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# Stable Isotope Probing for Microbial Iron Reduction in Chocolate Pots Hot Spring, Yellowstone National Park

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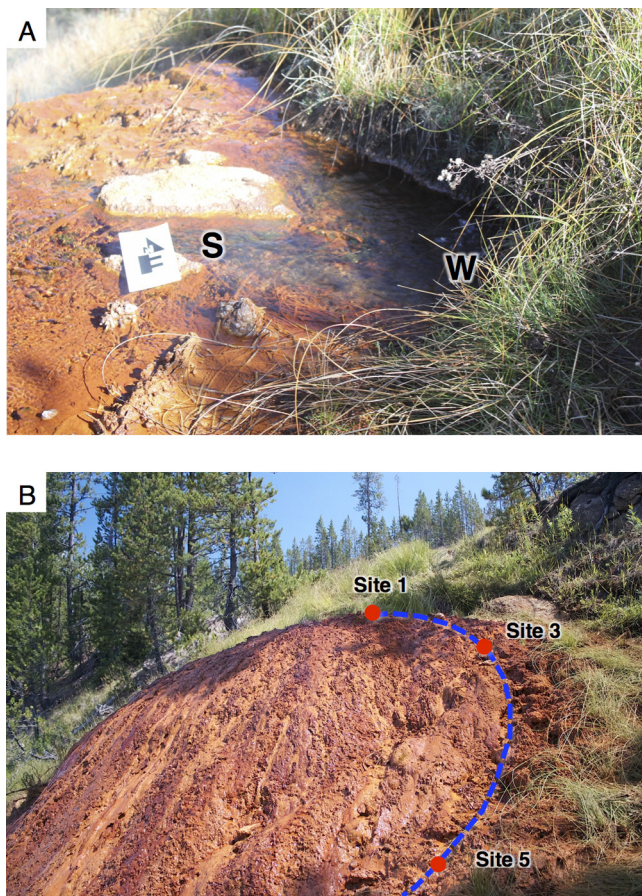
**ABSTRACT** Chocolate Pots hot springs (CP) is a circumneutral-pH Fe-rich geothermal feature located in Yellowstone National Park. Previous Fe(III)-reducing enrichment culture studies with CP sediments identified close relatives of known dissimilatory Fe(III)-reducing bacterial (FeRB) taxa, including *Geobacter* and *Melioribacter*. However, the abundances and activities of such organisms in the native microbial community are unknown. Here, we used stable isotope probing experiments combined with 16S rRNA gene amplicon and shotgun metagenomic sequencing to gain an understanding of the *in situ* Fe(III)-reducing microbial community at CP. Fe-Si oxide precipitates collected near the hot spring vent were incubated with unlabeled and <sup>13</sup>C-labeled acetate to target active FeRB. We searched reconstructed genomes for homologs of genes involved in known extracellular electron transfer (EET) systems to identify the taxa involved in Fe redox transformations. Known FeRB taxa containing putative EET systems (*Geobacter*, *Ignavibacteria*) increased in abundance under acetate-amended conditions, whereas genomes related to *Ignavibacterium* and *Thermodesulfovibrio* that contained putative EET systems were recovered from incubations without electron donor. Our results suggest that FeRB play an active role in Fe redox cycling within Fe-Si oxide-rich deposits located at the hot spring vent.

**IMPORTANCE** The identification of past near-surface hydrothermal environments on Mars emphasizes the importance of using modern Earth environments, such as CP, to gain insight into potential Fe-based microbial life on other rocky worlds, as well as ancient Fe-rich Earth ecosystems. By combining stable carbon isotope probing techniques and DNA sequencing technology, we gained insight into the pathways of microbial Fe redox cycling at CP. The results suggest that microbial Fe(III) oxide reduction is prominent *in situ*, with important implications for the generation of geochemical and stable Fe isotopic signatures of microbial Fe redox metabolism within Fe-rich circumneutral-pH thermal spring environments on Earth and Mars.

**KEYWORDS** Yellowstone National Park, metagenomics, microbial iron reduction, stable isotope probing

Iron (Fe) is the most abundant redox-active element in Earth's crust and is also present in significant quantities on other rocky worlds, such as Mars (1, 2). In microbial energy metabolism, Fe can serve as an electron acceptor in the form of ferric iron [Fe(III)] (3) or as an electron donor in the form of ferrous iron [Fe(II)] (4, 5). Researchers have suggested that microbial Fe cycling, Fe(III) reduction and Fe(II) oxidation, have both been active microbial metabolic processes since the Archean eon (6–8).

Oxidized and reduced Fe minerals on the Martian crust form a redox gradient



**FIG 1** (A) Vent pool of the main mound hot spring at Chocolate Pots looking north toward the Gibbon River. Sediment used to initiate Fe(III)-reducing incubations was collected from the bottom of the pool, labeled with an S. Spring water mixed with sediments to create a slurry for inoculating the Fe(III)-reducing incubations was collected near the hot spring source, labeled with a W. (B) Oblique view of the main mound vent of Chocolate Pots hot springs looking southeast toward Grand Loop Road. The approximate flow path of the hot spring water is indicated with a blue dashed line. Locations of the sampling sites of the sediment cores are labeled (sites 1, 3, and 5). The distance between site 1 (hot spring vent pool) and site 3 is 2.1 m, and the distance between sites 3 and 5 is 4.7 m.

between the surface and subsurface (9, 10), and such gradients can serve as a potential source of oxidants/reductants supporting microbial life. Recent mineralogical studies of deposits in the Endeavor and Gale craters by the Opportunity and Curiosity rovers have identified Fe(III)-rich smectites and other clay minerals, suggesting formation in a circumneutral-pH environment (9, 10). These results are counter to the previous identification of Fe(III)-sulfate minerals (e.g., jarosite) in other regions of the Meridiani Planum, such as the Burns Formation, which are suggestive of a more acidic environment formation (11). The identification of potentially habitable circumneutral-pH environments on Mars necessitates the study of modern analogue environments on Earth.

Chocolate Pots hot springs (CP) are located approximately 5 km southwest of the Norris Geyser Basin in Yellowstone National Park and comprise a series of warm Fe- and Si-rich circumneutral-pH springs. One of the most studied features, and the subject of this research, is located along the southeastern bank of the Gibbon River. This hot spring consists of a primary hot spring vent, referred to here as CP (Fig. 1A), and two smaller satellite vents (not pictured) located several meters below and to either side of the main vent. CP has been studied for the better part of the past century in regard to properties of the Fe-Si precipitates (12), groundwater chemistry (13–16), indirect photosynthetically mediated Fe(II) oxidation (15–18), stable Fe isotope geochemistry (19), and more recently, microbial dissimilatory iron reduction (DIR) (20). DIR is of particular

interest at CP, as it has the potential to produce geochemical and stable isotopic signatures of microbial activity (21), with important implications for the detection of past or even present Fe-based microbial life in astrobiologically relevant places, such as Mars (22).

