

CHEMOSYNTHETIC CARBON METABOLISM IN THERMOPHILES

by

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DEDICATION

For my wife Janna, who never stopped believing in me.

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ABSTRACT

Microbial communities inhabiting high temperature (>73°C) environments are supported by chemical energy, providing a unique opportunity to investigate the processes that supported life prior to the advent of photosynthesis. Previous work has focused on the importance of autotrophy in supporting such communities, and recent reports of organic substrate utilization in several high temperature springs in Yellowstone National Park (YNP), Wyoming, USA suggest that chemosynthetic populations are facultatively autotrophic. Nevertheless, little is known about the factors influencing relative rates of autotrophy and heterotrophy in these systems, and few studies have addressed the potential role of facultative autotrophs in supporting these ecosystems.

This work addressed these compelling questions using *in situ* microcosm assays to directly quantify organic and inorganic substrate transformation rates in 13 geochemically diverse YNP chemosynthetic communities. The results provide the first conclusive evidence that dominant autotrophs in these ecosystems are facultative, and can alter their metabolism over short time spans to preferentially exploit more thermodynamically favorable organic substrates at rates comparable to or exceeding those of inorganic substrate utilization. Multivariate statistical analysis of co-registered substrate transformation rates, geochemical measurements, and phylogenetic data collected from these communities suggests an important relationship between environmental variation, community composition, and the relative importance of autotrophic and heterotrophic metabolisms supporting these communities. Elevated formate utilization rates in crenarchaea-dominated chemosynthetic communities inhabiting acidic, sulfur-rich geothermal springs motivated the isolation of the first hyperthermophilic crenarchaeon (*Thermoproteus* sp CP80) capable of coupling formate oxidation to elemental sulfur reduction. Physiological characterization demonstrated that CP80 is a facultative autotroph that alters its metabolism to preferentially utilize formate over CO₂. Similar formate utilization characteristics by CP80 and its native population strongly suggests that this and other sulfur reducing crenarchaea may be responsible for high rates of formate utilization in high temperature, sulfur rich YNP systems. Overall, these results indicate an important, previously-underestimated role for organic substrates in supporting chemosynthetic communities inhabiting geochemically diverse YNP hot springs. Future work should focus on identifying additional organic carbon sources, measurement of carbon flux through chemosynthetic communities, and further characterization of the biochemical mechanisms underlying organic and inorganic substrate metabolism in *Thermoproteus* sp. CP80.

CHAPTER 1

GENERAL INTRODUCTION

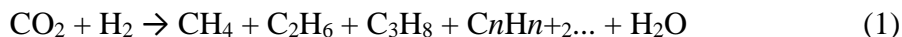
Microbial communities residing in hydrothermal environments with a temperature exceeding approximately 73°C are supported by chemical energy (chemosynthesis), rather than energy from light (photosynthesis) (1-5). Many of these hydrothermal environments support abundant microbial biomass (3, 6-8), indicating that the microbial assemblages in these communities must obtain their energy from chemical sources. Previous studies have suggested that chemolithoautotrophic metabolisms are the dominant primary producers in these systems (7). The prevalence of chemolithotrophic metabolism, or the assimilation of CO₂ is driven by energy obtained from the oxidation of H₂, sulfide (S²⁻), thiosulfate (S₂O₃²⁻), or elemental sulfur (S₈⁰) under aerobic or anaerobic conditions, based primarily on the known metabolic capabilities of organisms closely affiliated with the numerically-dominant organisms in these habitats (e.g. organisms from the bacterial order *Aquificales*)(6-11). However, physiological evidence from cultivated representatives of *Aquificales* indicates that some of these organisms are facultative autotrophs, capable of heterotrophic growth on organic acids such as formate, acetate and formamide, in addition to autotrophic growth on CO₂ (12, 13). Moreover, recent reports indicate that organic substrates may be used to a greater extent than CO₂ in chemosynthetic communities in some hydrothermal environments (14, 15), consistent with thermodynamic calculations which predict that energy derived from the oxidation of organic substrates, such as formate, is comparable to, or higher than, that derived from

the oxidation of hydrogen in many Yellowstone National Park (YNP) hot spring ecosystems (16). Taken together, these results indicate that chemosynthetic communities in hydrothermal environments may be facultative autotrophs and capable of utilizing organic substrates, in addition to inorganic substrates, as they become available in their habitats. Key remaining questions include what the source of organic carbon is in high temperature environments and whether microorganisms are capable of preferentially utilizing these organic carbon sources.

Abundance, Composition, and Possible Origin of Organic Substrates in High Temperature Hydrothermal Systems

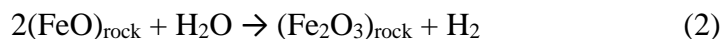
Organic matter in geological systems, including hydrothermal systems such as those present in YNP, typically arises from one of three different mechanisms of synthesis: *i*) “abiotic” organic matter synthesis results entirely from chemical processes that are not directly associated with the activity of biological organisms, *ii*) “biogenic” organic matter synthesis (e.g. lipids, fermentation products, methane, etc.) results directly from the metabolic and biosynthetic activity of one or more biological organisms, and *iii*) “thermogenic” organic matter synthesis results from the thermal decomposition of living organisms and/or biologically-derived compounds (17). For the purposes of this discussion, “biogenic” and “thermogenic” organic matter synthesis will both be treated as one in the same, since both of these processes involve the initial formation of organic compounds from biosynthetic activity. Much of the recent research on the abiotic synthesis of organic matter in hydrothermal environments has been focused on deep sea hydrothermal vent systems (17-28), but the relevant geochemical processes leading to

such synthesis reactions are expected to be similar in any hydrothermal environment with similar physical and chemical properties. In general, the abiotic synthesis of organic substrates in hydrothermal and other geological systems is driven by highly reducing conditions created as the result of interaction of water with rocks containing minerals with high concentrations of reduced iron (e.g., ultramafic and mafic rocks). These water rock interactions can generate large amounts of molecular hydrogen (H_2) through the process known as serpentinization (17), which at high enough concentrations can drive the reduction of carbon dioxide (CO_2) and/or other inorganic carbon compounds, such as carbon monoxide (CO) and bicarbonate (HCO_3^-) (17). The generalized overall chemical reaction for this process is:



Where C_nH_{n+2} can be a simple hydrocarbon, such as formate (CH_2O_2) (17, 29), or a complex organic molecule, such as an amino acid (30) or acylglycerol (31).

The highly reducing conditions required to drive the abiotic synthesis of organic compounds in hydrothermal environments via the generalized reaction given above are thought to result primarily from chemical reactions between ferrous iron-bearing minerals (e.g. pyrrhothite, olivine, pyroxene, etc) in the Earth's crust and underlying mantle with water descending from the surface (17, 32). These reactions involve the reduction of water to molecular hydrogen (H_2) via the oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}), resulting in the precipitation of ferric minerals, such as hematite and magnetite. A general chemical equation for this type of reaction is described by:



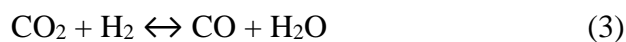
Where $(\text{FeO})_{\text{rock}}$ and $(\text{Fe}_2\text{O}_3)_{\text{rock}}$ refer to the ferrous and ferric components of oxidized and reduced (ultra)mafic minerals, respectively. The quantity of H_2 produced by this type of reaction depends largely on the silica content of the rock in which iron bearing minerals form. Ferrous iron tends to be excluded from the metal sites of minerals formed in low silica (ultramafic) rocks (e.g. brucite and serpentine), resulting in the exposure of more Fe^{2+} ions to descending H_2O , and thus the reduction of more water to H_2 . In contrast, iron-bearing minerals formed in rocks with a higher silica content, such as basalts, incorporate more Fe^{2+} ions into their metal sites, thus preventing the oxidation of these ions by H_2O and resulting in lower rates of H_2 production. Consequently, hydrothermal systems that form in locations with ultramafic bedrock tend to exhibit more strongly reducing conditions, and are therefore thought to be more favorable to the abiotic synthesis of organic substrates (32-36). While YNP is primarily hosted in a systems with silica rich and iron poor rhyllite, the presence of basalt in several of the hydrothermal areas in YNP (37) indicates that the reduction of water to H_2 can take place in these systems (38).

Simple organic compounds such as methane (CH_4) and HCOOH resulting from abiotic synthesis via the chemical mechanisms discussed above can be further reduced via sequential formation of carbon-carbon (C-C) bonds to form more complex hydrocarbons, such as lipids (39), amino acids (30) and other organic molecules (31). The mechanism most often proposed for the abiotic synthesis of more complex organic substrates in hydrothermal environments is known as Fischer-Tropsch synthesis (FTS). The FTS mechanism was initially developed as an industrial process for the synthesis of

hydrocarbon-based fuels (40), but has more recently been suggested to be involved in complex carbon compound synthesis in hydrothermal and subsurface settings. The general FTS reaction mechanism involves conversion of CO into organic compounds via sequential reduction and polymerization of carbon (41, 42). Initiation of this reaction involves the binding of a molecule of CO to the surface of a solid catalyst, thus forming a carbonyl group. Sequential reduction of this carbonyl group by hydrogen atoms (also bound to the catalytic surface) results in the formation of a surface-bound carbide group, followed by a methylene group (-CH₂). This methylene group may be reduced further to form a methyl group (-CH₃), depending on the specific conditions present in the environment in which the reaction proceeds. The bound methylene groups then serve as monomers which are subsequently polymerized via C-C bonds to form an alkyl chain. The final length of this chain is determined by the number of methylene groups polymerized before chain growth is terminated by the bonding of a methyl group or surface-bound hydrogen atom, rather than an additional methylene group. The binding of the alkyl chain to a surface-bound methyl group or hydrogen atom results in the release of the final hydrocarbon chain from the catalytic surface, making it available as a potential carbon and energy source to microorganisms in the surrounding bulk fluid.

The process described above can also result in the formation of more complex organic compounds, such as carboxylic acids and/or alcohols, by the polymerization of other surface-bound chemical groups, such as hydroxyl (-OH) or carbonyl groups, to the initial hydrocarbon chain. The relative abundance of different organic substrates produced from FTS synthesis has been demonstrated to be dependent both on the

probability that the growing catalyst-bound hydrocarbon chain will be polymerized to a chemical group other than methylene, as well as the nature of the catalytic surface upon which the reaction proceeds (41-43). Thus, the nature of the organic substrates produced in a particular hydrothermal environment depends on the relative abundance of chemical groups that can act as monomers for FTS synthesis, as well as the types of catalytic surfaces present. While CO is required to initiate the FTS reaction, it can also proceed from CO₂ with the addition of a first step involving the reversal of what is sometimes referred to as the “water-gas shift reaction”:



This reaction has been demonstrated to occur in laboratory experiments under conditions similar to those found in hydrothermal environments, therefore allowing CO₂, which is typically abundant in hydrothermal settings (44-47) to serve as the “raw material” for the abiotic synthesis of organic substrates (48-51).

Potential biogenic sources of organic carbon in high temperature, chemosynthetic communities are numerous, and not completely understood. However, in general, organisms associated with the trophic levels known to exist in mesophilic environments (52, 53) also appear to be present in hydrothermal habitats (54, 55). This suggests that the organic substrates utilized and produced by microorganisms occupying the functional niches present in mesophilic microbial communities should also be produced by hyperthermophilic organisms occupying similar functional niches in chemosynthetic communities in hydrothermal environments. In mesophilic microbial habitats, basic biopolymers (e.g. cellulose, hemicellulose, chitin, keratin, etc.) passed into the microbial

food web from higher plants and animals are degraded via microbial activity to their respective monomers, such as glucose, xylose and N-acetylglucosamine (52, 56, 57). These monomers then serve as carbon and/or energy sources for other microorganisms (facultative aerobes, acetogens, primary/secondary fermenters, etc.) residing in the same habitat, which further degrade these organic compounds to yet simpler organic molecules (e.g. formate, acetate, and other organic acids, alcohols, etc). In this way, exogenously sourced organic carbon initially produced from CO₂ by photosynthetic or other primary producers is degraded by microbial activity in a step-by-step process by which each subsequent trophic level utilizes the metabolic products of the previous level as energy and or carbon sources. The metabolic products resulting from the metabolism of these energy and/or carbon sources are then passed on to the next level, ultimately leading to the return of fixed carbon to CO₂, which once again becomes available for reuptake by primary producers (52, 53).

Recent studies have proposed that high temperature, chemosynthetic communities in terrestrial hydrothermal environments, such as YNP, likely receive intermittent pulses of exogenous organic carbon from their immediate environment as the result of meteoric activity (i.e. rain) (15, 58), suggesting that the basic biopolymers available to microbial communities in mesophilic environments are also available to chemosynthetic microbial communities residing in these high temperature habitats. Many bacterial and archaeal hyperthermophiles known to occur in hydrothermal environments, including YNP, have been demonstrated to be capable of degrading basic biopolymers, such as cellulose and hemicellulose (59-65), chitin (66-70), xylan (71), and many others. Genes for the

cellulolytic enzymes cellulase, xylanase, endoglucanase, exoglucanase, and cellobiohydrolase, required for the breakdown of cellulose, hemicellulose from plant material into monomers such as glucose, xylose, and cellobiose, are widely distributed amongst hyperthermophilic organisms, including bacteria (65) and archaea (71). For example, the hyperthermophilic bacterium *Thermoanaerobacter tengcongensis* MB4, isolated from a high temperature geothermal spring in a hot spring in the Tengcong volcanic area (Yunnan, China), has been demonstrated to possess a gene encoding a novel endoglucanase enzyme designated Cel5A (72). Cel5A was found to specifically cleave the β -1,4-glycosidic linkage in cellulose, and had optimum activity at temperatures between 75 and 80 °C, suggesting that this enzyme may be involved in the degradation of cellulose by *T. tengcongensis* MB4 in its native, high temperature environment.

Thermostable endoglucanases which remain active at temperatures as high as 100 °C have also been characterized in hyperthermophilic archaea, including the euryarchaeotes *Pyrococcus furiosus* and *Pyrococcus horikoshii* and the crenarchaeote *Sulfolobus solfataricus* (73), and putative homologs of the enzyme xylanase, which functions in the degradation of hemicellulose, have also been identified in the genomes of other hyperthermophilic crenarchaeota, including *Thermoproteus tenax* (74). In addition, active chitinase enzymes, responsible for the breakdown of the biopolymer chitin into glucosamine monomers, have been characterized from a number of hyperthermophilic archaea (67-69, 75). A novel chitinase from the hyperthermophilic, deep-sea hydrothermal vent archaeon *Thermococcus chitonophagus*, for example, has been shown to maintain up to 50% activity, even after exposure to temperatures as high as 120 °C for

a duration of 1 hour, and is capable of releasing chitobiose from colloidal chitin (68, 69). Based on the presence of so many hyperthermophilic microorganisms with the capability to degrade basic biopolymers in a wide variety of hydrothermal environments, it seems probable that the products of such degradation (e.g. glucose, xylose, cellobiose, N-acetylglucosamine, etc) are likely to be available in significant quantities as potential carbon and/or energy sources to chemosynthetic communities residing in these habitats.

The monomers produced by the microbial-driven degradation of biopolymers (e.g. glucose and xylose, N-Acetyl-D-glucosamine, cellobiose, etc) are utilized in mesophilic environments by a wide range of microorganisms, including primary fermenters, facultative aerobes and acetogens (52). As with the degradation of biopolymers, a number of hyperthermophilic organisms occupying the same functional niches as their mesophilic counterparts (with respect to the utilization of sugars and other monomers) have been identified (76-79). The ability to metabolize numerous sugars is widely distributed amongst hyperthermophilic archaea, including organisms from several orders of creanarchaea, including *Sulfolobales*, *Thermoproteales*, and *Desulfurococeales*, as well as the euryarchaeal orders *Thermococcales*, and *Archaeoglobales*, and also occurs in hyperthermophilic bacteria of the order *Thermotogales*, among others (76, 79). Many of these organisms metabolize sugars such as glucose, xylose, and other hexose and pentose sugars via fermentative pathways resulting in end products including as formate, acetate, lactate, butyrate, isobutyrate, isovalerate and propionate, as well as ethanol, H₂, and CO₂ (refs). For example, the hyperthermophilic archaeon *Pyrococcus furiosus*, which was isolated from geothermally-heated marine sediments, utilizes a modified

version of the Embden-Meyerhof Pathway to produce ATP, reduced equivalents, and precursor metabolites for cellular processes via the fermentation of glucose to acetate, alanine, H₂ and CO₂ (80). This pathway involves an ADP-dependent hexokinase in the first step of the reaction (phosphorylation of glucose to glucose-6-phosphate) and an ADP-dependent phosphofructokinase in the third reaction step (phosphorylation of fructose-6-P to fructose-1,6-bis-P), rather than the ATP-dependent kinases typically utilized in other organisms (76, 80). *P. furiosus* has also been demonstrated to ferment starch, glycogen, maltose, cellobiose, and pyruvate (76). Strains of the hyperthermophilic, acetogenic bacterium *Clostridium thermocellum* isolated from an Icelandic hot spring are also capable of fermenting the cellulolytic end product cellobiose, to produce acetate, CO₂, hydrogen, and small amounts of butyric acid (butyrate) (Stainthorpe and Williams, 1988). In addition to the fermentation of sugars, a number of hyperthermophilic archaea, including organisms from orders *Sulfolobales* and *Thermoproteales*, are known to oxidize glucose and other sugars aerobically or anaerobically, conserving energy from the coupling of sugar oxidation to the reduction of O₂, S⁰, or other sulfur compounds (79), and typically resulting in the formation of acetate, formate, and/or CO₂. The end products of the fermentative and respiratory metabolisms discussed above can also serve as additional carbon and energy sources for other organisms residing in high temperature, chemosynthetic communities.

The thermal fluids from several hydrothermal systems, including YNP, have been shown to often contain substantial concentrations of the organic substrates (16, 51, 81-83). Martins et al, 1990 (83) reported that concentrations of the organic acids acetate and

propionate increased with sediment depth from 0 to ~1.1 mM at depths of ~13 and ~42 cm, respectively, for acetate, and from 0 to ~220 μM , respectively, for butyrate at the same depths in pore water samples extracted from high temperature ($> 100^\circ\text{C}$), hydrothermal sediment cores hosting a chemosynthetic, deep-sea hydrothermal vent community in the Guaymas Basin of the Gulf of California. The authors attributed the presence of these organic acids to the alteration of biogenic organic matter through contact with hydrothermal solutions (i.e. thermogenesis), and noted that concentrations of both substrates were elevated in relatively high temperature sediments ($\leq 125^\circ\text{C}$), as compared to relatively low temperature sediments ($\leq 50^\circ\text{C}$). Isotopic analysis of n-alkanes and carboxylic acids, such as methane, ethane, propane and butane, in fluids sampled from the ultramafic-hosted Lost City and Rainbow hydrothermal vent communities located on the Mid-Atlantic Ridge (MAR), demonstrated that these organic compounds exhibited isotopic characteristics ($\delta^{13}\text{C}$ values) consistent with both an abiotic and abiogenic origin (81), leading the authors to conclude that both processes were likely contributing to the formation of organic substrates in this hydrothermal environment. Interestingly, the authors note that the $\delta^{13}\text{C}$ values exhibited by organic acids in these habitats were enriched in ^{13}C , relative to alkanes of the same chain length, which is consistent with previous $\delta^{13}\text{C}$ values observed in organic acids of biogenic origin (84, 85). This suggests a possible role for biology in the generation of organic acids in deep-sea, hydrothermal communities. In contrast, earlier reports from Proskurowski et al (2008) (51) observed an “inverse” trend in stable carbon and hydrogen isotopic data (i.e. $\delta^{13}\text{C}$ and δD values became more negative with increasing hydrocarbon chain length)

resulting from analysis of organic compounds sampled from Lost City hydrothermal fluids. The authors point out that these trends are opposite those observed in other organic molecules and gases known to be generated by “thermogenesis” (i.e. via the heat alteration of biomass) (86, 87), and are consistent with a similar increase in the negativity of $\delta^{13}\text{C}$ values observed in laboratory experiments involving the abiotic generation of organic compounds via FTS/FTT synthesis reactions (88). Although less attention has been paid to the distribution and origin of organic compounds in terrestrial hydrothermal systems than to that in their marine counterparts, organic substrates such as formate, acetate, lactate and others have been detected in terrestrial geothermal features (16, 89, 90). For example, Windman et al, (2007) (16) reported detectable levels of formate in the hydrothermal fluids of 47 out of 56 sampled geothermal springs in YNP with pH and temperature ranging from 1.91 to 8.85 and 35.3 °C to 92.6 °C, respectively. Formate concentrations in these geothermal features ranged from ~161 nM to ~11 μM . Taken together, the results above demonstrate that a wide range of organic substrates suitable for supporting chemosynthetic communities can be synthesized in hydrothermal systems by both biogenic and abiotic processes, and that many of these compounds are present in significant quantities in a number of these systems, including YNP. This suggests that organic substrates may play a more important role than previously supposed in supporting chemosynthetic microbial communities in these habitats.

Utilization of Organic Substrates by Predominantly Autotrophic Chemosynthetic_ Microbial Communities in High Temperature Hydrothermal Systems

Initial studies of the phylogenetic composition of chemosynthetic populations in high temperature (>73 °C) geothermal features in YNP indicated that many of these systems are numerically dominated by hyperthermophilic bacteria from order *Aquificales* (6-11, 91, 92), all known members of which have been demonstrated to grow autotrophically using H₂ and CO₂, respectively, as energy and carbon source. These results, combined with *in situ* concentrations of H₂ and CO₂ in excess of 300 nM and 20 mM, respectively (91), have been used to suggest that these chemosynthetic communities are predominantly supported by autotrophic metabolism. For example, Spear et al (2005) (91) reported that 77% of the 16s rRNA sequences amplified using universal primers from DNA extracted from the sediments of 5 high temperature YNP springs were most closely affiliated with known species of the *Hydrogenobacter* genus from order *Aquificales*, with another 3% of sequences being most closely related to *Aquificales* members of the genus *Hydrogenobaculum* and *Hydrogenothermus*. Similar studies have also demonstrated the presence of additional representatives of order *Aquificales* in other YNP chemosynthetic communities, including *Thermocrinis* spp. (8, 91, 93) and *Sulfurihydrogenibium* spp. (9). Thermodynamic modeling results predicting the energetic favorability of H₂ oxidation under the conditions present in the habitats from which they were isolated were offered as further evidence that these communities were predominantly supported by autotrophic CO₂ fixation using H₂ as an energy source (8, 91). These modeling efforts involved the comparison of the theoretical maximum energy

yield (ΔG_r) across a range of pH values under the extant geochemical conditions from redox reactions coupling the oxidation of H_2 , and various other inorganic electron donors commonly occurring in high temperature habitats of YNP (e.g. H_2S , S^0 , CH_4 , Fe^{2+} , CO , pyrite and goethite), to the reduction of O_2 , S_8^0 , and SO_4^{2-} . The predicted energy yields indicated that, under the conditions present in the springs studied, aerobic H_2 oxidation yielded more energy than anaerobic H_2 oxidation, or the aerobic or anaerobic oxidation of the majority of other inorganic electron sources considered, with the exception of carbon monoxide (CO).

In support of the predictions of these initial studies, a subsequent study utilized a radiotracer approach in combination with in situ microcosm incubations amended with radiolabeled bicarbonate ($H^{14}CO_3^-$) to directly measure the in situ rate of CO_2 uptake and assimilation in an S_8^0 -floc-associated, chemosynthetic community in the YNP geothermal spring known as Dragon Spring (73°C, pH 3.1) (6). The results of this experiment demonstrated that the CO_2 uptake rate exhibited by the chemosynthetic community in Dragon Spring exceeded that measured by a previous study in an alkaline, photosynthetic mat community of similar temperature dominated by the cyanobacterium *Synechococcus*. Moreover, the results of additional experiments showed no significant difference in CO_2 fixation rate between light and dark-incubated microcosms, conclusively demonstrating that the S_8^0 -floc-associated community in Dragon Spring was fixing carbon from CO_2 via chemosynthesis, rather than photosynthesis. In addition, clone libraries constructed from 16S rRNA genes amplified from S_8^0 floc confirmed the presence of organisms closely affiliated with *Hydrogenobaculum* spp., from order

Aquificales, and stimulation of CO₂ fixation activity upon the addition of O₂ to microcosms indicated that CO₂ fixation was likely being driven by the aerobic oxidation of available energy sources. Unexpectedly, however, amendment of S₈⁰-floc microcosms with H₂ resulted in an apparent decrease in the rate of CO₂ fixation, a result that is in direct opposition to the proposal of previous studies that chemosynthetic communities in YNP utilize H₂ as their primary energy source. Consistent with this result, D'Imperio et al (2009) (11) reported rates of H₂S consumption by microbial mat samples from Dragon Spring that were 4 orders of magnitude higher than rates of H₂ consumption in the same samples. Furthermore, results of culture experiments from the same study demonstrated that cultures inoculated with cells from Dragon Spring mat samples exhibited higher growth rates on H₂S, as compared to H₂. Physiological experiments with a pure culture of an isolated representative of the dominant phylotype in Dragon Spring indicated that this organism was capable of alternating between the utilization of H₂ and H₂S as energy source. The immediate utilization of H₂ and H₂S exhibited by the organism, regardless of which substrate it was pre-conditioned to, was interpreted as evidence that the metabolic switch between these substrates was not the result of enzyme induction or catabolite repression, but indicated that the organism was metabolically poised to immediately utilize either or both of the two potential energy sources as they became available in its environment. Overall, the results discussed above indicate that autotrophy is an important metabolic strategy utilized by chemosynthetic communities in YNP, but that the organisms comprising these populations also exhibit metabolic flexibility with respect to the electron donors they utilize.

The metabolic flexibility with respect to inorganic energy sources exhibited by some organisms isolated from chemosynthetic communities in high temperature geothermal features suggests that these populations may also be capable of utilizing organic compounds, depending on the availability of those compounds in their habitat. Several recent studies in YNP and other hydrothermal environments offer supporting evidence for this suggestion (14, 15, 58, 94). For example, a recent study used various mass spectrometry methods to characterize *Aquificales*-specific intact polar lipids (IPLs) isolated from streamer biofilm communities (SBCs) in three siliceous hot springs in Yellowstone National Park with respect their carbon isotope signatures ($\delta^{13}\text{C}$) (15). The authors reported that *Aquificales*-specific lipids isolated from the YNP feature known as ‘Bison Pool’ (BP) and ‘Flat Cone’ (FC) exhibited $\delta^{13}\text{C}$ values that were more similar to those measured in the springs’ dissolved inorganic carbon pool (DIC), than to the $\delta^{13}\text{C}$ values measured in the springs’ dissolved organic carbon (DOC) pool. In contrast, the $\delta^{13}\text{C}$ values of *Aquificales*-specific lipids isolated from another hydrothermal feature, known as ‘Octopus Spring’ (OS), were closer to that of the DOC isotopic signature in this spring. These results were interpreted to be indicative of a more autotrophic lifestyle in *Aquificales*-affiliated organisms residing in the chemosynthetic communities of BP and FC, while similar organisms in OS apparently relied more heavily on heterotrophic metabolisms. The apparent difference between the predominant metabolic strategies utilized by *Aquificales*-affiliated organisms in FC and OS was attributed to a difference in the availability of exogenous organic carbon in each habitat, possibly due to the latter being situated in a topographic low at the base of a heavily-vegetated hillside, thereby

potentially receiving a higher influx of organic carbon from meteoric runoff. A subsequent study utilized a continuous flow reactor approach to determine relative rates of uptake of several ^{13}C -labeled substrates (bicarbonate, formate, acetate, and glucose) by the SBCs in OS and BP (58). The authors reported the highest levels of C uptake were from acetate in both springs, with lower levels of glucose uptake and only very small amounts formate uptake. Significant bicarbonate uptake was observed only in BP, consistent with the results of the previous study indicating a more autotrophic lifestyle in SBC communities in this spring, as compared to OS. However, the low rates of formate uptake reported in this study are in disagreement with the results of a previous isotopic fractionation study which reported the depletion of ^{13}C in membrane lipids extracted from SBCs in OS, relative to $\delta^{13}\text{C}$ values of the DIC pool in fluids sampled from the spring's source (14). These results were found to be consistent with $\delta^{13}\text{C}$ values observed in membrane lipids extracted from laboratory cultures of *Thermocrinis* (a bacterium of order *Aquificales* previously shown to be numerically dominant in OS SBCs (92) grown on formate, as opposed to H_2 and CO_2 , indicating that this organism was likely utilizing formate in its natural habitat. Evidence for the in situ utilization of organic substrates by chemosynthetic communities has also been reported in hydrothermal systems other than YNP (94). Experiments investigating the effect of the amendment of several different organic and inorganic electron donors (including organic acids) on O_2 consumption by chemosynthetic communities in water and sediment slurries, taken directly from two geothermal features in the Great Basin, Nevada demonstrated that the consumption of O_2 was stimulated to a larger degree by amendment with organic acids, as compared to

amendment with yeast extract and peptone, NH_4^+ , $\text{S}_2\text{O}_3^{2-}$, H_2 or CH_4 . The authors noted that these results were not consistent with previous suggestions that H_2 is a key electron donor in relatively oxidized or aerobic terrestrial geothermal features (7, 91).

Facultative Autotrophy and Mixotrophy in Laboratory
Cultivars Isolated from High Temperature Hydrothermal Systems

The observed utilization of organic substrates, in addition to H_2 and CO_2 , by chemosynthetic communities in high temperature geothermal systems suggests that the organisms inhabiting these communities may be facultative autotrophs capable of altering their metabolic strategy to take advantage of organic compounds as they become available in their habitat. In support of this suggestion, a number of hyperthermophilic archaea and bacteria isolated from high temperature hydrothermal environments have been demonstrated to be facultative autotrophs (9, 12, 13, 95-99). For example, a novel crenarchaeote named *Pyrobaculum islandicum* and most closely affiliated with crenarchaeota of the order *Thermoproteales*, was isolated from an Icelandic Solfatara and demonstrated to be a strict anaerobe that grew optimally at 100 °C (95). The authors reported that this organism grew lithoautotrophically on H_2 , CO_2 and S_8^0 , as well as on various organic substrates, such as peptone, meat extract, and archaeal- and bacterial cell homogenates by respiration of S_8^0 and other sulfur compounds. A subsequent study reported the isolation of a hyperthermophilic, facultatively autotrophic bacterium representing a new genus in order *Aquificales*, designated *Thermocrinis ruber* (12). This organism was isolated from pink filamentous streamers in a high temperature (>80 °C), chemosynthetic SBC community in Octopus Spring, YNP, which is one of the

chemosynthetic communities from which evidence for facultative autotrophy has been obtained in the field studies discussed above (15, 58). *Thermocrinis ruber* was demonstrated to grow chemolithoautotrophically by coupling the oxidation of hydrogen, thiosulfate, and elemental sulfur with the reduction of O₂. The organism also grew aerobically on the organic compounds formate and formamide, indicating that it is indeed a facultative autotroph, and suggesting that it may play a role in the utilization of organic compounds in its natural habitat.

Facultatively autotrophic microorganisms have also been isolated from more alkaline features in YNP, including the hyperthermophilic, sulfur-oxidizing bacterium *Sulfurihydrogenibium yellowstonense* isolated from Calcite Hot Springs (74 °C and pH 8.3) (99). This bacterium was demonstrated to utilize various organic carbon sources such as yeast extract, tryptone, sugars, amino acids and organic acids, and also grew on S₈⁰ or thiosulfate as an electron donor and O₂ as an electron acceptor. Moreover, it was demonstrated in the same study that two previously-isolated members of the *Sulfurihydrogenibium* species (*Sulfurihydrogenibium subterraneum* (100) and *Sulfurihydrogenibium azorense* (101)), initially thought to be obligately autotrophic, are in fact facultative autotrophs, capable of growth on acetate (*S. subterraneum*) and yeast extract, Bacto peptone, trypticase peptone, and Casamino acids (*S. azorense*), respectively.

