Carbon source preference in chemosynthetic hot spring communities

Matthew R. Urschel¹ ², Michael D. Kubo³, Tori M. Hoehler³, John W. Peters² ⁴, and Eric S. Boyd¹ ²⁺

¹Department of Microbiology and Immunology, Montana State University, Bozeman, MT 59717
²Thermal Biology Institute, Montana State University, Bozeman, MT 59717
³NASA Ames Research Center, Mountain View, CA 94035
⁴Department of Chemistry and Biochemistry, Montana State University, Bozeman, MT 59717

*Corresponding Author: Eric S. Boyd
eboyd@montana.edu

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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 (Km) 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 (~73°C) is supported by chemical energy (1-5). In the case of high temperature (>73°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 (6-11). Cultivated representatives from the order Aquificales assimilate dissolved inorganic carbon (DIC) using energy derived from the oxidation of hydrogen (H₂), sulfide (S²⁻), thiosulfate (S₂O₃²⁻), or elemental sulfur (S₈) under aerobic conditions (7) or with H₂, S₈, or S₂O₃²⁻ under anaerobic conditions (12, 13). 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 (14, 15).

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. (16, 17)], including those in YNP where formate was measured in 56 hot springs at concentrations of up to 10 µM (18). The low concentrations of formate measured in some YNP hot springs (18) 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. (14, 19)]. More direct evidence for the utilization of formate in hot spring communities comes from a ¹³C-formate labeling study which showed documented incorporation into fatty acids at a single hot
spring in Yellowstone National Park (YNP), albeit at low levels (20). 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 O$_2$ 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 (21).
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 O$_2$ 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 (21). 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 (14,
22).

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., H$_2$, H$_2$S, Fe$^{2+}$, S$_8$) under the geochemical conditions that prevail
in most hydrothermal systems (18). 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* (23), were found to be depleted in $^{13}$C relative to DIC in hydrothermal fluids sampled from the spring’s source (24). Based on a comparison of these membrane lipid carbon isotope signatures with those obtained from cultures of *Thermocrinis ruber* [originally isolated from Octopus Spring (14)] 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 (24).

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 (25). 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’ (8) 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: $\text{CO}_2 + \text{bicarbonate}$), 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 (Fe$^{2+}$) and total sulfide (S$^{2-}$) 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 [NH$_4$(T)] were determined with AccuVac® ampuls for nitrate or nitrite and an AmVer® ammonia reagent set for total ammonia (Hach Company). NH$_4$(T) refers to the sum of the dissolved species of aqueous NH$_3$ and NH$_4^+$ as measured by colorimetry (26). 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 (27). 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 (28).

**Microcosm preparation.** Rates of DIC, formate, and acetate assimilation and mineralization were quantified using a previously described microcosm-based approach (4, 11). 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 N₂ 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 (NaH¹⁴CO₃) for DIC assays, 7.5 μCi (14.4 μM final concentration) of sodium formate (H¹⁴COONa) for formate assays, 5 μCi (9.4 μM final concentration) of 1-[¹⁴C] sodium acetate (CH₃¹⁴COONa), or 5 μCi (8.9 μM final concentration) of 2-[¹⁴C] sodium acetate (¹⁴CH₃COONa) for acetate assays. Data from individual 1-[¹⁴C]- and 2-[¹⁴C]-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 CO₂ 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 (11).

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 NaH₁⁴CO₃ 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 (Kₘ) (the concentration at which formate conversion velocity reaches ½ 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 (H₁²COONa) stock solution was prepared that contained 50 µCi (0.96 µmol) of radiolabeled formate (H₁⁴COONa) 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 (29) 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 µM) 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 volatize 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, N$_2$ purged serum bottles (12 ml) containing 1 ml of the CO$_2$-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 $^{14}$C 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 $^{14}$C tracers were determined using the methods of Lizotte et al, 1996 (30). 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 (31)] 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 (30). 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 (32). 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 (33). 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) (34) as previously described (33) 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 (35) as implemented in PhyML (version 3.0) (35). Bacterial 16S rRNA gene phylogenies were rooted with SSU rRNA genes from the Crenarchaeotes *Acidilobus sulfurreducens* 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) (36). Phylograms were rate-smoothed using the multidimensional version of Rambaut’s parameterization as implemented in PAUP (ver. 4.0) (37). 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) (38).

**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) (39) 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 alakaline (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 exceeded 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...
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 14C 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 µM 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 (*Ignsphaera 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(EH), 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°C) hot springs are supported by chemolithoautotrophic metabolism (6, 8, 11, 40, 41). 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 (20, 25).
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 (20). 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 (20, 25). 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 (42-44). 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 \textsuperscript{12}C-formate to \textsuperscript{12}CO\textsubscript{2}, or to production of \textsuperscript{12}CO\textsubscript{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 \textsuperscript{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 \textsuperscript{14}C-bicarbonate pool had been diluted by \textsuperscript{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 \textless{}0.08\% suppression of activity. This strongly indicates that the observed suppression is not due to isotopic exchange.