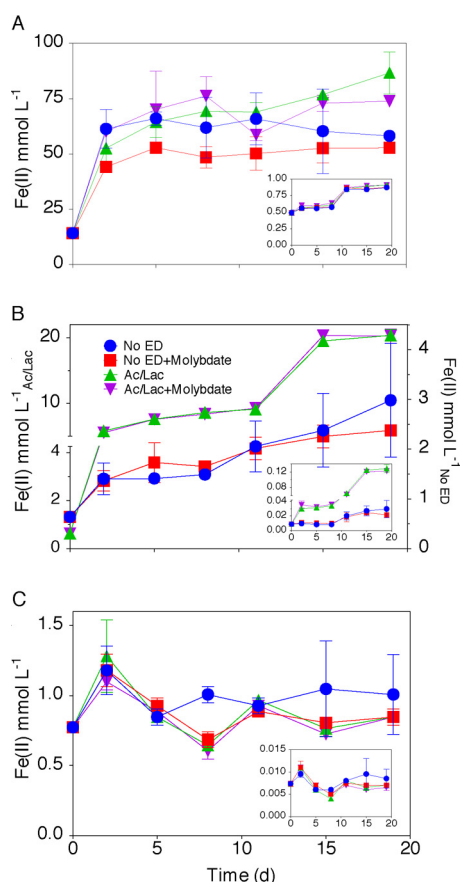
Enrichment culture studies with Fe-Si oxide material from CP successfully demonstrated the potential for reduction of these materials by microorganisms recovered from the *in situ* microbial community (20). 16S rRNA gene amplicon and metagenomic sequences from the enrichment cultures were related to known and potential dissimilatory iron-reducing bacteria (FeRB). Additionally, gene sequences corresponding to putative extracellular electron transfer (EET) protein complexes potentially responsible for DIR were identified in enrichment culture metagenomic libraries. Recent geochemical analyses have provided evidence for active DIR in sediments from the vicinity of the hot spring vent (N. Fortney, unpublished data), but the question remains as to which taxa are driving DIR *in situ* in CP. This study sought to identify active FeRB in CP by way of short-term  $^{13}\text{C}$ -labeled stable isotope probing (SIP) experiments with native CP materials. Metagenomic analysis of community DNA was undertaken to identify and make inferences about the metabolic potential of Fe(III)-reducing organisms.

## RESULTS

***In vitro* Fe(III) reduction experiments.** Anoxic incubation experiments were conducted with native CP materials collected on two occasions to assess *in situ* Fe(III) reduction potential (see Materials and Methods). Fe(II) ( $>50 \text{ mmol} \cdot \text{liter}^{-1}$ ) was produced in all treatment groups of the 2013 Fe(III)-reducing incubations of material collected from core sampling site 1, where greater than 85% of the Fe(III) oxides were reduced by 11 days (Fig. 2A). A smaller, but measurable, level of Fe(III) reduction activity was observed in the incubations of the site 3 material, with greatly diminished levels of activity in the incubations without an exogenous electron donor (Fig. 2B). A negligible level of activity was measured in the incubations of the site 5 material, with or without an added electron donor (Fig. 2C). No difference in Fe(III) reduction activity was observed between incubations with and without sodium molybdate, indicating that sulfate reduction was minimal; thus, Fe(III) was the main electron acceptor in these incubations. Substantial amounts of Fe(II) (ca.  $40 \text{ mmol} \cdot \text{liter}^{-1}$ ) were produced in the SIP incubations amended with both unlabeled acetate and  $^{13}\text{C}$ acetate after 10 days (Fig. 3). A measurable, although smaller, amount of Fe(II) (ca.  $16 \text{ mmol} \cdot \text{liter}^{-1}$ ) was produced in the incubations without an additional electron donor (Fig. 3).

**Isopycnic centrifugation.** Approximately  $1 \mu\text{g}$  of DNA was extracted from each replicate of the sediment slurry from the Fe(III)-reducing following incubation for 10 days and was subjected to isopycnic separation. The buoyant densities of all low-density fractions were an average of  $1.698 \text{ g} \cdot \text{ml}^{-1} \pm 0.004 \text{ g} \cdot \text{ml}^{-1}$  and  $1.718 \text{ g} \cdot \text{ml}^{-1} \pm 0.005 \text{ g} \cdot \text{ml}^{-1}$  for all high-density fractions (Fig. 4; see also Fig. S1 in the supplemental material), indicating the successful separation of  $^{13}\text{C}$ -labeled DNA from unlabeled DNA. In all treatment groups, the concentrations and yields of DNA recovered from the low-density fractions were greater than those from the corresponding high-density fractions (Table S2).

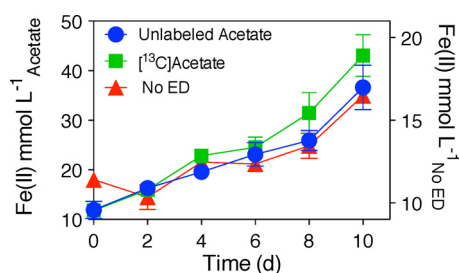
**Microbial community composition of Fe(III)-reducing incubations.** Bacterial 16S rRNA gene amplicon sequencing identified similar dominant taxa in the high- and low-density fractions from all acetate-amended Fe(III)-reducing treatment groups (Fig. 5 and Table 1; see also Table S3). The distinction between the 16S rRNA gene amplicon libraries from the different Fe(III)-reducing treatment groups was broken into the following three categories based on the analysis of similarity (ANOSIM) statistic  $R$ : indistinguishable ( $R = 0.00$  to  $0.25$ ), distinct with some overlap ( $R = 0.25$  to  $0.50$ ), and distinct ( $R > 0.50$ ). Using these criteria, only three pairs of 16S rRNA gene amplicon groups were significantly ( $P < 0.05$ ) distinct from one another. These three pairs are (i) low-density unlabeled acetate and high-density  $^{13}\text{C}$ acetate, (ii) low-density unlabeled acetate and low-density no-electron donor (ED), and (iii) high-density  $^{13}\text{C}$ acetate and low-density no-ED. The similarity percentage (SIMPER) dissimilarity percentage was also greatest (i.e., 80.1%) in the high-density  $^{13}\text{C}$ acetate and low-density no-ED pair.



**FIG 2** Fe(II) production for the 2013 Fe(III)-reducing incubations of Fe-Si oxide slurry prepared with CP spring water (pH 5.9). Insets, ratio of Fe(II) to total Fe at the same sampling time points. (A) Site 1 represents incubations of sediment samples collected from the hot spring vent pool. Sediment samples incubated from sites 3 (B) and 5 (C) were collected along the flow path approximately 2 and 7 m downstream from the spring source, respectively. Samples were incubated at *in situ* temperatures, which were 50°C for sites 1 and 3, and 43°C for site 5. Ac/Lac-amended treatments were dosed to a final concentration of 0.5 mM and given an additional dose at day 11. Treatments containing sodium molybdate (Na<sub>2</sub>MoO<sub>4</sub>) were dosed to a final concentration of 0.6 mM. Note different y axis scales for Fe(II) concentration measured in acetate-amended and no-ED incubations in panel B. Data points represent single measurements on duplicate serum bottles. Error bars represent 1 $\sigma$  variability in the measurements; error bars not shown are smaller than the symbol. No ED, no electron donor; Ac, acetate; Lac, lactate.

Pairwise comparisons of the remaining pairs indicated that they were not significantly distinct from each other (Table S4).

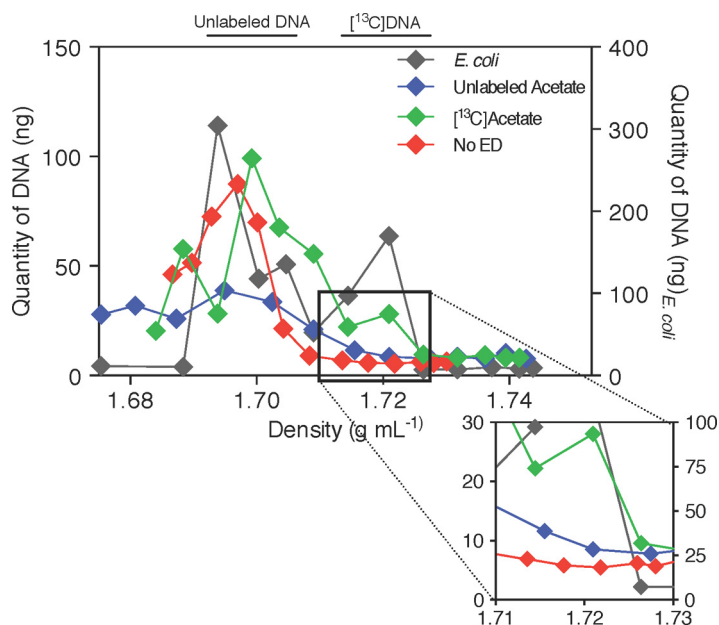
Dominant 16S rRNA gene operational taxonomic units (OTUs) from the high-density fractions of the [<sup>13</sup>C]acetate-amended and unlabeled-acetate-amended treatments were related to *Geobacter* spp. and the class *Ignavibacteria* (Fig. S3). No dominant *Geobacter*-related OTUs were identified in the no-ED treatment; however, several OTUs related to *Rhodocyclaceae*, *Thermodesulfovibrionaceae*, and *Ignavibacteria* were present. These same OTUs were present in the 16S rRNA gene amplicon libraries from the [<sup>13</sup>C]acetate-amended and unlabeled-acetate-amended incubations, although at lower abundance than that of the *Geobacter*-related OTUs. The dominant OTUs from the low-density fraction of the [<sup>13</sup>C]acetate-amended incubation were similarly represented in the no-ED high-density fraction, and the two contained the same relatives of *Ignavibacteria*, *Rhodocyclaceae*, *Thermodesulfovibrionaceae*, and *Comamonadaceae* (Tables 1 and S3b). The same OTUs related to *Geobacter* spp., *Ignavibacteria*, and *Rhodocyclaceae* were present between both the high- and low-density fractions of the unlabeled-acetate-treated incubation DNA pools (Tables 1 and S3a). The reads from the 10 most dominant OTUs comprised approximately 40 to 50% of all reads in these



