare occurrence due to the tendency for acidic water to mediate the dissolution of the constriction that is needed for pressure to develop and eject fluids from the subsurface reservoir.

In addition to chemical variation induced by subsurface boiling and phase separation, variation in subsurface hydrology results in differences in the fluid flow paths that allow for interaction with variable rock units. This interaction results in different fluid residence times which in turn are reflected by conductivity differences (**Figures 3B and 3C**) and different minerals being leached from the bedrock (**Figure 2**). Water-rock reactions change the composition of both the rocks and of the water as it flows through the subsurface resulting in fluid compositions that are extraordinarily diverse when compared to other natural surface waters. The chemical variability of the hydrothermal fluids is largely attributable to compositional differences between the underlying host rocks <sup>41</sup>. Thus, flow paths and the composition of rocks that hydrothermal fluids interact with play a key factor in determining fluid chemistry. Several different hot spring types are present in YNP hot springs; those that the authors are most familiar with are presented in **Figure 4** along with the ranges of substrates present in these systems. Differences between hot spring fluid compositions may include variations in the availability of metals, such as zinc, iron, molybdenum and manganese as well as phosphorous compounds like hypophosphite ( $\text{PO}_2^{3-}$ ) and/or phosphite ( $\text{PO}_3^{3-}$ ). Decreased density resulting from heating causes these ion-enriched hydrothermal fluids to ascend toward the surface. Importantly, near surface oxidation of dissolved chemicals such as



sulfide or ferrous iron can result in the deposition of different solid phases, such as elemental sulfur or iron oxyhydroxides, respectively <sup>42</sup>.

### **3. TAXONOMIC AND FUNCTIONAL DIVERSITY INCONTINENTAL HYDROTHERMAL ECOSYSTEMS.**

Microorganisms from both archaeal and bacterial domains have been detected in continental hydrothermal systems. Here, we will summarize several of the key chemical features that appear to exert strong controls on the composition of communities that inhabit these systems. Acidic continental hot springs often contain high concentrations of elemental sulfur, producing characteristic depositional zones <sup>42-44</sup>. As mentioned previously in this chapter, acidic hot springs develop due to phase separation, partitioning of sulfide into the vapor phase, and the near surface oxidation of this sulfide which generates sulfuric acid. Thus, acidic springs often have high abundances of sulfide, sulfur (intermediate oxidation product), and sulfate <sup>24,37,45-47</sup>, all of which have been shown to be metabolized by hyperthermophiles <sup>48-55</sup>. Moreover, a range of typically less stable intermediate sulfur species including thiosulfate, polysulfides, and polythionites have been detected in hot spring environments <sup>45-47,56</sup> and have been shown to be metabolized by biology <sup>56-58</sup>. As a result, sulfurous hot springs across the globe tend to harbor similar lineages of microorganisms <sup>59,60</sup>, underscoring the importance of sulfur in the metabolism and diversification of thermophilic microorganisms <sup>59,60</sup>.

Hydrogen is also likely to play a key role in defining the distribution of thermophilic organisms in hot spring environments <sup>28,48</sup>. Numerous H<sub>2</sub> oxidizing organisms, typically associated with the bacterial order *Aquificales* <sup>61,62</sup> and members of the euryarchaeota and crenarchaeota <sup>63-66</sup>, have been isolated from hot springs. In addition to H<sub>2</sub> oxidation, fermentative H<sub>2</sub> production appears to be an important process sustaining organisms inhabiting continental, magmatic hydrothermal environments <sup>7</sup>.

The discovery of ammonia oxidation by thermophilic Archaea <sup>67</sup> transformed understanding of the importance of the nitrogen cycle in hydrothermal environments. Since this initial discovery, molecular signatures of ammonia oxidizing archaea have been shown to be widespread in continental magmatic hydrothermal ecosystems <sup>68-70</sup>. Moreover, the process of ammonia oxidation and the depletion of the bioavailable ammonia pool has been suggested to represent a top-down control on the structure and composition of communities inhabiting circumneutral to alkaline continental hot springs, creating a strong selective pressure for inclusion of N<sub>2</sub> fixing populations <sup>71</sup>. Biological N<sub>2</sub> fixation is likely to play a key role in relieving fixed N limitation in continental hot spring environments and evidence for populations involved in this population have been identified by molecular approaches targeting the distribution and diversity of *nifH* genes <sup>8,72</sup> and their transcripts <sup>71,73,74</sup>. Incorporation of <sup>15</sup>N<sub>2</sub> into biomass in microcosm assays containing hot spring sediments indicate N<sub>2</sub> fixation in numerous continental hot spring environments with temperatures of up to 89°C <sup>72</sup>, which is close to the upper temperature limit for this process of 92°C which was demonstrated in a marine ecosystem <sup>75</sup>, provide additional evidence indicative of the role of N<sub>2</sub> fixation in relieving fixed N limitation.

Whereas a thermophilic bacterium distantly related to *Leptospirillum* spp. was responsible for the N<sub>2</sub> fixation activity observed in the 89°C continental hot spring <sup>72</sup>, a thermophilic methanogen was responsible for this activity observed in the marine vent ecosystem <sup>75</sup>. Surprisingly, despite the extensive molecular-based studies of N<sub>2</sub> fixation potential and gene expression in continental hot springs, only evidence for bacterial N<sub>2</sub> fixation has been observed to date.

Several unique and deeply rooted lineages of Bacteria and Archaea have only been identified in high temperature environments. For example, the bacterial order Aquificales has never been observed in environments with temperatures < 55°C <sup>62</sup>. The aquificae genera *Hydrogenobacter*, *Hydrogenobaculum*, *Thermocrinis*, and *Sulfurihydrogenibium* spp. are typically observed in continental hot springs and solfataric fields <sup>58,76-79</sup>. The distribution of these genera in continental hot springs is driven by pH, suggesting diversification in response to gradients in acidity. Whereas *Hydrogenobaculum* predominates in acidic springs, *Thermocrinis* and *Hydrogenobacter* predominate in alkaline springs. *Sulfurihydrogenibium* tends to inhabit sulfide-rich alkaline springs <sup>62</sup>. Unlike the above strains, members of the genera *Aquifex* and *Persephonella* are routinely isolated from marine hydrothermal systems only <sup>58,61,80-82</sup>. Identifying the factors that led the unique pattern in the distribution of aquificae strains in marine and continental hydrothermal systems is currently unknown, but is likely to involve differences in fitness in their respective niches.

Several thermophilic *Bacteria* have also been isolated from terrestrial hydrothermal sites. Examples include strains of *Thermocrinis*, *Hydrogenobaculum*, *Hydrogenobacter*, and *Sulfurihydrogenibium* from the *Aquificales* order, which have been identified as important constituents of a variety of geochemically-diverse geothermal communities <sup>62</sup>.

*Aquificales* are chemolithoautotrophic thermophilic *Bacteria* that couple CO<sub>2</sub> fixation with the oxidation of reduced compounds such as hydrogen, sulfide, or elemental sulfur <sup>58,61,83</sup>. Owing to their chemolithoautotrophic mode of metabolism, they are often the dominant primary producers of biomass within high temperature ecosystems <sup>61,83</sup>.

Together with members of the order *Thermotogales*, these microorganisms represent the *Bacteria* with the highest growth temperatures currently known <sup>58</sup>.

Other thermophilic *Bacteria* isolated from terrestrial hot springs include members of the *Desulfurella* genus. Examples of this genus include *Desulfurella multipotens*, *D. kamchatkensis*, *D. acetivorans*, and *D. propionica*. These strains were isolated from cyanobacterial mats and sediments collected from thermal areas with neutral pH (6.0-7.0) in New Zealand and Kamchatka, Russia. All are capable of chemoorganotrophic growth using elemental sulfur as a terminal electron acceptor; however, *D. multipotens* can also grow chemolithotrophically with hydrogen <sup>84-86</sup>.

