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Formation of zero-valent iron in iron-reducing cultures of *Methanosarcina barkeri*

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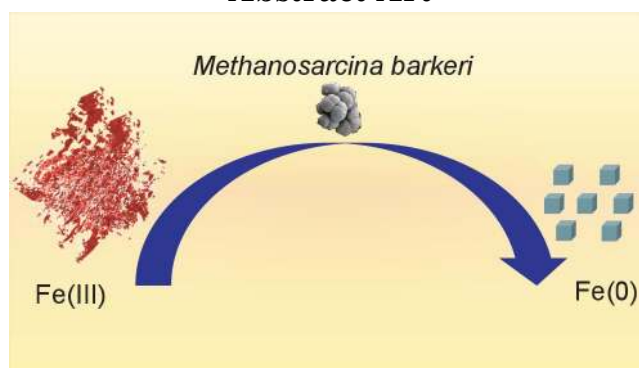
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Abstract

Methanogenic archaea have been shown to reduce iron from ferric [Fe(III)] to ferrous [Fe(II)] state, but minerals that form during iron reduction by different methanogens remain to be characterized. Here, we show that zero-valent iron (ZVI) minerals, ferrite [α -Fe(0)] and austenite [γ -Fe(0)], appear in the X-ray diffraction spectra minutes after the addition of ferrihydrite to the cultures of the methanogenic archaeon *Methanosarcina barkeri* (*M. barkeri*). *M. barkeri* cells and redox-active, non-enzymatic soluble organic compounds in organic-rich spent culture supernatants can promote the formation of ZVI; the latter compounds also likely stabilize ZVI. Methanogenic microbes that inhabit organic- and Fe(III)-rich anaerobic environments may similarly reduce oxidized iron to Fe(II) and ZVI, with implications for the preservation of paleomagnetic signals during sediment diagenesis and potential applications in the protection of iron metals against corrosion and in the green synthesis of ZVI.

Abstract Art



1 Introduction

Microorganisms mediate numerous redox transformations of iron in natural environments and couple the biogeochemical cycles of iron, carbon, oxygen, sulfur, nitrogen and other elements [1–4]. In soils and sediments, microbes can utilize ferric iron [Fe(III)] as the electron acceptor for dissimilatory iron reduction [1,4,5]. The reduction of Fe(III) produces ferrous iron [Fe(II)] that can be incorporated into different Fe(II)-containing minerals such as magnetite [6,7], vivianite [8,9] and siderite [10,11]. The formation of specific mineral phases is thought to depend on pH, electron

36 donors, $p\text{CO}_2$ and other environmental factors [11].

37 *Methanosarcina barkeri* (*M. barkeri*), a coccoid methanogen, can grow on methanol, acetate
38 and carbon dioxide/hydrogen [12,13]. The growth physiology of *M. barkeri* depends on the redox
39 potential of the ambient environment: this microbe is able to survive high redox-potential
40 conditions by generating its own low potential environment [14]. When *M. barkeri* produces
41 methane (CH_4) with hydrogen gas (H_2) as the electron donor, it uses several electron carriers with
42 low redox-potentials such as ferredoxin ($E^{\circ} = -500$ mV), coenzyme F_{420} ($E^{\circ} = -360$ mV), coenzyme
43 B ($E^{\circ} = -140$ mV) and methanophenazine ($E^{\circ} = -165$ mV) [15]. These enable *M. barkeri* to reduce
44 a range of oxidized compounds including ferrihydrite [$\text{FeOOH}(\text{am}) \rightarrow \text{Fe}(\text{II})$, $E^{\circ} = -50$ mV [16]]
45 into products with rather low reduction potentials. *M. barkeri* was shown to reduce amorphous
46 [17–19] and crystalline [20,21] Fe(III) to Fe(II). Some of these studies also reported the formation
47 of iron minerals such as magnetite [19] and vivianite [20]. However, only mineral phases that
48 contain Fe(III) and/or Fe(II) have been reported, although other minerals, such as ilmenite, were
49 hypothesized as well [17].

50 The low redox-potential environments where *M. barkeri* grows and persists may support the
51 formation of other iron phases with low reduction potential, such as zero-valent iron (ZVI) [$\text{Fe}(\text{III})$
52 $+ 3e^- \rightarrow \text{Fe}(0)$, $E^{\circ} = -37$ mV [22]]. ZVI is unstable in most surface environments because it is
53 easily oxidized to Fe(II) or Fe(III) by both abiotic [23] and biological [24–27] reactions in a
54 process known as iron corrosion. The reverse process – that is, the reduction of oxidized iron to
55 ZVI – has also been observed in some abiotic reactions [28–31]. One example is the formation of
56 Fe(0) in awaruite, a nickel and iron-containing alloy, in serpentinizing environments [31]. Several
57 studies have also reported the reduction of Fe(III) in aqueous tea-leaf extracts to ZVI [32–34].
58 However, to the best of our knowledge, only one study reported the presence of small X-ray
59 diffraction peaks of Fe(0) in microbial enrichment cultures of *Geobacter sulfurreducens* and
60 *Shewanella denitrificans* that grew on ochre pigment [35].

61 Here, we explore the biomineralization of iron in low-potential environments that support
62 microbial methanogenesis. This is done by characterizing minerals that form in iron-reducing
63 cultures of *M. barkeri* and exploring mechanisms that produce and stabilize these minerals. Our
64 results demonstrate the formation of titanomagnetite (or magnetite) and ZVI in active *M. barkeri*
65 cultures and spent culture supernatants. The ability of *M. barkeri* to reduce oxidized iron to its
66 metallic state may influence the cycling of nutrients and toxins in the environments, with potential
67 applications in the protection against iron corrosion and in the green synthesis of ZVI.
68

69 **2 Materials and Methods**

70 **2.1 Cell Incubation**

71 *M. barkeri* (DSM 800) was obtained from Deutsche Sammlung von Mikroorganismen und
72 Zellkulturen (DSMZ, Braunschweig, Germany). All serum bottles (160 mL) were autoclaved at
73 120 °C for 30 mins. Media were prepared according to modified medium recipe (Oregon
74 Collection of Methanogens Medium for Methanogens [17]) (Supporting Information, Table S1).
75 The medium contained either a low organic content (1.0 g/L of 50/50 wt% yeast extract and
76 casitone, DIFCO) or a high organic content (4.0 g/L of 50/50 wt% yeast extract and casitone,
77 DIFCO). Organic-free medium was prepared according to the same recipe, but without yeast
78 extract and casitone. The media were titrated with a saturated NaHCO_3 solution to pH 6.8. All
79 media were prepared anaerobically, filter sterilized and added into the autoclaved serum bottles.

80 *M. barkeri* cannot grow in the presence of O₂, so the vacuum-vortex technique [36] was used to
81 generate anaerobic conditions in the serum bottles. Gas mixture of H₂/CO₂ (80%/20%) was added
82 into serum bottles as the headspace atmosphere. Each 160 mL serum bottle contained 50 mL of
83 liquid and 110 mL of headspace gas. The final pressure of headspace atmosphere was 100 kPa.
84 Either Ti(III)-citrate (2.56 mM final concentration) or L-cysteine (0.5 mM final concentration)
85 were used as reducing agents. Sulfide was not used as a reducing agent to avoid reactions with
86 iron species and the formation of sulfide minerals. Preliminary experiments used different final
87 concentrations of Ti(III)-citrate (0.85 mM, 2.56 mM and 7.67 mM) and found that 2.56 mM
88 Ti(III)-citrate was optimal for *M. barkeri* to grow and produce CH₄. All cultures and controls
89 were incubated at 37 °C.