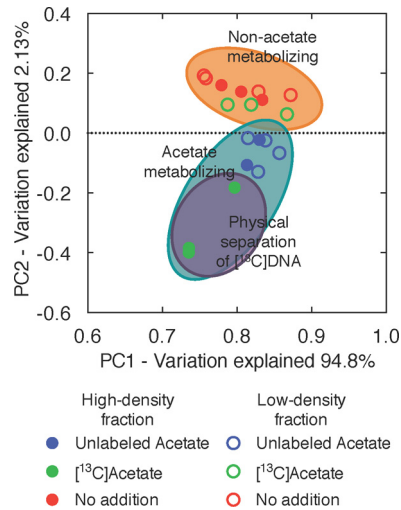
**FIG 3** Fe(II) production in the Fe(III)-reducing incubations of Fe-Si oxide slurry prepared with CP sediments and spring water collected from the CP vent pool in October 2014. Reactor vessels were incubated at an *in situ* pH of 5.8 and temperature of 50°C. Acetate-amended incubations were amended with 0.5 mM unlabeled acetate or [<sup>13</sup>C]acetate at time zero and were provided with an additional 0.5 mM unlabeled acetate or [<sup>13</sup>C]acetate every 2 days. Values represent the average of single measurements from triplicate incubations. Error bars represent 1 $\sigma$  variability in the measurements; error bars not shown are smaller than the symbol. Note different y axis scales for Fe(II) concentration measured in acetate-amended and no-ED incubations. The final Fe(II)/Fe total ratios were approximately 0.45 for the acetate-amended samples and 0.30 for the no-ED samples.

sequences, with the exception of the high-density fraction from the [<sup>13</sup>C]acetate-amended incubations, where ca. 80% of all reads were affiliated with the 10 dominant taxa (Table 1).

The dominant OTUs from the archaeal 16S rRNA gene amplicon libraries were similar between all treatment groups and were distantly related to a euryarchaeote from the family Methanomassiliicoccaceae and a crenarchaeote from the class Aigarchaeota (Table S5a to f). Neither of these taxa has been implicated in DIR (23, 24). The remaining dominant taxa were most closely related to two methanogenic archaeal clones (25). Reads from the 10 most dominant OTUs comprised greater than 75% of all reads in the treatment groups.



**FIG 4** DNA quantity collected from gradient fraction replicate B following isopycnic ultracentrifugation. Densities corresponding to the fractions of greatest DNA concentration in the high- and low-density *E. coli* DNA, labeled as “Unlabeled DNA” and “[<sup>13</sup>C]DNA”, respectively, were used to identify high- and low-density fractions in the DNA samples from the Fe(III)-reducing incubations. Zoomed-in panel highlights the gradient fraction (1.72 g · ml<sup>-1</sup>) with the peak concentration of high-density DNA collected from samples from the [<sup>13</sup>C]acetate-amended incubations. Note the different scales for quantity of DNA measured in samples from the Fe(III)-reducing incubation (left y axis scale) and *E. coli* standard (right y axis scale).



**FIG 5** Principal-component analysis of pairwise 16S rRNA gene community dissimilarity calculated using weighted UniFrac metrics of the high- and low-density fractions (containing  $[^{13}\text{C}]$ DNA and unlabeled DNA, respectively), from the Fe(III)-reducing SIP incubations. PC2 separates the non-acetate-metabolizing bacterial population (orange oval) from acetate-metabolizing bacterial population (teal oval, data points <0.0). Physical separation of the  $[^{13}\text{C}]$ acetate-metabolizing bacterial population (filled green symbols, purple oval), primarily represented by OTUs related to *Geobacter* are demarcated from the unlabeled-acetate-metabolizing bacterial populations.

**Metagenomic analysis of Fe(III)-reducing SIP incubations.** Shotgun metagenomic sequencing libraries were obtained from the low-density fraction of DNA from the Fe(III)-reducing incubations due to insufficient yield from the high-density fractions. The fact that we obtained shotgun metagenomic sequences from the low-density DNA pools from the Fe(III)-reducing incubations seems inconsequential to the answers we sought in this experiment. However, while the abundances of certain taxa differ between the 16S rRNA gene amplicon libraries of the different Fe(III)-reducing incubations, most notably the separation of libraries based on the metabolism of acetate and the physical separation of taxa that incorporated  $[^{13}\text{C}]$  into biomass, the dominant taxa are identical between high- and low-density DNA pools (Table 1).

Paired-end Illumina MiSeq shotgun sequencing produced a total of 7,408,844, 8,020,977, and 8,593,381 reads for the unlabeled-acetate,  $[^{13}\text{C}]$ acetate, and no-ED Fe(III)-reducing incubations, respectively. The combined metagenomic assembly (coassembly) of the Fe(III)-reducing treatment groups contained 24,023,202 reads, following trimming and merging. The coassembled Fe(III)-reducing metagenome contained 681,666 contigs, with an average length of 958 bp and an  $N_{50}$  of 1,257 bp.

The CONCOCT algorithm identified 132 bins in the Fe(III)-reducing metagenomic coassembly; one bin was manually split, for a total of 133 bins (Fig. S4). One bin was a composite of two bacterial phyla and could not be separated based on GC content, coverage, or binning algorithm. The composite bin was primarily composed of *Acidobacteria* and *Ignavibacteriae*. Sixty-four bins had completeness of greater than 50% and contamination that was less than 10% (Fig. S5). Twelve bins contained a putative EET system.

Sequences encoding putative porin-like structures homologous to the porin identified in the *Geobacter*-like porin-cytochrome-complex (*pcc*) gene cluster (26, 27) and that were proximal to a multiheme cytochrome *c* on assembled contigs were identified in three bins related to *Chlorobi*, *Geobacter* spp., and *Deltaproteobacteria* in the Fe(III)-reducing metagenomic coassembly (Fig. 6 and S6). No homologs of *Shewanella*-like *mtrABC* genes (28) were identified in the metagenomic coassembly.