Like the distribution of aquificae discussed above, the distribution of archaeal phyla appears to be dependent on environmental characteristics and selection for specific metabolic activities that track phylogenetically. Among the *Crenarchaeota*, all members of the *Sulfolobales* and *Acidolobales* orders and several representatives of the *Thermoproteales* order are acidophilic <sup>63,64,87</sup>. Among the *Euryarchaeota*, acidophily is

restricted to members of the order *Thermoplasmales*<sup>66</sup>. Moreover, a number of metabolic properties track phylogenetically at the phylum or domain level. For example, all thermophilic methanogens and halophiles belong to the phylum *Euryarchaeota*. In contrast, thermophilic organisms capable of reducing sulfate are known to be widely distributed among the *Bacteria* [e.g.,  $\delta$ -*Proteobacteria*, *Thermodesulfobacteria*, *Nitrospira*, and *Firmicutes*<sup>49,88</sup>] but are narrowly distributed among *Archaea*. The only archaeal sulfate-reducers described so far belong to the genera *Archaeoglobus* and *Caldivirga*<sup>89,90</sup>. It is unclear if sulfate-reduction was a character of the Last Universal Common Ancestor, especially considering that sulfate was unlikely to be an abundant anion prior to the rise of oxygenic photosynthesis<sup>91</sup>. An alternative hypothesis is that the genes required for sulfate reduction in either *Archaea* or *Bacteria* were acquired by horizontal gene transfer (discussed in more detail in section 6). For example, it has been suggested that the dissimilatory sulfite reductase (*dsrAB*) genes in *Archaeoglobus* species are the result of an ancient horizontal transfer from a bacterial donor, and that microbial sulfate respiration may have a bacterial origin<sup>92</sup>. Moreover, it appears that the patchy distribution of sulfate respiration among *Bacteria* is due to multiple horizontal gene transfer events as well as gene loss<sup>92,93</sup>.

Heterotrophic, thermoacidophilic *Archaea* that couple the oxidation of organic acids, carbohydrates, and/or complex peptides to the reduction of elemental sulfur have been isolated from various acidic hot springs. Example organisms include the anaerobic, thermoacidophilic crenarchaeotes from the order *Acidilobales*. Members of this lineage include *Acidilobus aceticus* and *Acidilobus saccharovorans*, both isolated from an acidic

hot spring of Kamchatka, Russia<sup>87,94</sup> and *Acidilobus sulfurireducens* isolated from an acidic hot spring in YNP<sup>54</sup>. Molecular analyses indicate that *A. sulfurireducens* is a numerically dominant organism in acidic, high-temperature YNP springs<sup>13,54</sup>, and that closely related strains of *Acidilobales* have been detected in acidic terrestrial geothermal environments around the world, illustrating the wide distribution of this genus<sup>60,87,94</sup>. Other organotrophic thermoacidophilic crenarchaeota include *Caldisphaera lagunensis* and *Caldisphaera draconis* isolated from acidic hot springs in the Philippines and YNP, respectively<sup>54,95</sup>. Additional organotrophic and thermoacidophilic *Archaea* typical of acidic hot springs include *Thermocladium modestius*<sup>96</sup>, *Caldivirga maquilingensis*<sup>89</sup>, *Vulcanisaeta distributa*<sup>97</sup>, and *Vulcanisaeta souniana*<sup>97</sup>. Unlike members of the *Acidilobales* (*Acidilobus* and *Caldisphaera* spp.) which are strict anaerobes, these *Archaea* are able to tolerate low levels of oxygen, suggesting a role for oxygen gradients in the diversification of thermoacidiphilic crenarchaeota.

Members of the order *Sulfolobales* represent another group of thermoacidophilic *Archaea* that are commonly detected in continental acidic hot springs<sup>59,98,99</sup>. This order is comprised of members of the genus *Sulfolobus*, *Metallosphaera* and *Sulfurococcus*, which are obligate aerobes, members of the genus *Stygiolobus*, which are strict anaerobes, and members of the *Acidianus* and *Sulfurisphaera* genera, which are facultative anaerobes<sup>64,65</sup>, along with *Sulfolobus* species, which are facultative autotrophs. During autotrophic growth, elemental sulfur or hydrogen may be oxidized to yield sulfuric acid or water as end products with carbon dioxide serving as a carbon source<sup>64,65</sup>. Sugars, yeast extract, and peptone may serve as electron and carbon sources

during heterotrophic growth of isolates in the lab, but these may not be the complex sources of carbon that support these organisms in situ <sup>99</sup>. To date several members of this genus have been isolated from various continental and acidic hot springs around the world. Some representative species of this genus include *S. acidocaldarius*, *S. metallicus*, *S. solfataricus*, *S. tokodaii*, *S. islandicus*, *S. shibatae* and *S. yangmingensis*, among others <sup>99-103</sup>. *Metallosphaera* and *Sulfurococcus* are also facultative autotrophs capable of oxidizing elemental sulfur or organic compounds. Three species of the *Metallosphaera* genus including *M. sedula*, *M. prunae*, and *M. hokonensis* have been isolated from terrestrial hot springs <sup>104-106</sup>. *Sulfurococcus* species have also been isolated from terrestrial sites. *Sulfurococcus mirabilis* and *Sulfurococcus yellowstonensis* were isolated from Kamchatka, Russia and from a hot spring in YNP, respectively <sup>107,108</sup>. In contrast, *Sulfurisphaera ohwakuensis*, the only species from the *Sulfurisphaera* genus, grows mixotrophically and was isolated from an acidic hot spring in Japan <sup>109</sup>. In addition to being capable of autotrophic and facultative heterotrophic growth, *Acidianus* species are physiologically versatile and grow as facultative aerobes with the ability to oxidize or reduce elemental sulfur depending on oxygen availability <sup>110</sup>. Some representative species of this genus include *A. ambivalens*, *A. manzaensis*, *A. infernus* and *A. brierleyi*, among others <sup>111-114</sup>. *Acidianus* species have been isolated primarily from terrestrial hot springs sites, but also occur in shallow submarine hydrothermal systems <sup>114</sup>. The capacity to inhabit both terrestrial and marine hydrothermal systems likely reflects the broad physiological diversity of this genus. Members of the *Acidianus* genus are also metabolically diverse being able to grow under a number of distinct conditions. One

unique *Acidianus* species, *A. manzaensis*, can couple grow chemolithoautotrophically by coupling the reduction of  $\text{Fe}^{3+}$  to the oxidation of  $\text{H}_2$  or elemental sulfur under anoxic conditions <sup>113</sup>.

Archaeal autotrophs that couple hydrogen oxidation with the reduction of elemental sulfur have also been isolated from acidic terrestrial hot springs. One example of this group of organisms is *Stygiolobus azoricus*, which is the only strictly anaerobic chemolithotrophic thermoacidophile from the *Sulfolobales* order. The type strain was isolated from solfataric fields in Azores, Portugal <sup>115</sup>. Arguably the most important metabolism discovered in continental magmatic hot springs over the past decade is the oxidation of ammonia coupled to the reduction of  $\text{O}_2$ . The first cultivated member of this genus, *Candidatus Nitrosocaldus yellowstonii*, is a chemoautotroph <sup>67</sup> and is widely distributed in circumneutral to alkaline hot spring environments <sup>68,70,71,116</sup>.

#### **4. APPLICATION OF PHYLOGENETIC APPROACHES TO MAP TAXONOMIC AND FUNCTIONAL DIVERSITY ON SPATIAL GEOCHEMICAL LANDSCAPES**

The accessibility of continental hydrothermal systems coupled with their extensive geochemical variability and comparatively simple taxonomic diversity (when compared to non-thermal environments) makes them ideal targets for applying phylogenetic and community ecology approaches to generate insights into the role of geochemical variation in dictating taxonomic and functional diversification. The application of phylogenetic ecology tools, while common in studies of the distribution and



diversification in plant communities <sup>1,2,5,6</sup>, is relatively new in microbial studies.

Phylogenetic ecology is in essence a fusion of molecular microbial ecology techniques and phylogenetics. Here, molecular sequence (gene, transcript, genomic, etc.) data are obtained across spatial geochemical or geographic gradients. This collection of sequence data is then used to generate a phylogeny of those sequences. Geochemical or geographic data obtained from the environments where the sequences were obtained is then quantitatively mapped on the phylogeny and used to assess and rank the extent to which variation in physical or chemical measurements can explain the topology of the phylogeny. A schematic of a hypothetical phylogenetic reconstruction is presented in **Figure 5**. Here, temperature explains the topology of the tree well, suggesting that temperature played a role in the diversification of this hypothetical sequence.

