



# Microbial Fe(III) oxide reduction potential in Chocolate Pots hot spring, Yellowstone National Park

Authors: N.W. Fortney, S. He, B.J. Converse, B.L.  
Beard, C.M. Johnson, E.S. Boyd, & E.E. Roden

This is the peer reviewed version of the following article: [Fortney, N. W., S. He, B. J. Converse, B. L. Beard, C. M. Johnson, E. S. Boyd, and E. E. Roden. "Microbial Fe(III) oxide reduction potential in Chocolate Pots hot spring, Yellowstone National Park ." [Geobiology](#) 14, no. 3 (January 2016): 255-275.], which has been published in final form at <https://dx.doi.org/10.1111/gbi.12173>. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving.

Fortney, N. W., S. He, B. J. Converse, B. L. Beard, C. M. Johnson, E. S. Boyd, and E. E. Roden. "Microbial Fe(III) oxide reduction potential in Chocolate Pots hot spring, Yellowstone National Park ." *Geobiology* 14, no. 3 (January 2016): 255-275. DOI: 10.1111/gbi.12173.

Made available through Montana State University's [ScholarWorks](https://scholarworks.montana.edu)  
[scholarworks.montana.edu](https://scholarworks.montana.edu)

# Microbial Fe(III) oxide reduction potential in Chocolate Pots hot spring, Yellowstone National Park

N. W. Fortney,<sup>1</sup> S. He,<sup>1</sup> B. J. Converse,<sup>1</sup> B. L. Beard,<sup>1</sup> C. M. Johnson,<sup>1</sup> E. S. Boyd,<sup>2</sup> and E. E. Roden<sup>1</sup>

<sup>1</sup>Department of Geoscience, NASA Astrobiology Institute, University of Wisconsin-Madison, Madison, WI, USA

<sup>2</sup>Department of Microbiology and Immunology, NASA Astrobiology Institute, University of Montana, Bozeman, MT, USA

## ABSTRACT

Chocolate Pots hot springs (CP) is a unique, circumneutral pH, iron-rich, geothermal feature in Yellowstone National Park. Prior research at CP has focused on photosynthetically driven Fe(II) oxidation as a model for mineralization of microbial mats and deposition of Archean banded iron formations. However, geochemical and stable Fe isotopic data have suggested that dissimilatory microbial iron reduction (DIR) may be active within CP deposits. In this study, the potential for microbial reduction of native CP Fe(III) oxides was investigated, using a combination of cultivation dependent and independent approaches, to assess the potential involvement of DIR in Fe redox cycling and associated stable Fe isotope fractionation in the CP hot springs. Endogenous microbial communities were able to reduce native CP Fe(III) oxides, as documented by most probable number enumerations and enrichment culture studies. Enrichment cultures demonstrated sustained DIR driven by oxidation of acetate, lactate, and H<sub>2</sub>. Inhibitor studies and molecular analyses indicate that sulfate reduction did not contribute to observed rates of DIR in the enrichment cultures through abiotic reaction pathways. Enrichment cultures produced isotopically light Fe(II) during DIR relative to the bulk solid-phase Fe(III) oxides. Pyrosequencing of 16S rRNA genes from enrichment cultures showed dominant sequences closely affiliated with *Geobacter metallireducens*, a mesophilic Fe(III) oxide reducer. Shotgun metagenomic analysis of enrichment cultures confirmed the presence of a dominant *G. metallireducens*-like population and other less dominant populations from the phylum *Ignavibacteriae*, which appear to be capable of DIR. Gene (protein) searches revealed the presence of heat-shock proteins that may be involved in increased thermotolerance in the organisms present in the enrichments as well as porin-cytochrome complexes previously shown to be involved in extracellular electron transport. This analysis offers the first detailed insight into how DIR may impact the Fe geochemistry and isotope composition of a Fe-rich, circumneutral pH geothermal environment.

## INTRODUCTION

Investigations that seek to unravel how geochemical variation shapes the structure, function, and evolution of microbial communities are critical to improving our understanding of modern and past environments on Earth and the potential for life on other rocky planets such as Mars. One goal of such studies is to quantify the relationships between the distribution, diversity, and metabolic composition of microbial life and the signatures of this life that may be recorded in the rock record (Cavicchioli, 2002).

Iron is the fourth most abundant element in the Earth's crust and the most abundant redox-sensitive element (Taylor & McLennan, 1985). There is great interest in Fe-based microbial systems in light of the wide range of microbial metabolisms that are dependent on Fe redox transformations on Earth (Bird *et al.*, 2011) and due to the potential for Fe redox transformations to generate isotopic signatures of past and present microbial life (Johnson *et al.*, 2008). Ferric iron [Fe(III)] reduction can be an abiotic process (Poulton, 2003) or proceed through microbial dissimilatory iron reduction (DIR), a process in which

reduction of Fe(III) (both aqueous and solid-phase forms) is coupled to oxidation of organic carbon or H<sub>2</sub> (Lovley *et al.*, 2004). Bacterial and archaeal organisms capable of DIR are highly represented in lineages that branch at the base of phylogenetic reconstructions of taxonomic genes, suggesting that DIR is potentially an ancient respiratory pathway (Vargas *et al.*, 1998; Weber *et al.*, 2006).

While several recent studies have examined microbial Fe redox cycling in high-temperature, acidic hot springs in Yellowstone National Park (YNP) [see Kozubal *et al.* (2012) and references cited therein], Fe cycling in lower temperature, and circumneutral pH geothermal environments, in particular cycling driven by DIR, remain largely unexplored. Chocolate Pots hot spring (CP) is a Fe-rich, circumneutral pH environment in YNP located approximately 5 km south of the Norris Geyser Basin along the Gibbon River in the northwestern portion of the park (McCleskey *et al.*, 2010). The most commonly studied spring in the Chocolate Pots thermal area consists of one main hot spring vent and mound (Fig. S1) and two smaller vents to either side. This hydrothermal feature was characterized in the early 20th century by Allen and Day who noted the unique properties of the non-crystalline and fine-grained Fe(III) oxide and silica precipitates unseen in other features in YNP (Allen & Day, 1935). Additional studies of the groundwater chemistry of CP found it to have remained fairly consistent through recent history (Rowe *et al.*, 1973; Thompson & Yadav, 1979; Pierson & Parenteau, 2000).

Previous research at CP has focused on Fe(II) oxidation, by way of both abiotic and photosynthetically driven Fe(II) oxidation reactions (Pierson *et al.*, 1999; Pierson & Parenteau, 2000; Trouwborst *et al.*, 2007; Parenteau & Cady, 2010). A key motivation for these studies was the concept of CP as a model for photosynthetically driven Fe(II) oxidation and associated mineralization of microbial mats and deposition of Archean banded iron formations. Geochemical data (presence of Fe(II) in vent deposits), however, suggest that microbial DIR could also play an active role in geochemical cycling of Fe in the CP deposits (Pierson *et al.*, 1999; Pierson & Parenteau, 2000). Studies of other freshwater circumneutral pH Fe redox cycling environments have suggested a microbially mediated coupling of Fe reduction and oxidation cycles (Emerson & Revsbech, 1994; Sobolev & Roden, 2002; Roden *et al.*, 2004, 2012; Blöthe & Roden, 2009), and a similarly coupled Fe redox-based microbial community may be present at CP.

Recent studies of Fe isotope geochemistry are also suggestive of a reductive iron cycle at CP (Wu *et al.*, 2011, 2013). Iron occurs as four stable isotopes, where <sup>54</sup>Fe and <sup>56</sup>Fe are the most abundant at 5.84% and 91.76%, respectively (Beard & Johnson, 1999). Iron isotope compositions for igneous rocks are relatively restricted, and match those of bulk crust (Beard & Johnson, 2004). Deviation from

average crustal compositions can occur when Fe undergoes redox cycling, and these fractionations can be used to infer redox history and potential biological involvement (Johnson *et al.*, 2008). Complete oxidation of Fe(II) to Fe(III), as occurs when Fe(II) is fully oxidized by atmospheric oxygen, results in no net Fe isotope fractionation (Beard & Johnson, 2004). Processes resulting in partial redox transformation, however, such as DIR, can result in a measurable isotopic fractionation (Beard *et al.*, 1999, 2003; Crosby *et al.*, 2005, 2007; Tangalos *et al.*, 2010; Percak-Dennett *et al.*, 2011). The use of stable Fe isotopes can be used to better understand the redox history of many environments on Earth and are applicable to better understanding the potential for identifying such processes on other planetary bodies, such as Mars (Johnson *et al.*, 2008; Dauphas *et al.*, 2009). Analyses of modern environments like CP will lay the groundwork required to gain insight into what isotopic signatures that are preserved in the rock record can reveal about the microbial and geochemical processes that were involved in their formation.

## MATERIALS AND METHODS

### Sample collection

Bulk solid-phase materials were obtained from a portion of the hot spring deposit away from the main mound at CP, in an area that had been previously disturbed by wildlife (e.g., bison), exposing the top 1–2 cm of Fe(III) oxide deposits. The solids were collected with a sterile spatula and stored in a sterile canning jar. Additional small samples of the deposits were collected from the spring source of the main mound (Vent) and midway down the main flow path (Mid) (see Fig. S1); these served as inocula for Fe(III) reduction experiments. Vent and Mid samples were collected from the oxide sediment–spring water interface. All samples were collected from locations not covered by photosynthetic mat communities. Hot spring fluid was collected from the spring vent in sterile, 1 L Nalgene bottles. Temperature and pH were measured at each sample location using a thermistor and combination electrode. The concentration of bicarbonate was determined by room temperature titration of 100 mL of spring water to a pH of 4 with 0.5 M HCl (Stumm & Morgan, 1996). A summary of the concentration of aqueous chemical species, temperature, and pH of the CP hot spring is provided in Table 1.

### Fe(III) reducing culture medium

Bulk solids consisting of Fe(III) oxide/silica co-precipitates were crushed using a mortar and pestle, suspended in distilled water, and passed through a 0.5 mm sieve; these materials are hereafter referred to as CP oxides. The CP oxide suspension was concentrated by centrifugation to

**Table 1** Fluid temperature and chemistry (mg L<sup>-1</sup>) of spring water at CP vent. These parameters were used to create ASW for use in enrichment culturing of Fe reducing organisms from the Vent and mid-locations

Measurement	Parenteau & Cady (2010)	Field campaign Sept., 2012		Enrichment culture medium					
		V	M	Natural CP Media		ASW + Sulfate		ASW – Sulfate	
				V	M	V	M	V	M
Temp. (°C)	51.8	50.7	42.8	45	40	45	40	45	40
pH	5.7	5.9	7.2	6.1	6.7	6.1	6.7	6.1	6.7
Aluminum	0.1			0.1	0.1				
Ammonium nitrogen	ND			140	140	140	140	140	140
Bicarbonate	ND	366		366	1281	305	1220	305	1220
Boron	0.5			0.51	0.51	0.01	0.01	0.01	0.01
Calcium	21			21	21	20	20	20	20
Carbonate	ND								
Chloride	29			455	455	469	469	490	490
Fluorine	4.5			4.5	4.5				
Iron(II)	5.5			61.4	61.4	55.9	55.9	55.9	55.9
Iron(III)	ND								
Total iron	5.4								
Lithium	0.8			0.8	0.8				
Magnesium	2			2	2	2.43	2.43	2.43	2.43
Manganese	1.4			1.7	1.7	0.34	0.34	0.34	0.34
Nitrate nitrogen	ND								
Nitrite nitrogen	ND								
Potassium	23			62.1	62.1	62.6	62.6	62.6	62.6
Phosphorous	<0.1			31.0	31.0	31.0	31.0	31.0	31.0
Silica	141			141	141	138	138	138	138
Sodium	115			115	460	184	529	184	529
Sulfate	25			25.5	25.5	29.3	29.3	0.47	0.47
Sulfide	ND								

ND, Not determined; V, Vent; M, Mid.

produce a thick slurry which served as a stock for Fe(III) reduction experiments. Portions of the slurry (0.5 mL) were added to 5 mL of 0.5 M HCl and agitated for 1 h. An aliquot of the extract was added to *Ferrozine* colorimetric reagent (Stookey, 1970) with and without the addition of 10% hydroxylamine hydrochloride to quantify total Fe and Fe(II), respectively. The amount of Fe(III) was determined by the difference between total solubilized Fe and Fe(II). The HCl-extractable Fe(III) content of the stock suspension was ca. 330  $\mu\text{mol g dry mass}^{-1}$ .

Fe(III)-reducing culture medium consisted of CP spring water (obtained during the sampling campaign and stored at room temperature) amended with 10 mM NH<sub>4</sub>Cl, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mL L<sup>-1</sup> of SL-10 trace metals (Widdel *et al.*, 1983), and 1 mL L<sup>-1</sup> of Wolfe's vitamin solution (Balch & Wolfe, 1976). Medium for use in experiments with the Mid samples was supplemented with 15 mM NaHCO<sub>3</sub> to achieve a pH value (ca. 6.7) comparable to that measured *in situ*, whereas medium (pH 6.1) for use in experiments with the Vent samples was not further buffered. Tubes of Mid and Vent medium (9 mL in 25 mL anaerobic pressure tubes) were amended with a sufficient quantity (ca. 0.25 mL) of CP oxides to achieve a concentration of approximately 40 mmol L<sup>-1</sup> of 0.5 M HCl-extractable Fe

(III). The tubes were sparged with O<sub>2</sub>-free N<sub>2</sub>:CO<sub>2</sub> (80:20%) (ca. 300 mL min<sup>-1</sup> for 12 min), capped with butyl rubber stoppers, and sterilized by autoclaving (121 °C, 15 min.).

### Most probable number determinations

Three-tube most probable number (MPN) series were prepared (using the medium described above) to estimate the abundance of dissimilatory iron-reducing bacteria (DIRB) in the Vent and Mid samples. The first dilution (10-fold) was inoculated inside an anaerobic chamber (Coy Products, Grass Lake, MI; 95:5%; N<sub>2</sub>:H<sub>2</sub>) with 1 g of homogenized hot spring material, and the tubes were removed from the chamber and flushed with N<sub>2</sub>:CO<sub>2</sub> (80:20%) to remove H<sub>2</sub> from the headspace. The samples were then serially diluted to 10<sup>-8</sup> in medium that contained a mixture of either 2 mM acetate and 2 mM lactate (Ac/Lac) or H<sub>2</sub> (10% in the headspace) as the electron donor. The Vent and Mid cultures were incubated at 45° and 40°C, respectively. Assessment of reduction activity was made by visual inspection (darkening of the solids) 3 and 5 days post-inoculation; no changes in activity were noted after 5 days. MPN values were calculated using a standard MPN table (Woormer, 1994).

## Enrichment culturing

Enrichment cultures were prepared identically to the MPN cultures described above. The cultures were inoculated (10% vol/vol) with material from the  $10^{-1}$  dilution of the MPN tubes. Reduction of Fe(III) in the enrichments was determined by quantification of Fe(II). The cultures were sampled weekly by collecting 0.5 mL of culture using a sterile,  $N_2$ -flushed syringe and needle followed by a 1 h, 0.5 M HCl extraction and the ferrozine assay as described above. Cultures were transferred to new medium every 2 weeks to assess the potential for continuous growth coupled to Fe(III) reduction. Each transfer is referred to as a new generation of the enrichment. A mixture of 2 mM Ac/Lac or  $H_2$  (10% in the headspace) was supplied as the electron donor. After one generation, cultures with  $H_2$  as electron donor failed to grow; the cultures were given an addition of 0.5 mM acetate which did not stimulate Fe reduction activity. Hence, a new set of enrichments with  $H_2$  and 0.5 mM acetate ( $H_2$ /Ac) was initiated by re-inoculating the tubes with 1 mL of a  $10^{-1}$  Ac/Lac MPN tube. After four generations of culturing, additional transfers were made from the Ac/Lac enrichments into medium containing only 2 mM acetate (Ac) or 2 mM lactate (Lac) as the electron donor.