A search of the metagenomic coassembly for multiheme *c*-type cytochromes (*c*-cyts) proximal to putative outer membrane (OM) porins revealed the presence of several additional potential EET. The Fe(III)-reducing metagenomic coassembly contained five

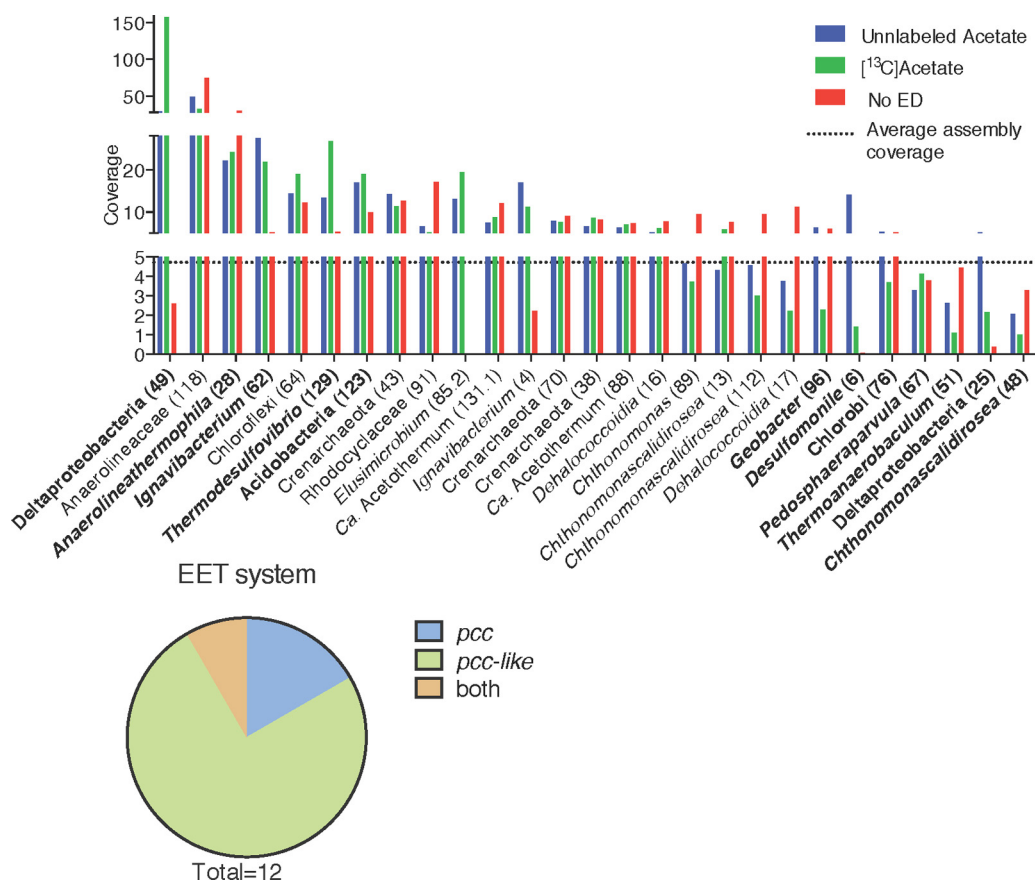
**TABLE 1** Bacterial community composition of the Fe(III)-reducing incubations

| DNA fraction                        | SILVA taxonomic assignment <sup>a</sup> | % of total reads for the sample | Representative species match <sup>b</sup>                  | GenBank accession no. | Similarity (%) | Inferred physiology                                            | Reference                                                  |
|-------------------------------------|-----------------------------------------|---------------------------------|------------------------------------------------------------|-----------------------|----------------|----------------------------------------------------------------|------------------------------------------------------------|
| High-density<br>[13C]acetate        | Geobacter (g)                           | 40.2                            | <i>Geobacter hephaestus</i>                                | AY737507.1            | 95             | Fe(III) reduction                                              | P. H. Janssen, unpublished data                            |
|                                     | Geobacter (g)                           | 12.5                            | <i>G. hephaestus</i>                                       | AY737507.1            | 93             | Fe(III) reduction                                              | P. H. Janssen, unpublished data                            |
|                                     | Geobacter (g)                           | 8.4                             | <i>G. hephaestus</i>                                       | AY737507.1            | 93             | Fe(III) reduction                                              | P. H. Janssen, unpublished data                            |
|                                     | Geobacter (g)                           | 3.7                             | <i>G. hephaestus</i>                                       | AY737507.1            | 93             | Fe(III) reduction                                              | P. H. Janssen, unpublished data                            |
|                                     | Geobacter (g)                           | 3.3                             | <i>G. hephaestus</i>                                       | AY737507.1            | 93             | Fe(III) reduction                                              | P. H. Janssen, unpublished data                            |
|                                     | <i>Deitaproteobacteria</i> (c)          | 2.4                             | <i>Cystobacter armeniacae</i> strain DSM 14710             | NR_043939.1           | 89             | Uncharacterized                                                | H. Reichenbach, unpublished data                           |
|                                     | <i>Ignavibacteria</i> (c)               | 2.3                             | Bacterium YC-Z55-LKJ31                                     | KP174519.1            | 88             | Uncharacterized moderate halophile                             | B. Zhao, K. Li, & K. Liu, unpublished data                 |
|                                     | <i>Syntrophobacteraceae</i> (f)         | 1.6                             | Bacterium YC-Z55-LKJ94                                     | KP174423.1            | 87             | Uncharacterized moderate halophile                             | B. Zhao, K. Li, & K. Liu, unpublished data                 |
|                                     | Geobacter (g)                           | 1.4                             | <i>G. hephaestus</i>                                       | AY737507.1            | 93             | Fe(III) reduction                                              | P. H. Janssen, unpublished data                            |
|                                     | <i>Elusimicrobiales</i> (o)             | 1.0                             | <i>Elusimicrobium minutum</i> strain Pei191                | NR_074114.1           | 88             | Fermenter                                                      | Herlemann et al. (60)                                      |
| Low-density<br>[13C]acetate         | <i>Rhodocyclaceae</i> (f)               | 5.9                             | Betaproteobacterium Rufe9b                                 | AY235688.1            | 94             | N <sub>2</sub> fixation                                        | Z. Y. Tan, T. Hurek, & B. Reinhold-Hurek, unpublished data |
|                                     | <i>Ignavibacteria</i> (c)               | 5.6                             | Bacterium YC-Z55-LKJ31                                     | KP174519.1            | 88             | Uncharacterized moderate halophile                             | B. Zhao, K. Li, & K. Liu, unpublished data                 |
|                                     | <i>Syntrophobacteraceae</i> (f)         | 5.4                             | Bacterium YC-Z55-LKJ94                                     | KP174423.1            | 87             | Uncharacterized                                                | B. Zhao, K. Li, & K. Liu, unpublished data                 |
|                                     | <i>Anaerolineae</i> (c)                 | 5.1                             | <i>Caldilinea aerophila</i> strain DSM 14535               | NR_074397.1           | 86             | Facultatively anaerobic heterotroph/fermentor                  | Sekiguchi et al. (61)                                      |
|                                     | <i>Thermodesulfobivibrionaceae</i> (f)  | 4.6                             | " <i>Candidatus</i> Magnetovum mohavensis" strain LO-1     | GU979422.1            | 89             | Oxidation of reduced S species                                 | Lefèvre et al. (62)                                        |
|                                     | Unassigned                              | 4.3                             | Bacterium YC-LK-LKJ27                                      | KP174640.1            | 87             | Uncharacterized moderate halophile                             | B. Zhao, K. Li, & K. Liu, unpublished data                 |
|                                     | <i>Thermodesulfobivibrionaceae</i> (f)  | 4.1                             | <i>Thermodesulfobivibrio yellowstonii</i> strain DSM 11347 | NR_074345.1           | 91             | Fe(III)/SO <sub>4</sub> <sup>2-</sup> reduction                | Sekiguchi et al. (32) and Henry et al. (63)                |
|                                     | <i>Thermodesulfobivibrionaceae</i> (f)  | 3.8                             | <i>T. yellowstonii</i> strain DSM 11347                    | NR_074345.1           | 91             | Fe(III)/SO <sub>4</sub> <sup>2-</sup> reduction                | Sekiguchi et al. (32) and Henry et al. (63)                |
|                                     | <i>Elusimicrobiales</i> (o)             | 2.9                             | <i>E. minutum</i> strain Pei191                            | NR_074114.1           | 88             | Fermenter                                                      | Herlemann et al. (60)                                      |
|                                     | <i>Bacteria</i> (k)                     | 2.3                             | <i>Moorella humifera</i> strain 64-FGQ                     | NR_108634.1           | 87             | Indirect Fe(III) reduction via humic acids                     | Nepomnyashchaya et al. (64)                                |
| Low-density<br>unlabeled<br>acetate | Total <sup>c</sup>                      | 43.8                            |                                                            |                       |                |                                                                |                                                            |
|                                     | Geobacter (g)                           | 13.5                            | <i>G. hephaestus</i>                                       | AY737507.1            | 95             | Fe(III) reduction                                              | P. H. Janssen, unpublished data                            |
|                                     | <i>Ignavibacteria</i> (c)               | 5.4                             | Bacterium YC-Z55-LKJ31                                     | KP174519.1            | 88             | Uncharacterized moderate halophile                             | B. Zhao, K. Li, & K. Liu, unpublished data                 |
|                                     | <i>Rhodocyclaceae</i> (f)               | 5.3                             | Betaproteobacterium Rufe9b                                 | AY235688.1            | 94             | N <sub>2</sub> fixation                                        | Z. Y. Tan, T. Hurek, & B. Reinhold-Hurek, unpublished data |
|                                     | Geobacter (g)                           | 3.9                             | <i>G. hephaestus</i>                                       | AY737507.1            | 93             | Fe(III) reduction                                              | P. H. Janssen, unpublished data                            |
|                                     | <i>Thermodesulfobivibrionaceae</i> (f)  | 3.4                             | <i>T. yellowstonii</i> strain DSM 11347                    | NR_074345.1           | 91             | Fe(III)/SO <sub>4</sub> <sup>2-</sup> reduction                | Sekiguchi et al. (32) and Henry et al. (63)                |
|                                     | <i>Thermodesulfobivibrionaceae</i> (f)  | 3.3                             | <i>T. yellowstonii</i> strain DSM 11347                    | NR_074345.1           | 91             | Fe(III)/SO <sub>4</sub> <sup>2-</sup> reduction                | Sekiguchi et al. (32) and Henry et al. (63)                |
|                                     | Unassigned                              | 3.1                             | Bacterium YC-LK-LKJ27                                      | KP174640.1            | 87             | Uncharacterized moderate halophile                             | B. Zhao, K. Li, & K. Liu, unpublished data                 |
|                                     | <i>Anaerolineae</i> (c)                 | 2.6                             | <i>C. aerophila</i> strain DSM 14535                       | NR_074397.1           | 86             | Facultatively anaerobic heterotroph/fermentor                  | Sekiguchi et al. (61)                                      |
|                                     | <i>Elusimicrobiales</i> (o)             | 2.2                             | <i>E. minutum</i> strain Pei191                            | NR_074114.1           | 88             | Fermenter                                                      | Herlemann et al. (60)                                      |
| Low-density<br>no-electron<br>donor | Geobacter (g)                           | 2.1                             | <i>G. hephaestus</i>                                       | AY737507.1            | 93             | Fe(III) reduction                                              | P. H. Janssen, unpublished data                            |
|                                     | Total <sup>c</sup>                      | 44.8                            |                                                            |                       |                |                                                                |                                                            |
|                                     | <i>Rhodocyclaceae</i> (f)               | 13.1                            | Betaproteobacterium Rufe9b                                 | AY235688.1            | 94             | N <sub>2</sub> fixation                                        | Z. Y. Tan, T. Hurek, & B. Reinhold-Hurek, unpublished data |
|                                     | Unassigned                              | 5.4                             | Bacterium YC-LK-LKJ27                                      | KP174640.1            | 87             | Uncharacterized moderate halophile                             | B. Zhao, K. Li, & K. Liu, unpublished data                 |
|                                     | <i>Anaerolineae</i> (c)                 | 4.3                             | <i>C. aerophila</i> strain DSM 14535                       | NR_074397.1           | 86             | Facultatively anaerobic heterotroph/fermentor                  | Sekiguchi et al. (61)                                      |
|                                     | <i>Chloroflexi</i> (p)                  | 3.1                             | Bacterium YC-LK-LKJ27                                      | KP174640.1            | 89             | Uncharacterized moderate halophile                             | B. Zhao, K. Li, & K. Liu, unpublished data                 |
|                                     | <i>Chloroflexi</i> (p)                  | 2.9                             | Uncultured bacterium clone RUGL1-593                       | GO420994.2            | 90             | Uncharacterized                                                | Pradhan et al. (65)                                        |
|                                     | <i>Thermodesulfobivibrionaceae</i> (f)  | 2.8                             | " <i>Candidatus</i> Magnetobacterium bavaricum"            | FP929063.1            | 89             | Sulfur oxidation                                               | Jogler et al. (66)                                         |
|                                     | <i>Bacteria</i> (k)                     | 2.5                             | " <i>Candidatus</i> Methylophilis oxyfera"                 | NR_102979.1           | 86             | NO <sub>2</sub> <sup>-</sup> -driven CH <sub>4</sub> oxidation | Ettwig et al. (67)                                         |
|                                     | <i>Ignavibacteria</i> (c)               | 2.1                             | Bacterium YC-Z55-LKJ31                                     | KP174519.1            | 88             | Uncharacterized moderate halophile                             | B. Zhao, K. Li, & K. Liu, unpublished data                 |
| Total <sup>c</sup>                  | <i>Comamonadaceae</i> (f)               | 1.7                             | <i>Curvibacter delicatus</i> strain DHW-S121               | HG974535.1            | 98             | Uncharacterized                                                | Anda et al. (68)                                           |
|                                     | <i>Bacteria</i> (k)                     | 1.7                             | <i>M. humifera</i> strain 64-FGQ                           | NR_108634.1           | 87             | Indirect Fe(III) reduction via humic acids                     | Nepomnyashchaya et al. (64)                                |
|                                     | Total <sup>c</sup>                      | 39.7                            |                                                            |                       |                |                                                                |                                                            |