Incorporation of additional geochemical variables or combinations therein into explanatory models, as outlined in the examples below, can be used to gauge the role of a particular parameter(s) in driving the diversification of the sequence(s) in consideration. Numerous studies of targeted single gene loci or community genomes in hydrothermal ecosystems reveal that variation in the composition of geothermal fluids strongly influences both the types of organisms present and their functional diversity <sup>7-11</sup>. As an example, Alsop et al., 2014 <sup>11</sup> compiled metagenomic sequence data from 28 continental hot springs in YNP and the Great Basin and subjected these data to a novel bioinformatics approach termed Markov clustering <sup>117,118</sup>. Markov clustering represents a robust mechanism to “bin” metagenomic sequence reads based on phylogenetic similarity (e.g., using BLAST expectancy values or e values). Once reads are binned, they can be

subjected to a number of multivariate statistical analyses aimed at uncovering relationships between differences in the genomic and functional composition of communities and the geochemical characteristics of their local environment. The results of the work of Alsop et al., 2014<sup>11</sup> suggest that high temperature ecosystems harbor lower taxonomic, phylogenetic, and functional diversity when compared to lower temperature environments. Likewise, acidic environments harbor lower taxonomic, phylogenetic, and functional diversity when compared to circumneutral to alkaline environments. Importantly, the genomic composition of the communities inhabiting hot springs characterized by extremes of acidity and temperature were substantially different from those inhabiting hot springs characterized by higher pH and lower temperature and show a strict delineation in the functional composition of communities supported by photosynthesis and those supported by chemosynthesis. In this case, communities supported by photosynthesis exhibit substantially greater taxonomic, phylogenetic, and functional diversity when compared to communities supported by chemosynthesis. These results show that hydrothermal communities are structured by interactions with the geologic processes that shape an environment's geochemical composition.

Targeted studies of the distribution of taxonomic and functional gene lineages across spatial geochemical gradients in hydrothermal systems reveal more intricate details of the role of environment as well as geographic isolation in shaping their diversification. For example, studies aimed at understanding the role of environmental variation on the genomic evolution of specific groups of organisms have also been conducted in hydrothermal environments. In particular, the work of Whitaker et al., 2003<sup>59</sup> found

evidence, at the genomic level, for barriers to the dispersal of *Sulfolobus* spp. Similar studies targeting thermophilic cyanobacteria<sup>119</sup> and thermophilic aquificae<sup>120</sup> also suggest that geographic barriers limit the dispersal of these lineages and allow divergence through local adaptation or random genetic drift (i.e., allopatry).

While the afore mentioned studies suggest a role for geographic isolation in the diversification of thermophiles, other studies indicate that the signal for geographic isolation is diluted by environmental parameters. As an example, the phylogenetic similarity of archaeal 16S rRNA genes sampled across geochemical gradients in YNP could best be explained by variation in spring pH, suggesting pH to be the primary driver of the diversification of these 16S rRNA gene lineages<sup>68</sup>. Geographic distance between hot springs (as a proxy for dispersal limitation) failed to explain a significant amount of the variation in the data. This suggests that in some cases, the probability and rate of successful dispersal is higher than the rate of local evolution. Similar studies have been conducted on hot springs in China, which found that a shift in highly structured assemblages toward less structured assemblages was associated with seasonal patterns in hot spring recharge and input of exogenous substrates (e.g., due to climatic events), suggesting that hot spring temporal dynamics are also an important contributor to the diversification of life in these systems<sup>121</sup>.

The non-random patterns in the distribution of taxonomic lineages across spatial geochemical gradients in continental hot springs suggests a role for geochemical variation in shaping the distribution and diversification of functional components of these assemblages. By focusing on a gene required for the synthesis of (bacterio)chlorophyll as

a marker for photosynthesis, it was shown that a combination of temperature and pH restrict the distribution of light-driven primary production in YNP hot springs<sup>3,7,9</sup>. While the upper temperature limit for photosynthesis of 73°C was confirmed in circumneutral to alkaline systems<sup>122</sup>, it was shown that in acidic systems (pH <4.0) photosynthesis was restricted to environments with temperatures of less than 56°C. A subsequent study that focused on the distribution of pigments across pH and temperature gradients in YNP confirmed these results and indicated an important and previously unrecognized role for sulfide in shaping the distribution of photosynthesis in the Park, with springs containing >5 µM sulfide being devoid of photosynthetic pigments. A follow-up study revealed that the sulfide-dependent distribution of photosynthesis in acidic environments was due to its inhibitory effect on CO<sub>2</sub> assimilation in algae [which predominate among phototrophs in hot springs with pH < 4.0<sup>123</sup>], but not in cyanobacterial dominated systems (pH > 4.0)<sup>3</sup>. These observations, coupled with observations from metagenomic sequencing efforts indicating that phototrophic systems support greater taxonomic and functional diversity, suggest that the lack of outward radiation of phototrophs due to the inhibitory effects of sulfide may have impacted the functional diversification of populations/communities inhabiting acidic and sulfide-rich high temperature ecosystems.

Phylogenetic ecology studies focused on other functional genes as proxies for key metabolic processes in high temperature environments, including those involved in the conversion of dinitrogen gas (N<sub>2</sub>) to bioavailable ammonia (i.e., nitrogenase; nitrogenase iron protein encoded by *nifH*<sup>8</sup>), the reversible oxidation of H<sub>2</sub> (i.e., [FeFe]-hydrogenase; large subunit encoded by *hydA*<sup>7</sup>), and the detoxification of mercury (i.e., mercuric

reductase encoded by *merA*<sup>10</sup>) further implicate the role of geological processes in shaping the distribution and functional diversification of microbes in these ecosystems. For example, the availability of fixed forms of nitrogen (e.g., ammonia) required by all forms of life is non-uniform in hydrothermal environments, as evinced by the generally lower availabilities of ammonia in circumneutral to high pH environments. The low concentrations of bioavailable ammonia in circumneutral to alkaline geothermal springs is due, in part, to geological and chemical factors, most notably the equilibration of aqueous  $\text{NH}_4^+_{(\text{aq})}$  with  $\text{NH}_{3(\text{g})}$  ( $\text{pK} = 7.6$  at  $90^\circ\text{C}$ ) and the subsequent volatilization of  $\text{NH}_{3(\text{g})}$  out of the system<sup>71,124</sup>. The abundance of *nifH* genes as a marker for nitrogenase, which functions to relieve fixed nitrogen limitation in ecosystems through the conversion of dinitrogen gas to ammonia, has been found to track with the availability of ammonia (Boyd et al., unpublished). Moreover, the diversification of NifH in hot springs in YNP was shown to be driven primarily by variation in pH<sup>8</sup>, which is consistent with the pH-dependent availability of fixed forms of nitrogen in YNP hot spring ecosystems.

The distribution of *hydA*, which encodes the large subunit of [FeFe]-hydrogenase that typically functions in the reduction of protons to generate  $\text{H}_2$  in fermentative *Bacteria*, was found to be pH dependent in hot spring ecosystems<sup>7</sup>. Here, *hydA* exhibited a greater representation in circumneutral to alkaline environments and those with lower temperatures when compared to acidic high temperature hot springs. The constrained distribution of *hydA* to environments with lower temperature and alkaline pH was hypothesized to reflect the unfavorable thermodynamics of organic carbon fermentation in the presence of high  $\text{H}_2$  partial pressure<sup>125</sup>. Elevated concentrations of  $\text{H}_2$  are

routinely measured in springs in YNP where temperatures exceed 65°C<sup>126,127</sup>. H<sub>2</sub> in these systems is likely of abiotic origin, derived from subsurface iron-catalyzed water hydrolysis (radiolytic and/or serpentinization)<sup>127</sup>. Iron-catalyzed H<sub>2</sub> production is sensitive to both solution pH and reaction temperature, with a near doubling of H<sub>2</sub> production rates resulting from the doubling of incubation temperature<sup>128</sup>. Similarly, the rate of basalt-catalyzed H<sub>2</sub> production increases with increasing water acidity (lower pH)<sup>128</sup>. Coincidentally, *hydA* was not detected in YNP springs predicted to have elevated geological H<sub>2</sub> production (e.g., acidic pH, >36°C; alkaline pH, >65°C). Thus, environments with elevated inputs of geological H<sub>2</sub> might select against bacterial fermentative metabolisms which in turn might constrain the distribution of organisms dependent on this metabolism in hot spring ecosystems.