90

91 **2.2 Experimental Design**

92 The experimental design is summarized in Table 1. Initially, we explored the biomineralization in
93 *M. barkeri* cultures in the presence of ferrihydrite as a function of: (1) the content of organic
94 additives in medium, (2) the timing of ferrihydrite addition, and (3) the composition of headspace
95 gas in serum bottles. The *M. barkeri* inocula for all experiments were grown in the medium with
96 1 g/L organic additives, these cultures were inoculated at 1:10 v/v into media that contained
97 either 1 g/L or 4 g/L organic additives for growth and/or iron-reduction experiments. Most
98 experiments described in what follows used 1 g/L of these additives (yeast extract and casitone),
99 a fourfold reduction relative to 4 g/L in the original recipe [17]. Poorly crystalline ferrihydrite
100 was prepared by titrating FeCl₃ with 10N NaOH to pH 7 to a final concentration of 7.5 mM of
101 ferrihydrite. Before the addition of ferrihydrite, the headspaces of triplicate *M. barkeri* cultures
102 and triplicate sterile controls were flushed by N₂/CO₂ (80%/20%) for 1 hour. To test if the
103 headspace gas composition influences the precipitation of Fe(0), the headspaces of additional
104 serum bottles with triplicate *M. barkeri* cultures or sterile media were not flushed to remove H₂
105 and CH₄. Aqueous FeCl₃ (7.5 mM final concentration) was added to the *M. barkeri* cultures to
106 test the influence of different iron sources on the production of Fe(0).

107 Precipitates formed in *M. barkeri* cultures and sterile controls were sampled 30 mins, 28
108 days and 42 days after the addition of ferrihydrite. X-ray powder diffraction (XRD) and micro-
109 focused X-ray diffraction (μ XRD) were used to characterize the mineral phases in the
110 precipitates. Scanning electron microscopy (SEM) was used to observe the morphology of *M.*
111 *barkeri* cells, minerals and characterize the association of cells and minerals. Energy-dispersive
112 X-ray spectroscopy (EDS) was used to determine the elemental composition of solids in the
113 cultures. The redox state of iron was measured by X-ray photoelectron spectroscopy (XPS). The
114 concentrations of Fe(II) were quantified by ferrozine assay [36]. The composition of headspace
115 gases was characterized using gas chromatography.

116 To test the effects of soluble electron donors on the reduction of iron from ferric to metallic
117 state, we separated *M. barkeri* cells and supernatants before the addition of ferrihydrite. This
118 procedure consisted of the following steps: (1) cells were separated from medium by filtration
119 using 0.1 μ m pore-size filters (glass microfiber, Cole Parmer, IL, USA), (2) spent supernatants
120 were placed into clean, autoclaved and anaerobic serum bottles, (3) cells were washed with
121 anoxic nanopure water, (4) cells were transferred into fresh organic-free medium in autoclaved
122 and anaerobic serum bottles, (5) headspaces were flushed by N₂/CO₂ (80%/20%) for 1 hour, and
123 (6) ferrihydrite was added to the serum bottles that contained either filtered spent supernatants or

124 cells in fresh organic-free medium. All steps in this procedure were performed using the sterile
125 technique in an anaerobic glove box. To ascertain that the separation process did not influence
126 the physiology of *M. barkeri*, procedure control cultures contained cells that were added back to
127 the filtered spent supernatants after the filtration and before the addition of ferrihydrite.

128 To determine whether any transient mineral phases formed in the early stages of these
129 experiments, precipitates from cells and supernatants were sampled for XRD characterization at
130 1 min, 5 mins, 10 mins and 30 mins after the addition of ferrihydrite. After 30 mins, the
131 precipitates were sampled for analysis every 14 days until 42 days after the addition of
132 ferrihydrite. Iron concentrations were measured at each sampling point.

133
134
135

Table 1: Experimental Design

# Bottle	<i>M.barkeri</i>	Fe(III) Source	Addition of Fe(III)	Headspace	Organics	Reductant	Separation	Heating
1, 2, 3	Inoculated	Ferrihydrite	Exponential phase	N ₂ /CO ₂	1.0 g/L	Ti(III)-citrate	No	No
4, 5, 6	Inoculated	Ferrihydrite	Stationary phase	N ₂ /CO ₂	1.0 g/L	Ti(III)-citrate	No	No
7, 8, 9	Inoculated	Ferrihydrite	Exponential phase	CH ₄ /H ₂ /CO ₂	1.0 g/L	Ti(III)-citrate	No	No
10, 11, 12	Inoculated	Ferrihydrite	Stationary phase	CH ₄ /H ₂ /CO ₂	1.0 g/L	Ti(III)-citrate	No	No
13, 14, 15	Inoculated	Ferrihydrite	Exponential phase	N ₂ /CO ₂	4.0 g/L	Ti(III)-citrate	No	No
16, 17, 18	Inoculated	Ferrihydrite	Stationary phase	N ₂ /CO ₂	4.0 g/L	Ti(III)-citrate	No	No
19, 20, 21	Sterile control	Ferrihydrite	–	N ₂ /CO ₂	1.0 g/L	Ti(III)-citrate	No	No
22, 23, 24	Sterile control	Ferrihydrite	–	CH ₄ /H ₂ /CO ₂	1.0 g/L	Ti(III)-citrate	No	No
25, 26, 27	Sterile control	Ferrihydrite	–	N ₂ /CO ₂	4.0 g/L	Ti(III)-citrate	No	No
28, 29, 30	Inoculated	Ferrihydrite	Exponential phase	N ₂ /CO ₂	1.0 g/L	L-cysteine	No	No
31, 32, 33	Sterile control	Ferrihydrite	–	N ₂ /CO ₂	1.0 g/L	L-cysteine	No	No
34, 35, 36	Procedure control	Ferrihydrite	Exponential phase	N ₂ /CO ₂	1.0 g/L	Ti(III)-citrate	Yes	No
37, 38, 39	Inoculated	Ferrihydrite	Exponential phase	N ₂ /CO ₂	organic-free	Ti(III)-citrate	Yes	No
40, 41, 42	Filtrate (no cells)	Ferrihydrite	Exponential phase	N ₂ /CO ₂	1.0 g/L	Ti(III)-citrate	Yes	No
43, 44, 45	Inoculated	Ferrihydrite	Exponential phase	N ₂ /CO ₂	organic-free	Ti(III)-citrate	Yes	Yes
46, 47, 48	Filtrate (no cells)	Ferrihydrite	Exponential phase	N ₂ /CO ₂	1.0 g/L	Ti(III)-citrate	Yes	Yes
49, 50, 51	Sterile control	Ferrihydrite	–	N ₂ /CO ₂	organic-free	Ti(III)-citrate	Yes	No
52, 53, 54	Sterile control	Ferrihydrite	–	N ₂ /CO ₂	organic-free	Ti(III)-citrate	Yes	Yes

55, 57	56,	Inoculated	FeCl ₃	Exponential phase	N ₂ /CO ₂	1.0 g/L	Ti(III)-citrate	No	No
58, 60	59,	Sterile control	FeCl ₃	–	N ₂ /CO ₂	1.0 g/L	Ti(III)-citrate	No	No

136 To explore the roles of extracellular and cell-associated enzymes in the reduction of ferrihydrite
137 and the production of Fe(0), we followed above procedure [steps (1) to (4)] to separate *M. barkeri*
138 cells and supernatants, flushed the headspaces by N₂/CO₂ (80%/20%) for 1 hour, heated all serum
139 bottles and organic-free sterile controls to 120 °C for 4 hours and allowed them to cool down for 5
140 hours to room temperature (20 °C) before the addition of ferrihydrite.

141 Because some reducing agents such as Ti(III)-citrate were previously hypothesized to influence
142 the redox transformations of iron [17], we also tested the importance of this reductant for the
143 formation of Fe(0). This was done by replacing Ti(III)-citrate by L-cysteine (0.5 mM final
144 concentration) as the reducing agent in the medium with low organic content (1 g/L).

145

146 **2.3 Analytical Methods**

147 The concentrations of headspace gases, including CH₄, CO₂ and H₂, were measured in triplicates by a
148 Shimadzu GC-2014 gas chromatograph configured with a packed column (Carboxen-1000, 5' × 1/8'',
149 Supelco, Bellefonte, Pennsylvania, USA). The temperature was set to 140 °C, and argon was used as
150 the carrier gas. CH₄ and CO₂ were measured by the methanizer-flame ionization detector (FID) and H₂
151 was measured by the thermal conductivity detector (TCD). The concentrations of gases were
152 calculated from their partial pressures based on the standards calibrated with the SCOTTY Specialty
153 Gas (T237-14, Sigma-Aldrich Corporation, MO, USA).