<sup>a</sup>Letters in parentheses indicate taxonomic level: k, kingdom; p, phylum; c, class; o, order; f, family; g, genus.

<sup>b</sup>As determined by NCBI BLASTn.

<sup>c</sup>Total percentage of reads comprising the 10 most dominant OTUs.



**FIG 6** Rank abundance plot of the 20 most abundant metagenomic bins and all bins containing putative genes of interest from the Fe(III)-reducing metagenomic coassembly ( $n = 27$ ). Bins containing genes involved in putative extracellular electron transport (EET) pathways (*pcc*, EET genes homologous to *Geobacter*-like porin cytochrome complex; *pcc*-like, no homology to the *pcc* system but contains all genes necessary for a putative porin cytochrome complex EET system) are indicated in bold. The consensus taxonomic classification of each bin is listed along with the corresponding bin number in parentheses.

putative OM porins and associated *c*-cyts in bins identified as *Chthonomonas calidirosea*, *Pedospaera parvula*, *Ignavibacterium* spp., *Thermoanaerobaculum* spp., and *Desulfomonile* spp., which were not previously identified in the search for other EET gene homologs (Fig. 6 and S6). Four high-coverage bins identified as *Deltaproteobacteria*, *Anaerolinea thermophila*, *Acidobacteria*, and *Thermodesulfobivrio* contained a putative OM porin but an incomplete set of proximal or supplemental *c*-cyts (Text S2.1 for details). An additional putative OM porin accompanied by proximal multiheme *c*-cyts was identified in the *pcc*-containing *Geobacter* bin in the Fe(III)-reducing metagenomic coassembly.

## DISCUSSION

**Fe(III)-reducing SIP incubations. (i) 16S rRNA gene amplicon libraries.** Relatives of *Geobacter* spp. were the dominant taxa in 16S rRNA gene amplicon libraries from both the [<sup>13</sup>C]acetate and unlabeled-acetate incubations. The acetate stimulation can be seen by the representation of similar taxa (e.g., *Geobacter*) in all acetate-amended treatments, with the exception of the low-density [<sup>13</sup>C]acetate incubations (Table 1; see also Fig. S3 and Table S3a). 16S rRNA gene amplicon libraries from the high- and low-density DNA from the unlabeled-acetate incubations are largely indistinguishable in terms of the dominant OTUs (Fig. 5). These results were not surprising and have been observed previously in acetate-amended Fe(III)-reducing incubations of CP materials and in other environments (3, 20), and they are most likely a result of *Geobacter* spp. outcompeting other native Fe reducers. A previous study demonstrated that *Geobacter*

spp. outcompeted *Rhodospirillum rubrum* spp. under conditions of acetate stimulation because of their higher growth rate (29). It seems likely that a similar phenomenon is responsible for the predominance of *Geobacter* spp. in our acetate-amended incubations.