## **5. MOLECULAR ADAPTATION TO HIGH TEMPERATURE.**

The patterns in the distribution of individual gene lineages in hydrothermal environments points to the presence of specific adaptations that facilitate life under the extreme temperature and pH conditions present in these systems. Thermophiles have a number of unique traits to cope with their high temperature habitats that include the production of structurally more stable enzymes and proteins, different mechanisms of motility, and adjustment of membrane lipid compositions.

## 5.1 LIPIDS.

The lipid membrane plays a fundamental role in energy conservation and in the maintenance of intercellular homeostasis by acting as a barrier between the cellular cytoplasm and the external environment. Microorganisms synthesize diverse lipid structures with widely varying biophysical properties <sup>129-131</sup> that have facilitated their diversification into environments with wide ranging geochemical conditions, including hydrothermal environments with extremes of temperature and pH <sup>68,132,133</sup>. The ether bonds of archaeal lipids are thought to be more resistant to acid and thermal stress as indicated by the observation that they are not broken down under conditions in which ester linkages are completely methanolized (5% HCl/MeOH, 100°C) <sup>134</sup>. The predominant membrane lipids of thermophilic *Archaea* are isoprenoid glycerol dialkyl glycerol tetraethers (iGDGTs) <sup>135-138</sup>, which consist of two ether-linked C<sub>40</sub> polyisoprenoid (i.e., biphytanyl) chains with zero up to eight cyclopentyl rings and up to one cyclohexyl ring (i.e., crenarchaeol). The internal cyclopentyl rings are thought to increase the packing density of the membranes and thus enhance their thermal stability <sup>139,140</sup> and decrease their permeability to ions <sup>141</sup>. Both pure culture <sup>132,142</sup> and environmental surveys <sup>68,133</sup> indicate that the number of cyclopentyl rings per iGDGT positively correlates with the temperature and the acidity of the system suggesting that *Archaea* acclimate to shifts in pH or temperature by adjusting the cyclopentyl ring composition of their GDGT lipids. *Candidatus Nitrosocaldus yellowstonii* <sup>67</sup> is the only archaeon identified to date that synthesizes Crenarchaeol and the function and competitive advantage of synthesizing this unique lipid remains unknown.

Thermophilic *Archaea* have also recently been shown to synthesize a variant of iGDGTs termed H-shaped GDGTs (H-GDGTs), also known as glycerol monoalkyl glycerol tetraethers (GMGT) <sup>135,143</sup>. H-GDGTs, which are structurally similar to iGDGTs but contain a C–C bond between the two C<sub>40</sub> biphytanyl chains, have been detected in both thermophilic *Crenarchaeota* <sup>143</sup> and *Euryarchaeota* <sup>144-147</sup> inhabiting high temperature continental hot springs and marine hydrothermal vents <sup>148-151</sup>. These lipids may function to further enhance the thermal stability of the lipid membrane and reduce its permeability to protons. In support of these hypotheses, the fractional abundance of H-GDGTs to total iGDGTs was found to be inversely correlated with pH but not with temperature in a survey of biomass sampled from YNP hot springs <sup>151</sup>.

Although the predominant lipids synthesized by *Bacteria* are glycerol fatty acyl diesters, *Bacteria* also acclimate to changing temperature conditions by adjusting the composition of their lipid membranes in order to maintain a liquid crystalline state suitable for embedding of membrane proteins <sup>152,153</sup>. The temperature of hot springs can be dynamic and is modulated by processes that operate over time scales ranging from seconds (e.g., earthquakes), to seasonal (e.g., precipitation), to decadal or even millennial (e.g., caldera inflation, glacial dynamics) <sup>154</sup>. *Bacteria* can acclimate to thermal stress imposed by rapidly increasing temperatures due to the aforementioned factors and decrease the permeability of the membrane while still maintaining a liquid crystalline state by increasing the length of acyl chains, increasing the saturation of acyl lipids, and increasing the ratio of iso/anteiso composition of their fatty acids <sup>155-158</sup>. Interestingly,



some bacterial thermophiles have been demonstrated to increase the amount of lipid produced during acclimatization to thermal stress <sup>159</sup>.

Recently, it was shown that some thermophilic *Bacteria* may also produce branched chain GDGT lipids (i.e., bGDGTs), which vary in the number of cyclopentyl moieties and in the degree of methylation of the alkyl chains <sup>160</sup>. Like GDGTs, bGDGTs have ether linkages, however, they are not isoprenoidal and differ in the stereochemical configuration of the second carbon position of the glycerol backbone <sup>161</sup>. Several recent studies have detected bGDGTs in biomass collected from hot springs spanning a wide temperature gradient <sup>162-166</sup>. However, the abundance of bGDGT lipids was shown to be lower in hot spring biomass sampled from environments with temperatures >70°C, suggesting that these lipids may not be synthesized by hyperthermophilic *Bacteria* <sup>164</sup>.

## 5.2 PROTEIN STABILITY.

A number of complex and interacting features have been identified as possible contributors to increased thermostability of proteins which have been subjects of extensive reviews and thus will only be briefly mentioned here <sup>167-170</sup>. A partial list of these features include *i*) helix dipole stabilization by negatively charged residues near their N-terminus and positively charged residues near the C-terminus, *ii*) intersubunit interaction and oligomerization, *iii*) a relatively small solvent-exposed hydrophobic surface area, *iv*) an increased packing density, *v*) an increase in core hydrophobicity, and *vi*) a deletion and/or shortening of surface loops. In general, the same forces that contribute to protein folding such as hydrophobic effects, disulfide bridges, hydrophobic

interactions, aromatic interactions, hydrogen bonding, and ionic interactions also may act as potential stabilization mechanisms. However, as outlined briefly below, different thermophilic enzymes appear to utilize different combinations of the aforementioned strategies to tolerate high temperature.

Numerous studies have highlighted the importance of ionic interactions as key structural determinants of thermal stability at high temperatures. For example, Russell et al., reported an increase in the number and extent of ion-pair networks in the thermostable enzymes glutamate dehydrogenase and citrate synthase from the hyperthermophilic archaeon *Pyrococcus furiosus*<sup>171,172</sup>. Citrate synthase from *P. furiosus* also exhibits more intimate association of the subunits, an increase in intersubunit interactions, and a reduction in thermolabile residues relative to non-thermophilic enzymes<sup>171</sup>.

The relationship between an increase in ionic interactions and thermostability is not universal among thermophile proteins. For example, the glutamate dehydrogenase from *Thermotoga maritima* has fewer intersubunit ion pairs and an increased number of hydrophobic interactions when compared with the enzyme from *P. furiosus*<sup>173</sup>. In contrast, adenylate kinase from *Methanococcus jannaschii* has a larger and more hydrophobic core, an increased number of hydrophobic and aromatic interactions, shorter loops, and helix dipole stabilization which together are thought to confer thermal stability<sup>174</sup>.

The mechanism of thermal adaptation has also been studied in the two component enzyme Nitrogenase (Fe protein and MoFe protein) from the thermophile *Methanobacter thermoautotrophicus*. The Fe protein component of this enzyme possesses shorter loop

regions and lower random coil content than its mesophilic counterpart from *Azotobacter vinelandii*. Both of these features have also been observed in several other thermostable enzymes. Moreover, there are also a greater number of ion-pairing interactions between the two components of this enzyme, suggesting the existence of dynamic protein–protein interactions at higher temperatures <sup>175</sup>. Other protein features that could enhance thermostability include extrinsic parameters such as the presence of high concentrations of intracellular compatible solutes (discussed in section 4.3), elevated concentrations of proteins, the use of molecular chaperones, or variations in environmental factors such as pressure <sup>167,176</sup>.