True mixotrophic growth (i.e. the simultaneous operation of both autotrophic and heterotrophic metabolisms) has also been demonstrated in thermophilic organisms. The moderately thermophilic bacterium *Nautilia profundicola*, a sulfur-reducing

epsilonproteobacterium isolated from deep-sea hydrothermal vents, grew on H_2 , CO_2 and S_8^0 as sole energy/carbon source and electron acceptor, respectively. Experiments aimed at determining the potential for heterotrophic growth of *N. profundicola* demonstrated that the organism grew only on formate as sole carbon and energy source. Moreover, when the organism was cultivated under mixotrophic conditions, with an H_2/CO_2 headspace and the addition of formate, acetate or pyruvate to the culture medium, it was observed that growth was significantly stimulated (i.e. specific growth rate increased and doubling time decreased compared to autotrophic growth) only in mixotrophic cultures to which formate had been added. The stimulation of growth by formate in the presence of H_2/CO_2 strongly suggests that *N. profundicola* is metabolically poised to allow the immediate exploitation of formate as the organic acid becomes available in its environment. This result further suggests the possibility that other thermophiles living in hydrothermal environments are similarly poised to alter their metabolic strategy over short time scales in order to take advantage of changing concentrations of organic substrates, as has been suggested based on the field studies previously discussed.

The preferential utilization of organic substrates such as formate by chemosynthetic populations suggested by the results of the field and laboratory investigations discussed above is consistent with the thermodynamic favorability of many redox reactions coupling the oxidation of organic substrates to the reduction of O_2 and/or anaerobic electron acceptors, such as S_8^0 and S-based compounds. Windman et al (2007) (16) calculated and compared the total Gibbs energy available (ΔG_r) from the oxidation of formate to that available from H_2 oxidation in a number of high temperature

geothermal features in YNP based on *in situ* measurements of spring fluid composition, including the concentrations of formate and H₂. The results of these calculations demonstrated that formate oxidation yielded more free energy than H₂ oxidation in all geothermal features sampled. These results suggest that facultative autotrophy could be an adaptive advantage for chemosynthetic populations living in hydrothermal environments that receive intermittent input of formate or other organic substrates, as these organisms would have access to more energy for use in cell maintenance and growth than would an obligate autotroph. In addition to the decreased energy yield from the oxidation of H₂, compared to formate and/or other organic compounds, evidence from studies comparing the growth kinetics of organisms grown on organic acids and other organic substrates to those of organisms grown autotrophically on H₂/CO₂ indicate that autotrophic growth may be less energetically efficient than growth on organic compounds (102-104). For example, the bacterium *Pseudomonas oxalaticus* OX1 was observed to require 4.4- and 1.9-fold more ATP per new cell produced when grown on CO₂, compared to growth on glucose and oxalate, respectively (103). In a similar finding, growth of the acetogenic bacterium *Acetobacterium woodii* on H₂/CO₂ required an 8.8-fold larger maintenance energy (m_E), as compared to growth on lactate (102). A possible mechanism for the decreased efficiency of autotrophic growth in some organisms was proposed by Ishaque et al (1971) (104), who reported lower ATP phosphorylation per unit O₂ consumed (P/O ratio) in the bacterium *Pseudomonas saccharophila* during autotrophic vs heterotrophic growth. This discrepancy was attributed to poor coupling of substrate oxidation to ATP phosphorylation in the electron transport system used by *P.*

saccharophila during autotrophic growth, as opposed to that utilized for heterotrophic growth. Overall, the combination of evidence indicating superior energetic yield and metabolic efficiency of organic versus inorganic substrate utilization provides support for the hypothesis that facultative autotrophs in high temperature ($>73^{\circ}\text{C}$) hot springs are likely poised to readily exploit rapid, intermittent influxes of organic substrates as they become available in their habitat.

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CHAPTER TWO

CARBON SOURCE PREFERENCE IN CHEMOSYNTHETIC HOT SPRING
COMMUNITIES

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Contributions: Principal investigator and corresponding author. Advised conception and implementation of experimental design. Collection and analysis of experimental data. Manuscript editing, approval and submission of final manuscript.

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Carbon source preference in chemosynthetic hot spring communities

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ABSTRACT (223 words)

Rates of dissolved inorganic carbon (DIC), formate, and acetate mineralization and assimilation were determined in 13 high temperature (>73°C) hot springs in Yellowstone National Park (YNP) in order to evaluate their relative importance in supporting microbial metabolism. While 9 of the hot spring communities exhibited rates of DIC assimilation that were greater than that of formate and acetate, 2 exhibited rates of formate and/or acetate assimilation that exceeded that of DIC assimilation. Overall rates of DIC, formate, and acetate mineralization and assimilation were positively correlated with spring pH but showed little correlation with temperature. Communities sampled from hot springs with similar geochemistry generally exhibited similar rates of substrate transformation which is consistent with similar community compositions springs with similar geochemistry as revealed by 16S rRNA gene tagged sequencing. Amendment of microcosms with low (μM) amounts of formate suppressed DIC assimilation in short term (<45 min.) incubations, despite native DIC concentrations that exceeded that of added formate by 2 to 3 orders of magnitude. The concentration of added formate required to suppress DIC assimilation was similar to the affinity constant (K_m) for formate transformation as determined by community kinetic assays. These results suggest that dominant chemoautotrophs in high temperature communities are facultatively autotrophic or mixotrophic, adapted to fluctuating nutrient availabilities, and are capable of taking advantage of energy-rich organic substrates when they become available.

INTRODUCTION

Life in environments with a temperature that exceeds the upper limit of photosynthesis ($\sim 73^{\circ}\text{C}$) is supported by chemical energy. In the case of high temperature ($>73^{\circ}\text{C}$) terrestrial hot spring environments, the prevalence of the bacterial phylum *Aquificales* has been interpreted to reflect the importance of lithoautotrophic metabolisms in supporting communities inhabiting these systems. Cultivated representatives from the order *Aquificales* assimilate dissolved inorganic carbon (DIC) using energy derived from the oxidation of hydrogen (H_2), sulfide (S^{2-}), thiosulfate ($\text{S}_2\text{O}_3^{2-}$), or elemental sulfur (S_8) under aerobic conditions or with H_2 , S_8 , or $\text{S}_2\text{O}_3^{2-}$ under anaerobic conditions. In addition, some members of the *Aquificales* (i.e., *Thermocrinis* and *Hydrogenobacter* spp.) are facultative autotrophs capable of growing heterotrophically on organic acids or amides such as formate or formamide, respectively, as their sole carbon and energy source.

The presence of organic acids at concentrations capable of supporting growth of organisms has been reported in many marine and continental hydrothermal environments [e.g.], including those in YNP where formate was measured in 56 hot springs at concentrations of up to $10\ \mu\text{M}$. The low concentrations of formate measured in some YNP hot springs may reflect preferential utilization of this substrate by endogenous populations or a low influx of this substrate to the system. In support of the former, numerous thermophilic organisms capable of growth on formate have been isolated from hot springs [e.g.]. More direct evidence for the utilization of formate in hot spring communities comes from a ^{13}C -formate labeling study which showed documented

incorporation into fatty acids at a single hot spring in Yellowstone National Park (YNP), albeit at low levels . Indirect evidence for organic acid utilization by hot spring communities comes from a recent study that found 7- to 49-fold increases in the rate of O₂ consumption when microcosms containing sediments sampled from two >73°C springs in the Great Basin of Nevada were amended with an equimolar mixture of formate, lactate, acetate, and propionate, when compared to unamended controls . However, it is not clear which of these four organic acids were utilized by the microbial populations in these incubations. Nonetheless, the short incubation time associated with the aforementioned study (<50 min.) suggests that the enhanced consumption of O₂ in the presence of organic acids is unlikely to be the result of enrichment of specific populations capable of this activity but rather suggests relative increases in the heterotrophic activity of facultatively autotrophic or heterotrophic populations . The dominant populations associated with the sediments used to inoculate the aforementioned microcosms were closely affiliated with the aquificae genus *Thermocrinis*, members of which have been shown to utilize DIC or formate .

The energy yield associated with aerobic organic acid oxidation, in particular formate oxidation, is predicted to be equal to or greater than that derived from the aerobic oxidation of other available reductants (e.g., H₂, H₂S, Fe²⁺, S₈) under the geochemical conditions that prevail in most hydrothermal systems . This indicates that formate may be preferentially metabolized by facultative autotrophic populations, such as *Thermocrinis* or *Hydrogenobacter* spp., when it is available. Consistent with this hypothesis, carbon isotopic analysis of membrane lipids extracted from pink streamer

communities sampled from Octopus Spring (84-88°C), Yellowstone National Park (YNP), Wyoming, shown previously to be dominated by 16S rRNA gene sequences affiliated with *Thermocrinis*, were found to be depleted in ^{13}C relative to DIC in hydrothermal fluids sampled from the spring's source. Based on a comparison of these membrane lipid carbon isotope signatures with those obtained from cultures of *Thermocrinis ruber* [originally isolated from Octopus Spring] grown autotrophically with H_2 or heterotrophically with formate, it was concluded that *Thermocrinis* inhabiting Octopus Spring were most likely growing heterotrophically and may be metabolizing formate. Likewise, a recent compound-specific analysis of aquificae lipid biomarkers recovered from filamentous communities inhabiting Octopus Spring revealed carbon isotopic compositions that were more similar to that of the dissolved organic carbon (DOC) pool as opposed to the dissolved inorganic carbon (DIC) pool. This finding was interpreted to reflect a predominantly heterotrophic lifestyle of these organisms. In contrast, the carbon isotopic compositions of these same aquificae-specific lipids recovered from sediments or filaments collected from Flat Cone (74°C) and 'Bison Pool' (74-86°C), YNP revealed values that were more similar to that of DIC present in spring waters, indicating a predominantly autotrophic lifestyle. Previous studies have shown that filament- or sediment-associated communities obtained from the source vents of Flat Cone (E.S. Boyd, *unpublished data*) and 'Bison Pool' are dominated by aquificae closely affiliated with *Thermocrinis* spp. Taken together, such observations may allude to the importance of intermittent surface input of organic carbon to these systems from precipitation runoff, aeolian deposition, or other exogenous sources. Exogenous input of

organic carbon by any of these mechanisms could initiate a shift in the metabolism of facultative autotrophic aquificae toward heterotrophy in order to maximize energy conservation.

In the present study, we compared rates of C assimilation or mineralization from dissolved inorganic carbon (DIC: CO₂ + bicarbonate), formate, and acetate in 13 chemotrophic communities that span large geochemical gradients in YNP to evaluate the hypothesis that non-phototrophic microbial communities inhabiting high temperature (>73°C) hot springs are supported primarily by autotrophic assimilation of inorganic carbon. To determine carbon source preference, we evaluated the extent to which amendment with low (μM) levels of formate suppresses DIC assimilation. These data were combined with taxonomic profiling of archaeal and bacterial 16S rRNA gene sequences and geochemical measurements in order to identify *i*) populations putatively involved in substrate transformations and *ii*) geochemical regimes that may influence potential rates of substrate transformation.

MATERIALS AND METHODS

Physical and chemical measurements. Samples used for chemical and biological measurements were collected between July and October, 2012. The pH of hot spring fluids was measured on site with a YSI pH100CC-01 pH meter. Conductivity and temperature were measured using a YSI EC300 Conductivity Meter (YSI, Inc., USA). Ferrous iron (Fe²⁺) and total sulfide (S²⁻) were quantified using Hach ferrozine pillows and Hach sulfide reagents 1 and 2, respectively, and a Hach DR/890 Spectrophotometer

(Hach Company, Loveland, CO). Dissolved nitrate (NO_3^-), nitrite (NO_2^-), and total ammonia [$\text{NH}_4(\text{T})$] were determined with AccuVac® ampuls for nitrate or nitrite and an AmVer® ammonia reagent set for total ammonia (Hach Company). $\text{NH}_4(\text{T})$ refers to the sum of the dissolved species of aqueous NH_3 and NH_4^+ as measured by colorimetry. For organic acid analyses, 10 mL of spring water was filtered through 0.2 μm polyethersulfone syringe filters into pre-combusted glass vials with polytetrafluoroethylene lined silicone septa. Vials were frozen and stored at -20°C until analysis via high performance liquid chromatography using a previously described alternate injection procedure. Hot spring water for dissolved inorganic carbon (DIC) was filtered (0.2 μm) and frozen at -20°C prior to acidification and quantification via a gas chromatograph as previously described.

Microcosm preparation. Rates of DIC, formate, and acetate assimilation and mineralization were quantified using a previously described microcosm-based approach. Microcosms were prepared in pre-sterilized 24 mL serum bottles. Roughly 100 mg of sediment was added to each vial and the bottle was capped with a butyl rubber stopper and purged with N_2 for ~5 minutes. Sediments were overlain with 10 mL of spring water sampled directly from the spring using a syringe and needle. The gas phase of all microcosms was equalized to atmospheric pressure using a sterile needle prior to injection of 10.0 μCi (20 μM final concentration) of sodium bicarbonate ($\text{NaH}^{14}\text{CO}_3$) for DIC assays, 7.5 μCi (14.4 μM final concentration) of sodium formate ($\text{H}^{14}\text{COONa}$) for formate assays, 5 μCi (9.4 μM final concentration) of 1- ^{14}C sodium acetate ($\text{CH}_3^{14}\text{COONa}$), or 5 μCi (8.9 μM final concentration) of 2- ^{14}C sodium acetate

($^{14}\text{CH}_3\text{COONa}$) for acetate assays. Data from individual 1- ^{14}C - and 2- ^{14}C -acetate assays were combined to quantify the rate of acetate assimilation or transformation.

All microcosms were wrapped in aluminum foil to eliminate light, placed in a sealed bag (secondary containment), and incubated in the source of the spring for ~30-45 min. Triplicate microcosms for each assay condition were terminated by freezing on dry ice and were stored at -20°C until processed (described below). The authors acknowledge that it is not possible to precisely mimic the natural hydrological conditions in a geothermal spring using a microcosm-based approach. However, the rapid addition of sediment and fluids taken directly from each spring, along with the short incubation times (30-45 minutes) used in these experiments, were aimed at minimizing variation between microcosm conditions and actual spring conditions that could potentially arise from outgassing, contact with atmospheric gases, and/or nutrient limitation in a closed system. Our previous supports the effectiveness of this approach to minimize the “bottle effect”, as rates of CO_2 fixation associated with a thermoacidiphilic community in a continuous flow reactor system were shown to be statistically indistinguishable from those associated with short term (<2 hr) microcosm incubations .

Assays were developed to investigate the response of DIC assimilation to amendment with formate in select hot spring ecosystems. Microcosms were prepared in triplicate as described above using $\text{NaH}^{14}\text{CO}_3$ as the radiotracer for DIC assimilation. Microcosms were then amended with 0, 5, 10, or 20 μM unlabeled formate, incubated for 45 minutes, and quenched by freezing as described above. Assays for suppression of DIC

assimilation were carried out in A(DS), MA(CP), MA(EP), NA(PS) and NA(BP) in order to capture a subset of the geochemical regimes selected for examination in this study.

Microcosm assays were conducted to determine the affinity constant (K_m) (the concentration at which formate conversion velocity reaches $\frac{1}{2}$ of its maximum rate) by chemosynthetic microbial communities in the same 5 YNP hot springs in which substrate suppression assays were carried out. A 10 mM sodium formate ($H^{12}COONa$) stock solution was prepared that contained 50 μCi (0.96 μmol) of radiolabeled formate ($H^{14}COONa$) as a tracer. Microcosms were prepared with final formate concentrations (i.e., $^{12}C + ^{14}C$ -formate) of 1.25, 2.50, 5.00 or 10.0 μM . Microcosms were prepared, incubated, and analyzed for formate conversion activity as described below. To determine formate conversion rates, the rate of CO_2 produced from formate oxidation (methods described below) was combined with the rate of C assimilation from formate (i.e., total formate transformation rate). The equations of Hobbie and Wright were used to determine the K_m of formate conversion because they allow for the calculation of kinetic parameters at low substrate concentrations (e.g., $<20 \mu M$) and when the natural substrate concentration is not known, as was the case in many of the springs analyzed (Table 2).

Determination of ^{14}C in microcosm headspace or filtered biomass. In the laboratory, sealed microcosm assays were thawed at room temperature for approximately 2 hours followed by acidification to pH ~ 2.0 by injection of 1.0 mL of 1N HCl to volatilize unreacted CO_2 into headspace and to protonate organic acids thereby minimizing adsorption through electrostatic interactions. After acidification, microcosms were

allowed to equilibrate for an additional 2 hrs. To estimate formate and acetate mineralization rates, N₂ purged serum bottles (12 ml) containing 1 ml of the CO₂-absorbing solution Carbo-Sorb[®] E (PerkinElmer, Inc., Santa Clara, CA, USA) were prepared. The gas phase in the Carbo-Sorb[®] E serum bottles was removed by vacuum to -10 torr, and 5 mL of the gas phase from each microcosm (sampled using a 10 mL syringe and stopcock) was injected into the bottle containing the Carbo-Sorb[®] E solution. The potential for confounding effects due to the development of a partial vacuum upon removal of the gas phase in microcosms could not be accounted for in the experimental design. Carbo-Sorb[®] E was allowed to react with sampled gas at room temperature (~22°C) for approximately 2 hours. Following incubation, the vials were opened and the Carbo-Sorb[®] E solution was removed with a 1 ml pipette and discharged into 10 mL of CytoScint ES[™] liquid scintillation fluid (MP Biomedicals, USA) for use in liquid scintillation counting (LSC) as described below.

To determine the amount of ¹⁴C assimilated into biomass from DIC, formate, and acetate, acidified samples were filtered onto 0.22 μm polycarbonate membranes. Filtered samples were washed with 5 mL of sterile deionized water, dried over night at 80°C, and weighed to determine the grams dry mass (gdm) of the filtrate. Dried filters were placed in scintillation vials and overlain with 10 ml of CytoScint ES[™] liquid scintillation fluid. Radioactivity associated with each of the samples (Carbo-Sorb[®] E solution and filtered sediment) was measured on a Beckman LS 6500 liquid scintillation counter (Beckman Coulter, Inc., Indianapolis, IN). Rates of carbon (C) assimilation and mineralization based on ¹⁴C tracers were determined using the methods of Lizotte et al, 1996 . Briefly,

uptake rates were calculated by multiplying the uptake of ^{14}C labeled substrate by the total effective concentration of the substrate (^{14}C labeled substrate + native substrate). In cases where the concentration of native formate or acetate was below the detection limit, a concentration corresponding to the detection limit was used. Thus, rates of formate or acetate assimilation/mineralization may be overestimated in these systems and may be more appropriately considered as rate potentials (see discussion) rather than absolute rates. Recognizing that isotopic discrimination factors differ for different autotrophic processes [as summarized in Havig et al, 2011] and are likely to differ for different modes of formate and acetate metabolism, we adopt the uniform isotopic discrimination factor of 1.06, as described previously . The data derived from 1- ^{14}C] and 2- ^{14}C]-acetate assays was combined prior to calculation of the overall rates of acetate assimilation and mineralization. The mean and standard deviation of the rates of substrate transformation rates, as normalized to grams dry mass per hour (gdm hr^{-1}), are presented.

Sequencing of bacterial and archaeal 16S rRNA genes. Genomic DNA was extracted in duplicate from ~ 250 mg of hot spring sediment as previously described . Equal volumes of replicate extractions were pooled and quantified using the Qubit DNA Assay (Life Technologies, Grand Island, NY) and a Qubit 2.0 Fluorometer (Life Technologies). Thirty five cycles of PCR were conducted using either bacteria-specific primers (1100F/1492R) or archaea-specific primers (344F/915R) with reaction and cycling conditions as previously described . PCR products were purified using the Promega Wizard PCR purification system (Madison, WI) and were quantified via Qubit

as described above. Amplicons were submitted to MrDNA (Shallowater, TX) for barcoding and multiplex sequencing via the 454 Titanium sequencing platform (Roche, Indianapolis, IN). Post sequencing processing was performed with Mothur (ver. 1.25.1) as previously described after removing reads of less than 225 base pairs. Raw untrimmed sequence and quality score files along with a mapping file have been deposited in the NCBI SRA database under the accession number SRR1042042.

Phylogenetic analysis of archaeal and bacterial 16s rRNA genes for each OTU was evaluated by approximate likelihood-ratio tests as implemented in PhyML (version 3.0). Bacterial 16S rRNA gene phylogenies were rooted with SSU rRNA genes from the Crenarchaeotes *Acidilobus sulfurireducens* str. 18D70 (EF057391) and *Caldisphaera draconis* str. 18U65 (EF057392). Archaeal 16S rRNA gene phylogenies were rooted with SSU rRNA genes from *Clostridium acetobutylicum* ATCC 824 (AE001437) and *Caldicellulosiruptor saccharolyticus* DSM 8903 (CP000679). Phylogenies were constructed using the General Time Reversible (GTR) substitution model with a proportion of invariable sites and gamma-distributed rate variation as recommended by Modeltest (ver. 3.8). Phylograms were rate-smoothed using the multidimensional version of Rambaut's parameterization as implemented in PAUP (ver. 4.0). Rate-smoothing for each phylogram was performed according to the parameters identified using Modeltest. This included the identification of the substitution model, the gamma distribution of rate variation across sites, the proportion of invariant sites, nucleobase frequencies, and the rate matrix for each phylogram. The rate-smoothed cladograms

were used to construct phylogenetic distance matrices for each cladogram with the program Phylocom (ver. 4.0.1) .

Statistical analyses. For the purposes of statistical analysis, all environmental measurements that were below detection were assigned a value equal to the detection limit for that particular measurement (Table 1). Relationships between measured DIC, formate, and acetate assimilation rates and geochemical variables for each of the hot spring environments were evaluated using a multivariate ordination method known as redundancy analysis (RDA). RDA analysis was performed with vegan 2.0.3 (<http://vegan.r-forge.r-project.org/>) as implemented within the R statistical computing package (ver 2.15.0). All rate measurements were log (base 10) transformed prior to analysis in order to preserve overall trends as a function of geochemical measurements and to allow visualization of DIC assimilation data alongside formate and acetate assimilation data despite differences in these measurements as large as 1 order of magnitude. RDA plots were scaled symmetrically by taking the square root of eigenvalues for both vectors. Cluster analysis was also used to visualize patterns in the cumulative rates of DIC, formate, and acetate mineralization and/or assimilation. PAST (ver. 1.72) was used to generate cluster dendograms specifying paired linkage and Bray-Curtis distances. Bootstrap values correspond to the frequency of observation for each node in a given position out of 100 replicates. Principle coordinates (PCO) analysis was used to visually identify patterns of community clustering using the Rao phylogenetic distance matrix. PCO ordination and cluster analysis were performed using Vegan as implemented in R (ver. 2.10.1).

RESULTS

Overview of hot spring sampling sites. The 13 geothermal springs selected for analysis were located in one of seven distinct thermal areas in YNP which includes Norris Geyser Basin (NGB), Crater Hills (CT), Geyser Creek (GC), Mud Volcano (MV), Rabbit Creek (RC), Sentinel Meadows (SM), and Sylvan Spring (SS) (Tables 1 and 2, Supp. Figures 1 and 2). For the purposes of this study, we organized experimental field sites into three groups based on spring pH: acidic (pH < 4.0), moderately to slightly acidic (pH 4.0-6.9), and neutral to alkaline (pH > 6.9) which are represented by the prefixes 'A', 'MA' or 'NA', respectively, followed by the spring name abbreviation in parenthesis (Tables 1 and 2). The A group includes the three low pH springs 'Lobster Claw' [A(LC)], 'Dragon Spring' [A(DS)], and 'Alice Spring' [A(AS)]. These springs are characterized as having elevated sulfide, ferrous iron, and ammonia (Table 1). The MA group includes Cinder Pool [MA(CP)], 'Hell's Gate' [MA(HG)], Evening Primrose [MA(EP)], Obsidian Pool [MA(OP)], and 'Corner Thing' [MA(CT)]. These springs are characterized by moderate concentrations of sulfide and ammonia. Springs in both the A and MA groups often contained deposits of solid phase S₈ [A(DS), MA(CP), MA(EP)], iron oxides [A(LC), MA(HG), MA(CT)], or were clay-rich [A(LC), A(AS), MA(CP), MA(EP)] (Supp. Fig. 1). The NA group consists of Perpetual Spouter [NA(PS)], Bison Pool [NA(BP)], 'Rabbit Creek South' [NA(RCS)], Flat Cone [NA(FC)] and 'Rabbit Creek North' [NA(RCN)]. These springs tended to have low concentrations of ferrous iron and ammonia when compared to the other groups and at the time of sampling not have visual deposits of iron or sulfur (Supp. Fig. 1).

Rates of carbon transformation by chemotrophic communities. DIC, formate, and acetate assimilation or mineralization was detected in all 13 hot spring communities examined in this study (Fig. 1 and Supp. Table 1). The rate of C assimilation from DIC exceeded that of C assimilation from formate (Fig. 1A) in 9 of the 13 communities examined. Similarly, the rate of C assimilation from DIC exceeded that of C assimilation from acetate in 11 of the 13 communities examined (Fig. 1B). Assimilation of C from either formate or acetate was detected in all 13 of the hot spring communities analyzed (Fig. 1C), with the rate of C assimilation from formate being greater than that for acetate in 8 of the 13 hot spring communities. Mineralization of C from either formate or acetate was also detected in all 13 springs examined (Fig. 1D) with the rate of formate mineralization exceeding that of acetate mineralization in 12 of the communities. Importantly, the data presented here were normalized to moles of C assimilated from DIC, formate, or acetate. A true comparison of the turnover of DIC and formate molecules relative to acetate molecules requires that the rate of C assimilation or mineralization from acetate be divided by two (2 C atoms per mol acetate). Applying this conversion factor reveals that acetate turnover is greater than that of DIC in only 1 of the springs examined, while acetate turnover exceeded that of formate in 2 of the springs examined.

Cluster analysis was performed to elucidate patterns in cumulative rates (DIC + formate + acetate) of C assimilation and mineralization in relation to hot spring pH and temperature (Fig. 2A). Cumulative substrate transformation activities in the 13 hot spring communities clustered primarily due to variation in temperature and pH. For example,

rates of DIC, formate, and acetate transformation were similar in NA(RCS) (88.3°C, pH 8.2) and NA5-RCN (88.6°C, pH 9.2). RDA analysis revealed similar patterns of clustering and further allowed for the elucidation of patterns in cumulative rates (DIC + formate + acetate) of C transformation in relation to individual rates of assimilation and mineralization from DIC, formate, and acetate, as well as hot spring geochemistry (Fig. 2B). The centroid of the RDA ordination plot represents the average cumulative rate of C assimilation and mineralization for the combined 13 hot spring communities. For example, the cumulative assimilation rate for the NA(RCN) and NA(RCS) communities were close to the average value for all communities examined, as exhibited by the clustering of this site near the centroid. Hot spring communities that plot away from the centroid exhibit rate(s) of C assimilation or mineralization from DIC, formate, and/or acetate that deviate from the average. The first two RDA axes explained 74.3% of the cumulative variation in the measured rates, with RDA axis 1 accounting for 50.9% of the total variance and RDA axis 2 accounting for 23.4% of the variance. Regression analysis indicates that RDA axis 1 was positively correlated with the rate of C assimilation from DIC (Pearson $R = 0.68$, $P = 0.01$), C assimilation and mineralization from acetate (Pearson $R = 0.80$ and 0.90 , respectively, $P = 0.01$ and <0.01 , respectively), and C mineralization from formate (Pearson $R = 0.58$, $P = 0.03$) (Supp. Table 2). In contrast RDA axis 2 was positively correlated with the rate of C assimilation and mineralization from formate (Pearson $R = 0.73$ and 0.76 , respectively, $P < 0.01$ and <0.01 , respectively). Thus, RDA axis 1 is separating communities primarily on the basis of differences in rates of DIC and acetate metabolism and to a lesser extent formate metabolism. In contrast,

RDA axis 2 is separating communities primarily on the basis of differences in rates of formate metabolism.

Similar to the results of the cluster analysis described above, RDA analysis indicated that rates of C assimilation and mineralization in communities residing in hot springs with similar geochemical conditions often formed clusters. For example, cumulative rates of C assimilation and mineralization in MA(CP), MA(CT), and MA(CP), which are all high temperature and slightly acidic pH, formed a cluster near the centroid of the RDA plot. This cluster was oriented along the individual vectors associated with rates of C assimilation and mineralization from formate. Cumulative rates of C assimilation and mineralization in NA(FC), NA(PS), A(DS), and NA(BP) also formed a cluster that trended in the direction of vectors describing the individual rates of C assimilation from DIC and acetate as well as acetate mineralization.

An overlay of measured geochemical variables reveals that C assimilation and mineralization from DIC, acetate and, to a lesser extent, formate are positively correlated with pH over the range of springs studied (1.9 to 9.2). This indicates that rates of transformation of these substrates are, on average, greater in systems with circumneutral to alkaline pH [e.g., NA(PS), NA(BP)] than in moderately acidic to acidic systems [e.g., A(AS), MA(CT)]. Rates of C assimilation and mineralization from DIC and acetate were inversely correlated with temperature, whereas C assimilation and mineralization from formate were positively correlated with temperature.

Rates of assimilation and mineralization of 1-[¹⁴C]- and 2-[¹⁴C]-acetate.

Separate microcosm assays were conducted for acetate labeled singly at the carboxyl position (1-[¹⁴C]-acetate) or singly at the methyl position (2-[¹⁴C]-acetate) (Supp. Table 3), thus allowing for a comparison of rates of assimilation and mineralization at these positions (Fig. 3). For the carboxy position, rates of mineralization were greater than those of assimilation in 3 of the 13 communities analyzed, less than those of assimilation in 8 of the communities, and not significant different from those of assimilation in 2 of the communities. For the methyl position, the rates of mineralization of were greater than those of assimilation in 7 of the 13 communities analyzed, less than those of assimilation in 2 of the communities, and not significant different in 4 of the communities. A comparison of the rate of assimilation from the carboxyl and methyl carbon of acetate indicated that the carboxyl carbon was preferentially assimilated in 3 communities while the methyl carbon was preferentially assimilated in 3 communities; 7 of the communities did not exhibit significant differences in assimilation of the carboxyl or methyl carbon of acetate. The carboxyl carbon was preferentially mineralized in 8 communities while the methyl carbon was preferentially mineralized in 4 communities; 1 community did not exhibit a significant difference in mineralization of the carboxyl or methyl carbon of acetate.

Suppression of DIC assimilation by formate. A series of microcosm experiments was conducted to investigate whether hot spring populations are capable of simultaneous utilization of these substrates or were capable of shifting their metabolism from CO₂ assimilation to formate uptake in order to take advantage of formate as it

became available in their environment. The amount of ^{14}C incorporated into biomass was monitored in microcosms amended with increasing concentrations of unlabeled formate, for five hot springs [A(DS), MA(CP), MA(EP), NA(PS) and NA(BP)]. These springs were selected to span the full range of pH represented among the 13 sites examined, and to target sites in which both DIC and formate metabolism had been detected. In all five springs, assimilation of DIC was systematically suppressed by amendment with increasing concentrations of formate (Fig. 4). The concentration of formate required to suppress DIC assimilation to a level that was significantly lower than that of the unamended control varied among the hot spring communities analyzed. Whereas 5 μM of formate was required to significantly (student T test: $P < 0.05$) suppress DIC assimilation relative to unamended controls in A(DS), NA(BP), and NA(PS), a similar response was not observed in MA(EP) and MA(CP) communities until 10 and 20 μM of formate was added, respectively. In addition to the observed difference in formate concentration required to significantly suppress DIC assimilation, the magnitude of the suppression response induced by formate amendment (5, 10, and 20 μM) also differed between the microbial assemblages.