From generation eight onward, all enrichment cultures were transferred into CP artificial spring water (ASW), with natural CP oxides still serving as the electron acceptor. The ASW composition was based on observed CP fluid composition (Parenteau & Cady, 2010) (Table 1). The medium consisted of  $CaCl_2 \cdot 2H_2O$  (0.2 mM),  $CaSO_4 \cdot 2H_2O$  (0.3 mM),  $MgCl_2 \cdot 6H_2O$  (0.1 mM),  $NaSiO_3 \cdot 9H_2O$  (1.5 mM), KCl (0.6 mM), and  $NaHCO_3$  (Vent 5 mM, Mid, 20 mM). Other components of the medium, the medium preparation procedure, and the incubation conditions were as described above. Fe(III) reduction in the ASW medium was compared to natural growth medium and transfers to ASW continued for an additional 26 generations. Enrichment culturing concluded with the freezing of aliquots of Vent and Mid cultures at  $-80^\circ C$ . The viability of the cultures after freezing was determined prior to terminating the enrichment culturing experiments.

## Potential influence of bacterial sulfate reduction on Fe(III) reduction

Sulfate-free medium was prepared identical to the ASW described above, replacing the  $CaSO_4 \cdot 2H_2O$  with  $CaCl_2 \cdot 2H_2O$ . Parallel sets of sulfate-containing and sulfate-free cultures were inoculated from the generation nine sulfate-containing enrichment cultures. Molybdate [a specific inhibitor of microbial sulfate reduction; (Oremland & Capone, 1988)] was added to sulfate-containing ASW media at a concentration equivalent to the sulfate concentration, ca. 0.3 mM. Molybdate amended cultures were transferred for five generations. Transfers to sulfate-free media were made for an additional 17 generations.

Samples (1 mL) were collected from the generation nine cultures weekly using a sterile  $N_2$ -flushed syringe and needle. Samples containing sulfate were inserted into a rubber stopper to prevent gas exchange and transferred immediately into the anaerobic chamber to minimize Fe(II) oxidation. Samples were transferred to 1.5-mL microcentrifuge tubes and spun down. The supernatant was collected for ion chromatographic (IC) analysis of sulfate using a Dionex ICS-1000 (Dionex Corporation, Sunnyvale, CA, USA) with an IonPac AS14A  $4 \times 250$  mm column and Chromeleon software (Thermo Fisher Scientific Inc., Sunnyvale, CA, USA). Sulfate standards were run with samples and prepared with ASW at concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5 mM sulfate. The previously described procedure for 0.5 M HCl extraction and ferrozine assay was used to quantify Fe(II) in the sulfate-free samples and the residual pellet from the sulfate-containing samples. Sulfate and Fe(III) reduction were tracked over one culture generation, spanning 2 weeks, and sampled at 0, 1, 2, 4, 7, 11, and 14 days. Following the experiment duplicate, 1 mL aliquots of the cultures was frozen for DNA extraction.

## XRD analysis and Mössbauer analysis

Samples for X-ray diffraction (XRD) analysis were prepared with 0.5 mL aliquots of non-reduced CP oxides, pre- and post-autoclaving, and microbially reduced slurry from generations 8 and 26 of the ASW Vent and Mid enrichment cultures. In an anaerobic chamber, microbially reduced samples were washed twice using anoxic distilled  $H_2O$ , dried under an anoxic  $N_2$  stream overnight, and mounted in thin-walled capillary tubes. Diffraction spectra were collected using a Rigaku Rapid II X-ray diffractometer with a two-dimensional image plate (Mo  $K\alpha$  radiation). Two-dimensional images were integrated to produce conventional intensity patterns using the Rigaku 2DP software (Rigaku Americas Corporation, The Woodlands, TX, USA).

Non-reduced CP oxides were diluted in distilled  $H_2O$  to an equivalent concentration to the culture media and added drop-wise to a petrographic slide and allowed to air-dry. The non-reduced materials were analyzed on a Scintag Pad V X-ray diffractometer (Cu  $K\alpha$  radiation).

Solids were collected from generation 9 ASW Vent and Mid enrichment cultures and dried anaerobically. Non-reduced CP oxides were similarly prepared. The samples were analyzed by Mössbauer spectroscopy at Penn State University using a SVT400 cryogenic Mössbauer system (SEE Co., Minneapolis, MN, USA) as previously described (Luan *et al.*, 2014).

## Reduced inorganic sulfur determination

A single-step reduced chromium reduction assay (Fossing & Jørgensen, 1989) was used to determine the concentration



of total reduced inorganic sulfur in the third and eleventh generations of the Vent and Mid ASW enrichment cultures. Samples (2 mL) from the cultures were added to three-arm flasks connected to a condenser. The flasks were continuously flushed with a stream of N<sub>2</sub> gas. Five mL of 1M CrCl<sub>2</sub> and 5 mL of 6 M HCl were added, and the materials were boiled for 1 h. Gaseous H<sub>2</sub>S liberated from the sample was trapped in a zinc acetate solution (5%), and the resulting ZnS precipitate was analyzed colorimetrically using Cline reagent (Cline, 1969).

### Fe isotope fractionation experiment

A separate experiment was conducted to assess how Fe isotope compositions changed during microbial reduction of CP oxides. Duplicate serum bottles were prepared using 90 mL ASW containing 2 mM Ac/Lac and 2.5 mL of the CP oxides stock suspension. Each bottle was inoculated with 10 mL of a 1-week-old enrichment culture, grown in parallel with generation 28 cultures, and incubated at identical conditions. Aliquots (10 mL) were collected from each bottle at 0, 4, 8, 12, 18, 24, 32, 48, and 72 h. Samples were centrifuged, and the aqueous phase was filtered (0.2 µm nylon filter) and acidified to a final concentration of 0.5 M HCl using 8 M HCl. The residual solid pellet was subjected to a 1-h extraction with 0.01 M HCl, followed by a 23 h extraction with 0.5 M HCl, with centrifugation and supernatant filtration between extractions, in order to separate the loosely associated and total HCl-extractable Fe components. *Ferrozine* analysis was used to quantify Fe(II) and total Fe, as described above.

Aliquots of Fe for isotopic analysis were purified through two rounds of anion-exchange chromatography (Beard *et al.*, 2003). Purified samples were analyzed as 600 ppb Fe using a MC-ICP-MS on a Micromass IsoProbe (Micromass Ltd, Wythenshawe, Manchester, UK) following previously published protocols (Beard *et al.*, 2003).

Data are reported as the <sup>56</sup>Fe/<sup>54</sup>Fe ratios relative to the average of igneous rocks using the standard δ notation in units of per mil (‰):

$$\delta^{56}\text{Fe} = \left[ \frac{(^{56}\text{Fe}/^{54}\text{Fe})_{\text{sample}}}{(^{56}\text{Fe}/^{54}\text{Fe})_{\text{Ig Rx}}} - 1 \right] \times 1000.$$

The Fe isotope fractionation between two phases is defined as:

$$\Delta^{56}\text{Fe}_{\text{A-B}} = \delta^{56}\text{Fe}_{\text{A}} - \delta^{56}\text{Fe}_{\text{B}}.$$

External precision of δ<sup>56</sup>Fe values was estimated to be ±0.08‰ (2 standard deviations) based on replicate analyses of in-house standards, test solutions using standards of a known Fe isotopic composition (HPS-I and -II) that were

passed through the entire analytical procedure, and unknown duplicates of samples. The following Fe isotope compositions of the standard, including external reproducibility, were obtained: J-M Fe: δ<sup>56</sup>Fe = 0.28 ± 0.12‰ (2 SD, *n* = 10), IRMM-014: δ<sup>56</sup>Fe = −0.04 ± 0.09‰ (2 SD, *n* = 9), HPS-I: δ<sup>56</sup>Fe = 0.50 ± 0.12‰ (2 SD, *n* = 20), and HPS-II: δ<sup>56</sup>Fe = 0.51 ± 0.09‰ (2 SD, *n* = 16) (Beard *et al.*, 2003). A total of 48 samples were analyzed, and 10 replicates with an average reproducibility of ±0.03‰.

### DNA extraction and 16S rRNA gene amplicon sequencing

Aliquots (1 mL) were collected from generations 7, 15, 23, and 33 of the enrichment cultures and frozen at −20 °C for subsequent DNA extraction and 454 pyrosequencing of 16S rRNA gene amplicons. Additional samples were collected from generation 33 for metagenomic analysis. All DNA samples were extracted using the MoBio PowerSoil® DNA Isolation Kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's instructions with the following exceptions: PowerBead tubes containing PowerBeads and buffer were decanted into sterile 2 mL centrifuge tubes, and 1 mL of the enrichment culture slurry was added to empty PowerBead tubes and centrifuged at maximum speed for 10 min to remove excess water from the samples, and to pellet the cells. Bead-beating beads and buffer were returned to tubes containing the culture pellet. Following addition of solution C1, samples were heated and agitated (60 °C, 1400 rpm) using a Thermixer (Eppendorf, Hamburg, Germany). Solutions C2 and C3 (0.1 mL each) were added in one step, with vortexing in between (recommended by MoBio when DNA yields are anticipated to be low). DNA was eluted in 40 µL of solution C6 and stored at −20 °C until used.

To prepare samples for 16S rRNA gene amplicon sequencing, extracted DNA was PCR amplified in duplicate using Invitrogen™ Platinum® Taq Supermix (Life Technologies, Grand Island, NY, USA) using the universal primers 515f/806r (Caporaso *et al.*, 2011), which target the V3-V4 region of the 16S rRNA gene. Samples were multiplexed using standard Roche MID primer tags. Amplicons were generated using the following PCR protocol: 94 °C for 2 min (initial denaturation) followed by 35 cycles of 94 °C for 30 s (denaturing), 55 °C for 30 s (annealing), 68 °C for 1 min (extension), and a final extension at 68 °C for 6 min. Successful generation of amplicons was verified by gel electrophoresis using a 1% agarose gel. Extracts were quantified using a Qubit® fluorometer (Version 1.0; Life Technologies, Inc., Madison, WI, USA). After quantitation, amplicons were pooled in an equimolar ratio, and sequenced at the University of Wisconsin-Madison Biotechnology Center (UWMBC) using the Roche 454 FLX+ pyrosequencing platform.

Microbial community sequence data were processed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (<http://www.qiime.org>, version 1.7.0) (Caporaso *et al.*, 2010b). QIIME allows for de-multiplexing of bar-coded sequences, *de novo* operational taxonomic unit (OTU) picking based on sequence similarity within samples, sequence alignment, and taxonomic assignment using the Greengenes database. Raw data was de-multiplexed using MID tags, and processed with default QIIME parameters to remove sequences of low quality: sequences <200 bp, with quality scores below 25, and with more than six ambiguous bases in a row were discarded. After library splitting and quality filtering, sequences were de-noised using default QIIME settings (Reeder & Knight, 2010). After de-noising, OTUs were clustered *de novo* using the default uclust algorithm (Edgar, 2010) and a representative set of OTUs was generated. Representative sequences were aligned to the Greengenes database using PyNast (Caporaso *et al.*, 2010a). Chimeric sequences were identified and removed using ChimeraSlayer (Haas *et al.*, 2011). Taxonomy was assigned to OTUs using the Greengenes reference database (McDonald *et al.*, 2012; Werner *et al.*, 2012) and Ribosomal Database Project classifier (Wang *et al.*, 2007). Sequences from the ten most abundant OTUs from each enrichment culture were analyzed using NCBI BLASTn search algorithm excluding models and uncultured/environmental sample sequences (Altschul *et al.*, 1990). BLASTn results were compared to the Greengenes taxonomies identified using QIIME to potentially gather additional information about the most abundant taxa.

### Metagenomic sequencing and analysis

Samples from generation 33 of the Vent and Mid enrichment cultures were submitted to the UWMBC for paired-end Illumina MiSeq shotgun metagenomic sequencing. Raw sequence data was processed using CLC Genomics Workbench 6.0.2 (<http://www.clcbio.com>). Adapters were removed from sequence data using the Trim Sequences function within CLC Genomics Workbench. In both Vent and Mid samples, overlapping pairs of 2 × 250 bp sequences were merged prior to quality trimming. Adapters were removed using the General Adapter List library and adapter usage information provided by UWMBC. Merged sequences of <50 bp or quality scores <95% were removed. Contigs were assembled using an overlapping word length of 63 bp and minimum length of 200 bp. Reads were mapped back to contigs based on 90% length and 95% similarity. Assemblies from both samples were uploaded to the Integrated Microbial Genomes with Microbiome Expert Reviewer (IMG/M ER) system ([img.jgi.doe.gov/mer](http://img.jgi.doe.gov/mer)) for gene calling using IMG/M ER Metagenome Gene Calling method and function annotation (Mavromatis *et al.*, 2009).

Metagenomes from Vent and Mid enrichments were assigned coarse taxonomic bins based on GC content and coverage using contigs 3 kb and greater. GC content for each assembled contig was determined using the Bioperl Toolkit (Stajich *et al.*, 2002) and a custom GC content script (Xiaodong Bai, Ohio State University, <http://www.oardc.ohio-state.edu/tomato/HCS806/GC-script.txt>). Identity of the bins was further refined by sequence composition and coverage using the automated clustering tool, CONCOCT (Alneberg *et al.*, 2014). Bin quality was determined by counting how many unique copies of the 111 conserved bacterial housekeeping genes were present in each bin (Albertsen *et al.*, 2013), hereafter referred to as ‘completeness’. Bins with a completeness of <50% were not considered for further analysis. Bins containing no conserved bacterial housekeeping genes and one or two contigs were immediately discarded. Taxonomic bins produced in CONCOCT were visualized using Databionics ESOM Tools (Ultsch & Moerchen, 2005). ESOM maps were produced using tetranucleotide frequencies of the metagenome assemblies as determined using a custom Bioperl script (Dick *et al.*, 2009). Raw tetranucleotide frequencies were transformed using the Robust ZT option in ESOM and K-batch training algorithm according to previously described methodology (Dick *et al.*, 2009). A window size of 5 kb was used on all contigs >3 kb. Maps generated were borderless and with dimensions of 150 × 246, greater resolution than the default settings of 50 × 82. Taxonomic identity of the bins was assigned using PhyloSift (Darling *et al.*, 2014).