### 5.3 CYTOPLASMIC OSMOLYTES.

Many microorganisms accumulate compatible solutes in response to increases in the levels of salts or sugars in the environment. Thermophilic and hyperthermophilic organisms are exposed to dynamically changing geochemical and physical conditions, such as changes in temperature, pH, and solute concentrations due to differences in mixing of hydrothermal and near surface waters. Mechanisms for adjusting and accumulating osmolytes are therefore necessary to survive such dramatic shifts. A survey of osmolytes produced in several species of *Archaea* found that *Pyrobaculum aerophilum*, *Thermoproteus tenax*, *Thermoplasma acidophilum*, and a suite of members of the order *Sulfolobales* accumulated the common osmolyte trehalose <sup>177</sup>. However, other thermophilic taxa were found to produce several unique osmolytes that have only been identified in thermophiles, suggesting that the biosynthesis of these osmolytes

represents an adaptation to high temperature environments. For example, the accumulation of mannosylglycerate has been reported in the bacterium *Rhodothermus marinus*<sup>178,179</sup> and the archaeon *Pyrococcus furiosus*<sup>177,180</sup> while, *Pyrodictium occultum* accumulates di-myoinositol-1,1'(3,3')-phosphate<sup>177</sup>. The unusual osmolyte cyclic-2,3-bisphosphoglycerate was found to accumulate to high concentrations in cells of the hyperthermophilic methanogen *Methanopyrus kandleri* while *Archaeoglobus fulgidus* accumulates diglycerolphosphate, in response to increasing salinity of growth medium or incubation temperature<sup>177</sup>. Additionally, both *P. furiosus*<sup>180</sup> and *Methanococcus igneus*<sup>181</sup> produce higher concentrations of the unusual phosphorous-containing solute di-myoinositol-1,1'-inositol-phosphate when grown at elevated temperatures. Likewise, *R. marinus* has been shown to accumulate higher concentrations of mannosylglycerate with increasing incubation temperature<sup>182</sup>. Interestingly, a characterization of intracellular organic solutes in several members of the *Thermotogales* revealed large differences in the solutes detected in each strain when grown under optimal conditions<sup>183</sup>, indicating that osmolyte production can vary even between closely related strains. This difference may be related to selective pressures imposed by the environment from which they were originally isolated. Similar findings were reported in the two closely related strains *Rhodothermus Marinus* and *Rhodothermus obamensis*. At several growth temperatures and salinities, the major compatible solutes in *R. marinus* were alpha-mannosylglycerate and alpha-mannosylglyceramide, whereas *R. obamensis* only accumulated alpha-mannosylglycerate. The absence of the amide solute in

*R. obamensis* at the several growth conditions represented the most pronounced difference in the compatible solute accumulation profile by the two strains <sup>182</sup>.

The observations outlined above indicate that specific compatible solutes may have been selected for due to their ability to also protect intracellular molecules against thermal denaturation <sup>184</sup>. Indeed, a comparison of the thermostabilizing effects of a number of organic osmolytes found that mannosylglycerate and trehalose increased the thermotolerance of lactate dehydrogenase and glucose oxidase enzymes *in vitro* <sup>185</sup>.

Likewise, the thermolabile enzymes glyceraldehyde-3-phosphate dehydrogenase and malate dehydrogenase from *Methanothermus fervidus* were found to be stabilized by the potassium salt of the osmolyte cyclic diphosphoglycerate *in vitro* <sup>186</sup>. Diglycerol phosphate, the predominant organic osmolyte synthesized by *A. fulgidus* <sup>177</sup>, was found to stabilize lactate dehydrogenase purified from rabbit muscle, alcohol dehydrogenase purified from yeast, and glutamate dehydrogenase purified from *Thermococcus litoralis* at elevated temperatures. This same study found that diglycerol phosphate stabilized purified rubredoxin, a low-molecular-weight iron-containing protein, from a number of anaerobic bacterial strains <sup>187</sup>. Of particular significance is the intracellular concentrations of cyclic 2,3-diphosphoglycerate and potassium in the methanogen *Methanopyrus kandleri*, which have been reported to be as high as 1.1M and 3M, respectively <sup>188</sup>. These salts have been shown to play an important role in the thermal stability of cyclohydrolase and formyltransferase from *M. kandleri* <sup>189</sup>, which is the most thermal tolerant methanogen currently known <sup>190</sup>.

## 5.4 MOTILITY.

Thermophiles have evolved a number of lifestyle strategies to cope with stress imposed by dynamic chemical and physical conditions. For example, to survive the geochemical variations that occur with the mixing of hydrothermal and near surface waters, many hyperthermophiles have been shown to develop surface-adherent, biofilm communities. The production of extracellular polymeric substances (EPS) not only facilitates attachment to surfaces as the first step in biofilm formation, but is also associated with enhanced survival during periods of environmental stress <sup>191</sup>. Cultivated members of the *Thermococcales* are capable of forming copious amounts of capsular polysaccharides and EPS in laboratory cultures, often under conditions of chemical or physical stress <sup>193</sup>, suggesting that this might also be a predominant mode of growth for these organisms in natural environments.

In particular, biofilms are common in areas of hot springs with geochemical conditions that allow for photosynthesis <sup>3,4</sup>. The structure and composition of naturally formed and laminated biofilms inhabiting alkaline siliceous hot springs has been subject to decades of study by a number of researchers, perhaps most notably Ward, Brock, and Castenholz [as reviewed in <sup>195</sup>]. Using glass rods suspended at the air-water interface in the runoff channel of a photosynthetic alkaline hot spring, Boomer and colleagues studied the successional development of multilayered mat (i.e., biofilm) communities in a photosynthetic (60-70°C) alkaline hot spring <sup>196</sup>. This study showed that the pioneer populations in the formation of the photosynthetic biofilm were cyanobacteria closely affiliated with *Synechococcus* spp. as well as *Thermus* spp. With additional incubation

time, a red layer developed deeper in the mat (under the green layer) which was attributed to the establishment of the anoxygenic phototroph *Roseiflexus* in the biofilm community. In this same study, the communities that formed the biofilm were compared with the planktonic populations emanating from the geyser vent, revealing similar taxa. This finding is consistent with the idea that many thermophilic populations have evolved to attach to surfaces in order to proliferate, concentrate nutrients, and resist environmental stress <sup>191</sup>.

Biofilms also appear to provide an adaptive advantage for chemosynthetic microbial communities that inhabit high temperature (>70°C) transects of continental hot springs. By placing sterile glass slides or cover slips in the outflow channels of several hot springs in YNP with temperatures that range from 80 to 90°C, attachment of microbial cells and growth of filamentous structures was observed <sup>197-199</sup>. Remarkably, the generation times of these surface associated filaments (estimated by dividing the cells produced along a filament structure by incubation time) were found to range from 2 to 7 hrs, which are similar to those of populations inhabiting non-thermal environments <sup>197</sup>. The substantial numbers of cells that accumulate on the surfaces when compared to the nearly devoid microbial titer in spring waters emanating from the source of the hot spring suggests that attachment to surfaces is pervasive in high temperature continental hot springs and may represent an evolutionary strategy for populations to distribute themselves at specific thermal and/or geochemical transects <sup>199</sup>. More recently, glass slides have been incubated in hot spring effluent channels and pools at high temperature to promote colonization and growth of hyperthermophiles for use in molecular-based studies <sup>28</sup>. Intriguingly, the

populations that attached to these surfaces were similar in composition to those present in sediments, supporting the notion that hyperthermophiles preferentially attach to surfaces. In a separate study, the composition of native elemental sulfur flocs in an acidic hot spring (73°C) were shown to harbor similar communities to those that emanated from the hot spring source, suggesting a role for these organisms in the formation and transformation of the substrate <sup>83</sup>.

Motility is another important mechanism used by thermophiles to respond to dynamic conditions. Chemotaxis refers to the movement toward or away from chemicals and has been observed in a wide variety of *Bacteria* and *Archaea* <sup>200,201</sup>. Chemotaxis represents a survival mechanism used by microorganisms to search for local environments favorable for colonization. The basis of chemotaxis is a two-component signal transduction pathway where by the phosphorylation of a histidine autokinase that senses environmental parameters signals the subsequent phosphorylation of a response regulator <sup>202</sup>. The response regulator then controls diverse processes such as chemotaxis. While the majority of chemotactic research has been performed on the model bacterium *E. coli*, some research exists on thermophile chemotaxis. For example, cells of an uncharacterized thermophilic bacterium termed “PS-3”, which grows optimally between 60 and 70°C, were shown to be attracted to a variety of amino acids and carbohydrates <sup>203</sup>. However, the absence of genes required for chemotactic behavior in the genomes of other representative hyperthermophiles such as *Pyrococcus furiosus*, *Pyrococcus horikoshii*, *Methanocaldococcus jannachii* and *Aquifex aeolicus* <sup>204-206</sup> suggests that



chemotactic behavior may be more restricted in hyperthermophiles than in their mesophilic counterparts.

Motility allows thermophiles to respond and reposition themselves in response to changing geochemical or thermal gradients. However, it is possible that chemotaxis is less important to hyperthermophiles than their positioning in the thermal gradient <sup>207</sup>.