Formate transformation kinetics. Community kinetic assays were conducted to estimate the affinity for formate in the 5 communities where DIC suppression assays were conducted (Fig. 5). To avoid the potential confounding effects of formate toxicity at concentrations above 100 μM (see discussion), we amended assays with $<20 \mu\text{M}$ formate. Hobbie-Wright plots indicated community formate uptake affinities (K_m) of 14.0, 36.9, 2.3, 6.3 and 7.8 μM in NA(BP), MA(CP), A(DS), MA(EP), and NA(PS),

respectively (Fig. 5). These values were broadly similar to the concentration of formate required to significantly suppress the DIC assimilation rate in these same communities (Fig. 4). Importantly, the kinetic values reported here should be regarded as conservative estimates since rate data used for kinetic determination was normalized to grams dry mass of sediments rather than a measurement of biomass (total protein) and the communities may comprise more than one formate-utilizing organism.

Archaeal and bacterial 16S rRNA gene composition. The taxonomic composition of bacterial and archaeal assemblages in the 13 hot springs was examined in order to identify putative taxa responsible for the measured C transformation activities (Fig. 6A and 6B). Bacterial amplicons were obtained from sediment DNA extracts from 8 of the 13 springs and archaeal amplicons were obtained from sediment DNA extracts in 10 of the 13 springs.

Archaea. Following normalization, a total of 1008 archaeal 16S rRNA gene sequences belonging to 562 distinct OTUs (defined at 3.0% sequence dissimilarities) were identified in the 10 hot spring communities where amplicons were obtained (Fig. 6A). Rarefaction analysis indicated that between 84.6 and 99.8% of the predicted 16S rRNA gene diversity was sampled at this depth of sequencing in these 10 communities (data not shown). Principle coordinates (PCO) analysis was used to identify relationships between archaeal community composition, environmental characteristics, and carbon transformation activities (Fig. 6C). Both the acidic to slightly acidic hot springs of A(AS), MA(EP), MA(CP), A(DS), and MA(OP) and the alkaline hot springs NA(RCS) and NA(FC) formed clusters, indicating similar community compositions in hot springs

with similar geochemistry. PCO axis 1 (24.6% of variance explained) was not significantly correlated with any of the environmental variables measured. However, this axis was significantly correlated with the rate of DIC assimilation (Pearson $R = 0.92$, $P < 0.01$), acetate assimilation (Pearson $R = 0.76$, $P = 0.01$), and acetate mineralization (Pearson $R = 0.69$, $P = 0.03$) (Supp. Table 3). PCO axis 2 (23.7% of variance explained) was significantly correlated with spring pH (Pearson $R = 0.77$, $P < 0.01$) which helps to explain the overall pattern of clustering based on pH.

The abundance of a number of the 20 most abundant OTUs varied significantly with rates of substrate transformation (Supp. Table 4). The abundance of Otu0001 (*Ignisphaera aggregans*; 94% sequence identities) and Otu0018 (*I. aggregans*; 90% sequence identities) were positively correlated with the rate of DIC assimilation (Pearson $R = 0.91$ for both), acetate assimilation (Pearson $R = 0.81$ and 0.87 , respectively), and acetate mineralization (Pearson $R = 0.76$ and 0.82 , respectively). The abundances of Otu0005 (*Acidilobus sulfurireducens*; 95% sequence identities), Otu0014 (*Caldisphaera draconis*; 99% sequence identities), and Otu0015 (*Thermogladius shockii*; 98% sequence identities) exhibited strong positive correlations with the rate of formate mineralization (Pearson $R = 0.86$, 0.78 , and 0.86 , respectively) and low or inverse correlations with the rate of formate assimilation (Pearson $R = 0.01$, -0.15 , and -0.10 , respectively). In contrast, the abundance of Otu0017 (*Geoglobus acetivorans*; 93% sequence identities) exhibited a strong correlation with the rate of formate assimilation (Pearson $R = 0.80$) but no correlation with the rate of formate mineralization (Pearson $R = 0.01$).

Bacteria. Following normalization, a total of 656 bacterial 16S rRNA gene sequences belonging to 389 distinct OTUs (defined at 3.0% sequence dissimilarities) were identified in the 8 hot spring communities where amplicons were obtained (Fig. 6B). Rarefaction analysis indicated that between 87.9 and 99.8% of the predicted 16S rRNA gene diversity was sampled at this depth of sequencing in these 9 communities (data not shown). PCO analysis was used to identify relationships between bacterial community composition, environmental characteristics, and carbon transformation activities (Fig. 6D). Communities inhabiting the acidic hot spring A(DS) clustered distinctly with respect to those from a cluster comprising the slightly acidic hot springs MA(CT), MA(HG), MA(EP), MA(CP), and MA(OP). The pattern of clustering in A(DS) was driven by the dominance of sequences affiliated with *Hydrogenobaculum* sp. NOR3L3B, which was rarely identified in the other springs. In contrast, the community inhabiting the circumneutral hot spring NA(PS) and the alkaline hot spring NA(FC) did not form a cluster. PCO axis 1 (62.2% of variance explained) was significantly correlated to the rate of acetate mineralization (Pearson $R = 0.94$, $P < 0.01$) and with concentrations of sulfide (Pearson $R = 0.90$, $P < 0.01$), ferrous iron (Pearson $R = 0.89$, $P < 0.01$), and nitrate (Pearson $R = 0.74$, $P = 0.03$) (Supp. Table 6). PCO axis 2 (24.3% of variance explained) was significantly correlated to the rate of acetate assimilation (Pearson $R = 0.95$, $P < 0.01$), with hot spring pH (Pearson $R = 0.72$, $P = 0.05$), and the concentration of DIC (Pearson $R = 0.76$, $P = 0.03$).

Linear regression indicated significant or strong relationships between the abundance of a number of the 20 most abundant bacterial OTUs and rates of individual substrate transformation (Supp. Table 5). The abundance of Otu0002 (*Thermodesulfobium narugense* Na82; 98% sequence identities) exhibited a strong, positive correlation (Pearson $R = 0.60$, $P = 0.12$). The rate of formate assimilation was significantly correlated with the abundance of Otu0017 (*Methylobacterium* sp. A4; 96% sequence identities; Pearson $R = 0.98$, $P < 0.01$) and the abundance of Otu0020 (*Geothermobacterium ferrireducens* FW-1a; 99% sequence identities; Pearson $R = 0.98$, $P < 0.01$) while the rate of formate mineralization was significantly correlated with the abundance of Otu0004 (*Paludibacter propionicigenes* WB4; 93% sequence identities; Pearson $R = 0.90$, $P < 0.12$). The rate of acetate assimilation was significantly correlated with the abundance of Otu0005 (*Thermocrinis* sp. P2L2B; 98% sequence identities; Pearson $R = 1.00$, $P < 0.01$) and the abundance of Otu0011 (*Thermus aquaticus*; 99% sequence identities; Pearson $R = 1.00$, $P < 0.01$) while the rate of acetate mineralization was significantly correlated with the abundance of Otu0001 (*Hydrogenobaculum* sp. NOR3L3B; 99% sequence identities; Pearson $R = 0.93$, $P < 0.01$).

DISCUSSION

Molecular and thermodynamic data suggest that non-photosynthetic microbial communities inhabiting high temperature ($>73^{\circ}\text{C}$) hot springs are supported by chemolithoautotrophic metabolism. The higher rates of C assimilation from DIC than the common heterotrophic substrates formate and acetate in the majority of the hot

springs examined herein provides the first empirical evidence supporting these predictions and indicates that autotrophic metabolism may predominate in high temperature geothermal communities. However, the generalization that all communities of this type are supported by chemoautotrophic metabolism is not supported by our data since rates of C assimilation from formate and acetate were found to exceed that of DIC in 2 of 13 hot spring communities [A(AS) and MA(HG)]. Similar findings indicating a greater extent of labeling of lipids by amendment with organic acid substrates when compared to bicarbonate in two alkaline hot springs in YNP further substantiate the claim that organic carbon may play an important and previously overlooked role in supporting these communities .

Individual rates of formate and acetate utilization generally exhibited an inverse correlation with the concentrations of these substrates in the environment suggesting a potential role for biological activity in depleting these organic acid pools. A previous study documented assimilation of DIC, formate, acetate, and glucose in two alkaline hot spring communities . Incorporation of DIC and organic substrates into the same diagnostic lipids suggested that the populations assimilating these substrates may be facultatively autotrophic. It is also possible that these populations may be capable of the simultaneous utilization of these substrates (mixotrophic), although the experimental design in the aforementioned study did not allow for testing this possibility. A facultative autotrophic or mixotrophic nature for the dominant autotrophic populations is supported by the rapid (<45 min.) suppression of DIC assimilation observed herein in the presence of low (μM) concentrations of formate. This would allow populations to take advantage

of temporal and abrupt input of exogenous organic materials from surrounding areas (e.g. aeolian deposition or surface runoff from precipitation). Such an explanation is consistent with a previous compound-specific isotopic analyses of lipid carbon in biomass sampled from several alkaline hot springs in YNP over a period of several years which revealed evidence for a shift between autotrophic and heterotrophic metabolisms in several aquificae biomarker lipids . The potential for input of exogenous organic materials from surrounding areas is also supported by the recovery of bacterial 16S rRNA genes from several of the chemosynthetic communities studied herein [MA(CP), MA(EP), MA(CT), NA(PS), and NA(BP)] that were closely affiliated with orders (*Enterobacteriales*, *Bacteroidales*, *Rhizobiales* and *Burkholderiales*) that are typically associated with feces-contaminated waters, soils, or root nodules . Intriguingly, some of the highest rates of organic acid assimilation and mineralization were observed in springs in which sequences affiliated with these lineages were present. This result is consistent with a recent input of exogenous organic material in these springs.

The suppression of DIC assimilation in the presence of formate could also be attributed to FDH-promoted isotopic exchange between ^{12}C -formate to $^{12}\text{CO}_2$, or to production of $^{12}\text{CO}_2$ as the product of formate oxidation by a separate population of heterotrophic organisms. Either of these scenarios would in effect dilute the spring water DIC pool, resulting in a smaller ratio of ^{14}C -DIC to unlabeled DIC and thus an apparent decrease in the rate of DIC assimilation. However, if the suppression of DIC assimilation was due to dilution of the labeled DIC pool by either of these mechanisms, it would also be expected that the quantity of the observed suppression would closely match the factor

by which the ^{14}C -bicarbonate pool had been diluted by ^{12}C -bicarbonate from oxidized formate. In all environments assayed, the concentration of DIC was at a minimum 125-fold greater than that of formate indicating that isotopic exchange could account for <0.08% suppression of activity. This strongly indicates that the observed suppression is not due to isotopic exchange.

The low concentrations of ^{14}C -labeled formate and acetate radiotracers used in our microcosm assays were chosen to closely match previously reported concentrations of formate (acetate concentrations have yet to be reported) in YNP hot springs [$< 10 \mu\text{M}$] and thus minimize induction of microbial processes above their basal or *in situ* rate. Moreover, formate occurs increasingly in the protonated form as pH decreases ($\text{pK}_a = 3.75$ at 80°C , meaning that half of all formate is protonated at $\text{pH}=3.75$). This neutral form will readily diffuse into the cell and deprotonate at the intracellular pH, thereby decreasing the membrane potential. Previous studies have shown that concentrations of formate as low as $100 \mu\text{M}$ inhibit the growth of the acidiphile *Thiobacillus* (*Acidithiobacillus*) *ferrooxidans* when grown in medium with a pH of 1.6. Since the highest concentration of formate that has been measured in YNP hot springs to date is $10 \mu\text{M}$ and since many of the springs subject to study have $\text{pH} < 3.75$ (Table 1), we attempted to avoid the confounding effects of formate toxicity by amending assays with $<20 \mu\text{M}$ formate. In addition, to avoid potential error resulting from low substrate concentration in our calculations of the K_m of formate conversion, we used Hobbie-Wright equations that allow for the more accurate calculation of kinetic parameters at low

substrate concentrations (e.g., $<20 \mu\text{M}$) or when the natural substrate concentration is not known .

The concentration of formate and acetate in spring waters sampled from our study sites at the time of our microcosm incubations were lower than anticipated and often times were below the detection limit (Table 2). Consequently, the final concentrations of radiolabeled formate and acetate in our microcosm assays were higher than native concentrations of these substrates in most cases. Since formate and acetate transformation rates were calculated using native concentrations or the detection limit concentration for those substrates, their rates can be considered to reflect upper limits. As a result, the formate and acetate assimilation and mineralization activities reported here may be more appropriately referred to as rate potentials, rather than absolute rates. A further consideration which is supported by repeated sampling of hot springs over seasonal or annual cycles, is that concentrations of organic substrates vary temporally . This variability makes the estimation of representative substrate transformation rates more difficult and potentially less meaningful, as these rates will likely vary to a large degree in accordance with variations in the mode and magnitude of delivery of organic substrates to these systems.

The cumulative rate of measured activities (DIC + formate + acetate mineralization and assimilation) and the individual rates of C assimilation from DIC, formate, and acetate were generally higher in circumneutral to alkaline low temperature springs when compared to acidic high temperature springs. Low rates of metabolic activity or productivity would be expected to translate to lower biomass abundances in

acidic high temperature hot springs relative to alkaline hot springs. ATP has been used as a measure of biomass in a number of environmental systems [e.g.], including hot springs. Atkinson et al., 2000 reported 3 to 4 order of magnitude lower ATP concentrations in hot spring sediments sampled from low pH environments when compared to those sampled from high pH environments . This result would be consistent with the low metabolic activities that are reported here for acidic hot spring communities. Alternatively, considering that not all ATP in a cell is directed toward the production of biomass but rather can also be directed toward maintenance functions , higher rates of catabolic reactions relative to anabolic reactions might be required in acidic and high temperature conditions to counteract the energetic stress imposed on cells from their surrounding environment , perhaps leading to lower biomass production. While Atkinson et al. (2000) only reported ATP contents and not ATP/ADP ratios, previous studies indicate that the cellular abundance of ATP and the ATP/ADP ratio varies by less than an order of magnitude in *Esheria coli* cells during various stages of growth and when grown under different metabolic regimes . Thus, the variation in ATP contents in hot springs observed by Atkinson et al., 2000 is unlikely to be due solely to potential differences in metabolic state or stress levels of the cells in acidic versus circumneutral to alkaline springs and instead is interpreted to reflect differences in biomass levels in these springs.

The use of 1-[¹⁴C]- and 2-[¹⁴C]-acetate labels (carboxyl and methyl groups, respectively) in microcosm assays provided an opportunity to examine potential differences in the metabolic state of acetate-utilizing populations. Entry of acetate in the form of acetyl CoA into the TCA cycle should result in a faster rate of CO₂ release from

the carbonyl carbon of acetyl CoA (the carboxyl group of acetate), since this carbon is only retained through the first turn of the TCA cycle and is completely lost as CO₂ during the second turn of the cycle. In contrast, the methyl group of acetyl CoA (the methyl group of acetate) is retained through the first two turns of the TCA cycle and is only lost as CO₂ in the third and fourth turns of the cycle. Siphoning off TCA cycle intermediates for use in amino acid and lipid biosynthesis would lead to higher rates of assimilation when compared to oxidation. In contrast, cells that are energy-limited might maximize oxidation of acetate, leading to higher levels of NADH/FADH₂ production, and a release of acetate carbon as CO₂. The rate of CO₂ production from the methyl and carboxyl carbon of acetate was greater than that assimilated in both NA(RCS) and MA(EP) while the rate of assimilation of methyl and carboxyl carbon positions of acetate were higher than that being released as CO₂ in NA(FC), A(AS), and MA(OP). This suggests that cells comprising the communities in NA(RCS) and MA(EP) may be, on average, in a more energy-limited state than cells comprising the communities in NA(FC), A(AS), and MA(OP), which appear to be in a state of active biosynthesis. Eight of the 13 communities exhibited rates of CO₂ production from the carboxyl carbon of acetate that exceeded the rate of CO₂ production from the methyl group, which would be consistent for oxidation of acetate via TCA enzymes if this pathway is the primary mechanism for acetate metabolism in these communities. Four of the remaining 5 communities exhibited rates of CO₂ production from the methyl carbon that were statistically indistinguishable or very similar to that of the carboxyl carbon, which may be attributable to full oxidation of acetate via the TCA cycle (>4 turns). However, a single community,

A(AS), exhibited a rate of CO₂ production from the methyl position of acetate that exceeded that from the carboxyl position. Acetate metabolism in this community is not easily explained by any of the known mechanisms of acetate metabolism.

The inferred physiology of a number of archaeal and bacterial 16S rRNA sequences recovered from the 13 hot spring communities examined were consistent with their potential role in the transformation of DIC, formate, and acetate. For example, the abundance of sequences affiliated with *Thermodesulfobium narugense*, a thermophilic and autotrophic sulfate reducing bacterium, were positively correlated with rates of DIC assimilation. Likewise, the abundance of sequences affiliated with several crenarchaeotes including *A. sulfurireducens* and *C. draconis* and the euryarchaeote *G. acetivorans* were positively correlated with the rate of formate mineralization. *G. acetivorans*, an iron reducing facultative chemolithoautotroph, has been shown to be capable of using formate as an electron donor. While a cultivation-based study of *A. sulfurireducens* 18D70 and *C. draconis* 18U65 did not reveal the ability to grow via formate oxidation at a concentration of 5 mM, the partial genome of *A. sulfurireducens* (locus tags: Asul_00004590 and Asul_00004590) and the complete genomes of the closely related strains *Acidilobus saccharovorans* 345-15 (locus tags: ASAC_0614 and ASAC_0615) and *Caldisphaera lagunensis* IC-154 (locus tags: Calag_1200 and Calag_1201) all encode for homologs of archaeal FDH enzymes (data not shown). The rate of acetate transformation was positively correlated with the abundance of the aquificae *Thermocrinis* sp. P2L2B and *T. aquaticus*. Several characterized strains of *Thermocrinis* and the type strain of *T. aquaticus* have been shown to use acetate,

consistent with the prevalence of these sequences in springs with high rates of acetate metabolism.

In summary, the results presented here substantiate previous suggestions for the importance of chemolithoautotrophy in sustaining high temperature, non-photosynthetic hot spring communities. However, carbon assimilation rates from a limited array of organic substrates tested here (formate and acetate) reveal rates of assimilation that in some cases exceed that of DIC, suggesting that non-phototrophic communities in hot springs may be reliant on or able to take advantage of organic carbon to support their metabolism. These findings are consistent with recent work by Schubotz et al. , which documented significant assimilation of organic substrates into lipid biomarkers in two hot spring communities. In support of a potential for facultative autotrophy in spring populations, amendment with successively higher concentrations of formate was found to systematically suppress DIC assimilation in springs with high rates of DIC assimilation. This indicates that organics are not only usable but may be actively selected for use over DIC when both substrates are available. Together, these results indicate an important and previously underestimated role for organic substrates in supporting non-phototrophic communities that inhabit geochemically diverse hot springs in YNP.

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Table 1. Location and field measurements for selected YNP hot springs. Several of the features sampled do not have official YNP names. Unofficial names are denoted with apostrophes.

YNP Thermal Inventory ID	Spring Name	Spring Abbreviation	Thermal Area [‡]	GPS Coordinates	Conductivity (mS)	Temperature (°C)	pH
Acidic (<4.0)							
GSSG060	'Lobster Claw'	A(LC)	SS	N44°41'58.00", W110°46'05.70"	7.1	85.2	1.9
CHA043	'Alice Spring'	A(AS)	CH	N44°39'11.82", W110°29'05.06"	5.3	77.5	2.3
NHSP106	'Dragon Spring'	A(DS)	NGB	N44°43'54.60", W110°42'39.20"	3.3	78.0	2.5
oderately to slightly acidic (4.0-6.9)							
NHSP103	Cinder Pool	MA(CP)	NGB	N44°43'56.89", W110°42'35.40"	5.0	88.7	4.0
N/A	'Hell's Gate'	MA(HG)	RC	N44°30'31.44", W110°48'32.46"	0.4	88.0	4.4
GSSG038	Evening Primrose	MA(EP)	SS	N44°41'57.70", W110°46'01.80"	4.8	78.4	5.1
MV007	Obsidian Pool	MA(OP)	MV	N44°36'36.50", W110°26'19.50"	0.2	74.5	5.4
N/A	'Corner Thing'	MA(CT)	GC	N44°41'26.52", W110°43'46.50"	0.2	85.8	5.9

Table 1. , Cont.

YNP Thermal Inventory ID	Spring Name	Spring Abbreviation	Thermal Area[‡]	GPS Coordinates	Conductivity (mS)	Temperature (°C)	pH
Neutral to alkaline (> 6.9)							
NBB113	Perpetual Spouter	NA(PS)	NGB	N44°43'35.80", W110°42'32.80"	6.0	84.3	7.1
LSMG013	'Bison Pool'	NA(BP)	SM	N44°34'10.67", W110°51'54.65"	3.2	80.0	7.8
N/A	'Rabbit Creek South'	NA(RCS)	RC	N44°30'31.74", W110°48'32.46"	0.5	88.3	8.2
LSMG004	Flat Cone	NA(FC)	SM	N44°34'06.59", W110°51'48.70"	1.6	77.1	8.2
N/A	'Rabbit Creek North'	NA(RCN)	RC	N44°31'11.40", W110°48'38.88"	2.1	88.6	9.2

[‡]NGB = Norris Geyser Basin, CH = Crater Hills, GC = Geyser Creek, MV = Mud Volcano, RC = Rabbit Creek, SM = Sentinel Meadows, SS= Sylvan Springs

Table 2. Concentration of DIC, organic acids, and selected ions in source waters sampled from 13 YNP hot springs. The unit on all ion and organic acid measurements is μM . The unit on DIC is mM.

Site Abbreviation	DIC	Formate	Acetate	S_2^{2-}	Fe^{2+}	NH_4 (T)	NO_2^-	NO_3^-
Acidic (<4.0)								
A(LC)	0.7	1.6	BD (0.8)	14.0	7.2	261.3	BD (0.1)	1.6
A(AS)	37.1	26.6	20.2	BD (0.3)	207.7	552.0	BD (0.1)	BD (0.2)
A(DS)	0.3	0.8	BD (0.8)	24.9	21.8	67.5	0.8	43.5
Moderately to slightly acidic (4.0-6.9)								
MA(CP)	1.2	BD (0.1)	BD (0.8)	6.2	4.7	392.2	BD (0.1)	9.7
MA(HG)	0.1	0.8	BD (0.8)	3.7	9.7	134.5	2.5	BD (0.2)
MA(EP)	2.4	BD (0.1)	BD (0.8)	BD (0.3)	1.4	66.4	BD (0.1)	BD (0.2)
MA(OP)	1.0	0.2	BD (0.8)	0.3	3.2	6.5	BD (0.1)	BD (0.2)
MA(CT)	1.7	BD (0.1)	BD (0.8)	5.3	1.6	28.2	BD (0.1)	29.0
Neutral to alkaline (> 6.9)								
NA(PS)	0.1	BD (0.1)	BD (0.8)	1.6	1.2	15.3	BD (0.1)	BD (0.2)
NA(BP)	6.0	BD (0.1)	BD (0.8)	BD (0.3)	BD (1.0)	BD (1.2)	BD (0.1)	19.4
NA(RCS)	2.8	0.9	2.0	BD (0.3)	BD (1.0)	5.9	16.4	BD (0.2)
NA(FC)	4.9	BD (0.1)	BD (0.8)	11.8	BD (1.0)	BD (1.2)	BD (0.1)	27.4
NA(RCN)	2.8	1.3	2.1	16.2	BD (1.0)	10.0	5.4	38.7

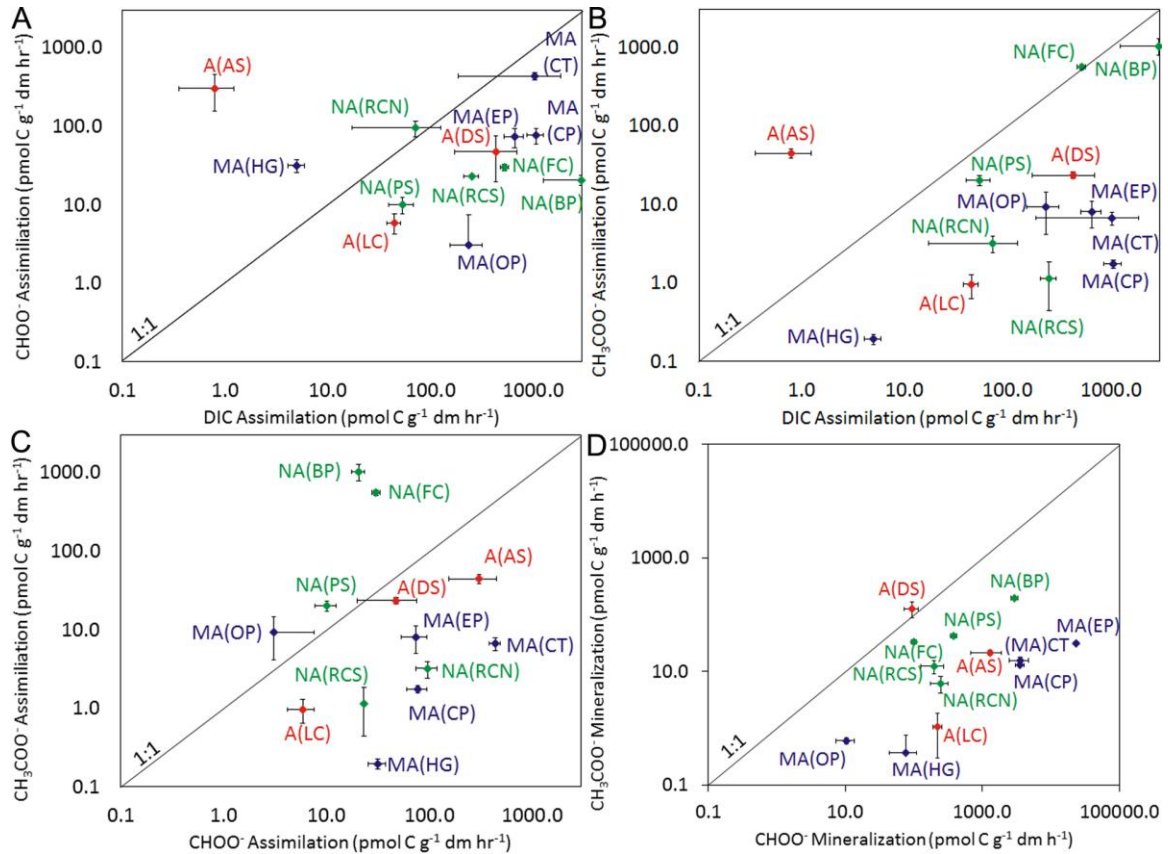


Figure 1. Rate of C assimilation or mineralization from DIC, formate, and acetate in microbial communities sampled from the source of 13 YNP hot springs (A-D). Site labels correspond to those presented in Tables 1 and 2. A 1:1 line is presented to facilitate comparison of rates of substrate transformation. Rates of individual substrate transformation for each assemblage are reported in Supp. Table S1. Acidic springs (pH < 4.0) are colored red, moderately acidic springs (pH 4.0 to 6.0) are colored navy blue, and neutral to alkaline springs (pH >7.0) are colored green.

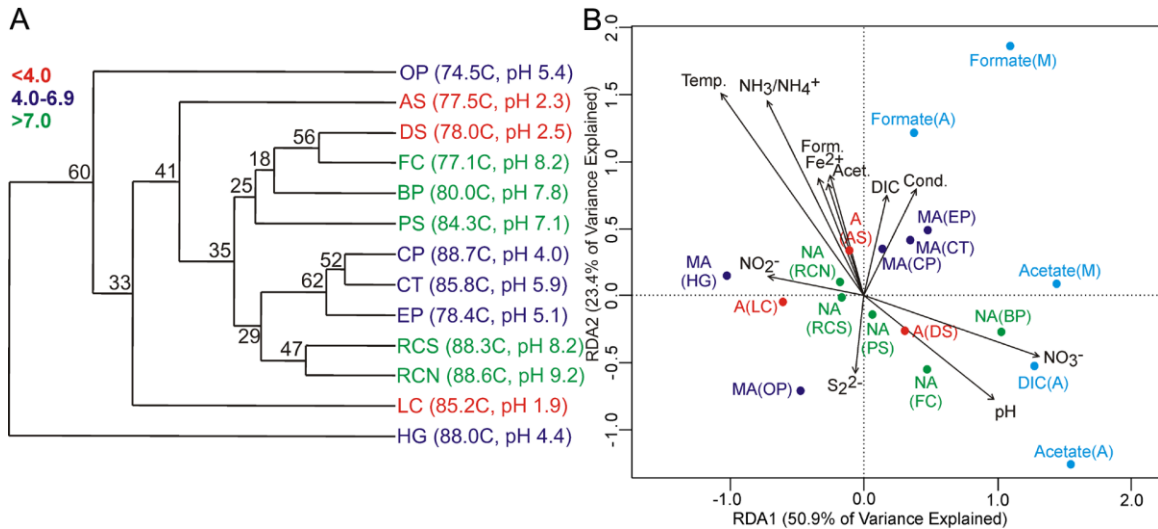


Figure 2. (A) Paired group cluster analysis depicting the Bray-Curtis similarity in cumulative rates of C assimilation and mineralization from DIC, formate, and acetate in 13 YNP hydrothermal communities. The temperature and pH of springs is indicated. Spring labels are color coded based on similarity in spring pH. The cophenetic correlation coefficient for the reconstructed dendrogram was 0.89 indicating a good fit of the 2 dimensional model to the data. (B) RDA ordination of the cumulative rates of C assimilation and mineralization from DIC, formate and acetate in 13 hot spring communities. The cumulative activities for communities (labels in black) were ordinated and their positions in relation to individual rates of substrate transformation (labels in light blue) are indicated. The plot was overlain with measured geochemical variables (black) with the direction of the vectors indicating the relationship to cumulative and individual rates of substrate transformation. Site labels correspond with those in Tables 1 and 2 where acidic springs (pH < 4.0) are colored red, moderately acidic springs (pH 4.0 to 6.0) are colored navy blue, and neutral to alkaline springs (pH > 7.0) are colored green.