154 The concentrations of Fe(II) were measured by a microplate reader (BioTek, Synergy™ 2, VT,
155 USA) at 562 nm in 200 μL triplicate samples of mixtures obtained by mixing 1 mL subsamples of
156 media that had been filtered through 0.2 μm pore-size filters (Acrodisc 25 mm syringe filter, PALL
157 Corporation, MA, USA) and fixed immediately with 100 μL ferrozine solution. This solution was
158 prepared by dissolving 0.01 M ferrozine (FW 492.47, 97%) in 0.1 M ammonium acetate
159 (CH₃COONH₄, 99.99%) solution [37]. The concentrations of Fe(II) were determined using standards
160 that contained solutions of Fe(II) with known concentrations from 0 to 0.36 mM and analytical
161 standard deviation of ~0.001 mM. The reported errors in this paper are the standard deviations from
162 the measured triplicates.

163 Precipitated minerals were characterized using XRD on an X'Pert PRO diffractometer
164 (PANalytical manufacturer) equipped with an X'Celerator detector. The precipitates were collected by
165 centrifugation at 14,000 rpm for 5 mins, smeared on zero diffraction disk (23.6 mm diameter x 2.0
166 mm thickness, Si Crystal, MTI Corporation, CA, USA) and dried in an anaerobic glove box. The
167 samples were analyzed inside an anaerobic dome to maintain the anoxic conditions during the XRD
168 analyses. The XRD patterns were measured in reflection mode with nickel-filtered copper K α
169 radiation ($\lambda = 1.5406 \text{ \AA}$) as the X-ray source. The X-ray energy and the wavelength, respectively,
170 were set with a Si(III) double-crystal monochromator to be, respectively, 10 keV and 1.2404 (± 0.001)
171 \AA . The 2θ angle ranged from 3° to 90° with a scanning step of 0.008°. The fixed counting time was set
172 as 1000 s at 45 kV and 40 mA. Wavelength and 2θ calibrations were maintained by frequently
173 measuring intensity data from an aluminum foil (transmission geometry) or LaB6 powder (reflection
174 geometry). A platinum-coated toroidal focusing mirror with a step size of 0.04° was used to reject X-
175 rays with the energies >11 keV and to produce a focused beam of dimensions 1 mm × 4 mm.
176 Transmission data were corrected for θ -dependent attenuation of incident and scattered X-rays. XRD
177 spectra were analyzed with the High Score Plus program (version 4.5, Malvern Panalytical
178 Incorporated, Netherlands). Rietveld fitting method [38] was used to refine crystals and to determine
179 the atomic coordinates and lattice parameters of different mineral phases. Iron phases were determined
180 according to the Miller indices (hkl) that denote planes orthogonal to the reciprocal crystal lattice
181 vector. The presence of superstructure reflections (hkls of 111 and 200) at $2\theta = 42.94^\circ$ and 50.01°

182 indicated austenite [γ -Fe(0)] with the d-spacing of 2.104 Å. The presence of superstructure reflections
183 (hkls of 110, 200 and 211) at $2\theta = 45.32^\circ$, 66.03° and 83.72° indicated ferrite [α -Fe(0)] with the d-
184 spacing of 1.999 Å. Rietveld fitting was also used to quantify the phases in the fitting phase mixture
185 and determine the percentages of austenite and/or ferrite.

186 Minerals were also analyzed using *in situ* synchrotron μ XRD at the Advanced Light Source at
187 the beamline 12.3.2. Samples were collected on site and the sample paste was loaded into
188 transmission sample XRD cells. The transmission synchrotron diffraction data were collected using a
189 DECTRIS Pilatus 1M hybrid pixel area detector placed at $2\theta = 35^\circ$ at approximately 170 mm from the
190 sample. The 4-bounce monochromator was set to 10 keV ($\lambda = 1.239842$ Å). The sample geometry
191 with respect to the incident beam and the detector was calibrated using Al_2O_3 powder. The 2D
192 diffraction patterns were analyzed and integrated along the azimuthal direction into 1D diffractograms
193 using the X-ray microdiffraction analysis software (version 6, XMAS) developed at the Advanced
194 Light Source for the beamline 12.3.2, and MATLAB R2017a.

195 XPS was performed on a K-AlphaTM X-ray photoelectron spectrometer (Thermo Fisher
196 Scientific, MA, USA). All samples were fractured in high vacuum (3×10^{-8} Torr) in the Kratos outer
197 pressure chamber and then moved directly into the main XPS measurement chamber. An incident
198 monochromatic X-ray beam from the Al K Alpha target (10 kV, 10 mA) was focused on a 0.4 mm \times
199 0.3 mm area at a 45° angle with respect to the sample surface. The electron energy analyzer
200 perpendicular to the sample surface was operated with a pass energy of 50 eV to obtain XPS spectra at
201 a 0.1 eV step size and a dwell time of 50 ms. Each peak was scanned 15 times. To ensure
202 representative data from heterogeneous samples, we probed a total of 50-70 points per sample. XPS
203 data were treated and analyzed using CasaXPS curve resolution software package. Spectra were best
204 fit after Shirley background subtractions by non-linear least squares CasaXPS curve resolution
205 software package. Gaussian/Lorentzian (G/L) contributions to the line shapes were numerically
206 convoluted using a Voigt function. The different XPS lines with sets of Gaussian and Lorentzian
207 peaks were empirically fitted with different standards corresponding to different oxidation states –
208 Fe(0), Fe(II) and Fe(III).

209 Thermomagnetic measurements were attempted to quantify the amount of ZVI in the samples.
210 This was done by heating the samples to a temperature at which only ZVI contributes significantly to
211 the magnetization. In particular, if the samples are heated up to the titanomagnetite Curie temperature
212 ($< 580^\circ\text{C}$), the saturation magnetic moment at this temperature could be used to infer the amount of
213 ZVI given knowledge of ZVI's temperature-dependent saturation magnetization [equation (2) in [39]].
214 With this goal, we sampled the precipitates from exponential-phase *M. barkeri* cultures incubated in
215 high organic medium (4 g/L) under a N_2/CO_2 headspace and placed ~ 0.1 mL samples on top of a
216 MicroSense quartz perpendicular sample holder (blank saturation magnetic moment of 4×10^{-9} Am²).
217 The samples were left to dry inside an anaerobic glove box. We then acquired saturation
218 magnetization thermomagnetic measurements using an ADE model 1660 vibrating sample
219 magnetometer with an applied field of 1 T at intervals of 50°C from room temperature up to 350°C
220 (in the laboratory of C. Ross in the Department of Materials Science and Engineering at MIT). Both
221 heating and cooling curves were obtained to check for thermochemical alteration.

222 SEM was used to image microbial and mineral morphologies, and microbe-mineral associations.
223 Scanning electron micrographs were acquired by a Zeiss Merlin GEMINI II column high-resolution
224 scanning electron microscope (Carl Zeiss microscopy, CA, USA) equipped with a field gun emission
225 and EDS (EDAX detector; EDAX, NJ, USA) operating at an accelerating voltage of 5-15 kV, probe
226 current of 100 pA and a working distance of 8.5 mm. On-axis in-lens secondary electron (SE-mode)
227 detector was used during imaging. The samples were fixed by 0.2 M sodium cacodylate, 0.1% CaCl_2