Metagenomes annotated in IMG were examined for genes of interest including those involved in thermotolerance and extracellular electron transfer (EET). Gene search was based on pfam functional units of genes encoding molecular chaperones known to be involved with thermotolerance in organisms. A broad EET gene search was conducted using functional units of genes encoding for c-type cytochromes and multiheme-binding motifs (Table S8). In-depth EET searches were limited to the known pathways including *mtrABC* as in *Shewanella*, (Hartshorne *et al.*, 2009), and the recently identified porin–cytochrome complex (PCC) found in all *Geobacter* spp. and several other genera (Liu *et al.*, 2014; Shi *et al.*, 2014). A total of 29 gene homologs to the porin in PCC, taken from the organisms identified in Shi *et al.* (2014), were used to generate a hidden Markov model (Eddy, 2009) using the hmmbuild function in HMMER 3.0 (Finn *et al.*, 2011). Candidate PCC porin homologs in the enrichment metagenomes were identified by searching all protein sequences from the metagenomes against the HMM using the hmmsearch function. Identified genes were further investigated to predict the porin secondary structure and the presence of an adjacent multiheme c-type cytochrome. Homologs of genes involved in dissimilatory sulfate reduction (DSR),

including Adenosine-5'-phosphosulfate reductase (*apsAB*), sulfate adenyltransferase (*sat*), and dissimilatory sulfite reductase (*dsrAB*), were identified using the BLASTp search tool on IMG.

Candidate genes of interest were compared between the bins in the Vent and Mid metagenomes, and the type strains *Geobacter metallireducens* GS-15, *Geobacter sulfurreducens* PCA, *Ignavibacterium album* Mat9-16, JCM 16511, and *Melioribacter roseus* P3M using gene copy number and organization. Identification of the location of the genes of interest was achieved by cross-referencing the contig Scaffold ID from IMG with the contig and bin taxonomy assigned during binning in CONCOCT.

## RESULTS

### Description and chemistry of Chocolate Pots

CP is located approximately 5 km south of the Norris Geyser Basin along the Gibbon River at the northern edge of the Yellowstone caldera. This circumneutral hot spring had a pH of 5.9 at the Vent sample site and 7.2 at the Mid sampling site. Temperatures at these two sample sites were 50.7 and 42.8 °C, respectively. The slightly acidic spring water emerging from the CP vent is thought to be the result of mixing of subsurface acid-sulfate hydrothermal waters with alluvial groundwater (McCleskey *et al.*, 2010; Parenteau & Cady, 2010). CP spring water is anoxic at the vent and contains high concentrations of Fe(II) at ca. 0.1 mM and dissolved silica close to amorphous Si saturation at ca. 150 ppm (Parenteau & Cady, 2010). Concentrations of other major ions are reported in Table 1. The iron-silica sinter comprising the hot spring mound at CP has been previously reported as 55% ferric oxides, 17% silica, and 19% water, Fe:Si = 3.2:1 (Allen & Day, 1935). Processing of the bulk solids (see Materials and Methods) for use in the culture medium resulted in removal of almost all of the silica. The Fe:Si ratio of the CP oxides in the culture medium was approximately 188:1.

### MPN enumerations

The MPN enumerations revealed the presence of substantial populations of Ac/Lac-utilizing DIRB in both the Vent (ca.  $10^5$  cells g<sup>-1</sup>) and Mid (ca.  $10^8$  cells g<sup>-1</sup>) materials (Table 2). Lower but detectable numbers of H<sub>2</sub> oxidizing DIRB ( $10^1$ – $10^3$  cells g<sup>-1</sup>) were observed.

### Fe(III)-reducing enrichment cultures

The enrichment cultures demonstrated continued growth and Fe(II) production for the duration (33 transfers) of the ca. 450-day culturing campaign (Fig. 1). Enrichment cultures using H<sub>2</sub> as an electron source failed to grow after

**Table 2** Most probable number enumerations for Fe(III)-reducing organisms in Mid and Vent materials collected from Chocolate Pots, YNP in September 2012

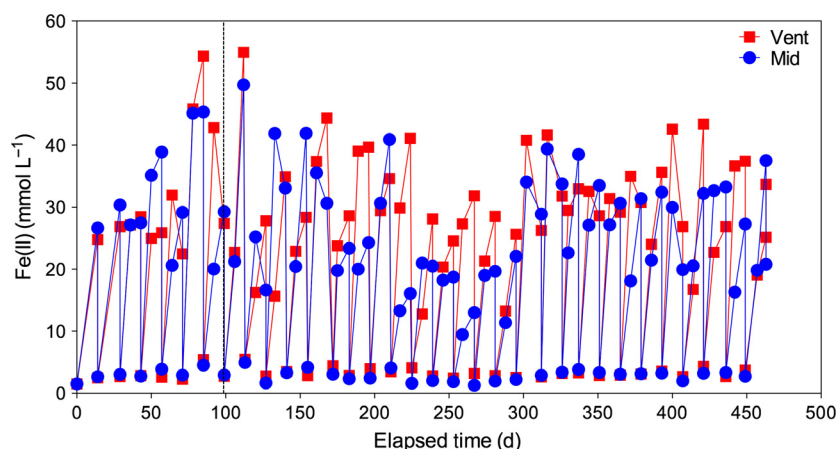
Sample site and e <sup>-</sup> donor	MPN (cells g <sup>-1</sup> wet weight)	
	Day 3	Day 5
Mid acetate/lactate	$1.5 \times 10^4$	$2.8 \times 10^8$
Mid H <sub>2</sub>	$4.3 \times 10^1$	$4.3 \times 10^1$
Vent acetate/lactate	$1.5 \times 10^5$	$1.5 \times 10^5$
Vent H <sub>2</sub>	$2.1 \times 10^3$	$2.1 \times 10^3$

inoculation from the 10<sup>-1</sup> dilution of an H<sub>2</sub> MPN tube. Culture tubes spiked with a small amount (0.5 mM) of acetate in an attempt to stimulate growth showed no observable increase in activity. Subsequent cultures, grown in the presence of H<sub>2</sub> and 0.5 mM acetate and inoculated from 10<sup>-1</sup> dilution of the Ac/Lac MPN tube, were able to sustain Fe(III) reduction activity. While at a lower yield than observed in 2 mM Ac/Lac cultures, H<sub>2</sub>/Ac enrichments showed greater activity than can be accounted for by the oxidation of 0.5 mM acetate alone, suggesting that the organisms were obtaining reductant from oxidation of H<sub>2</sub> as well (Fig. S2). After five generations, acetate and lactate were tested independently as energy sources in the Ac/Lac enrichments. Fe(III) reduction was maintained with either acetate or lactate in cultures over five generations with comparable levels of Fe(III) reduction observed in cultures containing either acetate or lactate alone (Fig. S2).

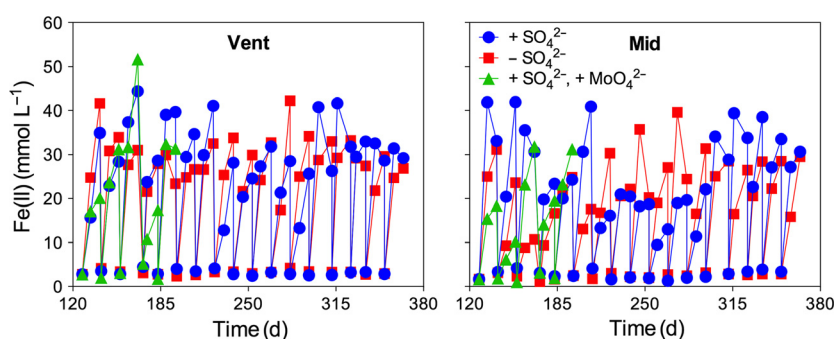
After seven generations, enrichments were transferred to culture medium prepared with ASW and CP oxides, allowing for adjustment of growth medium components, for example, sulfate concentration. The ASW cultures were successfully grown for 26 generations and maintained a level of Fe(III) reduction similar to that observed prior to transferring to artificial medium (Fig. 1). Enrichment cultures containing molybdate and those lacking sulfate were transferred along with unaltered Ac/Lac cultures for five successive generations. Sustained Fe(III) reduction took place in cultures both without sulfate and in those containing molybdate, albeit at a slightly reduced rate compared to the Ac/Lac cultures (Fig. 2). Cultures containing no sulfate were transferred for an additional ten generations with levels of Fe(III) reduction equivalent to that of the unaltered, sulfate-containing cultures (Fig. 2).

The concentration of sulfate decreased from ca. 0.3 mM to ca. 0.1 mM over the course of a 2-week incubation in the 9th-generation ASW cultures. Fe(III) reduction occurred more quickly in Vent cultures, producing 30.6 mmol L<sup>-1</sup> and 33.7 mmol L<sup>-1</sup> Fe(II) in the sulfate-containing and no-sulfate cultures, respectively, by Day 2. By Day 4, all four cultures had reduced about the same amount of Fe(III), producing  $30.8 \pm 2.8$  mmol L<sup>-1</sup> Fe(II) (Fig. 3). Sulfide production as determined by the





**Fig. 1** Fe(II) production in Vent and Mid enrichment cultures cultivated at 45° and 40 °C, respectively, in natural spring water medium (pH = 6.1 and 6.7, respectively) amended with processed Fe(III) oxides native to CP. Each low Fe(II) value (ca. 2 mmol L<sup>-1</sup>) represents the time zero value after inoculation or transfer of the culture. Enrichments contained a mixture of 2 mM acetate and 2 mM lactate as the electron donor and were grown in the presence of ca. 0.3 mM sulfate. The dashed line indicates the transition to ASW medium containing processed Fe(III) oxides native to CP. Values represent single measurements on a single enrichment culture for the Vent and Mid systems.



**Fig. 2** Fe(II) production in enrichments from the Vent and Mid sampling sites in the presence and absence of 0.3 mM sulfate in ASW medium, and in sulfate-containing ASW medium with 0.3 mM molybdate, an inhibitor of biological sulfate reduction. Each low Fe(II) value (ca. 2 mmol L<sup>-1</sup>) represents the value at time zero after transfer of the culture. Values represent a single measurement on a single enrichment culture.

reduced chromium assay showed a slight increase from 0.124 to 0.148 mmol L<sup>-1</sup> between the 3rd and 11th generation of the Mid enrichment cultures. The concentration of sulfide in the Vent enrichments was below detection (data not shown). This likely indicates that sulfate depletion was due to its use in assimilatory pathways.

### XRD analysis

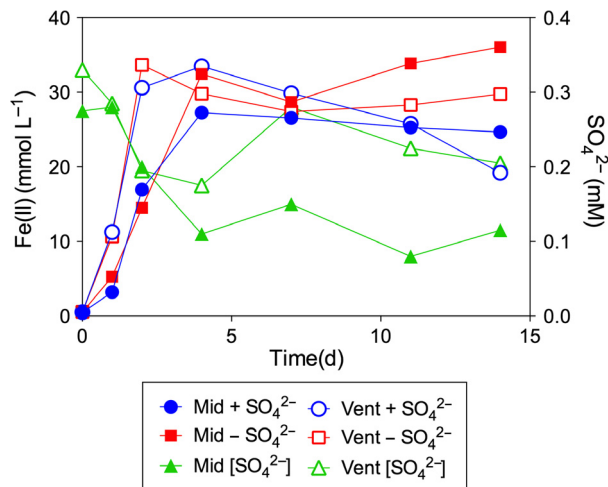
Non-reduced CP oxides consisted of amorphous Fe, and no increase in crystallinity was observed as a result of autoclaving (Fig. S3). Following microbial reduction, a small amount of siderite was observed in solids collected from both the Vent and Mid enrichment cultures (Fig. S3).

### Fe isotope fractionation experiment

Fe(III) oxide reduction activity in the Fe isotope fractionation experiment plateaued by the second day of incubation (Fig. 4A,B). Sequential extraction of samples from this experiment was used to separate dissolved (aqueous) Fe

from two parts of solid-phase Fe, that is, 0.01 M HCl- and 0.5 M HCl-extractable Fe, for Fe isotope analysis. The 0.01 M HCl-extractable phase consisted of almost entirely Fe(II) after hour 12 of the experiment, with final Fe(II)/total Fe ratios of 0.996 (Vent) and 0.999 ± 0.009 (Mid). The 0.5 M HCl-extractable phase initially consisted mainly of Fe(III), and a greater amount of Fe(II) at later time points [final Fe(II)/total Fe = 0.140 ± 0.001 (Vent) and 0.122 ± 0.006 (Mid)].

$\delta^{56}\text{Fe}$  values for aqueous and 0.01 M HCl-extractable phases were ca. 1–3‰ lower than bulk 0.5 M HCl-extractable Fe (Fig. 4C,D). A mass-balance approach was taken to estimate the  $\delta^{56}\text{Fe}$  value of the pure Fe(III) component that was sampled from the solid in the 0.5 M HCl extractions, which accounted for most of the Fe in the extraction. Calculations are based on the assumption that the  $\delta^{56}\text{Fe}$  value for Fe(II) in the 0.01M HCl extract, after the time (ca. 12 h) when the Fe(II)/total Fe ratio was approximately equal to 1.0, was equivalent to the isotopic composition of Fe(II) in the 0.5 M HCl phase, following previous work (Crosby *et al.*, 2005; Tangelos *et al.*, 2010; Percak-



**Fig. 3** Fe(II) production (circles and squares) and sulfate consumption (green triangles) during a 2-week culture in ASW medium with and without sulfate. Values represent the average of single measurements from duplicate cultures.

Dennett *et al.*, 2011). Mass balance for the solid phase sampled by 0.5 M HCl extraction is represented by the following equation.

$$\delta^{56}\text{Fe}_{\text{Fe(II)0.5 M HCl}} = X_{\text{Fe(II)0.5 M HCl}}\delta^{56}\text{Fe}_{\text{Fe(II)0.5 M HCl}} + X_{\text{Fe(III)0.5 M HCl}}\delta^{56}\text{Fe}_{\text{Fe(III)0.5 M HCl}} \quad (1)$$

Using  $\delta^{56}\text{Fe}_{\text{Fe(II)0.01 M HCl}}$  in place of  $\delta^{56}\text{Fe}_{\text{Fe(II)0.5 M HCl}}$  and rearranging the equation,  $\delta^{56}\text{Fe}_{\text{Fe(III)0.5 M HCl}}$  can be calculated using:

$$\delta^{56}\text{Fe}_{\text{Fe(III)0.5 M HCl}} = [\delta^{56}\text{Fe}_{\text{Fe(II)0.5 M HCl}} - (X_{\text{Fe(II)0.5 M HCl}}\delta^{56}\text{Fe}_{\text{Fe(II)0.01 M HCl}})] / X_{\text{Fe(III)0.5 M HCl}} \quad (2)$$

The calculations indicated that  $\delta^{56}\text{Fe}$  values for the solid-phase Fe(III) component sampled by the 0.5M HCl extraction increased slightly during Fe(III) reduction (Fig. 2C,D), as expected for Fe redox-driven Fe isotope fractionation where a greater proportion of Fe would exist in Fe(III) phases. Following Crosby *et al.* (2005, 2007), the solid Fe(III) component sampled in the 0.5 M HCl extraction can be considered the reactive Fe(III) pool in the solid that underwent at least partial Fe isotope exchange with aqueous and sorbed Fe(II).

Iron isotope fractionation factors between aqueous Fe (II) and HCl-extractable phases followed similar trends in both the Vent and Mid enrichments (Fig. 4E,F). The large fluctuations in the first 12–24 h correspond to periods of low extent of reduction (Fig. 4A,B). The ending fractionation factor between the Fe(II) components ( $\Delta^{56}\text{Fe}_{\text{Fe(II)aq-Fe(II)0.01}}$ ) was  $-0.69 \pm 0.13\text{‰}$  and  $-1.47 \pm 0.13\text{‰}$  for Vent and Mid, respectively. A larger ending fractionation occurred between the Fe(II) aq and Fe(III) phases

( $\Delta^{56}\text{Fe}_{\text{Fe(II)aq-Fe(III)0.5}}$ ) with values of  $-2.68 \pm 0.29\text{‰}$  and  $-3.37 \pm 0.30\text{‰}$  for Vent and Mid, respectively.