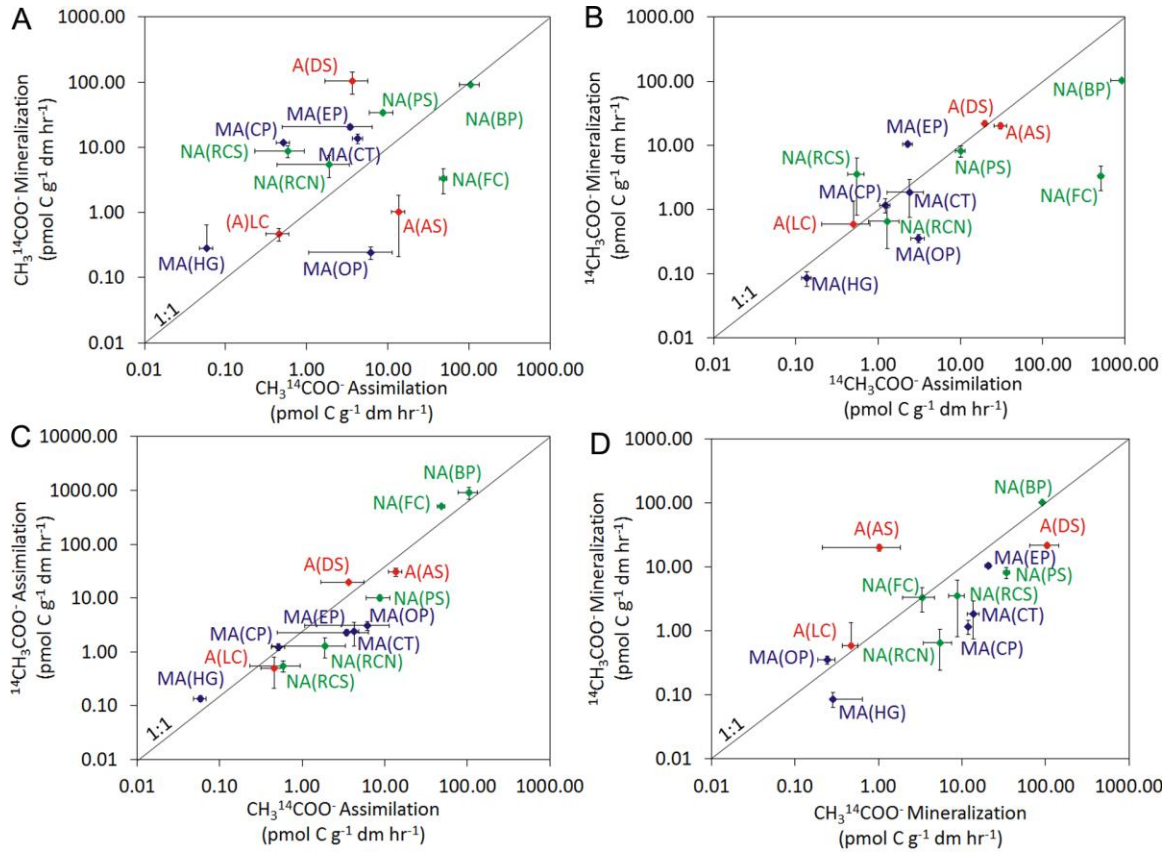


Figure 3. Rate of C assimilation or mineralization from $\text{CH}_3\text{-}^{14}\text{COO}^-$ (1- ^{14}C) acetate) and $^{14}\text{CH}_3\text{COO}^-$ (2- ^{14}C) acetate) in microbial communities sampled from the source of 13 YNP hot springs (A-D). Site labels correspond to those presented in Table 1 where acidic springs (pH < 4.0) are colored red, moderately acidic springs (pH 4.0 to 6.0) are colored navy blue, and neutral to alkaline springs (pH > 7.0) are colored green. A 1:1 line is presented to facilitate comparison of rates of substrate transformation. Rates of individual substrate transformation for each assemblage are reported in Supp. Table 1.

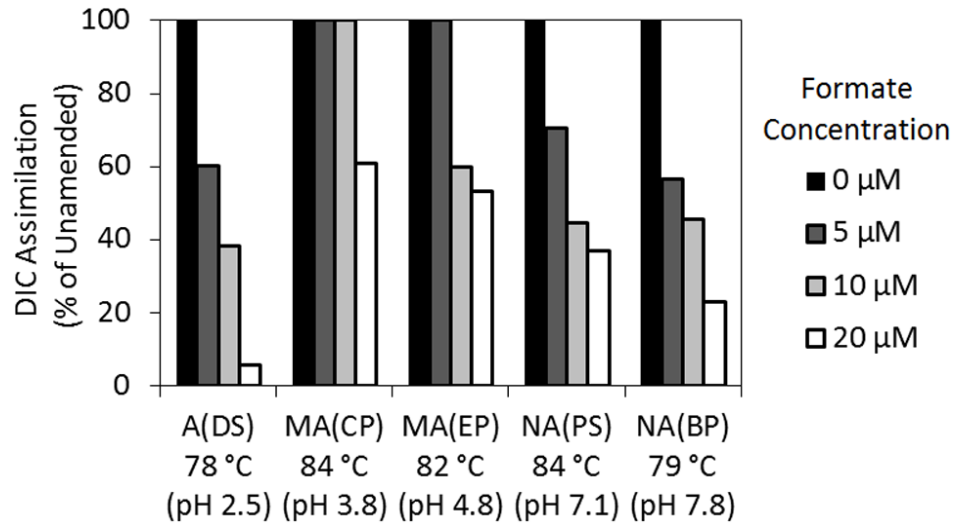


Figure 4. Suppression of DIC assimilation by amendment with varying concentrations of formate in microbial communities sampled from the source of five YNP hot springs. DIC assimilation is depicted as the percent of unamended (0 μM formate) controls. Site labels correspond with those presented in Tables 1 and 2.

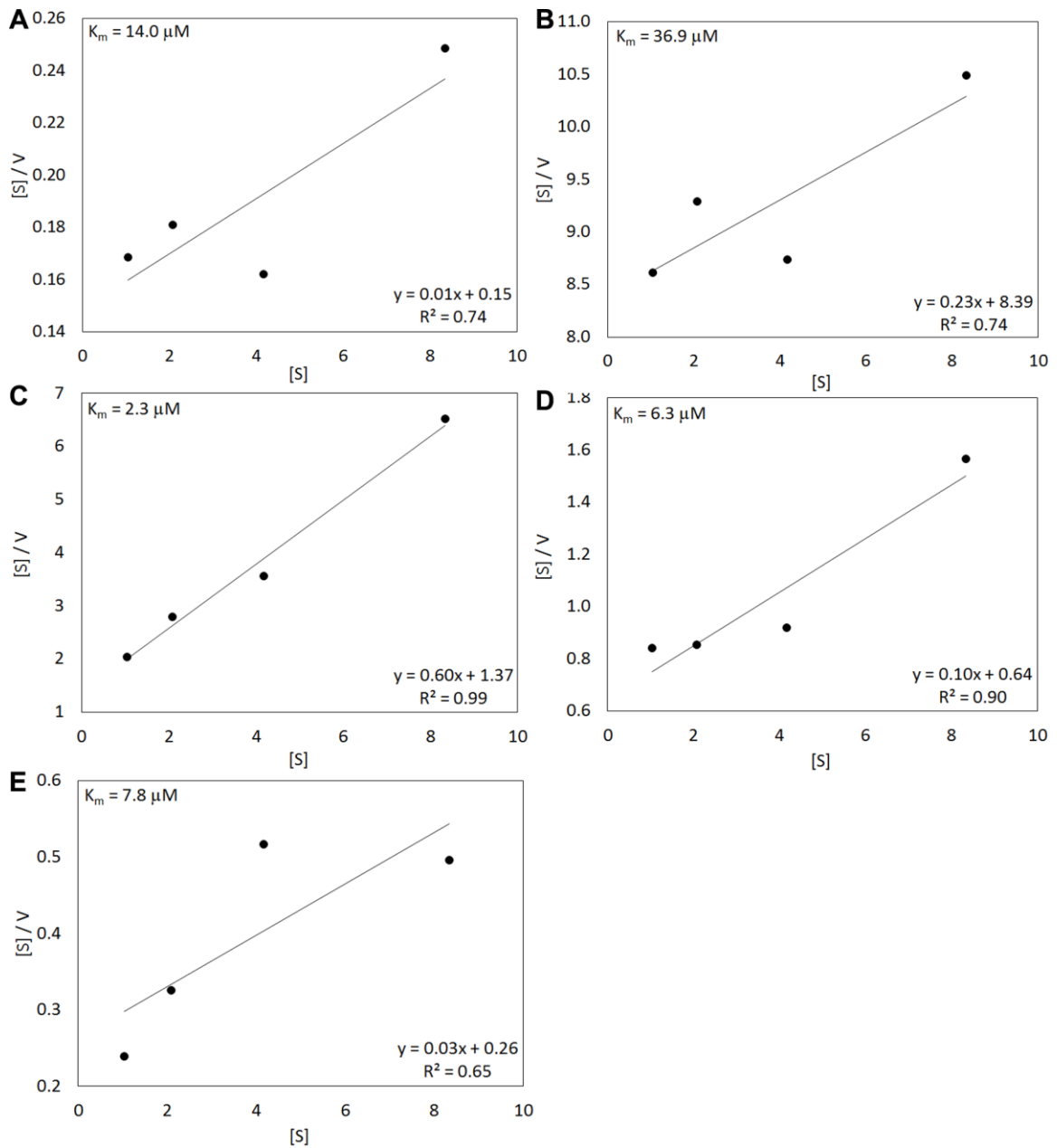


Figure 5. Results of formate kinetic assays in 5 select YNP geothermal features. The equations of Hobbie and Wright 1966 were used to determine affinity constants (K_m). The calculated K_m value for Bison Pool (A), Cinder Pool (B), Dragon Spring (C), Evening Primrose (D), and Perpetual Spouter (E) is presented in each panel.

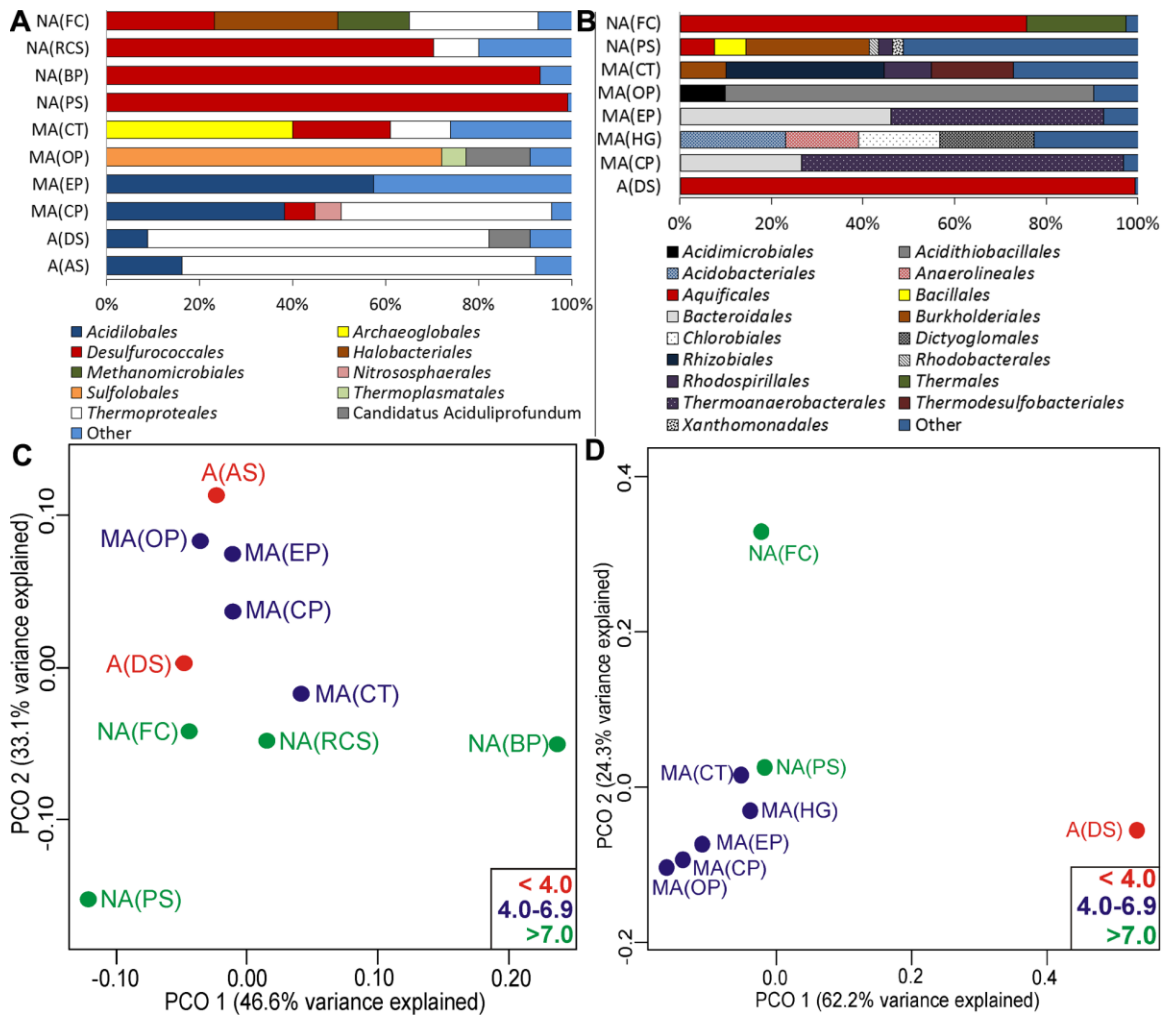
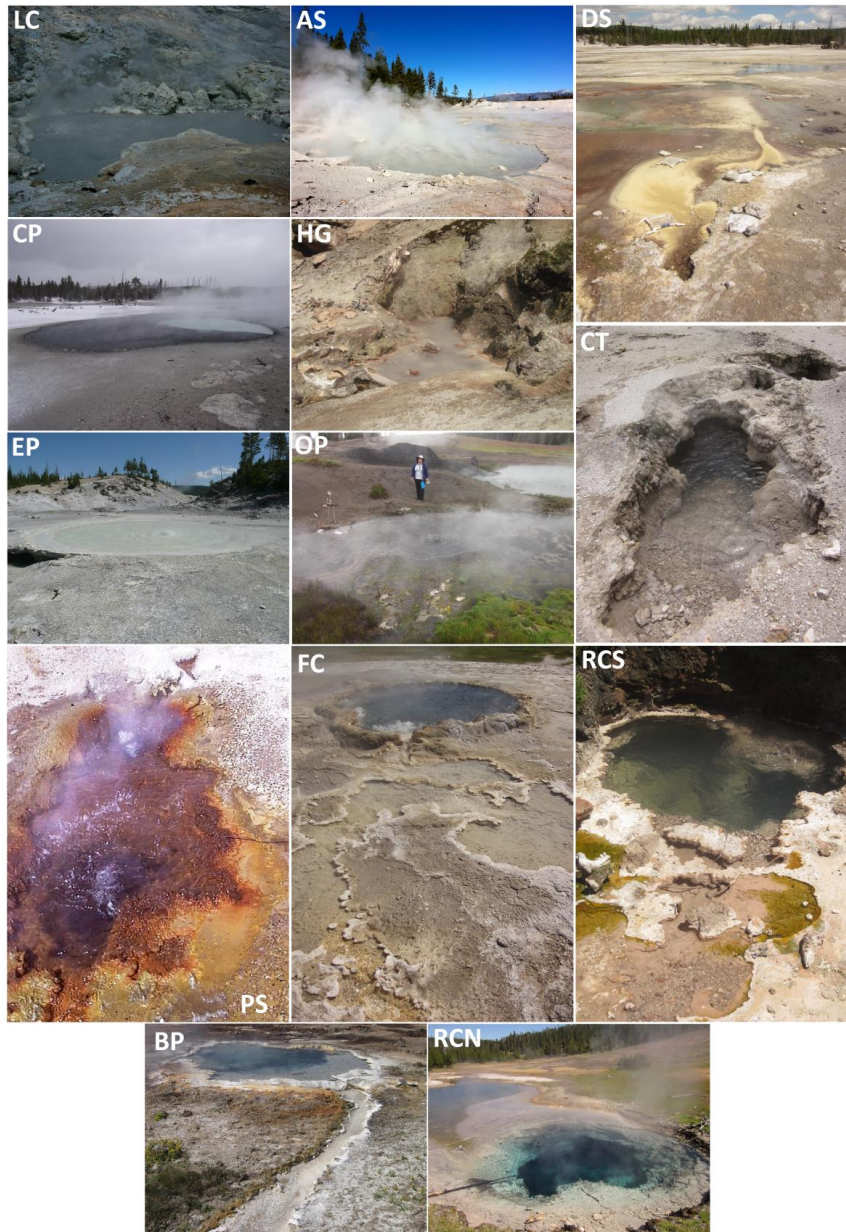


Figure 6. Composition of archaeal (A) and bacterial (B) 16S rRNA genes in DNA extracted from sediments sampled from the source of hot springs listed in Tables 1 and 2. More detailed taxonomic affiliations for archaeal and bacterial 16S rRNA genes are listed in Supplementary Tables 3 and 4, respectively. PCO ordination of the Rao phylogenetic dissimilarity associated with archaeal (C) and bacterial (D) 16S rRNA gene assemblages. Acidic springs (pH < 4.0) are colored red, moderately acidic springs (pH 4.0 to 6.0) are colored navy blue, and neutral to alkaline springs (pH > 7.0) are colored green.

SUPPLEMENTAL ONLINE MATERIAL



Supp. Figure 1. Photographs of field sites: Lobster Claw (LC), Alice Spring (AS), Dragon Spring (DS), Cinder Pool (CP), Hell's Gate (HG), Evening Primrose (EP), Obsidian Pool (OP), Corner Thing (CT), Perpetual Spouter (PS), Flat Cone (FC), Rabbit Creek South (RCS), Bison Pool (BP), and Rabbit Creek North (RCN).

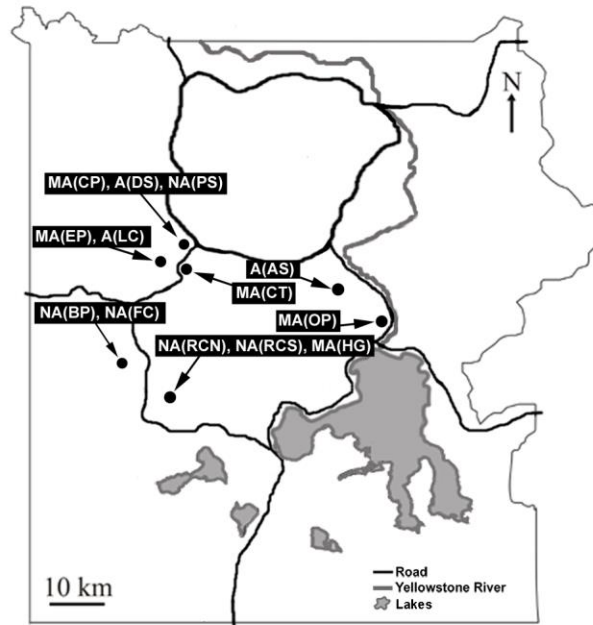


Figure S2. Map of Yellowstone National Park (YNP), Wyoming, USA indicating the general location of hot springs selected for study. GPS coordinates for individual hot springs are presented in Table 1

Supp. Table 1. Rate^a of DIC, formate, and acetate assimilation or mineralization attributable to sediment-associated microbial communities inhabiting 13 YNP hot springs. Site labels correspond with those presented in Table 1.

Site	DIC (A)		Form (A)		Form (M)		Acet (A)		Acet (M)	
	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD
LC	44.4	7.1	5.9	1.7	220.6	32.5	1.0	0.3	1.1	0.8
AS	0.8	0.4	302.6	148.8	1294.9	616.9	44.5	5.9	21.3	2.5
DS	432.8	260.1	47.2	27.6	93.6	21.9	23.4	2.2	126.3	39.5
CP	1061.0	196.2	76.9	17.3	3523.8	531.1	1.7	0.2	13.0	0.3
HG	5.0	0.9	31.4	6.1	75.9	32.1	0.2	0.0	0.4	0.4
EP	658.7	144.7	73.8	20.7	23293.3	1037.5	8.0	3.0	31.1	2.0
OP	234.6	80.0	3.1	4.4	10.3	3.1	9.3	5.1	0.6	0.1
CT	1029.1	842.1	436.2	51.2	3563.2	1104.8	6.7	1.3	15.5	2.5
PS	53.3	14.1	10.0	2.3	377.6	38.8	20.2	3.0	42.3	3.2
BP	2939.9	1696.3	20.5	2.9	2923.7	346.1	1021.9	238.7	194.4	4.6
RCS	252.3	41.9	23.0	0.4	197.3	70.1	1.1	0.7	12.3	3.3
FC	525.3	48.2	30.1	2.8	99.9	9.6	557.9	42.2	33.1	3.0
RCN	71.3	54.3	95.6	21.5	245.4	70.7	3.2	0.8	6.1	2.0

^a All units are pmol C gdm⁻¹ hr⁻¹

Abbreviations: DIC (A), DIC assimilation; Form (A), formate assimilation; Form (M), formate mineralization; Acet (A), acetate assimilation; Acet (M), acetate mineralization

Supp. Table 2. Pearson correlation coefficients (Pearson *R*) for RDA axes 1 and 2 in relation to substrate transformation Rates and geochemical measurements. Values in bold were significant at a $P < 0.05$.

Variables	RDA1 (50.9% of variance explained)	RDA2 (23.4% of variance explained)
DIC(A)	0.68	-0.19
Formate (A)	0.29	0.73
Formate (M)	0.58	0.76
Acetate (A)	0.80	-0.43
Acetate (M)	0.90	0.05
Cond.	0.13	0.26
Temp.	-0.35	0.49
pH	0.32	-0.25
DIC	0.06	0.24
Formate	-0.11	0.28
Acetate	-0.08	0.29
S ₂ ²⁻	-0.02	-0.19
Fe ²⁺	-0.09	0.27
NH ₄ (T)	-0.23	0.47
NO ₂ ⁻	-0.23	0.05
NO ₃ ⁻	0.43	-0.15

Abbreviations: DIC (A), DIC assimilation; Form (A), formate assimilation; Form (M), formate mineralization; Acet (A), acetate assimilation; Acet (M), acetate mineralization; Cond., conductivity; Temp., temperature; DIC, dissolved inorganic carbon.

Supp. Table 3. Rates ^a of acetate transformation determined with position specific labels. Site labels correspond with those presented in Table 1.

Site	1-[¹⁴ C]-acetate (CH ₃ ¹⁴ COO ⁻)				2-[¹⁴ C]-acetate (¹⁴ CH ₃ COO ⁻)			
	Assimilation		Mineralization		Assimilation		Mineralization	
	Avg	SD	Avg	SD	Avg	SD	Avg	SD
LC	0.46	0.14	0.47	0.10	0.50	0.29	0.59	0.76
AS	13.67	2.51	1.02	0.81	30.83	5.37	20.23	2.33
DS	3.66	1.98	104.50	39.41	19.74	1.01	21.84	2.09
CP	0.52	0.10	11.82	0.16	1.22	0.18	1.17	0.29
HG	0.06	0.01	0.28	0.36	0.14	0.02	0.09	0.02
EP	3.44	2.94	20.59	1.75	2.29	0.31	10.50	1.04
OP	6.18	5.11	0.24	0.06	3.09	0.58	0.35	0.05
CT	4.25	0.60	13.70	2.22	2.41	1.13	1.85	1.11
PS	8.72	2.76	34.10	2.72	10.08	1.29	8.20	1.63
BP	105.30	28.16	91.51	2.31	916.64	237.05	102.90	3.98
RCS	0.59	0.36	8.76	1.85	0.55	0.13	3.56	2.74
FC	48.71	4.92	3.33	1.39	509.19	37.24	3.33	1.39
RCN	1.89	1.46	5.44	1.99	1.29	0.52	0.65	0.41

^a All units are pmol C gdm⁻¹ hr⁻¹

Supp. Table 4. Pearson correlation coefficient (R) between the abundance of archaeal 16S rRNA gene OTUs and rates of substrate transformation. **Bold-faced values are significant with a $P < 0.05$.**

OTU No.	Taxonomic Affiliation	% ID	Substrate Transformation				
			DIC (A)	Form (A)	Form (M)	Acet (A)	Acet (M)
Otu0001	<i>Ignasphaera aggregans</i> <i>Candidatus</i> Caldiarchaeum	94%	0.91	-0.06	-0.06	0.81	0.76
Otu0002	subterraneum <i>Aciduliprofundum boonei</i>	84%	-0.36	-0.33	-0.26	-0.12	-0.14
Otu0003	T469	87%	-0.24	-0.28	-0.25	-0.23	0.01
Otu0004	<i>Thermogladius shockii</i> WB1 <i>Acidilobus sulfurireducens</i>	82%	-0.10	-0.14	-0.17	-0.16	0.42
Otu0005	18D70	95%	-0.04	0.01	0.86	-0.30	-0.18
Otu0006	<i>Vulcanisaeta distributa</i> IC- 124 <i>Thermoproteus neutrophilus</i>	99%	-0.30	0.48	-0.08	-0.14	-0.16
Otu0007	JCM9278 <i>Stygiolobus azoricus</i>	100%	0.01	-0.23	0.03	0.24	-0.22
Otu0008	DSM6296 <i>Halorubrum distributum</i>	100%	-0.20	-0.24	-0.18	-0.16	-0.27
Otu0009	JCM9100	80%	-0.08	-0.17	-0.17	0.40	-0.09
Otu0010	<i>Thermogladius shockii</i> WB1	83%	-0.27	-0.22	-0.16	-0.15	-0.04
Otu0011	<i>Ignisphaera</i> sp. Tok37.S1 <i>Candidatus</i> Caldiarchaeum	87%	0.02	-0.17	0.39	-0.31	0.08
Otu0012	subterraneum	89%	-0.08	-0.18	-0.17	0.39	-0.09

Supp. Table 4., Cont.

OTU No.	Taxonomic Affiliation	% ID	Substrate Transformation				
			DIC (A)	Form (A)	Form (M)	Acet (A)	Acet (M)
Otu0013	<i>Thermoproteus</i> sp. IC-061	99%	0.12	-0.09	0.48	-0.24	-0.24
Otu0014	<i>Caldisphaera draconis</i> ; 18U65	99%	0.01	-0.15	0.78	-0.30	-0.02
Otu0015	<i>Thermogladius shockii</i> strain WB1	98%	0.06	-0.10	0.86	-0.24	-0.21
Otu0016	<i>Candidatus Caldiarchaeum subterraneum</i>	83%	-0.08	0.35	-0.14	-0.25	-0.30
Otu0017	<i>Geoglobus acetivorans</i> SBH6	93%	0.13	0.80	0.00	-0.17	-0.19
Otu0018	<i>Ignisphaera aggregans</i> DSM 17230	90%	0.91	-0.20	-0.03	0.87	0.82
Otu0019	<i>Thermogladius shockii</i> WB1	97%	-0.19	-0.14	-0.17	-0.17	-0.23
Otu0020	<i>Candidatus Nitrosocaldus yellowstonii</i> HL72	87%	0.14	-0.06	0.00	-0.17	-0.20
Otu0021	<i>Pyrobaculum calidifontis</i> VA1	94%	0.91	-0.20	-0.03	0.87	0.82
Otu0022	<i>Thermofilum</i> sp. 1505	99%	0.15	-0.09	0.44	-0.21	-0.22

Supp. Table 4., Cont.

OTU No.	Taxonomic Affiliation	% ID	Substrate Transformation				
			DIC (A)	Form (A)	Form (M)	Acet (A)	Acet (M)
Otu0023	<i>Thermocodium modestius</i> IC-125	96%	-0.12	-0.13	-0.17	-0.15	0.44
Otu0024	<i>Methanomassiliicoccus luminyensis</i> B10	88%	-0.20	-0.24	-0.17	-0.16	-0.27
Otu0025	<i>Thermogymnomonas acidicola</i> JCM 13583	91%	-0.20	-0.24	-0.17	-0.16	-0.27

Abbreviations: DIC (A), DIC assimilation; Form (A), formate assimilation; Form (M), formate mineralization; Acet (A), acetate assimilation; Acet (M), acetate mineralization.

Supp. Table 5. Pearson correlation coefficient (*R*) between the abundance of bacterial 16S rRNA gene OTUs and rates of substrate transformation. Bold-faced values are significant with a *P* < 0.05.

	Substrate Transformations						Substrate Transformations				
	DIC (A)	Form (A)	Acet (A)	Form (M)	Acet (M)		DIC (A)	Form (A)	Acet (A)	Form (M)	Acet (M)
Otu001	-0.07	-0.12	-0.12	-0.19	0.93	Otu026	0.15	-0.05	-0.16	0.97	0.08
Otu002	-0.27	-0.24	-0.14	-0.20	-0.32	Otu028	0.53	0.98	-0.15	-0.02	-0.17
Otu003	0.60	-0.05	-0.23	0.57	-0.19	Otu029	0.53	0.98	-0.15	-0.02	-0.17
Otu004	0.45	-0.06	-0.22	0.90	-0.12	Otu030	-0.27	-0.24	-0.14	-0.20	-0.32
Otu005	0.01	-0.16	1.00	-0.20	0.01	Otu031	-0.45	-0.22	-0.12	-0.18	0.09
Otu006	-0.19	0.25	-0.22	-0.10	0.01	Otu032	-0.50	-0.16	-0.16	-0.19	-0.32
Otu007	-0.27	-0.25	-0.15	-0.20	-0.32	Otu033	0.53	0.98	-0.15	-0.02	-0.17
Otu008	-0.50	-0.16	-0.16	-0.19	-0.32	Otu034	-0.27	-0.24	-0.14	-0.20	-0.32
Otu009	-0.59	-0.18	-0.28	-0.34	-0.29	Otu035	-0.50	-0.16	-0.16	-0.19	-0.32
Otu010	-0.50	-0.16	-0.16	-0.19	-0.32	Otu036	-0.27	-0.24	-0.14	-0.20	-0.32
Otu011	0.02	-0.17	1.00	-0.20	0.00	Otu037	-0.27	-0.24	-0.14	-0.20	-0.32
Otu012	-0.50	-0.16	-0.16	-0.19	-0.32	Otu038	-0.45	-0.22	-0.12	-0.18	0.09
Otu013	-0.50	-0.16	-0.16	-0.19	-0.32	Otu039	-0.27	-0.24	-0.14	-0.20	-0.32
Otu015	-0.27	-0.24	-0.14	-0.20	-0.32	Otu040	-0.50	-0.16	-0.16	-0.19	-0.32
Otu017	0.51	0.98	-0.16	-0.03	-0.17	Otu041	-0.45	-0.22	-0.12	-0.18	0.09
Otu019	-0.27	-0.24	-0.14	-0.20	-0.32	Otu042	-0.50	-0.16	-0.16	-0.19	-0.32
Otu020	0.53	0.98	-0.15	-0.02	-0.17	Otu043	0.32	-0.05	-0.19	0.98	-0.07
Otu021	0.07	-0.09	-0.17	0.95	0.00	Otu044	-0.45	-0.22	-0.12	-0.18	0.09
Otu022	-0.45	-0.22	-0.12	-0.18	0.09	Otu045	-0.45	-0.22	-0.12	-0.18	0.09
Otu023	-0.27	-0.24	-0.14	-0.20	-0.32	Otu046	-0.50	-0.16	-0.16	-0.19	-0.32
Otu024	-0.27	-0.24	-0.14	-0.20	-0.32	Otu048	0.49	0.97	-0.16	-0.03	-0.16
Otu025	-0.50	-0.16	-0.16	-0.19	-0.32	Otu049	-0.45	-0.22	-0.12	-0.18	0.09

Abbreviations: DIC (A), DIC assimilation; Form (A), formate assimilation; Form (M), formate mineralization; Acet (A), acetate assimilation; Acet (M), acetate mineralization

Supp. Table 6. Pearson correlation coefficients between PCO vector scores, rates of substrate transformation, and environmental variables. A dash indicates that an assessment of correlation was not possible due to that compound being below detection

	Archaea			Bacteria			
	PCO Axis 1	PCO Axis 2	PCO Axis 3	PCO Axis 1	PCO Axis 2	PCO Axis 3	
DIC (A)	0.92	-0.16	-0.12	DIC (A)	-0.14	-0.06	-0.55
Form (A)	0.08	0.29	0.39	Form (A)	-0.07	-0.04	-0.09
Form (M)	0.08	0.34	0.06	Form (M)	-0.02	0.95	0.00
Acet (A)	0.73	-0.29	0.04	Acet (A)	-0.25	-0.26	-0.52
A (M)	0.63	-0.29	-0.16	A (M)	0.94	0.01	-0.13
Cond.	-0.22	0.00	0.49	Cond.	0.08	-0.21	-0.63
Temp.	0.06	-0.44	0.23	Temp.	-0.20	-0.19	-0.47
pH	0.29	-0.68	-0.03	pH	-0.51	0.72	0.19
DIC	0.04	0.48	0.74	DIC	-0.25	0.76	-0.18
Formate	-0.09	0.50	0.71	Formate	0.68	-0.17	0.15
Acetate	-0.08	0.49	0.72	Acetate	-	-	-
S ₂ ²⁻	-0.26	-0.08	-0.39	S ₂ ²⁻	0.91	0.23	-0.11
Fe ²⁺	-0.11	0.51	0.67	Fe ²⁺	0.89	-0.26	0.02
NH ₄ (T)	-0.13	0.57	0.60	NH ₄ ⁺	-0.16	-0.37	-0.63
NO ₂ ⁻	0.05	-0.21	0.11	NO ₂ ⁻	0.23	-0.06	0.07
NO ₃ ⁻	0.17	-0.19	-0.37	NO ₃ ⁻	0.74	0.34	-0.06

Abbreviations: DIC (A), DIC assimilation; Form (A), formate assimilation; Form (M), formate mineralization; Acet (A), acetate assimilation; Acet (M), acetate mineralization; Cond, conductivity; Temp, temperature

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CHAPTER THREE

SUBSTRATE PREFERENCE AND UPTAKE KINETICS IN A FACULTATIVELY
AUTOTROPHIC AND HYPERTHERMOPHILIC CRENARCHAEOTE

Contribution Of Authors And Co-Authors

Manuscript in Chapter 3

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Contributions: Conception and implementation of experimental design. Collection and analysis of experimental data. Wrote first draft of manuscript.