Thermotaxis, or the sensing and motile response to thermal gradients, has been demonstrated in nematodes <sup>208</sup> and *Escherichia coli* <sup>209</sup> but has not been robustly studied in thermophiles. Nevertheless, thermotaxis may be of equal importance to chemotaxis for thermophiles, because maintaining a position in a thermal gradient is likely critical to the functionality of their biomolecules <sup>207</sup>. Indeed, the distribution of numerous thermophiles in natural transects has been shown to closely correspond to their optimal growth temperatures. For example, while it is not known if *Synechococcus* are thermotactic, ecotypes of closely-related and thermophilic strains have been shown to have remarkably different cardinal growth temperatures <sup>210</sup> and distributions along a hot spring thermal gradient <sup>211</sup>. Likewise, the maximum rate of acetylene reduction activity (proxy for N<sub>2</sub> fixation) observed in an enrichment of a thermoacidiphilic diazotroph was shown to correspond to the temperature of the environment where it was isolated <sup>72</sup>. In this latter study, the enrichment was from a dynamic and poorly mixed pool containing diffuse elemental sulfur flocs. Such conditions might select for thermotactic behavior in hyperthermophiles.

## 6. MECHANISMS OF EVOLUTION IN HIGH TEMPERATURE ENVIRONMENTS.

Differences in the genomic compositions and organization of closely related taxa indicate that vertical descent alone cannot account for the evolution of these lineages. For example, analysis of the genome sequences of the three closely related thermophilic *Archaea* *Pyrococcus abyssi*, *Pyrococcus horikoshii*, and *Pyrococcus furiosus* revealed different levels of conservation among four regions of their chromosomes containing genetic elements that likely mediated chromosomal reorganization, along with a substantial degree of divergence<sup>212</sup>. Divergence of *P. furiosus* from the ancestral *Pyrococcus* strain might have occurred through Darwinian evolutionary processes (i.e., positive selection) acting on genes involved in translation and/or due to loss of some genes involved in signal transduction or cell motility. In the case of *P. horikoshii* and *P. abyssi*, positive selection was found to operate primarily on the transcription machinery and on the genes involved in inorganic ion transport<sup>213</sup>. At the proteomic level, the comparison of the three *Pyrococcus* species revealed substantial differences in their proteolytic enzymes<sup>212</sup>, implying these closely related *Archaea* may have different fitness in diverse hydrothermal environments depending on carbon source availability. This may suggest that genetically related but physiologically distinct *Thermococcales* spp. evolved to occupy slightly different niches<sup>207</sup>. The resulting polymorphism is probably linked to an adaptation of these thermophiles to differential environmental constraints. Gunbin et al., 2009 suggested hydrostatic pressure as one of the environmental factors that played a key role as an evolutionary divergence of *P.*

*furiosus*, *P. abyssi*, and *P. horikoshii* from their common ancestor given differences in cardinal pressures for growth of the three strains <sup>213</sup>. However, as pointed out by Gunbi et al., 2009, adaptation to pressure is not the sole causative factor, as there are also differences in the metabolisms of these strains, in particular at the level of carbohydrate utilization and amino acid auxotrophy. For example, while *P. furiosus* and *P. abyssi* can utilize maltose and pyruvate for growth, growth of *P. horikoshii* is inhibited by these substrates. Likewise, whereas *P. furiosus* and *P. abyssican* synthesize tryptophan, *P. horikoshii* is auxotrophic for this amino acid. For these reasons, a secondary driver in the diversification of *Pyrococcus* strains is suggested to be at the level of nutrient partitioning and to occupy different trophic level positions within the food web.

Horizontal gene transfer associated with CRISPR (clustered regularly interspaced short palindrome repeat) elements has also been observed in *P. furiosus* <sup>214</sup>. The CRISPR system constitutes a microbial immune system that functions to target and neutralize foreign DNA in a manner similar to eukaryotic RNA interference <sup>215,216</sup>. Horizontal gene transfer associated with CRISPR elements likely involves the insertion of DNA fragments from other organisms into archaeal genomes, thus contributing to genomic and physiological diversification of hyperthermophiles <sup>214</sup>.

Although horizontal gene transfer events are difficult to prove unambiguously, one potential method for doing so involves placing genes or proteins within a phylogenetic context and comparing them to taxonomic trees that are constructed through analysis of ribosomal RNA genes. Phylogenetic analyses of genes or proteins that are differentially represented in the genomes of closely related strains can be used to determine whether

these genetic differences are due to gene loss or acquisition through the process of horizontal gene transfer. For example, genomic analysis of bacteria belonging to the thermophilic order *Thermotogales* has revealed extensive evidence for horizontal gene transfer with other thermophilic Archaea likely occupying partially overlapping niches.<sup>217-220</sup> Many of the genes that have been horizontally transferred with *Archaea* encode ATP binding cassette (ABC) transporters. Analysis of the substrate binding affinities of the proteins encoded by these operons indicate that oligosaccharides are their likely substrates and not oligopeptides as was originally suggested<sup>221</sup>.

A recent screening of two representative *Aquificales* genomes [*Thermocrinis albus*<sup>222</sup> and *Hydrogenobacter thermophilus*<sup>223</sup>] revealed the presence of nitrogenase gene clusters<sup>224</sup>. Maximum likelihood-based phylogenetic reconstructions of a concatenation of the nitrogenase structural protein sequences, NifHDK, indicates that Nif proteins in these two *Aquificales* genomes were acquired recently through a lateral gene transfer with a more recently evolved and thermophilic member of the bacterial phylum *Deferribacteres* (e.g., *Calditerrivibrio nitroreducens* or *Denitrovibrio acetiphilus*). This suggests that *Aquificales* acquired *nif* comparatively recently from an exchange with a bacterial partner in a thermal environment. In further support of this hypothesis, numerous *Aquificales* genera (e.g., *Hydrogenobaculum*) do not encode *nif*<sup>225</sup>, despite branching more basal than *Thermocrinis* and *Hydrogenobacter* in taxonomic trees<sup>58</sup>. *Hydrogenobaculum* spp. tend to populate acidic geothermal environments where  $\text{NH}_4^+$  produced from magmatic degassing is in much higher supply<sup>124</sup> whereas *Thermocrinis* and *Hydrogenobacter* tend to populate circumneutral to alkaline environments that are N limited<sup>62</sup>. Thus, the recent

diversification of *Aquificales* into N limited environments may have been facilitated by acquisition of *nif*. Together, these findings add to a growing body of evidence suggesting that lateral gene transfer has played a significant role in expanding the taxonomic and ecological distribution of N<sub>2</sub> fixation<sup>226-228</sup>. Moreover, this analysis illustrates how the nuances of an environment (e.g., N limitation) can select for horizontal gene transfer events that increase fitness and also illustrates how environmental characteristics shape the co-distribution of lineages (e.g., *Thermocrinis/Hydrogenobacter* and *Deferribacteres*), which then allows for or promotes horizontal gene transfer to occur between these species<sup>229</sup>.

This same phenomenon also likely explains “phylogenetic barriers” to horizontal gene transfer, which as discussed above, probably reflect different evolutionary paths for specific lineages to occupy different and non-overlapping environmental niches<sup>230</sup>. For example, the genomes of the hyperthermophilic bacteria *Aquifex aeolicus* and *Thermotoga maritima* were shown to contain a considerably greater fraction of archaeal genes than any of the other, non-thermophilic bacterial genomes. This suggests a connection between the similarity in growth conditions of donor/recipient strains, their ecological niches, and the apparent rate of horizontal gene exchange between them. *A. aeolicus* and *T. maritima* likely acquired their genes by horizontal gene transfer from hyperthermophilic *Archaea*, perhaps the euryarchaeote *P. horikoshii*, which co-inhabit similar high temperature environmental niches<sup>217,231,232</sup>. Conversely, the thermoacidophilic archaeon *Thermoplasma acidophilum* and the moderate thermophile

*Halobacterium* sp. appear to possess a significantly greater number of genes that were horizontally transferred from *Bacteria*, when compared to other archaeal species <sup>230,233</sup>. The probability of horizontal gene transfer varies among genes in a given genome. Generally, informational genes (those involved in transcription, translation, and related processes) are less prone to horizontal transfer than are other categories of genes, such as operational genes (those involved in housekeeping). This difference may be due to the fact that informational genes are typically members of large and complex systems, whereas operational genes are not thereby making horizontal transfer of informational genes less probable. <sup>234</sup>. Interesting exceptions include aminoacyl-tRNA synthetases, which are key components of the protein translation machinery whose evolution involves horizontal gene transfer <sup>235-237</sup>. Nearly all of the 20 aminoacyl-tRNA synthetases are ubiquitous and essential for cell growth in all living organisms (i.e., informational genes). However, unlike most informational genes, they do not have multiple protein partners and their interactions are limited to their contacts with their cognate tRNA, ATP, and amino acid. Furthermore, although aminoacyl-tRNA synthetases belong to the translational (informational) machinery, they function in isolation from this complex <sup>238</sup> and due to their central role in protein translation, there is strong purifying selective pressure acting on the evolution of aminoacyl-tRNA synthetases and therefore it is unlikely to be lost during cell replication <sup>239,240</sup>.