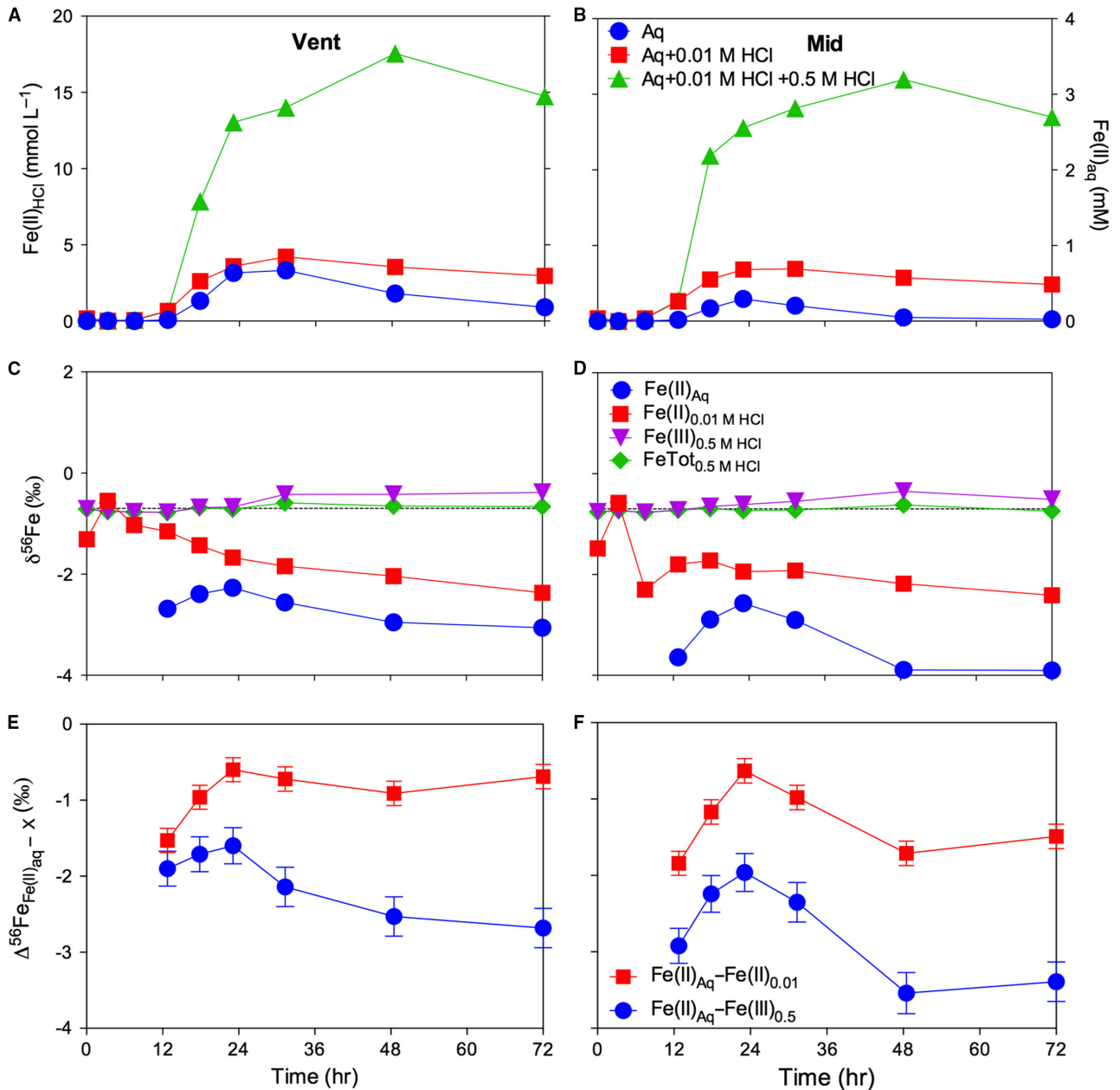
### Composition of enrichment cultures

DNA sequences from 16S rRNA gene amplicon pyrosequencing showed a similar assemblage of taxa in the Vent and Mid enrichment cultures. Over time, both cultures experienced a decrease in diversity where the 10 most dominant OTUs represented a greater proportion of all OTUs present in the cultures, reaching 80–90% by the end of the enrichment culturing experiment (Tables S1–S4). A noticeable shift in dominant taxa toward well-known and potential DIRB also occurred, with these taxa increasing dramatically in abundance relative to the corresponding identical (or near-identical) reads in the inoculum libraries (Table 3). The dominant OTU, comprising ca. 70% of all reads in Vent and 50% in Mid enrichment cultures after 46 weeks of transfers, was *Geobacter metallireducens* GS-15 [99% identity (Lovley *et al.*, 1993a)] and *Geobacter* sp. IST-3 [97% identity (Blöthe & Roden, 2009)], respectively (Fig. S4A,B, Table S5). In total, 26 and 51 different *Geobacter*-related OTUs were recovered from the 46 week Vent and Mid enrichment cultures, respectively.

An organism present in the inoculum sources, which remained prominent throughout the enrichment culturing campaign, is a distant relative of *Ignavibacterium* ( $t = 0$ ; 88% identity) or *Melioribacter* ( $t = 14, 30, 46$  week.; 92% identity) (Tables S1–S5) of the recently defined phylum *Ignavibacteriiae* within the Bacteroidetes/Chlorobi group (Podosokorskaya *et al.*, 2013). Previous research on *Ignavibacterium* showed it to be a complex heterotroph capable of fermentation and  $\text{H}_2$  oxidation (Liu *et al.*, 2012); however, recent studies have identified PCC in *Ignavibacterium* that are homologous to those found in *Geobacter* species (Liu *et al.*, 2014; Shi *et al.*, 2014). Initial studies of *Melioribacter* identified it as being able to use Fe(III) as a terminal electron acceptor (Kadnikov *et al.*, 2013; Podosokorskaya *et al.*, 2013). In total, one and two OTUs related to the families *Ignavibacteriaceae* or *Melioribacteriaceae*, respectively, were recovered from the 46 week Vent enrichment cultures, which together accounted for 1.47% of all reads; the corresponding values for the Mid enrichment were 1 and 9 OTUs which accounted for 3.14% of all reads. BLASTN results showed remaining dominant organisms to have relatively low percent similarity to other taxa including Fe(III)- and sulfate-reducing bacteria, for example, *Desulfohalobium*, *Anaeromyxobacter*, and *Thermodesulfovibrio* (Fig. S4A,B, Tables S4a,b).

### Metagenomic analysis of enrichment cultures

Illumina MiSeq shotgun metagenomic sequencing produced a total of 2 610 792 and 2 563 206 paired reads



**Fig. 4** (A, B) Aqueous and HCl-extractable Fe(II) production during the Fe isotope fractionation experiment. Red symbols represent the sum of aqueous and 0.01 M HCl-extractable Fe(II); green symbols represent the sum of aqueous, 0.01 M HCl-extractable, and 0.5 M HCl-extractable Fe(II). Note different Y-axis scales for aqueous Fe (right scale) and HCl extractions (left scale). (C, D)  $\delta^{56}\text{Fe}$  values for different Fe pools collected during the Fe isotope fractionation experiment. Fe(II)<sub>0.01</sub> represents  $\delta^{56}\text{Fe}$  for the 0.01 M HCl extraction, which was composed solely of Fe(II) after 12 h of incubation; FeTot<sub>0.5</sub> represents the measured  $\delta^{56}\text{Fe}$  for the 0.5 M HCl extraction, which was composed of a mixture of Fe(II) and Fe(III); Fe(III)<sub>0.5</sub> represents calculated  $\delta^{56}\text{Fe}$  of the pure Fe(III) component in the 0.5 M HCl extraction, inferred from mass balance, as described in the text. (E, F) Temporal variation in fractionation factor between aqueous Fe(II) (Fe(II)<sub>aq</sub>) and the 0.01 M HCl extraction (Fe(II)<sub>0.01</sub>), interpreted to reflect sorbed Fe(II), or the solid Fe(III) component contained in the 0.5 M HCl extraction (Fe(III)<sub>0.5</sub>). Error bars reflect 2 $\sigma$  uncertainty in measured Fe(II)<sub>aq</sub> and Fe(II)<sub>0.01 M HCl</sub>, and extrapolated uncertainty calculated for Fe(III)<sub>0.5 M HCl</sub>. All values represent a single measurement on a single enrichment culture.

from the Vent and Mid enrichment cultures, respectively, following trimming and merging. Seventy-five percent of reads from the Vent enrichment culture had an average

PHRED score above 35 and above 30 from the Mid enrichment culture. The Vent metagenome was assembled into 35 690 contigs with an average length of 1180 bp

**Table 3** Comparison of dominant taxa following 46-week enrichment in the presence of 2 mM acetate and 2 mM lactate when compared to the abundance of these taxa in Fe oxides used to inoculate Mid and Vent enrichments

Site	Taxa	Abundance in inoculum (number of reads)	Abundance in 46 week enrichment (number of reads)	% Identity*
Vent	<i>Geobacter metallireducens</i> strain GS-15	2 (<0.1%)	3033 (69.7%)	100.0
	<i>Geobacter daltonii</i> strain FRC-32	2 (<0.1%)	228 (5.25%)	96.88
	<i>Desulfohalobium retbaense</i> strain DSM 5692	25 (0.33%)	163 (3.75%)	99.63
	<i>Curvibacter delicatus</i> strain DHW-S121	7 (<0.1%)	123 (2.83%)	98.88
	<i>Clostridium</i> sp. 6-16	1 (<0.1%)	96 (2.21%)	98.88
	<i>Geobacter hydrogenophilus</i>	2 (<0.1%)	93 (2.14%)	97.44
	<i>Melioribacter roseus</i> strain P3M-2	10 (0.13%)	59 (1.36%)	99.63
	<i>Thermanaerotherix daxensis</i> strain GNS-1	260 (3.45%)	47 (1.08%)	98.52
	Anaerolineae bacterium SW7	1 (<0.1%)	36 (0.83%)	99.26
	Anaerolineae bacterium SW7	0	35 (0.81%)	N/A†
	<i>Ignavibacterium album</i> strain JCM 16511	86 (1.14%)	6 (0.14%)	98.88
	<i>Ignavibacterium album</i> strain JCM 16511	74 (0.98%)	1 (<0.1%)	85.71
	<i>Geobacter</i> sp. IST-3	4 (<0.1%)	4479 (52.0%)	100.0
	Anaerolineae bacterium SW7	2 (<0.1%)	618 (7.17%)	99.26
Mid	<i>Geobacter metallireducens</i> strain GS-15	2 (<0.1%)	414 (4.80%)	100.0
	<i>Thermodesulfovibrio hydrogenophilus</i> strain Hbr5	1 (<0.1%)	302 (3.50%)	99.63
	Bacterium sp. OF1	0	258 (2.99%)	N/A
	<i>Desulfohalobium retbaense</i> strain DSM 5692	5 (<0.1%)	245 (2.84%)	99.63
	<i>Treponema primitia</i> strain ZAS-2	0	217 (2.52%)	N/A
	<i>Melioribacter roseus</i> strain P3M-2	0	206 2.39%	N/A
	<i>Geobacter</i> sp OR-1	0	203 (2.36%)	N/A
	<i>Anaeromyxobacter</i> sp. Only E-B1	42 (0.66%)	156 (1.81%)	99.63
	<i>Ignavibacterium album</i> strain JCM 16511	68 (1.06%)	0	N/A

\*Pairwise alignments were used to calculate the percent identity between OTU sequences present in the inoculum and in the enrichment cultures.

†N/A = Not applicable, no corresponding read in inoculum or at 46 week.

and an N50 of 2072 bp. The Mid metagenome was assembled into 93 531 contigs with an average length of 920 bp and an N50 of 1149 bp.

Coarse bin assignments comparing coverage vs. %GC identified 11 taxonomic bins in the Vent metagenome and 13 in the Mid metagenome. Binning assignments were refined using CONCOCT, maintaining eight bins in the Vent metagenome and identifying additional bins in the Mid metagenome for a total of 15 (Fig. 5). The taxonomic identity of some bins remains poorly resolved due to uncertainties in putative taxonomy as identified using PhyloSift, and thus, the highest taxonomic rank identified for some bins was only class level. There is a high level of confidence in the identity of the bins of interest in both metagenomes (i.e., *Geobacter metallireducens* and *Melioribacter roseus*), based on high probability mass scores from the PhyloSift output.

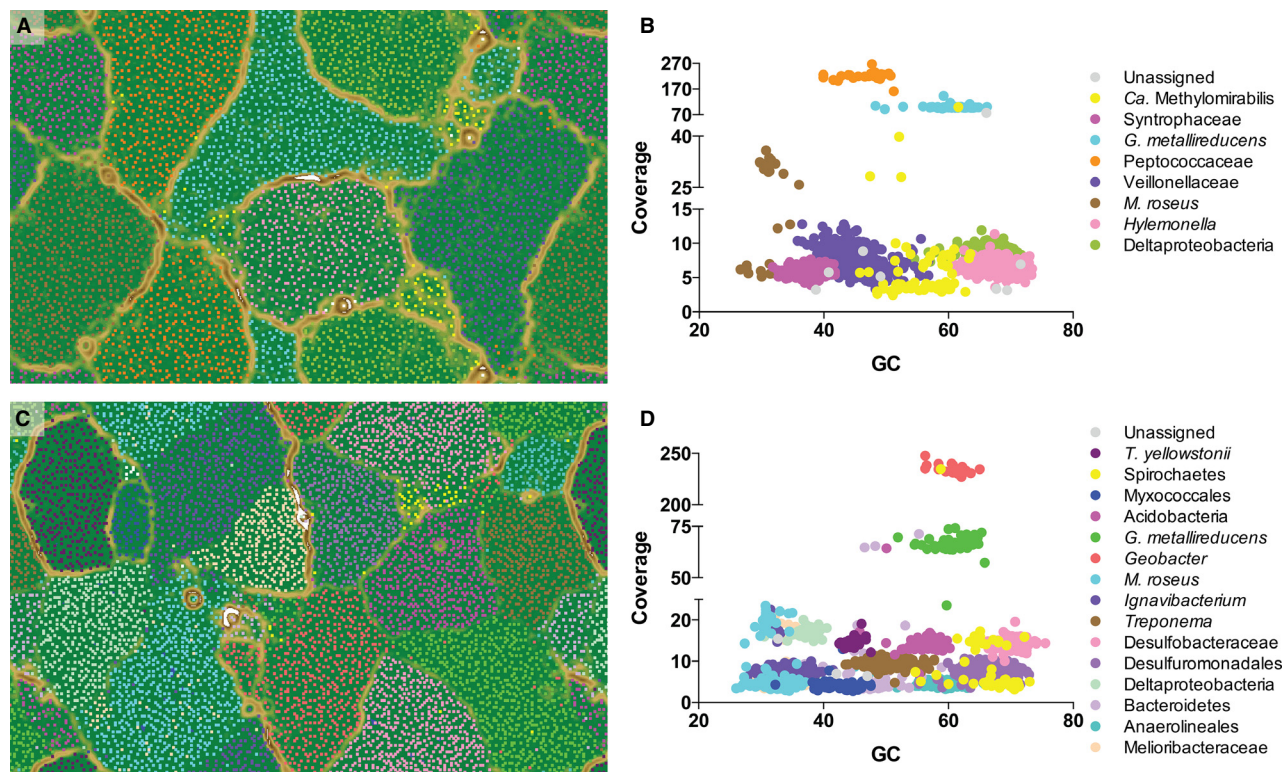
Four and seven nearly complete bins (completeness > 85%) were recovered from the Vent and Mid metagenomes (Table 4). Of these, two bins in the Vent metagenome and four in Mid metagenome contained putative 16S rRNA genes that corresponded to reads from the 46-week 16S rRNA gene amplicon library with an identity > 98%. With the exception of the *Geobacter metallireducens* bin in the Vent metagenome, the nearly complete bins containing 16S rRNA genes

were not closely matched to organisms within the GenBank database.