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Contributions: Partial genome assembly, annotation and analysis. Manuscript editing.

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Contributions: Partial genome sequencing. Manuscript editing.

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Contributions: Principle investigator and corresponding author. Advised conception and implementation of experimental design. Editing, approval and submission of final manuscript.

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Substrate Preference, Uptake Kinetics, and Energetics in a Facultatively Autotrophic, Hyperthermophilic Crenarchaeote

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ABSTRACT (272 WORDS)

A thermophilic elemental sulfur (S_8^0) reducing facultative autotroph (strain CP80) was isolated from Cinder Pool (CP, 88.7°C, pH 4.0), Yellowstone National Park, and characterized with respect to the preference, uptake kinetics, and energetics associated with various carbon sources. The 16S rRNA gene sequence from CP80 was 99% identical to *Thermoproteus uzonensis* and was identical to the dominant archaeon identified in CP sediments. Hydrogen-dependent autotrophic activity in cells of *Thermoproteus* strain CP80 was suppressed by amendment with formate at concentrations between 20 to 40 μ M, which was similar to the Michaelis constant for formate (ca. 30 μ M) as determined from whole cell kinetic assays. Cellular yields during S_8^0 -dependent growth with formate were $4.62 \times 10^9 \pm 9.0 \times 10^8$ cells kJ^{-1} whereas they were $5.35 \times 10^8 \pm 7.46 \times 10^7$ cells kJ^{-1} when the cells were grown autotrophically with hydrogen, indicating preferential use of formate for thermodynamic reasons. The formate concentration supporting optimal growth was 100 μ M; concentrations above this resulted in slower generation times. The total amount of sulfide produced from S_8^0 grown cultures decreased with increasing starting concentrations of formate and the amount of formate oxidized decreased in cultures amended with increasing initial concentrations of sulfide. This indicates that both formate and sulfide are cytotoxic to this organism. Genomic analysis reveals the presence of an uptake [NiFe]-hydrogenase, an “O”-type formate dehydrogenase, and a complete dicarboxylate/4-hydroxybutyrate pathway that allow for hydrogen, formate, and autotrophic metabolism, respectively. Collectively,

these results further out understanding of the factors that influence the physiology and ecology of thermoacidiphiles and for the first time demonstrate formate metabolism in a thermoacidiphilic archaeon.

INTRODUCTION

Crenarchaeota are common inhabitants of acidic, high temperature hot spring environments (1-4). Cultivated thermoacidiphilic crenarchaeotes include members of the orders Acidilobales (5) Thermoproteales (6) as well as a diversity of Sulfolobales (7). Common to many of these strains is the ability to grow via oxidation of complex organic carbon sources such as extracts of yeast and protein (i.e., tryptone, peptone) coupled to the reduction of elemental sulfur (S_8^0). Attempts to cultivate crenarchaeotes on defined carbon sources have been less successful. For example a variety of organic acids, carbohydrates, and alcohols were shown to not support the growth of the thermoacidiphilic crenarchaeotes *Acidilobus sulfurireducens* (Acidilobales) and *Caldisphaera draconis* (Acidilobales) (8). Similarly, *Vulcanisaeta* spp., which are common components of acidic hot springs communities, were shown to be unable to grow on a variety of organic acids, amides, amines, or alcohols (9).

Intriguingly, the genomes of a number of cultivated (10, 11) and uncultivated (2, 3) thermoacidiphilic crenarchaeotes including *Acidilobus* spp., *Vulcanisaeta* spp., and *Thermoproteus* spp. reveal the presence of homologs of enzymes that should allow for growth on simple organic carbon sources, such as organic acids. For example, formate

dehydrogenase (FDH) which catalyzes the oxidation of formate to CO₂ while catalyzing the reduction of NAD⁺ to NADH, is encoded by a number of acidophilic crenarchaeota. The reduced NADH produced through formate oxidation can then serve as reductant for electron transfer chains thereby allowing energy conservation through transmembrane proton translocation. It is possible that the lack of success in past attempts to cultivate acidophilic crenarchaeota on organic acids, including formate, is due to amendment of these substrates at concentrations of up to 10 mM formate (8, 12). This is problematic, since the concentration of protonated formate increases as pH decreases (pK_a = 3.75 at 80°C), reaching ½ the total formate concentration at pH=3.75. This uncharged form will readily diffuse into the cell and deprotonate at intracellular pH, thereby effectively decoupling proton translocation from ATP production. Previous studies have shown that concentrations of formate as low as 100 μM inhibit the growth of the acidophile *Thiobacillus (Acidithiobacillus) ferrooxidans* when grown in medium with a pH of 1.6 (13). Thus, it is possible that strategies to cultivate acidophiles using organic acids including formate have been compromised by supplying these substrates at cytotoxic concentrations.

We recently observed high rates of formate oxidation and assimilation in a number of archaea-dominated acidic hot springs in YNP using a microcosm-based approach amended with low (<10 μM) concentrations of formate (14). Kinetic experiments indicated that the formate utilizing populations of these springs were adapted to use formate at low concentration. For example, formate uptake affinities (K_m) ranging from 2.3 to 36.9 μM were determined for communities inhabiting acidic, sulfur (S₈⁰) rich

Dragon Spring (78.0°C, pH 2.5), Cinder Pool (88.7°C, pH 4.0), and Evening Primrose (78.4°C, pH 5.1). Moreover, it was shown that formate suppressed autotrophic carbon assimilation at concentrations (5 to 20 μM) that were similar to the K_m for formate transformation. Given the short incubation times (<45 minutes) of these assays, it was concluded that the populations that were involved in CO_2 and formate metabolism in these hot springs are facultatively autotrophic, and are capable of switching their metabolic strategy to preferentially utilize the more thermodynamically-favorable substrate formate, when both formate and inorganic carbon are available.

The dominant archaeon present in sediments sampled from Cinder Pool was closely affiliated (99% sequence identity) with *Thermoproteus uzonensis* 768-20. *Thermoproteus* species are rod shaped, sulfur-dependent, hyperthermophilic, obligate or facultative autotrophs which grow in hot springs with temperatures and pH ranging from 85°C to 105°C and 5.2 to 6.8, respectively (15). The physiology of *Thermoproteus* strains is variable. For example, *T. tenax* (16, 17) is capable of growing on organic substrates such as glucose, casamino acids, ethanol, malate or formamide whereas *T. neutrophilus* (15) is incapable of heterotrophic growth and is rather an obligate H_2 -dependent autotroph. *T. uzonensis* grows via the fermentation of complex organic carbon such as peptone and tryptone but growth is not supported by more simple organic substrates such as formate and acetate and this strain has not been demonstrated to grow autotrophically (12). Intriguingly, the recently completed genome sequence of *T. uzonensis* 768-20 (10) revealed the presence of putative homologous genes coding for formate dehydrogenase

(FDH), enzymes required to reduce S_8^0 via the Sre system (18), and a complete dicarboxylate/4-hydroxybutyrate pathway capable of supporting autotrophic growth.

In the present study, we enriched for a formate dependent, sulfur reducing strain from CP, in particular the close relative of *Thermoproteus*, for use in further investigation of metabolic switching and substrate preference in acidophilic Crenarchaea. Our enrichment strategy was designed to avoid the potential pitfalls associated with formate toxicity by supplying enrichments with a low concentration of formate (50 μ M). Application of this approach to sediments sampled from Cinder Pool (pH 4.0, 85°C) resulted in the isolation of a crenarchaeon with a 16S rRNA gene sequence that was 99% identical to that in the genome of *T. uzonensis* 768-20 and 100% identical to the dominant sequence present in sediments sampled from Cinder Pool (14). Strain CP80 is a facultative autotroph that preferentially metabolizes formate over CO_2 . Cells were capable of switching between autotrophic and heterotrophic metabolic strategies in response to changing organic carbon concentrations. Similar formate kinetic parameters measured for cells of CP80 and in our previous in situ experiments using sediments from Cinder Pool (14) suggest that CP80 may be responsible for the metabolism of formate in this spring. Moreover, the genome of strain CP80 encoded for proteins required to conserve energy from the coupling of formate or hydrogen oxidation to S_8^0 reduction, as well as a full complement of enzymes for the dicarboxylate/4-hydroxybutyrate pathway for CO_2 fixation. Comparison of these results to cultivars with available genomic data suggests that facultative autotrophy and preferential utilization of organic acids may be

widespread metabolic strategies employed by a diversity of hyperthermophilic crenarchaeotes in high temperature hydrothermal systems.

MATERIALS AND METHODS

Enrichment and Isolation of strain CP80. Clay-rich sediments were sampled from the hydrothermal feature known as Cinder Pool (N 44°43'56.8", W 110°42'35.1"), in Norris Geyser Basin, Yellowstone National Park, Wyoming in May, 2012. Sediments were sampled aseptically with a flame-sterilized spatula and were placed in a sterile serum bottle along with a small amount (~ 5 mL) of spring water collected using a sterile 10 mL syringe. Serum bottles and their contents were capped with a butyl rubber stopper and an aluminum crimp seal, briefly purged with N₂, and transported back to the lab at MSU where they were stored in an incubator at 80°C for ≤ 24 hours before being used for enrichment and isolation.

A base salts enrichment medium consisting of MgSO₄ (0.33 g L⁻¹), NH₄Cl (0.33 g L⁻¹), CaCl₂ (0.33 g L⁻¹), KCl (0.33 g L⁻¹), and KH₂PO₄ (0.33 g L⁻¹) (8) was prepared for use in enrichment and isolation of organisms capable of growth on formate (50 μM final concentration) as the sole carbon and electron source. The pH of the medium was adjusted to 4.0 with concentrated hydrochloric acid. Orthorhombic elemental sulfur flower (S₈⁰; 5 g L⁻¹) was provided as the sole terminal electron acceptor. SL-10 trace elements and Wolfe's vitamins were added at a final concentration of 1 mL L⁻¹ as previously described (8). This medium will be referred to as S₈⁰-base salts for the remainder of this communication.

Thirty five mL of S_8^0 -base salts medium was dispensed into several 70 mL serum bottles. Serum bottles and their contents were purged for ≥ 45 minutes with nitrogen (N_2) gas passed over heated (210°C) and reduced copper shavings to remove oxygen, capped with butyl rubber septa, and autoclave sterilized. Following sterilization, S_8^0 (autoclaved dry under an N_2 atmosphere at 110°C for 1 hr) was added to each serum bottle under a stream of sterile N_2 gas. S_8^0 -base salts medium was inoculated with 100 μL of the sediment/water slurry collected from Cinder Pool, and the cultures were incubated at 80°C . Cultures were amended every 3 days with an additional 50 μM formate. S_8^0 reduction activity in cultures was monitored by measurement of the production of sulfide (S^{2-}) via the methylene blue reduction method (19). Cultures exhibiting S_8^0 reduction activity that was significantly greater than that observed in un-inoculated controls were used in a subsequent dilution to extinction cultivation assay in attempt to isolate a pure culture. Briefly, 8 groups of serum bottles were prepared with 45 mL of S_8^0 -base salts medium amended with formate. Triplicate batches of medium were inoculated with 5 mL of inoculum taken from active enrichment cultures, for an initial dilution factor of 10^{-1} ; triplicate un-inoculated batches of medium were included to monitor abiotic production of S_2^{2-} . A dilution to extinction isolation strategy was employed from this first dilution to a final dilution factor of 10^{-8} . Cultures were incubated at 80°C for ~ 7 days, followed by the inoculation of a newly-prepared dilution series from the most dilute culture group showing S_8^0 reduction activity in the previous dilution series. Progress toward enrichment of a population with a single morphotype was monitored in each dilution to extinction

culture series by epifluorescence microscopy using SYBR Gold Nucleic Acid Gel Stain (Life Technologies, Inc., Grand Island, NY) as previously described (8).

Genomic characterization of strain CP80. Following two rounds of dilution to extinction a single morphotype was observed. DNA was extracted from a single culture for use in genomic sequencing. Three 5 mL subsamples of this culture were subjected to an initial centrifugation to remove S_8^0 (2000 x g, 1 min, 4°C). The supernatant from each subsample was then concentrated via centrifugation (14,000 x g, 15 min., 4 °C) and total DNA was isolated from the cell pellets using the FastDNA Spin Kit for soils (MP Biomedicals, LLC). Equal volumes of replicate extractions were pooled and quantified using the Qubit DNA Assay kit (Life Technologies, Grand Island, NY) and a Qubit 2.0 Fluorometer (Life Technologies). Thirty five cycles of PCR were conducted using bacterial- (1100F/1492R) or archaeal-specific (344F/915R) 16S rRNA gene primers with reaction and cycling conditions as previously described (20, 21). The resulting archaeal 16S rRNA gene amplicon (amplicons were not generated using bacterial specific PCR primers) were sent to MR DNA (Shallowater, TX, USA) for paired-end Illumina MiSeq sequencing. Post sequencing processing was performed with Mothur (ver. 1.25.1) (22) as previously described (21) after removing reads of less than 300 base pairs. The raw sequence libraries and quality score files have been deposited in the NCBI SRA database under accession number (SRR1812888).

Total genomic DNA was sequenced at the Genomics Core Facility at the University of Wisconsin-Madison using the Illumina MiSeq platform (Illumina, San Diego, CA). DNA fragments were prepared according to the manufacturer's protocol.

Quality of the reads was checked with FastQC (bioinformatics.babraham.ac.uk/projects/fastqc). Reads were quality trimmed from both ends using Trimmomatic (23). Reads containing more than 3 N's were removed and reads with an average quality score of less than Q20 or a sequence length less than 50 bps were removed. The trimmed paired-end reads were assembled using SPAdes version 3.5.0 (24).

The archaeal 16S rRNA gene Illumina tags revealed the presence of three different 16S rRNA gene phlotypes related to the crenarchaeotes *Thermoproteus*, *Caldivirga*, and *Vulcanisaeta* (described in more detail below). Thus, assembled contigs were binned using tetranucleotide frequency and coverage, resulting in taxonomically resolved clusters of contigs containing individual genomes (25) as previously described (26). Briefly, tetranucleotide frequency was calculated for both the forward and reverse-complement of each contig using a custom python script (26) and ordinated using Principal Component Analysis (PCA). PCA was performed using the `prcomp()` function in R (27) and further improved by extracting and rotating the first six principal components by varimax rotation (28). Contig coverage was incorporated to improve clustering of the contig scores (26). To improve assembly, paired reads mapping to each cluster of contigs (genomic bin) were reassembled using Velvet (29) as previously described (30). The kmer length resulting in the best assembly—the longest contigs, highest N50—was chosen for the final assembly. The resulting three bins were annotated using the RAST server (31). The genomes were assigned to taxa using the taxonomic affiliation of predicted genes based on the best BLASTx match. The three bins

corresponded to *Thermoproteus* sp., *Caldivirga* sp., and *Vulcanisaeta* sp. (data not shown). The raw sequence files can be found in the RAST annotation files have

Because our 16S rRNA and genomic characterization revealed the presence of three closely related crenarchaeal phylotypes, an additional 8 rounds of dilution to extinction was performed. A single culture (designated as CP80) with a single morphotype was selected and three 5 mL subsamples of this culture were concentrated and subjected to DNA extraction as described above. PCR amplification, sequencing, and analysis, as described above, revealed the presence of a single archaeal 16S rRNA phylotype. The raw paired end metagenomic sequence files for the early enrichment as well as the raw paired end 16S rRNA gene tag sequence files for the purified culture have been deposited in the NCBI SRA database under accession number (SRR1812888).

Growth of strain CP80 on formate or H₂/CO₂. Strain CP80 was grown in S₈⁰-base salts medium with formate as sole carbon source and electron donor or CO₂ as carbon source with hydrogen (H₂) as the electron donor. Triplicate cultures were prepared for both biological and abiological (un-inoculated) incubations as described above with the following exception for cells grown on 20% CO₂/ 80% H₂: the N₂ headspace was purged for 1 minute with sterile H₂ passed over heated (210°C) copper shavings. Ten mM stock solutions containing a 170:1 ratio of ¹²C to ¹⁴C formate or bicarbonate were used to amend media with 50 μM formate or bicarbonate. Given that the pK_a for bicarbonate to CO_{2(aq)} at 80°C is ~6.4 (32), it was assumed that the added bicarbonate dissociated to CO₂ when added to S₈⁰-base salts medium with the pH adjusted to 4.0 and equilibrated with headspace CO₂.

Formate or bicarbonate/H₂ amended cultures were inoculated to an initial cell density of 10⁴ to 10⁵ cells mL⁻¹ from mid log phase stock cultures grown on formate/ S₈⁰ or H₂/CO₂/S₈⁰, respectively. All cultures were incubated at 80°C for the duration of the experiment. Subsamples were collected every 24 to 48 hours for cell counts, oxidation or assimilation of formate or CO₂ (described below), and production of total S²⁻. Total S²⁻ concentration was determined by summing the concentrations of gaseous S²⁻ and aqueous S²⁻ in each culture [as calculated using Henry's Law (33)]. Cell counts were performed using epifluorescence microscopy as described above.

Determination of rates of substrate transformation. To quantify rates of CO₂ production in the gas phase of cultures grown on formate, N₂ purged serum bottles (12 ml) containing 1 ml of the CO₂-absorbing solution Carbo-Sorb[®] E (PerkinElmer, Inc., Santa Clara, CA, USA) were evacuated to a final pressure of -10 torr using a vacuum pump. One mL of the gas phase was removed from the headspace of each culture using a 1 mL syringe and stopcock and immediately injected into one of the bottles containing Carbo-Sorb[®] E solution.

To determine the concentration of dissolved CO₂ produced during growth on formate, 2 mL of culture was removed from each microcosm and injected into a 12 mL serum bottle prepared as described above, but without Carbo-Sorb[®] E solution. The sealed 2 mL subsamples were acidified by the addition of 0.2 mL 12N hydrochloric acid (HCl) to ensure that all dissolved CO₂ was driven out of solution. Following 2 hrs equilibration 5 mL of the headspace was removed using a 10 mL syringe and stopcock and injected into a separate 12 mL serum bottle containing 1 ml Carbo-Sorb[®] E solution,

prepared as described above. Serum bottles containing gas samples and Carbo-Sorb[®] E solution were allowed to react at room temperature (~22°C) for approximately 2 hours. Following incubation, the vials were opened and the Carbo-Sorb[®] E solution was removed and transferred to a 20 mL liquid scintillation vial containing 10 mL CytoScint ES[™] liquid scintillation fluid (MP Biomedicals, USA) in preparation for liquid scintillation counting (LSC), as described below.

The amount of ¹⁴C assimilated during growth of strain CP80 on either labeled formate or CO₂ was determined by removal of 2 mL subsamples from each culture. Samples were acidified as described above and filtered onto a 0.22 μm white polycarbonate membrane. Filtered samples were washed with 5 mL of culture medium (pH 4, no carbon source) and dried for 1-2 hours at 80°C. Dried filters were placed in scintillation vials and overlain with 10 ml of CytoScint ES[™] liquid scintillation fluid. Radioactivity associated with each of the samples (Carbo-Sorb[®] E solution and filtered biomass) was measured on a Beckman LS 6500 liquid scintillation counter (Beckman Coulter, Inc., Indianapolis, IN). Rates of oxidation and/or assimilation of formate and CO₂ were calculated from LSC counts using previously described methods (14). The mean and standard error of the rates of substrate transformation attributable to biology was calculated as the difference between substrate transformation rates in 3 biological replicates and 3 abiological (un-innoculated) controls.

Determination of the Michaelis constant (K_m) of formate conversion. Serum bottles containing 50 mL S₈⁰ base salts medium were amended with 10 mM ¹²C+¹⁴C-formate stock solution (prepared as described above) to achieve a final formate

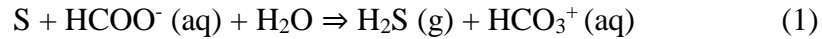
concentration of 1.25, 2.5, 5, 10, 20, or 40 μM . Triplicate inoculated and un-inoculated controls were prepared for each condition. Cells for use as inoculum were prepared by filtration of 50 mL of mid-log phase formate grown cultures onto a sterile, 0.2 μM polycarbonate filter. Filtered cells were washed with 20 mL of base salts medium lacking a carbon source and S_8^0 in order to remove residual formate that might be carried over from the inoculum. Filtered cells were re-suspended in 20 mL sterile, anaerobic S_8^0 base salts medium with no carbon source, and 1 mL of this inoculum was added to each microcosm to achieve an initial cell density of $\sim 10^5$ cells mL^{-1} . Cultures were incubated at 80°C for 24 hours, and sub-samples of the gas and aqueous phases of cultures were taken at intervals of 8 hours as described above. The amount of CO_2 produced via formate oxidation was calculated by summing the aqueous and gas phase CO_2 concentrations determined by LSC as described above. The K_m of formate conversion to CO_2 was estimated from a plot of the formate conversion rate during log phase growth versus substrate concentration using the KaleidaGraphTM software package (Synergy Software, Reading, PA, USA). Reported values reflect the average and standard deviation of three replicate biological cultures minus values from three replicate un-inoculated cultures.

Formate suppression of CO_2 assimilation. Serum bottles containing 10 mL of S_8^0 base salts medium were prepared as described above. Following removal of O_2 via N_2 sparging, the headspace of each culture was purged for 1 minute with sterile hydrogen (H_2) followed by the addition of CO_2 to achieve a final concentration of 20% CO_2 to 80% H_2 . Five μCi (0.1 μmol) of ^{14}C -bicarbonate was added. Medium was then amended with

0, 5, 10, 20, 40, or 80 μM ^{12}C -formate. Cultures were inoculated from H_2/CO_2 grown log-phase cultures to an initial cell density of 10^6 cells mL^{-1} . Following 4 hrs incubation at 80°C , S_8^0 base salts medium was acidified to a $\text{pH} < 2.0$ using HCl as described above to volatilize $^{14}\text{CO}_2$. Bottles were allowed to degas for 1 hour in a fume hood followed by filtration of cells onto $0.2 \mu\text{m}$ white polycarbonate membrane filters. Membranes were dried at 80°C for 24 hrs, and the ^{14}C activity in the biomass of each culture was determined by LSC as described above. The percent suppression of CO_2 assimilation activity at each formate concentration was determined by normalization of the ^{14}C activity in the biomass of the formate-amended cultures to that in the unamended cultures.

Determination of organic acid and sulfide toxicity. Strain CP80 was grown in S_8^0 -base salts medium in the presence of 25, 50, 100, 200, or 300 μM formate. Triplicate replicates were inoculated to an initial cell density of 10^5 cells mL^{-1} from mid-log phase stock cultures of CP80 grown on formate/ S_8^0 . Cultures were incubated at 80°C for 7 days and subsamples of medium were collected for cell counts and S^{2-} production at intervals of 24 – 48 hours. Growth curves were constructed and generation times (T_n) for cultures grown under each formate treatment were calculated during log phase growth.

Cell yields of strain CP80 when grown on formate and H_2/CO_2 , with S_8^0 as terminal electron acceptor. The coupling of formate (HCOO^-) or H_2 oxidation, respectively, with the reduction of elemental sulfur (simplified to indicate reduction of a single atom of S_8^0) should follow the reaction stoichiometries as depicted in equation 1 and 2 below, respectively:



The ratio of total formate transformed to total S_8^0 reduced was determined by dividing the amount of formate transformed by the amount of S_8^0 reduced (derived from total S^{2-} produced) at each time point, followed by calculation of the mean and standard deviation of this ratio across all time points. The ratio of total H_2 oxidized to total S_8^0 reduced could not be calculated because changes in H_2 concentration was not monitored during growth. The cell yield per mol C assimilated ($Y_{\text{cells/mol C}}$) and the cell yield per mol S_8^0 reduced ($Y_{\text{cells/mol S}}$) during growth on formate or CO_2 , was calculated by dividing the number of new cells produced at each time point by the total C assimilated from formate or CO_2 at each time point, or the total S_8^0 reduced at each time point, respectively, followed by calculation of the mean and standard deviation of these ratios across all time points.

The total amount of energy available to an organism catalyzing a redox reaction in a non-standard state displaced from equilibrium is given by equation 3:

$$\Delta_r G = \Delta_r G^\circ + RT \ln Q_r \quad (3)$$

where $\Delta_r G$ is the Gibbs free energy of reaction, $\Delta_r G^\circ$ is the standard Gibbs free energy of reaction, R is the ideal gas constant ($\sim 8.314 \text{ J mol}^{-1} \text{ K}^{-1}$), T is the temperature in Kelvin (K), and Q_r is the activity product (the product of the activities of each reactant and product, where reactant activities are negative, and product activities are positive). The activity (α) 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 (34).

For the purposes of this study, reactant and product activities at each sampling time during growth of strain CP80 on formate and H_2/CO_2 were calculated based on the ionic strength and composition of the S_8^0 -base salts medium as described above, as well as the measured or estimated concentration of each reactant and product, using the freeware chemical equilibrium modeling application Visual MINTEQ 3.0 (35). Reactant and product activities were then used to compute $\Delta_r G^\circ$ and $\ln Q_r$ using the *subcrt* command in the thermodynamic modeling package CHNOSZ (36), developed for use with the statistical computing language R (27). Calculated $\Delta_r G^\circ$ and $\ln Q_r$ values were then used to calculate $\Delta_r G$ with equation (3) above, and the resulting $\Delta_r G$ 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^{-1}). At each time point, the energy available to cultures of CP80 was calculated from the Gibbs energy calculations as described above and the total moles of electrons transferred from the reductant to the oxidant at that time point (equal to twice the number of mols of substrate transformed, as 2 moles of electrons are transferred per mole of substrate transformed). The number of new cells produced at each time point was then divided by the total energy available from the individual catalyzed redox reaction at that time point to compute the number of cells produced per unit of energy (cells kJ^{-1}). Since we did not quantify differences in H_2 concentration during the growth of strain CP80, we assumed a constant aqueous H_2 concentration equal to the saturation concentration of H_2 in H_2O at 80°C and 1 atmosphere of partial pressure ($\sim 768\ \mu\text{M}$).

RESULTS

Site Description. Cinder Pool (CP) is a high temperature, sulfur-rich, acid-sulfate-chloride (ACS) hot spring located in Norris Geyser Basin, Yellowstone National Park (YNP), Wyoming. The authors have observed the temperature and pH of the spring to be fairly constant, ranging from 84 °C to 89 °C and 4.0 to 4.4, respectively, over the past 12 years of sampling (data not shown). CP is a unique feature in YNP in that a reservoir of molten elemental sulfur resides at a depth of ~18 m, while its surface is partially covered with black, hollow spherules comprised of ~99.0% S_8^0 and ~1.0% pyrite (37). At the time when samples were collected for enrichment and isolation of strain CP80, the temperature, pH and conductivity in CP source waters were 88.7°C, 4.0, and 4.8 mS, respectively. Total sulfide and ferrous iron concentrations at the site were 0.2 mg L⁻¹ and 0.084 mg L⁻¹, respectively.

Enrichment and isolation of strain CP80. Samples of clay-rich sediment collected from the edge of CP were used to inoculate S_8^0 base salts media containing 50 µM formate as the sole carbon and energy source. Incubation of the cultures at 80°C resulted in sulfide formation within 5 days, indicating S_8^0 reduction activity. Eight rounds of dilution to extinction cultivation resulted in the isolation of a single morphotype. The 16S rRNA gene from strain CP80 was closely affiliated (99.0% sequence identities) with *Thermoproteus uzoniensis* 768-20 within the crenarchaeal order *Thermoproteales*. For the remainder of this communication, the strain will be referred to as *Thermoproteus* sp. CP80 or simply as strain CP80. Cells of strain CP80 are rod-shaped, with a diameter of

~0.4 μm and cell lengths that ranged between 1 μm and 10 μm . S_8^0 reduction activity was also observed in microcosms amended with acetate as sole carbon and energy source, although an increase in cell number was not observed in these cultures.

Growth Parameters of *Thermoproteus* sp. CP80 on formate. When grown on formate and S_8^0 , the generation time (T_n) of strain CP80 was 33.9 ± 2.1 hours (Fig. 1A). The maximum rate of formate mineralization and assimilation during log phase growth was 10.8 ± 2.0 nmol hr^{-1} and 0.7 ± 0.1 nmol hr^{-1} , respectively, resulting in an overall maximum formate transformation rate of 11.5 ± 2.0 nmol hr^{-1} . The maximum rate of S_8^0 reduction, as assessed by S^{2-} production, was 19.1 ± 6.1 nmol hr^{-1} . Formate mineralization and assimilation activity and S_8^0 reduction activity decreased as the remaining formate concentration approached ~ 30 μM (Fig. 1B) at which point the amount of sulfide decreased substantially (Fig. 1C). When additional formate was added ($t = 168$ hours) to a final concentration of approximately 65 μM , the rate of formate transformation increased to 6.8 ± 0.8 nmol hr^{-1} which was roughly half the maximum formate transformation rate observed in our studies. Again, while the concentration of formate increased following addition of formate (Fig. 1C), it decreased again once the rate of formate oxidation decreased (Fig. 1B).

The cell yield per pmol carbon assimilated, per pmol formate oxidized, and per pmol S_8^0 reduced was determined from growth data (Table 2). The ratio of total formate transformed to total S_8^0 reduced was 0.78 ± 0.23 , which is not statistically different from the expected 1:1 molar ratio of formate oxidized to S_8^0 reduced according to the reaction stoichiometry depicted by equation 1. The cell yield per mole electrons transferred from

formate to S_8^0 was $1.15 \times 10^5 \pm 2.41 \times 10^4$ cells mol e^-^{-1} , which translated to a yield of $4.62 \times 10^9 \pm 9.0 \times 10^8$ cells kJ^{-1} .

Growth Parameters of *Thermoproteus* sp. CP80 on H_2 and CO_2 . When CP80 cells were grown autotrophically with H_2 and S_8^0 , the T_n was 85.5 ± 4.7 hours, which is roughly 2.5-fold longer than when grown on formate and S_8^0 . Moreover, a lag phase of 96 hrs was observed which is roughly 4-fold longer than the lag phase of CP80 cells when grown with formate (data not shown). The maximum rate of C assimilation from CO_2 was 1.0 ± 0.1 nmol hr^{-1} , which is similar to the maximum rate of C assimilation observed when cells of CP80 were grown on formate (0.7 ± 0.1 nmol hr^{-1}) (Table 1). The maximum rate of S_8^0 reduction, as assessed by sulfide production, was 58.7 ± 7.4 nmol hr^{-1} . The cell yield per pmol carbon assimilated (Table 2) was 283 ± 107 cells $pmol^{-1}$. The cell yield per mol electron transferred from H_2 to S^0 (estimated from total S_8^0 reduced) was $2.22 \times 10^4 \pm 2.84 \times 10^3$ cells mol e^-^{-1} , which translated to a yield of $5.35 \times 10^8 \pm 7.46 \times 10^7$ cells kJ^{-1} .