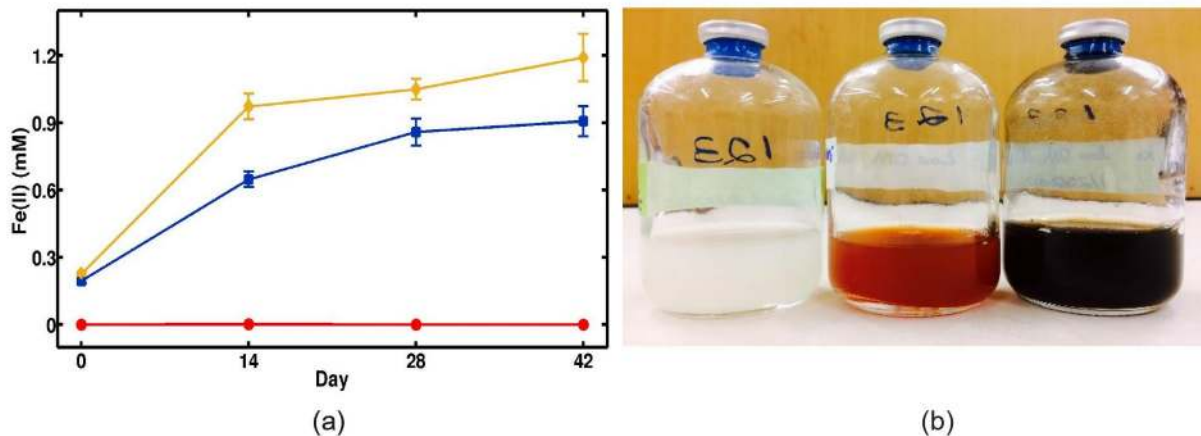
228 and 2.5% glutaraldehyde in anaerobic water for 2-3 days at 4 °C. The fixed samples were washed by
229 0.1 M sodium cacodylate, followed by a wash in nanopure water. After washing, the samples were
230 dehydrated with a series of ethanol-water solutions consisting of 30% (20 mins), 50% (20 mins), 70%
231 (20 mins), 80% (20 mins), 90% (20 mins) and 100% (20 mins) of 200 proof ethanol. After air-drying,
232 the samples were mounted on double-sided carbon tape and coated with a thin layer 5 nm of Au/Pd or
233 10 nm of carbon before imaging using Hummer V sputter coater. EDS spectra were treated and
234 analyzed using TEAM™ EDS software (version 2.0, EDAX Incorporated, NJ, USA) and Microsoft
235 Excel 2016.

236

237 3 Results

238 Because ferrihydrite is a common form of Fe(III) in the environment [40], we used this phase as the
239 solid electron acceptor in most of our experiments. We explored the formation of iron minerals in *M.*
240 *barkeri* cultures by adding ferrihydrite to the cultures initially grown on H₂/CO₂. The concentration of
241 Fe(II) in cultures increased with the incubation time due to the continuous reduction of ferrihydrite
242 [Figure 1(a)], regardless of the growth phase and the composition of the headspace gases (Table 1).
243 The color of the medium darkened with the incubation time and black precipitates formed [Figure
244 1(b)].

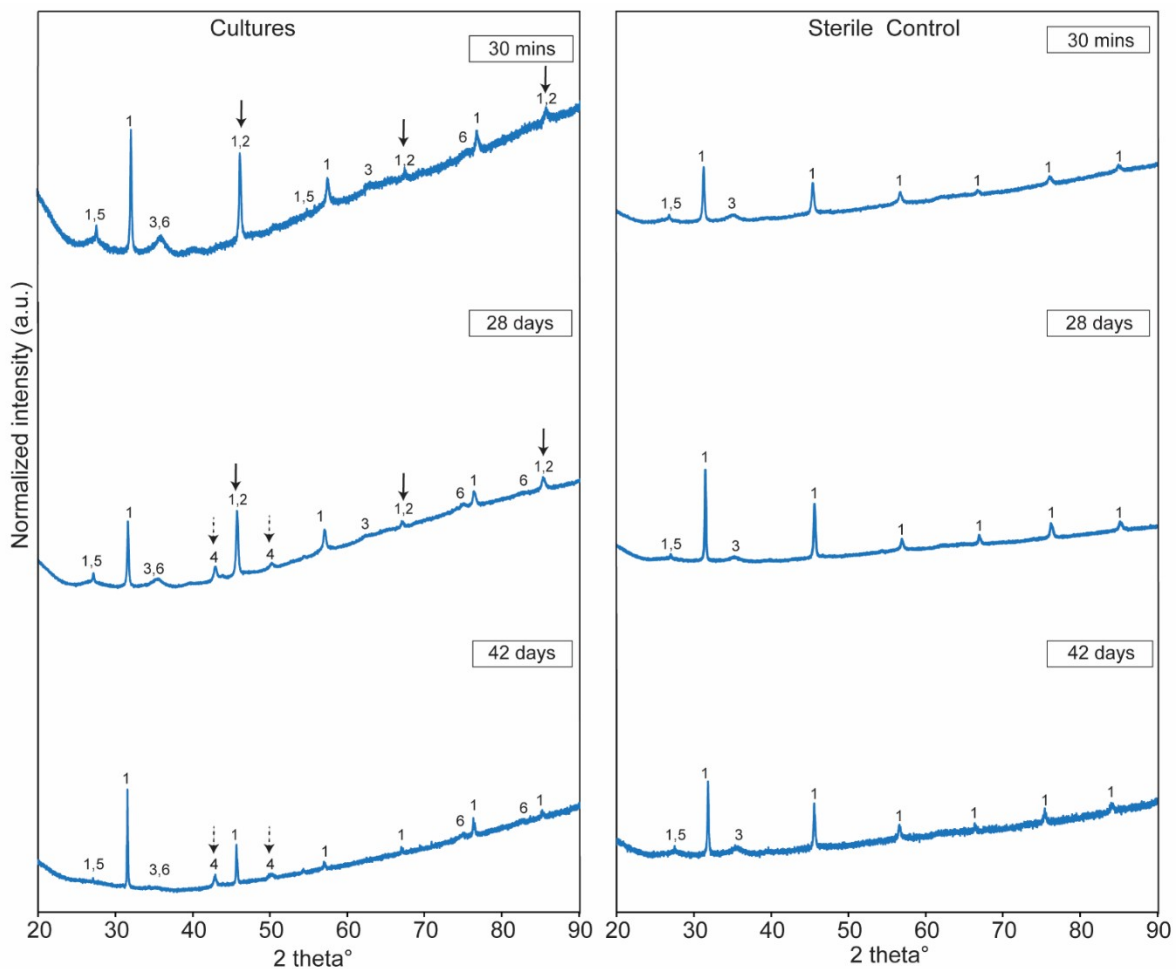
245



246 Figure 1: Representative changes of Fe(II) concentration and color in iron-reducing cultures of *M. barkeri* and sterile
247 controls. The headspaces of all cultures and controls shown here contained N₂/CO₂. The medium in all *M. barkeri* cultures
248 and controls contained 1.0 g/L of yeast extract and casitone. (a) Fe(II) concentration. Blue line and squares show
249 measurements from *M. barkeri* cultures to which ferrihydrite was added in exponential phase. Yellow line and diamonds
250 show measurements from *M. barkeri* cultures to which ferrihydrite was added in stationary phase. Red line and circles show
251 that Fe(II) was not produced in sterile controls. Each time point shows the average concentration from triplicate bottles and
252 the error bars show the standard deviation. (b) Representative color changes in cultures and sterile controls. Left (white)
253 bottle contains *M. barkeri* without ferrihydrite, the middle (reddish) bottle is the sterile control and the right (black) bottle
254 contains *M. barkeri* incubated with ferrihydrite for 28 days.
255
256

257 Precipitates were first sampled from cultures and sterile controls 30 mins after the addition of
258 ferrihydrite. Figure 2 shows representative XRD data. At this time point, halite (NaCl) and rutile (TiO₂)
259 were present in the XRD spectra of both *M. barkeri* cultures and sterile controls. This showed that rutile
260 formed by the abiotic oxidation of Ti(III)-citrate. Fe(II)-containing titanomagnetite (Ti₂Fe₃O₄) was
261 observed in *M. barkeri* cultures at all sampling time points, but never in sterile controls. Surprisingly,
262 the *M. barkeri* cultures at 30 mins also contained ferrite [α -Fe(0)] with d-spacing of 1.999 Å. Because
263 the α -Fe(0) and halite peaks overlapped, we used the Rietveld refinement to show that α -Fe(0) was
264 indeed present (Supporting Information, Table S2). After 28 days, the peaks of austenite [γ -Fe(0)] with