Another unexpected case of a likely horizontal gene transfer between eukaryotes and *Archaea* is the Trp-tRNA synthetase from the hyperthermophilic archaeal genus *Pyrococcus* <sup>236</sup>. Given the high temperatures at which *Pyrococcus* species grow, the

presence of a eukaryotic aminoacyl-tRNA synthetase in these hyperthermophilic *Archaea* is surprising and may suggest that *Pyrococcus* acquired this gene from a thermophilic eukaryote such as a polychaete annelid<sup>230,241</sup>. This apparent horizontal transfer of eukaryotic Trp-tRNA synthetase gene into *Pyrococcus* most likely involved a xenologous gene displacement, where the ancestral Trp-tRNA synthetase from *Pyrococcus* is replaced by the eukaryotic version<sup>236</sup>.

Other evolutionary mechanisms that can account for divergences between genomes are “gene duplications”, “gene loss”, and “gene fusions”. As previously mentioned analysis of the genomes of the closely related hyperthermophiles *Pyrococcus furiosus* and *Pyrococcus horikoshii* revealed extensive differences in their composition. Both genomes differ considerably in gene order, displaying displacements and inversions. Gene composition also differed between genomes, suggesting genomic rearrangements and gene loss. On the other hand, the occurrence of two paralogous families of ferredoxin oxidoreductases in both *Pyrococcus* genomes provides evidence for gene duplication preceding the divergence of the *Pyrococcus* species<sup>204</sup>.

Gene duplications, losses, and fusions were prevalent in the evolution of molybdenum (Mo)-nitrogenase, which has its origins in hyperthermophilic methanogens and which provides the majority of biologically fixed N on the planet<sup>242</sup>. While *nif* emerged in hydrogenotrophic methanogens and was likely acquired vertically in other members of the *Methanobacteriales*, *Methanomicrobiales*, and *Methanococcales*<sup>142, 242</sup>, not all members of these orders encode Mo-nitrogenase. This indicates gene loss among these members. Moreover, several of the structural proteins of Mo-nitrogenase encoded by

*nifDK* are homologous to *nifEN*, which encodes proteins required to synthesize the Mo-based cofactor of this enzyme<sup>243-246</sup>. Phylogenetic evidence indicates that *nifEN* are clearly the result of a in tandem duplication of *nifDK*<sup>226,242,247,248</sup>. NifB, another protein required for the synthesis of the active site cofactor of Mo-nitrogenase<sup>249,250</sup>, is a fusion protein consisting of an amino terminal domain belonging to the radical S-adenosyl methionine (SAM) family of proteins and a carboxy terminal domain belonging to the NifX/NafY family of proteins in most organisms<sup>251</sup>. However, in early evolving thermophilic methanogens, NifB is not fused<sup>242</sup>, indicating that the fusion event took place later in the diversification of this metabolic process.

## 7. CONCLUDING REMARKS.

The early evolutionary events that led to the spectrum of functional diversity present in contemporary microorganisms has been suggested to trace back to a hydrothermal heritage<sup>14,16</sup>. While, this appears to be the case for several processes that are considered by many to have been key to the origin of life such as hydrogen oxidation<sup>252</sup> as well as processes that were key to the emergence of higher forms of life such as nitrogen fixation<sup>224,242,253</sup>, it is less clear in the case of taxonomic diversification. In particular, phylogenetic reconstructions of taxonomic genes samples across environmental gradients often fail to show high temperature environments as harboring the most basal branching members<sup>68</sup>. This may suggest that evidence for a high temperature origin of life has been obscured by subsequent diversification in response to other environmental drivers such as pH and oxygen. Alternatively, such results may point to a mesophilic origin of



life<sup>21,22</sup>. Additional studies that incorporate more spring environments and more robust genetic datasets (e.g., whole genomes or concatenations of house-keeping genes) may provide a more comprehensive picture of the characteristics of the environment most likely to have supported early evolving lineages.

The taxonomic and functional diversity of microbial life in modern hydrothermal systems, in particular those that are too hot for photosynthesis ( $>73^{\circ}\text{C}$ ) has been suggested to be the result of the underlying geological processes that create extensive geochemical variation in these systems<sup>25,38</sup>. The integration of phylogenetic tools and molecular microbial ecology approaches (i.e., phylogenetic ecology) now permit these hypotheses to be quantitatively evaluated. Significant questions remain, however, including the extent to which interspecies interactions (facilitative, competitive) influence the evolutionary trajectory of key biological processes and whether these interactions overwhelm the geochemical influences on the diversification of these processes. Indeed attempts to explain the diversification of key proteins in hydrothermal environments based on geochemical data using phylogenetic tools often fail to explain the majority of the variation in the sequence dataset<sup>7-9,11</sup>. This suggests that other unaccounted variables, such as interspecies interactions, or additional geochemical variables need to be included in such analyses. Omics approaches provide a potential mechanism to begin to unravel the nature and extent of such interactions which may provide a path forward to furthering our understanding of the processes that have influenced the evolution of thermophilic microorganisms.

Thermophiles have a number of unique adaptations that enable their persistence in high temperature environments. However, it is likely that adaptations allowing hyperthermophiles to persist in high temperature environments remain to be discovered. The development and application of next generation sequencing technologies continue to reveal new protein encoding genes that cannot be classified based on homology to biochemically characterized proteins. As an example, annotations of thermophile genome sequences typically result in functional assignment of only a small fraction (<40%) of the encoded open reading frames (ORFs). The function of the remaining ORFs and their role in thermophile physiology are a frontier in our understanding the evolution of thermophiles. Despite the success of applying molecular tools to characterize the taxonomic and functional diversity of microbial communities in hydrothermal systems, much of this diversity is only reported in sequence datasets and is not represented in culture collections preventing physiological and biochemical studies. Continued effort to bring representatives of ecologically relevant (dominant) lineages into culture, in conjunction with development of new genetic systems suitable for heterologous expression of thermophile genes, is needed to begin to close the gap in our understanding of the functional role of poorly characterized genes identified through genome sequencing efforts.

The development and application of next generation sequencing tools also permit analyses of the rate of evolutionary change in thermophiles under the natural conditions of their environment. Similar approaches have been applied in studies of rates of evolutionary change in other extreme environments, such as acid mine drainage<sup>254</sup>.

Application of such techniques to communities that span the geochemical gradients present in continental hydrothermal systems offers the unique opportunity to examine questions related to the role of environmental variation, both spatial and temporal, in constraining or promoting evolutionary change.

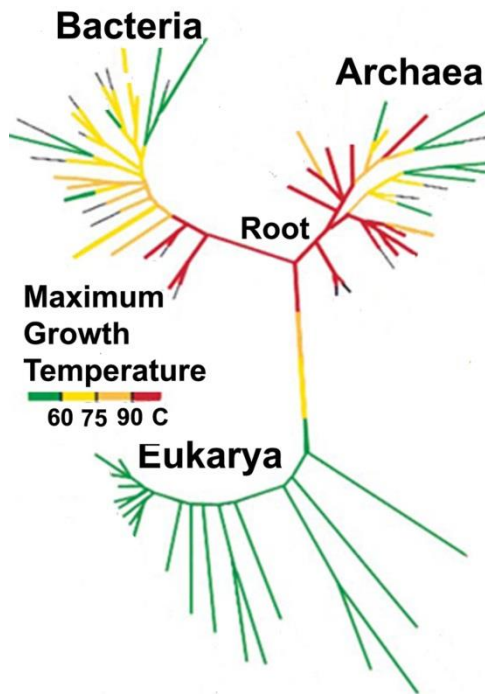


**Figure 1.** (A) Universal tree of life with an overlay of maximum growth temperature implies a thermophilic origin of life [Figure adapted from Lineweaver and Schwartzman, 2004].