Formate transformation kinetics and substrate preference in *Thermoproteus* sp. CP80. The Michaelis-Menten constant of formate conversion (K_m) of strain CP80 was 31.2 ± 7.3 μM and the maximum velocity of formate conversion (V_{max}) was 0.036 ± 0.005 μmol hr^{-1} (Fig. 3). As mentioned previously, the rate of CO_2 assimilation was not significantly suppressed in the presence of 5 or 10 μM formate when compared to that of the unamended control (0 μM formate), but decreased by approximately 22% in the presence of 20 μM formate and was below the limits of detection in the presence of 40 and 80 μM formate (Fig. 4). Together, these results suggest a reduced efficiency for

Thermoproteus sp. CP80 to utilize formate at low concentrations and help to explain the near cessation of formate utilization in this isolate when the formate concentration in the bulk medium dropped below values of 30 μM (Fig. 1B).

Organic acid toxicity. Generation times (T_n) of *Thermoproteus* sp. CP80 cells grown on a range of formate concentrations were determined to address the possibility that the observed suppression of CO_2 assimilation activity by formate may be due to its potential cytotoxicity. Calculated T_n values were 75.8 ± 15.9 hours, 61.3 ± 15.3 hours, 38.1 ± 0.9 hours, 57.4 ± 1.3 hours, and 73.8 ± 9.1 hours when formate was provided at an initial concentration of 50, 100, 200, 400, and 1000 μM , respectively. The formate concentration at which T_n values transitioned from being inversely correlated with formate concentration to being positively correlated with formate concentration was 100 μM , indicating that formate concentrations at or below 100 μM do not suppress the growth of *Thermoproteus* sp. CP80 when grown with S_8^0 .

Genomic characterization of *Thermoproteus* sp. CP80. Pair-ended Illumina MiSeq libraries were generated, assembled, and annotated to generate new insight into formate and H_2/CO_2 metabolism in strain CP80. Assembly of the CP80 genome yielded 187 contigs with a predicted size of 2.35 mega base pairs and a GC content of 46.5%. The partial genome sequence of CP80 encodes a single putative formate dehydrogenase (FDH) homolog which consists of the alpha, beta, and gamma, subunits of the FDH-O complex organized in an apparent operon. The alpha subunit of the FDH-O complex encodes a twin arginine motif indicating that it is likely localized to the outer membrane

of the plasma membrane. The alpha, beta, and gamma subunit encoding genes exhibit 94, 94, and 79% sequence identities to FDH-O homologs in *Thermoproteus uzoniensis*.

A single, trimeric [NiFe]-hydrogenase complex was identified in the genome of *Thermoproteus* sp. CP80. The large subunit of this complex exhibits 97% sequence identities to the large subunit of a hydrogenase (YP_004338974) in *T. uzoniensis* 768-20. Phylogenetic analysis reveals that the large subunit of this hydrogenase clusters with known group 1 [NiFe] uptake hydrogenase proteins (Supp. Fig. X). Group I [NiFe]-hydrogenases are multimeric membrane-bound complexes that are involved in coupling of H₂ oxidation to the reduction of anaerobic electron acceptors such as fumarate, NO₃⁻, SO₄²⁻ and O₂ (38). The large subunit houses the [NiFe] active site cluster, while a small subunit and a cytochrome *b* subunit act to shuttle electrons through a series of Fe-S clusters in the small subunit and heme groups in the cytochrome *b* subunit (39). The cytochrome *b* subunit anchors the complex to the membrane, with the small and large subunits oriented toward the exterior of the plasma membrane. These proteins are targeted for transport across the membrane by a twin-arginine motif present in the small subunit (39). In the genome of CP80, two genes located immediately upstream of the large subunit are homologous to the small subunit (95% sequence identities; YP_004338976) and the cytochrome *b* containing subunit (91% sequence identities; YP_004338975) to subunits comprising the complex in the genome of *T. uzoniensis* 768-20.

In addition to putative FDH and [NiFe]-hydrogenase genes, homologs of a multimeric sulfur reductase (Sre) complex putatively involved in sulfur reduction were

identified. The three subunits (A, B, C) that comprise this complex were co-localized and exhibited 90, 94, and 88% percent identities to SreABC subunits present in the genome of *T. uzoniensis* 768-20 (YP_004336866 to YP_004336868). Additional genes of direct relevance to energy conservation identified in the genome of strain CP80 include a multimeric V-type ATP synthase complex as well as a number of proteins that are likely to be involved in electron transport chain including homologs of NADH dehydrogenase and NADH-ubiquinone oxidoreductase.

A complete complement of gene homologs encoding enzymes involved in the dicarboxylate/4-hydroxybutyrate pathway of CO₂ fixation were identified in the genome of strain CP80. The presence of homologous genes encoding for the enzymes in this pathway in the genome of strain CP80 is consistent with the known distribution of the pathway in the archaeal orders *Desulfurococcales* and *Thermoproteales*, including *Thermoproteus* spp. (40, 41). The CP80 genome encodes for the key enzymes of this pathway, as illustrated in Figure 7. These enzymes include 3-hydroxybutyryl-CoA dehydrogenase, 4-hydroxybutyrate-CoA ligase (synthetase), 4-hydroxybutyryl-CoA dehydratase, acetyl-CoA C-acetyltransferase (acetoacetyl-CoA thiolase), crotonyl-CoA hydratase, fumarate hydratase, fumarate reductase, malate dehydrogenase, phosphoenolpyruvate carboxylase, pyruvate synthase, pyruvate-water dikinase (Phosphoenolpyruvate synthase), succinate semialdehyde reductase (NADPH), succinyl-CoA reductase, succinyl-CoA synthetase (succinate thiokinase), and Acetyl-CoA C-acyltransferase (Acetoacetyl-CoA β -ketothiolase).

DISCUSSION

Strain CP80 was isolated from the sulfur-rich, high temperature (84°C) hydrothermal feature known as Cinder Pool, in Yellowstone National Park using formate as the electron donor and carbon source and S_8^0 as the electron acceptor. The 16S rRNA gene sequence from strain CP80, which exhibited 100% sequence identity with the numerically dominate archaeal sequence obtained previously from Cinder Pool sediments (14), was 99% identical to that from *T. uzoniensis* 768-20, an anaerobic heterotroph that is capable of reducing S_8^0 (12).

Thermoproteus sp CP80 couples formate oxidation to S_8^0 reduction at temperatures and pH conditions similar to those present in Cinder Pool (80 °C, pH 4.0). The cessation of formate metabolism by CP80 at a remaining formate concentration of 27 μ M is consistent with the Michaelis-Menten constant (K_m) of formate conversion calculated from a subsequent experiment to determine the kinetics of formate conversion by CP80 ($31.2 \pm 7.3 \mu$ M, see Fig.3). Since the conversion rate of a particular substrate at a particular concentration is expected to be directly related to the Michaelis-Menten constant (K_m) of the organism for the substrate, the cessation of formate metabolism when concentrations of substrate fall below 30 μ M may be due to inefficient mechanisms for uptake or substrate transformation. Similar sharp drops in substrate conversion velocity have were observed in mixed cultures of thermophilic butyrate-utilizing bacterium together with *Methanobacterium thermoautotrophicum* and a thermophilic acetate-utilizing methanogenic rod, known as the TAM (thermophilic acetate-utilizing

methanogenic) organism, when the concentration of butyrate and acetate fell below the K_m for their utilization (42). The observed increase in formate oxidation and mineralization rates after additional formate was added at $t = 168$ hours, along with a significant increase in cell density by 192 hours, as compared to that at $t = 96$ hours (when formate conversion ceased), indicates that formate was a limiting nutrient in the CP80 microcosms. Interestingly, the cessation in formate metabolism in strain CP80 between 96 hrs and 168 hrs was accompanied by a lack of cell production, consistent with the predictions of the Monod equation relating microbial specific growth rate in an aqueous environment to the concentration of a limiting nutrient, in this case formate (43, 44).

Growth and formate metabolism in cultures of CP80 ceased at $t = 196$ hours, despite a remaining formate concentration of $\sim 60 \mu\text{M}$, indicating that the growth and metabolism of CP80 was limited by factors other than formate concentration. One such factor may be buildup of hydrogen sulfide (H_2S) during S_8^0 -dependent growth. Sulfide toxicity in microbes is well documented (45-50) and has been shown to inhibit a number of processes including photosynthesis (51, 52), sulfate reduction and methanogenesis (53), and the anaerobic degradation of the organic acids acetate, lactate and propionate (45-49). The toxicity of sulfide may stem from inhibition of key enzymes, such as cytochrome c oxidase (54), photosystem II (52), or other oxidase enzymes (55). Alternatively, the toxicity of sulfide in microbes may be due the ability of its protonated form (H_2S) to penetrate the cell membrane and subsequently deprotonate in the cytosol. The production of protons in the cytosol by deprotonation uncouples the electromotive

force (proton or sodium ion gradient) from ATP production and other essential cellular processes (49). Since the pKa for the dissociation of H₂S to HS⁻ is 6.6 at 80°C (32), the majority of sulfide produced during S₈⁰-dependent growth in medium with a pH of 4.0 would be in the protonated form, which may impart additional energetic demands on cells of CP80. Indeed, in a number of cultures of thermophilic microorganisms the concentration of sulfide is positively correlated with doubling (generation) time and cellular maintenance energy (46).

Interestingly, when grown on H₂/CO₂/S⁰, CP80 cells did not exhibit a slowing or cessation of H₂ oxidation (as evidenced by S⁰ reduction) activity, C assimilation activity, or growth, despite a final maximum sulfide concentration that was more than 3-fold larger than that observed when CP80 was grown on formate/S⁰. One possible explanation for this result is that the cessation of formate metabolism, and the lower cell yields, observed in formate-grown CP80 at high H₂S concentration are due to the combined toxicity of formate and H₂S (both of which are cytotoxic in their protonated forms) rather than the toxicity of H₂S alone. Similar results have been observed in sulfate-reducing bacteria (SRB) grown on the organic acids butyrate, acetate and propionate, which were demonstrated to be up to more than 2-fold more sensitive to increased sulfide concentrations and pH changes (which effect the toxicity of organic acids and sulfide), compared to SRB grown on H₂/CO₂ (56).

The cell yield per pmol carbon assimilated in cultures of CP80 during growth on formate was 499 ± 69 cells pmol⁻¹, which translates to a dry weight mass of approximately 1.5 g mol⁻¹ assuming a conservative estimate of cell mass of 3 fg (57).

This yield is slightly lower than that observed during formate dependent growth of the bacterium *Wolinella succinogenes* on S_8^0 , which exhibited a cell yield per mol formate of 3.5 g mol^{-1} (58-60). The slightly lower cell yield per mol formate oxidized in cultures of CP80 when compared to that of *W. succinogenes* may also be attributable to the buildup of sulfide, as high sulfide concentrations have been shown to decrease cell yields in other thermophiles growing on organic acids and S_8^0 (46). The calculated Gibbs free energy of reaction for the formate/ S_8^0 redox couple under the CP80 culture conditions at t_1 ($-121.9 \text{ kJ mol e}^{-1}$, Table 2) translates to approximately $-29.2 \text{ kcal mol e}^{-1}$ transferred to S_8^0 . This value is in agreement with those calculated by Windman et al, 2007 (61) using geochemical measurements taken from a number of YNP hydrothermal features ($\sim -24 \text{ kcal mol e}^{-1}$ to $\sim -26 \text{ kcal mol e}^{-1}$), indicating that the thermodynamics associated with formate dependent S_8^0 reduction in cultures of CP80 are similar to those likely present in YNP springs.

The generation time exhibited by CP80 during growth on H_2 , CO_2 and S^0 was 85.5 ± 4.7 hours, which is between 5- and 10-fold longer than has been previously observed in *Thermoproteus neutrophilus* growing on H_2 , CO_2 and S_8^0 (15, 62, 63). The calculated Gibbs free energy of reaction for the H_2/S^0 redox couple under CP80 culture conditions at t_1 ($-92.9 \text{ kJ mol e}^{-1}$, Table 2) translates to approximately $-22.2 \text{ kcal mole e}^{-1}$ transferred from H_2 to S_8^0 . which is $\sim 7 \text{ kcal mol e}^{-1}$ less than that available to CP80 grown on formate/ S_8^0 ($\sim 29 \text{ kJ mol e}^{-1}$), indicating that formate is a more thermodynamically-favorable energy source than H_2 under the conditions present in CP80 microcosms. The calculated cell yields in autotrophically grown CP80 cells per

pmol C assimilated and per pmol S_8^0 reduced were significantly lower than those observed during growth on formate/ S_8^0 . Moreover, the cell yield per kJ of electrons transferred was a full order of magnitude lower in $H_2/CO_2/S_8^0$ grown cultures, as compared to that observed in formate/ S_8^0 grown cultures, despite the fact that the maximum rate of carbon assimilation was not significantly different between the two substrates. These results indicate that, in addition to the lower energy yield available from the oxidation of H_2 with S_8^0 , growth of CP80 on $H_2/CO_2/S_8^0$ is a less efficient, more energy-intensive process than growth on formate. Decreased efficiency of growth on H_2/CO_2 , compared to that on the organic acid lactate, was also observed in the acetogenic bacterium *Acetobacterium woodii* (38, 64), in which the maintenance energy of growth (m_E) was 8.8-fold larger during growth on H_2/CO_2 compared to growth on lactate. Similarly, the ATP required to produce a cell of the organism *Pseudomonas oxalaticus* OX1 was demonstrated to be 4.4- and 1.9-fold higher when grown on CO_2 when compared to growth on glucose and oxalate, respectively (65). A possible explanation for this phenomenon was proposed by (66), who attributed lower ATP phosphorylation per unit O_2 consumed (P/O ratio) in the bacterium *Pseudomonas saccharophila* during autotrophic growth, as compared to heterotrophic growth, to be an indicator of poor coupling of substrate oxidation to ATP phosphorylation in the electron transport system used during autotrophic growth. Additional investigation is required to conclusively explain the observed differences in yields during growth on H_2/CO_2 versus formate.

The lower energy available and the decreased efficiency of growth on $H_2/CO_2/S^0$ strongly suggest that growth on formate/ S^0 should be preferred by CP80 over growth on

H₂/CO₂. To investigate this hypothesis, we examined the ability of formate to suppress DIC (CO₂) assimilation in cells of CP80 growing with H₂ and S₈⁰. The observed suppression of DIC assimilation by CP80 in the presence of 20 μM formate is consistent with the concentration of formate required to suppress DIC assimilation in sediment-associated communities sampled from Cinder Pool (14). The observed similarity between kinetic properties of formate/S⁰ grown CP80 in culture and the formate uptake kinetics exhibited by the chemosynthetic microbial population in its native habitat, Cinder Pool, indicates that the environmental conditions influencing formate utilization kinetics in Cinder Pool are likely also similar to those in CP80 microcosms, and that the kinetic properties of formate utilization exhibited by CP80 in culture are likely similar those exhibited by this organism in its natural habitat. Further support for this conclusion comes from the measured K_m of formate conversion by CP80 in culture (31.2 ± 7.3 μM), which is consistent with that exhibited by the microbial community in CP (36.9 μM). Taken together, these results indicate that CP80 is likely an important driver of formate transformation in the CP chemosynthetic community.

Another possible explanation for the observed suppression of CO₂ assimilation by CP80 in the presence of sufficient concentrations of formate is that this suppression resulted from formate toxicity by way of the mechanism discussed above, rather than a metabolic shift from H₂ oxidation to formate oxidation. To address this possibility, we investigated the generation time (T_n) of cultures of strain CP80 in the presence of subsequently higher formate concentrations (Fig. 5A). The growth rate of CP80 was not significantly suppressed at formate concentrations of 100 μM or less, suggesting that the

suppression of CO₂ assimilation activity by CP80 observed at formate concentrations between 20 μM and 80 μM was not caused by formate toxicity. This result is consistent with reports of formate toxicity occurring at concentrations at or above 100 μM in the acidophilic proteobacterium *Acidithiobacillus ferrooxidans* (syn. *Thiobacillus ferrooxidans*) (13). Interestingly, although the sulfide concentration in formate/S₈⁰ microcosms at which formate mineralization and assimilation by CP80 slowed or ceased was positively correlated with initial formate concentration, this correlation was not 1:1 above an initial formate concentration of 25 μM (Fig. 5B), indicating that the maximum amount of formate that CP80 was able to metabolize was limited both by the initial formate concentration and the concentration of accumulated sulfide. The positive (but less than 1:1) correlation between the maximum sulfide concentration tolerated by CP80 and the initial formate concentration strongly suggests that the increased energy yield (ΔG_r) from formate oxidation afforded by higher formate concentrations allowed CP80 to more effectively compensate for the increased energy demand of maintaining a proton gradient against the decoupling effect caused by the deprotonation of both formate and H₂S in the cytosol, thereby allowing the organism to continue utilizing formate at higher concentrations of H₂S than those that were tolerated at lower initial formate concentrations. These results suggest that sulfide concentration may be a limiting factor for formate utilization by chemosynthetic communities in natural environments where the sulfide concentration reaches levels higher than ~25 μM.

To investigate the possible genetic mechanisms used by CP80 to grow on formate/S⁰ or H₂/CO₂/S⁰, we generated a partial genome sequence for this organism.

CP80's genome encodes for a putative group I, membrane-bound, periplasmic nickel-iron (NiFe) uptake hydrogenase composed of three subunits (large, small and membrane-bound cytochrome b). NiFe hydrogenases of this group are known to catalyze respiratory H₂ oxidation coupled via a quinone pool to the reduction of a number of potential anaerobic electron acceptors, such as SO₄²⁻, NO₃⁻, CO₂, or fumarate, as well as O₂, while ultimately conserving energy by creation of a proton motive force (67). The membrane-bound subunit of the group I hydrogenase is composed of a di-heme cytochrome b that is coupled to a membrane-bound electron transport chain via a quinone pool. This complex, together with the C-terminus of the small subunit, links the group I hydrogenase complex to the cytoplasmic membrane. Although the group I uptake hydrogenases from proteobacteria have been most intensively studied (67, 68), these H₂ consuming complexes also occur in hyperthermophilic, sulfur-respiring organisms, such as the bacterium *Aquifex aeolicus* (18, 69), and archaea such as *Pyrodictium abyssi* (70), *Acidianus ambivalens* (71), and *Thermoproteus uzoniensis* (10). Although the enzyme complex has not been characterized in *Thermoproteus uzoniensis*, *A. aeolicus*, *P. abyssi* and *A. ambivalens* possess enzyme complexes composed of a group I hydrogenase in complex with a sulfur reductase and, in some cases, an electron transfer component required to couple the oxidation of H₂ to the reduction of S⁰ to H₂S via an intermediate quinone pool. The genome of CP80 encoded for a putative trimeric sulfur reductase complex similar to putative sulfur reductase complexes present in other Sulfur reducing organisms (18, 69-71) as well as a multimeric archaeal ATP synthase (A-ATPase, (72)). A mechanism for energy conservation from hydrogen oxidation is proposed whereby

electrons liberated from the oxidation of extracellular H₂ catalyzed by the putative group I hydrogenase are transferred to an intermediate quinone pool via the membrane-bound cytochrome b subunit of the hydrogenase. Oxidation and reduction of quinone drives translocation of protons from the cytoplasm to the exterior of the cells, which can then be used to drive ATP synthesis.

Also encoded in the genome is an “O” type formate dehydrogenase (FDH-O) (73, 74). FDH-O from *E. coli* is a multimeric, membrane-anchored protein consisting of an α subunit (FdoG) that contains the molybdopterin active site co-factor, a β subunit (FdoH) located on the periplasmic side of the cytoplasmic membrane that functions in electron-transport, and a γ subunit (FdoI) which functions as a membrane-anchor (75-77). The FdoI subunit contains a quinone binding site that allows electrons from formate oxidation to enter the electron transport chain (77, 78). FDH-O is constitutively-expressed at low levels under aerobic, fermentative, and nitrate-reducing (anaerobic) conditions (73, 79). FDH-O has been proposed to function as a means to oxidize formate during the transition from aerobic growth to anaerobic, nitrate-reducing conditions (76). It is likely that FDH-O functions to couple formate oxidation to the reduction of quinones and ultimately S₈⁰ through the activity of Sre. FDH-O is constitutively-expressed in *E. coli* (73, 79) suggesting that this may also be the case in CP80, which would be consistent with this organism’s ability to “switch” between H₂/CO₂ utilization and formate/S₈⁰ utilization on a short time scale. Based on these results, we propose a model for the conservation of energy in CP80 from the oxidation of formate whereby extracellular formate is oxidized by the putative FDH-O, liberating 2 electrons that are subsequently transferred to the

quinone pool via the quinone binding site in the membrane-bound gamma subunit. Subsequent transfer of electrons from the quinone pool to the putative sulfur reductase discussed above yields energy that is conserved via the translocation of protons from the cytosolic to the periplasmic face of the cytosolic membrane, establishing a proton gradient which drives ATP production and other essential cellular processes. Finally, as with the proposed mechanism for energy conservation from hydrogen, electrons are transferred from the sulfur reductase to S_8^0 in the cytosol, producing H_2S .

In addition to the pathways described above for the conservation of energy from formate and H_2 oxidation with S_8^0 , the genome of *Thermoproteus* sp CP80 also codes for a full complement of putative genes comprising the dicarboxylate/4-hydroxybutyrate pathway of carbon fixation. This pathway was first characterized in the hyperthermophilic crenarchaeon *Ignicoccus hospitalis* (80), and has since been identified in the genomes of a number of crenarchaeotes from the orders *Desulfurococcales* and *Thermoproteales* (81). This pathway functions to generate acetyl-CoA from the fixation of one molecule of CO_2 and one molecule of HCO_3^- , while consuming 5 molecules of ATP (81). The pathway can be divided into two separate cycles, the first of which starts with one molecule of acetyl-CoA, one molecule of bicarbonate, and one molecule of CO_2 and converts these substrates to succinyl-CoA via pyruvate, phosphoenolpyruvate, and oxaloacetate. The second part of the cycle involves conversion of succinyl-CoA into two molecules of acetyl-CoA via 4-hydroxybutyrate. Interestingly, due to the oxygen sensitivity of the key enzyme of this pathway (4-hydroxybutyryl-CoA dehydratase) (82), the dicarboxylate/4-hydroxybutyrate pathway is typically used only by anaerobic

organisms, with some exceptions in the order *Desulfurococcales* which operate this pathway under microaerobic conditions (83). We propose that CO₂ generated by CP80 from formate oxidation (in the case of growth on formate as sole carbon source), or CO₂ present in the media (in the case of growth on CO₂ as the sole carbon source) diffuses across the cytoplasmic membrane and is fixed into biomass via acetyl-CoA generated by this pathway.

In conclusion, our results demonstrate that the hyperthermophilic crenarchaeon *Thermoproteus* sp CP80 is a facultative autotroph capable of energy conservation and growth on both formate and H₂, using S₈⁰ as sole electron acceptor. To our knowledge, this is the first reported example of the coupling of formate oxidation to S₈⁰ reduction in a hyperthermophilic crenarchaeote. Moreover, the results of H₂/CO₂ growth experiments, combined with the observed suppression of CO₂ assimilation in CP80 in the presence of formate, demonstrate that CP80 is a facultative autotroph capable of altering its metabolism in response to increased availability of thermodynamically more favorable organic substrates. Sequencing and analysis of CP80's genome revealed the presence of metabolic pathways that are likely utilized by this organism to conserve energy from both formate and H₂/CO₂, using S₈⁰ as sole electron acceptor. In addition, the remarkable similarity between the kinetic properties of formate utilization exhibited by CP80 in culture and those exhibited by the chemosynthetic community in Cinder Pool, indicate that this organism is well adapted to its native habitat where it likely plays an important role as a primary producer and heterotroph. The presence of FDH homologs in numerous previously characterized autotrophic and heterotrophic crenarchaeaota suggests that the

ability to utilize formate as an energy and carbon source with S_8^0 as an electron acceptor is likely more widespread than previously thought.

Table 1. Generation times and substrate utilization rates by *Thermoproteus* sp. CP80 during incubation at 80 °C in S_8^0 base salts medium (pH 4.0) with formate or H_2/CO_2 as sole carbon source and S_8^0 as sole electron acceptor.

Substrate	T_n (Hours)	Max. substrate oxidation rate	Max. rate of C assimilation	Max. rate of total substrate transformation	Max. S_8^0 reduction rate
CHOO ⁻	33.9 ± 2.1	10.8 ± 2.0	0.7 ± 0.1	11.5 ± 2.0	19.1 ± 6.1
CO ₂ /H ₂	85.5 ± 4.7	N.D.	1.0 ± 0.1	N.D.	58.7 ± 7.4

N.D. = No data

Max. = Maximum

Error reported as standard deviation.

Units are nmol hr⁻¹ unless otherwise noted.

Table 2. Cell yields (Y), stoichiometry and energetics of *Thermoproteus* sp. CP80 incubated at 80 °C in S₈⁰-base salts medium (pH 4.0) with formate or H₂/CO₂ as sole carbon/energy source and S₈⁰ as sole electron acceptor.

Redox reaction	e- Transferred	ΔG_r at t₁ (kJ mol e⁻¹)	Y_{cells/sulfur} reduced*	Y_{cells/C} assimilated*	H₂S / mol substrate	Y_{cells/mol e-}	Y_{cells/kJ}
S (s) + HCO ₂ ⁻ (aq) + H ₂ O (l) → H ₂ S (g) + HCO ₃ ⁻ (aq)	2	-121.9	21.0 ± 5.0	499 ± 69	0.8 ± 0.2	1.2 x 10 ⁵ ± 2.4 x 10 ⁴	4.6 x 10 ⁹ ± 9.0 x 10 ⁸
H ₂ (g) + S (s) → H ₂ S (aq)	2	-45.6	7.9 ± 2.7	283 ± 107	N.D.	2.2 X 10 ⁴ ± 4.9 X 10 ³	5.4 X 10 ⁸ ± 1.3 X 10 ⁸

* = substrate units are pmol

N.D. = No data

Error reported as standard

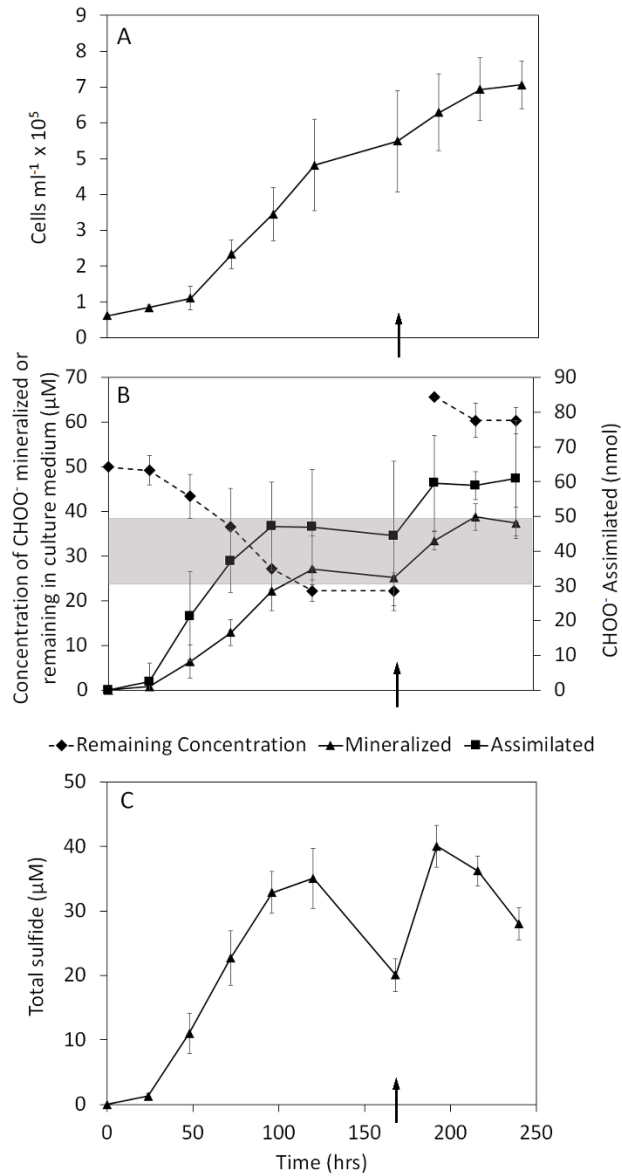


Figure 1. Concentration of cells (A), concentrations of formate oxidized (as determined from CO_2 produced), formate assimilated, and formate remaining in culture medium (B), and sulfide produced (C) by *Thermoproteus* sp. CP80 incubated at 80°C in S_8^0 base salts medium (pH 4.0) with and formate and S_8^0 as the sole electron donor and electron acceptor, respectively. The gray box in figure B delineates the average (center of vertical box) and standard error (width of vertical box) of determinations of the K_m ($31.3 \pm 7.3 \mu\text{M}$) for formate transformation (see Fig. 3). Arrows indicate the time point at which cultures were amended with additional formate ($50 \mu\text{mol L}^{-1}$). The average and standard error of measurement (SEM) of three replicate cultures is presented.

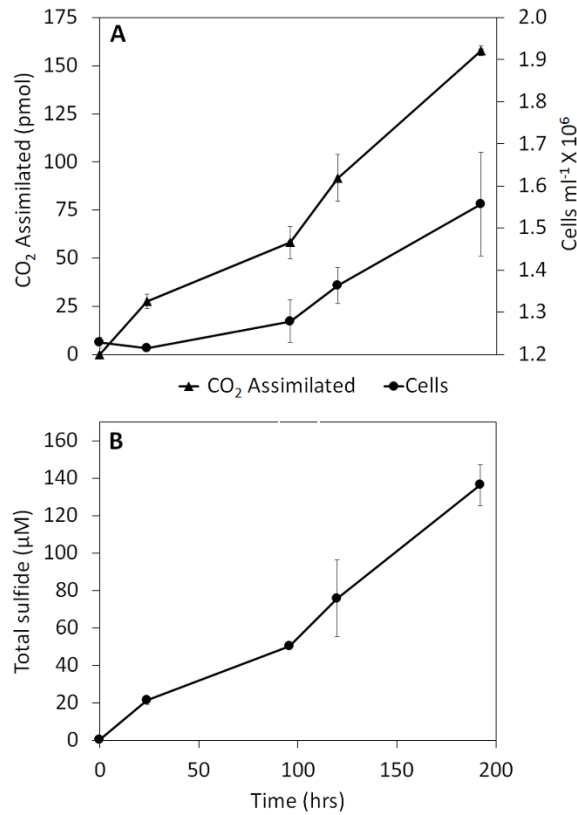


Figure 2. Concentration of cells and the amount of carbon assimilated from CO_2 (A), and total sulfide produced (B), by *Thermoproteus* sp. CP80 during incubation at 80°C and in S_8^0 base salts medium (pH 4.0) with CO_2 as the sole carbon source, hydrogen (H_2) as the sole electron donor, and elemental sulfur (S_8^0) as sole electron acceptor. The average and standard error of measurement (SEM) of three replicate cultures is presented.