265 d-spacing of 2.104 Å appeared in *M. barkeri* cultures. After 42 days, the peaks of α -Fe(0) disappeared
 266 from the XRD spectra, but the peaks of γ -Fe(0) were still present. γ -Fe(0) was also detected by the
 267 μ XRD (Supporting Information, Figure S1) and XPS (Supporting Information, Figure S2). To confirm
 268 that Fe(0) was not present in the original iron source, we characterized ferrihydrite samples by XRD
 269 and found no Fe(0) peaks. These observations showed that ferrihydrite-reducing *M. barkeri* produced
 270 titanomagnetite and mediated the precipitation of Fe(0). The same phases were detected in *M. barkeri*
 271 cultures in stationary phase (Supporting Information, Figure S3), under a CH₄/H₂/CO₂ atmosphere
 272 (Supporting Information, Figure S4) and in the high-organic (4 g/L) medium (Supporting Information,
 273 Figure S5). α -Fe(0) was absent from sterile controls at all times, whereas some low-intensity XRD
 274 peaks – either a single peak or two small peaks – matching γ -Fe(0) appeared in some spectra of sterile
 275 controls 28 days after the addition of ferrihydrite Supporting Information, Figure S4 and S5). These
 276 Fe(0) peaks were detected in two-thirds of all independent experiments under these conditions and were
 277 not detectable 42 days after the addition of ferrihydrite. In contrast, γ -Fe(0) was always identifiable by
 278 two distinct peaks in the XRD spectra of *M. barkeri* cultures and remained detectable 42 days after the
 279 addition of ferrihydrite. If γ -Fe(0) was indeed present in sterile controls, then some components in the
 280 sterile media can contribute to the formation of Fe(0), but are not effective at stabilizing it over long
 281 incubation times.
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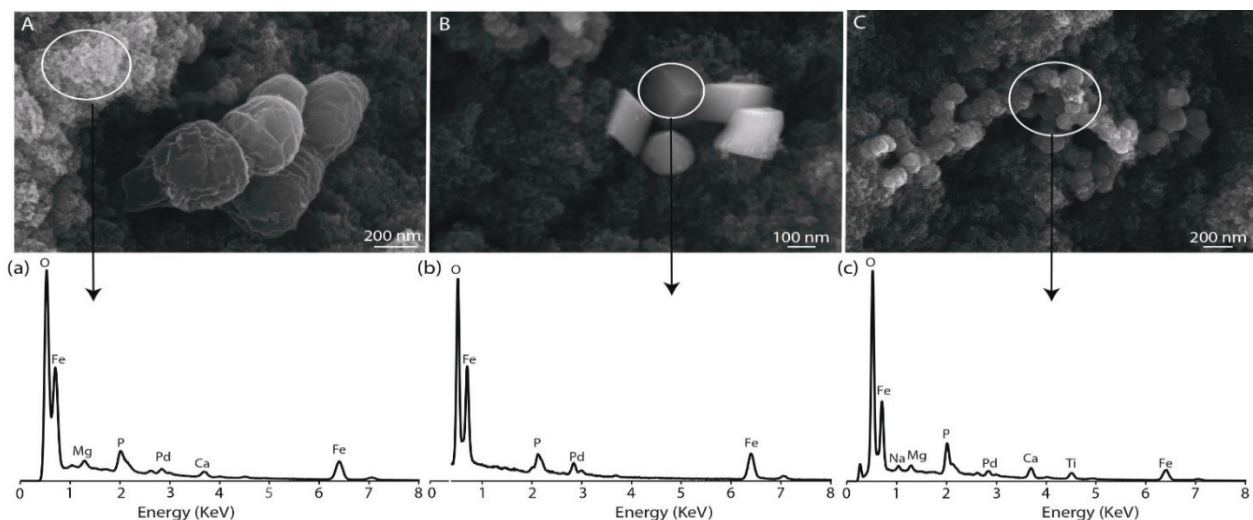
283
 284 Figure 2: XRD spectra of minerals sampled from *M. barkeri* cultures (left column) and sterile controls (right column). The
 285 medium was reduced by Ti(III)-citrate and contained 1 g/L of organic additives. The headspace gas was replaced by N₂/CO₂
 286 before the addition of ferrihydrite in exponential phase. The samples were collected at 30 mins, 28 days and 42 days after

287 the addition of ferrihydrite. XRD peak assignments: (1) Halite (NaCl); (2) Ferrite [α -Fe(0)]; (3) Ferrihydrite (Fe₂O₃); (4)
288 Austenite [γ -Fe(0)]; (5) Rutile (TiO₂); (6) Titanomagnetite (Ti₂Fe₃O₄).
289

290 The stoichiometry of the reduction of Fe(III) to Fe(II) and Fe(0) remains to be elucidated. Under
291 our experimental conditions, titanomagnetite formed readily, so we were not able to separate various
292 solid phases, quantify the relative amounts of reduced iron present in titanomagnetite and ZVI and
293 compare them to the concentrations of Fe(II) in the solution. Thermomagnetic measurements were used
294 to determine the quantities of magnetite and Fe(0) (Supporting Information, Figure S6); however, these
295 attempts were not successful due to the production of new magnetic materials and changes in magnetic
296 mineralogy during the laboratory heating cycle.

297 SEM imaging revealed morphological changes of *M. barkeri* and the morphologies of minerals
298 that formed during the incubation with ferrihydrite. Initially, the rounded *M. barkeri* cells had smooth
299 surfaces and formed large aggregates (Supporting Information, Figure S7). After the addition of
300 ferrihydrite, the diameter of *M. barkeri* cells and the sizes of cell aggregates decreased with the
301 incubation time and the cells exhibited wrinkled surfaces [Figure 3(A)]. Cubic minerals with the EDS
302 spectra consistent with ZVI phases and spherical minerals with the EDS spectra consistent with
303 titanomagnetite were not associated with cells or cell aggregates [Figure 3(B) and (C)], suggesting that
304 the formation of these minerals did not require direct cell-mineral contact.

305 To understand whether the precipitation of Fe(0) was a function of the Fe(III) source, we added
306 aqueous FeCl₃ (7.5 mM final concentration) to the triplicate cultures of exponential-phase *M. barkeri*
307 and triplicate sterile controls. All bottles contained medium with a low content (1 g/L) of organic
308 additives and was reduced by Ti(III)-citrate. All headspaces were replaced by N₂/CO₂ before the
309 addition of FeCl₃. Again, halite and rutile peaks were present in the XRD spectra of both *M. barkeri*
310 cultures and sterile controls (Supporting Information, Figure S8) at all time points. The precipitates in
311 *M. barkeri* cultures also contained titanomagnetite, but α -Fe(0) peaks only appeared in these cultures 42
312 days after the addition of FeCl₃. ZVI was absent from the sterile controls at all time points. Thus, ZVI
313 formed much more slowly when FeCl₃ was used instead of ferrihydrite as the source of ferric iron.
314