Heat-shock proteins (HSP) and molecular chaperone proteins involved with maintaining structure and function of other proteins under heat-stress were identified in several bins in both metagenomes (Table S7). The copy numbers of the small HSP, *hsp20*, in the *Geobacter metallireducens* bins from both the Vent and Mid metagenomes were greater and occurred as a tandem repeat with three copies arranged in a row. This arrangement is not seen in the type strain *Geobacter metallireducens* GS-15; however, it is seen in the *Geobacter sulfurreducens* PCA type strain (Fig. S5). An alternating arrangement of *hsp20*, *hsp70*, and *hsp20* was observed in both the Vent and Mid metagenomic bins, and in the type strain *Melioribacter roseus* (Fig. S6).

*mtrABC* genes were not identified in the metagenomic libraries. However, porins structurally homologous to the porin in PCC and in close proximity to a multiheme cytochrome were identified in both metagenomes and all type strains referenced including *Melioribacter roseus* P3M, which has not previously been investigated for this gene cluster (Fig. 6). PCC was present in bins identified as *Geobacter metallireducens* and *Melioribacter roseus* from the Vent and Mid metagenomes. It was also discovered in bins identified as Desulfobacteraceae, *Ignavibac-*





**Fig. 5** ESOM of metagenomic fragments from DNA extracted from Fe oxides sampled from the Vent (A) and Mid (C) sampling locations using CONCOCT binning approaches (5 kb window size, all contigs >3 kb were considered). Coverage vs. %GC content for bins identified in CONCOCT from the Vent (B) and Mid (D) metagenomes. Taxonomic assignments were made using PhyloSift. *Geobacter* and *G. metallireducens* from Mid (C, D) were binned manually using %GC and coverage due to being binned together in CONCOCT.

*terium*, and *Thermodesulfobivrio yellowstonii* in the Mid metagenome.

## DISCUSSION

Previous studies of other thermal springs in YNP have identified Fe-cycling microorganisms, including those involved in DIR (Kashefi *et al.*, 2002a; Kozubal *et al.*, 2012). Although prior studies have suggested that DIR could play a role in Fe redox cycling in the Fe(III) oxide deposits at CP hot springs, (Pierson *et al.*, 1999; Pierson & Parenteau, 2000; Wu *et al.*, 2013), to date there have been no explicit studies of organisms capable of DIR in this particular hydrothermal environment. The goal of this study was to obtain a first look at the potential for DIR in CP materials through a combination of MPN enumerations and enrichment culturing, and to assess the potential for Fe isotope fractionation during microbial reduction of the siliceous Fe(III) oxides native to CP. In addition, phylogenetic and metagenomic analysis of microorganisms in the enrichment cultures as well as from the natural environment revealed the presence of both well-known and novel putative Fe(III)-reducing taxa.

## Culture-based analysis of Fe(III) reduction potential

The MPN results were suggestive of an active heterotrophic Fe(III)-reducing community at CP (Table 1). The comparatively low estimates of H<sub>2</sub>-oxidizing DIRB and the failure of initial enrichment cultures to grow using only H<sub>2</sub> as an electron donor indicated an absence of culturable autotrophic DIR organisms. Nevertheless, DIR was active in cultures where H<sub>2</sub> served as the main electron donor with small quantities of acetate provided as a carbon source. Dominant taxa identified in the H<sub>2</sub>/Ac cultures were different from the other electron donor treatments (Fig. S4C). However, OTUs related to organisms previously shown to be capable of coupling H<sub>2</sub> oxidation to Fe(III) or sulfate reduction, including *Geobacter* sp. IST-3 (97% identity (Blöthe & Roden, 2009)) and *Desulfobalobium retbaense* [87% identity (Spring *et al.*, 2010)], were identified in enrichments both with and without H<sub>2</sub> as an electron donor (Tables S2a–d, Fig. S4C). No known autotrophic Fe(III) reducers or close relatives thereof were identified in any of the enrichments. This is perhaps not surprising given that the only known autotrophic H<sub>2</sub>-oxidizing Fe(III) reducers are hyperthermophilic Bacteria and Archaea (Vargas *et al.*, 1998; Kashefi *et al.*, 2002b, 2008;



**Table 4** Taxonomic assignment of enrichment culture metagenome bins

Site	CONCOCT Bin	PhyloSift phylogeny	Average coverage	Bin size (kbp)	Completeness (%)	Similarity to 16S amplicon library	%Identity to GenBank
Vent	1	<i>Candidatus Methyloirabilis</i>	8.48	377.4	5.4	N/A*	
	2	Syntrophaceae	5.86	1523.5	65.8	N/A	
	3	<i>Geobacter metallireducens</i>	100.07	3726.1	94.6	99.3	99
	4	Peptococcaceae	233.03	3326.8	95.5	N/A	
	5	Veillonellaceae	7.60	3205.5	67.6	N/A	
	6	<i>Melioribacter roseus</i>	19.62	3052.3	95.5	N/A	
	7	<i>Hylemonella</i>	6.54	1938.7	62.2	N/A	
Mid	8	Deltaproteobacteria	8.43	2332.0	89.2	100.00	92
	1	<i>Thermodesulfovibrio yellowstonii</i>	14.68	1965.0	92.8	98.9	89
	2	Spirochaetales	13.71	222.2	9.0	N/A	
	3	Myxococcales	4.21	318.3	8.1	N/A	
	4	Acidobacteria	14.74	2700.4	93.7	N/A	
	5	<i>Geobacter metallireducens</i>	231.50	2932.1	72.1	N/A	
	6	<i>Geobacter</i>	65.03	3209.4	95.5	N/A	
	7	<i>Melioribacter roseus</i>	8.32	3295.9	94.6	100.00	93
	8	<i>Ignavibacterium</i>	7.48	2849.3	83.8	N/A	
	9	<i>Treponema</i>	9.55	2259.9	86.5	N/A	
	10	Desulfobacteraceae	14.24	3927.0	93.7	98.90	82
	11	Desulfuromonadales	7.76	1939.9	78.4	N/A	
	12	Deltaproteobacteria	16.89	2250.5	95.5	98.9	87
	13	Bacteroidetes	5.46	546.3	29.7	N/A	
	14	Anaerolineales	4.73	476.3	17.1	N/A	
	15	Melioribacteraceae	5.53	1584.4	55.0	N/A	

\*N/A = Not applicable, metagenomic 16S rRNA gene sequence present on contig <3 kb and not binned.

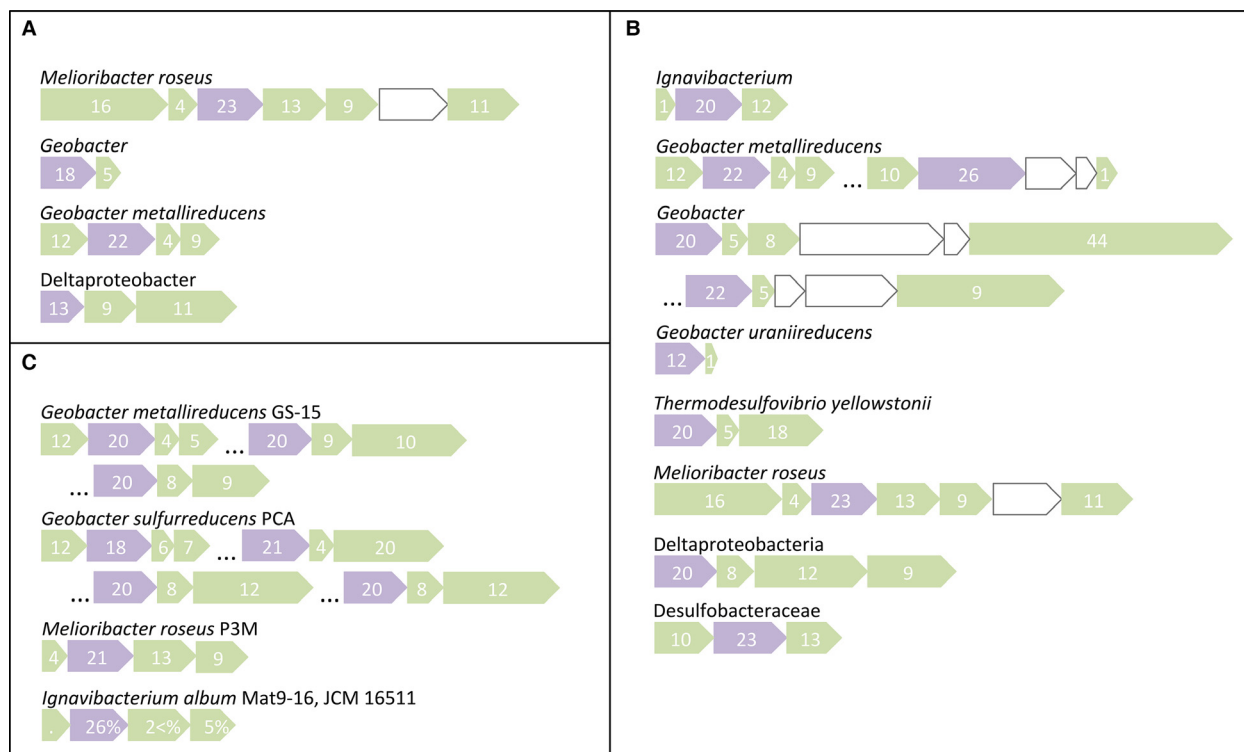
Kashefi & Lovley, 2003). Although methanogens have previously been implicated in reducing amorphous Fe(III) oxides (Van Bodegom *et al.*, 2004), and an OTU related the methanogen, *Methanobacterium oryzae* (98% identity (Joulian *et al.*, 2000)) was recovered in the H<sub>2</sub>-containing enrichment culture, this particular organism has not been reported as capable of reducing Fe(III) oxides.

Electron donor studies of acetate-only and lactate-only enrichments revealed similar levels of reduction between the two treatments, suggesting that both carbon sources were capable of serving as electron donor and carbon source in the cultures (Fig. 1). Interestingly, the microbial community was very similar between the Ac/Lac, Ac, and Lac treatments. Lactate is a commonly metabolized substrate by DIRB (Lovley *et al.*, 2004) and SRB (Fauque & Ollivier, 2004), both of which oxidize lactate partially to acetate followed by utilization of acetate. Organisms related to the known lactate oxidizing DIRB *Geobacter argillaceus* [98% identity (Shelobolina *et al.*, 2007)] were identified in the Lac enrichments, and were likely a major contributor to Fe(III) reduction observed in those cultures. OTUs related to organisms known to be unable to metabolize lactate [e.g., *Melioribacter roseus*, 92% identity (Podosokorskaya *et al.*, 2013)] were present in all enrichments (14 weeks onward) regardless of electron donor (Fig. S4A–C). It is likely that such organisms utilized acetate produced via partial lactate oxidation.

The appearance of *Geobacter metallireducens* as a dominant organism in the enrichments amended with acetate

(Fig. S4A,B) is not surprising given the prevalence of Geobacteraceae in Fe(III)-reducing environments worldwide (Lovley *et al.*, 2004). Moreover, the stimulation of *Geobacter* populations with the addition of acetate as an electron source has been well documented (Lovley *et al.*, 2004). The OTUs in the inoculum sources that corresponded to the *G. metallireducens* strain in the Vent and Mid enrichment cultures comprised only <0.1% (2/7538) and (2/6412) of the total microbial community, respectively. Additional abundant *Geobacter*-related OTUs (*G. daltonii* and *G. hydrogenophilus*) identified in the 46-week Vent enrichment culture were both present in the inocula source at <0.1% (2/7538). Other *Geobacter*-related OTUs that were abundant in the Mid enrichment culture were present in trace numbers (<0.1%, 4/6412 for *Geobacter* sp. IST-3), or absent altogether (*Geobacter* sp. OR-1) in the inoculum library. This suggests that the enrichment culturing selected for *Geobacter* and it was able to thrive and outcompete other DIRB that may have been present.

Another DIRB taxon that comprised a smaller but significant fraction of the enrichment culture communities was related to *Melioribacter roseus* (92% identity). *Melioribacter* is a member of the class *Ignavibacteria*, the sole taxonomic class in the newly identified phylum *Ignavibacteriae* within the Bacteroidetes–Chlorobi group (Podosokorskaya *et al.*, 2013). *Ignavibacteria* relatives were present in the inoculum sources and throughout the 46-week Fe(III)-reducing enrichment incubation. Although this population



**Fig. 6** Gene map of porin–cytochrome complex (PCC) in bins from the Vent (A) and Mid (B) metagenomes, and genomes of the type strains (C). Porins are denoted in purple with the number of transmembrane domains indicated by white numbering. Cytochromes are in green with the number of heme-binding sites indicated by white numbering. The taxonomic identity of contigs containing PCC correspond to taxonomic identity of bins as identified by Phylosift. The contigs labeled as *Geobacter* from the Vent metagenome (A), and *Geobacter uraniireducens* from the Mid metagenome (B) were <3 kb and not binned; taxonomic assignments were made using DOE-IMG. Ellipsis denotes spaces in bin where gene clusters are coded for on the same genome, but potentially as part of a different operon. Outlined genes in white are genes not involved with PCC.

was sustained through the enrichment culturing, there was a noticeable change in the abundance of OTUs representing this group during enrichment as compared to the inocula sources. Vent and Mid inocula contained abundant OTUs most closely related to the species *Ignavibacterium album* which decreased to a trace portion of the population by 46 weeks. Conversely, 46-week enrichments contained OTUs most closely related to the species *Melioribacter roseus* which was present in only trace numbers in the inoculum or absent altogether. This shift in diversity from the inoculum source to 46-week enrichment shows a replacement of the *Ignavibacterium* relatives by *Melioribacter* relatives. The presence of relatives of *Ignavibacteria* in the inocula and enrichment cultures as well as in recent un-enriched, *in vitro* Fe(III) reduction assays suggests that these organisms may be responsible for DIR *in situ* at CP (N.W. Fortney, S. He, B.J. Converse, B.L. Beard, C.M. Johnson, E.S. Boyd & E.E. Roden in prep.).