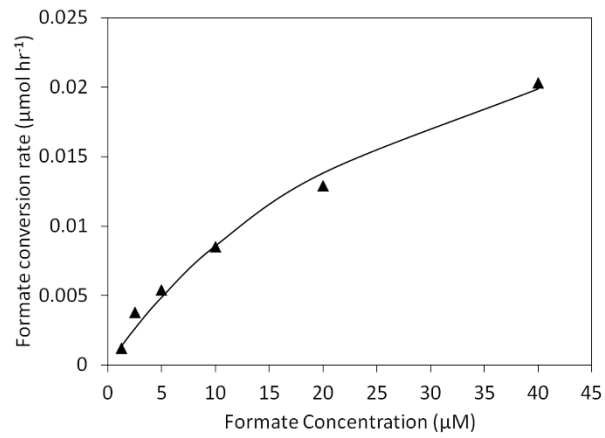


Figure 3. Formate conversion rate by *Thermoproteus* sp. CP80 at 80°C and in S_8^0 base salts medium (pH 4.0) plotted as a function of formate concentration ($K_m = 31.2 \pm 7.3$ µM, $V_{max} = 0.036 \pm 0.005$ µmol hr⁻¹, $\chi^2 = 2.71 \times 10^{-6}$, $R = 0.99$). The data presented reflects the average of three replicate cultures.

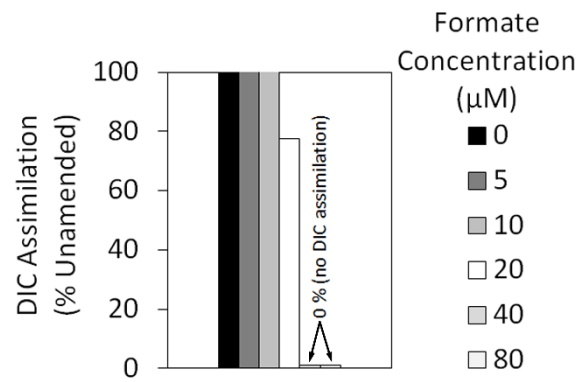


Figure 4. Suppression of DIC assimilation by *Thermoproteus* sp. CP80 at 80°C in S_8^0 base salts medium (pH 4.0) amended with 0, 5, 10, 20, 40 or 80 µM formate. The data presented reflects the average of three replicate cultures.

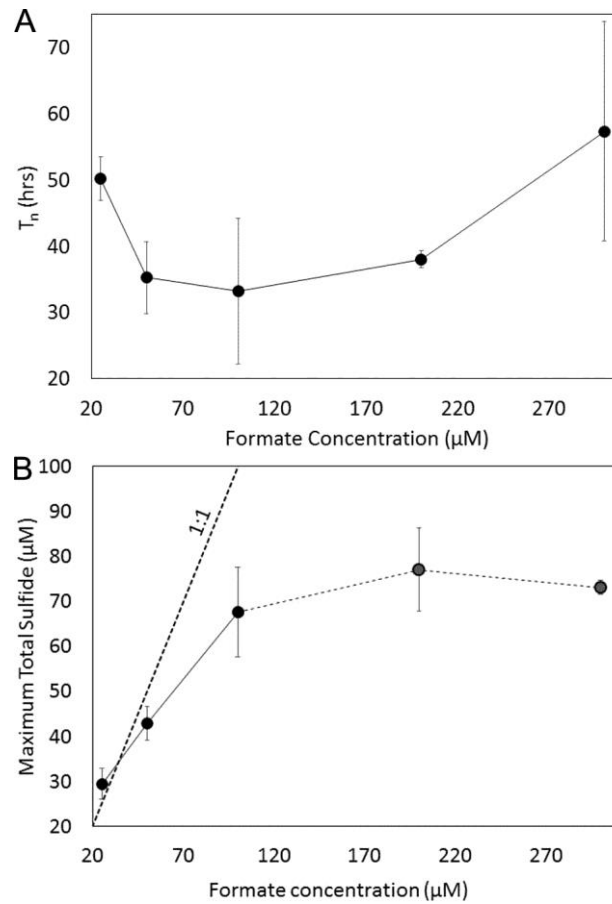


Figure 5. (A) Generation time (T_n) of *Thermoproteus* sp. CP80 grown at 80°C in S_8^0 base salts medium (pH 4.0) with formate as sole carbon source and S_8^0 as sole electron acceptor plotted as a function of formate concentration. The average and standard error of measurement (SEM) of three replicate cultures is presented. (B) The maximum total sulfide concentration achieved in cultures of *Thermoproteus* sp. CP80 grown at 80°C in S_8^0 base salts medium (pH 4.0) plotted as a function of the initial concentration of formate in culture medium. Values represented by gray dots were collected during late log growth since cultures were not monitored beyond this time point. A 1:1 line depicting the predicted stoichiometry of formate utilized per mol of sulfide produced is indicated. The average and standard error of measurement (SEM) of three replicate cultures is presented.

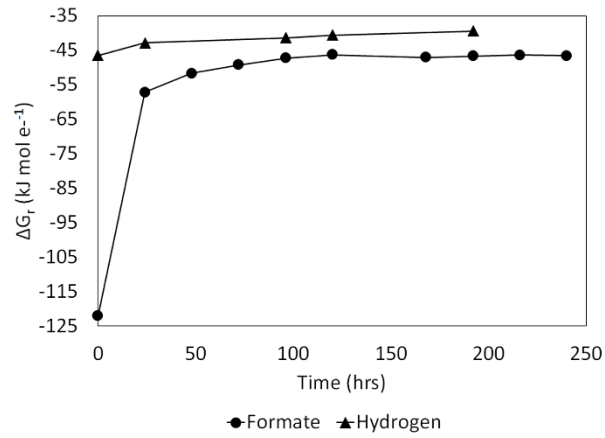


Figure 6. Gibbs energy of reaction (ΔG_r) per mol electrons available to *Thermoproteus* sp. CP80 catalyzing redox reactions involving formate (circles) or hydrogen (triangles) oxidation coupled to the the reduction of S_8^0 at each time point during growth in S_8^0 base salts medium (pH 4.0) when incubated at 80°C .

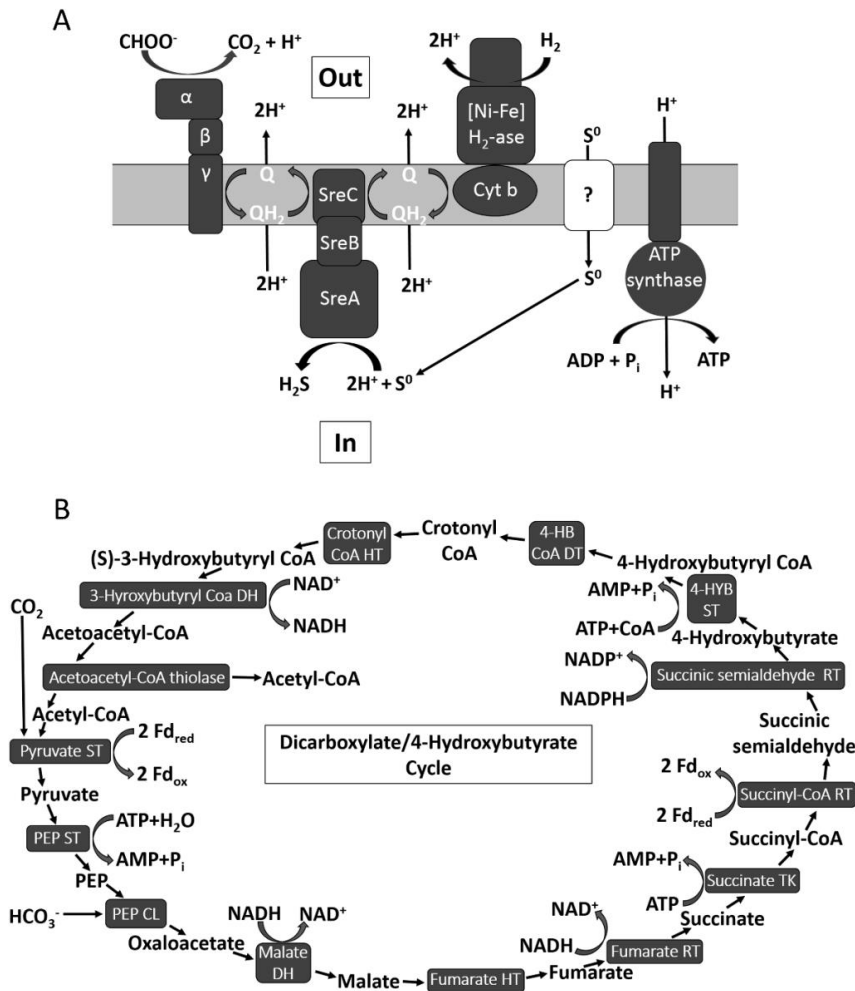


Figure 7. Proposed mechanism for energy conservation in the presence of various electron donors/carbon sources in cells of *Thermoproteus* sp. CP80. Inferences were based on the identification of gene homologs in the partial genome sequence of *Thermoproteus* sp. CP80 in combination with results from physiological characterization. Dark colored enzymes indicate those for which a gene encoding a homologous enzyme is present in the genome of *Thermoproteus* sp. CP80, while light colored enzymes indicate that a homologous enzyme with the proposed function has not been identified in the partial genome. FDH: formate dehydrogenase (O-type; NAD-dependent), ETC: electron transport chain, Sre: sulfur reductase, H₂-ase: group 1 nickel-iron [NiFe]-hydrogenase, Cyt b: [NiFe]-hydrogenase cytochrome b subunit, ?: unknown S₀ transport protein, Q/QH₂: oxidized/reduced quinone (quinone pool of ETC), DH: dehydrogenase, ST: synthase, CL: carboxylase, DT: dehydratase HT: hydratase, RT: reductase, TK: thiokinase, PEP: phosphoenolpyruvate, HYB: hydroxybutyrate, HB: hydroxybutyryl, CoA: Coenzyme A, Fd_{red}/Fd_{ox}: reduced/oxidized ferredoxin, NAD⁺/NADH: oxidized/reduced nicotinamide adenine dinucleotide, NADP⁺/NADPH: oxidized/reduced nicotinamide adenine dinucleotide phosphate, ATP: adenosine triphosphate, AMP: adenosine monophosphate, P_i: pyrophosphate.

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CHAPTER 4

GENERAL CONCLUSION

The presented dissertation was focused on the potential for organic and inorganic carbon and energy source utilization (autotrophy and heterotrophy) in chemosynthetic microbial communities residing in high temperature ($> 73^{\circ}\text{C}$) geothermal features in Yellowstone National Park, as well as on the identification of biogeochemical factors that may drive differential rates of utilization of organic and inorganic carbon and energy sources in these systems. Gaining a more complete understanding of these factors could yield important clues about the evolution of autotrophic and heterotrophic metabolisms before the advent of photosynthesis, as well as the selective pressures that may have influenced this evolution. In addition, gaining a deeper insight into the environmental influences that are important in shaping the substrate utilization patterns of extremophile chemosynthetic communities on Earth could contribute to the construction of a better conceptual framework with which to evaluate the potential for life that may exist under similar conditions elsewhere in the universe. To accomplish this goal, several different lines of evidence were presented resulting from the analysis of co-registered, multivariate field and laboratory data collected using a number of different geochemical, radioisotope probing, phylogenetic and genomic analysis, and laboratory culturing methods. While several previous studies have concluded that chemosynthetic communities in hydrothermal environments, such as YNP, are supported primarily by chemolithoautotrophic metabolisms, such as the utilization of H_2 and CO_2 as an energy

and carbon source, respectively, these conclusions were based entirely on the inference of physiology from the close affiliation of SSU rRNA gene sequences present in these communities to those of organisms from the predominantly-autotrophic bacterial order *Aquificales* (1, 2). To the authors' knowledge, the body of work contained in chapter 1 of this thesis represents the first direct evidence of the predominance of autotrophy in YNP chemosynthetic communities with a broad range of geochemical regimes. Moreover, our results demonstrate for the first time that many of these chemosynthetic communities are capable of utilizing organic compounds, such as formate and acetate, as energy and carbon source at rates that are comparable to, and may actually exceed, rates of autotrophy in the same communities.

Even more intriguingly, the demonstrated suppression of DIC assimilation in the presence of sufficient concentrations of formate in several YNP chemosynthetic communities with varying pH and temperature regimes indicates that microorganisms living in these chemosynthetic communities are likely facultative autotrophs, capable of undergoing metabolic 'switching' between autotrophy and heterotrophy when suitable organic substrates become available in the habitat. Although this metabolic switching behavior has been suggested by past studies (3), and has been demonstrated in the limited setting of two streamer biofilm communities (SBCs) in the YNP springs 'Bison Pool' and 'Octopus Spring' (4), the results of the suppression experiments in chapter 1 represent the first direct evidence that metabolic switching behavior is likely a widespread phenomenon in chemosynthetic communities residing in high temperature YNP geothermal features. This suggestion is also consistent with the demonstration of

facultative autotrophy and mixotrophy in a number of hyperthermophilic microorganisms (5-11), some of which have also been demonstrated to preferentially utilize organic over inorganic compounds (10).

The results of multivariate analyses of the relationship between measured geochemical parameters and observed metabolic activity performed in chapter 1 revealed an inverse relationship between the concentrations of formate and acetate in the environment and the measured rates of their utilization. This suggests that chemosynthetic populations are poised to readily take advantage of organic substrates as they appear in their habitat, thereby keeping their concentrations low. Furthermore, rates of inorganic and organic substrate utilization were generally inversely correlated with temperature, and positively correlated with pH, in good agreement with the results of previous studies demonstrating the necessity of increased maintenance energy expenditure in cells growing in low pH, high temperature habitats, compared to those growing in high pH, low temperature habitats (12). Rates of mineralization and/or assimilation of particular substrates were also positively correlated with the presence of taxa known to be capable of utilizing the respective substrate, indicating that community composition, in addition to metabolic switching, likely plays a role in determining differential rates of autotrophy and heterotrophy in chemosynthetic communities.

Interestingly, the positive correlation observed between pH and substrate utilization rate was much stronger for acetate transformation rates, as compared to formate transformation rates. One possible explanation for this phenomenon may come from the fact that the negative log of the acid dissociation constant (pKa, or the pH at

which exactly $\frac{1}{2}$ of an acid will exist in the protonated form) of formate (3.77) is significantly lower than that of the pKa for the first deprotonation of acetate (4.76), meaning that less formate will exist in the protonated form at a given pH. Protonated organic acids are uncharged, and can therefore freely diffuse across the cytoplasmic membrane into the cytoplasm, where their deprotonation upon exposure to higher pH conditions can lead to the dissipation of the proton motive force (13). Therefore, the lower pKa of formate, relative to acetate, should render it less toxic to chemosynthetic populations inhabiting low pH springs, possibly resulting in the preferential utilization of formate by these populations. Consistent with this suggestion, generation times (T_n) of the novel crenarchaeon *Thermoproteus* sp. CP80 (discussed in chapter 3) grown at pH 4.0 and 80 °C with sulfur as the terminal electron acceptor, and formate, pyruvate or acetate as sole carbon and energy source, were observed to be positively correlated with pKa (Fig. S1 in Appendix B), with the shortest generation times being observed in CP80 grown on pyruvate (pKa = 2.5), and no significant growth being observed in CP80 grown on acetate (pKa = 4.76). Moreover, the concentration at which optimum growth (as indicated by minimum T_n) occurred was inversely correlated with pKa, with 400 μ M and 200 μ M being the optimum growth concentrations for CP80 grown on pyruvate (pKa = 2.5) and formate (pKa = 3.77), respectively. Taken together, these results suggest that the interplay between pKa and pH may be an important factor influencing the relative rates of organic acid utilization by chemosynthetic populations.

The weak correlation between pH and formate utilization rates observed in YNP chemosynthetic communities may also be the result of the interplay between pH and the

thermodynamic characteristics of formate oxidation. Calculations of the theoretical total Gibbs energy (ΔG_r) of formate oxidation with O_2 , based on geochemical conditions in a number of YNP springs, revealed an inverse relationship between pH and ΔG_r , evidenced by the decrease of estimated ΔG_r of formate oxidation from $\sim -24 \text{ kcal mol e}^{-1}$ at pH 10, to $\sim -26 \text{ kcal mol e}^{-1}$ at pH 0 (14). Moreover, the ratio of formate concentration to acetate concentration also tended to be higher in lower pH springs, despite the well-documented increase in formate decomposition rates with decreasing pH. Although it is not possible to determine the biogeochemical drivers of higher formate concentrations in the lower pH springs based on the data collected in this study, this could be indicative of either increased rates of abiotic or biogenic formate production, or increased stability of formate (due to factors other than pH), relative to acetate, in lower pH springs. This suggestion is consistent both with explanations offered in previous studies noting the occurrence of higher formate concentration, compared to that of acetate, in hydrothermal fluids (15, 16), and with the increased rates of formate transformation observed in the low pH chemosynthetic communities investigated in this study. Taken together with the demonstrated preferential utilization of formate over less thermodynamically favorable DIC assimilation by YNP chemosynthetic communities in several acidic to moderately acidic YNP geothermal features, as well as the lower potential of formate to be cytotoxic at low pH, these results strongly suggest that preferential utilization of formate at low pH and high temperature may be responsible for higher rates of formate utilization, relative to acetate utilization, in low pH systems.

Chapter 2 presents the results of a series of physiological experiments aimed at characterizing the ability of a novel crenarchaeote (designated *Thermoproteus* sp. CP80), isolated from the high temperature chemosynthetic community hosted by the sulfur-rich geothermal feature Cinder Pool (CP, pH 4.0, 88.7 °C) (17), in the Norris Geyser Basin thermal area of Yellowstone National Park, to grow on formate and elemental sulfur (S_8^0) as sole carbon and energy source and sole electron acceptor, respectively. To the authors' knowledge, this organism is first to be isolated from CP. Even more intriguingly, *Thermoproteus* sp. CP80 is also the first crenarchaeon affiliated with order *Thermoproteales* that has been demonstrated to be capable of growth using formate as sole electron donor and carbon source, and S_8^0 as sole electron acceptor, despite the fact that the ability to reduce S_8^0 and the possession of genes coding for a putative homolog of the formate dehydrogenase (FDH) enzyme is common in crenarchaea of orders *Thermoproteales* and *Desulfurococcales* (18-21). Additional experiments were also carried out to investigate the ability of CP80 to grow using H_2 and CO_2 as sole energy and carbon source, respectively, and S_8^0 as sole electron source. The results of these experiments demonstrated that *Thermoproteus* sp. CP80 is a facultative autotroph capable of both autotrophic and heterotrophic metabolic strategies involving S_8^0 as an electron acceptor. These results are also consistent with the utilization of both formate and DIC exhibited by the chemosynthetic community in CP80's sulfur-rich native habitat (CP) (22).

Elemental sulfur (S^0) respiration is known to be a broadly-distributed metabolic strategy amongst hyperthermophilic crenarchaeaota inhabiting high temperature,

chemosynthetic communities in YNP and other hydrothermal systems (23-26), including facultatively autotrophic *Thermoproteus* sp. (27, 28). Elemental sulfur is thought to be formed in acidic environments by the disproportionation of thiosulfate ($S_2O_3^{2-}$) with hydrogen sulfide (H_2S) to form sulfite (SO_3^{2-}) and S_8^0 rings (29-32). Aggregation of nanocrystals formed from these S_8^0 rings eventually results in the precipitation of bulk elemental sulfur (33). S_8^0 can further react with H_2S to form linear chains of S^0 terminated by sulfhydryl groups (-SH) (34). These linear chains, called polysulfides (S_x^{2-}), are more stable under conditions of circumneutral pH, and have been suggested to serve as an important electron acceptor for sulfur-reducers in circumneutral pH environments (35). However, low concentrations of S_x^{2-} resulting from its instability at low pH (35, 36) suggest that this compound is not likely to be an important electron acceptor in more acidic geochemical regimes, such as that found in Cinder Pool (pH 4.0). Similarly, the low solubility in water of S_8^0 itself results in concentrations that are too low to account for the observed growth of sulfur reducing cultivars in the laboratory (35, 37), suggesting that physical contact with aggregate precipitated bulk S_8^0 may be necessary for its utilization as an electron acceptor. Intriguingly, however, recent reports demonstrating sulfur reduction at acidic pH by the hyperthermophilic crenarchaeon *Acidolobus sulfurireducens* in experimental microcosms in which S_8^0 was sequestered in dialysis tubing refute this suggestion (38). The authors concluded that utilization of S_8^0 as an electron acceptor proceeded instead via the reduction of suspended S_8^0 nanocrystals which were observed to accumulate in the medium as the result of the hydrolysis of S_x^{2-} . Interestingly, Boyd and Druschel (2013) (38) also observed an inverse correlation

between aggregation rates of nanoparticulate S_8^0 and pH, with final S_8^0 particle diameters observed at pH 2.0 being more than 2-fold larger than that observed at pH 4.0 after 2 hours. This suggests that nanoparticulate S_8^0 may be a more bioavailable electron acceptor in moderately-acidic systems, such as Cinder Pool, as compared to more acidic systems, due to a slower rate of precipitation of S_8^0 nanocrystals as bulk S_8^0 . Taken together, these results indicate that S_8^0 is likely to be of particular importance as an electron acceptor supporting transformation of organic and inorganic compounds by *Thermoproteus* sp. CP80 and other sulfur reducing crenarchaea residing in the Cinder Pool chemosynthetic community, and those of other moderately acidic, sulfur-rich features. Future work could further investigate whether coupling of the oxidation of organic substrates to S_8^0 reduction is more prevalent in moderately acidic features, as compared to highly acidic features.

The results of an experiment investigating the effect of amendment of progressively higher concentrations of formate on the CO_2 assimilation activity of *Thermoproteus* sp. CP80 grown on H_2/CO_2 demonstrated that this organism preferentially utilizes formate over CO_2 , and is capable of metabolic ‘switching’ between growth on formate and growth on H_2/CO_2 , with S_8^0 as sole electron acceptor. In support of this conclusion, the total Gibbs energy (ΔG_r) theoretically yielded by the coupling of formate oxidation to S_8^0 reduction in CP80 microcosms, estimated by computational geochemical modeling methods based on the physical and chemical conditions present in these cultures, was higher than that yielded by the coupling of H_2 oxidation to S_8^0 reduction under culture conditions that were identical to those in formate microcosms, with the

exception of the carbon and energy source provide to CP80. This result, along with lower cell yields per unit energy consumed exhibited by CP80 when grown on H₂/CO₂, as opposed to formate, strongly suggests that the preferential utilization of formate over CO₂ observed in CP80 is driven by the greater thermodynamic favorability of formate utilization, compared to H₂/CO₂ utilization. The estimates of theoretical energy yield from both formate and H₂ oxidation with S₈⁰ in CP80 microcosms were also consistent with previous estimates of ΔG_r for formate and H₂ oxidation calculated based on the physical and geochemical conditions present in several high temperature geothermal springs in YNP (14). This suggests that the prevailing conditions in our experimental microcosms with respect to formate and H₂ oxidation were similar to those present in some YNP geothermal features, and that the preferential utilization of formate over DIC observed in these chemosynthetic communities during the field experiments described in chapter 1, is likely also driven by the greater thermodynamic favorability of formate oxidation, as opposed to H₂ oxidation.

Intriguingly, the minimum formate concentration required to suppress CO₂ assimilation by CP80 in the laboratory was identical to that required to suppress DIC assimilation by the chemosynthetic community present in CP (20 μ M formate) (22). Consistent with this result, the Michaelis-Menten constant of formate conversion calculated from a plot of formate conversion rate by CP80 as a function of formate concentration ($K_m = 31.2 \pm 7.3 \mu\text{M}$), was also remarkably similar to that observed in the CP chemosynthetic community ($K_m = 36.9 \mu\text{M}$). Taken together, these results strongly

suggest that *Thermoproteus* sp. CP80 likely plays a role in the biogeochemical cycling of formate in its native habitat.

In support of the above conclusions from the results of physiological experimentation with *Thermoproteus* sp. CP80, the body of work described in chapter 2 also included the sequencing and analysis of a partial genome from this organism which revealed the presence of putative gene homologs for subunits of enzymes likely involved in energy conservation and C fixation from formate and H₂/CO₂, using S₈⁰ as an electron acceptor. These included putative homologs for subunits of formate dehydrogenase (FDH), a group I Ni-Fe uptake hydrogenase, a sulfur reductase (Sre), and a full complement of genes for enzymes involved in the dicarboxylate/4-hydroxybutyrate cycles of CO₂ fixation, which is widely distributed amongst creanarchaeota of order *Thermoproteales*, including organisms of genus *Thermoproteus* (39). Based on the presence of these putative gene homologs, combined with the observed results of the physiological experiments described above, we proposed a mechanism of energy conservation from the oxidation of formate involving the transfer of electrons from formate, to FDH, to a proton-translocating quinone pool, and finally to the putative Sre homolog that likely reduces S₈⁰ that has moved into the cytosol via simple diffusion or active transport (19) to H₂S, which then diffuses across the cytoplasmic membrane to the outside of the cell. Energy conserved by the formation of a proton gradient as the result of the proton-translocating activity of the quinone pool can then be utilized to generate ATP via a putative, membrane-bound, V-ATPase of the type which is thought to function in the synthesis of ATP in archaea (40-42). We propose a similar mechanism for energy

conservation and ATP production from the oxidation of H₂, with formate oxidation via FDH being replaced with H₂ oxidation by a membrane-bound [NiFe] hydrogenase similar to that which has been previously described in S₈⁰ reducing hyperthermophiles, such as *Aquifex aeolicus* (43, 44). Carbon fixation from CO₂ diffused into the cytosol from the surrounding bulk fluid, or resulting from formate oxidation, is likely assimilated via the dicarboxylate/4-hydroxybutyrate cycle, which generates a single molecule of acetyl-CoA from the fixation of one molecule of CO₂ and one molecule of HCO₃⁻, while consuming 5 molecules of ATP (45). However, sequencing of the complete genome of *Thermoproteus* sp. CP80, combined with investigation of the differential expression of the putative genes involved under conditions of heterotrophic growth on formate, and autotrophic growth on H₂ and CO₂, is required to conclusively demonstrate the existence and proposed function of these mechanisms.

The putative FDH genes identified in the CP80 genome appear to code for a homolog of the aerobic type FDH (FDH-O), first identified and characterized in *Eschericia coli* (46-49). Homologous genes putatively coding for FDH-O homologs have also been identified in other archaea (50), including *Thermoproteus uzoniensis* (18). Although the 3D structure of FDH-O is not currently known, it is thought to be similar to that of the FDH-N enzyme (51), which is expressed under anaerobic conditions in the presence of nitrate (NO₃⁻), and functions in the anaerobic coupling of formate oxidation to NO₃⁻ reduction. In *E. coli*, FDH-O is a heterotrimeric, membrane-bound, periplasm-facing enzyme consisting of a periplasmic formate oxidizing alpha (α) subunit, containing a 4-iron, 4-sulfur cluster [4Fe-4S] and a molybdopterin (Mo) cofactor in which the active

site is located, a periplasmic beta (β) electron-transfer subunit, containing 4 [4Fe-4S] clusters, and a membrane-bound, menaquinone-reducing gamma (γ) subunit, containing two b-type bis-histidinyll-coordinated haems (51-53). These subunits are co-located on the *fdoGHI* operon in *E. coli*, which also codes for a membrane integration protein (*fdoF*) and a maturation protease (*fdoE*) that have been demonstrated to be essential for the formation of the FDH-O enzyme (51). A putative *fdoGHI* operon is also coded for in the genome of *Thermoproteus uzoniensis* (18), and alignment of the putative homologs of the *fdoGHI*, *fdoF* and *fdoE* genes identified in the partial genome of CP80 with those in the *T. uzoniensis* genome reveal that the gene order of the two operons are identical. In *E. coli*, expression of *fdoGHI* operon genes was observed to increase 3-fold under aerobic conditions, and 2-fold under anaerobic conditions in the presence of NO_3^- (51). In the latter case, FDH-O is co-expressed with the dissimilatory nitrate reductase enzyme, NarZ/WV, allowing it to function in NO_3^- reduction via formate oxidation during the transition from aerobic to anaerobic conditions, under which expression of the NO_3^- -reducing FDH-N is induced (51, 54). Interestingly, however, the regulation of FDH-O gene expression in *E. coli* is not under the control of the fumarate and nitrate reductase transcriptional regulator FNR, nor the two-component regulatory system ArcB/ArcA, which control anaerobic and aerobic gene expression, respectively, in *E. coli* (47, 55). Instead, FDH-O expression appears to be regulated by the action of the nucleoid-associated, DNA binding, transcriptional repression protein H-NS (56), and the transcriptional regulator CRP, which functions in catabolite repression (51, 57). This relationship was evidenced by a significant reduction of *fdoGHI* operon expression in *E.*

coli upon mutation of the genes coding for H-NS and CRP, along with the presence of a CRP binding site in the 5' regulatory region of the *E. coli* *fdoGHI* operon (51).

The ability of CP80 to alter its metabolism from DIC assimilation to formate utilization under anaerobic conditions is consistent with the constitutive expression of the FDH-O enzyme in *E. coli* under both aerobic and anaerobic conditions. Such constitutive expression would make such a metabolic switch possible without the necessity of further enzyme induction. This strategy would allow the organism to take advantage of temporally-variable formate concentrations in its native habitat, thereby maximizing energy conservation during periods of elevated formate concentration. Interestingly, however, the S_8^0 base-salts medium used during CP80 physiological experiments contains no added NO_3^- , indicating that the presence of NO_3^- is not a prerequisite for anaerobic expression of this putative crenarchaeal FDH-O homolog, in contrast to what has been observed in *E. coli*. This also suggests the tantalizing possibility that, under anaerobic conditions with S_8^0 as the sole terminal electron acceptor, the crenarchaeal FDH-O homolog may be co-expressed with the putative sulfur reductase (Sre) enzyme also coded for in the partial CP80 genome, similar to the co-expression of FDH-O and the NarZ/WV nitrate reductase observed in *E. coli* in the presence of NO_3^- . Such co-expression has been observed in other hyperthermophilic crenarchaeota, such as *Pyrodictium abyssi* (58) and *Acidianus ambivalens* (44, 59), both of which have been demonstrated to co-express an Sre homolog with a Ni-Fe uptake hydrogenase when coupling H_2 oxidation to S_8^0 reduction. Also intriguing is the apparent absence from the CP80 genome of genes coding for either the transcriptional repression protein H-NS, or

the transcriptional regulator CRP, which are key players in the transcriptional regulation of FDH-O expression in *E. coli*. While it is possible that the absence of gene homologs for these regulatory proteins may be due to the incomplete nature of the genome obtained from CP80, this seems unlikely, given that neither of these proteins are coded for in the genome of *Thermoproteus uzoniensis* (18), which is the *Thermoproteus* cultivar most closely affiliated with CP80. This suggests the intriguing possibility that FDH-O expression in CP80, and perhaps other crenarchaeota, is controlled by a novel regulatory mechanism, which may involve proteins that are functionally-homologous to H-NS and CRP. One such protein may be the acetylation-modulated nucleoid-associated protein (NAP) known as 'Alba' (60). Similar to H-NS (61), Alba is thought to function in gene regulation via the suppression (silencing) of transcription (62, 63). Given the presence of a putative Alba homolog in the partial genome of CP80 (Urschel, unpublished data), together with the fact that Alba is the most widely distributed NAP so far identified in crenarchaea (including *Thermoproteus uzoniensis* and *Thermoproteus tenax*), it seems likely that Alba plays a central role in the regulation of gene expression via the suppression of transcription, suggesting it as a possible functional homolog for H-NS in the regulation of FDH-O expression. Further characterization of the CP80 genome, combined with investigation of expression under anaerobic conditions in which the putative crenarchaeal FDH-O gene is also expressed, may demonstrate a role for Alba or other currently-unknown genes involved in the regulation of FDH-O expression in *Thermoproteus* sp. CP80 and other sulfur reducing, hyperthermophilic crenarchaeota.