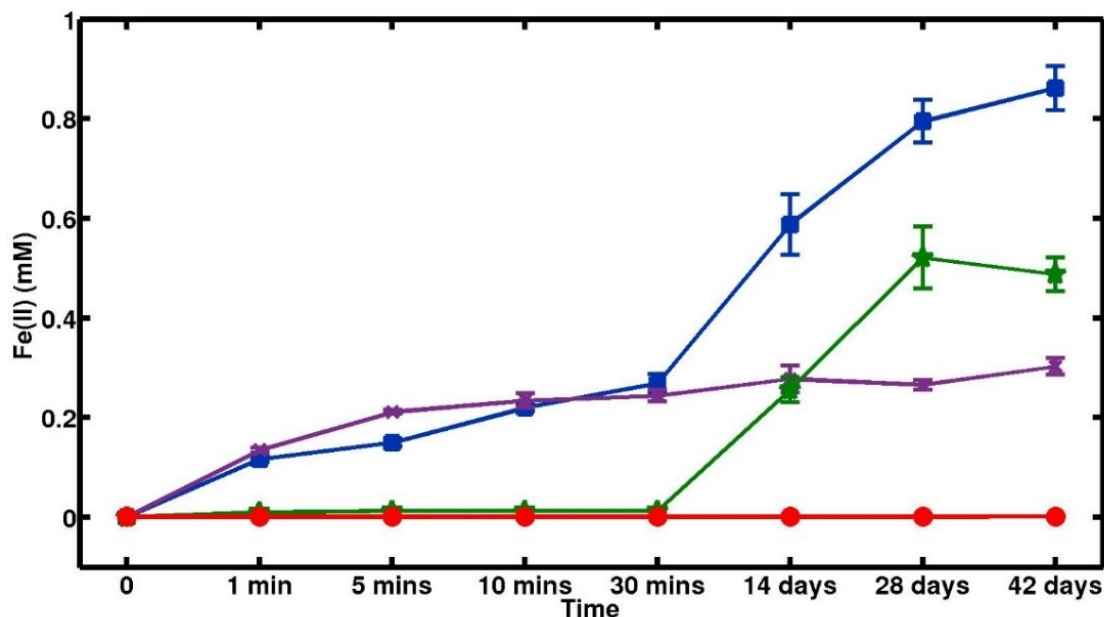
315
316 Figure 3: SEM images and EDS spectra of *M. barkeri* cultures and minerals 28 days after the addition of ferrihydrite. (A)
317 Wrinkled surfaces of *M. barkeri*. (a) EDS spectrum of amorphous ferrihydrite. The peak of phosphorus (labeled as P) was
318 also present in the EDS spectra of ferrihydrite from sterile controls. (B) Cubic-shaped ZVI. (b) EDS spectrum of (B). (C)
319 Titanomagnetite. (c) EDS spectrum of (C).
320

321 To determine the influence of reducing agents on the formation of Fe(0), we added 0.5 mM L-

322 cysteine instead of Ti(III)-citrate to the medium with a low organic content (1 g/L). The headspace was
323 replaced by N₂/CO₂ before the addition of ferrihydrite to exponential-phase cultures and sterile controls.
324 The XRD spectra (Supporting Information, Figure S9) showed Fe(0), magnetite and halite in the solids
325 from the cysteine-reduced cultures. As expected, rutile and titanomagnetite were absent due to the lack
326 of Ti(III). Thus, the formation of Fe(0) did not depend on the presence of Ti(III) as the reducing agent.
327 The XRD spectra of cysteine-reduced sterile controls (Supporting Information, Figure S9) contained α -
328 Fe(0) at 28 days and γ -Fe(0) phase at 42 days after the addition of ferrihydrite. This observation was
329 consistent with the previously reported ability of L-cysteine to precipitate and stabilize ZVI [41].

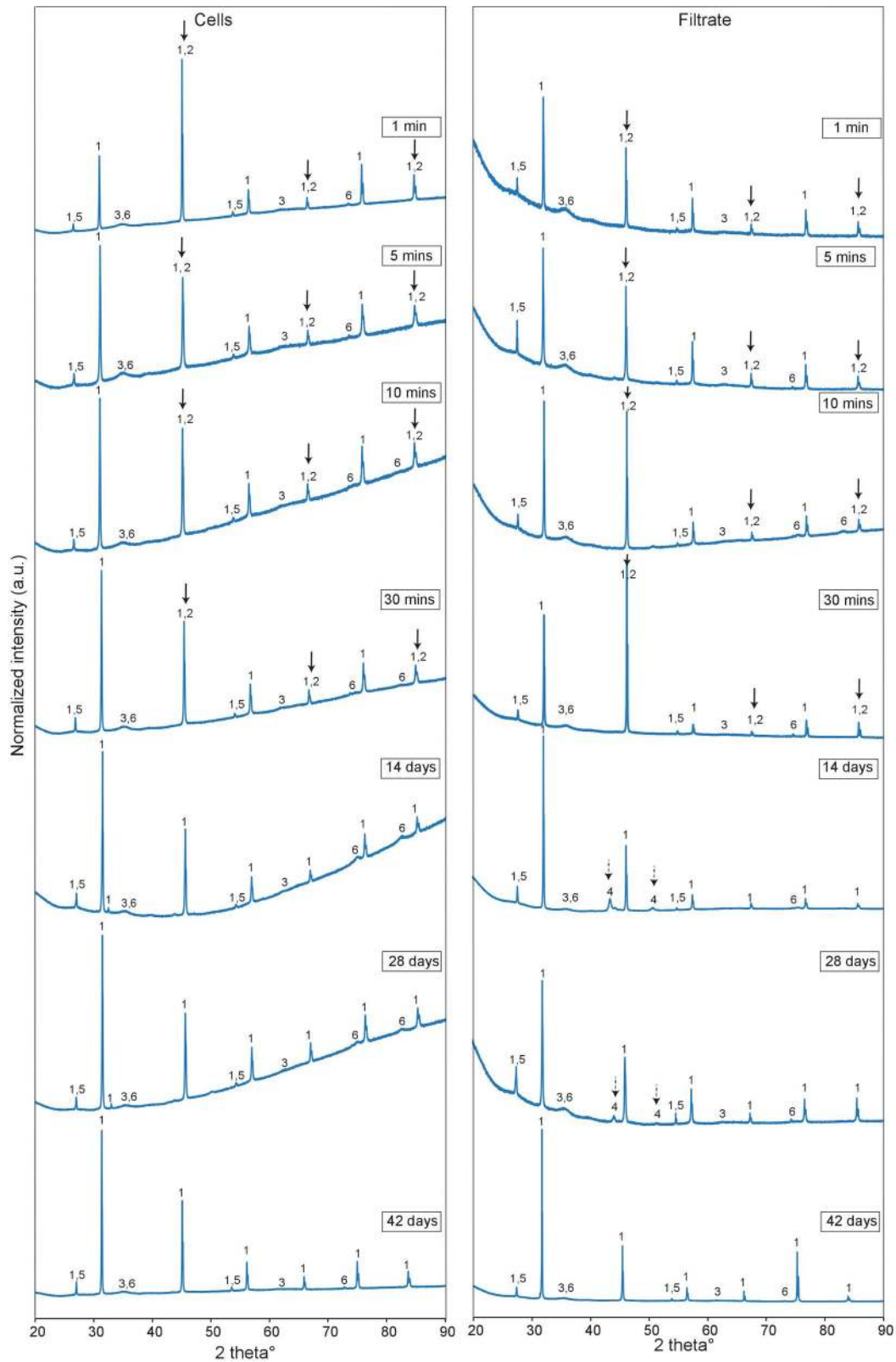
330 We hypothesized that both *M. barkeri* cultures and dissolved electron donor(s) reduced iron [17].
331 To understand the relative importance of either mechanism in the reduction of ferric iron and the
332 formation of ZVI, we separated *M. barkeri* cells in exponential phase from the liquid medium by
333 filtration. The organic-free medium in sterile serum bottles was inoculated by these filter-separated
334 cells. Equal volumes of organic-containing spent culture supernatants were added to three clean,
335 autoclaved and anaerobic sterile serum bottles. The headspaces of all bottles were then flushed by
336 N₂/CO₂ before the addition of ferrihydrite (Table 1). To confirm that the filtration did not influence the
337 physiology of *M. barkeri*, we recombined the filtered *M. barkeri* cells and the filtered supernatants in a
338 separate procedure control (Table 1). The final Fe(II) concentration in this procedure control was 0.86
339 mM. This recovered around 95% of the Fe(II) concentration – 0.91 mM – measured in a previous
340 experiment where the cells were not separated from the supernatants [Figure 1(a)] and showed that the
341 filtration and separation of cells from the spent culture supernatants did not influence the physiology of
342 *M. barkeri*. Iron reduction occurred both in the organic-free medium with *M. barkeri* cells and in filter-
343 sterilized spent supernatants that lacked cells (Figure 4), but cells and organic additives reduce iron by
344 different mechanisms and at different rates. Namely, the concentration of Fe(II) in the organic-free
345 medium inoculated by *M. barkeri* increased to about 0.3 mM during the first 30 mins after the addition
346 of ferrihydrite (Figure 4) and remained unchanged afterwards. In contrast, the filtered supernatants
347 contained only 0.013 mM Fe(II) at 30 min after the addition of ferrihydrite, but the concentration of
348 Fe(II) increased to 0.52 mM after 28 days (Figure 4). These differences between organic-free *M.*
349 *barkeri* cultures and cell-free spent culture supernatants were accompanied by different trends in the
350 formation and stabilization of ZVI.