High-density DNA was collected from the no-ED Fe(III)-reducing incubations to (i) identify which members of the microbial community might naturally fall in that range due to higher-density DNA (e.g., by virtue of having higher GC content), and (ii) demonstrate that the organisms identified in the [ $^{13}\text{C}$ ]acetate-amended incubations were not simply carryover from this naturally higher-density population but were indeed from organisms metabolizing the [ $^{13}\text{C}$ ]acetate and incorporating it into biomass. The clear separation of the high-density DNA from the [ $^{13}\text{C}$ ]acetate treatment group and high-density DNA from the no-ED treatments demonstrates that the SIP incubation and subsequent gradient density separation successfully captured organisms that metabolized and incorporated [ $^{13}\text{C}$ ] into biomass (Fig. 5). This separation is also reflected in a notable difference in the abundant OTUs from these samples (Table 1), where the 16S rRNA gene library from the [ $^{13}\text{C}$ ]acetate treatments is composed almost entirely of *Geobacter* OTUs, which are absent from the no-ED 16S rRNA gene amplicon libraries.

16S rRNA gene sequences from the low-density DNA pool from the [ $^{13}\text{C}$ ]acetate incubations clustered with the no-ED samples (Fig. 5), suggesting that this DNA pool represents the non-[ $^{13}\text{C}$ ]-incorporating portion of the microbial community. In summary, the response of the microbial community to the different incubation conditions for the SIP experiment resulted in a clear separation based on the ability of the organisms to metabolize acetate or not, followed by a clustering within the acetate-metabolizing population based on the physical separation of the [ $^{13}\text{C}$ ]DNA from the unlabeled DNA (Fig. 5).

**(ii) Metagenomic libraries.** SIP enabled the recovery of DNA from organisms in the Fe(III)-reducing incubation that metabolized [ $^{13}\text{C}$ ]acetate and incorporated the heavy isotope into DNA. Unfortunately, the quantity of [ $^{13}\text{C}$ ]DNA from the Fe(III)-reducing incubations was insufficient for shotgun metagenomic sequencing. However, as mentioned above, similar taxa were recovered in the low-density unlabeled-acetate and high-density [ $^{13}\text{C}$ ]acetate 16S rRNA gene amplicon libraries. Thus, the same organisms were sequenced as part of a metagenomic library, regardless of the DNA pool from which the sequences originated, such that we were still able to assess *in situ* taxa and metabolic pathways in CP vent materials.

The metagenomic libraries from the Fe(III)-reducing incubations reflect the striking stimulation of certain members of the microbial community with the addition of acetate. This is most apparent in the dominant *Deltaproteobacteria* bin in the metagenomic coassembly. The *Deltaproteobacteria* bin was a minor member of the microbial community in the no-ED treatment metagenomic library, yet it increased in abundance (i.e., read coverage) by up to two orders of magnitude in the presence of acetate (2.6 $\times$ , 29.5 $\times$ , and 157.2 $\times$  coverage of the *Deltaproteobacteria* bin in the no-ED, unlabeled-acetate, and [ $^{13}\text{C}$ ]acetate libraries, respectively). This response is similar to what was observed for *Geobacter* OTUs in the 16S rRNA gene amplicon libraries. The competition among acetate-utilizing members of the microbial community is reflected by the fact that more-abundant members of the microbial community under no-ED conditions decreased in abundance under acetate-amended conditions, presumably because they were outcompeted by other acetate-utilizing taxa (e.g., *Deltaproteobacteria* or *Geobacter*).

Principal-component analyses did not show a distinct pattern of clustering of the archaeal members of the microbial community based on the unlabeled-acetate, [ $^{13}\text{C}$ ]acetate, and no-ED treatment groups (Fig. S2). Conversely, the bacterial portion of the microbial community had more separation between samples from the high- and low-density DNA pools from the different incubation treatments (Fig. 5). A contributing factor could be whether or not the archaeal microbial community is capable of utilizing acetate. Although many archaeal OTUs were (distantly) related to methanogenic species (Table S5a to f), it has been reported that the optimal pH for acetotrophic

methanogenesis is greater than the average pH (ca. 5.8) of the vent pool at CP (30, 31). Given this information, it is likely that the archaeal microbial community does not utilize acetate and was therefore unaffected by the addition of acetate to the incubations, resulting in nearly indistinguishable populations between the treatment groups. The lack of response to acetate stimulation in the archaeal community is also reflected in the metagenomic library. The three Crenarchaeota bins are of roughly equal read coverage under all incubation conditions (Fig. 6).

**Relative abundances of known and potential FeRB under Fe(III)-reducing conditions.** Average differential coverage of the metagenomic bins was used as an estimate of which taxa were more abundant in the incubations under Fe(III)-reducing conditions, with or without additional electron donor. Because Fe(III) reduction was observed under all incubation conditions, we hypothesized that the more abundant taxa containing a putative EET system would be involved in this metabolic process. The average coverage per treatment group of all binned contigs in the Fe(III)-reducing metagenomic coassembly was 4.7, and bins with an average coverage greater than this value were considered to be dominant members of the microbial community. While the dominant *Deltaproteobacteria* bin cannot be conclusively identified as a *Geobacter* relative, this is a likely possibility given the extremely high coverage of the metagenomic bin (Fig. 6) and the dominating presence of *Geobacter* spp. in the acetate-amended samples from the 16S rRNA gene amplicon library (Fig. S2). Given these assumptions, the *Geobacter* relatives contributed extensively to DIR under acetate-amended Fe(III)-reducing conditions. Although not previously documented as FeRB, bins related to the genus *Ignavibacterium* ( $n = 2$ ) had greatly increased coverage in the acetate-amended incubations. A similar increase in coverage was observed in the *Thermodesulfovibrio*-related bin. Canonically, *Thermodesulfovibrio* spp. are documented as being sulfate reducers; however, they have also been shown to be able to reduce Fe(III) (32). The *Geobacter*-like *pcc* EET system has been identified in *Ignavibacterium album* (26), although, as described above, no putative EET systems have been identified in any of the isolated *Thermodesulfovibrio* genomes. The increased read coverage along with the presence of putative EET systems in the genomes, and in the case of the *Thermodesulfovibrio* bin, previously documented Fe(III) reduction, all support the idea that these organisms contributed to acetate-stimulated Fe(III) reduction. The *Acidobacteria*-related bin had high coverage under all incubation conditions and showed much less stimulation in the presence of acetate than the more abundant bins described above. This suggests that the *Acidobacteria* relative, while still a potentially active FeRB in the incubations, is less competitive than other FeRB in the microbial community. Interestingly, the *Acidobacteria*-related bin was the highest coverage putative FeRB in the no-ED incubations, a possible indication of its function as the dominant FeRB *in situ*.

**EET pathways involved in Fe(III) reduction present at CP.** The presence of the *pcc* EET system in bins related to *Geobacter*, *Ignavibacterium*, *Melioribacter*, and *Deltaproteobacteria* in the Fe(III)-reducing metagenomic coassembly (Fig. 6 and S6) was expected based on previous comparative genomic analyses (26, 27), as well as recent CP FeRB enrichment culture experiments that revealed the presence of *pcc* in reconstructed genomes of *Geobacter* and *Ignavibacteriae* (20). Both *Geobacter* spp. and *Melioribacter* spp. are documented FeRB (33–35), and it is reasonable to expect that metagenomic bins belonging to relatives of these organisms reflect the same metabolic capacity under the imposed Fe(III)-reducing conditions. Although poorly resolved phylogenetically, the presence of *pcc* homologs in a bin identified as *Deltaproteobacteria* is also not surprising. *Ignavibacterium* and *Melioribacter* relatives were previously classified under the phylum *Chlorobi* (36), and both taxa have been documented as having *pcc*, so it is possible that the poorly resolved *Chlorobi* bin from the Fe(III)-reducing metagenomic coassembly is related to these genera and possesses a similar genomic makeup.