Identification of microorganisms capable of fermentation such as relatives of *Anaerolineae*, *Thermanacrotrix*, and *Clostridium* (89–92%, 92%, 85% identity, respectively; see Table S4) is not surprising given the *in situ* environment where organic matter from the cyanobacterial mat commu-

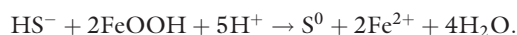
nities or exogenous carbon influx could be broken down into organic acids capable of supporting heterotrophic DIRB community members (Schubotz *et al.*, 2013, 2015; Urschel *et al.*, 2015). However, in the organic acid-amended enrichment culture media, their role is less obvious. Although many fermentative organisms are capable of diverting small amounts of reducing equivalents to Fe(III) reduction during fermentative growth (Lovley *et al.*, 2004), it is unknown whether the putative fermenters played a direct role in Fe(III) reduction in the enrichment cultures. The simplest explanation for the persistence of these organisms in the enrichments is that they were involved in depolymerization and fermentation of decaying microbial biomass.

### Potential role of sulfur cycling in Fe(III) reduction

A black, putative iron-monosulfide (FeS) precipitate was occasionally observed on the Fe oxide surface/spring water interface in the enrichment cultures. This, along with a distinct H<sub>2</sub>S odor observed during HCl extraction, was a possible indicator of sulfide-driven Fe(III) reduction mediated by SRB (Coleman *et al.*, 1993; Straub & Schink, 2004; Haveman *et al.*, 2008). These observations motivated an

in-depth study of the potential role of SRB in driving Fe(III) reduction in the cultures.

The sulfate present in the CP spring water used for the growth medium, ca. 0.3 mM, was relatively low and could have only accounted for <1 mmol L<sup>-1</sup> of Fe(II) produced by coupling abiotic reduction of Fe(III) to oxidation of sulfide through the well-known reaction (Poulton *et al.*, 2004):



Concentrations of reduced inorganic sulfur were very low in the Mid enrichments ( $\leq 0.15$  mmol L<sup>-1</sup>) and undetectable in Vent (data not shown), suggesting that large quantities of sulfide were not produced in the enrichment cultures. Although sulfur recycling linked to sulfur disproportionation (Thamdrup *et al.*, 1993) could have allowed for more Fe(III) reduction, 16S rRNA gene libraries did not support this possibility as sequences affiliated with putative elemental sulfur or thiosulfate disproportionators such as *Desulfocapsa* (Janssen *et al.*, 1996; Finster *et al.*, 1998) were not identified in the enrichment cultures. This conclusion was supported by XRD and Mossbauer analysis of culture solids which showed no detectable S<sup>0</sup> or iron-sulfide mineral phases (data not shown), the latter of which presumably would have ultimately been produced if reduced sulfur disproportionation had been primarily responsible for Fe(III) reduction (Thamdrup *et al.*, 1993).

In order to further constrain the possible contribution of DSR to overall Fe(III) reduction, transfers were made to ASW with and without 0.3 mM sulfate. In addition, a subset of cultures were amended with molybdate, a structural analog of sulfate that inhibits bacterial sulfate reduction (Peck, 1959). Differences in the Fe(II) produced between the sulfate-containing and no-sulfate cultures (Fig. 2) were minor in relation to variability observed in the earlier enrichment culture generations (Figs 1 and S2). In other words, the difference in the amount of Fe(II) produced with and without added sulfate suggests that DSR did not contribute substantially to overall Fe(III) reduction by means of abiotic sulfide-driven Fe(III) reduction. This conclusion is further confirmed by (i) the equivalent rates of Fe(III) reduction observed in sulfate-containing medium with and without molybdate (Fig. 2), and (ii) the relatively slow and incomplete consumption of sulfate over a 2-week culturing period (Fig. 3). In addition, microbial community composition was largely unchanged between sulfate-containing and no-sulfate cultures, although taxa related to known SRB (e.g., *Desulfobalobium*, *Thermodesulfovibrio*, and *Desulfosporosinus*) were present in both cultures (Tables S4 and S5). It should be noted that species of the genera *Desulfovibrio* (Lovley *et al.*, 1993b; Roden & Edmonds, 1997) and *Desulfosporosinus* (Ramamoorthy

*et al.*, 2006) have the ability to reduce Fe(III), and it is possible that the SRB relatives identified in the 16S rRNA gene amplicon libraries may have contributed directly to Fe(III) reduction in the enrichments.

### Genomic analysis of Fe(III)-reducing taxa

Assembly and binning of the Vent and Mid metagenomes produced four and seven nearly complete genomes, respectively. 16S rRNA genes are usually not included in metagenomic bins. This is due to poor assembly of 16S reads as a result of highly repetitive regions being co-assembled (Miller *et al.*, 2011; Treangen & Salzberg, 2012) and as a result the short length reads and contigs generally fall short of the 3 kb cutoff for binning. Longer length contigs can bin poorly due to differences in tetranucleotide frequency between the 16S rRNA gene and the rest of the genome (Kraft *et al.*, 2014). Two nearly complete bins from the Vent metagenome and four from the Mid metagenome contain 16S rRNA genes which allows for the comparison to earlier analysis of the 46-week 16S rRNA gene amplicon library. In all cases, the percent identity between the metagenomic and 16S rRNA gene amplicon reads were >98%, suggesting that the 16S rRNA genes recovered from the metagenomes were the same as in the amplicon library. Only the 16S rRNA genes from the *Geobacter metallireducens* bin and amplicon library 16S rRNA gene OTU were a close match (99% identity) to GenBank; all other sequences had identities <93%. This suggests that metagenomic sequencing was able to recover the nearly complete genome of five novel taxa from the Fe(III)-reducing enrichment cultures.

Type strains were picked because close relatives thereof were identified in the Vent and Mid 16S rRNA gene amplicon and metagenomic libraries and were of most interest in terms of their role in DIR and their potential role to inform understanding of the thermotolerant physiology they might possess at the mildly thermophilic temperatures of CP.

### Thermotolerance in Fe(III) reducing enrichment cultures

Even at the lowest incubation temperature (Mid, 40 °C), the environment at CP is warmer than the published optimal growth temperature for *G. metallireducens* of 30–35 °C (Lovley *et al.*, 1993a). Thermotolerance in microorganisms can be conferred in a variety of ways including possessing a genome with a higher %GC (Zheng & Wu, 2010) and by the expression of HSP chaperone proteins (Lindquist & Craig, 1988; Vabulas *et al.*, 2010) to assist in protein folding at elevated temperatures. Additional tolerance to heat-stress may be conferred by the amount of DNA supercoiling and DNA gyrase activity (Tse-Dinh *et al.*, 1997) or by increasing the concentration of cytoplasmic osmolytes (Amenabar *et al.*, 2015). Several adapta-

tions to life at higher temperatures including modifications to lipid [e.g., increased length of acyl chains or saturation of acyl lipids or the extent of isoprene lipid cyclization (Boyd *et al.*, 2011)] and protein structures (e.g., increased hydrophobicity of protein core) have been found in thermophilic microorganisms (Amenabar *et al.*, 2015). These adaptive mechanisms were not explored in detail in this study.

The metagenomic analysis of the enrichment cultures has provided information on how these organisms are able to survive and thrive at higher temperatures than previously reported for closely related strains. The %GC of *G. metallireducens* GS-15 upon isolation was reported as 56.6% (Lovley *et al.*, 1993a). 16S rRNA gene pyrosequencing revealed the OTUs of dominant organisms from the enrichment cultures to have a 99% identity to this strain. Shotgun metagenomic sequencing of the enrichments revealed a %GC of 61.4% and 60.3% of the contigs from the bins identified as *G. metallireducens* from the Vent and Mid enrichments, respectively. This is consistent with the idea that a higher %GC can confer a greater thermotolerance and suggests that the organism identified from the enrichments is a mildly thermophilic strain of *Geobacter metallireducens*.

Metagenomic analysis of the *Geobacter* bins revealed a greater number of HSPs than in the type strain of the organism (Table S7). Surprisingly, there were a greater number of these genes present in the bins from the Mid enrichment which was incubated at a lower temperature than the Vent enrichment. Binning was limited to contigs >3 kb in both enrichments, so it is possible that some of these HSP genes were encoded on smaller contigs and overlooked in the Vent bins. In the case of *hsp20*, three copies of the genes were arranged in a tandem repeat in both bins (Fig. S5). This arrangement is not present in the type strain, *G. metallireducens* GS-15; however, the same pattern is seen in the type strain *G. sulfurreducens* PCA (Fig. S5). It is possible that this arrangement of *hsp20* confers a greater level of heat tolerance in these organisms. Previous studies have shown that tandem gene duplications have allowed for increased utilization of a particular environmental constituent such as citrate (Blount *et al.*, 2012). Additionally, several studies of the model Eukaryotic organisms *Caenorhabditis elegans* and *Drosophila melanogaster* have demonstrated increased thermotolerance as a result of increased production of chaperone proteins due to increased gene copy number (Welte *et al.*, 1993; Lithgow *et al.*, 1995; Bettencourt *et al.*, 2008). The alternating arrangement of *hsp20* and *dnaK* in the *M. roseus* bins was the same as in the type strain *M. roseus* P3M (Fig. S6). *M. roseus* has been identified as a thermophile (Podosokorskaya *et al.*, 2013), making it reasonable to expect that the gene arrangement would be similar between metagenomic bins and the type strain.

### Extracellular electron transport (EET) in enrichment cultures

Interrogation of the enrichment culture metagenomes for the presence of genes involved in DIR, outer membrane cytochromes, and associated porins (Straub & Schink, 2003; Lovley *et al.*, 2004; Liu *et al.*, 2014; Shi *et al.*, 2014) was used to shed light on the potential role of these mechanisms in explaining DIR in the CP enrichment cultures. A number of genes encoding c-type cytochromes and proteins with heme-binding site motifs were identified in the enrichment culture metagenomes (Table S8) and have the potential to provide clues into the metabolism of poorly identified taxa and their role in metal cycling in the environment. While numerous taxa have been identified as DIRB (Lovley *et al.*, 2004), to date, the only two well-documented EET systems are *mtrABC* (Hartshorne *et al.*, 2009) and PCC (Liu *et al.*, 2014; Shi *et al.*, 2014). The focus of this investigation was on identifying genes involved with these two systems.

Neither the 16S rRNA gene amplicon libraries nor the metagenomic binning results suggested the presence of a *Shewanella* relative, and perhaps unsurprisingly *mtrABC* homologs were not identified in the Vent and Mid metagenomes. This is in contrast to PCC, which was identified in multiple bins in both metagenomes, in particular in genomes representing potentially new and novel taxa.

Porin–cytochrome complex porins were identified at four loci in the Vent metagenome and 10 in Mid metagenome (Fig. 6A,B). Genes were located on contigs in bins related to *G. metallireducens* and *M. roseus* in both metagenomes as was expected, as well as in other unexpected bins identified as Desulfobacteraceae, and *Thermodesulfobivibrio yellowstonii* in the Mid metagenome. *T. yellowstonii* and taxa from the order Desulfobacteraceae are documented sulfate reducers (Rabus *et al.*, 2006). Unsurprisingly, a homolog to the PCC porin was also identified in the bin related to *Ignavibacterium* in the Mid metagenome, and is consistent with previous studies (Liu *et al.*, 2014; Shi *et al.*, 2014). To date, none of these organisms have been reported as DIRB. The presence of PCC is not necessarily indicative of DIR as is the case for *Ignavibacterium album*, and PCC may be involved in EET with other substrates (Shi *et al.*, 2014). Arrangement of the porins and cytochromes in PCC, and the number of transmembrane regions and heme-binding sites differs in the metagenomic bins and their reference type strains. This is reasonable to expect as the organisms present in the enrichment cultures are only relatives of the type strains.

### Potential novel Fe(III)/sulfate-reducing organisms

Homologs of genes involved in DSR were present in two bins in the Vent metagenome, and five bins in the Mid



metagenome. A full complement of the genes and subunits comprising the DSR pathway, *apsAB*, *sat*, and *dsrAB*, was only identified in two bins in the Mid metagenome, *Thermodesulfovibrio yellowstonii* and *Ignavibacteriales*. This is reasonable to expect for *Thermodesulfovibrio*, a documented sulfate reducer (Rabus *et al.*, 2006); however, this pathway has not previously been observed in *Ignavibacterium*. Additionally, this is the first recorded occurrence of this pathway in the deeply branching Bacteroidetes/Chlorobi group. Although the ability of these organisms to reduce Fe(III) and sulfate were not interrogated directly, the presence of DSR pathways and PCC in both reconstructed genomes suggests the possibility that these relatives of *Thermodesulfovibrio* and *Ignavibacterium* identified in the Mid metagenome may be capable of both DIR and DSR.

### Implications for the *in situ* Fe redox cycle

The robust Fe(III) reduction activity observed in the MPN and subsequent enrichment culturing experiments clearly demonstrated the presence of DIRB native to CP in the enrichment inocula. Recent core samples collected from CP have shown significant quantities of Fe(II) in materials deposited in the vicinity of the Vent outflow. This, along with *in vitro* Fe(III) reduction assays showing similar levels of Fe(II) production in the presence and absence of added electron donor (N.W. Fortney *et al.*, in prep.), suggest that Fe(III) reduction is active *in situ* at CP. These results are consistent with previous speculation that DIR plays a role in the Fe redox cycle at CP (Pierson *et al.*, 1999; Pierson & Parenteau, 2000; Wu *et al.*, 2013). Although well-known DIRB were identified in the enrichment cultures, the enrichment conditions provided a selective advantage for these organisms, allowing them to outcompete the DIRB likely to be active *in situ*. The lack of putative DIRB in the enrichment inocula source (Tables S1a,b), and *in vitro* reduction assay communities (N.W. Fortney *et al.*, in prep.), support the idea of a novel DIR community at CP. The consistent representation of certain taxa previously unknown as DIRB in the enrichment inoculum sources (Tables S1a,b), 46-week enrichment (Tables S4a,b), and *in vitro* libraries (N.W. Fortney *et al.*, in prep.) suggest that relatives from the order *Ignavibacteriales* may be at least partly responsible for DIR *in situ*.

Aqueous and HCl-extractable Fe from the enrichment cultures demonstrated production of millimolar quantities of isotopically light Fe(II) (Fig. 4A–D). These large quantities of mobile (aqueous phase) and potentially mobile (0.01 M HCl extractable) Fe(II) have the potential to be preserved in the deposits at CP, leaving an isotopic signature of DIR activity. The inflections in  $\delta^{56}\text{Fe}$  values for Fe(II)<sub>aq</sub> for time periods <24 h, when the Fe(II) inventory is small, likely reflects kinetic Fe isotope fractionations prior

to establishment of Fe isotope equilibrium (Friedrich *et al.*, 2014). Over longer time periods, the isotopic fractionation between Fe(II)<sub>aq</sub> and “sorbed” Fe(II), as inferred from the 0.01 M HCl extractions, lies between  $-1$  and  $-1.5\text{‰}$ , which lies in the range measured by Wu *et al.* (2012) for Fe-Si co-precipitates, but is significantly larger than that measured during DIR of Fe-Si co-precipitates (Percak-Dennett *et al.*, 2011). It remains unclear whether these differences lie in the nature of the Fe-Si solids used in the current study relative to the previous work, or kinetic isotope effects. Over time, the isotopic fractionation between Fe(II)<sub>aq</sub> and the solid reactive Fe(III) component, as estimated using 0.5 M HCl extractions, averaged ca.  $-3\text{‰}$ , which lies within the range of those measured for Fe-Si co-precipitates by Wu *et al.* (2012), although variations in Fe:Si ratios can exert a secondary, but significant effect. Given these observations, production of Fe(II)<sub>aq</sub> by DIR would catalyze Fe isotope fractionations among the reactive Fe pools. Successful identification of Fe isotope fractionation patterns indicative of DIR in CP will aid in interpretation of isotopic signatures left in the rock record on Earth and potentially other rocky planets. Modern hydrothermal systems similar to CP have been used as analogs to ancient features on Mars where hydrothermal activity has resulted in sulfate mineral formation due to groundwater acidification from volcanic outgassing (Bishop *et al.*, 2004). Environments such as these are useful for gaining insight into ancient life on Earth and Mars (Shock, 1996). In particular, it seems feasible that signatures of DIR or other microbial Fe redox transformation processes could be preserved in relic (ancient) near-surface hydrothermal vent systems that have been identified on the Martian surface (e.g., see Marion *et al.*, 2011).