351



352
 353 Figure 4: The change of Fe(II) concentration in: organic-free medium with cells (purple line and crosses), filter-sterilized
 354 spent supernatants (green line and stars), sterile control (red line and circles) and procedure control that contained
 355 recombined filtered cells and spent supernatants (blue line and squares). Each point shows the average concentration from
 356 triplicate bottles and the error bars show the standard deviation.

357
 358 Previous studies identified green rust, an unstable precursor of several iron oxide minerals such as
 359 magnetite and hematite, as a transient phase during iron reduction under both biotic and abiotic
 360 experimental conditions [42–44]. To look for the presence of similar short-lived mineral phases in *M.*
 361 *barkeri* cultures, we sampled the precipitates from the organic-free medium inoculated by cells, filter-
 362 sterilized spent supernatants and organic-free sterile controls at 1 min, 5 mins, 10 mins and 30 mins
 363 after the addition of ferrihydrite. After that time, the samples were collected every 14 days. In short, we
 364 did not detect green rust in any of the analyzed precipitates, but did confirm various observations from
 365 our previous experiments. Fe(0) minerals were absent from organic-free sterile controls at all time
 366 points (Supporting Information, Figure S10). In the organic-free medium inoculated by cells (Figure 5),
 367 peaks of α -Fe(0) appeared in the precipitates at 1 min after the addition of ferrihydrite. Their intensity
 368 decreased



369
 370 Figure 5: XRD spectra of minerals sampled from serum bottles that contained cells in organic-free medium (left column)
 371 and filter-sterilized spent supernatants (right column). The medium was reduced by Ti(III)-citrate and the headspace gas was
 372 replaced by N_2/CO_2 before the addition of ferrihydrite. The solids were sampled at 1 min, 5 mins, and 10 mins, 30 mins, 14
 373 days, 28 days and 42 days after the addition of ferrihydrite. XRD peak assignments: (1) Halite (NaCl); (2) Ferrite [α -Fe(0)];
 374 (3) Ferrihydrite (Fe_2O_3); (4) Austenite [γ -Fe(0)]; (5) Rutile (TiO_2); (6) Titanomagnetite ($Ti_2Fe_3O_4$).

375 over the first 30 mins of the experiment to below the detection limit after 14 days. Comparatively,
376 smaller α -Fe(0) peaks appeared in the XRD spectra of the spent supernatants 1 min after the addition of
377 ferrihydrite and their intensity increased at 5, 10, and 30 mins (Figure 5). α -Fe(0) in the spent
378 supernatants transformed to a different phase of ZVI – γ -Fe(0) – after 14 days, but none of the Fe(0)
379 phases were detectable in 42-day-old samples. Again, because the α -Fe(0) and halite peaks overlapped,
380 the Rietveld refinement was used to demonstrate the presence of α -Fe(0) (Supporting Information,
381 Table S3). These experiments revealed the very rapid formation of Fe(0) when either *M. barkeri* cells
382 were present in the organic-free medium or organic compounds were present in the cell-free spent
383 supernatants. The stabilization of Fe(0) phases for more than 30 days required both cells and organic
384 additives. We detected no Fe(0) in the organic-free sterile controls, but found tentative peaks in the
385 sterile controls amended by organic compounds 28 days after the addition of ferrihydrite (Supporting
386 Information, Figure S4 and S5). Hence, yeast extract and casitone may promote the formation of ZVI,
387 but are not sufficient for its stabilization.

388 Our results so far indicated that *M. barkeri* cells and soluble organic compounds both promoted
389 the formation and stabilization of ZVI. To determine whether this process required some enzymatic
390 activity, we characterized the precipitates from the heat-treated spent supernatants and the organic-free
391 media inoculated by *M. barkeri* cells. Spent supernatants or organic-free *M. barkeri* cultures were
392 prepared as described in the previous paragraphs (also see Section 2.2). Triplicate sealed serum bottles
393 containing either separate were heated at 120 °C in an oven for 4 hours and allowed to cool at room
394 temperature for 5 hours before the addition of ferrihydrite. The heat-treated *M. barkeri* cultures reduced
395 0.007 mM of Fe(III) to Fe(II) 30 mins after the addition of ferrihydrite; this concentration remained
396 unchanged after 42 days (Supporting Information, Figure S11). The concentration of Fe(II) in heat-
397 treated spent supernatants increased from 0.005 mM at 30 mins after the addition of ferrihydrite to
398 0.313 mM after 42 days (Supporting Information, Figure S11). These concentrations were 2.5% and
399 64.3% of the respective Fe(II) concentrations measured in culture separates that did not undergo
400 heating (Figure 4). Thus, non-enzymatic redox-active compounds were responsible for around 37% of
401 the total activity that reduced ferric to ferrous iron in *M. barkeri* cultures. This is consistent with a
402 previous report, which measured 11% of this activity in unfiltered heat-treated cultures 14 days after the
403 addition of ferrihydrite [17]. The heat treatment did not prevent ZVI from forming. The peaks of Fe(0)
404 appeared in the XRD spectra from both heat-treated separates 30 mins after the addition of ferrihydrite
405 (Supporting Information, Figure S12). These peaks disappeared after 28 days in the presence of heat-
406 treated *M. barkeri* cells (in organic-free medium), but were still detectable after 42 days in heat-treated
407 spent supernatants. Given that ZVI was not detected in 42-day-old spent supernatants that did not
408 undergo any heat treatment, the heat treatment appeared to have increased the persistence of ZVI in
409 organic-containing medium.

410

411 4 Discussion

412 Various microorganisms, including *M. barkeri*, have been shown to reduce iron oxides and mediate the
413 formation of different Fe(II)-containing minerals [6–11,17,18]. This work provides the first evidence
414 that archaea can mediate the rapid precipitation of ZVI in the presence of Fe(III). The precipitation of
415 Fe(0) requires the presence of soluble, redox-active compounds that can interact with ferric iron and
416 stabilize ZVI and can occur in the absence of cells and enzymatic activity. At 37 °C, this reduced phase
417 was stabilized over periods longer than one month only in the presence of both *M. barkeri* and soluble
418 organic compounds.

419 The mechanism of electron transport during the formation of Fe(0) remains to be elucidated. The
420 detection of ZVI in spent supernatants and the lack of spatial associations among *M. barkeri* cells and

421 ferrihydrite, titanomagnetite and ZVI support the extracellular reduction of ferrihydrite to Fe(II) and
 422 ZVI. Previous studies have proposed several mechanisms of extracellular electron transport between
 423 microbes and minerals: (1) electrically conductive pili or nanowires, (2) chelating compounds that
 424 reduce and solubilize Fe(III) minerals and (3) soluble electron carriers [2,17]. We did not observe any
 425 pili or nanowires by SEM, so the last two mechanisms are more likely to occur under our experimental
 426 conditions.

427 *M. barkeri* synthesizes several electron carriers with low redox potentials, including ferredoxin,
 428 coenzyme F420, coenzyme B and methanophenazine (Table 2) [15]. According to the Eh-pH diagram
 429 for the Fe-CO₂-H₂O system [45,46], ZVI can be stable in the Eh range from about -450 mV to -650 mV
 430 at pH values 5-7 seen in our carbonate-buffered system. This is comparable to the redox potential of
 431 ferredoxin (-500 mV) (Table 2), although the roles of this and other carriers remain to be tested. As
 432 stated previously, enzymatic activity cannot account for the rapid formation of ZVI by the heat-treated
 433 *M. barkeri* cells and spent supernatants. Furthermore, due to the poor solubility of methanophenazine in
 434 water and its strong association with cell membranes [47], we suspect that this compound is not critical
 435 for the formation of ZVI in spent supernatants or at a distance from *M. barkeri* cells.