Our previous study of FeRB enrichment culture studies from CP identified homologs to the *pcc* EET system in metagenomic bins related to *Thermodesulfovibrio* (20). A *pcc*-like EET system also was identified in the *Thermodesulfovibrio* bin from the Fe(III)-reducing coassembly in this study, but the gene arrangement was not directly analogous to the canonical *pcc* system described by Liu et al. (27). Although this putative *pcc*-like EET system has not been scrutinized using genomic and physiological experiments, the gene arrangement and properties (e.g., number of heme-binding sites, predicted cellular location, and transmembrane [TM] domains) suggest a function similar to that of *pcc*. Curiously, although no putative EET systems have been identified in any of the published *Thermodesulfovibrio* sp. genomes available on IMG, the ability to reduce Fe(III) has been demonstrated previously (32), which might explain the presence of putative EET genes in the *Thermodesulfovibrio*-related metagenomic bin. It should be noted that the *Thermodesulfovibrio*-related bins from the previous enrichment culture metagenome (20) and the current SIP metagenomic analysis are only distantly related to each other and to the type strains of *Thermodesulfovibrio* (ca. 90% identification, based on the V4 region of 16S rRNA gene sequences; data not shown), suggesting that the bins from the two experiments, while both identified as being related to *Thermodesulfovibrio*, are in fact not the same organism; therefore, we should not expect them to possess the same metabolic potential. Of further note, the *Thermodesulfovibrio*-related bin from the present study is only partially complete (78%; Fig. S6).

Putative EET systems were also identified in bins belonging to several phyla, including *Acidobacteria*, *Chloroflexi*, *Armatimonadetes*, *Proteobacteria*, and *Verrucomicrobia*. Metagenomic analyses suggest that the *Acidobacteria* bins ( $n = 2$ ), the *Desulfomonile* bin (*Proteobacteria*), and the *Pedospaera* bin (*Verrucomicrobia*) are potentially involved in Fe(III) reduction (Text S3.1). However, neither the *Desulfomonile* nor *Pedospaera* bins are particularly abundant, especially *Desulfomonile* in the no-ED treatment, which suggests that if these taxa are indeed FeRB, they are unlikely to have a major contribution to overall Fe(III) reduction at CP (Fig. 6).

**Summary and conclusions.** The purpose of this study was to identify which members of the microbial community endemic to CP are actively involved in Fe(III) reduction under conditions meant to represent *in situ* conditions as best as possible. Previous incubation studies have demonstrated the ability of enrichment cultures of CP microorganisms to reduce Fe(III) oxides (20), but this is the first use of SIP to target the organisms responsible for this activity. By using  $^{13}\text{C}$ -labeled acetate and isopycnic centrifugation, we were able to separate the portion of the community that had incorporated  $^{13}\text{C}$  into its DNA by metabolizing acetate coupled to DIR. Acetate is a universal electron donor for FeRB communities (3), including thermophilic communities (37–39), and there is good reason to suspect that acetate is also a major electron donor for DIR at CP, especially given the immediate response of the microbial community to acetate addition. 16S rRNA gene amplicon sequencing identified OTUs related to known FeRB, and metagenomic sequencing identified genes within these taxa which are involved in EET, thereby strengthening the hypothesis that these organisms are involved in DIR.

*Geobacter* was a dominant taxon in the acetate-containing SIP incubations. However, *Geobacter* spp. were not detected in the no-ED incubations, which also showed significant DIR activity. This suggests that relatives of this taxon were stimulated by even a very small addition of acetate, which is consistent with previous enrichment culture experiments (20). The results from the SIP incubations also revealed the presence of other moderately abundant taxa that incorporated acetate into biomass (e.g., *Thermodesulfovibrio* and *Ignavibacteria*). Notably, these taxa were also significant members of the microbial community of the no-ED incubations. In addition, these taxa were also present, though in lower abundances, in previous enrichment culture experiments (20). The ability of *Thermodesulfovibrio* relatives native to CP to metabolize acetate and reduce Fe(III) is consistent with the abilities of isolated strains of this

organism (32). Together, these results suggest that moderately thermophilic taxa, such as *Thermodesulfovibrio* and *Ignavibacteria*, are responsible for *in situ* DIR at CP.

Forthcoming research is targeting the *in situ* microbial community at CP using sediment cores and vent pool fluid to assess the role of Fe-redox transformations in generating geochemical and stable Fe isotopic signatures of microbial Fe energy metabolism within this Fe-rich circumneutral-pH thermal spring. Delineation of such signatures in modern Earth environments is a prerequisite for detecting signs of ancient terrestrial and past or present Fe-based microbial life on Mars and other rocky planets.

## MATERIALS AND METHODS

**Description of Chocolate Pots hot springs.** CP (thermal ID: GCPNN002; 44.71008, -110.7413) is located approximately 5 km south of the Norris Geyser Basin along the southern bank of the Gibbon River, next to Grand Loop Road. The temperatures of the sampling locations where the sediment cores were collected in 2013 were 50.7°C at site 1 (hot spring vent), 48.4°C at site 3, and 42.7°C at site 5. The pHs of the sediment core sampling sites were 5.9, 6.5, and 7.8, respectively. The temperature and pH of the vent pool were 51.5°C and 5.8, respectively, in October 2014. Subsurface water emanating from the vent pool is anoxic and contains high levels of dissolved Fe(II) and Si, at ca. 0.1 and 5 mM, respectively (16).

**Sample collection.** Small sediment cores and spring water were collected from the CP vent and along the flow path in August 2013 (Fig. 1B). Water and sediment samples were collected near the vent in October 2014. Spring water was collected from the pool (Fig. 1A) at the vent of the main mound of CP using a peristaltic pump. Fe-Si oxide sediment was collected from the bottom of the vent pool using a plastic scoop. Water and sediment were stored in degassed and sterilized bottles. The bottles were fitted with a stopper to ensure an airtight seal. One bottle of spring water was kept anoxic by bubbling with N<sub>2</sub> for 15 min; the other bottle was kept partially oxic by including an air headspace. The sediment was overlain with an approximately equal volume of spring water and degassed with N<sub>2</sub> for 5 min with swirling to remove any traces of oxygen in the sediment and water. Temperature, pH, and conductivity were measured at the vent pool using a WTW pH 3310 ProfiLine meter with SenTix 51 electrode (Cole-Parmer, Vernon Hills, IL).

**Fe-Si oxide slurry preparation.** A portion of the sediment cores collected in 2013 was used to produce a Fe-Si oxide slurry for use in small-scale incubation experiments. In an anaerobic chamber (95:5% N<sub>2</sub>:H<sub>2</sub>; Coy Products, Grass Lake, MI), an additional volume of anoxic spring water was added to the Fe-Si sediment bottle for a final ratio of ca. 1:2 solid to liquid. The jar containing the sediment and water was swirled, and the suspended material was decanted into a beaker so that coarse sand grain-sized material remained in the bottle. Fine-grained material was withdrawn from the beaker using a needle and syringe. Fine-grained material, here referred to as CP slurry (CPS), was dispensed into a glass bottle fitted with a cap modified to hold the top of a crimped and stoppered anaerobic pressure tube. The bottle containing CPS was removed from the anaerobic chamber, and the headspace was degassed with N<sub>2</sub> to remove residual H<sub>2</sub>. CPS was prepared identically immediately upon return to the lab using the fresh sediment and spring water collected in 2014.

**Fe(III) reduction experiments.** CPS for the Fe(III)-reducing incubations was aliquoted into sterile anoxic serum bottles fitted with butyl rubber stoppers. All transfers were made using sterile N<sub>2</sub>-flushed syringes and needles. Four treatments were tested for the 2013 Fe(III)-reducing incubation experiments. Reactors were prepared in duplicate with a mixture of acetate and lactate as an additional electron donor (0.5 mM final concentration), without an electron donor added (no-ED), and each treatment with and without sodium molybdate (Na<sub>2</sub>MoO<sub>4</sub>) to a final concentration of 0.6 mM as a specific inhibitor of bacterial sulfate reduction (40). Treatment groups are here referred to as acetate/lactate (Ac/Lac), Ac/Lac plus molybdate, Ac/Lac, no-ED, and no-ED plus molybdate, respectively.