### ACKNOWLEDGMENTS

This work was supported by the NASA Astrobiology Institute. We thank H. Xu (University of Wisconsin-Madison) for XRD analysis, and C. Gorski (Pennsylvania State University) for Mossbauer analysis, of the CP materials.

### REFERENCES

- Albertsen M, Hugenholtz P, Skarshewski A, Nielsen KRL, Tyson GW, Nielsen PH (2013) Genome sequences of rare, uncultured bacteria obtained by differential coverage binning of multiple metagenomes. *Nature Biotechnology* **31**, 533–538.
- Allen ET, Day AL (1935) *Hot Springs of the Yellowstone National Park*. Carnegie Institution of Washington, Washington, DC.
- Alneberg J, Bjarnason BSR, De Bruijn I, Schirmer M, Quick J, Ijaz UZ, Lahti L, Loman NJ, Andersson AF, Quince C (2014) Binning metagenomic contigs by coverage and composition. *Nature Methods* **11**, 1144–1146.
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403–410.



- Amenabar MJ, Urschel MR, Boyd ES (2015) Metabolic and taxonomic diversification in continental magmatic hydrothermal systems. In *Microbial Evolution under Extreme Conditions* (ed. Bakermans C). Walter de Gruyter GmbH & Co KG, Boston, pp. 57–95.
- Balch WE, Wolfe RS (1976) New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of methanobacterium ruminantium in a pressurized atmosphere. *Applied and Environment Microbiology* **32**, 781–791.
- Beard BL, Johnson CM (1999) High precision iron isotope measurements of terrestrial and lunar materials. *Geochimica et Cosmochimica Acta* **63**, 1653–1660.
- Beard BL, Johnson CM (2004) Fe isotope variations in the modern and ancient earth and other planetary bodies. In: *Geochemistry of Non-Traditional Stable Isotopes* (eds Beard BL, Johnson CM, de Albarè F). Rev. in Mineralogy and Geochemistry 55, Mineralogical Society of America and Geochemical Society, Washington, DC, pp. 319–357.
- Beard BL, Johnson CM, Cox L, Sun H, Nealson KH, Aguilar C (1999) Iron isotope biosignatures. *Science* **285**, 1889–1892.
- Beard BL, Johnson CM, Skulan JL, Nealson KH, Cox L, Sun H (2003) Application of Fe isotopes to tracing the geochemical and biological cycling of Fe. *Chemical Geology* **195**, 87–117.
- Bettencourt BR, Hogan CC, Nimali M, Drohan BW (2008) Inducible and constitutive heat shock gene expression responds to modification of Hsp70 copy number in *Drosophila melanogaster* but does not compensate for loss of thermotolerance in Hsp70 null flies. *BMC Biology* **6**, 15.
- Bird LJ, Bonnefoy V, Newman DK (2011) Bioenergetic challenges of microbial iron metabolisms. *Trends in Microbiology* **19**, 330–340.
- Bishop JL, Murad E, Lane MD, Mancinelli RL (2004) Multiple techniques for mineral identification on Mars. *Icarus* **169**, 311–323.
- Blöthe M, Roden EE (2009) Microbial iron redox cycling in a circumneutral-pH groundwater seep. *Applied and Environment Microbiology* **75**, 468–473.
- Blount ZD, Barrick JE, Davidson CJ, Lenski RE (2012) Genomic analysis of a key innovation in an experimental *E. coli* population. *Nature* **489**, 513–518.
- Boyd ES, Pearson A, Pi Y, Li WJ, Zhang YG, He L, Zhang CL, Geesey GG (2011) Temperature and pH controls on glycerol dibiphytanyl glycerol tetraether lipid composition in the hyperthermophilic crenarchaeon *Acidilobus sulfurreducens*. *Extremophiles: Life under Extreme Conditions*, **15**, 59–65.
- Caporaso JG, Bittinger K, Bushman FD, Desantis TZ, Andersen GL, Knight R (2010a) PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* **26**, 266–267.
- Caporaso JG, Kuczynski J, Strombaugh J, Bittinger K, Bushman FD, Costello EK (2010b) QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* **7**, 335–336.
- Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences of the United States of America* **108**(Suppl 1), 4516–4522.
- Cavicchioli R (2002) Extremophiles and the search for extraterrestrial life. *Astrobiology* **2**, 281–292.
- Cline JD (1969) Spectrophotometric determination of hydrogen sulfide in natural waters. *Limnology and Oceanography*, **14**, 454–458.
- Coleman ML, Hedrick DB, Lovley DR, White DC, Pye K (1993) Reduction of Fe(III) in sediments by sulphate-reducing bacteria. *Nature* **361**, 436–438.
- Crosby HA, Johnson CM, Roden EE, Beard BL (2005) Coupled Fe(II)–Fe(III) electron and atom exchange as a mechanism for Fe isotope fractionation during dissimilatory iron oxide reduction. *Environmental Science and Technology* **39**, 6698–6704.
- Crosby HA, Roden EE, Johnson CM, Beard BL (2007) The mechanisms of iron isotope fractionation produced during dissimilatory Fe(III) reduction by *Shewanella putrefaciens* and *Geobacter sulfurreducens*. *Geobiology* **5**, 169–189.
- Darling AE, Jospin G, Lowe E, Matsen FAT, Bik HM, Eisen JA (2014) PhyloSift: phylogenetic analysis of genomes and metagenomes. *PeerJ* **2**, e243.
- Dauphas N, Craddock PR, Asimow PD, Bennett VC, Nutman AP, Ohnenstetter D (2009) Iron isotopes may reveal the redox conditions of mantle melting from Archean to Present. *Earth and Planetary Science Letters* **288**, 255–267.
- Dick GJ, Andersson AF, Baker BJ, Simmons SL, Thomas BC, Yelton AP, Banfield JF (2009) Community-wide analysis of microbial genome sequence signatures. *Genome Biology* **10**, R85.
- Eddy SR (2009) A new generation of homology search tools based on probabilistic inference. In: *Genome Informatics* (eds Morishita S, Lee SY, Sakakibara Y). Imperial College Press, London, pp. 205–211.
- Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**, 2460–2461.
- Emerson D, Revsbech NP (1994) Investigation of an iron-oxidizing microbial mat community located near Aarhus, Denmark: laboratory studies. *Applied and Environment Microbiology* **60**, 4032–4038.
- Fauque G, Ollivier B (2004) Anaerobes: the sulfate-reducing bacteria as an example of metabolic diversity. In *Microbial Diversity and Bioprospecting* (ed. Bull AT). ASM Press, Washington, D.C., pp. 169–176.
- Finn RD, Clements J, Eddy SR (2011) HMMER web server: interactive sequence similarity searching. *Nucleic Acids Research* **39**, W29–W37.
- Finster K, Liesack W, Thamdrup B (1998) Elemental sulfur and thiosulfate disproportionation by *Desulfocapsa sulfoexigens* sp. nov., a new anaerobic bacterium isolated from marine surface sediment. *Applied and Environment Microbiology* **64**, 119–125.
- Fossing H, Jørgensen BB (1989) Measurement of bacterial sulfate reduction in sediments: evaluation of a single-step chromium reduction method. *Biogeochemistry* **8**, 205–222.
- Friedrich AJ, Beard BL, Reddy TR, Scherer MM, Johnson CM (2014) Iron isotope fractionation between aqueous Fe(II) and goethite revisited: new insights based on a multi-direction approach to equilibrium and isotopic exchange rate modification. *Geochimica et Cosmochimica Acta* **139**, 383–398.
- Haas BJ, Gevers D, Earl AM, Feldgarden M, Ward DV, Giannoukos G, Ciulla D, Tabbaa D, Highlander SK, Sodergren E, Methe B, Desantis TZ, Human Microbiome C, Petrosino JF, Knight R, Birren BW (2011) Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Research* **21**, 494–504.
- Hartshorne RS, Reardon CL, Ross D, Nueter J, Clarke TA, Gates AJ, Mills PC, Fredrickson JK, Zachara JM, Shi L, Beliaev AS, Marshall MJ, Tien M, Brantley S, Butt JN, Richardson DJ (2009) Characterization of an electron conduit between bacteria and the extracellular environment. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 22169–22174.

- Haveman SA, Didonato RJ Jr, Villanueva L, Shelobolina ES, Postier BL, Xu B, Liu A, Lovley DR (2008) Genome-wide gene expression patterns and growth requirements suggest that *Pelobacter carbinolicus* reduces Fe(III) indirectly via sulfide production. *Applied and Environment Microbiology* **74**, 4277–4284.
- Janssen PH, Schuhmann A, Bak F, Liesack W (1996) Disproportionation of inorganic sulfur compounds by the sulfate-reducing bacterium *Desulfocapsa thiozymogenes* gen. nov., sp. nov. *Archives of Microbiology* **166**, 184–192.
- Johnson CM, Beard BL, Roden EE (2008) The iron isotope fingerprints of redox and biogeochemical cycling in modern and ancient earth. *Annual Review of Earth and Planetary Sciences* **36**, 457–493.
- Joulian C, Patel BKC, Ollivier B, Garcia J-L, Roger PA (2000) *Methanobacterium oryzae* sp. nov., a novel methanogenic rod isolated from a Philippines ricefield. *International Journal of Systematic and Evolutionary Microbiology* **50**, 525–528.
- Kadnikov VV, Mardanov AV, Podosokorskaya OA, Gavrillov SN, Kublanov IV, Beletsky AV, Bonch-Osmolovskaya EA, Ravin NV (2013) Genomic analysis of *Melioribacter roseus*, facultatively anaerobic organotrophic bacterium representing a novel deep lineage within Bacteroidetes/Chlorobi group. *PLoS One* **8**, e53047.
- Kashefi K, Lovley DR (2003) Extending the upper temperature limit for life. *Science* **301**, 934.
- Kashefi K, Holmes DE, Reysenbach AL, Lovley DR (2002a) Use of Fe(III) as an electron acceptor to recover previously uncultured hyperthermophiles: isolation and characterization of *Geothermobacterium ferrireducens* gen. nov., sp. nov. *Applied and Environment Microbiology* **68**, 1735–1742.
- Kashefi K, Tor JM, Holmes DE, Gaw Van Praagh CV, Reysenbach A-L, Lovley DR (2002b) *Geoglobus ahangari* gen. nov., sp. nov., a novel hyperthermophilic archaeon capable of oxidizing organic acids and growing autotrophically on hydrogen with Fe(III) serving as the sole electron acceptor. *International Journal of Systematic and Evolutionary Microbiology* **52**, 719–728.
- Kashefi K, Shelobolina ES, Elliott WC, Lovley DR (2008) Growth of thermophilic and hyperthermophilic Fe(III)-reducing microorganisms on a ferruginous smectite as the sole electron acceptor. *Applied and Environment Microbiology* **74**, 251–258.
- Kozubal MA, Macur RE, Jay ZJ, Beam JP, Malfatti SA, Tringe SG, Kocar BD, Borch T, Inskeep WP (2012) Microbial iron cycling in acidic geothermal springs of Yellowstone National Park: integrating molecular surveys, geochemical processes, and isolation of novel Fe-active microorganisms. *Frontiers in Microbiology* **3**, 109.
- Kraft B, Tegetmeyer HE, Sharma R, Klotz MG, Ferdelman TG, Hettich RL, Geelhoed JS, Strous M (2014) Nitrogen cycling. The environmental controls that govern the end product of bacterial nitrate respiration. *Science* **345**, 676–679.
- Lindquist S, Craig EA (1988) The heat-shock proteins. *Annual Review of Genetics* **22**, 631–677.
- Lithgow GJ, White TM, Melov S, Johnson TE (1995) Thermotolerance and extended life-span conferred by single-gene mutations and induced by thermal stress. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 7540–7544.
- Liu Z, Frigaard N-U, Vogl K, Iino T, Ohkuma M, Overmann JR, Bryant DA (2012) Complete genome of *Ignicoccus hospitalis*, a metabolically versatile, flagellated, facultative anaerobe from the phylum Chlorobi. *Frontiers in Microbiology* **3**, 1–15.
- Liu Y, Wang Z, Liu J, Levar C, Edwards MJ, Babauta JT, Kennedy DW, Shi Z, Beyenal H, Bond DR, Clarke TA, Butt JN, Richardson DJ, Rosso KM, Zachara JM, Fredrickson JK, Shi L (2014) A trans-outer membrane porin-cytochrome protein complex for extracellular electron transfer by *Geobacter sulfurreducens* PCA. *Environmental Microbiology Reports* **6**, 776–785.
- Lovley DR, Giovannoni SJ, White DC, Champine JE, Phillips EJP, Gorby YA, Goodwin S (1993a) *Geobacter metallireducens* gen. nov. sp. nov., a microorganism capable of coupling the complete oxidation of organic compounds to the reduction of iron and other metals. *Archives of Microbiology* **159**, 336–344.
- Lovley DR, Roden EE, Phillips EJP, Woodward JC (1993b) Enzymatic iron and uranium reduction by sulfate-reducing bacteria. *Marine Geology* **113**, 41–53.
- Lovley DR, Holmes DE, Nevin KP (2004) Dissimilatory Fe(III) and Mn(IV) reduction. *Advances in Microbial Physiology* **49**, 219–286.
- Luan F, Gorski CA, Burgos WD (2014) Thermodynamic controls on the microbial reduction of iron-bearing nontronite and uranium. *Environmental Science and Technology* **48**, 2750–2758.
- Marion GM, Catling DC, Crowley JK, Kargel JS (2011) Modeling hot spring chemistries with applications to martian silica formation. *Icarus* **212**, 629–642.
- Mavromatis K, Ivanova NN, Chen IM, Szeto E, Markowitz VM, Kyrpides NC (2009) The DOE-JGI standard operating procedure for the annotations of microbial genomes. *Standards in Genomic Sciences* **1**, 63–67.
- McCleskey RB, Nordstrom DK, Susong DD, Ball JW, Holloway JM (2010) Source and fate of inorganic solutes in the Gibbon River, Yellowstone National Park, Wyoming, USA. *Journal of Volcanology and Geothermal Research* **193**, 189–202.
- McDonald D, Price MN, Goodrich J, Nawrocki EP, Desantis TZ, Probst A, Andersen GL, Knight R, Hugenholtz P (2012) An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *The ISME Journal* **6**, 610–618.
- Miller CS, Baker BJ, Thomas BC, Singer SW, Banfield JF (2011) EMIRGE: reconstruction of full-length ribosomal genes from microbial community short read sequencing data. *Genome Biology* **12**, R44.
- Oremland RS, Capone DG (1988) Use of “specific” inhibitors in biogeochemistry and microbial ecology. In: *Advances in Microbial Ecology* (ed. Marshall KC). Plenum, New York, pp. 285–383.
- Parenteau MN, Cady SL (2010) Microbial biosignatures in iron-mineralized phototrophic mats at Chocolate Pots Hot Springs, Yellowstone National Park, United States. *Palaios* **25**, 97–111.
- Peck HD (1959) The ATP-dependent reduction of sulfate with hydrogen in extracts of *Desulfovibrio desulfuricans*. *Proceedings of the National Academy of Sciences of the United States of America* **45**, 701–708.
- Percak-Dennett EM, Beard BL, Xu H, Konishi H, Johnson CM, Roden EE (2011) Iron isotope fractionation during microbial dissimilatory iron oxide reduction in simulated Archean seawater. *Geobiology* **9**, 205–220.
- Pierson BK, Parenteau MN (2000) Phototrophs in high iron microbial mats: microstructure of mats in iron-depositing hot springs. *FEMS Microbiology Ecology* **32**, 181–196.
- Pierson BK, Parenteau MN, Griffin BM (1999) Phototrophs in high-iron-concentration microbial mats: physiological ecology of phototrophs in an iron-depositing hot spring. *Applied and Environment Microbiology* **65**, 5474–5483.