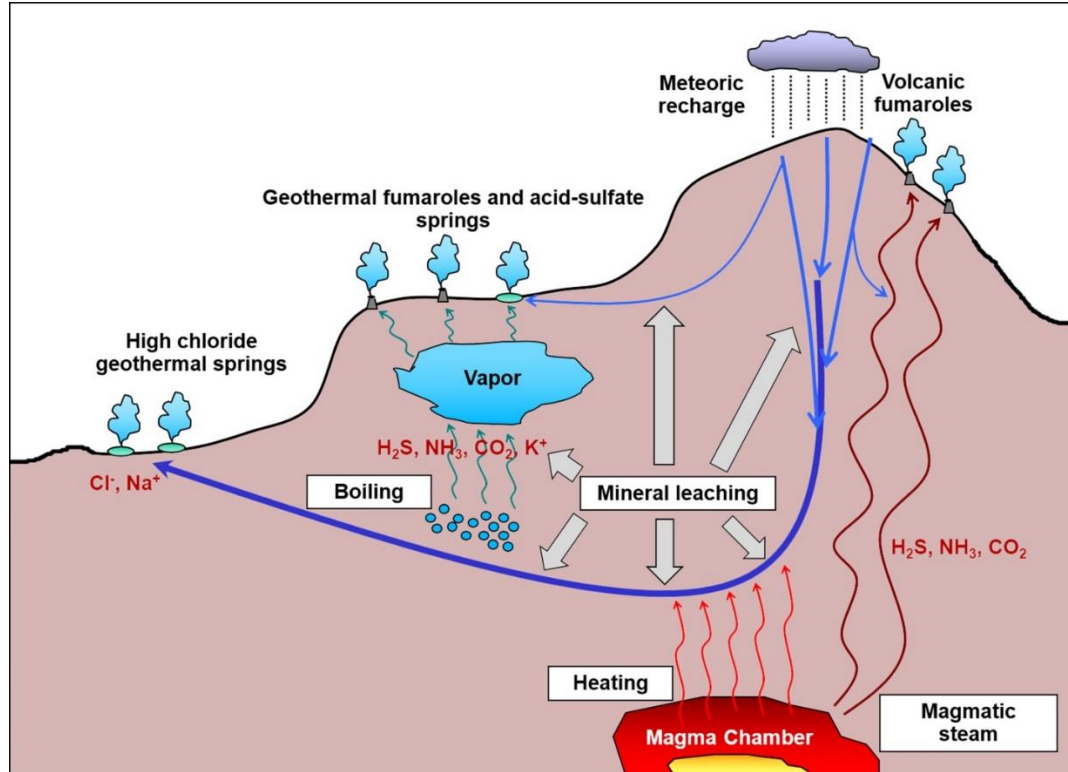


**Figure 2.** Schematic illustrating the hypothesized functioning of a continental magmatic hot spring system. Here, meteoric water infiltrates to the subsurface where it is heated by a magma chamber. Heated water returns to the surface due to density differences. During the flow path back to the surface, heated water interacts with different bedrock types, leaching different minerals. Ascending water can also undergo phase transitions leading to acidic fumaroles and acid-sulfate springs or alkaline high chloride springs.

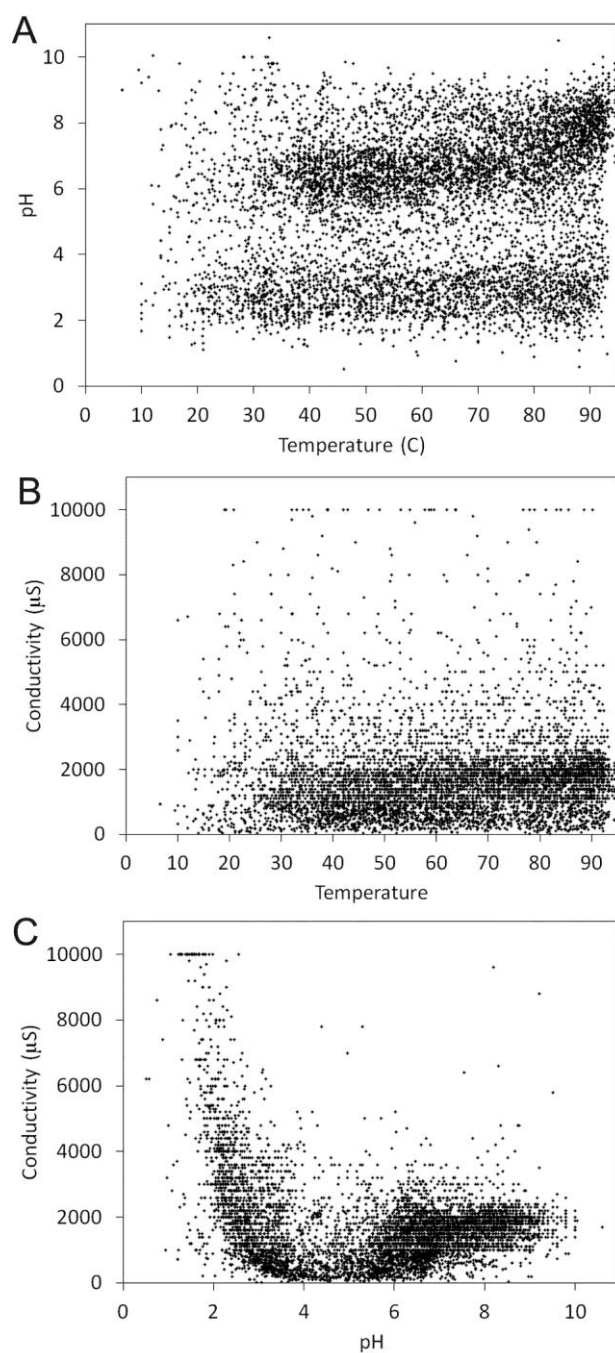


**Figure 3.** pH, temperature, and conductivity measurements plotted as a function of each other for ~7700 hot springs in Yellowstone National Park. Data was compiled from the Yellowstone Research Coordination Network website (<http://www.rcn.montana.edu/>).



**Figure 4.** Examples of several of the predominant spring types present in magmatic continental hot spring systems, in this case, Yellowstone National Park. The variation in physical and chemical properties of the hot spring types present in YNP is indicated.

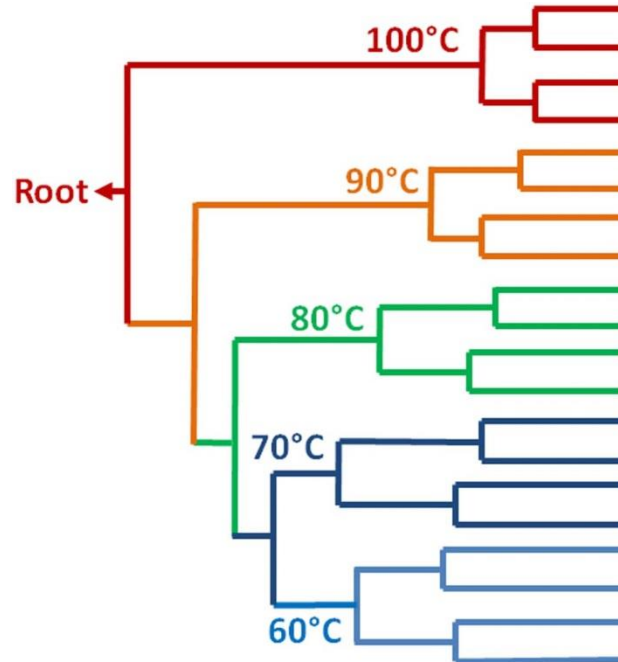


**Figure 5.** Schematic illustrating phylogenetic approaches to ecological studies. In this case, sequences were collected across a temperature gradient ranging from 60 to 100°C and were subjected to phylogenetic reconstruction. Using community ecology tools it is possible to map geochemical characteristics onto the tree and quantify the extent to which they influenced the diversification of a given gene, protein, or genomic tree.



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APPENDIX B

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**Title:** Microbial substrate preference dictated by energy demand rather than supply

**Author:** Maximiliano J. Amenabar, Everett L. Shock, Eric E. Roden, John W. Peters, Eric S. Boyd

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