Fe(III)-reducing incubations for the 2014 stable isotope probing (SIP) experiment were prepared in triplicate and contained 0.5 mM <sup>13</sup>C-labeled acetate ([<sup>13</sup>C]H<sub>3</sub>COONa, 99%; Cambridge Isotope Laboratories, Inc., Andover, MA), 0.5 mM unlabeled acetate, or no additional electron donor. The treatment groups are here referred to as [<sup>13</sup>C]acetate, unlabeled acetate, and no-ED, respectively. All incubations were conducted at 50°C in the dark.

Fe(III) reduction activity was determined by the accumulation of acid-soluble Fe(II). Subsamples were collected approximately every 2 days, added to 0.5 M HCl, and agitated for 1 h. An aliquot of the extract was added to ferrozine colorimetric reagent (41) 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). Acetate-amended incubations received an additional 0.5 mM acetate after each sampling, unless there was no increase in Fe(II) since the previous sampling.

The incubations from the 2013 Fe(III)-reducing experiments were terminated after 19 days. Fe(III)-reducing incubations from the 2014 SIP experiment were terminated after 10 days. The contents of the Fe(III)-reducing serum bottles were decanted into Falcon tubes in an anaerobic chamber and then frozen at -20°C.

**DNA extraction and stable isotope probing.** DNA was extracted from the frozen aliquots of sediment using the Mo Bio PowerSoil DNA isolation kit (Mo Bio, Carlsbad, CA), in accordance with previously published modifications (20). Replicate samples were kept separate during DNA extraction. DNA was not extracted from the azide-amended treatments from the Fe(II)-oxidizing incubations. High-density (<sup>13</sup>C-labeled) and low-density (<sup>12</sup>C-labeled) DNA from the 2014 Fe(III)-reducing SIP experiment was separated using isopycnic centrifugation, according to previously published methods (42–44), with modifications described in Text S1.1.

**16S rRNA gene amplicon sequencing and analysis.** Gradient fractions corresponding to high- and low-density pools of DNA were selected for 16S rRNA gene amplification based on fractions with the highest fluorescence measurement in the PicoGreen assay (Thermo Fisher Scientific, Darmstadt, Germany), according to the manufacturer's instructions ( $n = 19$ ). Buoyant densities of fully  $^{13}\text{C}$ -labeled and unlabeled *Escherichia coli* DNA fractions with high fluorescence were also used to select fractions for amplification and sequencing. DNA was amplified using universal 16S rRNA gene PCR primers specific to target bacteria and archaea. The PCR and amplification conditions are described elsewhere (Text S1.2).

PCR amplicons from bacterial and archaeal amplifications of the gradient-density-separated Fe(III)-reducing incubation DNA ( $n = 38$ ) were submitted to the University of Wisconsin Biotechnology Center (UWBC; <https://www.biotech.wisc.edu/>) for paired-end  $2 \times 300$ -bp Illumina MiSeq 16S rRNA gene amplicon sequencing. Microbial community sequence data were processed using the Quantitative Insights into Microbial Ecology (QIIME) pipeline version 1.8.0 (<http://www.qiime.org>) (45), following the protocol for handling paired-end Illumina sequence data (Text S1.2). Briefly, QIIME is used to identify and cluster reads into operational taxonomic units (OTUs) for each amplicon library, which are then subjected to homology-based analyses to obtain taxonomic information about the composition of the reads within each sample. The beta diversity of the samples was calculated using weighted UniFrac metrics (46, 47). The software package PRIMER (Primer-E [48]) was used to conduct an analysis of similarity (ANOSIM) and similarity percentage (SIMPER) to analyze the statistical significance of the differences between treatment groups in the Fe(III)-reducing incubation.

**Metagenomic sequencing and assembly.** The remaining non-PCR-amplified genomic DNA from the low-density fractions of the Fe(III)-reducing SIP incubations ( $n = 3$ ) was submitted to the UWBC for paired-end  $2 \times 300$ -bp Illumina MiSeq shotgun metagenomic sequencing. Raw sequence data were assembled and processed using CLC Genomics Workbench 7.5.1 (CLC bio) at the UWBC computer center (Text S1.3 for details).

Binning was accomplished using the automated clustering tool CONCOCT (49) on all contigs 2,500 bp and greater. Completeness, contamination, and bin heterogeneity were calculated using CheckM (50). "Contamination" is calculated by the number of multicopy marker genes identified in each bin, generally as a result of multiple closely related organisms (e.g., strains) being binned together (50). The strain heterogeneity measurement determines how closely related multicopy genes are. A low heterogeneity measurement suggests that genes are from closely related organisms and can be thought of as redundancy, whereas high strain heterogeneity suggests that genes came from unrelated organisms and can be thought of as true contamination in a given genome bin. Highly redundant bins with high variance in average coverage were manually split based on the fold coverage of the contigs.

Taxonomic bins were visualized using Databionic ESOM Tools (51), according to previously described methods (20). Prodigal (52) and HMMer (53) were used to identify copies of the 111 conserved essential bacterial housekeeping genes (54) within the metagenomic coassembly. Amino acid sequences of the genes were aligned to the BLAST database (current as of 8 June 2016) using the BLASTp function in command-line BLAST (55). BLAST searches were performed using the computational resources and assistance of the UW-Madison Center for High Throughput Computing (CHTC) in the Department of Computer Sciences (<http://chtc.cs.wisc.edu/>). Output files were uploaded to MEGAN (56), and taxonomic information was exported for viewing in Dendroscope (57). The taxonomic identification of the bins was determined (Table S6) using a consensus between PhyloSift (58), CheckM, and MEGAN with the BLASTp input.

Differential coverage of metagenomic reads was used to determine the response of the microbial community to treatments in the Fe(III)-reducing SIP incubation experiment. Briefly, raw metagenomic sequence reads from each treatment group were paired, and sequencing adapters removed and quality trimmed. Processed reads from each treatment group were individually mapped back to the coassembly using the read mapping function in CLC Genomics Workbench.

**Metagenomic sequence analysis.** The coassembly annotated in IMG/M ER was searched for genes of interest, i.e., genes involved in putative Fe cycling. A hidden Markov model (HMM) was created to search for the well-characterized *Geobacter*-like porin-cytochrome complex (*pcc*) (26, 27) EET system, as described previously (20). A list of candidate *pcc* homolog genes was refined (Text S1.4). Homologs of the well-characterized EET system in the known FeRB *Shewanella* sp. (*mtrABC*) (28) were searched for in the metagenomic coassembly using command-line BLAST and the BLASTp function in IMG. Putative EET systems which were not homologous to known model systems were identified as follows: a Python script was used to search for multiheme *c*-cyts ( $\geq 5$  heme-binding sites) in the amino acid assembly from the metagenome. Putative OM porins were identified by investigating genes proximal to aforementioned multiheme *c*-cyts for TM domains and a predicted OM location. Genes fitting these criteria were classified as *pcc*-like. Bins containing putative EET systems were investigated further for supplemental genes predicted to be involved in Fe(III) transformation, as previously described (Table S1) (59).

**Accession number(s).** All metagenomic contigs for the Fe(III)-reducing coassembly are available through IMG/M ER with taxon identification number [3300009943](https://img.jgi.doe.gov/data/record/3300009943). This targeted locus study project has been deposited at DDBJ/EMBL/GenBank under the accession no. [PRJNA438487](https://www.ncbi.nlm.nih.gov/nuccore/PRJNA438487). The version described in this paper is the first version, [KBW501000000](https://www.ncbi.nlm.nih.gov/nuccore/KBW501000000).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.02894-17>.

**SUPPLEMENTAL FILE 1**, PDF file, 1.6 MB.

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