- Podosokorskaya OA, Kadnikov VV, Gavrillov SN, Mardanov AV, Merkel AY, Karnachuk OV, Ravin NV, Bonch-Osmolovskaya EA, Kublanov IV (2013) Characterization of *Melioribacter roseus* gen. nov., sp. nov., a novel facultatively anaerobic thermophilic cellulolytic bacterium from the class Ignavibacteria, and a proposal of a novel bacterial phylum Ignavibacteriae. *Environmental Microbiology* **15**, 1759–1771.
- Poulton SW (2003) Sulfide oxidation and iron dissolution kinetics during the reaction of dissolved sulfide with ferrihydrite. *Chemical Geology* **202**, 79–94.
- Poulton SW, Krom MD, Raiswell R (2004) A revised scheme for the reactivity of iron (oxyhydr)oxide minerals towards dissolved sulfide. *Geochimica et Cosmochimica Acta* **68**, 3703–3715.
- Rabus R, Hansen TA, Widdel F (2006) Dissimilatory sulfate- and sulfur-reducing prokaryotes. In: *The Prokaryotes* (eds Dworkin M, Falkow S, Rosenberg E, Schliefer KH, Stackebrandt E). Springer, New York, pp. 659–768.
- Ramamoorthy S, Sass H, Langner H, Schumann P, Kroppenstedt RM, Spring S, Overmann J, Rosenzweig RF (2006) *Desulfosporosinus lacus* sp. nov., a sulfate-reducing bacterium isolated from pristine freshwater lake sediments. *International Journal of Systematic and Evolutionary Microbiology* **56**, 2729–2736.
- Reeder J, Knight R (2010) Rapidly denoising pyrosequencing amplicon reads by exploiting rank-abundance distributions. *Nature Methods* **7**, 668–669.
- Roden EE, Edmonds JW (1997) Phosphate mobilization in iron-rich anaerobic sediments: microbial Fe (III) oxide reduction versus iron-sulfide formation. *Archiv für Hydrobiologie* **139**, 347–378.
- Roden EE, Sobolev D, Glazer B, Luther GW (2004) Potential for microscale bacterial Fe redox cycling at the aerobic-anaerobic interface. *Geomicrobiology Journal* **21**, 379–391.
- Roden EE, Mcbeth JM, Blothe M, Percak-Dennett EM, Fleming EJ, Holyoke RR, Luther GW 3rd, Emerson D, Schieber J (2012) The microbial ferrous wheel in a neutral pH groundwater seep. *Frontiers in Microbiology* **3**, 172.
- Rowe JJ, Fournier RO, Morey GW (1973) Chemical analysis of thermal waters in Yellowstone National Park, Wyoming, 1960–65. *Geological Survey Bulletin* **1303**, 31.
- Schubotz F, Meyer-Dombard DR, Bradley AS, Fredricks HF, Hinrichs KU, Shock EL, Summons RE (2013) Spatial and temporal variability of biomarkers and microbial diversity reveal metabolic and community flexibility in Streamer Biofilm Communities in the Lower Geyser Basin, Yellowstone National Park. *Geobiology* **11**, 549–569.
- Schubotz F, Hays LE, Meyer-Dombard DR, Gillespie A, Shock EL, Summons RE (2015) Stable isotope labeling confirms mixotrophic nature of streamer biofilm communities at alkaline hot springs. *Frontiers in Microbiology* **6**, 42.
- Shelobolina ES, Nevin KP, Blakeney-Hayward JD, Johnsen CV, Plaia TW, Krader P, Woodard T, Holmes DE, Vanpraagh CG, Lovley DR (2007) *Geobacter pickeringii* sp. nov., *Geobacter argillaceus* sp. nov. and *Pelosinus fermentans* gen. nov., sp. nov., isolated from subsurface kaolin lenses. *International Journal of Systematic and Evolutionary Microbiology* **57**, 126–135.
- Shi L, Fredrickson JK, Zachara JM (2014) Genomic analyses of bacterial porin-cytochrome gene clusters. *Frontiers in Microbiology* **5**, 657.
- Shock EL (1996) Hydrothermal systems as environments for the emergence of life. In: *Evolution of Hydrothermal Ecosystems on Earth (and Mars?)* (eds Bock GR, Goode JA). John Wiley & Sons, Ltd., Chichester, UK, pp. 40–60.
- Sobolev D, Roden EE (2002) Evidence for rapid microscale bacterial redox cycling of iron in circumneutral environments. *Antonie van Leeuwenhoek* **81**, 587–597.
- Spring S, Nolan M, Lapidus A, Glavina Del Rio T, Copeland A, Tice H, Cheng JF, Lucas S, Land M, Chen F, Bruce D, Goodwin L, Pitluck S, Ivanova N, Mavromatis K, Mikhailova N, Pati A, Chen A, Palaniappan K, Hauser L, Chang YJ, Jeffries CD, Munk C, Kiss H, Chain P, Han C, Brettin T, Detter JC, Schuler E, Goker M, Rohde M, Bristow J, Eisen JA, Markowitz V, Hugenholtz P, Kyrpides NC, Klenk HP (2010) Complete genome sequence of *Desulfohalobium retbaense* type strain (HR(100)). *Standards in Genomic Sciences* **2**, 38–48.
- Stajich JE, Block D, Boulez K, Brenner SE, Chervitz SA, Dagdigan C, Fuellen G, Gilbert JGR, Korf I, Lapp H, Lehväslaiho H, Matsalla C, Mungall CJ, Osborne BI, Pocock MR, Schattner P, Senger M, Stein LD, Stupka E, Wilkinson MD, Birney E (2002) The Bioperl toolkit: perl modules for the life sciences. *Genome Research* **12**, 1611–1618.
- Stookey LL (1970) Ferrozine – a new spectrophotometric reagent for iron. *Analytical Chemistry* **42**, 779–781.
- Straub KL, Schink B (2003) Evaluation of electron-shuttling compounds in microbial ferric iron reduction. *FEMS Microbiology Letters* **220**, 229–233.
- Straub KL, Schink B (2004) Ferrihydrite-dependent growth of *Sulfurospirillum deleyianum* through electron transfer via sulfur cycling. *Applied and Environment Microbiology* **70**, 5744–5749.
- Stumm W, Morgan JJ (1996) *Aquatic Chemistry*. John Wiley and Sons, New York.
- Tangalos GE, Beard BL, Johnson CM, Alpers CN, Shelobolina ES, Xu H, Konishi H, Roden EE (2010) Microbial production of isotopically light iron(II) in a modern chemically precipitated sediment and implications for isotopic variations in ancient rocks. *Geobiology* **8**, 197–208.
- Taylor SR, McLennan SM (1985) *The Continental Crust: Its Composition and Evolution*. Blackwell Scientific Publications, Oxford.
- Thamdrup B, Finster K, Hansen JW, Bak F (1993) Bacterial disproportionation of elemental sulfur coupled to chemical reduction of iron or manganese. *Applied and Environment Microbiology* **59**, 101–108.
- Thompson JM, Yadav S (1979) Chemical Analysis of Waters from Geysers, Hot Springs, and Pools in Yellowstone National Park, Wyoming from 1974 to 1978. U.S. Geological Survey, Menlo Park, CA, pp. 49.
- Treangen TJ, Salzberg SL (2012) Repetitive DNA and next-generation sequencing: computational challenges and solutions. *Nature Reviews Genetics* **13**, 36–46.
- Trouwborst RE, Johnston A, Koch G, Luther GW, Pierson BK (2007) Biogeochemistry of Fe(II) oxidation in a photosynthetic microbial mat: Implications for Precambrian Fe (II) oxidation. *Geochimica et Cosmochimica Acta* **71**, 4629–4643.
- Tse-Dinh Y-C, Qi H, Menzel R (1997) DNA supercoiling and bacterial adaptation: thermotolerance and thermoresistance. *Trends in Microbiology* **5**, 323–326.
- Ullsch A, Moerchen F (2005) ESOM-Maps: tools for clustering, visualization, and classification with Emergent SOM. In: Technical Report. Dept. of Mathematics and Computer Science. University of Marburg, Germany.
- Urschel MR, Kubo MD, Hoehler TM, Peters JW, Boyd ES (2015) Carbon source preference in chemosynthetic hot spring communities. *Applied and Environment Microbiology* **81**, 3834–3847.



- Vabulas RM, Raychaudhuri S, Hayer-Hartl M, Hartl FU (2010) Protein folding in the cytoplasm and the heat shock response. *Cold Spring Harbor Perspectives in Biology*, **2**, a004390.
- Van Bodegom PM, Scholten JC, Stams AJM (2004) Direct inhibition of methanogenesis by ferric iron. *FEMS Microbiology Ecology* **49**, 261–268.
- Vargas M, Kashefi K, Blunt-Harris EL, Lovley DR (1998) Microbiological evidence for Fe(III) reduction on early Earth. *Nature* **395**, 65–67.
- Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environment Microbiology* **73**, 5261–5267.
- Weber KA, Achenbach LA, Coates JD (2006) Microorganisms pumping iron: anaerobic microbial iron oxidation and reduction. *Nature Reviews Microbiology* **4**, 752–764.
- Welte MA, Tetrault JM, Dellavalle RP, Lindquist SL (1993) A new method for manipulating transgenes: engineering heat tolerance in a complex, multicellular organism. *Current Biology* **3**, 842–853.
- Werner JJ, Koren O, Hugenholtz P, Desantis TZ, Walters WA, Caporaso JG, Angenent LT, Knight R, Ley RE (2012) Impact of training sets on classification of high-throughput bacterial 16S rRNA gene surveys. *The ISME Journal* **6**, 94–103.
- Widdel F, Kohring G-W, Mayer F (1983) Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids III. Characterization of the filamentous gliding *Desulfonema limicola* gen. nov. sp. nov., and *Desulfonema magnum* sp. nov. *Archives of Microbiology* **134**, 286–294.
- Woomer PL (1994) Most probable number counts. In *Methods of Soil Analysis, Part 2* (eds Weaver RW, Angle S, Bottomley P, Bezdicsek D, Smith S, Tabatabai A, Wollum A). Soil Science Society of America, Madison, Wisconsin, pp. 59–79.
- Wu L, Beard BL, Roden EE, Johnson CM (2011) Stable iron isotope fractionation between aqueous Fe(II) and hydrous ferric oxide. *Environmental Science and Technology* **45**, 1847–1852.
- Wu L, Percak-Dennett EM, Beard BL, Roden EE, Johnson CM (2012) Stable iron isotope fractionation between aqueous Fe(II) and model Archean ocean Fe–Si coprecipitates and implications for iron isotope variations in the ancient rock record. *Geochimica et Cosmochimica Acta* **84**, 14–28.
- Wu L, Brucker RP, Beard BL, Roden EE, Johnson CM (2013) Iron isotope characteristics of hot springs at chocolate pots, Yellowstone National Park. *Astrobiology* **13**, 1091–1101.
- Zheng H, Wu H (2010) Gene-centric association analysis for the correlation between the guanine-cytosine content levels and temperature range conditions of prokaryotic species. *BMC Bioinformatics* **11**(Suppl 11), S7.

## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Table S1** Microbial community diversity of the Vent (a) and Mid (b) enrichment culture inoculum.

**Table S2** Microbial community diversity of enrichment cultures after (a) 14 weeks with 2 mM acetate and 2 mM lactate as electron donors; (b) 14 weeks with 2 mM acetate as electron donor; (c) 14 weeks with 2 mM lactate as electron donor; (d) 14 weeks with 10% H<sub>2</sub> and 0.5 mM acetate as electron donors.

**Table S3** Microbial community diversity of the Vent (a) and Mid (b) enrichment cultures after 30 weeks with 2 mM acetate and 2 mM lactate as electron donors.

**Table S4** Microbial community diversity of the Vent (a) and Mid (b) enrichment cultures after 46 weeks with 2 mM acetate and 2 mM lactate as electron donors.

**Table S5** Microbial community diversity of the Vent (a) and Mid (b) no-sulfate iron reducing enrichment cultures after 30 weeks with 2 mM acetate and 2 mM lactate as electron donors.

**Table S6** Raw isotopic data for sequential HCl extraction of samples from enrichment cultures tracking Fe isotope fractionation during Fe(III)-oxide reduction activity.

**Table S7** Genes potentially involved in conferring thermotolerance in Vent and Mid bins and type strains.

**Table S8** Total copy number of c-type cytochromes and genes containing multi-heme binding domains from enrichment culture metagenomes, type strain genomes.

**Fig. S1** Chocolate Pots hot spring viewed from the Gibbon River looking east toward the Grand Loop Road.

**Fig. S2** Fe(II) production in enrichments from the Vent and Mid sampling sites in natural spring water medium (pH = 6.1 and 6.7, respectively) amended with various combinations of electron donor/carbon source.

**Fig. S3** (a) Comparison of non-reduced CP oxides before autoclaving and after enrichment culture media was sterilized by autoclaving. (b) Comparison of non-reduced and bio-reduced (by DIRB) CP oxides.

**Fig. S4** Composition of 16S rRNA genes recovered from (a) Vent and Mid (b) Fe-oxides used to inoculate enrichments (0 week) and from materials sampled from acetate/lactate-containing enrichment cultures over time. (c) Composition of 16S rRNA genes in enrichment cultures 14 weeks after inoculation with Fe-oxides sampled from the Mid sampling location under different electron donor treatments.

**Fig. S5** Arrangement of multiple *hsp20* homologs in *Geobacter metallireducens* bins obtained from a metagenome of DNA extracted from the Vent (a) and Mid (b) enrichment cultures compared to the gene arrangement in the genomes of type strains *Geobacter metallireducens* GS-15 (c) and *Geobacter sulfurreducens* PCA (d).

**Fig. S6** Arrangement of *hsp20* (red) and *dnaK* (blue) in the *Melioribacter roseus* bins obtained from a metagenome of DNA extracted from the Vent (a) and Mid (b) enrichment cultures compared to the arrangement of the genes in the genome of type strain *Melioribacter roseus* P3M (c).