436 Our experimental results do not directly link the formation of ZVI to the interactions between
 437 cellular metabolism and Fe cations. In fact, our observations suggest that *M. barkeri* cells and/or any
 438 cell-associated surface organics that are not removed during the washing procedure and/or the heat
 439 treatment can promote the formation of ZVI and that active metabolism is not essential. Potential
 440 electron donors for the reduction of ferric iron to ZVI in *M. barkeri* cultures include membrane-
 441 associated or water-soluble redox-active compounds such as enzymes, coenzymes or even amino acids
 442 such as L-cysteine. Although the tentative appearance of ZVI in a few organic-amended sterile controls
 443 suggested that the organic additives (yeast extract and casitone) might promote the production of ZVI,
 444 they were not effective at stabilizing it under our experimental conditions. Given that Fe(0) formed in
 445 the heat-treated organic-free cell cultures and heat-treated spent supernatants, a major contribution of
 446 heat-destabilized enzymes is also unlikely. Instead, the formation of ZVI depends primarily on non-
 447 enzymatic and soluble metabolites produced by *M. barkeri* in organic-replete media.

448 The long-term persistence of ZVI in *M. barkeri* cultures requires soluble organic compounds. In
 449 keeping with this, Fe(0) peaks persist longer in the XRD spectra of spent supernatants compared to
 450 those of the cells incubated in the organic-free medium (Figure 5). The metabolite(s) produced by *M.*
 451 *barkeri* may be similar to the ZVI-stabilizing L-cysteine [41]. Previous attempts to synthesize ZVI in an
 452 ecologically friendly manner have produced Fe(0) by adding Fe(III) as FeNO₃ or FeCl₃ to plant extracts
 453 [32,33]. The formation of ZVI in the presence of polyphenols from plant extracts contributed to the
 454 blackening of solutions [32,33] in a manner similar to that shown in [Figure 1(b)]. The E^0 of these
 455 compounds range from 300 mV to 800 mV, which is higher compared to the potentials in *M. barkeri*
 456 cultures. It is unclear whether the cultures of *M. barkeri* contain phenolic compounds, but we speculate
 457 that the low redox potential in the methanogen cultures (Table 2) further facilitates the reduction of iron
 458 from ferric to metallic state and the stabilization of ZVI.

459
 460 Table 2: Relevant redox half-reactions at pH=7

Possible Redox Half Reaction	E^0 (mV)
Ferredoxin (ox) → Ferredoxin (red)	-500 [15]
Coenzyme F ₄₂₀ (ox) → Coenzyme F ₄₂₀ (red)	-360 [15]
Coenzyme B (ox) → Coenzyme B (red)	-140 [15]

466	Methanophenazine (ox) → Methanophenazine (red)	-165 [15]
467	Fe(III) → Fe(0)	-37 [22]

468
469

470 5 Environmental Implications

471 Microbially mediated iron corrosion is a known natural process and a major industrial and engineering
472 concern [48–50]. Methanogenic archaea, sulfate reducing bacteria, acetogenic bacteria and nitrate
473 reducing bacteria are all able to oxidize Fe(0) under anaerobic conditions [50]. Our results demonstrate
474 that *M. barkeri* can mediate the reverse process, wherein Fe(III) in ferrihydrite is reduced and stabilized
475 as ZVI. This expands the list of microbially mediated iron reduction reactions and biomineralization
476 processes. In the light of our results, ZVI may form and become stabilized in reducing and organic-rich
477 anaerobic environments. If ZVI produced in this manner can interact with other elemental cycles, it can
478 be readily oxidized to Fe(II)/Fe(III) and therefore not detectable.

479 Biogenic magnetite is widespread in marine [51,52] and riverine sediments [19,53]. This mineral
480 can be produced by different microorganisms including bacteria (e.g., magnetotactic bacteria [51,52],
481 *Geobacter* [54,55] and *Shewanella* [56]), archaea (e.g., *Methanosarcinaceae* [19]) and fungi (e.g.,
482 *Fusarium oxysporum* and *Verticillium sp.* [57]). Recent studies have shown that electrically conductive
483 substances such as magnetite, hematite and graphite facilitate direct interspecies electron transfer
484 (DIET) [19,54] and promote syntrophic cooperation in microbial communities [58–60]. For example,
485 magnetite can enhance the electron transfer from *G. sulfurreducens* to *T. denitrificans* and facilitate
486 nitrate reduction by *T. denitrificans* [58]. Magnetic minerals in deep sediments carry paleomagnetic
487 information that can be altered by phase transformations during diagenesis [61,62]. Magnetite and ZVI
488 produced by anaerobic microorganisms such as methanogens are examples of phases that can influence
489 the paleomagnetic records. Ferrihydrite is ubiquitous in nature [40]; its delivery to reducing and
490 organic-rich anaerobic environments may enable microbial precipitation of ZVI (i.e., a conductive and
491 reactive material) that then promotes DIET in the communities of anaerobes [63,64]. Fe(0) peaks were
492 not reported in the XRD spectra in the studies of DIET in the presence of microbial iron reduction [19,
493 55–57]. Their absence might indicate that the redox conditions or organic compounds in these studies
494 did not support the formation and/or stabilization of ZVI or that the produced ZVI was oxidized during
495 the processes of mineral characterization. Our experiments tested for Fe(0) production by *M. barkeri*
496 only, so it remains to be seen whether ZVI formation by methanogens is a ubiquitous phenomenon.

497 The impact of exogenously added ZVI on the structure of microbial communities in aquifer
498 sediments [65] and soils [66] is an area of active research. For example, several studies of pure cultures
499 have reported that ZVIs are toxic to *Escherichia coli* [67,68], *Dehalococcoides* spp. [69] and
500 *Desulfosporosinus* spp. [65,70]. Thus, ZVI particles produced naturally by *M. barkeri* or other
501 methanogens may influence the ecology of anaerobic environments by inhibiting the growth of some
502 microbes and promoting the growth of Fe(0)-resistant anaerobes (e.g., *Raoultella planticola* [71] and
503 *Alcaligenes eutrophus* [72]). Previous studies have shown that the reduction of ferric to ferrous iron by
504 methanogens can compete with methanogenesis [17,18]. Any ZVI particles produced by methanogenic
505 archaea might also intersect with the biogeochemical cycles of carbon and sulfur by either enhancing
506 [73,74] or inhibiting [75] methanogenesis and inhibiting biological sulfate reduction [65,70]. These
507 results imply that the production of Fe(0) by methanogens may affect both the reduction of CO₂ and the
508 accumulation of CH₄ in anaerobic environments and CH₄-consuming microbes. In our experiments and
509 other studies [17,18], methanogenesis was inhibited after the addition of ferrihydrite into the *M. barkeri*
510 cultures. However, the main goal of our experiments was to characterize minerals in pure cultures of

511 iron-reducing *M. barkeri* as a function of the reductants and cellular and enzymatic activity. Further
512 experiments are needed to determine the impact of ZVI formation on methanogenesis and the anaerobic
513 oxidation of CH₄ in natural communities.

514 The microbially mediated production of ZVI may find industrial applications in metal protection
515 and green synthesis. Microorganisms can inhibit iron corrosion by several indirect mechanisms, such as
516 the formation of protective films and consumption of corrosive substances [47,76]. The reverse iron
517 corrosion in *M. barkeri* cultures shows that at least this microbe can form and stabilize ZVI in
518 anaerobic and organic-rich environments that receive labile forms of ferric iron such as ferrihydrite.
519 These conditions can be explored further to identify the range of chemical conditions and more
520 complex microbial communities that protect steel. Given that microbial methanogenesis is widespread
521 in various terrestrial or marine environments and industrial or water-treatment facilities [77–80], the
522 availability of methanogenic enrichments and organic-rich fluids from these systems should be
523 conducive to cost-effective green synthesis of ZVI.

524

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534

535 **Supporting Information.** Medium recipe; X-ray diffraction (XRD) spectra under different
536 experimental conditions; Rietveld analyses of XRD spectra; micro-focused X-ray diffraction;
537 thermomagnetic measurements; scanning electron microscope images; Fe(II) concentration
538 measurements

539

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