The low concentrations of \textsuperscript{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 \textless{} 10 \, \mu\text{M} (18) and thus minimize induction of microbial processes above their basal or \textit{in situ} rate. Moreover, formate occurs increasingly in the protonated form as pH decreases (pKa = 3.75 at 80°C, meaning that half of all formate is protonated at pH=3.75). This neutral form will readily diffuse into the cell and deprotonate at the intracellular pH, thereby decreasing the membrane potential (45).
Previous studies have shown that concentrations of formate as low as 100 µM inhibit the growth of the acidophile *Thiobacillus (Acidithiobacillus) ferroxidans* when grown in medium with a pH of 1.6 (46). Since the highest concentration of formate that has been measured in YNP hot springs to date is 10 µM (18) and since many of the springs subject to study have pH < 3.75 (Table 1), we attempted to avoid the confounding effects of formate toxicity by amending assays with <20 µM 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 µM) or when the natural substrate concentration is not known (29).

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 (25, 47). 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. (48)], including hot springs. Atkinson et al., 2000 (49) 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 (49). 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 (50), 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 (51), 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 *Esherichia coli* cells during various stages of growth and when grown under different metabolic regimes (52). Thus, the variation in ATP contents in hot springs observed by Atkinson et al., 2000 (49) 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 $1\text{-}[^{14}\text{C}]$- and $2\text{-}[^{14}\text{C}]$-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 CO$_2$ 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 CO$_2$ 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 CO$_2$ 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/FADH$_2$ production, and a release of acetate carbon as CO$_2$. The rate of CO$_2$ 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 CO$_2$ 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 CO$_2$ production from the carboxyl carbon of acetate that exceeded the rate of CO$_2$ 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 the Thermodesulfobium narugense, a thermophilic and autotrophic sulfate reducing bacterium (53), 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 (54). 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 (32), 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* (7, 14, 15) and the type strain of *T. aquaticus* (55) 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 (6-8, 40, 41, 56) 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. (20, 25), 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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FIGURE LEGENDS

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 dendogram 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 CH$_3^{14}$COO$^-$ (1-[14C] acetate) and 14CH$_3$COO$^-$ (2-[14C] 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.
Table 1. Location and field measurements for YNP hot springs sampled in this study. Several of the features sampled do not have official YNP names. Unofficial names are denoted with apostrophes.

<table>
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<th>pH Grouping</th>
<th>YNP Thermal Inventory ID</th>
<th>Spring Name</th>
<th>Spring Abbreviation</th>
<th>Thermal Area*</th>
<th>GPS Coordinates</th>
<th>Cond. (mS)</th>
<th>Temp. (°C)</th>
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<td></td>
<td>GSSG060</td>
<td>'Lobster Claw'</td>
<td>A(LC)</td>
<td>SS</td>
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<td>CHA043</td>
<td>'Alice Spring'</td>
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<td>'Dragon Spring'</td>
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<td>NA(RCS)</td>
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<td>88.3</td>
<td>8.2</td>
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<td>NA(RCN)</td>
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<td>2.1</td>
<td>86.6</td>
<td>9.2</td>
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* 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.

<table>
<thead>
<tr>
<th>pH Grouping</th>
<th>Site Abbreviation</th>
<th>DIC</th>
<th>Formate</th>
<th>Acetate</th>
<th>S\textsuperscript{2} \textsuperscript{-}</th>
<th>Fe\textsuperscript{2+}</th>
<th>NH\textsubscript{4}(T)</th>
<th>NO\textsubscript{2} \textsuperscript{-}</th>
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<td>BD (0.8)</td>
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<td>7.2</td>
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<td>A(AS)</td>
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<td>26.6</td>
<td>20.2</td>
<td>BD (0.3)</td>
<td>207.7</td>
<td>552.0</td>
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<td>A(DS)</td>
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<td>0.8</td>
<td>BD (0.8)</td>
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<td>21.8</td>
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<tr>
<td>Moderately to slightly acidic (pH 4.0-6.0)</td>
<td>MA(CP)</td>
<td>1.2</td>
<td>BD (0.1)</td>
<td>BD (0.8)</td>
<td>6.2</td>
<td>4.7</td>
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<td>BD (0.1)</td>
<td>9.7</td>
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<tr>
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<td>MA(HG)</td>
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<td>0.8</td>
<td>BD (0.8)</td>
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<td>9.7</td>
<td>134.5</td>
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<td>BD (0.2)</td>
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<td>MA(EP)</td>
<td>2.4</td>
<td>BD (0.1)</td>
<td>BD (0.8)</td>
<td>BD (0.3)</td>
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<td>66.4</td>
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<td>BD (0.2)</td>
</tr>
<tr>
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<td>MA(OP)</td>
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<td>0.2</td>
<td>BD (0.8)</td>
<td>0.3</td>
<td>3.2</td>
<td>6.5</td>
<td>BD (0.1)</td>
<td>BD (0.2)</td>
</tr>
<tr>
<td>Neutral to alkaline (pH &gt; 7.0)</td>
<td>MA(CT)</td>
<td>1.7</td>
<td>BD (0.1)</td>
<td>BD (0.8)</td>
<td>5.3</td>
<td>1.6</td>
<td>28.2</td>
<td>BD (0.1)</td>
<td>29.0</td>
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<tr>
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<td>NA(PS)</td>
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<td>BD (0.1)</td>
<td>BD (0.8)</td>
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<td>15.3</td>
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<td>NA(BP)</td>
<td>6.0</td>
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<td>BD (0.8)</td>
<td>BD (0.3)</td>
<td>BD (1.0)</td>
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<td>NA(RCS)</td>
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<td>0.9</td>
<td>2.0</td>
<td>BD (0.3)</td>
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<td>NA(FC)</td>
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<td>10.0</td>
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BD: Below detection (detection limit in parentheses).