Overall, the body of work contained in this thesis represents the first direct evidence for the predominance of autotrophic metabolism in supporting chemosynthetic microbial communities living in hydrothermal environments at temperatures above $\sim 73^{\circ}\text{C}$ in YNP, and suggests that a similar pattern is likely to be observed in other high temperature hydrothermal environments. Moreover, this work also demonstrates for the first time that many chemosynthetic communities are facultative, and capable of utilizing organic substrates at rates that are comparable to, and in some cases exceed, that of DIC assimilation. Moreover, the observed preferential utilization of formate over DIC by several chemosynthetic communities indicates that these populations are capable of metabolic ‘switching’ between autotrophy and heterotrophy in order to take advantage of more energetically favorable organic substrates as they become available in their habitat. This conclusion is supported by an inverse correlation between the concentrations of formate and acetate and rates of uptake and/or assimilation of these substrates, which likely indicates that chemosynthetic populations in YNP are metabolically poised to immediately utilize these organic substrates as they appear, thus keeping the concentration of these compounds at a minimal level. In addition, the isolation of a facultatively autotrophic crenarchaeote capable of coupling the oxidation of formate to the reduction of S_8^0 indicates that this and other crenarchaeota from order *Thermoproteales* known to be the numerically dominant archaea in many high temperature, sulfur-rich chemosynthetic communities in YNP are likely involved in the biogeochemical cycling of formate, and possibly other organic substrates, in their native habitats. In sum, these results indicate a more prominent role for heterotrophy in

supporting chemosynthetic microbial communities in YNP and other hydrothermal systems.

Future Directions

To gain a complete understanding of the biogeochemical factors driving relative rates of inorganic and organic substrate utilization by chemosynthetic communities, future work should focus both on the determination of the relative contribution of abiotic and biogenic generation of organic carbon to the support of chemosynthetic communities in hydrothermal environments, and on the measurement of the flux of organic and inorganic substrates through these communities. This objective could be accomplished via a more comprehensive application of the isotopic methods employed by Schubotz (2013), involving determination of the ratio of ^{13}C to ^{12}C ($\delta^{13}\text{C}$) of intact polar lipids (IPLs) in microbial biomass of chemosynthetic communities. Lipids from chemosynthetic communities supported primarily by biogenically-generated carbon sources would be expected to be “isotopically light” with respect to incorporated C, compared to those supported by abiotically-generated carbon sources, due to the well-documented preferential utilization of ^{12}C over ^{13}C by living organisms (64). Similar isotopic methods have been used to evaluate relative contributions of biogenic and abiogenic C in other hydrothermal environments (65, 66). The application of this method to samples taken from a wide range of geochemically-diverse geothermal features, combined with parallel SSU rRNA gene sequencing and measurement of geochemical parameters in each feature, would allow investigation of the potential biogeochemical drivers influencing the relative contribution of biogenic and abiogenic carbon to the support of chemosynthetic

communities. The flux of biogenic and abiogenic C through chemosynthetic communities could be determined by application of the same isotopic method to the measurement of the ratio of biogenic to abiogenic carbon in the dissolved organic and inorganic carbon pool (DOC/DIC) of the same geothermal features sampled repeatedly over a period of time.

The lower cell yield per unit energy conserved in *Thermoproteus* sp. CP80 when grown on H_2/CO_2 and S_8^0 , as compared to growth on formate and S_8^0 , suggests that CP80 expends a larger percentage of conserved energy on cell maintenance (m_E) when grown autotrophically. Maintenance energy can be calculated using the following equation (67, 68):

$$m_E = m_D \gamma_D \times \Delta G_{av}^{01} \quad (1)$$

where m_E is the maintenance energy, m_D is the maintenance coefficient, γ_D is the degree of reduction (number of equivalents of available electrons per gram atom C) for growth on the substrate in question, and ΔG_{av}^{01} is the available standard

Gibbs free energy (calculated from ΔG^0 of the reaction divided by the number of C atoms and the degree of reduction). Determination of m_D is accomplished via conversion of the maintenance coefficient in millimoles of substrate per hour per gram [dw] (m_S) to moles of substrate C per mole of biomass C per hour, where m_S is calculated via the following equation:

$$1/Y = 1/Y^{max} + m_S \times \mu \quad (2)$$

where Y is the apparent growth yield at different dilution rates ($\mu = D$) as determined by chemostat growth on the substrate in question, Y^{max} is the growth yield at infinite dilution

rate (determined via regression analysis of Y at different dilution rates), and μ is the specific growth rate (dilution rate $[D]$ in chemostat growth). Alternatively, maintenance energy can also be directly measured via Isothermal microcalorimetry (IMC), which measures the waste heat (i.e. energy that does not contribute to growth) produced by an organism growing on a particular substrate (69). This method typically involves measuring heat produced by a microbial culture grown in a closed ampule using a channel microcalorimeter, which measures heat produced by the organism in culture relative to a reference material (e.g. water) with a heat capacity similar to that of the specimen. Future experiments could involve combining the methods described above to determine and compare the maintenance energy expended by CP80 during growth on formate or H_2/CO_2 with S_8^0 as terminal electron acceptor. The application of these methods could be combined with gene expression experiments comparing the gene expression profiles of CP80 grown on formate and H_2/CO_2 with the aim of identifying potential differences in the electron transport pathways utilized for energy conservation during growth on these substrates.

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APPENDICES

APPENDIX A

METABOLIC AND TAXONOMIC DIVERSIFICATION IN CONTINENTAL
MAGMATIC HYDROTHERMAL SYSTEMS

Contribution Of Authors And Co-Authors

Manuscript in Appendix A

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Contributions: Co-first author. Figure design, manuscript writing and editing.

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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 frameworks through approaches commonly referred to as phylogenetic ecology, 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. 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. 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 composition¹. 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 function⁷⁻¹³.

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**)¹⁴ and many of the physiological functions that sustained it [e.g., hydrogen oxidation¹⁵] 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 ¹⁶. 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) ¹⁷⁻¹⁹. However, more recent studies based on phosphate isotopic data suggest that Archaean oceans were clement (<40°C) ²⁰. 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*. 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 ²³, 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). 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.

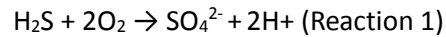
Hydrothermal systems form as the result of interaction of magmatic fluid with a heat source. Three key components are required for the formation of a hydrothermal system: a source of water, a source of heat, and permeability of the rock strata overlying the heat source²⁶. Although water sources, heat 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 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 ²⁹, 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 ³⁰⁻³³. 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 ³⁴⁻³⁷. 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 ($\text{pK}_a \sim 6.4$ at 20°C and 6.6 at 100°C)³⁸. 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 springs³⁹ as well as in a focused study of Yellowstone National Park (YNP), Wyoming⁴⁰. 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 rocks⁴¹. 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 (PO_2^{3-}) and/or phosphite (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 ⁴².

3. TAXONOMIC AND FUNCTIONAL DIVERSITY IN CONTINENTAL 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 ⁴²⁻⁴⁴. 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, all of which have been shown to be metabolized by hyperthermophiles ⁴⁸⁻⁵⁵. Moreover, a range of typically less stable intermediate sulfur species including thiosulfate, polysulfides, and polythionites have been detected in hot spring environments and have been shown to be metabolized by biology ⁵⁶⁻⁵⁸. As a result, sulfurous hot springs across the globe tend to harbor similar lineages of microorganisms, underscoring the importance of sulfur in the metabolism and diversification of thermophilic microorganisms.

Hydrogen is also likely to play a key role in defining the distribution of thermophilic organisms in hot spring environments. Numerous H₂ oxidizing organisms, typically associated with the bacterial order *Aquificales* and members of the euryarchaeota and crenarchaeota ⁶³⁻⁶⁶, have been isolated from hot springs. In addition to H₂ oxidation, fermentative H₂ production

appears to be an important process sustaining organisms inhabiting continental, magmatic hydrothermal environments ⁷.

The discovery of ammonia oxidation by thermophilic Archaea ⁶⁷ 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 ⁶⁸⁻⁷⁰. 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 N₂ fixing populations ⁷¹. Biological N₂ 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 and their transcripts. Incorporation of ¹⁵N₂ into biomass in microcosm assays containing hot spring sediments indicate N₂ fixation in numerous continental hot spring environments with temperatures of up to 89°C ⁷², which is close to the upper temperature limit for this process of 92°C which was demonstrated in a marine ecosystem ⁷⁵, provide additional evidence indicative of the role of N₂ fixation in relieving fixed N limitation. Whereas a thermophilic bacterium distantly related to *Leptospirillum* spp. was responsible for the N₂ fixation activity observed in the 89C continental hot spring ⁷², a thermophilic methanogen was responsible for this activity observed in the marine vent ecosystem ⁷⁵. Surprisingly, despite the extensive molecular-based studies of N₂ fixation potential and gene expression in continental hot springs, only evidence for bacterial N₂ 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 $< 55^{\circ}\text{C}$ ⁶². The aquificae genera *Hydrogenobacter*, *Hydrogenobaculum*, *Thermocrinis*, and *Sulfurihydrogenibium* spp. are typically observed in continental hot springs and solfataric fields. 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 springs⁶². Unlike the above strains, members of the genera *Aquifex* and *Persephonella* are routinely isolated from marine hydrothermal systems only. 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 communities⁶². *Aquificales* are chemolithoautotrophic thermophilic *Bacteria* that couple CO_2 fixation with the oxidation of reduced compounds such as hydrogen, sulfide, or elemental sulfur. Owing to their chemolithoautotrophic mode of metabolism, they are often the dominant primary producers of biomass within high temperature ecosystems. Together with members of the order *Thermotogales*, these microorganisms represent the *Bacteria* with the highest growth temperatures currently known⁵⁸.

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 hydrogen⁸⁴⁻⁸⁶.

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. Among the *Euryarchaeota*, acidophily is restricted to members of the order *Thermoplasmatales*⁶⁶. 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., *δ-Proteobacteria*, *Thermodesulfobacteria*, *Nitrospira*, and *Firmicutes*] but are narrowly distributed among *Archaea*. The only archaeal sulfate-reducers described so far belong to the genera *Archaeoglobus* and *Caldivirga*. 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 photosynthesis⁹¹. 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 origin⁹². 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 .

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 and *Acidilobus sulfurireducens* isolated from an acidic hot spring in YNP⁵⁴. Molecular analyses indicate that *A. sulfurireducens* is a numerically dominant organism in acidic, high-temperature YNP springs , 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 . Other organotrophic thermoacidophilic crenarchaeota include *Caldisphaera lagunensis* and *Caldisphaera draconis* isolated from acidic hot springs in the Philippines and YNP, respectively . Additional organotrophic and thermoacidophilic *Archaea* typical of acidic hot springs include *Thermocodium modestius*⁹⁶, *Caldivirga maquilingensis*⁸⁹, *Vulcanisaeta distributa*⁹⁷, and *Vulcanisaeta souniana*⁹⁷. 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 thermoacidophilic crenarchaeota.

Members of the order *Sulfolobales* represent another group of thermoacidophilic *Archaea* that are commonly detected in continental acidic hot springs . 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, 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. 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 situ⁹⁹. 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 others⁹⁹⁻¹⁰³. *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 springs¹⁰⁴⁻¹⁰⁶. *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. In contrast, *Sulfurisphaera ohwakuensis*, the only species from the *Sulfurisphaera* genus, grows mixotrophically and was isolated from an acidic hot spring in Japan¹⁰⁹. 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 availability¹¹⁰. Some representative species of this genus include *A. ambivalens*, *A. manzaensis*, *A. infernus* and *A. brierleyi*, among others¹¹¹⁻¹¹⁴. *Acidianus* species have been isolated primarily from terrestrial hot springs sites, but also occur in shallow

submarine hydrothermal systems ¹¹⁴. 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 Fe³⁺ to the oxidation of H₂ or elemental sulfur under anoxic conditions ¹¹³.

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 ¹¹⁵. 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 O₂. The first cultivated member of this genus, *Candidatus Nitrosocaldus yellowstonii*, is a chemoautotroph ⁶⁷ and is widely distributed in circumneutral to alkaline hot spring environments .

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, 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 diversity⁷⁻¹¹. As an example, Alsop et al., 2014¹¹ 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. 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., 2014¹¹ 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., 2003⁵⁹ found evidence, at the genomic level, for barriers to the dispersal of *Sulfolobus* spp. Similar studies targeting thermophilic cyanobacteria¹¹⁹ and thermoophilic aquificae¹²⁰ 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 aforementioned 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 lineages⁶⁸. 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 systems¹²¹.

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. While the upper temperature limit for photosynthesis of 73°C was confirmed in circumneutral to alkaline systems¹²², 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 $>5 \mu\text{M}$ 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_2 assimilation in algae [which predominate among phototrophs in hot springs with $\text{pH} < 4.0$ ¹²³], but not in cyanobacterial dominated systems ($\text{pH} > 4.0$) ³. 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_2) to bioavailable ammonia (i.e., nitrogenase; nitrogenase iron protein encoded by *nifH* ⁸), the reversible oxidation of H_2 (i.e., [FeFe]-hydrogenase; large subunit encoded by *hydA* ⁷), and the detoxification of mercury (i.e., mercuric reductase encoded by *merA* ¹⁰) 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°C) and the subsequent volatilization of $\text{NH}_{3(\text{g})}$ out of the system. 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 ⁸, 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 H₂ in fermentative *Bacteria*, was found to be pH dependent in hot spring ecosystems ⁷. 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 H₂ partial pressure ¹²⁵. Elevated concentrations of H₂ are routinely measured in springs in YNP where temperatures exceed 65°C. H₂ in these systems is likely of abiotic origin, derived from subsurface iron-catalyzed water hydrolysis (radiolytic and/or serpentinization) ¹²⁷. Iron-catalyzed H₂ production is sensitive to both solution pH and reaction temperature, with a near doubling of H₂ production rates resulting from the doubling of incubation temperature ¹²⁸. Similarly, the rate of basalt-catalyzed H₂ production increases with increasing water acidity (lower pH) ¹²⁸. Coincidentally, *hydA* was not detected in YNP springs predicted to have elevated geological H₂ production (e.g., acidic pH, >36°C; alkaline pH, >65°C). Thus, environments with elevated inputs of geological H₂ 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 properties¹²⁹⁻¹³¹ that have facilitated their diversification into environments with wide ranging geochemical conditions, including hydrothermal environments with extremes of temperature and pH. 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)¹³⁴. The predominant membrane lipids of thermophilic *Archaea* are isoprenoid glycerol dialkyl glycerol tetraethers (iGDGTs)¹³⁵⁻¹³⁸, which consist of two ether-linked C₄₀ 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 and decrease their permeability to ions¹⁴¹. Both pure culture and environmental surveys 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*⁶⁷ 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). H-GDGTs, which are structurally similar to iGDGTs but contain a C–C bond between the two C₄₀ biphytanyl chains, have been detected in both thermophilic *Crenarchaeota*¹⁴³ and *Euryarchaeota*¹⁴⁴⁻¹⁴⁷ inhabiting high temperature continental hot springs and marine hydrothermal vents¹⁴⁸⁻¹⁵¹. 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 springs¹⁵¹.

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. 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)¹⁵⁴. *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 ¹⁵⁵⁻¹⁵⁸. Interestingly, some bacterial thermophiles have been demonstrated to increase the amount of lipid produced during acclimatization to thermal stress ¹⁵⁹.

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 ¹⁶⁰. 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 ¹⁶¹. Several recent studies have detected bGDGTs in biomass collected from hot springs spanning a wide temperature gradient ¹⁶²⁻¹⁶⁶. 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* ¹⁶⁴.

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 ¹⁶⁷⁻¹⁷⁰. 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*. 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 enzymes¹⁷¹.

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*¹⁷³. 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 stability¹⁷⁴.

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 ¹⁷⁵. 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.

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 ¹⁷⁷. 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* and the archaeon *Pyrococcus furiosus* while, *Pyrodictium occultum* accumulates di-*myo*-inositol-1,1'(3,3')-phosphate ¹⁷⁷. 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 ¹⁷⁷. Additionally, both *P. furiosus* ¹⁸⁰ and *Methanococcus igneus* ¹⁸¹ produce higher concentrations of the unusual phosphorous-containing solute di-myo-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 ¹⁸². 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 ¹⁸³, 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 ¹⁸².

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 ¹⁸⁴. 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* ¹⁸⁵. 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* ¹⁸⁶. Diglycerol phosphate, the predominant organic osmolyte

synthesized by *A. fulgidus*¹⁷⁷, 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 strains¹⁸⁷. 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, respectively¹⁸⁸. These salts have been shown to play an important role in the thermal stability of cyclohydrolase and formyltransferase from *M. kandleri*¹⁸⁹, which is the most thermal tolerant methanogen currently known¹⁹⁰.

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 stress¹⁹¹. 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 stress¹⁹³, 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 . 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 ¹⁹⁵]. 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 ¹⁹⁶. 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 ¹⁹¹.

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 ¹⁹⁷⁻¹⁹⁹. 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 ¹⁹⁷. 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 ¹⁹⁹. 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 ²⁸. 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 ⁸³.

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*. 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 whereby the phosphorylation of a histidine autokinase that senses environmental parameters signals the subsequent phosphorylation of a response regulator ²⁰². 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 carbohydrates²⁰³. 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*²⁰⁴⁻²⁰⁶ 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 gradient²⁰⁷. Thermotaxis, or the sensing and motile response to thermal gradients, has been demonstrated in nematodes²⁰⁸ and *Escherichia coli*²⁰⁹ 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 biomolecules²⁰⁷. 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 temperatures²¹⁰ and distributions along a hot spring thermal gradient²¹¹. Likewise, the maximum rate of acetylene reduction activity (proxy for N₂ fixation) observed in an enrichment of a thermoacidiphilic diazotroph was shown to correspond to the temperature of the environment where it was isolated⁷². 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 divergence²¹². 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 transport²¹³. At the proteomic level, the comparison of the three *Pyrococcus* species revealed substantial differences in their proteolytic enzymes²¹², 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 niches²⁰⁷. 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 strains²¹³. 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. abyssi* can 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* ²¹⁴. The CRISPR system constitutes a microbial immune system that functions to target and neutralize foreign DNA in a manner similar to eukaryotic RNA interference. 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 ²¹⁴.

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. ²¹⁷⁻²²⁰. 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 ²²¹.

A recent screening of two representative *Aquificales* genomes [*Thermocrinis albus* ²²² and *Hydrogenobacter thermophilus* ²²³] revealed the presence of nitrogenase gene clusters ²²⁴. 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* ²²⁵, despite branching more basal than *Thermocrinis* and *Hydrogenobacter* in taxonomic trees ⁵⁸. *Hydrogenobaculum* spp. tend to populate acidic geothermal environments where NH_4^+ produced from magmatic degassing is in much higher supply ¹²⁴ whereas *Thermocrinis* and *Hydrogenobacter* tend to populate circumneutral to alkaline environments that are N limited ⁶². 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_2 fixation ²²⁶⁻²²⁸. 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 ²²⁹.

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 ²³⁰. 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. 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.

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. ²³⁴. Interesting exceptions include aminoacyl-tRNA synthetases, which are key components of the protein translation machinery whose evolution involves horizontal gene transfer ²³⁵⁻²³⁷. . 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 complex²³⁸) 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 .

Another unexpected case of a likely horizontal gene transfer between eukaryotes and *Archaea* is the Trp-tRNA synthetase from the hyperthermophilic archaeal genus *Pyrococcus*²³⁶. 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 . 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 version²³⁶.

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* species²⁰⁴.

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 ²⁴². While *nif* emerged in hydrogenotrophic methanogens and was likely acquired vertically in other members of the *Methanobacteriales*, *Methanomicrobiales*, and *Methanococcales* ^{142 242}, 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 ²⁴³⁻²⁴⁶. Phylogenetic evidence indicates that *nifEN* are clearly the result of a tandem duplication of *nifDK*. NifB, another protein required for the synthesis of the active site cofactor of Mo-nitrogenase, 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 ²⁵¹. However, in early evolving thermophilic methanogens, NifB is not fused ²⁴², 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. 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 ²⁵² as well as processes that were key to the emergence of higher forms of life such as nitrogen fixation, 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 members⁶⁸. 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. 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°C) has been suggested to be the result of the underlying geological processes that create extensive geochemical variation in these systems. 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. 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 drainage²⁵⁴. 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 Legends

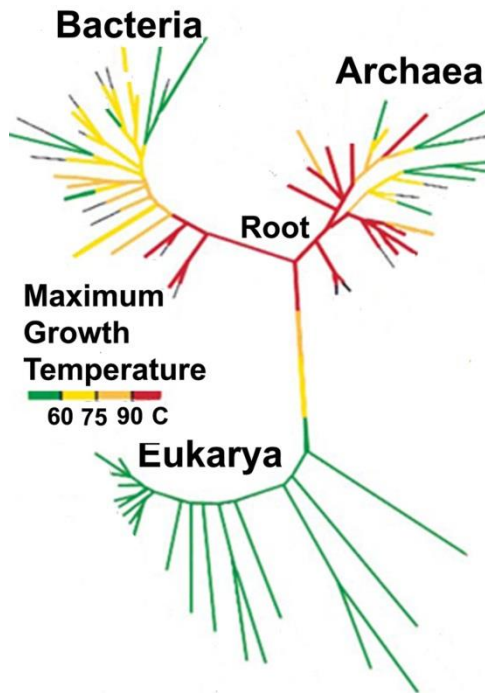


Figure 1. (A) Universal tree of life with an overlay of maximum growth temperature implies a thermophilic origin of life [Figure adapted from Lineweaver and Schwartzman, 2004].

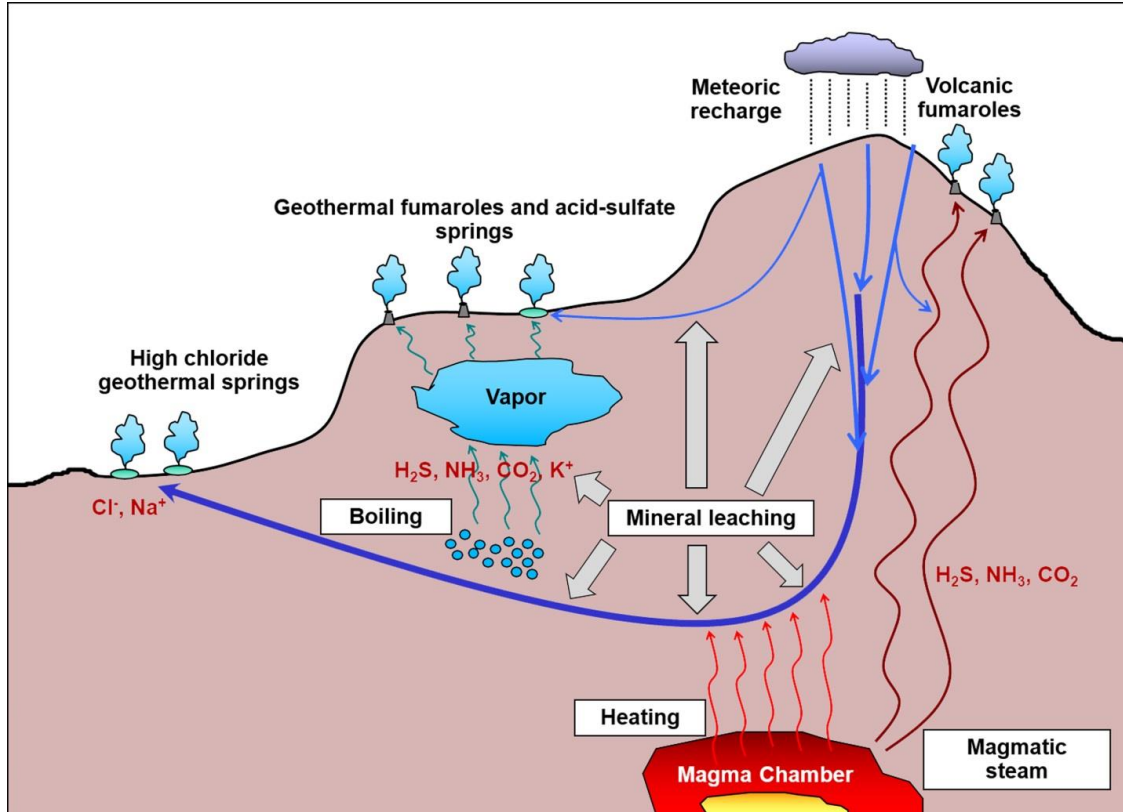


Figure 2. Schematic illustrating the hypothesized functioning of a continental magmatic hot spring system. Here, meteoric water infiltrates to the subsurface where it is heated by a magma chamber. Heated water returns to the surface due to density differences. During the flow path back to the surface, 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.

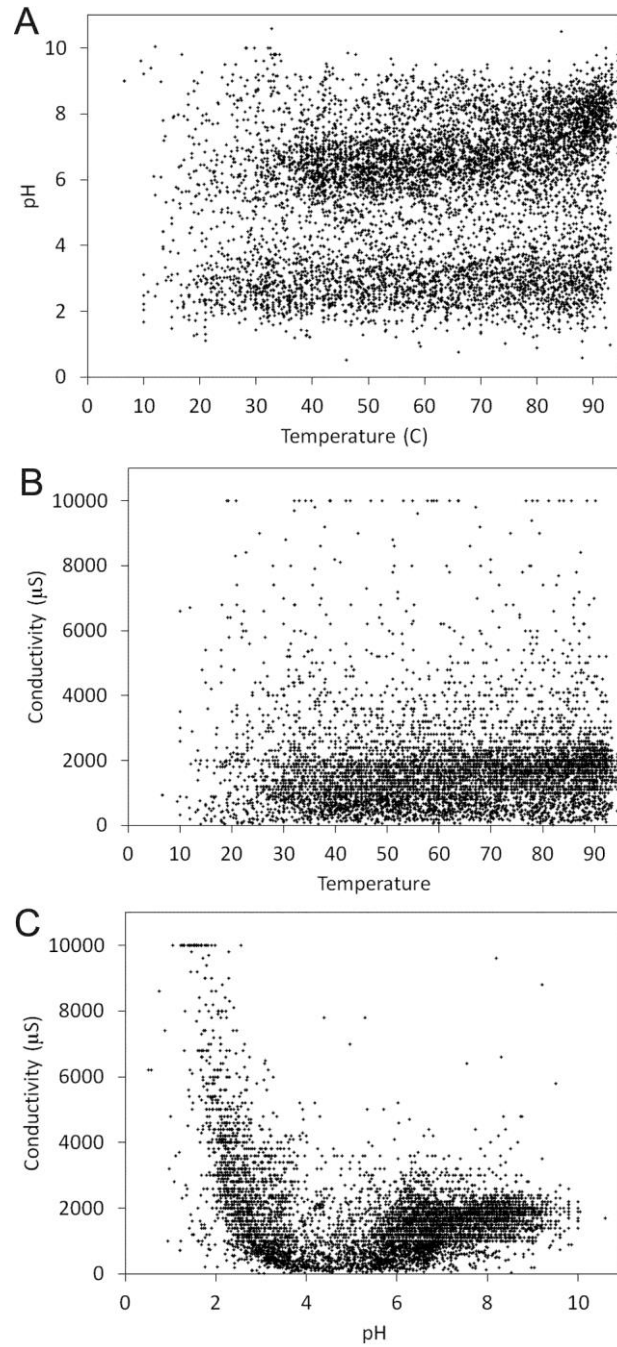


Figure 3. pH, temperature, and conductivity measurements plotted as a function of each other for ~7700 hot springs in Yellowstone National Park. Data was compiled from the Yellowstone Research Coordination Network website (<http://www.rcn.montana.edu/>).



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.

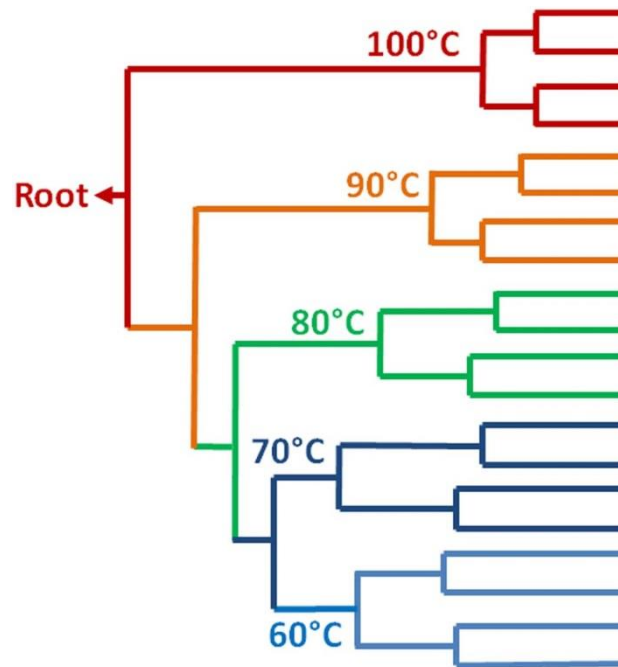


Figure 5. Schematic illustrating phylogenetic approaches to ecological studies. In this case, sequences were collected across a temperature gradient ranging from 60 to 100C and were subjected to phylogenetic reconstruction. Using community ecology tools it is possible to map geochemical characteristics 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

COPY OF PERMISSION TO REPRINT

From: Julia.Reindlmeier@degruyter.com [mailto:Julia.Reindlmeier@degruyter.com]
Sent: Thursday, February 12, 2015 3:02 AM
To: matthewur@gmail.com; eboyd@montana.edu
Cc: Margarete.Mischorr@degruyter.com
Subject: WG: Your contribution to "Microbial Evolution under Extreme Conditions" by Corien Bakermans

Dear Dr. Urschel,

We herewith confirm that the chapter in question ("**Metabolic and taxonomic diversification in continental magmatic hydrothermal systems**" to be published 2015 in: **Bakermans (Ed.)/ Microbial Evolution under Extreme Conditions**) may be included as a chapter in your doctoral thesis. Please make sure that the complete bibliographic details of the original publication are given at an appropriate location in the dissertation.

With kind regards

Julia Reindlmeier

Project Editor STM

APPENDIX C

SUPPORTING INFORMATION FOR CHAPTER 4

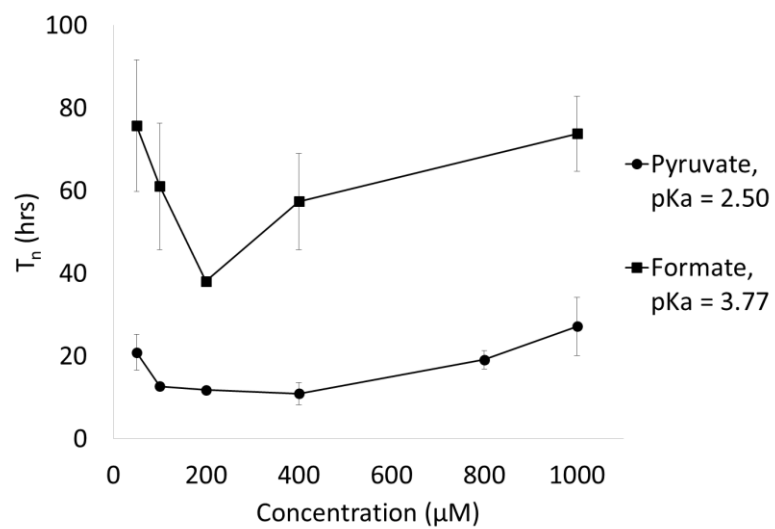


Figure S1. Generation times of *Thermoproteus* sp. CP80 during growth at 80 °C and pH 4.0, with either pyruvate or formate as sole electron donor and S_8^0 as sole electron acceptor.

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